Mikhail V. Blagosklonny

Cell Cycle Checkpoints and Cancer

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PREFACE

Advances in Research and Challenges in Therapy

Mikhail V. Blagosklonny

The ultimate goal of cancer research is the development of effective anticancer therapy. During the last several decades, the discovery of oncogenes, tumor suppressors, growth factors, signal transduction pathways has dramatically escalated our understanding of cancer cell biology and mechanisms of cell transformation.[1-3] Hundreds of cellular proteins and pathways have been logically considered as molecular targets in a mechanism-based approaches of anticancer drug development.[4-6]

Yet, the progress in cancer treatment has not paralleled these dramatic achievements in basic research. Certainly, a delay must exist between identification of molecular targets and their clinical applications. However in many other fields of medicine, effective drugs had been found prior to identification of their molecular mechanisms, such as aspirin, anti-malaria drugs and antibiotics. Vaccination against viruses such as smallpox had been developed almost two centuries before the immune system and viruses were described. The most relevant parallel to anticancer drug development is the discovery of antibiotics. Penicillin had revolutionized the treatment of bacterial diseases long before its molecular target was identified. The bacterial wall, a structure that does not exist in human cells, allows penicillin to kill a bacteria without affecting a human cell, thus exercising dramatic selectivity. In this light, the absence of the magic bullet against cancer is consistent with the lack of a cancer-selective target. We have learned that there are very few molecules in cancer cells that are dispensable or absent in normal cells. One of these few, Bcr-Abl, is a selective target in Bcr-Abl-positive leukemia, even though molecular therapeutics that inactivate Bcr-Abl have additional targets [7] Mutant p53 is another example of a potential cancer-selective target.[8, 9] Although telomerase exists in normal cells, its functional significance in cancer cells allows us to consider this enzyme as a reasonably-selective target. [10] However, these and other examples do not alter the general conclusion: proto-oncogenes and signal transduction molecules are required for proliferation and survival of normal cells and therefore most mechanism-based therapeutics (e.g., inhibitors of kinases) will be also toxic for certain normal cells. The absence of cancer-selective targets is the most important problem of the anticancer drug-screen, because compounds toxic to cancer cells also kill normal cells, therefore side-effects are inevitable. Besides, natural compounds are synthesized by microorganisms, plants and animals in order to kill other organisms. They are not intended to discriminate between normal and cancer cells and cannot selectively kill cancer cells.

In light of the low probability of finding a "magic bullet", it is not surprising that alternative approaches emerge. These range from the targeting of endothelial cells to protection of normal cells, from a selective delivery of drugs using tissue-specific markers to exploiting hypoxia and drug-resistance.

Loss of cell cycle checkpoints is the most universal alteration in human cancer.[11-13] Furthermore, as emphasized by Hanahan and Weinberg, the large and diverse collection of cancer-associated genes can be tied to the operations of a small group of regulatory circuits. [2] In other words, although numerous genetic alterations may cause loss of normal checkpoints, common strategies might be developed against a wide variety of cancers. As suggested by Paul Nurse, this would present a more promising approach than unspecific attempts to block cell cycle progression, which are less likely to distinguish between cancerous and normal cells.[14] Aiming at defective cell cycle checkpoints is different from targeting cancer-specific molecules. In the checkpoint approach, it is not necessary to target cancer-promoting or key-functional molecules (e.g., CDK), nor the molecule which is altered in cancer (mutated, overexpressed, etc). A target may lie upstream of the affected function or may belong to parallel pathways. Although the same molecule will be targeted in both cancer and normal cells, the functional outcome can be different in cells with defective checkpoints. For example, loss of the G1 checkpoint is common in cancer cells with mutant p53. In response to DNA damage, such cancer cells are arrested in G2. The arrest at G2/M is dramatically sensitive to even one double strand break because failure to arrest would lead to the irreversible loss of chromosome fragments.[15] Since G2 arrest in cells lacking p53 depends on the Chk1 kinase, inhibition of this kinase results in abrogation of the G2 checkpoint exclusively in cancer cells lacking p53.[16-18] Following treatment with DNA damaging drugs, mitotic progression of cancer cells will result in selective killing of cells with defective checkpoints.[19, 20]

Even currently used chemotherapy, such as DNA-damaging and microtubule active drugs, is effective in the treatment of some malignancies, especially of apoptosis-prone leukemia and lymphomas, and some solid tumors such as testicular cancer. Of course, as expected, the toxicity to normal cells limits effectiveness of chemotherapy in many cases. More intriguingly however is the question of why these drugs are useful and in some cases may cure the disease. Although most of these drugs target nonselective and even nonmechanism-based targets, such as DNA, topoisomerases, or tubulin, their ultimate effects converge on targeting checkpoints. These drugs indirectly target checkpoints.

Modulation of cell cycle checkpoints may result in treatment regimens with improved therapeutic indices by exploiting the disruption of checkpoints in tumor cells.[21, 22] Loss of the G2/M delay might be more consequential to a cell carrying a defect in a G1/S checkpoint than to an otherwise wild-type cell.[15] Pharmacological abrogation of the G2 checkpoint can increase sensitivity to chemotherapy in G1-checkpoint-deficient cells, whereas cells with normal checkpoints may take refuge in G1. Furthermore, loss of checkpoints could be used for selective protection of normal cells.[23-27] Recently it has been shown that inhibitors of CDK can prevent chemotherapy-induced hair loss in rats.[28]

Exploiting defective checkpoints is only in its infancy of development. However, as is often in the history of medicine, unintentional exploitation of checkpoint loss in cancer might be responsible for the effectiveness of standard therapies. The link between checkpoint control and apoptosis also tempts novel therapeutic approaches.[29-31] Rational design based on the understanding of cell cycle control coupled with utilizing novel mechanism-based therapeutics for manipulating the cell cycle will bring anticancer chemotherapy to a new level.[32]

The Book Overview

This book "Cell Cycle Checkpoints and Cancer" addresses mechanisms of normal and cancer cell cycling, checkpoint control, the link of mitogenic signaling and cell cycle machinery. Considerable attention is devoted to the analysis of checkpoint mechanisms from yeast to man allowing us to understand the logic of the cell cycle. Applications to current and future anticancer therapies is discussed throughout the book and especially in last Chapters.

Mitogenic signaling is normally initiated on the cellular membrane by mitogens, growth factors and cytokines.[33] Not surprisingly, autocrine production of mitogens is common in malignant transformation. Autocrine and paracrine growth factor synthesis contribute to angiogenic and metastatic properties of transformed cells. In the following Chapter, James McCubrey et al review the mechanisms of autocrine production of cytokines and growth factors. The cytokine model illustrates deregulation of autocrine cytokine expression on several levels with potential therapeutic approaches. In additional Chapter, McCubrey et al discuss signal transduction from cytokine receptors to cell cycle machinery via Ras/Raf-1/MEK/ERK, PI3K/Akt, Jak-STAT and other pathways. The Chapter spotlights links between mitogenic signaling and apoptotic machinery and mechanisms that allow cancer cell to evade apoptosis.

In normal cells, growth factors are necessary to initiate and maintain the transition through G1 phase leading to S phase. The point at G1 at which commitment occurs and a cell no longer requires growth factors to complete the cell cycle has been termed the restriction point by Arthur Pardee in 1974. This discovery shaped the main direction of the research in cell cycle regulation culminating in the discovery of cyclins and cyclin-dependent kinases. It is important that following growth-regulating stimuli, both inhibitors and stimulators of CDKs are simultaneously induced. The choice between proliferation and growth arrest is determined by the state of the restriction point. The Chapter discusses that the restriction point could be considered as a prototype of cell cycle checkpoints.

By arresting the cell cycle, activation of checkpoints presumably allows cells to repair DNA. In "DNA damage, cell cycle control, and cancer" Jens Oliver Funk et al describes series of events that is triggered in cells upon DNA damage as well as a framework for the understanding of the functions of the core components involved in the cell cycle response to DNA damage.

Cell cycle checkpoints are not restricted to DNA damage.[34] As discussed by Dunkan Clarke et al, checkpoints are mechanisms that establish dependence relationships between biochemically unrelated cellular processes. For example, the S-phase checkpoint ensures that genome duplication is completed before cell division. The topoisomerase II-dependent checkpoint ensures that the topology of the newly replicated DNA has been correctly organized before cells begin mitosis. Distinct checkpoints monitor mitotic spindle assembly, preventing the onset of chromosome segregation until all the chromosomes are correctly aligned, and prevent exit from mitosis until anaphase chromosome segregation has been completed.

The p53 tumor suppressor play a key role in checkpoint control in mammalian cells. Levels of p53 are regulated by the Mdm-2-dependent protein degradation.[35] p53 can induce growth arrest and/or apoptosis. Intriguingly, p53-mediated

apoptosis involves both transcription-dependent and independent mechanisms.[36] In this book, R. Vogt Sionov, I. L. Hayon and Ygal Haupt discuss mechanisms of p53 induction and its effect on cell cycle checkpoints.

As emphasized, p21 is an important regulator of cell cycle checkpoints. The identification of p21 (also named WAF1 by Wafik S.El-Deiry) as a p53-inducible protein had culminated the search for a mediator of the p53 tumor suppressor by Bert Vogelstein and his colleagues.[37] Later, Wafik S. El-Deiry and coauthors have demonstrated that p21 is transactivated by another tumor suppressor, BRCA1.[38] In famalies that inherit breast and ovarian cancer, BRCA1 mutations account for close to 100% of resultant cancers. As discussed in this book by Timothy MacLachlan and Wafik El-Deiry, among other qualities of BRCA1, it is influenced by and affects directly the position of the cell cycle and the transition from phase to phase in the cell cycle intimately involves BRCA1. Yet, many functions of BRCA1 are not clear. The authors summarize recent advances leading to new hypothesis.

Discovered in 1994, BCRA1 is not the last tumor suppressor identified to date. The logic of the discovery of tumor suppressors is illustrated in the Chapter by Carlo M. Croce and coauthors. The novel tumor suppressor FHIT, fragile histidine triad protein, is normally expressed in epithelial tissues and is inactivated in most common cancers including lung and breast cancer. It is inactivated in more than 50% of these tumors. FHIT is the most common genetic alteration in human cancer.

Amato J. Giaccia and his colleagues discuss hypoxia and the cell cycle. When tumors are more than 150 μm or approximately ten cells in diameter, they exceed their ability to obtain sufficient oxygen by diffusion alone; and hypoxia develops. As hypoxia plays important roles in both tumor response to therapy and malignant progression, it is essential to understand how hypoxia affects cell cycle and molecular mechanisms involved in this process. The Chapter provides insights in the cell cycle control by hypoxia.

Recent studies spotlight the importance of G2 checkpoint.[39] Stewart and Pietenpol discuss DNA-damage induced G2 checkpoint signaling pathways. The Chapter discuss mechanism of G2 checkpoint activation and G2 checkpoint maintenance. The authors analyzed how knowledge of these signaling pathways may lead to more efficient use of current anticancer therapies and the development of novel agents.

As emphasized by Rosandra Kaplan and David E. Fisher in "p53, apoptosis, and cancer therapy", the challenge in cancer therapy focuses fundamentally on the paucity of therapeutic exploitable differences between cancer cells and normal cells. The actions of p53 likely mediates the successful treatment responses in those few tumors in which chemotherapy produces durable cures.

p53 and p21 act as positive regulators of accelerated senescence in tumor cells, but they are not absolutely required for this response.[40] By contrasting the functions of p53 as a positive regulator of apoptosis and as a negative regulator of mitotic catastrophe with secondary cell death, Igor B. Roninson et al explain conflicting and paradoxical results in the literature. In their provocative Chapter, the authors raised a prospect that induction of program of accelerated senescence in tumor cells may be a feasible and biologically justified approach to cancer therapy and that the induction of permanent cytostatic arrest could be the primary mode of treatment response in certain clinical cases.

Geoffrey I. Shapiro reviews preclinical and clinical development of small molecule inhibitors of cyclin-dependent kinases. As more potent and selective CDK inhibitors are now eagerly anticipated, it is important to review the preclinical and clinical results with the agents presently under development. According to Dr. Shapiro, as novel CDK inhibitors are developed, with improved potency and selectivity, it will be critical to determine whether they induce cytotoxicity, or whether they are primarily cytostatic, and to continually evaluate the selectivity of CDK inhibition for transformed cell types.

In the final Chapter "Cell Cycle Molecular Targets and Drug Discovery" John K. Buolamwini focuses on potential molecular targets in cell cycle regulatory pathways and their exploitation for small molecule drug design and discovery. The inhibition of kinase catalytic activity has been successfully achieved with small molecules that have advanced into clinical trials for cancer therapy. More potential anticancer molecular targets are emerging including critical oncogenic kinases and regulatory proteins identified in the progression through mitosis. These include aurora kinases, polo-like kinases, and the anti-apoptotic protein survivin.

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CHAPTER 1

Autocrine Transformation: Cytokine Model

James A. McCubrey, Xiao-Yang Wang, Paul A. Algate, William L. Blalock and Linda S. Steelman

Abstract

Autocrine growth factor secretion by cells is a frequent event involved in malignant transformation. Constitutive growth factor gene expression can in turn result in the deregulation of survival. Furthermore, autocrine and paracrine growth factor synthesis can also contribute to the enhanced angiogenic and metatastatic properties of transformed cells converting them into more malignant tumors. We will discuss three fundamental mechanisms which can result in autocrine transformation; first, mutations of the cytokine or growth factor genes themselves, second, the aberrant expression of upstream receptors, kinases, or downstream transcription factors which can induce autocrine growth factor synthesis and third, retrovirally induced cytokine gene expression. We will discuss possible therapeutic strategies designed to inhibit these events. We will use as a model the interleukin-3 (*IL-3*) gene and discuss how the aberrant regulation of this gene can result in the prevention of apoptosis and lead to autocrine transformation.

Cytokine Regulation of Growth

Cytokine usually refers to growth factors which often affect the hematopoietic system. Some cytokines were initially called lymphokines because they were produced by lymphocytes and often, but not always, functioned on lymphocytes. Even though some cytokines such as IL-3 and granulocyte/macrophage colony stimulating factor (GM-CSF) have quite different sounding names, they share many properties, are closely genetically linked, and were most likely derived from a common ancestral gene which underwent tandem duplication. In this Chapter we will use the term cytokine more frequently than growth factor. However, it should be kept in mind that IL-3 and GM-CSF are often referred to interchangeably as lymphokines, cytokines, and growth factors.

Cytokines can stimulate cell cycle progression, proliferation, and differentiation, as well as, inhibit apoptosis of hematopoietic and other types of cells.[1-3] Peripheral blood cells are generated from self-renewable, pluripotential hematopoietic stem cells in the bone marrow. Cytokines such as IL-3, GM-CSF, stem cell factor (SCF, a.k.a. steel factor, c-Kit-L, macrophage growth factor), FL (a.k.a. Flt-3L, the ligand for the flt2/3 receptor), erythropoietin (EPO), and others affect the growth and differentiation of these early hematopoietic precursor cells into cells of the myeloid, lymphoid, and erythroid lineages.[1-4] This Chapter will concentrate on the regulation of IL-3 since much of the knowledge of how cytokines affect cell growth, signal transduction, cell cycle progression, and apoptosis has been elucidated from research with IL-3 and IL-3-dependent cell lines. IL-3 was initially defined over 20 years ago by its ability to induce the enzyme 20-α-hydroxysteroid dehydrogenase in cultures of splenic lymphocytes from nude mice.[5] However, it soon became apparent that IL-3 was being studied by a number of

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investigators under a variety of aliases. It was called persisting cell-stimulating factor (PSF),[6] mast cell growth factor (MCGF),[7] hematopoietic cell growth factor (HCGF),[8] histamine-producing cell-stimulating factor,[9] multi-colony stimulating factor (Multi-CSF),[10] Thy-1-inducing factor,[5] and burst promoting activity (BPA).[11] All of these growth stimulatory activities were subsequently identified as the same protein and renamed IL-3. It is apparent that the many names by which this cytokine was known reflected its diverse biological properties. There are over 7000 citations in the Medline® database which use IL-3 as a keyword. Interestingly, IL-3 has remained one of the most intensively studied growth factors for over 20 years. This may be due, in part, to its strong anti-apoptotic activities.

IL-3 acts on both myeloid and lymphoid lineages. In vivo administration of pharmacological doses of recombinant IL-3 to mice resulted in the increased production of red blood cells, leukocytes, and platelets.[12] Moreover, over-expression of the *IL-3* gene in hematopoietic progenitors via retroviral transduction of bone marrow cells resulted in a noneoplastic, myeloproliferative syndrome in vivo.[13] Infection of primary hematopoietic cells with retroviruses encoding IL-3 does not normally result in malignant transformation, as over-expression of a second gene is often required which will synergize with IL-3 and lead to autonomous growth. Experimentally, the *hox2.4* gene product has been shown to synergize with IL-3 and result in the transformation of certain hematopoeitic cells.[14]

In addition to stimulating proliferation and differentiation of hematopoietic cells, cytokines such as IL-3 also promote cell survival. IL-3-dependent cells undergo apoptosis after withdrawal of IL-3 for a prolonged period of time (12 to 48 hours, depending upon the cell type and species of origin).[15] However, addition of IL-3 to IL-3-deprived cells can prevent apoptosis in a significant proportion of these cells.[15] This anti-apoptotic function of IL-3 remains intensively studied today. Investigation of the effects of IL-3 on apoptosis has contributed significantly to the apoptosis/programmed cell death field. In fact, the initial clues to the function of Bcl-2 came after the observation that over-expression of Bcl-2 prolonged the survival of IL-3-dependent cells cultured in the absence of cytokines.[16]

Regulation of IL-3 Expression: TCR Ligation and Mitogen Induced IL-3 Expression

Most hematopoietic cells do not usually synthesize the 26-kDa IL-3 protein. In those cells that do express the IL-3 gene, the gene is normally under stringent controls.[17-43] In peripheral blood, activated T cells, natural killer cells, mast cells and some megakaryocytic cells can synthesize IL-3.[17-19] For optimal IL-3 expression, T cells must be activated via the T-cell receptor (TCR)/CD3 pathway or by agents that mimic this pathway, e.g., the combination of the phorbol ester, phorbol 12-myristate 13-acetate (PMA) and calcium ionophores.[17-19] When a T cell is activated, aggregation of the TCR/CD3 complex occurs. Receptor aggregation is followed by a complex series of biochemical events leading to the activation of protein kinase C (PKC) and a rise in the intracellular concentration of Ca^{2+}.[1,20] The activated serine/threonine PKC kinase can phosphorylate the repressor protein, inhibitor κB (I-κB), which is subsequently ubiquitinated and degraded, thus permitting I-κB to disassociate from nuclear factor-κB (NF-κB).[21] The unmasked NF-κB nuclear localization signals present on NF-κB allow it to enters the nucleus and transactivate cytokine gene expression.

In addition, there is a complex of proteins which also phosphorylates I-κB: the I-κB kinases α and β (I-κKα and I-κKβ).[22] I-κK phosphorylates I-κB on serine residues. The I-κB kinases can be also activated through serine/threonine phosphorylation by the NF-κB inducing kinase (NIK), the mitogen-activated protein kinase kinase kinase-1 (MEKK1), and Akt (see below).[23] In summary, there are multiple mechanisms to activate I-κK, which in turn regulates I-κB and subsequently NF-κB. An illustration of the regulation of IL-3 gene expression is presented in Figure 1. Similar mechanisms mediate the expression of IL-2, GM-CSF, and other T-cell derived cytokines (Fig. 1).

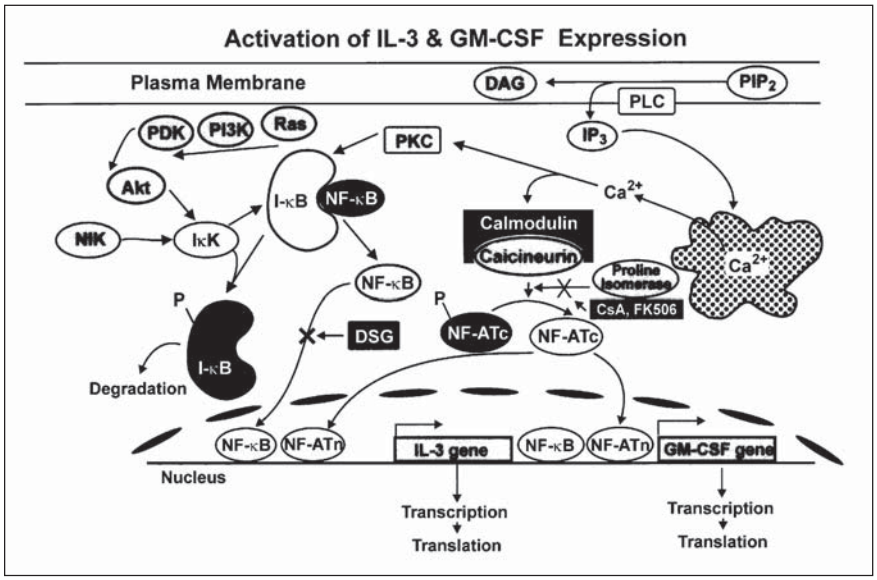

Fig. 1 Activation of IL-3 and GM-CSF expression. The effects of diacylglycerol (DAG) and Ca^{2+} on PKC activation and the subsequent activation of calmodulin, calcineurin, NF-κB and NF-AT. Moreover the effects of activation of the Ras/PI3K anti-apoptotic cascade are indicated. NF-κB can also be activated by NIK. I-κB can be targeted for degradation by serine/threonine phosphorylation mediated by PKC Akt, I-κKα and I-κKβ. The activated transcription factors are indicated in clear ovals. Once activated, NF-κB and NF-AT enter the nucleus and stimulate IL-3 and GM-CSF expression. The sites of inhibition by the immunosuppressive drugs CsA, FK506 and DSG are also shown in black on this diagram.

There are other transcription factors which modulate the expression of cytokine genes. Increased levels of intracellular Ca^{2+} following TCR aggregation allows calmodulin to activate calcineurin, a serine/threonine phosphatase.[20] Activated calcineurin dephosphorylates the cytoplasmic (c) form of the transcription factor, NF-ATc (nuclear factor of activated T cells) enabling NF-AT to translocate to the nucleus (n). This results in the transactivation of cytokine gene expression, including IL-3 and GM-CSF whose promoters contain NF-AT binding sites.[24-42]

There are also additional kinase cascades which regulate cytokine gene expression. PKC can also activate the Ras pathway by inactivating the GTPase activating protein (GAP), a negative regulator of Ras.[26,35-37] Ras is a member of a large multi-gene family, which encodes small GTP-binding proteins that serve as molecular switches. Inactivation of GAP stimulates Ras activity, which results in the enhancement of activator-protein-1 (AP-1) binding activity as discussed below.[26, 35-37] A diagram of where some of these transcription factors bind the IL-3 promoter region is presented in Figure 2. AP-1 can then stimulate cytokine gene expression, including IL-3. Interestingly, the neurofibromatosis-1 (NF1) gene, a tumor suppressor frequently lost in juvenile chronic myelogenous leukemia (CML), is functionally related to GAP.[44,45] NF1 likely serves to block Ras activation, thus its loss leads to constitutive Ras activation and contributes to the generation of CML.

Regulation of IL-3 Expression: Transcription Factor Binding Sites

The cis-acting elements of the human IL-3 promoter include two activation regions separated by an inhibitory region.[24-43] These genetic elements lie within a region that extends to

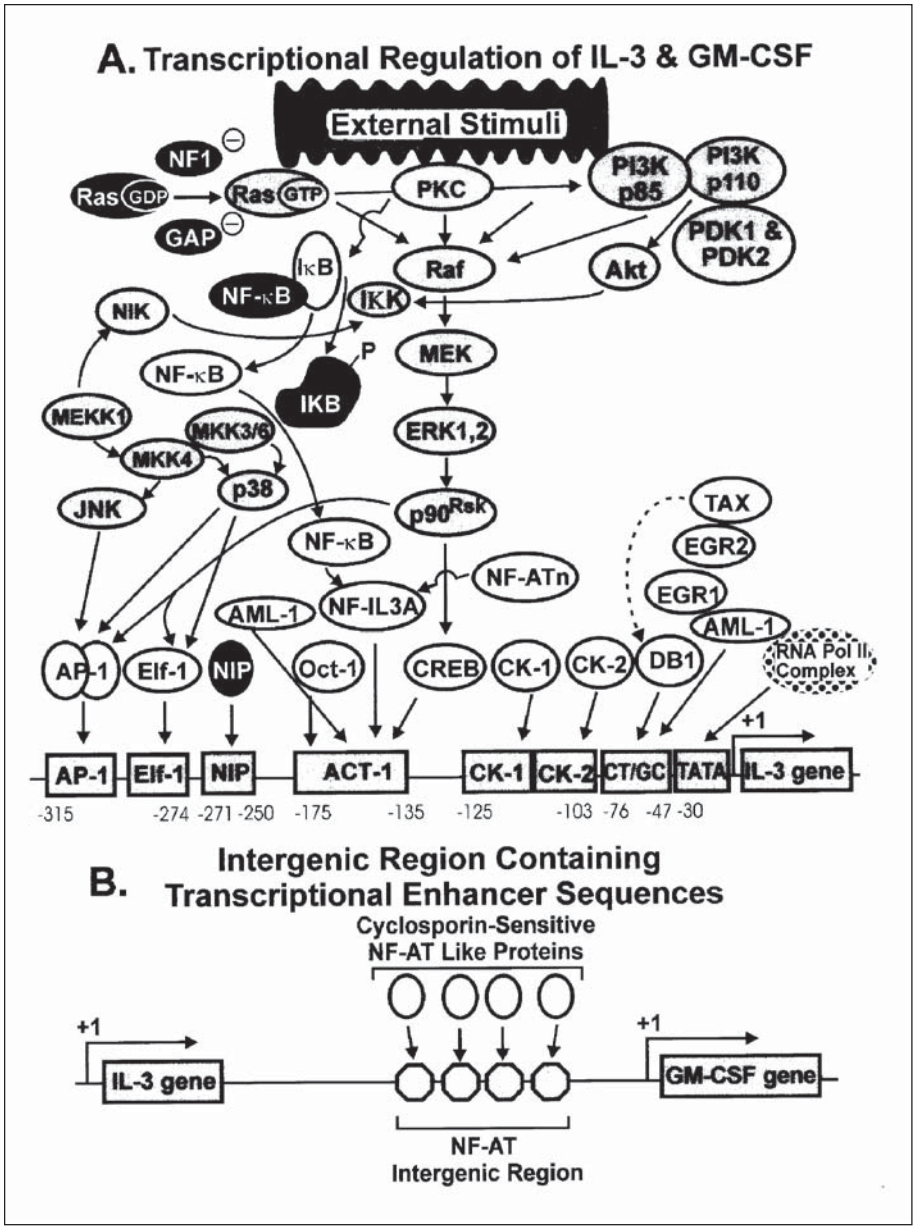

Fig. 2 (See Figure legend on opposite page)

~300 bp upstream of the transcription start site (Fig. 2, Panel A). An inhibitory element, nuclear inhibitory protein (NIP), has been described that binds to the IL-3 promoter, which suppresses IL-3 transcription. This binding site for this transcription factor is located between bp -271 to -250.[24] Unfortunately no further information has been provided about this factor and the role that deletion of this transcription factor binding site plays in leukemia.

Fig. 2. (opposite page) Transcription regulation of IL-3 and GM-CSF. Panel A, The effects of activated Ras, PKC, KSR and Src family kinases on the Raf/MEK/ERK signal transduction pathway and IL-3 expression. Ras activation can be induced by external stimuli but inhibited by NF1 or GAP (black ovals). Active Ras and downstream kinases are indicated in gray ovals. Ras can further transmit the signal to Raf, MEK, ERK and $p90^{Rsk}$ which can result in the activation of the AP-1 and CREB transcription factors (shown in clear ovals) which bind the promoter region of the *IL-3* and *GM-CSF* genes. Activation of Ras can also result in the activation of PI3K and the subsequent activation of PDK1, PDK2 and Akt which can phosphorylate and activate IκK. Raf can also be activated by KSR and Src family tyrosine kinases. Other transcription factors (e.g., c-Jun, Elf-1) are activated by other kinases such as JNK and p38 that in turn are activated by MKK4, MEKK1 and MKK3/6 and SEK. The NF-κB and NF-AT as well as the Oct-1, AML1 and CREB transcription proteins bind to the ACT-1 region. Possible control mechanisms for NF-κB, I-κB and NF-AT (NIK, IκK and calcineurin) were presented in Figure 1. Also shown in this picture are the negative effects of the NIP protein which binds the NIP region and suppresses transcription. In addition, there are CK1 and CK2 transcription factors, which bind to the CK1 and CK2 regions, as well as the Tax, EGR1, EGR2, DB1 and AML1 transcription factors, which bind to the CT/GC rich region. Panel B, This panel depicts the binding of NF-AT molecules to the intergenic region between the *IL-3* and *GM-CSF* genes. The binding of these proteins to the intergenic region influences the chromatin configuration of this gene cluster.

The IL-3 promoter region contains sequence motifs common to many cytokine promoters, including CK (cytokine)-1 and CK-2/GC elements. These sequences appear to be dispensable for the activity of the IL-3 promoter.[26-30] A CT/GC-rich region located between bp -76 to -47 confers a basal transcriptional activity to the IL-3 promoter and responds to *trans*-activation by the human T-cell leukemia virus type I-encoded Tax protein.[31] At least four transcription factors, (AML1, EGR1, EGR2 and DB1) have been shown to bind to this region.[28-31,40-42] The binding of EGR1 and EGR2 to these sites increases IL-3 promoter activity when an appropriate cell is activated.[34] In contrast to EGR1 and EGR2, DB1 binds constitutively and enhances the transcriptional activity of the IL-3 promoter when trans-activated by Tax.[31] The AML1 transcription factor also binds this region, but it has a higher affinity binding site in the -175 to -135 region.[40-42]

Two regions of the IL-3 promoter play important positive regulatory roles in the response of T cells to mitogens. One region is called Act-1 and is located between -175 to -135 bp.[27, 28] The 5′ part of this region binds a mitogen-inducible, T-cell specific, octamer-1-associated protein (Oct-1).[27] Nuclear factor-IL3A (NF-IL3A) binds the middle region, while the 3′ Act-1 sequence contains a consensus-binding site for a cAMP responsive element binding protein (CREB).[30,38] The role of the Act-1 region is to coordinate the functions of several *cis*-acting transcription factors, which leads to a maximal effect upon IL-3 transcription.[27,28] The AML1 transcription factor also binds to this region.[40-42] The other important transcriptional regulatory region is located between bp -315 to -274 and contains AP-1 and Elf-1 binding sites.[19,25] The c-Jun and c-Fo*s* heterodimer (AP-1) binds to the AP-1 site,[19] while the Elf-1 site is bound by an Ets-related transcription factor, Elf-1.[25,35] Tissue specific expression of the *IL-3* gene may result from interactions between the Act-1 and this latter region.[35-37]

There are many kinases which regulate the activity of the transcription factors that bind the IL-3 promoter region. Signal transduction cascades originating from extracellular signals (including cell stress) often regulate the activities of these kinases (MEKK1, MKK4, JNK, ERK, MKK3/6, $p38^{MAPK}$, $p90^{Rsk}$, and others) which in turn can regulate IL-3 expression.[34, 45-47]

In addition to the cis-acting elements 5′ to the IL-3 transcription start site, there is another set of cis-acting elements found in the intergenic region between the IL-3 and GM-CSF genes which are sensitive to the immunosuppressive drugs CsA and FK-506 (Fig. 2, Panel B).[39] This region contains four NF-AT sites, which are bound by NF-AT transcription factors upon mitogen activation.

Proto-oncogenes, which are sometimes mutated in human cancer, (e.g., c-Fos, c-Jun NF-κB, EGR, and Ets-related transcription factors) can bind the IL-3 promoter region.[45, 46] This suggests that abnormal expression of these oncoproteins may result in autocrine transformation and

lead to leukemia. In many transformed cells, the pathways controlling the activities of these transcription factors are dysregulated.[45,46] For example, constitutive activation of the Ras/Raf/MEK/ERK (extracellular regulated kinase) cascade can alter the activity of transcription factors, to induce autocrine growth factor synthesis.[48,49]

Genetic Influences on IL-3 Expression: DNA Methylation

Genomic DNA demethylation is also believed to influence the propagation of specific T-cell cytokine profiles. The extent of methylation of certain cytokine genes, such as IL-3 and interferon-γ (IFN-γ), may contribute to distinct patterns of cytokine gene expression in T-cell clones. Demethylation of the IL-3 promoter was shown to be confined to specific CpG sites within the same clones.[50] This is a potential mechanism that could lead to the ability of certain T-cell clones to express specific cytokines.

Therapeutic Approaches Based Upon Reducing Cytokine Gene Expression

We have discussed the mechanisms by which T-cell activation can result in the transcription of the IL-3 gene. Now we will discuss therapies that exploit the inhibition of IL-3 transcription. There may be therapeutic approaches to inhibit the activity of NF-κB, which will decrease cytokine gene expression. 15-Deoxyspergualin (DSG), an immunosuppressive drug which has been through Phase I/II clinical trials, inhibits the localization of heat shock protein 70 (Hsp 70) to the nucleus in response to heat stress, as well as the intranuclear activation of NF-κB, through its interaction with Hsp70.[51] Another approach to inhibiting NF–κB activation involves introducing adenoviral vectors into leukemic cells which overexpress I-κB.[52] This would result in a decrease in NF-κB activity and cytokine gene synthesis. This gene therapeutic approach may prove beneficial in the suppression of tumor growth. Decreasing the levels of NF-ATc would suppress cytokine gene expression. The immunosuppressive drugs cyclosporin A (CsA) and FK506 mediate their activity by inhibiting calcineurin activation, thereby preventing the dephosphorylation of NF-ATc.[39,53] Another approach is to treat leukemic patients with immunosuppressive drugs. This approach would have to be carefully monitored as it could render the patient susceptible to life-threatening microbiological infections.

Other targets to inhibit cytokine gene synthesis include the upstream signal transduction cascades. Ras is frequently targeted by anti-neoplastic drugs including farnesyl transferase (FT) inhibitors (see below). Addition of a farnesyl group is necessary for Ras localization to the cytoplasmic membrane. Drugs, which block Ras farnesylation, are currently being developed by pharmaceutical companies for therapeutic use (e.g., Janssen, Merck).[54] Inhibiting Ras could decrease cytokine gene expression.

Pharmacological companies have developed inhibitors to some of the kinases involved in regulation of cytokine gene expression (e.g., SB203580 is a $p38^{MAPK}$ inhibitor developed by the Smith Klein Beecham Company).[55] Blocking $p38^{MAPK}$ activity would suppress some of the transcription factors involved in cytokine synthesis. The critical question remains: How do we target these inhibitors exclusively to malignant cells rather than normal cells? It may be possible to control, either directly or indirectly to control the activities of these important regulatory molecules in transformed cells.[26,34,45,51,56]

Regulation of IL-3 Expression: Post-Transcription Regulation

We have described how TCR ligation and mitogen stimulation can activate kinase pathways resulting in the activation of transcription factors, which bind the IL-3 promoter region and induce expression of the *IL-3* gene. The next point of IL-3 regulation to be discussed is the control of IL-3 synthesis due to post-transcriptional mechanisms. IL-3 mRNAs are very unstable and decay within one-half to one hour after their synthesis.[57-75] This property appears to be critical for their normal function, since the degradation of IL-3 mRNA, as well as other cytokine mRNAs, is stringently controlled.[57-75] An AU-rich element (ARE) found within the 3' untranslated region (UTR) of the IL-3 and other cytokine mRNAs is involved in the regulation of IL-3 mRNA stability (Fig. 3).[58-63] These evolutionarily conserved ARE sequences

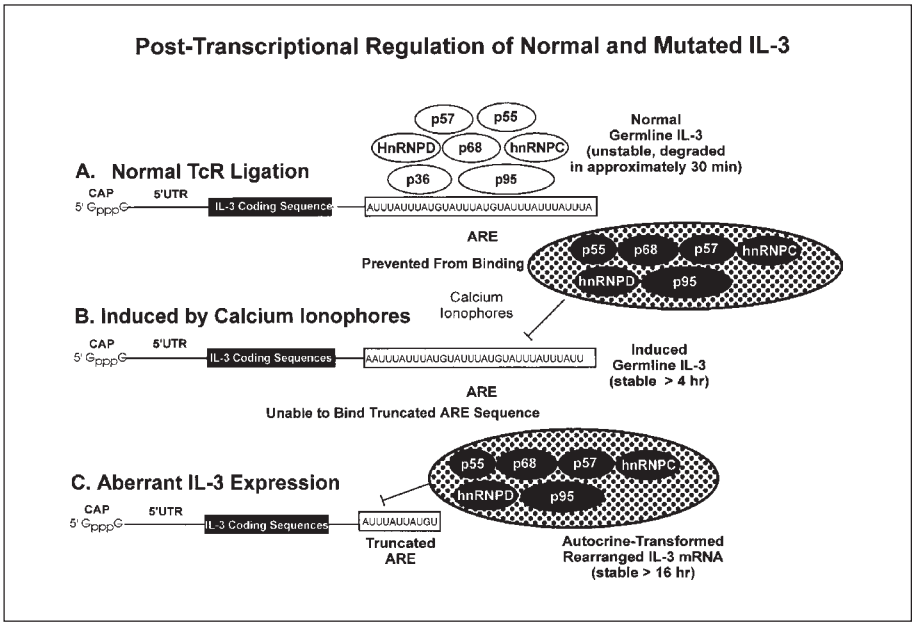

Fig. 3. Post-transcriptional regulation of normal and mutated IL-3 expression. Panel A, The wild-type IL-3 ARE is shown which binds the indicated proteins. This IL-3 mRNA would be induced in T cells after TCR ligation. The binding of these proteins results in mRNA with a short mRNA half-life. p36 and p95 are the only proteins that were demonstrated by northwestern analysis to bind directly to the IL-3 gene.[3,53,55] p95 is depicted as a larger sphere due to artistic constraints. The exact sequences where p36 and p95 bind the IL-3 UTR are not known, nor is the stoichiometry of binding. Panel B, Calcium ionophores disrupt the binding of RNA- binding proteins to the IL-3 ARE resulting in conditional stabilization of IL-3 mRNA. This stabilized IL-3 mRNA would be detected after treatment of T-cell lines with calcium ionophores. Panel C, The RNA-binding proteins are prevented from binding the truncated IL-3 gene in tumorigenic autocrine transformed cells which contain an IAP provirus inserted into the IL-3 ARE.[3,58-62] The prevention of binding of these proteins results in the continuous stabilization of IL-3 mRNA. The sizes of the coding and noncoding IL-3 sequences are not drawn to scale.

serve to tightly regulate cytokine expression, a critical function due to the potent growth stimulatory and anti-apoptotic effects of cytokines. A diagram of the post-transcriptional regulation of IL-3 is presented in Figure 3.

Electrophoretic mobility shift assays (EMSAs) and UV-crosslinking experiments have identified the proteins that bind to cytokine AREs (62, 64-71). These included proteins with apparent molecular weights of 36-, 40-, 43-, 46-, 55-, 57-, 68- and 95-kDa. The adenine/uridine binding protein (AUF1, also known as heterogeneous nuclear ribonuclear protein D [hnRNP D]) was shown to bind to the IL-3 ARE, by an EMSA followed by immunoprecipitation of IL-3 ARE binding proteins with a specific α-AUF1 antibody.[62]

All three isoforms of hnRNP D, which exhibit apparent molecular weights of 40-, 43-, and 46-kDa, bind to the IL-3 ARE.[62] hnRNP C also binds to the IL-3 ARE region.[62] Calcium ionophore treatment prevents/reverses binding of these proteins to the IL-3 ARE and results in stabilized IL-3 mRNA[62] (Fig. 3, Panel B). The affinities of the hnRNP D proteins for their RNA substrates were shown to be negatively correlated with mRNA stability.[69]

Therapeutic Approaches Based Upon Decreasing Cytokine mRNA Stability

CsA and FK506 decrease IL-3 production by certain mast cells via mRNA destabilization as well as affecting NF-ATc activation.[74] These results suggest three possibilities: 1) CsA and FK506 may have additional targets besides calcineurin which regulate mRNA stability, 2) calcineurin may have other targets besides NF-AT which regulate mRNA stability or 3) NF-AT may regulate the expression of additional genes besides cytokines, which regulate mRNA stability.

The immunosuppressive drug rapamycin, which has a different biochemical target than CsA, also destabilizes IL-3 mRNA in certain autocrine transformed cells.[75] Rapamycin is primarily thought to affect p70 ribosomal S6 kinase (p70S6K) phosphorylation, which subsequently modulates the efficiency of protein translation (see below). This is believed to result from rapamycin inhibiting the mammalian target of rapamycin (mTor), which is downstream of phosphatidylinositol-3 kinase (PI3K) but upstream of p70S6K. The mechanisms by which the immunosuppressive drugs CsA, FK506, and rapamycin prevent the binding of proteins to the IL-3 ARE are unknown. The drugs may alter the phosphorylation states of ARE binding proteins, preventing them from interacting with the IL-3 ARE.

Chromosomal Translocations which may Inhibit IL-3 Expression

We have discussed how IL-3 mRNA is synthesized and regulated in normal cells. Now we will discuss how IL-3 can be abnormally expressed in certain leukemias and lymphomas. Chromosomal translocations have been linked to aberrant IL-3 expression. In certain human B-cell lymphomas, chromosomal translocations between the immunoglobulin heavy chain (IgH) locus on chromosome 14 and the *IL-3* gene on chromosome 5 [t (5; 14)(q31; q32)] were detected.[76,77] The IgH enhancer, a strong tissue specific enhancer involved in many chromosomal translocations in hematopoietic cells (e.g., Burkitt's lymphoma, follicular B-cell lymphomas involving Bcl-2) induces the transcriptional activation of the *IL-3* gene. The role IL-3 plays in the growth of B-cells remains controversial. IL-3-dependent pro-B cell lines have been available since 1985. These cell lines offer support to show that IL-3 can play a role in the growth of some early hematopoietic cells.[4] It is also possible that in human B-cell lymphomas containing a translocated *IL-3* gene, IL-3 serves as a paracrine growth factor to support the growth of neighboring cells. This, in turn, provides the necessary growth factors for the B-cell lymphoma. For example, the IL-3 produced by the B-cell lymphoma may stimulate the expression of: IL-4, IL-5, IL-6, IL-7, in either neighboring cells or the B-cell lymphoma, which in turn supports the lymphoma growth. Such complicated cytokine circuitry is common, although often we do not know which growth factor plays the critical role.

Abnormal IL-3 Expression: Inhibition Due to Chromosomal Translocations

The AML1 transcription factor is normally a transcriptional activator which binds the promoter region of genes such as IL-3 and stimulates its expression.[40-42] Some chromosomal translocations may result in the creation of chimeric transcription factors which suppress IL-3 expression. The AML-ETO fusion protein that is generated by the t:(8; 21) chromosomal translocation encodes a transcriptional repressor which has been shown to suppress IL-3 promoter activity in in vitro promoter activity assays. Moreover, the t:(12: 21) translocation encodes the chimeric TEL-AML protein which also represses transcription of the IL-3 and other genes as measured by in vitro promoter activity assays. This chromosomal translocation is the most commonly identified molecular abnormality in childhood acute lymphoblastic leukemia (ALL). In freshly isolated human ALL cells which have the TEL-AML1 fusion protein, no IL-3 was detected.[40-42] Thus some chromosomal translocations appear to suppress IL-3. The roles that these chimeric transcription factors play in leukemogenesis are being investigated in "knock-in" mice.[43] It may be that suppression of IL-3 synthesis is unrelated to the leukemic properties of the cells and that the real targets of suppression by these chimeric transcription factors are other genes involved in the induction of differentiation.

Abnormal Cytokine Gene Expression Due to Retroviral Infection

Retroviruses, such as human T-cell leukemia virus-I (HTLV-I), encode sequences which can regulate gene expression. The tax gene product of HTLV-I is a transactivator which can induce the expression of many genes including: IL-2, IL-3, IL-15, GM-CSF, TNF, c-*fos,* c-*jun*, and IL-2Rα chain.[31] The tax protein can induce genes whose promoter regions contain CREB, Ap-1, and serum responsive element (SRE) sequences. Although most studies on HTLV-I infection of hematopoietic cells have focused primarily upon IL-2 and IL-15 expression, there may be some cases where HTLV-I infection can result in abnormal autocrine IL-3 expression in certain early hematopoietic cells which lead to autocrine transformation. Recent studies have shown that both IL-2 and IL-15 expression are necessary for autocrine transformation; as treatment of cells with antibodies to either cytokine by themselves did not fully inhibit the growth of the HTLV-I-infected T cells.[79] In contrast, when antibodies to both IL-2 and IL-15 were added, a greater level growth inhibition was observed.[78,79]

Autocrine Cytokine Gene Expression Due to Activated Raf and MEK1 Expression

We have observed that introduction of activated Raf and MEK1 genes into cytokine-dependent cells resulted in autocrine transformation.[80-89] Initially, we observed the synthesis of GM-CSF, but not IL-3 transcripts, in cells which would grow in response to either Raf or MEK1 expression. Moreover, the GM-CSF cytokine was detected in the supernatants, which supported the proliferation of the parental cells. However, when we treated the autocrine-transformed cells with neutralizing antibodies to GM-CSF, the highest level of growth inhibition observed was approximately 50%.[80,81] When we examined the expression of other cytokines, we noticed that mRNAs encoding additional cytokines were detected in the Raf and MEK1 transformed cells including IL-5, IL-6, IL-10, and IL-12. Some of these cytokine transcripts (e.g., IL-5 & IL-6) were detected in uninfected cells and were observed to be regulated in the cells by the addition of either GM-CSF or IL-3 to the growth medium. In contrast, IL-10 and IL-12 were only detected in the cells which grew in response to activated Raf and MEK1. The contribution of these additional cytokines to autocrine responsive growth is being determined. Thus, the activation of downstream signal transduction cascades by Raf and MEK1 resulted in the activation of cytokine genes that were not detected in the parental cytokine-dependent cells.

Abnormal Regulation of IL-3 Expression: Biological Consequences of IL-3 ARE Disruption

We have characterized autocrine-transformed cells, which secrete IL-3 and have an intracisternal type A particle (IAP) transposed into the IL-3 ARE.[58,59,62] In these autocrine-transformed FL-*IL-3R* cells, only two AUUUA motifs adjacent to the IL-3 gene remain intact due to the IAP transposition in the parental FL5.12 cell line (Fig. 4, Panel A). IL-3 mRNA isolated from these cells has a much longer half-life ($T_{1/2}$ = 16 to 24 hours) than wild-type IL-3 mRNA ($T_{1/2}$ = 0.5 to 1 hour). Moreover, the IL-3-secreting hematopoietic cells expressing the mutated IL-3 mRNAs were tumorigenic upon injection into immunocompromised nude mice (Fig. 4).[57, 59-61]

To determine the regions of the rearranged IL-3 gene that were responsible for the abrogation of cytokine-dependency, chimeric IL-3 gene constructs containing portions of the wild-type and IAP-disrupted IL-3 genes were made, transfected into IL-3-dependent parental FL5.12 cells, and examined for their ability to abrogate cytokine-dependency. The resulting factor-independent cells were then examined for their tumorigenicity upon injection into immunocompromised mice.[59-61] Recombinant IL-3 constructs were also made which tested the abilities of various IAP-LTRs and exogenous retroviral LTRs (e.g., Moloney-Murine Leukemia Virus, Mo-MuLV) to affect IL-3 expression and factor-dependency. In Figure 4, Panel B, we

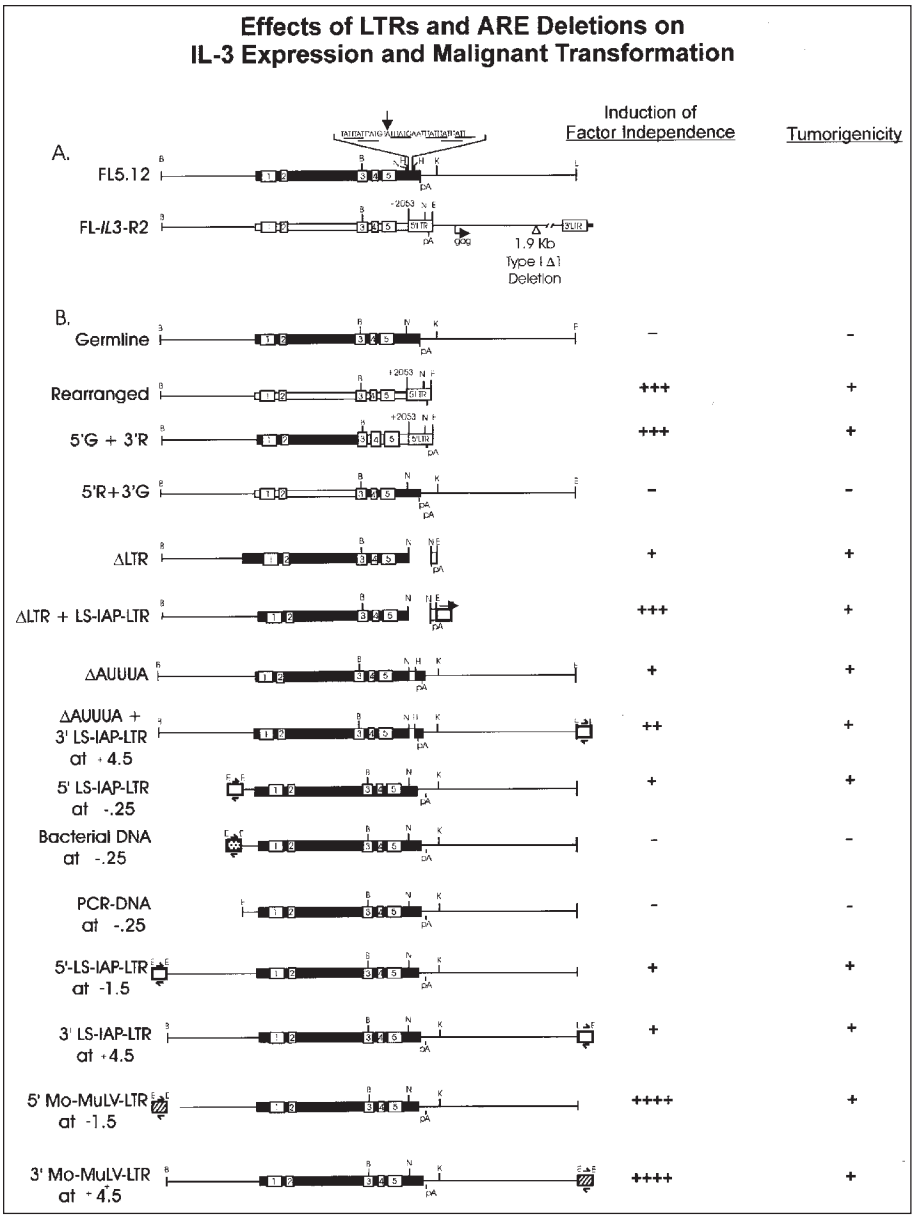

Fig. 4. (see figure legend on opposite page)

have illustrated the recombinant IL-3 constructs and their abilities to abrogate the cytokine-dependency of the parental FL5.12 cells.

Transfection of cells with a germline IL-3 gene did not result in the frequent isolation of factor-independent cells. In those cells that were factor-independent, amplification of the introduced GIL3 construct was detected.[60,61] In contrast after transfection with the RIL3 construct factor-independent cells were detected. These transfected cells had not inherited a

Fig. 4. (opposite page) Effects of LTRs and ARE deletions on IL-3 expression and tumorigenicity. Structures of germline and rearranged *IL-3* genes that are contained in the FL-*IL3*-R2 cell line and modified *IL-3* genes. Panel A. Maps of the germline (G) *IL-3* locus present in FL5.12 cells and the rearranged (R)IL-3 locus contained in FL-IL3-R cells. The black thick line is the germline IL-3 locus from start of transcription to termination of transcription. The open thick line is the rearranged *IL-3* gene from start to termination of transcription. Boxes indicate the five IL-3 exons. Panel B. The germline and rearranged *IL-3* genes as well as various constructs containing deletions of the AUUUA regions as well as additions of different LTR and other genetic sequences were inserted into the pSV2neo expression vector.[59-61] The constructs were transfected into IL-3 dependent FL5.12 cells, and their abilities to abrogate cytokine dependence were determined and compared. Relevant restriction sites are indicated (E = EcoRI, B = Bam HI, N = NcoI, H = Hae III, K = KstI). LS-IAP-LTR = Lymphocyte specific IAP-LTR (identical to the IAP-LTR contained in the rIL3 gene), Bacterial DNA = insertion of a 450 bp piece of *Bacteriodes fragilis* DNA. Mo-MuLV -LTR = Moloney Murine Leukemia Virus LTR, PCR-DNA at -0.2 is the parent construct for the other LTR insertion constructs which contain the different LTRs at -0.25. The 5′G + 3′R and 5′R + 3′G are chimeric IL-3 constructs which contain respective portions of the germline and rearranged *IL-3* genes. Key to induction of factor independence following transfection of IL-3 constructs: (–) = no or very low level of factor-independence, ++ moderate level of factor-independence, +++ = higher level of factor-independence, ++++ = highest level of induction of factor-independence. Key to tumorgenicity: (–) no tumors or very few (sporadic) tumors, (+) tumors in all mice examined.

high copy number of the rIL3 construct indicating that inheritance of a single rIL3 construct was sufficient to abrogate cytokine-dependency.

The effects of the 5′ and 3′ regions of the germline and rearranged regions of the *IL-3* genes were examined by creating chimeras of these genes by cleaving them in the middle with the Bam HI (B) restriction endonuclease. This resulted in two constructs, 5′R + 3′G and 5′G + 3′R. The 5′R + 3′G construct, which contained the wild-type ARE sequence, did not readily abrogate the cytokine-dependency of FL5.12 cells, whereas the construct (5′G + 3′R) which contained the IAP-truncated ARE sequence did. These results indicated that the promoter region of the RIL3 gene did not have any mutant elements (DNA sequences) in it which resulted in abrogation of cytokine-dependency and the 3′R region of the RIL3 gene was responsible for abrogation of cytokine-dependency. The DNA sequence of the RIL3 promoter region was determined and confirmed that there were no differences in the promoter regions of the GIL3 and RIL3 genes.

To determine whether deletion of the ARE region of the IL-3 gene was sufficient for abrogation of cytokine-dependency, an IL-3 construct was made lacking the AUUUA region. Transfection of cells with the GIL3 + ΔAUUUA construct did not result in the frequent isolation of factor-independent cells. To determine if an LTR region was also required to abrogate cytokine-dependency, an IAP-LTR was added to the gIL3 + ΔAUUUA construct. Transfection of cells with this construct resulted in the isolation of factor-independent cells. These results indicated that addition of the IAP-LTR was necessary for the transcription of the *IL-3* gene.

Additional IL-3 constructs were made containing the various LTRs inserted in different positions. The exogenous Moloney Murine Leukemia Virus (Mo-MuLV) LTR was more effective in inducing the expression of the *IL-3* gene than the endogenous IAP-LTR. As a control, bacterial DNA was inserted where the various LTRs were positioned. Transfection of IL-3 dependent cells with this control construct did not result in the isolation of factor-independent cells. LTR-CAT transient transfection experiments indicated that the LS-IAP-LTR contained in the rIL3 gene was weaker than other LTRs and enhancer regions, thus IAP transpositions involving this class of IAP-LTR may require additional mutagenic events to stimulate sufficient gene transcription to induce malignant transformation.

The effects of the retroviral LTRs and the presence of the ARE on the levels of IL-3 expression in the transfected factor-independent cells are illustrated in Figure 5. This was determined by purifying supernatants from the various cell lines and then titering them on the factor-dependent parental cell line. The activity in the supernatants was determined to be IL-3 as treatment of the supernatants with an α-IL-3 Ab inhibited their abilities to stimulate

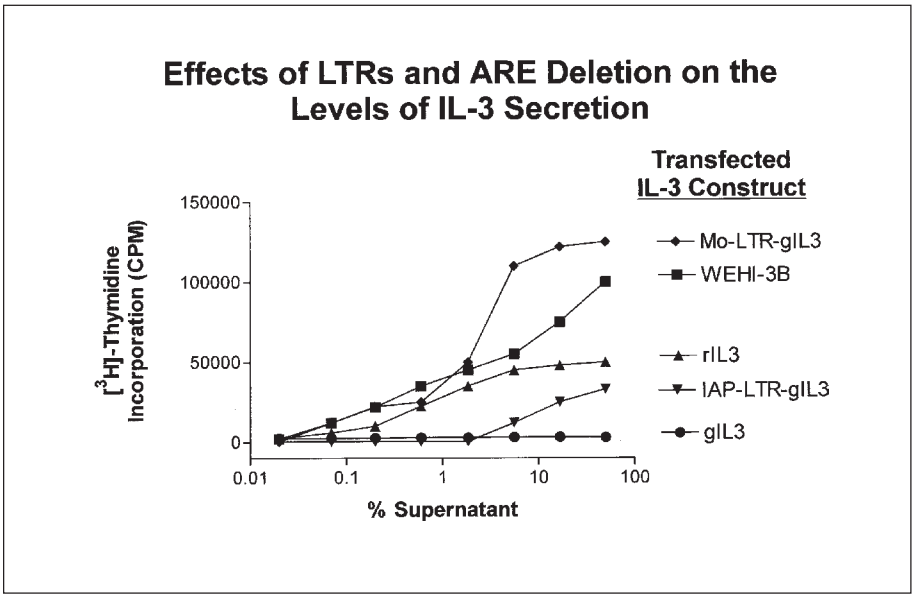

Fig. 5. Effects of LTRs on the levels of IL-3 secretion. The levels of IL-3 secreted in the various transfected cell lines were determined by preparing supernatants from some factor-independent cells transfected with some of the IL-3 constructs presented in Figure 4. The level of [^{3}H]-thymidine incorporation is a measure of DNA synthesis and a marker of proliferation. The WEHI-3B supernatant is a control since it is prepared from the WEHI-3B cell line which produces a large amount of murine IL-3 and is a source of IL-3 for the growth of murine IL-3 dependent cells. The gIL3 supernatant was prepared from a rare factor-independent FL5.12 cell line transfected with the germline IL-3.[59-61]

[^{3}H]-thymidine incorporation. In contrast, when the supernatants were incubated with an α-GM-CSF Ab, there was no inhibition of [^{3}H]-thymidine incorporation. Transfection of the parental FL5.12 cells with a germline IL-3 construct ligated to a strong LTR (e.g., Mo-MuLV LTR) led to the highest level of IL-3 secretion detected. In contrast transfection of FL5.12 cells with a LTR with a low level of activity (e.g., IAP-LTR) led to a lower level of IL-3 synthesis. Transfection of FL5.12 cells with a rearranged *IL-3* gene which had a deletion of the mRNA stability region and an IAP LTR resulted in an intermediate amount of IL-3 expression.

These studies indicated that the IAP transposition stabilized IL-3 mRNA. The remaining two AUUUA motifs could not efficiently destabilize IL-3 mRNA, and hence, the transfected cells were autocrine-transformed and tumorigenic. Site-directed mutagenesis studies indicated that destabilization of IL-3 mRNA requires a clustering of either the three 5′ or the distal three 3' AUUUA motifs present in the IL-3 ARE. However, the cluster of the three 3′ AUUUA motifs was a stronger destabilizer.[63]

In order to determine how the IAP transposition altered the binding of proteins to the IL-3 ARE, EMSAs were performed. Proteins were specifically bound to the wild-type IL-3 mRNA ARE region, whereas no protein binding was detected to the RNA which had only two AUUUA motifs, nor to an artificial RNA probe which did not contain any AUUUA motifs (Fig. 3, Panel C).[62] Thus, certain IAP transpositions disrupt IL-3 AREs and prevent the binding of proteins to this region. These mutations result in autocrine growth stimulation leading to malignant transformation.

Conclusions

This Chapter has examined the mechanisms of regulation of IL-3 expression in normal and autocrine transformed cells. We have also described therapeutic approaches which might be effective in treating autocrine tumors. Clearly the aberrant expression of growth promoting cytokines represents a significant challenge in cancer therapeutics because they can be activated by diverse mechanisms.

Acknowledgments

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CHAPTER 2

Signal Transduction Pathways: Cytokine Model

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Abstract

Growth factors (GF) initiate and maintain transition through G1 to S phase. GF-dependence ends with phosphorylation of Rb by cyclin-dependent kinases (CDKs), enabling cells to pass through the restriction (R) point and to complete the remaining phases of the cell cycle. Cyclin D-dependent kinase phosphorylates Rb leading to induction of cyclin E which in turn activates CDK2 and collaborates with cyclin D-CDKs to complete Rb phosphorylation. GF simultaneously induce cyclins and CDK inhibitors. Not only their ratio but also cellular context determines response: proliferation vs arrest. R-point, a prototype of cell cycle checkpoints, is usually lost in cancer. Loss of R-point can be exploited for selective killing of cancer cells by cycle-dependent chemotherapy.

Cytokine-Induced Signal Transduction Resulting in Growth and the Prevention of Apoptosis

In the previous Chapter, we discussed the mechanisms by which IL-3 is synthesized after T-cell activation, mitogen stimulation, chromosomal translocations, and retroviral infection. Next, it is logical to consider the effects of the synthesized IL-3 on signal transduction pathways leading to growth and the prevention of apoptosis. The intracellular signal transducing machinery induced by cytokines, such as IL-3 and granulocyte/macrophage colony stimulating factor (GM-CSF), represents a promising area to exploit for the therapy of leukemia. The ultimate goal of many of these studies described below is the development of specific compounds or therapies, which will modulate key intermediates in signal transduction pathways. An overview of some of the growth and anti-apoptotic pathways induced by IL-3 is presented in Figure 1.

Neither the α nor the β chains of the specific receptors for IL-3 (IL-3R) has any obvious homology to known signaling molecules, such as kinases, phosphatases, nucleotide binding proteins, or src homology (SH)-containing proteins.[1-36] However, the IL-3 β_c-chain functions in the activation of signal transduction pathways by recruiting the necessary kinases.[10-36] An immediate response of cells upon IL-3 activation is the tyrosine phosphorylation of Jak and STAT proteins[1,2,8,10-32] and the activation of Ras, Raf, MEK, and MAPK (mitogen-activated protein kinase, ERK1/2). MAP kinase is a generic name referring to a group of three serine-threonine MAP kinases (ERK, p38, and JNK).[1,2,15-20] Subsequently, these signals are

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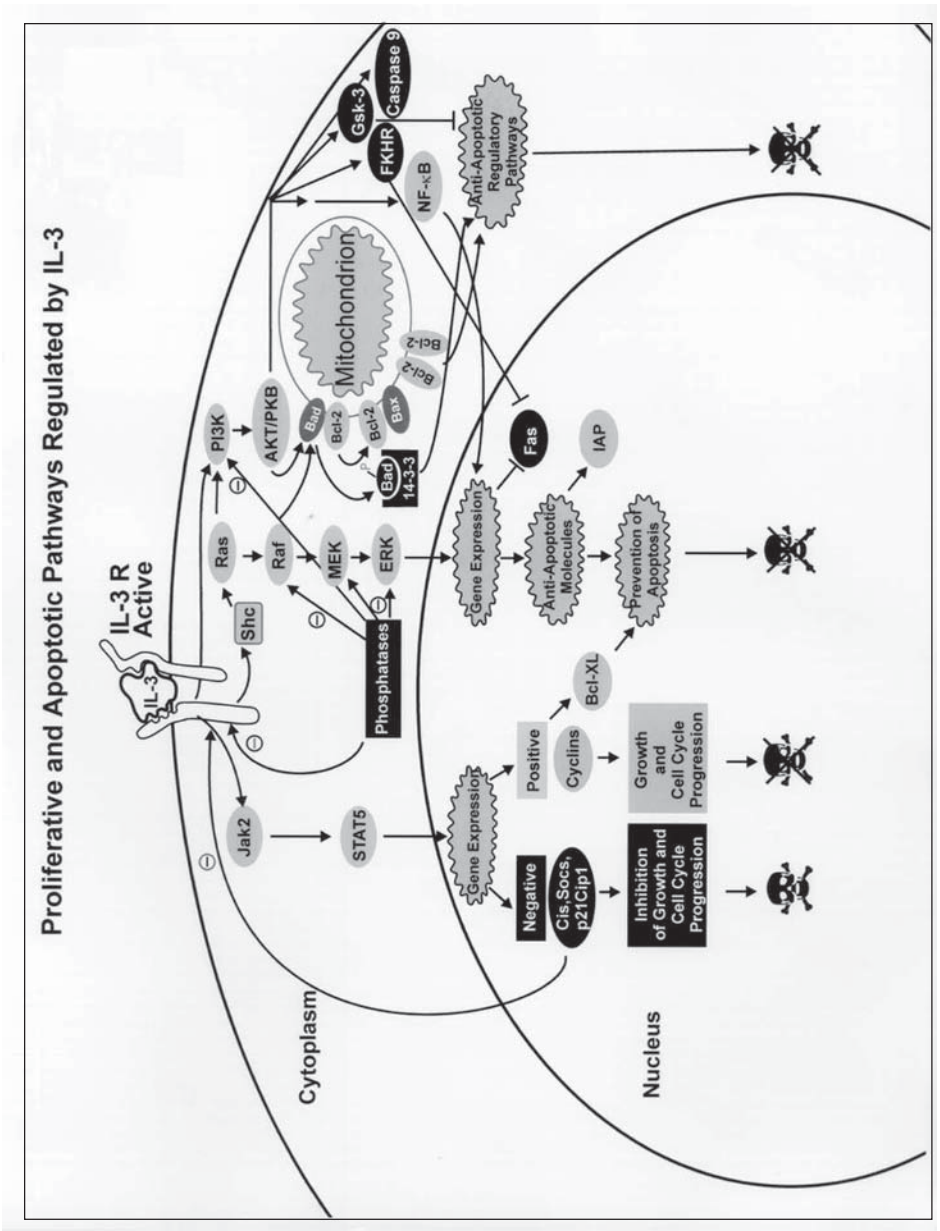

Fig. 1. Proliferative and apoptotic pathways regulated by IL-3. This diagram is an overview of the different effects which IL-3 has on cell growth and the prevention of apoptosis. IL-3 can stimulate Jak kinases, which activate gene expression through STAT proteins. Some of the genes that are induced by STAT stimulate proliferation (e.g., cyclins) or prevent apoptosis (e.g., Bcl-X_L), whereas others (e.g., Cis and Socs), serve to inhibit the Jak/STAT signal transduction pathway or regulate cell cycle progression (e.g., $p21^{CIP1}$). IL-3 can also induce anti-apoptotic pathways by stimulating the Ras, or PI3K pathways, which can result in the phosphorylation of the pro-apoptotic Bad, Gsk-3, FKHR and caspase 9 proteins. Also shown are the negative effects of phosphatases, which can dampen IL-3 mediated signal transduction.

transduced to the nucleus resulting in the transcriptional induction of proto-oncogenes such as c-*myc* and c-*fos*.[21-29]

Adaptor Proteins that Couple Receptors with Downstream Pathways

Upon IL-3 stimulation, the adapter molecule Shc is also rapidly phosphorylated and associates with the phosphorylated β_c chain.[30,36-40] Shc contains two domains that are capable of interacting with tyrosine-phosphorylated proteins: an N-terminal phosphotyrosine-binding (PTB) domain and a C-terminal SH2 domain.[40] The PTB domain is responsible for the physical association of the Shc protein to the receptor β_c chain.[40]

Phosphorylated Shc protein binds to another adapter protein, Grb2 (growth-factor-receptor-bound protein-2), which in turn associates with the GTP exchange factor, mSos (mammalian son of sevenless homologue), to activate Ras.[38,39] The protein tyrosine kinases responsible for the phosphorylation of Shc have been suggested, but not exclusively identified. The kinase which phosphorylates Shc is proposed to be Jak2.[35,38]

IL-3 stimulation also results in tyrosine phosphorylation of an SH2-containing inositol phosphatase (SHIP), which forms a complex with Shc, Grb2, and SOS and may act to regulate this pathway.[41] Phosphorylation of SHIP does not appear to be necessary for its IP_3-phosphatase activity; rather, it may be involved in the binding of proteins necessary for targeting SHIP to its correct subcellular component where its catalytic activity is necessary. Indeed, phosphorylated SHIP is found predominantely in the membrane fraction of cells.[41]

The Vav protein is yet another adaptor/signaling molecule activated by IL-3/GM-CSF stimulation.[42,45] Vav contains a single SH2 domain and two SH3 domains.[44,45] The SH2 domain mediates the interaction of Vav with Jak2, which has been proposed to be responsible for the phosphorylation and activation of Vav.[43] Once phosphorylated, Vav can interact with the Tec protein kinase through Tec's SH2 domains. Tec can then bind phosphatidylinositol 3-kinase (PI3K) and initiate additional signal transduction cascades. In addition, PKC can also activate Vav leading to Ras/Raf or potentially Ras/PI3K activation.[45]

The Jak-STAT Pathway

Upon binding of IL-3 to the IL-3 receptor, the IL-3 receptor α and β chains heterodimerize, and the entire receptor oligomerizes with other IL-3 receptors.[1,30-32] The association of Jak2 with the cytoplasmic membrane-proximal region of the β_c chain allows for the subsequent oligomerization, phosphorylation, and activation of Jak2 upon IL-3 receptor aggregation.[32] A diagram of IL-3 mediated signal transduction pathways is presented in Figure 2.

Jak2 is a member of a multi-gene family including Jak1, Jak2, Jak3, and Tyk2.[14,32-34] A unique characteristic of Jak family members is that they contain two tyrosine kinase domains: a carboxy-terminal catalytic domain and an amino-terminal pseudo-kinase domain.[32-34] Jak2 has been demonstrated to be the molecule responsible for some of the immediate responses of IL-3 stimulation and is required for mitogenesis.[14,32,46]

Jak activity is necessary for STAT activation by non-tyrosine kinase receptors, such as the IL-3 receptor.[12-32,36,40,46-63] IL-3 receptor binding leads to the activation of Jak2 and the recruitment and subsequent tyrosine phosphorylation of STAT5 (Y694 of STAT5a and Y699 of STAT5b).[32] Tyrosine phosphorylation of STATs leads to their dimerization and activation.[32] These STAT dimers then translocate to the nucleus where they act as transcription factors by binding regulatory sites within the promoter region of immediate-early genes such as c-myc, β-casein, Osm, and Bcl-X_L,[32,47] as well as feedback inhibitors of the JAK/STAT pathway (e.g., Cis).[47] Although STAT activation requires tyrosine phosphorylation by Jak kinases, STAT translocation to the nucleus is enhanced by threonine phosphorylation via Raf/MEK/ERK activation.[2,64-66] The consequences of activation of this pathway will be discussed later.

Constitutive activation of members of the Jak-STAT pathway has been associated with the onset of HTLV-induced adult T-cell leukemia[67] as well as v-Abl,[68] BCR-ABL[69] and v-Src[70-72] mediated transformation of various hematopoietic cells. Mutant Jak proteins have

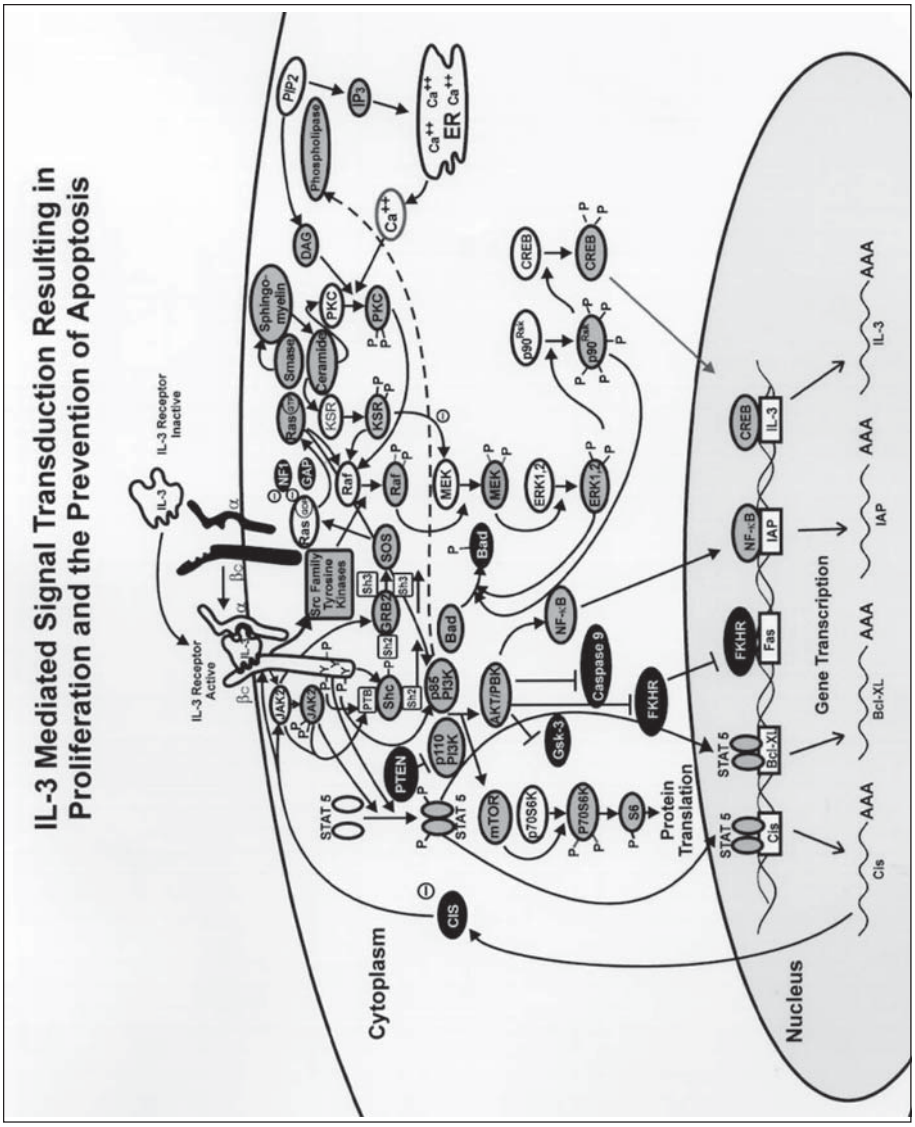

Fig. 2. IL-3 mediated signal transduction resulting in proliferation and the prevention of apoptosis. IL-3 mediates activation of the Jak/STAT and Ras/Raf/MEK/ERK signal transduction pathways. IL-3 can also affect apoptosis by inducing the PI3K pathway which can be regulated by the PTEN tumor suppressor gene which functions as a phosphatase. Also shown are the activation of PKC and kinase suppressor of Ras (KSR), which can also activate the Raf pathway. Inactivated proteins are depicted in clear ovals whereas the activated forms are depicted in grey ovals. Proteins which have a negative role on cell growth are indicated in black ovals. ER = endoplasmic reticulum.

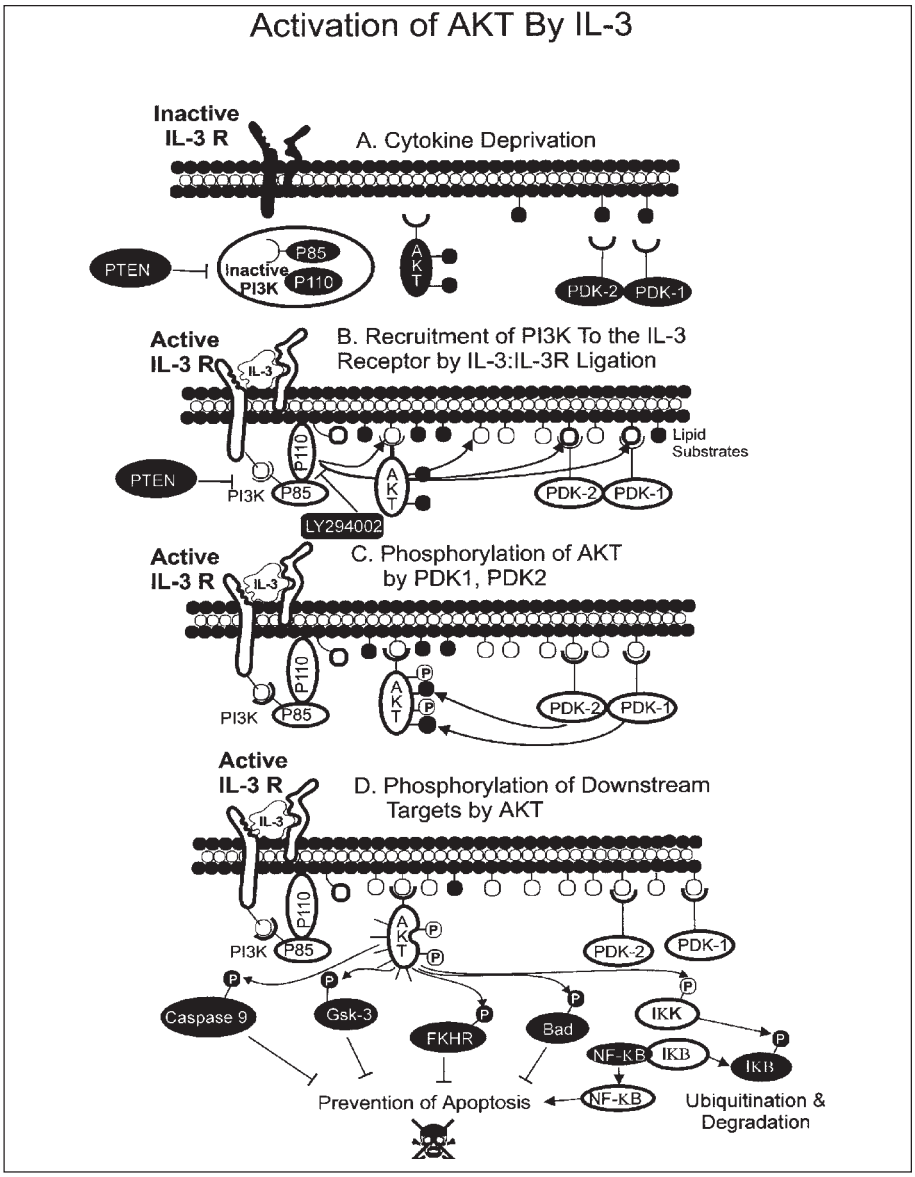

Fig. 3 Activation of Akt by IL-3. A) In cytokine-deprived cells, Akt is not localized to plasma membrane. Also the phosphatase encoded by the PTEN tumor suppressor can result in the inactivation of PI3K. The LY294002 drug inhibits the catalytic activity of the p110 kinase. B) When IL-3 binds the receptor, phosphorylation of a tryosine residue on the IL-3β chain occurs creating a binding site for the PI3K p85 regulatory subunit. This results in the recruitment of p85 via an Sh2 domain. P85 in turn activates the PI3K p110 catalytic site. p110 PI3K then phosphorylates certain membrane lipids which result in the activation of PDK-1 and PDK-2 which occurs via their plextrin homology domains. C) PDK-1 and PDK-2 phosphorylate Akt on two different serine/threonine residues which results in activation of Akt. D) Akt can phosphorylate many downstream targets which result in their activation/inactivation and the prevention of apoptosis.

also been observed in certain patients with immunodeficiencies.[73-79] Thus, modulation of Jak and STAT activities may be a method of therapeutic intervention in HTLV-I-induced leukemias, chronic myelogenous leukemia, immunodeficiency and other diseases which rely upon Jak/STAT mediated signal transduction.

The PI3K/Akt Pathway

The stimulation of appropriate target cells by IL-3 also leads to the rapid activation of PI3K.[2,80] PI3K is a heterodimeric protein consisting of an 85 kDa regulatory subunit, which contains SH2 and SH3 domains and a 110 kDa catalytic subunit.[2,80-85] IL-3 stimulation leads to the creation of a binding site for PI3K on the IL-3R. The SH2 domain of the p85 subunit associates with this site on the receptor β_c chain.[51,83] The p85 subunit is then phosphorylated which subsequently leads to the activation of the p110 catalytic subunit that in turn activates the downstream targets p70 S6 kinase (p70S6K) and protein kinase B (PKB), also known as Akt.[86-90]

The kinase which phosphorylates PI3K may be a member of the Src tyrosine kinase family, which includes Fgr, Fyn, Hck, Lyn, Src, Syk, Tec, and Yes in hematopoietic cells.[91-94] In addition, Ras, as well as other Rac and Rho family proteins, can activate or enhance PI3K activity.[2,80-95]

Activated PI3K phosphorylates certain membrane lipids which serve to activate the phosphoinositide kinase dependent kinases (PDK-1 and PDK-2). PDK-1 and PDK-2 then phosphorylate the Akt kinase (aka protein kinase B, PKB) on a serine and a threonine residue.[2,96] A model of IL-3 induced Akt activity and the subsequent effects on the prevention of apoptosis is presented in Figure 3.

Activated Akt can further transduce the signal to other targets (e.g., glycogen synthase-3 [Gsk-3] and Tec family kinases) and mediate anti-apoptotic functions by phosphorylating the pro-apoptotic Bad protein and the regulatory caspase, caspase 9 (See below).[2,9,88,89,95-107] In contrast to the inactivation of the previous molecules by Akt phosphorylation, Akt can also phosphorylate I-κK, which phosphorylates I-κB, resulting in its ubiquitination and subsequent degradation in the proteasome.[101-108] Since I-κB is disassociated from NF-κB, NF-κB can then enter the nucleus and transactivate gene expression. NF-κB can promote gene expression that, under certain circumstances, stimulates growth as well as prevents apoptosis.[101-108,181-187,121] Akt can also phosphorylate certain transcription factors such as the Forkhead family of transcription factors (FKHR).[97,98] Phosphorylation of the FKHR family of transcription factors prevents their ability to transactivate the expression of certain pro-apoptotic genes including Fas.

The PI3K pathway can also result in the activation of ribosomal protein kinases. The p70S6K is an S6 ribosomal protein kinase that phosphorylates S6 in vitro and enhances protein translation of certain mRNAs.[109] The inhibitors wortmannin and LY294002 suppress the activity of PI3K and rapamycin can inhibit the activity of p70S6K (see Fig. 7). Alternatively, p70S6K can be activated by PI3K-independent means as well.

The PI3K pathway is also regulated by phosphatases which serve to decrease the activity of PI3K. The phosphatase PTEN (phosphatase and tensin homologue deleted on chromosome ten, aka MMAC1 mutated in multiple advanced cancers) has been proposed as a tumor suppressor gene. PTEN is a dual specificity lipid and protein phosphatase that can remove the phosphates on PI3K-phosphorylated substrates. PTEN downregulates events catalyzed in response to Shc, Ras and ERK activation.[5]

The Ras/Raf/MEK/ERK Signal Transduction Pathway

The Ras/Raf/MEK/ERK cascade is perhaps one of the best-studied signal transduction pathways. It is centrally involved in the transmission of mitogenic and anti-apoptotic signals as it couples information initiating from membrane receptors to transcription factors which control gene expression. Many of the members of this pathway (e.g., Ras, Raf, MEK), as well as additional downstream targets (e.g., c-Fos, c-Jun, and Ets) are proto-oncogenes. One important reason why

this pathway was one of the better studied cascades is that certain transforming retroviruses contained activated oncogenes encoding viral homologues of some of these genes. In contrast, only the Akt gene and the downstream NF-κB gene have been shown to have viral counterparts in the PI3K/Akt cascade and no viral counterparts have been detected in the Jak/STAT pathway. The Ras/Raf/MEK/ERK pathway is often aberrantly regulated in transformed cells. Thus, elucidation of the regulation of this pathway may aid in the development of drugs which will be useful in the treatment of various malignancies.

Ras is a small monomeric GTP-binding protein whose GTP-bound form can associate with its downstream target, which in some cases is Raf.[110-123] Because Ras is ubiquitously expressed and often mutated in human cancer, Ras was one of the first oncogenes identified as a potential chemotherapeutic target by pharmaceutical companies.[117-122] For Ras to be functional, it must be farnesylated by the enzyme farnesyltransferase (FT) which attaches a 15-chain fatty acid to Ras. This modification allows Ras to be tethered to the plasma membrane. There is a large family of Ras-related proteins, including Rho and Rac.[117] The roles of these Ras-related proteins in the growth and transformation of hematopoietic cells remains undefined, but they may serve as potential targets for the FT inhibitors as well.

Ras frequently passes its mitogenic signal onto the Raf proteins, a family of three serine/threonine kinases (Raf-1, A-Raf and B-Raf) which contain binding sites for interaction with Ras.[110-116] Activated Ras will induce the translocation of Raf from the cytosol to the plasma membrane.[110-116] Thus, mutations which alter Ras activity may also perturb the actions of Raf and the downstream cascade. Evidence suggests that this pathway is intimately associated with the control of apoptotic machinery in myelo-monocytic cells.[124]

Activation of the Raf-1 pathway is essential for growth factor-induced proliferation during hematopoiesis.[125] The events that lead to activation of Raf-1 at the plasma membrane are not fully understood. However Raf-1 activation often occurs in the presence of GTP-Ras. Inactive Raf-1 proteins are present in the cytosol bound to 14-3-3 chaperonin proteins. The 14-3-3 proteins may bind to a cysteine-rich domain (CRD) present in Raf.[126] Cytosolic Raf-1 is translocated to the plasma membrane through interactions with GTP-Ras. This occurs between the Ras binding domain (RBD) on Raf-1 (aa 55 to 131) and the switch region of GTP-Ras.[116, 127] Once Raf-1 is localized to the plasma membrane, Ras can interact with the Raf-CRD via the Ras switch-2 region.[116,127] These interactions between Ras and the Raf-CRD serve to displace the 14-3-3 proteins from Raf-1 and uncover its kinase domain, allowing the phosphorylation of two regulatory tyrosine residues (Y340 and Y341 on Raf-1) by a Src-related protein-tyrosine kinase.[127-145]

Displacement of the 14-3-3 proteins from the Raf-CRD also permits the dephosphorylation of two regulatory serine residues on Raf-1 (S259 and S621). Once all of these changes in phosphorylation have occurred, Raf-1 is fully activated. It has also been noted that partial activation of Raf-1 can also be achieved through phosphorylation by other membrane-associated kinases. There is some evidence for direct activation of Raf-1 by certain protein kinase C (PKC) isotypes.[208,214-217] The α, δ, and ε isoforms of PKC will lead to phosphorylation of Raf-1; however, only PKC e may functionally activate Raf-1.[130,137-139] Alternatively, different PKC isoforms may stimulate autocrine growth factor loops that, in turn, activate Raf-1.[146]

There is evidence for crosstalk between the Jak/STAT and the Ras/Raf pathways. Activation of Raf-1 by Jak is dependent upon recruitment of Raf-1 to the plasma membrane by Ras and occurs by phosphorylation of Raf-1 at Y340 and Y341.[67,71,130,140] Moreover, Ras may activate both Raf and PI3K.

Certain Raf proteins appear to promote cell cycle arrest. High levels of B-Raf and Raf-1 induce $p21^{Cip1}$ expression, which inhibit the kinase activities of CDK4/6 and CDK2, thereby preventing cell cycle progression.[147-150] $p21^{Cip1}$ functions by binding CDK/cyclin And blocking the phosphorylation of inhibitor pocket proteins, such as Rb. Many of the effects of the Raf and downstream MEK1 proteins have been elucidated by conditionally-active DRaf:ER and ΔMEK1:ER constructs.[2-9,150-152] These constructs have been developed by Dr. Martin McMahon

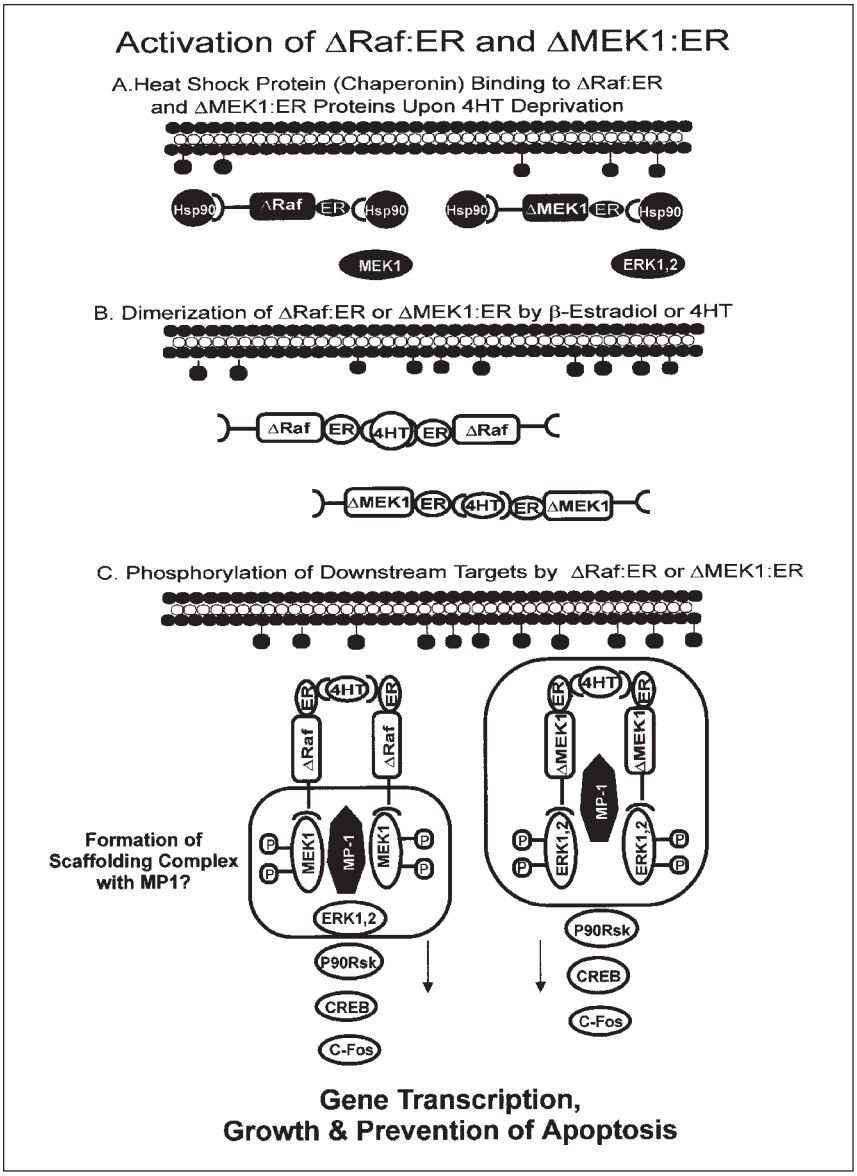

Fig. 4. Activation of the ΔRaf:ER and ΔMEK1:ER constructs. The ΔRaf:ER and ΔMEK1:ER retroviruses have been used to evaluate the interactions between different signaling pathways in the abrogation of the cytokine-dependency of hematopoietic cells. A) In the absence of either β-estradiol or 4-HT, the ΔRaf:ER, and ΔMEK1:ER proteins molecules are believed to be complexed with heat shock proteins and present in monomeric forms. B) Upon addition of β-estradiol or the estrogen receptor antagonist, 4-HT, dimerization of the ΔRaf:ER or ΔMEK:ER constructs occurs as well as disassociation of the heat shock proteins. C) The MP-1 scaffolding protein may interact with the dimerized ΔRaf:ER and ΔMEK1:ER constructs creating a more efficient signalling complex within the cell. One means to activate Raf in cells is by cross-linking two Raf proteins together. Thus the dimerization of the ΔRaf:ER molecules by either β-estradiol or 4HT may resemble a natural mechanism by which Raf molecules are activated. Activated Raf and MEK are then believed to phosphorylate their respective targets and induce gene expression in the nucleus.

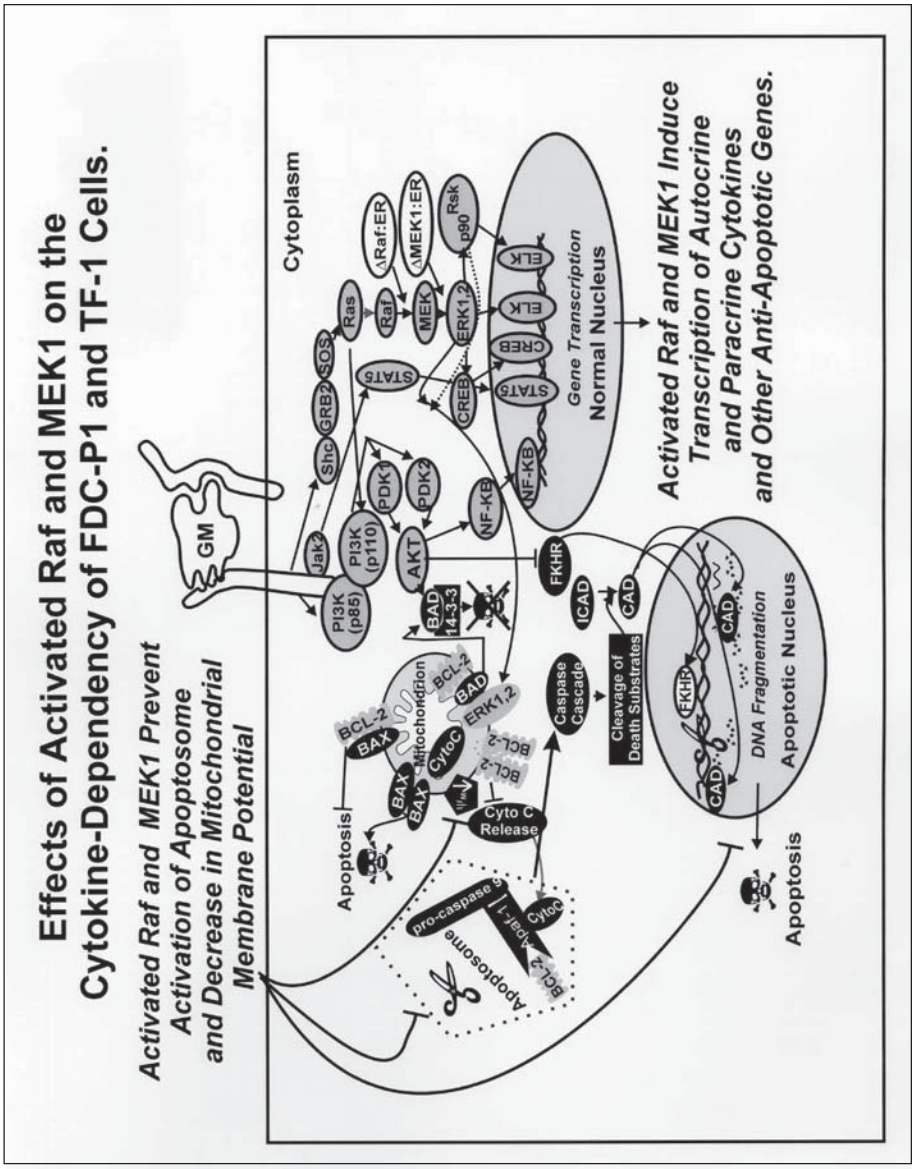

Fig. 5. Effects of activated Raf and MEK1 on the cytokine-dependency of FDC-P1 and TF-1 cells. An outline of the effects of activated Raf and MEK1 expression on the cytokine—dependency of the FDC—P1 and TF-1 cell lines is presented in this figure. Factor-independent FDC-P1 and TF-1 cells can be isolated after infection with retroviruses encoding activated forms of Raf (ΔA-Raf:ER, ΔB-Raf:ER or ΔRaf-1:ER) or MEK-1 (ΔMEK1:ER). This results in the activation of downstream ERK activity and the secretion of an autocrine growth factor (GM-CSF) as well as other cytokines (e.g., IL-5, IL-6). In the presence of ΔRaf:ER or ΔMEK1:ER and autocrine cytokines, a decrease in the mitochondrial membrane potential does not occur and neither downstream activation of the caspases nor fragmentation of cellular DNA occurs. Active kinases, transcription factors and anti-apoptotic molecules are shown in gray ovals, whereas pro-apoptotic molecules are shown in black ovals. IL-3 and the ΔRaf-1:ER and ΔMEK1:ER are shown in clear ovals.

(University of California San Francisco) and can be related by the addition of β-estradiol or the estrogen receptor antagonist 4-hydroxy-tamoxifen (4HT). A model for the activation of the ΔRaf:ER and ΔMEK1:ER constructs is presented in Figure 4.

We have observed that the introduction of activated Raf and MEK1 oncogenes into the FDC-P1 and TF-1 hematopoietic cells resulted in the abrogation of the dependency upon exogenous growth factors in some of the cells. There was a hierarchy in terms of the ability of the different Raf genes to abrogate cytokine-dependency as the ΔA-Raf:ER was more efficient than the ΔRaf-1:ER which in turn was more efficient than either ΔMEK1:ER or ΔB-Raf:ER.[151,152] Thus the weakest Raf kinase, ΔA-Raf:ER was more efficient in abrogating the cytokine-dependency of these hematopoietic cells. In contrast, ΔB-Raf:ER, the strongest Raf isoform, was more efficient in relieving contact inhibition in fibroblast NIH-3T3 cells. The hematopoietic cells which grew in response to Raf and MEK1 activation synthesized sufficient GM-CSF to promote autocrine growth. A model for the effects of Raf and MEK1 on the downstream signal transduction and apoptotic pathways in FDC-P1 and TF-1 cells is presented in Figure 5.

It is conceivable that there are specific interactions between certain Raf and 14-3-3 family members.[153-163] These interactions may modulate the activity of Raf proteins and regulate their ability to lead, either directly or indirectly to the phosphorylation of the pro-apoptotic Bad protein. Phosphorylation of Bad, which leads to sequestering of Bad by 14-3-3, can also inhibit apoptosis (see below). We have shown that the B-Raf oncoprotein was the least efficient Raf oncoprotein in abrogating the cytokine-dependency of human hematopoietic cells.[151,152] This may be a reflection of the enhanced capacity of the B-Raf oncoprotein to induce the expression of cell cycle inhibitory proteins or prevent the phosphorylation of the Bad protein.

In summary, there may be a delicate balance between inducing cell growth and inducing cell cycle arrest in hematopoietic cells. A Raf oncoprotein with high kinase activity may actually be the least efficient protein in terms of abrogating cytokine dependency. These studies indicate that results obtained with fibroblastic models may not always be relevant for other cell systems (e.g., hematopoietic cells).

The Ras/Raf/MEK/ERK Pathway: Downstream Kinase Activation

Raf activates the dual specific serine/threonine and tyrosine kinase, MEK1, which, in turn, activates the MAP kinases ERK1 and ERK2 (p42/p44). In cells expressing normal MEK1, the kinase appears as a 45-kDa protein.[164-173] The amino terminal end of the kinase has a negative-regulatory domain, since deletion of these residues results in constitutive activation of MEK1, while the catalytic activity is localized to the carboxyl terminus of the protein.[174,178] Proline-rich sequences between kinase domains IX and X of MEK1 are required for Raf-1 binding and subsequent activation of MEK1.[164]

Raf-1-mediated activation of human MEK1 requires the phosphorylation of MEK1 serine residues 218 and 222.[165-168] Substitution of either of these residues with aspartic or glutamic acid results in a 10- to 50-fold increase in MEK1 activity.[177,178] When both serines are replaced with aspartic acid or aspartic and glutamic acid (218 & 222), MEK1 activity was 400-to 6000-fold greater.[164-168,177,178] These substitutions are believed to confer to the MEK1 protein a configuration that is constitutively active.[167,168] When these MEK1 mutants were transfected into NIH3T3 cells, constitutive activation of p42/p44 ERKs occurred as well as foci formation.[164-168] This suggests ERK activation through Raf requires MEK1. In support of this hypothesis, PD98059, a MEK1 inhibitor developed by Parke-Davis, prevents ERK activation mediated by activated Raf constructs.[151,152,179,180]

In addition to the three Raf kinases, several other kinases influence MEK activity. One such kinase is the oocyte-expressed proto-oncogene, Mos.[169,170] Constitutively active forms of Mos (v-Mos) transform fibroblasts via a MEK1-ERK-dependent pathway implicating Mos as a MEK kinase.[170] Mos preferentially phosphorylates MEK1 on serine 222.[169,170] In contrast, the MEK kinase-1 (MEKK1), which is associated with stress-activated pathways (SAPK) and whose

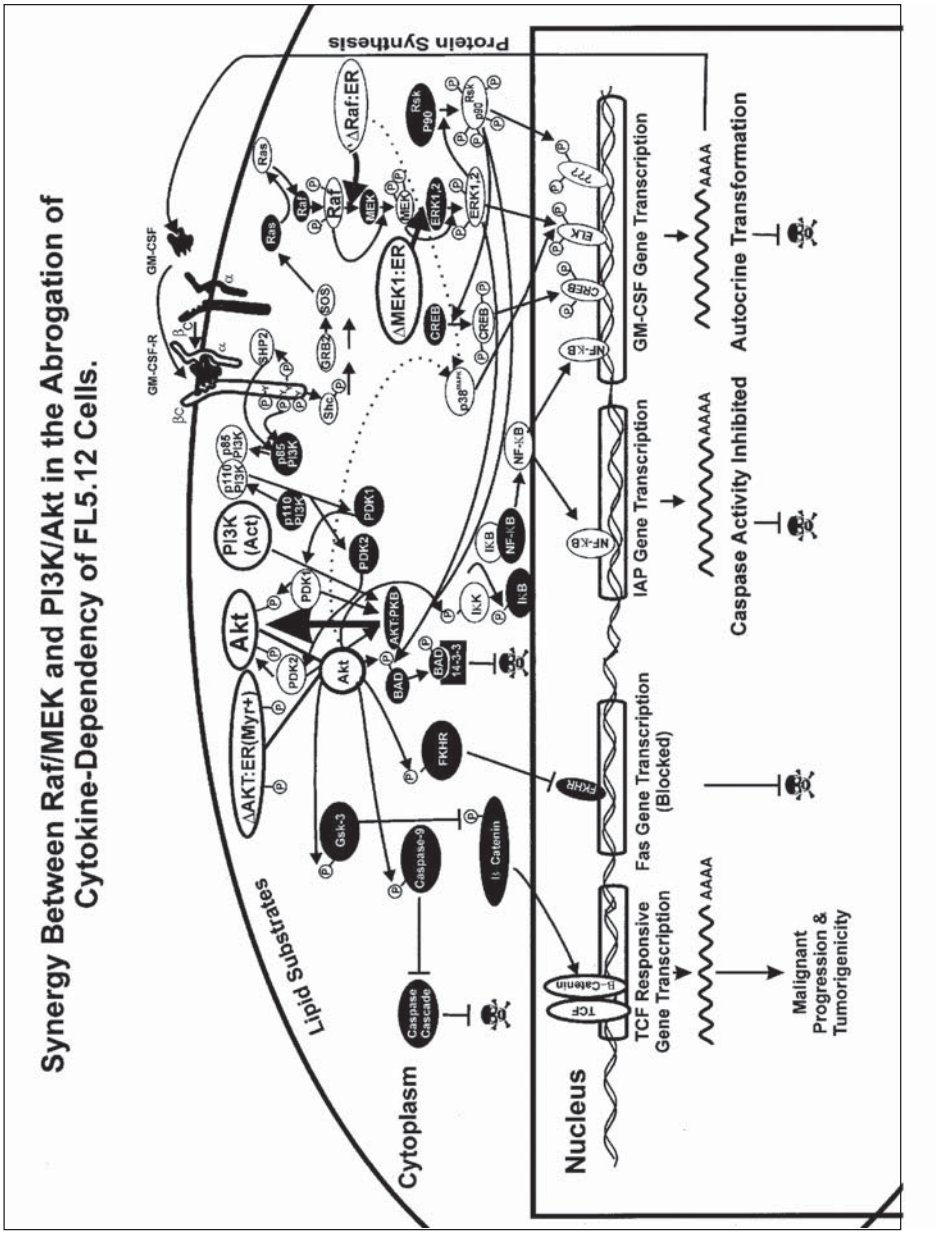

Fig. 6. Synergy between Raf/MEK and PI3K/Akt in the abrogation of cytokine-dependency of FL5.12 cells. The stimulation of the Raf/MEK/ERK and Akt/PI3K pathways can lead to autocrine transformation of FL5.12 cells. Inactive kinases are depicted in black ovals. Activated kinases, phosphatases and transcription factors are depicted in clear ovals. Induced expression of ΔRaf:ER and Akt:ER may lead to GM-CSF expression transcription and autocrine transformation. The steps after MEK1 activation, which result in GM-CSF transcription, are not known at the present time, although a plausible pathway is indicated. Potential effects of Raf and Akt on $p38^{MAPK}$ activation are indicated in dotted lines. Also shown are additional pathways regulated by Akt which may be activated (TCF Responsive gene transcription) or inactivated (Fas).

activation is associated with the induction of apoptosis, phosphorylates MEK1 on serine 218.[179] Thus, MEK1 may represent a common intermediate in pathways that exert anti- and pro-apoptotic effects.

Interactions Between the Raf/MEK/ERK and the PI3K/Akt Pathways

Another potential means of activating MEK1 is via the PI3K pathway.[171] The effector directly leading to MEK1 activation in this pathway has not been determined but does not appear to be Raf-1.[171] Active PI3K leads to the induction of ERK1 and ERK2 and may be responsible for the prolonged ERK activity observed in certain cytokine-stimulated cells.[171] Other activators of Raf, such as PKC, can also result in MEK1 and ERK activation.[172,173] We have recently observed that activated PI3K or Akt expression will synergize with activated Raf/MEK1 expression and result in the abrogation of the cytokine-dependency of the FL5.12 hematopoietic cell line. In contrast, activated Raf, MEK1, PI3K, or Akt expression by themselves was sufficient to abrogate the cytokine-dependency of the other cells. In these cells, higher levels of activated ERK1,2, $p38^{MAPK}$ and JNK were detected than in their cytokine-dependent counterparts. These results suggest that activation of both Raf/MEK1 and PI3K/Akt enhance the expression of signal transduction pathways leading to uncontrolled growth and the prevention of apoptosis. These cells also expressed autocrine growth factors (see below). A model for the interactions between these two signaling pathways is presented in Figure 6.

The Ras/Raf/MEK/ERK Pathway: A Tether Enhancing Signal Transduction

Recently, a MEK1 binding partner, MP-1, was identified and shown to enhance the enzymatic activation of the MAP kinase cascade.[181]MP-1 is a nonenzymatic, scaffolding protein that serves to anchor both the MEK and ERK proteins facilitating the activation of both proteins. Raf has not been demonstrated to be present in this scaffolding complex. It was suggested that MP1 functions as an adapter to enhance the efficiency of the Ras/Raf/MEK/ERK cascade. A functionally similar protein (JIP-1) has also been identified, which binds another MAP kinase family member, JNK.[182] The JIP-1 protein also serves as a scaffolding protein to bind the MLK and MKK7 proteins, upstream activators of JNK, in a complex with JNK (see below). Thus, these signal transduction cascades also contain other matrix proteins that serve to form scaffolding devices, which can enhance sequential activation of downstream substrates.

The Ras/Raf/MEK/ERK Pathway: Regulation of Downstream Transcription Factors

Ultimately, the signals generated by the Raf/MEK/ERK pathway are transmitted to the nucleus where they lead to activation of various transcription factors necessary for the regulation of cell growth and differentiation.[183-194] Raf-1 can activate the c-Jun transcription factor. [191] c-Jun and c-Fos heterodimers bind AP-1 driven elements which are contained in promoter regions of many cytokine and immediate-early genes. Raf activity is also linked to activation of another AP-1-like site by phosphorylation of the TAR, ATF3, and c-Jun transcription factors.[161,163,164] This protein complex binds a response element and leads to cell survival. The removal of growth factors leads to formation of a JunD/ATF3 complex, which acts as a repressor of this response.[191,194]

Activated ERK can enter the nucleus where it acts as a kinase and phosphorylates certain key regulatory proteins. For example, activation of Elk-1 by ERK occurs in the nucleus where active Elk-1 binds the serum response element (SRE) contained in the promoter regions of certain cytokine and immediate-early genes (i.e., c-fos).[185,186] In addition, ERK can also directly activate $p90^{Rsk}$, an S6 kinase family member. $p90^{Rsk}$ activates the cyclic adenosine monophosphate response element (CRE) binding protein (CREB) which binds CRE response

elements in cytokine and immediate-early gene promoters (See Chapter 1).[188,190,195] Thus, induction of the Raf pathway leads to activation of at least three transcription factors that bind elements contained in some cytokine gene promoter regions (CREB, c-Fos and c-Jun).

Induction of Autocrine Gene Expression by Altered Raf/MEK and PI3K/Akt Expression

An area which has not been well investigated is the mechanism by which the Raf/MEK/ERK signal transduction pathway stimulates cytokine gene expression. Is this through the activation of transcription factors such as Elk-1 and CREB or by Raf activating the PI3K/Akt pathway, which can in turn activate p70S6 kinase that leads to protein stabilization? We have observed enhanced PI3K activity after activation of Raf in Raf-responsive hematopoietic cells (See Figs. 5 and 6).[9] In these cells, Raf also induced p70S6 kinase activity. An additional mechanism by which Raf could enhance cytokine gene expression is by increased protein synthesis due to elevated p70S6K activity. The effects of signal transduction pathways on protein translational efficiency leading to increased levels of cytokine expression have not been well investigated.

Mutations of Ras/Raf/MEK/ERK Cascade which Result in Neoplasia

Ras is one of the most frequently mutated oncogenes in human cancer.[110,111] There are three Ras related genes: Ha-Ras, Ki-Ras and N-Ras.[110, 111] Mutations in Ras are observed in 10 to 50% of patients with myelodysplastic syndrome and acute myelogenous leukemia.[196-200] These lesions often result from point mutations in three conserved codons, 12 (Ha-Ras, Ki-Ras), 13 (Ki-Ras), or 61 (Ha-Ras, N-Ras), which convert all three Ras proteins into constitutively active proteins.[196-200] Deregulated Ras often has downstream effects which results in altered Raf activity.

Deregulated Raf expression has been observed in diverse neoplasias including hematopoietic, breast, cervical, renal, laryngeal, hepatocellular, small cell lung carcinomas, and in lung biopsies recovered from cigarette smokers.[201-230] However, the role(s) of Raf in the initial transformation events are not clear, since the biopsied cells were established tumors.[219] Mechanisms responsible for activated Raf expression include point mutations, deletions, gene rearrangements, and gene amplifications.[221-228]

Constitutive activation of MEK1 has been associated with a variety of neoplasias including hepatocellular carcinoma, renal cell carcinoma, breast cancer, squamous cell carcinoma, AIDS-related Kaposi's sarcoma, acute myelogenous leukemia, and chronic myelogenous leukemia.[219,227-243] In addition, constitutive activation of other downstream members of this pathway has been implicated in oncogenic processes such as invasion, metastases, angiogenesis, and radioresistance.[236-240] One factor involved in invasiveness of cancers is the urokinase-plasminogen activator gene whose synthesis is increased following ERK activation.[210,211]

Regulatory Phosphatases of the Ras/Raf/MEK/ERK Pathway

MAP kinase phosphatase-1 (MKP-1) is a phosphatase that is activated by mitogenic signals and calcium.[242,243] This phosphatase serves to turn off activated ERK in a negative feedback manner and inhibits ERK-stimulated DNA synthesis.[242] Alterations in MKP-1 expression have been observed in prostate, colon, and bladder cancer where MKP-1 is over-expressed in the early stages, but progressively diminishes with higher histological grade and metastases.[242,243] Moreover, MKP-1 over-expression inhibits the differentiation of myoblasts.[244] In v-Raf-transformed cells, MKP-1 expression appears to be suppressed.[245] This suppression has been speculated to occur via feedback regulation of MKP-1 by v-Raf.[245] MKP-1 might be a target for gene therapy in aggressive hematopoietic tumors, which have lost MKP-1 expression. The involvement of this and other phosphatases in hematopoietic malignancies is an area of research in its infancy and requires further investigation.

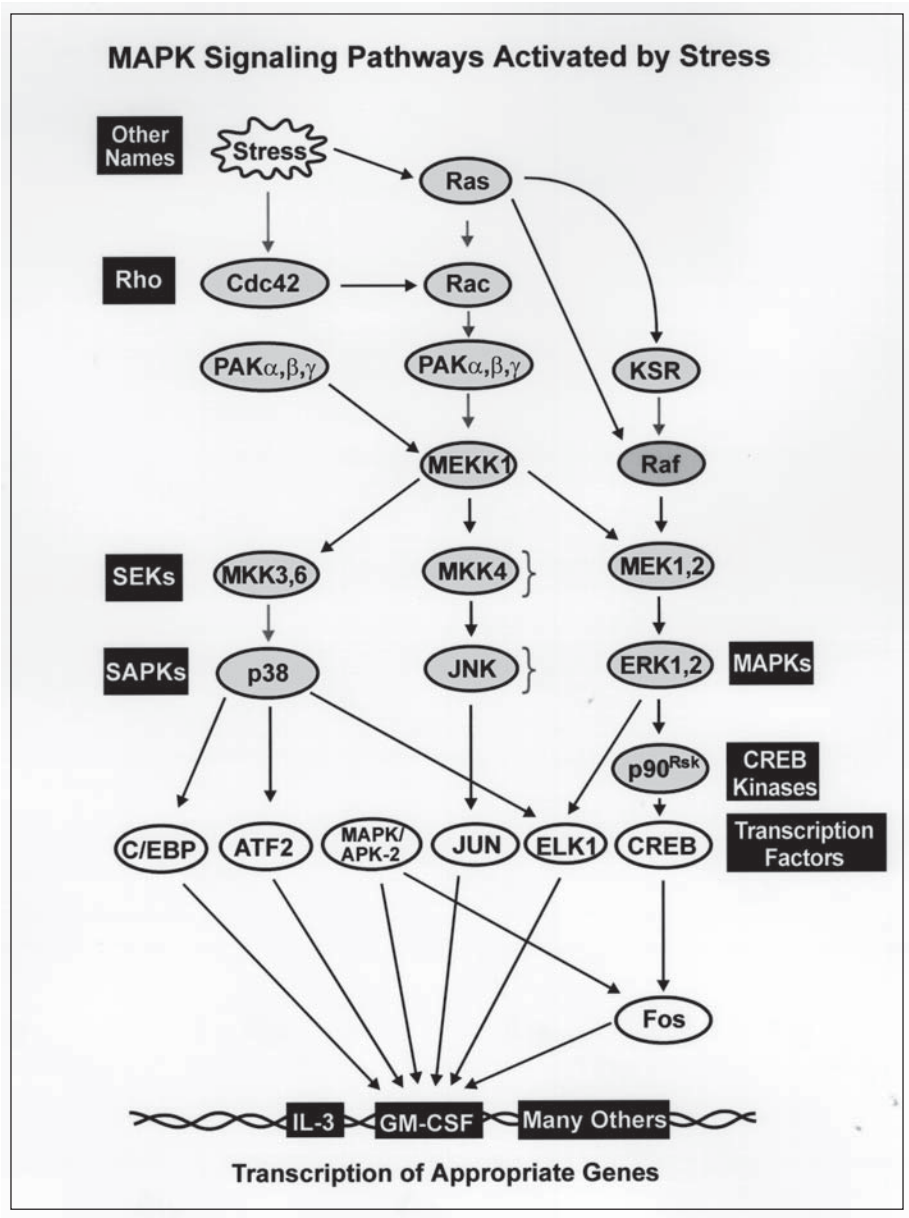

Fig. 7. MAPK signaling pathways activated by stress. In addition to the Ras/Raf/MEK/ERK pathway, there are other signal transduction pathways which may crosstalk (interact) with each other. These pathways can be induced by many different stimuli that induce cell stress. The kinases, which are functionally similar, are at the same horizontal positions in the pathway. Additional related molecules and other names are shown on the left and right hand sides of the figure. Kinases are indicated in gray ovals and transcription factors are indicated in clear ovals.

Alternative MAPK Pathways Activated by Stress

In addition to the Jak/STAT, PI3K/AKT, and Raf/MEK/ERK pathways, there are alternative signal transduction cascades activated by Ras-dependent and independent mechanisms.[246-253] A diagram illustrating these pathways is presented in Figure 7. These related pathways can interact with the Raf/MEK/ERK pathway. Ras-dependent activation of the MEK1 kinase (MEKK1), a serine/threonine kinase, is responsible for activating three MAPK pathways.[247-249] MEKK1 phosphorylates and activates MEK1, MEK2,[192,250] and the dual serine/threonine and tyrosine stress/extracellular regulated kinases (SEKs).[250-255] Activation of the SEKs (MKK4 and MKK3/MKK6) results in the activation of the SAPKs, JNK, and $p38^{MAPK}$.[250-254] JNK is a 46-kDa MAP kinase responsible for activating the AP-1 transcription factor component c-Jun by phosphorylating it on serine residues 63 and 73.[258] $p38^{MAPK}$ is responsible for phosphorylating and activating certain members of the AP-1 transcription factor family, a C/EBP transcription factor family member (CHOP), and the MAPK-associated protein kinase (MAPK-AP).[251,252,256] We have observed that both of these kinases are activated after IL-3 and hydrogen peroxide treatment of certain hematopoietic cells (JTL, JAM and RAF manuscript in preparation). Activation of MEKK1 preferentially leads to the activation of JNK > $p38^{MAPK}$ > ERK1 and ERK2.[251] On the other hand, Raf-1 leads to the activation of ERK1 and ERK2 but does not result in activated JNK or $p38^{MAPK}$.[175] Although pharmaceutical companies have produced specific inhibitors to $p38^{MAPK}$, direct inhibitors to JNK and ERK have not yet been developed.[65]

Recent evidence suggests that a significant amount of crosstalk occurs among the PI3K, MEKK/SEK/SAPK, and Raf/MEK/ERK pathways.[109,257-267] PI3K has been implicated in MEKK1 activation as well as MEK1/ERK activation,[171,260,262] whereas oncogenic Raf-1 and MEK1 have also been reported to activate p70S6K in a PI3K-independent way.[261]

Default Pathways which Dampen Signaling

Signal transduction pathways are also under the regulation of phosphatases which serve to dampen cytokine stimulated signaling.[268-279] p145 SHIP is purported to be an inhibitory growth regulator which down-regulates PI3K and Ras signaling activities. SHIP contains a conserved SH2 domain and an inositol polyphosphate-5-phosphatase domain.[39,83,268-283] Targeted disruption of SHIP has suggested an important role of this molecule in controlling cytokine signaling. SHIP knockout mice have increased numbers of granulocyte-macrophage progenitors, possibly as a consequence of hyper-responsiveness to stimulation with IL-3 and other cytokines.[275] Thus, we see the consequences of a deregulated IL-3 signal transduction pathway on the growth of hematopoietic cells. In addition to the association of the Shc protein with SHIP, IL-3 induces the transient association of SHIP with another phosphatase, SHP-2.[39,269-275] The intracellular levels of this complex may influence whether a cell proliferates or undergoes apoptosis.

Binding sites are also present on the IL-3β_c chain for SHP-1, a tyrosine phosphatase bearing two SH2 domains.[274-276] SHP-1 is also known as HCP, SH-PTP1, and PTP1C. SHP-1 associates with the β_c chain via its N-terminal SH2 domain.[284] SHP-1 is preferentially expressed in hematopoietic cells[275] and negatively regulates the growth stimulation induced by hematopoietic growth factors.[284] SHP-1 activity is an essential component for controlling the events of cell activation induced by hematopoietic growth factors as evidenced by a mutation at the SHP-1 gene, which is responsible for the moth-eaten phenotype observed in mice.[277] Moth-eaten mice have severe immunodeficiency and autoimmune syndromes. SHP-1 negatively regulates erythropoiesis based upon the observations that erythroid cells from these mice are hypersensitive to Epo.[275] Clearly, cytokines induce phosphatases which play critical roles in regulating signal transduction. Thus, cytokines also induce inhibitory molecules that serve to dampen the effects of cytokines. Through the studies of knockout or naturally occurring mutant mice, it is becoming apparent what devastating effects dysregulation of these phosphatases can have on the development of the hematopoietic system, as well as viability and health.

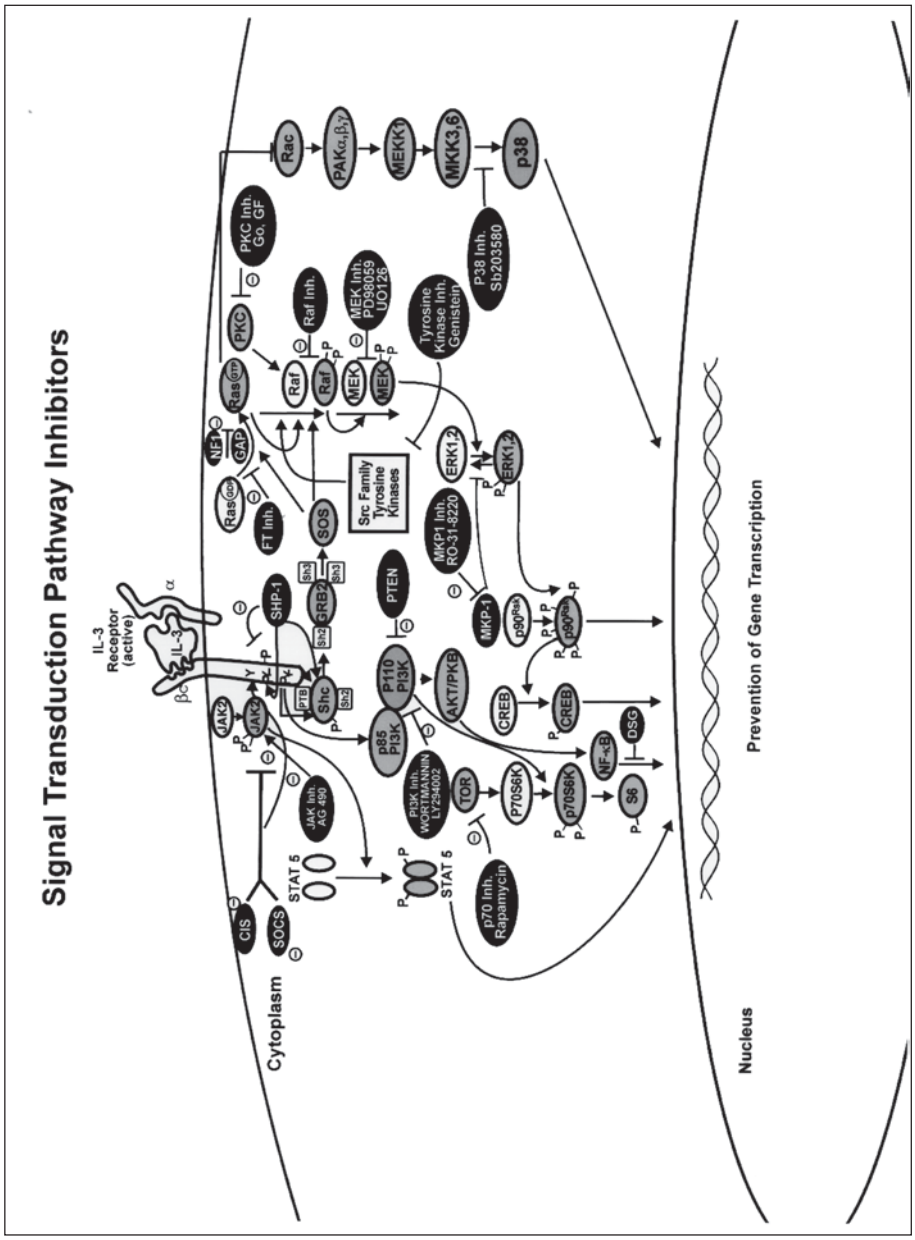

Fig. 8. Signal transduction pathway inhibitors. Sites of intervention of pharmaceutically-derived chemical drugs and naturally-derived proteins (in black). Deletion or mutation of the genes encoding the PTEN, MKP-1 and SHP-1 proteins shown in black can contribute to hematological defects and in some cases malignant transformation.

Jak/STAT Inhibitors

Recently a family of proteins which inhibits the activation and/or function of Jaks and STATs has been described. This family is referred to as the Cis family of proteins and consists of Cis, Soc1, Soc2, and Soc3, as well as other proteins.[48,285-289] Some of these proteins inhibit multiple Jak and STAT proteins, whereas others only suppress a specific Jak or STAT protein.[48,285-289] In addition, expression of Soc proteins appears to be tissue-specific. These inhibitors can function either by binding the Jak protein and blocking its activity or by binding the cytokine receptors to prevent Jak binding and subsequent activation of the signal transduction cascade. Moreover, there are naturally occurring STAT isoforms, which can function as dominant-negative mutants.[296] Thus, it may be possible to target the Jak-STAT pathway in certain cells by introducing vectors which over-express these natural inhibitory proteins. Finally, certain drugs, such as the tyrphostins AG490, AG198, and AG6450, specifically inhibit Jak kinases as well as the progression of lympoblastic leukemia.[365,366] Thus, there exists both natural and chemical means to inhibit Jak and STAT activities. A diagram of potential regulatory sites of these inhibitors is presented in Figure 8.

PI3K/p70S6K Inhibitors

Wortmannin, LY294002 and rapamycin exert their effects at different points in the PI3K/p70S6K pathway.[263,264,292] Wortmannin and LY294002 inhibit PI3K, while the immunosuppressive drug rapamycin inhibits p70S6K via an upstream target of rapamycin (mTOR) (Fig. 8). However, once cells have entered the cell cycle, inhibition of p70S6K does not stop proliferation.[259,267] Use of these inhibitors has documented the PI3K-independent activation of p70S6K.[266] Rapamycin has been recognized for years as a potentially useful immunosuppressive drug.[293] Thus, derivatives of rapamycin may be useful in the treatment of patients with certain leukemias.

Ras/Raf/MEK/ERK Pathway Inhibitors

Companies, including Janssen Pharmaceutica, Schering Plough, Merck & Company, and Parke-Davis either plan to perform clinical trials or have clinical trials in progress with FT inhibitors.[117,122,294] In addition, there are also mutations that eliminate Raf-1 kinase activity. Site-directed mutations, which change the lysine in the ATP-binding site of the catalytic domain, render the kinase inactive and can serve as dominant-negative mutations.[295,296] Kinase-inactive mutants of Raf-1 inhibit Ha-Ras-mediated cell transformation although Ha-Ras can, in some cells, signal through other molecules that will allow growth. Use of dominant-negative mutants of upstream activators of MEK1, as well as the MEK-specific inhibitor PD98059, blocked MEK1 and ERK activation.[179,180] The MEK1 inhibitor, in turn, blocked Ras- and Raf-mediated transformation and cytokine-stimulated growth.[180] PD98059 and related drugs that inhibit MEK1 activity may be useful in turning off this major pathway in rapidly proliferating malignant cells. Inhibitors to MKP-1 (e.g., Ro-31-8220) or related phosphatases have been isolated, suggesting that it may be possible to modulate the activity of these proteins in certain leukemias.[297] At first glance, it may appear that this type of inhibitor might enhance proliferation.

PKC Inhibitors

Several pharmaceutical companies have focused on modifying PKC activity as a potential therapeutic treatment. Many PKC inhibitors (G_0, G_F) as well as activators, including Bryostatin, are being used in clinical trials with patients who suffer from certain types of cancer.[2,297-300] Prolonged activation of PKC by inducers like Bryostatin result in PKC downregulation.[2] Thus, activation of PKC by such differentiation inducing compounds as Byrostatin may be a viable therapeutic approach. An obvious problem with PKC inhibitors is that they may affect multiple signal transduction pathways leading to growth.

Cytokine Regulation of Cell Cycle Progression

We have addressed control of IL-3 expression and the effects of IL-3 on signal transduction cascades. An IL-3-dependent cell in G_0 (a quiescent resting stage theoretically out of the cell cycle)/G_1 (Gap_1) receives positive (presence of IL-3) and negative (absence of IL-3) stimuli. These signals are integrated to decide whether or not the cell should enter the cell cycle.[23,26,301,302] The decision whether to progress from G_1 into S phase is a position in the cell cycle termed the restriction (R) point.[302] Coordination of cell cycle progression is achieved through the activity of the cyclins and their associated cyclin-dependent kinases (CDKs), as well as interactions with tumor suppressor genes (e.g., pRb, p53, $p21^{Cip1}$) that regulate the activity of these complexes.

Links Between the Ras/Raf/MEK/ERK Pathway and Cell Cycle Proteins

The Ras-activated signal transduction pathway provides a link between IL-3 and stimulation of cell cycle machinery.[303-309] Ras is required for cell cycle progression and activation of both CDK2 and CDK4 before the G_1/S transition. Ectopic overexpression of Ras or its downstream molecules, such as ERK or Ets-2,[305] can lead to the induction of cyclin D but has little or no effect on cyclin E or A. [303,305-309] The regulation of cyclin D is thought to be the more critical and limiting step because of its rapid degradation. Its expression is dependent on continued growth factor stimulation until cells pass the G_1 restriction point. The cell cycle will also be discussed in more detail by Drs. Blagosklonny and Pardee.

Cytokine Regulation of Apoptosis and Cell Death

The final regulatory aspect of IL-3 to be discussed in this chapter is the role of IL-3 in the modulation of apoptosis. Many cytokines such as IL-3 act to prevent apoptosis. Research with IL-3-dependent cell lines has proven very rewarding in terms of our understanding of the mechanisms of apoptosis since removal of IL-3 from the culture medium causes the cells to undergo apoptosis 24 to 48 hrs later. Apoptosis can be suppressed in these cells for approximately 3 to 6 days by the introduction of constitutively expressed anti-apoptotic genes such as Bcl-2.

Apoptotic Mediators: The Caspases

The caspase family of proteases comprise the effector arm of the apoptotic pathway.[310-312] At least 15 caspases, have been identified thus far.[313,314] These cysteine proteases cleave substrates carboxy-terminal to an aspartate residue, the P1 site.[315] However, caspases can be divided into three subgroups based on differences in substrate preference dictated by the residues immediately amino-terminal to the P1 site.[316]

Caspases are synthesized as inactive proenzymes consisting of an amino-terminal prodomain and large and small subunits. Caspase prodomains contain protein-protein interaction modules, which facilitate the association of multiple factors required for caspase activation in response to apoptosis-inducing stimuli. Activation requires proteolytic removal of the prodomain and formation of a heterodimer between the large and small subunits. Upon tetramer formation, the caspase complex is activated.[317,318]

Some caspase prodomains contain a death effector domain (DED) through which they bind to other DED domains contained on adapter proteins (e.g., FADD/MORT1). This targets the caspase to ligand-activated death receptors at the cell membrane where caspase activation occurs in the death-inducing signaling complex (DISC).[319-324] Formation of DISCs is triggered by binding of death ligands such as TNF-α, Fas, and TRAIL to their respective death receptors. Other caspase prodomains contain a caspase recruitment domain (CARD) through which they bind to the apoptotic protease activating factor-1 (Apaf-1) resulting in the formation of a cytoplasmic caspase-activating complex, the apoptosome.[325-328] In both cases, multiple factors are required for caspase activation. Caspases are activated in a cascade in which "upstream" caspases are activated by cleavage in the apoptosome or DISC. These caspases, in

turn, cleave and activate "downstream" caspases whose substrates are cellular components critical to the life of the cell.

Death receptors contain cytoplasmic death domains (DD) which, when bound by their respective death ligand, recruit adapter proteins containing DD and DED domains.[329,330] The DED domains of adapter and pro-caspase proteins interact leading to autoproteolytic activation of the upstream caspases and priming of the caspase cascade. Apoptosome formation in some cells can be triggered by the release of cytochrome c from mitochondria in response to apoptosis-inducing stimuli.[325,331] Cytochrome c binds to Apaf-1, which can interact with an upstream caspase, procaspase-9, and an apoptosis inhibitor, Bcl-X_L.[326] Binding of dATP to Apaf-1 in some cells is required for cleavage of procaspase-9, while binding of Bcl-X_L can inhibit this cleavage.[332] Once activated, caspase-9 then proteolytically activates downstream caspases, such as caspase-3, which directly cleaves life-sustaining cellular proteins. As stated earlier, phosphorylation of caspase 9 by Akt results in its inactivation.

A caspase-3-like protease is also responsible for activating the cytoplasmic endonuclease, CAD (caspase-activated deoxyribonuclease) that generates the oligonucleosomal DNA fragments indicative of apoptosis.[333] CAD is rendered inactive by its association with two isoforms of a chaperone-like protein, ICAD/DFF-45 (inhibitor of CAD/DNA fragmentation factor-45), which inhibit CAD endonuclease activity by concealing the CAD nuclear localization sequence.[331,333] Induction of apoptosis results in the cleavage and inactivation of ICAD/DFF-45, the activation and nuclear translocation of CAD, and fragmentation of DNA into oligonucleosomes. Another endonuclease, cyclophilin C, may be involved in generating the 50- to 200-kbp-sized DNA fragments.[334]

Roles of Bcl-2 Family Members in Cytokine-Mediated Regulation of Apoptosis

The *Bcl-2* gene was identified at the chromosomal breakpoints of t(14;18)-bearing follicular B cell lymphomas. Having been translocated to a location near the enhancer elements of the immunoglobulin heavy chain locus, the *Bcl-2* gene was transcriptionally enhanced and the 26-kDa Bcl-2 protein was overexpressed, contributing to malignant transformation.[335-337] Most importantly for this chapter, Bcl-2 protects hematopoietic cell lines from apoptosis following growth factor withdrawal.[338] Bcl-2 is a member of a growing family of related proteins that play pivotal roles in determining whether or not apoptosis will proceed to completion.

The Bcl-2-related proteins share some structural similarities but are divided into subgroups based on their structural differences and pro- versus anti-apoptotic activities.[339] Bcl-2 contains four conserved motifs, the Bcl-2 homology (BH) domains BH1 to BH4, as well as a transmembrane domain. The most closely related Bcl-2 proteins contain 2-4 of the BH domains (at least BH1 and BH2) and are anti-apoptotic rather than pro-apoptotic. These include Bcl-2, Bcl-X_L, Bcl-W, Mcl-1, and A1. The BAX subfamily is comprised of three proteins, BAX, Bak, and Bok, which are structurally similar to Bcl-2, but promote apoptosis rather than survival. The BH3 subfamily includes proteins that contain only the BH3 domain and also promote apoptosis: Bik, Blk, Hrk, Bim_L, Bad, Bid, and BNIP3.[340-343]

The Bcl-2 proteins function in a concentration-dependent manner by forming homo- and heterodimers with other family members via the BH domains.[334] For example, when Bcl-2 is in excess, Bcl-2/BAX heterodimers are formed and apoptosis is inhibited; when BAX predominates, BAX homodimers are formed and the cells are susceptible to apoptosis. The formation of dimers among family members appears to be somewhat discriminate since some members form dimers with any of the other members while some exhibit more limited associations.[343-353]

Mitochondrial Regulated Apoptosis

Many of the Bcl-2 related proteins, including Bcl-2, Bcl-X_L, and BAX are localized in the outer mitochondrial membrane. Bcl-2 and related, pro-survival proteins can inhibit apoptosis upstream of caspase activation, presumably by binding Apaf-1 during formation of the

apoptosome, thus preventing caspase activation.[326] However, these pro-survival proteins do not inhibit apoptosis induced by death receptor activation.[354-357] Apoptosis is associated with distinct changes in the mitochondrion including a reduction of ψ_m and the release of cytochrome c and apoptosis inducing factor (AIF).[358] Bcl-2-related proteins are thought to be involved in regulating these changes by forming pores (channels) in the membrane which allow the passage of cytochrome c and AIF.[358-360] Both pro-apoptotic BAX and pro-survival Bcl-2-related proteins form pores in vitro.[361-367] However, whereas Bcl-2 and Bcl-X_L have been shown to inhibit cytochrome c release, BAX stimulates the release of cytochrome c. [365,367] Furthermore, Bcl-2 can inhibit the capacity of BAX to form pores.[362-364] The functional significance of the mitochondrial pores in the initiation or progression of apoptosis is still controversial. Bcl-2 and Bcl-X_L can bind cytochrome c directly, suggesting that Bcl-2/Bcl-X_L inhibition of apoptosis is accomplished by sequestering cytochrome c from apoptosomes and preventing caspase activation.[368, 369] Certain pro-apoptotic Bcl-2 family members become activated during the apoptotic response by caspase cleavage.[370,371] Bid is a pro-apoptotic family member, which is cleaved from a 22 kDa inactive protein to a 15 kDa active protein by caspase 8. [368,371] Active p15 Bid then translocates to the mitochondria, oligomerizes with the pro-apoptotic Bak molecule, and results in the release of cytochrome c.[370,371] The mechanism of Bcl-2-mediated regulation of apoptosis is still unclear, but like the regulation of caspases, it is likely to be complex with multiple checkpoints.

Interactions Between Cytokine Signaling Pathways and Apoptosis

Cytokines can prevent apoptosis via different mechanisms.[124] The carboxy region of the IL-3Rβ_c chain has been associated with the IL-3-mediated prevention of apoptosis. Stimulation of appropriate target cells by IL-3 leads to phosphorylation of Bad and its sequesterization by 14-3-3 proteins.[372-374] The unphosphorylated form of Bad normally forms heterodimers with anti-apoptotic factors, such as Bcl-2 or Bcl-X_L, to induce cell death.[372,374] Phosphorylation of Bad induced by IL-3 stimulation releases Bad from Bcl-2 and Bcl-X_L. The kinase(s), which directly phosphorylates Bad, is unknown; however, ERK, Akt, and PKA have been speculated to be involved.[105-107,372-374] Sequestering of Bad by chaperonin 14-3-3 proteins allows Bcl-2 and Bcl-X_L to bind BAX, resulting in the prevention of apoptosis. However this is not the only mechanism by which cytokines suppress apoptosis. Expression of DNp85, a dominant-negative version of the regulatory PI3K subunit, in BAF/3 cells suppressed apoptosis, but resulted in similar levels of Bad phosphorylation as observed in unstimulated cells even when these cells were treated with IL-3.[375] Thus, there are other kinases besides PI3K, which phosphorylate Bad. Even so, these cells were still resistant to apoptosis indicating at least one additional mechanism by which IL-3 prevents apoptosis. This secondary means may be a result of Bcl-2 or Bcl-X_L phosphorylation.

Phosphorylation of Bcl-2: Positive and Negative Effects

Chemotherapeutic drugs and cytokines induce phosphorylation of Bcl-2, which is associated with pro-apoptotic and anti-apoptotic activities respectively.[2,376-381] Some evidence has shown that Bcl-2 phosphorylation by chemotherapeutic drugs, such as Taxol, results in inactivation or cleavage by caspases generating a truncated Bcl-2 protein which has thereby gained pro-apoptotic properties. Other evidence suggest phosphorylation of the S70 residue of Bcl-2 by the Ras/Raf/MAPK pathway is anti-apoptotic.[380,381] This phosphorylation is correlated with enhanced Bcl-2 anti-apoptotic activity and the subsequent association of protein phosphatase 2A (PP2A) with Bcl-2. PP2A dephosphorylates S70 to return Bcl-2 anti-apoptotic activity to basal levels.[380,381]

Future Remarks

More recently studies of the ability of oncogenes to either abrogate the cytokine-dependency of hematopoietic cells or to induce various signal transduction pathways have been performed with activated cellular or viral oncogenes inserted into retroviral vectors containing

either the oncogene-estrogen receptor (ER) or the oncogene-androgen receptor (AR).[2,9,65,149-152,221-223] Studies performed in FDC-P1 and TF-1 cells with either ΔRaf:ER or ΔMEK1:ER inducible constructs have demonstrated that the activated viral oncogene is necessary for abrogation of cytokine-dependency since the cells reverted to cytokine-dependency when the steroid hormone was removed.[2,9,151,152] These conditional constructs enable investigators to distinguish between biochemical effects, which are due to the activated oncogene, and effects which result from the cytokine. Moreover, studies performed with FL5.12 cells have indicated that both activation of Raf/MEK1 and PI3K/Akt pathways were required for efficient abrogation of cytokine-dependency.[9] Given that some activated oncogene constructs contain the androgen receptor, it is possible to activate one oncogene in the absence of the other oncogene by adding testosterone in the absence of exogenous estrogen. We have determined that human TF-1 as well as murine FDC-P1 and FL5.12 cells all infected with ΔRaf:ER oncogenes secrete GM-CSF at sufficient levels to promote autocrine growth. Moreover the autocrine cytokine production and the activated oncogene expression were both required for the proliferation, providing further evidence for the theory that oncogenes induce autocrine cytokine gene expression which is required for transformation. With conditionally active oncogenes it is possible to directly monitor the effects of certain oncogenes on signal transduction cascades as well as other pathways, which may be induced by the oncogene and determine the signal transduction pathways involved in inducing autocrine transformation and promoting tumorigenicity. These oncogene:ER or oncogene:AR constructs will aid us in determining which signal transduction, cell cycle regulatory and apoptotic pathways are induced by oncogenes. Knowledge of such information may allow the rational design of drugs which will affect multiple points in different signal transduction cascades.

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CHAPTER 3

The Restriction Point of the Cell Cycle

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Introduction: Mitogen-Dependent and -Independent Phases of the Cell Cycle

Production of two cells from one requires duplication of all molecules and organelles that compose each cell. DNA does not duplicate throughout the cycle but only during several hours in S-phase (Fig. 1). During S phase, the DNAs of the 100,000 genes located on 23 pairs (in humans) of chromosomes are replicated, each in a timely fashion. This requires the ordered assembly of many proteins at the origins of DNA replication to form a competent, prereplicative chromosomal state. All eukaryotes use similar proteins to license replication origins.[1] After formation, competent origins are activated by cyclin-dependent kinases. Once started, DNA replication must be finished. Therefore, extracellular signals such as growth factors (GF) must not and do not control S-phase progression. Although S-phase is independent of growth factors, massive DNA damage or deprivation of nucleotides forces a cell to be arrested in S-phase, but such arrest is usually accompanied by cell death.

After successful completion of DNA synthesis, cells enter G2 phase in preparation for mitosis. Protein kinases that are activated in G2 phase prevent rereplication of the DNA. Quiescent mammalian cells are usually diploid, so after DNA duplication a cell must divide. Therefore, control by growth factors is also unnecessary in the G2 phase. This is a time to check the internal signaling with events like DNA damage.

At the onset of mitosis, cyclin B/cdc2 phosphorylates laminin, a nuclear membrane protein, thus dissolving the nuclear envelope. In metaphase of mitosis, condensing of chromosomes associates with forming of mitotic spindles. The endoplasmic reticulum and the Golgi complex break down into small vesicles. During mitosis, chromosomes are condensed, proteins hyperphosphorylated and transcription and biosynthesis are reduced or absent. Progression of mitosis is determined by monitoring of the microtubule function to ensure chromosomal fidelity. External regulation by growth factors would be detrimental for a cell, and consequently, mitosis is a growth factor-independent phase of the cell cycle. In anaphase of mitosis the two sets of chromosomes are pulled apart. During telophase and cytokinesis the preceding fragmentation process of intercellular membranes is reversed. The nuclear envelope reappears around the chromosomes, endoplasmic reticulum is rebuilt and cytoplasmic microtubules reassemble. The transcription and biosynthetic functions of the cell are normalized and intracellular membrane traffic is resumed.[2] As cells exit mitosis, the cell cycle is reset, allowing the establishment of a new, competent replication state in G1 phase (Fig. 1). Therefore, as the logic dictates, G1 phase is the only part of the cell cycle that can and must be growth factor-dependent.

The Restriction Point

The major regulatory events leading to proliferation occur in the G1-phase of the cell cycle. In vivo as well as in cell culture, most quiescent cells have a G1 DNA content. The

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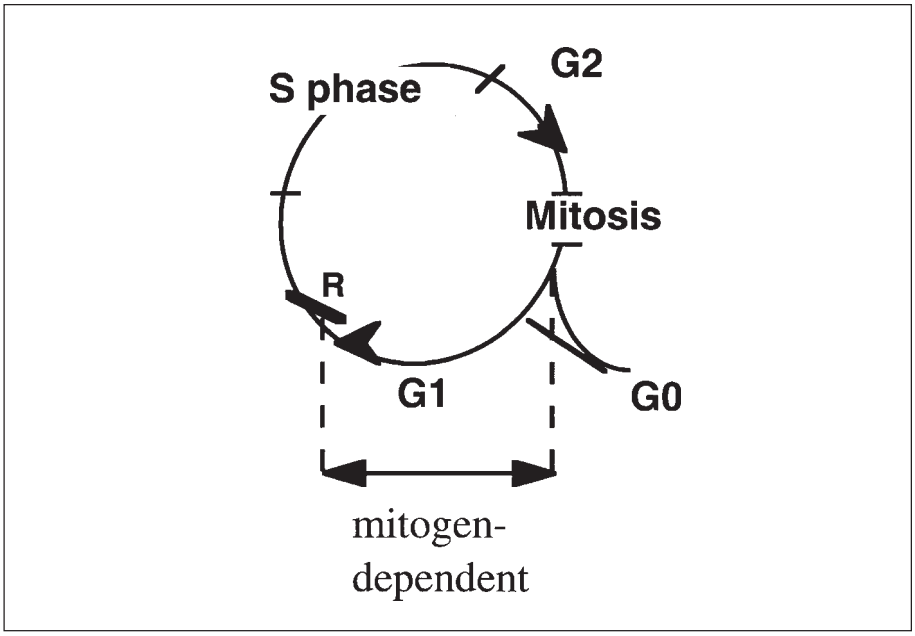

Fig. 1. Mitogen-dependent and -independent phases of the cell cycle.

growth of normal cells in culture is regulated by complex interactions between growth factors, cell density, and attachment to substrate.[3] Growth factors are necessary to initiate and maintain the transition through G1 phase leading to S phase. In normal cells, withdrawal of growth factors prevents the onset of S phase. The point at G1 at which commitment occurs and the cell no longer requires growth factors to complete the cell cycle has been termed the restriction (R) point.[4] The R point has been temporally mapped at 2-3 hours prior to the onset of DNA synthesis.[5] Once beyond the R point, or point of no return, cells are committed to DNA synthesis and they no longer require the extracellular growth factors during the remainder of the cell cycle. Transition of the restriction point was proposed to be determined by accumulation of a labile protein.[4]

In Search of Mediators of the Restriction Point

In 1974, comparison of growth of cancer and normal cells revealed that restriction point is lost in cancer.[4,6] Identification of the R-point provided the difference between normal and cancer cell cycles. But what is the biochemical nature of the point of no return? R-protein is a functionally short-lived (labile) regulatory protein, whose synthesis is sensitive to growth factors, and needs to accumulate to a critical amount before a cell can pass the R point and proceed towards DNA synthesis.[5] When this question had been asked, oncogenes and signal transduction pathways were not known. Many of the later discovered proteins were evaluated for meeting the criteria of the R-protein.

Search for proteins that are differentially expressed in the late G1 phase versus resting cells identified numerous proteins.[7] In order to effectively identify and isolate the late G1 expressing genes, Liang and Pardee developed a method known as differential display.[8] The differential display detects mRNA species that are different between sets of mammalian cells, permitting their recovery and cloning of the corresponding cDNAs. Differential display allows one to separate and clone individual mRNAs by PCR. The key element is to use a set of oligonucleotide primers, one being anchored to the polyadenylate tail of a subset of mRNAs, the other

being short and arbitrary in sequence so that it anneals at different positions relative to the first primer. The mRNA subpopulations defined by these primer pairs were amplified after reverse transcription and resolved on a DNA sequencing gel. When multiple primer sets were used, reproducible patterns of amplified complementary DNA fragments were obtained that showed strong dependence on sequence specificity of either primer. Many late-G1 expressing genes were isolated by differential display.[9] However, R protein is also accumulated by the mechanism of protein stabilization, but protein stabilization cannot be detected by the differential display method.

R-protein is a labile protein which is induced, stabilized and accumulated in response to growth factors leading to growth factor independence. The discovery of G1-phase cyclins (D and E) was an important breakthrough. Cyclin D1, a labile nuclear protein, accumulates following growth factor stimulation.[10-15] It is commonly overexpressed in human cancer. Cyclin E was discovered in the course of a screen for human complementary DNAs that rescue a deficiency of G1 cyclin function in budding yeast. The amounts of both the cyclin E protein and an associated protein kinase activity (cyclin E-CDK2 complexes) fluctuated periodically through the human cell cycle; both were maximal in late G1 and early S phases.[16] In 1993, Pardee and his colleagues suggested that the molecular basis of the R protein could be production of cyclin E[17,18] because cyclins satisfy all of the criteria for the R protein, which includes a late G1 phase increase, a delay of appearance after inhibition of protein synthesis in nontransformed cells, and a faster recovery in transformed cells.

Cyclins: From Mitogen Signaling to the Restriction Point

The progression from one phase to another is driven by enzymes named cyclin-dependent kinases (CDK). Their activators, the cyclins, however are unstable and are "cycling" during the cell cycle.[19] Thus, cyclins control the activities of cyclin-dependent protein kinases (CDKs) and play a key role in cell cycle regulation. As cells proceed through the cycle, four major cyclins are produced sequentially (D, E, A, and B), and they activate CDKs (Fig. 2). B-type cyclins associate with p34cdc2 to trigger mitosis. Progression through S phase requires cyclin A, presumably in association with p33CDK2. Cyclins D and E drive a cell into S-phase. The three D-type cyclins (cyclin D1, D2, D3) are very similar but they share very little homology with cyclin E. During cell cycle progression, D cyclins start accumulating at mid-G1, whereas cyclin E appears later, just prior to the G1/S transition.

Mitogen-dependent progression through G1 phase is mediated by induction of the cyclin D family. Growth factors regulate cyclin D1 by four simultaneous mechanisms (Fig. 3):

1. transcriptional induction,
2. stabilization of the cyclin D protein,
3. its translocation to the nucleus, and
4. assembly with their catalytic partners, CDK-4 and CDK-6.[14,15]

The promoters of D-type cyclins respond to a variety of mitogen-activated signals such as Ras, β-catenin-Tcf/lef pathways.[13,20] The transcriptional induction of cyclin D1 by growth factors is dependent on the Ras/Raf-1/Mek/ ERK pathway.[21-23] However, cyclin D1 protein is rapidly degraded and therefore it has a short half-life. It is important to emphasize that not only cyclin D and E but other proteins that regulate or affect the restriction point can be increased by protein stabilization. Normally, wt p53 is rapidly degraded by the proteasome, but it is accumulated after DNA damage, almost exclusively by protein stabilization. Similarly the CDK inhibitors p27 and p21, as well as cyclin E and the E2F-1 transcription factor are degraded by the proteasome.[24-28] Cyclin D1 turnover is governed by ubiquitination and proteasomal degradation, which are stimulated by cyclin D1 phosphorylation on threonine-286.[29] This phosphorylation can be inhibited by signaling through a pathway that sequentially involves Ras, phosphatidylinositol-3-OH kinase (PI3K), and protein kinase B (Akt). Thus turnover of cyclin D1, like its assembly, is mitogen dependent.[30] Nuclear localization and assembly of newly synthesized cyclin D1 with CDK-4 is also GF-dependent.[31]

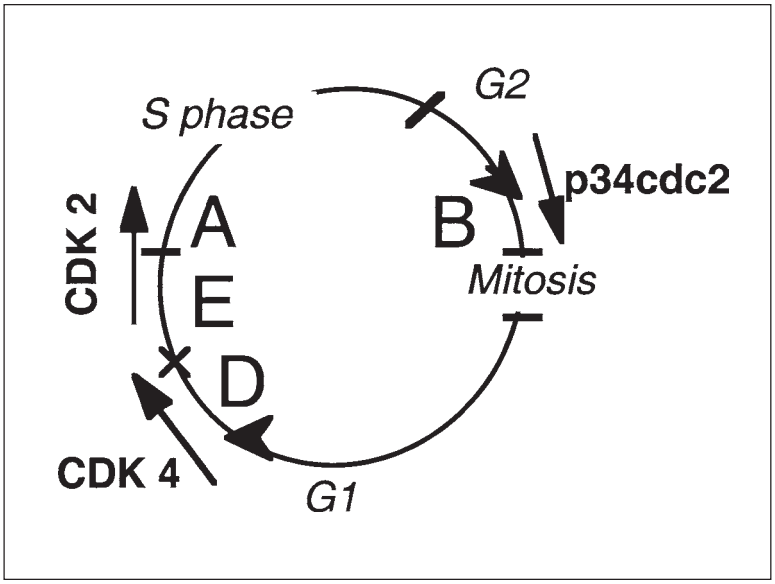

Fig. 2. Cyclin-dependent kinases (CDKs) and cyclins.

After active cyclin D-dependent kinase is assembled in the nucleus, it phosphorylates the retinoblastoma (Rb) protein,[32] preventing its binding to E2F thus activating E2F-1 mediated transcription.[33] The E2F transcription factor activates genes whose products are involved in nucleotide metabolism and DNA synthesis.[34] For understanding the restriction point, it is important to emphasize that E2F transactivates cyclins E and A.[35] Cyclin E enters into a complex with CDK2 and collaborates with cyclin D-CDKs to complete Rb phosphorylation. Cyclin E-CDK has broader specificity than cyclin D-CDK. For example, cyclin E-CDK phosphorylates the CDK inhibitor, p27, causing its degradation.[36] The activity of cyclin E-CDK is inhibited by p21, p27 and p57.[15] In contrast, these CDK inhibitors, at least at low concentrations, coactivate cyclin D-CDKs (Fig. 4). Another class of CDK inhibitors, p15, p16, and p18, specifically inhibits cyclin D-CDKs.

The Restriction Point: A Knot of Mitogen and Inhibitory Signaling

Growth factors activate their receptors and other tyrosine kinases, Ras, and mitogen activated pathways culminating in transcriptional induction of numerous genes, including protooncogenes. Similarly, genes coding many growth factors, receptors, receptor-associated proteins and kinases are all protooncogenes.[37,38]

One of the greatest breakthrough in the understanding of the regulation of cell cycle is the connection of mitogenic stimulation to the cell cycle machinery. Expression of cyclins D, their nuclear location, stability, and their assembly with CDK-4 and CDK-6 into active kinase complexes are regulated by growth factors.[15] Therefore, cyclins D are growth factor sensors. In turn, the ability of cyclin D-dependent kinases to trigger phosphorylation of Rb in the mid- to late G1 phase of the cell cycle makes inactivation of the growth suppressive function of Rb to be a mitogen-dependent step. Rb participates in controlling the G1/S-phase transition, by binding E2F transcription factor family members (Fig. 5). Absence of functional Rb is sufficient for S-phase entry under growth-limiting conditions.[39] E2F-1 accumulation bypasses a G1 arrest resulting from the inhibition of G1 cyclin-dependent kinase activity.[40-42] GF-dependency ends with the phosphorylation of Rb, enabling cells to pass through the restriction point at the end of mid-G1 phase and to commit to completing the remaining phases of the growth cycle.[43,44] Given their

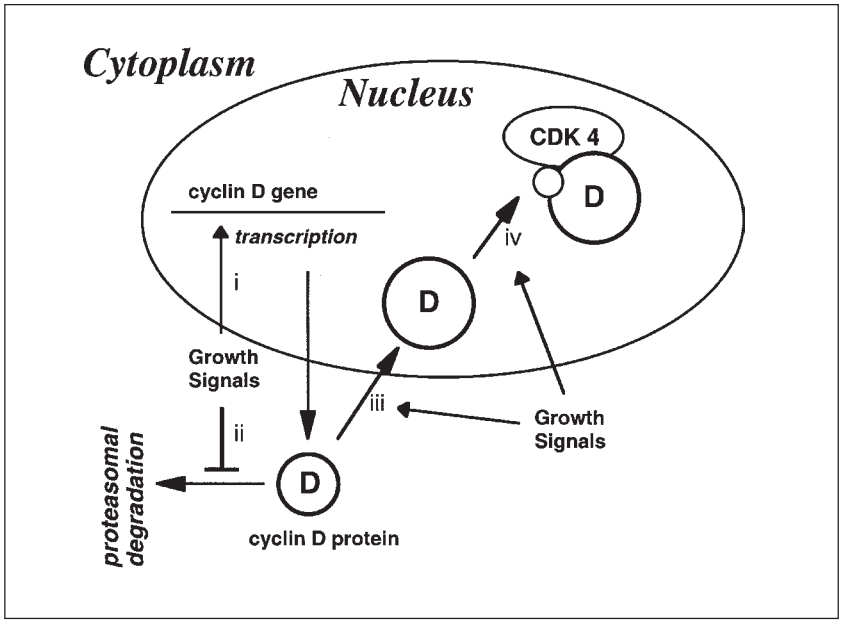

Fig. 3. Cyclins D are growth factor sensors. Expression of cyclins D, their nuclear location, stability, and their assembly with CDK4 and CDK6 into active kinase complexes are regulated by growth factors.

ability to inactivate Rb, do cyclins D meet the criteria of R-protein? Not completely. Their ability to act as growth factor sensors depends not only on their rapid induction by mitogens but also on their protein instability, which ensures their precipitous degradation in cells deprived of growth factors. The fact that D-type cyclins are labile proteins guarantees that the subunit pool shrinks rapidly when cells are deprived of mitogens. Induction of cyclin D1 is not sufficient for the transition from quiescence through G1 into S phase.[20]

Cyclin E in complex with CDK2 is downstream from cyclin D-CDK4. Cyclin E/CDK2 completes Rb phosphorylation (Fig. 5). This shift from cyclin D-CDK-4 to cyclin E-CDK2 accounts for the loss of dependency from growth factor. Precisely, the restriction point lies between cyclins D and E (Fig. 5). As emphasized by Kohn, the mammalian G1/S cell cycle phase transition comprise a highly nonlinear network that produces seemingly paradoxical results and makes intuitive interpretations unreliable.[45] Numerous feedback loops lead to a situation that downstream events lie upstream of themselves. For example, pRb2/p130 and p27 both are involved in a negative feedback regulatory loop with cyclin E.[46] Also, c-myc expression is downstream from p21, CDKs, and E2F,[47,48] yet c-myc is also an upstream regulator of p21 and CDKs.[49]

Similarly, cyclin E is located downstream from Rb and E2F-1 because cyclin E is transactivated by E2F-1 (Fig. 5). Yet, it is cyclin E that inactivates Rb and releases E2F (Fig. 5). Positive loops ensure irreversibility of commitments. Once expressed, cyclin E become independent of downstream GF-dependent cyclin D1. Therefore, cyclin E is a better candidate for the R-protein than cyclin D. Dependence on GF ends with the phosphorylation of Rb, enabling the cells to pass through the restriction point and to commit to completing the remaining phases of the growth cycle.[43] Yet, both cyclin D and E, as well as cyclin A, phosphorylate Rb. The restriction "protein" splits into at least two slices (Fig. 6).

Furthermore, E2F-1 partially expresses functional features of the R-protein. In fact, E2F is sufficient to drive a cell into S-phase.[41] Under enlargement, the restriction “point” looks like a restriction “knot” (Fig. 5 and 6).

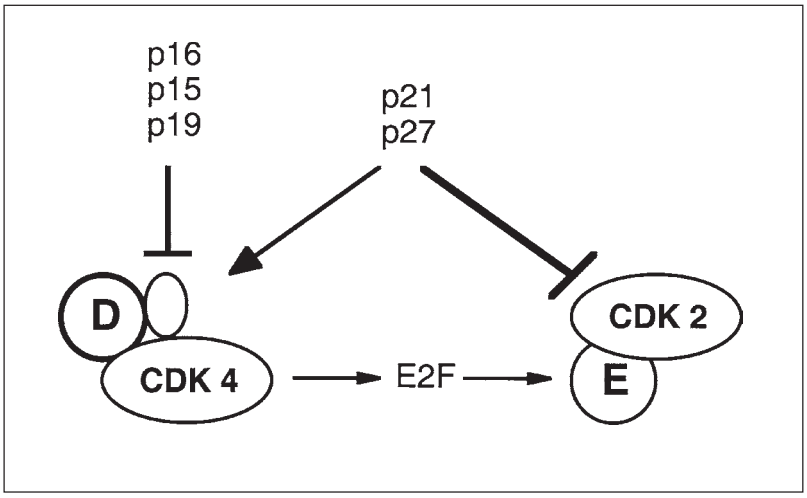

Fig. 4. Effects of "CDK inhibitors".

Growth Arrest versus Proliferation

An isolated activation of signaling, which is downstream of the restriction point, induces S phase entry. However, apoptosis may occur.[50, 51] Apoptosis is suppressed by GF-dependent events upstream of the restriction point. On the other hand, the choice between proliferation and growth arrest in response to growth factors and inhibitors is determined by the state of the restriction knot.

It is well known that exogenous stimuli such as EGF, TGFβ, CSF-1, interleukins, phorbol esters and endogenous transduction molecules such as Ras, PKC, Raf-1, ERK can induce either growth arrest or cycle progression (Fig. 7).[52-61] Yet, one consideration is so simple that it is often overlooked: growth stimulation can be detected only when an agent is applied to a resting cell, whereas to detect growth inhibition the agent must be applied to a proliferating cell.[51]

There is increasing evidence that mitogenic signaling induces cyclin D and p21 simultaneously. [51] Not only their ratio but a proliferative status of a cell may determine the response. In a resting cell, one can expect to find low basal levels of cyclins D1 and E but high levels of the CDK inhibitor p27. In a proliferating cell, one would expect to find high levels of cyclin D1 and E with low levels of p27. In resting fibroblasts, low levels of Raf-1 activity induces cyclin D1 and proliferation, whereas, in cycling fibroblasts, high levels of Raf-1 activity leads to growth arrest due to p21 induction. [57-59] Treatment with phorbol ester increased both cyclin D1 and p21.[60, 62, 63] While the growth of malignant melanoma cells was inhibited by TPA, the growth of normal melanocytes was stimulated. [62] Similarly, stimuli that are mitogenic for mature (resting) T-cells induce arrest in proliferating tumor T-cells, inducing both p21 and cyclin D. [63, 60] E2F-1 and H-Ras can activate the p21 promoter, and induction of the p21 promoter by activated Ras is mediated at least in part by E2F-1.[64]

The same stimuli can induce different outcomes depending on the Rb status. Cytokine response gene induced p21 that blocked either G1/S or G2/M transition, but did not prevent G1/S transition in Rb-negative (Rb-) cells, causing endoreduplication.[65] The same stimuli caused proliferation in quiescent normal human T cells.[65] Levels of p21 are also important. Thus, in HCT116 cells, low levels of endogenous p21 caused G2 but did not cause G1 arrest following DNA damage.[66,67] However, much higher levels of exogenous p21 arrested these cells in G1 phase.[67] In primary cells, Ras is initially mitogenic but eventually induces premature senescence. Constitutive activation of MEK induces both p53 and p16 and is required for Ras-induced senescence of normal human fibroblasts.[68] Furthermore, activated MEK permanently arrests primary murine

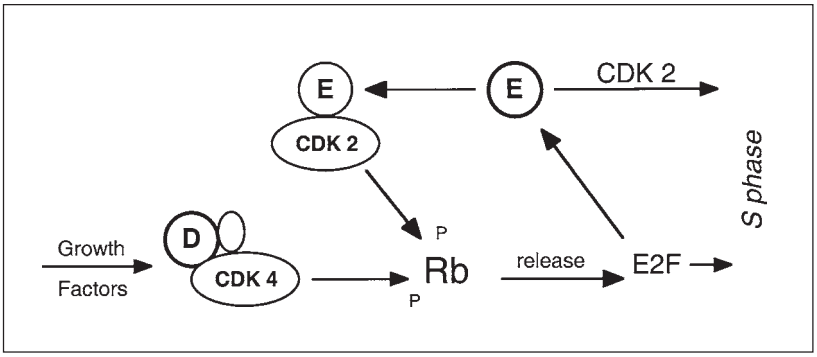

Fig. 5. The restriction knot.

fibroblasts but forces proliferation in cells lacking either p53 or p16. This may explain the opposite response of normal and immortalized cells to constitutive activation of MEK.[68]

It is very important that not only inhibitors and stimulators of CDKs are induced simultaneously, but some of these molecules may act as both an inhibitor and a stimulator (Fig. 7). While inhibiting cyclin-E-CDKs, p21 and p27 (at least at low levels) activate cyclin D1-CDKs complexes.[69] For example, p21 permits growth factor-induced cell cycle progression of vascular smooth muscle.[70] PKC alpha controls glioma cell cycle progression via upregulation of p21, which facilitates active cyclin-CDK complex formation.[71] In contrast, in many cell lines, PKC induces growth arrest due to p21 induction,[48] but the sensitivity to p21 may be lost in very aggressive cancers.[72] Increased expression of ectopic cyclin E in a mouse mammary epithelial cell line inhibits rather than stimulates growth, and this may be due to increased expression of the inhibitor p27.[73]

In addition to p21, p16(INK4a) and p15(INK4b), which are pure inhibitors of cyclin D CDK-4 and -6, are induced through the MAPK pathway (Fig. 7). This pathway is involved in p21 and p15 induction by TGF-β.[74] Like contact inhibition, TGF-β can arrest the cell cycle in G1 by inducing p27.[75] Oncogenic Ras inhibits growth of primary cells due to induction of p16 and p19(ARF).[68,76] p15 is induced by oncogenic Ras to an extent similar to that of p16, and expression of both is associated with G1 arrest and senescence.[77] Ras-dependent induction of these two INK4 genes is mediated mainly by the Raf-MEK-ERK pathway. 77 In addition, high expression levels of both wild-type and oncogenic Ras inhibit growth of K562 leukemia cells, which are deficient for p53, p16, p15, and p19 (ARF) genes. H-Ras increases p21 and causes growth arrest in these cells.[78]

From Restriction- to "Check"-Points

The restriction point could be considered as a prototype of cell cycle checkpoints. The restriction point is related to mitogen deprivation. Checkpoints are mostly related to DNA damage[79] and mitotic progression. Following DNA damage, a cell undergoes growth arrest to repair DNA. Damaged DNA can propagate during S-phase and mitosis. Therefore, arrest occurs in G1 (before S), and in G2 (before mitosis). Arrest in G1 prevents aberrant replication of damaged DNA and arrest in G2 allows cells to avoid segregation of defective chromosomes. Some mechanisms of G1 and G2 checkpoints are similar, including p53-dependent p21 induction and Rb dephosphorylation.[66,80] Other mechanisms are distinct because different sets of cyclins and kinases are activated in G1 and G2 phases. Massive DNA damage or deprivation of nucleotides forces a cell to be arrested in S-phase.[81,82] In the G2 checkpoint, p21 and the 14-3-3 protein play distinct but complementary roles and cooperate to achieve arrest following DNA damage.[83] p21 overexpression may result in senescence-like growth arrest and abnormal mitosis.[84,85]

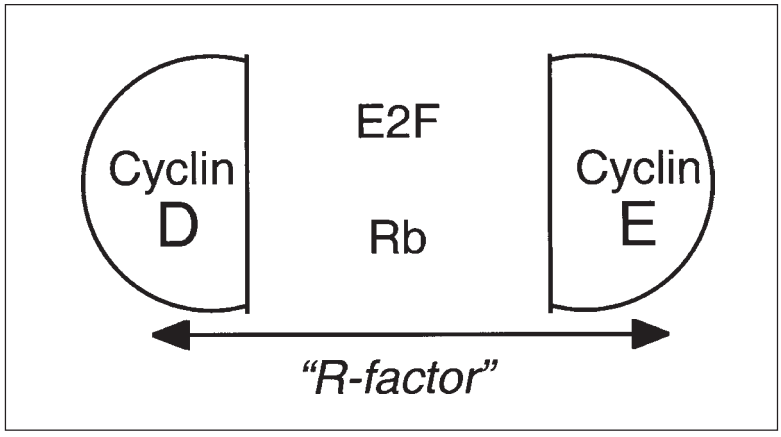

Fig. 6. "R-factor".

In order to ensure faithful chromosome segregation, cells have evolved mechanisms that delay progress into and out of mitosis until certain events are completed.[86] A spindle checkpoint in mitosis ensures equal chromosome segregation. The spindle checkpoint inhibits cell-cycle progression in response to a signal generated by mitotic spindle damage or by chromosomes that have not attached to microtubules. This prevents chromosome dysfunction and exit from mitosis until all of the kinetochores (and thus chromosomes) are attached to the spindle. A canonical example is the mitotic arrest by microtubule-active drugs such as Paclitaxel.[87]

The Restriction Point and G1 Checkpoint

As discussed, the restriction "point" lies between the accumulation of cyclins D and E and the activation of cyclin E-CDK2. What is the topological relationship of this GF-dependent restriction point and DNA-damage-induced G1 checkpoint?

DNA damage targets CDKs by two mechanisms. First is rapid and transient disappearance of cyclin D1. DNA damage-induced pathways target cyclin D and then cyclin E-CDK2. Following DNA damage, cyclin D1 is rapidly degraded by the proteasome.[88] Cyclin D1 degradation initiates a specific release of p21 from CDK4 complexes, leading to CDK2 inactivation. p21 may be redistributed without an increase in total amount. This is a p53-independent process. (Note: the restriction point control is also p53-independent). Second is a p53-dependent induction of p21 resulting in inhibition of cyclin E-CDK2. The escape of cells with nonfunctional p53 from the initial G1 arrest probably stems from the fact that the reservoir of p21 held by cyclin D1/CDK4 complex is quickly exhausted after DNA damage.[88] Simultaneously, p53 protein is accumulated. This results in p21 induction and sustained growth arrest. Importantly, pRB is a critical component of this DNA damage checkpoint. The checkpoint pathway uses the normal cell cycle regulatory machinery to induce the accumulation of the growth suppressive form of pRb.[89]

Not only is the DNA damage checkpoint located nearby the restriction point, but many regulators cause growth arrest by affecting the restriction point. Progesterone, a steroid hormone, regulates proliferation and differentiation in the mammary gland and uterus and induces p27 and p18 to inhibit cyclin E-CDK2 and CDK4.[90] Since similar models have been developed for growth inhibition by TGF-β and during adipogenesis, interaction between the Cip/Kip and INK4 families of inhibitors may be a common theme in physiological growth arrest.[90] The protein kinase inhibitor staurosporine stops progression of normal nontransformed cells in the G1 phase between cyclin D and cyclin E activities.[91] In contrast, the histone deacetylase inhibitor FR901228 causes nonphysiological G1 arrest with downregulation of cyclin D1 and upregulation of cyclin E in cancer cells lacking cycle control.[92]

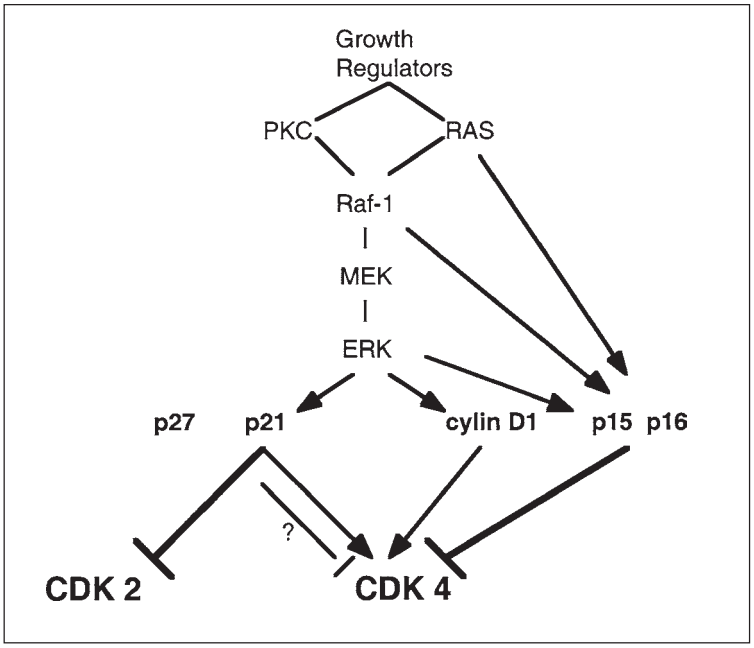

Fig. 7. Proliferation vs growth arrest.

The Restriction Point and Therapy

Selective killing of malignant cells without killing of normal cells is the ultimate goal of cancer therapy. Loss of the restriction point predisposes selective killing of cancer cells by chemotherapy.[93] Autonomous growth of cancer cells lacking the restriction point can be exploited to selectively arrest growth of normal cells.[94] For example, malignant transformation is associated with loss of dependency on epidermal growth factor (EGF).[95] MCF-10A, EGF-dependent immortalized breast cells, underwent G0/G1 growth arrest following EGF withdrawal. In contrast, MCF-7 cancer cells were not affected by EGF withdrawal. By using inhibitors of the EGF receptor kinase, this dependence on EGF can be exploited for protection of MCF-10A cells from microtubule-active drugs.[96] Microtubule-active drugs, such as Paclitaxel (Taxol) and vinblastine, kill cells in a cycle-dependent manner, in mitosis. Paclitaxel causes microtubule dysfunction leading to mitotic arrest of cells that entered mitosis.[97,98] Mitotic arrest is accompanied by phosphorylation of serine in multiple regulatory proteins, including Bcl-2, and this precedes cell death.[87] A cell arrested in interphase by a nontoxic agent cannot enter mitosis and therefore cannot undergo mitotic arrest when exposed to Paclitaxel. Hence, arresting normal cells in interphase prior to treatment with Paclitaxel could increase the selectivity of microtubule-active drugs against cancer cells by minimizing undesired toxicity to normal cells. Low doses of AG1478, an inhibitor of the EGF receptor kinase, arrested the MCF-10 breast cell line but not the MCF-7 cancer cell line.[96] In fact, AG1478 abrogated Paclitaxel effects in MCF-10A cells but not in MCF-7 cells.[96] Also, by arresting growth, CDK inhibitors, such as p21 and p16, can protect cells from apoptosis caused by cell-cycle dependent chemotherapy.[67, 99, 100] Targeting pathways that are not essential for cancer cells which lack the restriction point can selectively protect normal cells against chemotherapy.

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CHAPTER 4

DNA Damage, Cell Cycle Control and Cancer

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Abstract

Cell cycle checkpoints constitute a network of signal transduction mechanisms to monitor DNA damage and regulate progression through the cell cycle. A series of events is triggered in cells upon DNA damage. Here we describe a framework for the understanding of the functions of the core components involved in the cell cycle response to DNA damage and the relevance to the origin of cancer.

Introduction

Various mechanisms exist to maintain genetic stability of cells facing DNA damage. A state of genetic instability may be seen at the chromosomal level, at the nucleotide level, or be reflected by chromosomal translocations and gene amplifications.[1] Decisions to enter S phase and proliferate, arrest in the G_0/G_1 phase of the cell cycle or differentiate are based on multiple internal and external environmental stimuli. These processes are tightly regulated and well balanced. A hallmark of cancer cells is that the normal balance of these processes is perturbed, that they are prone to uncontrolled proliferation, and that they may further display progressive genetic instability.[2]

Multiple pathways are involved in the maintainance of genetic integrity, most of which link to the cell cycle.[3] The inactivation of these pathways as part of a multi-step process contributes significantly to the origin of tumors. By arresting the cell cycle, checkpoints presumably allow cells to repair DNA. Checkpoints can be seen as a network of surveillance systems, i.e., signal transduction systems, that interrupt cell cycle progression, when damage to the genome or failure of a previous activity in the cell cycle is detected.[3,4] Some cell types may primarily undergo apoptosis, avoiding the risk of generating genetically altered progeny.

In order to signal cell cycle arrest, checkpoint control pathways must sense DNA damage and then transduce the signal. To delay cell cycle progression after DNA damage, these mechanisms affect the activity of critical cell cycle regulators. Conceptually, checkpoint control pathways consist of three elements: stimuli (i.e., different types of DNA damage), a signal transduction machinery, and its targets (i.e., different basal cell cycle regulators). The DNA damage checkpoint[5,6] can be regarded as a coherent signal transduction system which allows cells to transfer the information from a DNA lesion to the cell cycle. This signal affects at least three stages in the cell cycle: the G_1/S transition, S phase progression and the G_2/M boundary. Although there are many points of cell cycle arrest, the DNA damage surveillance system can be regarded as one checkpoint because all forms of arrest are signaled by DNA damage, often simultaneously, and many genes are involved in more than one stage of the arrest.

In this Chapter, we focus primarily on the main signal transduction pathways and targets and describe recent concepts, as to how mammalian cells arrest the cell cycle after DNA damage, and to what extent some of these scenarios might be involved in the generation of cancer.

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Origins of DNA Damage

DNA damage could broadly be understood as any major change in the basic structure and function of the DNA double helix that needs repair. Although any of the polynucleotide structures can be damaged, the genomic DNA is of particular interest due to heritability of such changes.

DNA damage can originate from internal or external causes. Damage from internal sources may arise from biochemical products and replication errors, which occur during cellular metabolism and division. External causes include irradiation and mutagenic chemicals. Importantly, different types of DNA damage can occur during different cell cycle phases, e.g., oxidative damage mostly accumulates in G_1, incomplete replication or nucleotide misincorporation occurs in S phase, and cells in mitosis may be prone to chromosome breakage during chromatid segregation.

DNA Damage of Intrinsic Origin

Oxygen and its metabolic products, like reactive oxygen species, are one major source of intrinsic DNA damage. The high reactivity of DNA as a biomolecule renders it particularly susceptible to this process. A mammalian genome may undergo over 10^5 modifications a day,[7] and it is estimated that mitochondrial DNA is oxidized at the frequency of 1 base per 100kb.[8] Certain physiological processes may also constitute a form of DNA damage. Recombination during VDJ immunoglobulin synthesis or meiosis, and the DNA replication process by itself incorporate steps of DNA breakage, which can be considered as DNA damage.[9]

DNA Damage of External Origin

DNA damaging agents may interfere with DNA structure and function in various ways, some of which are still incompletely understood. The type, extent, and outcome of these types of damage depend on the dose and duration of the damaging stimuli, as well as the sensitivity of a cell. For example irradiation of cells by ultraviolet (UV) or ionizing radiation (IR) may cause cross-links, double strand breaks, or may just knock off sub-atomic particles to generate radicals in the DNA structure. Isolated nucleotide crystals have been irradiated to induce free radical formation on both base and sugar moieties,[10] indicating that the damage caused by radiation can affect both the bases and the backbone of the DNA. Moreover, clustered damage during IR resulted from both low and high radiation doses,[11] implying that the outcome depends on the type of radiation rather than on its dose. Evidence for the variability of cellular response to the type of irradiation stems mostly from the analysis of ataxia telangiectasia (AT) cells which are exquisitely sensitive to IR but not UVR (see below).[12,13]

Chemicals may interfere with DNA integrity and replication fidelity by reacting with atoms in the DNA molecule and by modifying nucleotides, thereby distorting the normal base-pairing pattern. Similar to the damage by IR, the nature and hence the response to DNA damage induced by chemical agents is variable. Multiple chemical agents that are known to be mutagenic/carcinogenic or to damage DNA are used for the induction of (programmed) cell death, e.g., in anticancer therapy. Given the variability of DNA damaging agents, mechanisms of damage, and their cellular responses it is necessary to consider DNA damage not as a single entity, but a whole spectrum of insults to a cell which in turn may react with a spectrum of cellular responses.

Upstream DNA Damage Signaling

In response to DNA damage, especially IR and UVR, two phosphatidyl-inositol kinase (PIK)-related proteins, ataxia telangiectasia mutated (ATM) and ATM-rad3-related (ATR), play an important role. They are characterized by similar structure and have sequence homologies to PI3K; however, they are not lipid but protein kinases. After DNA damage, they function as the most proximal signal transducers. Although ATR appears to be particularly important in the cellular response to UVR, it also participates in other DNA damage pathways, since

cells expressing a dominant-negative ATR are sensitive to many forms of DNA damage.[14] So far, no human disease is known to involve exclusively ATR, and $ATR^{-/-}$ mice are not viable.[15] Therefore less is known about ATR function, but available data collectively indicates that ATR operates in pathways parallel to ATM. Here, we focus on signalling of ATM and its downstream targets, CHK2 and p53 (Fig. 1).

ATM-Dependent Signaling Pathways

The ATM gene was found to be inactivated in the autosomal recessive disorder AT.[16] AT is characterized by diverse symptoms, such as cerebellar degeneration, oculocutaneous telangiectasia, immunodeficiency, hypogonadism, chromosomal instability, premature aging, cancer predisposition, extreme IR sensitivity, and cell cycle abnormalities.[17,18] Therefore, ATM must have multiple cellular functions. In the majority of AT patients ATM is inactivated by mutations. The product of the ATM gene is a 370 kD phosphoprotein of variable cellular localization.

Most notably, ATM is involved in the regulation of cellular responses to IR, while it appears to be less important for signaling after UVR, damage by alkylating agents, or intrinsic damage. It appears that ultimately the vast majority of cellular responses to DNA double strand breaks (of different origin, including IR, radiomimetic agents, or topoisomerase inhibitors) depends on intact ATM. ATM is a serine/threonine protein kinase whose phosphorylation targets include p53, MDM2, CHK2, NBS1, and BRCA1. The functional interaction with p53 (see below) is intriguing due to p53's well known and universal relevance in DNA damage scenarios. The observation that some of these targets are still phosphorylated in IR-treated AT cells, albeit with delayed kinetics, points to the existence of additional pathways responding to IR.

Importantly, features of $ATM^{-/-}$ mice closely resemble the human disease.[19] The mice are viable and show growth retardation and infertility. This further shows that ATM also functions in development since double strand breaks may occur as a part of physiological processes.[20]

Cells derived from AT patients are defective in G_1, S and G_2 arrest following IR.[21,22] The ATM kinase activity increases after IR by mechanisms poorly understood. Probably double strand breaks induced by IR damage either directly or indirectly signal to ATM. Extensive studies have been carried out on the mechanisms by which ATM might transduce signals from damaged DNA to other cellular partners.

The exact nature of the exquisite radiosensitivity of AT cells is debated. While there is no solid evidence for a deficient double strand break repair in AT cells, there is evidence that defects in cell cycle checkpoints are responsible for this radiosensitivity. However, several arguments point to an opposite functional relationship:[23,24] Radiosensitivity and cell cycle defects are separable phenotypes, and there is no correlation between radiosensitivity and increased apoptosis induced by IR in AT cells. Finally, even under experimental conditions with additional time for repair of DNA damage, the survival of AT cells is not increased. Instead, it is assumed that subtle alterations in double strand break rejoining activity and chromatin, or other cellular activities of ATM, such as induction of NF-κB, could be involved in the radiosensitivity.[13,25]

CHK2—The Next Line of Defense

CHK2 is a protein kinase which is activated, by post-translational modifications, after DNA damage. CHK2 is phosphorylated in an ATM-dependent manner after IR and in an ATM-independent manner after UVR or stalled replication (Fig. 1). Another homolog, CHK1, is phosphorylated after DNA damage in an ATR-dependent manner, but its precise role in mediating downstream signals remaines elusive. Therefore, here we will discuss only CHK2 activities and refer to these in the context of the G_1/S and G_2/M checkpoints (see below). The best known targets identified in vitro are p53 and CDC25C; however, the in vivo relevance of the mechanisms involved in the DNA damage reponse remains to be shown.

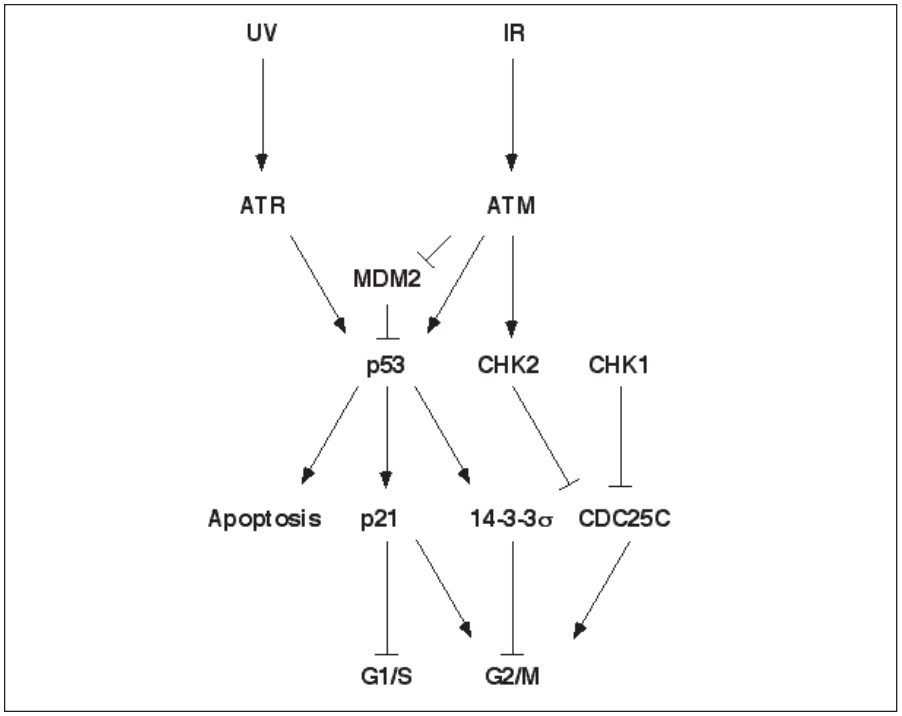

Fig. 1. Simplified model of the core cell cycle pathways induced by DNA damage, such as UVR or IR.

p53—The Core of the DNA Damage Pathways

The p53 tumor suppressor protein is a transcription factor with "sensor functions" integrating signals from the external and internal environment.[26,27] Biochemically, p53 is a sequence-specific transcriptional activator and a nonspecific transcriptional repressor. It has been known for years that the half-life of the otherwise short-lived p53 is increased several fold in cells treated with DNA-damaging reagents.[28-30] This correlates well with cellular responses such as cell cycle arrest or apoptosis, depending on the particular cell type and damaging agent.[26,31] However, it is still poorly understood why one cell type undergoes cell cycle arrest whereas another cell type dies by apoptosis.

There are currently two models that try to explain these phenomena.[32] According to one model, activated p53 always sends the same signals of cell cycle arrest and apoptosis, but apoptosis occurs only if other pathways, e.g., survival or oncogenic pathways, either augment or impede apoptosis, possibly incorporating also p53-independent signals. The other model suggests that the choice lies mainly within the activities of p53 itself, either determined by p53 protein abundance-low protein level leading to cell cycle arrest, high protein level leading to apoptosis- or specific posttranslational modifications. These models are not mutually exclusive. In both cases p53 is likely regulated by other cellular partners in a specific manner to tightly control its possibly deadly effect on the cell.

Thus, *p53* is a central DNA damage checkpoint gene and is clearly required for a complete DNA damage response. However, cells with mutant p53 grow normally and $p53^{-/-}$ mice are normal, indicating that p53 is not essential for cell cycle progression.[33] Importantly, the fidelity of mitotic chromosome transmission is reduced in p53 mutant cells. Furthermore, the rate of gene amplification is several orders of magnitude higher as compared to p53 wild-type

cells,[34] and $p53^{-/-}$ mice exhibit an abnormally high frequency of spontaneous cancers. As with the other checkpoint genes in this pathway, such as the downstream genes p21 and 14-3-3σ,[35] this clearly points to subtle defects accumulating in mutant cells, even in the absence of external DNA damage, which likely contribute to genetic instability. If an additional disturbance intervenes, even if not primarily inducing DNA damage, a defect phenotype may then be obvious.

Regulatory Effects Converging on p53

Multiple, mainly posttranslational, mechanisms regulate p53 activity. The best characterized mechanisms include the regulation of p53 protein half-life, phosphorylation, and acetylation events, all of which contribute to influence p53 stability.[36] The stability can also be regulated by the interaction with cellular partners of p53.

The MDM2 oncoprotein is a crucial player in the regulation of p53 by targeting p53 for degradation in the ubiquitin-dependent proteosome pathway.[37-39] Central to this degradation process is the direct binding of MDM2 to p53. In addition, MDM2 is also able to directly inhibit the transactivational activity of p53.[40-42] Since MDM2 is transactivated by p53[39] this constitutes a tightly regulated feedback loop between both partners which likely reflects a well-balanced equilibrium.

The importance of the MDM2/p53 interaction in the regulation of the p53 protein levels and functions is also evident from the fact that different pathways involve MDM2 or the MDM2/p53 complex. A product from the INK4a/ARF tumor suppressor gene locus, $p14^{ARF}$, is a cell cycle inhibitor[43] whose functions are, at least in part, p53-dependent. Upon oncogenic signals such as c-MYC and E1A,[44,45] ARF binds to MDM2 and inhibits the ability of MDM2 to promote p53 degradation, thus increasing its half-life and transactivation of p53-responsible genes.[46-49] It is open as to what extent ARF might also contribute to DNA-damage responses.[50] Recently, ARF was shown to be implicated in p53-dependent cell cycle arrest after IR exposure and mictrotubular disruption, while it did not affect arrest after ribonucleotide depletion or actinomycin D treatment.[51]

p53 is also regulated by its phosphorylation and acetylation. This includes the phosphorylation of serine 6/9,[52] serine 15,[53] serine 20,[54] serine 33,[55] serine 37,[56] serine 315,[57] serine 378,[58] serine 392,[59] as well as acetylations at lysine 382 and 320.[60] The appearance of so many regulatory phosphorylation/acetylation sites on p53 suggest a network of different signals integrating into p53.[36] Indeed many kinases have been connected to specific phosphorylation events on the p53 protein, including CKI, CAK, CDK2, CDK1, pCAF, PKC, and p300.

IR leads to the phosphorylation of serines 15/20, both of which have been shown to negatively influence the binding of MDM2 to p53 leading to stabilization of p53.[53,61,62] Serine 15 is phosphorylated following IR by ATM placing p53 in an IR-induced DNA damage pathway downstream of ATM (Fig. 1). ATR is also able to phosphorylate serine 15. However, this occurs following UVR and not IR exposure.[14] In contrast, serine 20 is phosphorylated following IR in an ATM-dependent manner, not directly by ATM but indirectly through the CHK2 kinase.[63] ATM is able to phosphorylate CHK2, which in turn phosphorylates serine 20 on p53 leading to stabilization of p53.[63-65] In addition, the activation of CHK2 by ATM-mediated phosphorylation leads to phosphorylation of CDC25C (see below).

Many phosphorylation/acetylation events affect the carboxy-terminal end of p53 which is implicated in the regulation of the DNA binding capacities of p53. Phosphorylations at carboxyterminal amino acids, binding with a specific antibody directed against the carboxy terminus, and acetylations at lysine 320/382 have been shown to lead to an increased specific DNA-binding function of p53.[60,66,67] It appears, that many phosphorylation/acetylation events of p53 reported in the last years have a predominantly cooperating effect in regulating p53 stability and transcriptional activity.[68,69]

The G1/S Checkpoint

At the G_1/S boundary the prerequisites for continuation with DNA replication are checked. DNA damage leads to arrest in G_1 via p53-dependent transactivation of genes, primarily p21. Importantly, even in the absence of extrinsic DNA damage, unregulated S phase entry can lead to genetic instability or apoptosis. The exact nature of DNA damage associated with unregulated progression into S phase is unknown. Examples include experiments with overexpressed G_1 cyclins.[70-72] Such cells enter S phase prematurely, after a shortened G_1, exhibit genetic instability,[73] and an enhanced dependence on checkpoint functions for survival.[9] This might be explained by DNA damage occurring before S phase that cannot be repaired during replication. On the other hand, premature entry into S phase could be an additional cause of DNA damage, e.g., either due to failure to complete replication before entry into mitosis, or due to abnormal nucleotide pools as a result of shortened G_1. The importance of maintaining sufficient nucleotide pools before entering S phase is reflected in the fact that cells presumably have a mechanism to monitor nucleotide pools and arrest in a p53-dependent manner if these pools are insufficient.[74]

$p21^{CIP1}$—A Two-Tailed Cell Cycle Regulator

Among the transcriptional targets of p53, the CDK inhibitor $p21^{CIP1}$ plays a key role in mediating G_1 arrest. Cells lacking p21 have a defect in the DNA damage-induced G_1/S arrest.[75,76] The inhibition of CDK activities by CDK inhibitors constitutes a powerful cell cycle control mechanism and provides an important link to other signaling pathways during proliferation, differentiation, and senescence.[77] The CIP/KIP family members $p21^{CIP1}$, $p27^{KIP1}$ and $p57^{KIP2}$ share broad specificity for binding to and inhibition of most CDK/cyclin complexes through conserved motifs for CDK and cyclin Binding in the amino termini of the inhibitors,[78-80] but only p21 is directly involved in the DNA damage-induced cell cycle arrest. The identification of similar cyclin Binding motifs in several unrelated proteins, which may function as inhibitors, substrates, or associated regulators of CDK/cyclin complexes, stresses the importance of these motives for various cell cycle-related activities.[81] The CIP/KIP inhibitors can also function as adaptors to promote CDK/cyclin complex assembly.[82]

p21 is distinguished from the other CDK inhibitors by several unique features (Fig. 2). The carboxy terminus of p21 binds to proliferating cell nuclear antigen (PCNA) and thereby inhibits PCNA-dependent DNA replication but not PCNA-dependent nucleotide excision repair.[83,84] A second cyclin-binding motif overlaps with the PCNA binding motif and can inhibit CDK activity in vitro[79,80,85] and induce G_1 arrest in vivo,[85] though its precise role in modulating the cell cycle is unclear. This indicates that inhibition of PCNA is not the sole activity of the p21 carboxy terminus that is required for the cell cycle arrest.[86] Inactivation of the p21 inhibitory activities could occur by several mechanisms,[81] which might be involved in tumorigenesis.[87] The carboxy-terminal nuclear localization signal in p21 also serves to target CDK/cyclin complexes to the nucleus. We have further proposed that proteins which bind to the carboxy terminus of p21 could also target p21/CDK/cyclin complexes to other subcellular locations or substrates.[81]

Given the critical role p21 plays in coordinating cell cycle and G1 transition, DNA replication, terminal differentiation and senescence, it was surprising that its elimination did not result in a tumorigenic phenotype in mice.[88] Furthermore, mutations in p21 are rarely found in human tumors. Perhaps the positive requirement for p21 in CDK/cyclin Assembly or other cell cycle-related functions precludes mutations. Also, other proteins that modulate p21 activity may be mutated in cancer.

Recently, a role for p21 in the G_2/M phase of the cell cycle was identified;[89-92] however, the mechanisms underlying this function remaine elusive. Though supraphysiological levels of p21 can inhibit the activity of CDK1/cyclin B1 complexes in vitro,[85] it is doubtful that this is the primary mechansim by which p21 regulates G_2/M. Disruption of the p21/p53 pathway has been associated with mitotic spindle pole defects and the appearance of multiple centrosomes,[93,94] and

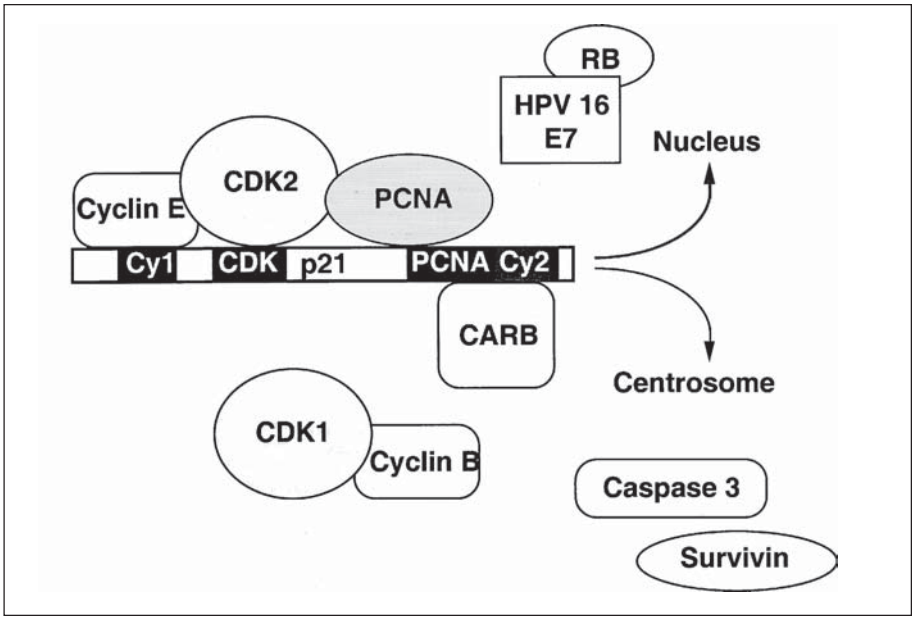

Fig. 2. Schematic model of the p21 protein and its key functional activities. Apparently, p21 can localize either to the nuclear or the centrosomal compartment.

$p21^{-/-}$ cells showed S/M uncoupling with subsequent polyploidy after treatment with DNA damaging agents.[95] In the absence of any direct mechanistic link to the mitotic spindle poles and centrosome, recent evidence has suggested that these abnormalities may arise as a result of alterations in CDK2/cyclin E activity at the time of centrosome duplication in S phase.[96] A recent model further illuminating the function of p21 in G_2/M is discussed below.

The G_2/M Checkpoint

The G_2/M checkpoint pathways converge mainly on activity and intracellular localization of the CDK1/cyclin B1 complex. Activation of this complex is essential for cells to enter mitosis. Just prior to mitosis, CDK1/cyclin B1 complexes translocate to the nucleus and trigger the initiation of mitotic changes like chromosome condensation and nuclear membrane breakdown.

Control of the Unperturbed G_2/M Transition

The entry into mitosis is regulated by the spatial distribution of the CDK1 kinase complex: during interphase, CDK1 is sequestered in the cytoplasm.[97] Also, cyclin B1 is predominantly cytoplasmic due to CRM-1-mediated export of cyclin B1 out of the nucleus.[98,99] The cytosolic retention of the complex is also complemented by the inactivating phosphorylation of CDK1 (see below).

The activation of the CDK1/cyclin B1 complex requires that the inhibitory phosphorylations at threonine 14 and tyrosine 15 of CDK1 are removed by a phosphatase. The known phosphatase for this activating dephosphorylation is CDC25C, while the inhibitory phosphorylation is performed by the kinases Weel[100] and myt1.[101] Activation of CDK1 by dephosphorylation is associated with entry of cells into mitosis and is accompanied by chromosome condensation and nuclear membrane breakdown. Some reports indicate, however, that early nuclear mitotic events take place before CDK1/cyclin B1 complexes are intranuclear.[102]

CARB (CIP1-associated regulator of cyclin B1), a protein functioning to regulate cyclin B1 in a p21-dependent manner, has also been recently suggested to contribute to the cytosolic

retention of cyclin B1 proximal to the centrosome.[103] Cyclin B1 is subject to tight control of subcellular localization.[92,99,104,105] Sequestration of cyclin B1 to centrosomal CARB may form a storage site for cyclin B1 from which it can be rapidly mobilized, or may protect cyclin B1 from proteolytic degradation. Consistent for a role for p21 in CARB regulation, the cyclin B1-CARB association is potently inhibited by the carboxy terminus of p21, suggesting that free p21 is able to dissociate this complex under certain conditions. Since CARB is found associated with either p21 or cyclin B1 but not with both, it seems unlikely that the association of CARB and cyclin B1 is mediated by p21.[103] CARB sequesters cyclin B1 in the cytoplasm close to the nuclear membrane, and thus may regulate the availability of cyclin B1 for nuclear import (Fig. 2 and 3). A similar suggestion that cyclin B1 is retained in the cytoplasm by an active mechanism has been proposed previously[106] but a candidate molecule was not identified. Sequestration by CARB complements mechanisms for the CDK1/cyclin B1 regulation. CARB-dependent regulation of cyclin B1 bioavailability may regulate the onset of prophase, since availability of p21 for CARB/cyclin B1 complex disruption could only occur if p21 was not required for nucleotide excision repair or affecting other growth arrest stimuli.

This suggests that the p21 carboxy terminus can act in a manner to promote release of cyclin B1 from CARB. This function is distinct from the potent inhibitory activities of p21, and one interpretation of the data to date would be that p21 actually promotes the progression into M phase by increasing cyclin B1 bioavailability. As a consequence, the evolution of such bifunctionality would confer no selective advantage to cells which completely eliminate p21 since under DNA damaged conditions they would be able to abrogate the G_1/S damage checkpoint but be unable to efficiently initiate mitosis. Such a possibility may explain the lack of sporadic tumor formation in $p21^{-/-}$ mice and the very low frequency of p21 inactivations in human cancers.[107,108]

Regulation of the CDC25C Phosphatase

Although the regulation of CDK1 by CDC25C phosphatase is well established,[109-111] the dynamics and regulation of CDC25C remain to be clearly shown. CDC25C can be phosphorylated at two major sites, at serine 216 and at its uncharacterized amino-terminal end. Serine 216, which appears to be the major regulatory site, is phosphorylated by CHK1 and CHK2.[64,111,112] C-TAK1 is another kinase that has been shown to phosphorylate serine 216 in vitro,[113] although the relevance of this event in vivo is unknown. The amino terminus is phosphorylated by CDK1[114] and the polo-like kinase Plk.[115] Whereas serine 216 phosphorylation inhibits CDC25C phosphtase activity, aminoterminal phosphorylation enhances the activity.

Serine 216 of CDC25C is also a regulatory residue which upon phosphorylation dictates the localization of the protein (Fig. 3). Phosphorylation of this residue, as it occurs in interphase or after DNA damage, may regulate its subcellular localization by creating a 14-3-3 binding site.[113,116] The binding to 14-3-3 proteins prevents the nuclear import of CDC25C[117] and hence prevents the G_2/M transition. On the other hand, in a feedback loop, CDK1 phosphorylates the activating amino-terminal region of CDC25C during mitotic progression, thereby amplifying its own activation.[118] The role of CDC25C phosphorylation by Plk in vivo is not known.

DNA Damage and the G_2/M Transition

CHK1, CHK2, and ultimately p53 are phosphorylated upon DNA damage. In addition to phosphorylating p53, both CHK1 and CHK2 also directly phosphorylate CDC25C at serine 216, leading to its inactivation. This inactivation could lead to a p53-independent G2 arrest by blocking CDK1 activation.[119] Since serine 216-phosphorylated CDC25C binds to 14-3-3 proteins, remains cytoplasmic,[116,117] and is probably unable to activate CDK1, the exit from G_2 and hence entry into mitosis is prevented after DNA damage (Fig. 3). This implies that the DNA damage-induced G_2 arrest is doubly secured through p53-dependent and -independent mechanisms, by which CDK1 and CDC25C are sequestered by 14-3-3 proteins and kept inactive due to sustained phosphorylation.[97,116,117]

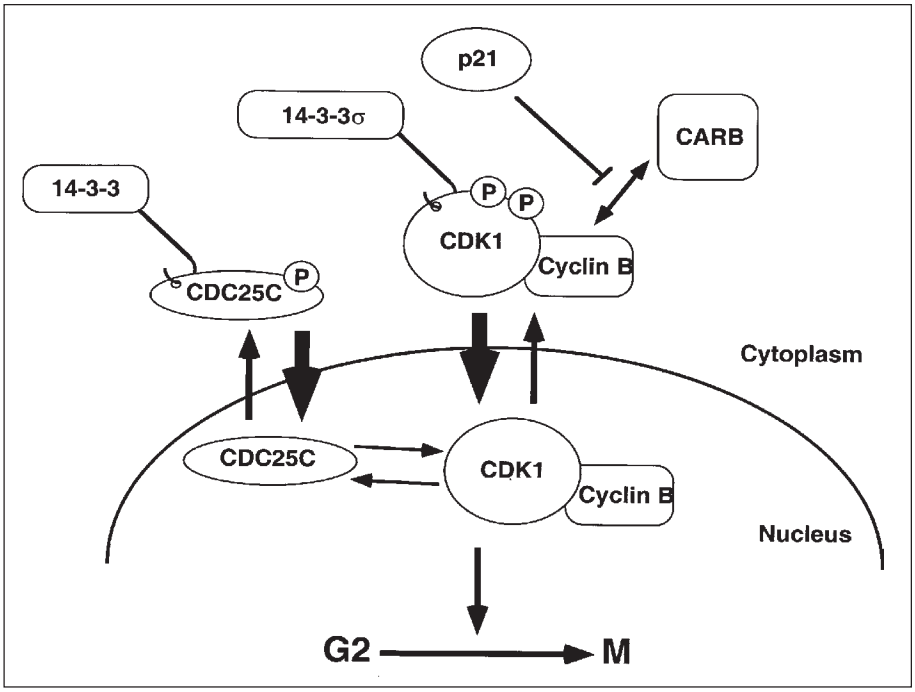

Fig. 3. Simplified model of the G2/M transition and the key proteins involved in the regulation of the CDK1/cyclin B1 complex.

14-3-3σ is upregulated in a p53-dependent manner and is involved in the G_2 checkpoint arrest.[120] Cells lacking 14-3-3σ are defective in G_2 arrest and undergo mitotic catastrophe upon DNA damage.[97] Cells lacking both 14-3-3σ and p21 are even more sensitive to DNA damage[35] indicating the cooperative role of these inhibitors in the G_2 arrest. However, some evidences also indicate a positive or negative role of p21 in G_2/M (see above). The nuclear accumulation of p21 at the onset of mitosis,[89] as well as the regulation of the CARB-cyclin B1 interaction by p21,[103] hint at the positive regulation of p21 during G_2/M. On the other hand the observation that p21 inhibits phosphorylation of CDK1 at threonine161[121] contributing to the G_2 arrest, indicates a negative role for p21 at G_2/M. Further work is needed to clarify the contribution of each of these mechanisms to G_2 arrest. Finally, it is debated, to what extent the upregulation of apoptotic regulators, such as BAX,[122] may favor an apoptotic outcome, even in cells overcoming G_2 arrest.

Links to Cancer and Genetic Instability

Loss of cell cycle checkpoint control has emerged as a central cause of genetic instability.[3,6] Consequently, chances that these unstable cells progress to cancer are increased. This notion has several important implications:

1. Since checkpoints may determine the ultimate response (arrest vs. apoptosis), the integrity of these checkpoints influences the susceptibility of cells to DNA damage. This is relevant either to the cells' fate after accumulation of undesired DNA damage or to the cells' sensitivity to desired damage during chemo-/radiotherapy.
2. Exploring the early checkpoint defects in cancerous or precancerous lesions may serve as a prognostic or, in certain tissues, as an additional diagnostic marker.

3. Furthermore, known defects of pivotal checkpoint genes may help to predict treatment outcome or to design more specific therapeutic strategies. In addition, checkpoint components which are defective in certain cancer cells may be targeted during therapy to enhance the anti-tumor effect, e.g., by preventing arrest and/or by forcing cells into apoptosis. Work is in progress to develop novel therapeutic strategies with an increased therapeutic index.
4. Moreover, strategies could be considered to restore missing or dysfunctional checkpoints in order to provide additional time for DNA repair and delay the onset of cancer.
5. Finally, since some of the components that are involved in the DNA damage checkpoint are also involved in other cellular regulatory activities, e.g., during senescence, differentiation, or certain immunological responses, this could lead to cross-signalling into other pathways and might permit new strategies to influence related cellular functions.

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CHAPTER 5

DNA-Damage-Independent Checkpoints from Yeast to Man

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Introduction

Checkpoints are mechanisms that establish dependence relationships between biochemically unrelated cellular processes. The temporal order of many critical cell cycle events must be strictly maintained to ensure cell survival and integrity. A simple example is that of genome duplication which must be completed before cell division. The relationship between these processes is controlled by the S-phase checkpoint. After S-phase, the Topoisomerase II-dependent checkpoint ensures that the topology of the newly replicated DNA has been correctly organized before cells begin mitosis. During mitosis itself, distinct checkpoints monitor mitotic spindle assembly, preventing the onset of chromosome segregation until all the chromosomes are correctly aligned on the mitotic spindle, and prevent exit from mitosis until anaphase chromosome segregation has been completed. In this Chapter, we discuss these checkpoint control systems (Fig. 1).

An elegant way to define checkpoint pathways has been by analysis of loss-of-function mutants in the genetically manipulable yeast systems. For example, the S-phase checkpoint was described in budding yeast by the isolation of mutants that initiate mitosis despite a replication block enforced by the ribonucleotide reductase inhibitor hydroxyurea (HU). Other checkpoint systems were originally described in mammalian cells. Indeed, the existence of checkpoint controls had been inferred from mammalian cell-fusion studies earlier than the genetic analyses performed in yeast (Fig. 2).[1] More recently, mammalian checkpoints have been investigated by demonstrating that some checkpoint arrests can be overridden by drugs such as caffeine.[2-9] Caffeine inhibits the kinase ATM, a key component of eukaryotic checkpoint pathways.[10-13] Although mammalian cells are less amenable to genetic studies than are yeast, they have proven to be important for the study of checkpoint biology. Our descriptions of checkpoint controls use a commonly adopted format which divides the pathways into (1) the sensor, (2) the transducer and (3) the target. Checkpoints do not necessarily follow simple linear pathways, however. Many of the sensor and transducer components are likely to be assembled into large complexes. Still, this nomenclature allows for a framework to be drawn up, on which the details can be built. The sensor components are those that monitor completion of the relevant process, for example DNA replication. The transducer transmits the signal from the sensor to the target of the checkpoint. It is the activity of the target that controls cell cycle progression.

Budding Yeast versus Higher Eukaryotes

Since checkpoint pathways in both budding yeast and higher eukaryotes will be discussed in this Chapter, it is important to describe a fundamental difference in budding yeast cell cycle organization that sets it apart from other species. In budding yeast, checkpoints promote the activity of anaphase inhibitors, whereas in most eukaryotes, checkpoints inhibit the activity of

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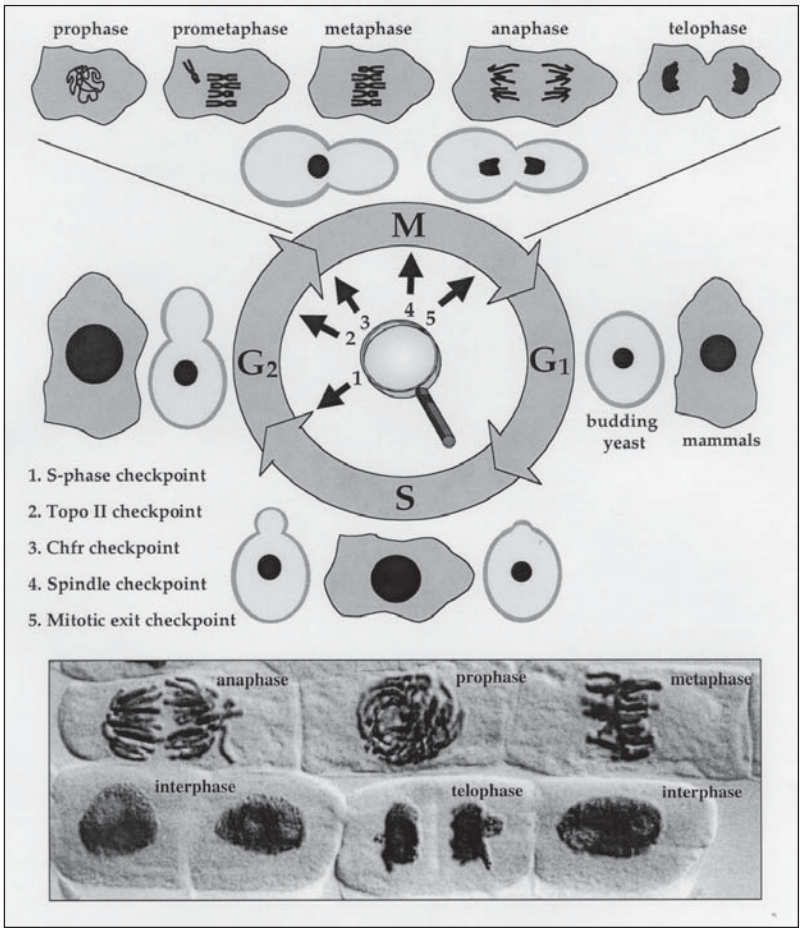

Fig. 1. DNA-damage-independent checkpoints. A summary of the five checkpoints that are discussed in detail (see magnifying glass). In mammals the G2/M transition is regulated by at least 2 non-DNA damage checkpoint pathways (1 and 2). These prevent the initiation of mitosis until DNA replication is complete (1, S-phase checkpoint), and until DNA is sufficiently decatenated (2, Topoisomerase II-dependent checkpoint). The Chfr checkpoint (3) appears to restrict chromosome condensation when spindle assembly is perturbed. Also within mitosis the spindle assembly checkpoint (4) delays the onset of anaphase until all the chromosomes of the karyotype have been correctly arranged on the mitotic spindle. In budding yeast, similar checkpoint controls inhibit the onset of anaphase rather than preventing passage beyond the G2/M transition. Therefore the G2/M transition in mammals and the metaphase-anaphase transition in budding yeast are somewhat analogous. Indeed, sensor and signaling components of these checkpoint pathways are conserved. However, the checkpoint targets differ between mammals and budding yeast, and the topoisomerase II-dependent checkpoint is not functional in budding yeast. Exit from mitosis (5) is also under checkpoint control, to ensure that anaphase has been completed before cell division. Photomicrographs of onion root meristematic cells depict the mitotic stages. The cartoons compare cell cycle stages in budding yeast and mammals.

the mitotic kinase (cyclin/CDK), required for passage through the G2/M transition. A need for distinct modes of control is related to differences in the spindle assembly pathway. In many eukaryotes, including mammals, the mitotic spindle does not assemble until mitosis. However,

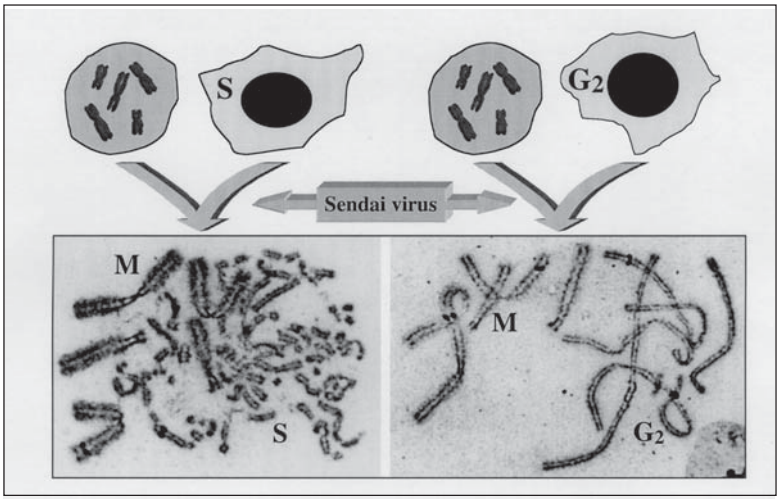

Fig. 2. Premature chromosome condensation (PCC). Interphase cells can be fused with mitotic cells by Sendai virus treatment. This induces PCC, suggesting that interphase checkpoints might normally inhibit the onset of chromosome condensation. Photomicrographs show fusions of S—phase/G2 with M—phase *Muntiacus muntjak* cells.

budding yeast spindles assemble during S-phase; checkpoints must inhibit spindle elongation even while DNA is being replicated. In addition, sister chromatid cohesion, established during DNA replication, must be maintained until the onset of anaphase. In budding yeast, an inhibitor of anaphase, Pds1, can perform both of these tasks.[14-18] Before anaphase, Pds1 binds to protease Esp1 and thereby inhibits the anaphase-promoting activity of Esp1.[18] During an unperturbed cell cycle, Pds1 becomes poly-ubiquitinated at the metaphase to anaphase transition by a multi-subunit enzyme complex known as the APC (Anaphase Promoting Complex); the modified forms are recognized and degraded by 26S proteasomes.[16] Once released from Pds1, Esp1 induces cleavage of Scc1, a cohesin required to maintain cohesion between sister chromatids.[18-21] Concurrently with loss of sister cohesion, Esp1 induces spindle elongation.[22] Not surprisingly, Pds1 is a major target of checkpoints controlling anaphase onset. Vertebrate proteins named securins, that are at least partial functional homologues of Pds1, have been identified,[23] making the study of checkpoints in budding yeast highly relevant. Moreover, budding yeast and higher eukaryotes employ a common strategy for controlling exit from mitosis. In this case, regulation of cyclin/CDK activity appears to be a universally adopted mode of control.

S-Phase Checkpoint

The S-phase checkpoint ensures that the onset of mitosis is dependent on the completion of DNA replication.[24-26] Since little is known about S-phase checkpoint control in mammals, the components of this pathway in budding yeast will be described (Fig. 3). As mentioned above, budding yeast cells initiate DNA replication and mitotic spindle formation at a common cell cycle point, early in S-phase. To prevent the generation of aneuploid daughter cells that are inviable, it is essential that the mitotic spindle does not elongate before DNA replication has been completed. The order of these two processes is normally maintained by a timing mechanism rather than a checkpoint control: DNA replication takes only 20-30 minutes and spindle formation takes around 60 minutes; thus, spindle assembly is not completed before genome replication. This example illustrates how the temporal order of two events can be maintained independently of checkpoint controls, i.e., if the processes have a common starting point and each require a differing minimum amount of time for their completion.

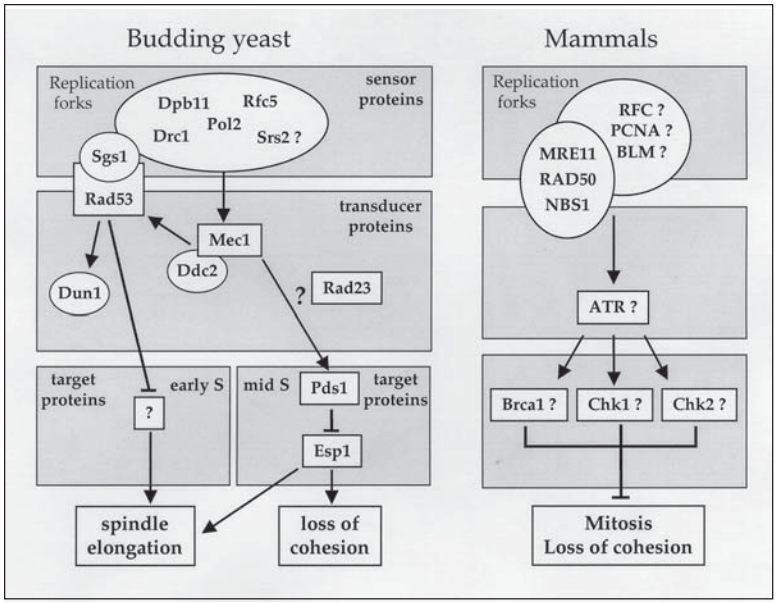

Fig. 3. S-phase checkpoint. Linear pathways are drawn for simplicity though more complex interactions between the checkpoint components are likely. In budding yeast, a signal generated by replication forks is transmitted by kinases Mec1 and Rad53. Rad53 activation is dependent on the Mec1-Ddc2 complex. Downstream of Rad53 and Mec1, sequential pathways operate: a Mec1-Rad53 pathway is required early in S phase, and a Mec1-Pds1 pathway is required part way through S phase. The Mec1-Rad53 pathway also induces a Dun1-dependent transcriptional response which protects cells from replicative stress. The mammalian pathway drawn on the right is speculative, based mainly on HU-induced phosphorylation events and HU-induced localization of proteins at replication foci.

A checkpoint pathway does exist, however, to ensure that the dependence between spindle elongation and DNA replication is always maintained. If DNA replication is inhibited with HU,[27] the cells arrest with fully assembled short G2 spindles. After removal of the HU, spindle elongation is delayed until replication is complete. The S-phase checkpoint does not only control the mitotic spindle, however. All eukaryotes establish sister cohesion during DNA replication, and it must be maintained until the onset of anaphase.[20,21] Maintenance of sister chromatid cohesion is of great importance to mammals and yeast alike, and is a function of the S-phase checkpoint. At least in yeast, cohesion is established at some loci very early in S-phase and must therefore be maintained for the remainder of the S-phase period as well as during G2 and until the moment of anaphase onset. The homologs of yeast S-phase checkpoint components are therefore likely to be important regulators of mammalian sister chromatid cohesion.

To define the budding yeast S-phase checkpoint, loss-of-function mutations causing sensitivity to HU were identified. The proteins encoded by these genes were determined to have S-phase checkpoint functions by showing that the loss-of-function mutations allowed entry into mitosis when DNA replication was blocked with HU. Thus the S-phase checkpoint is defined as that which restrains entry into mitosis when replication is blocked. However, kinetic analyses of various checkpoint mutants, grown in the presence of a concentration of HU that allows replication to proceed, but more slowly than in an unperturbed cell cycle, have revealed genetically distinct S-phase checkpoint systems (see below).

To monitor ongoing DNA replication, cells seem to have replication sensors that reside at replication forks. In budding yeast, the putative sensor components include Pol2, Rfc5, Dpb11, Drc1 and Sgs1 (Refs. 28-32). POL2 encodes the replicative DNA polymerase, Polε and Rfc5 is a replication factor C subunit involved in recruiting polymerases to replication forks. Dpb11 is

also required for DNA replication; it can bind to Polε and is thought to help recruit Polα-primase complexes to ARS sequences at replication origins.[33] Dpb11 also binds to Drc1, which is itself essential for DNA replication.[31] Together with Srs2, Sgs1 has a redundant but essential role in DNA replication.[34]

Evidently, the sensor proteins also play important roles in DNA replication itself, and with hindsight it might seem elementary that components of the replication fork machinery are involved in generating the checkpoint signal. For each of these components, it was important to know that their checkpoint functions could be distinguished from their roles in DNA replication. This is the case because the S-phase checkpoint cannot be activated until DNA replication has begun,[35] a fact illustrated by the phenotype of cells carrying a heat-inducible cdc45 degron mutant.[36] Cdc45 binds to replication origins before S-phase in budding yeast and is required for origin firing. The cdc45 degron mutant is rapidly degraded at the restrictive temperature. When degradation was induced in G1 of the cell cycle, replication origins could not fire and the cells entered mitosis without replicating any DNA. When the temperature shift was performed within S-phase, DNA replication was immediately inhibited because Cdc45 is also needed for elongation of replication forks, but in this case mitotic progression was inhibited. Thus, the S-phase checkpoint signal requires the presence of replication forks that have fired, and Cdc45 is not a component of the checkpoint response.

Mec1 and Rad53 kinases are traditionally described as components of the signal transduction element of the S-phase checkpoint.[24,25,37] When replication is perturbed, these checkpoint kinases are activated in a manner dependent on the sensor components. Exactly how Rad53 and Mec1 activation occurs is not known, but several physical interactions have been identified that may represent key steps. Sgs1 was found to co-localize with Rad53 in discrete nuclear foci during S-phase. Intriguingly, Sgs1 is reported to interact with the FHA domain of Rad53,[38] the domain required for the formation of the Rad53-Rad9 complex, required for DNA damage checkpoint signaling.[39] Rad53-Sgs1 association may have revealed an Sgs1-dependent loading of Rad53 onto specific chromatin regions that might be involved in monitoring replication. That Sgs1 is involved in S-phase checkpoint control,[32] adds weight to this model. But, the S-phase checkpoint defect of *sgs1* mutants is rather weak, not nearly as substantial as other S-phase checkpoint mutants. This suggests that Sgs1 has a redundant checkpoint function, perhaps with another helicase such as Srs2.

Another activator of signal transduction may be Ddc2 (also known as Lcd1), a component that physically associates with Mec1 and is phosphorylated by Mec1.[40,41] Phosphorylated Ddc2 is present in unperturbed S-phase cells and in HU-treated cells, and Ddc2 is required for cell cycle arrest in the presence of HU. Phosphorylation and activation of Rad53 in response to replication arrest is Ddc2-dependent. Therefore, Ddc2 appears to mediate between Mec1 and Rad53 in response to ongoing DNA replication and when fork progression is blocked. It remains to be tested whether the association of Ddc2 with Mec1, or the phosphorylation of Ddc2 by Mec1, depends on the sensor components, and how these events are regulated.

In response to replication inhibition, Mec1 and Rad53 enforce cell cycle arrest partly by blocking Pds1 degradation. In addition, these kinases induce transcription of genes involved in DNA repair and that help deal with the perturbed replication process.[24] This safety system most likely protects stalled replication forks, allowing them to be reinitiated when conditions have improved. The transcription pathway depends on Rad53-dependent phosphorylation of the kinase Dun1 (damage uninducible).[24,42] Activation of Dun1, in response to DNA damage or DNA replication blocks, induces transcription of genes that promote efficient DNA repair.[24] This transcription response is partially initiated by Crt1 hyperphosphorylation.[42] Crt1 represses transcription of DNA damage-inducible genes by binding to their promoter regions; binding is prevented by hyperphosphorylation. Activation of a transcription program clearly has the potential to enforce a wide range of Mec1-and Rad53-dependent functions, and a growing literature has made clear that Mec1 and Rad53 are involved in numerous cellular processes. For example, Mec1 and Rad53 inhibit the firing of late replication origins during early S-phase. Eukaryotic cells replicate their genomes by initiating DNA synthesis from mul-

tiple replication origins. Some fire early in S-phase, others are initiated part way through S-phase. When cells are arrested in early S-phase with HU, late firing origins are kept dormant by a dominant process that requires the Rad53-Mec1 pathway.[43] Mec1 and Rad53 are also involved in the regulation of telomere length and in transcriptional silencing at telomeres.[44-46]

It is not clear, however, whether the Mec1/Rad53/Dun1-dependent transcriptional response contributes to cell cycle arrest in the presence of HU, since *dun1* null mutants are not S-phase checkpoint defective. In agreement with this, the cell cycle checkpoint defects of *mec1* and *rad53* mutants are somewhat different. Both mutants elongate their mitotic spindles when DNA replication is blocked with HU, so it seems that no checkpoint response remains in these cells. However, *rad53* mutants delay in anaphase, while *mec1* mutants exit mitosis. Thus some aspects of mitotic progression are inhibited in *rad53* mutants. Light was shed on the basis of this difference by analysis of *pds1* mutants, revealing that there are several S-phase checkpoint targets. Pds1 is not an essential target in early S-phase because *pds1* mutants can inhibit spindle elongation when replication is blocked with HU in early S. However, kinetic analyses determined that, part-way through S-phase, a critical point is reached where Pds1 becomes essential: *pds1* mutants elongate spindles and lose sister chromatid cohesion when roughly 2/3 of the genome has been replicated.[47] These experiments were performed in the presence of a concentration of HU that does not fully block replication, but instead, slows the rate of DNA replication. In these experiments, *mec1* or *rad53* mutant cells began anaphase when very little DNA had been replicated (as is the case when replication is blocked). Therefore, a Pds1-independent system restrains spindle elongation in early S-phase, but later in S-phase, Pds1 is required. A reasonable prediction is that Pds1 and Rad53 function downstream of Mec1 in the context of S-phase checkpoint control, and that these pathways run in parallel, and are temporally regulated; one necessary in early S-phase, the other part way through S-phase. Presumably, Mec1 controls Pds1 stability in late S-phase. Several details remain unresolved, however. For example, the fact that *pds1* null mutants are able to restrain spindle elongation and prevent premature loss of sister chromatid cohesion in early S-phase necessitates a novel Mec1/Rad53 target at that point in the cell cycle.

An explanation for the duality of S-phase checkpoint control in budding yeast is the linkage of spindle elongation with regulation of sister cohesion. Sister cohesion is established during DNA replication, and must be maintained until the onset of anaphase.[20,21] Once cohesion is established, part way through S-phase,[48] checkpoint control of anaphase must coordinate release of cohesion with spindle elongation. Early in S-phase, prior to replication of critical cohesion regions and concomitant establishment of cohesion, spindle elongation might be regulated independently of cohesion. Therefore, the switch in the mode of checkpoint control from the Mec1-Rad53 pathway to the Mec1-Pds1 pathway may be controlled by the establishment of sister chromatid cohesion.

It remains to be determined how Pds1 levels are controlled in late S-phase when DNA replication is perturbed. Recent evidence has linked two yeast genes to regulation of Pds1 in this context.[49] Rad23 or Ddi1 overproduction was found to rescue the sensitivity of *pds1* mutant cells to HU. Rad23 is a nucleotide excision repair protein, but recent studies suggest a novel role of Rad23 in ubiquitin-dependent proteolysis.[50] Rad23 binds to mono- or di-ubiquitinated proteins, but cannot bind when the ubiquitin chains have been elongated. Crucially, Rad23 blocks extension of the ubiquitin chains. For most ubiquitinated proteins that are targeted for degradation, ubiquitin chain elongation is critical for efficient recognition by the 26S proteasome. Therefore, Rad23 might have an important function in preventing or delaying the degradation of proteasome targets. Although this mechanism has not been tested directly in the context of S-phase checkpoint control, overexpression of Rad23 is able to stabilize a mutant *pds1* protein, suggesting that Rad23 might play a role in S-phase checkpoint signaling.[49] A role of Rad23 in checkpoint signaling may be utilized by virally expressed proteins. The HIV-1 encoded protein Vpr has been shown to bind to the C-terminal UBA (Ubiquitin associated) domain of human Rad23 (HHR23A).[51-53] This interaction is needed for one of the

cellular functions of Vpr – the ability of Vpr to induce G2 cell cycle arrest which allows time for viral replication. It seems plausible that Vpr mimics an endogenous cellular checkpoint response that involves binding of the Rad23 UBA to an unknown protein, inducing G2 arrest.

Mammalian cells also need an S-phase checkpoint. The initiation of mitosis must be prevented during S-phase and chromatid cohesion must be maintained. Mammalian Sgs1 homologs are clearly important for S-phase regulation.[38] Sgs1 is a budding yeast member of the *Escherichia coli* recQ helicase family, and *sgs1* mutants are genomically unstable.[54] Mammalian recQ helicase family members include WRN (mutated in Werner's syndrome patients)[55] and BLM (mutated in Bloom's syndrome patients).[56] Bloom's syndrome is characterized by genomic instability and a high incidence of cancer, whereas Werner's syndrome causes premature ageing. The BLM protein was recently identified as a component of a large complex that includes tumor suppressor proteins, DNA repair and checkpoint proteins that localize to nuclear foci when cells are treated with HU.[57] Indeed, cultured cells from Bloom's syndrome patients have S-phase defects, but it is not clear whether these abnormalities include checkpoint abrogation. In general, there are mammalian homologs of all the budding yeast checkpoint proteins, but their potential roles in S-phase checkpoint control have not been thoroughly investigated.

The Mec1 homologs are ATM and ATR.[58,59] ATM, the gene mutated in ataxia telangiectasia patients who have an increased incidence of cancer, is a nuclear protein kinase. ATR (ataxia telangiectasia and rad3 related) is also a protein kinase, and is structurally more homologous to Mec1, than is ATM. Both ATM and ATR are clearly involved in DNA damage checkpoint signaling,[60,61] but do not seem to be required for preventing the onset of mitosis during S-phase. Whether these proteins have roles in regulating cohesion has not been addressed. In the context of the DNA damage checkpoint, the tumor suppressor proteins p53 and BRCA1 (breast cancer gene 1) seem to be targets of ATM/ATR, but again there is little evidence for roles in S-phase.[62-68] BRCA1 is, however, phosphorylated by ATR in response to HU treatment.[69]

The mammalian Rad53 homolog, kinase Chk2 (Checkpoint kinase),[70] and the mammalian homolog of budding and fission yeast Chk1 (also named Chk1), are required for the ATM-dependent DNA damage checkpoint.[71,72] After γ-irradiation, mammalian Chk2 phosphorylation (on thr-68, within the serine/threonine cluster domain of Chk2) and activation is ATM-dependent.[73] Chk2 kinase also becomes phosphorylated and activated upon HU treatment, but in an ATM-independent manner, and not on thr-68 (Refs.70,73,74) If the HU-induced phosphorylation is relevant for S-phase checkpoint control, it might be that the mammalian S-phase checkpoint operates by a kinase distinct from ATM.

Chk1 is not needed for S-phase checkpoint control in budding yeast, but does seem to be required in some higher eukaryotes. Xenopus Chk1 is activated in post-MBT (mid-blastula transition) embryonic cells treated with HU.[75] Similarly, there is good evidence for a role of Drosophila Chk1 (named Grapes) in coordinating embryonic DNA replication with the onset of mitosis.[76,77] In Xenopus egg extracts, immunodepletion of Chk1 impairs an ability to delay cell cycle progression in response to replication blocks.[78] Immunodepletion of ATR has the same effect since Chk1 activity depends on phosphorylation by ATR when unreplicated DNA is present.[79] Human ATR has been implicated in Chk1 phosphorylation in response to HU treatment, but it is not known if cells lacking Chk1 or ATR have defective S-phase checkpoint controls.[80] In Xenopus, Chk1 activation also depends on a protein named Claspin which has a close human homolog. Xenopus egg extracts depleted of Claspin are S-phase checkpoint deficient.[81]

Both Chk1 and Chk2 can phosphorylate Cdc25C on ser-216 in humans and it is thought that this phosphorylation prevents Cdc25C from activating the mitotic kinase, cyclinB1/Cdc2.[70,71] Cdc25C is a protein phosphatase that promotes entry into mitosis by dephosphorylating Cdc2. This phosphorylated residue creates a binding site for a 14-3-3 protein, resulting in Cdc25C inhibition.[72] Interestingly, expression of a mutant Cdc25C that cannot be phosphorylated on ser-216 induces mitosis in the presence of unreplicated DNA, suggesting that this pathways may be important for S-phase checkpoint control.[72]

Topoisomerase II-Dependent Checkpoint

Topoisomerase II (topo II) is required for chromosome condensation and segregation in eukaryotes.[3,82-87] Although these are mitotic processes, their successful completion depends partly on topo II activity during DNA replication and in G2 phase (Fig. 4). Chromosome replication creates two identical sister DNA molecules that are knotted together (catenated). Topo II removes the catenations; in higher eukaryotes, the majority must be resolved before entry into mitosis to allow accurate chromosome condensation.[3,88] A G2 checkpoint ensures that DNA catenations have been sufficiently resolved before mammalian cells enter mitosis.[3] It had been known for some time that topo II inhibitors block or delay mammalian cell cycle progression in G2, but the inhibitors used were also known to cause DNA damage; it was assumed that the G2 arrest was due to activation of the DNA damage checkpoint. That cells also need to monitor topo II activity in G2 was an attractive hypothesis, however, and the characterization of novel topo II inhibitors that do not damage DNA allowed this theory to be tested. A variety of assays that measured DNA damage were used to assess the effects of bisdioxopiperazine topo II inhibitors on mammalian cells and on isolated DNA. Bisdioxopiperazine were found not to induce DNA breaks in vivo or in vitro (Ref. 3 and refs. therein), but did block mammalian cells in G2. In these studies, entry into mitosis was assessed based on the onset of chromosome condensation. Yet, topo II is needed for chromosome condensation, albeit a requirement at late steps in the process.[3,88] It was therefore necessary to prove that cells were physically capable of initiating chromosome condensation when topo II activity was absent. Such evidence could not be sought by isolating loss-of-function budding or fission yeast mutants, since the topo II-dependent checkpoint seems to be absent in yeast.[85,89] Therefore the checkpoint was first described in mammals, by demonstrating checkpoint bypass induced with caffeine or kinase and phosphatase inhibitors.[3] Cells treated with ICRF-193, the most potent of the bisdioxopiperazines, could be forced into mitosis with caffeine; the cells began to condense chromosomes without delay. Fully condensed chromosomes were not formed, consistent with the essential role of topo II late in the condensation process. Thus, the topo II-dependent checkpoint prevents the onset of chromosome condensation, a process which the cells can begin, but cannot complete in the absence of topo II activity. Although the evidence for this checkpoint in mammals is substantial, its absence in yeast has prevented the checkpoint components from being rapidly identified. Indeed, it is not known whether topo II levels are sensed directly, or if physical structures within chromosomes, such as catenations (Fig. 5), are monitored. Some data suggest the latter is more likely. Replicative catenations are introduced between daughter DNA duplexes during S-phase. Disentangling daughter duplexes is of crucial importance since they otherwise could not separate and segregate during mitosis. Most replicative catenations are resolved in G2, when cellular topo II activity increases, but since the decatenation reaction is reversible, topo II activity inevitably promotes some catenation. This generates nonreplicative catenations, that can involve distant regions of chromatin, and can join different chromosomes together.[90,91] Cells inhibited for topo II activity late in G2 and forced to enter mitosis with caffeine, have striking chromosome aberrations caused by persistent nonreplicative catenations that join chromosomes together and create o figures within individual chromosomes (Figs. 4 and 5).[92] Circumstantial evidence suggests that the removal of nonreplicative catenations in G2, the process that promotes chromosome individualization, may be monitored by the topo II-dependent checkpoint.[92]

Another question is what is the nature of the topo II-dependent checkpoint sensor? It might be that sensors bind to sites of DNA catenation. Though purely speculative, there is a precedent for such a proposal, that a signaling cascade might be activated by protein complexes at sites of DNA crossover. In bacteria, stable maintenance of the natural multicopy plasmid CoIE1 requires a *cer* sequence element (Fig. 6). *cer* is necessary for recombination that converts unstable plasmid multimers to monomers.[93,94] The expression of Rcd, a transcript encoded from within *cer*, is specifically expressed in cells containing multimers. Rcd1 enforces a cell cycle checkpoint that inhibits cell division when multimers are present,[95] thus allowing time for site-

specific recombination to occur. An interesting observation is that the Rcd1 promoter resides within the *cer* sequence, and that the topology of *cer* is likely to be altered in multimers that assemble recombination complexes at the *cer* sites.[96] This difference in topology might influence Rcd1 transcription, providing an elegant mechanism to activate the checkpoint in the presence of multimers. The parallel between this phenomenon in bacteria, and the topo II-dependent checkpoint signal generated by persistent DNA catenations in mammalian cells is remarkable.

The target of the topo II-dependent checkpoint is presumed to be mitotic cyclin/CDK activity: recent work has shown that a topo II-dependent checkpoint exists in plant cells that can be overridden by overexpressing a mitotic cyclin (JFGA, unpublished data). How the mitotic kinase is regulated in response to topo II-dependent checkpoint activation is not known, although some data give clues as to the upstream components of the pathway. The topo II inhibitor genistein arrests mammalian cells in G2 by activating Chk2 kinase, which in turn leads to the inhibition of Cdc25C-dependent tyr-15 dephosphorylation of CDK1.[97] Activation of Chk2 occurs very efficiently at genistein doses that inhibit topo II but cause minimal DNA damage compared to other topo II inhibitors such as etoposide. In the case of genistein, ATR might be the kinase upstream of Chk2, since caffeine overrides the G2 arrest whereas Wartmannin does not. In contrast to ATM, which is inhibited by caffeine and Wartmannin, ATR is only efficiently inhibited by caffeine.[97] The topo II-dependent checkpoint and DNA damage checkpoint might be regulated primarily by ATR and ATM respectively. Although these checkpoints are distinct, the possibility remains that they are closely linked pathways. One way to address this issue will be to test whether other components of the DNA damage checkpoint, such as Chk1, p53 and 14-3-3, are activated in the context of ICRF-193-induced G2 arrest.

Checkpoint Control in Prophase

A recent study identified a novel mammalian checkpoint protein Chfr (Checkpoint with FHA and Ring finger) apparently acting to slow chromosomal condensation in prophase and prometaphase when microtubule polymerization is perturbed.[98] In a cohort of 8 human tumor cell lines, three were identified that failed to express Chfr at the transcriptional level, although this was not due to loss of both gene copies. Furthermore, a mutation was identified in a fourth cell line leading to loss of Chfr function. In tumor cell lines lacking Chfr function, mitotic chromosome condensation occurred at the same rate in the presence or absence of nocodazole (or Taxol). In cells with functional Chfr, or cells lacking Chfr but transiently transfected with a functional copy, chromosome condensation occurred at a reduced rate in nocodazole treated cells, relative to cell cycle progression from G2 to metaphase based on the accumulation of cyclin/CDK activity and prophase separation of centrosomes. Examination of nuclear morphology and DNA content following 48 hours of microtubule depolymerizing treatment demonstrated that Chfr defective cells undergo aberrant mitosis, implicating this checkpoint in chromosome instability. While there is no definitive yeast homolog for Chfr, two *S. cerevisiae* open reading frames and one *S. pombe* gene, defective in mitotic arrest (Dma1),[99] appear closely related. Clearly study of a larger cohort of tumor cell lines and further mechanistic studies need to be performed to fully assess the import of this checkpoint in tumorigenesis. These may also substantiate the authors claims that Chfr is inactivated more frequently than 'all known spindle checkpoint proteins combined'.[98]

Spindle Assembly Checkpoint

The spindle assembly checkpoint is an example of how a combination of yeast genetics and cell biology in higher eukaryotes has rapidly expanded our knowledge of a biological system.[26,100-104] Eukaryotic cells arrest in metaphase when microtubule polymerization is disturbed. Higher eukaryotic cells, with normal mitotic spindles, also arrest if chromosomes fail to become bioriented on the spindle and have not congressed to the metaphase plate.[105]

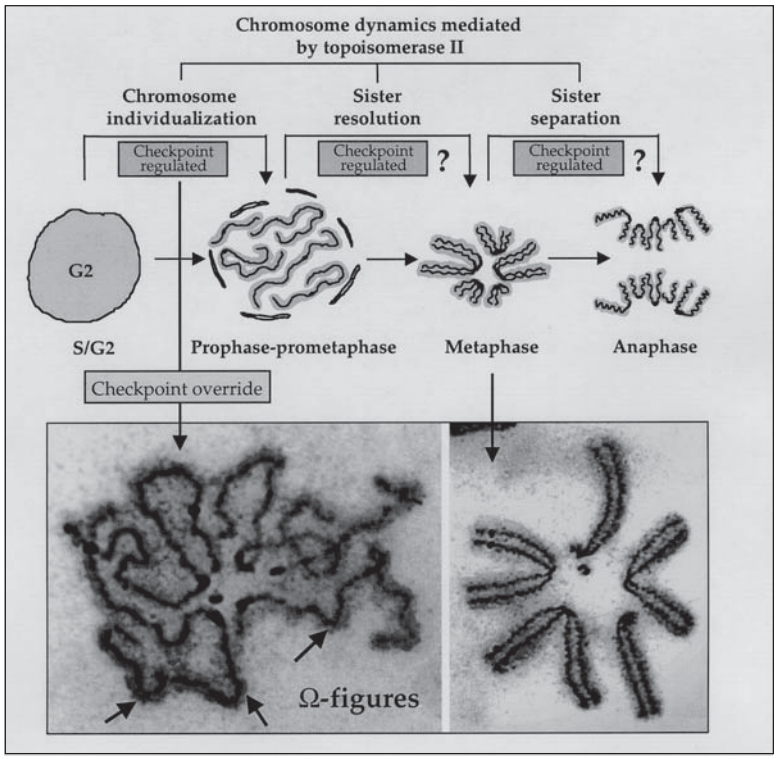

Fig. 4. Chromosome dynamics mediated by Topoisomerase II (Topo II). Topo II decatenation reactions are needed for various mitotic steps in mammalian cells. Before mitosis, chromosome individualization is promoted by the resolution of nonreplicative catenations (left). This process may be monitored by the G2 topo II-dependent checkpoint since forcing G2 cells into mitosis in the absence of topo II activity produces o figures (see Checkpoint override). Topo II is also needed for sister chromatid resolution in prometaphase (middle) and sister separation in anaphase (right). Whether checkpoints controls these processes has not been fully investigated. The photomicrographs show *Muntiacus muntjak* chromosomes.

Other defects such as spindle pole body (SPB), kinetochore and centromere abnormalities also activate the checkpoint,[100] and it is known that incorrect spindle orientation (relative to the cell axis) can delay the onset of anaphase.[106] Therefore, the spindle assembly checkpoint monitors the process of bipolar attachment of all the chromosomes to the mitotic spindle and ensures that the spindle is correctly positioned.

Chromosomes become bioriented by amphitelic attachment of their kinetochores to spindle microtubules.[100,107] The process of chromosome capture by the spindle occurs more or less randomly:[107] within the same species and cell type, it is accomplished quickly in some cells, but takes much longer in others.[105] Therefore, in animals and in yeast, the checkpoint is needed every cell cycle.[108-110] However, biorientation of the chromosomes on the mitotic spindle forms a stable structure,[107,111] thus the correct alignment of chromosomes, creating the metaphase plate, is favored. Once the last chromosome becomes bioriented, the spindle assembly checkpoint signal diminishes and anaphase is initiated in a highly regimented manner.[105] At least one facet of the checkpoint signal emanates from kinetochores that have not attached to the spindle.[112] In higher eukaryotes, a phospho-epitope (recognized by the 3F3/2 antibody) is present on unattached kinetochores (Fig. 7).[107,113] Attachment of microtubules to a kinetochore induces dephosphorylation of the 3F3/2 phospho-epitopes at that kinetochore. As chromosomes

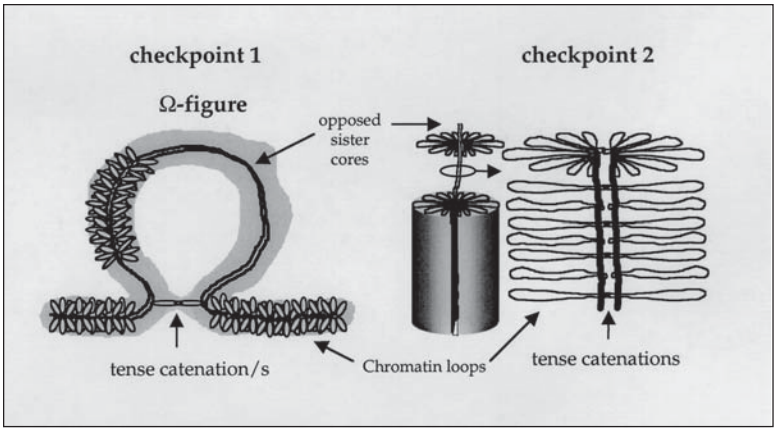

Fig. 5. Location of putative topoisomerase II-dependent checkpoint sensors. The cartoons depict a model of chromosome structure showing loops of chromatin attached to a chromosome core or scaffold, and the location of nonreplicative and long-lived replicative catenations. (Checkpoint 1) Perturbed topo II activity in G2 results in persistent nonreplicative catenations between chromatin loops, positioned at distant points within the same metaphase chromosome but fortuitously closer during interphase (left). Nonreplicative catenations produce a cytologically observable chromosome aberration named o-figures. When these catenations involve different chromosomes they promote interchromosomal recombination. o-figures are likely to generate tension at the base of the loops at sites of catenation. Such regions might have the potential to produce a checkpoint response in G2. (Checkpoint 2) Long lived replicative catenations are present at the bases of chromatin loops, in the regions where loops are attached to the closely opposed sister chromatid cores, until prometaphase (right). These replicative catenations might help maintain sister chromatid cohesion until prometaphase. Tension at sites of replicative catenation, created by ongoing chromosome condensation, might generate a checkpoint response during mitosis.

attach to the spindle, the 3F3/2 epitopes are dephosphorylated, and the checkpoint becomes inactive . The molecular basis of this phosphorylation is not understood, but mechanistically, it is thought that checkpoint sensors, that are tension-sensitive complexes residing within the kinetochores, control the kinetochore phosphorylation status.[105,107,112,114] Elegant studies have shown that tension exerted on kinetochores, applied by manipulating chromosomes with a micro-needle, induces loss of the kinetochore 3F3/2 epitopes.[115] Therefore, it appears as though a lack of tension generates the checkpoint signal. The identity of the kinase which creates the 3F3/2 epitope is not known, but recent work indicates that it is an integral component of kinetochores. Cells lysed in detergent do not contain kinetochores that are reactive against the α-3F3/2 antibody, but the α-3F3/2 reactivity can be reinstated by the addition of ATP.[116,117] The activity of the kinase must be tightly associated with kinetochores. Furthermore, the substrate and kinase are likely to be associated. In theory, this 'in vitro' system could be used as a biochemical assay to identify the kinase. Genetic studies have revealed components of the yeast spindle assembly checkpoint (Fig. 8). Several groups of checkpoint proteins were identified in genetic screens designed to find mutants sensitive to microtubule antagonists. These are Mad1, Mad2, Mad3 (Mitotic Arrest Defective),[109] and Bub1, Bub2, Bub3 (Budding Uninhibited by Benzimidazole).[118] In addition, Mps1 is required.[119] Many of these proteins have homologs in higher eukaryotes (see Table 1). One of these proteins, Mad2, was shown to bind selectively to phosphorylated kinetochores in vertebrate cells.[117] Conversely, Mad2 binding was inhibited by kinetochore-microtubule attachment.[120] Therefore, phosphorylated components of attachment-sensitive or tension-sensitive complexes might be recognized by Mad2. The current hypothesis is that Mad2 binding to 3F3/2 positive epitopes leads to the formation of an active checkpoint complex. In this model, kinetochores are

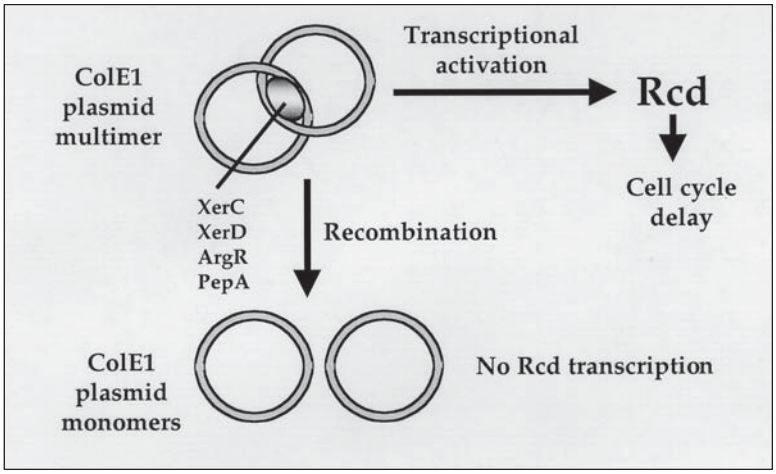

Fig. 6. A model for the bacterial Rcd checkpoint. Rcd imposes a cell cycle arrest before cell division that allows time for plasmid multimers to be recombined into stable monomers. Rcd is encoded from within the *cer* element of the recombination site. Transcription of Rcd occurs only in multimers and is induced by the topological environment created when the XerC/XerD/ArgR/PepA recombination complex forms. In theory, such a system could be utilized in mammalian cells to monitor the catenation state of DNA.

catalytic sites for formation of the checkpoint signaling element, namely the activated Mad2 complex.

The target of the activated checkpoint complex was revealed in key experiments demonstrating that cell cycle arrest is brought about by inhibition of APC activity, which in turn prevents Pds1 ubiquitination and subsequent degradation. Yeast Mad2 was shown to bind to Cdc20, a component of the APC required for Pds1 degradation,[121,122] and this binding can inhibit ubiquitination of APC substrates.[121] Overexpression of *CDC20*, or expression of a *cdc20* mutant that cannot bind to Mad2, bypasses the spindle assembly checkpoint arrest.[122] In the "catalytic kinetochore" model, unattached kinetochores might form an active site at which Mad2-Cdc20 complexes are assembled, then released, thereby excluding Cdc20 from APCs. Alternatively, active Mad2 complexes might be released from kinetochores allowing them to inhibit APC^{Cdc20} in other cellular locations. The latter model is supported by measurements of Mad2 localization dynamics in living cells; Mad2 is a transient component of unattached kinetochores, having a $t_{1/2}$ of roughly 25 seconds.[123]

But what is the nature and function of the active Mad2 complex? Studies in yeast have shown that spindle defects activate kinase Mps1, resulting in Mad1 hyperphosphorylation (perhaps directly by Mps1).[119,124] Overexpression of Mps1 alone can activate the checkpoint and this arrest is (at least partly) dependent on Mad1, Mad2, Mad3 and Bub1, Bub2, Bub3. Mad1 phosphorylation also requires Bub1, Bub3 and Mad2.[124,125] This modified form of Mad1 is required to mediate metaphase arrest. Significantly, Mad1 has been shown to bind to Mad2, and in this complex, Mad1 is a better substrate for Mps1 kinase than is unbound Mad1. At least in Xenopus egg extracts, Mad1 is required for the association of Mad2 to kinetochores that are not attached to the mitotic spindle.[126] Together, the yeast genetic data and studies in higher eukaryotes indicate that checkpoint activation relies on the recognition of unattached kinetochores by Mad2, and the formation of an activated Mad1-Mad2 complex in which Mad1 is hyperphosphorylated. But how do the other checkpoint components fit into this scheme? Somewhat parallel to the case of the Mad1-Mad2 complex, yeast Bub1 and Bub3 are tightly associated.[127] This is also the case in mammalian cells, and the Bub1 domain required for Bub3 binding is also needed for localization of Bub1 to kinetochores.[128] The implication is that

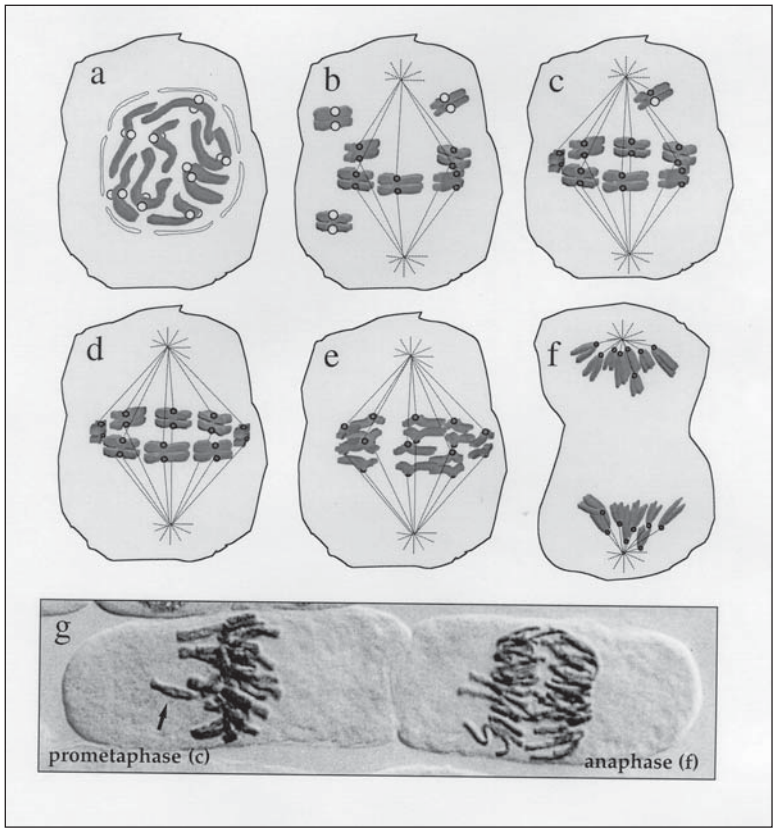

Fig. 7 Tension-sensitive kinetochore phosphorylation. The kinetochores of prometaphase chromosomes (a-c and the left cell in g) become phosphorylated forming a 3F3/2 antibody-reactive epitope (open circles). The phospho-epitope is lost (filled circles) as chromosomes attach to the mitotic spindle forming the metaphase plate (b-d). This inactivates the spindle assembly checkpoint and cells begin anaphase roughly 20 minutes later (e-f and the right cell in g). Micrographs show onion root meristematic cells. The left cell is represented schematically in c, in which one chromosome has not congressed (also see arrow in g).

Bub3 drives localization of Bub1 to kinetochores, as is the case for Mad1 and Mad2. A recent study of the budding yeast proteins sheds some light into how these complexes might be related.[108] Mad1 was shown to associate with Bub1 and Bub3 in unperturbed cell cycles and the amount of the complex in cells was increased in response to spindle checkpoint activation. Mad2 and Mps1 are required for the formation of the Bub1-Bub3-Mad1 complex in yeast.[108] A Mad1 mutation that abolished Bub1-Bub3-Mad1 complex formation, also led to a defective spindle checkpoint.

But how are the Mad1-Mad2 and Bub1-Bub3 complexes related? It may be the case that each complex becomes localized to kinetochores under slightly different conditions, in order to broaden the scope of defects that the checkpoint can detect. However, since deletion mutants of any one of these components results in a fully defective checkpoint, it is hard to argue that the complexes play entirely redundant roles. Instead, the different complexes might well detect different aberrations, but still all be needed for generating the active checkpoint complex that inhibits APC.[Cdc20] Recent studies have allowed a working model to explain such an interconnection between the Mad1-Mad2 and the Bub1-Bub3 complexes, and how APC[Cdc20] might be inhib-

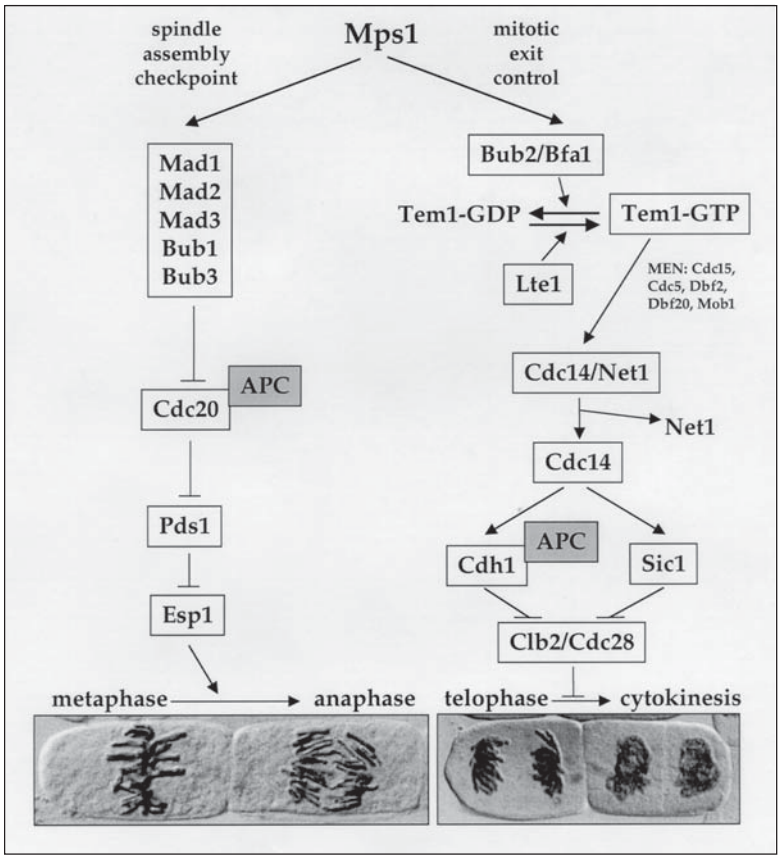

Fig. 8. Two branches of the spindle checkpoint. Mad2 and Bub2 are both activated by the kinase Mps1, but induce arrest at different stages of mitosis. The Mad2 (spindle assembly) pathway inhibits the metaphase to anaphase transition by preventing APC^{Cdc20}-dependent Pds1 ubiquitination while the Bub2 pathway inhibits mitotic exit by preventing APC^{Cdh1}-dependent B-type cyclin degradation and by maintaining CDK activity. Both branches are required for indefinite arrest although a significant delay in mitosis can be invoked when either pathway is activated. Photomicrographs of onion root meristematic cells depict the mitotic stages. See text for detailed descriptions.

ited.[108] Although Bub1-Bub3-Mad1 complexes exist in yeast, and this complex forms in a manner dependent on Mad2 and Mps1 kinase activity, Mad2 is not found in this complex.[108] Additionally, the Bub1-Bub3-Mad1 complex does not seem to be able to bind to Cdc20.[108] This might suggest that an exchange mechanism is necessary to generate the active checkpoint complex (Fig. 9). In such a model, Mad2 is displaced from Mad1-Mad2 complexes, induced by Mps1 kinase, simultaneously stimulating the formation of the Bub1-Bub3-Mad1 complex on the one hand and an active Mad2 complex on the other.[108,129] Mad1 phosphorylation, dependent on Mps1, Bub1, Bub3 and Mad2, may also be involved in this exchange. The nature of the activated Mad2 complex in not known, but may include Mad3.[129]

In support of this model, animal homologs of Mad1, Mad2, Mad3, Bub1 and Bub3 are found at the kinetochores of prophase and prometaphase (not yet bioriented) chromosomes. Following congression to the metaphase plate, these proteins seem to dissociate.[130] These local-

ization studies suggest that formation of an active checkpoint complex within kinetochores is likely to be a conserved mechanism that activates the checkpoint pathway. But how is the checkpoint signal mobilized? How does a single unattached kinetochore generate a signal that inhibits anaphase spindle elongation and prevents loss of sister chromatid cohesion of all the other chromosomes? One study has revealed important information that should help to resolve this question. Rieder et al, examined the timing of anaphase onset in cells that contain two functional and independent spindles.[131] Such polykaryons are generated by cell fusion. When two cells at different stages of the cell cycle are fused, cell cycle progression of their nuclei soon becomes synchronized, allowing measurements of anaphase timing in independent spindles that share a common cytoplasm. This analysis revealed that the inhibitor emanating from a single unattached kinetochore is not freely diffusible, but rather is likely to be associated with the spindle itself. Therefore activated complexes might track from kinetochores along spindles.

Checkpoint Control of Mitotic Exit

Many of the components of the mitotic exit machinery have been identified by the cell cycle phenotype of budding yeast mutants which arrest as a large dumbbells with elongated spindles (see Table 1 and 2). This phenotype is consistent with arrest at the anaphase/telophase transition. Mutants are unable to pass this arrest and the proteins are therefore essential for exit from mitosis. These genes appear to define a GTP-dependent kinase signaling cascade, ultimately releasing a phosphatase that induces spindle disassembly, cytokinesis and mitotic exit. Control of mitotic exit therefore resides in the inhibition of this essential pathway.

Exit from mitosis absolutely requires inhibition of B-type cyclin/CDK activity. Under normal circumstances this is mediated by both inhibition of CDK activity and by degradation of mitotic cyclins. Study of the *S. cerevisiae* spindle checkpoint has revealed that the 'spindle assembly' checkpoint is branched, inhibiting both the transition from metaphase to anaphase and mitotic exit (Fig. 8). The different functions of the two branches begs the question as to whether it is erudite to continue calling both branches by the term 'spindle assembly checkpoint'. Others have begun to call the two branches the 'spindle assembly' and 'spindle position' checkpoints.[132] For the purposes of this review we continue to use the term spindle assembly checkpoint for the inhibition of the metaphase-anaphase transition and the generic term 'mitotic exit control' for the branch that regulates the activity of the B-type cyclin/CDK activity. The mammalian machinery for mitotic exit control is currently being elucidated while substantial inroads into understanding the mechanism has been achieved in *S. cerevisiae*. Here we describe current knowledge in the *S. cerevisiae* checkpoint control of mitotic exit (Fig. 10). Comparison to mammalian homologs and discussion of their possible clinical importance in tumorigenesis is left for the next section.

Evidence that Bub2 operates in a separate checkpoint pathway to the Bub1, 3, Mad1-3 pathway (hereafter collectively referred to as the Mad2 pathway) came from studies of double mutants treated with antitubulin drugs.[133-138] Double mutant combinations that included *bub2* failed to arrest in nocodazole whilst double mutant combinations that did not include Bub2 retained a mitotic delay. However, in *mad2* cells treated with nocodazole the metaphase-anaphase transition occurs with kinetics comparable to those of untreated cells while *bub2* cells delay the metaphase-anaphase transition. In addition, delay of the cell cycle in *ctf13* mutants (limited for a key kinetochore component) requires Bub1 and 3 and Mad 1, 2 and 3 but is independent of Bub2.[139] In *cdc20* mutants, the mitotic arrest caused by maintained Pds1 levels is dependent on Bub2 but independent of the Mad2 pathway genes.[140] Inhibition of Dbf2 in late mitotic arrest requires Bub2 but not the Mad2 pathway proteins.[141] Together, these studies define distinct pathways. The Mad2 pathway ultimately targets Pds1, preventing spindle elongation and loss of sister chromatid cohesion at the metaphase to anaphase transition. The Bub2 pathway inhibits mitotic cyclin/CDK activity, and thus prevents spindle disassembly and exit from mitosis. Since the Bub2 pathway also monitors spindle integrity, and is triggered by microtubule depolymerizing agents, there is a common upstream element, kinase Mps1. However, in

Table 1. Mitotic checkpoint components

S. cerevisiae	*S. pombe*	*M. musculus*	*H. sapiens*	*Chromosomal location (H.s.)*
BFA1	*byr4*$^{+}$	—	—	—
BUB1	*bub1*$^{+}$	Bub1	BUB1	2q14
BUB2	*cdc16*$^{+}$	Mm29982 *	VRP*	2
BUB3	*spac23h3.08c* *	Bub3	BUB3	10q26
CDC14 †	*spac782.09c* *	Mm.6355 */Mm28909 *	CDC14A/CDC14B	1p21 / 9
CDC15 †	*cdc7*$^{+}$	Mess1 *	STK4 */STK3 *	20q11.2-13.2 / ?
CDC20 †	*slp1*$^{+}$	—	CDC20	9q13-q21
CDC5 †	*plo1*$^{+}$	Plk/Cnk/Stk18 *	PLK/CNK/SNK/STK18 *	16 / 1 / 5/ 4q27-q28
CDH1	*spbc1198.12* *	Mm.2440 *	hCDH1	19p13.3
DBF2	*sid2*$^{+}$*/spcc417.06* *	Lats1 */Lats2 *	LATS1 */LATS2 *	? / 13q11-q12
DBF20	*sid2*$^{+}$*/spcc417.06* *	Lats1 */Lats2 *	LATS1 */LATS2 *	? / 13q11-q12
ESP1 †	*cut1*$^{+}$	—	ESP1	8
LTE1	*efc25*$^{+}$ */ste6*$^{+}$ *	Rasgrf1 *	RASGRF1 *	15q24
MAD1	*spbcd6.04 */spac26a3.10 *	—	MAD1L1	7p22
MAD2	*mad2*$^{+}$	Mad2L1	MAD2L1/MAD2L2	4q27 / 1p36
MAD3	*bub1*$^{+}$ */spcc895.02* *	Bub1b	hBUB1R1	15q14-21
MOB1 †	*mob1*$^{+}$	—	FLJ10788 *	—
MPS1 †	*mph1*$^{+}$	Ttk	TTK	6q13-q21
NET1	*spbc1711.05* *	Mm.4480 *	LAD1 *	1q25.1-q32.3
PDS1	*cut2*$^{+}$	mSec	PTTG	5q35.1
SIC1	—	Nfatc3 *	—	—
TEM1 †	*spg1*$^{+}$	Rab5b *	RAB36 *	22q11.22
BIM1	*mal3*$^{+}$	Eb1	EB1	—

* denotes ortholog based solely upon sequence homology. † denotes essential gene in *S. cerevisiae*.

Table 2. Function/localization of mitotic checkpoint components

Gene	Function	Localization
BFA1	?	dSPB
BUB1	S/T kinase	nucleus/kinetochore
BUB2	GAP (GTPase activating protein)	dSPB
BUB3	?	nucleus/kinetochore
CDC14 †	Phosphoprotein phosphatase	nucleolus/nucleus
CDC15 †	S/T kinase	dSPB/bud neck
CDC20 †	APC specificity factor	kinetochores/SPB/spindle
CDC5 †	S/T kinase	SPB
CDH1	APC specificity factor	?
DBF2	S/T kinase	SPB/bud neck
DBF20	S/T kinase	nucleus
ESP1 †	Separin	nucleus/spindle
LTE1	GEF (GDP/GTP exchange factor)	bud
MAD1	?	nucleus
MAD2	Cdc20 inhibitor	kinetochores/SPB
MAD3	?	nucleus/kinetochore
MOB1 †	?	SPB
MPS1 †	S/T kinase	?
NET1	sequesters Cdc14	nucleolus
PDS1	sequesters ESP1; securin	nucleus/spindle
SIC1	B-type cyclin/CDK Inhibitor	?
TEM1 †	GTP binding memebr of RAS superfamily	dSPB
BIM1	?	?

† denotes essential gene in *S. cerevisiae*. dSPB denotes daughter-bound spindle pole body.
S/T kinase denotes serine/threonine protein kinase.

contrast to the inhibition of anaphase onset via APC^{Cdc20} regulation mediated by the Mad2 pathway, the Bub2 pathways appears to primarily act by inhibition of mitotic cyclin degradation and maintenance of mitotic cyclin dependent kinase activity. This is achieved by suppression of APC^{Cdh1} and Sic1 activity which promote Clb1/Clb2 degradation and inhibit mitotic cyclin dependent kinase activity respectively.

The mitotic exit branch of the checkpoint is essential if microtubule polymerization is perturbed during anaphase i.e., after APC^{Cdc20} dependent degradation of Pds1. Normal progression of the cell cycle ensures that Cdc20 remains active and bound to the APC until after the onset of anaphase when Cdh1 replaces Cdc20 as the APC specificity factor targeting B-type cyclins for degradation. However, deletion of *CDH1* is not sufficient to prevent mitotic exit since inactivation of mitotic cyclin dependent kinase activity by Sic1 is sufficient to allow exit from mitosis. The redundancy of APC^{Cdh1} and Sic1 activity ensures that cells may exit mitosis in the absence of checkpoint stimulation. In the presence of checkpoint stimulation the activity of both Sic1 and APC^{Cdh1} is inhibited by nucleolar sequestration of the phosphatase Cdc14. Indeed, it appears that release of Cdc14 from the nucleolus is a key event in mitotic exit. The mechanism of Cdc14-mediated exit from mitosis appears to be three-fold. First, by dephosphorylating Cdh1 the APC targets the B-type cyclins for degradation. Ordinarily, phosphorylation of Cdh1 by Cdc28 is inhibitory and thus is self protecting,[142] but when Cdh1

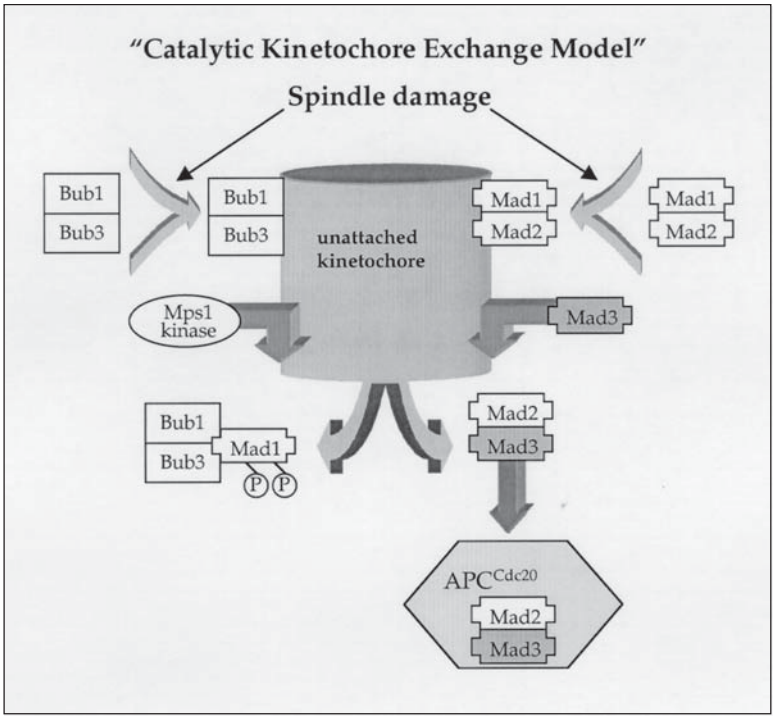

Fig. 9. Model for the formation of the activated spindle assembly checkpoint complex. Association of Mad1-Mad2 and Bub1-Bub3 complexes with kinetochores is stimulated by the onset of spindle assembly and/or spindle aberrations. Kinase Mps1 induces exchange between the complexes, forming an active Mad2 complex, perhaps containing Mad3. Exchange may be stimulated by Mad1 phosphorylation. Activated Mad2, released from kinetochores, binds to Cdc20 to inactivate the APC.

is dephosphorylated by Cdc14 this self-protection is removed. Second, by dephosphorylating Swi5, activating the transcription of Sic1, and third by directly dephosphorylating Sic1 itself. Thus Cdc14 both inhibits the activity of cyclin/CDK activity and induces the destruction of the cyclin components.[143]

How does the 'mitotic exit' checkpoint inhibit the release of Cdc14 from the nucleolus? Throughout most of the cell cycle Cdc14 is held inactive within the nucleolus in complex with Net1/Cfi1 (Fig. 11)[144,145] termed the RENT complex (regulator of nucleolar silencing and telophase). This inactive localization appears to be dependent upon Tem1;[145] a GTP binding protein localized to the daughter-bound spindle pole body (SPB). Cdc14 may also play a structural role in the nucleolus.[146] A recent paper proposes a mechanism for monitoring the completion of anaphase and presents a compelling model.[147] When SPB-associated Tem1-GDP locates to the bud at the end of anaphase it interacts with a cortical protein Lte1 which is a GDP/GTP exchange factor (GEF). Tem1-GDP is activated by conversion to Tem1-GTP triggering the release of Cdc14 via a kinase cascade termed the mitotic exit network (MEN see below). Even when localized to the bud cortex, Tem1 activation could be inhibited by GAP (GTPase activating protein) activity of Bub2, preventing exit from mitosis. There are several compelling reasons for supposing this to be the checkpoint mechanism. Bub2 has considerable sequence homology to cdc16$^+$ in *S. pombe* which is known to form a two component GAP with byr4$^+$. Together they activate the GTPase encoded by spg1$^+$, the *S. cerevisia*e homolog of Tem1. Furthermore, deletion of the *cerevisiae* homolog of byr4$^+$ (Bfa1) causes a phenotype similar to *bub2*, as does overexpression of Tem1. Finally, localization of Bub2 to the SPB and preferentially to that

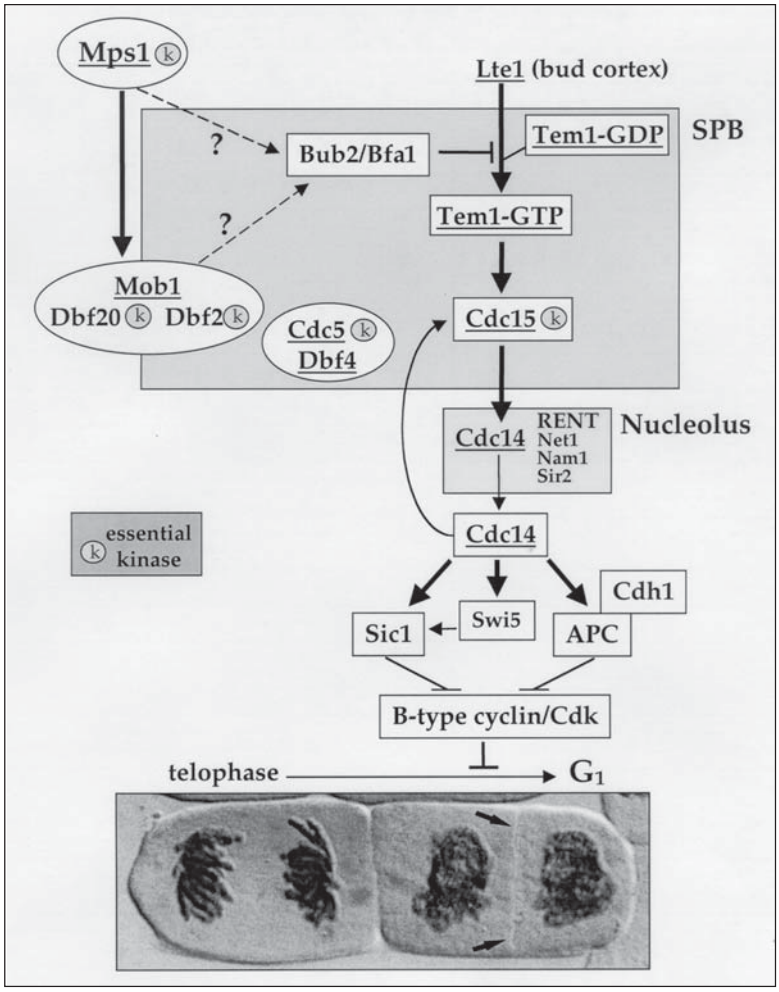

Fig. 10. Mitotic exit (Bub2) pathway: Many components of the MEN localize to the spindle pole bodies either symmetrically or asymmetrically with preference for that destined for the daughter cell. Components of the RENT complex localize mainly to the nucleolus. Release of Cdc14 from the nucleolus dephosphorylates Sic1, Swi5 and Cdh1 both activating APC dependent B-type cyclin degradation and inhibiting B-type cyclin-dependent kinase activity. Components underlined are essential for mitotic exit as mutants arrest in telophase with a 'dumbbell morphology'. The involvement of Cdc5 in MEN and interactions of the Mob1/Dbf2/Dbf20 complex in MEN have yet to be defined. Heavy arrows represent activation with demonstrated physical interaction.

destined for the daughter cell,[138,148,149] provides strong circumstantial evidence in support of this model.

The mechanism by which activation of Tem1 leads to the release of Cdc14 from the nucleolus via the MEN involves a number of key components including Cdc15, Cdc5, Dbf2, Dbf20 and Mob1.[150] Most appear essential for mitotic exit since single mutants are lethal, while in the case of Dbf2 and Dbf20, it is only the double mutant that is synthetically lethal.[151] Localization and phosphorylation of these proteins appear to be important factors during the cell cycle and probably contribute to their function (Table 2). In particular, many of the com-

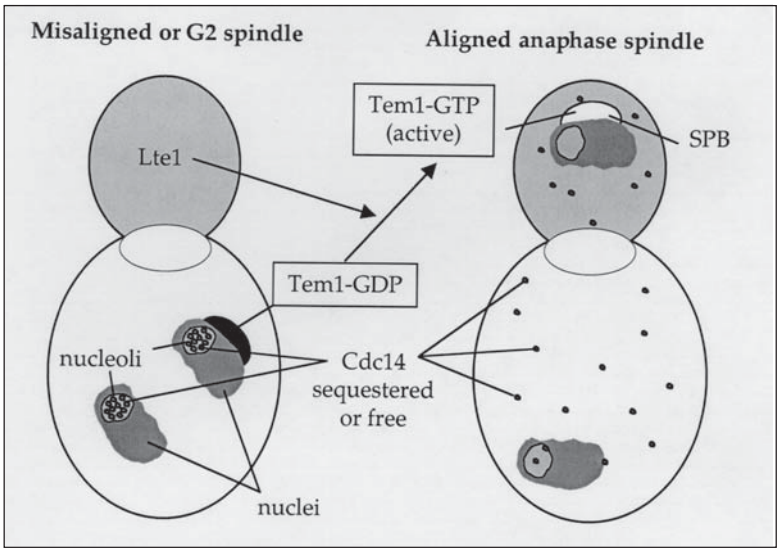

Fig. 11. Model for colocalization of Tem1/Lte1 inducing mitotic exit in budding yeast. Mitotic exit is triggered by Tem1-GTP that induces release of Cdc14 from the nucleolus. Inactive Tem1-GDP is bound to the SPB destined for the daughter cell. Once the spindle has elongated into the bud, the guanine nucleotide exchange factor Lte1 activates Tem1.

ponents localize to the nucleolus or are asymmetrically distributed between the SPBs, being preferentially bound to that destined for the daughter cell. Cdc15 localizes to the SPB during mitosis and relocates to the bud neck after telophase.[152,153] Furthermore, Cdc15 phosphorylation increases gradually during the cell cycle until it is rapidly dephosphorylated in late mitosis.[152,154] Like Cdc15, Dbf2 localizes to the SPB and moves to the bud neck in telophase.[155] While asymmetric localization to the daughter-bound SPB is true for some components, others such as Mob1 (or at least the *S. pombe* homolog), localize symmetrically to both spindle pole bodies. Localization to the SPB and the relocation of these components to the bud neck in telophase mimics the localization in *S. pombe* in which their homologs principally act by regulating cytokinesis rather than mitotic exit per se.

While mechanistic details of the MEN/RENT complex are being reported, much work has still to be completed before a clear picture can be drawn. Some details of physical interactions and colocalization can at least allow some description of the cascade (Fig. 10). The rapid dephosphorylation of Cdc15 in late mitosis appears to be mediated by Cdc14.[153] However, there also appears to be a role for Cdc15 as an activator of Cdc14, and it is thus both an activator and substrate.[154] Also there appears to be a role for Pds1 as an inhibitor of B-type cyclin degradation independent of its role as a securin[156] thus forming potential 'crosstalk' between the Mad2 and Bub2 checkpoint branches. What is less clear is the role of Cdc5 which physically interacts with Dbf4 (part of the DNA replication machinery) but as yet has no clearly defined role in the mitotic exit pathway. As Cdc5 is a target of DNA damage checkpoint control, this component offers an attractive link between the damage checkpoint and mitotic exit control. Direct interactions have been established for a number of MEN components while the exact nature of many interactions remain unclear. For example, physical interaction between Mps1 and Mob1 has been demonstrated. Furthermore, interactions between Mob1 and Dbf2 and Dbf20 have been demonstrated. However, there has been no direct link established between these components and the remaining MEN components to date. Similarly, the role of Cdc5 in the MEN has yet to be elucidated. Nonetheless, we present an attempt to order the

events of mitotic exit regulation based upon physical interactions and localization in Figure 10. In this scheme we present Cdc15 'upstream' of Cdc14 as the former localizes to the SPB coincident with other upstream elements.

Evidence suggests that Bub2, and its associated partner Bfa1, participate in an essential checkpoint that is also activated by DNA damage.[157,158] Thus the maintenance of B-type cyclin/CDK activity by the Bub2 pathway may represent a universal mechanism that can respond to stress at any stage of G2 and M-phase. Other genetic interactions of Bub2 include synthetic lethality of a *bub2 arc35-1* mutant[159] and that Bub2 is essential for arrest in *tub4-1* cells.[160] Thus, the Bub2 pathway seems to respond to defects in spindle orientation, spindle localization, spindle damage and DNA damage.

Oncological Implications of Mitotic Checkpoint Homologs

The existence of numerous mitotic exit and spindle assembly checkpoint protein homologs in *S. pombe* and higher eukaryotes suggests that similar mechanisms regulate mitotic exit in all eukaryotes despite the fact that the asymmetric cytokinesis in *S. cerevisiae* appears to have fundamentally different spatial and temporal strategies. As aberrant mitosis frequently results in asymmetric distribution of the genetic material and aneuploid daughter cells, dysfunctional regulation of these checkpoints has become an attractive hypothetical mechanism for chromosomal instability in mammalian tumorigenesis. Indeed, some established tumor cell lines and tumors appear to have dysfunctional checkpoint controls[23,161,162] and some of the checkpoint proteins appear to be targets of oncogenic viral proteins.[163] However, screening of aneuploid colorectal tumor panels for such mutations revealed only mutations in the human hBUB1/hBUBR1 genes.[164] A similar study of 31 aneuploid lung, and head and neck, tumors showed no such mutations.[165] One hBUB1 somatic mutation that led to an amino acid substitution was found among 30 human primary lung cancer tumors.[166] hBUB1 and hBUBR1 mutants have also been found in adult T-cell leukemia's/lymphomas [167] and some colorectal tumor cell lines.[161] Perhaps significantly, one study has implicated Brca2, which is responsible for a fraction of the inherited susceptibilities to breast cancer, in the spindle assembly checkpoint.[168] Brca2 was found to interact with hBubR1, and was phosphorylated by hBubR1 in vitro, though no direct role in the checkpoint was demonstrated. Although inactivation of Bub1 appears to confer chromosomal instability,[161] more studies are required to determine whether mutations in BUB1 and other mitotic checkpoint proteins represent significant causative events or whether other checkpoints may account for aneuploid tumorigenesis.

In addition to the MAD2 and BUB2 pathway components, a number of other *S. cerevisiae* genes appear to have mammalian homologs which have been implicated as either protooncogenes or as tumor suppressor genes (see Table 1). Notably *S. cerevisiae* Pds1 may have two mammalian homologs, at least one of which is associated with pituitary tumors.[169-172] Most of the human genes have been localized at least to the chromosome level and recent publication of the human genome will therefore facilitate further study. Furthermore, many mouse homologs have been identified and murine models of tumorigenesis may further elucidate the contribution of these genes to tumorigenesis. At the present time only two knockout mouse models have been reported, MAD2 [173] and BUB3,[174] both of which are early embryonic lethals. In the BUB3 mouse, from day 3.5 onwards, embryonic cells display mitotic aberrations such as micronuclei, anaphase chromosome laggards and bridging. In the presence of microtubule antagonists, the cells fail to arrest in metaphase.

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CHAPTER 6

The Regulation of p53 Growth Suppression

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Abstract

The p53 tumor suppressor protein plays a pivotal role in the cellular response to stress. A variety of stress signals trigger accumulation and activation of p53 to halt the cell cycle and to prevent replication of damaged DNA. The p53 protein is required for a proper G1 arrest, it is essential for maintaining the G2 arrest, and it contributes to the mitotic spindle checkpoint. p53 exerts these actions by inducing multiple target genes. Under defined conditions, p53 induces programmed cell death by mechanisms that are partially understood and involve a combination of transcriptional dependent and –independent activities. The choice between arrest and cell death depends on the final integration of antagonistic signals. These include the type and intensity of the stress signal, the spectrum of the target genes induced, the type of cell and its oncogenic status, and the presence of growth and survival factors. The stability of the p53 protein and its activities are tightly regulated by many factors among which the Mdm2 proto-oncoprotein is the central player. Inhibitory effects of Mdm2 on p53 stability and activities are modulated by multiple mechanisms including post-translational modifications of p53 and Mdm2 and by other interacting proteins. Importantly, p53 is also regulated at the level of its sub-cellular localization. Sequestration of p53 into the cytoplasm is sufficient for its inhibition. In contrast, accumulation of p53 in the nucleus induces its transcriptional activity. This activity can be further enhanced by specific post-translational modifications and by recruitment of p53 into nuclear bodies. We discuss current views on the regulation of p53 and its growth inhibitory activities.

Introduction

The p53 tumor suppressor protein plays a key role in the regulation of the cell cycle and cell death. The p53 protein is also involved in cell differentiation, DNA repair, senescence and angiogenesis.[1-8] Wild type (wt) p53 and intact signaling pathways are essential for the prevention of cancer, consistent with a high tumor incidence observed in p53 null mice[9] and in p53-heterozygous Li-Fraumeni patients.[10] It is estimated that approximately one half of human cancers contain a mutation in p53.[11] It is predicted that in the majority of the remaining tumors the p53 signaling pathway is inactivated by up-regulation of p53 inhibitors, such as Mdm2, or by down-regulation of p53 cooperators, such as ARF.[12,13] The increased predisposition to tumor development in the absence of p53 is due to the accumulation of genetic alterations and failure to eliminate these defective cells.[14]

Wt p53 is a labile protein with a short half-life. Accumulation and activation of the protein can be triggered by a variety of stress signals including DNA damage, hypoxia, nucleotide deprivation, viral infection, heat shock, and mitogenic or oncogenic activation.[15-17] The specific activity of p53 is further enhanced by post-translational modifications and by a variety of positive and negative regulators.[6,18,19] Activated p53 elicits cellular responses that ultimately lead to growth arrest and/or programmed cell death (apoptosis).[3,8] In this Chapter we will

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focus on the regulation of p53, the growth inhibitory activities of p53 and the cellular choice to stall or die.

Regulation of p53

The p53 protein is subject to tight regulation at multiple levels. This is achieved by a variety of positive and negative regulators, often creating feedback loops. Three major levels of regulation are recognized: protein stability, protein activity, and subcellular distribution. New information has broadened our understanding of how p53 is regulated under normal and stress conditions, and the molecular mechanisms governing these regulatory processes are beginning to emerge. It is beyond the scope of this review to discuss all aspects of p53 regulation. Instead, we will focus on two major aspects: the regulation of p53 stability, with emphasis on the pivotal role of Mdm2, and the regulation of p53 subcellular localization. Comprehensive reviews on other aspects of p53 regulation, such as p53 post-translational modifications and their effects on p53 activities have been recently published.[6,18-20]

Mdm-2-Negative Autoregulatory Feedback Loop

The major negative regulator of p53 is the Mdm2 proto-oncogene.[12,21,22] Mdm2 is transcriptionally induced by p53, thus p53 triggers its own destruction through a negative feedback loop.[12] This feedback loop leads to oscillations in the expression of both proteins following DNA damage. These oscillations may enable a more effective execution of a reversible p53 response.[23] The importance of Mdm2 in this negative regulation is demonstrated by the lethal effect during early stages of embryonic development of mdm2 knockout mice. The early mortality of these mice is abolished by the simultaneous inactivation of p53.[24,25] Overexpression of Mdm2 in many cancers is often sufficient to inactivate p53 without further mutation.[12] Similarly, mere loss of Mdm2 is sufficient to induce p53-mediated apoptosis in vivo.[26] Therefore, Mdm2 is critical for keeping p53 in check. The following sections discuss the regulation of p53 by Mdm2 and how this regulation is modulated.

Mdm2 Signals p53 for Proteasomal Degradation

The finding that Mdm2 promotes p53 degradation through the ubiquitin-proteasome system[27,28] was central to our understanding of how p53 stability is regulated. It also helped to explain the elevated expression of p53 mutant proteins in cancer cells, which results from their inability to induce *mdm*2 expression.[29] Reduced affinity of p53 mutants for Mdm2 may contribute to this effect.[30] Mdm2 promotes p53 for degradation by acting as an E3 ubiquitin ligase,[31] even though it does not contain the HECT (Homologous to E6AP Carboxy Terminus) domain. The RING-finger domain of Mdm2 is crucial for the E3 activity of Mdm2.[32,33] The oligomerization of p53 appears to be essential for the degradation of p53 by Mdm2.[34,35] Removal of the last 30-40 C-terminal amino acids from p53 impairs its degradation by Mdm2,[34] but has minimal effect on the extent of p53 ubiquitination.[35] Within this region there are several lysines that serve as potential ubiquitination sites. Substitution of the 6 lysines between residues 370-386 markedly reduces the ubiquitination of p53 by Mdm2 and its susceptibility to Mdm2-mediated degradation.[36,37] In addition, two N-terminal regions have been recently shown to affect the susceptibility of p53 for destabilization by Mdm2. The polyproline region (residues 62-91) of p53 may provide protection for p53 from Mdm2. p53 lacking this region is excessively sensitive to inhibition and destabilization by Mdm2.[38] On the other hand, the adjacent region (residues 92-112) appears to be required for the degradation of p53 by Mdm2.[39] These findings suggest that additional proteins are probably involved in the ubiquitination of p53 by Mdm2 in vivo. A possible candidate is JNK that has been implicated in the ubiquitination and degradation of p53 in unstressed cells, and binds p53 between residues 97 and 116.[40]

Inhibition of p53 Activities by Mdm2

Mdm2 binds the transactivation domain of p53 (residues 18-28) in a region important for the interaction of p53 with components of the transcription machinery, such as TBP and its

associated factors (TAFs),[12] and with its transcriptional coactivator p300.[41] This observation prompted the 'masking model', whereby the binding of Mdm2 to p53 conceals its transactivation domain.[42] It is difficult to distinguish between effects of Mdm2 on p53 stability from its effect on p53 activity. Several studies have suggested that Mdm2 can inhibit p53-mediated apoptosis independent of protein degradation.[38,43,44] This has been demonstrated by flow cytometric measurement of the inhibitory effect of Mdm2 on p53 apoptotic activity, without reduction in p53 stability. This notion is supported by the fact that the Mdm2 analogue MdmX (Mdm4) inhibits p53 transcriptional activation without promoting its degradation.[45,46] Similarly, the inhibition of p53-dependent transcription by Mdm2 may not require p53 degradation.[47] In apparent conflict, recent studies suggested that in the absence of p53 ubiquitination, Mdm2 is unable to block p53 transcriptional activity,[37] and the degradation of p53 is obligatory for blocking p53 transrepression and apoptotic activities.[47] These findings are difficult to reconcile, especially because both p53 and Mdm2 were overexpressed and different ratios of these proteins were used. This important question needs to be addressed at physiological levels of p53 and Mdm2.

Modulation of the p53/Mdm2 Autoregulatory Loop

In order to exert its growth inhibitory activities, p53 degradation by Mdm2 should be regulated. Modulations of the autoregulatory feedback loop involving Mdm2 and p53 have been the subject of intensive study. In the following sections, we discuss the major mechanisms governing this important regulation.

Post-translational Modifications

The Mdm2-p53 interaction is obligatory for p53 degradation.[27,28] Preventing this interaction is sufficient to protect p53 from degradation.[48-51] Following various stress stimuli, such as γIR and UV, this interaction is interrupted. How is this interaction regulated in response to stress? Phosphorylation of p53 on serine 20 (Ser20), which resides within the Mdm2 binding domain, reduces the affinity of Mdm2 for p53,[44] and enhances the stabilization of p53 in response to γIR and UV light.[52,53] Checkpoint kinase 2 (Chk2) was identified as the kinase responsible for phosphorylation of Ser-20,[54] without which the stabilization of p53 in response to DNA damage is abrogated.[55] Hence, a cascade of DNA damage signaling from ATM to Chk2 to p53 phosphorylation leads to the accumulation of p53. In response to γIR, p53 is also phosphorylated on Thr18 by a CKI-like kinase, a modification that reduces the binding affinity between p53 and Mdm2.[56,57] However, the physiological relevance of the latter modification is yet to be explored.

In addition to these modifications, p53 is subjected to multiple phosphorylations at the N- and C- termini (Table 1). While modifications such as phosphorylation of Ser-15 or Ser-37,[57,58] do not appear to regulate p53 stability, they play important regulatory roles in p53 binding to DNA and p53 transcriptional activity (Table 1; reviewed in[6,18,19]). Similarly, acetylation of p53 on lysine residues within its C-terminus enhances its transcriptional activity (Table 1). Finally, p53 undergoes sumoylation, a modification that mildly enhances its activities without affecting p53 stability.[59-61]

The Mdm2 protein is also subjected to modifications that may affect its ability to regulate p53. For example, the sumoylation of Mdm2, which is reduced in response to DNA damage, enhances its ability to ubiquitinate p53.[62] Further, Mdm2 undergoes rapid phosphorylation in vivo by ATM in response to γIR or radiomimetic drugs. [63] The effect of this modification on the auto-regulatory loop is yet unclear. In response to DNA damage, Mdm2 is also phosphorylated at multiple sites by DNA-PK. This phosphorylation impairs p53/Mdm2 interaction,[64] but its relevance in vivo is currently unknown. The combined modification of Mdm2 and p53 may have synergistic effects on the auto-regulatory loop in response to stress signals.

Table 1. Posttranslational modifications of p53 and their effects on p53

Modifying enzymes	Stress signal	p53 modifications	Effectson p53	References
A. Phosphorylation				
ATM	IR	Ser-15	Enhanced SST	169,209,210
ATR	IR, UV	Ser-15, Ser-37	Enhanced SST	170
DNA-PK	IR, UV	Ser-15,Ser-37	Enhanced SST	168
Chk1	IR, UV	Ser-20	Reduced Mdm2 binding	211
Chk2	IR, UV	Ser-20	Reduced Mdm2 binding	54,55
JNK	IR, UV	Ser-33	Enhanced stability	212
CAK	ND	Ser-33, Ser-376, Ser-378	Enhanced SST	126,213
p38	UV	Ser-15, Ser-33, Ser-46, Ser-392	Enhanced SST and stability	214-216
CKII	UV	Ser-392	Enhanced SST	217
PKR	IFN, dsRNA	Ser-392	Enhanced SST	218
CKI	IR	Thr-18	Reduced Mdm2 binding	49, 57
CyclinA-CDK2	ND	Ser-315	Enhanced SST	219-220
PKC	UV	Ser-371, Ser-376, Ser-378	Enhanced SST	6
B. Dephosphorylation				
Unknown	IR	Ser-376	Increase binding to 14-3-3	221
Cdc14	ND	Ser-315	Unknown	222
C. Acetylation				
p300/CBP	IR, UV	Lys-373, Lys-382	Enhanced SST and stability	166,223
PCAF	UV	Lys-320	Enhanced SST	166
D. Sumoylation				
Unknown enzyme	ND	Lys-386	Enhanced SST	59-61
E. Ribosylation				
PARP	IR	Poly(ADP)ribosylation	Enhanced SST and stability	224-225

Abbreviation: SST – Sequence specific transactivation, IR – ionizing radiation, ND – not determined, IFN – interferon, dsRNA – double stranded RNA

Involvement of Interacting Proteins

The majority of the positive regulators of p53 can be classified into two general groups. The first group consists of proteins that activate and stabilize p53 by neutralizing the inhibitory effects of Mdm2 (e.g., ARF, c-Abl), and their contribution will be discussed below. The second group comprises proteins that bind the C-terminus of p53 and activate it by relieving p53 from the inhibitory effect of this region on DNA binding or by stabilizing the p53 tetramer (e.g., c-Abl, BRCA-1, 14-3-3-σ, Ref-1).

The p53-ARF axis is critical for eliminating potential tumor cells containing deregulated oncogene expression.[13,65] Either ARF or p53 is frequently inactivated during tumorigenesis.[66] However, the tumor spectrum differs in mice lacking either ARF or p53. Whereas p53-null mice develop predominantly T-cell lymphomas (~70%), ARF-null mice exhibit primarily poorly differentiated sarcomas (~50%).[9,67] This indicates that ARF is selectively activated by a subset of stress signals that potentially activate p53. ARF is activated by deregulation of proto-oncogenes, such as E2F-1, by mitogenic oncogenes such as c-myc or Ras and by the adenoviral E1A.[13] In turn, ARF triggers p53-dependent growth arrest in G1 and G2 phases. In the presence of appropriate signals, ARF sensitizes cells to apoptosis in a p53-dependent manner.[68] The activation of p53 by ARF is auto-regulated by a feedback loop. p53 down-regulates the expression of ARF by directly suppressing its promoter[69] and by blocking E2F-1 activation which induces ARF expression.[70,71] ARF activates p53 by neutralizing Mdm2-mediated ubiquitination and degradation of p53.[71-73] ARF sequesters Mdm2 into the nucleolus, thereby preventing the nuclear export of p53.[74-76] Two regions within ARF, the N-terminus and an Arg-rich C-terminus, contribute to the nucleolar import of Mdm2.[77,78] It is proposed that the binding of ARF to Mdm2 unmasks a cryptic nucleolar localization signal (NrLS; residues 466-473), which resides within the RING finger of Mdm2. This sequence promotes shuttling of the ARF/Mdm2 complex to the nucleolus.[78,79]

Another cooperator of p53 is c-Abl, a stress activated protein with multiple effects on cell cycle regulation.[80,81] Mice deficient for c-abl exhibit retarded growth,[82,83] whereas mice doubly deficient for p53 and c-abl are nonviable.[84] Under nonstressed conditions, c-Abl is required for the proliferation of cells that lack p53, but not normal cells.[84] These effects of c-Abl are poorly understood. On the other hand, in response to genotoxic stress c-Abl induces growth arrest or apoptosis (reviewed in references 80,81,85). c-Abl enhances the transcriptional activity of p53, and by binding to its C-terminus it stabilizes the specific interaction of p53 with DNA.[86] We have previously shown that cooperation between c-Abl with p53 is achieved by neutralizing the inhibitory effects of Mdm2 on p53 activity and stability.[87] These inhibitory activities of Mdm2 are also neutralized by other proteins. The retinoblastoma protein stabilizes p53 and relieves its apoptotic activity from inhibition by Mdm2.[88] The catenin protein, which is often deregulated in colon cancer, accumulates p53 and enhances its transcriptional activity. This presumably serves as a safeguard to protect cells from deregulated oncogenic expression.[89] While proteins such as ARF, protect p53 in response to mitogenic and oncogenic signals, other proteins, such as c-Abl, stabilize p53 in response to DNA damage.

Regulation of Intracellular Distribution of p53

The p53 protein shuttles between the cytoplasmic and the nuclear compartments in a cell cycle-dependent fashion.[98,99] The accumulation of p53 in the nucleus is crucial for its tumor suppressive activity. Prevention of nuclear accumulation provides an efficient mechanism by which tumor cells may continue to proliferate in the presence of wt p53. Indeed, cytoplasmic sequestration of p53 has been commonly observed in certain tumors, such as neuroblastomas, breast and colon cancer.[90-92] In at least a subset of these tumors, Mdm2 is responsible for the cytoplasmic accumulation of p53.[93] Several viral proteins also influence p53 localization. The E6 protein promotes the nuclear export of p53 in HPV-infected cervical carcinomas,[94] whereas the adenoviral E1B 55kD protein and the hepatitis B virus HBx protein keep p53 in cytoplasmic structures.[95-97] In addition, defects in the import/export machinery of p53 may alter its distribu-

tion. For example, truncation of importin-α was identified in breast cancer and this modification blocks the nuclear import of p53.[90] Therefore, abrogating the appropriate sub-cellular distribution of p53 is an important mechanism for eliminating p53 functions in cancer development.

The regulatory mechanism governing p53 import/export has begun to emerge only recently. The import of p53 into the nucleus is an active process involving the association of the importin complex with p53 via the nuclear localization signals (NLS). The major NLSI (residues 316-325) mediates the interaction between p53 and importin- and it is essential for the import.[90] Two minor sites, NLSII (residues 369-375) and NLSIII (residues 379-384), facilitate this import.[100] In addition to these sites, two adjacent basic residues, K305 and R306, are important for import, while the spacer between them and the NLSI (residues 326-355) is important for the cytoplasmic sequestration of p53.[101] Presumably this cytoplasmic region serves as an anchor for attachment by cytoplasmic factors that remain to be identified. Alternatively, protein binding to this region masks the NLSI.[100] p53 may be sequestered in the cytoplasm by associating with one or more of cytoplasmic proteins such as tubulin, hsc70, hsc84, F-actin and vimentin.[102] A recent study revealed that p53 is associated with microtubules and is transported to the nucleus by dynein, a motor protein. This facilitates nuclear accumulation of p53 in response to DNA damage.[103]

The nuclear export of p53 is essential for its proteasomal degradation.[104,105] Blocking p53 nuclear export by leptomycin B is sufficient to accumulate p53 in its active form in the nucleus.[104,106] Leptomycin B inhibits the nuclear exporting protein CRM1, which binds the nuclear export signals (NES) of proteins.[107] Yet the regulation of p53 nuclear export is still controversial. It is generally accepted that Mdm2 promotes the nuclear export of p53. However, it is debatable whether the NES of Mdm2,[105] the NES of p53,[108,109] or both are responsible for p53 export. Intriguingly, the NES of p53 resides within the p53 tetramerization domain. This raises the attractive model that NES is masked as long as p53 remains tetrameric, but becomes unmasked when p53 acquires dimeric or monomeric form.[109] However, oligomerization of p53 increases its affinity to Mdm2, and oligomerized p53 is ubiquitinated more efficiently than monomeric or oligomerization-defective mutant.[34,35,110] Nuclear ubiquitination of p53 seems to be a prerequisite for its nuclear export because Mdm2 lacking the RING finger harboring the E3 ligase activity is unable to promote p53 export.[111,112] This suggests that the ubiquitination of p53 exposes NES allowing p53 to interact with CRM1. Consequently, p53 is exported to the cytoplasm (Fig. 1). This may be a simplistic model, in particular because multiple lysines within the regulatory C-terminal region of p53 need to be ubiquitinated by Mdm2 for degradation.[36,37] Further, this model challenges the dogma that the ubiquitination of p53 by Mdm2 is important primarily for signaling p53 degradation. It is possible that the ubiquitination of p53 is primarily aimed at promoting export of p53 to the cytoplasm. Whether p53 is stored in a cytoplasmic pool, processed by the 26S proteasome or recycled back to the nucleus presumably depends on the regulation of p53 in the cytoplasm.

In the nucleus, p53 may be sequestered into small structures termed promyelocytic leukemia protein-nuclear bodies (PML-NB)[113,114] (also known as PML oncogenic domains [PODs] and nuclear domain 10 [ND10]). These structures contain several proteins, including PML, Sp100, Sumo-1, p300/CBP, and HMG1, and are thought to be involved in transcriptional regulation.[115,116] Some forms of PML (e.g., PML3) bind and sequester p53 to the PML-NBs, enhancing transcriptional activity of p53.[113,114] This regulation is important for a full p53 response to stress. In response to γIR, transcriptional and DNA binding activities of p53 are impaired in $PML^{\gamma IR}$ primary cells.[114] This explains thetolerance of $PML^{-/-}$ mice to lethal doses of γIR.[117] Moreover, oncogenic Ras upregulates PML and promotes the formation of a trimeric p53-PML-p300/CBP complex within PML-NB,[118] enhancing acetylation of p53 on Lys-382 by p300/CBP.[118] Thus, PML may activate p53 by recruiting its coactivator p300. Since overexpression of PML leads to apoptosis,[116] this protein may play a central regulatory role in p53 response to stress.

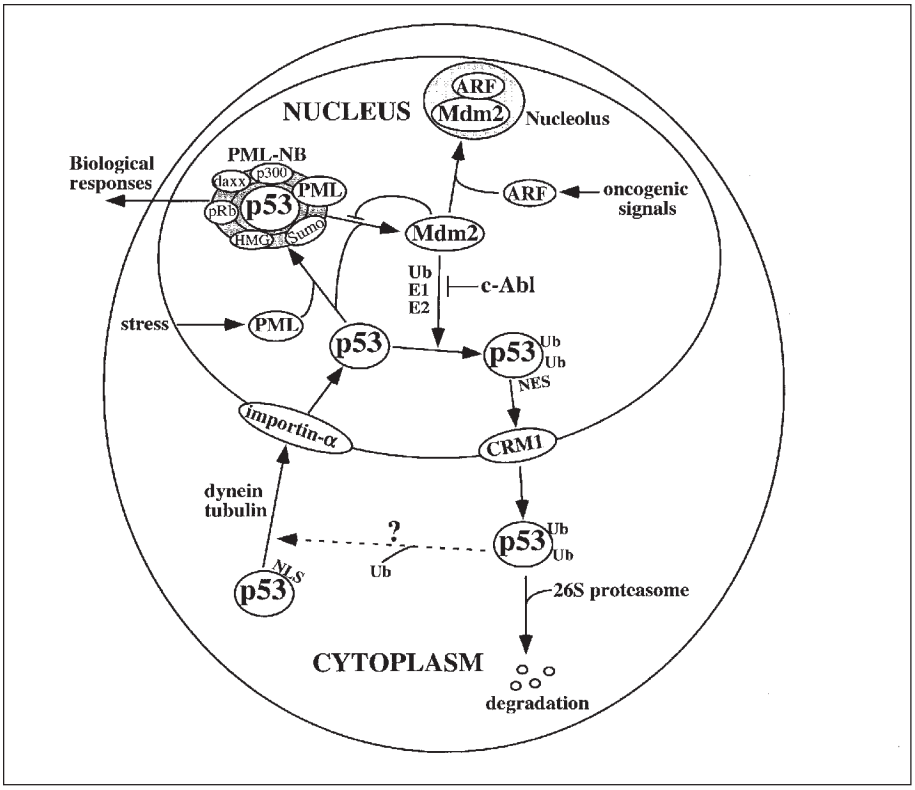

Fig. 1. A model for the regulation of p53 sub-cellular distribution. p53 is imported into the nucleus by interaction with microtubulin and dynein motor protein. This import is mediated through interaction of p53 nuclear localization signal (NLS) with importin-α. Following certain signals, p53 is transported into the PML-nuclear bodies (PML-NB) where it is further activated. Mdm2-mediated ubiquitination of p53 in the nucleus may expose the nuclear export signal (NES) of p53, thereby allowing its nuclear export via interaction with CRM1. In the cytoplasm p53 is degraded by the 26S proteasome system. Whether all the cytoplasmic p53 pool is being degraded, or some is recycled back into the nucleus, is not known. Activation of ARF by oncogenic stimuli protects p53 from Mdm2 by recruiting Mdm2 into the nucleolus. p53 is also protected from Mdm2 by c-Abl following DNA damage.

p53-Mediated Growth Regulatory Functions

Once activated, p53 triggers either growth arrest or apoptosis. Factors that influence this decision are discussed below. p53 is crucial for the induction of growth arrest by numerous stress signals, but it is dispensable for cell cycling under normal conditions.[14] The following sections describe the mechanisms of p53-mediated growth arrest.

p53-Mediated Cell Cycle Arrest

Activation of p53 may lead to growth arrest at both G1 and G2 phases of the cell cycle. Arrest in G1 prevents replication of damaged DNA, while arrest in G2 prevents improper segregation of chromosomes. p53 may also arrest DNA replication in S phase, which is usually masked by the prior G1 arrest.[119] Moreover, p53 is involved in a mitotic spindle checkpoint preventing endoreduplication of 4N cells.[120] The ability of p53 to arrest cells at multiple checkpoints is crucial for suppression of amplification of genetic alterations which otherwise can lead to cancer. Inactivation of wt p53 results in a loss of the DNA damage-induced G1/S

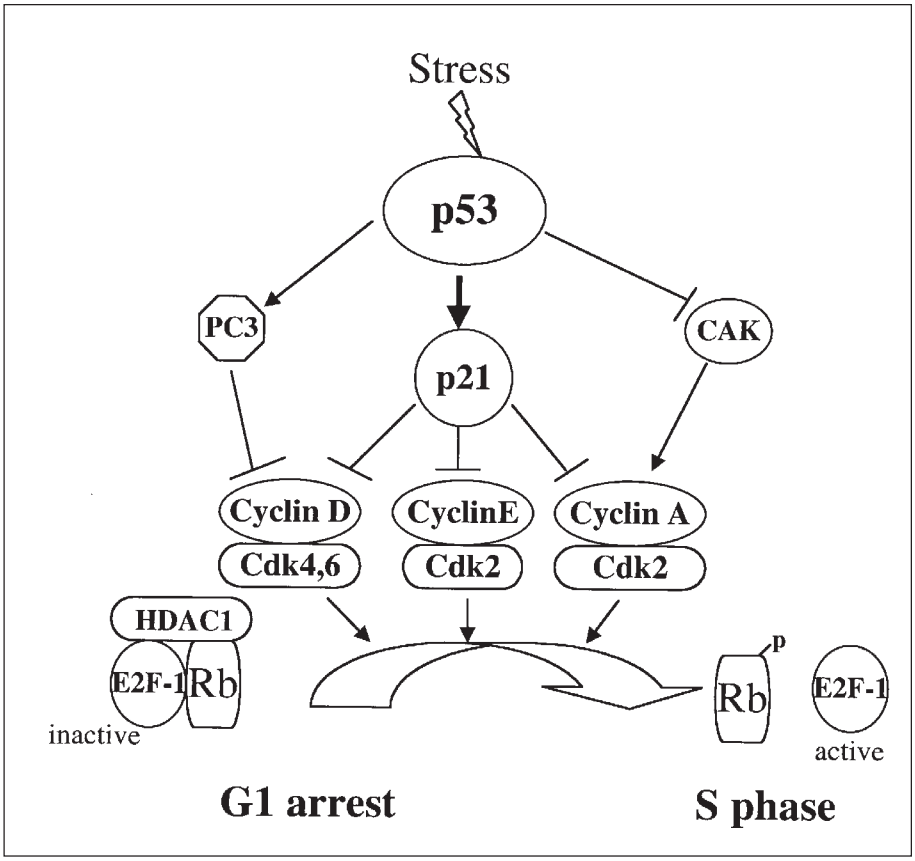

Fig. 2. Induction of G1 cell cycle arrest by p53. The activation of p21 is central for mediating G1 arrest by inhibition of multiple cyclin/CDK complexes. The induction of PC3 and inhibition of CAK activity contribute to this effect. As a result, pRb is not phosphorylated and it inactivates E2F-1 through recruitment of histone deacetylase (HDAC1).

checkpoint,[121] impaired G2 arrest,[122] and the appearance of aneuploid and polyploid cells.[120] While the pathway leading to G1 arrest is well established, the pathway leading to G2 arrest is beginning to emerge (discussed in this book by Stewart and Pietenpol).

p53-Mediated G1-Arrest

The p53-target gene, p21 (waf-1/cip-1), is the key player in G1 arrest. p21 inhibits different complexes of cyclin/cyclin-dependent kinases (CDKs) (Cyclin D-CDK4/6 and Cyclin A, E-CDK2) that sequentially phosphorylate the retinoblastoma (pRb) protein, and as a result release the S phase-promoting E2F-1 transcription factor.[123] Cells deficient in p21 show aberrant G1 arrest following radiation.[124] Unlike p53 null mice, those lacking p21 develop normally, exhibit normal apoptotic response and are not susceptible to spontaneous malignancies.[124] p53 also promotes G1 arrest by directly inhibiting the activity of the CDK-activating kinase (CAK) complex CDK7/CyclinH1/Mat1, which activates cyclin A-CDK2 by phosphorylation.[125] CAK is also a component of the TFIIH transcription factor complex controlling the transcriptional activity of RNA polymerase II. Hence, binding of p53 to CAK results in a strong reduction of

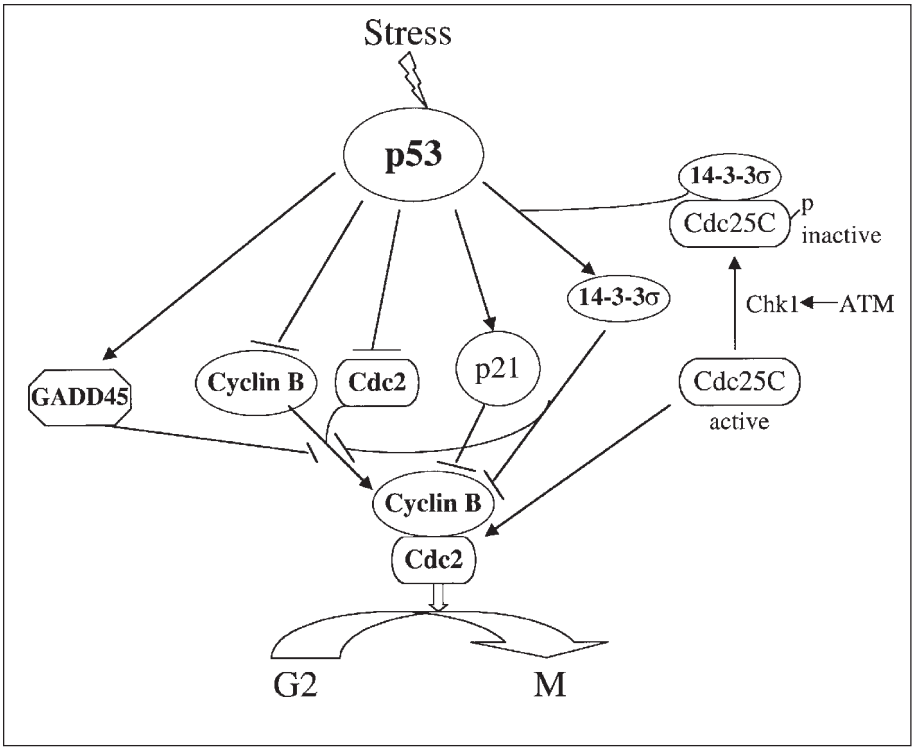

Fig. 3. Contribution of p53 to G2 cell cycle arrest. p53 triggers several parallel pathways that block the formation of the mitotic cyclinB/Cdc2 complex and inhibit its activity. The activation of Chk1 by ATM is also important for this effect. Defects in one of these pathways cause premature entry into M phase. This activity of p53 is essential for maintaining the G2 arrest.

CAK activity towards both CDK2 and the C-terminal repeat domain (CTD) of RNA polymerase II.[125,126] In addition, p53 may activate pRb via PC3 (TIS21,BTG2), a newly identified p53-target gene. The PC3 gene product promotes accumulation of hypophosphorylated pRb by reducing Cyclin D1 protein level and thereby inhibiting CDK4 activity.[127] These pathways are summarized in *Fig. 2*.

p53-Mediated G2-Arrest

Activation of p53 can promote and maintain G2 arrest. This depends on functional pRb[128] and is mediated by several target genes (Fig. 3). Acting in concert, p53 and its target genes efficiently inhibit the CyclinB1/Cdc2 activity, which is essential for cells to enter mitosis. p21 inhibits the activity of the CyclinB1/Cdc2 complex.[128] GADD45 binds Cdc2 and disrupts its ability to complex with Cyclin B.[129,130] The importance of GADD45 is manifested in GADD45$^{-/-}$ mice which exhibit both genetic instability, failure of G2 arrest and centrosome amplification.[131] The 14-3-3-σ protein sequesters and inhibits the phosphorylated form of Cdc25C.[132,133] The Cdc25C phosphatase dephosphorylates and thereby activates the CyclinB/Cdc2 complex.[134] Also, 14-3-3-σ sequesters Cdc2 in the cytoplasm, preventing it from translocating into the nucleus in the late G2.[135] These effects of p21, GADD45 and 14-3-3-σ are further compounded by the transcriptional repression of cdc2 and cyclin B1 by p53.[136-138] Although the role of p53 in triggering G2 arrest is still unclear, its ability to induce the expression of p21 and 14-3-3-σ is essential for sustaining this arrest.[122,135,139]

Mitotic Spindle Checkpoint

Loss of p53 function leads to genomic instability, abnormal centrosome duplication, and formation of aneuploid and polyploid cells. This suggests that p53 plays a role in the control of centrosome duplication and normal chromosomal segregation.[120,140,141] The mitotic spindle checkpoint proceeds normally in the absence of p53, but some responses to the mitotic spindle damage requires p53.[120,142] Induction of G1-like arrest prevents reentry into the S-phase, thereby avoiding 4N cell formation. This process depends on p21 which inhibits CyclinE-CDK2,[143] a complex that drives initiation of centrosome duplication and when constitutively expressed it uncouples the centrosome duplication from DNA replication.[144,145] This is consistent with the tendency of p21 deficient cells to undergo endoreduplication.[146-148] The GADD45$^{-/-}$ cells also show genomic instability, aneuploidy and centrosome amplification,[131] suggesting a complementary role for GADD45. This is consistent with the idea that proper G2 checkpoint is required for preventing premature entry into mitosis.

A cooperative role may exist between p53 and its interacting protein BRCA-1 in regulating chromosomal segregation. Both proteins associate with centrosomes in mitosis and bind to γ-tubulin.[103,120,149] BRCA-1 null cells resemble p53 null cells in genomic instability and abnormal centrosome duplication.[150,151] BRCA-1 deficient cells posses a defective G2/M checkpoint,[152] indicating the role of G2 checkpoint for proper chromosome segregation.

p53-Mediated Apoptosis

The apoptotic activity of p53 is crucial for eliminating defective and potentially carcinogenic cells. Accumulating evidence demonstrates that p53 induces apoptosis by multiple pathways.[3,8,153] While growth arrest largely depends on p53 target genes, a full apoptotic response requires both transcription and transcription-independent functions including repression of survival-promoting genes. p53-mediated apoptosis involves generation of reactive oxygen species (ROS),[154,155] and depolarization of the mitochondrial electropotential gradient ($\Delta\Psi_m$), thus releasing caspase-9 and Apaf-1 which trigger apoptosis through the activation of the caspase cascade.[156,157] The topic of p53-mediated apoptosis is covered elsewhere in this book and has been reviewed previously.[3,8,158]

The Choice Between Growth Arrest and Apoptosis

As discussed above, p53 is activated by a complex array of signals and in turn induces multiple biological responses, predominantly growth arrest and apoptosis. The choice between these responses is influenced by a variety of extracellular and intracellular signals. The major factors are outlined in the following sections.

The Type and Intensity of the Stress Signal

UV radiation and γIR cause different types of DNA damage, leading to different cellular responses.[6] For instance, the osteosarcoma U2OS cells undergo apoptosis in response to UV but undergo growth arrest upon γIR.[159] The accumulation of p53 in response to γIR is rapid and transient, whereas in response to UV it is slow, intense and persists for long period.[159-161] These differences can be explained, at least in part, by the different kinetics by which double strand breaks (DSB) versus bulky adducts are being repaired. The nucleotide excision repair (following UV) is slower than the DSB repair (following γIR).[6] Moreover, UV and γIR trigger different signal transduction pathways that ultimately lead to different pattern of post-translational modifications of p53.[6,18,19,162] This reflects differential activation of upstream kinases. ATM is the critical upstream effector of p53 after γIR whereas p38 is essential for the UV-induced p53 activation. ATR and DNA-PK phosphorylate p53 following both signals. Unlike γIR, UV triggers significant phosphorylation of Ser-392[163-165] and prolonged phosphorylation of Ser-37.[166] Acetylation of Lys-320 occurs early with UV but late with -IR.[166] In contrast, phosphorylation of Ser-15 and Ser-20 is faster after γIR than after UV.[53,167-170] After UV exposure, phosphorylation of Ser-46 appears to be a late event.[167] Furthermore, high doses of

UV are required to phosphorylate Ser-15 and 392, but low doses are sufficient for rapid phosphorylation of Ser 9, 20 and 372.[162] These differences are summarized in Table 1. Contribution of all of these modifications ultimately affects the outcome of p53 response.

The Spectrum of Target Genes Induced by p53

A particular repertoire of target genes which are activated by p53 may determine the cell fate. A different set of target genes are required for p53-mediated growth arrest and apoptosis. The repertoire is affected by many factors including the pattern of post-transcriptional modification of p53, type and strength of a stress signal, and expression of p53 regulatory proteins. p53 binds to its responsive promoters with different affinity due to sequence heterogeneity among the various responsive elements.[171-173] Moreover, the sequence specific DNA binding can be affected by changes in p53 conformation and by the conformation of cognate DNA sequences.[174,175] This is demonstrated by the identification of mutant p53 proteins, including tumor-derived mutants, which manifest distinct promoter specificities. p53 mutants (e.g., 143Ala, 120Arg, 175Pro, 181Leu, and 283His) that retain their ability to transactivate the high affinity promoter of p21, but have lost the ability to transactivate the lower affinity promoter of BAX, are of particular interest. As a result, these mutants induce growth arrest, but they fail to promote apoptosis.[8,29,176-178] The loss of binding to the BAX promoter can be "corrected" in some of these mutants (e.g., 175Cys and 181Leu) by creating multiple binding sites,[172] suggesting that these mutations reduced the affinity for specific sites.

The promoter specificity of p53 may be further defined. The p53 mutant M246I retains the ability to induce *mdm*2 but not PIG3,[179] whereas p53 S121F mutant specifically activates p53AIPI, but not p21, PIG3 or *mdm*2.[167] The promoter specificity of p53S121F explains the enhanced apoptotic activity of this p53 mutant.[167,180] Similarly, variation in promoter-specificity has been demonstrated in p53 mutant lacking the proline-rich region, which has impaired apoptotic activity.[179,181] The requirement for specific p53 conformation in the induction of p53AIP1 is further supported by the observation that this pro-apoptotic gene is only induced by p53 phosphorylated on Ser46.[167] These data demonstrate that post-translational modification of p53 can alter its promoter specificity. Finally, the promoter specificity may be altered under specified cellular conditions. The Pw1/Peg3 gene is induced during p53/c-myc or p53/E2F-1 mediated apoptosis, but neither during p53-mediated G1 growth arrest nor by c-myc alone.[182] Similarly, the TRAIL receptor KILLER/DR5 is induced during p53-dependent apoptosis, but not during growth arrest.[183] Mdm2 and p21 are induced by γIR but repressed by UV.[21,184]

Cell Type-Dependence

Different cell types respond differently to the activation of p53 by DNA-damage. Whereas T lymphocytes often undergo extensive apoptosis, fibroblasts undergo growth arrest.[3,8] This may reflect differential induction of relevant target genes. In lymphoid and myeloid cells but not in fibroblasts, γIR induces BAX and rapid apoptosis.[185]

Thymus, spleen and intestine cells which undergo apoptosis in response to γIR express similar levels of full-length (p90) and truncated (p76) Mdm2 [186]. However, testis, brain, heart and kidney cells, which respond to γIR by growth arrest, express predominantly the p90 form. The truncated form is induced by p53 in response to UV [187] and it antagonizes the inhibitory activity of p90.[186] Thus, the ratio of the two Mdm2 proteins may be decisive for the cellular response to DNA damage.

UV-induced p53 response is enhanced in XP-A and Cockayne's syndrome (CS-A; CS-B) in which transcription-coupled repair is defective.[188] Cells derived from these patients show increased sensitivity to UV radiation with rapid and prolonged accumulation of p53.[189,190] This may explain the increased susceptibility of these cells to p53-mediated apoptosis. Overall, it is consistent with the observations that (i) extensive DNA damage promotes apoptosis rather than growth arrest and (ii) high levels of p53 are required for apoptosis, whereas lower levels are sufficient for growth arrest.[191]

Deregulated Expression of Oncogenes

Activation of p53 in cells with intact stress signaling pathways, such as fibroblasts, causes G1 arrest. On the other hand, cells with defective checkpoints (e.g., cells deficient in ATM or pRb) tend to undergo apoptosis. Often in the latter cases E2F-1 is activated. Deregulated E2F-1 cooperates with p53 to promote apoptosis[192] through the induction of ARF[13] and p73.[193] Following p53 activation, fibroblasts overexpressing c-myc or E1A tend to undergo apoptosis, whereas fibroblasts expressing Ras or ARF enter premature senescence, [68] reflecting differential effects of these oncogenes on p53 activity. Both c-myc and E1A enhance p53 expression through upregulation of ARF.[13] In addition, E1A inhibits p53-induced *mdm*2 expression.[194] On the other hand, Ras causes upregulation of both ARF and Mdm2,[13,195] which have antagonistic effects on p53 expression levels. Consequently, unlike Myc and E1A, Ras does not induce p53 to the levels sufficient for triggering apoptosis. Similarly, loss of p21 or cleavage of the p21 protein renders cells prone to apoptosis in response to DNA damage.[196-200] This effect is further enhanced by the concurrent loss of 14-3-3-σ.[139] Reconstitution of these cells with growth arrest-promoting genes such as, p21, Rb, GADD45 and p53R2 can overcome apoptosis.[200-203] p21 not only promotes growth arrest, but also antogonizes apoptosis by inhibiting the apoptosis-regulating kinase 1 (ASK1) and JNK1/SAPK.[204] In overall, the status of the G1 and G2 checkpoints is critical for determining the cellular fate in response to stress.

Growth and Survival Factors

Induction of growth arrest rather than apoptosis is favored by the presence of survival factors, such as cytokines and growth factors.[8] Two major survival pathways have been implicated in prevention of apoptosis. First, a cross talk exists between growth signaling pathways and members of the Bcl-2 family. For instance, IL-3 dependent phosphorylation of Bad leads to its sequestration, with subsequent release of free Bcl-2 and Bcl-X_L.[205] The second mechanism involves the induction of *mdm*2 by growth factors, such as *b*FGF, IGF-1, and the thyroid hormone,[206-208] and by the Ras pathway.[195]

Concluding Remarks

A big leap has been made towards a deeper understanding of how p53 is regulated at the molecular level. The major mechanism for controlling the p53 response is at the level of protein stability. The tight inhibition of p53 by Mdm2 has to be relieved in order for p53 to accumulate and be activated. It is difficult to estimate the relative contribution of protein destabilization versus inhibition of activities, for the overall negative effect of Mdm2 on p53. Future experiments under physiological conditions are needed. Different pathways are triggered in response to different stresses, and the various steps in these pathways are constantly being dissected. Often multiple and parallel pathways are elicited in order to achieve a coordinated and rapid response. This concept is well exemplified by the activation of ATM in response to DNA damage, which not only phosphorylates p53 and Mdm2 directly, but also activates concurrently several positive regulators of p53 including Chk2, c-Abl, NBS1 and BRCA1. This amplifies the stress signal leading to p53 activation and ensures the transmission of the signal even if one or more pathways are defective. The post-translational modifications of p53 modulate the auto-regulatory feedback p53/Mdm2 loop, but also monitor the fine-tuning of p53 transcriptional activities. These modifications, among many other factors, influence the cellular decision to die or pause. In contrast to our increasing knowledge how p53 is triggered in response to stress, we know very little about how the signaling for p53 activation is being turned off. For example, how the completion of DNA repair signals for p53 deactivation? Does it involve specific phosphatases? How they are regulated and what are their targets?

Relatively little progress has been made towards the understanding of how p53 kills cells. No one major pathway has been defined, but rather combinations of many parallel pathways are probably required to fulfill this task. The ability of p53 to kill defective cells with genomic instability, or with aberrant oncogenic events, is probably the most critical function of p53 as a

tumor suppressor. The role of p53 in the G1 checkpoint, and in the maintenance of the G2 checkpoint, minimizes the accumulation of genetic abnormalities.

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CHAPTER 7

Functional Interactions Between BRCA1 and the Cell Cycle

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Introduction

The onset of breast cancer in women is one of the most devastating diseases known today, afflicting approximately one in nine women in Western countries.[1] In families that inherit breast and ovarian cancer, BRCA1 mutations account for close to 100% of resultant cancers, and in pedigrees that solely inherit breast cancer, BRCA1 alterations are present in nearly two-thirds of the families.[2] These findings have led to the terminology of BRCA1 as a true tumor suppressor. Its discovery in 1994 initially did not lead to any insights into its functions, as the domains of the 220kDa protein were not exceptionally homologous to any known proteins.[3] However, research into the function of BRCA1 has yielded several theories regarding its purpose in normal cells. Identification of interacting proteins, production of antibodies against the protein, development of knockout and transgenic mouse models and comparisons between BRCA1 wild type and mutant expressing cells has assisted in placing functional characteristics with the BRCA1 protein. Among other qualities of BRCA1, it has become clear that it is influenced by and affects directly the position of the cell cycle. From phosphorylation to subcellular localization, protein-protein interaction to transcription activation, it has become clear that BRCA1 activities are closely related with cell cycle events. The transition from phase to phase in the mammalian cell cycle intimately involves the BRCA1 tumor suppressor.

BRCA1 Protein and mRNA during the Cell Cycle

Detection of BRCA1 transcripts initially did not identify a cell cycle component to its regulation. However, a study looking specifically at the status of BRCA1 mRNA in G0 cells found that the transcript was greatly reduced[4]. While expression was high in exponentially growing cells, withdrawal of growth factor from human mammary epithelial cells resulted in a disappearance of BRCA1 altogether. In addition, senescent cells also had dramatically reduced BRCA1 transcript. The lack of BRCA1 transcript in non-dividing cells led to the notion that this may be a component of the cell division machinery. At least in the case of senescence, recent reports of the ability of p53 to repress the transcription of BRCA1 may underlie this result[5,6]. Another contributor to the regulation of BRCA1 mRNA expression is the pRb-E2F complex[7]. The promoter of BRCA1 contains several E2F binding sites and pRb is able to repress transcription from the BRCA1 promoter in an E2F dependent fashion.

Development of antibodies against the full length BRCA1 protein further established its role in the cell division process. Several papers described the shift of the BRCA1 protein band to a higher mobility upon the onset of DNA replication, or if cells had been arrested in S phase by such agents as hydroxyurea[8-11]. This shift was apparently due to phosphorylation, as addition of phosphatase to extracts resulted in a collapse of the band to its normal state, and

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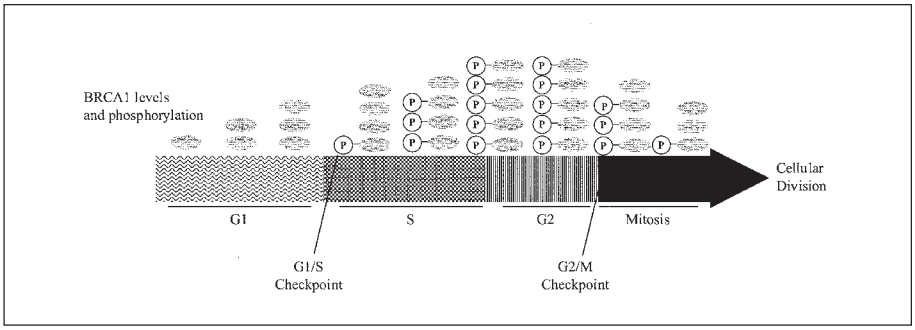

Fig. 1. The subcellular localization of BRCA1 changes with respect to the cell cycle In G1 phase of the cell cycle, BRCA1 is typically expressed in low amounts and what protein is Present, is distributed ubiquitously throughout the nucleus (Black is the cytoplasm, dark green represents the nucleus and a relatively low level of BRCA1 expression). As the cell progresses towards DNA replication, BRCA1 expression increases and forms foci along replication forks in the genomic DNA (bright green dots represents high concentration of BRCA1 protein isolated to regions of replication in the nucleus). Expression of BRCA1 remains high through G2 phase And then relocalizes to the centrosomes after nuclear envelope breakdown (bright green dots represent high concentration of BRCA1 protein at the centrosomes extending microtubules to the condensed chromatin). During spermatogenesis and oogenesis, cells enter meiosis at which time BRCA1 relocalizes to the synaptonemal complexes (bright green dots represent high concentration of BRCA1 protein on the condensed chromatin.

phosphorylation occurred predominantly on serine (Fig. 1). The phosphorylation of BRCA1 continued throughout S and onto the G2/M phases, after which it was progressively dephosphorylated. Blockage of cell cycle progression also resulted in phosphorylation, either at the G1/S border or at G2/M by treatment with colchicine. Later, BRCA1 was found to posses a cdk2 phosphorylation site at serine 1497.[12] This site was found to be efficiently phosphorylated in vitro by cdk2 complexed with either cyclin A or E. Kinases complexed with cyclin D have also been shown to phosphorylate BRCA1[9] Therefore, at least one of the kinases that force the hyperphosphorylation of BRCA1 at S phase is a major component of the cell cycle machinery. To date, there exist several other kinases that are capable of BRCA1 phosphorylation including casein kinase 2, DNA damage responsive kinases such as ATM, ATR, hCds1 and the AKT kinase as stimulated by heregulin.[13-20] Whether these kinases also play a role in cell cycle mediated alteration of BRCA1 remains to be tested. Also, it remains to be seen if cdk2 is capable of phosphorylating BRCA1 as late as G2/M phase—a phase that retains high levels of phosphorylated BRCA1 yet has classically been thought to possess low levels of cdk2 kinase activity. One report suggests that BRCA1 is actually predominantly tyrosine phosphorylated at G2/M stages, so there may be other cell cycle regulated kinases that are able to affect BRCA1 phosphorylation status at different stages of the cycle.[21] Thus far, the only concrete effect of phosphorylation on BRCA1 to be shown has been a change in protein-protein interaction with such regulators of its transcriptional activity as CtIP.[22] There exists correlative evidence suggesting that phosphorylation may affect subcellular localization and the conferring of sensitivity to DNA damage,[8,16,19] but no data thus far has placed a functional significance on cell cycle-dependent phosphorylation of BRCA1.

Subcellular Localization

One of the first reports describing a cell cycle-dependent phosphorylation phenotype of BRCA1 also noted a peculiar cellular distribution of BRCA1 (Fig. 2). In cells that were undergoing DNA synthesis, BRCA1 protein appeared as foci when immunofluorescence staining was performed.[8] When S phase was interrupted by treatment with hydroxyurea or DNA dam-

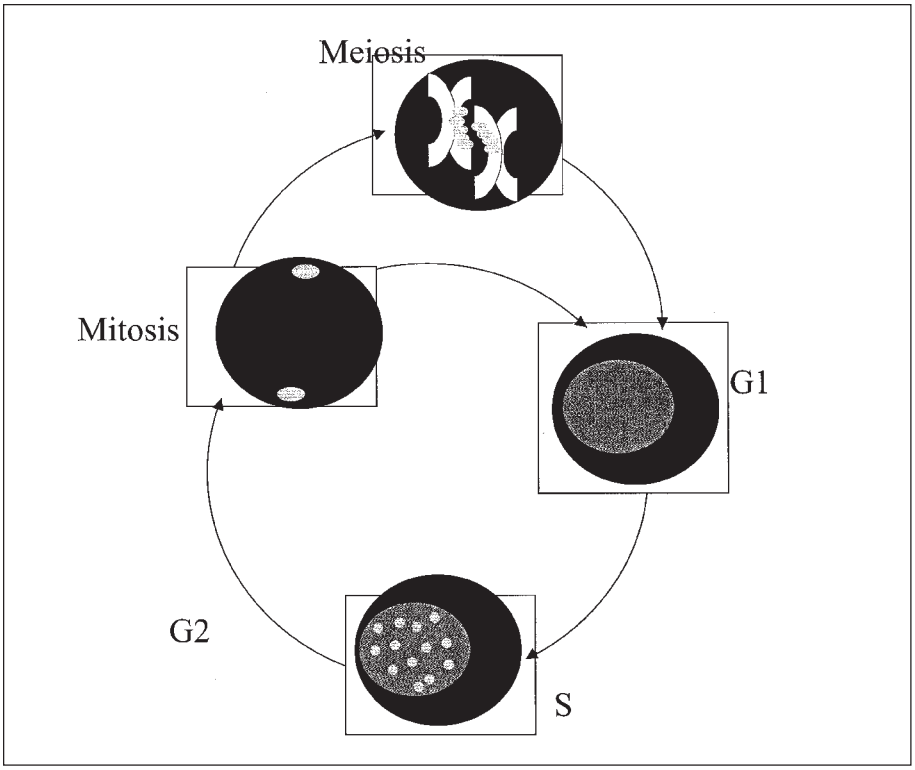

Fig. 2. Accumulation and phosphorylation of BRCA1 during the cell cycle. In early G1 phase, BRCA1 protein is expressed at very low levels and is at best underphosphorylated. As the cell progresses towards the DNA replication checkpoint, protein levels accumulate and begin to be phosphorylated. At the onset of S phase, BRCA1 becomes increasingly phosphorylated by, among other possible kinases, cdk2. This level of phosphorylation status remains high through G2 phase and then becomes progressively dephosphorylated during mitosis.

aging agents, these foci dispersed, indicating a correlation of the foci with ongoing DNA replication. Proteins that are known to associate with BRCA1 such as BARD1 and Rad51 colocalized with BRCA1 to these foci. Interestingly, during S phase these foci also colocalized with PCNA positive replication structures, suggesting that BRCA1 containing complexes are an integral part of the DNA replication machinery present at the replication fork. It may be that BRCA1 is essential for maintaining high fidelity replication in concert with its associated repair proteins such as Rad51.

At another phase of the cell cycle however, BRCA1 possesses an entirely different localization. In mitosis, the nuclear membrane breaks down, allowing the normally nuclear localized of BRCA1 to distribute itself around the cell. One area of concentrated BRCA1 protein is the centrosome.[23] Immunofluorescence localizes BRCA1 to the polar ends of the cell during mitosis and is able to be immunoprecipitated with antibodies against gamma tubulin, a centrosome component. Interestingly, the gamma tubulin precipitated form of BRCA1 is hypophosphorylated. This is at odds with previous data suggesting that phosphorylation of BRCA1 continues throughout S and into G2/M. Perhaps there exist multiple populations of BRCA1 in the cell. It is of note that microtubule destabilizing agents such as nocodazole induce a strong shift in phosphorylation of BRCA1,[10] raising the possibility that this phosphorylation induction may dissociate

BRCA1 from the microtubule organizing centers such as the centrosome. It will be interesting to see if BRCA1 remains associated with the polar ends of the cell after treatment with colchicine or nocodazole.

In meiotic cells, BRCA1 also has specific cellular localization. Along the chromosomes during a period when synaptonemal complexes form, BRCA1 is isolated to regions that could be undergoing recombination. [24]This is consistent with the induction of BRCA1 that is seen during this process. Interestingly, the DNA repair protein also colocalizes with BRCA1 during this meiotic process. Perhaps the two proteins are intimately involved in the proper exchange of genetic information between homologous chromosomes in spermatocytes or oocytes.

Activity at Cell Cycle Checkpoints

One aspect of subcellular localization of BRCA1 remains constant, regardless of cell cycle phase. It is always involved in the protection of genomic DNA. Two major cell cycle checkpoints that are required for genomic stability are DNA replication, where the lack of fidelity could result in a mutation that may be deleterious to the daughter cell, and mitosis, where the separation of sister chromatids must be performed carefully lest the distribution of unequal amounts of genetic information be passed on to each cell at division. As it turns out, through knockout and overexpression models, BRCA1 seems to be an integral component of both of these checkpoints.

The first attempt at eliminating BRCA1 from the genomes of mice resulted in very early embryonic lethality.[25] The complete lack of BRCA1 in developing mice forced death at embryonic day.[7-8] What was interesting about these embryos is that when checked for expression levels of p21WAF1, the amounts were staggeringly high, indicative of the activation of the G1/S checkpoint. This finding suggested that sufficient DNA damage had occurred in the absence of BRCA1 enough to activate the G1/S checkpoint. BRCA1 itself may also be involved in activating this checkpoint when it is present in cells. Overexpression of BRCA1 has been shown to cause cell cycle arrest, but this effect requires the presence of p21WAF1 or pRb, both proteins that are intimately involved in the G1/S checkpoint.[26,27] Therefore, while it is clear that the G1/S checkpoint is still intact in the absence of BRCA1, some events that are necessary to take place (or to avoid) require a functional BRCA1 protein.

After the disastrous effect deletion of BRCA1 had on embryogenesis, a few studies sought to find types of BRCA1 knockouts that would allow development of embryos, at least until fibroblasts could be harvested. It has long been known that a truncated form of BRCA1, lacking the coding sequence from exon 11, is expressed in cells, albeit to a much lower level than full length and is mostly only expressed in developing embryos.[28] One study developed a transgenic mouse that only expressed this truncated form of BRCA1, called BRCA1Δ11. Mouse embryo fibroblasts derived from these animals senesced much faster than wild-type cells and harbored a plethora of genomic abnormalities. The chromosomal alterations seemed to be the result of unequal recombination and breakage—two anomalies that predominantly occur during mitosis. Indeed, these fibroblasts treated with DNA damaging agents failed to enact their G2/M checkpoint, and progressed into mitosis as if there was no chromosomal damage. One possible factor in the increase of chromosomal breakage in BRCA1Δ11 MEFs was the amplification of centrosomes in mitotic cells. Pulling on chromatids in several directions as opposed to just to polar ends of the dividing cell could certainly have an effect on the state of the chromosomes after anaphase. As previously mentioned, the centrosomes are a prime location for BRCA1 during this process—perhaps the lack of full length protein at these complexes could account for the amplification. The overall lack of G2/M checkpoint control pointed clearly at the involvement of the exon 11 region of BRCA1 as an absolutely necessary factor in qualifying cells for division. The overexpression of the C-terminus of BRCA1 in normal breast epithelial cells has also been shown to adversely affect G2/M checkpoint control.[29]

From this data it is then clear that cells lacking a wild-type, DNA damage-responsive BRCA1 proceed throughout the cell cycle past mitosis regardless of the anomalies that occur during mitosis. These aberrations pile up and lead to a cell that is genetically incapable of

dividing. In the case of the G1/S checkpoint, BRCA1 is apparently not required to halt the cell cycle in the case of damage; however, it is necessary for proper repair of damage that may occur during or prior to S phase. On the other hand, in human cancer, mutations in BRCA1 lead to unrestricted cell growth, therefore, these transgenic and knockout models do not necessarily reflect the true in vivo nature of cancerous BRCA1 protein. In breast cancer, BRCA1 is not entirely deleted, usually mutations result in single amino acid changes in the N-terminal to middle portions of the protein or in small truncations at the C-terminus. Such miniscule mutations may permit semi-normal function of a particular cell, yet at the same time allow genetic alterations here and there to slip by the damage sensing that is otherwise detected by BRCA1. The right (or wrong, depending on your point of reference) mutations left undetected could cut the brakes on the speed of cell growth.

Interactions with Cell Cycle Proteins

While being post-translationally modified by the cell cycle machinery and also controlling critical steps of the checkpoint pathways, it seems logical that BRCA1 would associate with proteins that are part of this process. Following the findings that BRCA1 is phosphorylated in a cell cycle dependent manner, a number of reports detailed the interaction of BRCA1 with cyclins, cyclin-dependent kinases, E2Fs and the Rb protein. Not surprisingly, BRCA1 binds cyclin-dependent kinase 2, and interacts with it as an active kinase, as immunoprecipitation of BRCA1 co-precipitates with kinase activity[12]. The activating cyclin in this complex bound to BRCA1 appears to be cyclin A. Whether or not cyclin E, another cyclin Bound to cdk2 that allows phosphorylation of BRCA1 in vitro, is also bound to BRCA1 in cells remains to be seen. A slew of other cell cycle machinery proteins have also been described to bind BRCA1 including cdc2, cyclins B and D, cdks 2 and 4 and E2F4, however the functional significance of these interactions has yet to be determined.[30]

While BRCA1 is dependent, at least in some cell types, on the presence of pRb to arrest cell growth,[27] BRCA1 is also able to interact with pRb,[31] BRCA1 was found to interact directly with pRb as well as the pRb interacting proteins RbAP48/46. This binding also led to an indirect association with histone deacetylase 1 (HDAC1), significant in that there has been much speculation as to the involvement of BRCA1 in transcriptional control. This association provides at least an indirect link to an enzymatic process that has been definitively shown to be involved in transcription. The effect these interactions have on the progress of the cell cycle or with the activity of pRb has not been found. It will be interesting to see if the phosphorylation of pRb at the G1/S border, approximately the same temporal location as BRCA1 phosphorylation, has any effect on the interaction with BRCA1.

A few other proteins that were not originally thought to play a role in cell cycle control also appear to associate with BRCA1 in a cell cycle specific manner. BARD1, a protein that is structurally similar to BRCA1, was one of the first proteins found to bind directly to BRCA1.[32] The nuclear dot pattern that is characteristic of BRCA1 during S phase also is true for BARD1. This foci formation of BARD1 only occurs during S phase, and the dots are overlapping with BRCA1 foci, indicating that association of the two proteins may be cell cycle specific.[24,33] The overall expression of BARD1 however appears to be ubiquitous throughout the cell cycle, therefore it is likely that post-translational modifications such as phosphorylation may be a determining factor for this association. While clearly being a true binding partner to BRCA1 in vivo, the function of BARD1 and the significance of BRCA1 interaction remains elusive. The copurification of BARD1 with CstF-50, an mRNA stabilization factor, could be a lead on a possible involvement in BRCA1 transcriptional control.[34]

The BRCA1 binding protein CtIP has been characterized as a protein that is able to inhibit the transcriptional activation of promoters such as p21WAF1 by BRCA1.[35-37] It is in a complex with BRCA1 and BARD1, but in contrast to BARD1, CtIP is in fact expressed in a cell cycle dependent manner, roughly mirroring the expression pattern of BRCA1.[37] The interaction of the two proteins is therefore cell cycle specific. While inhibiting BRCA1's transcriptional

activity, the association has recently been shown to be broken by phosphorylation of BRCA1 by the gamma-irradiation responsive kinase ATM.[22] The removal of CtIP allows BRCA1 transcription activation to proceed. As this activation of BRCA1 is true for ionizing radiation, another study has found that the CtIP interaction is stable throughout several other DNA damaging stimuli such as UV, adriamycin or hydrogen peroxide.[37] Whether BRCA1 transcription activity is affected by these other treatments is yet to be known.

Transcription of Cell Cycle Genes

It is well established that BRCA1 is likely involved in cell cycle checkpoint maintenance and/or DNA damage sensing. However, another faction of BRCA1 research is the link of BRCA1 to transcription of specific genes (Fig. 3). Early on, BRCA1 was found by biochemical purification to be associated with the RNA polymerase holoenzyme and to induce transcription from a synthetic promoter when tethered to it.[38,39] Nevertheless, this data merely associated BRCA with general transcription, not differential specific activation or repression. Indeed, no evidence had been shown that BRCA1 possesses a promoter binding sequence nor had it been able to bind DNA directly. However, much work since then has suggested that the expression of BRCA1 forces changes in the expression patterns of certain genes that are not so coincidentally linked to processes it has been proven to be involved in such as DNA damage response and cell cycle progression. BRCA1 has been shown to be bound to such transcriptional regulators as p53, c-Myc, the estrogen receptor and p300.[40-43] Its effect on each of these proteins's activity is what one would expect from a tumor suppressor—co activation of p53, inhibition of c-Myc, etc.

Activation of the p21WAF1 gene expression was the first in a line of BRCA1 regulated genes to be discovered.[26] Overexpression of BRCA1 strongly activates the p21WAF1 promoter and interestingly is not dependent on p53. Expression of BRCA1 from an exogenous promoter, as previously mentioned, is able to cause growth arrest in most cell lines. This arrest in G1 phase is dependent on the presence of p21WAF1, as expression of BRCA1 in isogenic cell lines lack p21WAF1 are able to progress into DNA replication and eventually arrest in G2 phase.[48]

Two reports describing expression of BRCA1 in different cell lines identified the DNA damage response gene GADD45 as a strong target of BRCA1 as well. In both, an induction of GADD45 mRNA was clearly visible by northern blot analysis after BRCA1 transcript induction.

In one case, BRCA1 induction had an apoptotic effect in the U2OS cell line by use of a tetracycline inducible system.[44] As GADD45 had been previously shown to activate the proapoptotic c-Jun N-terminal kinase a link was drawn between JNK and subsequent apoptosis, by coexpression of a dominant negative mutant of JNK and a concomitant decrease in apoptosis seen by BRCA1 expression. However, recent evidence has suggested that GADD45 in fact is not involved in JNK activation and that GADD45 is not a component of apoptosis induction in vivo.[45-47] Therefore, whether BRCA1 causes apoptosis by activating GADD45 expression, or if in fact this is a cell-type specific effect has yet to be determined.

In another case, an adenovirus expressing BRCA1 was infected into several different cell lines with varying cell cycle changes, but no apoptosis.[48] A common theme among all the cell cycle changes in each cell type was an increase in the G2/M phase content. Interestingly, GADD45 knockout mice possess a defective G2/M checkpoint, and the involvement of BRCA1 in the maintenance of genomic stability at mitosis has already been established.[28,47] Therefore, it is possible that BRCA1 may in part induce the GADD45 protein in the interest of activation of the G2/M checkpoint.

Cyclin B1 is also an apparent target of BRCA1, however its expression levels are decreased by the exogenous expression of BRCA1.[48] Again, the links to the involvement in G2/M phase control are evident. Upon the detection of anomalies in mitosis, the first step in allowing repair to proceed is to halt progression of the cycle. The inactivation of cdc2 kinase by depletion of the activating cyclin B1 is one way to accomplish this goal. In this study, a G2/M arrest induced by BRCA1 overexpression was found to be abrogated by coexpression of exogenous cyclin B1, indicating that one way by which BRCA1 is able to arrest the cell cycle at G2/M

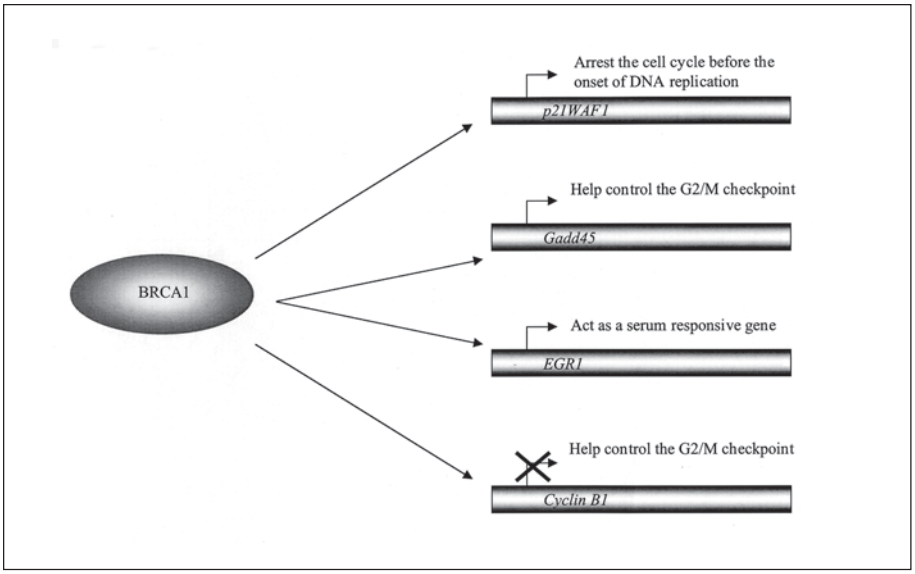

Fig. 3: Activation of genes by BRCA1 relevant to the cell cycle. BRCA1 has been shown to control the expression levels of four genes—p21WAF1, GADD45, Cyclin B1 and EGR1—that have implications on checkpoint activation. The p21WAF1 and EGR1 genes are involved in G1 phase of the cell cycle and may effect the onset of a G1 block prior to DNA replication in the face of DNA damage. The induction of GADD45 and repression of Cyclin B1 may represent an attempt by BRCA1 to halt progression towards mitosis.

phase is by repression of cyclin B1. It will be interesting to see the possible involvement of the histone deacetylase 1 enzyme bridged to BRCA1 by RbAP46/48 in this process.

The induction of the serum responsive, early response growth factor 1 (EGR1) has also been found to be induced by BRCA1 in array screening for BRCA1 targets,[44] EGR1 belongs to a group of proteins that are involved in the progress through G1 phase of the cell cycle following growth factor stimulation, which includes c-Myc, c-Jun and c-Fos. These other immediate early genes however are not induced by BRCA1. The induction of EGR1 by BRCA1 may be indirect, owing to its late upregulation and abrogation of the JNK pathway.

Finally, the physical link of BRCA1 to the regulatory regions of these genes that it is proposed to affect has been lacking. However, a recent finding of a protein, termed ZBRK1, has provided some insight into this necessary interaction,[49] BRCA1 binds directly to ZBRK1, which in turn binds to a consensus DNA sequence defined as GGGxxxCAGxxxTTT. Interestingly, this sequence resides in many of the genes described in recent papers to be up or down regulated as a result of BRCA1 expression. These include p21 (3 sites), GADD45, Gadd153, Ki-67 and EGR1. Co expression of ZBRK1 and BRCA1 were found to actually repress the GADD45 promoter, contrary to what one would expect given previous findings of activation of GADD45 expression by BRCA1. Thus far, it is hypothesized that overexpression of BRCA1 may titrate ZBRK1 away from the promoter, allowing transcription to occur. In vivo, the situations may be different, as they involve such other events as phosphorylation by a number of kinases, binding to other repressors such as CtIP and cell cycle specific localization.

Conclusion

The links between BRCA1 and the cell cycle have been made clear with solid data published over the last few years. Advances in the field have allowed visualization of BRCA1 localization during the cell cycle, during mitosis, meiosis and DNA replication that provide insight

into how BRCA1 functions with respect to the cell cycle. Expression at both the mRNA and protein levels have also been linked to this.

Further study into this association between BRCA1 and the cell cycle will allow delineation such issues as whether BRCA1 is a regulator or is regulated by the progression of the cell cycle, how phosphorylation affects the localization and function of BRCA1 during this progress and if transcriptional control by BRCA1 is a requirement for the normal transition from phase to phase of the mammalian cell cycle clock.

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CHAPTER 8

The Role of *FHIT* in Carcinogenesis

Yuri Pekarsky, Kay Huebner and Carlo M. Croce

Abstract

Tumor suppressor genes and oncogenes can be identified by positions of chromosomal translocations (in leukemia and lymphomas) and by detection of homozygous deletions and loss of heterozygosity (in solid tumors). Using these approaches, we identified a specific locus from the short arm of chromosome 3, cloned, and characterized the *FHIT* gene at 3p14.2 which is involved in chromosomal abnormalities in most common human tumors. In lung cancer associated with smoking, inactivation of *FHIT* occurs very early in tumor development. In other tumors, such as clear cell renal carcinoma and breast carcinoma, this inactivation occurs in later stages of tumor progression. Thus, evaluation of *FHIT* expression in premalignant lesions and tumors may be important in the diagnosis and prognosis of human cancer

Chromosomal Changes in Cancer

Carcinogenesis is a complex process which includes consecutive genetic changes affecting oncogenes and tumor suppressor genes. Most human malignancies show multiple genetic abnormalities and are heterogenous even if they originate from a single cell. Therefore, different cells of the same tumor may be affected differently by chemotherapy, gene therapy or radiation therapy.

Chromosomal rearrangements are associated with human malignancies involve most human chromosomes.[1,2] Four major types of such cytogenetic rearrangements have been observed: deletions, amplifications, translocations and inversions. Most human leukemias and lymphomas show consistent chromosomal rearrangements, mostly translocations or inversions, resulting in activation of oncogenes[3,4] or in loss of function of tumor suppressor genes,[5] causing malignant transformation.

In solid tumors, chromosomal deletions cause inactivation of tumor suppressor genes. This inactivation usually results in abnormal cell cycle control and/or increased cell survival leading to malignant transformation. A prototypical example of such loss is observed in human retinoblastomas where a deletion within the 13q14 region causes the inactivation of the retinoblastoma tumor suppressor gene.[1] Other such examples are deletions of the p53 gene and Wilm's tumor gene.[6] In many of these cases, the first copy of the gene is deleted while the second copy is mutated (mutations can be somatic or germline). These genetic changes result in inactivation of tumor suppressors in malignant cells.[7] Although the development of some solid tumors is caused by the inactivation of known tumor suppressor genes, the initiating events in the most common human cancers, including breast, prostate and lung cancer, are not known.

The identification and understanding of the earliest genetic changes that initiate transformation in solid tumors and detection of these events in premalignant lesions may result in the development of new therapeutic approaches (or novel drugs) to destroy premalignant cells providing new opportunities for cancer prevention. In addition, we could use the proteins

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encoded by the genes involved in these early steps of tumor development (or their biochemical pathways) as targets for novel therapeutical agents.

FHIT Loci is the Target of Chromosomal Abnormalities at 3p14.2

Chromosomal deletions and loss of heterozygosity involving the short arm of chromosome 3 (3p) have been described as a frequent event in most common epithelial tumors such as breast, lung and kidney cancers.[8-12] Cytogenetic analysis of bronchial dysplastic lesions[11] and lung tumors[10] has shown the frequent involvement of the short arm of chromosome 3 in these malignancies. Therefore it seemed possible that rearrangements in gene(s) on the short arm of chromosome 3p may occur in preneoplastic lesions (bronchial dysplasia) causing the development of malignant tumors. Although four major regions of 3p (3p25, 3p21.3, 3p14.2 and 3p12) are involved in allelic losses in cancer, the region 3p14.2 attracts most of the interest. In addition to its involvement in cancer deletions, this region contains a familial kidney cancer-associated 3p14.2 translocation break t(3;8)(p14.2;q24),[13] most common human fragile site, FRA3B,[14] and papilloma virus integration sites[15] (Fig. 1).

To identify gene(s) at 3p14.2 affected by these alterations, it was necessary to define precisely the region involved in deletions. First, we investigated the loss of heterozygosity (LOH) of gastrointestinal and kidney malignancies, evaluating the presence of markers on 3p in the DNA of tumor tissues and their normal counterparts. The 3p14.2 region is often involved in hemi-and homozygous deletions in human tumors, thus narrowing the region of interest to several hundred kilobases of DNA.[16] We also determined that this critical region overlapped with the one involved in homozygous deletions in tumor-derived cell lines and primary tumors and was located very close to the t(3;8)(p14.2;q24) chromosome translocation breakpoint in a hereditary renal cell carcinoma.[16] To identify gene(s) located in this narrowed region we constructed a cosmid contig covering the region and used this contig in exon trapping experiments. A single exon was identified then subjected to RACE (rapid amplification of cDNA ends) analysis leading to the discovery of 1.1 kb ubiquitously expressed FHIT. This 1.1 kb mRNA encoded a 17 kD FHIT protein containing 147 amino acids and showing ~70% similarity to a diadenosine 5′,5"'-P1,P4-tetraphosphate (Ap_4A) hydrolase, a member of a histidine triad protein (HIT) family from the fission yeast *Schizosaccaromyces pombe*.[16] This newly identified gene was named *FHIT* because it encodes a HIT family protein containing the most common human fragile site. Because of the similarity of FHIT and yeast (Ap_4A) hydrolase, *FHIT*t was tested for this activity. These experiments demonstrated that *FHIT* possesses Ap_3A (diadenosine 5′,5"'-P(1),P(3)-triphosphate) hydrolytic activity: it cleaved Ap_3A into ADP and AMP.[17] Physical mapping of 5′ and 3′ ends of *FHIT* mRNA revealed that *FHIT* is one of the largest human genes, it spans a 1-2 mb fragment of genomic DNA. Further genomic analysis revealed that *FHIT* contains 10 small exons, whereas exon 5 contains an initiating ATG codon (Fig.1). The most common human fragile site, FRA3B, a papilloma virus integration site and a translocation breakpoint of t(3;8)(p14.2;q24) associated with familial kidney cancer were mapped within the *FHIT* gene.[16] Some of the homozygous deletions within the *FHIT* locus observed in tumor derived cell lines are shown on Figure 1. Siha, a cervical carcinoma-derived cell line, has two overlapping allelic deletions, thus generating a small homozygous deletion in intron 4. Therefore, Siha cells do not encode a full-length *FHIT* mRNA nor protein. Similarly, Kato III, a gastric cancer cell line exhibits independent deletions in both alleles (Fig. 1), although these cells retain a copy of each *FHIT* exon. Thus, Kato III and Siha can not express a full-length *FHIT* mRNA or a FHIT protein.[18] Both, AGS, a stomach carcinoma-derived cell line and LS180, a colon carcinoma cell line exhibit three separate regions of homozygous deletions as shown on Figure 1.

Taking together the observation that some tumors and tumor-derived cell lines contain homozygous deletions in the *FHIT* gene and that translocation breakpoints of t(3;8)(p14.2;q24) are associated with familial kidney cancer and LOH in most common human malignancies, we conclude that *FHIT* indeed is a target of chromosomal abnormalities at 3p14.2 observed in human cancer.

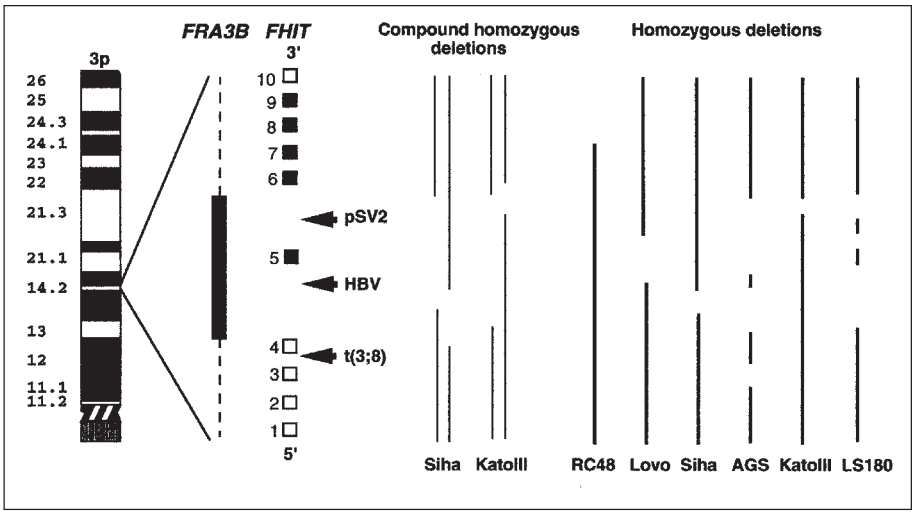

Fig. 1. The map of FRA3B/F*FHIT* loci at 3p14.2. The FRA3B region is showed by the solid area at p14.2. *FHIT* exons are numbered 1 through 10, coding exons are in black. Positions of viral integration sites and t(3;8) translocation are marked with arrows. Gaps in the lines represent *FHIT* locus in the tumor cell lines demonstrate deletions.

Inactivation of *FHIT* mRNA and Protein Expression in Cancer

Given that *FHIT* is involved in LOH and chromosomal deletions at 3p14.2, we carried out detailed studies of the *FHIT* mRNA and protein expression in cervical, stomach, lung, kidney, breast tumors, in both tumor-derived cell lines and primary tumors (Fig. 2). As determined by immunohistochemistry, FHIT is abundantly expressed in epithelial tissues of all human organs including lung, stomach and kidney.[19-21] As expected, immunohistochemical studies and immunoblot analysis of human malignancies demonstrated that tumors and cell lines expressing altered *FHIT* transcripts, with genomic rearrangements of the *FHIT* locus, usually do not express FHIT or express reduced levels of FHIT.[18-20] We investigated a large collection (474 cases) of stage 1 nonsmall cell lung cancers (NSCLC) by immunohistochemistry.[21] FHIT was not expressed in 73% of these tumors (Fig. 2), indicating a very high frequency of loss of FHIT expression in NSCLC (Table 1). A significant difference in the frequency of loss of its expression in adenocarcinoma (59%) versus squamous cell carcinoma (87%) has been found. Loss of FHIT expression was observed in 69% of large cell lung cancers. Interestingly, loss of FHIT expression was less frequent in tumors of nonsmokers than smokers (Table 1).

We also examined levels of FHIT in precancerous lesions by immunohistochemistry. Loss of FHIT was already apparent in bronchial dysplasia, a precancerous condition, indicating this is an early event in the development of lung cancer (Table 1). Interestingly, loss of the FHIT protein was more frequent and occurred earlier than alterations of the EGF receptor and more common than p53 mutation in lung cancer and preneoplastic lesions.[21] It has also been reported that allele loss at the *FHIT* locus is more frequent in the bronchial epithelium and tumors of smokers than nonsmokers and more frequent than loss at other tumor suppressor regions.[22]

Further studies have demonstrated that abnormalities in FHIT mRNA and protein in the most common human malignancies (Fig. 2). When compared to normal levels in epithelium, FHIT was absent or reduced in 67% of stomach tumors,[18, 20] 40-89% of kidney tumors,[16,18,23] 76% of cervical tumors,[24] 62% of pancreatic tumors[25] and 30%-70% of breast carcinomas.[26-28] Therefore, the *FHIT* gene is normally expressed almost exclusively in the epithelial tissues, an origin of most common human neoplasias. It is inactivated in more than 50% of these tumors, more frequently than any other known tumor suppressor gene.

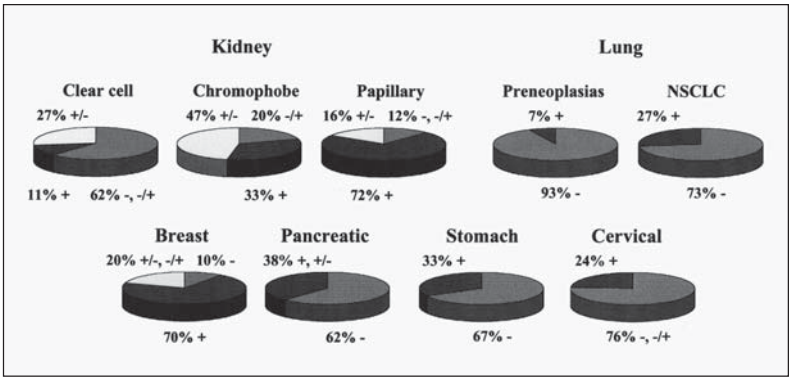

Fig. 2. Expression of *FHIT* in most common human tumors.

Despite the involvement of the *FHIT* gene in most common human cancers, molecular mechanisms of *FHIT* deletions remain unknown. To examine molecular basis of chromosomal fragility at 3p14.2, we sequenced 870 kilobases of the *FHIT*/FRA3B locus, spanning intron 3 through intron 7 of *FHIT*.[29,30] The sequence of the region surrounding exon 5, a center of the fragile site (Fig. 1), revealed that it is poor in Alu and rich in LINE 1 repeat elements. Interestingly, most of breakpoints that are involved in deletions in a variety of human tumor-derived cell lines occur in long terminal repeats (LTR) or LINE 1 elements larger than 1 kb suggesting these deletions may be caused by the homologous recombination between these elements.[29]

The Tumor Suppressor Activity of *FHIT*

Given that both *FHIT* alleles are frequently altered in human tumors and the translocation associated with hereditary kidney cancer disrupts one *FHIT* allele,[16] it is reasonable to consider *FHIT* as a tumor suppressor gene.[16]

To demonstrate that *FHIT* is a bona fide tumor suppressor,[31] the human *FHIT* cDNA was transfected into four *FHIT*-negative tumor cell. Then FHIT-expressing cells were implanted into nude mice. In these experiments, *FHIT*-expressing cells had lost their ability to form tumors, suggesting the tumor suppressor activity of *FHIT*.[31] Also, mutant *FHIT* protein with reduced enzymatic activity suppressed tumorigenicity, suggesting that FHIT enzymatic activity is not required for tumor suppression.[31]

Using adenovirus, *FHIT* cDNA was transduced into human lung cancer cells and head and neck carcinoma cells lacking FHIT expression.[32] In these cells but not in normal human bronchial epithelial cells, re-expression of FHIT inhibited cell growth, induced apoptosis and accumulation of cells in S phase.[32] Less than 20% of Ad-*FHIT*-infected cancer cells survived by day 7. Furthermore,in vivo tumorigenicity of H1299 human lung cancer cells was eliminated by infection with Ad-*FHIT* but not with the empty adenoviral vector. The expression of FHIT in H460, a FHIT-negative human lung cancer cell, inhibited cell growth and induced p53-independent apoptosis, suggesting proapoptotic functions of FHIT. [33]

The ultimate proof of a tumor suppressor activity is the development of neoplasias in knockout mouse models. Although it is still early to discuss the phenotype of *FHIT*-/- mouse, the encouraging results describing carcinogen induced tumors in *FHIT*+/- mice have recently been reported.[34] In this study FHIT +/+ and +/- mice were treated intragastrically with carcinogen nitrosomethylbenzylamine and observed for 10 wk posttreatment. A total of 25% of the +/+ mice developed adenoma or papilloma of the forestomach, whereas 100% of the +/- mice developed multiple tumors that were a mixture of adenomas, squamous papillomas, invasive carcinomas of the forestomach, as well as tumors of sebaceous glands. The visceral and sebaceous tumors, which lacked FHIT protein, were similar to those of Muir-Torre familial cancer syndrome.

Table 1. Loss of FHIT expression in stage 1 non small cell lung carcinomas and preinvasive bronchial lesions

Tumor Type	% FHIT negative	Total Cases
Bronchial lesions:		0
Dysplasia	85%	20
Carcinoma in situ	100%	25
Lung carcinoma:		0
Adenocarcinoma	57%	196
Squamous cell carcinoma	87%	233
Smokers	75%	451
Nonsmokers	39%	23

Taken together, results in vitro and in vivo imply that the *FHIT* gene is a tumor suppressor gene.

Toward *FHIT* Function

As discussed above, the FHIT protein in vitro possesses the Ap_3A hydrolase activity[17] and this activity is not required for tumor suppression.[31] Another study reported that mutant FHIT which can not cleave Ap_3A but suppresses tumorigenicity in vivo [31] still bind Ap_3A.[35] This suggests that the Ap_3A bound form of FHIT may be the active tumor suppressor.

To investigate whether the loss of the FHIT gene would cause any phenotype in *Drosophila* melanogaster and *C. elegans*, we cloned the *Drosophila* and *C. elegans* FHIT genes.[36] Although *Drosophila* and *C. elegans* FHIT mutants were not available, we discovered that both proteins occur as a fusion proteins of ~450 amino acids in which a C-terminal FHIT domain is fused with a novel 35 kD domain showing homology with yeast and bacterial proteins of unknown function and with plant nitrilases.[36] Therefore, the~450 amino acid *Drosophila* and *C. elegans* FHIT proteins that contain an N-terminal nitrilase domain and a C-terminal FHIT domain were designated as NitFHIT (Fig. 3). Like human FHIT, *Drosophila* NitFHIT possesses Ap_3A hydrolyzing activity.[36] Thus, in *Drosophila* and *C. elegans*. FHIT is a chimeric protein, possibly with a dual enzymatic function. We next isolated human and murine NIT homologs. These separate genes, designated as NIT1, are located on human chromosome 1q21 and mouse chromosome 1.

In several eukaryotic biosynthetic pathways, multiple steps are catalyzed by multi-enzymatic proteins containing two or more functional domains. In prokaryotes, the same steps are often carried out by a single enzyme with homology to individual domains of the corresponding eukaryotic protein.[37] For example, Gars, Gart and Airs are domains of the same protein in Drosophila and mammals. These domains catalyze different steps in de novo synthesis of purines. All three homologs (PurM, PurN and PurD) are separate proteins in bacteria. In yeast, Gars and Airs homologs (Ade5 and Ade7) are domains of a bi-enzymatic protein and the Gart homolog (Ade8) is a separate protein.[37] If domains of a multi-enzymatic protein are expressed as individual proteins in other organisms, these individual proteins participate in the same pathway.[38] This observation implies that FHIT and Nit1 may participate in the same molecular pathway in mammalian cells. Further functional studies are required to determine these pathway(s).

In a recent report,[39] the interaction between either wild-type or mutant FHIT and tubulin in vitrois described. Both wild-type and mutant forms of FHIT specifically bind to tubulin without causing nucleation or formation of microtubules, promoting their assembly to a greater extent than microtubule-associated proteins alone.[39]

In yeast two hybrid system, which identified proteins physically interacting with FHIT, the human ubiquitin-conjugating enzyme 9 (hUBC9) specifically interacted with FHIT.[40] The C-terminal region of FHIT is responsible for this interaction. Given that yeast UBC9 is involved in the degradation of S- and M-phase cyclins, FHIT may be also involved in cell cycle control through its interaction with hUBC9.[40] Additional biochemical studies are necessary to determine the exact role of FHIT in this molecular pathway.

Conclusions

To identify tumor suppressor genes and oncogenes involved in the development and/or progression of human cancer it is critical to determine their chromosomal regions. In leukemias and lymphomas, these regions can usually be determined by cytogenetic positions of chromosomal translocations. This approach was effective in the discovery of critical cancer genes.[3,4] In most solid tumors, chromosomal translocations are rare and different approaches such as detection of homozygous deletions and loss of heterozygosity are necessary.

By using a combination of these approaches, we identified a specific locus from the short arm of chromosome 3, cloned and characterized the *FHIT* gene at 3p14.2 involved in chromosomal abnormalities in most common human tumors. The *FHIT* gene is the second largest known human gene and contains a t(3;8) translocation in familial renal cell carcinoma and the FRA3B, the most common human fragile site.

In lung cancer associated with smoking, inactivation of *FHIT* occurs very early in tumor development.[21] In other tumors, such as clear cell renal carcinoma and breast carcinoma, its inactivation occurs in later stages of tumor progression.[23,28] Thus, evaluation of *FHIT* expression in premalignant lesions and tumors may be important for diagnosis and prognosis.

Re-expression of FHIT protein in human cancer cell lines causes apoptosis and suppresses tumorigenicity in vivo.[31-33] The study of the *FHIT* knockout mouse model proved that *FHIT* is indeed a tumor suppressor gene. Therefore, *FHIT* may be a candidate gene for gene therapy.

Future goals are to find biological functions of FHIT that affect cell growth, differentiation and cell death. This may lead to the development of novel approaches for diagnosis and treatment of common human cancers.

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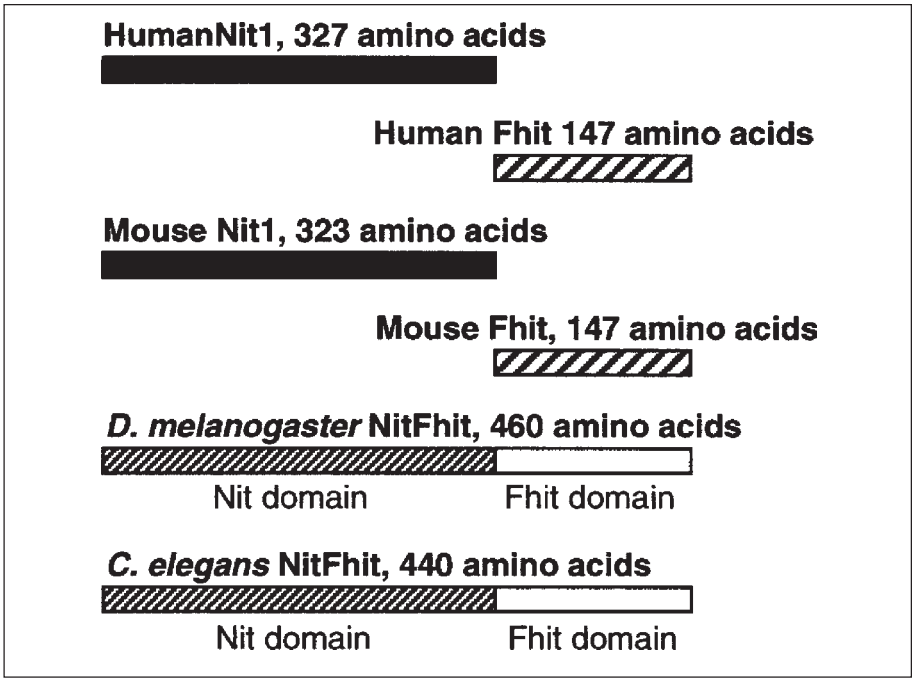

Fig. 3. Nitrilase and FHIT homologs are encoded as fusion proteins in Drosophila melanogaster and Caenorhabditis elegans.

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CHAPTER 9

Hypoxia and Cell Cycle

Rachel A. Freiberg, Susannah L. Green and Amato J. Giaccia

Introduction

Tumor initiation is dependent on several key changes in the requirements for cell growth. Three of the most important features that distinguish transformed cells from untransformed cells are the loss of senescence, anchorage independent growth, and loss of contact inhibition. Cells that adopt a transformed phenotype based on these criteria typically exhibit rapid growth as they have escaped regulation from both internal and external regulatory signals. This uncontrolled cellular growth leads to an accumulation of cells, which are initially genetically identical, but continued growth results in additional mutations which can lead to the outgrowth of transformed cell variants that possess a survival advantage in the context of the tumor microenvironment. When the tumor is less than 150 mM or approximately ten cells in diameter, the supply of oxygen and nutrients through passive diffusion is sufficient to support growth and metabolism of transformed cells. However, as tumor cells continue dividing unchecked, they exceed their ability to obtain sufficient nutrients and oxygen by diffusion alone from pre-existing blood vessels. As a result, regions of the tumor become hypoxic and start secreting mitogens such as VEGF (vascular endothelial cell growth factor) which can stimulate the migration of microvascular endothelial cells into the tumor region to form new microvessels. However, these new microvessels are often disorganized and malformed, resulting in inefficient oxygen and nutrient delivery. Ultimately, this process results in tumor containing areas that are either poorly perfused or subjected to cycling hypoxia through the opening and closing of blood vessels. Angiogenesis has now been recognized as a critical modulator of tumor cell expansion and angiogenic activity has also been implicated in the development of metastases.[1,2] Transformed cells unable to tolerate the nutrient and oxygen poor microenvironment become necrotic or apoptotic, and the cells that survive are selected to do so by their ability to resist apoptosis and reduce their oxygen requirements by cycling slowly or switching to glycolysis. In this way, hypoxia has been suggested to act as a selective pressure for the expansion of variants resistant to its growth restrictive conditions (Fig. 1).

A variety of publications have supported the hypothesis that human tumors possess hypoxic regions.[3-6] In addition to the increased likelihood of metastases, hypoxic tumors are associated with poor therapeutic response and poor prognosis for a patient regardless of the therapeutic modality.[4,6] For example, radiation therapy depends in part on the presence of molecular oxygen to mediate DNA damage.[7,8] Therefore the damage induced by radiation therapy in oxygen-deficient regions of a tumor is substantially reduced and can lead to a three-fold decrease in killing in response to radiation treatment. In contrast to radiation therapy, chemotherapeutic drugs are delivered systemically through the circulatory system and act best on rapidly dividing cells and those which are in close proximity to blood vessels. Since hypoxic tumor cells are poorly perfused and growth retarded, current chemotherapy protocols have exhibited limited therapeutic efficacy against hypoxic tumor cells.[9]

Cell Cycle Checkpoints and Cancer, edited by Mikhail V. Blagosklonny.

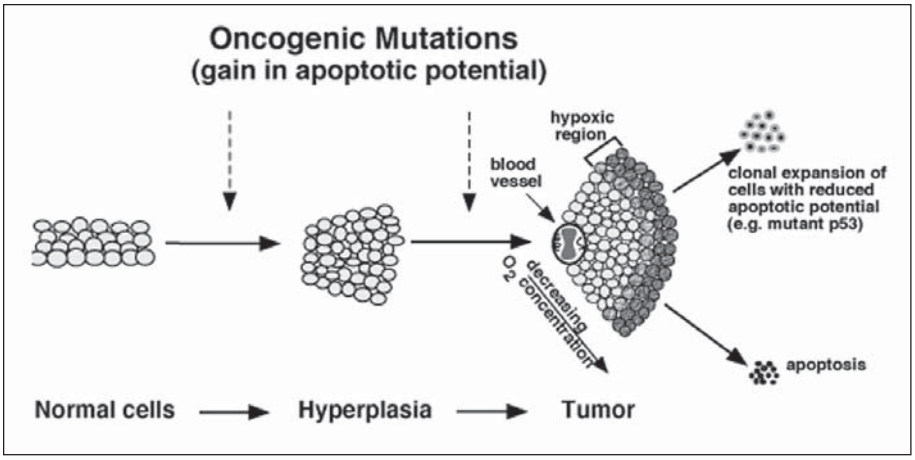

Fig. 1. Hypoxia as a selective pressure. Normal cells aquire mutations which allow some cells to escape cell cycle contol. Continued hyperplastic growth leads to accumulation of cells, some of which are too distant from blood vessels to obtain oxygen and nutrients through passive diffusion. Cells removed from proper growth conditions may either arrest and undergo apoptosis or continue growing at a reduced rate despite the lack of oxygen and other growth factors (reprinted with permission from Giaccia A. The influence of tumor hypoxia on malignant progression. In: Vaupel P, Kelleher D, eds. page 117).

Finally, cells located in hypoxic regions of tumors that are growth arrested possess varying abilities to re-enter the cell cycle upon reoxygenation, suggesting that growth arrest by hypoxia is reversible under certain conditions. As hypoxia plays important roles in both tumor response to therapy and malignant progression, it is essential to understand how hypoxia and reoxygenation modify cell cycle function and to identify the molecular mechanisms involved in this process. Although knowledge of the mechanisms by which the sensors of oxygen deprivation act to signal to cell-cycle checkpoint pathways is still incomplete, more extensive information exists on the downstream cell-cycle regulatory proteins that are modulated by a low oxygen environment.

Cell Cycle and Check Points

Untransformed cells progress through G_1, S, G_2 and M-phases of the cell cycle in a highly regulated manner. Starting in G_1, the cell undergoes a phase of growth in which it accumulates the necessary nucleotides and proteins needed for replication of DNA in S-phase. Upon the completion of DNA synthesis, cells enter the G_2-phase during which the cell continues growing and generating additional cellular components needed for cell division in M-phase or mitosis. During mitosis chromosomes condense, the nuclear membrane breaks down, chromosomes pair and align along the midline of the cell, and sister chromatids separate through the shortening action of the tubulin filaments attached to both the kinetochores of the chromosomes and the centrioles. Other cellular components such as the endoplasmic reticulum, Golgi apparatus and mitochondria divide somewhat randomly between the two daughter cells. This stage completes the cell cycle.

Cyclins and cyclin dependent kinases (CDKs) are the major effector proteins that are responsible for the progression through the stages of G_1, S, G_2 and M phases. (Fig. 2) Cyclins, as their name implies, are present at predictable and specific times in the cell cycle. CDKs are enzymes which are inactive as monomers, but become active subsequent to associating with a cyclin, being transported into the nucleus, and being phosphorylated by the CDK-activating kinase (CAK).[10] When the complex has carried out its function, it is transported from the nucleus to the cytoplasm and the cyclin is degraded via ubiquitin-mediated proteolysis. Other

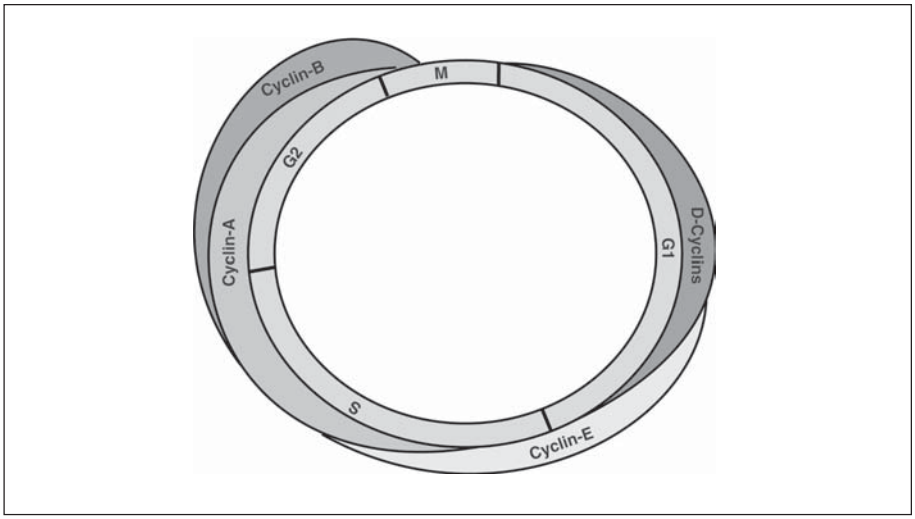

Fig. 2.Cyclin Expression. Cyclical expression of D, E, A, and B type cyclins allows precise control of the initiation and duration of different phases of the cell cycle. Cyclin D-family members are key to the progression through the G_1 phase of the cell cycle by facilitating the dephosphorylation of pRb. Continued depohsphorylation of pRb by Cyclin E is required for entry into S-phase of the cell cycle. Cyclin A and to a greater extent Cyclin B are both required for entry into mitosis.

important cell cycle regulators are the p53 and pRB tumor suppressor genes, which act through the transcriptional stimulation or inhibition of positive and negative growth regulatory genes.[11]

During the gap phases G_1 and G_2 preceding S and M phases respectively, the cell prepares itself by synthesizing many of the proteins needed to initiate and complete DNA synthesis and mitosis. Progression from one phase to the next is often dependent on the satisfaction of certain criteria; if the build-up or degradation of essential cell-cycle proteins does not proceed in a tightly regulated manner, a cell with all of its checkpoint machinery intact will remove itself from the cell cycle. Such cell cycle "checkpoints" are thought to have evolved to ensure proper replication and transmission of genetic material. For example, checkpoint pathways are triggered by nutrient deprivation, incomplete DNA replication, or improper chromosome segregation. Elegant studies from yeast to mammalian cells have demonstrated that "checkpoints" in the cell cycle occur in all phases, and typically act via the temporary inactivation of CDKs.

The role of checkpoint genes has been demonstrated fairly well in response to DNA damage. Two critical regulators of checkpoint response are the ATM and ATR proteins. ATM is triggered by DNA damage and phosphorylates p53 on serine 15. In contrast, ATR seems to be activated by perturbations in DNA replication, but can also phosphoylate many of the same substrates as ATM.[12-14] After cells are exposed to DNA damaging agents, p53 can either initiate cell cycle arrest through the transcriptional induction of the p21 cyclin kinase inhibitor or induce apoptosis.[15] In a low oxygen environment, checkpoint control is thought to play a pivotal role in ensuring that cell cycle progression does not occur under growth restrictive conditions until physiologic homeostasis (e.g., reoxygenation) is returned. Hypoxia, though a nongenotoxic stress, can lead to p53 phosphorylation on serine 15 and protein accumulation. This response is intact in cells deficient in ATM, suggesting that hypoxia does not utilize the same signaling pathway as DNA damage (E. Hammond, M. Kastan, and A. Giaccia, unpublished observations). Studies are currently ongoing to determine whether ATR plays a role in p53 regulation under hypoxic conditions. Interestingly, in cells exposed to hypoxic conditions, p53 is not necessary for, nor does it contribute to, cell cycle arrest.[16]

Although DNA damage can induce cell-cycle arrest in G_1, S and G_2 phases of the cell cycle through the activation of checkpoint pathways, it is unclear what the stimulus is for cell cycle arrest observed in response to hypoxia. While reports have proposed both biochemical and genetic mechanisms to explain cell-cycle arrest under hypoxic conditions, we still do not know how and if these mechanisms can be generalized or if they are specific for individual cell lines.

Hypoxia-Induced Arrest

In vivo, at the tumor level, there is conflicting evidence as to whether or not hypoxic regions of tumors exhibit a decrease in cell proliferation. In some cases, investigators have found that hypoxic tumor regions have fewer actively cycling cells[17] where others have found that hypoxic tumors proliferate at an increased rate.[18,19] Studies in which proliferation and hypoxia were simultaneously assessed in tumor sections showed that proliferating cells were proximal to blood vessels and hypoxic cells were found in regions that were distal to blood vessels.[17,20,21] Analysis of single cells from disaggregated tumors indicated that oxic regions exhibit substantially greater proliferation than hypoxic regions as assessed by in vivo incorporation of the thymidine nucleotide analog bromodeoxyuridine (BrdU).

Studies have also assessed the effect of hypoxic conditions on cellular proliferation rates in vitro by analyzing cellular incorporation of BrdU or ^{3}H-thymidine. These studies indicate that untransformed or transformed cells cultured under hypoxic conditions contain a greatly reduced number of BrdU positive S-phase cells or reduced amounts of ^{3}H-thymidine incorporation as compared to cells grown under oxic conditions.[22-24] Investigators utilizing both types of nucleotide incorporation have revealed that the stringency of hypoxia is an important determinant of the kinetics of cell cycle arrest. For example, mild hypoxia (i.e., 2% oxygen) has been found to enhance cell growth.[25] Additionally, there is little difference in cell cycle profile for some cells incubated in normoxic (21.0% oxygen) versus moderate hypoxic conditions (1.0% or 0.1% oxygen).[26] After 24 or 48 hours under moderate hypoxic conditions, immortalized mouse embryo fibroblasts (MEFs) exhibit no significant cell cycle inhibition as measured by BrdU or ^{3}H-thymidine incorporation. However, stringent hypoxia (oxygen tensions below 0.01% O_2) elicits a robust S-phase arrest response; under these conditions, DNA synthesis ceases within 5 hours and recovers within a few hours after reoxygenation. While cell cycle arrest induced by hypoxia is reversible, the extent of reversibility is reduced with longer or more stringent hypoxic treatments and is variable among different cell types.[27] In fact, most cells are able to resume DNA synthesis within 10 minutes to 3 hours after reoxygenation.[28]

Until recently, little has been published on the molecular mechanisms that govern the re-entry of hypoxic cells into the cell cycle. One report found that recovery of DNA synthesis after removal from hypoxic conditions is blocked by cycloheximide addition in Ehrlich ascites cells,[29,30] suggesting that synthesis of a rapidly degraded protein is necessary to resume DNA synthesis after arrest. In a separate study, re-entry into the cell cycle was associated with the reappearance of hyperphosphorylated pRb in T-47D breast cancer cells.[31] However, the investigators could only speculate whether the relationship between pRb phosphorylation and hypoxia-induced cell cycle arrest was causal or correlative.

Hypoxia-induced cell cycle arrest raises several important questions. What role if any does arrest play in protecting cells from the potentially deleterious effects of a low oxygen environment? Does hypoxia-induced arrest contribute to or protect the cell from genomic instability? Do hypoxia-arrested cells accumulate even more damage as they escape apoptosis and necrosis or sustain less damage because of the arrest? Reports in the literature have indicated that hypoxia and reoxygenation induce gene amplification. While this has been demonstrated in experimental systems, it is unknown whether the presence of hypoxia in human tumors is in any way related to increased genomic instability or gene amplification.[32,33] The implication of these studies is that S-phase arrest under hypoxic conditions may be somehow involved in gene amplification as well as in genomic instability. Additionally, hypoxia itself may not directly induce DNA damage, but may permit the accumulation or manifestation of DNA damage or

mutagenic events. Results recently reported by Denko et al[16] indicate that hypoxia induces cell-cycle arrest in human tumor cells through a different mechanism than DNA damage, and that loss of the p21 cyclin-CDK inhibitor does not lead to increased genomic instability under hypoxic conditions. The observation that loss of p21, which renders cells more sensitive to ionizing radiation induced genomic instability, does not increase genomic instability under hypoxic conditions is consistent with the finding in the same report that hypoxia induced G_1/S-phase arrest does not rely on the p53 tumor suppressor gene. Additional studies will be needed to more rigorously address the role of hypoxia-induced arrest in cellular protection against a low oxygen environment.

Mechanisms Underlying Cell Cycle Arrest by Hypoxia

Initial studies investigating the inhibition of cell proliferation seen in response to hypoxia focused on a biochemical mechanism for cell cycle arrest. Numerous biochemical studies were performed on Ehrlich ascites tumor cells, which are capable of proliferating in the peritoneal cavity of mice, an environment which is largely devoid of oxygen. When exposed to hypoxic conditions, Ehrlich ascites tumor cells that are in S-phase stop synthesizing DNA and cells in G_2 and M phase proceed into G_1-phase and undergo a G_1 arrest.[34-36] It has been proposed that these cells arrest in response to depletion of deoxynucleotides needed for the synthesis of DNA during S-phase. There are two oxygen-dependent enzymes, ribonucleotide reductase and dihydroorotate dehydrogenase, both of which are necessary for the completion of the deoxynucleotide biosynthetic pathway. The active site of ribonucleotide reductase, which converts ribonucleotides to deoxyribonucleotides, contains a tyrosyl radical that requires molecular oxygen for its regeneration. Dihydroorotate dehydrogenase is responsible for the conversion of dihydroorotate to orotate, an early step in the de novo synthesis of pyrimidines. The enzyme's activity is dependent on the mitochondrial respiratory chain and is thus linked to oxygen availability. Oxygen deprivation, therefore, could lead to S-phase arrest by directly preventing the synthesis of the building blocks needed for DNA replication. Further studies indicated that reduced DNA synthesis correlated with the suppression of replicon initiation and that chain elongation and maturation were both unaffected by hypoxia.[30, 37] Arrest found under hypoxic conditions could sometimes be observed at oxygen tensions as high as 2% and consistently at oxygen tensions below 0.2%.[30] However, it was found that the addition of exogenous deoxyribonucleosides (which can cross the cell membrane and be phosphorylated via an oxygen-independent pathway) allowed some cells to enter S-phase, but without normal progression of the cell cycle, strengthening the idea that hypoxia induced arrest may simply not be due to lack of nucleotides.[34,37-39] Taken together, these studies suggested that the arrest mechanism under hypoxic conditions was not totally biochemical in origin and may possess a genetic or epigenetic component.

In most cells that have been investigated, hypoxia causes a decrease in DNA synthesis, which may be accompanied by an accumulation of cells in the G_1-phase of the cell cycle. However, Chinese hamster fibroblasts, V79-379A, arrest in all phases of the cell cycle and thus its cell cycle profile under hypoxic conditions appears unchanged even when DNA synthesis has ceased.[22] CV-1P monkey kidney cells also arrest in all phases of the cell cycle.[40] More common is the cell-cycle profile displayed by many transformed and untransformed human and rodent cells in which the S-phase arrest is accompanied by an accumulation of cells in G_1.[27,41] Since G_1-and S-phase arrest are more commonly found in cells exposed to hypoxic conditions, current research has focused on the cell cycle components regulating the progression of cells through the G_1 and S-phases.

As discussed above, cyclins are responsible for progression through the cell cycle. In particular, the G_1-phase of the cell cycle is controlled by D-type cyclins. Expression of cyclin-D family members D_1, D_2 and D_3, can depend on the differentiation state of the cell as well as its histological origin.[42,43] Overexpression of Dcyclins causes the cell to progress through G_1 at an accelerated rate, and conversely, removal of these cyclins causes a G_1 arrest.[44,45] During early and mid G_1-phase, removal of growth stimulus can cause arrest prior to the initiation of DNA

synthesis. However, in late G_1 there is a point at which the cell is committed to progressing to S-phase, past which the removal of growth stimulus or the binding of cyclins will have no effect. This is known as the restriction point. A key component in the regulation of this restriction point is the retinoblastoma protein (pRB). The sequential phosphorylation of pRB by different cyclin-CDK complexes is essential for progression of the cell from G_1 into S-phase. During the G_1 phase, D cyclins associate with the cyclin dependent kinases CDK4 and CDK6 to form functional units that are capable of phosphorylating pRB and do so in early G_1. Further phosphorylation of pRb is mediated by cyclin E/CDK2, which begins functioning during mid G_1, and cyclin A/CDK2, which acts at the G_1/S-phase boundary and maintains pRb phosphorylation through S and G_2-phases.[46] Krtolica et al have observed decreased CDK4 and CDK2 activity in CV-1P monkey kidney cells treated with ~1% O_2, and this kinase inhibition is associated with an increase in $p27^{Kip1}$ and a decrease in CDK4, cyclin D and cyclin E protein levels. They propose that this decreased kinase activity, coupled with increased PP-1 phosphatase activity, leads to dephosphorylation of pRb and inhibition of cell cycle progression.[47] Similar results to those found in CV-1P cells were obtained in ovarian carcinoma cells, suggesting a possible common pathway.[48]

Unphosphorylated pRb binds to and inhibits the activity of the E2F family of proteins, which are transcriptional activators of genes required for entry into S-phase such as PCNA, Cdc2, dihydrofolate reductase, cyclin E, thymidine kinase, DNA polymerase and histone H2A, among others. Upon hyperphosphorylation of pRB, E2F is released from pRB and can function as a transcriptional activator.[49] Additional regulators of pRb include the CIP/KIP family of cyclin-CDK inhibitors, including p21, p27 and p57, which block phosphorylation of pRB through their interactions with CDK4, CDK6 and CDK2.[50] Another family of CDK4/CDK6 specific inhibitors known as the INK4 family is comprised of p15, p16, p18 and p19.[50] (Fig. 3) In a recent study, immortalized mouse embryo fibroblasts derived from mice deficient in either pRb, p130, $p21^{CIP1}$, $p27^{Kip1}$ or both $p21^{CIP1}$ and $p27^{Kip1}$, along with genetically matched wild type cell lines, were exposed to an oxygen tension of 0.01%.[26] Following BrdU incorporation under hypoxia, each of the cell types was analyzed by two-dimensional FACS analysis. Within a few hours, DNA synthesis ceased. These results indicate that pRB, p130, p21 and p27 are not needed for cell cycle arrest induced by hypoxia and do not contribute to this arrest.[26] Interestingly, when the cells were reoxygenated after hypoxic treatment, the p21-/- cells re-entered the cell cycle more rapidly than wild type cells, whereas there was no acceleration of the S-phase re-entry of the p27-/- cells as determined by both FACS analysis and ^3H incorporation assays. The double knockout p21-/-p27-/- cells resumed S-phase even more quickly than the p21-/- alone did. Thus, while p21 and p27 are not necessary for arrest under hypoxia, they do play a role in inhibiting cell cycle re-entry upon reoxygenation. In contrast to the studies by Green et al studies by Gardner et al[51] have resulted in the conclusion that p27 mediates hypoxia-induced arrest. According to their data, cells deficient in p27 were observed to resist hypoxia-induced arrest upon exposure to 0.1-0.5% oxygen for 32 hours. At present, the data published by Green and Gardner seem to be in conflict and can only be explained by the different experimental procedures and conditions used in each study. However, both groups agree that the kinase activity of CDK2 in cells was found to decrease dramatically under hypoxic conditions without a corresponding decrease in protein levels of CDK2 or a decrease in cyclin A or E abundance. Cyclin A and E immunoprecipitates from hypoxic cells exhibited decreased activity, but no change in CDK2 association.

There are several possible explanations for a decrease in cyclin Activity without a corresponding decrease in protein levels. (Fig. 4) One possibility is that if cyclin Activating kinase (CAK) fails to phosphorylate threonine 160 of CDK2, the cyclin/CDK complex will be inactive. This modification of threonine 160 results in increased mobility on SDS-PAGE, which is unaffected in both WT and p21-/-p27-/- cells exposed to hypoxic conditions. Thus, reduced T160 phosphorylation could not explain the decrease in kinase activity observed under hypoxia-treated cells. A second possibility is the binding of an inhibitory protein, such as the CIP/KIP or INK4 family members mentioned previously as well as the pRb family members

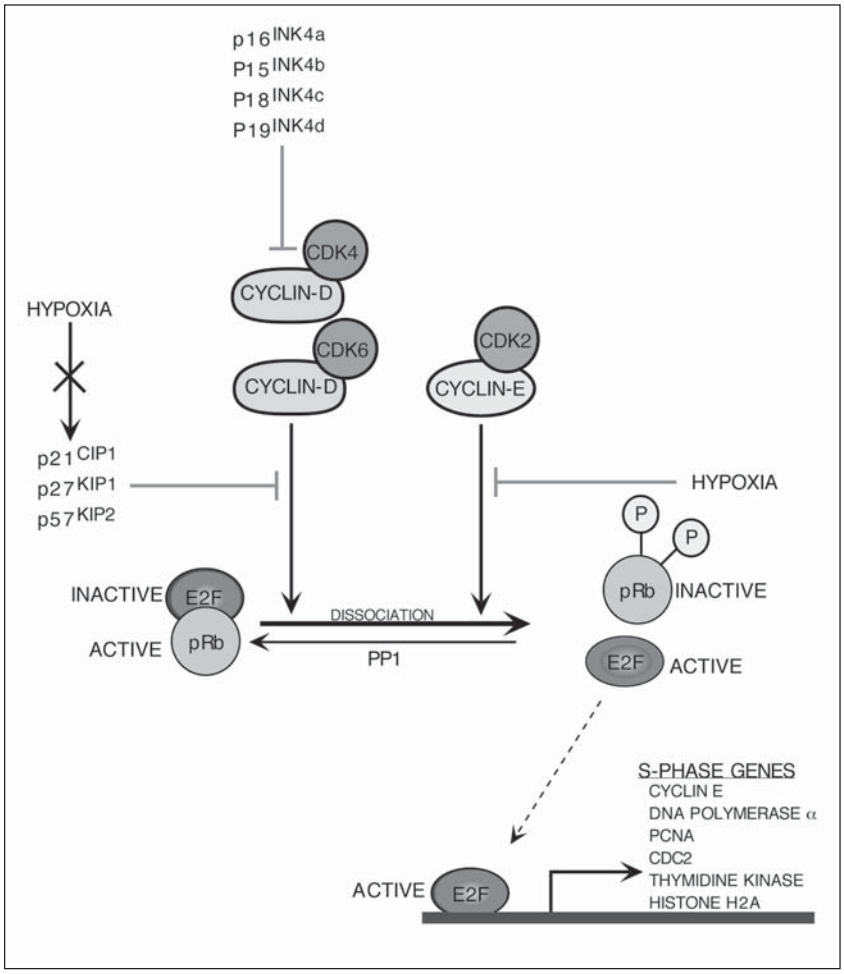

Fig. 3. Possible hypoxic effects on the progressive phosphorylation of pRb. INK family members negatively regulate Cyclin D and its partners CDK4 and CDK6, inhibiting their kinase activity on the pRb/E2F complex. Hypoxia does not induce the CyclinD/CDK2/4 inhibitors of the CIP/KIP family. The Cyclin E/CDK2 complex has been found to be inhibited by hypoxia.

p107 and p130 to the cyclin/CDK complex. The experiments described above eliminated p21, p27 and p130 as candidate effectors of arrest, as cells lacking these proteins arrested normally. Though the INK4 proteins do not interact with CDK2, they have been proposed to displace CIP/KIP proteins from CDK4/6 and thereby indirectly inactivate CDK2. Hypoxia, however, had little effect on protein levels of p16,^{INK4a} p15,^{INK4b} p18,^{INK4c} p19,^{INK4d} p130 and p107 in MEFs. A third possibility is that CDK2 can be inactivated by inhibitory phosphorylation on threonine 14 and tyrosine 15. To address this latter possibility CDC25B, a protein phosphatase known to dephosphorylate these residues in vitro was used to treat extracts from oxic and hypoxic cells. Treatment of CDK2 immunoprecipitates from control and hypoxic cells with GST tagged and purified CDC25B produced a significant activation of CDK2 complexes, particularly in the hypoxia treated samples. CDK2 immunoprecipitates from confluent and serum starved cells, by contrast, remained inactive upon CDC25B treatment. These results suggest that inhibitory

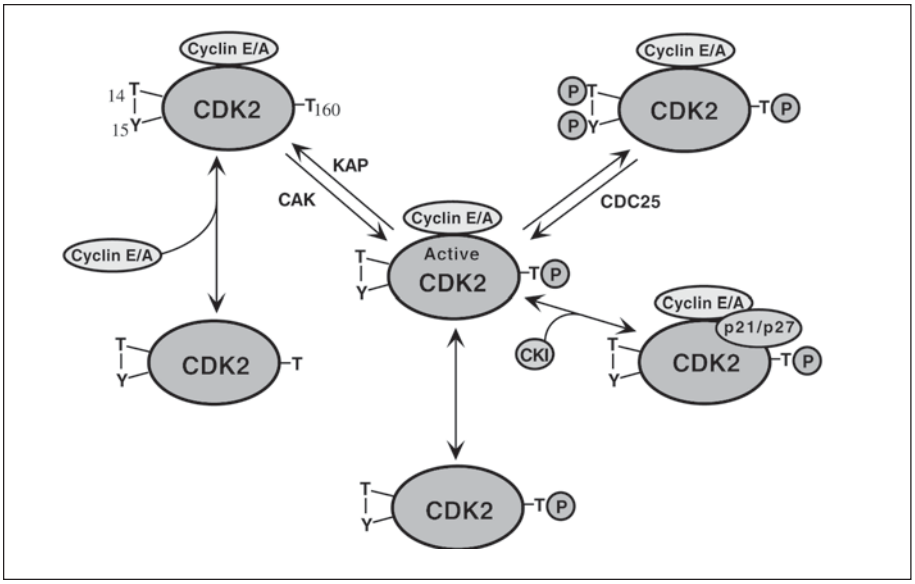

Fig 4.Mechanisms of CDK2 regulation: modified from Morgan.[57]

phosphorylation plays an important role in CDK2 inactivation under hypoxic conditions. Previous studies have indicated that Tyr15 is the more important of the two phosphorylation sites.[52] To directly analyze this residue, CDK2 was immunoprecipitated and subjected to SDS-PAGE and probing with phosphospecific antibody recognizing Tyr15-phosphorylated cdc2 and CDK2. Results revealed that phosphorylation was not increased by hypoxia, but was in fact mildly decreased. Given the evidence that CDC25B can restore activity, this would imply that phosphorylation of Thr14 could contribute to CDK2 inhibition by hypoxia. These results suggest that the phosphorylation of Tyr15 alone cannot account for the hypoxia-induced inhibition of CDK2, and further investigation to distinguish the difference between the two phosphorylation sites is needed.

Hypoxia Mimetics and Cell Cycle Arrest

The arrest seen in response to hypoxic stress seems independent of other pathways, such as those used by the cell in responses to ionizing radiation, ultraviolet light and aberrant growth signaling. As mentioned previously, early studies focused on a biochemical mechanism for cell cycle arrest, such as depletion of nucleotides or nucleotide precursors. Much of the current research does not support this hypothesis. A comparison between cell cycle arrest seen in response to hypoxia and that induced by hypoxia mimetic drugs addresses how cells sense hypoxia and to what extent the hypoxic response overlaps with other stress responses. While significant progress has been made towards answering this question with regards to other elements of the hypoxic response (i.e., gene induction and apoptosis), in the context of cell cycle arrest they remain largely unanswered.

The chemical hydroxyurea is a radical scavenger and is thought to inactivate ribonucleotide reductase in a manner similar to hypoxia. Mouse fibroblasts exposed to this chemical displayed a rapid cell cycle arrest in which there was negligible DNA synthesis 6 hours after treatment started. The S-phase accumulation of cells persisted for 12-24 hours and then declined to levels similar to the control sample. Kinase assays revealed that hydroxyurea did not affect CDK2 activity, as had been observed previously.[53] The distinct differences between the

cellcycle arrest responses to hypoxia and hydroxyurea indicate that the hypoxic cell cycle arrest cannot be due to ribonucleotide reductase inactivation alone.

Desferrioxamine (DFO) is an iron chelator that has been classified as chemical "hypoxia." Based on the observation that iron chelators or divalent metals can induce some aspects of the hypoxic response, in particular activation of the transcription factor HIF-1,[54] the hypoxic response is thought to rely partially on oxygen sensing by a heme-containing protein. DFO and cobalt chloride are commonly used in lieu of hypoxia due to the practical challenges posed by achieving hypoxia in the lab. However, these chemicals do not necessarily cause a response identical to that induced by true hypoxia. Cells exposed to DFO experience cell cycle arrest in mid S-phase in less than six hours, similar to that induced by hypoxia. However, cell division appears to continue as the G1 population increases significantly after 12 and 24 hours. Additionally, DFO causes a decrease in cyclin-E and cyclin-A associated kinase activities, without changing the protein levels of these two cyclins.[55] These data indicate a possible common pathway for hypoxia-and-DFO induced cell cycle arrest. Other pharmacologic and metabolic agents have also been examined for cell cycle inhibitors that include an inhibitor of uridine synthesis (N-phosphonacetyl-L-aspartate-PALA)), glucose deprivation, and an electron transport inhibitor (antimycin). Each of these treatments or agents inhibit cell cycle progression, but none were significantly similar to the hypoxic induced arrest. (Green et al unpublished observations)

Hypoxia-Induced Inhibition of CDK2 Activity and Resistance to Chemotherapy

The proliferation status of tumor cells has little effect on killing by radiotherapy. In contrast, many commonly used chemotherapeutic agents target rapidly proliferating cells. A recent study by Davis et al[56] has demonstrated that inhibition of CDK2 activity was sufficient to reduce the severity of chemotherapy-induced alopecia in 33 to 50% of the animals treated. This study makes a direct connection between the rate of proliferation of hair follicle cells and sensitivity to chemotherapy. Thus, if proliferation is a critical determinant for chemotherapeutic killing, inhibition of cell proliferation by hypoxia can explain the decreased efficacy of chemotherapeutic agents in killing hypoxic tumor cells. Furthermore, as multiple studies have demonstrated that hypoxia inhibits CDK2 activity, then restoration of CDK2 activity in hypoxic cells should increase the efficacy of chemotherapy. This hypothesis was tested by transfecting a mutant version of CDK2 (CDK2-A14F15) resistant to phosphorylation into oxic and hypoxic cells. The transiently transfected HA-tagged CDK2-A14F15 mutant was analyzed for kinase activity under various conditions. Anti-HA immunoprecipitates from transfected cells displayed substantial Histone H1 kinase activity whereas extracts from untransfected cells exhibited no HA-associated kinase activity. However, CDK2-AF transfected cells exposed to hypoxia displayed a progressive inhibition of kinase activity, reaching 60% inhibition by 12 hours. While this inhibition was slower and less severe than that of endogenous CDK2, the data indicates that there is still some other phosphorylation-independent means of CDK2 inhibition activated by hypoxia. If this inhibition is stoichiometric (i.e., inhibitor binding), then one should be able to compete out the inhibition. Therefore CDK2-AF was co-transfected with Cyclin E to generate more active complexes and thereby drive the cells into cycle. As expected, cells transfected with both molecules had a much higher kinase activity than CDK2 AF alone. But the activity was still inhibited by hypoxia, suggesting that the reduction in activity is not a result of inhibitor binding. Therefore, introduction of CDK2 or CDK2-A14F15 will not be sufficient to stimulate cells to proliferate under hypoxic conditions, and whether such stimulation would render hypoxic cells chemosensitive is still unknown.

These results underscore the importance of mechanistically understanding how hypoxia inhibits CDK2 activity in order to increase the sensitivity of hypoxic cells to killing by chemotherapy. The goal of future studies should be to determine how CDK2 is being inhibited under hypoxic conditions, and whether restoration of CDK2 activity will stimulate hypoxic cells to proliferate and become more chemosensitive.

Acknowledgments

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CHAPTER 10

G2 Checkpoint and Anticancer Therapy

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Abstract

Over the past two decades, the basic molecular events controlling eukaryotic G2 to M-phase cell cycle transition have been deciphered. Studies in a variety of organisms have identified an evolutionarily conserved system for controlling mitotic onset through regulation of Cdc2 kinase activity. Recently, investigations have focused on how the signaling pathways that mediate the G2 transition are regulated and modified after cellular stresses. In response to DNA damage, eukaryotic cells activate biochemical pathways, called checkpoints, to halt cell cycle progression and allow cellular damage to be repaired. Recent studies suggest that the DNA damage-induced G2 checkpoint is comprised of an early activation stage as well as a subsequent maintenance phase. In the absence of proper G2 checkpoint function, cells proceed to mitosis with damaged DNA, resulting in either apoptosis or permanent alteration of the genome that may contribute to tumorigenesis. The ability to manipulate G2 checkpoint signaling also has important clinical implications, as abrogation of the G2 checkpoint in human tumor cells can enhance cellular sensitivity to chemotherapeutic regimens that induce DNA damage. This Chapter will focus on

(i) eukaryotic DNA damage-induced G2 checkpoint signaling pathways and
(ii) how knowledge of these signaling pathways may lead to more efficient use of current anticancer therapies and the development of novel agents.

Introduction

The G2/M transition is regulated by the cyclin A/Cdc2 and cyclin B/Cdc2 kinase complexes.[1] Like other CDK molecules, Cdc2 function is tightly regulated by a complex system involving post-translational modifications and protein-protein interactions.[2] Cdc2 activation requires association with a cyclin partner, phosphorylation on Thr-161 by CAK ,[3,4] and dephosphorylation on Thr-14 and Tyr-15 by a Cdc25 phosphatase.[5,6] Several substrate proteins of Cdc2 have been identified, including the nuclear lamins A and B,[7] histone H1,[8] survivin,[9] and microtubule-associated proteins such as MAP4,[10] Eg5,[11] and stathmin.[12]

As previously noted, Cdc2 can interact with both cyclin A and cyclin B family members to regulate the G2/M transition. In mammalian cells, two cyclin A family members have been identified, cyclin A1 and cyclin A2. Mammalian cyclin A1 expression is restricted to the testis, brain, and hematopoietic cells.[13,14] Mice deficient for cyclin A1 develop normally but males are sterile due to defective spermatogenesis from impaired Cdc2 activation.[15,16] Cyclin A1 is frequently overexpressed in acute myelocytic leukemias, presumably through activation of the cyclin A1 promoter by fusion proteins created through chromosomal translocation.[13,17] Cyclin A2 is ubiquitously expressed and participates in both the G1/S and G2/M transitions by interacting with CDK2 and Cdc2.[18,19] Homozygous deletion of cyclin A2 in mice results in

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embryonic lethality, demonstrating the importance of this cyclin for normal development.[20] However, further studies are necessary to define the role of cyclin A2 kinase activity in G2/M transition.

Similar to the cyclin A family, mammalian cells contain two cyclin B family members, cyclin B1[21,22] and cyclin B2;[23] however, chickens, frogs, flies, and nematode worms possess a third, more distant relative, cyclin B3.[24,25] Both mammalian cyclin B family members are frequently co-expressed in proliferating cells, although tissue-specific temporal expression of cyclin B1 and cyclin B2 has been observed in murine germ cells.[23] Further, the two cyclin B family members have distinct subcellular localizations, with cyclin B2 strictly associated with intracellular membranes[26] while cyclin B1 is found both on intracellular membranes and in the cytoplasm.[10,27] Cyclin B2-null mice develop normally and both males and females are fertile.[28] In contrast, cyclin B1-deficient mice die in utero prior to embryonic day 10.[28] In late G2 phase of the cell cycle, cyclin B1 enters the nucleus, as nuclear localization of cyclin B1 is required during the G2/M transition.[27,29] Unlike cyclin B1, cyclin B2 does not relocate to the nucleus during the G2/M transition.[26] The nuclear localization of *Xenopus* cyclin B1 is mediated by phosphorylation of residues in the cytoplasmic retention signal sequence of the protein.[29] Several studies indicate that cyclin B1 is continuously exported from the nucleus by interaction with the nuclear exporter CRM1 during interphase, as the cytoplasmic retention signal of cyclin B1 contains a functional nuclear export signal.[30-32] Since the role of cyclin B1 in the G2/M transition has been the most extensively studied and the best defined of any eukaryotic Cdc2-associated cyclin, the subsequent discussion of Cdc2 activity will focus on regulation of the cyclin B1/Cdc2 complex.

During the G2-phase of the cell cycle, inactive cyclin B1/Cdc2 complexes accumulate in mammalian cells due to inhibitory phosphorylation of Cdc2 on Thr-14 and Tyr-15 by the Wee1 and Myt1 kinases (Fig. 1).[33,34] Wee1 is a dual-specificity protein kinase that was initially identified as a dose-dependent inhibitor of mitosis in *S. pombe*.[35] When Cdc2 is complexed with cyclin B1, recombinant Wee1 can phosphorylate Cdc2 on Tyr-15 and inhibit Cdc2 kinase activity.[33] Wee1 is hyperphosphorylated and degraded during mitosis, suggesting that negative regulation of Wee1 is part of the mechanism that mediates Cdc2 activation during the G2/M transition.[36-38] In support of this, studies in fission yeast have shown that Nim1 negatively regulates the S. *pombe* Wee1 by phosphorylation of its C-terminal catalytic region.[39-41] Further, *Xenopus* Wee1 is inhibited by phosphorylation in its N-terminal noncatalytic region by an unidentified protein kinase.[42] However, the kinases responsible for the phosphorylation and negative regulation of Wee1 in mammalian cells have not been determined.

The Wee1-related protein kinase, Myt1, is a cytoplasmic, membrane-bound kinase found in *Xenopus* and mammalian cells that can phosphorylate Cdc2 on both Thr-14 and Tyr-15, but has a strong preference for Thr-14.[34,43,44] Human Myt1 localizes to the endoplasmic reticulum and Golgi complex [45] and specifically phosphorylates and inactivates Cdc2 complexes to inhibit G2/M progression.[46] Myt1 interacts with Cdc2 complexes through its carboxy terminus and overexpression of human Myt1 prevents entry into mitosis by sequestering cyclin B1/cdc2 complexes in the cytoplasm.[47,48] Thus, Myt1 inhibits the G2/M transition by disrupting the nuclear localization of cyclin B1/Cdc2 complexes, as well as by phosphorylating Cdc2 on Thr-14 and Tyr-15. Another Wee1-related kinase, Mik1, has been identified in fission yeast. Mik1 acts redundantly with Wee1 in *S. pombe* to negatively regulate Cdc2 through phosphorylation of Tyr-15.[49] While a null allele of Mik1 has no discernible phenotype in fission yeast, a Mik1/Wee1 double mutant undergoes mitotic lethality that is correlated with loss of tyrosine phosphorylation on Cdc2.[49]

In eukaryotic cells, the Cdc25 family of dual-specificity phosphatases dephosphorylates CDKs to mediate CDK activation and cell cycle progression. Mammalian cells contain three isoforms of Cdc25 (A, B, and C) that share 40-50% amino acid identity and regulate distinct cyclin/CDK complexes throughout the cell cycle.[50,51] Cdc25A dephosphorylates cyclin E/CDK2 and cyclin A/CDK2 complexes to regulate the G1 and S-phase progression.[52] Cdc25B activity peaks during the G2-phase and ablation of Cdc25B activity prevents mitotic entry, suggesting

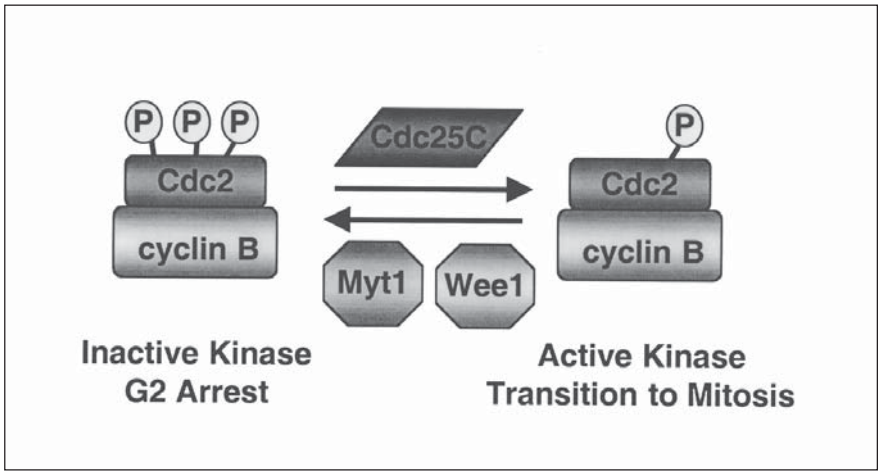

Fig. 1. G2/M Transition. The activity of Cdc2 is tightly regulated by phosphorylation and protein interactions. Cdc2 activation requires association with cyclin partners and phosphorylation by CAK. During S- and G2-phases, cells accumulate cyclin B1/Cdc2 in an inactive form due to inhibitory phosphorylations by Wee1 and Myt1 kinases. The conversion of Cdc2 from an inactive to active form is mediated by the Cdc25C phosphatase. Mitotic events are stimulated by active cyclin B/Cdc2 complexes.

Cdc25B is a positive mitotic regulator.[53-55] Further, Cdc25B dephosphorylates Cdc2 on Thr-14 and Tyr-15 *in vitro*[6] and overexpression of Cdc25B causes cells to prematurely enter mitosis.[56] Several studies implicate Cdc25A and Cdc25B as human oncogenes, as overexpression of these proteins is observed in primary breast tumors as well as head and neck cancers.[57,58]

Cdc25C is required for entry into mitosis and is believed to be the major phosphatase that dephosphorylates Thr-14 and Tyr-15 of Cdc2 *in vivo*.[5] In cycling *Xenopus* egg extracts, the activity of Cdc25 (biochemically equivalent to human Cdc25C) is low during S-phase and increases in mitosis,[51] and recombinant human Cdc25C dephosphorylates Cdc2 in vitro.[50,59] *Xenopus* Cdc25 is extensively phosphorylated in its N-terminal noncatalytic region during mitosis and active cyclin B/Cdc2 activates Cdc25 by phosphorylating the N-terminal region of Cdc25 in an autocatalytic loop.[60,61] Thus only a small amount of active cyclin B/Cdc2 is required for rapid activation of all the cyclin B/Cdc2 in the cell. In addition to cyclin B/Cdc2, other protein kinases are likely to be important for Cdc25 activation; these kinases may mediate the initial activation of cyclin B/Cdc2 during G2/M.[62] For example, the polo-like kinase, Plx1, is associated with Cdc25 in *Xenopus* egg extracts and phosphorylates Cdc25 on its amino terminus to stimulate its activity.[63] The mammalian Plx1 homolog, Plk1, also phosphorylates Cdc25C, and Plk1 activity is necessary for the functional maturation of centrosomes in late G2/early prophase and for the establishment of a bipolar spindle.[64,65] Plk1 may be a useful prognostic marker for head and neck,[66] esophageal,[67] and non-small cell lung[68] carcinomas, as overexpression in these tumors is correlated with poorer prognosis.

G2 Checkpoint Activation

At key transitions during eukaryotic cell cycle progression, signaling pathways monitor the successful completion of upstream events prior to proceeding to the next phase. These regulatory pathways are commonly referred to as cell cycle checkpoints.[69] Cells can arrest at cell cycle checkpoints temporarily to allow for

(1) the repair of cellular damage,

(2) the dissipation of exogenous cellular stressors, or

(3) the accumulation of essential nutrients and growth factors.

Checkpoint signaling may also activate pathways leading to programmed cell death if cellular damage cannot be properly repaired. Defects in cell cycle checkpoints can result in gene mutations, chromosome damage, and aneuploidy, all of which result in permanent alteration of the genome.[70] The checkpoint mechanisms that normally regulate the fidelity of cell cycle progression are frequently disrupted in tumor cells, verifying the importance of intact checkpoint signaling pathways for maintenance of the genome.[71]

In addition to controlling normal mitotic entry, regulation of Cdc2 phosphorylation plays a critical role in activating the G2 checkpoint after DNA damage. Genotoxic stress activates cell cycle checkpoint pathways that initiate DNA repair and arrest at the G2/M transition to prevent the propagation of DNA lesions during mitosis.[72] Initial experiments in the fission yeast *S. pombe* demonstrated that phosphorylation of Cdc2 on Tyr-15 is required for G2 arrest after ionizing radiation treatment.[73] Expression of a dominant Cdc2 mutant (Tyr-15 changed to phenylalanine) completely abolishes the mitotic delay observed after ionizing radiation exposure.[73] The mechanism by which Tyr-15 phosphorylation is upregulated in fission yeast after DNA damage involves both increased Wee1 protein levels and kinase activity, as well as decreased Cdc25 (biochemical equivalent of human Cdc25C) levels and activity.[73-75] The importance of this two-step mechanism to modulate Tyr-15 phosphorylation of Cdc2 is evidenced by the finding that while Wee1 inactivation in *S. pombe* increases sensitivity to both ionizing[76] and ultraviolet[77] radiation, Wee1-deficient yeast maintain G2 checkpoint control.[78] In contrast, inactivation of both Wee1- and Cdc25-dependent function completely abolishes checkpoint control in addition to sensitizing cells to ionizing and ultraviolet (UV) radiation.[75] Thus, upregulation of Cdc2 Tyr-15 phosphorylation after DNA damage requires both increased phosphorylation of Tyr-15 and a reduced rate of Tyr-15 dephosphorylation.[75] Recent studies indicate that Mik1 may also function during the G2 DNA damage checkpoint in fission yeast, as Mik1 protein levels are induced in response to ionizing radiation.[79,80]

Similar mechanisms appear to govern the DNA damage-induced G2 checkpoint arrest in mammalian cells, as phosphorylation of Cdc2 on Thr-14 and Tyr-15 plays a critical role in the DNA damage-induced G2 arrest (Fig. 2).[81] Treatment of Chinese hamster ovary cells with either etoposide or ionizing radiation results in rapid Cdc2 Tyr-15 phosphorylation and inhibition of cyclin B/Cdc2 kinase activity.[82,83] Increased Tyr-15 phosphorylation of Cdc2 is observed in HL-60 human myeloid leukemia cells after exposure to ionizing radiation.[84] Further, the overexpression of a non-phosphorylatable Cdc2 mutant (Thr-14 and Tyr-15 mutated to alanine and phenylalanine, respectively), significantly reduces the G2 delay observed in HeLa cells exposed to ionizing radiation.[85] Numerous studies have also shown that after genotoxic stress, the G2 checkpoint can be the predominant phase of cell cycle arrest in primary epithelial cells, including keratinocytes[86] as well as prostate,[87] and bronchial epithelial cells.[88]

While the biochemical pathways involved in the DNA damage-induced G2 arrest in mammalian cells entail signaling cascades that converge to inhibit Cdc2 activation[72], specific pathways that mediate G2 arrest after DNA damage in higher eukaryotes have only recently been elucidated. In human hematopoietic cells, ionizing radiation activates the Lyn kinase, a member of the Src family of protein tyrosine kinases.[89] Lyn localizes to the nucleus and directly binds to Cdc2 in hematopoietic cells exposed to ionizing radiation.[90] Further, recombinant Lyn phosphorylates Cdc2 on Tyr-15 in vitro, suggesting this kinase may be important in the G2 checkpoint response in hematopoietic cells.[90,91] In addition to upregulation of Cdc Tyr-15 phosphorylation, decreased dephosphorylation of Cdc2 may also be important in G2 arrest in mammalian cells, as impaired Cdc25C activation is observed in human lymphoma cells treated with nitrogen mustard,[92] as well as in HeLa cells treated with either ionizing[93] or UV [94] radiation.

Recent studies have identified critical evolutionarily conserved G2 checkpoint pathways mediated by members of the phosphoinositide-3 kinase (PI-3K) family in response to genotoxic stress. After DNA damage, the PI-3K family members ATM (Tel1 in fission and budding yeast) and ATR (Rad3 and Mec1 in fission and budding yeast, respectively), become activated and initiate specific signal transduction pathways that regulate DNA repair and cell cycle arrest

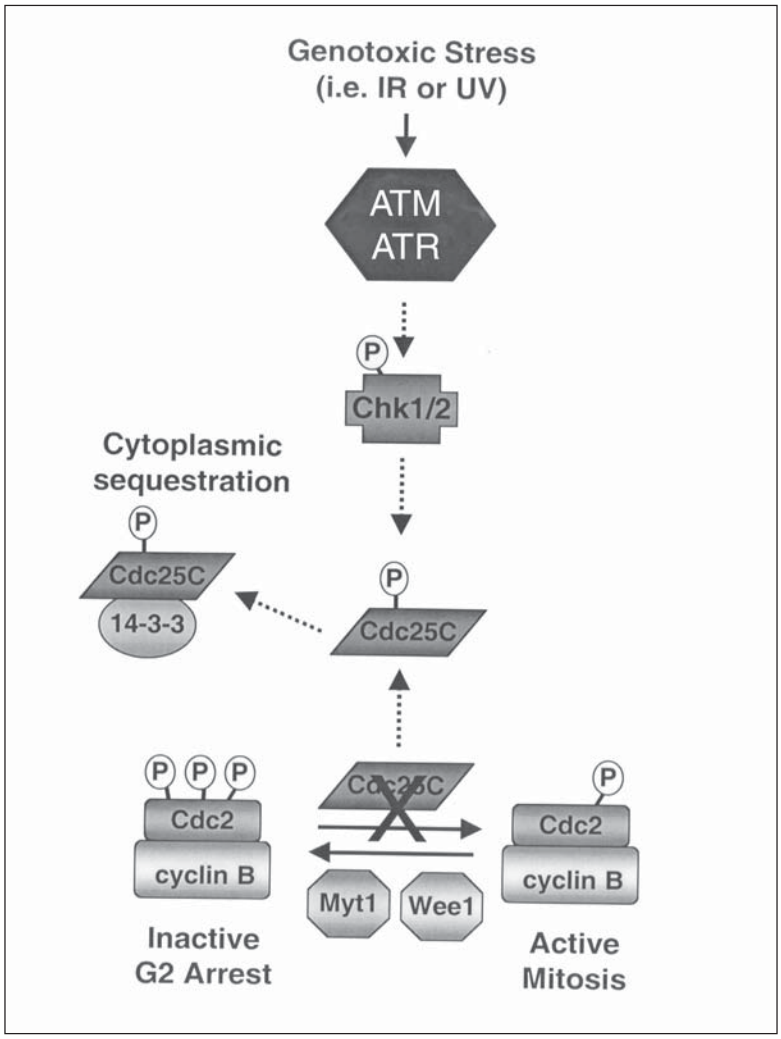

Fig. 2. G2 Checkpoint Activation After Genotoxic Stress. In response to genotoxic stress, the ATM/ATR signaling pathway is activated leading to phosphorylation and activation of Chk1 and Chk2 kinases and subsequent phosphorylation of Cdc25C. This latter phosphorylation promotes the interaction of Cdc25C with 14-3-3 adaptor proteins and inhibits the ability of Cdc25C to activate cyclin B1/Cdc2, resulting in G2 cell cycle arrest.

in yeast, *Xenopus*, and mammalian cells (Fig. 2). ATM phosphorylates and activates the Chk2 kinase (Cds1 and Rad53 in fission and budding yeast, respectively) in cells exposed to ionizing radiation.[95-98] Similarly, ATR-dependent signaling mediates activation of the Chk1 kinase (Chk1 in fission and budding yeast) in cells treated with UV radiation.[99,100] Activated Chk1 and Chk2 can phosphorylate Cdc25C on Ser-216, generating a consensus binding site for 14-3-3 proteins.[95,101-104] The binding of 14-3-3 proteins to Cdc25C results in the nuclear export of Cdc25C, cytoplasmic sequestration of the phosphatase, and G2 arrest due to inhibition of Cdc2 activity.[105]

In addition to phosphorylating Cdc25 on Ser-216, activated Chk1 also phosphorylates Wee1 in fission yeast, resulting in stabilization of the protein and thus prolonged Wee1 kinase activity during G2 checkpoint responses.[74] Further, Chk1 is essential for the DNA damage-induced G2 checkpoint in *S. pombe* as Chk1-deficient cells have increased sensitivity to ionizing radiation due to defective G2 arrest.[106] Thus Chk1 integrates Rad3-dependent sigaling in fission yeast to mediate the two-step mechanism to modulate Tyr-15 phosphorylation of Cdc2 through activation of the Wee1 kinase and inactivation the Cdc25 phosphatase.[75]

The importance of the PI-3K G2 checkpoint pathways is exemplified by the fact that yeast and mammalian cells deficient in function of these family members have defective G2 checkpoint responses after DNA damage resulting in enhanced cellular sensitivity to genotoxic stressors. Inactivation of Rad3[107,108] and Mec1[109] in yeast cells ablates G2 arrest after ionizing radiation, while the expression of a kinase-defective ATR in human fibroblasts abrogates the G2 checkpoint and sensitizes the cells to both ionizing and UV radiation.[110] Further, homozygous deletion of ATR in mice results in embryonic lethality, as cells die by chromosome fragmentation and apoptosis.[111,112]

There are overlapping functions between the ATR and ATM classes of PI-3K kinases in yeast. Deletion of the ATM homolog, Tel1, in fission[113] or budding[114] yeast has minimal effect on DNA damage-induced checkpoint responses, as Tel1 functions to maintain telomere integrity in yeast.[115] In contrast, loss of ATM in mammalian cells eliminates G2 checkpoint function and sensitizes cells to ionizing radiation,[116,117] but does not alter checkpoint maintenance or cellular sensitivity to UV radiation. ATM-null mice exhibit growth retardation, neurologic dysfunction, infertility, defective T lymphocyte maturation, and sensitivity to ionizing radiation.[117,118] The majority of ATM-deficient animals develop malignant lymphomas by 4 months of age, while ATM-/- fibroblasts have abnormal radiation checkpoint function after exposure to ionizing radiation.[117,118] ATM function is defective in patients with ataxia telangiectasia, a disorder in which patients have increased sensitivity to radiation[119] and are highly prone to the development of leukemias and lymphomas in childhood.[120] Further, individuals harboring a heterozygous germ line mutation of ATM have an elevated incidence of breast cancer.[120,121]

Chk1 and Chk2 proteins are also essential for maintenance of the DNA damage-induced G2 checkpoint in yeast and mammalian cells. Loss of Chk1 in fission yeast,[106] budding yeast,[122] and mammalian cells.[99,123] results in defective G2 checkpoint function after exposure to ionizing radiation. Abrogation of Chk1 in mice results in early embryonic lethality.[99,123] Similarly, Cds1[124] and Rad53[125,126] are essential for G2 checkpoint integrity after DNA damage in fission and budding yeast, respectively. Mammalian Chk2-/- embryonic stem cells also fail to undergo G2 arrest after ionizing radiation treatment, while Chk2-/- thymocytes are resistant to DNA damage-induced apoptosis.[127] Of note, heterozygous germline mutations of Chk2 occur in a subset of individuals with Li-Fraumeni syndrome, a highly penetrant familial cancer syndrome associated with significantly increased rates of brain tumors, breast cancers, and sarcomas that is typically associated with germline mutations in the tumor suppressor p53.[128]

While regulation of Cdc25C by PI-3K family members plays a critical role during G2 checkpoint responses, other kinases are capable of phosphorylating Cdc25C on Ser-216 in response to DNA damage in mammalian cells. For example, C-TAK1 is a kinase that is ubiquitously expressed in human cells and phosphorylates Cdc25C on Ser-216 to promote 14-3-3 protein binding.[129] Similarly, Prk is a kinase expressed in human ovary, placenta, and lung that also phosphorylates Cdc25C on Ser-216 in vitro.[130-132] Prk mRNA expression is downregulated in human lung tumors, suggesting that disruption of specific G2 checkpoint pathways may contribute to tumorigenesis.[130] However, a role for C-TAK1 and Prk-dependent phosphorylation of Cdc25C after DNA damage has not been established.

Recently, the Plk1 kinase (Cdc5 in fission and budding yeast) was linked to G2 DNA damage checkpoint signaling. Smits et al[65] reported that Plk1 activity is inhibited in the G2 phase of the cell cycle in human tumor cells exposed to ionizing radiation, camptothecin, and doxorubicin. The fission[133,134] and budding[135] yeast homologs of mammalian Plk, Cdc5, also

participate in G2 checkpoint signaling by preventing anaphase entry and mitotic exit after DNA damage. Expression of a mutant Plk1 in which residues necessary for Plk1 activation are altered, prevents Plk1 inactivation and leads to G2 override in cells treated with doxorubicin.[65] Similarly, Plk1 activity is persistent during abrogation of the G2 checkpoint in tumor cells by caffeine treatment.[65] Several independent studies demonstrate that normal epithelial cells[136] and fibroblasts[64] undergo a G2 arrest in response to Plk inactivation in the absence of DNA damage. In contrast, inhibition of Plk in human tumor cells under the same circumstances results in mitotic catastrophe, although this latter event is independent of Plk1-mediated phosphorylation and inactivation of Cdc25.[64,136]

In budding yeast, *Xenopus*, and mammalian cells, the G2 checkpoint response may also be regulated by the Pin1 protein. Pin1 is an essential peptidyl-prolyl isomerase that inhibits entry into mitosis and is also necessary for mitotic progression.[137] Depletion of Pin1 from yeast or mammalian cells induces mitotic arrest, while Pin1 overexpression in these cells results in a G2 arrest.[137] In both *Xenopus* and human cells, Pin1 directly interacts with Wee1, Myt1, Cdc25C, and Plk1 in a phosphorylation-dependent manner.[138,139] The binding of Pin1 to Cdc25C inhibits the phosphatase activity of the latter, thus accounting for the ability of Pin1 to block mitotic entry.[138] Further, Winkler et al[140] determined that Pin1 is essential for DNA replication checkpoint responses, as depletion of Pin1 from *Xenopus* extracts results in inappropriate G2 progression and mitotic entry in the presence of aphidocolin. Depletion of Pin1 activity from human tumor cells by multiple mechanisms, including overexpression of Pin1 antisense mRNA, overexpression of a dominant-negative Pin1, and treatment of cells with a chemical inhibitor of Pin1, juglone, confirms that Pin1 catalytic activity is essential for both tumor cell survival and mitotic entry.[141] However, further studies are necessary to determine if Pin1 function is required for the DNA damage-induced G2 checkpoint.

Proteins that mediate direct repair or detection of DNA damage may also be essential for proper G2 checkpoint activation. MLH1 is a necessary component of the DNA mismatch repair machinery and is thought to participate in the G2 checkpoint in human tumor cells. Ovarian tumor cells lacking MLH1 expression have defective G2 cell cycle arrest after cisplatin and 6-thioguanine treatment.[142] Further, MLH1-deficient human colon carcinoma cells also have decreased survival and concomitant G2 checkpoint deficiency after ionizing radiation or 6-thioguanine exposure as compared to genetically matched cells in which mismatch repair function has been restored.[143] Similarly, MLH1-/- mouse embryo fibroblasts (MEFs) are sensitized to ionizing radiation and 6-thioguanine and have impaired G2 arrest after exposure to these agents as compared to wild-type (wt) MEFs.[143] Interestingly, Brown et al[142] observed loss of MLH1 expression in 9 of 10 human ovarian cell lines after cisplatin treatment in vitro, as well as the loss of MLH1 expression in 4 of 11 tumors biopsied during second look laparotomy after chemotherapy. Since, MLH1-deficiency in human ovarian cells renders them resistant to numerous chemotherapeutic agents, including cisplatin, doxorubicin, 6-thioguanine, and N-methyl-N-nitrosourea, these latter findings have important therapeutic implications.[142] Taken together, these studies suggest that MLH1-mediated regulation of the G2 checkpoint is indispensable for proper DNA damage detection and repair, although the mechanism by which MLH1 enforces G2 checkpoint integrity has not been elucidated.

Members of the homeobox family of proteins have also been implicated in regulation of the G2 checkpoint. HSIX1 is a homeobox protein expressed during the S and G2 phases of the cell cycle.[144] Overexpression of HSIX1 in human breast cancer cells abrogates the G2 cell cycle checkpoint response after ionizing radiation.[144] Further, HSIX1 expression is absent or very low in normal mammary tissue but is elevated in nearly half of primary breast cancers and 90% of metastatic lesions.[144] HSIX1 expression is also elevated in a variety of human cancer cell lines, suggesting an important function for the protein in multiple tumor types.[144] Further studies are necessary to determine if HSIX1 function is mediated through regulation of the G2 checkpoint.

Finally, several members of the MAPK family are activated in response to ionizing radiation; however, the biological relevance of these kinases to the G2 checkpoint is uncertain. Recently,

the p38γ kinase was shown to have an essential function in the G2 checkpoint in human tumor cells exposed to ionizing radiation.[145] Activation of p38γ occurs in tumor cells treated with cisplatin, etoposide, or ionizing radiation.[145,146] Further, p38γ-dependent signaling is required for DNA damage-induced G2 arrest, as disruption of p38γ-mediated signaling abrogates the G2 arrest and enhances the cytotoxicity observed in human tumor cells and human fibroblasts treated with ionizing radiation.[145] The DNA damage-mediated activation of p38γ is ATM-dependent. ATM-deficient cells fail to induce p38γ activity after ionizing radiation treatment; however, the downstream targets of p38γ activity during G2 checkpoint signaling have not been elucidated.[145]

G2 Checkpoint Maintenance

Numerous studies indicate that the G2 arrest response is comprised of an early or activation stage as well as a subsequent maintenance phase, with p53 signaling implicated to play a role in the latter.[147-150] While the importance of p53-mediated signaling in G1/S checkpoint function is well documented, the role of p53 signaling at the G2 checkpoint has only recently been well defined. Early studies showing p53-deficient cells maintain the DNA damage-induced G2 arrest suggested that p53 does not function to regulate the G2 checkpoint.[151,152] However, expression of p53, in the absence of cellular stress, induces cell cycle arrest at both the G1 and G2 checkpoints, suggesting p53 signaling modulates the G2 checkpoint response.[153-155] Subsequent studies showed that p53 and $p21^{WAF1/Cip1}$ (p21) are necessary to maintain a G2 arrest following DNA damage, since tumor cells lacking these proteins enter into mitosis with accelerated kinetics.[150,156]

p53 utilizes multiple signaling pathways to modulate the G2 checkpoint (Fig. 3). One of the initial components of p53-dependent G2 checkpoint maintenance is the transcriptional upregulation of 14-3-3σ. 14-3-3sσ is induced in a p53-dependent manner by exposure to ionizing radiation and doxorubicin in colorectal carcinoma cells.[157] Further, overexpression of 14-3-3σ in proliferating cells induces a G2 arrest.[157] Deletion of both alleles of 14-3-3σ in colorectal carcinoma cells results in abrogation of G2 arrest and premature mitotic entry after exposure to ionizing radiation and doxorubicin.[158] The p53-dependent increase in 14-3-3σ is thought to modulate cyclin B1/Cdc2 signaling, as the binding of 14-3-3σ to Cdc2 results in cytoplasmic sequestration of the kinase.[158]

In addition to transcriptional upregulation of 14-3-3σ, the mechanism of p53-dependent G2 arrest involves inhibition of cyclin B1/Cdc2 activity by p21 and a subsequent reduction of cyclin B1 and Cdc2 protein levels.[156,159-161] Similar to its regulation of the cyclin E/CDK2 complex at the G1/S checkpoint, p21 binds to and inhibits the cyclin B1/Cdc2 complex in vitro by blocking the activating phosphorylation of Cdc2 on Thr-161,[162] although p21 has a significantly lower affinity for the cyclin B1/Cdc2 complex as compared to the G1 phase kinase complexes.[163] Thus, DNA damage-induced G2 delay is regulated by modulation of both the activating and inhibitory phosphorylations of Cdc2. The reduced expression of cyclin B1/Cdc2 is mediated in part by p53-dependent transcriptional repression of the cyclin B1 and Cdc2 promoters, although this transrepression is not due to direct interaction of p53 with these promoters.[156,164] The CCAAT-biding factor NF-Y was recently shown to mediate transcriptional inhibition of cyclin B1 and Cdc2 during p53-dependent G2 arrest.[165] Cdc2 transrepression may also result from the interaction of p130 and E2F4 with the Cdc2 promoter.[166] The importance of p53-dependent regulation of Cdc2 activity is exemplified by the finding that constitutive activation of cyclin B1/Cdc2 overrides p53-mediated G2 arrest.[167]

The p53-mediated decrease in cyclin B1 and Cdc2 transcription also requires the retinoblastoma protein (pRB). Abrogation of pRB function in cells containing wt p53 blocks the down regulation of cyclin B1 and Cdc2 expression and leads to an accelerated exit from G2 after genotoxic stress.[156] Thus, similar to what occurs in cells that are p21 and p53 deficient, pRB loss can uncouple S phase and mitosis after genotoxic stress in tumor cells. pRB is a transcription repressor that, in its hypophosphorylated state, binds to the E2F-family (E2F) of

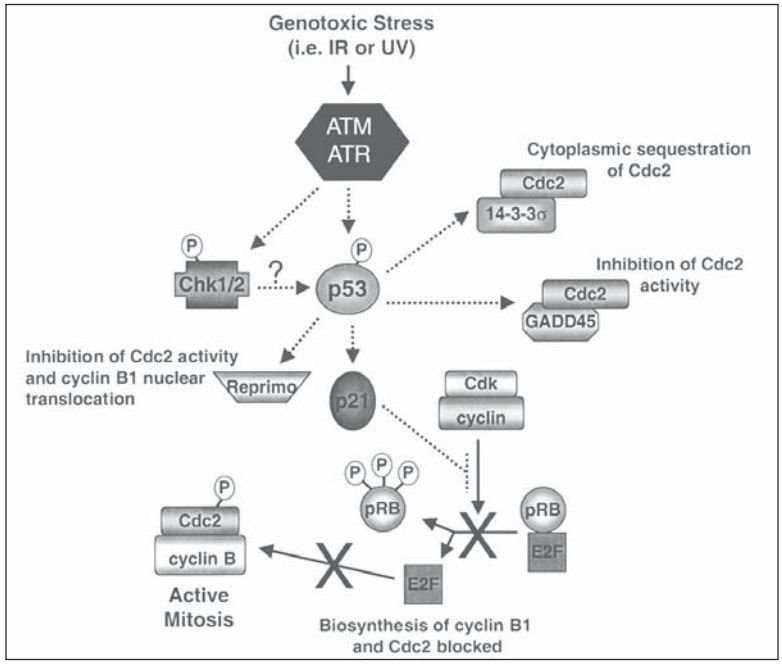

Fig. 3. G2 Checkpoint Maintenance After Genotoxic Stress. After DNA damage, activated ATM/ATR as well as Chk1 and Chk2 can phosphorylate p53, resulting in stabilization and activation of the tumor suppressor. p53-dependent signaling contributes to maintenance of the G2 cell cycle arrest by upregulating the 14-3-3σ protein that binds to Cdc2 and sequesters the kinase in the cytoplasm. p53-dependent transcription also elevates the CDK inhibitor p21, that binds to cyclin/CDK complexes to reduce phosphorylation of pRB. Hypophosphorylated pRB remains bound to E2F, preventing E2F from mediating the biosynthesis of cyclin B1 and Cdc2. p53 may also play a role in G2 checkpoint maintenance through transcriptional upregulation of GADD45 and Reprimo. GADD45 can directly impede cyclin B1/Cdc2 activity after UV radiation by binding to Cdc2. Reprimo can inhibit Cdc2 activity and inhibit nuclear translocation of cyclin B1.

transcription factors and blocks E2F-dependent transcription of genes whose products are necessary for S-phase entry, G2 progression, and M phase transition.[168] Activated G1-phase cyclin/CDK complexes phosphorylate pRB, resulting in the dissociation of E2F and pRB and cell cycle progression after E2F-mediated transcription.

In addition to modulation of cyclin B1/Cdc2 activity by indirect transcriptional mechanisms, p53 may also exert G2 checkpoint responses through transcriptional upregulation of the downstream target genes, GADD45, and Reprimo. Increased expression of GADD45 in primary fibroblasts results in a G2 arrest that can be abrogated by the overexpression of cyclin B1 or Cdc25C.[169] This GADD45-induced G2 arrest is p53-dependent since overexpression of GADD45 in p53-deficient fibroblasts fails to mediate a G2 arrest.[169] Also, GADD45 has been shown to directly inhibit the cyclin B1/Cdc2 complex after UV radiation by binding to Cdc2.[170] Of note, the GADD45-dependent G2 arrest is induced only after specific types of DNA damage. Lymphocytes from GADD45 knockout mice failed to arrest after exposure to UV radiation, but retained the G2 checkpoint initiated after exposure to ionizing radiation.[169] Reprimo was recently identified as a novel p53 downstream target that is induced in MEFs exposed to ionizing radiation.[171] Reprimo is a highly glycosylated protein that induces G2 arrest when overexpressed in human tumor cells regardless of p53 status.[171] Cdc2 and cyclin B1 protein

levels are unaffected by Reprimo overexpression, although Cdc2 activity and cyclin B1 nuclear translocation are inhibited.[171]

p53-mediated transrepression of target genes may also contribute to its role in G2 arrest. One critical cell cycle target of p53 transrepression is stathmin/oncoprotein 18.[172,173] Stathmin is frequently overexpressed in breast[174,175] and ovarian[176] cancers, as well as hematologic malignancies,[177,178] suggesting a critical function in cell cycle control. Stathmin is a microtubule-associated phosphoprotein that functions in the regulation of microtubule dynamics during mitosis.[179] Inhibition of stathmin by mutation of sites required activating phosphorylations by Cdc2 and MAPK prevents mitotic spindle formation.[12,180-182] Overexpression of stathmin can override p53-dependent G2 arrest in human tumor cells exposed to ionizing radiation, indicating that regulation of stathmin expression is critical to p53-regulated G2 checkpoint responses.[173]

In addition to their previously described role in G2 checkpoint function, the ATM, ATR, Chk1, and Chk2 kinases also directly phosphorylate the amino terminus of p53 after DNA damage.[183-186] ATM-and ATR-induced phosphorylation of human p53 on Ser-15 may be important for p53 activation after genotoxic stress.[183-187] Similarly, Chk1- and Chk2-mediated phosphorylation of human p53 on Ser-20 may contribute to p53 activation after DNA damage,[127,188,189] since substitution of Ser-20 with alanine abrogates p53 stabilization after exposure to either ionizing or UV radiation.[190] Further, as previously described, cells deficient in either ATM, ATR, Chk1, or Chk2 have defective G2 arrest after ionizing radiation, a phenotype similar to that of p53-/- cells.[150] However, p53 appears to function through the G1/S checkpoint after activation by these kinases in response to DNA damage;[189] although, a role for these kinases in p53-dependent G2 checkpoint responses cannot be excluded.

The p53-mediated G2 checkpoint also modulates genomic integrity after MYC oncogene overexpression. Felsher et al[191] recently reported that overexpression of MYC protein in human fibroblasts triggers aneuploidy through a mechanism involving p53-dependent G2 arrest. Loss of the p53-mediated G2 checkpoint decreases the number of aneuploid cells after MYC overexpression, as p53 inactivation reduces the population of G2-arrested cells with the potential to become aneuploid.[191] Thus, cells in the G2 or M phases of the cell cycle become aneuploid regardless of p53 status in the presence of activated MYC; however, the loss of p53-dependent cell cycle checkpoints and p53-mediated apoptosis enhances the ability of MYC-overexpressing cells to progress through the cell cycle and become aneuploid.

Experiments analyzing viral proteins that inactivate p53 or pRB demonstrate the importance of the p53-mediated, pRB-dependent G2 checkpoint after DNA damage. p53 function is disrupted in cells by the ectopic expression of the human papillomavirus E6 protein. E6 binds p53 and targets it for ubiquitin-mediated degradation, thus abrogating p53-dependent signaling.[192] Human fibroblasts and tumor cells expressing E6 have attenuated G2 checkpoint function after ionizing radiation exposure[148,156,193,194] or adriamycin,[156,195] as a significantly greater proportion of E6-containing cells enter mitosis as compared to control cells. Similarly, loss of the G2 checkpoint is observed after exposure to ionizing radiation in fibroblasts[196] and tumor cells[156] expressing the human papillomavirus E7 protein, as the E7 protein binds to and inactivates pRB. The adenovirus E1A protein is also capable of disrupting the DNA damage-induced G2 checkpoint.[197,198] Mouse keratinocytes and human tumor cells expressing the E1A protein are sensitized to treatment with cisplatin, adriamycin, and ionizing radiation due to defective G2 arrest.[199,200] However, it remains unclear if E1A-mediated disruption of the G2 checkpoint is p53-dependent. Sanchez-Prieto et al[199] failed to find a correlation between the presence or absence of p53 in keratinocytes and the ability of E1A to impair G2 checkpoint activation. In contrast, Bulavin et al[197] found that in E1A-expressing rat fibroblasts exposed to ionizing radiation, deregulation of p53-dependent signaling pathways contributed to defective G2 delay. Regardless of its ability to modulate p53-dependent G2 checkpoint signaling, E1A directly mediates transactivation of the Cdc2 promoter to facilitate G2 progression, although the contribution of E1A-mediated events to abrogation of the G2 checkpoint has not been elucidated.[201]

In some hematopoietic cell lines, p53 may accelerate the exit from G2 after DNA damage and this accelerated mitotic entry contributes to p53-mediated apoptosis. In murine myeloid leukemia cells bearing a temperature-sensitive p53 mutant, wt p53 positively modulates G2/M progression after etoposide or ionizing radiation exposure.[202,203] In these studies, the accelerated G2/M progression induced by p53 was associated with enhanced cytotoxicity and apoptosis. Similarly, myeloblast-enriched bone-marrow cells from p53+/+ mice have accelerated mitotic entry after ionizing radiation as compared to cells from p53-/- mice.[203] Thus, while p53 appears to be required for DNA damage-induced G2 arrest in epithelial cells, the G2 checkpoint in hematopoietic cells may be p53-independent.

Similar to hematopoietic cells, downregulation of Wee1 mRNA and protein expression and loss of Cdc2 Tyr-15 phosphorylation is observed after activation of p53 in rat embryo fibroblasts expressing the temperature-sensitive p53val135 mutant.[204] Downregulation of Wee1 also occurs in thymus of p53+/+ but not p53-/- mice after exposure of the animals to ionizing radiation.[204] The p53-mediated reduction in Wee1 is likely due to a transrepression mechanism similar to that discussed previously for cyclin B1 and Cdc2. Based upon the important roles of Wee1 and Cdc2 in regulating the G2/M transition, this mechanism may represent one biochemical pathway by which p53 modulates G2/M progression after DNA damage.

Modulation of the G2 Checkpoint—Therapeutic Implications

Since preclinical studies have shown that cells with defective checkpoint function are more vulnerable to anticancer agents, it is hypothesized that the same will hold true in the clinical setting. Indeed, numerous laboratories are now searching for compounds that interfere with and/or override cell cycle checkpoints, in hope that such agents may be more effective in anticancer therapy. A majority of tumor cells have defective G1 checkpoint function, making the G2 checkpoint their "last line of defense" after exposure to DNA damaging agents. Thus, the G2 checkpoint is a particularly attractive target for chemotherapeutic manipulation, since ablation of G2 checkpoint function in tumor cells may result in enhanced susceptibility to genotoxic anticancer drugs.

Chemical Approaches

There is strong evidence that abrogation of DNA damage-induced G2 arrest in human cancer cell lines results in higher rates of apoptosis. Exposure of cells to ionizing radiation in combination with caffeine or pentoxifylline, compounds which activate Cdc2 by activation of Cdc25C phosphatase, results in G2 checkpoint override and increased rates of apoptosis.[205-207] Caffeine disrupts the G2 checkpoint in p53-defective cells and results in radiosensitization of tumor cells;[205-207] however, the concentrations of caffeine required for abrogation of the G2 checkpoint in vitro are too cytotoxic for in vivo use. Nonetheless, elucidation of how caffeine overrides the G2 checkpoint has provided important mechanistic insight to how the G2 checkpoint can be modulated to enhance therapeutic efficacy. Caffeine inhibits the catalytic activity of both ATM and ATR at drug concentrations similar to those that induce radiosensitization.[184,208] Treatment of tumor cells with caffeine blocks ATM-mediated phosphorylation of Chk2 on Thr-68 after ionizing radiation.[209] ATR phosphorylates Chk1 on Ser-345 in human cells after ionizing and UV radiation exposure.[99] In *Xenopus*, DNA damage-induced phosphorylation of Chk1 is inhibited by caffeine,[210] suggesting that caffeine will also inhibit ATR-mediated phosphorylation of Chk1 in human cells. Caffeine also prevents the ionizing and UV radiation-induced phosphorylation of p53 on Ser-15, presumably by disrupting ATM and ATR function.[208] However, since caffeine preferentially sensitizes p53-deficient cells to DNA damage, the radiosensitizing effects of caffeine are most likely related to inhibition of ATM- and ATR-mediated activation of Chk2 and Chk1, respectively.

Pentoxifylline is a methylxanthine derivative that enhances the sensitivity of a wide variety of human tumor cells to DNA damaging agents.[211-213] While the precise mechanism of action of pentoxifylline has not been elucidated, several studies indicate that its ability to enhance

chemosensitivity is a direct result of G2 checkpoint abrogation.[211] Like caffeine, pentoxifylline preferentially radiosensitizes p53-deficient cells.[213,214] When used in combination with cisplatin, thiotepa, carboplatin, or cyclophosphamide, pentoxifylline enhances the ability of these compounds to inhibit the growth of murine mammary tumors in vivo.[215,216] Pentoxifylline also inhibits the growth of human bladder tumors[217] and human lung tumors[212] in mice xenograft tumor models. These promising preclinical results have prompted the evaluation of pentoxifylline in several clinical trials for efficacy against lung[218] and cervical[219] cancer in combination therapy with genotoxic chemotherapeutics such as ionizing radiation and cisplatin. Another related methylxanthine, lisofylline, also abrogates G2 checkpoint function and sensitizes p53-deficient human tumor cells to ionizing radiation[216,220] and cisplatin.[221] Further, lisofylline is more effective than pentoxifylline at enhancing the sensitivity of murine mammary tumor cells to ionizing radiation.[216,220]

Staurosporine is a non-specific protein kinase inhibitor that can override DNA damage-induced G2 delay in response to ionizing radiation.[222] However, the cytotoxicity of staurosporine has limited its potential clinical efficacy, leading to the development of staurosporine analogs with improved specificity and reduced cytotoxicity.[223] One such staurosporine derivative, UCN-01, is also a potent abrogator of the G2 cell cycle checkpoint and increases the cytotoxic effect of DNA-damaging agents in human tumor cells.[224,225] UCN-01 significantly inhibits the growth of a variety of human tumors in mice xenograft tumor models[226,227] and is currently in Phase I clinical trials showing promising results.[228] Preclinical studies have provided many mechanistic insights to UCN-01 activity. Treatment of tumor cells with UCN-01 results in Wee1 inactivation and Cdc25C activation, although these are indirect effects of UCN-01 inhibition of upstream checkpoint kinases.[229] UCN-01 specifically inhibits Chk1, as the related Chk2 kinase and the upstream ATM kinase are refractory to inhibition by UCN-01.[230] UCN-01 also inhibits C-TAK1 in vitro, although the contribution of this kinase to phosphorylation of Cdc25C during G2 checkpoint activation has not been fully elucidated.[231] Interestingly, UCN-01 selectively ablates the G2 checkpoint in cancer cells with defective p53 function.[224] Since ablation of G2 checkpoint function by UCN-01 can occur in the absence of p53, signaling to substrates other than p53 must be sufficient for G2 override.

Another approach to disrupt G2 checkpoint is the use of cell permeable peptides that can block specific G2 signaling components. For example, Suganuma et al[232] engineered short peptides corresponding to amino acids 211-221 of human Cdc25C fused with the retroviral TAT protein.[232] The TAT protein allows these fusion proteins to permeabilize the cell membrane and accumulate in excess of the endogenous Cdc25C, thus blocking Chk1 and Chk2 kinase activity toward the endogenous Cdc25C. Human tumor cells treated with these Cdc25C peptides are sensitized to DNA damage due to defective G2 checkpoint response, suggesting that Chk1 and Chk2 are effective targets to mediate abrogation of the G2 checkpoint.[232] Evidence supporting this latter strategy is provided by the recent report of SB-2180708, a selective Chk1 inhibitor structurally related to staurosporine that disrupts the G2 checkpoint.[233] In the presence of SB-2180708, HeLa cells exposed to ionizing radiation or topoisomerase I inhibitors fail to undergo a G2 arrest.[233] Further, inhibition of Chk1 activity in HeLa cells enhanced the cytotoxic effects of genotoxic agents, thus supporting the validity of Chk1 as a target for G2 checkpoint override.[233]

The p38γ cascade is another potential target for the development of radiosensitizing agents. Activation of p38γ is required for G2 arrest in tumor cells and fibroblasts exposed to ionizing radiation and disruption of p38γ-dependent signaling enhances the sensitivity of these cells to ionizing radiation.[145] Since p38γ is inactive under normal cellular growth conditions and elimination of p38γ-dependent signaling does not alter normal cell cycle progression,[145] the molecules in this pathway are potential targets for the development of inhibitors that will mediate increased sensitivity to radiation therapy. Thus, a further understanding of the mechanism by which p38γ regulates G2 arrest may lead to the development of novel strategies for the improvement of radiation therapy.

While the majority of anticancer drugs activate the G2 checkpoint after genotoxic stress by directly targeting kinases involved in DNA damage signaling pathways, alternative compounds that regulate the G2 checkpoint exist. For example, histone deacetylase inhibitors trigger a G2 arrest in normal human cells; however, this G2 arrest fails to occur in a diverse range of human tumor cells and they undergo mitotic catastrophe.[234] These compounds block histone deacetylase activity, increasing the acetylation state of the chromatin, altering chromatin structure and regulation of gene expression. These inhibitors may represent a novel mechanism of G2 override in tumor cells in the absence of concurrent DNA damage.[235] Of note, histone deacetylase inhibitors upregulate p21 to mediate cell cycle arrest at the G1/S and G2/M transitions,[236,237] as p21-/- colon carcinoma cells are resistant to these compounds.[238] The anticancer potential of histone deacetylase inhibitors has been demonstrated in both in vitro[236,239] and in vivo[240] model systems and several histone deacetylase inhibitors are currently being used in clinical trials with promising early results.[241]

In addition to providing insight to mechanisms that result in enhanced clinical efficacy, a further understanding of G2 checkpoint function may also lead to the identification of the signaling pathways that mediate tumor cell chemoresistance. For example, overexpression of the receptor tyrosine kinase, ERbB2 (HER2/neu), results in Paclitaxel resistance in breast cancers.[242] Yu et al[242] demonstrated that ERbB2 overexpression in breast cancer cells results in upregulation of p21, which binds cyclin B1/Cdc2 complexes and inhibits Paclitaxel-mediated Cdc2 activation and mitotic entry.[242] It is hypothesized that the ERbB2-mediated G2 arrest inhibits the action of Paclitaxel, which requires cell transition into mitosis and/or cyclin B1/Cdc2 activation.[243] Based on this latter study, it was hypothesized that the Paclitaxel resistance of ERbB2-overexpressing tumors could be eliminated by downregulation of ERb2 function. In support of this hypothesis, Baselga et al[244] recently demonstrated that combinatorial use of Paclitaxel and anti-HER2 antibodies results in significant growth inhibition of HER2 overexpressing human breast cancer xenograft tumors as compared to treatment with either agent alone.

Screens for New Compounds

In an effort to identify novel anticancer agents, including those which may abrogate G2 checkpoint function, the National Cancer Institute has utilized a panel of 60 human tumor cell lines in a drug screen to identify and characterize compounds with anticancer activity.[245] To date, 70,000 compounds have been tested in these cell lines and the results recorded in a database.[245,246] Of note, p53 gene mutations occur in a majority of the NCI drug screen cell lines, as 39 of 58 cell lines analyzed contain a mutant p53 sequence and have defective biochemical p53 activity.[247] Thus, many of the cell lines analyzed in the drug screen are predicted to have impaired G2 checkpoint maintenance following DNA damage. In support of this hypothesis, cell lines containing mutant p53 exhibit less growth inhibition in this screen than the wt p53 cell lines when treated with the majority of clinically used anticancer agents, including DNA cross-linking agents, antimetabolites, and topoisomerase I and II inhibitors.[247] This latter result suggests that disruption of the G2 checkpoint may enhance the clinical efficacy of anticancer reagents and exemplifies the need for the development of novel agents that might induce G2 checkpoint override. Amundson et al recently evaluated the basal expression levels of 10 transcripts from genes that participate in DNA damage signaling pathways in these same NCI cell lines and correlated this data with the sensitivity of the cells to a panel of 122 standard chemotherapy agents.[246] Further, cDNA microarray analyses have been used to assess gene expression profiles of these same 60 cancer cell lines in response to several standard chemotherapeutic drugs.[248] Grouping the cell lines by patterns of gene expression resulted in different relationships than those obtained by clustering the cell lines as a function of their chemosensitivity.[248] Analyses such as these may provide valuable insight as to how the transcription of specific genes relates to drug sensitivity. Similar strategies can be utilized in the future to examine the patterns of gene expression after treatment of cells with novel anticancer agents.

Investigators are also developing high-throughput screens to identify G2 checkpoint inhibitors. Roberge et al[249] used MCF-7 breast cancer cells that express a dominant-negative mutant p53 in a high-throughput screen for compounds that override ionizing radiation-induced G2 arrest and allow entry into mitosis. The loss of wt p53 function in these cells eliminates G1 checkpoint function and causes the majority of cells to arrest at the G2 checkpoint after exposure to ionizing radiation. To identify compounds that can ablate G2 checkpoint function, the mutant MCF-7 cells were grown in 96-well plates, irradiated to induce G2 arrest, and then co-treated with nocodazole and various extracts from marine invertebrates.[249] In the presence of a compound that could override the IR-induced G2 arrest, the cells were trapped in mitosis by the presence of the microtubule inhibitor nocodazole. The plates were then rapidly screened by use of an antibody that recognizes a phosphorylated form of nucleolin present only in mitotic cells. The reported screening process of 1300 extracts was validated by the isolation of staurosporine, a previously described G2 checkpoint inhibitor.[249] The screen identified one novel G2 inhibitor, isogranulatimide, a structurally unique compound. Isogranulatimide shows only mild toxicity to cells when used alone; however, treatment of the MCF-7 cells expressing mutant p53 with ionizing radiation and isogranulatimide results in synergistic cytotoxicity.[249] The use of this type of assay to identify G2 checkpoint inhibitors should allow further isolation of novel compounds that override G2 arrest.

Genetic Approaches

Since the major signaling pathways and cell cycle checkpoints are conserved between yeast and mammalian cells, yeast model systems may be manipulated to determine the molecular mechanism of anticancer drugs and to identify novel molecular targets for rational drug design. In contrast to mammalian cells, yeast offer the unique advantage of simple and rapid genetic manipulations that, coupled with the availability of the S. *cerevisiae* genomic sequence and the ongoing *S. pombe* genome sequencing project,[250] makes this organism an attractive model in which to evaluate chemotherapeutic agents. The Seattle Project encompassed multiple approaches for the discovery of anticancer targets and drugs through use of yeast genomics.[251] In one approach, a panel of isogenic yeast strains, each having single or multiple mutations in pathways involved in DNA repair, cell cycle checkpoint function, or cell cycle regulation were generated and used to screen new and existing anticancer drugs.[251] In a separate effort to identify novel cellular pathways to target for anticancer drug discovery, Norman et al[252] utilized budding yeast to genetically select peptide inhibitors. In this latter approach, peptides were selected based on phenotypic analyses and genetic dissections of candidate target pathways were performed to identify putative targets of the inhibitors.[252]

An additional example of the power of yeast genomics is the ability to perform cDNA microarray analyses to determine how genome-wide expression can be modulated in cells exposed to anticancer compounds.[253,254] This methodology was recently exploited by Jelinsky and Samson, as they used DNA chip technology (with the 6,200 *S. cerevisiae* genes represented) to compare the transcription profiles of *S. cerevisiae* treated with an alkylating agent, methyl methanesulfonate, to those of untreated cells.[254] The potential for microarray analyses to integrate existing regulatory networks is exemplified by the recent use of this technology to link the DNA excision repair pathway of *S. cerevisiae* to proteasome-associated control elements.[255] Further, Hughes et al[256] recently generated a database of expression profiles corresponding to 300 diverse mutations and chemical treatments in *S. cerevisiae* and used this database to identify novel genes required for various cellular functions as well as to identify novel target genes of known compounds. Of note, subsequent findings indicate that many yeast mutants exhibit chromosome-wide aneuploidy as compared to isogenic parental wt strains.[257] This observation has significant implications for interpreting whole-genome transcriptional expression profile data, particularly data obtained from malignant or immortalized cells that are known to be genetically unstable.[257] Genetic approaches such as those described

here will further validate the signaling pathways used by current chemotherapies, identify compounds with preferential lethality to cells with defective checkpoint function, and reveal additional signaling pathways to target for new drug discovery.

Future Directions

A fundamental challenge in the development of anticancer agents is the identification of molecular differences between cancer cells and normal cells that can be targeted for chemotherapeutic intervention to preferentially eliminate cancer cells while minimizing the toxicity to normal tissues. The drug discovery process for cancer will continue to be transformed by the wealth of information generated by the genome projects across many organisms, both prokaryotic and eukaryotic. As our understanding of cell cycle regulation and checkpoints increases so will the number of signaling molecules and pathways that can be used as targets for rational drug and therapy design. The hope is that from a detailed understanding of these processes, more incisive, mechanism-based approaches to cancer treatment will evolve that exploit the molecular defects in human tumors. To achieve this goal, we need to continue to develop

(1) technologies to precisely define the checkpoint defects in individual tumors,
(2) panels of anticancer agents that target cells with defined genetic alterations, and
(3) treatment regimens that are tailored to the resulting cell cycle phenotype.

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Chapter 11

p53, Apoptosis and Cancer Therapy

Rosandra Kaplan and David E. Fisher

Abstract

Several decades of genetic and molecular study have revealed enormous insights into the mechanistic underpinnings of cancer. From the identification of dominantly acting oncogenes to the signaling pathways which modulate the cell cycle, our understanding of the machinery of cell cycle progression as well as the regulatory circuits which control it have never been so detailed. However the translation of these discoveries into improved therapeutic approaches has been slow. The more recent appreciation of the pivotal role for cell survival pathways in both the genesis of malignancies as well as their response to treatment has created a burst of excitement for the prospect that the unraveling of these pathways stands to more directly impact on therapeutic strategies for cancer. This chapter focuses on one major regulator of cell survival in human cancer: the p53 tumor suppressor gene product. Abundant evidence suggests that the action(s) of this one gene serves to prevent formation of many human malignancies and likely mediates the successful treatment responses in those few tumors in which current/traditional chemotherapy produces durable cures. Through the study of p53's central roles in the cancer cell, molecular oncology has become inextricably linked to the quest for novel approaches to the therapy of cancer.

Introduction

The challenge in cancer therapy focuses fundamentally on the paucity of therapeutically exploitable differences between cancer cells and normal cells. This small margin is the therapeutic index, and it is the final endpoint for successful cancer therapy. Aside from the fact that cancer cells are so similar to normal cells, data accumulated over the past decade have suggested that most cancer cells are even more resistant than normal cells to a variety of death triggers. Dismantling the machinery for cell death confers an obvious survival advantage, potentially explaining a fundamental feature of neoplastic transformation. For this reason, cancer is now recognized to be a disease involving both uncontrolled proliferation and a lack of normal cell death.

There is a growing body of evidence that anticancer therapies kill not exclusively through the toxic effects of disrupting cellular metabolism, but also by triggering pathways that lead to the cell's suicide, also known as apoptosis. In both animal models and human cancers, cells with intact apoptotic death machinery are more susceptible to cancer therapies than those with disrupted apoptosis pathways. p53, the gene most commonly mutated in human cancer, is a major regulator of apoptosis. Among the few curable human cancers, even in advanced stages, are pediATRic acute lymphoblastic leukemia, testicular cancer, and Wilms tumor, which all show a relative paucity of p53 aberrations and remarkably intact apoptotic death machinery. In these responsive cancers, apoptosis is likely a central mechanism in the induction of remission.

Apoptosis is genetically encoded, evolutionarily conserved, and is intrinsically linked to mechanisms of cell growth and differentiation. The first genetic descriptions of apoptosis came

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from studies of death mutants in nematodes.[1,2] In these organisms three genes were discovered that are involved in cell survival and death decisions. One of these is homologous to mammalian caspases—a family of cysteine proteases that are essential to apoptosis. Another gene is homologous to mammalian Apaf-1, which participates in formation of a complex which relays death signals to the caspases. The third major death gene is homologous to the Bcl-2 family, members of which have either pro-apoptotic or anti-apoptotic activity. The identification of these genes initiated a research burst which has shed enormous light on our understanding of mechanisms of cell death both in normal development and under pathological conditions such as cancer.

Although apoptosis can involve a variety of diverse triggers and cellular intermediates, there are certain common features as well (Fig. 1). Apoptosis involves an initiator trigger such as DNA damage, osmotic stress or cues from the extracellular environment. Through either of several pathways, signaling events emanating from the trigger eventually lead to activation of upstream initiator caspases which, in turn, activate downstream, "executioner" caspases. These downstream caspases, such as caspases 3 and 7 cleave numerous cellular targets, resulting in profound changes in the protein, lipid, and DNA compartments. Regulation of apoptosis occurs at numerous levels within a cell. Of particular importance is the modulation of initial caspase activation by the triggering pathway. Many survival factors as well as death factors likely operate at this level, as described below.

Virtually all apoptotic triggers appear to employ one of two main pathways (Figs. 2 and 3). One pathway initiates from death receptors which may lead quite directly to the activation of caspases, or in certain cases may utilize mitochondria as intermediates. The other is a pathway that appears to depend upon release of key mitochondrial factors in order to activate cytosolic caspases in response to a large number of stress-like triggers.

Caspases exist in normal cells as inactive enzymes called pro-caspases, analogous to zymogens involved in the regulation of blood clotting. When activated by removal of the pro-domain, these proteases cleave proteins at aspartic acid residues contained within tetrapeptide recognition motifs. There are two general types of caspases: initiators, also known as upstream caspases, and downstream, or executioner, caspases. Caspases -8, -9, -10 are upstream caspases and are activated via auto-proteolysis upon recruitment into complexes in which high local concentrations are presumed to permit weak proteolytic activity of the pro-caspase to cleave a neighboring pro-caspase, thereby setting off an amplifying cascade. Once these upstream caspases are activated, they then activate the executioner caspases such as caspase -3, -6 and -7, which cleave numerous proteins throughout the cell and lead to membrane changes and DNA cleavage events which are pathognomonic of apoptosis.

There are important negative regulators of apoptosis that can act on the caspase cascade at several points. These inhibitors of apoptosis (IAPs) include c-IAP-1, c-IAP-2, XIAP, and survivin, and are thought to provide a safeguard mechanism which serves as an endogenous threshold regulator to temper minimal activation of the cascade. The overexpression of IAPs typically renders the cell resistant to a wide variety of apoptotic stimuli. It has been suggested that IAPs act by directly interfering with the catalytic activity or activation of certain caspases.[3-5] Cellular levels of IAPs may thus determine the difference in sensitivities of cells to apoptosis-inducing stimuli. The recent identification of Smac/Diablo as a mitochondrially released IAP antagonist has given rise to the concept that regulation of IAPs may represent a general means of modulating the apoptotic response.[6,7]

Fas is a death receptor which triggers an apoptotic pathway involved in creating tolerance to self in B and T cells by inducing death of autoreactive clones. Fas ligand binds to the Fas receptor causing oligomerization and conformational changes which lead to recruitment of the Fas-associated death-domain-containing molecule, FADD (Fig. 2). This in turn leads to binding of pro-caspase-8 oligomers to FADD and caspase-8 auto-activation[8] presumably through auto-cleavage due to the high local concentrations of both enzyme and substrate. Similar death receptor events are thought to occur for the Tumor Necrosis Factor (TNF) pathway and other related receptor family members.

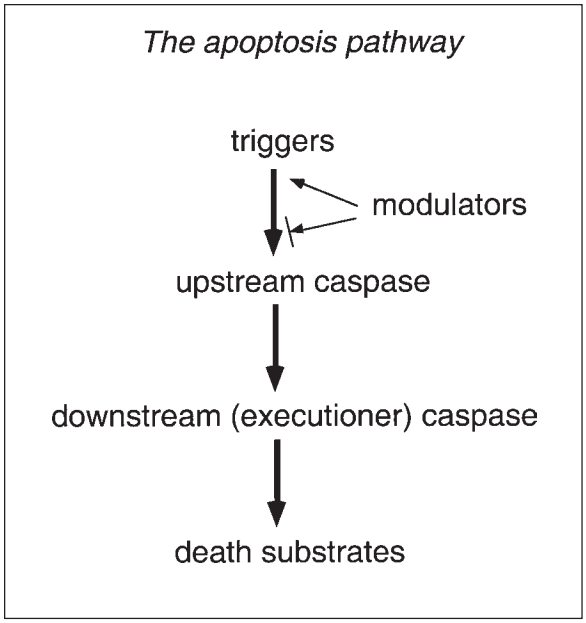

Fig. 1. Blueprint of the apoptosis pathway. A wide assortment of triggering events activate signaling cascades which direct the modification of upstream (or initiator) caspases, especially caspases 8 and 9, from their zymogens to the active proteases. In turn, these activated caspases cleave and activate downstream caspases such as caspase 3 and 7, which cleave numerous cellular targets known as death substrates.

The mitochondrial pathway of apoptosis (Fig. 3) involves a variety of signaling events, not entirely understood at present, which trigger release of various mitochondrial contents including cytochrome C into the cytosol. Cytochrome C, once released from the mitochondria, acts together with Apaf-1 and procaspase-9 to activate downstream executioner caspases-3, -6, and -79. A major regulatory step in this pathway is release of cytochrome C[10]as well as Smac/Diablo.[6,7] Antiapoptotic members of the Bcl-2 family such as Bcl-2 or Bcl-xL when overexpressed can prevent cytochrome C release.[11,12] The mitochondrial pathway is also strongly regulated by pro-apoptotic members of the Bcl-2 family, such as BAX and Bad. These proteins may dimerize with the anti-apoptotic Bcl-2 family members as well as form channels in the mitochondrial membrane or alter the activity of the existing channels allowing for cytochrome C release.

There is immense interconnectedness among these apoptotic pathways. p53 has been reported to induce apoptosis through several different points within these apoptotic pathways. p53 may transcriptionally activate genes that are essential to growth arrest and DNA damage such as GADD45, PA26, IGFBP-3, SIAH-1, and 14-3-3. It can also activate the pro-apoptotic Bcl-2 family member BAX.[13] In addition, separate experimental evidence has suggested that p53 might mediate death without transcriptional activity.[14-17] It may alter trafficking of Fas to the cell surface with activation of the receptor leading to activation of the caspase cascade.[18] p53 may also activate XPB and XPD DNA helicases which may, through an incompletely understood pathway, trigger caspase activation.[19] The mechanistic connection between p53 and caspase activation has remained incompletely understood, but remains of pivotal importance because of p53's apparently major role in modulating the decision to live or die in human cancer cells.

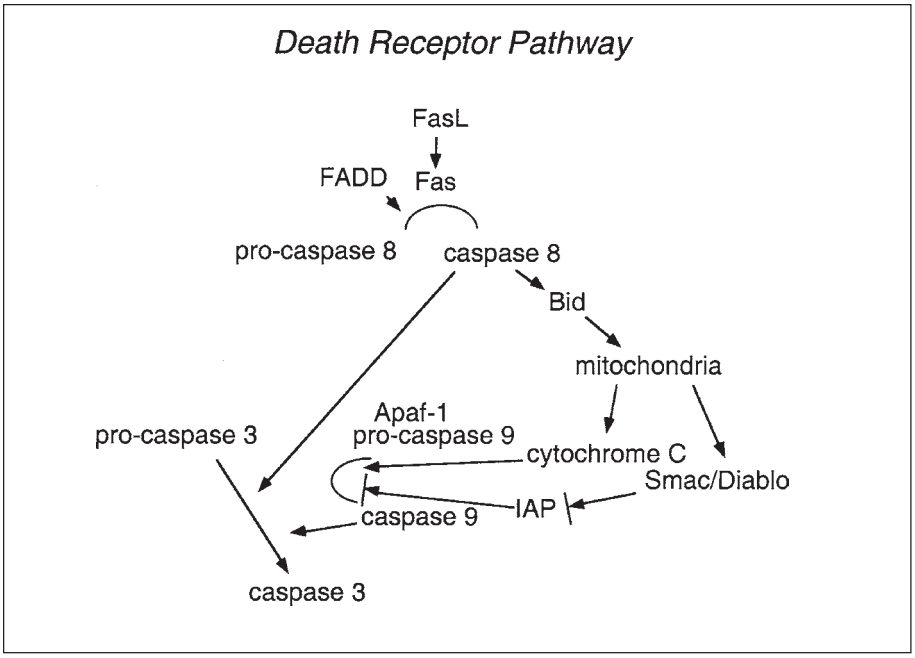

Fig. 2. The death receptor pathway of apoptosis. Shown here is a schematic of the pathway initiated by the Fas ligand (FasL). Similar events are thought to occur for the other members of the TNF receptor family of death receptors. In this pathway FasL induces trimerization of the Fas receptor, thereby recruiting FADD which binds pro-caspase 8. High local concentrations of pro-caspase 8 oligomers result in auto-proteolysis and activation. Active caspase 8 may either directly activate pro-caspase 3 or alternatively cleave Bid, resulting in stimulation of the mitochondrial apoptosis pathway.

p53's Emergence as a Key Death Regulator

p53 was discovered in 1979 when it was found as a -53kd cellular protein associated with the tumor (T) antigen present in SV40 transformed cells.[20,21] The protein was found to vary somewhat in size, but retain homology in hamster, monkey and human cells. Its presence in murine embryonal cell lines as well as SV-40 infected or transformed cells led to the conclusion that the protein is encoded by the host genome. It was believed that p53 played a role in modulation of the transformed state.

In the 1980s p53 was thought to be a dominant nuclear oncogene.[22-26] Evidence for dominant transforming activities of p53 resulted from the fact that the cDNAs and p53 genomic clones used in previous studies possessed dominant negative mutations.[27-29] p53 was definitively demonstrated to be a tumor suppressor gene in 1989 as evidence of loss of wildtype p53 expression in many tumor types became apparent.[30-34] Wildtype p53 showed the ability to produce a marked reduction in the number of transformed foci of rat embryo fibroblasts.[35] The p53 gene was localized to chromosome 17 band p13.[36] p53 germline mutations were identified in the rare autosomal dominant cancer predisposition condition known as Li- Fraumeni syndrome.[37] Li-Fraumeni syndrome is characterized by early onset tumors including breast, pancreatic, adrenocortical, and prostate carcinomas, soft tissue sarcomas, brain tumors, osteosarcoma, and leukemia.

Over the past decade p53's molecular functions began to be elucidated. p53 protein was discovered to be a transcription factor that enhances transcription of many genes thought to integrate the cellular responses to stress. Protein levels are increased in response to double stranded breaks in DNA and the presence of DNA repair intermediates after ultraviolet radia-

tion exposure or chemical damage to DNA.[38-42] Increased p53 activity in damaged cells can lead to either cell cycle arrest or apoptosis.

The original observation which linked p53 protein to induction of the apoptotic response involved an elegant experiment using temperature-sensitive mutant p53. In this study, the ts-p53 gene was expressed in myeloid leukemia cells. At the permissive temperature, p53 triggered massive apoptosis in the tumor cell population.[43] Subsequent studies demonstrated that p53 wildtype mouse thymocytes undergo apoptosis in response to ionizing radiation whereas thymocytes from p53 -/- mice do not undergo apoptosis when similarly irradiated.[44,45] The ability of p53 to trigger apoptosis in response to DNA damage was further highlighted by the findings that multiple chemotherapeutic agents triggered p53-dependent apoptosis in murine oncogene-transformed fibroblasts both in vitro and in a mouse sarcoma model.[46,47]

Clinical Aspects of p53

Functional loss of p53 appears to be a very frequent, and possibly essential, prerequisite during tumorigenesis. p53 mutations are found in over 50% of all human cancers. There are currently over 2,000 literature reports documenting p53 mutations in diverse human tumor types[48] with p53 mutation being the most frequent genetic event in human cancer demonstrated to date.[49] The most common mutations are missense point mutations located in four of the five evolutionarily conserved domains between amino acids 120 and 300.[50] p53 mutations have been found in all major histologic groups, including cancers of the colon (60%), stomach (60%), breast (20%), lung (70%), brain (40%), and esophagus (60%).[51] Mutations have been analyzed by several different methods including DNA analytical methods, immunohistochemical analyses (which show accumulation of mutant p53), and functional analyses which can be used to assess transactivating activity.[52]

There has been a significant literature addressing clinical correlations between p53 and prognosis. Some studies have found that p53 mutations in tumor cells have been associated with a worse prognosis.[53-55] Those malignancies with the best clinical outcomes such as testis cancer, pediATRic acute lymphoblastic leukemia, and Wilms tumor have a paucity of p53 mutations and are quite responsive to therapy.[56-58] When these tumors relapse, ineffectiveness of therapy has been correlated with the acquisition of p53 mutations.[59,60]

Complicating these analyses, there are malignancies with wildtype p53 that exhibit poor prognosis. Initially this finding gave pause to the idea that p53 aberrations played a significant role in therapeutic responsiveness. However these examples have proven insightful in suggesting that p53 function, rather than gene mutation per se, is the key measure of its role in the therapeutic response. For example in cervical and anogenital cancers, p53 has been found to be genetically wildtype, but the protein is rapidly degraded via a mechanism triggered by the E6 protein encoded by human papillomovirus.[61-63] Similarly, p53 is both functionally inhibited and degraded by the action of the Mdm2 oncoprotein. Mdm2 amplification is seen in a variety of poor prognosis tumors that retain wildtype p53.[64-67] Multiple recent studies show that p14 ARF (the gene encoded by the alternative reading frame at the p16/Ink4a locus) regulates p53 interactions with Mdm2.[68,69] The genetic locus for this protein is frequently mutated, deleted, or hypermethylated in a wide variety of cancers. Another example in which wildtype p53 is present but lacks functionality is seen in neuroblastoma and certain breast carcinomas in which p53 protein is sequestered in the cytoplasm, preventing proper nuclear localization.[70-72]

p53 Actions: Arrest vs. Death

Far beyond its early suspected role in viral transformation, p53 has subsequently been termed "the guardian of the genome" and "the gatekeeper for cellular growth and division" because of its vital function in tumor suppression and growth regulation.[60,73] Normally p53 is present at low concentration with a short half-life. However, in response to multiple signals including DNA damage, oncogene activation, hypoxia/acidosis, stimulation or deprivation by cytokines, as well as depletion of nucleoside triphosphate pools, p53 protein levels surge in the cell.[41, 74-77] Upon activation, p53 can induce a variety of cellular responses, most notably cell

cycle arrest or apoptosis. Additionally, p53 has been implicated in prevention of embryonic malformations despite the fact that p53 null mice display a relatively normal pattern of development.[78,79] p53 is involved in cellular senescence and participates in the regulation of centrosome number.[80-82] The regulation of cell cycle arrest and apoptosis likely underlie p53's role in modulating cancer therapy and are explored here in further detail.

Cell Cycle Arrest

p53 intervenes at several points in the cell cycle. It is thought to do so by transcriptionally activating genes that are key to the cellular replication machinery. p53's sequence-specific DNA binding domain is localized between amino acid residues 102 and 292 where hydrogen bonds contact both the minor and major groove of the double helix.[83] p53 can mediate both G1 and G2/M cell cycle arrest in response to DNA damage. It likely performs these functions by activating target genes that contain p53-dependent, cis-acting, DNA- responsive elements namely p21/WAF1/CIP1, GADD45 and IGF-BP3.[39,60,84]

p21/WAF1/CIP1 is a potent mediator of the G1 cell cycle checkpoint because it is able to associate with and inhibit G1 cyclin/cyclin-dependent kinase complexes, specifically cyclin D1-CDK4, cyclin E-CDK2, cyclin A-CDK2, and cyclin A-cdc2.[84-87] p21/WAF1/CIP1 has been shown to bind directly to proliferating cell nuclear antigen (PCNA) and inhibit PCNA-dependent DNA replication.[88-90] Mice deficient in the p21 gene develop normally but fibroblasts derived from these mice have a limited ability to arrest in G1 following DNA damage.[91] Nonetheless, p21/WAF1/CIP1 is not the only p53 mediated mechanism to arrest cells in G1, since p21 null fibroblasts retain some of the p53-dependent G1 checkpoint activity. GADD45 expression has been associated with both G1 and G2/M arrest.[92,93] GADD45 can stimulate excision repair and like p21WAF1/CIP1 also binds to PCNA and may play a backup role for p21.[94] IGF-BP3 gene codes for a protein that inhibits insulin-like growth factor, a mitogenic factor. p53 has been shown to activate IGF-BP3 thereby limiting the mitogenic response.[95]

p53 may also interact via protein-protein interactions with other tumor related factors including the Wilm's tumor gene (WT1) and the Gas1 gene. WT1 gene when coexpressed with p53 results in higher steady state levels of p53, enhanced transcriptional activity of p53, and increased levels of p53 DNA sequence-specific binding.[96] The Gas 1 gene encodes a membrane protein associated with Go arrest which functions when p53 is present. Interestingly p53's transcriptional function appears dispensable for Gas 1 induction because p53 with a mutation preventing p53 transcriptional activity can still induce Gas1.[97]

Chk2, a DNA damage-responsive kinase, can phosphorylate Cdc25C causing binding to 14-3-3 protein and inhibition of its ability to promote cell cycle progression via cdc2.[98] In addition, Chk2 has another important role in cell cycle regulation which is mediated by its ability to phosphorylate p53 on serine 20. This phosphorylation prevents Mdm2 binding and results in p53 stabilization.[98] ATM (the gene defective in ataxia-telangectasia) can also phosphorylate p53 on serine 15 following ionizing radiation. This phosphorylation is important for activation of p53 as a transcription factor and may act synergistically with serine 20 phosphorylation.[99,100] ATM, Chk1 and Chk2 are all activated following DNA damage, though with differential responsiveness depending on the agent inducing the DNA damage as well as the particular cell type and the cell cycle stage during which the damage occurs.[101] Chk2 is now recognized as a tumor suppressor; germline mutations in Chk2 occur in Li-Fraumeni-like syndrome patients who lack the more common germline p53 mutations.[98]

Apoptosis

p53's role in modulating the apoptotic response likely plays an important role in p53's tumor suppressor activity as well as its ability to mediate therapy-directed tumor cell death. Although its ability to trigger apoptosis following appropriate signals is profound, the mechanistic pathway(s) connecting p53 to the apoptotic death machinery have remained complex and elusive. In contrast to the cell cycle activity of p53 which is largely (though not entirely)

attributable to the transcriptional activation of the p21 CDK-inhibitor, no single essential mediator of p53's apoptotic activity has emerged thus far. In fact, a variety of experimental systems have suggested that p53 may fundamentally utilize at least two apoptotic pathways, one involving transcription of specific target death genes, and another which is a transcription-independent road to apoptotic death. Although the road to apoptosis may traverse distinct paths, the common endpoint is activation of executioner caspases. The two pathways leading to such caspase activation are the death receptor and the mitochondrial pathways (Figs. 2 and 3).

p53 Mediated Transcription Dependent Apoptosis

There has been a growing list of p53 target genes involved in apoptosis. These include death-promoting genes such as BAX, CD95/Fas/Apo1, the PIG genes involved in redox regulation, and the TNF-receptor family of genes containing death domains. BAX is the best known pro-apoptotic p53 target and member of the bcl-2 family. Its promoter contains p53-binding sites and is upregulated by p53 in response to DNA damage.[13,102] BAX can function as an effector of p53, allowing for chemotherapy-induced apoptosis and suppression of oncogenic transformation.[103] The precise mechanism by which BAX activation leads to apoptosis has been somewhat unclear. BAX is found both in the cytosol and mitochondria, but its translocation from cytosol to the mitochondria seems to be essential for BAX function.[104] This translocation of BAX may require the presence of a newly discovered mediator of apoptosis known as Peg3/Pw1.[104,105] Once localized to mitochondria BAX may participate in generation of membrane channels leading to the release of cytochrome C to the cytosol where it binds with Apaf-1 and activates procaspase 9.[104,106] Thus BAX clearly links p53 with the apoptotic machinery of the mitochondria. However, despite the importance of BAX as a p53 target gene, the observation that BAX-deficient thymocytes retain intact radiation induced apoptosis[107] suggests that BAX cannot be the only mediator of p53-dependent apoptosis.

PIGs are p53-induced genes identified using SAGE analysis from colon carcinoma cells.[108] Most were predicted to be involved in modulating the redox state of the cell. These genes encode proteins which may directly or indirectly stimulate the production of reactive oxygen species which signal mitochondrial release of cytochrome C or other caspase activators.

Another group of p53-targeted apoptosis modulators consists of members of the TNF family of transmembrane death receptors. These include Fas/APO-1/CD95, TNFR1, DR-3/APO-3/WSL-1/TRAMP, DR4/TRAIL-R1, and DR5/TRAIL-R2/KILLER. These receptors share a conserved cysteine-rich repeat at their extracellular domains. Fas, TNFR1, DR3, DR5/KILLER, and CAR1 carry a region of homology in the cytoplasmic tail called the death domain, which is essential to its ability to transduce an apoptotic signal.[109] The activating ligands for the death receptors are structurally related and belong to the TNF gene superfamily. Fas ligand (FasL) binds to Fas, Apo3 ligand (Apo3L) binds to DR3 and Apo2 ligand (Apo2L or TRAIL) binds to DR5/Killler.[110] When Fas binds to its ligand (FasL) it induces trimerization of Fas and the cytoplasmic region of Fas, which contains the death domain, recruits FADD. FADD also contains a death domain and it is the interaction between the Fas and FADD death domains that propogate the death signal inside the cell.[106] A single point mutation in the death domain abrogates the apoptotic signal.[111,112] Physical association of FADD with Fas leads to oligomerization of pro-caspase 8 within the DISC complex and generation of active caspase 8 which propagates subsequent activation of the executioner caspases. DR5/KILLER has a similar mechanism of action after binding by the ligand TRAIL (APO2L). Both TRAIL and KILLER/DR5 when overexpressed can act independently to induce apoptosis. However there are decoy receptors for TRAIL (DcR1 and DcR2) which can block TRAIL function.[113] p53 may transcriptionally activate Fas and FasL expression.[114-116] It has also recently been found that KILLER/DR5 is induced by DNA damage selectively in wild type p53 cells, and p53-dependent transcriptional induction of KILLER/DR5 death receptor may be restricted to cells undergoing apoptosis rather than growth arrest.[117]

IGF-BP3, in addition to its anti-mitotic function, can bind to IGF-1 and thereby antagonize its anti-apoptotic influence. A number of cytokine growth factors display strong

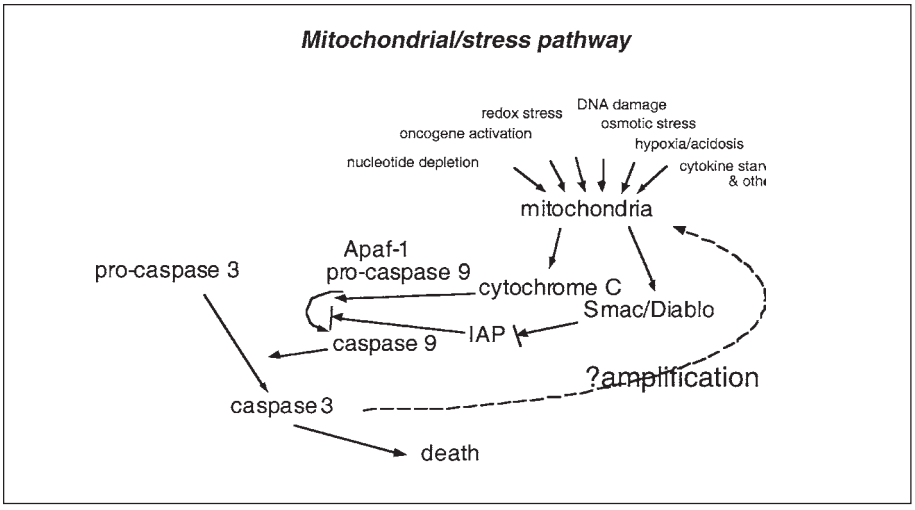

Fig. 3. The mitochondrial apoptosis pathway. A wide variety of cellular stresses trigger signaling cascades which produce mitochondrial release of cytochrome C and Smac/Diablo. These mitochondrial factors then assist in the activation of pro-caspase 9 in the cytosol. Cytochrome C forms an activating complex with Apaf-1 and pro-caspase 9, while Smac/Diably displaces the IAP apoptosis inhibitor molecules from caspase 9.

anti-apoptotic influences. p53 may act through induction of IGF-BP3 to promote apoptosis to disrupt the anti-apoptotic signaling.[95,118]

There are several novel p53 target genes believed to be mediators of apoptosis about which relatively little mechanism of action is known. PAG608 is a zinc finger protein that induces apoptosis when introduced into human cancer cells.[119] PERP is a recently identified p53 gene target in the gas3 family which is specifically induced upon DNA damage during apoptosis and is another candidate effector in the p53 transcription dependent apoptotic pathway[120]. DRAL, a p53 responsive gene, has been implicated in the apoptotic pathway because its expression efficiently triggers apoptosis in multiple cell lines of different origins.[121] This protein also contains a zinc finger domain similar to PAG608. Potential functions for this protein include a scaffolding role or as a modulator of transcription.[122,123] Noxa encodes a protein containing the Bcl-2 homology 3 (BH3) motif seen in the Bcl-2 family of proteins. The promoter region of the Noxa gene contains a p53 response element and increased expression of Noxa mRNA was observed after cells were infected with adenovirus overexpressing p53.[124] Noxa protein localizes to the mitochondrial membrane.

p53 can also transcriptionally repress certain genes, particularly in the context of promoters lacking p53 consensus DNA binding elements. For example, bcl-2 expression may be decreased in the presence of p53-mediated apoptosis.[125] p53 also negatively regulates expression of MAP4 a microtubule stabilizing protein.[126]

p53 Mediated, Transcription-Independent Apoptosis

A major puzzle has emerged from studies which suggested that p53 may, in certain contexts, trigger apoptosis without the apparent need for its transcriptional activity.[14,15,127,128] The original data supporting this possibility arose from the observation that p53's apoptotic activity was resistant to actinomycin D or cycloheximide treatment. Additional evidence came from mutational studies in which overexpression of transcriptionally incompetent p53 mutants produced clear apoptotic activity.[16] These observations as well as others which followed, effectively uncoupled p53's apoptotic activity from its transcriptional function.

The proline rich region of p53 contains several SH3 binding PXXP motifs and displays SH3 binding activity. This area may serve as a docking site for other SH3-containing proteins. A human p53 mutant derived from familial breast cancer destroys one of these proline rich binding motifs suggesting that the proline rich domain is important to p53's apoptotic function.[129,130] A Li-Fraumeni syndrome family was found to harbor a p53 hinge domain mutation which retained the ability to arrest the cell cycle but not trigger apoptosis when overexpressed.[131] Perhaps this hinge region serves as a regulatory domain for apoptosis. The most common mutations seen in human cancers occur in the central DNA-binding domain of p53 that lead to a loss in the DNA binding function of the p53 protein or a change in confirmation of the protein.[51,83] There is evidence that this central domain can bind 53BP2 protein that interacts with bcl-2.[132] Finally, a cell-free apoptosis system was devised in which p53 protein appears to modulate the activation of caspase 8 via as yet undefined intermediates.[17, 133]

Regulating p53 Activation in the Stress Response

Regulation of p53 occurs predominantly through control of its protein stability. Mdm2 directly binds residues within the N-terminal transactivation domain of p53 thereby repressing transcriptional activation of p53 and targeting it for degradation.[64,65,134] p53 transcriptionally activates Mdm2 expression thus providing negative autoregulation. Mdm2 acts as ubiquitin ligase for p53.[135,136] The high levels of mutant p53 in many tumors is probably related to lack of Mdm2-mediated p53 turnover.[137] Other factors can act to degrade p53 such as papillomavirus E6 and adenovirus E1B proteins.[138,139] Onocogenes are potent stabilizers of p53, potentially providing the organism with a mechanism to curb malignant transformation. Myc, Ras and E1A are thought to stabilize p53 via E2F-mediate upregulation of p19ARF (P14ARF in humans), which inhibits Mdm2 and therefore decreases p53 degradation.[140-142]

p53 activity is significantly affected by acetylation and phosphorylation. p53 is phosphorylated on Ser 15 by the ATM protein kinase in response to DNA damage.[99] This phosphorylation leads to protein accumulation as well as the increased ability of p53 to transactivate downstream target genes.[100] Phosphorylation of p53 can also take place on Ser 20 by Chk2, which results in p53 stabilization.[98] It appears that phosphorylation at Ser 15 acts synergistically with Ser 20 phosphorylation.[143] Acetylation of p53 at the C terminus promotes DNA binding activity as well as proper oligomerization and subcellular localization.[144,145] In addition, phosphorylation at the N terminus mediates acetylation at the C terminus and can enhance DNA binding.[127,146]

Cell Cycle Arrest vs Death

The decision of arrest vs. death is potentially of enormous clinical importance in cancer therapy. Studies in fibroblasts have demonstrated that in the oncogene-transformed setting, ionizing radiation triggers p53-dependent apoptosis whereas in primary, untransformed fibroblasts ionizing radiation triggers p53-dependent cell cycle arrest.[46,47] The relevance of these behaviors to cancer therapy stems from both the selective sensitization towards death in the oncogene-transformed setting, as well as the protective effects of cell cycle arrest on the ability of normal cells to repair DNA damage. Thus an apparent therapeutic index is created in which p53 simultaneously protects normal cells via its cell cycle activity while targeting tumor cells for apoptotic death (Fig. 4).

How might p53 selectively trigger death vs. arrest? One hypothesis proposes that the quantity of DNA damage and, by extension, the overall level of p53 protein induction may determine the outcome. In this system, higher levels of p53 protein were associated with apoptosis.[147] The oncogene-specific death response in primary fibroblasts could also theoretically arise via Ink4a/ARF effects. Since oncogenic upregulation of ARF is predicted to repress Mdm2's downregulation of p53, oncogene-transformed cells are predicted to contain higher basal p53 levels. In certain settings evidence suggested that both cell cycle arrest genes and apoptosis mediating genes are activated in cells undergoing arrest alone or apoptosis.[108,142]

Such data could still be consistent with the above hypothesis, if the decision were also determined by cellular context. In fact, whereas primary fibroblasts arrest following ionizing radiation, certain primary cell types such as thymocytes undergo p53-dependent apoptosis instead. Still, it is unclear whether p53 levels correlate in different cell systems with the death vs. arrest decision. For these reasons it is also plausible that a cellular setpoint or apoptosis threshold determines whether p53's output will result in death.

Another important determinant of arrest versus death may be the presence or absence of cytokines. For example in the hematopoietic Baf/3 cell system, irradiation in the presence of IL-3 produces cell cycle arrest whereas irradiation in the absence of IL-3 produces rapid apoptosis. In this system, p53-independent regulation of Gadd45 and p21/Waf1/Cip1 could play a role in modulating the apoptosis vs. arrest threshold.[148] Alternatively, it is plausible that activation of the PI3 kinase-Akt survival pathway by growth factors or Ras-mediated signaling may "set" the apoptotic threshold to respond differently to identical p53 dependent apoptosis triggers.

Finally, the KILLER/DR5 receptor is a TNF receptor family member whose expression is upregulated by DNA damage in a p53 dependent fashion.[117,149,150] Activation of this pathway by the ligand TRAIL results in death receptor-mediated apoptosis. Interestingly, a decoy receptor which binds the same ligand (TRAIL) is often expressed in non-tumor cells, thereby potentially conferring tumor selectivity to TRAIL as a therapeutic. However recent evidence suggests that p53 may upregulate expression of decoy receptors as well, thereby potentially blunting the apoptotic response to TRAIL.[151,152] This pathway, as well as the Fas/FasL pathway which is also transcriptionally regulated by p53, may help explain p53's apoptotic activity and may simultaneously be exploitable for clinical benefit if tumor selectivity were consistently revealed.

Therapy

Numerous well-known variables determine therapeutic efficacy in cancer including drug delivery, selectivity (therapeutic index), and mechanisms of intrinsic or acquired resistance. A number of experimental therapeutic strategies for cancer have revolved around modulating p53's apoptotic activity presumably because of p53's ability to selectively instruct tumor cells to undergo apoptosis. While most of these approaches have theoretical pitfalls, the creative evolution of this field lends optimism to the prospect that improved therapies may eventually emerge.

One treatment strategy is reintroduction of wildtype p53 itself into p53 deficient tumor cells.[153-155] Concerns of delivery, normal cellular toxicity, and overcoming dominant negative effects of mutant p53 could limit the applicability of this approach, but potential benefits may be seen in specific clinical settings. It is likely that p53 reintroduction would be especially useful in tumors harboring dysregulated E2F-1 (the majority of human malignancies) since such cells should be hypersensitive to p53-dependent apoptosis if the Ink4a/ARF pathway is intact.

Another strategy employs a C-terminal fragment of p53 peptide to reactivate endogenous mutant p53.[156] This approach is based upon the known inhibitory role of the endogenous C-terminal region of p53. Displacement of that motif by exogenous peptide may permit reactivation of the mutant protein, and since the mutant protein is often present at significantly higher levels, the result could be tumor-selective apoptosis as described by Fine and colleagues.[157] In the long term, it is likely that small molecule drug discovery or peptido-mimetic strategies will be necessary to translate this approach into an in vivo therapy.

There are certain tumors in which p53 is wildtype, but its function is apparently deficient. For example in cervical carcinoma cells, disruption of E6 without compromising E7 can lead to extensive apoptosis.[158,159] Although the generality of these findings remain to be confirmed, one group has found that certain neuroblastomas express genomic sequences from human polyomavirus BK (BKV). Neuroblastomas typically lack p53 mutations, although p53 tends to be cytoplasmically sequestered and therefore less than fully functional. In this setting, treatment of neuroblastoma cells with BKV large T Ag antisense was reported to correct the translocation of p53 to the nucleus with rescue of wildtype p53 function including apoptosis.[160]

Another approach focuses on altering p53 in those cancer cells with mutated p53. Trans-splicing ribozymes were used that can simultaneously reduce mutant p53 expression and

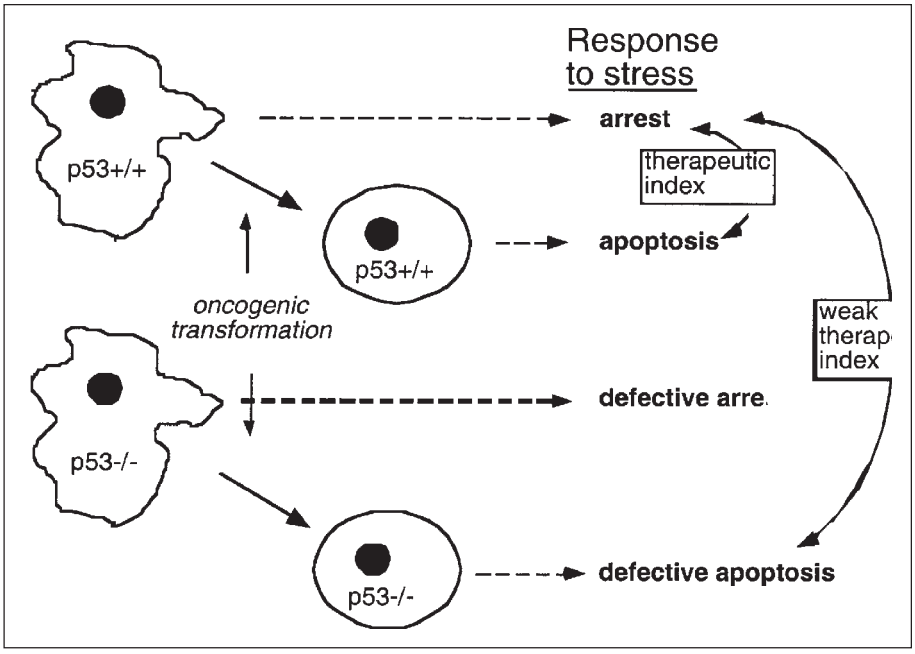

Fig. 4. Role of p53 in the cellular stress response. In fibroblasts, primary cells undergo cell cycle arrest, whereas oncogene-transformed cells undergo apoptosis in the setting of wildtype p53. Where p53 is deficient, primary fibroblasts display aberrant G0/G1 cell cycle arrest, and oncogene transformed fibroblasts are significantly resistant to the induction of apoptosis, thereby diminishing the potential therapeutic index conferred by wildtype p53.

restore wildtype p53 activity in various human cancers. The ribozyme acts to repair mutant p53 mRNAs with high fidelity and specificity. Results showed transactivation of p53-responsive promoters and down-regulated expression of the multidrug resistant gene promoter.[161] One novel therapeutic approach involves exploiting the ATM pathway of p53 activation. Although ATM deficiency significantly delays p53 activation following ionizing radiation,[162] ATM deficiency also confers profound radiation sensitivity to multiple organ systems in ATM knockout mice and this radiosensitivity is largely p53-independent.[163-165] Therefore ATM may serve as a significant radiosensitizing drug target for p53-deficient cancers, although tumor selectivity is a potential problem (which might be at least partially overcome through localized radiation treatment).

An alternate strategy for exploiting p53 in cancer therapy focuses on targeting p53 as a radio-or chemo-protective treatment. Since most incurable human tumors are p53 deficient, it was proposed that interfering with host p53 may diminish normal cellular toxicity during chemotherapy, while having minimal effect on the tumor population (which is already p53 deficient). This strategy proved promising in recent studies,[166] although it carries a theoretical risk of facilitating oncogenic transformation, particularly during chemotherapy much of which is mutagenic. However no increased tumor incidence was reported in the initial work, so transient p53 inhibition may prove to be clinically useful.

Clearly much remains to be learned about the mechanisms which underlie p53's tumor selective activities. Specifically, it is still incompletely understood how the death vs. arrest decision is made, and whether this pathway may be amenable to drug targeting for therapeutic benefit in p53 deficient tumors. The explosion of information surrounding the numerous pathways with which p53 interacts suggests that a clearer understanding of its activities may

not be far away. Moreover the frequency of its inactivation in human cancer and the potency of its activities in experimental models suggest that the payoff may be well worth the wait.

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CHAPTER 12

Non-Apoptotic Responses to Anticancer Agents: Mitotic Catastrophe, Senescence and the Role of p53 and p21

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Abstract

Chemotherapeutic drugs and radiation inhibit tumor cell proliferation by inducing growth arrest and cell death. The best-studied antiproliferative response to anticancer agents is programmed cell death or apoptosis. Inhibition of apoptosis, however, has been frequently found to have little or no effect on clonogenic survival of human tumor cells. This apparent paradox is explained by an increase in the fraction of damaged cells that undergo senescence-like irreversible growth arrest or die through mitotic catastrophe (aberrant mitosis that leads to the formation of multiple micronuclei) rather than apoptosis. This review discusses the phenotypic changes and molecular mechanisms of mitotic catastrophe and damage-induced senescence, and the role of these processes in the treatment response. It also describes the roles of p53 and its downstream effector p21 as negative regulators of mitotic catastrophe, positive regulators of senescence, and mediators of the paracrine effects of cellular damage. Mitotic catastrophe and senescence provide promising targets for augmentation and modulation in cancer treatment.

Can Apoptosis Account for Tumor Cell Response to Anticancer Agents?

Over the past decade, the major effort in the study of cellular responses to chemotherapy and radiation has been concentrated on apoptosis, the general physiological program of cell suicide, which is induced by different cytotoxic agents in normal and tumor cells.[1,2] The significance of apoptosis in the antiproliferative effects of anticancer drugs and radiation has been investigated by modulating the expression of apoptosis-regulating genes, such as apoptosis inhibitor BC-L2, or p53 that usually acts as a positive regulator of apoptosis. The role of apoptosis as a determinant of treatment response in solid tumors has been suggested in part by the works of Lowe et al.[3,4] These studies demonstrated that the knockout of p53 rendered transformed mouse fibroblast cell lines more resistant to drugs and radiation, in vitro and in vivo, and that this resistance was associated with a decreased induction of apoptosis in p53-/- cells. Decreased induction of apoptosis was also associated with increased radiation and drug resistance in normal mouse tissues[5] and in murine lymphomas,[6] as well as in human leukemia and lymphoma cells in vitro.[1] In contrast, studies with cell lines derived from human solid tumors failed to produce an unambiguous correlation between the p53 status, propensity to

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undergo apoptosis and treatment sensitivity (see ref. 7 for a recent discussion). The role of apoptosis in determining the treatment outcome was also questioned by a series of studies that examined the effects of BCL2 on the response of human solid tumor cell lines to aphidicolin,[8] etoposide[9] and ionizing radiation.[10,11] In all of these studies, ectopic overexpression of BCL2 inhibited the induction of apoptosis, but it had little or no effect on the clonogenic survival of treated cells. In particular, Wouters et al[11] found that BCL2 expression in a derivative of HCT116 colon carcinoma cells strongly inhibited radiation-induced apoptosis in vitro and in vivo, but apoptosis-resistant xenograft tumors turned out to be more sensitive rather than more resistant to radiation treatment.

The lack of correlation between apoptosis and treatment survival can be understood, however, if one considers that apoptosis is not the only mechanism of terminal proliferative death in tumor cells. Two other known mechanisms, which are the subject of the present Chapter, are mitotic catastrophe and senescence-like terminal proliferation arrest. The role of these non-apoptotic mechanisms in treatment response is illustrated by an example from our recent study.[12] This work investigated how the MDR1 P-glycoprotein, which blocks apoptosis through an as yet undefined mechanism distinct from its well-known function as a multidrug transporter,[13] affects cellular responses to ionizing radiation. Figure 1 shows the time course of morphological changes induced by 9 Gy of radiation in HeLa-derived HtTA cells with tetracycline-regulated MDR1 expression. When MDR1 expression is turned off, morphologically detectable apoptosis is the most prominent response to radiation in this cell line. Upon MDR1 induction, apoptosis is greatly decreased (Fig. 1a), but this decrease is followed by a compensating increase in the fractions of cells that undergo mitotic catastrophe (Fig. 1b) or senescence (Fig. 1c). As a result, MDR1 induction has only a minimal effect on clonogenic survival after radiation (Fig. 1d). Similar results were obtained through radiation dose response analysis and with another cell line.[12] Thus, although the damaged tumor cells may be dying through apoptosis, a functional apoptotic program is not required for their proliferative death, which in the absence of apoptosis occurs through the processes of senescence and mitotic catastrophe.

Mitotic Catastrophe

Mitotic cell death, a.k.a. mitotic catastrophe, has been observed in cells treated with all the major classes of anticancer drugs or radiation[2,14] when such cells enter mitosis with unrepaired DNA damage or in the presence of mitotic spindle-disrupting agents. The resulting mitosis is aberrant and incomplete. Depending on the cell line and the nature of damage, the process of mitosis may terminate at the stage of abnormal metaphase[15] or at abnormal anaphase or late prophase.[16,17] This abortive mitosis does not produce proper chromosome segregation and cell division but results in the formation of large non-viable cells with two or more micronuclei that are completely or partially separated from each other (Fig. 2a). In the case of partial separation, the cell displays a large multilobulated nucleus. Micronuclei arise through the formation of multiple nuclear envelopes around chromatin clusters,[18] with chromosomes showing random distribution among the micronuclei.[14,19] Micronucleated cells arising from mitotic catastrophe can be easily distinguished from apoptotic cells by their morphology (Fig. 2a). While apoptotic cells may also have fragmented nuclei, they are characterized by shrunken cytoplasm and condensed chromatin, whereas micronucleated cells are large and contain uncondensed chromosomes. Cells that undergo mitotic catastrophe do not usually show DNA ladder formation or DNA breaks that are detectable by TUNEL staining in apoptotic cells.

Mitotic catastrophe has been studied most extensively in the field of ionizing radiation, where it has been characterized as the main form of radiation-induced cell death.[2] Aberrant mitosis and micronucleation have been observed in irradiated cells both in vitro and in vivo;[20] in fact, micronuclei formation is a common indicator of radiation damage.[21] Mitotic death was also identified as a prominent response to different anticancer drugs, such as etoposide, Taxol,

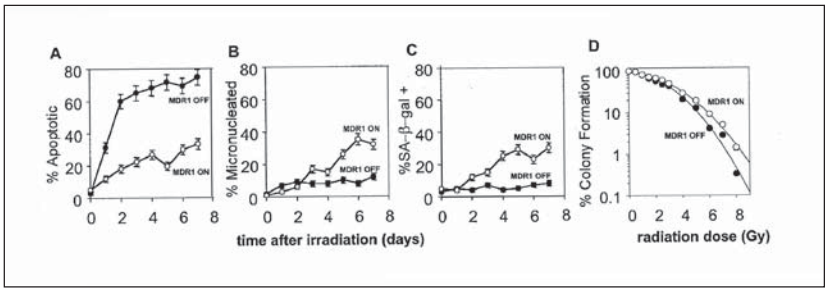

Fig. 1. Effects of MDR1 on radiation-induced apoptosis (A), mitotic catastrophe (B), senescence (C) and clonogenic survival (D) (reproduced with permission from: Ruth, A., Roninson, I.B. Cancer Res. 2000; 60:2576-2579).
HtTA-MDR1 cells that express MDR1 from a tetracycline-inhibited promoter were irradiated with a single dose or different doses of radiation. The fractions of cells that undergo apoptosis, mitotic catastrophe or senescence were determined by morphological assays and changes in cell proliferation were analyzed by clonogenic assays. Left to right: Time course of changes in the percentages of apoptotic, micronucleated and SA-β-gal+ after irradiation with 9 Gy, and clonogenic survival of different doses of radiation. MDR1 was turned off (•) or turned on (o).

cisplatin or bleomycin.[9,22,23] In our study,[14] treatment of HT1080 fibrosarcoma cells with equitoxic (ID_{85}) doses of seven different chemotherapeutic drugs and ionizing radiation invariably induced mitotic catastrophe in a high fraction (45-66%) of treated cells. Furthermore, moderate doses of doxorubicin induced mitotic catastrophe in 11 other solid tumor cell lines, including those that were resistant to apoptosis.[14] Mitotic catastrophe and apoptosis often arise in the same population of irradiated cells; as a general trend, lower doses of cytotoxins preferentially induce mitotic catastrophe while higher doses induce apoptosis.[22,23] The onset of mitotic catastrophe may be followed in some cases by the activation of the apoptotic program.[24,19,15] For this reason, mitotic catastrophe is viewed sometimes as an early stage of apoptosis. The apoptotic program, however, is not needed for the lethal effect of mitotic catastrophe. This was shown not only in the above-described work of Ruth and Roninson[12] (Fig. 1), but also in an earlier study by Lock and Stribinskiene.[9] When etoposide-induced apoptosis of HeLa cells was suppressed by BCL2, drug-treated tumor cells died through mitotic catastrophe, and there was no significant change in the clonogenic survival.[9]

The mechanisms that lead to mitotic abnormalities in cells that enter mitosis without repairing the damage are still poorly understood. In yeast cells, mitotic catastrophe was shown to result from premature cell entry into mitosis before the completion of DNA replication.[25] The notion of mitotic catastrophe as premature mitosis has also been promulgated by the analysis of human tumor cells treated with hyperthermia[18] or ionizing radiation.[16] In these studies, mitotic catastrophe has been described as starting with uneven chromatin condensation around nucleoli, which resembled premature chromosome condensation originally described in the fusions of interphase cells (in S or G2) with mitotic cells.[26] Based on this similarity and on the observations of increased cellular levels of cyclin B1 and elevated Cdc2 kinase activity at the onset of mitotic catastrophe, this process was proposed to represent premature induction of mitosis in cells that are damaged in S or G2.[16] Uneven chromosome condensation, however, may result not only from PCC but also from localized defects in the assembly of chromosome-condensing proteins (condensins and cohesins) in the damaged regions of DNA. It is still unclear therefore if mitotic catastrophe in mammalian cells represents premature mitosis.

Another key feature of mitotic catastrophe is the amplification of centrosomes in cells with fragmented or multilobulated nuclei.[17,27] Centrosome amplification leads to multipolar mitosis, which appears to be the most common type of mitotic abnormality in drug-treated tumor cells.[28] The role of the centrosome as a potentially critical target in mitotic catastrophe is

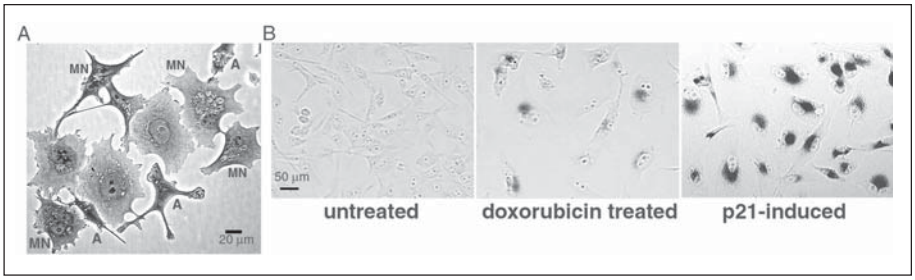

Fig. 2. Morphology of mitotic catastrophe (A) and senescence (B) (reproduced with permission from: Chang BD et al. 1999; Oncogene 18:4808-4818 and Chang BD et al. 1999; Cancer Res 59:3761-3767). A. Cells with multiple micronuclei (mn) and apoptotic cells (a) in doxorubicin-treated HCT116 colon carcinoma cells. B. Expression of the SA-β-gal marker of senescence in HT1080 fibrosarcoma cells, untreated (left), treated with 50 nM doxorubicin (middle), or following ectopic expression of p21 (right).[5]

suggested by a study of Sibon et al.[29] These authors found that treatment of *Drosophila* embryos with several DNA-damaging agents resulted in a loss of γ-tubulin from centrosomes, anastral spindle formation and failure of chromosome segregation. Since centrosome duplication occurs prior to mitosis, this process, may represent an early event of mitotic catastrophe.

Mitotic catastrophe can be induced not only by DNA damage but also by genetic means, through untimely activation of the cyclinB/Cdc2 kinase complex. Ectopic coexpression of cyclin B1 and Cdc2, cyclin A and Cdc2, and cyclin B1 and Cdc25C (an enzyme that dephosphorylates and activates Cdc2) were shown to induce premature mitosis and subsequent mitotic catastrophe in mammalian cells.[30] Mitotic catastrophe was also produced by perturbing the expression of genes that control early stages of mitosis, such as RCC1 involved in chromosome condensation[31] and centrosome-associated proteins NuMA (nuclear-mitotic apparatus)[32] and survivin.[33] Survivin is best known as an inhibitor of apoptosis, and the loss or displacement of survivin from centrosomes may potentially account for the induction of apoptosis in cells undergoing mitotic catastrophe. These observations outline a general picture of mitotic catastrophe as a process that occurs either when a cell initiates mitosis prematurely, before all the required mitosis-control mechanisms are in place, or when the proper course of mitosis is hindered by a deficiency in some of these control mechanisms.

p53 as a Negative Regulator of Mitotic Catastrophe

The entry of damaged cells into mitosis is delayed by growth arrest at the G2 checkpoint, and this checkpoint was shown to play a key role in preventing mitotic catastrophe. Agents that abrogate the G2 checkpoint, such as caffeine, okadaic acid or staurosporine, promote mitotic death in irradiated or drug-treated cells.[34-36] Mitotic catastrophe is also enhanced by genetic inactivation of proteins that control the G2/M checkpoint. As demonstrated by a number of studies, p53 is an essential regulator of the G2/M checkpoint in human cells.[17,37] The first p53 target gene that was shown to play a role in this checkpoint is $p21^{WAF1/Cip1/Sdi1}$, a pleiotropic inhibitor of different cyclin/CDK complexes and a regulator of several transcription factors and cofactors.[38] The role of p21 in G2 arrest was demonstrated by the observations that p21 knockout in human HCT116 colon carcinoma cells greatly diminishes damage-induced G2 arrest,[17] that p21-/- mouse fibroblasts show more rapid entry into mitosis,[39] and that ectopic overexpression of p21 induces growth arrest in both G1 and G2.[40] In addition to p21, another p53-inducible protein, 14-3-3-σ, was shown to play a role in G2 arrest of damaged cells by maintaining cytoplasmic sequestration of cyclin B1 and Cdc2.[41] The knockout of p53, p21 or 14-3-3-σ in HCT116 cells drastically increased the induction of morphologically detectable

cell death by doxorubicin or radiation,[17,19,41] including both mitotic catastrophe and apoptosis.[42] In contrast, the induction of cell death by 5-fluorouracyl was decreased by p53 knockout in the same cells.[43] These opposite effects of p53 on apoptosis can be explained by contrasting the function of p53 as a positive regulator of the primary (mitosis-independent) apoptotic response with its role as a negative regulator of mitotic cell death and apoptosis that develops as a consequence of mitotic catastrophe. These opposite roles of p53 in drug-and radiation-induced cell death, together with its role in different forms of damage-induced growth arrest (see below), are illustrated in Figure 3.

Induction of Senescence by DNA-Damaging Agents

Exposure of tumor cells to chemotherapeutic drugs or radiation induces not only mitotic death and apoptosis but also cell cycle arrest, which is mediated in part by a direct effect of the drugs (e.g., G2 arrest by topoisomerase II inhibitors) and in part by the activation of cellular G1 and G2 checkpoints. Damage-induced growth arrest may be reversible, allowing the cells to repair the damage and reenter the cycle, or irreversible, with the damaged cells unable to divide but remaining physiologically functional. Studies on normal fibroblasts that underwent DNA damage showed a striking phenotypic resemblance between irreversibly arrested cells and cells that undergo replicative senescence, the normal physiological program of terminal growth arrest.[44,45] The phenotypic markers of senescence include enlarged and flattened morphology, increased granularity, and expression of a relatively specific marker of senescence, senescence-associated β-galactosidase (SA-β-gal), which is detectable by X-gal staining at pH6.0 (ref. 46) (Fig. 2b). The rapid senescence-like growth arrest which can be induced not only by DNA-damaging agents but also by mutant RAS[47] has been termed accelerated senescence. Accelerated senescence, like apoptosis, was proposed to be a programmed protective response of the organism to potentially carcinogenic damage.[48] In contrast to replicative senescence, a slow process that is mediated by telomere shortening,[49,50] accelerated senescence does not appear to involve changes in the telomere length,[51-53] and it is not prevented by constitutive activation of telomerase.[54] However, the programs that execute terminal growth arrest in replicative and accelerated senescence of normal cells appear to be identical. Both telomere shortening and DNA damage activate p53, which then induces p21 that arrests the cell cycle in G1 and G2. p21 activation in senescent cells (which can also be induced by p53-independent mechanisms) is transient, and it is followed by stable activation of another CDK inhibitor, $p16^{INK4A}$, which maintains the growth arrest of senescent cells after the shutoff of p21.[55,56] p53 and p16 (but not p21) are two of the most commonly mutated tumor suppressor genes in human cancer,[57] and it has long been thought that tumor cells, which are often deficient in one or both of these genes, can no longer undergo senescence.

Over the past several years, it has become apparent, however, that immortal tumor-derived cell lines, which tend to have short telomeres and to carry mutations that trigger accelerated senescence in normal cells (such as oncogenic RAS mutations), can be forced into senescence. This was first suggested by the reports that a senescent phenotype could be induced in tumor cells by ectopic overexpression of tumor suppressor genes, such as p53, RB, p16 or p21.[42,58-63] Perhaps more significantly, inhibition of papillomavirus oncoproteins E6 and E7 in cervical carcinoma cell lines was recently shown to induce rapid senescence in almost 100% of tumor cells.[52] The latter result suggested that tumor cells may be "primed" to senesce once senescence-restraining mechanisms (in this case E6 and E7) have been removed.[52] These findings raised a prospect that the enhancement of the extant program of accelerated senescence in tumor cells may be a feasible and biologically justified approach to cancer therapy.

We and others have found that accelerated senescence can be readily induced in a sizable fraction of cells in different tumor cell lines after treatment with different chemotherapeutic drugs, ionizing radiation, or differentiating agents.[14,53] A phenotype characterized by senescence-specific morphological changes and SA-β-gal expression (Fig. 2b) was induced by cytotoxic agents in a dose-dependent manner, along with mitotic catastrophe, and its induction was

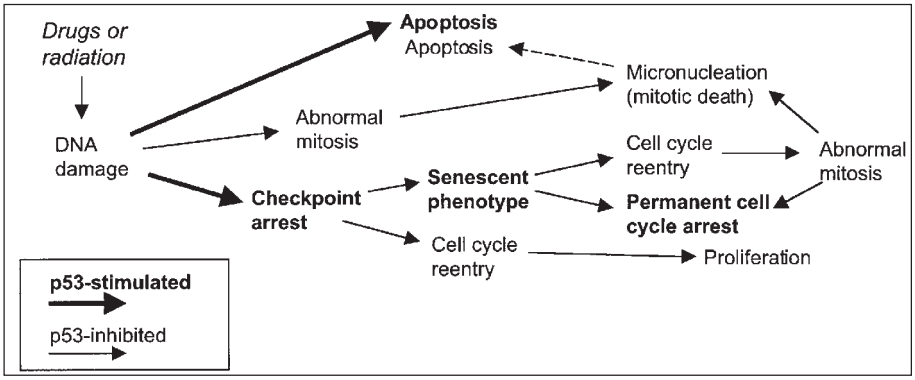

Fig. 3. Cellular responses to DNA damage and the effects of p53. Thick arrows and boldface indicate the events that are positively regulated by p53, and thin arrows and regular fonts indicate events that are negatively regulated by p53.

detectable even at the lowest drug doses.[14] The senescent phenotype was also efficiently induced in breast carcinoma cells that were treated with retinoids, in vitro or in vivo, under the conditions of minimal cytotoxicity. Using a FACS-based procedure that separates proliferating from growth-inhibited cells, we have found that SA-β-gal expression and the senescent morphology distinguish drug-treated cells that are physically intact but are no longer clonogenic from the cells that recover and form colonies after drug treatment.[14] This result indicates that detection of senescence markers in treated tumors may be useful as a diagnostic procedure indicative of treatment response.

While the senescent phenotype is associated with a loss of clonogenicity, the mechanisms of the clonogenic failure of senescent cells differ among tumor cell lines (see Fig. 3). In some lines, such as HCT116, the senescent cells do not divide and maintain permanent cell cycle arrest, despite the lack of p16.[19,42] The nature of the genes that maintain permanent growth arrest in such cells remains to be determined. In another class of tumor cell lines, typified by HT1080 fibrosarcoma, most of the cells that develop the senescent phenotype eventually reenter the cycle, but they stop dividing or die after 1-3 cell divisions.[14] The proliferative failure in these cells is accompanied by an increase in DNA ploidy[14] and numerous mitotic abnormalities (our unpublished data). These events closely parallel our observations in HT1080 cells where growth arrest and the senescent phenotype were produced by expressing p21 from a regulated promoter[42] (see below).

The induction of the senescent phenotype by anticancer agents has been observed not only in vitro but also in vivo, in xenograft tumors that were stained for SA-β-gal after treatment with different drugs (ref. 14 and our unpublished data). While it is difficult to discern the effects of accelerated senescence from the more rapid cytotoxic action of chemotherapy or radiation in the clinics, some observations in radiation therapy suggest that the induction of permanent cytostatic arrest could be the primary mode of treatment response in certain clinical cases. In particular, complete regression of prostate cancers was reported in some patients to take more than a year[64] and of desmoid tumors up to two years[65] after radiation treatment. Stimulation of senescence in normal tissues may also represent an adverse clinical effect of cancer therapy. For example, radiation-induced damage to normal tissues frequently develops late after treatment, and such late effects are considered the dose-limiting factor in radiation therapy.[66]

Role of p53 and p21 in Damage-Induced Senescence and Abnormal Mitosis

As mentioned above, two of the key regulators of senescence in normal cells, p53 and p16, are frequently inactivated in cancers. Moderate doses of doxorubicin, however, induced the senescent phenotype in all tested cell lines with wild-type p53 and in one half of p53-deficient lines; this response was also readily observed in p16-deficient tumor cells.[14] To investigate the role of p53 and p21 in the induction of senescence-like growth arrest, we have analyzed HT1080 fibrosarcoma cells where p53 function and p21 expression were blocked by a p53-derived genetic suppressor element, and HCT116 colon carcinoma cells with homozygous knockout of p53 or p21 (both HT1080 and HCT116 lines are p16-deficient and carry different RAS mutations). In both cell lines, the inhibition or knockout of p53 or p21 strongly decreased but did not completely abolish drug- or radiation-induced senescence. These results have indicated that p53 and p21 act as positive regulators of accelerated senescence in tumor cells, but they are not absolutely required for this response.[42] The opposite roles of p21 as a positive regulator of damage-induced senescence and a negative regulator of mitotic catastrophe help to explain the paradoxical results of Waldman et al[67] and Wouters et al,[68] who found that the wild-type and p21-/- HCT116 cell lines form a similar number of colonies after radiation or doxorubicin treatment, despite the much higher degree of cell death observed after treatment in p21-/- cells. The role of p53 as a positive regulator of both reversible and irreversible growth arrest (senescence) is illustrated in Figure 3.

To investigate the role of p21 in drug-induced senescence, we have analyzed the effects of p21 expression from a promoter inducible by a physiologically neutral β-galactoside IPTG in HT1080 fibrosarcoma cells. p21 induction in these cells leads to growth arrest in both G1 and G2, accompanied by markers of senescence (Fig. 2b). Concurrent inhibition of p53 in these cells had no significant effect on the induction of the senescent phenotype by p21, indicating that p21 function was sufficient for this phenotype. On the other hand, comparison of the percentages of senescent cells and p21 protein levels between IPTG-induced and doxorubicin-treated cells showed that p21 induction was insufficient to account for the full extent of drug-induced senescent response. At the same level of senescence, IPTG-induced cells expressed four times more p21 than did doxorubicin-treated cells, indicating that some other factors in drug-treated cells cooperated with p21 in producing the senescent phenotype.[42]

The development of the senescent phenotype in HT1080 cells with IPTG-inducible p21 expression was accompanied by gradual loss of cellular clonogenic capacity upon release from p21. This loss of clonogenicity correlated with the extent and duration of p21 induction, and it was not due to a failure of the cells to reenter the cycle upon release from p21. When p21 was induced by IPTG for three or more days and then turned off, all the cells reentered the cycle but most of them either died (with features of mitotic catastrophe) or underwent terminal arrest after a few cell divisions and failed to form colonies. This proliferative failure was associated with the predominance of abnormal mitotic figures during the first cell division after release from p21. We have found that this abnormal mitosis was associated with an imbalance in the intracellular pools of mitosis-initiating proteins (such as cyclin B1, Cdc2, or polo-like kinase (Plk1)) and mitosis-controlling proteins (such as spindle checkpoint control proteins Mad2 or BubR1 or aurora kinase family of proteins that control centrosome function). Transcription of the genes encoding such proteins was inhibited upon the induction of p21, and the intracellular pools of these proteins decayed after two or more days of p21 induction. Once p21 was turned off, these proteins were resynthesized asynchronously. As a result, cells acquired sufficient amounts of mitosis-initiating proteins and entered mitosis with only a minimal level of Mad2 and possibly other mitosis-control proteins, resulting in abnormal mitosis.[69] It remains to be determined whether the same or a similar mechanism is responsible for the abnormal mitosis that occurs in drug-treated cells that reenter the cycle after prolonged growth arrest.

Paracrine Activities of Senescent Cells: Implications for Treatment Outcome and Side Effects of Cancer Therapy

Terminal proliferative arrest is not the only aspect of accelerated senescence that has important clinical implications. Senescent cells, while not dividing, are not physiologically neutral and produce secreted proteins with clinically important functions. Many far-ranging paracrine activities have been associated with normal senescent cells.[50,70,71] Some of the proteins overexpressed in senescent cells are proteases and protease inhibitors, which have tissue-reorganizing effects and may promote metastasis, whereas others are paracrine growth factors that affect the growth of non-senescent tumor cells or de novo carcinogenesis. [50,70,71] The same tumor-promoting activities have also been associated with tumor-derived stromal fibroblasts.[72] The tumor-promoting effect of the stromal tissue was recently shown in a mouse mammary carcinogenesis model to be induced by ionizing radiation.[73] Induction of mitogenic and angiogenic factors by ionizing radiation was also detected by gene expression profiling studies.[74]

One of the strongest effects of ionizing radiation in mammary stromal fibroblasts[75] and many other cell types is the induction of p21. As discussed above, p21 induction is an essential component of cell senescence. While the best-known function of p21 is the inhibition of cyclin/cyclin-dependent kinase (CDK) complexes, this protein also binds and modulates the activity of many transcription factors and cofactors.[38] Through cDNA microarray analysis, we[76] have found that p21 induction in HT1080 fibrosarcoma cells upregulates the genes for many secreted proteins, including senescence-associated extracellular matrix (ECM) proteins and plaque-forming proteins that have been implicated in age-related amyloid diseases (e.g., Alzheimer's amyloid precursor protein, serum amyloid A, tissue transglutaminase). p21 also upregulates the expression of $p66^{Shc}$, a mediator of oxidative damage that was shown to be a negative determinant of lifespan in mice.[77] Furthermore, p21 induces several antiapoptotic, mitogenic and angiogenic secreted factors, and conditioned media from p21-induced cells shows antiapoptotic and mitogenic activities.[76]

While DNA damage induces the expression of growth-promoting factors, this effect may be counterbalanced by the concurrent induction of secreted growth inhibitors, such as TNFα, serine protease inhibitors or IGF-binding protein 3.[74] The induction of both growth-promoting and growth-inhibiting factors in damaged cells was found to depend on the presence of wild-type p53, and ectopic overexpression of p53 induces transcription of such genes.[74] The induction of growth-promoting factors by p53 is likely to be mediated at least in part through p21. p21, however, was not found to upregulate any growth-inhibitory factors,[76] suggesting that the induction of secreted growth inhibitors is mediated by other downstream effectors of p53. Conditioned media from irradiated or p53-induced cells was shown to be growth-inhibitory,[74] in contrast to the growth promoting effect of the media conditioned by p21-induced cells.[76] It remains to be determined whether secreted growth-inhibiting factors are expressed by senescent cells along with the growth-stimulating factors. The diverse paracrine effects associated with drug-induced senescence may have important consequences for the long-term outcome and side effects of cancer therapy.

Mitotic Catastrophe and Senescence as Target Responses in Cancer Treatment

The above described non-apoptotic responses to cancer therapeutic agents, i.e., mitotic catastrophe and senescence, provide promising directions of research into improving the efficacy of cancer treatment. Apoptosis is a powerful control mechanism that eliminates normal cells with potential oncogenic changes, and therefore mutations that disable the program of apoptosis are commonly selected in the course of carcinogenesis. These mutations include those that inactivate p53, a positive regulator of the primary (mitosis-independent) apoptotic response. In normal cells, however, the program of apoptosis is fully functional, and it accounts largely for treatment-induced damage to normal tissues. Inhibition of p53 and p53-mediated apoptotic

program has been shown to provide a striking increase in the survival of irradiated animals.[5] In contrast to apoptosis, mitotic catastrophe is potentiated by p53 mutations and by the weakening of G2 and mitotic checkpoints, which are characteristic of many human tumors.[79,80] Tumor cells therefore are likely to be more susceptible to mitotic catastrophe than normal cells. Another potential advantage of mitotic cell death lies in the observations that mitotic catastrophe in vivo leads to necrosis, with its associated inflammatory effects.[2] The inflammatory response may conceivably increase the efficacy of treatment, in contrast to the non-inflammatory nature of apoptotic cell death. This difference may potentially explain a paradoxical observation of Wouters et al[11] that the inhibition of apoptosis in a BCL2-transduced derivative of HCT116 cells has made this cell line more sensitive rather than more resistant to radiation in vivo. Elucidation of the molecular mechanisms of mitotic catastrophe may help to exploit this preferred mode of tumor cell death in a therapeutic setting.

While cell senescence, like apoptosis, represents a protective mechanism of eliminating cells that have experienced potentially carcinogenic events,[48] studies reviewed in the present Chapter have demonstrated that many and perhaps most tumor cells have retained the ability to undergo senescence. The program of senescence can be activated in tumor cells either by inhibiting senescence-overriding proteins (such as E6 and E7 oncoproteins of papilloma virus),[52] or by treatment with chemotherapeutic drugs, radiation, or "differentiating" agents.[14] The induction of tumor senescence is a significant antiproliferative effect of anticancer agents, and its augmentation may increase the efficacy of cancer therapy. On the other hand, the senescent phenotype of tumor or normal cells is accompanied by paracrine side effects that may interfere with the success of treatment and increase its toxicity to normal tissues. For example, secretion of antiapoptotic/mitogenic factors or proteases may promote tumor growth and metastasis, and increased expression of amyloid proteins may contribute to the eventual development of different amyloid-associated diseases. Elucidation of the molecular mechanisms that are responsible for permanent growth arrest in drug-induced senescence and for the induction of different factors secreted by senescent cells should make it possible to develop strategies that would promote tumor cell senescence or inhibit its undesirable side effects.

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CHAPTER 13

Small Molecule Inhibitors of Cyclin-Dependent Kinases

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Introduction

Cyclin-dependent kinases (CDKs) are core components of the cell cycle machinery. Orderly transition between cell cycle phases requires the scheduled activity of the CDKs, governed in part by their associations with cyclins and CDK inhibitors, as well as by their state of phosphorylation. In malignant cells, altered expression of CDKs and their modulators, including overexpression of cyclins and loss of expression of CDK inhibitors, results in deregulated CDK activity, providing a selective growth advantage. Because of their critical role in cell cycle progression, as well as the association of their activities with the processes of differentiation and apoptosis, the CDKs comprise an attractive set of targets for novel antineoplastics.

Multiple strategies can be used to modulate cellular CDK activity, including the genetic replacement of endogenous CDK inhibitors or the introduction of small peptides that reproduce their effects. In addition, several known anticancer agents cause reduction in expression of D-type cyclins, and other drugs under development reduce CDK stability or inactivate the proteasome-mediated degradation of endogenous CDK inhibitors (reviewed in refs. 1-3). This Chapter will focus on small molecules that directly modulate CDK activity.

The classes of small molecule CDK inhibitors are shown in Table 1. In addition to their chemical categorization, they can be further classified based on their overall specificity for CDKs and their ability to inhibit CDK4.[4-6] Most of these drugs inhibit tumor growth in preclinical models by inducing cell cycle arrest or apoptosis. Several have profound effects on cellular transcription or have been shown to cause differentiation. To date, flavopiridol and UCN-01 have advanced the furthest in clinical application, and both have demonstrated significant synergy with standard chemotherapy drugs. Although not all of the biologic effects of flavopiridol have been definitively linked to CDK modulation, it serves as a model for the potential antitumor activity of these agents. In addition, preclinical data exist for new generations of purine analogs as well as newer classes of CDK inhibitors, such as the paullones. As more potent and selective CDK inhibitors are now eagerly anticipated, it is useful to review the preclinical and clinical results with the agents presently under development.

Flavopiridol

Antiproliferative Mechanisms

Early Studies

Flavopiridol, also known as L86-8275 or HMR 1275, is the first CDK inhibitor to enter clinical trials. The drug is a semi-synthetic flavonoid derived from rohitukine, an alkaloid

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isolated from the stem bark of *Dysoxylum binectariferum*, a plant native to India. Flavopiridol was initially selected for further study because it demonstrated modest inhibition of the epidermal growth factor receptor (EGFR) tyrosine kinase (IC_{50} = 21 μM), relatively specific compared to its inhibition of other kinases, such as protein kinase A (IC_{50} = 122 μM).[7] However, early studies showed that flavopiridol inhibited the proliferation of exponentially growing human breast and lung carcinoma cells at concentrations as low as 25-160 nM.[8] In addition, the mean IC_{50} across the 60-cell line NCI anticancer drug screen panel was 66 nM. Furthermore, antiproliferative effects were independent of EGFR status, indicating alternative cellular targets.

Cell Cycle Arrest

Flavopiridol was subsequently shown to block cell cycle progression. For example, when MDA-MB-468 breast cancer cells are released from an aphidicolin-induced block at the G1/S boundary in the presence of flavopiridol, they ultimately arrest in G2. When the same cells are released from a nocodazole-induced block in the presence of flavopiridol, they complete mitosis but arrest in G1,[8] with an accumulation of the hypophosphorylated form of the retinoblastoma-susceptibility gene product, Rb.[9] Consistent with G1 and G2 arrest, in studies using purified CDKs, flavopiridol has been shown to directly inhibit the activities of CDK1 (cdc2), CDK2 and CDK4, with IC_{50} values in the 40-400 nM range.[7,9,10] Recently, inhibition of CDK6 has also been demonstrated.[11] Inhibition is competitively blocked by ATP, with a K_i range of 41-65 nM. [9,10]

The biochemical data are supported by structural studies, in which the deschloro derivative of flavopiridol has been co-crystallized with CDK2. This work demonstrated that the aromatic portion of flavopiridol binds to the ATP-binding pocket of CDK2 with good complementarity, as the size of buried surfaces of the inhibitor and the target kinase are similar upon complex formation.[12] In addition, the phenyl group of flavopiridol allows contact points with the enzyme not seen in the CDK2-ATP complex. The stretch of amino acids contacting the phenyl ring of flavopiridol is well conserved among the CDKs, but is not present in kinases less potently inhibited, explaining the relative specificity of flavopiridol for CDKs.[12]

Flavopiridol may also induce cell cycle arrest by other mechanisms. For example, in addition to direct inhibition of CDKs 1, 2, 4 and 6, flavopiridol also inhibits CDK7, the CDK-activating kinase, (IC_{50} ~ 300 nM),[13] resulting in decreased phosphorylation of CDKs at threonine 160/161, necessary for their activation.[14] Finally, transcriptional repression of cyclin D1 by flavopiridol may also contribute to G1 arrest. This has been demonstrated following flavopiridol exposure in MCF-7 breast cancer cells, in which decreases in cyclin D1 mRNA levels precede the loss of CDK4 activity. The depletion of cyclin D1 mRNA was associated with a decline in cyclin D1 promoter activity, measured by a luciferase reporter assay.[15]

While the effects of flavopiridol on CDK4 activity are specific for cells retaining wild-type Rb, inhibition of CDK2 by flavopiridol also allows G1 arrest in cells lacking Rb.[8,9] Cell cycle arrest mediated by flavopiridol is also independent of p53, as it occurs in cell lines in which p53 is inactivated by mutation or deletion.[8,9]

Differentiation

In some instances, growth arrest via CDK inhibition has been associated with cellular differentiation. For example, treatment of NCI-358 non-small cell lung cancer (NSCLC) cells with flavopiridol resulted in growth arrest and the induction of mucinous differentiation. In these experiments, the onset of differentiation coincided temporally with the loss of CDK2 activity. [16]

Apoptosis

In addition to cell cycle arrest, flavopiridol can also induce p53-independent apoptotic cell death, especially in cells of hematopoietic origin.[17,18] Apoptosis has also been observed in solid tumor cell lines, including those derived from head and neck cancers,[19] as well as those

derived from carcinomas of the esophagus, lung and breast.[20-22] The mechanisms by which flavopiridol mediates apoptosis have not yet been elucidated. Specifically, the contribution of CDK inhibition to apoptotic cell death has not been defined. For example, in lung cancer cell lines, CDK inhibition and cell cycle arrest frequently occur at concentrations of drug lower than those required for apoptosis.[21] In many solid tumor cell lines, prolonged exposure (i.e., 72 hours) to high concentrations of flavopiridol are also required to achieve apoptosis. In addition, cytotoxicity has been shown to occur even in non-cycling cells, including starved or confluent A549 NSCLC cells,[21,23] resting lymphoid cells in treated mice,[18] and human endothelial cells.[24]

These findings raise the possibility that effects of flavopiridol on additional cellular targets may contribute to apoptotic cell death. Several lines of evidence indicate that flavopiridol most likely interacts with DNA.[25] For example, in a recent study, reverse-phase high-performance liquid chromatography demonstrated that flavopiridol binds to genomic DNA to a similar extent as ethidium bromide and Hoechst 33258. Nuclear magnetic resonance (NMR) spectroscopy revealed that DNA caused extreme broadening of flavopiridol ^{1}H NMR signals that could be reversed by addition of ethidium bromide or by DNA melting, suggesting that flavopiridol binds to and likely intercalates into duplex DNA. Equilibrium dialysis demonstrated that the dissociation constant of the flavopiridol-DNA complex was in the same range observed for binding of intercalators such as doxorubicin and pyrazoloacridine to DNA. Futhermore, analysis of the cytotoxic effects of flavopiridol (i.e., LC_{50} data) in the NCI tumor cell line panel using the COMPARE algorithm[26] demonstrated that flavopiridol most closely resembles cytotoxic antineoplastic intercalators.[25] In addition, clinically achievable concentrations of flavopiridol result in induction of p53 in A549 NSCLC cells.[21,25] While this supports an interaction of flavopiridol with DNA, p53 induction is not required for apoptosis,[21] and it remains unclear whether flavopiridol induces DNA damage. Flavopiridol does not inhibit topoisomerases,[17, 25] as has been shown for other flavones. In addition, the flavopiridol-DNA interaction has only been demonstrated at very high flavopiridol concentrations (10-50 μM), well beyond those required for antiproliferative effects.[25] Nonetheless, the data collectively suggest that DNA represents another cellular target that could be responsible for the cytotoxic effects of flavopiridol, especially in non-cycling cells.

While flavopiridol-mediated apoptosis is clearly independent of p53, the role of Bcl-2 and other anti-apoptotic proteins remains controversial, and may be cell type dependent. For example, in some hematopoietic cell lines, down-regulation of anti-apoptotic proteins (including Mcl-1, XIAP, Bag-1 and Bcl-2) has been observed.[27, 28] However, in other cell lines, alterations in Bcl-2 and BAX levels in response to flavopiridol do not occur.[17,29] Furthermore, in a recent study of HeLa cell and small cell lung cancer (SCLC) cell lines, flavopiridol-mediated apoptosis was clearly independent of Bcl-2.[30] In these cell lines, neither overexpression nor antisense oligonucleotide-mediated down-regulation of Bcl-2 had any effect on flavopiridol-induced apoptosis. Pathways involving activation of caspase 8, followed by activation of other caspases, such as caspase 3, appeared to play a major role in the apoptotic response to flavopiridol. In contrast, caspase 8-independent induction of mitochondrial cytochrome c release (modulated by Bcl-2 family members) followed by caspase 9 activation did not contribute significantly. Interestingly, in cell lines lacking caspase 8, flavopiridol triggered mitochondrial depolarization in the absence of cytochrome c release, followed by caspase 3 activation and cell death. In summary, flavopiridol is able to kill tumor cells that are normally resistant to chemotherapeutic agents due to Bcl-2 overexpression or the absence of caspase 8.[30]

Transcriptional Effects

Two studies have demonstrated an effect of flavopiridol on cellular transcription. As already indicated, human breast carcinoma cells treated with flavopiridol showed decreased transcription of the gene encoding cyclin D1.[15] In addition, high concentrations of flavopiridol affected the level of 63 different mRNAs in *Saccharomyces cerevisiae,* including mRNA encoding proteins regulating cell cycle progression and phosphate and cellular energy metabolism.[31]

Recently, it has been demonstrated that flavopiridol inhibits P-TEFβ, a protein kinase Composed of CDK9 and a cyclin subunit derived from one of three different genes. P-TEFβ controls the elongation phase of transcription by RNA polymerase II. The ability of flavopiridol to inhibit P-TEFβ-mediated phosphorylation of the carboxyl-terminal domain of the large subunit of RNA polymerase II is particularly potent with an IC_{50} ~ 6 nM, non-competitive with ATP.[32]

P-TEFβ, composed of CDK9 and cyclin T1 is a required cellular cofactor for the human immunodeficiency virus (HIV-1) transactivator, Tat. Consistent with its ability to inhibit P-TEFβ, flavopiridol blocked Tat transactivation of the viral promoter in vitro, and blocked HIV-1 replication in both single-round and viral spread assays with an IC_{50} < 10 nM.[32]

Antiangiogenic and Antimetastatic Properties

Antiangiogenic and antimetastatic effects may also contribute to the antitumor activity of flavopiridol. In vitro, incubation of human umbilical vein endothelial cells (HUVECs) with flavopiridol results in apoptotic cell death, even in non-cycling populations.[24] In vivo, flavopiridol has been found to decrease blood vessel formation in a mouse Matrigel model of angiogenesis.[33] In addition, at low nM concentrations, flavopiridol has been shown to inhibit the induction of vascular endothelial growth factor (VEGF) by hypoxia in human monocytes.[34] Finally, flavopiridol has been shown to inhibit the secretion of matrix metalloproteinases by breast cancer cells, resulting in decreased invasiveness in a Matrigel cell invasion assay. Decreased invasiveness was noted in parental cells, as well as those engineered to overexpress c-eRB-2.[22]

Other Enzyme Targets

Although flavopiridol is relatively specific for CDKs compared to other serine and threonine kinases, recent data have also implicated other possible enzyme targets. For example, immobilized flavopiridol binds not only to CDK4 in tumor cell extracts, but also to cytosolic aldehyde dehydrogenase 1.[35] In addition, flavopiridol inhibits glycogen phosphorylase, most potently in the presence of glucose, suggesting that it may affect cancer cells by starving them of glycolytic intermediates.[36,37]

Summary

Some of the biologic effects of flavopiridol in preclinical models are clearly explained by its ability to inhibit CDKs, including cell cycle arrest and differentiation. In addition, CDK inhibition probably contributes to apoptosis as well. However, the ability of flavopiridol to cause apoptosis in non-cycling cells, interact with DNA, modulate transcription and P-TEFb activity, affect endothelial cells in biological models and inhibit glycogen phosphorylase, all suggest that antitumor effects seen in in vivo models are indeed due to the effect of the drug on multiple cellular targets.

Preclinical Antitumor Activity and Pharmacology

Xenograft Models

Initial studies in tumor-bearing mice used both the sub-renal capsule assay as well as subcutaneously implanted human tumor xenografts. Scheduling experiments demonstrated that the drug was more active when administered frequently than intermittently (i.e., daily versus weekly).[7,38] Similar effects were seen with IV and oral dosing. When administered over protracted periods, flavopiridol was cytostatic in most xenograft models. Flavopiridol significantly inhibited growth under the renal capsule of human mammary, lung, ovarian and colon carcinoma, as well as glioblastoma with T/C ratios from 31 to 77% (ratio of the median tumor weight of treated animals over the median tumor weight of control animals). In subcutaneously growing xenograft tumor models, it similarly inhibited growth of human lung, colon, gastric and mammary carcinomas as well as melanoma and glioblastoma with T/C values ranging from 29 to 86%.[7] Some of the most pronounced effects were seen against prostate carci-

noma xenografts. At the maximal tolerated dose of 10 mg/kg/day administered orally on days 1-4 and 7-11, flavopiridol caused tumor stasis lasting 4 weeks in one prostate xenograft model and tumor regression of a second prostate xenograft.[39]

Preclinical Pharmacokinetics and Metabolism

Murine plasma concentration-time profiles for flavopiridol exhibited biexponential behavior with mean α and β half-lives of 16.4 and 201.0 minutes, respectively. The mean total-body plasma clearance was 22.6 ml/min/kg, and the mean oral bioavailability is ~20% in mice and rats and ~35% in dogs.[7] The metabolism of flavopiridol has been determined using isolated rat liver perfusion models. Flavopiridol undergoes glucuronidation in the liver.[40] Monoglucuronides may be released into the systemic circulation but are predominantly excreted into the biliary tract, resulting in the propensity of flavopiridol for enterohepatic circulation. Preclinical pharmacologic and toxicologic evaluation identified dose-limiting toxicities as reversible hematopoietic and gastrointestinal effects.[7]

Phase I Studies

72-Hour Continuous Infusion Schedule

The demonstration that preclinical antitumor activity was superior with frequent administration over a prolonged period led to phase I trials in which flavopiridol was given as a 72-hour continuous infusion every 2 weeks. In the NCI phase I trial, 76 patients were treated. Dose-limiting toxicity was secretory diarrhea with a maximal tolerated dose of 50 mg/M^2/ day .[41] In the presence of antidiarrheal prophylaxis, including a combination of cholestyramine and loperamide, patients tolerated higher doses, defining a second maximal tolerated dose of 78 mg/M^2/day for 3 days. The dose-limiting toxicity observed at the higher dose level was reversible hypotension and a pro-inflammatory syndrome, including fever, fatigue, local tumor pain and increases in acute phase reactants that made it difficult to administer drug chronically.[41]

One patient with renal cell carcinoma achieved a partial response (shrinkage of ≥50% of tumor masses) and three other patients had minor responses (shrinkage of <50%), including patients with colon carcinoma, renal cell carcinoma and non-Hodgkin's lymphoma. Fourteen patients received flavopiridol for more than 6 months, including five who received drug for more than 1 year and one patient who received drug for more than 2 years, indicating that disease stabilization for protracted periods is possible.[41]

The mean plasma flavopiridol concentration was 271 nM at the 50 mg/M^2/day x 3 dose level and 344 nM at the 78 mg/M^2/day x 3-dose level, within range for inhibition of CDKs in preclinical in vitro systems. Total clearance ranged from 11.5-27.3 l/hr/M^2 (range 1.3-29.1) and apparent volume of distribution at steady state was 131.16 l/M^2 (range 24.3-516.7). Pharmacokinetics appeared linear with dose, although there was patient heterogeneity with respect to concentration and total plasma clearance for a given dose.[41]

In a second phase I study using the same 72-hour continuous infusion schedule, performed at the University of Wisconsin, secretory diarrhea defined a maximal tolerated dose of 40 mg/M^2/ day x 3. Similar plasma concentrations of flavopiridol were observed.[42] One patient in this trial had gastric cancer metastatic to the liver and had failed to respond to 5-fluorouracil. Following treatment with flavopiridol, this patient achieved a sustained complete response with no evidence of disease more than 2 years after treatment was completed.

Phase II Studies

Overview

Phase II studies of infusional flavopiridol have been completed or are ongoing in a number of disease types, including chronic lymphocytic leukemia, non-Hodgkin's lymphoma, as well as prostate, colon, gastric, renal and non-small cell lung cancer (NSCLC).[43] In general, these trials employ the schedule of 50 mg/M^2/day as a 72-hour infusion every 14 days; in some trials, higher

doses have been permitted with anti-diarrheal prophylaxis in patients who have no toxicity. To date, extensive data are available from trials of patients with renal cell carcinoma,[44] gastric carcinoma[45] and NSCLC.[46] Several themes have emerged from the results of these three studies.

Toxicities

First, the toxicity profiles in the three trials were similar. The major toxicities reported included fatigue/asthenia, diarrhea, and clotting events. Diarrhea was an expected toxicity, based on the preclinical and phase I data. In the renal cell trial, diarrhea occurred in 27/35 (77%) of patients and was severe in 7/35 (20%). Similar toxicity occurred among 16 patients enrolled in the gastric cancer trial. In the NSCLC trial, diarrhea occurred in 11/20 (55%) of patients but no severe toxicity was reported.

In the renal cell cancer trial, the plasma concentrations of flavopiridol and flavopiridol glucuronide were measured at 23, 47 and 71 hours during the flavopiridol infusion and the metabolic ratios of flavopiridol glucuronide: flavopiridol were determined. At 71 hours, the metabolic ratios showed a bimodal distribution with an antimode of 1.2. Thirteen patients experienced diarrhea and had lower metabolic ratios [0.72 (0.53-0.86)] at 71 hours than patients without diarrhea [2.25 (1.76-2.3); P = 0.002]. Eight of 11 extensive glucuronidators (ratio > 1.2) did not develop diarrhea, whereas 10 of 11 poor glucuronidators (ratio <1.2) developed diarrhea (P = 0.008). Thus, the glucuronidation of flavopiridol is apparently polymorphic, suggesting a genetic etiology, and is inversely associated with the risk of developing diarrhea.[47]

Fatigue and asthenia were common in the three trials as well, reported in 88% and 93% of patients in the renal cell carcinoma and gastric carcinoma trials, respectively. Although this was also noted in phase I trials, it seemed to be more distressing to the presumably better functioning patients in these phase II studies. Half of the patients in the lung cancer trial experienced fatigue (10/20), although it was only rated as severe in three patients. The symptom is difficult to interpret in lung cancer patients, as fatigue occurs commonly in this population, and has been reported as severe in significant percentages of patients in the best supportive care arm of randomized trials.[48] Therefore, it is difficult to definitively conclude that the fatigue observed in the NSCLC trial was entirely drug-related.

A serious toxicity that was not appreciated in the phase I studies was the occurrence of venous and arterial clotting events. In the renal cancer trial, six patients developed venous thrombosis; two events were catheter related, two occurred in the lower extremities and two patients suffered probably pulmonary emboli. In addition, in this trial, three patients experienced arterial vascular adverse events, including myocardial infarction, transient ischemic attack and transient vision loss. In the trials of gastric and lung cancer, only venous clotting events were reported. Five patients in the gastric cancer study developed catheter-related thrombosis. Venous thromboses occurred in 7/20 (35%) patients in the NSCLC trial. While the majority of these events occurred at the catheter site, lower extremity deep venous thromboses and probably pulmonary embolism also occurred.

The incidence of venous thromboses does not appear to be affected by the concentration at which flavopiridol is administered. Although a high concentration of 2.5 mg/ml was used in some patients in the gastric cancer trial, the renal cell cancer trial administered drug at a concentration of 0.1-0.5 mg/ml and in the NSCLC trial, all patients received flavopiridol at a maximal concentration of 0.2 mg/ml. Despite the presumed higher volume and flow rate of infusion associated with the administration of lower drug concentrations, catheter-related thromboses were common.

Although patients with metastatic gastric, renal or lung cancer have high incidences of deep venous thrombosis, the frequency of clotting events was higher than expected compared with other clinical trials in comparable cohorts.[44,49] Nonetheless, whether flavopiridol truly causes a clotting diathesis remains unclear. For example, in the phase I trial, only five instances of catheter related thrombosis occurred among 76 patients, and spontaneous thrombosis in the absence of foreign bodies was not observed, despite alterations in plasma fibrinogen levels.[41]

Similarly, in a preliminary report of the phase II trial in patients with metastatic colorectal cancer, only 1 of 14 patients developed a catheter-related subclavian thrombosis.[50] Measurement of multiple clotting events in future trials may help assess the etiology of these events.

Single Agent Activity of Flavopiridol

A second theme emerging is the limited single agent activity of flavopiridol in these diseases, despite the promise of phase I results in patients with renal cell or gastric cancer. Among the 35 minimally pre-treated renal cell cancer patients, two objective responses were observed. In the trial designed for advanced gastric cancer 14/16 enrolled patients were evaluable for response; only one minor response was achieved. Necrosis was noted in one selected post-treatment tumor biopsy, although the patient had progression of disease shortly after. Among the 20 patients in the NSCLC study, 18 patients were evaluable for response. Although three patients had minor responses, again there were no major objective responses.

Despite the lack of responses, several patients in these trials did achieve stable disease. For example, three patients with renal cell cancer remained on trial for >30 weeks, and one patient reported remained on flavopiridol more than 1 year. Similarly, in the NSCLC trial, four patients (including the three patients with minor responses) achieved stable disease for 4, 5, 10 and 16 months respectively. In addition, the median survival of 7.5 months achieved with flavopiridol was similar to the range of 7.4-8.2 months recently reported in a randomized trial of four chemotherapy regimens containing platinum analogs in combination with taxanes or gemcitabine.[46,51] These trials were not designed to evaluate cytostatic effects of flavopiridol, and it is possible that the tumors of patients remaining on flavopiridol for long periods had biologic characteristics predicting a more indolent growth rate. Nonetheless, the results of phase I and II trials thus far suggest that protracted disease stability with acceptable toxicity can be achieved in some patients.

Available pharmacokinetic data indicate that plasma levels of flavopiridol achieved in these studies were in the range for CDK inhibition. In the renal cell trial, flavopiridol concentrations were 389 nM (296-567 nM), 412 nM (297-566 nM) and 397 nM (196-553 nM) at 23, 47 and 71 hours during the infusion, respectively.[47] Levels were somewhat lower in the NSCLC trial, where mean steady state concentrations (C_{ss}), defined as the geometric mean of the daily drug levels, were reported for the first two infusions. During the first infusion, the mean C_{ss} ($\pm$ SD) was 200 $\pm$ 90 nM; during the second infusion the mean was 225 $\pm$ 124 nM. Preclinical work in NSCLC cell lines had indicated that concentrations of drug of at least 300 nM were required for cytotoxic effects, whether mediated by apoptosis or necrosis.[21,23] In addition, in preclinical models, cytotoxicity was far more likely when drug concentrations were $\geq$500 nM. Importantly, only six patients in the NSCLC achieved a C_{ss} exceeding 300 nM during either infusion, and no patient achieved a C_{ss} above 500 nM, consistent with the absence of major responses. Interestingly, in this trial, there was a correlation between plasma flavopiridol level and stable disease. Plasma concentrations of flavopiridol were determined in 9 of 10 patients with stable disease at 8 weeks, including two with minor response. Among these nine patients, seven achieved a C_{ss} that was one standard deviation or more above 150 nM (the minimum concentration producing clear growth arrest in the majority of NSCLC cell lines) during either the first or second infusion. In contrast, among the eight patients with disease progression at 8 weeks, only 2 achieved similar plasma concentrations. Among the two groups of patients, there was a statistically significant difference in the highest C_{ss} of flavopiridol achieved.[46]

Correlative Studies

Another similarity among these trials has been the lack of utility of in vitro cell cycle studies performed on peripheral blood mononuclear cells (PBMCs). In the renal cell trial, PBMCs were collected at baseline and 71 hours into the infusion. It was postulated that cells collected at the end of the infusion would be inhibited from entering the cell cycle when stimulated with phytohemagglutinin A (PHA) and interleukin-2 (IL-2). However, there was no difference in cell cycle parameters of PHA/IL-2-stimulated lymphocytes collected at baseline

or at the end of the flavopiridol infusion. The lack of observed effect was likely because cells need to be continuously exposed to flavopiridol in order for cell cycle arrest to become manifest. Thus, cells that are exposed to flavopiridol for 72 hours and then undergo exvivo growth stimulation do not experience any permanent cell cycle perturbations. Similarly, in the gastric cell trial, no evidence of cell cycle effects or apoptosis were seen in circulating lymphocytes examined by TdT or 7-AAD labeling with flow cytometry; evidence of PARP cleavage and caspase-3 activation was also absent.

While the concentrations of flavopiridol observed in these studies should be capable of achieving CDK inhibition in cycling tumor cells, this has not yet been confirmed in clinical tumor specimens. The importance of this is underscored by the recent reports that immobilized flavopiridol can bind to cytosolic aldehyde dehydrogenase 1[35] and glycogen phosphorylase[36] present in various cell lines, which may reduce its ability to compete with cellular ATP for CDK binding. Currently, phosphospecific Rb antibodies exist, directed against sites specifically phosphorylated by either CDK2 or CDK4. CDK inhibition by flavopiridol should cause decreased phosphorylation of Rb at these sites. Adaptation of these antibodies for immunohistochemical studies should allow confirmation of CDK inhibition in selected biopsies in future studies. Similarly, determination of cyclin D1 levels by immunohistochemistry may also confirm the biologic activity of flavopiridol in primary tumor specimens.

Alternative Strategies for the Development of Flavopiridol

Bolus Administration

Preclinical Data

Recently, promising activity has been reported in human leukemia and lymphoma xenografts, using bolus-dosing schedules. For example, after treatment with 7.5 mg/kg flavopiridol bolus (either IV or intraperitoneal) on each of 5 consecutive days, 11/12 advanced stage HL-60 xenografts underwent complete regressions, and animals remained disease-free several months after one course of flavopiridol treatment.[18] Similar results were seen in two lymphoma xenograft models, with complete and major regressions noted.[18] Evidence of apoptosis was seen both in the xenografts and in normal hematopoietic organs. In contrast, continuous infusion of flavopiridol for 3 days, which results in plasma levels >400 nM, resulted in only modest antitumor effect in animals bearing HL-60 xenografts. In a similar study against a head and neck squamous cell carcinoma xenograft, flavopiridol administered intraperitoneally at 5 mg/kg per day for 5 consecutive days induced a 60-70% reduction in tumor size, which was sustained for 10 weeks. Apoptotic cell death and cyclin D1 depletion were observed in tissues from xenografts treated with flavopiridol at peak plasma concentrations of 5-8 μM.[19] In summary, the best antitumor effects in xenografted animals have been observed after daily bolus IV or IP administration of flavopiridol that results in peak plasma levels of approximately 7 μM, followed by a progressive decline to 100 nM in 8 hours. Achievement of relatively short-lived, but repetitive high plasma levels of flavopiridol in the μM range appears to be an effective way to produce the maximum antitumor effect with flavopiridol at least in hematopoietic malignancy and head and neck cancer models.

Phase I Trial

Based on these results, the NCI initiated a phase I trial of a daily 1-hour bolus of flavopiridol for 5 consecutive days every 3 weeks.[52] Initially, 24 patients received doses ranging from 12-52.5 mg/M^2/day x 5. Dose-limiting toxicities of nausea, neutropenia and fatigue were reached at the 52.5 dose level, resulting in a recommended phase II dose of 37.5 mg/M^2/day x 5. The C_{max} of flavopiridol at this dose level was 1.4 $\pm$ 0.1 μM. To attempt to achieve higher concentrations, 12 patients received 1-hour flavopiridol for 3 consecutive days every 3 weeks. Dose-limiting toxicity was reached at 62.5 mg/M^2/day, including severe hepatotoxicity, resulting in a recommended phase II dose of 50 mg/M^2/day for 3 consecutive days. At this dose level, the

median C_{max} of flavopiridol reached 3.2 ± 1.2 μM. The elimination half-life of flavopiridol was shorter with IV bolus (5.5 ± 5.2 hours) than with 72-hour infusion, perhaps because enterohepatic circulation was not observed.[53] Three patients on trial (with NSCLC, mantle-cell lymphoma and melanoma) have had stable disease >6 months.[52] Phase II trials with this schedule are currently under development.

Combinations with Standard Chemotherapy Agents

It is possible that CDK inhibitor drugs will be primarily cytostatic when used alone, especially against solid tumors derived from cells not typically predisposed to apoptotic responses. Although flavopiridol may not have impressive antitumor activity when used as a single agent, it may have great utility when combined with standard chemotherapy. Several preclinical studies have indicated that flavopiridol produces additive and synergistic cytotoxicity when administered in combination with many chemotherapy agents used in standard practice.[54,55] In most cases, this is a schedule-dependent phenomenon in which synergy is seen when standard chemotherapy drugs are administered before flavopiridol.

Taxol

Several studies have explored the interaction of flavopiridol with Taxol. If flavopiridol is used first, CDK1 (cdc2) inhibition and G2 arrest prevents entry into mitosis, inhibiting Taxol-mediated cell death, which depends at least in part on mitotic arrest. However, if flavopiridol is used following Taxol, significant synergism is seen.[54,56] The activation of caspases, specifically caspase 3, is enhanced by the presence of flavopiridol in Taxol-treated cells.[56] The events that occur in cells treated sequentially with Taxol followed by either no further treatment or by flavopiridol have been analyzed. When Taxol is used alone, a transient mitotic block is induced, after which cells exit mitosis without undergoing cytokinesis. Although they retain 4N DNA content, they express G1 markers, a stage referred to as pseudo-G1. Cell death most likely occurs in relation to both the mitotic block and the exit from an abnormal mitosis. Addition of flavopiridol after Taxol treatment appears to accelerate the exit from mitotic block, with a more rapid decline of mitotic cell markers, such as MPM-2.[56] It is possible that the more rapid emergence from an abnormal mitosis, caused by the appropriately timed inhibition of CDK1 (cdc2) is associated with enhanced cell death. The data demonstrated synergism with a concentration of flavopiridol of 300 nM administered for 24 hours. To date, the synergism of Taxol and flavopiridol has only formally reported in cell lines expressing wild-type p53, including A549 NSCLC cells, MKN-74 gastric cancer cells and MCF-7 breast cancer cells,[54, 56] although a recent report suggests synergism in Calu-1 cells, which lack p53.[57] It will be of interest to determine whether the same degree of synergism occurs in cells lacking p53, which are often more sensitive to taxane therapy.

A phase I trial of sequential Taxol and flavopiridol has been completed. The recommended phase II dose is 175 mg/M^2 Taxol, administered over 3 hours on day 1, followed by a 24-hr infusion of 80 mg/M^2 flavopiridol, beginning on day 2.[58] Promising clinical activity has been reported, including responses in some patients with taxane refractory tumors. This has prompted a phase II study of the combination in patients with esophageal cancer that is refractory to prior Taxol. However, phase III trials will be required to assess the true impact of flavopiridol in the combination.

Combinations with Agents Damaging DNA and Inhibiting DNA Synthesis

Cytotoxic synergy has also been observed when flavopiridol follows

1. S-phase specific agents, such as cytarabine, gemcitabine and 5-fluorouracil;[54, 59, 60]
2. topoisomeraseI inhibitors, such as topotecan and CPT-11, which also work primarily during S phase;[54,61]
3. topoisomerase II inhibitors such as doxorubicin and etoposide, which work in G1 and G2, as well as in S phase;[54] and

4. mitomycin C, an alkylating agent.[55]

All of these agents can cause retardation of S phase progression when used alone in vitro at non-cytotoxic doses, resulting in populations of cells effectively recruited to S phase. The known activity of CDK2 during S phase suggests a possible mechanism for flavopiridol-mediated apoptosis following S phase recruitment by standard chemotherapy drugs.

The initiation of S phase requires activation of the E2F-1 transcription factor, which occurs at the G1/S transition following CDK-mediated phosphorylation of Rb. However, proper S phase progression also requires the appropriately timed deactivation of E2F-1, which is in part the result of cyclin A-CDK2-mediated phosphorylation of E2F-1 and its heterodimeric partner, DP-1.[62-65] Inhibition of CDK2 activity during S phase would be expected to result in inappropriately persistent E2F-1 activity, which can drive cells down both p53-dependent and independent apoptotic pathways (reviewed in ref. 66; Fig. 1). Recently, peptides that block the interaction of cyclin A-CDK2 with substrates such as E2F-1 have been shown to induce S phase arrest and abrupt apoptosis.[67] Chemotherapy agents may effectively recruit cycling cells to S phase by imposing an S phase delay. Flavopiridol-mediated cyclin A-CDK2 inhibition during such a delay may account for enhanced cytotoxicity.

This mechanism suggests several testable hypotheses. For example, cells recruited to S phase by other mechanisms should be sensitized to flavopiridol treatment. This has been shown in cells released from a hydroxyurea-induced block at the G1/S boundary[68] and in cells engineered to express an antisense oligodeoxynucleotide that inhibits expression of $p27^{Kip1}$.[69] Furthermore, in cells treated sequentially with chemotherapy followed by flavopiridol, cyclin A-CDK2 inhibition should result in decreased phosphorylation of E2F-1/DP-1, and E2F-1 DNA-binding and transactivation activities should be increased. In this sense, flavopiridol used during S phase should be an E2F-1-metic drug. In addition, apoptosis mediated by chemotherapy/flavopiridol combinations may very well depend on the level of cellular E2F-1 activity.[68] Cells with higher baseline levels of E2F-1 activity will also have higher E2F-1 activity following CDK2 inhibition, capable of generating an apoptotic response that might not occur in cells with lower baseline levels of E2F-1 activity. In this regard, tumor cells may be selectively sensitive to chemotherapy/flavopiridol combinations because they have high levels of E2F-1 activity compared to normal cells because of disruption of the Rb-axis. Indeed, peptide CDK2 antagonists have been shown to selectively kill tumor cells,[67] and chemotherapy/flavopiridol combinations have been shown to be selectively toxic to T-antigen transformed fibroblasts compared to their normal counterparts.[60]

An NCI-sponsored phase I trial of gemcitabine, followed by flavopiridol, is currently underway in patients with solid tumors. Gemcitabine is administered on day 1, and a 24-hour infusion of flavopiridol is administered beginning on day 2. This trial should lay the groundwork for phase III trials in which the true contribution of flavopiridol can be assessed.

Cisplatin

In the study of NSCLC cell lines, the combination of cisplatin and flavopiridol is also synergistic,[54,68] although synergism has not been detected in bladder carcinoma models.[70] In NSCLC cells, the most pronounced effects occur when cisplatin precedes flavopiridol. However, a synergistic interaction has also been demonstrated when flavopiridol is administered first or concomitantly with cisplatin.[54] Recently, it has been reported that flavopiridol can enhance cisplatin accumulation in an ovarian cancer cell line, providing a possible explanation for the sequence-independent synergy.[71] Currently, a phase I trial is in progress assessing doses of cisplatin immediately followed by a 24-hour infusion of flavopiridol. In addition, cisplatin has recently been added directly prior to flavopiridol in the Taxol/flavopiridol combination, in order to exploit the synergy demonstrated in in vitro systems.

Delayed Administration of Antimetabolites

Use of a CDK inhibitor before or concomitant with most DNA-damaging agents would be expected to cause G1 arrest and therefore to decrease the cytotoxicity of such drugs, and

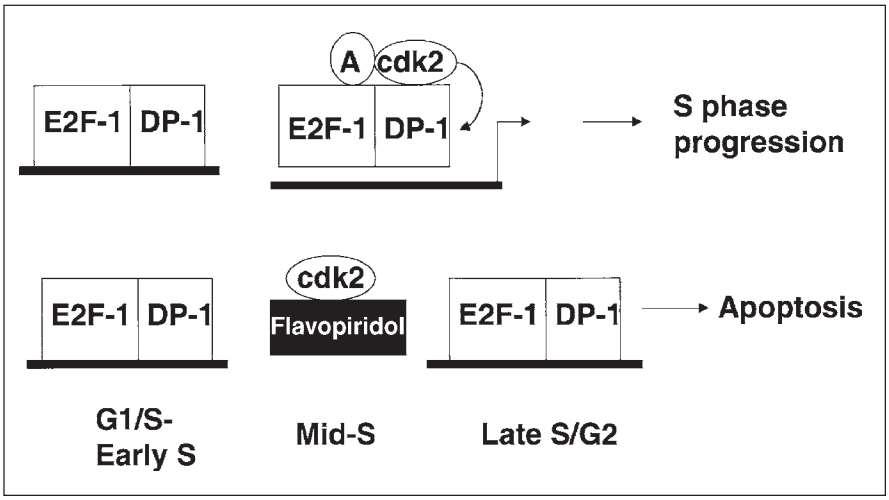

Fig. 1. CDK2 inhibition during S phase can sensitize cells to E2F-1-dependent apoptosis. Following the G1/S transition, E2F-1 activity is derepressed and E2F-1 is released from Rb. E2F-1, bound to its heterodimeric partner, DP-1, directs transcription of genes required for S phase. Importantly, however, this transcription is activated only transiently. Orderly S phase progression requires the down regulation of E2F-1 activity, accomplished in part by cyclin A-CDK2-mediated phosphorylation. E2F-1 forms stable complexes with cyclin A-CDK2, which phosphorylates both E2F-1 and DP-1, resulting in inhibition of the transactivation and DNA binding activities of E2F-1 (upper row). Inhibition of CDK2 by flavopiridol (or any other CDK2 inhibitor) during S phase would be expected to result in inappropriately persistent E2F-1 activity, which is known to result in S phase delay and apoptosis (lower row).

indeed, most sequence specific interactions require that chemotherapy be administered before flavopiridol. However, a CDK inhibitor might also be used to synchronize cells at the G1/S boundary. After a sufficient interval to allow CDK activity to resume and for cells to enter S phase in a synchronized fashion, administration of an S phase-specific agent may be predicted to cause greater cytotoxicity than when used on an asynchronous tumor population. In vitro, following G1 arrest by flavopiridol, cytarabine or 5-FU was administered following a 48-72 hour delay, with markedly enhanced cytotoxicity.[54] In the case of 5-fluorouracil, synergy is also seen without a long delay between the two drugs (and is greater than the degree of synergy seen when 5-fluorouracil is used first).[54] It is possible that the G1/S block imposed by flavopiridol is accompanied by a down-regulation of thymidylate synthetase levels.[72] Following removal of flavopiridol, subsequent cytotoxicity in response to 5-FU would be enhanced. Thus, the interaction of flavopiridol with antimetabolites suggests additional strategies for clinical trial.

Prevention of Endoreduplication by Flavopiridol

In addition to the therapeutic strategies for the further development of flavopiridol, one recent report suggests the possibility that CDK inhibition may be appropriate as chemoprevention. Cells defective in checkpoint control tend to undergo endoreduplication in response to damage to DNA or the spindle apparatus. In the case of premalignant cells, entrance into S phase prior to completing the previous mitosis leads to aneuploidy and genomic instability, resulting in eventual emergence of the malignant clone. Recently, it has been shown that following microtubule inhibition, the endoreduplication (i.e., progression to 8N) of checkpoint deficient cells lacking p53 or $p21^{WAF1/Cip1}$ can be prevented by flavopiridol, which causes an arrest of 4N cells in a pseudo-G1 state.[73] While the current formulation and toxicities of flavopiridol

do not make it suitable for use as a chemopreventive agent, oral preparations of newer CDK inhibitors may be effective for chemoprevention in selected high-risk populations.

Resistance to Flavopiridol

If flavopiridol used alone is largely cytostatic, its use over long periods of time can be anticipated in some patients. It is noteworthy that it has been difficult to generate resistant cell lines thus far. The first resistant cell line recently reported was an ovarian carcinoma cell line that developed spontaneous resistance to both flavopiridol and cisplatin upon repeated passage in the absence of drug exposure. At least part of the resistance appeared attributable to reduced intracellular accumulation of the two drugs.[71] Although one study has demonstrated that flavopiridol may interact with the multi-drug resistance efflux protein, MRP1,[74] additional studies indicate that expression of MDR1 or MRP1 does not affect sensitivity to flavopiridol. For example, the resistant ovarian carcinoma cell line displayed no resistance to substrates of the P-glycoprotein drug efflux pump such as Taxol, etoposide or doxorubicin. Similarly, a colon carcinoma cell line 9-fold resistant to flavopiridol, established in vitro following exposure to increasing concentrations of drug, demonstrated only low level cross-resistance to Taxol, not attributable to elevated levels of P-glycoprotein.[75] Importantly, cells overexpressing P-glycoprotein do not have increased flavopiridol resistance.[70,76,77]

Recently, it has been shown that overexpression of the ATP-binding cassette half transporter, ABCG2, confers resistance to flavopiridol.[77,78] In addition, a 24-fold resistant MCF-7 breast carcinoma cell subline, maintained in 1 μM flavopiridol, overexpresses ABCG2 mRNA and protein. These cells display cross-resistance to mitoxantrone and camptothecins, also handled by this transporter.[77] To date, no resistant cell lines based on alterations of CDKs or their endogenous modulators have been reported.

The Paullones

Discovery

Several members of the paullone family were identified using the COMPARE algorithm to detect compounds from the database tested in the NCI Human Tumor Cell Line Anti-Cancer Drug Screen that had similar biochemical targets and cellular mechanism to flavopiridol.[26] Kenpaullone is a potent inhibitor of cyclin B-cdc2 (IC_{50} = 0.4 μM), cyclin A-CDK2 (IC_{50} = 0.68 μM), cyclin E-CDK2 (IC_{50} = 7.5 μM) and CDK5/p35 (IC_{50} = .85 μM), and has much lower activity against cyclin D1/CDK4 (IC_{50} = > 100 μM) or other kinases.[79] Kenpaullone competitively inhibits the binding of ATP, and molecular modeling has demonstrated that it binds to the ATP binding site with residue contacts similar to other CDK2 inhibitors.

Cell Cycle Effects

The cell cycle effects of kenpaullone were characterized in the MCF10A breast epithelial cell line. In exponentially growing cells, a small increase in S phase fraction is seen, perhaps consistent with inhibition of cyclin A/CDK2. In subsequent experiments, cells were synchronized in G0/G1 by serum starvation, and then stimulated to re-enter the cell cycle in the presence of vehicle or kenpaullone at its approximate IC_{50} concentration of 30 μM. After 20 hrs, vehicle-treated cells entered S phase, while kenpaullone-treated cells were arrested at the G1/S boundary, consistent with CDK2 inhibition.[79]

Kenpaullone Analogues and Other Paullones

Kenpaullone contains a 9-bromo substituent. Its replacement with either a 9-cyano or 9-nitro group results in significantly enhanced inhibition of CDK1 (cdc2).[80] The same is true of 2-substituted paullones.[81] 10-bromo-paullone (NSC 672234) inhibits various protein kinases in addition to CDKs 1, 2 and 5, including several protein kinase C isoenzymes. Interestingly, it is less selective between cyclin A/CDK2 and cyclin E/CDK2, and also causes down-regulation

of CDK4 expression.[82] G1 arrest can be detected following drug exposure in exponentially growing cells or in cells synchronized in G0/G1 by serum starvation.[79]

Purine Derivatives

First Generation Compounds

Purine derivatives were among the first CDK inhibitors discovered. Initially, 6-dimethylaminopurine was shown to inhibit mitosis in sea urchin embryos without inhibiting protein synthesis.[83,84] It was subsequently shown to inhibit CDK1 (cdc2) activity with an IC_{50} = 120 μM, but its specificity was poor.[5] Further studies identified substituted purine derivatives, including isopentenyladenine (IC_{50} = 55 μM) and olomoucine (IC_{50} = 7 μM) capable of inhibiting cyclin B-CDK1 (cdc2) in an in vitro kinase assay.[5,85,86] Roscovitine, a derivative of olomoucine, is an even more potent CDK1 (cdc2) inhibitor (IC_{50} = 0.45 μM).[5] Olomoucine and roscovitine demonstrate good selectivity for CDKs.

Isopentyladenine, olomucine and roscovitine all inhibit CDK2 as well and have been crystallized in complex with CDK2.[87-89] All of these compounds bind to the ATP site in the deep groove between the N- and C-terminal domains of CDK2, usually occupied by the adenine ring of ATP. Analysis of the ATP-CDK2 structure indicates that the N6 amino group acts a hydrogen- bond donor to the backbone carbonyl of Glu-81, and N1 accepts a hydrogen bond from the backbone nitrogen of Leu-83. The presence of a hydrogen-bond acceptor for the α-amine of Leu-83 is a common feature of all known CDK2-inhibitor structures. For example, the purine rings of isopentenyladenine and olomoucine are oriented so that the N3 and N7 positions, respectively, accept a hydrogen bond from the backbone nitrogen of Leu-83. Their respective N2 and N6 positions also form novel interactions with the peptide oxygen of Leu-83. Alteration of these positions by methylation results in loss of CDK inhibitory activity, confirming the importance of the interaction of CDK inhibitors with Leu-83.

In the isopentyladenine-CDK2 complex, the N9 acts as a hydrogen-bond donor to the carbonyl oxygen group of Glu-81. In contrast, the orientation of the purine ring of olomoucine and roscovitine within the ATP-binding site of CDK2 is rotated nearly 160° relative to that of the adenosine ring of ATP, such that there is no equivalent interaction to that of ATP with the backbone carbonyl of Glu-81. However, olomoucine and roscovitine interact with a region of CDK2 outside of the ATP binding pocket; this region is also involved in complexes of flavopiridol with CDK2 and is likely responsible for the relative selectivity of olomoucine, roscovitine and flavopiridol for CDKs.[12]

Second Generation Compounds

The rotated orientation of the purine ring of olomoucine and roscovitine compared with ATP suggested that new substituents at the 2, 6 and 9 positions of the purine ring might enhance binding affinity, as well as selectivity. Compounds derived from combinatorial libraries of 2, 6, 9-trisubstituted purines were screened in in vitro kinase assays, identifying several novel CDK inhibitors. The most potent of these compounds, purvalanol B, has an IC_{50} against cyclin A-CDK2 of 6 nM, corresponding to a 1000-fold increase over olomoucine.[31] The overall geometry of purvalanol B bound to CDK2 resembles that of the related CDK2-olomoucine and CDK2-roscovitine complexes. However, purvalanol B is able to hydrogen bond to the backbone oxygen of Glu-81 via the acidic C8 atoms of its purine ring. In addition, the increased affinity for CDK2 results from improved hydrophobic contact sites within the active site and the inhibitor, and steric constraints of the ring systems that limit the number of conformations of the inhibitor.

The combinatorial library strategy has generated a large number of additional potent purine analog inhibitors directed against the ATP binding site of CDK2, including CVT-313 (IC_{50} = 0.5 μM).[90] CVT-313 is relatively selective for CDK2; when added to CDK1 (cdc2) or CDK4, an 8.5- and 430-fold higher concentration of drug is required for half-maximal inhibition of enzyme activity. In addition, there is no effect on other, non-related ATP-dependent serine/threonine kinases.

Cellular Effects

Consistent with inhibition of CDK2 and cdc2 (CDK1), olomoucine, roscovitine, CVT-313 and purvalanol A (a more membrane-permeable relative of purvalanol B) cause cell cycle arrest at the G1/S and G2/M boundaries in a variety of transformed and non-transformed cell types.[31, 90-93] In NSCLC and neuroblastoma cell lines, cell cycle arrest mediated by olomoucine and roscovitine is also accompanied by apoptosis.[94]

In addition to cell cycle arrest, purine derivatives have recently been implicated in differentiation processes. In one study, a library of 2, 6,9-substitute purine derivatives was screened for the ability to promote the differentiation of a myeloid leukemic cell line in culture. From this screen, aminopurvalanol (AP) was identified; in the presence of this compound, cells acquire phenotypic characteristics of differentiated macrophages and became arrested in the cell cycle with a 4N DNA content.[95] AP also inhibited mitosis in *Xenopus* egg extracts and affinity chromatography and biochemical reconstitution experiments with these extracts identified cyclin B-cdc2 (CDK1) as a target of the compound. AP inhibited immunoprecipitates of both cdc2 and CDK2 in human leukemic cell extracts, although the data suggested that the compound preferentially targets the G2/M-phase transition in vivo.[95]

UCN-01

Mechanisms of Action

Protein Kinase C Inhibition

UCN-01 (7-hydroxystaurosporine), a staurosporine analogue, is a relatively non-specific CDK inhibitor, and also has activity against several protein kinase C (PKC) isoenzymes.[96] It is most specific for the calcium-dependent protein kinase C (IC_{50} ~30 nM) and less potent against calcium-independent isoenzymes (IC_{50} ~500 nM).[97-99] In a study of A549 lung adenocarcinoma cells with acquired resistance to UCN-01 (A549/UCN cells), derived following long-term culture in medium containing UCN-01, expression of the PKC-α, -ε and -θ isoenzymes was drastically reduced. On subsequent culture in drug-free medium, levels of these isoenzymes returned to baseline, and drug sensitivity was restored.[100]

However, the antiproliferative activity of UCN-01 against multiple human tumor cell lines is far greater than that of other equipotent specific protein kinase C inhibitors, suggesting alternative cellular targets.[101] In addition, cells resistant to other protein kinase C-selective staurosporine analogues (CGP 41251 and Ro 31-8220) that also expressed markedly reduced levels of several PKC isoenzymes retained sensitivity to UCN-01.[100]

G1 Arrest

The effect of UCN-01 on cell cycle progression was first studied in several breast carcinoma cell lines. Following release from a nocodazole-induced mitotic block, cells were arrested in G1, and entry into S phase was inhibited.[102] Although progression through S phase was retarded in a dose-dependent fashion following release from an aphidicolin-induced block at the G1/ S boundary, entry into and completion of M phase were not affected by UCN-01. G1 arrest by UCN-01 was confirmed in A431 epidermoid carcinoma cells[103] and in A549 NSCLC cells[104] by simultaneous analysis of DNA content and 5-bromo-2-deoxyuridine (BrdU) incorporation. In both cell lines, there was accumulation of the hypophosphorylated form of Rb. When UCN-01 was added to CDK2 immune precipitates, histone H1 and Rb kinase activities were inhibited in vitro (IC_{50} = 530 and 640 nM, respectively), indicating that UCN-01 can directly inhibit CDK2 with moderate potency.[105] In addition, CDK2 activities of A431 cells pretreated with UCN-01 for 24 hrs at 260 and 520 nM were markedly inhibited (with IC_{50}'s of far less than 260 nM). When the same cell lysates were analyzed by Western blotting for CDK2, the active, phosphorylated form of the kinase was significantly reduced,[105] suggesting that UCN-01 may act on CDK-activating kinase. Furthermore, treatment with UCN-01

induced expression of $p21^{WAF1/Cip1}$ and its complex formation with CDK2. Induction of $p21^{WAF1/Cip1}$ occurred at the transcriptional level. Expression of $p27^{Kip1}$ was also increased following UCN-01 exposure. Therefore, both direct and indirect inhibition of CDK2 appears to contribute to UCN-01-mediated G1 arrest.[105]

The importance of CDK2 inhibition in UCN-01-mediated G1 arrest was confirmed in a recent analysis of the A549/UCN NSCLC subline, derived following long term exposure of the parent cells to UCN-01. Under the treatment conditions used, parental cells were capable of undergoing apoptosis; the subline was resistant to apoptosis and displayed enhanced G1 arrest. While the subline had altered expression of multiple cell cycle related proteins, it most notably had significantly lower levels of CDK2 compared to parental cells. Enhanced G1 arrest in response to UCN-01 was associated with a significantly greater degree of inhibition of CDK2 activity following treatment.[106]

G1 arrest mediated by UCN-01 is clearly independent of p53, as it has been demonstrated in cell lines in which p53 has been mutated or deleted.[102,103] Initially, it also appeared that G1 arrest could occur in cells lacking Rb as well, such as the MDA-MB468 cells used in the analysis of breast carcinoma cell lines.[102] However, following the nocodazole-induced mitotic block, inhibition of S phase entry in these cells was incomplete. In addition, it was noted that the growth inhibitory IC_{50} for MDA-MB468 cells was significantly higher than that for other breast cancer cell lines that retained wild-type Rb. More recent studies in both breast and lung carcinoma lines indicate that Rb is an important determinant of UCN-01-mediated G1 arrest.[107,108] In the absence of Rb, G1 arrest is either absent or less pronounced, and S phase arrest occurs. Furthermore, in isogenic models, Rb-expressing derivatives of Rb-null and mutant bladder and prostate carcinoma cell lines were capable of G1 arrest following UCN-01 treatment, while G1 arrest in the parent cells was markedly compromised.[107]

The Rb-dependence of the G1 arrest suggests that components of the CDK4/6 axis also comprise critical targets of UCN-01. Direct inhibition of CDK4 has been described in in vitro assays with purified CDK components, although the IC_{50} value was similar to that seen for CDK2.[104] In A431 cells, exposure to UCN-01 results in significant diminution of cyclin D1 levels.[105] In breast epithelial cells, decreased CDK4 levels have been described, with redistribution of $p27^{Kip1}$ from CDK4 to CDK2, resulting in CDK2 inhibition as well.[108] Such events may dominate the response of certain cell types. They either do not occur or produce little impact in cells lacking Rb.

Apoptosis

In some cell lines, including hematopoietic cell lines,[101] a colon carcinoma cell line[109] and several breast cancer cell lines,[110] UCN-01 was shown to induce apoptosis. Cell cycle analysis of leukemic T cell lines treated with UCN-01 demonstrated concomitant loss of cells with G2/M DNA content and an increase in the proportion of cells with either a G1/S or a sub-G1 DNA content.[101] These events coincided with the induction of apoptosis. Importantly, apoptosis correlated with decreased inhibitory tyrosine phosphorylation of CDK1 (cdc2) and CDK2, as well as *activation* of the histone H1 kinase activity of CDK1 (cdc2) and to a lesser degree, CDK2.[101] Inappropriate activation of CDK1 (cdc2) has been associated with apoptosis in a number of model systems, inducing premature chromosome condensation in interphase cell.[111] These data raise the possibility that modulators of CDK1 (cdc2) phosphorylation, such as wee1 kinase or the Cdc25C phosphatase, are important targets in cells undergoing apoptosis in response to UCN-01.

G2 Checkpoint Abrogation

The activation of CDK1 (cdc2) during the apoptotic response in hematopoietic cell lines led to the hypothesis that UCN-01 could be used to prevent G2 arrest following DNA damage in cell lines of diverse origin. During the cellular response to DNA damage, CDK1 (cdc2) is maintained in an inactive state through phosphorylation at Thr-14 and Tyr-15. This results in G2 checkpoint arrest, in part to allow time for repair so that damaged cells do not enter mitosis.

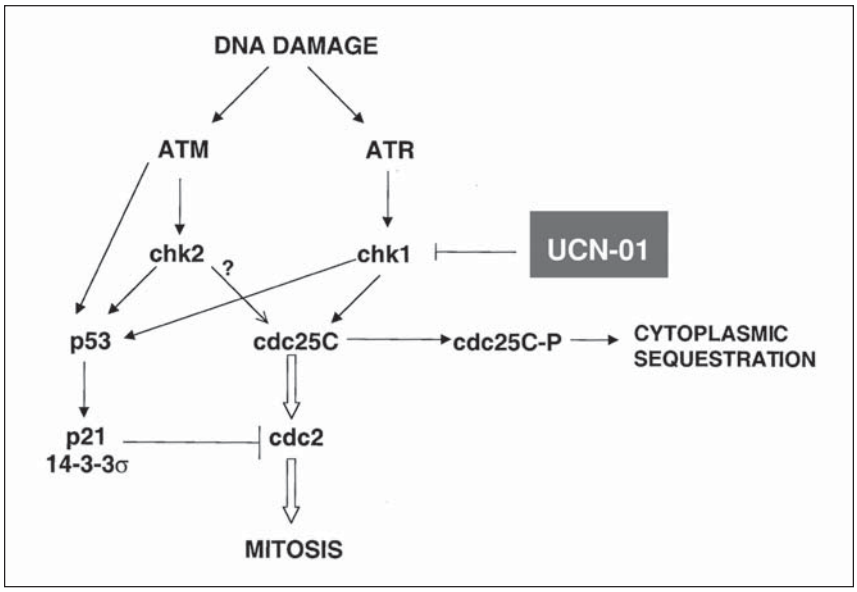

Fig. 2. UCN-01 abrogates G2 checkpoint control following DNA damage by inhibiting Chk1. Following DNA damage, activation of the ATM/ATR kinases occurs, resulting in phosphorylation and activation of the checkpoint kinases, Chk1 and Chk2. Chk1 (and perhaps Chk2) phosphorylate Cdc25C on Ser-216, resulting in its binding to 14-3-3 proteins and ultimate cytoplasmic sequestration. This prevents it from removing inhibitory phosphates from CDK1 (cdc2); the latter remains inactive, preventing the entry of damaged cells into mitosis. (The activities that Cdc25C cannot accomplish following DNA damage because of its cytoplasmic sequestration are indicated with white arrows). UCN-01 causes loss of Ser-216 phosphorylation and 14-3-3 binding of Cdc25C in DNA-damaged cells, allowing dephosphorylation and activation of CDK1 (cdc2), abrogation of the G2 checkpoint, and entry into mitosis.

The ATM, Chk2 and Chk1 kinases also phosphorylate p53 following DNA damage, contributing to its stabilization. Stabilization of p53 following DNA damage occurs even in the presence of UCN-01, and can mediate G2 arrest via transcriptional induction of $p21^{WAF1/Cip1}$ and 14-3-3σ, which contribute to CDK1 (cdc2) inhibition. Therefore, in the presence of p53, UCN-01 does not prevent the arrest of cells in G2 following DNA damage. In the absence of p53, the checkpoint is completely dependent on the Cdc25C pathway, and is severely compromised by UCN-01-mediated Chk1 inhibition. Inappropriate entry into mitosis following DNA damage is frequently lethal. Thus, UCN-01 should enhance the cytotoxicity of DNA damaging agents selectively in cells deficient in p53.

Following treatment with DNA damaging agents such as γ-irradiation or cisplatin, treatment with UCN-01 prevents G2 arrest.[112,113] The abrogation of the G2 checkpoint, resulting in the mitotic entry of damaged cells, is frequently associated with increased cytotoxicity.

The events that mediate G2 checkpoint control in response to DNA damage are shown in Figure 2. Following DNA damage, activation of the ATM and ATR phosphatidylinositol 3-kinase-related kinase family members result in phosphorylation of the checkpoint kinases, Chk1 and Chk2. Chk1 phosphorylates Cdc25C at Ser-216, which promotes binding of Cdc25C to 14-3-3 proteins, resulting in its cytoplasmic sequestration, where it cannot dephosphorylate and activate CDK1 (cdc2).[114-117] As the inhibitory phosphorylation of CDK1 (cdc2) is maintained, cells undergo G2 arrest. Recently, it has been demonstrated that UCN-01 causes loss of Ser-216 phosphorylation and 14-3-3 binding to Cdc25C in DNA-damaged cells. In addition, UCN-01 potently inhibits the ability of Chk1 to phosphorylate Cdc25C in vitro.[118,119] These results identify Chk1 (and the Cdc25C pathway) as potential targets of G2 checkpoint abrogation by UCN-01. In contrast, Chk2 was refractory to inhibition by UCN-01 in vitro and was

still phosphorylated in irradiated cells treated with UCN-01, excluding Chk2 and ATM as in vivo targets of UCN-01.

It has been postulated that UCN-01 must affect other pathways in addition to the Cdc25C-Ser-216 regulatory pathway, because the checkpoint abrogation observed after UCN-01 treatment is much more severe than that observed after expression of a 14-3-3-binding mutant of Cdc25C.[118] Premature mitotic entry following treatment with UCN-01 indicates that active CDK1 (cdc2) is accumulating in the nucleus. Disruption of the Cdc25C pathway explains activation of CDK1 (cdc2), but does not address its nuclear accumulation. Throughout interphase, and during the cellular response to DNA damage, cyclin B-CDK1 (cdc2) complexes shuttle between nucleus and cytoplasm. Nuclear export of cyclin B-CDK1 (cdc2) complexes is regulated in part by phosphorylation of a nuclear export signal (NES) in cyclin B; therefore, it has been proposed that kinases regulating cyclin B NES function could also be potential targets inhibited by UCN-01.[118]

Abrogation of the G2 checkpoint by UCN-01 in DNA-damaged cells occurs selectively in cells that lack p53. This was confirmed using parental and HPV16E6-expressing breast carcinoma cells; absence of G2 arrest, sensitization to DNA damage and increased cytotoxicity occurred primarily in E6 expressing cells.[112] This is because p53-mediated events, including the transcriptional induction of $p21^{WAF1/Cip1}$ and 14-3-3σ results in CDK1 (cdc2) inhibition and cytoplasmic sequestration,[120,121] causing G2 arrest even with abrogation of the Chk1-Cdc25C-cdc2 pathway in UCN-01 treated cells. Therefore, treatment with UCN-01 should result in the selective enhancement of p53-deficient cells to DNA damaging treatments, an attractive feature for its therapeutic use.

S Phase Checkpoint Abrogation

In addition to arrest at the G1/S or G2/M boundaries following DNA damage, S phase retardation and arrest may occur, comprising an S phase checkpoint. Manifestation of the S phase checkpoint most likely depends on several factors, including the cell type undergoing damage and its expression of Rb,[122] as well as on the type and dose of DNA damage used. Two systems that have been studied include colon carcinoma cells, treated with camptothecin (CPT-11), a topoisomerase I inhibitor,[123] and CHO cells, treated with cisplatin.[124] Both cell types undergo S phase delay and arrest in response to the respective treatments. In both cases, UCN-01 has been found to abrogate the S phase arrest and enhance chemotherapy-mediated cytotoxicity. In the case of camptothecin-treated colon carcinoma cells, this occurs in concert with a modulation of cyclin A-CDK2 activity as well as activation of CDK1 (cdc2) activity, an effect that is selective for p53-deficient cells. In cisplatin-treated CHO cells, UCN-01 induces a redistribution of PCNA to the detergent-insoluble, DNA-bound fraction, which presumably contributes to accelerated DNA synthesis. The UCN-01-mediated redistribution of PCNA following cisplatin treatment may be more likely to occur in p53-deficient cells, in which PCNA is not sequestered by high levels of $p21^{WAF1/Cip1}$. Importantly, in both systems, the effects were associated with an enhancement of chemotherapy-induced cytotoxicity at concentrations of UCN-01 that alone were not cytotoxic and had no detectable effect on cell cycle progression.

In the above in vitro models, cell death was presumably enhanced by the mitotic entry of damaged cells, allowed by combined abrogation of the S and G2 checkpoints. In studies of hematopoietic cells, UCN-01 has also resulted directly in the death of S phase arrested cells. Recently, it has been shown that the PI 3-Kinase-AKT-BAD pathway is inhibited by UCN-01 in gemcitabine-treated human myeloid leukemia ML-1 cells. Although neither gemcitabine nor UCN-01 alone had any effect of PI 3-Kinase activity, sequential gemcitabine-UCN-01 treatment resulted in significant diminution in this activity. As a consequence, the combination treatment resulted in decreased phosphorylation of AKT as well as decreased phosphorylation of BAD, permitting an apoptotic response.[125]

Effects on E2F-1 Protein Expression

Orderly progression through S phase requires appropriately timed diminution of E2F-1 activity. The diminution in E2F-1 activity occurs by phosphorylation mediated by cyclin A-CDK2, which disrupts DNA binding and transactivation activities, followed by ubiquitin-proteasome-dependent degradation. It has recently been shown that UCN-01 enhances degradation of E2F-1, which perhaps could contribute to abrogation of an S phase checkpoint.[72,126] One consequence of decreased E2F-1 activity is decreased thymidylate synthetase gene expression, resulting in enhanced sensitivity to 5-fluorouracil.

Inhibition of DNA Repair Signaling

In addition to its effects as a checkpoint abrogator, UCN-01 may also directly inhibit DNA repair. Interestingly, in studies of CHO cells, UCN-01 was capable of preventing cisplatin induced G2 arrest in all CHO lines, regardless of their DNA repair status. However, UCN-01 enhanced cisplatin-mediated cytotoxicity only in repair proficient CHO/AA8 cells, and not in repair-deficient CHO/UV41 cells (which are incapable of recovery from cisplatin-mediated DNA damage).[113] While it does not physically inhibit the repair apparatus, UCN-01 inhibits repair signaling, resulting in the attenuation of the interaction of XPA and ERCC, two components of the nucleotide excision repair (NER) pathway; the phosphorylation of an XPA-associated protein is also inhibited in UCN-01 treated cells.[127]

Preclinical Antitumor Activity and Pharmacology

Administration of UCN-01 by an intravenous or intraperitoneal route resulted in antitumor activity in several xenograft model systems, including breast carcinoma, renal carcinoma, pancreatic carcinoma and leukemia.[2,128] Both in vitro and in vivo, the antitumor effects were greatest when UCN-01 was given over a protracted period, usually greater than 72 hours.[2,102] Pharmacokinetics and toxicologic studies using several schedules were completed in rats and dogs. Following a 72-hour continuous infusion of UCN-01 in beagle dogs, local reactions at the site of injection and gastrointestinal toxic effects were dose limiting. A steady-state plasma concentration of 330 nM was achieved. Pharmacokinetic parameters included a volume of distribution of 6.09 L/kg, and a total clearance of 0.6 L/kg per hour with a β elimination half-life of ~12 hours.[129]

Phase I Studies

UCN-01 as a Single Agent

The first phase I trial of UCN-01 utilized a 72-hour continuous infusion every 2 weeks.[130] In this trial, an unexpectedly long half-life of approximately 4 weeks was observed, approximately 100 times longer than the half-life predicted from preclinical models. This was most likely caused by the avid binding of UCN-01 to α_1-acid glycoprotein.[131] For this reason, the protocol was amended to retreat every 4 weeks, and the continuous infusion duration was reduced to 36 hours during the second and subsequent courses. In this trial, there was a relative lack of myelotoxicity or gastrointestinal toxicity, which were also predicted based on experience in animal models. Instead, prominent toxicities included nausea and vomiting, symptomatic hyperglycemia associated with an insulin-resistance state, and pulmonary toxicity, characterized by substantial hypoxia without obvious radiologic changes. Other toxicities included myalgias, headaches and elevation of transaminases. Pharmacokinetic parameters included a clearance of .014 L/hour and a terminal half-life of 574.4 (199-4099) hours. The concentration of free UCN-01 was assessed in saliva. At the maximal tolerated dose of 42.5 mg/M^2/day, the median free concentration was 111 nM, which is associated with G2 checkpoint abrogation in in vitro models.

One patient with refractory metastatic melanoma achieved a partial response that lasted 6 months. Several other patients with leiomyosarcoma, non-Hodgkin's lymphoma and lung cancer achieved disease stabilization for 6 months or greater.

Because of the long half-life, two phase I trials have addressed administration of UCN-01 as a short infusion, given over either 1 hour or 3 hours. In the American trial, at doses up to 68 mg/M^2 given over 1 hour, toxicity was mild and reversible, including fatigue, fever, hyperglycemia, and elevated transaminases and creatinine.[132] At higher doses given over 1 hour, dose-limiting hypotension occurred and accrual continues with a 3-hour infusion. Mean half-life was 534 ± 580 hours. In this study, no responses were seen among the first 15 patients, although one patient with cervical cancer has remained on study for more than 1 year with stable disease. Using a 3-hour infusion, the Japanese trial has reported on patients receiving up to 51.1 mg/M^2 with only mild diarrhea, nausea and arrhythmia reported. Pharmacokinetic studies have confirmed the low systemic clearance and distribution volume, as well as the extremely long half-life.[133]

Combinations with Standard Chemotherapy Agents

Several phase I studies are planned or in progress in which UCN-01 is administered in combination with standard chemotherapy drugs. For example, a trial in which UCN-01 follows fludarabine is in progress in patients with refractory low-grade lymphoid malignancies. Trials in which UCN-01 follows cisplatin have been initiated in patients with solid tumors. In in vitro models, sequential cisplatin-UCN-01 treatment results in decreased levels of cyclin A and a phosphorylation pattern of CDK1 (cdc2) consistent with activated kinase. Serial biopsies of tumors are planned in patients; immunostaining with cyclin A antibodies and appropriate phosphospecific CDK1 (cdc2) antibodies should provide confirmation of UCN-01 mediated abrogation of cisplatin-induced G2 arrest.[134] Finally, a trial in which 5-fluorouracil and UCN-01 are combined sequentially is also in progress, with the hope that decreased thymidylate synthetase levels mediated by UCN-01 will enhance 5-fluorouracil-induced cytotoxicity.

Novel Selective CDK Inhibitors

Inhibitors of CDK2

Several selective CDK2 inhibitors are currently under development. The most extensive reporting to date has been on a series of compounds developed at Glaxo Wellcome. Several of these compounds have demonstrated cell cycle arrest in normal cells, and selective killing of transformed cells. For example, it has been possible to kill colon carcinoma cells under the same conditions that only slow proliferation of human diploid fibroblasts.[135] Thus, these compounds appear to mimic the killing of transformed cells by cell-permeable CDK2 inhibitory peptides, perhaps by a mechanism dependent on the high levels of E2F-1 activity in transformed cells.[67]

Another series of substituted oxindole inhibitors of the ATP binding site of the cyclin A-CDK2 have also been synthesized. Compound 4 is a potent and selective inhibitor of CDK2 (IC_{50} = 10 nM), and a weaker inhibitor of cyclin B-CDK1 (cdc2) and cyclin D1-CDK4 (IC_{50}s = 110 and 130 nM, respectively), and showed an average IC_{50} of 2 μM against a panel of 12 other protein kinases.[136] This drug produced the expected reversible cell cycle arrest in human diploid fibroblasts. In addition, when mink lung epithelial cells were exposed to compound 4 first, followed by treatment with several standard cytotoxic chemotherapy agents, significant inhibition of cytotoxicity was observed, as expected when a CDK inhibitor is used first in the sequence.

Compound 4 has been formulated for topical application, and has recently been shown to confer protection from chemotherapy-induced alopecia in rodent models. In one set of experiments, newborn rats received chemotherapy with or without topical compound 4. While control animals lost all of their hair, -30-70% of those treated with compound 4 had at least partial protection at the application site. These data suggest that CDK inhibition may not only be useful as antitumor therapy, but may also be useful in the protection of normal cells.[136]

Table 1. Families of Small Molecule Inhibitors of Cyclin-dependent kinases

Family	Agent	IC50 (μM) against cdc2 (CDK1)	Relative specificity for CDKs	CDK4 inhibition	Ref.
Purine Derivatives	Dimethylaminopurine	120	-	-	83, 84
	Isopentyladenine	55	-	-	85
	Olomoucine	7	+	-	148
	Roscovitine	0.45	+	-	5, 88
	CVT-313	4.2	+	-	90
	Purvalanol Derivatives	0.004	+	-	31
Flavopiridols	Flavopiridol	0.4	+	+	10
	Deschloroflavopiridol				12
Staurosporines	Staurosporine	0.004	-	-	85
	UCN-01	0.03	-	+	104
—	Butyrolactone I	0.6	+	-	149
—	9-hydroellipticine	1	ND	ND	150
Polysulfates	Suramin	4	-	ND	151
—	Toyocamycin	0.88	+	ND	152
Paullones	Kenpaullone	0.4	+	-	79
	10-bromopaullone	1.3	+	ND	79
Indirubins (Active constituent of a Chinese herbal medicine)	Indirubin	10	+	+	153
	Indirubin-3′-monoxime	0.18	+	+	153

Chemical CDK inhibitors can be divided into nine families. However, not all are specific for CDKs. Staurosporine, UCN-01, suramin, 6-methylaminopurine, and isopentenyladenine are relatively non-specific protein kinase inhibitors. In contrast, olomoucine, roscovitine, CVT-313, purvalanol derivatives, flavopiridol, butryolactone I, paullones and indirubins are more selective for CDKs. Buryrolactone I, olomoucine, roscovitine, CVT-313, purvalanol and the paullones are relatively selective for CDK1 (cdc2), CDK2 and CDK5, but are relatively inactive against CDK4 and CDK6. Flavopiridol, staurosporine, UCN-01 and the indirubins can inhibit CDK4; flavopiridol has also been shown to inhibit CDK6, CDK7 and P-TEFb (containing CDK9). ND- not determined.

E7070

Another CDK2 inhibitor under investigation in Europe and Japan is E7070, a novel chloroindolyl-sulphonamide, which demonstrated a cytotoxicity profile suggesting a unique mechanism of action using the NCI COMPARE program.[137] The primary mechanism of this drug may be indirect inhibition of CDK2 activity, as it demonstrates affects on CDK2 phosphorylation as well as on cyclin E transcription.[138] Xenograft and orthotopic transplantation studies have indicated suppression of tumor growth and reduction of tumor volume in both colon and lung cancer models.[139,140] Recently, the combined results of four phase I studies utilizing different schedules were reported; in all schedules, dose-limiting toxicities were hematologic. Partial and minor responses among 127 total patients have been observed in

patients with breast, endometrial, renal and ovarian carcinoma.[141] The drug displays a half-life ranging 10-31 hours, with a large volume of distribution.[142] Initiation of phase II studies is anticipated. In human tumor xenograft models, E7070 appears synergistic with camptothecin (CPT-11).[143]

Inhibitors of CDK4

The universal involvement of the Rb-axis in human tumors has also motivated the development of compounds specific for the ATP-binding site of CDK4, with the hope of achieving selectivity for transformed cells. Some concern for this strategy has been raised by the effects of a dominant-negative CDK4 mutant on the cell cycle profile of transformed cells retaining wild-type Rb. Expression of this mutant, which fails to bind ATP, in U2OS osteosarcoma cells, inhibits cyclin D1-CDK4 activity but does not cause G1 arrest.[144] Unlike ectopic expression of $p16^{INK4A}$, it does not cause redistribution of Cip/Kip proteins from CDK4 to CDK2, and hence does not cause concomitant inhibition of CDK2. Nevertheless, several compounds, including diaminothiazoles[145] and [2,3-d] pyridopyrimidines,[146,147] aimed at the ATP binding site of CDK4, have been reported to result in Rb-dependent G1 arrest, and inhibit tumor cell proliferation in vitro at submicromolar concentrations. In clonogenic survival assays, irreversible inhibition of colony formation has been observed. Finally, delayed tumor growth and time to progression in human colon tumor xenografts has been observed in vivo. Currently, these compounds are under investigation for their ability to induce differentiation or apoptosis, or produce cytotoxic synergy in a sequence-dependent fashion with standard chemotherapy agents.

Conclusion

The universal deregulation of CDKs in human cancer makes these enzymes extremely attractive targets for antineoplastic drug development. The first two CDK modulators to reach clinical trial, flavopiridol and UCN-01, may exert their effects on a number of biologic pathways. Data thus far indicate acceptable toxicity profiles, and some patients have achieved impressive disease stabilization. Nonetheless, the optimal schedules for administration of these drugs remain to be determined. Although plasma concentrations achieved appear sufficient for CDK modulation, confirmation of the intended targets has not yet been demonstrated in tumor biopsies from treated patients.

As novel CDK inhibitors are developed, with improved potency and selectivity, it will be critical to determine whether they induce cytotoxicity, or whether they are primarily cytostatic. These agents have the potential for significant synergism with standard chemotherapy drugs, where they may ultimately find their place in the anticancer armamentarium. Such combinations may be very sequence-dependent; in most cases maximal effects can be expected if CDK inhibition follows exposure to DNA or microtubule damage.

Finally, it will be important to continually evaluate the selectivity of CDK inhibition for transformed cell types. In this regard, several strategies of CDK modulation, including increasing E2F-1 activity during S phase via CDK2 inhibition, or abrogating G2 checkpoint control via CDK1 (cdc2) activation, have demonstrated selectivity for transformed cells in vitro. In addition, appropriate use of CDK inhibition may confer protection on normal cells. Thus, small molecule CDK inhibitors have great promise for increasing both the efficacy and safety of current cancer treatment.

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CHAPTER 14

Cell Cycle Molecular Targets and Drug Discovery

John K. Buolamwini

Abstract

There have emerged, within the aberrant cell cycle regulatory pathways frequently encountered in cancer cells, several potential targets for novel anticancer drug discovery.Cyclin-dependent kinases (CDKs) and their regulatory units, cyclins, play a central role in cell cycle progression, and several have been shown to be viable anticancer targets. The inhibition of kinase catalytic activity has been successfully achieved with small molecules that have advanced into clinical trials for cancer therapy. As the molecular players in the pathways and cascades involved in progression through the cell cycle are uncovered, more potential anticancer molecular targets are emerging including critical oncogenic kinases and regulatory proteins identified in the progression through mitosis. These include aurora kinases, polo-like kinases, and the anti-apoptotic protein survivin.

Introduction

The cell cycle comprises a complex set of sequential, well coordinated specific events that result in cell division,[1-3] and is therefore central to proliferative diseases such as cancer. Although the final stages of the cell cycle, mitosis, has been the target of conventional chemotherapy with drugs like the vinca alkaloids and taxanes, this has been largely nonspecific and cytotoxic. Recent advances in the molecular characterization of cell cycle regulatory pathways have uncovered component oncogenic and tumor suppressor genes that are mutated and/or aberrantly expressed in many human cancers and show potential as targets for novel cancer therapies. This Chapter focuses on the emerging and potential molecular targets in cell cycle regulatory pathways and their exploitation for small molecule drug design and discovery.

Cell cycle progression is generally divided into four phases: G1, S, G2 and M2 phases as depicted in Figure 1. The progression of the cell division cycle depends on the catalytic activity of cyclin-dependent kinases, a class of serine threonine kinases that require binding to regulatory subunits known as cyclins for their activation. It is these cyclin-CDK complexes that constitute the central players ("work horses") that drive cell division cycle. Whereas the cellular levels of CDKs remain relatively constant throughout the cell cycle, the levels of cyclins fluctuate in a periodic and sequential manner, with particular cyclins expressed at specific stages of the cell cycle. Progress has been made in exploiting the new cell cycle molecular targets, primarily cyclin-dependent kinases for cancer chemotherapy. Clinical trials are on-going with two small molecule drug candidates in this regard. The control of cell cycle progression is achieved primarily by a sequential expression and degradation of cyclins, which bind to specific cyclin-dependent kinases (CDKs) or groups thereof. CDK-cyclin Binding interactions that drive cell cycle progression are as follows: CDK1 (or CDC2) binds cyclins A and B to modulate G2-M

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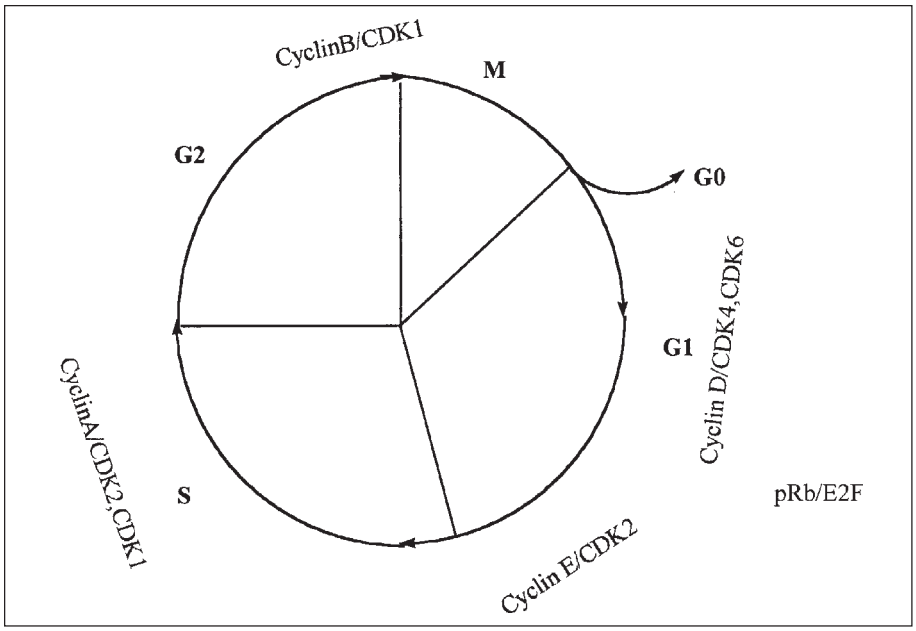

Fig. 1. A simplified representation of the phases and molecular players in cell cycle progression.

transition, CDK2 binds to cyclins A, D, and E to drive the G1-S transition and S phase, CDK4 and CDK6 bind cyclin D for progression through G1, whereas CDK7 binds cyclin H, interestingly, for the activation of other cyclin-CDK complexes.[4,5] Some CDKs may bind non-cyclin counterparts, such as the binding of CDK5 to p35, a protein that shares no sequence homology to the cyclins. CDK7 has been found to be a component of the basal transcription factor TFIIH as well. Interestingly, it has been shown that cyclin D binds to and activates the estrogen receptor. Other non-cell cycle associations of CDKs and cyclins are the following: CDK 8 binds to cyclin C, and CDK9 binds cyclin T in transcription regulation.

Events in Cell Cycle Progression

A simplified outline of the cell cycle progression is depicted in Figure 1. Quiescent cells in (G0) enter the G1 phase to be prepared for the DNA synthesis as a result of mitogenic signals.[6] At this initial stage, D cyclins are synthesized, which bind to and activate CDK4 and CDK6 to begin the phosphorylation of the retinoblastoma gene product, pRb. Subsequently, cyclin E is synthesized to activate CDK2 for further phosphorylation of pRb to move the cell cycle through late G1 and carry it through the G1-S phase transition. Hyperphosphorylated pRb releases transcription factor E2F and associated proteins for the transcription of genes necessary for cell cycle progression. The cell then enters the S phase for DNA synthesis, and cyclin A is expressed to activate its associated CDKs, CDK2 and CDK1 (Cdc2), to take the cell through the S and G2 phases. Following the completion of the G2 phase, cyclin B-CDK1 complexes carry the cell through mitosis (M phase) to complete the division cycle.[7,8]

In addition to the central players in cell cycle progression, there are complex auxiliary networks of kinases, phosphatases and proteases as well as regulatory proteins[5] that feed into the mainstream to promote progression or to halt it (checkpoints). For example CDK activating protein kinases (CAKs) such as cyclin H-CDK7 and protein phosphatases, such as the dual specific Cdc25 protein phosphatase family, serve to activate CDKs and thereby promote cell cycle progression.[9-12] CAKs activate CDKs by phosphorylation of threonine residues (Thr160

or 161 in CDK1 and CDK2, respectively) in the T-loop of CDKs, whereas Cdc25 dual-specific phosphatase family activates CDKs by dephosphorylation of Thr14 and Tyr15[9].

Built-in surveillance mechanisms termed checkpoints exit in cell cycle transition phases to ensure that the integrity of the genome is maintained, as well as its proper apportioning between the two daughter cells. The G1 and G2 checkpoints monitor DNA status and prevent inappropriate entry into the phase or mitosis, respectively.[13,14] The spindle checkpoint ensures correct chromatid alignment on the spindle prior to the beginning of anaphase.[15,16] Cell cycle checkpoint abrogation is an emerging therapeutic strategy for sensitizing cancer cells to DNA damaging agents.[17]

Regulatory Pathways

To keep the wheels of cell division cycle spinning, CDKs must be activated. Several positive and negative CDK regulatory proteins and enzymes have been identified, and the list is sure to continue as much still remains to be known about the mechanisms that control the cell cycle. Some of these regulatory mechanisms will be highlighted and potential cancer molecular targets pointed out. Figure 2 shows a simple representation of the fate that may befall cyclin-CDK complexes.[7,18,19] Following the binding of an appropriate cyclin to its target CDK(s), the complex is mostly activated through phosphorylation by a CDK activating kinase such as the cyclin H-CDK7 complex.[9] Two families of cell cycle regulatory proteins have been found to exert their effects primarily by binding to and inactivating cyclin dependent kinases in the G1 phase of the cell cycle,[20] and have therefore been termed cyclin-dependent kinase inhibitors (CKIs). CKIs are classified into the Cip/Kip and Ink4 families.

The Cip/Kip family was the first to be recognized and currently consists of three proteins, p21Cip/WAF1 (the first CKI,[21-24] p27Kip1[25] and p57Kip2.[21] In vivo, they act primarily on CDK2 complexes.[20] It has been shown, however, that CDK4 complexes sequester p27Kip1. CKI $p21^{WAF1/CIP1}$ is induced by the p53 tumor suppressor gene to cause G1 arrest upon DNA damage.[27,28]

Four proteins have been identified in the Ink4 family, namely p16Ink4a,[29] p15Ink4b, p18Ink4c and p19Ink4d.[20] They are basically inhibitors of cyclin D-CDK complexes and are expressed in a cell-type specific manner. Ink4a has been classified as a tumor suppressor gene[29,30] and has been found to be frequently mutated in melanomas.[31] X-ray crystallographic studies have indicated that CKIs such as $p21^{WAF1/CIP1}$ inhibit CDKs by binding to the cyclin-CDK complexes and using their 3_{10} helix to block the ATP-binding site.[32] Recent studies show an increasingly more complex interplay among cell cycle regulating proteins than was thought previously, especially the interaction between CDKs and CKIs.[5] Another negative factor in the CDK regulation is phosphorylation at the G2 checkpoint. Phosphorylation of amino acid residues Thr 14 and Tyr 15 on CDK1 by Myt1 and Wee1 kinases[18,33] keeps it inactive to prevent mitosis until earlier cell cycle events are properly completed. This inactivation is reversed by dephosphorylation of Thr 14 and Tyr 15 by the Cdc25 protein phosphatase family to generate an active CDK.[7] The activity of Cdc25 is kept in check through phosphorylation at its Ser 216 residue by Ch1 kinase. This is thought to be the reason that G2 checkpoint abrogation is achieved by inhibiting Chk1 kinase with the staurosporine derivative UCN-01 (1).[5,27] The phosphorylation of Cdc25 allows binding to the 14-3-3 proteins, which sequester Cdc25 and keep it away from cyclinB-CDK1.[34] It has also recently been demonstrated that 14-3-3 sigma, a major p53-induced protein following irradiation, sequesters cyclin B1 and cdc2/CDK1 and prevents them from entering the nucleus. These results may indicate a mechanism for maintaining the G2 checkpoint and preventing mitotic death.[35] Furthermore, it has been shown that polo-like kinases are part of the team that activates Cdc25 phosphatase[36] in a loop leading to cyclin B degradation by the proteasome complex that is also activated by polo-like kinases.[37] Activated G1 cyclin-CDK complexes target the retinoblastoma tumor suppressor gene product, pRb and related "pocket proteins' p103 (pRb2) and p107[2,38-40] which they phosphorylate and cause to dissociate from their complexes with E2F and related transcription factors freeing them to induce the transcription of genes to drive the cell division

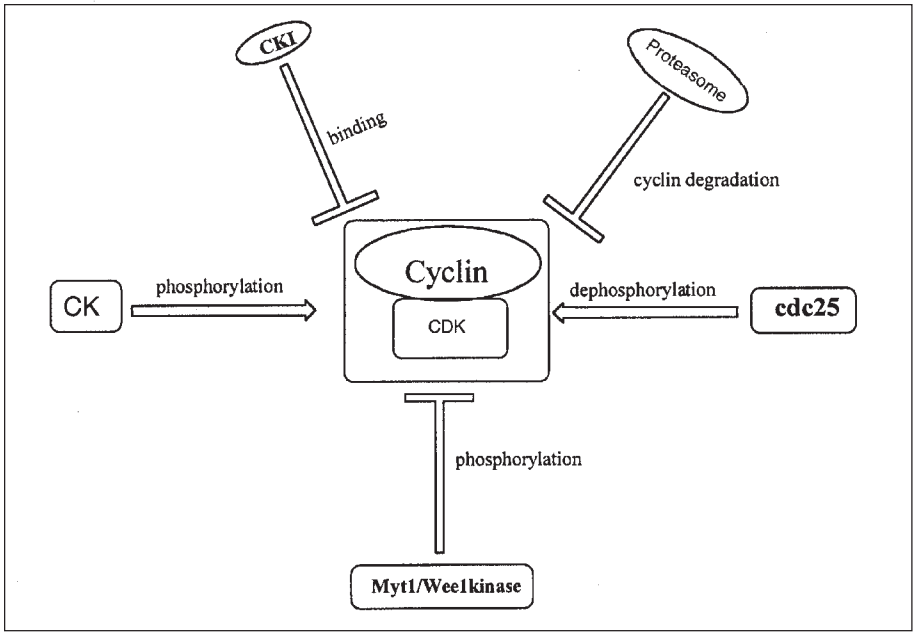

Fig. 2. Regulation of CDK-cyclin complexes. Arrow shows positive and hammer shows negative regulation, respectively.

cycle.[41] It appears that pRb interacts also with histone deacetylases (HDAC1) to repress transcription.[42-44] It has recently been shown that p130 (pRb2), which complexes with E2F-4, may cooperate with p27Kip1 in regulating cell proliferation in a negative feedback loop with cyclin E.[45] In a complex relationship, which is yet to be fully elucidated, p130 (pRb2) and p107, both inhibit CDK2 kinase activity.

Although elucidation of the mechanisms and molecular players of M-phase progression has lagged behind that of earlier events of the cell cycle, it is now progressing rapidly and uncovering potential cancer intervention targets.[15,16,33] At the onset of mitosis, cyclin B is degraded by the proteasome. The mitotic spindle then begins to form with chromosome condensation in prophase. Following this, chromosomes line up at the equatorial plate attached to the mitotic spindle in metaphase. The sister chromatids then begin to migrate to opposite poles of the parent cell in the metaphase to anaphase transition. The anaphase-promoting complex/cyclosome (APC/C) plays a major role in this metaphase to anaphase transition,[33-46] which it does by ubiquitnating mainly the checkpoint protein Pds1, which functions to prevent sister chromatids from separating. Polo-like kinases have been shown to play an extensive role in mitosis, beginning from the onset of M phase, at spindle formation, during the metaphase-anaphase transition (affecting the APC complex) and at late M phase.[36] Mitotic progression from anaphase to telophase involves the disappearance of the mitotic spindle and the formation of nucleoli and a new nuclear membrane. Cytokinesis ensues after that to divide the parent cell into two daughter cells. The aurora/Ipl1p family of serine/threonine kinases is expressed in a cell-cycle regulated fashion, and is a key promoter of chromosome segregation and cytokinesis.[47] Survivin, a newly identified inhibitor of apoptosis (IAP) protein,[48,49] is expressed in the G2/M phase in a cell cycle-regulated fashion and associates with microtubules of the mitotic spindle at the beginning of mitosis.[50] Survivin has also been shown to control the mitotic spindle checkpoint,[51] and was recently shown to have a similar cell-cycle localization and gene knockout phenotype with the inner centromere protein INCENP.[52]

Oncogenic Cell Cycle Targets

Table 1 gives a summary of cell cycle target aberrations in human tumors. Oncogenic expression and/or mutation of cyclins and CDKs, expecially those involved in the G1 phase, have been found frequently in many human cancers.[1-3,7,53,54] Common cancers in which oncogenic expression of cyclins and CDKs has been demonstrated include B- and T-cell lymphomas, esophageal cancer, breast cancer, bladder cancer, small cell lung cancer, prostrate cancer, colon cancer, glioblastoma, neuroblastoma, leukemias, retinoblastoma, melanoma and adenomatous polyposis.[25,55-57] The D and E families[25,58] of G1 cyclins are the ones most frequently associated with oncogenic expression.[40,57] The overexpression of cyclin D1 causes cell type dependent transformation,[58-60] sometimes in cooperation with the myc oncogene.[61,62] Overexpression or gene amplification of D cyclins is particularly prevalent in breast cancer, with cyclin D1 overexpressed or amplified in more than 50% of breast cancers.[41,63-65] Cyclin D1 overexpression has also been suggested to play a role in drug resistance to antifolates in a human fibrosarcoma cell line.[66] Amplification of cyclin E is also rampant in breast cancers[67,68] and shows a strong correlation with tumor aggressiveness.[57] Cyclin E2 has recently been identified and shown to be overexpressed in human cancers including breast, lung and cervical cancers, and has been proposed to be a potential cancer-specific molecular target.[69] Oncogenic amplification and overexpression of CDKs have also been reported in gliomas and soft tissue sarcomas,[70] as well as other cancers.[31,55,56] The E2F transcription factor gene family has also been shown to be oncogenic.[71]

The oncogenic potential of Cdc25 proteins has also been demonstrated. Overexpression of Cdc25B has been shown in 32% of human primary breast cancers.[72] It is interesting that specific Cdc25B protein phosphatase inhibitors discovered recently were shown to inhibit tumor growth.[73] Chk1 kinase, an important mediator of cell cycle arrest in G2 following DNA damage, is becoming an important cancer therapeutic target.[17,74] A 1.7 A resolution X-ray crystal structure human Chk1 kinase domain in complex with an ATP analog has recently been published, providing a template for structure-based drug design.[75]

New potential oncogenic targets are emerging from the end stage of the cell cycle, i.e., mitosis. Disruption of survivin-microtubule interactions results in loss of survivin's anti-apoptosis function and increased caspase-3 activity. The overexpression of survivin in cancer is thought to overcome apoptosis and favor aberrant progression of transformed cells through mitosis.[51] In one study, survivin messenger RNA was shown to be overexpressed in 85% of non-small cell lung tumors.[76] Polo-like kinases are also mitotic oncogenic targets found to be overexpressed in non-small cell lung cancer. Aurora 2 kinase amplification has been shown in more that 50% of colorectal cancers[77] and in 12% of primary breast cancer samples.[78] The overexpression of polo-like kinase has been demonstrated in non-small cell lung cancer.[79]

Cell Cycle Molecular Target-Based Cancer Drug Discovery

Oncogenic G1 cyclin interactions have not been successfully disrupted, but down regulation of these cyclins by antisense oligonucleotides to achieve an anticancer effect has been demonstrated.[80,81] Inhibition of the catalytic activity of CDKs has been a successful strategy for the discovery of novel anticancer agents, some of which are under clinical development[82,83] such as flavopiridol (2). This strategy has produced a large number of potent small molecule CDK inhibitors,[4,7,55,56,82,84-92] which will be highlighted in this section.

The CDK inhibitors discovered to date are all essentially ATP-binding competitors at the kinase domain. The ATP competitive activity was an initial concern for selectivity, since there is a plethora of kinases in the cell all using ATP. Surprisingly, however, it appears this fear was unwarranted as excellent selectivity has been demonstrated not only in discrimination between serine/threonine kinases on the one hand and tyrosine kinases on the other, but also among very closely related serine/threonine kinases. This selectivity has been attributed to interaction of inhibitors with regions in the vicinity of the binding site that do not interact with bound ATP, and where the amino acid residues are not conserved among the CDKs.[85] This finding

Table 1. Oncogenic cell cycle molecular targets

Oncogenic Target	Oncogenic Changes	Major Tumors
Cyclin D1	Gene amplification	
and/or overexpression	breast cancer (40-80%)	
	Familial Polyposis (~70%)	
B-Cell Lymphoma (50%)		
NSC Lung Cancer (~50%)		
Head and neck cancer (~35%)		
Esophageal cancer (25-50%)		
Bladder cancer (~25%)		
Cyclin K (cyclin D-like)	KSHV Infection	Kaposi's sarcoma
Cyclin E	Amplification	Breast cancer (30-80%)
Prostate cancer (~70%)		
Ovarian cancer (~18%)		
Gastric cancer		
Cervical cancer		
Cyclin E2	Overexpression	Breast cancer
Small cell lung cancer		
	Cervical cancer	
Cyclin B1	Overexpression	Colorectal Cancer (~90%)
Cyclin A	Stabilization	Hepatocellular carcinomas
CDK2	Overexpression	Colorectal cancer
CDK4	Amplification	Sarcomas and gliomas
	Point Mutation	Familial melanoma
Cdc25	Overexpression	Breast cancer (32%)
Polo-like kinases	Overexpression	Non-small cell lung cancer
Aurora 2	Amplification	Colorectal cancer (>50%)
	Breast cancer (12%)	
Survivin	Overexpression	Non-small cell lung (85%)

has advanced the structure-based drug design efforts on CDK inhibitors. The structure-activity relationships of CDK inhibitors have been reviewed recently.[91] The many small molecule CDK inhibitors discovered to date represent a chemically group of compounds including the following major ones (structures shown in Fig. 3):

i) flavopiridol (2), which represents flavone class is the most extensively studied selective CDK inhibitor, and now in clinical trials,
ii) purine derivatives introduced with olomoucine (3) and for which extensive structure-activity relationship studies have been conducted using state-of-the art drug discovery technologies including X-ray crystal structure-based computer-aided drug design, combinatorial chemistry and high throughput screening, as well as functional genomics,[93]
iii) 7-hydroxy staurosporine derivative UCN-01 (compound 1), which also inhibits G2 check point kinase Chk1, as well as protein kinase C, represents the bisindolylmaleimide class and has entered clinical trials,
iv) the dihydroindolo[3,2-d][1]benzazepin-6(5H)-ones also known as paullones, represented by kenpaullone, 4, are a more recent addition,[83,94,95] as well as

v) the indigoids derived from a Chinese antileukemia herbs,[96] and represented by indirubin-3′-monoxime (compound 5), and
vi) a new diaminothiazoles series represented by compound 6.[97,98]

The staurosporine class is the least selective, inhibiting protein kinase C and Chk1 kinase activity as well.[17,85] Flavopiridol is a relatively general CDK inhibitor that is active against CDK1, CDK2 and CDK4, whereas the purine analogs olomoucine and congeners inhibit CDK1 and CDK2 but are much less potent against CDK4.[99] The paullone class of CDK inhibitors, like flavopiridol, is selective towards CDK1 and CDK2, as well as CDK5.[83] Additionally, kenpaullone is relatively inactive against the protein kinase C (PKC) family, compared to flavopiridol.[95] The indigoid series have a more broad selectivity, inhibiting CDK1, CDK2, CDK4, and CDK5.[96] The most active compound in that report, indirubin-5-sulfonic acid exhibited IC_{50} values of 0.055, 0.035, 0.15 and 0.3 μM against CDK1/cyclin B, CDK2/cyclin A, CDK2/cyclin E, CDK4/cyclin D1 and CDK5-p35, respectively.

Cancer Drug Development of Small Molecule CDK Inhibitors

In in vitro studies flavopiridol inhibits CDK2 and CDK4 and causes cell cycle arrest in G1 phase that is independent of p53 or Rb states,[100] as well as G2 arrest.[101] Flavopiridol has been shown to inhibit human tumor xenografts of lung, colon, stomach, breast and brain cancers. Flavopiridol has been in clinical development sponsored by the National Cancer Institute for sometime now.[101] Dose-limiting toxicities of flavopiridol did not include leukopenia, anemia or thrombocytopenia,[102] but instead have included diarrhea, hypotension and a pro-inflammatory syndrome.[82] Clinical response has been observed in some solid tumors and lymphomas.[82] UCN-01 causes cell growth arrest in G1 by a mechanism involving dephosphorylation of the pRb and CDK2, as well as induction of p21WAF1/CIP1/SDI1.[103] It has been shown to potently inhibit the phosphorylation of Cdc25C by Chk1 in vitro, and to abrogate the G2 checkpoint and sensitize cancer cells to DNA damaging agents.[17] It is also currently undergoing clinical trials.[82,101] Dose-limiting toxicities include hyperglycemia, lactic acidosis, nausea and vomiting, as well as pulmonary toxicity. Among the purine derivatives, olomoucine inhibits the proliferation of human breast, gastric and pancreatic cancers, and lymphoma.[105] Lymphoma cells were shown to be arrested in both G1 and G2 phases by this agent related to its inhibition of cyclin E/CDK2 and cyclin B/CDK1 complexes.[104] Other cellular targets of the purine class of CDK inhibitors are nucleoside transporters, which they inhibit at micromolar concentrations.[105-107] Olomoucine and roscovitine are said to be possible clinical trials candidates.[108] The paulones, which in addition to inhibiting CDKs have also recently been shown to inhibit glycogen synthase kinase-3 beta,[109] cause cell cycle arrest in the G1 phase and have shown anticancer activity in the NCI tumor cell line screen.[94,95] Induribine-3′-monoxime, one of the recently identified indigoid compounds, has been shown to inhibit the proliferation of cancer cells including MCF-7 human breast cancer and human K562 leukemia cells causing cell cycle arrest in at G2/M.[96] Much less is still known about the diaminothiazoles, but they show anti-tumor activity at submicromolar concentrations and cause cell cylce arrest in both in G1 and G2 phases, which is associated with the phosphorylation status of pRb.[110]

Other Targets

The recent discovery of Cdc25 specific small molecule inhibitors[73] opens another window of opportunity for exploiting cell cycle targets in cancer therapy. Fy21-aa09 (7), one of the Cdc25B inhibitors in the above series, exhibited a Ki of 7.6μM and caused growth arrest of tsFT210 cells at G2/M phase of the cell cycle. Chk1 kinase inhibition is also an interesting area to enhance the chemosensitivity of tumors by abrogating the G2 check point. Proteasome inhibitors have been reported that may be useful for inhibiting CKI or cyclin degradation to halt cell cycle progression in cancer.[111] Other potentially important points of intervention in cell cycle pathways yet to be exploited are CAK or Cdc25 activation of cyclin-CDK complexes

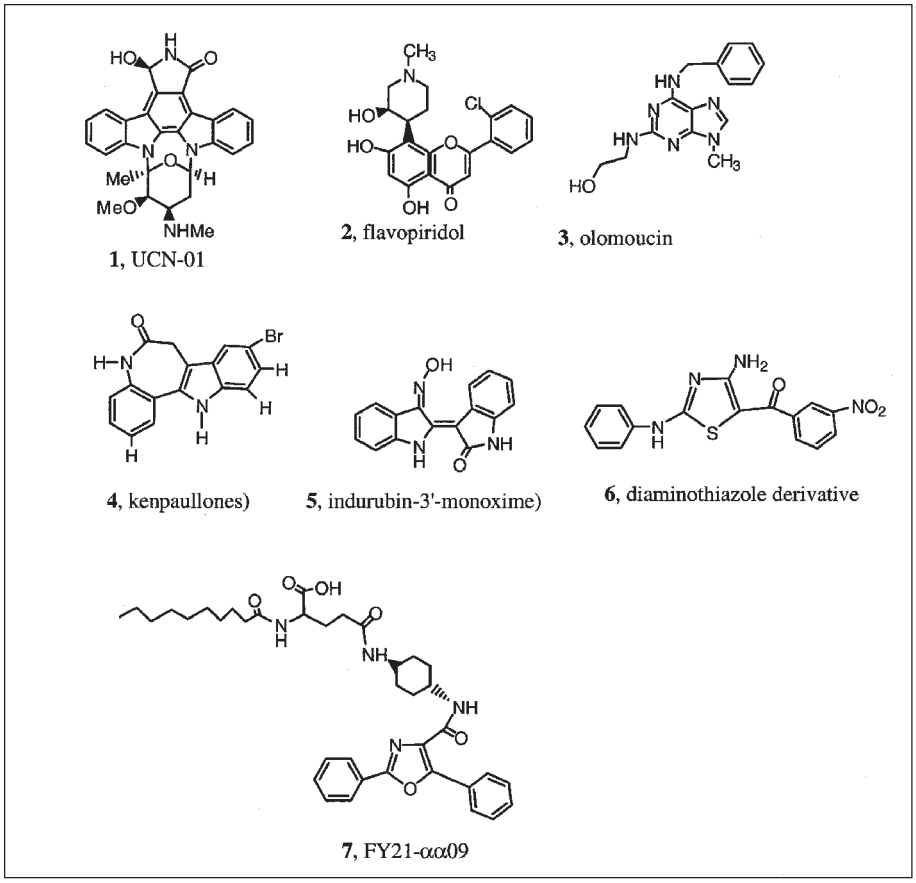

Fig. 3. Chemical structures of representative cell cycle target inhibitors

and the catalytic activity of proteins such as aurora 2 and polo-like kinases.[7,33] Polo kinases contain a distinct region of homology in the C-terminal non-catalytic domain, termed the polo-box, which is thought to be critical for their oncogenic properties. The development of chemical compounds that will interact with and disrupt the polo-box of polo-like kinases has also been envisioned as a potential strategy to for cancer therapy.[112,113]

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