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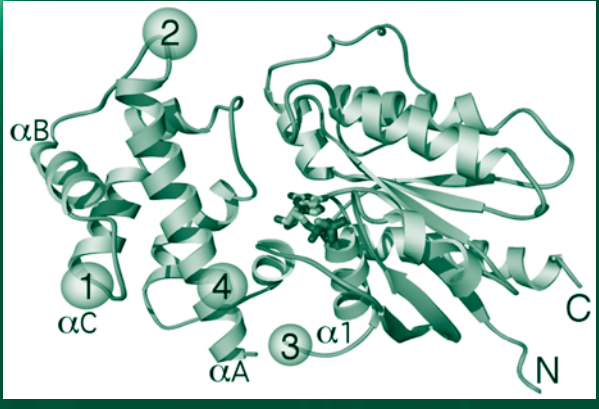

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Purification of Recombinant G Protein α Subunits from *Escherichia coli*

Wendy K. Greentree and Maurine E. Linder

Summary

The purification of recombinant G protein a subunits expressed in *Escherichia coli* (*E. coli*) is a convenient and inexpensive method to obtain homogeneous preparations of protein for biochemical and biophysical analyses. Wild-type and mutant forms of Gα are easily produced for analysis of their intrinsic biochemical properties, as well as for reconstitution with receptors, effectors, regulators, and G protein βγ subunits. Methods are described for the expression of $G_i\alpha$ and $G_s\alpha$ proteins in *E. coli*. Protocols are provided for the purification of untagged G protein a subunits using conventional chromatography and histidine (His)-tagged subunits using metal chelate chromatography. Modification of Gα with myristate can be recapitulated in *E. coli* by expressing *N*-myristoyltransferase (NMT) with its G protein substrate. Protocols for the production and purification of myristoylated Gα are presented.

Key Words: G protein; α subunit; signal transduction; protein purification; affinity chromatography; GTPase; membrane protein; myristoylation; *N*-myristoyltransferase.

1. Introduction

Heterotrimeric G proteins are localized at the inner leaflet of the plasma membrane where they convey signals from cell-surface receptors to intracellular effectors ***(1)***. G proteins function as dimers of an α subunit and a tightly associated βγ complex. The α subunit harbors the guanine nucleotide-binding site. In the inactive guanosine diphosphate (GDP)-bound state, Gα is associated with the βγ complex. Exchange of GDP for guanosine triphosphate (GTP) on Gα results in a conformational change that causes the subunits to dissociate. Both α-GTP and βγ interact with downstream effectors and regulate their

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activity. The intrinsic GTP hydrolase activity of the α subunit returns the protein to the GDP-bound state, thereby increasing its affinity for Gβγ, and the subunits reassociate.

To date, 17 genes that encode G protein α subunits have been identified and they can be grouped into four subfamilies: G_s, G_i, G_q, and $G_{12/13}$. Significant advances in our understanding of the structure and function of G protein α subunits have been made possible by the availability of purified recombinant proteins produced using bacterial expression systems. However, a significant limitation in using bacteria to prepare purified recombinant Gα is that not all G protein α subunits are amenable to purification after expression in *Escherichia coli* (*E. coli*). The criterion for successful purification from bacteria is the presence of Gα in the soluble fraction of cell lysates. Efforts to solubilize and/or refold Gα associated with the particulate fraction have not been successful. Wild-type and mutant forms of $G_s\alpha$, $G_i\alpha_1$, $G_i\alpha_2$, $G_i\alpha_3$, and $G_o\alpha$ are soluble and easily purified in active form after expression in *E. coli*. Small quantities of recombinant $G_z\alpha$ have been purified from *E. coli* ***(2)***, but expression in insect cells using recombinant $G_z\alpha$ Baculovirus is the method currently used by most investigators. $G_t\alpha$ is expressed in *E. coli*, but the protein is insoluble. Hamm and coworkers, noting that $G_t\alpha$ is 68% identical to $G_i\alpha_1$ at the amino acid level, constructed chimeric molecules of $G_t\alpha$ and $G_i\alpha_1$ ***(3)***. Regions of $G_t\alpha$ were systematically replaced with the corresponding $G_i\alpha_1$ region in an effort to create a $G_t\alpha$-like molecule that would fold properly in *E. coli*. A chimeric protein containing only 11 amino acids different from native $G_t\alpha$ functioned essentially the same as native $G_t\alpha$ and could be purified in large quantities ***(4)***. Members of the G_q and G_{12} families of α subunits have not been successfully purified in active form after expression in *E. coli*, but they can be produced in insect cells using recombinant Baculovirus ***(5–7)***.

Initial protocols for the purification of G protein α subunits utilized conventional chromatography. However, the use of affinity tags on Gα to simplify purification has been adopted. This chapter describes how to purify Gα subunits using an affinity tag that consists of six consecutive histidine residues (6-His-Gα). This tag results in high-affinity binding of the protein to a resin-containing chelated Ni^{2+}. Most of the contaminating proteins in the *E. coli* extract either fail to bind or bind with low affinity and can be washed off the matrix with solutions of increasing ionic strength. 6-His-Gα is eluted with a buffered solution of imidazole, which competes for Ni^{2+}-binding sites on the resin. This method provides a simple and rapid method for purification of Gα in an active form ***(8)***.

Addition of hexahistidine tags to proteins is typically at the N- or C-terminus. 6-His-$G_i\alpha_1$ or 6-His-$G_s\alpha$-tagged at the N-terminus (Met-Ala-6-His-Ala-$G_s\alpha$ or -$G_i\alpha_1$ sequence) behaves similarly to the untagged recombinant protein in assays

of guanine nucleotide binding and hydrolysis, effector interactions, and receptor interactions (Linder, M. E., unpublished results). However, addition of the N-terminal tag replaces the consensus sequence for NMT and is therefore incompatible with the coexpression system described below for producing myristoylated recombinant Gα. Expression of a myristoylated His-tagged $G_i\alpha_1$ has been achieved by insertion of a hexahistidine tag at an internal site (position 121, where the yeast α subunit Gpa1p has a long insert when compared to the mammalian protein) ***(5)***. It should also be possible to produce a C-terminal His-tagged protein that is myristoylated. $G_s\alpha$ has been tagged at the C-terminus ***(9)*** and purified in large quantities for structural analysis ***(10)***. Because the C-terminus is an important site for interaction of Gα with receptor, an N-terminal or internal tag may be a better choice when the recombinant protein is used to study interactions between receptor and G protein ***(11)***. Hexahistidine tags have also been inserted into $G_s\alpha$ in exon 3 where splice variants are produced ***(9)***. Although the internally tagged 6-His-$G_i\alpha_1$ and 6-His-$G_s\alpha$ proteins are active in many assays of G protein activity, detailed side-by-side comparisons of their activity in comparison to untagged proteins have not been published.

A typical problem with eukaryotic proteins expressed in bacteria is the lack of posttranslational modifications. G protein subunits are fatty acylated with amide-linked myristate, thioester-linked palmitate, or both (reviewed in **ref. *12***). Members of the $G_i\alpha$ family ($G_o\alpha$, $G_i\alpha$, $G_z\alpha$, $G_t\alpha$, and gustducin) are cotranslationally modified with myristate at Gly2, following cleavage of the initiator methionine. The process of *N*-myristoylation of $G_o\alpha$ and $G_i\alpha$ can be recapitulated in *E. coli* by coexpressing NMT ***(13)***. Stoichiometrically myristoylated $G_o\alpha$, $G_i\alpha_1$, $G_i\alpha_2$, and $G_i\alpha_3$ have been purified from *E. coli* using the coexpression system ***(14)***. Unmodified $G_o\alpha$ and $G_i\alpha$ produced in *E. coli* have reduced affinity for βγ subunits ***(15)*** and adenylyl cyclase ***(16)***. In contrast, the recombinant myristoylated proteins are indistinguishable from $G_o\alpha$ and $G_i\alpha$ purified from tissues with respect to their subunit ***(15)*** or effector interactions ***(16)***.

To produce *N*-myristoylated Gα subunits in *E. coli*, the cDNAs for NMT and Gα are cloned into separate plasmids, each under the regulation of a promoter inducible with isopropyl-1-β-D-galactopyranoside (IPTG). The plasmids carry either kanamycin or ampicillin resistance markers and different (but compatible) origins of replication. The *Saccharomyces cerevisiae NMT1* gene is subcloned into a plasmid designated pBB131 ***(17)***. The promoter for *NMT* (P_{tac}) is fused to a translational "enhancer" derived from the gene 10 leader region of bacteriophage T7 ***(18)***. The cDNA for Gα is expressed using pQE-60. Both plasmids are transformed into bacterial strain JM109. When protein expression is induced by adding IPTG, NMT is synthesized and folds into an active enzyme that is able to *N*-myristoylate $G_i\alpha$ or $G_o\alpha$ cotranslationally. This system is very efficient, approx 90% of the soluble pool of Gα is *N*-myristoylated ***(14)***.

This chapter describes the protocols for the purification of *N*-myristoylated G protein α subunits using conventional chromatography, which can be used for $G_i\alpha$, $G_o\alpha$, or $G_s\alpha$ that is expressed in its native form (i.e., lacking any tags for affinity chromatography). Purification of hexahistidine-tagged G protein α subunits is also described.

2. Materials

2.1. Bacterial Culture and Preparation of Cell Extracts for Myristoylated Gα

2.1.1. Bacterial Strains and Plasmids (see ***Notes 1*** *and* ***2****)*

1. Plasmid pQE6 containing $G_o\alpha$, $G_i\alpha_1$, $G_i\alpha_2$, or $G_i\alpha_3$ ***(14)***.
2. Plasmid pBB131 ***(17)***.
3. JM109 bacteria (New England Biolabs E4107S).

2.1.2. Culture Media

1. Stock solutions and powders.
   a. Tryptone (Difco, cat. no. DF0123-17-3): store at room temperature.
   b. Yeast extract (Difco, cat. no. DF0127-17-9): store at room temperature.
   c. Sodium chloride.
   d. Ampicillin (Fisher, cat. no. BP1760-5): store powder at 4°C; 50 mg/mL stock made in water, store at –20°C.
   e. Kanamycin (Fisher, cat. no. BP906-5): store powder at room temperature; 50 mg/mL stock made in water, store at –20°C.
   f. IPTG (Sigma, cat. no. I-5502): 1 *M* stock made in water, store at –20°C.
   g. Chloramphenicol (Sigma, cat. no. C-7795): store powder at 4°C; 20 mg/mL stock made in ethanol, store at 4°C.
2. Luria-Bertani (LB) plates: 1% (w/v) tryptone, 0.5% (w/v) yeast extract, 1% (w/v) NaCl, 1.5% (w/v) Bacto Agar (Difco, cat. no. DF0140-01-0).
3. Enriched medium: 2% (w/v) tryptone, 1% (w/v) yeast extract, 0.5% (w/v) NaCl, 0.2% (w/v) glycerol, 50 m*M* potassium KH_2PO_4, pH 7.2 (*see* **Note 3**).

2.1.3. Cell Lysis

1. Dithiothreitol (DTT, Amresco, cat. no. 0281): store powder dessicated at –20°C; 1 *M* stock made in water, store in aliquots at –20°C.
2. Phenylmethylsulfonyl fluoride (PMSF, Sigma, cat. no. P-7626): store powder at room temperature; 100 m*M* stock made in ethanol, store at –20°C (*see* **Note 4**).
3. Lysozyme (Sigma, cat. no. L-6876): store powder at –20°C; make fresh 10 mg/mL stock in water.
4. DNAse I (Sigma, cat. no. D-5025): store powder at –20°C.
5. 1 *M* Magnesium sulfate ($MgSO_4$).
6. TEDP: 50 m*M* Tris-HCl, pH 8.0, 1 m*M* EDTA, 1 m*M* DTT, 0.1 m*M* PMSF (*see* **Note 4**).

2.2. Bacterial Culture and Preparation of Cell Extracts for His-Tagged Gα

2.2.1. Bacterial Strains and Plasmids

1. 6-His-$G_i\alpha_1$ in pQE60 ***(8)*** (*see* **Note 5**).
2. Plasmid pREP4 available in M15 bacteria (Qiagen, cat. no. 34210).
3. BL21(DE3) bacteria (Novagen, cat. no. 69387-3).
4. Culture medium (*see* **Subheading 2.1.2.**).

2.2.2. Cell Lysis

1. TBP: 50 m*M* Tris-HCl, pH 8.0, 10 m*M* β-mercaptoethanol (Sigma, cat. no. M-6250) (*see* **Note 6**), 0.1 m*M* PMSF (*see* **Subheading 2.1.3.**).
2. Lysozyme (*see* **Subheading 2.1.3.**).
3. DNAse I (*see* **Subheading 2.1.3.**).

2.3. Purification of Myristoylated Gα Using Conventional Chromatography

2.3.1. Batch Diethylaminoethyl (DEAE) Chromatography

1. DEAE-Sephacel resin (200 mL) (Amersham Biosciences, cat. no. 17-0709-01) store at 4°C .
2. TEDP (*see* **Subheading 2.1.3.**).
3. 300 m*M* NaCl TEDP.
4. 1 *M* NaCl TEDP.
5. Buchner funnel: capacity for 200 mL DEAE resin.
6. Whatman 4 filter paper.

2.3.2. Phenyl Sepharose Chromatography

1. 100 mL Resin phenyl Sepharose (PS) (Amersham Biosciences, cat. no. 17-0973-05): store at 4°C.
2. C26/40 column (Amersham Biosciences, cat. no. 19-5201-01).
3. 3.6 *M* Ammonium sulfate $(NH_4)_2SO_4$.
4. 25 m*M* GDP (Sigma, cat. no. G-7127): store powder at –20°C; 25 m*M* stock in water; pH should be 6.0–8.0, store in aliquots at –20°C.
5. PS equilibration buffer: 50 m*M* Tris-HCl, pH 8.0, 1 m*M* EDTA, 1 m*M* DTT, 1.2 *M* ammonium sulfate, 25 μ*M* GDP.
6. PS elution buffer: 50 m*M* Tris-HCl, pH 8.0, 1 m*M* EDTA, 1 m*M* DTT, 35% glycerol (*see* **Note 7**), 25 μ*M* GDP.
7. PS bump buffer: 50 m*M* Tris-HCl, pH 8.0, 1 m*M* EDTA, 1 m*M* DTT, 25 μ*M* GDP.
8. 400 mL vol Amicon stirred cell (Fisher, cat. no. 5124).
9. Filter: 30,000 molecular weight (MW) cutoff, 76 mm (Amicon, cat. no. PM-30, Fisher, cat. no. 13242 or PBTK-30,000 high flow polyether sulfone (PES) filter, Fisher, cat. no. PBTK-076-10).
10. Desalting buffer: 50 m*M* Tris-HCl, pH 8.0, 1 m*M* EDTA, 1 m*M* DTT.

2.3.3. Q Sepharose Chromatography

1. Q Sepharose (QS) Fast Flow (Amersham Biosciences, cat. no. 17-0510-01): store at 4°C.
2. C26/40 column (Amersham Biosciences, cat. no. 19-5201-01).
3. QS equlibration buffer: 50 m*M* Tris-HCl, pH 8.0, 1 m*M* EDTA, 1 m*M* DTT.
4. QS elution buffer: 50 m*M* Tris-HCl, pH 8.0, 1 m*M* EDTA, 1 m*M* DTT, 250 m*M* NaCl.

2.3.4. Hydroxyapatite Chromatography

1. Biogel Hydroxyapatite (Hap) resin (Bio-Rad, cat. no 130-0420): store powder at room temperature (*see* **Note 8**).
2. 2.5 × 10-cm column (Bio-Rad, cat. no. 737-2512).
3. 1 *M* potassium phosphate buffer (pH 8.0) (*see* **Note 9**).
4. Hap equilibration buffer: 10 m*M* Tris-HCl, pH 8.0, 10 m*M* potassium phosphate buffer, pH 8.0, 1 m*M* DTT.
5. Hap elution buffer: 10 m*M* Tris-HCl, pH 8.0, 300 m*M* potassium phosphate buffer, pH 8.0, 1 m*M* DTT.
6. HED: 50 m*M* NaHEPES, pH 8.0, 1 m*M* EDTA, 1 m*M* DTT.
7. Concentration of Gα pool. 50 mL-vol Amicon stirred cell (Fisher, cat. no. 5122). 44.5-mm Amicon filters (Fisher, cat. no. PBTK-043-10).

2.4. Purification of Hexahistidine-Tagged Gα Using Metal Chelate Chromatography

2.4.1. Ni^{2+} Chromatography

1. 50-mL Ni^{2+} resin (Qiagen, cat. no. 30230).
2. 2.5 × 10-cm column (Bio-Rad, cat. no. 737-2512).

2.4.2. Buffers for Ni Column Chromatography

1. Lysis buffer: 50 m*M* Tris-HCl, pH 8.0, 20 m*M* β-mercaptoethanol (*see* **Note 6**), 0.1 m*M* PMSF (*see* **Note 4**).
2. Column equilibration buffer: 50 m*M* Tris-HCl, pH 8.0, 20 m*M* β-mercaptoethanol (*see* **Note 6**), 0.1 m*M* PMSF (*see* **Note 4**), 100 m*M* NaCl.
3. Wash buffer: 50 m*M* Tris-HCl, pH 8.0, 20 m*M* β-mercaptoethanol (*see* **Note 6**), 0.1 m*M* PMSF (*see* **Note 4**), 500 m*M* NaCl, 10 m*M* imidazole.
4. Elution buffer: 50 m*M* Tris-HCl, pH 8.0, 20 m*M* β-mercaptoethanol (*see* **Note 6**), 150 m*M* imidazole, 10% glycerol (*see* **Note 10**).

2.4.3. Concentration of Gα Pool

1. Amicon stirred cell and filter (*see* **Subheading 2.3.4., step 7**).
2. TEDG: 50 m*M* Tris-HCl, 1 m*M* EDTA, 1 m*M* DTT, 10% glycerol.

2.5. GTPgS-Binding Assay (see *Note 11*)

2.5.1. Stock Solutions

1. 1 *M* Na HEPES, pH 8.0: store at 4°C.
2. 0.1 *M* EDTA, pH 8.0: store at 4°C.
3. 1 *M* DTT: store at –20°C.
4. 4 *M* NaCl: store at 4°C.
5. 1 *M* $MgCl_2$: store at 4°C.
6. 10% Polyoxyethylene-10-lauryl ether (Sigma, cat. no. P9769): prepared as a 10% solution (v/v) and deionized with mixed-bed resin AG501 (Bio-Rad, cat. no. 143-6424); store at 4°C.
7. 10 m*M* GTPγS (Roche, Indianapolis, IN, cat. no. 220-647): store powder dessicated at –20°C, dissolve powder in a solution of 2 m*M* DTT. Store in aliquots at –70°C.
8. [^{35}S]GTPγS 1500 Ci/mmol (DuPont NEN).
9. Filters BA85 (Schleicher and Schuell, Keene, NH, cat. no. 20340).

2.5.2. Working Solutions

1. Dilution buffer: 50 m*M* NaHEPES, pH 8.0, 1 m*M* EDTA, 1 m*M* DTT, 0.1% polyoxyethylene-10-lauryl ether.
2. 100 μ*M* GTPγS stock: dilute 10 m*M* stock 1:100 in water.
3. GTPγS filtration buffer: 20 m*M* Tris-HCl, pH 8.0, 100 m*M* NaCl, 25 m*M* $MgCl_2$.
4. GTPγS binding mix (1.5 μL for 60-tube assay): 75 μL 1 *M* NaHEPES, pH 8.0, 15 μL 0.1 *M* EDTA, 1.5 μL 1 *M* DTT, 15 μL 10% polyoxyethylene-10-lauryl ether, 30 μL 1 *M* $MgCl_2$, 60 μL 100 μ*M* GTPγS, [^{35}S]GTPγS, 1.5×10^7 cpm (specific activity 2500 cpm/pmol), water to make 1.5 mL.

3. Methods

3.1. Bacterial Culture and Preparation of Cell Extracts for Myristoylated Gα (see *Note 12*)

3.1.1. Large-Scale Culture

1. Prepare 10.2 L enriched medium. Dispense 10×1 L in 2-L Erlenmeyer flasks and 150 mL in a 500-mL Erlenmeyer flask. Dispense the remaining medium in a 100-mL bottle for small-scale cultures. Autoclave.
2. Inoculate a culture from a frozen glycerol stock (*see* **Note 2**). Quickly transfer a few crystals of the frozen glycerol stock using a sterile toothpick to a LB agar plate containing 50 μg/mL kanamycin and 50 μg/mL ampicillin. Streak for single colonies and incubate the plate overnight at 37°C.
3. Pick a single colony from the fresh plate and inoculate a 3-mL culture of enriched medium containing 50 μg/mL ampicillin and 50 μg/mL kanamycin.
4. Incubate 8–20 h overnight at 37°C.
5. Transfer the 3-mL overnight culture to a flask containing 150 mL enriched medium with 50 μg/mL ampicillin and 50 μg/mL kanamycin and incubate overnight at 37°C.

6. Add 10 mL of the 150-mL overnight culture to each 10 L of medium.
7. Grow the cells at 30°C until the OD_{600} reaches 0.5–0.7.
8. Add IPTG to a final concentration of 100 μ*M* and chloramphenicol to a final concentration of 1 μg/mL.
9. Grow the cells for the appropriate period depending on the Gα subunit expressed at 30°C with gentle shaking at 200 rpm. *See* **Table 1** for induction times.
10. Harvest the cells by centrifugation at 9000*g* in a Beckman JA-10 rotor or equivalent for 10 min at 4°C.
11. Discard the medium and scrape the cell pellet directly into liquid N_2. Once frozen, transfer to a plastic container and store at –70°C.

*3.1.2. Cell Lysis (*see ***Note 13****)*

The following steps are all performed at 4°C.

1. Thaw the cell paste in a beaker containing 1.8 L TEDP with gentle stirring.
2. Disrupt any clumps with a syringe and 18-gauge cannula.
3. Add lysozyme to a final concentration of 0.2 mg/mL and incubate for 30 min on ice. The solution should become viscous.
4. Add $MgSO_4$ to a final concentration of 5 m*M* and 20 mg DNAse I in powder form.
5. Incubate for 30 min. The viscosity of the solution should diminish.
6. Remove insoluble material from the lysate by centrifugation in a Beckman JA-14 rotor or equivalent at 30,000*g* for 1 h at 4°C. Collect the supernatant fraction.

3.2. Bacterial Culture and Preparation of Cell Extracts for His-Tagged Gα

3.2.1. Large-Scale Culture

Optimal expression of His-tagged G protein α subunits occurs under conditions that are identical to those described for unmodified proteins. The cell culture procedures are the same as those described previously.

3.2.2. Cell Lysis

The following steps are all performed at 4°C.

1. Thaw the cell paste in a beaker containing 1.8 L of TBP with gentle stirring.
2. Disrupt any clumps with a syringe and 18-gauge cannula.
3. Add lysozyme to a final concentration of 0.2 mg/mL and incubate for 30 min on ice. The solution should become viscous.
4. Add $MgSO_4$ to a final concentration of 5 m*M* and 20 mg DNAse I in powder form.
5. Incubate for 30 min. The viscosity of the solution should diminish.
6. Remove insoluble material from the soluble fraction by centrifugation at 4°C in a Beckman Ti45 ultracentrifuge rotor for 30 min at 100,000*g*. Collect the supernatant fraction (*see* **Note 14**).

Table 1
Incubation Times for Expression of Gα in *E. coli*

	Postinduction time (h)	
Gα	Unmodified[a]	*N*-myristoylated[b]
$G_i\alpha_1$	9–12	16–18
$G_i\alpha_2$	16–18	16–18
$G_i\alpha_3$	16–18	6
$G_o\alpha$	16–18	16–18
$G_s\alpha$	12–15	Not applicable

[a] Taken from **ref. *8***.
[b] Taken from **ref. *14***.

3.3. Purification of Myristoylated Gα Using Conventional Chromatography

3.3.1. Batch DEAE Chromatography

Perform all steps at 4°C.

1. Fit a Buchner funnel on a vacuum flask with Whatman 4 filter paper. Add 200 mL DEAE-Sephacel. Wash with 1 L TEDP. Remove excess buffer by vacuum suction.
2. Transfer resin to a plastic beaker containing the supernatant fraction (**Subheading 3.1.2.**). Incubate the extract with the resin for 20 min with occasional stirring.
3. Collect on a Whatman no. 4 filter in the Buchner funnel. Wash the resin with 1.5 L TEDP. Elute protein with three 200-mL vol TEDP containing 300 m*M* NaCl. Collect in a plastic flask without vacuum.

3.3.2. PS Chromatography

Perform all steps at 4°C.

1. Prepare a 100-mL PS column (2.6 × 40 cm) by washing the resin with 1 L PS equilibration buffer.
2. Adjust the DEAE eluate to 1.2 *M* ammonium sulfate by the addition of 0.5 vol (300 mL) of 3.6 *M* ammonium sulfate. Add GDP to a concentration of 25 μ*M* (*see* **Note 15**).
3. Incubate the mixture on ice for 10 min and remove any precipitated protein by centrifugation at 11,000*g* for 10 min in a Beckman JA-14 rotor.
4. Apply the supernatant fraction to the column and collect the flow-through.
5. Elute protein with a 1-L descending gradient of ammonium sulfate (1.2 to 0 *M*). The 500-mL starting buffer for the gradient is PS equilibration buffer. The 500-mL diluting buffer for the gradient is PS elution buffer. Wash the column with 250 mL PS bump buffer. Collect 15-mL fractions across the gradient and the final wash step.

6. Assay 2.5-µL aliquots of the fractions from the PS column by GTPγS binding (**Subheading 3.5.**). Unmodified Gα will elute as a single peak of activity in the later fractions of the gradient. When purifying myristoylated Gα, the myristoylated protein will resolve from the unmodified protein at this step, eluting very late in the gradient or in the no-salt wash of the column (*see* **Fig. 1** and **Note 16**).
7. Pool the peak fractions (typically 100–125 mL) from the PS column. Desalt the pooled fractions using an Amicon ultrafiltration stirred cell. Use desalting buffer as the diluent (**Subheading 2.3.2., step 10**). Take the protein through successive concentration and dilution cycles until the ammonium sulfate concentration is reduced below 20 m*M*.

3.3.3. QS Chromatography

Perform all steps at 4°C.

1. Prepare a 2.6 × 40-cm column of QS by equilibrating 100-mL resin with 500-mL QS equilibration buffer.
2. Apply the desalted PS pool to the column. Collect the flow-through. Wash the column with 100-mL QS equilibration buffer and elute protein with a 500-mL gradient of NaCl (0–250 m*M*). Generate the gradient with 250 mL QS equilibration buffer and 250-mL QS elution buffer. Collect 8-mL fractions.
3. Assay 2.5-µL aliquots by GTPγS binding.

3.3.4. Hydroxylapatite Chromatography

Perform all steps at 4°C.

1. Prepare a 2.5 × 10-cm 20-mL column of Hap by equilibrating the resin with 100-mL Hap equilibration buffer (*see* **Note 8**).
2. Pool the peak fractions (usually 25 mL) from the QS column and adjust to a phosphate concentration of 10 m*M* by the addition of 1/100 vol of 1 *M* potassium phosphate buffer, pH 8.0. Dilute the protein solution with an equal volume of Hap equilibration buffer.
3. Apply the protein to the Hap column and collect the flow-through. Wash the column with 25 mL Hap buffer, and elute protein with a 200-mL gradient of phosphate (10–300 m*M*). Generate the gradient with 100-mL Hap equilibration buffer and 100-mL Hap elution buffer. Collect the gradient in 4-mL fractions.
4. Assay the fractions by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and GTPγS binding. Pool fractions according to activity and purity.
5. Concentrate the pool using an Amicon ultrafiltration device to a protein concentration of 1 mg/mL or more. During the concentration step, the buffer should be exchanged into HED. The final pool should be stored at –70°C in aliquots to avoid repeated freeze-thaws.

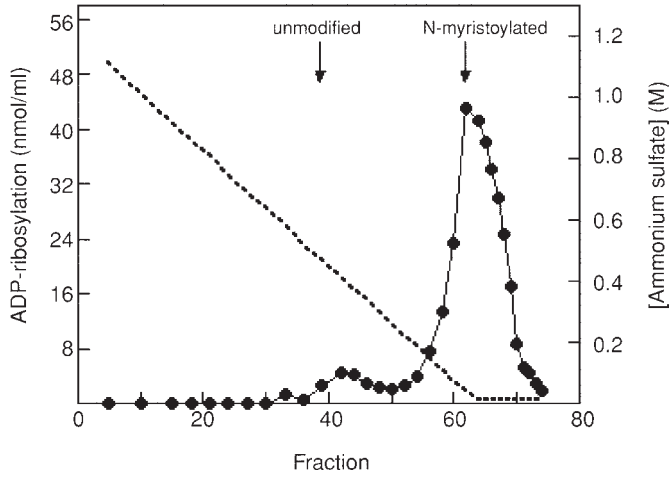

Fig. 1. PS chromatography of recombinant $G_i\alpha_2$ co-expressed with NMT. Cell lysates from *E. coli* cultures coexpressing $G_i\alpha_2$ and NMT were prepared and processed by diethylaminoethyl (DEAE) chromatography as described in **Subheading 3.3.1.** The DEAE eluate was applied to a column of PS. Protein was eluted by a descending gradient of ammonium sulfate (dashed line). Fractions containing $G_i\alpha_2$ were detected by adenosine diphosphate (ADP)-ribosylation (*see* **Note 16**; closed circles). Unmodified $G_i\alpha_2$ elutes in the first peak in fractions 43–49. Myristoylated $G_i\alpha_2$ elutes in fractions 60–70. (From **ref. *18a***. Reprinted with permission.)

6. Preparations of myristoylated and nonmyristoylated $G_i\alpha_1$ are purified to near homogeneity at this step. However, other Gα subunits are not expressed as well and may require additional steps of purification (*see* **Notes 17** and **18**).

3.3.5. Characterization of the Final Pool

1. Measure the protein concentration of the final pool using standard techniques.
2. Determine the GTPγS-binding activity of the final pool as described in **Subheading 3.5.** GTPγS-binding stoichiometries typically exceed 0.8 mol GTPγS-binding sites/mol protein. However, measurements can range from 0.4 to 1.1 mol GTPγS-binding sites/mol protein.

3.4. Purification of Hexahistidine-Tagged Gα Using Metal Chelate Chromatography

Perform all steps at 4°C.

1. Prepare a 50-mL column (2.5 × 10 cm) of Ni^{2+}-agarose column by equilibrating the resin with TBP containing 100 m*M* NaCl.
2. Apply the crude supernatant directly to the Ni^{2+}-agarose column and collect the flow-through.

3. Wash the column with 125 mL TBP containing 500 m*M* NaCl and 10 m*M* imidazole, pH 8.0.
4. Elute protein with a 600-mL linear gradient of 0–150 m*M* imidazole in TBP containing 100 m*M* NaCl and 10% glycerol. Collect 8-mL fractions (*see* **Note 10**).
5. Identify fractions containing Gα by SDS-PAGE; assay 10-µL aliquots.
6. Pool the fractions, desalt, and concentrate using an Amicon ultrafiltration device. The dilution buffer is TEDG. The final pool should be stored at –70°C in aliquots to avoid repeated freeze-thaws.
7. Recombinant Gα is often purified to near homogeneity at this step. If contaminating proteins are still present, they are usually removed by further chromatography on QS. The protocol for QS chromatography described in **Subheading 3.3.3.** can be used, but should be scaled according to the amount of protein in the pool. A good rule of thumb is 10-mg protein/mL QS resin.

3.5. GTPγS Binding as an Assay of G Protein Activity

1. Prepare GTPγS-binding cocktail and dilution buffers as described in **Subheading 2.5.3., step 4**.
2. Dilute samples to be assayed in dilution buffer.
3. Add 25 µL diluted protein to 25 µL-binding mix.
4. Mix well and incubate at 30°C for $G_i\alpha$ and 20°C for $G_o\alpha$ and $G_s\alpha$. The time course of the incubation also varies with the subunit assayed; a 30-min incubation is sufficient for $G_o\alpha$ and $G_s\alpha$; a 90-min incubation is appropriate for $G_i\alpha$.
5. At the end of the incubation, dilute the binding reactions with 2 mL ice-cold filtration buffer and filter through BA85 nitrocellulose filters. Wash the filters with a total volume of 12 mL of the same buffer and dry completely.
6. Suspend the filters in liquid scintillation cocktail and quantitate using liquid scintillation spectrometry.
7. Determine the specific activity of the [^{35}S]GTPγS by counting 5 µL of the binding mix (5 µL = 20 pmol GTPγS).

4. Notes

1. A number of bacterial expression vectors have been used to express Gα subunits in *E. coli*. The pQE vector series from Qiagen (Chatsworth, CA) has been particularly useful for production of large quantities of Gα for structural studies and this system will now be described in detail. Expression of Gα using T7 RNA polymerase-driven vectors has also been successful, but expression levels for some Gα are not as high as with the pQE vectors ***(8)***.

   The prokaryotic expression vector pQE-60 contains a very strong coliphage T5 promoter upstream of two *lac* operators. Transcription of genes subcloned into pQE-60 is induced with IPTG, which relieves repression by binding to the *lac* repressor and clearing it from the promoter. Efficient transcriptional termination is mediated by the terminator, t_o, from phage l. Translation of the recombinant protein is initiated by the binding of ribosomes to the synthetic ribosomal binding site (RBS II). Gα cDNAs are usually subcloned into pQE-60 as *Nco*I-

*Hin*dIII fragments, where the *Nco*I site is at the codon for the initiator methionine of Gα ***(8,14)***, which results in the production of Gα with native protein sequence. Construction of plasmids to express Gα is performed using standard molecular biological procedures as described by Sambrook and colleagues ***(19)***.

The pQE-60 plasmid must be maintained in a host strain that expresses *lac* repressor (*lac*I gene). It is convenient to carry out subcloning procedures using the bacterial strains JM109 or TG1, as these strains carry the mutated gene *lac*I^q and produce up to tenfold more *lac* repressor than strains carrying the wild-type *lac*I ***(20)***. The pQE-60/Gα plasmid is then transformed into the appropriate expression host. In cases where the host strain expresses either low levels or no *lac* repressor, cotransformation of the pREP4 plasmid, which carries the *lac*I gene, is performed. The pREP4 plasmid contains a kanamycin resistance marker and is compatible with pQE-60. Double transformants containing both plasmids are selected with LB plates containing 50 μg/mL kanamycin and 50 μg/mL ampicillin.

Selection of a suitable host strain for expression of Gα subunits is determined empirically. Various host strains have been tested for the ability to accumulate high levels of G protein a subunits in the soluble fraction. BL21/DE3, a protease deficient strain of *E. coli*, is able to accumulate high levels of $G_i\alpha_1$, $G_i\alpha_2$, $G_i\alpha_3$, and $G_s\alpha$. However, greater expression of $G_o\alpha$ can be obtained in strain M15 than in BL21/DE3. Because *lac* repressor is absent in M15, cotransformation with the plasmid pREP4 is required to maintain the $G_o\alpha$ plasmid. For expression of myristoylated Gα subunits, JM109 is the bacterial strain that gives the highest levels of soluble myristoylated protein.

2. Glycerol stocks of the bacterial strain harboring the expression plasmid should be prepared and stored at –70°C. To prepare glycerol stocks, mix equal volumes of a fresh overnight culture and sterile 40% glycerol and aliquot into 1-mL aliquots. To inoculate a culture from the frozen stock, quickly transfer a few crystals of the frozen glycerol stock using a sterile toothpick to an LB agar plate containing the appropriate antibiotics. Streak for single colonies and incubate the plate overnight. The glycerol stock can be returned to –70°C if it has not completely thawed during the transfer process. We have found that glycerol stocks are stable for years at –70°C. However, permanent storage of the expression plasmid as purified DNA at –20°C is strongly recommended.
3. The 50 m*M* KH_2PO_4 solution is brought to pH 7.2 using NaOH.
4. PMSF should be added to prechilled buffers immediately before use.
5. Expression of the plasmid 6-His-$G_i\alpha_1$ ***(8)*** in pQE60 results in production of a protein with a noncleavable hexahistidine tag. A vector containing a cleavable hexahistidine tag has been constructed by Lee and Gilman ***(8)***. The vector is designed with an N-terminal sequence Met-6-His-Ala-Glu-Asn-Leu-Tyr-Phe-Gln-Gly-Ala. Cleavage of the H_6TEVGα fusion protein by tobacco etch virus (TEV) protease results in the removal of the hexahistidine sequence and most of the TEV cleavage sequence. Details regarding the vector and the cleavage protocol are given elsewhere ***(8)***. TEV protease tagged with histidine residues (rTEV-6-His) is commercially available from Invitrogen (Carlsbad, CA).

6. β-Mercaptoethanol (Sigma, cat. no. M-6250) is sold as a 14.3 *M* solution; store at room temperature.
7. Glycerol is included to increase the density of the dilution buffer to stabilize gradient formation and slow the rate of dissociation of GDP from the α subunit ***(21)***.
8. To prepare powdered Hap resin for chromatography, mix resin with 2 vol water. Let settle for 5 min, then pour off fines. Repeat three to four times.
9. To prepare a 1 *M* stock of potassium phosphate buffer, pH 8.0, mix 6 mL 1 *M* KH_2PO_4 with 94 mL 1 *M* K_2HPO_4. Check pH of a 1:100 dilution and adjust as necessary to pH 8.0.
10. Glycerol is included in the buffer to prevent precipitation of the His-tagged proteins. Solubility is a particular problem following elution from the Ni^{2+} column.
11. The GTPγS-binding assay is a modification of the method described by Sternweis and Robishaw ***(22)***.
12. The key to a high yield of purified recombinant Gα is to optimize the accumulation of soluble protein. Standard protocols for induction of protein expression call for cell growth at 37°C and 1–2 m*M* concentrations of IPTG. Higher levels of soluble Gα accumulate with cell culture at 30°C and induction of protein with low concentrations of 30–100 μ*M* IPTG. For some Gα subunits, including a 1 μg/mL concentration of chloramphenicol during the induction period, increases the yield of soluble protein. There are no deleterious effects associated with including chloramphenicol at this concentration; therefore, we routinely include it when expressing all Gα subunits. The time period postinduction for peak accumulation of protein varies with the Gα expressed and is another important variable to optimize. In **Table 1**, the peak expression times are shown for unmodified and myristoylated Gα subunits.

    A frequently encountered problem is that Gα is expressed but is insoluble. The conditions of cell culture and induction of the protein can be modified as described previously. Reducing temperatures below 30°C with longer times of induction may permit the accumulation of soluble protein. Yields of soluble protein have also been increased by using a French Press to lyse the bacteria ***(2)***. The chimera strategy used by Hamm and colleagues to express a $G_t\alpha$-like molecule was discussed in the **Introduction** ***(3)***.
13. Alternative lysis protocols include the use of a French Press or sonication of the lysate following treatment with lysozyme. Cells are sonicated (5 × 30 s, on ice) using a probe-tip sonicator (Heat Systems Ultrasonics, Farmingdale, NY).
14. The high-speed centrifugation removes aggregates that interfere with the binding of the His-tagged protein to the chelated Ni^{2+} resin.
15. GDP is included in the buffers during this stage of purification because high ionic strength facilitates dissociation of the nucleotide from G protein α subunits. The protein is more sensitive to denaturation when in the nucleotide-free form ***(21)***.
16. A commonly encountered problem is that there are multiple peaks of GTPγS-binding activity eluting from the column. GTPγS binding provides a rapid means of screening column fractions throughout the purification. However,

there are *E. coli* proteins that will bind GTPγS, which are usually resolved from Gα in the PS chromatography step. If Gα expression is high, the signal associated with recombinant Gα will be the predominate signal and minor peaks of activity resulting from endogenous *E. coli* proteins that bind GTPγS can be ignored. However, if purifying a Gα that expresses at low levels, it may be more difficult to identify the peak of activity that corresponds to Gα. In that case, several alternative methods are available to assay for Gα.

Western blots using G protein antibodies provide a simple and specific method for identifying fractions that contain Gα, but does not discriminate between active and denatured protein. However, used in combination with GTPγS binding, it will not be difficult to identify fractions with active Gα protein. Immunoblots are not a rapid assay, but after the PS step, most Gα are stable at 4°C for several days.

Pertussis toxin-catalyzed adenosine diphosphate (ADP)-ribosylation is a rapid and very specific assay for $G_o\alpha$ and $G_i\alpha$ subtypes. $G_s\alpha$ is not a substrate for pertussis toxin-catalyzed ADP-ribosylation. When soluble lysates containing $G_o\alpha$ or $G_i\alpha$ are subjected to ADP-ribosylation by pertussis toxin in the presence of [^{32}P] nicotinamide adenine dinucleotide (NAD) and analyzed by SDS-PAGE and autoradiography, the predominant-labeled band seen is recombinant Gα. Because *E. coli* proteins are not labeled significantly, the presence of recombinant $G_o\alpha$ or $G_i\alpha$ can be easily identified in column fractions using a rapid precipitation and filtration assay. The disadvantages of this assay are the expense and requirement for a source of purified G protein βγ subunits. ADP-ribosylation is carried out as described by Bokoch et al. ***(23)*** with minor modifications ***(24)***.

Another problem that may be encountered is that myristoylated Gα does not elute from the PS column. Unmodified Gα typically elutes from the PS column as a uniform peak during the gradient. Myristoylated Gα should begin to elute from the column before the end of the gradient, but the peak elution is often during the final TEDP wash at the end of the gradient. The myristoylated protein is more hydrophobic than the unmodified Gα and binds tightly to the resin. If the myristoylated protein does not completely elute from the column, wash the column with additional TEDP buffer and continue to collect fractions. Alternatively, myristoylated Gα can be eluted with TEDP buffer containing 1% sodium cholate. However, this has the undesirable consequence of eluting more contaminants.

Occasionally, myristoylated Gα does not resolve from the unmodified Gα on the PS column. A typical PS elution profile is shown in **Fig. 1**. However, there may not be a well-resolved peak of unmodified protein preceding the myristoylated protein. Under this circumstance, avoid pooling the initial fractions that have Gα activity. The electrophoretic mobility difference between myristoylated and unmodified Gα can be detected on immunoblots and used to identify fractions that contain exclusively the myristoylated form. NMT of Gα results in a faster electrophoretic mobility (*see* **Fig. 2**). Although this difference can sometimes be detected by standard SDS-PAGE, the mobility shift is exaggerated when urea is added to a final concentration of 4 *M* in the resolving gel mix ***(15)***. The difference is also more apparent on longer resolving gels (~12 cm).

Fig. 2. Unmodified and myristoylated $G_i\alpha_2$ can be distinguished by electrophoretic mobility. Recombinant 2 μg $G_i\alpha_2$ was purified from *E. coli* in the absence (left lane) or presence (right lane) of NMT and resolved by SDS-PAGE in gels supplemented with 4 *M* urea. Protein was detected by staining with Coomassie blue. (From **ref. *18a***. Reprinted with permission.)

17. If the preparation of Gα is not sufficiently purified after Hap chromatography, additional steps may be used. The most commonly used method is a second round of hydrophobic interaction chromatography (PS). If possible, a high resolution PS column on a fast protein liquid chromatography (FPLC) system (Amersham Biosciences) should be used as described by Lee et al. ***(8)***. However, if that system is not available, conventional chromatography using PS is a suitable substitute and is described here.

    Pool the fractions containing Gα after Hap chromatography (**Subheading 2.3.4.**) and add ammonium sulfate and GDP to final concentrations of 1.2 *M* and 50 μ*M*, respectively. Apply the pool to a 10-mL PS column that has been equilibrated in PS equilibration buffer. Elute protein with a 120-mL gradient of decreasing ammonium sulfate from 1.2 to 0 *M*. Include 35% glycerol (v/v) in the gradient diluting buffer (**Subheading 2.3.2., step 5**). Collect 2-mL fractions and assay for Gα by SDS-PAGE for purity. Pool the peak fractions and process for storage as described in **Subheading 3.3.4., step 5.**

    Another method to remove contaminating proteins from the Hap pool is gel-filtration chromatography, but this method is only useful when the contaminating proteins are significantly different in size from Gα.

18. Yields reported for *N*-myristoylated Gα are 60, 8, 4, and 15 mg for $G_i\alpha_1$, $G_{i}\alpha_2$, $G_i\alpha_3$, and $G_o\alpha$, respectively, from a 10-L preparation ***(14)***. Yields reported for unmodified Gα subunits are 400, 40, 65, and 35 mg for $G_i\alpha_1$, $G_i\alpha_2$, $G_o\alpha$, and $G_s\alpha$, respectively ***(8)***.

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2

Purification of G Protein Subunits from Sf9 Insect Cells Using Hexahistidine-Tagged α and βγ Subunits

Tohru Kozasa

Summary

G protein-mediated pathways are the most fundamental mechanisms of cell signaling. In order to analyze these pathways, the availability of purified recombinant G proteins are critically important. Using Sf9-Baculovirus expression system, a general and simplified method to purify various G protein subunits is described in this chapter. This method is useful for purification of most of G protein subunits.

Key Words: G protein; recombinant protein; Sf9-Baculovirus expression system.

1. Introduction

G protein-mediated signal transduction is a fundamental mechanism of cell communication, being involved in various cellular functions ***(1–3)***. G proteins receive signals from a large number of heptahelical cell surface receptors, and they transmit these signals to various intracellular effectors. Each heterotrimeric G protein is composed of a guanine nucleotide-binding α subunit and a high-affinity dimer of β and γ subunits. Agonist-bound receptor activates G protein to facilitate guanosine diphosphate–guanosine triphosphate (GDP–GTP) exchange on Gα subunit, which induces subunit dissociation to generate GTP-bound α and free βγ subunits. Both of these molecules are able to regulate the activity of downstream effectors. GTP on Gα is hydrolyzed to GDP by its own GTPase activity as well as GTPase-activating proteins, such as regulator of G protein signaling (RGS) proteins. GDP-bound Gα reassociates with βγ subunit to form an inactive heterotrimer to complete the G protein cycle.

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Gα subunits are commonly classified into four subfamilies based on their amino-acid sequence homology and function: G_s family ($G_s\alpha$ and $G_{olf}\alpha$; activate adenylyl cyclase); G_i family ($G_{i1}\alpha$, $G_{i2}\alpha$, $G_{i3}\alpha$, $G_o\alpha$, $G_t\alpha$, $G_z\alpha$, and $G_g\alpha$; substrate for pertussis toxin-catalyzed adenosine-5'-diphosphate (ADP) ribosylation except for $G_z\alpha$, inhibit adenylyl cyclase, or stimulate guanosine-2',3'-cyclic phosphate (cGMP) phosphodiesterase, and so forth); G_q family ($G_q\alpha$, $G_{11}\alpha$, $G_{14}\alpha$, $G_{15}\alpha$, and $G_{16}\alpha$; stimulate phospholipase C-β isozymes); and G_{12} subfamily ($G_{12}\alpha$ and $G_{13}\alpha$; regulate Rho guanine nucleotide exchange factor [GEF] activity) ***(4–6)***. Five β subunits and 13 γ subunits have been identified in mammals. βγ subunits directly regulate several effectors, such as adenylyl cyclase, phospholipase Cβ, K^+ channels, Ca^{2+} channels, or PI3 kinase ***(7)***. Functional specificity of different combinations of βγ subunit have been shown for several cases, particularly for the combination with β_5 subunits.

Purification of G proteins from natural tissue requires lengthy procedures, and quantity is often limiting. It is also difficult to resolve closely related members of Gα subunits or practically impossible to purify specific combinations of βγ subunit. Expression of $G_s\alpha$, $G_i\alpha$, and $G_o\alpha$ in *Escherichia coli* (*E. coli*) yields large amounts of protein that can be myristoylated where appropriate ($G_i\alpha$ and $G_o\alpha$) ***(8)***, but the proteins are not palmitoylated and may be missing some other unknown modifications. Alpha subunits of G_q and G_{12} subfamilies and the βγ complex have not been successfully expressed in *E. coli* as active proteins.

The Sf9-Baculovirus expression system has advantages to overcome these problems. First, a variety of posttranslational modification mechanisms, especially lipid modifications, such as palmitoylation, myristoylation, and prenylation, are present in Sf9 cells. These lipid modifications are critically important for the interactions of G protein subunits with receptors, RGS proteins, or effectors. With these modifications present, the recombinant G protein subunits from Sf9 expression system are almost as active as native proteins ***(9,10)***. Second, we can coinfect multiple viruses encoding α, β, and γ subunits to express desired G protein heterotrimer or βγ complex on Sf9 cells. The inactive heterotrimer is the stable structure for Gα subunits. Coexpression of βγ was particularly required to purify properly folded α subunits of G_q subfamily ***(10,11)***. Without βγ subunit, these α subunits aggregated in Sf9 cells and could not be purified. It was also shown that the amount of membrane-bound Gα increases by coexpressing of βγ subunit. In spite of these advantages, the yield of recombinant G protein subunit from Sf9 cells was often low, and the purification procedure was laborious with conventional purification methods.

A general and simple method for purification of G proteins from Sf9 cells is described in this chapter. The G protein subunit to be purified is coexpressed with an associated hexahistidine-tagged subunit. The oligomer is adsorbed to a

Ni^{2+}-containing resin and the desired untagged protein is eluted with aluminum tetrafluoride $(AlF)_4^-$, which reversibly activates the α subunits of G proteins and causes dissociation of α from βγ. This method takes advantage of the high affinity and large capacity of Ni-NTA resin for the hexahistidine tag, as well as the extremely specific elution of the untagged subunit with AlF_4^-. It is especially useful for purification of Gα subunits that can not be purified using *E. coli* expression system ($G_z\alpha$, $G_q\alpha$, $G_{11}\alpha$, $G_{12}\alpha$, and $G_{13}\alpha$) and for the purification of defined combinations of βγ subunits. The detailed procedures to purify each of these subunits are described in the following sections.

2. Materials

2.1. Sf9 Cells, Baculoviruses, and Culture Supplies

1. Baculoviruses for expression of the appropriate G protein combination: For all α subunit purifications, 6-His-γ_2 or with hexahistidine tag at the N-terminus, a wild-type β_1 subunit and the appropriate wild-type α subunit are needed. For βγ subunit purification, 6-His-$G_{i1}\alpha$ (hexahistidine tag is inserted at position 121 of $G_{i1}\alpha$) and the appropriate wild-type β and γ subunit combinations are necessary. Recombinant viruses encoding each G protein subunit have already been described ***(9,10,12–14)***. General methods for construction, isolation, and amplification of recombinant viruses are described in **refs. *15*** and ***16*** (*see* **Note 1**).
2. Frozen stock of Sf9 cells (Invitrogen/LifeTechnologies, American Type Culture Collection, or Pharmingen).
3. IPL-41 medium (*see* **Note 2**).
4. 10% Heat-inactivated fetal bovine serum (FBS): heat-inactivated at 55°C for 30 min.
5. 10 mL to 2 L Glass culture flasks with steel closure (BELLCO).
6. Chemically defined lipid concentrate (Invitrogen/LifeTechnologies).
7. 0.1% Pluronic F-68 (Invitrogen/LifeTechnologies).

2.2. Chromatography Supplies and Solutions

Different supplies are required for different G protein subunit preparations. Check the specific G protein purification protocol outlined in **Subheading 3.4.** for the columns and solutions that will be required.

1. Ni^{2+} containing resin (Ni-NTA agarose; Qiagen, cat. no. 30230).
2. Ceramic hydroxyapatite (macroprep; Bio-Rad, cat. no. 158-4000).
3. 2.5 × 5-cm Econo chromatography column (Bio-Rad).
4. Mono Q HR5/5 anion-exchange column (Amersham/Pharmacia).
5. Mono S HR5/5 cation-exchange column (Amersham/Pharmacia).
6. Fast Protein Liquid Chromatography (FPLC) system (Amersham/Pharmacia).
7. Centricon YM30 centrifugal concentration devices (Millipore/Amicon).
8. Cholic acid (Sigma). Make a 20% stock solution in water and store at 4°C. Sodium cholate is purified from cholic acid by diethylaminoethyl (DEAE) Sepharose column as described ***(17)***.

9. Polyoxyethylene-10-lauryl ether ($C_{12}E_{10}$; Sigma). Make a 10% stock solution in water and store at 4°C.
10. CHAPS (Calbiochem or Sigma). Make a 0.1 *M* stock solution in water and store at 4°C.
11. *N*-octyl-β-D-glucopyranoside (octylglucoside). Prepare a fresh 10% stock solution in water.
12. *N*-dodecyl-β-D-maltoside (dodecylmaltoside; Calbiochem). Prepare a fresh 10% stock solution in water.
13. The stock solutions of proteinase inhibitors (Sigma) are prepared as follows; 800 mg each of phenylmethylsulfonyl fluoride (PMSF), *N*-tosyl-L-phenylalanine-chloromethyl ketone (TPCK), and *N*α-p-tosyl-L-lysine chloromethyl ketone (TLCK) are dissolved in 50 mL of 50% dimethylsulfoxide (DMSO)/50% isopropanol. 160 mg each leupeptin and lima bean trypsin inhibitor are dissolved in 50 mL H_2O. The stock solutions of proteinase inhibitors are stored at –20°C and used as 1000X stock.
14. The following solutions are used to prepare the buffers for purification; 1 *M* HEPES-NaOH, pH 8.0; 1 *M* HEPES-NaOH, pH 7.4; 0.1 *M* EDTA, pH 8.0; 4 *M* NaCl; 1 *M* $MgCl_2$; 1 *M* KPi, pH 8.0; 14 *M* 2-mercaptoethanol; 1 *M* DTT (store frozen); 2 *M* imidazole-HCl, pH 8.0; 1 *M* NaF, 10 m*M* $AlCl_3$; 50 m*M* GDP (store frozen); and 10 m*M* GTPγS (purified over Mono Q column, store frozen). The compositions of the purification solutions are shown in **Tables 1** and **2**.

3. Methods

The methods outlined in **Subheadings 3.1.–3.3.** are general methods required for purification of all G protein subunits. **Subheading 3.4.** gives specfic protocols required for the individual G protein subunit desired.

3.1. Sf9 Cell Culture (see Note 3)

1. Sf9 cells are grown and maintained in IPL-41 medium supplemented with 10% heat-inactivated FBS heat-inactivated at 55°C for 30 min and 0.1% pluronic F-68.
2. Freshly thawed Sf9 cells are cultured in a 25-mL tissue culture flask at 27°C for about 1 wk to recover.
3. Then, they are transferred to suspension culture at 27°C with constant shaking at 125 rpm.
4. Stock culture, usually 50 mL in 100-mL flask, are passaged every 3 d and maintained at a density between 0.5 and 3×10^6 cells/mL.

3.2. Infection of Sf9 Cells and Membrane Preparation

The membrane preparation procedure from 4 L of Sf9 cell culture is described.

1. Sf9 cells are expanded from 50-mL stock culture to 250 mL ($0.5–1 \times 10^6$ cells/mL) by IPL-41 medium with 10% FBS and 0.1% pluronic F-68 in a 500-mL flask.
2. After 2–3 d, they are further expanded to 1 L ($\sim 1 \times 10^6$ cells/mL) with IPL-41 containing 10% FBS and are divided into four 500-mL flasks.

Table 1
Solutions for Sf9 Membrane Preparation

Stocks	Lysis buffer	Wash buffer
1 *M* HEPES-NaOH, pH 8.0	20	20
4 *M* NaCl	25	25
100 m*M* EDTA	1	0
1 *M* $MgCl_2$	2	1
14 *M* 2-mercaptoethanol	0.7	0.7
50 m*M* GDP	0.2	0.2
Total volume (mL)	1000	1000

Addition in milliliters. Adjust volume of solutions to the indicated final volume. Stock solution of 1000X proteinase inhibitors are added before use.

3. After 2 d, cells are transferred to four 2-L flasks and diluted with 750 mL of IPL-41 medium containing 1% FBS and 1% lipid concentrate and 0.1% pluronic F-68.
4. The following day, cells (usual density of 1.5–2 × 10^6 cells/mL) are infected with amplified recombinant Baculoviruses encoding the desired combination of G protein subunits. For purification of Gα subunit, viruses encoding Gα, β_1, and 6-His-γ_2 are infected. For purification of βγ subunit, 6-His-$G_{i1}\alpha$ is coinfected with the desired combination of β and γ viruses. The typical infection is 15 mL of α, 10 mL of β, and 7.5 mL of γ amplified viruses to 1 L Sf9 culture (*see* **Note 4**).
5. After 48 h of infection, cells are harvested by centrifugation at 500*g* for 15 min in a JLA-10 rotor (Beckman Coulter). Cell pellets can be frozen in liquid nitrogen and stored at –80°C, or they can be further processed for membrane preparation.
6. Cell pellets from 4 L of Sf9 cells are resuspended in 600 mL ice-cold lysis buffer: 20 m*M* HEPES, pH 8.0, 0.1 m*M* EDTA, 2 m*M* $MgCl_2$, 10 m*M* 2-mercaptoethanol, 100 m*M* NaCl, 10 μ*M* GDP; with fresh proteinase inhibitors.
7. Cells are lysed by nitrogen cavitation (Parr bomb) at 500 psi for 30 min at 4°C.
8. The lysates are collected and centrifuged at 500*g* for 10 min in a JLA-10 rotor to remove intact cells and nuclei.
9. The supernatants are collected and centrifuged at 35,000 rpm for 30 min in a Ti-45 rotor (Beckman Coulter).
10. The pellets are resuspended in 300 mL wash buffer: 20 m*M* HEPES, pH 8.0, 100 m*M* NaCl, 1 m*M* $MgCl_2$, 10 m*M* 2-mercaptoethanol, 10 μ*M* GDP, and fresh proteinase inhibitors. Then, the pellets are centrifuged again as above.
11. The pellets (cell membranes) are resuspended in 200 mL wash buffer and the protein concentration is determined using a Bradford protein assay (Coommassie Protein Assay Reagent, Pierce).
12. The membranes are frozen by slowly pouring into a container of liquid nitrogen to form small chunks that are similar to popcorn and stored at –80°C. The amount of membrane protein from 4 L of Sf9 cells is 1.2–2 g.

Table 2
Solutions for G Protein Subunit Purification

Stocks	A	B	C	D	E	F	G	H	I
1 *M* HEPES-NaOH, pH 8.0	30	2	0.6	0.6	0.6	0.6	4	1	0.6
4 *M* NaCl	37.5	7.5	0.375	0.375	0.375	0.375	5	0.625	0.75
1 *M* $MgCl_2$	1.5	0.3	0.09	1.5	0.09	0.006	0.2	2.5	0.03
14 *M* 2-mercaptoethanol	1.05	0.07	0.021	0.021	0.021	0.021	0.14	0.035	0.021
50 m*M* GDP	0.3	0.02	0.006	0.006	0.006		0.04	0.02	0.012
1 m*M* GTPγS						0.15			
2 *M* Imidazol-HCl, pH 8.0		0.5	0.15	0.15	2.25	0.15	1.5	0.25	2.25
1 *M* NaF				0.3				0.5	
10 m*M* $AlCl_3$				0.09				0.15	
10% $C_{12}E_{10}$	75	5					10	2.5	1.5
20% Sodium cholate			0.3	1.5	1.5	1.5			
Total volume (mL)	1500	100	30	30	30	30	200	50	30

Stocks	J	K	L	M	O	P	Q
1 *M* HEPES-NaOH, pH 8.0	0.6	0.6	0.6	0.6	0.4	0.4	0.4
4 *M* NaCl	0.75	0.75	0.375	0.375	0.5	0.5	0.5
1 *M* $MgCl_2$	0.03	0.03	1.5	0.03	0.02	1	0.02
14 *M* 2-mercaptoethanol	0.021	0.021	0.021	0.021	0.014	0.014	0.014
50 m*M* GDP	0.012	0.012	0.012	0.012	0.004	0.004	0.004
2 *M* Imidazol-HCl, pH 8.0	0.15	0.15	0.15	2.25	0.1	0.1	1.5
1 *M* NaF			0.3			0.2	
10 m*M* $AlCl_3$			0.09			0.06	
10% Dodecylmaltoside	0.18	0.6	0.6	0.6			
10% Octylglucoside					0.6	2	2
Total volume (mL)	30	30	30	30	20	20	20

Table 2 (Continued)
Solutions for G Protein Subunit Purification

Stocks	R	R1000	S	S1000	T	T150	T300
1 *M* HEPES-NaOH, pH 8.0			8	2	0.8	0.4	0.4
1 *M* HEPES-NaOH, pH 7.4	8	2					
4 *M* NaCl		25		25	0.5	0.25	0.25
0.1 *M* EDTA	2	0.5	2	0.5			
1 *M* $MgCl_2$	0.8	0.2	0.8	0.2	0.04	0.02	0.02
1 *M* DTT	0.4	0.1	0.4	0.1	0.04	0.02	0.02
0.1 *M* CHAPS	44	11	44	11			
10% Dodecylmaltoside					0.8	0.4	0.4
1 *M* KPi, pH 8.0						3	6
Total volume (mL)	400	100	400	100	40	20	20

Additions are in milliliters. Adjust volume of solutions to the indicated total volume.

3.3. Detergent Extraction of SF9 Cell Membranes and Loading onto Ni-NTA Agarose

1. 1500-mg Frozen cell membranes are thawed and diluted to 5 mg/mL with wash buffer containing fresh proteinase inhibitors.
2. 20% Sodium cholate stock solution is added to a final concentration of 1% (w/v), and the mixture is stirred on ice for 1 h prior to centrifugation at 9600*g* for 30 min in a Ti-45 rotor.
3. The supernatants (membrane extracts) are collected, diluted threefold with buffer A: 20 m*M* HEPES, pH 8.0, 100 m*M* NaCl, 1 m*M* $MgCl_2$, 10 m*M* 2-mercaptoethanol, 10 μ*M* GDP, 0.5% $C_{12}E_{10}$, and loaded onto a 2.5 × 5-cm Econo Column packed with 4 mL Ni-NTA agarose and equilibrated with 20 mL buffer A. The loading of approx 1 L of diluted membrane extract onto 4 mL Ni-NTA resin usually takes 6–7 h. After loading, the Ni-NTA column is processed differently according to the subunit to be purified as described in **Subheading 3.4.**

3.4. Purification Procedures for Individual G Protein Subunits

3.4.1. $G_z\alpha$

1. Ni-NTA column is washed with 100 mL buffer B: 20 m*M* HEPES, pH 8.0, 300 m*M* NaCl, 3 m*M* $MgCl_2$, 10 m*M* 2-mercaptoethanol, 10 μ*M* GDP, 10 m*M* imidazole, 0.5% $C_{12}E_{10}$. This is followed by 12 mL buffer C: 20 m*M* HEPES, pH 8.0, 50 m*M* NaCl, 3 m*M* $MgCl_2$, 10 m*M* 2-mercaptoethanol, 10 μ*M* GDP, 10 m*M* imidazole, 0.2% sodium cholate, at 4°C.
2. The column is incubated at room temperature for 15 min, then washed with 12 mL buffer C at 30°C.
3. The $G_z\alpha$ protein is eluted with 32 mL buffer D: 20 m*M* HEPES, pH 8.0, 50 m*M* NaCl, 10 m*M* 2-mercaptoethanol, 10 μ*M* GDP, 30 μ*M* $AlCl_3$, 50 m*M* $MgCl_2$, 10 m*M* NaF, 10 m*M* imidazole, 1% sodium cholate, at 30°C and 4-mL fractions are collected (**Fig. 1A**; *see* **Note 5**).
4. Finally, β_1 6-His-γ_2 is eluted with 12 mL buffer E: buffer D without $AlCl_3$, $MgCl_2$, NaF (AMF) and containing 150 m*M* imidazole. 4-mL Fractions are collected (**Fig. 1A**).
5. The peak fractions containing $G_z\alpha$ from the Ni-NTA column are diluted threefold with buffer R: 20 m*M* HEPES, pH 7.4, 0.5 m*M* EDTA, 2 m*M* $MgCl_2$, 1 m*M* DTT, 0.7% CHAPS. The fractions are loaded onto Mono S HR5/5 column, which was equilibrated with buffer R at a flow rate of 0.5 mL/min using an FPLC system (Amersham Pharmacia).
6. $G_z\alpha$ is eluted with a 25-mL gradient of 0–550 m*M* NaCl. Fractions of 0.5 mL are collected and assayed for protein staining after sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and GTPγS-binding activity.
7. $G_z\alpha$ elutes as a broad peak in fractions approx 400–450 m*M* NaCl (**Fig. 1B**). The peak fractions are concentrated and the buffer is exchanged into buffer R with 100 m*M* NaCl and 5 μ*M* GDP by repeated concentration and dilution in Centricon 30 concentration device (*see* **Note 6**).

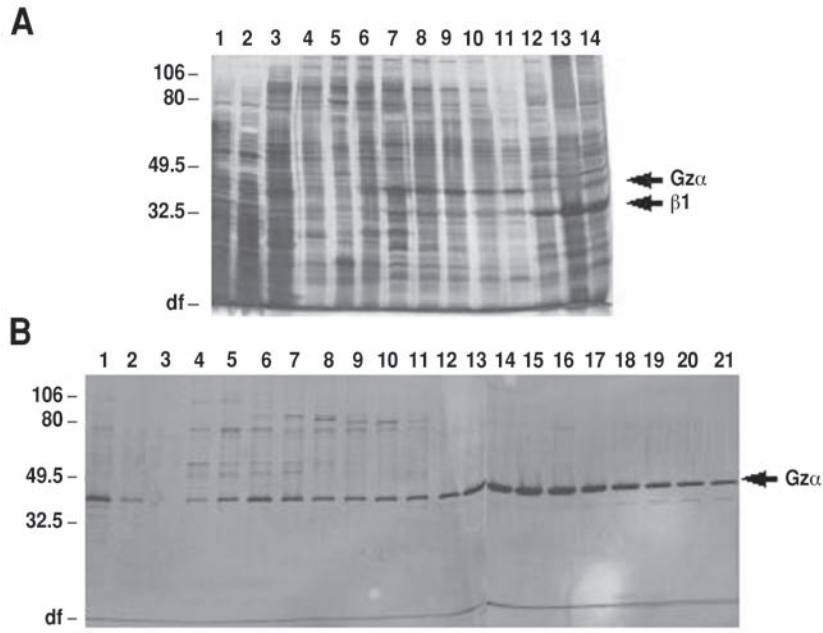

Fig. 1. Purification of $G_z\alpha$. (**A**) Ni-NTA column for purification of $G_z\alpha$. Fractions of 4 µL were subjected to SDS-PAGE and stained by silver nitrate. Lane 1, load; lane 2, flow-through; lanes 3–5, wash with high salt and low imidazole; lanes 6–11, elution with AMF; lanes 12–14, elution with 150 m*M* imidazole. (**B**) Mono S chromatography of $G_z\alpha$. The peak fractions from the Ni-NTA column were loaded onto a Mono S column and chromatographed as described. Fractions of 4 µL were subjected to SDS-PAGE and stained by silver nitrate. Lane 1, load; lane 2, flow-through, lanes 3–21, NaCl gradient 0–500 m*M*.

3.4.2. $G_q\alpha$

1. After loading the membrane extract from Sf9 cells expressing $G_q\alpha$, β_1 and 6-His-γ_2, the Ni-NTA column is washed with 100 mL buffer B and 12 mL buffer C.
2. The column is incubated at room temperature for 15 min and is further washed with 12 mL buffer C at room temperature.
3. The column is then incubated with buffer F: 20 m*M* HEPES, pH 8.0, 50 m*M* NaCl, 10 m*M* 2-mercaptoethanol, 0.2 m*M* $MgCl_2$, 5 µ*M* GTPγS, 10 m*M* imidazole, and 0.2% sodium cholate, for 15 min at room temperature and washed with 32 mL the same buffer. Endogenous $Sf9G_i\alpha$-like protein is removed from the column at this step (*see* **Note 7**).
4. Recombinant $G_q\alpha$ is eluted from Ni-NTA column by washing with 24 mL buffer D at room temperature and collecting 4-mL fractions (**Fig. 2**).

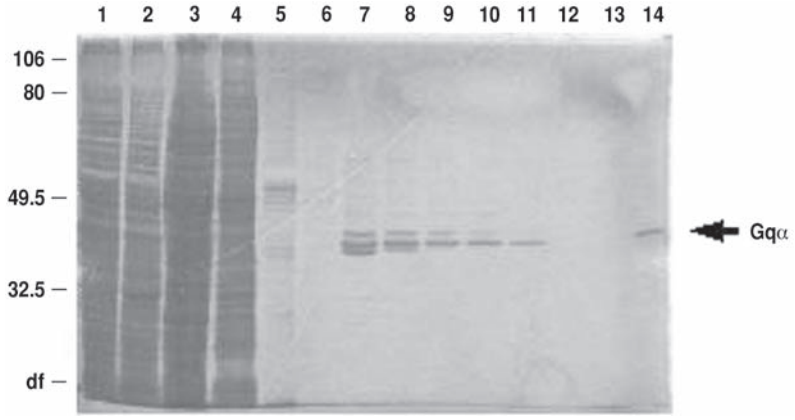

Fig. 2. Purification of $G_q\alpha$. Ni-NTA column for purification of $G_q\alpha$. Fractions of 4 µL were subjected to SDS-PAGE and stained by silver nitrate. In this purification, $G_q\alpha$ was eluted with two steps; first with 1% cholate containing buffer, then with AMF containing buffer. Lane 1, load; lane 2, flow-through; lanes 3–5, wash with high salt and low imidazole; lanes 6–12, elution with 1% cholate; lanes 13 and 14, elution with AMF.

5. The peak fractions containing $G_q\alpha$ from the Ni-NTA column are diluted threefold with buffer S and loaded onto a Mono Q HR5/5 column that was equilibrated with buffer S. CHAPS in buffer S can be replaced with 1% octylglucoside.
6. $G_q\alpha$ is eluted with a linear gradient of 0–400 m*M* NaCl 20-mL gradient with a flow rate of 0.5 mL/min: collecting 0.5-mL fractions. Fractions are assayed by immunoblotting with $G_q\alpha/G_{11}\alpha$ antiserum Z811 and $G_{11}\alpha$ antiserum B825 ***(18)***.
7. Recombinant $G_q\alpha$ is recognized by both Z811 and B825 and eluted in fractions containing approx 220 m*M* NaCl. An endogenous Sf9 $G_q\alpha$-like protein is recognized by Z811, but not by B825 ***(10)***, and eluted later in the gradient (~ 280 m*M* NaCl).
8. The peak fractions that mainly contain recombinant $G_q\alpha$ are pooled. The sample is concentrated and the buffer is changed into buffer S containing 100 m*M* NaCl and 5 µ*M* GDP by repeated dilution and concentration using Centricon YM30 (*see* **Notes 8** and **9**).

3.4.3. $G_{12}\alpha$

$G_{12}\alpha$ has several different biochemical properties from other $G\alpha$ subunits, and the purification method for $G_z\alpha$ or $G_q\alpha$ cannot be used for $G_{12}\alpha$. The high-salt wash on Ni-NTA column disrupts the interaction between $G_{12}\alpha$ and $\beta_1\gamma_2$. Also, $G_{12}\alpha$ cannot be eluted from Ni-NTA column with buffers containing sodium cholate.

1. After loading the extract expressing $G_{12}\alpha$, β_1, and 6-His-γ_2, the Ni-NTA column is washed with 100 mL buffer G: buffer A containing 15 m*M* imidazole and 100 m*M* NaCl.

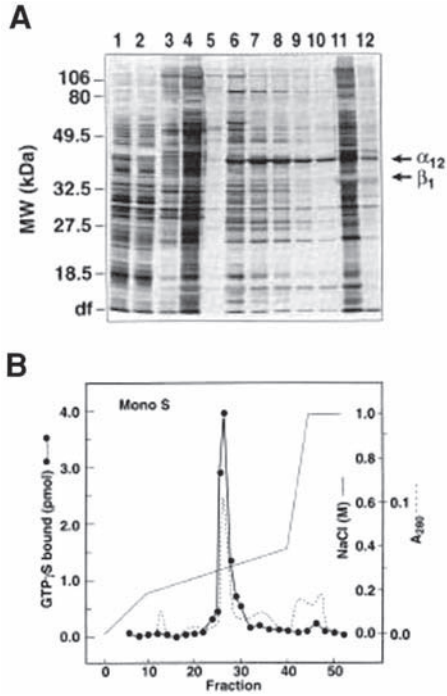

Fig. 3. Purification of $G_{12}\alpha$. **(A)** Ni-NTA column for purification of $G_{12}\alpha$. Fractions of 4 µL were subjected to SDS-PAGE and stained by silver nitrate. Lane 1, load; lane 2, flow-through; lanes 3–5, wash with low imidazole; lanes 6–10, elution with AMF; lanes 11 and 12, elution with 150 m*M* imidazole. **(B)** Mono S chromatography of $G_{12}\alpha$. The peak fractions from the Ni-NTA column were loaded onto a Mono S column and chromatographed with NaCl gradient of 200–400 m*M*. Fractions of 3 µL were assayed for GTPγS binding activity with 5 µ*M* GTPγS and 10 m*M* $MgSO_4$ for 90 min at 30°C. (Reproduced from **ref. *12*** with permission.)

2. The column is incubated at room temperature for 15 min. Then the column is washed with 12 mL buffer G and 32 mL buffer H: 20 m*M* HEPES, pH 8.0, 50 m*M* NaCl, 10 m*M* 2-mercaptoethanol, 20 µ*M* GDP, 50 m*M* $MgCl_2$, 10 m*M* NaF, 30 m*M* $AlCl_3$, 10 m*M* imidazole, and 0.5% $C_{12}E_{10}$, at 33°C (**Fig. 3A**) collecting 4-mL fractions.
3. The dissociation of $G_{12}\alpha$ from $\beta\gamma$ on Ni-NTA column is usually incomplete. Approximately 50% of $G_{12}\alpha$ remains on the column after AMF elution based on Western blot. The temperature of the AMF elution buffer can be raised to 37°C to further facilitate the dissociation.
4. The peak fractions of $G_{12}\alpha$ are diluted threefold with buffer S and loaded onto Mono S HR5/5 column, which was equilibrated with buffer S and chromatographed with the gradient of 200–400 m*M* NaCl at 0.5 mL/min, and 0.5-mL frac-

tions are collected. 0.7% CHAPS in buffer S can be replaced by 1% octylglucoside. $G_{12}\alpha$ elutes from Mono S column as a sharp peak near 250 m*M* NaCl (**Fig. 3B**).

5. The peak fractions containing $G_{12}\alpha$ are concentrated and the buffer is exchanged into buffer S containing 100 m*M* NaCl, 1 μ*M* GDP, and 10% glycerol using a Centricon YM30 and repeated dilution and concentration (*see* **Note 10**).

3.4.4. $G_{13}\alpha$

1. After loading the membrane extract from Sf9 cells expressing $G_{13}\alpha$, β_1 and 6-His-γ_2, the Ni-NTA column is washed with 100 mL buffer B, then is warmed up to room temperature for 15 min.
2. Then the column is washed with 12 mL buffer J: 20 m*M* HEPES, pH 8.0, 100 m*M* NaCl, 1 m*M* $MgCl_2$, 10 m*M* 2-mercaptoethanol, 20 μ*M* GDP, 10 m*M* imidazole, 0.06% dodecylmaltoside.
3. Wash with 12 mL buffer K: buffer J containing 0.2% dodecylmaltoside.
4. 24 mL buffer L: buffer K containing 50 m*M* NaCl, 30 μ*M* $AlCl_3$, 50 m*M* $MgCl_2$, and 10 m*M* NaF. 4-mL Fractions are collected. As shown in **Fig. 4A**, the peak fractions are almost pure on SDS-PAGE.
5. The column is then eluted with12 mL buffer M:buffer K containing 50 m*M* NaCl and 150 m*M* imidazole, at 30°C to elute βγ and to check for any remaining $G_{13}\alpha$.
6. The fractions from the elution containing relatively pure $G_{13}\alpha$ are combined and applied onto 1 mL of ceramic hydroxyapatite column equilibrated with buffer U: 20 m*M* HEPES, pH 8.0, 50 m*M* NaCl, 1 m*M* $MgCl_2$, 1 m*M* DTT, 0.2% dodecylmaltoside, 10% glycerol.
7. The flow-through is collected and applied again onto the column.
8. The column is then washed with 4 mL buffer T, 4 mL T150 (buffer T containing 150 m*M* KPi, pH 8.0), and 4 mL T300 (buffer T containing 300 m*M* KPi, pH 8.0). 1-mL fractions are collected. $G_{13}\alpha$ elutes in fractions of T150 (**Fig. 4B**).
9. The peak fractions are combined and the buffer is exchanged to buffer T containing 100 m*M* NaCl, 1 m*M* EDTA, 1 m*M* DTT, 1 μ*M* GDP and 10% glycerol by repeated dilution and concentration with a Centricon YM30 (*see* **Note 11**).

3.4.5. Wild-Type βγ Subunit

Because βγ subunit expresses at much higher level than the α subunits described previously, the purification from 1 L of Sf9 cells is usually enough for most of the experimental purposes.

1. To purify wild-type $\beta_1\gamma_2$ subunit , 1 L of Sf9 cells are infected with recombinant baculoviruses encoding β_1, γ_2, and 6-His-$G_{i1}\alpha$, the membrane extracts are prepared and loaded onto 1 mL Ni-NTA agarose column following the same procedure as described in **Subheadings 3.2.** and **3.3.**
2. Then the column is washed with 25 mL buffer B and 10 mL buffer O: 20 m*M* HEPES, pH 8.0, 100 m*M* NaCl, 1 m*M* $MgCl_2$, 10 m*M* 2-mercaptoethanol, 10 μ*M* GDP, 10 m*M* imidazole, 0.3% octyglucoside.

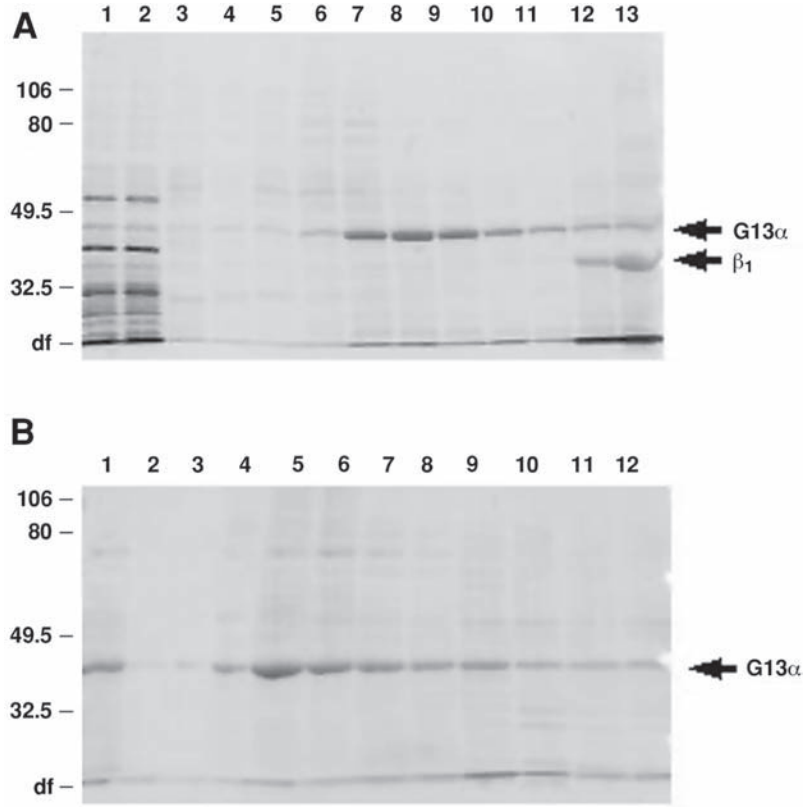

Fig. 4. Purification of $G_{13}\alpha$. **(A)** Ni-NTA column for purification of $G_{13}\alpha$. Fractions of 4 µL were subjected to SDS-PAGE and stained by silver nitrate. Lane 1, load; lane 2, flow-through; lanes 3–5, wash with low imidazole; lanes 6–11, elution with AMF; lanes 12 and 13, elution with 150 m*M* imidazole. **B.** Hydroxyapatite chromatography of $G_{13}\alpha$. The peak fractions from the Ni-NTA column were loaded onto a hydroxyapatite column and chromatographed as described. Fractions of 5 µL were subjected to SDS-PAGE and stained by silver nitrate. Lane 1, load; lane 2, flow-through; lane 3, wash ; lanes 4–8, elution with 150 m*M* KPi; lanes 9–11, elution with 300 m*M* KPi.

3. The column is incubated at room temperature for 15 min and washed with 4 mL buffer P (buffer O containing AMF and 1% octylglucoside) and 4 mL buffer Q (buffer O containing 150 m*M* imidazole and 1% octylglucoside).
4. 1-mL Fractions are collected. $\beta_1\gamma_2$ or 6-His-$G_{i1}\alpha$ elutes in fractions with buffer P or buffer Q, respectively (**Fig. 5A**).
5. The elution fractions from Ni-NTA column can be further purified by Mono Q chromatography. The peak fractions are diluted threefold with buffer S: 20 m*M*

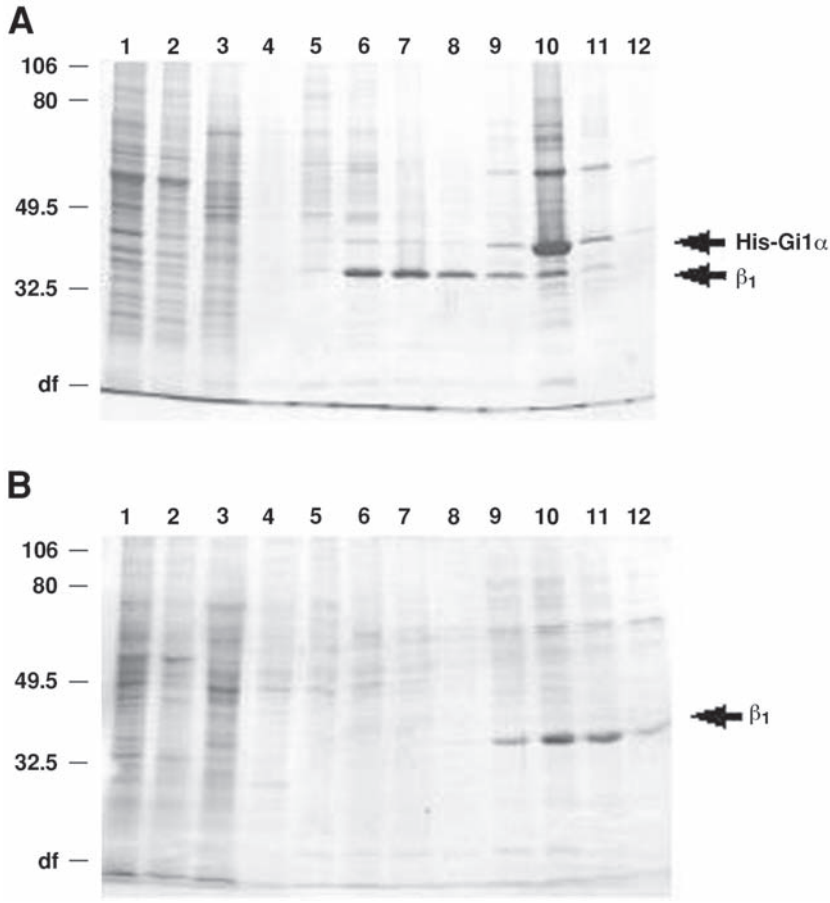

Fig. 5. Purification of βγ subunit.Ni-NTA column for purification of wild-type $\beta_1\gamma_2$ **(A)** or 6-His-$\beta_1\gamma_2$ **(B)** Fractions of 4 μL were subjected to SDS-PAGE and stained by silver nitrate. Lane 1, load; lane 2, flowthrough; lanes 3–5, wash with high salt and low imidazole; lanes 6–11, elution with AMF; lanes 12 and 13, elution with 150 m*M* imidazole.

HEPES, pH 8.0, 0.5 m*M* EDTA, 2 m*M* $MgCl_2$, 1 m*M* DTT, 0.7% CHAPS. The fractions are loaded onto Mono Q HR5/5 column that was equilibrated with buffer S. 0.7% CHAPS in buffer S can be replaced with 1% octylglucoside.

6. $\beta_1\gamma_2$ elutes from the column with a 20-mL gradient of 0–400 m*M* NaCl. Fractions of 0.5 mL are collected. Peak fractions at approx 200 m*M* NaCl are concentrated and the buffer is exchanged into buffer S containing 100 m*M* NaCl by Centricon 30 (*see* **Note 12**).

3.4.6. Purification of 6-His-Tagged βγ Dimers

Functionally active βγ subunit can also be purified without Gα coexpression using either 6-His-tagged β or γ subunits ***(19,20)***.

1. Sf9 cells are infected with Baculoviruses encoding β_1 and 6-His-γ_2.
2. Membrane extracts are loaded on Ni-NTA column as described previously.
3. The column is processed with the same procedure for wild-type βγ. Washing with AMF-containing buffer removes endogenous Sf9 Gα subunits. However, this step can be omitted, and the entire procedure can be performed at 4°C. **Figure 5B** shows the elution profile.
4. The peak fractions are concentrated and the buffer is exchanged into buffer S containing 100 m*M* NaCl by Centricon-30 (*see* **Note 13**).

4. Notes

1. We keep low passage viruses as the progenitor stocks at 4°C and at –80°C. The operating virus stocks (usually 200–500 mL/batch) are amplified from these stocks and are stored at 4°C. These viral stocks are stable for at least several months.
2. Preparation of IPL-41 medium from powder (JRH) is more cost effective for large-scale cultures.
3. Early passage cells are frozen in medium containing 10% FBS and 10% DMSO and can also be used as cell stocks. Cells from one vial of frozen stock can be maintained for approx 4–6 mo. We change to a new frozen stock when we start to see cells with irregular shapes, a decrease of growth rate, or a reduction in the expression level of recombinant proteins. For large-scale culture >1 L, IPL-41 medium containing 1% FBS, 1% chemically defined lipid concentrate, and 0.1% pluronic F-68 is used.
4. To increase the yield of G protein subunit, the freshly amplified recombinant viruses are recommended. Among three G protein subunits, γ subunit usually expresses most efficiently. The excess expression of γ subunit may inhibit the expression of Gα subunit. Therefore, if the expression level of the Gα subunit to be purified is low, infecting less amount of 6-His-γ_2 (and/ or β_1) virus may be helpful to increase the Gα expression.
5. The presence of AMF and the increase in cholate concentration facilitate the dissociation of a subunit from βγ subunit on the column.
6. The yield of $G_z\alpha$ from 1500-mg membrane protein is approx 300 µg. It was demonstrated that $G_z\alpha$ purified with this method directly inhibited specific subtypes of adenylyl cyclases ***(12)***. Such activity could not be detected using $G_z\alpha$ produced in *E. coli*. It is likely that the myristoylation that is present in $G_z\alpha$ from Sf9 cells, but not from *E. coli*, is critical for the interaction with adenylyl cyclase.
7. $G_q\alpha$ has slow GDP–GTP exchange rate and cannot be activated by incubation with buffer F. In contrast, endogenous $G_i\alpha$ subunit has faster GDP–GTP exchange rate and is activated with this procedure. Contamination of Sf9 $G_i\alpha$ increases the GTPase activity of the purified $G_q\alpha$ sample and interferes with GTPase assays, especially assays with receptor reconstitution.

8. The final yield of recombinant $G_q\alpha$ is approx 200 mg from 1500-mg membrane protein. The same procedure can be applied for the purification of $G_{11}\alpha$. However, the yield of $G_{11}\alpha$ is much less than that of $G_q\alpha$. $G_{15/16}\alpha$ could not be purified using this method because of its low expression in Sf9 cells and its inability to be activated by AMF ***(11)***.
9. In order to perform GAP assays for $G\alpha$, GTP-bound form of $G\alpha$ has to be prepared as a substrate for the reaction. Because the intrinsic GDP–GTP exchange rate of $G_q\alpha$ is low, it is quite difficult to generate enough amount of GTP-bound $G_q\alpha$ by simply incubating $G_q\alpha$ with GTP. The agonist-activated receptor is usually required to facilitate the GDP–GTP exchange of $G_q\alpha$ ***(21)***. $G_q\alpha$R183C mutant in which arginine183 in switch I region is mutated to cysteine has much reduced GTP hydrolysis rate than wild-type. This helps to increase the fraction of GTP-bound $G_q\alpha$ subunit during incubation with GTP. Furthermore, this mutant can respond to GAP activities of RGS proteins. Since the receptor reconstitution system takes a lot of effort to establish, $G_q\alpha$R183C mutant has been used as a convenient alternative tool to detect GAP activity of $G_q\alpha$ ***(22,23)***. Although the affinity of $G_q\alpha$R183C to $\beta\gamma$ is lower than wild-type $G_q\alpha$, it can be purified on Ni-NTA column using the method as described previously for wild-type $G_q\alpha$. After the elution with AMF and 1% cholate containing buffer D, the peak elution fractions are concentrated, and the buffer was exchanged into buffer S with 100 m*M* NaCl and 5 m*M* GDP to remove AMF (final volume is <1 mL from 4-L culture). The final sample is not pure as wild-type $G_q\alpha$, but GAP assays of approx 20 tubes can usually be performed using 20–30 µL of the purified sample.
10. The presence of glycerol prevents the aggregation of the protein during concentration. It is also recommended to occasionally mix the sample gently during concentration by Centricon. The yield of $G_{12}\alpha$ from 1500-mg membrane is approx 200 µg.
11. Becuase $G_{13}\alpha$ tends to aggregate during concentration, it is also recommended to occasionally mix the sample gently during concentration by Centricon. The yield is approx 200 µg from 4 L of Sf9 culture.
12. The yield of $\beta_1\gamma_2$ from 1200-mg membrane is 1–2 mg. The same protocol can be applied to purify different combinations of $\beta\gamma$ subunits, such as $\beta_2\gamma_2$, $\beta_1\gamma_1$, $\beta_1\gamma_3$, $\beta_1\gamma_5$, or $\beta_1\gamma_7$. The methods to purify β_5 subunit complexed with γ_2 or RGS proteins with G protein γ subunit-like domain have been described in **refs. *24–26***.
13. Hexahistidine-tagged $\beta_1\gamma_2$ can be further purified over Mono Q column as described for wild-type $\beta\gamma$ subunit. The same procedure can be applied to purify the combination of 6-His-β_1 and γ_2. In both cases, the yield of hexahistidine-tagged $\beta_1\gamma_2$ is 1–2 mg from 1-L culture. The presence of hexahistidine tag at the amino terminus of β_1 or γ_2 does not interfere with the interactions with effectors. Mutant $\beta\gamma$ subunits that have less affinity for $G\alpha$ subunits can be purified with this method. Wild-type, nontagged $\beta\gamma$ subunit is recommended for receptor reconstitution assays. The imidazole bump fractions from Ni-NTA column of $G\alpha$ purification that contain hexahistidine tagged $\beta_1\gamma_2$ can also be used as sources to purify 6-His-$\beta_1\gamma_2$ with Mono Q column.

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3

Expression and Purification of Soluble Adenylyl Cyclase from *Escherichia coli*

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Summary

This chapter outlines methods to purify soluble adenylyl cyclase (AC7) expressed in an *Escherichia coli* (*E. coli*) heterologous expression system. Guidelines are provided for constructing the expression plasmids, optimizing expression, culturing, and purifying the proteins. Purification requires two chromatographic steps. A histidine tag (H6) is incorporated into the expression vector and utilized for affinity purification on a Ni-NTA column. Subsequently, an anion exchange column is employed to further purify the protein.

Key Words: Mammalian membrane-bound adenylyl cyclase; engineered soluble adenylyl cyclase; protein expression in *E. coli*; 3-5-cyclic adenosine monophosphate; Ni-NTA affinity purification.

1. Introduction

Adenylyl cyclase (AC), the enzyme that catalyzes the conversion of adenosine 5-triphosphate (ATP) to 3-5-cyclic adenosine monophosphate (cAMP), is a prototypical cell signaling molecule involved in regulating numerous physiological processes, including sugar and lipid metabolism, cardiac function, memory formation, olfaction, and cell growth and differentiation. Biochemical characterization of the catalysis mechanism and regulation of mammalian membrane-bound AC has proven challenging because purifying functional AC is difficult. AC is an integral membrane protein and labile, requiring detergent for purification.

A significant advance in the field was the development of soluble AC systems ***(1)***. All nine isoforms of mammalian membrane-bound AC share a common structure of a short cytoplasmic N-terminus, followed by a six-transmembrane spanning region (M1), a cytoplasmic loop (C1), another six-transmembrane spanning region (M2), and a second cytoplasmic loop (C2). The two cytoplasmic

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loops, C1 and C2, each contain a region (C1a and C2a, respectively) that is homologous with each other (~50% identity) and highly conserved across cyclases (50–90% identity) ***(2)***. When expressed as recombinant soluble proteins, C1a and C2a can be mixed together to reconstitute AC activity that exhibits many of the key properties of the full-length enzyme, including stimulation by $G_s\alpha$, Fsk, calcium/calmodulin, and inhibition by $G_i\alpha$ and P-site inhibitors ***(3–13)***.

Soluble AC systems have also enabled important advances in the study of the adenylyl cyclase enzyme, including a crystallographic structural model of the enzyme ***(14–19)***. Although a number of soluble AC systems have been developed ***(1,3,5,9–11,20)***, the construction of such a system is still not routine. One of the primary challenges is determining the initiation and termination points for C1a and C2. If C1 and C2 are constructed solely on sequence homology, they are typically subject to significant problems in proteolysis and/or folding, making it difficult to obtain a stable, functional, and uniform soluble protein. The method described here identifies the initiation and termination points we have arrived at for stable 7C1a and 7C2 proteins. Constructing other C1a and/or C2 isoforms would require making an initial hypothesis based on alignment of the target cyclase sequence with previously successful soluble C1a or C2 proteins, followed by empirically testing various constructs using functional assays in combination with mass spectrometry to arrive at an optimal soluble protein ***(21)***.

A more difficult issue is the degree to which a soluble protein accurately reflects the properties of the full-length enzyme. In particular, the nonconserved region of C1, designated as C1b, is generally not included in soluble C1 proteins because it is labile and can significantly reduce the expression of C1 protein. However, considerable evidence suggests that the C1b region plays a crucial role in isoform-specific regulation of AC ***(3,4,8,9,11,22–26)***. In an effort to construct a soluble system with more verisimilitude with the full-length enzyme, we have constructed a soluble 7C1b protein that consists of C-terminal two-thirds of C1b, designated as 7C1b-4h. Initial analysis indicates that this protein has activity when added to 7C1a and 7C2, exhibiting a capacity to both stimulate and inhibit activity depending on conditions (Beeler, J. A. and Tang, W.-J., unpublished observations).

The method that is outlined focuses on purifying 7C1a, 7C2, and 7C1b-4h as model systems. To purify soluble proteins from isoforms other than AC7, this method could serve as a starting point, but would need to be modified to optimize expression and purification.

2. Materials

2.1. Constructing Expression Plasmid

1. pProEX-1 and the modified version of this plasmid, pProEx-H6, pProEx-HAH6, and pProEx-H6EE prokaryotic expression system (Invitrogen, cat. no.

10711-018; pProEx-HT, newer version of pProEx-1, www.invitrogen.com) (*see* **Note 1**).
2. Vent DNA polymerase (New England Biolabs, cat. no. M0254S, www.neb.com).
3. Thermal cycler.
4. Restriction enzymes (New England Biolabs, www.neb.com).
5. 10X DNA loading dye: 20% Ficoll 400, 0.1 *M* EDTA pH 8.0, 1% sodium dodecyl sulfate (SDS), 0.25% bromophenol blue, 0.25% xylene cyanol.
6. Agarose.
7. 10 mg/mL ethidium bromide in ddH_2O.
8. 1X TAE buffer: 40 m*M* Tris-acetate, 1 m*M* EDTA. 50X Stock solution: 242 g Tris-base, 57.1 mL glacial acetic acid, 100 mL 0.5 *M* EDTA, pH 8.0, ddH_2O to 1 L.
9. 1-Kb Plus DNA Ladder (Invitrogen, cat. no. 10787-018, www.invitrogen.com).
10. Wizard DNA Clean-up System (Promega, cat. no. A7260).
11. QIAEX II Gel Extraction Kit (Qiagen, cat. no. 20021).
12. T4 DNA Ligase (New England Biolabs, cat. no. M0202S, www.neb.com).
13. XL10Gold competent cells (Stratagene, www.stratagene.com)
14. SOB medium: 20 g Bacto-tryptone, 5 g yeast extract, 0.5 g NaCl, ddH_2O to approx 800 mL. Adjust pH to 7.5 using 1 *M* KOH. Add ddH_2O to 780 mL. Autoclave. Add 20 mL filtered (0.2 µm) 1 *M* $MgSO_4$.
15. SOC medium: Add 0.5 mL 2 *M* sterile glucose to 49.5 mL SOB.
16. Ampicillin (AMP): 100 mg/mL in water, filtered with 0.2 µm filter.
17. Kanamycin (KAN): 100 mg/mL in water, filtered with 0.2 µm filter.
18. Luria-Bertani (LB) medium: 10 g Bacto tryptone, 5 g yeast extract, 10 g NaCl, ddH_2O to 1 L. Autoclave.
19. LB-AMP agar plates.
20. DNA sequencing kit BigDye Terminator Cycle Sequencing Ready Reaction (Applied Biosystems, cat. no. 4303149, Foster City, CA).
21. Wizard Plus Midipreps DNA Purification System (Promega, cat. no. A7640, www.promega.com).

2.2. Optimizing Expression of Soluble Protein

1. BL21(DE3) competent cells (Stratagene, cat. no. 200131, www.stratagene.com) (*see* **Note 2**).
2. SOB medium, SOC medium, LB medium, LB-AMP plates (*see* **Subheading 2.1.**).
3. 0.1 *M* 3-Isobutyl-1-methylxanthine (IBMX) in double-distilled water (ddH_2O), filtered (0.2 µm filter).
4. 14-mL Falcon polypropylene round-bottom tubes.
5. T7 medium: 80 g Bacto-tryptone, 40 g yeast extract, 20 g NaCl, 16 mL 50% glycerol, 200 mL potassium phosphate buffer (720 mL 1 *M* K_sHPO_4 + 280 mL 1 *M* KH_2PO_4). Adjust pH to 7.3 with 10 *N* NaOH. Autoclave.
6. 96-Well plate reader with 595- or 600-nm wavelength filter or spectrophotometer.
7. Refrigerated shaker/incubator.
8. 1.5-mL Polyallomer microfuge tubes to spin at 200,000*g* (Beckman, cat. no. 357448).

9. Table-top ultracentrifuge with fixed angle rotor for 1.5-mL microfuge tubes.
10. Lysis buffer for small samples: 20 m*M* Tris-HCl, pH 8.0, 10 m*M* β-mercaptoethanol, 0.1 mg/mL lysozyme, 100 μ*M* phenylmethanesulfonyl fluoride (PMSF; *see* **Note 3**).
11. Equipment to cast and run SDS-polyacrylamide gels (Amersham Biosciences, www.amershambiosciences.com).
12. Reagents for SDS-polyacrylamide gels: 30% acrylamide/bis solution, 1 *M* Tris-HCl, pH 8.6 and 0.5 *M* Tris-HCl, pH 6.8, TEMED, 10% ammonium persulfate, 10% SDS.
13. 4X SDS-polyacrylamide gel electrophoresis (PAGE) loading buffer: for 10 mL, 2.5 mL 0.5 *M* Tris-HCl, pH 6.8, 4 mL glycerol, 0.8 g SDS, 0.1 mg Bromophenol blue, ddH_2O to 10 mL. Aliquot 500 μL and store at –20°C. Prior to use, add 100 μL/mL dithiothreitol (DTT) and 40 μL/mL β-mercaptoethanol.
14. SDS-PAGE running buffer: 25 m*M* Tris-base, 250 m*M* glycine, pH 8.3, 0.1% SDS.
15. Optitran supported nitrocellulose transfer and immobilization membrane (Schleicher & Schuell, cat. no. 10439380, Keene, NH).
16. Equipment for gel transfer (Amersham Biosciences, www.amershambiosciences.com).
17. Anti-H5 monoclonal antibody (Qiagen, cat. no. 34660, www.qiagen.com).
18. Horseradish peroxidase (HRP)-conjugated anti-mouse IgG (γ chain) monoclonal antibody (Sigma-Aldrich, cat. no. A3673, St. Louis, MO).
19. 1X Transfer buffer: 25 m*M* Tris base, 250 m*M* glycine, electrophoresis grade, pH 8.3, 0.1% SDS, 20% methanol.
20. PBST: 80 m*M* Na_2HPO_4, 20 m*M* KH_2PO_4, 100 m*M* NaCl, 0.1% Tween-20, pH 7.5.
21. Fat-free powdered milk.
22. Supersignal West Pico Chemiluminescent Substrate (Pierce, cat. no. 34080, www.piercenet.com).
23. Digital imaging system or film for visualizing enhanced chemiluminesence (ECL) blots (Kodak Image Station, cat. no. 440CF).

2.3. Culturing and Purifying Protein

1. BL21(DE3) competent cells, isopropyl β-D-thiogalactopyranoside (IPTG), T7 medium, LB medium, and AMP (*see* **Subheading 2.2.**).
2. PMSF: 0.1 *M* stock in 100% ethanol.
3. Four 500-mL or 1-L centrifuge bottles for super-speed centrifuge.
4. Super-speed refrigerated centrifuge.
5. Lysis buffer for purification: 20 m*M* Tris-HCl, pH 8.0, 100 μ*M* PMSF, 5 m*M* β-mercaptoethanol, 100 m*M* NaCl (*see* **Note 4**).
6. Lysozyme lyophilized, from chicken egg white (Sigma-Aldrich, cat. no. L6876, St. Louis, MO).
7. 18-Gauge hypodermic needle and 30cc syringe.
8. 50-mL Polystyrene Falcon tubes.
9. Sonic dismembrator, probe size appropriate for 50-mL vol (i.e., 1-cm tip probe).
10. Ultracentrifuge with fixed angle rotor and centrifuge tubes.

11. Equipment and columns for protein purification capable of regulating flow rate, fractionation and buffer gradients (e.g., fast protein liquid chromatography [FPLC], Gradi-fract, Akta-prime; consult Amersham Biosciences, www.amershambiosciences.com).
12. Ni-NTA Superflow resin (Qiagen, cat. no. 30410, www.qiagen.com).
13. Q Sepharose High Performance resin (Amersham Biosciences, cat. no. 17-1014-01, www.amershambiosciences.com) (*see* **Note 5**).
14. Ni-NTA buffer 1 (equilibration buffer): 20 m*M* Tris-HCl, pH 8.0, 100 m*M* NaCl, 100 µ*M* PMSF, 5 m*M* β-mercaptoethanol.
15. Ni-NTA buffer 2 (high-salt wash): 20 m*M* Tris-HCl, pH 8.0, 500 m*M* NaCl, 100 µ*M* PMSF, 5 m*M* β-mercaptoethanol.
16. Ni-NTA buffer 3 (low-imidazole wash): 20 m*M* Tris-HCl, pH 8.0, 100 m*M* NaCl, 100 µ*M* PMSF, 5 m*M* β-mercaptoethanol, 20 mM imidazole, pH 7.0.
17. Ni-NTA buffer 4 (elution buffer): 20 m*M* Tris-HCl, pH 8.0, 100 m*M* NaCl, 100 µ*M* PMSF, 5 m*M* β-mercaptoethanol, 200 m*M* imidazole, pH 7.0.
18. Q-Sepharose buffer A (no salt): 20 m*M* Tris-HCl, pH 8.0, 1 m*M* DTT, 100 µ*M* PMSF, 1 m*M* EDTA.
19. Q-Ssepharose buffer B (1 *M* NaCl): 20 m*M* Tris-HCl, pH 8.0, 1 m*M* DTT, 100 µ*M* PMSF, 1 m*M* EDTA, 1 *M* NaCl.
20. SDS-PAGE equipment and reagents as in **Subheading 2.1.**
21. Coomassie blue staining solution: for 1 L, 300 mL isopropanol, 100 mL glacial acetic acid, 2.5 g Coomassie Brilliant blue R-250, 600 mL H_2O.
22. Coomassie blue destaining solution: 300 mL methanol, 100 mL glacial acetic acid, 600 mL H_2O.
23. $H_{2O}M_2E_1$ buffer: 20 m*M* HEPES, pH 8.0, 2 m*M* $MgCl_2$, 1 m*M* EDTA.
24. Recombinant $G_s\alpha$ protein ***(27)***.
25. 1 mg/mL Pyruvate kinase (Roche Diagnostics, cat. no. 0128155, Indianapolis, IN).
26. 10 m*M* Forskolin in dimethylsulfoxide (DMSO).
27. 10 m*M* $AlCl_3$.
28. 0.5 *M* NaF.
29. ^{32}P-ATP (NEN, cat. no. NEG003X, http://lifesciences.perkinelmer.com).
30. ^{3}HcAMP (NEN, cat. no. NET275, http://lifesciences.perkinelmer.com).
31. Stop solution for assay: 2.5% SDS, 50 m*M* ATP, 1.75 m*M* cAMP.
32. 0.15 *M* K_2PEP: 0.15 *M* potassium phosphenolpyruvate (Sigma-Aldrich, cat. no. P7127, St. Louis, MO) brought to pH 7.0 with 5 *M* potassium hydroxide (potassium ion is required).
33. Basic cocktail mix (BCT): 1.67 m*M* ATP, 33.3 m*M* $MgCl_2$, 3.3 m*M* EDTA, 0.33 mg/mL bovine serum albumin (BSA), 166.66 m*M* Na-HEPES, pH 8.0, 10 m*M* K_2PEP (**step 32**), 666.67 µ*M* 4-(3-butoxy-4-methoxybenzyl)-2-imidazdidinone (BMI, Calbiochem, cat. no. 557502, La Jolla, CA).
34. Columns for assay: large plastic pipet tips as columns (Baxter Scientific Products, cat. no. P5079-5 [for dowex] and cat. no. P5079-6 [for alumina], McGaw Park, IL).
35. AG501-X8 (dowex) resin (Bio-Rad Laboratories, cat. no. 142-6424, Hercules, CA).

36. Alumina (Sigma-Aldrich, cat. no. A9003, St. Louis, MO)
37. 1 *N* HCl.
38. Imidazole buffer for assay: 200 m*M* NaCl, 20 m*M* imidazole, pH 7.5.
39. Biosafe II scintillation cocktail (Research Products International, cat. no. 111195).
40. 50-mL capacity Amicon-stirred cell filtration device (Model 8050) and associated equipment (Millipore, www.millipore.com/amicon).
41. Amicon YM10 and YM3 ultrafiltration discs (NMWL 10,000) 44.5-mm diameter (Millipore, cat. no. 13622 and 13422, www.millipore.com/amicon).
42. Centricon YM10 and YM3 centrifugal filter units (Millipore, www.millipore.com/amicon).
43. Protein assay dye reagent (Bio-Rad, cat. no. 500-0006, Hercules, CA).

3. Methods

3.1. Constructing Expression Plasmid

1. Select restriction sites for subcloning target sequence in pProExH6 vector (*see* **Note 1**).
2. Amplify target sequence from full-length AC cDNA using polymerase chain reaction (PCR, 100 µL reaction; *see* **Note 6**).
3. Set up restriction digest of pProExH6 vector and PCR product, with selected restriction enzymes (*see* **Note 7**).
4. Take 2–5 µL samples (approx 100–300 ng) of digested PCR product, digested vector, undigested PCR product and undigested vector and add 1 µL 10X bromophenol blue-loading dye and ddH_2O to a final volume of 10 µL for each sample.
5. Load samples on a 1% agarose gel (0.5 µg/mL ethidium bromide in gel) in 1X TAE buffer. Load 100 ng 1 kb + DNA ladder and run gel at 96 V for approx 30 min.
6. Visualize gel bands on ultraviolet illuminator to verify digestion.
7. Clean digested DNAs using a clean up kit (e.g., Promega Wizard DNA Clean-up system, cat. no. A7260).
8. Add 5 µL bromophenol blue-loading dye to 45 µL each of digested and cleaned insert and vector and load onto a 1.0% agarose gel, without ethidium bromide, and run at 50 V for 2–3 h to obtain maximum separation of DNA bands.
9. Stain gel in 50 mL of 1X TAE buffer with 0.5 µg/mL ethidium bromide for 10–15 min. Place stained gel on ultraviolet illuminator, excise DNA bands, and recover DNA from gel using DNA extracting kit (e.g., QIAEX II Gel Extraction Kit, cat. no. 20021).
10. Again load approx 5 µL purified insert and vector as in **step 4**, visualize gel under ultraviolet light, and estimate concentrations of DNA.
11. Set up ligation reaction using T4 ligase. Total volume should be 30 µL with total DNA in reaction at approx 100 ng in a 10:1 molar ratio of insert:vector. Reaction includes 0.75 µL T4 ligase (300 enzyme units), 3 µL 10X T4 ligase buffer, DNA, and ddH_2O to 30 µL. Incubate reaction overnight at 16°C. Set up a vector-only ligation as a control.
12. Transform ligation reactions into *E. coli*-competent cells (e.g., XL1Blue or XL10Gold) using 20 µL ligation reaction, including vector-only control, and

200 µL-competent cells. After thawing competent cells on ice and adding DNA, incubate them on ice for 1 h, then heat shock cells at 42°C for 1.5 min, followed immediately by 2 min on ice. Add 500 µL SOC medium and place in 37°C shaking incubator for 1 h. Plate on LB-AMP plates at various volumes (i.e., plate 50, 100, 250, 500 µL) and place in 37°C incubator overnight.

13. Ligation reaction of vector and insert should produce colonies in considerable excess of vector-only control. Inoculate 10–20 colonies from the cells transformed with the ligation mix containing insert and vector into 3 mL LB-AMP and shake overnight at 37°C.
14. From each inoculation, remove 500 µL cells and place in microfuge tube with 500 µL 50% sterile glycerol and freeze at –80°C for frozen stock for future use.
15. Prepare mini-prep DNA from inoculated colonies using method of choice (*see* **Note 8**) and load 5-µL miniprep DNA from each colony onto an agarose gel as in **step 4** to estimate DNA concentration.
16. Set up restriction enzyme digestion to test for positive clones. Use approx 100–300 ng DNA and visualize bands with ultraviolet (UV) light (*see* **Note 9**).
17. Verify correct sequence of positive clones by DNA sequencing (e.g., BigDye Terminator Cycle Sequencing Ready Reaction or other methods of choice).

3.2. Optimizing Expression of Soluble Protein

1. Transform expression plasmid purified previously into *E. coli*-competent cells (e.g., BL21[DE3]) (*see* **Note 2**) using 10–50 ng of midiprep DNA to 100 µL of cells. After thawing cells on ice and adding DNA, incubate them on ice for 1 h. Heat shock at 42°C for 1.5 min and immediately place on ice for 2 min. Add 0.8 mL SOC medium and place in shaking incubator at 37°C for 1 h. Plate on LB-AMP plates (LB-AMP-KAN if pUBS520 plasmid present), plating 50 and 200 µL. Incubate overnight at 37°C. Transform the expression vector alone as well as a negative control.
2. Select three colonies and inoculate into 3 mL LB-AMP (+KAN if using pUBS520) overnight at 30°C. Select one colony from vector control and also inoculate.
3. Set up screen to test various expression conditions. Typically, we initially test two inductions temperatures (30° and 25°C), two IPTG concentrations (30 µ*M* and 100 µ*M*) and three induction times (4 and 8 h and overnight), and all three colonies (*see* **Note 10**). Using 14-mL Falcon round bottom polyethelene tubes for 6-mL cultures. Each colony using the previous conditions would require four cultures (i.e., two at 30°C for each IPTG concentration and two at 25°C for each IPTG concentration). Thus, testing three colonies necessitates twelve 6-mL cultures (the time points are drawn from the same cultures). A culture should be set up for the vector control as well, however, testing only 30°C and 100 µ*M* is sufficient for the control.
4. Add 6-mL warm T7 media into each culture tube and transfer 60 µL of cells from the overnight inoculation to the appropriate cultures (i.e., colony 1, 2, 3, and vector only). Place all cultures in 30°C shaking incubator. Grow cultures for 2 h, then remove from each culture 250-µL sample and place in 96-well plate reader

and check OD_{600}. Target density for induction is 0.4 OD_{600}. Continue growing, checking OD as needed to determine when cells are between 0.4 and 0.45 OD_{600}.

5. When cells have reached appropriate density, add IPTG at concentrations being tested. Place cultures to be grown at 25°C in refrigerated incubator.
6. At 4 h, remove from each culture 1.5-mL cells and place in clearly labeled microfuge tubes (use hard-walled Beckman tubes, cat. no. 357448, appropriate for ultracentrifuge in later steps). Pellet cells with microcentrifuge at maximum speed for 1 min. Aspirate all medium and freeze samples at –80°C. Repeat this process for all cultures at 8 h and overnight time points.
7. To prepare lysates, resuspend thawed cell pellets in 150 µL lysis buffer. Incubate on ice for 30 min. Centrifuge samples at 200,000*g* for 20 min at 4°C. Transfer supernatant into appropriately labeled microfuge tubes and freeze on dry ice. Store at –80°C.
8. Prepare SDS-PAGE mini-gel (11% for C1a and C2, 15% for C1b) using 15 µL wells (15 wells per gel). Prepare samples (*see* **Note 11**) using 10 µL lysate and 3.3 µL 4X SDS-PAGE loading buffer (with freshly added 100 m*M* DTT and 0.5 *M* β-mercaptoethanol. Denature protein in 100°C heat block for 5 min. Load entire sample onto gel and run gel at 250 V until dye front has reached bottom of gel.
9. Transfer protein to nitrocellulose blot. Place transfer cassette and foam sponges into dish with sufficient 1X transfer buffer so that the apparatus and blot may be kept under the buffer. Remove gel from plate assembly and place on blotting paper moistened in 1X transfer buffer. Moisten nitrocellulose blot of appropriate size and place on gel, being careful not to introduce air bubbles. Place a second moistened blotting paper on top of the nitrocellulose. Ensure that no air bubbles have been introduced, then close and clip the transfer cassette, placing it in the transfer tank unit, orienting the gel toward the negative pole and the nitrocellulose toward the positive. Transfer at 80 V for 2 h.
10. Remove blot and place in 5% fat-free milk made in PBST and block overnight at 4°C.
11. Perform Western blot analysis using chemiluminescent kit of choice following manufacturer's instructions (e.g., Pierce SuperSignal West Pico Chemiluminescent Substrate, cat. no. 34080). Our standard protocol is to wash the blot once for 15 min in PBST, then twice for 5 min in PBST. We then apply the primary antibody diluted in PBST for 1 h, repeat wash, apply the secondary HRP-conjugated antibody diluted in PBST for 1 h, then repeat wash (*see* **Note 12**). Chemiluminescent reagents are then applied and gel visualized using either film or digital imager (e.g., Kodak Image Station 440CF).
12. Analyze gel to determine which conditions yield the greatest expression.

3.3. Culturing and Purifying Protein

1. Inoculate the appropriate BL21(DE3) frozen cell stock into 3 mL LB-AMP (include KAN if pUBS520 plasmid present) and grow overnight at 30°C. Make 4 L (*see* **Note 13**) of T7 medium and autoclave. Prepare columns according to manufacturer's instructions (manuals can be found online at www.apbiotech.com and www.qiagen.com).

2. Add ampicillin to medium (final concentration of 50 μg/mL) (*see* **Note 14**). Take 1-mL sample of medium to blank OD readings later. Add 1 mL of inoculated cells to each liter of T7 and incubate at 30°C in shaking incubator. Grow to OD_{600} of 0.4–0.45 (typically 4–6 h). Add IPTG at concentration determined to be optimal in expression test and grow at the optimal temperature and time.
3. To harvest cells, place cultures on ice and add PMSF to final concentration of 100 μ*M* (i.e., 1:1000 dilution of 100 m*M* stock). Place in two to four centrifuge bottles (smaller bottles may require pouring out supernatant, refilling with additional culture, and centrifuging again, perhaps multiple times) and spin for 15–20 min at 12,000*g*. Remove all supernatant and freeze/store pellet at –80°C.
4. To prepare lysate from 4 L culture, thaw cell pellets on ice for approx 10–15 min and resuspend in 200 mL lysis buffer, aliquoting buffer between all bottles saving approx 50 mL of buffer for later step. Combine resuspended cells into one bottle and pass through 18-gauge syringe to further resuspend cells. Add 0.2 mg of lysozyme to unused buffer and add to preparation and stir. Aliquot preparation into 50-mL Falcon tubes, adding approx 40–45 mL to each tube. Keep on ice.
5. Sonicate preparation on ice at maximum level using a cycle of 1 s on and 3 s off to prevent overheating of preparation for a total of 1 min sonication. Typically, the preparation will turn darker after sonication. After sonication, incubate on ice for 30 min.
6. Combine preparation into tubes for ultracentrifuge (prechill tubes and rotor) and centrifuge for 30 min at 95,000*g*. Collect and combine supernatant for loading onto Ni-NTA column. Add additional 200 μL PMSF and keep at 4°C.
7. Attach Ni-NTA column to purification equipment and equilibrate column with Ni-NTA buffer 1 (pump 1–2 column volumes). Zero chart recorder. Load lysate at 3 mL/min flow rate, saving approx 1 mL for subsequent analysis. Collect flow-through.
8. Wash column with Ni-NTA buffer 2 (high salt) and buffer 3 (low imidazole) at 2 mL/min flowrate for approx 12 and 6 column volumes, respectively collect flow-through.
9. Elute with Ni-NTA buffer 4 (high -imidazole) into twenty 6-mL fractions.
10. Analyze 10 μL lysate, flow through, washes, and fractions by SDS-PAGE and Coomassie blue staining (staining gel with Coomassie blue stain for 25–30 min and destaining for additional 30 min; *see* **Note 15**). Pool the fractions containing the expressed protein (typically from fractions 3–12. Dilute 1:5 in Q-Sepharose buffer A to reduce salt concentration to 20 m*M*. Load onto Q-Sepharose column using 3 mL/min flow rate. Collect flow-through.
11. Set up elution gradient using buffer A (no NaCl) and Buffer B (1 *M* NaCl). Elute protein from Q-Sepharose column using a 2 mL/min flow rate and 3-min fractions (*see* **Note 16**). Using chart recording from UV reading, identify peaks. Using 10-μL samples as in **step 9**, run peak fractions, Q-Sepharose load, and Q-Sepharose flow-through on SDS-PAGE to determine fractions containing expressed protein.

12. Conduct AC assay to determine/confirm activity of expressed protein. Include in assay samples from: lysate, Ni-NTA flow-through, Ni-NTA flow-through from wash 1 and 2, Q-Sepharose load, Q-Sepharose flow-through, and all Q-Sepharose fractions that potentially have protein (*see* **Notes 17** and **18**). Use 10 mL of sample for each 100-mL assay reaction. For C1a and C2, add 1–2 ug of the complementary soluble protein (i.e., C1a complementary to C2) for each assay reaction (*see* **Note 19**). We typically perform 100-µL reactions comprised of 50 µL of assay mix and 50 µL of sample preparation per reaction. Per reaction, the assay mix contains 30 µL BCT (final concentrations: 0.5 m*M* ATP, 10 m*M* $MgCl_2$, 1 m*M* Na-EDTA, 0.1 mg/mL BSA, 50 m*M* HEPES, pH 8.0, 3 m*M* K_2PEP and 200 µ*M* BMI) and 1 µg pyruvate kinase, plus activators, ^{32}P-labeled ATP and water to 50 µL/per reaction. We typically stimulate with final concentrations of 1 µ*M* $G_s\alpha$, 50 µ*M* $AlCl_3$, 10 m*M* NaF, and 100 µ*M* Forskolin. ^{32}P-ATP should yield approx 100,000 cpm/5 µL of assay mix. All preparation should be done on ice and the assay conducted at 30°C for 20 min. Activity should confirm the presence of expressed protein in fractions identified in previous step through Coomassie staining. For detailed instructions on setting up AC assay, *see* **ref. *28***.
13. Pool fractions that show activity (*see* **Note 20**) and concentrate to 10 mL using Amicon filtration device at 40–55 psi (maximum is 70 psi). Use YM10 filters for C1a and C2 (YM3 filters for C1b). Based on chart recorder graph, calculate an estimated salt concentration based on where gradient protein eluted. Dilute and concentrate using Amicon (repeated cycles may be required) so that salt concentration is approx 20 m*M* or less. Final volume should be 5–10 mL (*see* **Note 21**).
14. Prepare two centricon filtration devices (10 C1a and C2, 3 C1b) by adding 500 µL buffer A and centrifuging at recommended speed for 5 min at 4°C. Empty wash and load protein from Amicon into two centricon devices and centrifuge at recommended speed for 30–45 min until volume reduced to approx 500 µL. Repeat process as necessary until all of protein solution from Amicon device has been loaded into centricon device. Final concentration should result in 0.8–1 mL protein solution. Make 50- or 100-µL aliquots and freeze protein on dry ice. Store at –80°C.
15. Determine protein concentration using method of choice (e.g., Bradford method; *see* **Note 22**).

4. Notes

1. We use a pProEx-1 prokaryotic expression vector in which we have inserted either an H6, HAH6, or H6EE tag immediately upstream the multiple cloning site (MCS) ***(5)***.
2. Codon bias may affect expression if AGA and AGG codons, which are commonly used in eukaryotic genes, but rare in *E. coli*, constituting more than 2% of codons in cDNA of target protein. To improve expression in these cases, we use BL21(DE3)-competent cells transformed with pUBS520, which expresses dnaY that enhances expression of protein with high-codon bias ***(29)***. pUBS520 con-

tains a p15α origin of replication and is compatible with the pBR322-based pProEx-1. pUBS520 encodes a KAN selection marker, necessitating the use of KAN along with AMP (pProEx-1 antibiotic-resistant marker) to ensure propogation of both plasmids. Use of pUBS520 will enhance 7C2 expression.

3. Additional protease inhibitors may be used. For purification of 7C1b-4h, we include 0.5 μg/mL aprotinin, 0.1 μ*M* pepstatin A, and 0.1 m*M* benzamidine.
4. Again additional protease inhibitors may be used (i.e., for 7C1b-4h). If used for purification, we typically include the additional protease inhibitors in the lysis buffer and all buffers for the first purification step (Ni-NTA), then use only PMSF and EDTA during subsequent steps (Q-Sepharose).
5. Both Ni-NTA and Q-Sepharose columns may be regenerated multiple times.
6. Selecting a functional initiation and termination point for C1a, C2, and C1b has to be empirically determined. The N-terminus of C2 ***(3,8)***, as defined by sequence homology, is subject to proteolysis. Consequently we initiate our soluble 7C2 at amino acid (aa) 864, which excludes 50 residues from the N-terminus of the highly conserved 7C2 region. Similarly, we find that 7C1a expresses stably from aa 263–476, which excludes 66 residues from the N-terminus of the highly conserved 7C1a region and adds 21 residues to the C-terminus that is typically considered to be within the 7C1b region. 7C1b domain when expressed as a soluble protein is highly unstable. We have expressed a stable 7C1b protein, named 7C1b-4h, which is comprised of aa 506–584. 7C1b-4h exhibits activity when added to 7C1a/7C2, showing both stimulation and inhibition depending on conditions (Beeler, J. A. and Tang, W.-J., unpublished observations). All AC7 soluble proteins were constructed from human AC7.
7. 5 μL PCR product may be run on 1% agarose gel to estimate concentration and confirm PCR reaction before proceeding to the next step.
8. We generally use either alkali lysis or boiling method. Consult any molecular biology protocol book ***(30)***.
9. For C1a and C2, digesting with the sites used for cloning will yield a fragment of sufficient size to clearly differentiate positive and negative clones. Given the small size of C1b (249 bp), we typically select restriction sites that yield a 600–800-bp fragment. This makes screening for positive clones simpler as the difference between 800- and 1050-bp fragments is easily observed.
10. Additional parameters to vary would include cell density prior to induction (i.e., 0.4–1.5) and ± chloramphenicol ***(27)***, as well as higher and lower temperatures (i.e., 37°C or 22°C).
11. Exhaustive testing of conditions may yield a high number of samples to be checked. Typically, only a subset at a time is examined, such as all conditions for one colony. Subsequently, we would select the conditions that show better expression and compare those among colonies. Generally, thorough expression testing will involve some sequential strategy of checking samples as opposed to running innumerable gels and all samples at once.
12. The antibodies used for blotting depend on initial expression plasmid. Anti-H5 antibody will always work for His-tagged proteins. We also use anti-EE mono-

clonal antibodies and 12CA5, which targets the glu-glu (EE) and hemagglutinin (HA) epitope tags, respectively.

13. 8- or 12-L cultures may also be used without requiring a commensurate increase in lysis buffer. For 8-L cultures 200–300 mL lysis buffer is adequate. For 12-L cultures, use 300–400 mL lysis buffer. Four liters provide reasonable yield for 7C1a and 7C2. Generally, 8 L is necessary for 7C1b.
14. For large cultures of BL21(DE3) cells with pUBS520, we generally discontinue the KAN after the inoculation step.
15. Sampling strategy alternatives include sampling only peaks, every other fraction, or the first 10–12 only. Generally, His-tagged proteins primarily elute from the Ni-NTA column (200 m*M* imidazole elution buffer) in the second or third fraction with diminished elution continuing up to 12 additional fractions.
16. 7C1a and 7C2 are eluted with a 500-mL linear gradient of 0–500 m*M* NaCl. 7C1b is eluted with a 500-mL linear gradient of 100–400 m*M* NaCl.
17. Again, a variety of sampling strategies may be employed, including every other fraction, sampling peak fractions or a combination of these.
18. Assaying each step (e.g., lysate, washes, Q-Sepharose load, flow-through) allows the calculation of total initial activity (i.e., activity from lysate multiplied by starting volume of lysate) and estimation in percentages of where that activity went throughout the purification process. Additionally, specific activity may be calculated at each purification step to quantitatively determine the increase in purified activity ***(21)***.
19. If purified complementary protein is not available, 10 µL lysate prepared from cultures expressing the complementary protein may be used instead and should be prepared in advance.
20. Both the Coomassie blue-stained gel and the assay should be examined to determine fractions to pool and concentrate. In some instances, fraction exhibiting activity may be excluded as they contain less target protein and more contamination.
21. 7C1b-4h precipitates easily when salt concentration is reduced. Consequently, we maintain a 50 m*M* NaCl concentration in all subsequent buffers. After pooling the Q-Sepharose fractions, we use the Amicon ultrafiltration device to concentrate to approx 10 mL without diluting, then use dialysis to set the NaCl concentration to 50 m*M* (i.e., dialyze against buffer A with 50 m*M* NaCl). We use Spectra/Por 1 membrane, molecular weight cut-off (MWCO) 6000–8000. After the dialysis, we continue on with the centricon step.
22. Typically, 7C1a will yield approx 5 mg/L of *E. coli* culture; 10 mg/L 7C2; 0.3–0.8 mg/L 7C1b (**Fig. 1**).

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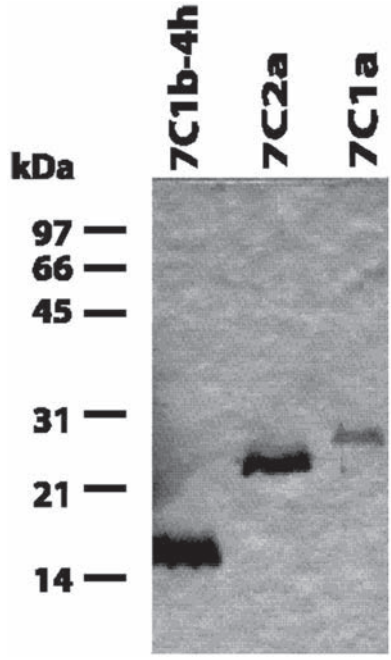

Fig. 1. Purified 7C1a, 7C2a, and 7C1b-4h. 1 µg 7C1a, 2 µg 7C2, and 7C1b-4h were run on 15% SDS-PAGE gel and stained with Coomassie blue.

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4

Purification of Phospholipase C β and Phospholipase C ε from Sf9 Cells

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Summary

Phosphatidylinositol-specific phospholipase C (PLC) enzymes catalyze hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP_2) generating diacylglycerol (DAG) and inositol 1,4,5-triphosphate (IP_3). The PLCβ isoforms of PLCs are activated by G proteins after hormone or neurotransmitter stimulation of G protein-coupled receptors (GPCR). PLCε is a recently identified PLC isoform that is activated by Ras and Gβγ subunit although the physiological role of this enzyme is not well understood. Methods for purification of PLCβ and PLCε from Sf9 cells are described. In the case of hexahistidine (6-His)-tagged PLC β the purification involves two steps, affinity chromatography with Ni-NTA agarose followed by heparin Sepharose chromatography. 6-His-tagged PLCε can be purified in a single step with nickel nitrilotriacetic acid-agarose (Ni-NTA) affinity chromatography.

Key Words: Phospholipase C β; phospholipase C ε; purification; G protein; affinity chromatography; Sf9 cells.

1. Introduction

Phosphoinositide-specific phospholipase C (PLC) enzymes carry out the hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP_2) to generate the two second messengers, diacylglycerol (DAG) and inositol-1,4,5-triphosphate (IP_3), which activate protein kinase C and cause release of intracellular Ca^{2+}, respectively ***(1)***. The PLCβ isoforms of PLC are activated by heterotrimeric G proteins in response to the extracellular signal-mediated activation of G protein-coupled receptors (GPCR). This activation can be reconstituted in an in vitro assay with purified PLC and G proteins, which is described in Chapter 5.

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PLCε is a novel member of the PLC isoform family. It has homology to other PLC isoforms in the catalytic X and Y domains, but differs from these other isoforms in that it contains two Ras-binding domains (RA domains) at the C-terminus and a Ras guanine nucleotide exchange factor-like domain in the N-terminal domain ***(2)***. When transfected into cells, PLCε activity is increased by the cotransfection of a variety of proteins, including Ras, G protein βγ subunits and $G\alpha_{12}$ subunits. The physiological significance of activation by these regulators has not been clearly defined. For reasons that remain unclear, regulation of PLCε by protein activators has not been reconstituted in vitro as has been done for the PLCβ isoforms.

This chapter describes the methods for purification of $PLC\beta_1$, $_2$, and $_3$ isoforms and PLCε using a Baculovirus insect cell expression system. Attempts to purify PLCβ using bacteria have been relatively unsuccessful because of the high degree of proteolysis and difficulty in obtaining pure protein. On the other hand, the insect cell expression system yields large quantities of pure protein. For these purifications, the PLCβ isoforms were modified with a hexahistidine (6-His) tag at the amino-terminus and PLCε was modified with a 6-His tag at both the N- and C-termini to facilitate purification. The PLC enzymes are highly soluble, but a significant portion of the expressed protein is associated with the insoluble membrane fraction. The enzyme associated with the particulate fraction is readily extracted with salt and combined with cytosolic fraction. Both PLCβ and PLCε enzymes are purified using nickel-nitrilotriacetic acid-agarose (Ni-NTA) affinity chromatography. PLCβ isoforms are further purified by heparin Sepharose chromatography. Both PLCβ and PLCε are highly susceptible to proteolysis, so extra care is taken to ensure that cleavage does not occur during the purification.

2. Materials

2.1. Insect Cell Culture Reagents and Baculoviruses

1. Sf9 cells (Invitrogen/Life Technologies) (*see* **Note 1**).
2. Sf-900 II media (Invitrogen/Life Technologies).
3. Recombinant Baculoviruses directing the expression of 6-His-tagged PLCβ or PLCε enzymes (*see* **Note 1**).

2.2. Stock Solutions (see Note 2)

1. Phosphate-buffered saline (PBS): 1.5 m*M* KH_2PO_4, 8.1 m*M* Na_2HPO_4, 140 m*M* NaCl, 2.7 m*M* KCl.
2. 1 *M* Na-HEPES, pH 7.0.
3. 1 *M* Na-HEPES, pH 7.4.
4. 1 *M* Na-HEPES, pH 8.0.
5. 4 *M* NaCl.

6. 0.1 *M* Dithiothreitol (DTT) stored at –20°C.
7. 0.1 *M* Ethylenediamine tetra-acetic acid (Na-EDTA), pH 8.0.
8. 0.1 *M* Ethylenebis(oxyethylenenitrilo)tetra-acetic acid (Na-EGTA), pH 8.0.
9. 10% Polyoxyethylene-10-lauryl ether ($C_{12}E_{10}$) (Sigma, cat. no. P-9769).
10. Protease inhibitors: stored as stocks at –20°C.
    a. PMSF/TPCK/TLCK. We make a 1000X stock that combines Phenylmethylsulfonyl floride (PMSF), *N*α-p-tosyl-L-lysine chloromethyl ketone (TLCK) and *N*-tosylphenylalanine chloromethyl ketone (TPCK). 160 mg Each of TPCK and TLCK are dissolved in 2.5 mL dimethylsulfoxide (DMSO). 1 mL of 1 *M* PMSF in DMSO is added to this solution followed by 4 mL 95% ethanol (EtOH) for a total of 7.5 mL 1000X stock. PMSF is a potent neurotoxin. Always wear a laboratory coat and gloves when handling this stock solution.
    b. 1 mg/mL 1000X Aprotonin dissolved in 0.1 *M* Tris-HCl, pH 8.0.
    c. 10 mg/mL 5000X Leupeptin dissolved in water.
    d. 1 mg/mL 1000X Pepstatin A dissolved in DMSO.
    e. 21 mg/mL 1000X Tosylarginine methyl ester (TAME) dissolved in DMSO.
    f. 10 mg/mL 1000X Soybean trypsin inhibitor (SBTI) in water.
11. 10 mg/mL Deoxyribonuclease 1.

2.3. Chromatography Resins

1. Ni-NTA resin (QIAGEN).
2. Prepacked Hi Trap Heparin Sepharose HP column (Amersham Pharmacia Biotech AB).

2.4. Cell Lysis and Chromatography Buffers (see *Note 3*)

The buffers described in this section are made on the day of the purification using the stock solutions described in **Subheading 2.4.** and are chilled to 4°C prior to using.

2.4.1. Buffers for PLCβ Purification (see *Note 4*)

1. PBS with 0.1 m*M* EGTA, 0.1 m*M* EDTA, and 1X PMSF/TPCK/TLCK.
2. Sf9 cell Lysis buffer: 50 m*M* Na-HEPES, pH 7.4, 0.1 m*M* EGTA, 0.1 m*M* EDTA, 0.1 m*M* DTT, 100 m*M* NaCl, and protease inhibitors (133 μ*M* PMSF, 21 μg/mL TLCK, 21 μg/mL TPCK, 1 μg/mL aprotonin, 2 μg/mL leupeptin, 1 μg/mL pepstatin A, 21 μg/mL TAME, and 10 μg/mL SBTI).
3. Dilution buffer: 10 m*M* Na-HEPES, pH 8.0, 10 m*M* β-mercaptoethanol, 0.1 m*M* EGTA, 0.1 m*M* EDTA, 0.5% lubrol, and protease inhibitors.
4. Wash buffer 1: 10 m*M* Na-HEPES, pH 8.0, 0.1 m*M* EGTA, 0.1 m*M* EDTA, 800 m*M* NaCl, 0.5% lubrol, 15 m*M* imidazole, and protease inhibitors.
5. Wash buffer 2: 10 m*M* Na-HEPES, pH 8.0, 0.1 m*M* EGTA, 0.1 m*M* EDTA, 100 m*M* NaCl, 15 m*M* imidazole, and protease inhibitors with no SBTI.
6. Elution buffer: 10 m*M* Na-HEPES, pH 8.0, 0.1 m*M* EGTA, 0.1 m*M* EDTA, 50 m*M* NaCl, 125 m*M* imidazole, and protease inhibitors without SBTI.

7. Heparin Sepharose buffer A (low-salt gradient buffer): 20 m*M* Na-HEPES, pH 8.0, 1 m*M* EDTA, 1 m*M* EGTA, 100 m*M* NaCl, 1 m*M* DTT and protease inhibitors without SBTI.
8. Heparin Sepharose buffer B (high-salt gradient buffer): 20 m*M* Na-HEPES, pH 8.0, 1 m*M* EDTA, 1 m*M* EGTA, 800 m*M* NaCl, 1 m*M* DTT and protease inhibitors without SBTI.

2.4.2. Buffers for PLCε Purification

1. Lysis buffer: 50 m*M* Na-HEPES, pH 8.0, 50 m*M* NaCl, 5% glycerol, 0.3 m*M* EDTA, 0.3 m*M* EGTA, 2 m*M* β-mercaptoethanol, and protease inhibitors.
2. Membrane suspension buffer: 50 m*M* Na-HEPES, pH 8.0, 800 m*M* NaCl, 5% glycerol, 2 m*M* β-mercaptoethanol, and protease inhibitors.
3. ε Ni-NTA equilibration buffer: 50 m*M* Na-HEPES, pH 8.0, 300 m*M* NaCl, 5% glycerol, 2 m*M* β-mercaptoethanol, 10 m*M* imidazole, 0.2% $C_{12}E_{10}$, and protease inhibitors.
4. ε Wash buffer 1: 50 m*M* Na-HEPES, pH 8.0, 1 *M* NaCl, 5% glycerol, 2 m*M* β-mercaptoethanol, 30 m*M* imidazole, 0.2% $C_{12}E_{10}$, and protease inhibitors.
5. ε Wash buffer 2: 5 0 m*M* Na-HEPES, pH 7.0, 400 m*M* NaCl, 5% glycerol, 2 m*M* β-mercaptoethanol, 50 m*M* imidazole, and protease inhibitors without SBTI.
6. ε Elution buffer: 50 m*M* Na-HEPES, pH 7.0, 400 m*M* NaCl, 5% glycerol, 2 m*M* β-mercaptoethanol, 400 m*M* imidazole, and protease inhibitors without SBTI.

3. Method

3.1. Purification of PLCβ

The method described here is essentially the same for purification of $PLC\beta_1$, $PLC\beta_2$, or $PLC\beta_3$.

*3.1.1. Expression of Recombinant 6-His PLCβ in Insect Cells (*see ***Note 5****)*

1. Infect 1 L of Sf9 cells at a density of $1.5–2 \times 10^6$ cells/mL with 10 mL 6-His-PLCβ Baculovirus and incubate with continuous shaking at 125 rpm for 48 h at 27°C.
2. Harvest the cells by centrifugation at 5000*g* for 20 min (Beckman Centrifuge, JA-10 rotor).
3. Resuspend the cells in 40 mL PBS containing protease inhibitors PMSF/TPCK/TLCK, 0.1 m*M* EDTA, and 0.1 m*M* EGTA; transfer to a 50-mL conical tube and centrifuge again at 1000*g* for 20 min at 4°C.
4. Discard the supernatant and continue as in **Subheading 3.1.2.** or rapidly freeze the pellet by submerging the tube in liquid N_2 until the pellet is frozen. Store at –70°C until further processing is done.

*3.1.2. Cell Lysis (*see ***Note 6****)*

1. Suspend Sf9 cells expressing 6-His-PLCβ pellet in 25 mL Sf9 cell lysis buffer. If the cells have been stored at –70°C use room temperature lysis buffer, otherwise, use buffer that has been chilled to 4°C.

2. Freeze the suspended cells in liquid N_2 and thaw in a 37°C water bath until only a small amount of ice remains (*see* **Note 6**). Repeat three more times.
3. Adjust the volume of the thawed lysate to 45 mL with ice-cold lysis buffer and add 15 mL of ice cold 4 *M* NaCl to obtain a final concentration of 1 *M* NaCl.
4. Centrifuge the cell extract at 40,000 rpm (100,000*g*) in a Beckman Ti60 rotor for 45 min and collect the supernatant.
5. Dilute the supernatant by adding 240 mL dilution buffer to obtain a fivefold dilution.
6. Centrifuge the diluted extract at 40,000 rpm (100,000*g*) in a Ti45 rotor for 45 min at 4°C and collect the supernatant. Discard the pellets. Save 100 µL supernatant in an Eppendorf tube as "load" for later analysis.
7. Add 300 µg deoxyribonuclease1 to the supernatant.

3.1.3. Affinity Chromatography Using Ni-NTA Agarose

1. Pack a 2 × 8-cm glass column with 4 mL Ni-NTA resin and equilibrate with 10 bed-volumes of dilution buffer (*see* **Note 7**).
2. Load the supernatant onto the column at a flow rate of 2–2.5 mL/min and collect the flow-through for later analysis.
3. Wash the column with 80 mL of wash buffer 1.
4. Wash with the column with 12 mL of wash buffer 2.
5. Elute the protein with six successive 4-mL applications of the elution buffer, collecting each elution in a separate tube.
6. Analyze the load, flow-through, wash, and elution fractions of 10 µL each on a 9% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gel followed by staining with Coomassie blue.
7. Pool the fractions containing PLCβ from the Ni-NTA column based on the extent of purity as seen on the SDS-PAGE gel. PLCβ can be identified as a strongly staining band running at between 130 and 150 kDa. At this point, the intact PLCβ is usually the most intensely staining band, but a number of contaminants, some of which may be proteolytic products, will be visible. Store at 4°C until ready to be loaded onto a heparin Sepharose column. Preferably, this column is run on the same day, but the sample can be saved on ice overnight.

3.1.4. Heparin Sepharose Chromatography

1. Equilibrate a 5-mL prepacked Hi-Trap heparin Sepharose column with 20 mL heparin Sepharose buffer A using a 10-mL syringe. Be sure not to push air into the column during this procedure.
2. Load the pooled fractions on the column using a syringe and collect the flow-through and store at 4°C for later analysis.
3. Wash the column with 25 mL of heparin Sepharose buffer A using a 10-mL syringe.
4. Elute with a 150-mL linear gradient from heparin Sepharose buffer A to heparin Sepharose buffer B (100–800 m*M* NaCl), collecting 6-mL fractions (*see* **Notes 8** and **9**).
5. Analyze each of the fractions for PLCβ by running on a 9% SDS-PAGE gel and monitoring by Coomassie blue staining.

6. Pool in all the fractions containing pure PLCβ_2 enzyme running at between 130 and 150 kDa and eluting at approx 300 m*M* NaCl (**Fig. 1**).
7. Concentrate the pooled fractions to 1–2 mL using a 10-mL Millipore diafiltration device with a PM 30 membrane filter at 4°C or with a Centriprep centrifuge-based concentrator following the manufacturer's instructions.
8. Estimate the yield of the protein by determining the protein concentration by Amido black protein assay ***(11)*** or Bradford protein assay. In general, we obtain 1–5 mg of protein from 1 L of Sf9 cells.
9. Make small aliquots of the concentrated sample in Eppendorf tubes and rapidly freeze in liquid N_2 before storing at –70°C. Usually the aliquots of PLC are thawed immediately before use and then discarded, and very small amounts of protein (1–500 ng) are needed for a full set of PLC assays. Thus, the smaller the aliquots that are made, the further the prep will go.

3.2. Purification of PLCε

PLCε expression virus is constructed with 6-His tags on both N-terminal and C-terminal of the protein. Sf9 cells are infected and harvested in the same manner as PLCβ. We use nitrogen cavitation to disrupt the cells instead of freeze-thaw, and some of the chromatography buffer conditions are changed to get the nearly pure protein after a single-affinity column.

3.2.1. Lyse the Cells with Parr Bomb

1. Chill a 500-mL capacity Parr N_2 cavitation bomb in cold room for 2 h or overnight.
2. Resuspend frozen Sf9 cell pellet from 1–5 L cells with room temperature lysis buffer at ratio 50 mL/L of culture. Swirl it in 37°C water bath to melt the pellet, but do not allow to thaw completely.
3. Put the cells in the Parr Bomb and add a stir bar. Check that the bar works properly at low speed.
4. Equilibrate the bomb by connecting to a N_2 tank and raising the pressure to 600 psi. Seal the bomb, disconnect from the N_2 tank, transfer to the cold room and stir for 30 min.
5. Place the outlet tube into a flask and release the pressure to allow the cells to flow smoothly out of the outlet into the flask (*see* **Note 10**).
6. Transfer the lysate to the appropriate-sized centrifuge bottles and centrifuge the lysate at 1000*g* for 10 min to remove intact nuclei. The pellet is often quite large at this stage.
7. Transfer the supernatant to tubes for ultracentrifugation and centrifuge at approx 100,000*g* in Ti45 rotor for 1 h.
8. Decant the supernatant into a graduated cylinder and measure the volume (X mL).
9. Resuspend the pellet in 1/2 X mL buffer salt extraction buffer with a tissue homogenizer.
10. Put in ice for 30 min and centrifuge again at approx 100,000*g* in Ti45 for 45 min.
11. Take the supernatant and combine with the supernatant of previous centrifugation.
12. The combined extracts can be frozen in liquid N_2 and stored at –80°C.

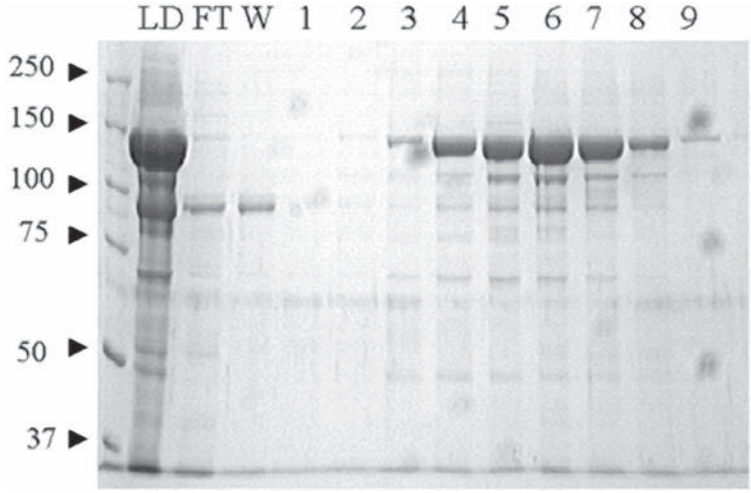

Fig. 1. Purification of PLCβ_2 with heparin Sepharose chromatography. Fractions are as indicated: LD, loaded lysate; FT, column flow-through; W, column washes; numbers are fraction numbers. Molecular weights in kDa.

3.2.2. Ni-NTA Affinity Chromatography

1. Pack 2 mL Ni-NTA agarose into either a glass column or a disposable plastic poly-prep column (Bio-Rad).
2. Equilibrate the column with 10 mL buffer Ni equilibration buffer.
3. To the combined extracts from **steps 11** or **12** in **Subheading 3.2.1.**, add imidazole to 10 m*M*, $C_{12}E_{10}$ to 0.2%, and all the protease inhibitors listed in **Subheading 2.2.**
4. Suspend the 2 mL Ni resin in 10 mL of equilibration buffer and add 5 mL of the suspended resin to each of two 50-mL Falcon tubes.
5. Add 40 mL cell lysate to each tube containing the Ni resin and rotate in the cold room for 4 h.
6. Combine the two tubes and pour the lysate and the resin through an empty column, allowing the resin to pack into the column and the remaining lysate to flow through the column. Save the flow-through for later analysis.
7. Wash the packed resin with 30 mL wash buffer 1 at 4°C and save the wash.
8. Wash with 30 mL wash buffer 2 at 4°C.
9. Elute with five successive applications of 2 mL elution buffer. Collect each fraction separately.
10. Run 1 µL of the load, flow-through, washes and elutions on 7% SDS-PAGE gel and stain with silver stain. Use Bio-Rad, Unstained and Broad Range Precision Protein Standards as molecular weight standards. PLCε runs near the uppermost standard at 250 kDa. Fractions 2 and 3 usually contain most of the protein (**Fig. 2**).

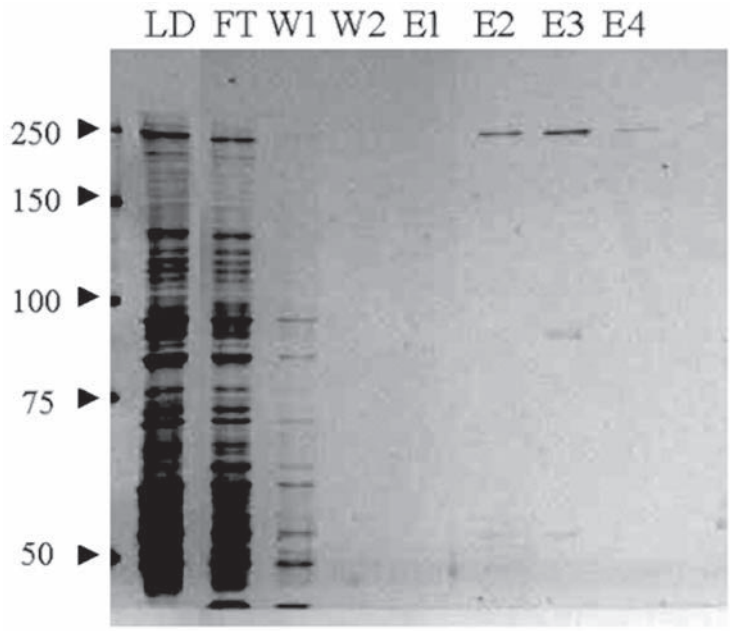

Fig. 2. Purification of PLCε using Ni-NTA agarose chromatography. Fractions are as indicated: LD, loaded lysate; FT, column flow-through; W, column washes; E, column elutions. Molecular weights in kDa.

11. The yield from 1 L of Sf9 cells is approx 200 µg. We have experienced significant loss of protein during concentration by Centricon, so we aliquot the protein and freeze in liquid N_2 in the Ni column elution buffer. If necessary, we change the buffer of PLCβ samples by dialysis of small aliquots in Slide-A-Lyzer mini-dialysis units (Pierce) with 10–100-µL capacity and a 10,000 molecular weight cut-off.
12. The protein can be assayed for activity as described in Chapter 5.

4. Notes

1. Baculoviruses have been generated for expression of amino-terminally 6-His-tagged PLCβ_1, PLCβ_2, and PLCβ_3. Here the sequence, MHHHHHHG, was cloned in frame immediately upstream of the PLCβ-coding sequences starting with the amino acids immediately following the endogenous methionine, resulting in the deletion of this methionine. For PLCε, 6-His tags were cloned at the amino- and C-termini. Both clones were inserted into pFastbac for production of Baculoviruses using the Bac-to-Bac system (Invitrogen/Life Technologies) following the manufacturer's instructions or the Baculoviruses can be obtained from our laboratory.

2. We grow Sf9 cells in suspension shaking at 125 rpm in Bellco flasks (50 mL flasks for 10 mL, 250 mL for 50 mL, 1 L for 200 mL, and 2 L for 1 L of cell culture) at 27–28°C. Cells are maintained between 0.5 to 8×10^6 cells/mL and have a doubling time of 24 h. Flasks are washed with 0.1 *M* NaOH, then by water, followed by 0.1 *M* HCl followed by water. Flasks are autoclaved, then baked overnight at 190°C. Sf9 cell cultures are successively scaled up to a 200-mL culture with a cell count of approx 8×10^6 cells/mL. This 200 mL cells are then poured into a 2-L flask containing 800 mL of Sf900 II media of the same to obtain 1 L of the culture with a cell count of approx $1.5–2 \times 10^6$ just prior to infection with Baculoviruses. Maximal expression of the protein is obtained when the Sf9 cells are maintained up to approx 25–28 cell passages.
3. Stock solutions are stored at 4°C unless otherwise indicated. The Ni-NTA resin and the prepacked heparin Sepharose columns are stored at 4°C.
4. The buffers are made just prior to use. Overnight storage results in the inactivation of some of the protease inhibitors and oxidation of the DTT.
5. Maximal expression of PLCβ is at 48 h. Once the cells are harvested, all processing from cell lysis, and until the purified protein is obtained, is done at 4°C to minimize any proteolytic degradation of the enzyme.
6. During cell lysis, the freeze-thawing process should be done rapidly to prevent any degradation of the protein either because of proteolytic cleavage or from changes in the temperature. During the process of thawing, the temperature of the lysate should not exceed 4°C. All the centrifugation steps are also done at 4°C.
7. All the steps for affinity chromatography are done at 4°C. Loading of the supernatant onto the column should not be at a flow rate greater than 2–2.5 mL/min because this may prevent binding of the protein to the matrix. An overnight loading at a slower flow rate could also be done for loading the sample.
8. We use fast protein liquid chromatography (FPLC) to control the gradient more accurately. This procedure can also be readily accomplished using a gradient maker and a peristaltic pump to deliver the gradient.
9. The heparin Sepharose column may be reused for several purifications if regenerated with 1 *M* NaCl.
10. The cells are broken in the discharge tube. During the discharging, temperature of the solution will drop and sometimes ice will form. Try to prevent ice to form by using a warm flask.

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5

Assay for G Protein-Dependent Activation of Phospholipase C β Using Purified Protein Components

Mousumi Ghosh and Alan V. Smrcka

Summary

The activity of mammalian phosphoinositide-specific phospholipase C β (PLCβ) is regulated by the α_q family of G protein α subunits and by βγ subunits thought to be released from G_i. Interactions between G protein subunits and PLCβ can be assayed by measuring the stimulation of PLCβ enzymatic activity on reconstituting the purified G protein subunits with purified PLCβ on artificial phospholipid vesicles containing the substrate, phosphatidylinositol-4,5-bisphosphate (PIP_2). These vesicles are doped with [^{3}H]-inositol PIP_2 and the rate of hydrolysis is determined by quantitating the amount of [^{3}H]-inositol triphosphate (IP_3) released from the vesicle into the aqueous phase. This assay provides a relatively simple method for assessing the activity PLC activity and its ability to be regulated by βγ and α_q subunits. It can also be used to assess the functionality of the components after modification by mutagenesis, chemical modification, or in the presence of competing molecules.

Key Words: Phospholipase regulation; PLCβ; G protein; $G\alpha_q$, Gβγ.

1. Introduction

A large number of extracellular signals stimulate the hydrolysis of the minor membrane phospholipid, phosphatidylinositol 4,5-bisphosphate (PIP_2), by phosphoinositide-specific phospholipase C (PLC) ***(1–4)***. This is one of the earliest key events in the regulation of various cell functions initiated by a wide range of hormones, neurotransmitters, and chemoattractants that bind to transmembrane receptors coupled to heterotrimeric G proteins (αβγ). G protein-coupled receptors (GPCRs) regulate PLC by stimulating the activation of specific G protein α or βγ subunits ***(5–8)***. These activated subunits, in turn, can

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independently activate PLC through direct protein–protein interactions, resulting in the production of the two intracellular second messengers, inositol triphosphate (IP_3), and diacylglycerol (DAG).

Mammalian PLC enzymes have been grouped into four classes: β, γ, δ, and ε, based on sequence homology with multiple isoforms in each class ***(3)***. Lower eukaryotes, such as yeast and slime moulds, contain only the δ type isozymes. The three-dimensional crystal structure of PLCδ1 has been resolved, recently providing insights into the multidomain structure of the enzyme ***(9,10)***. On the basis of the structural information, a catalytic mechanism comprising two steps—tether and fix—was proposed. The pleckstrin homology (PH) domain of the enzyme would tether to the membrane by specific binding to PIP_2, and the C2 domain would fix the catalytic domain in a productive orientation on the membrane. The EF domain serves as a flexible link between the PH domain and the rest of the enzyme. Calcium is required for the function of the C2 domain, and another Ca^{2+} ion located at the active site participates directly in enzyme catalysis.

PLCβ isozymes are activated through interactions with the α subunits of the pertussis toxin-insensitive α_q family of heterotrimeric G proteins ***(11,12)***. The GPCRs that are known to utilize this Gqα/ PLCβ pathway include the bradykinin, bombesin, angiotensin, histamine, vasopressin, muscarinic, α_1 adrenergic, thyroid-stimulating hormone, and endothelin-1 receptors. The GTPγS/AlF_4-activated $G\alpha_q$ or $G\alpha_{11}$ subunits stimulate PLCβ isoforms with the rank order of potency: $PLC\beta_4 \geq PLC\beta_1 \geq PLC\beta_3 > PLC\beta_2$.

PLCβ isozymes are also activated by the Gβγ subunits ***(4)***. The sensitivity of PLCβ isozymes to Gβγ subunits differs in contrast to $G\alpha_q$ and decreases in the order $PLC\beta_3 > PLC\beta_2 > PLC\beta_1$, whereas $PLC\beta_4$ is not activated to any extent. The M2 and M4 muscarinic acetylcholine receptors, interleukin-8 receptor, V2 vasopressin receptor, and the luteinizing hormone receptor are involved with this pathway mediated by Gβγ subunits.

Although the concentrations of Gβγ that are required for maximal activation of PLCβ isoforms in vitro are much larger than that of $G\alpha_q$, the final extents of activation are similar. Thus, both $G\alpha_q$ and Gβγ are transducers of PLCβ signaling, however, the region of PLCβ that interacts with $G\alpha_q$ differs from the region responsible for interaction with Gβγ. The COOH-terminal end, downstream of the Y domain, is essential for binding and activation of PLCβ by $G\alpha_q$ subunit ***(12,13)***, whereas the site of interaction of Gβγ in $PLC\beta_2$ is localized in the PH domain and in the Y domain of the catalytic region of the enzyme ***(14,15)***. By use of synthetic peptides derived from the region Leu580-Val641 from the Y domain of the catalytic region in PLCβ, the Gβγ-binding domain was further localized to amino acids Glu574-Lys583 of $PLC\beta_2$ ***(16)***. Unlike the regulation of PLCβ by G protein subunits, members of the PLCγ

family are activated by receptor and nonreceptor protein tyrosine kinases, whereas the activation of PLCε is regulated by the small G protein Ras and possibly many other small G proteins. The regulation of the members of PLCδ family is not yet clearly understood.

G protein βγ-subunits activate the PLCβ enzymes both in vitro and in transfection assays ***(17–21)***. In the in vitro assays, artificial bilayers composed of a mixture of 1:4 PIP_2: L-α-phosphatidylethanolamine (PE) in sonicated vesicles are used ***(22,23)***. Vesicles are doped with approx 8000 cpm/sample [^{3}H-inositol] PIP_2 to follow the PLC catalyzed hydrolysis of PIP_2 and release of ^{3}H-IP_3.

2. Materials

2.1. Lipids (see *Note 1)*

1. Brain PIP_2 (Avanti Polar-Lipids): 1 m*M* stock solution in chloroform stored in a desiccator in glass vials with PTFE lined caps under N_2 at –20°C.
2. Liver PE (Avanti Polar-Lipids): 13 m*M* stock solution in chloroform stored in a desiccator in glass vials under N_2 at –20°C.
3. [^{3}H]-labeled PIP_2 (Dupont, New England Nuclear [NEN]) aliquoted into Nensure vials.

2.2. Purified Proteins (see *Note 2)*

1. Purified PLCβ enzyme stored in aliquots at –70°C.
2. Purified $G\alpha_q$ subunit stored at –70°C.
3. Purified Gβγ subunit stored at –70°C.

2.3. Stock Solutions (see *Note 3)*

1. 2X Assay buffer: 100 m*M* HEPES, pH 7.2, 6 m*M* EGTA, 160 m*M* KCl.
2. 2X Incubation buffer: 100 m*M* HEPES, pH 7.2, 6 m*M* EGTA, 2 m*M* EDTA, 200 m*M* NaCl, 10 m*M* $MgCl_2$.
3. 10% Octyl β-D-glucopyranoside (OG) (Calbiochem).
4. 0.1 *M* $CaCl_2$ stock.
5. 10% Trichloroacetic acid (TCA).
6. 10 mg/mL Bovine serum albumin (BSA).
7. 100 mg/mL BSA.
8. Gβγ blank (solution in which purified Gβγ is stored).
9. $G\alpha_q$ blank (solution in which purified $G\alpha_q$ is stored).
10. 0.1 *M* Dithiothreitol (DTT).
11. 1X Sonication buffer: 500 µL 2X assay buffer, 10 µL 0.1 *M* DTT, 490 µL water.
12. $CaCl_2$ Solution: 500 µL 2X assay buffer, 60–180 µL 0.1 *M* $CaCl_2$, 10 µL 0.1 *M* DTT, and balance water up to 1 mL.
13. 10X AlF_4^- solution: 100 µL 1 *M* NaF, 30 µL 10 m*M* $AlCl_3$, 870 µL water.

3. Methods

This assay is performed in a final volume of 60 µL with four different components added:

1. 20 µL Sonicated phospholipid vesicles containing the substrate.
2. 20 µL Solution containing the PLC.
3. 10 µL Solution containing the G protein subunit.
4. 10 µL $CaCl_2$ solution.

Each of these solutions is prepared separately and added to the final assay mixture. Because the components of the solutions will be diluted when added to the assay, they are made at 3× or 6× the final concentration. The following describes the preparation of each of the first three components from the stocks described previously, followed by the assay itself.

3.1. Preparation of Lipid Vesicles (see Note 4)

1. Calculate the volume of lipid vesicles that will be needed by counting the number of individual enzyme reactions with each measurement using 20 µL of sonicated vesicles. Do not make too much more than necessary for the assay because the lipids are expensive.
2. Calculate the volume of the lipid stock solutions necessary to make the total volume of lipid vesicles calculated in **step 1**, at 3× the final concentration of the lipids in the assay. The final concentration of the lipids in the assay are 50 µ*M* PIP_2 and 200 µ*M* PE, so the concentration in the lipid vesicle solution will be 150 µ*M* PIP_2 and 600 µ*M* PE.
3. Calculate the amount of [^{3}H]-labeled PIP_2 needed to give 6000–8000 cpm/assay. This can be calculated based on the total volume of lipid vesicles necessary (calculated in **step 1**) and multiplying by 0.03.
4. Transfer the amounts of lipid stock solutions in chloroform calculated in **steps 2** and **3** into a 10-mL glass Pyrex tube using a 50–100-µL glass Hamilton syringe. Rinse the syringe at least 3× with chloroform between transfer of each of the different lipids.
5. Dry down the lipid mixture under N_2 for 15 min. Connect a hose between a regulator on a tank of nitrogen gas and a glass Pasteur pipet mounted on a ring stand. The glass pipet is lowered about one-fourth of the way down the tube containing the lipids. The flow of nitrogen is very slowly increased until it can be seen that the surface of the chloroform solution is gently moving. If the flow is increased too much the chloroform solutions will be blown out of the tube, or worse, the glass pipet could blow off the end of the tubing and cause injury. The dried lipids will form a film at the bottom of the tube.
6. Add the calculated volume of sonication buffer from **step 1** and sonicate in a bath sonicator for 5 min. The sonicated vesicles should form a homogeneous translucent suspension with no particulate matter.

7. Count 20 µL lipid solution by liquid scintillation counting. This will convey how much radioactivity is being added to each assay, serving as an indicator that the vesicles were sonicated successfully. There should be between 4000 and 8000 counts per minute (cpm) in 20 µL vesicle solution. If the cpm is significantly lower than this, the vesicle preparation was incorrect.
8. Store in ice until ready to be added to the reaction.

3.2. Purified PLCβ Enzyme Solution (see Note 5)

1. The PLCβ enzyme is used at a final concentration of 1–10 ng/reaction, depending on the type of the assay. Determine the number of reactions that will be performed to calculate the volume of PLC solution needed for the assay, given that each assay will get 20 µL PLC solution.
2. For Gβγ activation of PLCβ, make the PLC enzyme in 1X assay buffer, 3 m*M* DTT, 3 mg/mL BSA. Add the purified PLCβ, such that 1–10 ng are added in the 20 µL solution that is added to each reaction. For $G_q\alpha$ activation of PLCβ, the enzyme is prepared in a similar fashion, except AlF_4 is added from the 10X stock, such that it is 2.5× the final concentration in the reaction.
   For example, for 10 reactions, 200 µL are needed, plus a little extra so the mixture would be made as follows for a βγ activation assay: 125 µL 2X assay buffer, 7.5 µL 100 mg/mL BSA, 7.5 µL 0.1 *M* DTT, 62.5 ng PLC, and H_2O to 250 µL total volume for a final of 5 ng PLC/assay. For an assay of PLC activation by α_q, 62.5 µL 10X AlF_4^- would be substituted for 62.5 µL H_2O.

3.3. Purified Gβγ Subunit Solution (see Note 6)

1. Dilute the Gβγ stock in βγ blank, if necessary, prior to addition to the incubation buffer.
2. Mix the βγ in 1X incubation buffer at 6× the final concentration in the assay. Add DTT to make the solution 1 m*M*. Make enough of the solution so that each assay can be done in duplicate (*see* **Table 1**).
3. A control reaction is always performed where a volume of βγ blank solution is added to the incubation buffer to equal that containing the βγ subunit protein to control for detergent or other solution artifacts.
4. When performing titrations of varying amounts of βγ or α_q protein, we calculate the volume of βγ containing solution that will be required for the highest concentration of βγ (fixed value A in the example in **step 5**). For each of the lower amounts of protein, we balance the volume of βγ added with βγ blank, such that the βγ + βγ blank volume equals the volume of the highest concentration of βγ. This ensures that all reactions have an equal amount of detergent solution. A similar approach is used for other titrations.
5. For example, if you need 50 µL each of βγ solution at different concentrations, all tubes will get 25 µL 2X incubation buffer, 5 µL 10 m*M* DTT, βγ stock (to give 6× the final concentration) + βγ blank = the fixed volume A in **step 4**, and water to bring to a total of 50 µL (*see* **Table 1**).

Table 1
Setup of βγ Subunit Dilutions

Final βγ concentration in assay (n*M*)	2X Incubation buffer (μL)	2 μ*M* βγ stock (μL)	βγ blank (μL)	10 m*M* DTT (μL)	Water (μL)
0	25	0	15	5	5
10	25	1.5	13.5	5	5
30	25	4.5	10.5	5	5
100	25	15	0	5	5

3.4. Purified Gα$_q$ Subunit (see *Note 7)*

1. Dilute the stock Gα$_q$ into α$_q$ blank solution, if necessary, prior to addition to the incubation buffer.
2. Mix Gα$_q$ in 1X incubation buffer at 6× the final concentration in the assay with 1 m*M* DTT and 1X AlF_4^-. The final concentration of α$_q$ in the assay should be between 1 and 60 n*M*.
3. A control reaction is always performed where a volume of α$_q$ blank solution is added to the incubation buffer to equal that containing the α$_q$ subunit protein to control for detergent or other solution artifacts.

3.5. PLC Assay (see *Note 8)*

1. Add 20 μL sonicated phospholipid vesicles to individual 5-mL polypropylene tubes (VWR, cat. no. 60818-383).
2. Add 10 μL α$_q$/βγ or blank solutions to the vesicles.
3. Incubate the reaction tubes in an ice bath for 30 min.
4. Add 20 μL PLCβ solution to all tubes.
5. Add 10 μL calcium solution to the reaction tubes, resulting in a final volume of 60 μL. A set of duplicate blank reactions with no Ca^{2+} added should be left on ice to account for free [^{3}H]-IP$_3$ in the stock lipid.
6. Transfer the rack of tubes from the ice bath to a 30°C water bath to initiate the enzyme reaction. Leave the blanks on ice.
7. Incubate for 5 min and transfer back to the ice bath.
8. Add 200 μL ice-cold 10% TCA to each tube, including the blanks, to terminate the reaction.
9. Add 100 μL 10 mg/mL BSA to each tube.
10. Remove precipitated proteins and lipids by centrifuging for 5 min at 2000*g*.
11. Pipet out 300 μL supernatant with a bent pipet tip and transfer to a 6-mL capacity scintillation vial. Tilt the pellet so that it is up and direct the bent tip so that it will only touch the supernatant (*see* **Note 9**).
12. Add 4 mL scintillation fluid, shake, then place in scintillation counter.

13. Do a 5-min reading by liquid scintillation counting to measure the [^{3}H]-IP_3 released.

3.6. Calculations

Each assay gets 3 nmol of PIP_2. The cpm from the counting of the lipid vesicles (**Subheading 3.1., step 7**) is divided by 3 nmol to give the specific radioactivity in cpm/nmol.

Enzymatic specific activity (nmol/mg PLC/min) = (assay cpm – blank cpm)/specific radioactivity (cpm/nmol)/mg PLC/min of reaction.

4. Notes

1. These lipids are stored in chloroform solution at –20°C in glass vials. Before beginning each experiment, the vials containing the lipids are warmed to room temperature in a dessicator before opening to prevent the absorption of any moisture.
2. Purified proteins are stored in –70°C in small aliquots and rapidly thawed at 37°C immediately before the experiment. PLCβ is generally not frozen and thawed more than once. Being comparatively stable to denaturation because of repeated freeze-thawing, G protein subunits can be used several times if rapidly frozen in liquid nitrogen and stored at –70°C immediately after use.
3. Solutions 1–6 can be stored at 4°C for 2 mo. Solutions 7–10 should be stored at –20°C. Solutions 11–13 are made fresh each time before the experiment.
4. The concentrations of the lipids can be varied, but usually 50 μ*M* PIP_2 is used as the final concentration for each reaction. The PE:PIP_2 is added in the ratio of 4:1 in order to obtain a lipid bilayer. Therefore, PE is added at a final concentration of 200 μ*M*. The lipid vesicles are stable for approx 4 h at 4°C.
5. The amount of OG that will be added to each reaction will depend on the amount of βγ or α_q blank added because both contain 1% OG. In general, the final concentration of octylglucoside is kept below 0.1%. AlF_4^- is included in the reaction mixture for $G\alpha_q$ activation.
6. Make the initial concentration of βγ 6× of the final concentration that is desired in the reaction. Dilute the βγ as needed in βγ blank before adding to the incubation buffer. βγ blank is the same as the solution in which the βγ protein is stored. All tubes should get an equal amount of βγ solution. A final concentration of 100 n*M* βγ is sufficient to obtain βγ-mediated in vitro PLCβ activation. The concentration range that we usually prefer is 3–300 n*M* βγ with saturation occurring at 100 n*M*.
7. The α_q blank may or may not contain OG. OG is not an obligate requirement for the $G\alpha_q$ activation of PLCβ. The initial concentration of α_q, like that for βγ, is made 6× the desired concentration required in the reaction mixture.
8. This assay is used to determine the G protein-mediated activation of PLC enzyme. The basic procedure is similar for both $G\alpha_q$ and Gβγ activation, except that AlF_4^- is included in the reaction when $G\alpha_q$ regulation is studied.
9. While removing the supernatant, care has to be taken to ensure that the bent tip of the pipet tip does not touch the pellet.

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6

Assays of Recombinant Adenylyl Cyclases Expressed in Sf9 Cells

Ronald Taussig

Summary

This chapter outlines procedures for the expression of mammalian membrane-bound adenylyl cyclases (AC) in Sf9 cells and subsequent in vitro methods for assessing the activity of these cyclases. Membrane preparations derived from this overexpression system provide homogeneous sources of mammalian AC because AC that are endogenously expressed in Sf9 cells contribute low amounts of activity relative to the overexpressed enzyme. These approaches are applicable to all nine isoforms of mammalian membrane-bound AC isoforms.

Key Words: Adenyly cyclase; cAMP; heterotrimeric G proteins; Baculovirus; Sf9 cells; recombinant proteins; membrane-bound isoforms.

1. Introduction

Regulation of intracellular 3-5-cyclic adenosine monophosphate (cAMP) concentrations is principally controlled at the level of its synthesis through the hormonal regulation of adenylyl cyclase (AC), the enzyme responsible for the conversion of adenosine 5-triphosphate (ATP) into cAMP. Currently, 10 isoforms of AC have been identified by molecular genetic approaches ***(1,2)***. Nine of these contain two structural regions each that are comprised of six stretches of hydrophobic amino acids that anchor the proteins to the plasma membrane. These AC systems are comprised of three components: seven-transmembrane-spanning receptors for a variety of hormones and neurotransmitters; heterotrimeric G proteins; and the catalytic entity itself. Studies of these nine enzymes reveal that these isoforms are regulated by subunits of heterotrimeric G proteins ***(3–6)*** and demonstrate both common and unique regulatory features. Regulation of the activity of membrane-bound AC is not solely limited to the

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actions of heterotrimeric G proteins; in addition, AC activity has been shown to be directly regulated in an isoform-dependent manner by calmodulin, protein kinases and phosphatases ***(7–9)***, and small molecules, such as calcium, adenosine analogs (P-site inhibitors), and the plant diterpine, forskolin ***(10–12)***.

Native cells typically express multiple isoforms of AC; therefore, it is difficult to assess the relative contributions that each isoform makes to the overall regulation of intracellular cAMP in these systems. Preparations derived from cells expressing a single isoform of AC are clearly valuable reagents whereby the regulation of each isoform can be directly assessed. The Baculovirus/Sf9 cell system has been widely used for the production of large quantities of proteins suitable for biochemical analysis ***(13)*** and has been employed for the expression and characterization of the membrane-bound forms of AC ***(14–17)***. These preparations provide a homogeneous source of single isoforms of mammalian AC, containing minimal amounts of interference from the endogenous cyclase present in Sf9 cells. This chapter describes procedures for the isolation of membrane preparation derived from Sf9 cells overexpressing single isoforms of mammalian AC as well as methods for assessing the activity of these cyclases in vitro.

2. Materials

2.1. Construction and Generation of Baculovirus

1. pFastBac plasmids (Invitrogen/Life Technologies).
2. Competent cells (e.g., DH5α).
3. DH10Bac-competent cells (Invitrogen/Life Technologies).
4. Sf21 cells, SFM-adapted cells (Invitrogen/Life Technologies). Sf21 cells are a serum-adapted version of Sf9 cells.
5. CellFECTIN reagent (Invitrogen/Life Technologies).
6. Sf-900 II SFM media (Invitrogen/Life Technologies).
7. Ampicillin.
8. Kanamycin.
9. Gentamicin.
10. Tetracycline.
11. X-gal.
12. Isopropyl β-D-thiogalactopyranoside (IPTG).

2.2. Preparation of Membrane Fractions

1. Lysis buffer: 20 m*M* HEPES, pH 8.0, 150 m*M* NaCl, 5 m*M* EDTA, and 1 m*M* EGTA, 2 m*M* dithiothreitol (DTT).
2. Membrane storage buffer: 20 m*M* HEPES, pH 8.0, 200 m*M* sucrose, and 1 m*M* DTT.
3. Nitrogen cavitation apparatus (Parr Instrument).
4. Protease inhibitors (final concentrations): 16 µg/mL each of L-1-tosylamido-2-phenylethyl ketone, 1-chloro-3-tosylamido-7-amino-2-heptone, and phenyl-

methylsulfonyl fluoride (PMSF); 3.2 µg/mL each of leupeptin and lima bean trypsin inhibitor; and 2 µg/mL aprotinin.
5. Potter glass homogenizer.
6. Bio-Rad Protein Assay (Bio-Rad).

2.3. Assay of AC Activity

1. HMED: 10 m*M* HEPES, pH 8.0, 2 m*M* $MgCl_2$, 1 m*M* EDTA, pH 7.0, and 1 m*M* DTT.
2. Stop solution: 0.25% sodium dodecyl sulfate (SDS), 5 m*M* ATP, and 0.175 m*M* cAMP, pH 7.5.
3. [^{3}H] cAMP (American Radiolabeled Chemicals, St. Louis, MO).
4. [α-^{32}P] ATP (Perkin Elmer Life Sciences, Boston, MA).
5. Dowex 50AG-WX4 resin (200–400 mesh; Bio-Rad Laboratories, Hercules, CA).
6. Alumina (Sigma).
7. 10 × 75-Siliconized glass tubes.
8. 1 *M* HEPES, pH 8.0.
9. 500 m*M* EDTA, pH 7.0.
10. 10 mg/mL Bovine serum albumin (BSA).
11. 150 m*M* Phosphoenolpyruvate (potassium salt), pH 7.0, with KOH.
12. 50 m*M* ATP.
13. 1 *M* $MgCl_2$.
14. 1 mg/mL Pyruvate kinase.
15. Polyoxethylene-10-lauryl ether ($C_{12}E_{10}$). A detergent that can be substituted for lubrol that is no longer sold, but is often seen in the literature.
16. 10 m*M* 3-Isobutyl-L-methylxanthine (IBMX).
17. 1 *N* HCl.
18. 1 *N* NaOH.
19. Imidazole buffer: 200 m*M* NaCl, 20 m*M* imidazole.
20. 20 mL Scintillation vials.
21. Cytoscint scintillation cocktail (ICN, Costa Mesa, CA).
22. Scintillation counter.

3. Methods

3.1. Construction and Generation of Baculovirus (see *Note 1*)

1. cDNAs encoding mammalian AC are ligated into the multicloning site of the pfastbac transfer vector (*see* **Notes 2** and **3**).
2. Ligation reactions are transformed into competent bacterial cells (DH5) and plated on Luria-Bertani (LB) plates containing 50 µg/mL ampicillin.
3. Colonies are grown in 2 mL LB medium containing 50 µg/mL ampicilin, and plasmids are prepared by the use of commercially available kits (Qiagen).
4. Recombinant plasmids are verified by restriction mapping and sequencing.
5. To generate recombinant bacmids, 5 µL miniprep pfastbac DNA is transformed into 100 µL competent DH10Bac cells. Transformation mixes are plated on LB

plates containing 50 µg/mL kanamycin, 7 µg/mL gentamicin, 10 µg/mL tetracycline, 300 µg/mL X-gal, and 40 µg/mL IPTG (*see* **Note 4**). Incubate plates for at least 24 h at 37°C.

6. Select isolated white colonies from a plate and streak each onto LB plates containing 50 µg/mL kanamycin, 7 µg/mL gentamicin, 10 µg/mL tetracycline, 300 µg/mL X-gal, and 40 µg/mL IPTG to ensure they do not contain any blue cells (*see* **Note 5**).
7. Inoculate a single isolated bacterial colony into 2 mL LB medium containing with 50 µg/mL kanamycin, 7 µg/mL gentamicin, and 10 µg/mL tetracycline. Grow at 37°C to stationary phase up to 16 h, shaking at 250–300 rpm.
8. Bacmid DNA for transfection of Sf9 cells and subsequent Baculovirus production is prepared from the bacterial culture using alkaline lysis methods, followed by isopropanol precipitation and ethanol washing. The final DNA pellet is resuspended in 100 µL sterile water (*see* **Note 6**).
9. To generate recombinant baculovirus, transfect the isolated bacmid DNA into Sf9 cells that have been plated in 2 mL Sf-900 II SFM medium (2×10^6 cells per 350-mm well) using CellFECTIN reagent as outlined by the manufacturer.
10. The media, which contains a low titer of recombinant Baculovirus, is harvested 72 h posttransfection (*see* **Note 7**).
11. Virus stocks are amplified by sequential infection of Sf9 cells with Baculovirus. For the initial amplification, 10 mL cells (1×10^6 cells/mL) are infected with 100 µL harvested virus stock. Following 6 d in culture, the culture is centrifuged at 2000*g* for 5 min, and 1 mL of the recovered supernatant is used to infect a second culture of Sf9 cells (50 mL, 1×10^6 cells/mL). Following 6 d in culture, the supernatant-containing Baculovirus is recovered by centrifuging the culture at 2000*g* for 5 min (*see* **Note 8**).

3.2. Preparation of Membrane Fractions

1. 1 L Sf21 cells are grown to a density of 2×10^6 cells/mL in SF900 II medium, and infected with recombinant baculovirus at an MOI = 2 (*see* **Note 9**).
2. 46–52 Hours postinfection, cells are harvested by centrifugation at 2000*g* for 5 min at 4°C (*see* **Note 10**).
3. Cell pellet is suspended in 75 mL lysis buffer containing protease inhibitors and lysed by nitrogen cavitation at 600 psi for 30 min on ice.
4. Nuclei are removed by centrifugation at 500*g* for 30 min at 4°C.
5. Membranes are recovered from the supernatant by centrifugation at 70,000*g* for 30 min at 4°C.
6. Membranes are washed by suspending the pellet in 85 mL membranes storage buffer containing protease inhibitors and recovered by centrifugation at 70,000*g* for 30 min at 4°C.
7. The final membrane pellet is suspended in 10 mL membrane storage buffer containing protease inhibitors (*see* **Notes 11** and **12**).
8. Protein concentration is determined using Bio-Rad Protein Assay (*see* **Note 13**).

3.3. Assay of AC Activity

1. On ice, add 5–20 µg membranes, and G protein subunits to the reaction tube. HMED buffer is added to bring the volume to 60 µL (*see* **Notes 14–16**).
2. The reactions are sequentially initiated by the addition of 40 µL cocktail (*see* **Note 17**).
3. Reactions are incubated at 30°C for 10–20 min (*see* **Note 18**).
4. The reactions are sequentially terminated by the addition of 0.8 mL stop solution.
5. 100 µL Solution containing 1–2 × 10^4 cpm of [^{3}H] cAMP in water are added to each tube (*see* **Note 19**).
6. The contents of each reaction tube are poured onto a column containing Dowex 50AG-WX4 resin (*see* **Note 20**). The flow-through is allowed to drain to waste.
7. Add 2 mL water to each column; discard the flow-through to waste.
8. Elute the cAMP directly into a second set of columns containing alumina with 2 mL water (*see* **Note 21**). Allow the flow-through to drain to waste.
9. Elute the cAMP from the alumina columns directly into scintillation vials using 3 mL imidazole buffer.
10. Add 8–10 mL scintillation fluid to each vial.
11. Prepare controls for scintillation counting. [^{3}H] controls vials contain 100 µL [^{3}H] cAMP solution (1–2 × 10^4 cpm). [^{32}P] vials contain 10 mL dilution of the assay cocktail (1:20 dilution). All vials also contain 3 mL imidazole buffer and 8–10 mL scintillation cocktail.
12. Samples are counted on a scintillation counter set to capture [^{3}H] and [^{32}P] in separate channels.
13. Calculate the activity of the AC preparation in each assay (*see* **Note 22** and **Fig. 1**).

4. Notes

1. Recombinant Baculoviruses are prepared using the Bac-to-Bac Baculovirus Expression Systems (Invitrogen/Life Technologies). A manual outlining detailed procedures is available at: http://www.invitrogen.com/content/sfs/manuals/bac.pdf.
2. A restriction map of pfastbac is available at http://www.invitrogen.com/content/sfs/vectors/pfastbac1.pdf.
3. To aid in subsequent identification and purification of the expressed AC protein, sequences encoding small-affinity tags can be included on the amino- or carboxyl-termini of the protein without altering the activity of the cyclases ***(6,15,18)***.
4. As the goal is to isolate single isolated colonies, appropriate dilutions of cells must be plated to account for differences in transformation efficiencies obtained with different preparations of competent DH10Bac cells. Cells are diluted (1:10, 1:100, 1:1000) with SOC medium.
5. Most white cells (>80%) contain bacmids that have recombined with the recombinant pfastbac plasmid; however, some colonies contain sectors of blue cells that are not readily visible. Additionally, the development of the blue color usually takes 36–48 h. It is therefore advisable to process multiple colonies that have been derived from independent pfastbac miniprep DNAs and to avoid colonies that have any blue sectors.

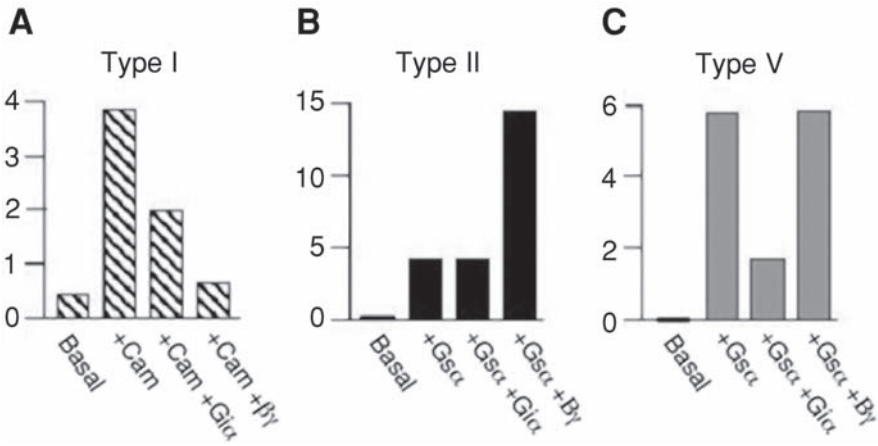

Fig. 1. Activities of mammalian AC. Representative activities determined from membrane preparations derived from Sf9 cells infected with recombinant Baculoviruses-encoding type I **(A)**, type II **(B)**, or type V **(C)** AC are shown. Activities were performed in the absence (Basal) or presence of the following regulators: calmodulin (CAM), recombinant $G_s\alpha$ ($+G_s\alpha$), recombinant myristoylated $G_i\alpha$ ($+G_s\alpha$), recombinant $G\beta\gamma$ ($+\beta\gamma$), and a combination of regulators.

6. Bacmid DNA is too large to prepare using commercially available miniprep kits. Repeated freezing and thawing of the isolated bacmid DNA should be avoided, and it is advisable to use the DNA in transfections immediately after isolation.
7. The titer of this initial stock of Baculovirus isolated from the transfection plates is estimate to be 1×10^7 pfu/mL. At this stage, it is difficult to determine if the virus has actually been produced. If the virus has been produced, infection of subsequent suspension cultures with the supernatant (as outlined in **Subheading 3.1., step 11**) should significantly slow or stop doubling of the cells.
8. The titer of this stock of baculovirus is estimate to be 1×10^8 pfu/mL. The actual titer of the stock can be determined using procedures outlined in the Bac-to-Bac Baculovirus Expression Systems manual.
9. We grow Sf9 cells in suspension shaking at 125 rpm in Bellco flasks (20-mL flasks for 10 mL, 125-mL for 50 mL, 500-mL for 200 mL and 2-L for 1 L cell culture) at 27–28°C. Cells are maintained between 0.5 to 3×10^6 cells/mL and have a doubling time of 24 h. Flasks are washed with 0.1 *M* NaOH, followed by water, then by 0.1 *M* HCl, and again followed by water. Flasks are then autoclaved and then baked overnight at 190°C. SF9 cell cultures are successively scaled up to a 200 mL culture with a cell count of approx 3×10^6 cells/mL. This 200 mL cells is then poured into a 2-L flask containing 800 mL Sf900 II media of the same and grown to obtain 1 L culture with a cell count of approx $1.5–2 \times 10^6$ just prior to infection with Baculoviruses. Maximal expression of the protein is

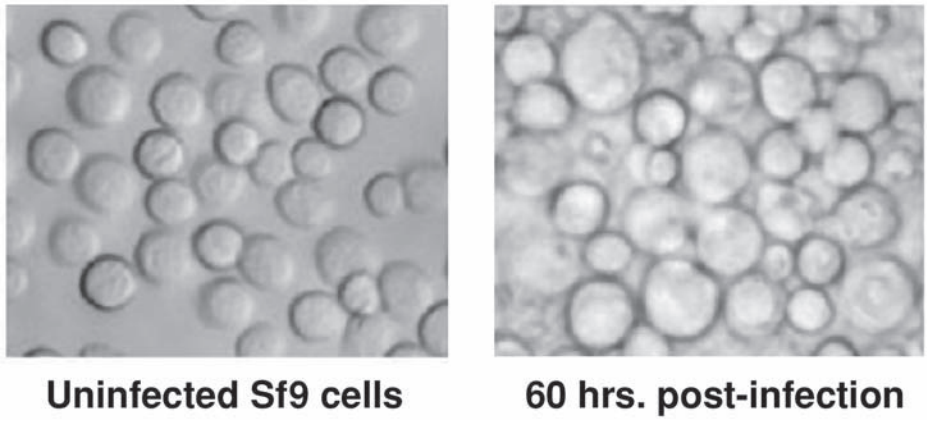

Fig. 2. View of Sf9 cells infect with Baculovirus-encoding type V AC. **(A)** Uninfected Sf9 cells. **(B)** Cells (initially grown to a density of 2×10^6 cells/mL) were visualized 60 h postinfection with a recombinant Baculovirus-encoding type V AC (MOI = 2).

obtained when the Sf9 cells are maintained up to approx 25–28 cell passages. Inspection of cells at the time of harvest is recommended. Cells become enlarged 48 h after infection with Baculovirus (*see* **Fig. 2**).

10. It is important to fully suspend the membranes to an even consistency; clumps of membrane in the suspension will lead to inaccurate determination of AC activity when assayed. The use of a glass potter homogenizer (20–30 strokes) is recommended for this step.
11. Membranes are stored at centrifugation at –80°C. Avoid repeated freezing and thawing of the preparation; AC activity is lost during this process.
12. The preparation yields 50–100 mg of membranes/L of culture.
13. The reactions are assembled in 10×75 glass tubes that have been treated with a siliconizing agent.
14. Membranes and recombinant G protein α subunits (purified from bacteria) are diluted in HMED; G protein $\beta\gamma$ subunits are diluted into HMED containing 0.05% $C_{12}E_{10}$ detergent and should be diluted in the assay 10-fold. The presence of detergents may affect the activity of AC in this assay. If G protein subunits solutions are used that contain other detergents, it is advisable to test the effect of these detergents and to utilize appropriate boiled preparations as controls.
15. Duplicate samples are prepared for each condition to be tested.
16. All reactions contain the following components (final concentrations): 50 m*M* HEPES, pH 8.0, 0.6 m*M* EDTA, pH 7.0, 100 µg/mL BSA, 3 m*M* potassium salt, 0.5 m*M* ATP, 10 m*M* $MgCl_2$, 10 µg/mL pyruvate kinase, 100 µ*M* IBMX, and 10^6 cpm [α-^{32}P] ATP. A solution containing the first five components can

be prepared in advance and stored at –20°C. The remaining components are to be added fresh. For assays that utilize $G_i\alpha$ subunits, the $MgCl_2$ concentration is reduced to 4 m*M*.

17. The assay is linear with time up to 20–30 min.
18. [^{3}H] cAMP is added in order to monitor sample loss during subsequent column chromatography.
19. The dowex resin is prepared by defining the resin, washing with 3 vol 1 *N* HCl, followed by three washes with 5 vol water, and three washes with 3 vol 1 *N* NaOH. The resin is then resuspended in water (1:1 slurry), and 2 mL are added to each column. Prior to use, the columns are washed with 3 mL 1 *N* HCl, and followed by an additional wash with 10 mL water. The columns can be regenerated for reuse by washing with 3 mL 1 *N* HCl, followed by an additional wash with 10 mL water.
20. Alumina columns are prepared by equilibrating 0.75 g resin with 3 mL 100 m*M* imidazole (overnight). Prior to use, the columns are washed with 20 mL imidazole buffer, followed by an additional wash with 10 mL water. The columns can be regenerated for reuse by washing with 20 mL imidazole buffer, followed by an additional wash with 10 mL water.
21. The activity of each sample is calculated by first determining the amount of cAMP that is recovered from each of the alumina columns; then, the specific activity of the cAMP in the assay is determined from the [^{32}P] control vials (approx 20 cpm·pMol^{-1}). This value is then adjusted for the losses incurred during the dowex/alumina chromatography steps; this is determined by calculating the percentage of the [^{3}H] cAMP that was recovered for each of the alumina columns. The activities are expressed as pmol cAMP formed·(min^{-1})·(mg of cyclase membrane protein^{-1}).

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7

Measurement of G Protein-Coupled Receptor-Stimulated Phospholipase D Activity in Intact Cells

Stephanie J. Walker and H. Alex Brown

Summary

Mammalian phospholipase D (PLD) activity hydrolyzes phosphatidylcholine (PC) into phosphatidic acid (PA) and free choline. This activity can be stimulated by a wide variety of extracellular agonists, including those for G protein-coupled receptors (GPCRs). This chapter outlines a protocol for the measurement of PLD activity in intact cells following stimulation by an extracellular agonist. The protocol takes advantage of a unique property of mammalian PLDs—the ability to substitute a primary alcohol for water in the hydrolytic reaction. This transphosphatidylation reaction results in the formation of a phosphatidylalcohol, which is a specific and unique marker for PLD activity. This protocol is highly sensitive for the detection of PLD activity following the stimulation of intact cells, being a valuable method for studying the regulation of PLD activity in vivo.

Key Words: Phospholipase D; G protein; lipid signaling; transphosphatidylation; phosphatidic acid.

1. Introduction

Phospholipase D (PLD) enzymes catalyze the hydrolysis of phospholipids at the distal phosphodiester bond. Mammalian PLDs are primarily phosphatidylcholine (PC)-specific; hydrolysis of PC by these enzymes generates phosphatidic acid (PA) and choline. PA is a second messenger involved in membrane-remodeling events that are critical to cell growth (e.g., vesicle trafficking and regulated secretion). PLD-produced PA levels are transient; dephosphorylation of PA by lipid phosphate phosphatase (LPP) is a major pathway for diacylgycerol (DAG) production. Because of the important cellular

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functions of PLD and its products, the enzymatic activity of PLD is tightly regulated by a variety of hormones, growth factors, cytokines, and other agonists involved in cellular signaling ***(1–3)***.

The ability to measure PLD activity in cells is critical to understanding this regulation. The protocol described here outlines an endogenous substrate assay for measuring stimulated PLD activity in intact cells, taking advantage of the unique ability of mammalian PLD enzymes to carry out a transphosphatidylation reaction. In the presence of sufficient concentrations of primary alcohols (e.g., ethanol [EtOH]), PLD activity will produce phosphatidylethanol (PtdEtOH) at the expense of PA. The PtdEtOH product is metabolically more stable than PA and serves as a unique and specific marker of PLD activity.

Determination of PLD activity in intact cells requires the labeling of the endogenous PC substrate with a radioactive tracer. In this protocol, cells are incubated with a radiolabeled fatty acid ([^{3}H]-myristic acid), which incorporates into PC—the mammalian PLD substrate. Labeling of the cells is carried out under conditions of serum starvation so that cells may be stimulated with extracellular agonist following incorporation of the label. During the agonist stimulation, the cells are incubated with a primary alcohol (i.e., EtOH), for the conversion of radiolabeled substrate into PtdEtOH by PLD. Following stimulation, the cellular phospholipids are extracted using a modified Bligh and Dyer protocol ***(4,5)***, resolved by thin layer chromatography (TLC) using a solvent system developed in the Brindley laboratory ***(6)***, and visualized by exposure to a tritium phosphor screen for subsequent scanning on a Phosphoimager. The PLD activity is measured using imaging and quantitation software specific to the Phosphoimager. An alternative method for visualization using indirect autoradiography is noted.

This chapter describes the labeling and stimulation of PLD activity in intact (i.e., PC12, pheochromocytoma cells) cells by agonists for G protein-coupled receptors (GPCR). This protocol can be easily adapted for use with other cell types and other signaling systems, such as growth factor activation. It should be noted that this protocol does not distinguish between the mammalian PC-specific PLD activities, PLD1 and PLD2, nor does it give any information as to the acyl chain composition of the PC species hydrolyzed to form PA and PtdEtOH. It is a highly sensitive technique for the detection of PLD activity following the stimulation of intact cells and is considered a valuable method for studying the regulation of PLD activity in vivo.

2. Materials

2.1. Plating of PC12 Cells for Endogenous Assay (see Note 1)

1. Dulbecco's modified Eagle's medium (DMEM) with 10% calf serum (CS) and 5% fetal bovine serum (FBS; complete medium).

2. 1X Phosphate-buffered saline (PBS): 140 m*M* NaCl, 2.7 m*M* KCl, 10 m*M* Na_2HPO_4, 1.8 m*M* KH_2PO_4, in ddH_2O.
3. Trypsin-EDTA: 0.05% trypsin, 1X PBS 0.5 m*M* EDTA.
4. 12-Well tissue culture plates (Costar).

2.2. Labeling of Cellular Phospholipids

1. Chloroform ($CHCl_3$) (*see* **Note 2**).
2. 25 µL Hamilton syringe.
3. Phosphatidylethanolamine (PE): 10 mg/mL in $CHCl_3$, stored at –20°C (Avanti Polar Lipids).
4. [9,10]-^{3}H–myristic acid: 1 mCi/mL in EtOH, stored at –20°C (New England Nuclear [NEN]/Perkin-Elmer; *see* **Note 3**).
5. Compressed nitrogen tank with tubing and a Pasteur pipet attached to the regulator.
6. Serum-free DMEM with 0.2 mg/mL bovine serum ablumin (BSA), sterile-filtered for use in tissue culture.
7. 13 × 100-mm Pyrex, rimless glass tubes.
8. Water bath sonicator.
9. Scintillation vials.
10. Scintillation fluid.

2.3. Stimulation of PLD Activity

1. Serum-free DMEM.
2. Serum-free DMEM + 2% EtOH (*see* **Note 4**).
3. Carbachol: 10 m*M* stock in H_2O (Calbiochem; *see* **Note 5**).
4. ATPγS: 10 m*M* stock in H_2O (Roche/Boehringer Mannheim; *see* **Note 5**).

2.4. Extraction of Cellular Phospholipids

1. 1X PBS.
2. Ice-cold acidified methanol: 1 part methanol (MeOH) and 1 part 0.1 *M* HCl.
3. Cell scrapers.
4. $CHCl_3$.

2.5. Thin-Layer Chromatography of Cellular Phospholipids

1. 10 × 30 × 27-cm large TLC tank with latch lid (Kontes).
2. Cellulose chromatography paper (46 × 57-cm, 0.35-mm thick), cut to 3/4 height of TLC tank.
3. Brindley solvent system ***(6)***, freshly made: $CHCl_3$/MeOH/acetic acid/acetone/H_2O (10:2:2:4:1) (*see* **Note 6**).
4. 25 µL Hamilton syringe.
5. Compressed nitrogen with tubing and a Pasteur pipet attached to the regulator OR speed vacuum.
6. TLC plates: Whatman LK6DF silica gel 60, 20 × 20-cm, with 19 channels/plate.
7. PtdEtOH (18:1): 25 mg/mL in $CHCl_3$, stored at –20°C (Avanti Polar Lipids).
8. PA (16:0–18:1): 25 mg/mL in $CHCl_3$, stored at –20°C (Avanti Polar Lipids).

2.6. Visualization and Quantitation of Cellular Phospholipids (see Note 7)

1. Tritium [^{3}H] Phosphor screen with cassette (Molecular Dynamics; *see* **Note 8**).
2. Phosphoimager.
3. TLC tank with iodine crystals covering the bottom surface.

3. Methods

3.1. Plating of PC12 Cells for Endogenous Assay (see Note 9)

1. Aspirate growth medium from a confluent 100-mm plate of cells.
2. Wash cells with 4–5 mL 1X PBS.
3. Trypsinize cells with 1–2 mL trypsin-EDTA. Add volume of trypsinized cells to an equal volume of complete medium.
4. Count cells.
5. Seed cells at 5×10^5 cells/well in 12-well plates containing 0.5 mL complete medium/well. Plate sufficient wells to analyze each test condition with and without EtOH. Each experiment should be carried out in duplicate.
6. Incubate cells in a humidified environment at 37°C with 5% CO_2 for 16–20 h.

3.2. Labeling of Cellular Phospholipids (see Note 10)

1. Remove PE and [^{3}H]-myristic acid from the freezer and thaw at room temperature for approx 30 min (*see* **Note 11**).
2. Rinse the inside of a glass tube several times with $CHCl_3$ and discard the washes. Using a Hamilton syringe, add 10 µL PE to the bottom of the tube and swirl to coat the bottom and sides of the tube (*see* **Note 12**).
3. With a pipettor, add the appropriate volume of [^{3}H] myristic acid to the glass tube. The final labeling medium should be approx 10 µCi/mL (*see* **Note 13**).
4. Dry the lipids under a stream of N_2. Parafilm the tube and move to sterile tissue culture hood.
5. Under sterile conditions, add 1 mL of DMEM + 0.2 mg/mL BSA to the dried lipids. Parafilm the tube.
6. Sonicate the tube for 2–5 min. Vortex the sample one or two times during sonication.
7. Under sterile conditions, add the sonicated lipid mixture to the rest of the sterile DMEM + 0.2 mg/mL BSA. Mix well and remove a 10 µL aliquot to a scintillation vial. Add scintillation fluid, count, and confirm that the labeling media is in the appropriate range (2–10 µCi/mL) for labeling.
8. Remove growth medium from cells and wash each well twice with 1 mL serum-free media.
9. Add 0.5 mL labeling media to each well. Incubate for 16–20 h. Approximately 70–80% of the label is incorporated into the cells during this incubation.

3.3. Stimulation of PLD Activity

1. Remove cells from the incubator and wash each well twice with warm serum-free DMEM at 37°C.
2. Add 0.5 mL serum-free DMEM to half of the wells; add 0.5 mL serum-free DMEM + 2% EtOH to the other half. Incubate for 10 min at 37°C.

3. Remove plates from the incubator and add 5 μL of each agonist, for a final concentration of 100 μ*M*, to the appropriate wells. Incubate for 20 min at 37°C.
4. Stop the stimulation by placing the plates on ice.

3.4. Extraction of Cellular Phospholipids

1. Remove the media and add 0.5 mL ice-cold acidified MeOH to each well.
2. Scrape the cells from each well into labeled microfuge tubes.
3. Add 250 μL $CHCl_3$ to each microfuge tube and vortex vigorously. The vortexed mixture should look white and cloudy.
4. Centrifuge tubes at 14,000*g* for 5 min. After centrifugation, two phases divided by a tight white band of precipitated protein should be apparent. The upper portion is the aqueous phase, and the lower portion is the $CHCl_3$ phase containing cellular phospholipids (*see* **Notes 14** and **15**).

3.5. TLC of Cellular Phospholipids (see Note 16)

1. In a dry TLC tank, place chromatography paper so that it covers all four sides of the tank to approximately three-quarters of the tank's height.
2. Using approx 100 mL, add freshly made Brindley solvent to cover the bottom of the tank. Cover the tank and equilibrate for 1 h or until the chromatography paper is completely wet. While the tank is equilibrating, load the samples onto the TLC plate, following **steps 3–8**.
3. Using a Hamilton syringe, remove the $CHCl_3$ layer from each microfuge tube and place into a clean microfuge tube. Rinse the syringe in $CHCl_3$ between each sample. Keep the tubes closed until all the samples are in clean tubes.
4. Open the tubes and dry under N_2 or in a speed vacuum (*see* **Note 17**).
5. One tube at a time, resuspend the sample in 25 μL $CHCl_3$. Use the syringe to mix with approx 15 strokes.
6. Load the total 25 μL on the TLC plate. Start approx 0.5 cm from the origin and streak sample onto the preadsorbent area to approx 0.5 cm from the bottom of the plate (*see* **Note 18**).
7. Rinse the syringe in $CHCl_3$ and repeat **steps 5** and **6** until all of the samples are loaded on the plate. Each plate contains 19 channels. Be sure to leave at least two channels for the lipid standards (*see* **Note 19**).
8. Load 10–25 μg of each lipid standard (PtdEtOH and PA) using a single channel for each standard (*see* **Note 20**).
9. Let plate dry for at least 10 min.
10. Place the plate into the pre-equilibrated TLC tank.
11. Run plate until the solvent front is approx 1 cm from the top of the plate. This typically takes 1.5–2 h.
12. Dry the plate in a hood for several hours (or overnight) (*see* **Note 21**).

3.6. Visualization and Quantitation of Cellular Phospholipids (see Note 22)

1. Place dry TLC plate face-up in an 8 × 10 cassette.
2. Place [^{3}H] screen down on TLC plate and close cassette (*see* **Note 23**).

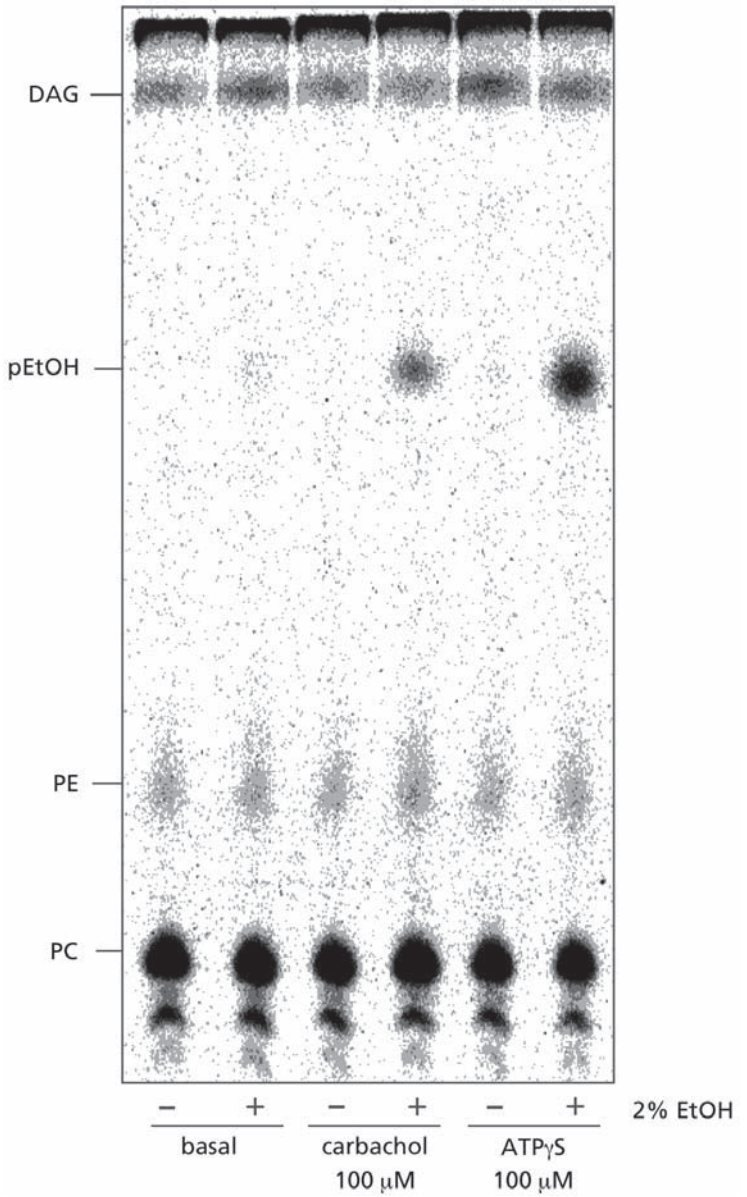

Fig. 1. Thin-layer chromatogram of radiolabeled phospholipids from PC12 cells following stimulation with carbachol and ATPγS, two different GPCR agonists. PC12 cells were labeled with [^{3}H]-myristic acid under serum-free conditions for 16 h. Washed cells were stimulated with agonists as indicated in the absence or presence of 2% EtOH. Radiolabeled phospholipids were extracted, resolved using TLC, and visualized using a [^{3}H] Phosphor screen and a phosphoimager.

3. Expose plate to screen 3–4 d in a light-tight drawer or box.
4. Carefully remove the screen from the cassette. Be sure to keep face-down away from light.
5. Immediately place the screen on the Phosphoimager.
6. Using instructions specific to the Phosphoimager, scan the screen and save the image. A TLC of radiolabeled cellular phospholipids from PC12 cells following stimulations with carbachol and ATPγS is shown in **Fig. 1** (*see* **Note 24**).
7. To visualize the lipid standards, place the TLC plate in a TLC tank with iodine crystals. The stained phospholipids will appear over several hours as brown spots (*see* **Note 22**).
8. Use the iodine-stained PtdEtOH standard to identify the corresponding radiolabeled PtdEtOH on the TLC plate. Using software specific to the Phosphoimager, quantitate the relative amount of PtdEtOH in each channel.

4. Notes

1. While this protocol was written specifically for the stimulation of PC12 cells, PLD activity in all cell types can be measured using the protocol. Use growth media and reagents specific to the cell type of interest.
2. $CHCl_3$ is hazardous (refer to a MSDS for details). Be sure to wear appropriate protection (gloves and laboratory coat) and to work in a fume hood.
3. Cells can also be labeled with [^{3}H]-palmitic acid, [^{3}H]-oleic acid, or [^{3}H]-arachidonic acid.
4. One to three percent EtOH is used for the transphosphatidylation reaction (production of PtdEtOH). Alternatively, 0.1–0.5% primary butanol may be used.
5. These agonists were used here to stimulate PLD activity downstream of GPCRs (carbachol: muscarinic acetylcholine receptors; ATPγS: purinergic receptor subtypes). This same protocol may be used to study other signaling systems (e.g., growth factor stimulation). Adjust the agonist and stimulation conditions accordingly.
6. It is important to make the solvent system fresh prior to use to prevent evaporation of the components and a subsequent change in the composition. It is also important that the acetic acid is of high quality and relatively new; using old acetic acid changes the polarity of the solvent and affects the resolution of the lipids. Purchasing the acetic acid in 100-mL bottles and using it within 6 mo is a good rule of thumb to follow.
7. If a Phosphoimager is unavailable, the radiolabeled phospholipids can be visualized using indirect autoradiography, which requires EN^{3}HANCE scintillation spray (NEN/Perkin-Elmer) and blue-sensitive X-ray film.
8. ^{3}H Phosphor screens should be handled carefully and always while wearing gloves. Protect screens from liquids and excess moisture. Do not clean with water or solvents; remove particulates with a gentle air stream. Store the screen in a protective box or face-down in an exposure cassette.
9. This section contains details specific to the plating of PC12 cells. For other cell types, adjust the seeding density and incubation time so that the cells will be 80% confluent at the time of labeling.

Table 1
R_f values for Phospholipids Resolved Using the Brindley Solvent System *(6)*: Chloroform/Methanol/Acetic Acid/Acetone/H_2O (10:2:2:4:1)

Phospholipid	R_f value
Phosphatidylinositol (bovine liver)	0.15
Phosphatidylserine (bovine brain)	0.19
Phosphatidylcholine (dipalmitoyl)	0.22
Phosphatidylcholine (palmitoyl, arachidonyl)	0.24
Phosphatidylethanolamine (palmitoyl, arachidonyl)	0.36
Phosphatidic acid (dipalmitoyl)	0.40
Phosphatidylethanol	0.58
Myristic acid	0.85
Diacylglycerol (crude)	0.9
Diacyglycerol (dipalmitoyl)	1.0

10. Labeling media can be made ahead of time and stored at 4°C or –20°C. Warm to 37°C prior to use.
11. Warm vials to room temperature before opening to prevent oxidation of the lipids.
12. An applied film of PE on the glass tube increases the recovery of [^{3}H]-myristic acid during sonication (**Subheading 3.3., step 6**).
13. Calculate the amount of [^{3}H]-myristic acid required to make a 10 μCi/mL stock of labeling media. The actual amount of radioactivity in the labeling media will be less than 10 μCi/mL, as some lipid is lost in the drying and sonication steps (**Subheading 3.2.**).
14. If phase separation does not occur, add $CHCl_3$ dropwise to each tube and repeat **steps 3** and **4** from **Subheading 3.4.**
15. At this point, it is best to finish processing the samples as described in the rest of the protocol. However, the samples with both phases may be stored at 4°C up to 1 wk. Begin at **Subheading 3.5.** to continue.
16. Perform equilibration and separation at room temperature.
17. A speed vacuum is the easiest and fastest way to process multiple samples.
18. The origin is the silica gel/preadsorbent interface. The optimal volume for loading is 20–30 μL and should not exceed 50 μL. It is extremely important not to load any sample to the bottom 0.5 cm of the plate.
19. It is necessary to run a PA and a PtdEtOH standard to identify the lipid products of PLD activity in the samples. If desired, other lipid standards (i.e., PC, PE, PI) can be run in additional channels.
20. The vials containing the lipid standards should be warmed to room temperature prior to opening.

21. Tritium Phosphor screens are extremely sensitive to moisture and organic solvents; both can damage the surface of the screen. It is imperative that the TLC plate be completely dry before exposing it to the screen.
22. To visualize the radiolabeled phospholipids using indirect autoradiography, spray the TLC plate with three even coats of EN^{3}HANCE spray. Allow 5–10 min between coats for drying. All spraying should be done in a fume hood. Allow plate to dry thoroughly and immediately expose to blue-sensitve film. Exposure times will vary depending on the signal; a minimum of 24 h is usually required. The optimal exposure temperature is –70°C. Following exposure, develop the film and use densitometry to quanitate the lipids. The lipid standards (PA and PtdEtOH) can be subsequently visualized using iodine staining (*see* **Subheading 3.6., step 7**).
23. Blank the screen immediately before exposure by placing it on a clean light box for 10–15 min. This erases any accumulated energy from radioactive decay and reduces the background on the exposed image.
24. R_f values for phospholipids resolved using the Brindley solvent system are shown in **Table 1**.

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8

Analysis of G Protein-Mediated Activation of Phospholipase C in Cultured Cells

Bo Liu and Dianqing Wu

Summary

Inositide-specific phospholipase C (PLC) β can be activated by the G_q family of Gα subunits and by the Gβγ subunits of heterotrimeric G proteins. PLC catalyzes the hydrolysis of phosphotidylinositol 4,5-bisphosphates (PIP_2) to inositol trisphosphate (IP_3) and diacylglycerol (DAG). This chapter describes a cell-based assay system to determine the activity of PLC by monitoring the levels of IPs in cultured cells. This assay system can be used to examine the activation of G protein-coupled receptors (GPCRs), coupling of GPCRs to G proteins, and the interactions of G proteins to PLCβ.

Key Words: G proteins; PLC; inositol phosphates.

1. Introduction

Many G protein coupled receptors (GPCRs) transduce signals through the activation of the β isoforms of phospholipase C (PLC). Four isoforms of PLCβ have been cloned. The Gα subunits of G_q class proteins can activate all four PLCβ isoforms ***(1)***. GPCRs, such as α_1-adrenergic receptor and m1-muscarinic receptor, activate PLC mainly through the G_q proteins ***(2)***. It was found that the Gβγ subunits released from heterotrimeric G proteins could also activate PLCβ proteins, primarily $PLC\beta_2$ and β_3 ***(3,4)***. As higher concentrations of Gβγ are required for activation of PLCβ than the α subunits of the G_q proteins, the Gβγ-mediated activation of PLC may only be relevant where high levels of G proteins can be activated to release sufficient amounts of Gβγ. Ample evidence has indicated that chemoattractant receptors, which primarily couple to the G_i proteins that are abundant in leukocytes, activate PLC through the Gβγ subunits ***(5,6)***.

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We have been using a cell-based assay system to study the activation of PLCβ by G proteins and GPCRs in COS7 cells ***(2,5,7–12)***. The accumulation of inositol phosphates (IPs) is used as an indicator for PLC activation. It should be noted that COS7 cells contain only certain components of the G protein-PLC pathway. For example, COS7 cells contain $G\alpha_q$, $G\alpha_{11}$, and $PLC\beta_1$, but not $G\alpha_{14}$, $G\alpha_{15}$, $G\alpha_{16}$, or $PLC\beta_2$ ***(5,7,12)***. Thus, GPCRs that induce accumulation of IPs in COS7 cells should act through the $G_{q/11}$ proteins. Gβγ showed very little effect on the accumulation of IPs in COS7 cells probably because of the lack of sufficient levels of $PLC\beta_2$ and $PLC\beta_3$. To detect the effects of Gβγ on PLC activation in these cells, $PLC\beta_2$ needs to be coexpressed ***(5,7)***. We have used this cotranfection system to successfully detect the coupling of G_i coupled receptors, mainly chemoattractant receptors, to G_i and $PLC\beta_2$ in COS7 cells ***(5,6,11)***.

2. Materials

2.1. Cell Culture, Transfection, and Cell Labeling

1. COS7 cells (ATCC, Rockville, MD).
2. 1X Dulbecco's modified Eagle's medium (DMEM) (Life Technologies, Rockville, MD): 4.5 g/L D-glucose, L-glutamine, 25 m*M* HEPES buffer, and pyridoxine hydrochloride, but no sodium pyruvate.
3. Fetal bovine serum (FBS; Hyclone Laboratories Inc., Logan, UT).
4. 24-Well cell culture plates (Corning, Corning, NY).
5. LipofectAMINE and PLUS reagent kit (Life Technologies).
6. LacZ expression plasmid.
7. Inositol-free DMEM 1X (Life Technologies): 4.5g/L D-glucose, L-glutamine, and pyridoxine hydrochloride, but no i-inositol or sodium pyruvate.
8. Myo-[2-^{3}H] inositol (Amersham, Piscataway, NJ), handled with caution for radioactive protection.

2.2. Measurement of the Level of IPs

1. 1X phosphate-buffered saline (PBS) buffer: 0.137 *M* NaCl, 2.7 m*M* KCl, 1.4 m*M* KH_2PO_4, 0.01 *M* Na_2HPO_4.
2. Inositol-free DMEM 1X (Life Technologies): 4.5 g/L D-glucose, L-glutamine, and pyridoxine hydrochloride, but no i-inositol or sodium pyruvate.
3. 500 m*M* LiCl.
4. 1.5 mL Microcentrifuge tubes.
5. pHydrion dye (VWR).
6. 0.5 *M* EDTA, pH 8.2.
7. 2 *M* KOH.
8. 10% Perchloric acid (Sigma).
9. 20 mg/mL Phytic acid (Sigma).
10. 1 *M* Tris-HCl, pH 8.0.
11. AG1-X8 resins (Bio-Rad, Hercules, CA).

12. Washing buffer: 5 m*M* borax, 60 m*M* Na formate.
13. Elution buffer: 1 *M* ammonium formate, 0.1 *M* formic acid.
14. Liquid scintillation cocktail (Wallac, Turku, Finland).

3. Methods (*see* Note 1)

3.1. Cell Culture and Transfection

1. COS7 cells are maintained in DMEM containing 10% FBS at 37°C under 5% CO_2.
2. The day before transfection, cells are seeded at 5×10^4 cells/well into a 24-well cell culture plate.
3. Transfection procedures are the same as described in Chapter 13.
4. The PLC assay is carried out 24–48 h after transfection (*see* **Note 2**).

3.2. Cell Labeling

1. The day before the PLC assay, cells are labeled with [^{3}H] inositol.
2. For 24-well plates, 0.5 mL inositol-free DMEM containing 10% dialyzed fetal calf serum (FCS) and 0.5 µCi of [^{3}H] myoinositol is added to each well.

3.3. Measurement of the Level of IPs

1. Aspirate the cell media.
2. Wash the cells twice with 1X PBS, 250 µL/well.
3. Add 200 µL/well inositol-free DMEM containing 10 m*M* LiCl (*see* **Note 3**). If ligand-stimulated PLC activity is measured, proceed to **step 4**. Otherwise, proceed to **step 5**.
4. Add ligand at desired concentrations into wells after 5-min incubation after the addition of LiCl at room temperature.
5. Incubate the cells at 37°C for 1 h.
6. Prepare microcentrifuge tubes for each well and put them on ice. Add into each tube 5 µL pHdrion dye, 5 µL 0.5 *M* EDTA, pH 8.2, and 150 µL 2 *M* KOH.
7. Put the cell plates on ice (*see* **Note 4**). Add into each well 200 µL 10% perchloric acid and 20 µL phytic acid (20 mg/mL). Incubate on ice for 10 min.
8. Take 350 µL cell lysate from each well and add into the microtubes containing the dye. Add 75 µL 1 *M* Tris-HCl, pH 8.0, (*see* **Note 5**). Vortex briefly and centrifuge in a microcentrifuge at 10,000*g* for 5 min.
9. Transfer the supernatant into another set of microtubes containing 0.2 mL AG1-X8 resins. Vortex and centrifuge for 5 min. Discard the supernatant.
10. Wash the resins with 1 mL/tube of the washing buffer 3×.
11. Elute with 1 mL/tube elution buffer. Vortex and spin for 20 s.
12. Take 0.5 mL supernatant and mix with 5 mL liquid scintillation cocktail. Count in a scintillation counter.

4. Notes

1. As opposed to in vitro reconstitution assays, the cotransfection method for PLC assay described in this chapter make it easy and possible to study the ligand-

induced effector activation through their specific GPCRs. Moreover, G protein-PLC coupling can be investigated in detail by selectively introducing certain proteins into cells.
2. The PLC assay is usually performed at 24 h after transfection. However, the protein expression duration should also be adjusted according to the cell types and strength of the promoters in the plasmids.
3. LiCl inhibits inositol monophosphatase activity, thus leading to the accumulation of IPs in cells.
4. All reactions starting from this step need to be carried out on ice.
5. If the color is yellow, add NaOH until it turns green. If the color is purple, add HCl until it is green.

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9

Intensive Mutational Analysis of G Protein-Coupled Receptors in Yeast

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Summary

Expression of G protein-coupled receptors (GPCRs) in yeast makes possible a genetic procedure for determining the range of amino-acid substitutions that are compatible with function in particular receptor regions. The regions of interest are targeted for intensive random mutagenesis, providing multiple amino-acid substitutions per allele. Genetic screening of the mutagenized receptors in yeast allows the identification of rare functional alleles, which can then be recovered, sequenced, and further characterized. Procedures for random oligonucleotide-directed mutagenesis, creation, and screening of mutational libraries in yeast, as well as quantitative assay of receptor function, are described.

Key Words: G protein-coupled receptors, mutagenesis, pheromone, receptors, membrane proteins, genetic screening, β-galactosidase assay.

1. Introduction

Despite the importance of the large superfamily of G protein-coupled receptors (GPCRs) in cell signaling and medicine, there is still insuffient understanding of the mechanism by which binding of ligands at the cell surface of a GPCR leads to the activation of an intracellular trimeric G protein. The three-dimensional structure of the unactivated form of one GPCR, rhodopsin, is now known ***(1)***, and there is evidence that the activation of GPCRs involves relative motions of transmembrane segments ***(2)***. However, the particular molecular rearrangements of GPCRs accompanying this activation remain obscure. One way of acquiring information about the structure and activation of the hundreds of GPCRs is to evaluate the spectrum of allowed amino-acid substitutions that are compatible with receptor function. This is often attempted in mammalian GPCRs through the site-directed mutagenesis of particular resi-

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dues for which there is some evidence of their significance in receptor structure and function. However, examination of the allowed sequence space compatible with receptor function is a slow process when mutations are created one at a time. Furthermore, the information obtained by this approach tends to be model-dependent because investigators generally only introduce mutations at positions that they believe will be functionally important.

An alternative approach that allows a more general characterization of the spectrum of amino-acid side chains compatible with signaling function was initially applied to GPCRs by Baranski, Bourne, and coworkers ***(3,4)*** and has been previously used in studies of other transmembrane proteins ***(5,6)***. This approach makes use of extensive random mutagenesis to introduce a large number of base substitutions targeted to particular regions of a protein. The frequency of amino-acid substitutions is high enough to cause loss of function in the large majority of individual mutant alleles. However, if cells expressing mutagenized receptors can be subjected to a screen or selection for function, the small number of mutant alleles that retain function can be identified. Because each functional allele identified in this way will contain multiple-base substitutions, this provides an expedient way to map the spectrum of amino-acid substitutions that are compatible with function in any given region of the targeted receptor.

Effective use of this type of mutagenesis requires a system in which base substitutions can be introduced with high efficiency to produce a large number of mutant alleles against a background devoid of normal alleles. It also requires a system where an efficient screen or selection for function can be performed. In the case of GPCRs, these requirements are most effectively met using yeast cells. Baker's yeast, *Saccharomyces cerevisiae*, normally exists in either a haploid or diploid state. There are two haploid cell types, *MAT***a**, and *MAT*α, that can fuse to form a diploid zygote. Detection of cells of the opposite-mating type is mediated by secreted mating pheromones, α-factor and **a**-factor, short peptides, that bind to the surface of *MAT***a** and *MAT*α cells, respectively. The cell surface receptors for these pheromones are GPCRs that activate a cytoplasmic heterotrimeric G protein consisting of subunits that exhibit a high degree of sequence similarity to mammalian G protein subunits. Ligand binding to the pheromone receptors causes release of guanosine diphosphate (GDP) from the G protein α subunit, followed by binding of guanosine triphosphate (GTP) and dissociation of the subunits. Downstream signaling in the pheromone response pathway appears to be mediated exclusively by the interaction of released Gβγ subunits with downstream effectors, resulting in the activation of a mitogen-activated protein (MAP) kinase cascade that causes cell-cycle arrest, activation of transcription of a number of genes, and physiological changes that prepare cells for mating (*see* **ref. *7***).

Extensive genetic analysis of the yeast mating pheromone pathway has led to the development of convenient genetic screens and selections that can be used to monitor activation of the pheromone response (*see* ***8***,***9***). The most common readout for detecting cells expressing functional receptors involves activation of transcription of *FUS1*, a gene involved in cell fusion that is strongly induced at the transcriptional level in response to pheromone binding. Yeast colonies of strains expressing functional receptors can be detected on culture plates if the strains also express a reporter construct in which the *FUS1* promoter is fused to the *Escherichia coli* gene encoding β-galactosidase. Signaling-competent colonies of such strains take on a blue color on culture plates containing mating pheromone and the colorimetric β-galactosidase substrate X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactoside). Reporter constructs in which the *FUS1* promoter is fused to the auxotrophic marker *HIS3* allow selection for cells expressing functional receptors on media containing mating pheromone and lacking histidine ***(4***,***10)***. In screening for cells displaying pheromone-dependent growth in the absence of histidine, it is important to use a yeast strain that does not undergo cell-cycle arrest in response to pheromone signaling, such as one containing a deletion of the *FAR1* gene. Cells that undergo pheromone-dependent cell-cycle arrest can be accommodated in screening for *FUS1-lacZ* induction by replica-plating pregrown colonies onto media containing pheromone and X-gal.

Although yeast pheromone receptors contain no significant sequence similarity to receptors found in mammalian cells, the mechanism of action of the pheromone receptors must be similar to their mammalian counterparts, because they are, in many cases, interchangeable. Mammalian receptors expressed in yeast can activate the pheromone response pathway ***(11–16)*** and, at least in one case, yeast receptors can activate signaling by a mammalian G protein ***(17)***. This makes it possible to screen for mutations that affect function of mammalian receptors by expressing the mutated receptors in yeast and using assays for the activation of the yeast pheromone response pathway, an approach taken by Baranski et al. ***(3***,***4)*** in screening for functional mutations of the human chemotactic C5a receptor. In this case, the cDNA encoding the C5a receptor was expressed in yeast that also expressed a chimeric yeast/human G protein α subunit to facilitate receptor G protein coupling, although other mammalian receptors have been shown to couple directly to the endogenous yeast G protein ***(14***,***15)***. The yeast-signaling system has also been previously used to analyze determinants of coupling specificity between mammalian receptors and mammalian G protein subunits ***(18)***.

Our laboratory has recently applied the technique of extensive mutagenesis to the endogenous yeast α-factor receptor encoded by the *STE2* gene, targeting all seven predicted transmembrane segments ***(19)*** and the third intracellular loop ***(20)*** (*see* **Fig. 1**). This was undertaken to examine the sequences important for

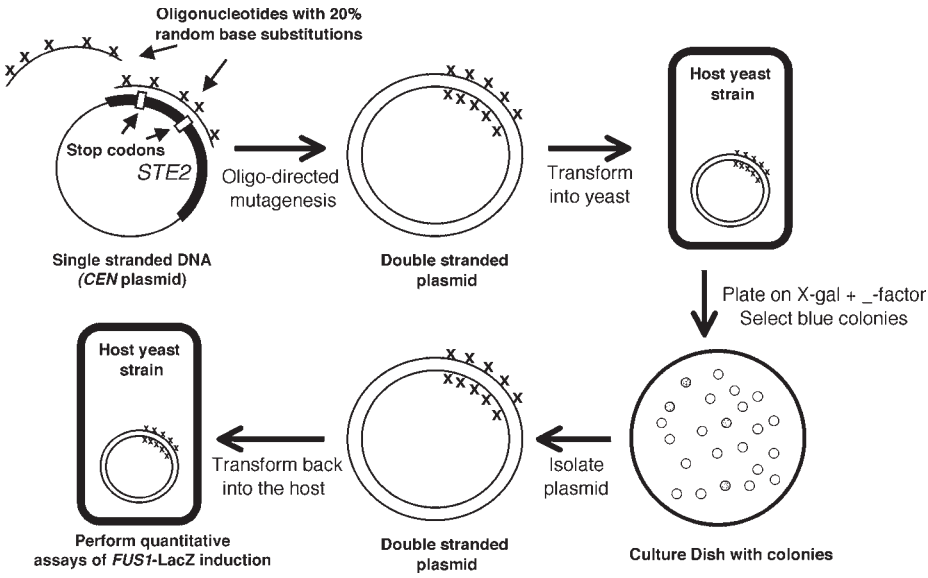

Fig. 1. Overall scheme for mutagenesis, screening and testing of GPCRs in yeast.

structure and function of the yeast receptor, as well as to provide a comparison with mammalian receptors, for which more structural information is available. In the absence of an experimental basis for comparison, the extreme sequence divergence between the α-factor receptor and mammalian receptor makes it difficult to extend the results obtained in one system to the other, in spite of their functional interchangeability. Even among endogenous yeast receptors, there is little or no sequence similarity between the receptors for α-factor and **a**-factor, despite the fact that both signal by activating the same G protein.

A particularly useful property of the yeast pheromone response pathway as a target for investigating sequence dependence of receptor function is that signaling is not significantly affected by drastic changes in levels of receptor expression ***(21,22)***. Therefore, mutations causing loss of signaling are likely to reflect direct effects on receptor structure or G protein activation, rather than changes in biosynthetic or degradation rates or alterations in intracellular targeting.

This chapter describes the procedures for creating libraries of extensively mutagenized alleles, screening these libraries for function, and quantitative characterization of functional alleles identified from these libraries. The approach is surprisingly rapid and complete. Application to the yeast α-factor receptor allowed characterization of 576 unique amino-acid substitutions, representing 61% of all possible single-base substitutions at 157 positions in the seven-transmembrane segments of the receptor in a period of less than 1 yr.

2. Materials

2.1. Creation of a Library of Genes Encoding Mutagenized Receptors

1. Oligonucleotide containing a mixture of bases at each position (*see* **Note 1**).
2. Single-stranded template DNA from a yeast-*E. coli* phagemid shuttle vector encoding a nonfunctional receptor allele (*see* **Note 2**).
3. T4 Polynucleotide kinase and supplied buffer (United States Biochemical).
4. T7 DNA polymerase (not Sequenase®), and supplied buffer (United States Biochemical).
5. EDTA: 0.1 *M* and 0.5 *M*, pH 8.0 stock.
6. ATP: 100 m*M* solution in water.
7. 20X SSC: 3 *M* NaCl, 300 m*M* sodium citrate, pH 7.0.
8. Competent *E. coli* cells (e.g., XL1-Blue), either purchased or prepared by standard techniques ***(23)***.
9. YT plates containing 100 µg/mL ampicillin ***(24)***.
10. Liquid Terrific Broth (TB) containing 100 µg/mL ampicillin ***(25)***.
11. Miniprep DNA isolation kit (e.g., Wizard kit from Promega Corp.).
12. Supplies for sequencing of double-stranded plasmids (*see* **Note 3**).

2.2. Screening Yeast Colonies for Signaling Function

1. Competent yeast cells from an appropriate strain (*see* **Note 4**).
2. Culture plates containing synthetic complete dropout medium (*see* **Note 4**). 10X Complete synthetic dropout medium powder consists of 1.67 g/L yeast nitrogen base (Difco), 5 g/L ammonium sulfate, amino-acid s and nitrogenous bases: 30 mg/L isoleucine, 150 mg/L valine, 20 mg/L adenine, 20 mg/L arginine, 20 mg/L histidine, 30 mg/L leucine, 30 mg/L lysine, 20 mg/L methionine, 50 mg/L phenylalanine, 20 mg/L tryptophan, 30 mg/L tyrosine, and 20 mg/L uracil. Leave out the appropriate combination of nitrogenous base and/or amino-acid s for selection of yeast transformants. To make plates, an appropriate amount of premixed powder (without the appropriate amino-acid or nitrogenous base) is dissolved in 1 L distilled water. 20 g/L Dextrose and 20 g/L agar are added and the mixture is autoclaved and allowed to cool to 65°C before pouring plates (*see* **ref. *25*** for a more detailed description).
3. Culture plates containing buffered synthetic complete dropout medium, ligand, and X-gal (*see* **Note 5**). Plates are made as in **step 2**, but to only 90% of the normal volume. After autoclaving and cooling to 65°C, 9 parts of the medium are mixed with 1 part 10X stock of phosphate buffer consisting of 1 *M* potassium phosphate, pH 7.0, then 1 mL 40 mg/mL X-gal in dimethyl formamide is added to each liter of medium. The 10X phosphate buffer is prepared by dissolving 136.1 g KH_2PO_4 and 42.1 g KOH in 950 mL distilled water, titrating to pH 7.0, and adjusting the volume to 1 L, followed by autoclaving. Synthetic α-factor is spread on pre-poured plates from delutions of a 1 mg/mL stock and allowed to dry just before use.
4. Velveteens for replica plating. Short-nap dense cotton fabrics most effectively preserve isolated colonies.

5. Acid-washed 500-micron glass beads (Sigma) are soaked in concentrated HCl for 15 min, then extensively rinsed with distilled water until they reach neutral pH.
6. Miniprep DNA isolation kit for isolation of yeast plasmids (*see* **Subheading 2.1., step 11**).
7. Toothpicks.
8. TE buffer: 10 m*M* Tris-HCl, 1 m*M* EDTA, pH 8.0.

2.3. Quantitation of Signaling Function

1. Glass tubes for culturing yeast.
2. Liquid synthetic complete dropout medium (*see* **Subheading 2.2., step 2**).
3. Ligand solution (varying concentrations, *see* **Subheading 3.3., step 3**) diluted into a solution of 5 µg/mL horse cytochrome-*c* in distilled water.
4. Flat-bottom 96-well microtiter plates (Nunc).
5. Purified β-galactosidase (Sigma-Aldrich) for standard curve.
6. Bovine serum albumin.
7. Fluorescein di-β-D-galactopyranoside (FDG) solution prepared by combining equal amounts of solution A (1.4 m*M* FDG [Molecular Probes] in 25 m*M* PIPES, pH 7.2) and solution B (5% Triton X-100 in 250 m*M* PIPES, pH 7.2). Solution A should be prepared fresh just before each use from a 10-m*M* FDG stock in dimethylsulfoxide (DMSO).
8. Microtiter plate readers for both absorbance (e.g., SpectraMax Plus, Molecular Devices Corp.) and fluorescence (e.g., SpectraMax Gemini, Molecular Devices Corp.).
9. 1 M Na_2CO_3 stop solution.

3. Methods

3.1. Creation of a Library of Genes Encoding Mutagenized Receptors

3.1.1. Kinasing Oligonucleotides and Annealing to Template

1. Prepare a kinase mix for each reaction that includes: 1 µL 10X T4 polynucleotide kinase buffer, 1 µL 10 m*M* ATP, 1 U T4 polynucleotide kinase, 1 µL mutagenic oligonucleotide (~ 5 ng/µL; *see* **Note 1**), and sterile distilled water to final volume of 10 µL.
2. Include a control reaction containing 1 µL water instead of oligonucleotide.
3. Incubate at 37°C for 1 h, then stop the reaction by adding 1.5 µL of 0.1 *M* EDTA and heating to 65°C for 10 min.
4. Add 1 mg single-stranded DNA (*see* **Note 2**) in approx 1 µL to each kinase reaction followed by enough 20X SSC to yield a final concentration of 1X. Use of an excess of template to oligonucleotides prevents the competition that could lead to selective incorporation of sequences with lower annealing temperatures, thereby reducing the diversity of the library. We find that a 3:1 molar ratio of DNA to oligonucleotides gives the highest number of mutations/allele.
5. Heat tubes to 75°C by placing in a beaker with 500 mL water.

6. Allow to cool to room temperature for 2.5–3 hr. Once at room temperature, transfer to ice bath.

3.1.2. Extension and Ligation

1. Prepare a DNA synthesis/ligation mix for each mutagenesis reaction and the control lacking an oligonucleotide by adding 1 µL 1 *M* Tris-HCl, pH 8.0, 0.5 µL 1 *M* $MgCl_2$, 1 µL 0.1 *M* DTT, 5 µL 10 m*M* ATP, pH 7.0, 2.5 µL mix containing dATP, dCTP, dGTP, and dTTP, each at 10 m*M*, 1.25 U T7 DNA polymerase, 2 U T4 DNA ligase, and sterile double-distilled water to a final volume of 40 µL.
2. Aliquot 40 µL DNA synthesis/ligation mix into each of the tubes containing annealed oligonucleotide and template.
3. Incubate on ice for 30 min, then at 18°C for 30 min, then at room temperature for 30 min, then at 37°C for 2 h. The gradual warming is designed to maintain the diversity of the hybridized products by allowing the polymerase to act initially under conditions where weakly hybridizing oligonucleotides will not dissociate from template.
4. Stop the reaction by adding 1.2 µL 0.5 *M* EDTA to each tube. Samples can be stored at –20°C or transformed immediately into *E. coli*.

3.1.3. E. coli *Transformation and Preparation of Library DNA*

1. Transform into a strain of *E. coli* used for cloning, such as XL1 blue, using a standard protocol. It is necessary at this point to empirically establish conditions that will lead to the desired number of transformants (*see* **Note 6**). Using homemade-competent cells produced using a RbCl treatment from a standard protocol ***(23)***, we typically conduct multiple transformations of 10 µL each into 150 µL competent cells plated on 6 YT plates with the appropriate antibiotic.
2. Pipet 5 mL TB liquid medium with antibiotic onto a plate containing colonies from the transformation. Using a sterile glass rod gently resuspend the colonies in the medium. Pipet the liquid from that first plate onto a second and resuspend the colonies from that plate. Repeat until colonies from all the plates are resuspended.
3. Dilute 1–2 mL of scraped cell suspension in 10–15mL fresh TB medium with antibiotic and culture for a few hours.
4. A small volume of the *E. coli* culture should be streaked or spread for single colonies on a fresh culture plate for use in testing the library. Individual colonies from this plate are picked the next day and cultured for preparation of plasmid DNA, which is then sequenced using standard protocols for double-stranded plasmids.
5. Prepare plasmid DNA from this culture using a miniprep protocol, such as the Promega Corp. Wizard. DNA from several minipreps can be pooled. The remainder of the culture can be frozen for future use following standard procedures for *E. coli* strain preservation.

3.2. Screening Yeast Colonies for Signaling Function

3.2.1. Transforming Yeast

1. Conduct transformations using a standard LiCl protocol ***(26)*** (*see also* the yeast transformation Website: http://www.umanitoba.ca/faculties/medicine/biochem/gietz/

Trafo.html). However, single-stranded carrier DNA is not included in the transformation mixtures, because the presence of this DNA may slightly increase the frequency of unwanted chromosomal mutations and its use is unnecessary to achieve the desired number of transformants. Use approx 10 µL of pooled *E. coli* miniprep/transformation as needed to obtain the requisite number of yeast colonies.

2. Plate the yeast transformation on agar with the appropriate auxotrophic selection. The number of plates to be used depends on the transformation efficiency and the desired number of colonies. To avoid a loss of diversity of the library, the number of yeast transformants should be greater than the original number of colonies obtained on transformation into *E. coli*. For screening for function based on *FUS1-lacZ* induction, there should be no more than 1000–2000 colonies/plate to allow examination of the color of individual colonies.

3.2.2. Screening Yeast Transformants for FUS1-LacZ Induction

1. About 3 d after transformation, yeast colonies can be replica-plated onto selective plates containing X-gal and α-factor. After replica plating using sterile velveteens, incubate plates at 30°C wrapped in aluminum foil (X-gal is light-sensitive). Colonies with a functional signaling pathway will turn blue 2–3 d after replica plating. Color development is variable over the surface of plates. Colonies on the perimeter of plates tend to turn blue spontaneously, so it is best to choose signaling-competent colonies that appear dark blue and are on a plate region in the vicinity of similar-sized white colonies.
2. Subclone each blue colony onto a fresh plate with the appropriate selective medium, but lacking X-gal and α-factor, to isolate single colonies. Pick a single colony from each isolate and streak across a sector of a plate with the same selective medium to create a master plate of homogeneous cells.

3.2.3. Confirming Plasmid-Dependence of the Signaling-Competent Phenotype

1. An efficient way to rescue the mutagenized plasmids from yeast exhibiting signaling function (*see* **Note 7**) is to use a miniprep designed for isolation of plasmids from bacteria. Start a yeast liquid culture using yeast cells from a master plate in 2 mL synthetic dropout medium lacking the appropriate marker and grow to a very high density (~24–36 h). Transfer this culture into a microcentrifuge tube and follow the instructions from Promega for use of the Wizard plasmid preparation kit with the following modifications. After adding resuspension and lysis buffer, add 100 mg acid-washed glass beads and vortex for 5 min. Spin at 12,000*g* in a microcentrifuge for 10 min. Transfer the supernatant to a new tube and add neutralization buffer. Continue with the normal Wizard protocol, but elute plasmid DNA in 35 µL TE buffer.
2. Transform all 35 µL yeast miniprep DNA into 100–150 µL of *E. coli*-competent cells. Pick a single colony from each transformation plate and grow to saturation. Prepare plasmid DNA from this culture using a miniprep protocol and transform back into yeast as described previously. The plasmids may be

sequenced either at this point or after the phenotype has been confirmed by retransformation (*see* **Note 7**).

3. Pick three yeast colonies from each transformation plate. Streak each colony onto a sector of a fresh plate to isolate single colonies. Prepare frozen stocks and/or master plates from each subcloned transformant. The signaling competence of three isolates of each mutant allele can be tested using the quantitative assay of β-galactosidase activity. In some cases, it is convenient to recheck the signaling competence on X-gal plates before conducting the quantitative assay.

3.3. Quantitation of Signaling Function Based on Induction of the FUS1-LacZ *Reporter*

1. Inoculate 4 mL of dropout medium lacking the appropriate marker with desired strain and grow overnight to an OD_{600} of 1.5–2.0.
2. Using cells from the first culture, inoculate a second 4-mL culture in the same medium. The number of cells should be adjusted based on the generation time of the relevant strain so that the culture will reach an OD_{600} of approx 0.4 at the time it is desired to start the assay after growing for at least two generations.
3. Prepare dilutions of ligand. Dilute α-factor from a 1-mg/mL stock in water into solutions containing 5 µg/mL final concentration of horse cytochrome c in water to prevent adsorption of low concentrations of α-factor to surfaces. The dilutions are prepared such that the desired final concentration of α-factor will be achieved following mixing of 10 µL diluted α-factor and 150 µL cells and media in each well of a microtiter plate. Final concentrations typically range from 0 to 2000 n*M* depending on the application and yeast strain.
4. Pipet 10 µL appropriately diluted α-factor into each well of a 96-well microtiter plate.
5. Pipet a range of concentrations of purified β-galactosidase (usually 0–60 ng diluted into 160 µL final volume of 1 mg/mL BSA) into a series of wells on each plate as a calibration curve.
6. Pipet 150 µL cell culture to each well, except those containing the calibration. Include control samples lacking cells and/or α-factor are also included.
7. Incubate the plate at 30°C with gentle shaking for 105 min.
8. Using an automated plate reader, determine the OD_{600} of each well of the microtiter plate relative to the sample lacking cells. Readings are more consistent without any automatic path-length correction. Also, it is advisable to avoid the rapid mixing of the plate provided by some readers, as this tends to distribute cells around the outside of the wells, rendering optical density readings unreliable.
9. Pipet 32 µL diluted FDG solution (*see* **Subheading 2.**) into each well.
10. Incubate 15–120 min at 37°C, depending on the particular application, then add 32 µL stop solution to each well. Longer incubation times provide a better signal-to-noise ratio for cells that signal weakly, however, it is important to ensure that the assay time is short enough so that β-galactosidase is assayed in a linear realm. This can be verified by comparison to the standard curve using purified β-galactosidase assayed concomitantly with the experimental samples.

11. Measure the fluorescence of each well in a fluorescent plate reader using an excitation wavelength of 485 nm and an emission wavelength of 530 nm (*see* **Note 8**).

3.4. Analysis of Functional Mutations

1. The completeness of the unselected mutational library can be estimated from the number of translationally silent mutations recovered among the functional alleles, making the assumption that most changes in codon usage do not affect signaling function. This is likely to be true in the case of the α-factor receptor, where the response to pheromone is insensitive to the level of receptor expression. Dividing the number of silent-single base substitutions that were recovered within the targeted region by the number of possible silent single-base substitutions within the region provides an estimate of the number of times each codon change would be expected to arise in the unselected library. This number can also be obtained directly by sequencing of random clones from the mutational library, although this requires a substantial amount of additional sequencing.
2. Enumeration of functional alleles in extensively mutagenized libraries provides direct information on the range of allowed substitutions in the mutagenized positions, subject only to the assumption that individual mutations do not interact in such a way as to allow one mutation to suppress the detrimental effects of another. Although such intragenic suppression events can occur in receptors, such as the α-factor receptor, we have previously found that they are rare ***(27)***.

   The range of tolerated mutations in receptors can be extremely broad. For example, in the α-factor receptor, our analysis recovered 61% of all possible single base substitutions among 390 functional alleles mutagenized over 157 residues of the transmembrane segments ***(19)***. Every position in the transmembrane segments could tolerate at least one substitution. Polar amino-acid side chains were recovered at 73% of all positions. Proline substitutions were recovered at 53% of all positions, where this change could arise from a single-base substitution, even though the introduction of prolines would be expected to distort the predicted helical structure of the transmembrane segments.
3. In screening or selecting for functional alleles from random libraries, the failure to recover a particular mutation at any given position cannot be regarded as definitive evidence that the particular substitution can not be tolerated, unless the mutation in question is actually created by site-directed mutagenesis and tested directly. On the other hand, given the knowledge of the extent of sequence space coverage by the library, the likelihood that a functional mutation would be missing from the library on a purely random basis can be calculated. The average number of times, n, that each individual codon change is recovered can be considered to be the mean of a Poisson distribution. Thus, the likelihood of failing to recover any particular functional change as a functional mutation is $p = e^{-n}$. For the libraries we screened in our analysis of the α-factor receptor, we recovered 1.1–3.3 substitutions per possible codon change, corresponding to probabilities of 4–33% for failure to recover any given functional mutation. Conclusions regarding positions where two or more possible mutations (e.g., from hydrophobic to hydrophilic residues) are not recov-

ered are more statistically significant because the probabilities of failure to recover these changes on a random basis are multiplied.

4. Notes

1. Our approach for mutagenizing receptors was to use oligonucleotide-directed mutagenesis of a single-stranded template to introduce the desired substitutions. A disadvantage of the use of this type of oligonucleotide-directed mutagenesis is the possibility of preferential selection of certain amino-acid substitutions or alleles encoded by oligonucleotides containing patterns of base substitutions that enhance hybridization to the template, which could result in a reduction of the number of substitutions per allele. An alternative approach is to engineer the target gene with appropriate restriction sites that allow mutagenized regions to be ligated as double-stranded fragments ***(3,4)***. The insertion of double-stranded inserts eliminates preferential hybridization by using a DNA polymerase to create a second strand that is exactly complementary to the randomly mutagenized synthetic oligonucleotide. However, we find that the use of oligonucleotide-directed mutagenesis as described here (with particular attention to details that minimize preferential hybridization of oligonucleotides to template), provides an adequate range and number of substitutions for the intensive mutational approach. This conclusion is supported by our recovery of all possible single-base substitutions at many positions in the mutagenized α-factor receptor and a large fraction of all possible single-base substitutions in targeted regions of the receptor ***(19)***. Furthermore, use of a method based on alteration of a single-stranded template does not require the introduction of restriction sites at the ends of the targeted regions and eliminates the ligation and cloning steps that could reduce the size of the mutagenized library.

   "Spiked" oligonucleotides used for extensive mutagenesis must be custom synthesized so as to contain a mixture of bases at each position. The exact proportions of the different bases to incorporate at each position are determined primarily by the choice of the desired level of mutagenesis. A high level of mutagenesis provides the fastest mapping of sequence space, making use of the smallest number of mutant alleles. On the other hand, the incorporation of a large number of substitutions per allele makes the frequency of functional alleles low, possibly making it difficult to recover them. Additionally, higher numbers of substitutions per allele increase the likelihood that the observed phenotype will reflect interactions between mutations (e.g., intragenic suppression), rather than direct compatibility of substitutions with receptor function.

   The optimum way to synthesize "spiked" oligonucleotides is by making mixtures of the different types of phosphoramidite nucleotide precursors in proportions that correspond to the desired final mix of bases. For example, if it is desired to make mutant alleles in which each position has a 9% chance of being mutated, each major type of nucleotide would be mixed with the other three in the proportion 91:3:3:3. The most reliable way to accomplish this type of synthesis is to make four such mixtures in different bottles, using them directly in the synthesis.

An alternative approach allowing the automated synthesizer to mix nucleotides by drawing from multiple bottles simultaneously is likely to give a less uniform frequency of mutations. In some cases, depending on the particular chemistry being used, a correction must be applied for different coupling efficiencies of different types of phosphoramidite during oligonucleotide synthesis.

The length of the oligonucleotide determines the extent of the regions targeted for mutagenesis. Given the degradation in oligonucleotide quality that occurs with longer lengths of synthesis, we have not attempted to use oligonucleotides longer than approx 70 bases. Although we incorporate mixed bases at all positions in the nucleotides, the efficiency of mutagenesis is low near the ends of the targeted regions. The most frequent incorrect products in oligonucleotide synthesis are those that lead to deletions following introduction into *E. coli*. Because frameshifts and other deletions are likely to lead to complete loss of function, which would not be seen in screens for functional alleles, no purification of olignucleotides is necessary prior to their use for this type of mutagenesis.

2. Oligonucleotide-directed mutagenesis often yields a significant number of unmutagenized alleles (>10%, depending on the exact conditions). Because the goal is to screen or select for functional alleles that likely represent the order of 0.1% of the total library, it is important to have a way of eliminating recovery of unmutagenized alleles. This can be readily accomplished by engineering a starting allele that is completely nonfunctional. Our studies have made use of a set of starting alleles that contain nonsense mutations within the region targeted for mutagenesis. Wherever possible, the nonsense codons are engineered into the starting alleles at sites where they can be introduced by making a single-base substitution. These mutations will be corrected in any functional allele that is recovered from the randomly mutated library. The use of multiple nonsense mutations can eliminate reversion events that could lead to functional alleles. To prevent partial annealing of oligonucleotides, when multiple nonsense codons are used, they are placed near the ends of the regions to be targeted for random mutagenesis. The fact that the starting alleles differ from the wild-type sequence at only a small number of bases makes it unlikely that they will introduce significant bias by preferential hybridization to mutant oligonucleotides.

   The shuttle vector that was used for mutagenesis incorporates the following useful features ***(19)***. (1) It contains a centromeric yeast origin of DNA replication to allow maintenance in yeast at a low-copy number. The low-copy number ensures that the level of expression can be significantly increased or decreased with minimal effects on signaling levels ***(21,22)***. (2) It contains an f1 origin of replication for bacteriophage M13, allowing efficient production of single-stranded DNA as a template for oligonucleotide-directed mutagenesis. (3) It encodes an allele of *STE2* containing a *c*-myc epitope tag at the carboxyl-terminal to facilitate quantitation of levels of receptors in cells. (4) It contains origins of DNA replication and ampicillin resistance allowing propagation in *E. coli*.

   The quality of the preparation of single-stranded DNA is important in obtaining efficient mutagenesis, because contaminating RNA and genomic DNA can

provide priming activity that increases the frequency of unmutagenized alleles. For preparation of single-stranded templates, we use a procedure similar to that described by Kunkel et al. ***(28)*** with particular attention to complete removal of supernatants during precipitation steps.

3. It is advisable to sequence a set of random clones from the mutagenized library to determine the average number of substitutions per allele and make sure that mutagenesis is working efficiently. This is most easily accomplished using a thermostable polymerase and fluorescent reagents such as the Big Dye kit from Applied Biosystems, analyzed by an automated sequencing facility. For reasons that are not entirely clear, we generally obtain a lower number of substitutions than would be expected based on the particular mixtures of incorrect and correct bases used during oligonucleotide synthesis. Factors influencing the efficiency of mutagenesis include the quality of single-stranded DNA preparations, oligonucleotide-to-template ratio, and the hybridization and extension conditions for T7 DNA polymerase.
4. The following are requirements for a yeast host strain: (a) For mutagenesis of the α-factor receptor encoded on a plasmid, the strain must contain a deletion of the chromosomal copy of *STE2*. This may be unnecessary for testing mutants of mammalian receptors expressed in yeast. (b) The strain should contain an appropriate reporter gene construct, such as *FUS1-lacZ* or *FUS1-HIS3* allowing selection or screening for functional alleles. Assays of signaling function tend to be most reproducible in strains where the reporter construct is integrated into the yeast chromosome, rather than maintained on a plasmid. (c) The strain should contain a deletion of the *FAR1* gene responsible for cell-cycle arrest in response to activation of the pheromone response pathway. This allows functional strains to grow well on plates and may be critical for the efficient recovery of constitutively active or hypersensitive receptor alleles, which can represent a significant fraction of all mutant receptors. (d) The strain should contain an auxotrophic marker that allows maintenance of a receptor-encoding plasmid on selectable medium. (e) For mutagenesis of the α-factor receptor, it is advantageous to use a yeast strain with a mutation in the *BAR1* gene encoding an enzyme that degrades α-factor. This allows for more reproducible assays of α-factor-induced signaling using lower levels of α-factor. (f) For expression of *STE2* under its normal promoter and to prevent autocrine responses, the strain should be of the *MAT***a** mating type.

   A standard protocol for high-efficiency yeast transformation is used for the introduction of receptor-encoding plasmids into yeast ***(26,29)***. Transformation mixtures are plated on yeast synthetic dropout medium, lacking the particular nutrient that is supplied by the auxotrophic marker gene encoded on the plasmid being transformed. Detailed instructions for preparing yeast culture media are available ***(25)***.
5. The β-galactosidase enzyme exhibits maximal activity near neutral pH and activity declines significantly at acid pH, such as is typically seen in yeast cultures. Because yeast do not grow well at neutral pH, *FUS1-lacZ* induction is

most easily monitored by replica plating of colonies from a plate containing normal synthetic complete medium to a buffered plate containing X-gal and, where appropriate, ligand (e.g., α-factor).

6. The size of mutational library needed for this type of analysis is a function of the desired coverage of sequence space, length of the mutated region, average number of substitutions per allele, percentage of amino-acid substitutions that are likely to be functional, and the efficiency of detection of colonies exhibiting signaling function. For example, if the mutated library contains a 10% base substitution rate at each position, it should only be necessary to examine hundreds of colonies to obtain a mutated library that contains every possible single-base change, allowing for oversampling to accomodate statistical variations in the representation of each substitution an the library. However, if each substitution has, for example, a 50% chance of causing loss of function, only 1 out of every 64 colonies will be functional in a mutagenized region 60 bases in length. This raises the number of colonies to be screened to approx 10,000. The mutational libraries we have created in the α-factor receptor have typically contained approx 30,000 *E. coli* transformants and 60,000 yeast transformants with less than1% exhibiting signaling function.
7. It is important to transform the mutated plasmid rescued from functional strains into a fresh yeast host to confirm that the observed mutations are compatible with signaling function. Some chromosomal mutations can increase ligand-dependent and ligand-independent activation of the pheromone response pathway. Thus, it is important to verify that the observed phenotype is plasmid-dependent. In addition, a small percentage of yeast cells can take up multiple plasmids during transformation. If this happens, there is a high probability that at least one plasmid in the strain will encode a nonfunctional receptor. It is therefore important to verify that the rescued plasmid is, in fact, the one responsible for detected signaling function. Plasmid rescue, followed by low-efficiency transformation into *E. coli*, provides a way of purifying the plasmid population.
8. Results of β-galactosidase assays are displayed as arbitrary units of β-galactosidase activity, normalized for the length of time of the actual enzymatic assay and for the number of cells in the culture (measured as optical density at 600 nm) immediately prior to permeabilization of cells and addition of substrate. A set of standard samples containing 0–60 μg purified β-galactosidase is included with every microtiter plate to facilitate standardization of results between different assays. A linear least-squares fit of fluorescence to the standard points is calculated for every plate and used to normalize the experimental data.

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10

Green Fluorescent Protein-Tagged β-Arrestin Translocation as a Measure of G Protein-Coupled Receptor Activation

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Summary

The G protein-coupled receptor (GPCR) superfamily is the largest family of integral membrane proteins. GPCRs respond to a wide variety of sensory and chemical stimuli and contribute directly to the regulation of all major organ systems. As such, GPCRs represent primary drug targets for therapeutic intervention. Although GPCRs respond to a diverse range of ligands and signal through multiple heterotrimeric G proteins, the inactivation of GPCR signaling is mediated by a limited set of proteins. In particular, the desensitization of the majority of GPCRs is mediated by the binding of two arrestin isoforms, β-arrestin1 and β-arrestin2, that exhibit overlapping substrate specificity. In response to GPCR activation and phosphorylation by GPCR kinases, β-arrestins redistribute from the cytosol to the plasma membrane to bind GPCRs and subsequently target the receptors for internalization via clathrin-coated vesicles. This property of β-arrestins has allowed the development of a green fluorescent protein (GFP)-based assay for detecting GPCR activation by confocal microscopy. This β-arrestin-GFP translocation methodology is described in detail in this chapter.

Key Words: β-arrestin; green fluorescent protein; G protein-coupled receptor; G protein-coupled receptor kinase; confocal microscopy; translocation; clathrin; dynamin; endocytosis; agonist; antagonist; plasma membrane.

1. Introduction

G protein-coupled receptors (GPCRs) are seven-transmembrane spanning receptor proteins that constitute the largest family of integral membrane proteins. Upon agonist binding, GPCRs modulate the activity of a diverse array of signal transduction pathways by coupling to heterotrimeric guanine nucleotide-binding proteins (G proteins). GPCRs respond to sensory and chemical stimuli,

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such as light, odor, taste, pheromones, peptides, hormones, and neurotransmitters *(1)*. The diversity of GPCR functions and ligand specificities, in combination with the cell surface accessibility of these receptors to exogenously administered drugs, has made GPCRs a primary target for therapeutic drug discovery. It is estimated that nearly 60% of all prescription drugs owe their therapeutic activity either directly or indirectly to the regulation of GPCRs *(2,3)*. The Human Genome Project predicted that more than 600 rhodopsin, secretin, and metabotropic glutamate class GPCRs exist, comprising 1.5–2% of the human genome *(4)*. However, the natural ligands and function of only approx 300 GPCRs are known *(5)*. Although many of these orphan receptors may be olfactory receptors, GPCRs remain a virtually untapped resource for drug discovery. Because GPCRs respond to a variety of different ligands and activate multiple heterotrimeric G protein subtypes and second-messenger pathways, biochemical methodologies for measuring the agonist activation of GPCRs are not only laborious and time-consuming, but must be customized for each individual receptor. Consequently, new methodologies for rapid drug screening and orphan receptor identification are in demand. One of these technologies involves the plasma membrane relocalization of green fluorescent protein (GFP)-tagged β-arrestin in response to GPCR activation *(6)*.

The majority of GPCRs utilize a common mechanism to either dampen or terminate receptor signaling in response to agonist activation *(1)*. Termed as desensitization, this mechanism involves a common set of proteins called GPCR kinases (GRKs) and arrestins *(1)*. Arrestins bind to agonist-activated and GRK-phosphorylated receptors, and both uncouple the receptors from G proteins and target receptors for internalization in clathrin-coated vesicles *(1)*. The binding of arrestins to GPCRs represents a convergent step in GPCR signaling, because the desensitization of the majority of GPCRs is mediated by only two arrestin isoforms that exhibit overlapping receptor specificity, β-arrestin1 and β-arrestin2. As a consequence, the β-arrestin2-GFP translocation assay described in this chapter represents a powerful and universal methodology for screening orphan GPCRs, GRK inhibitors, and novel GPCR ligands. An additional advantage of this technique is that it allows the individual investigator to examine: (1) the agonist-specificity of receptor β-arrestin–receptor interactions; (2) whether β-arrestins become compartmentalized with receptors in endocytic vesicles; (3) how drug treatments affect β-arrestin–receptor interactions; and (4) the mechanisms regulating the dissociation of the GPCR–β-arrestin endocytic complex *(6–9)*.

2. Materials

2.1. Cell Culture

1. Human embryonic kidney (HEK 293) cells (ATCC).
2. Minimal essential medium (MEM) (Life Technologies)

3. Fetal bovine serum (FBS) (Hyclone).
4. 0.25% Trypsin-EDTA (Life Technologies).
5. Gentamicin (Life Technologies).
6. Phosphate-buffered saline (PBS) 137 m*M* NaCl, 2,7 m*M* KCl, 10 m*M* Na_2HPO_4, pH 7.4.
7. T175 Tissue culture flasks.
8. 100-mm Tissue culture dish.
9. 35-mm Dishes containing 1-cm centered sealed glass coverslip (Matek Corp.).
10. Hanks' buffered saline solution (HBSS): 1.2 m*M* KH_2PO_4, 5 m*M* $NaHCO_3$, 20 m*M* HEPES, 11 m*M* glucose, 116 m*M* NaCl, 4.7 m*M* KCl, 1.2 m*M* $MgSO_4$, 2.5 m*M* $CaCl_2$.
11. 0.2-μm Bottle top filters.
12. 10-, 25-, and 50-mL sterile plastic serological pipets.
13. Type 1 collagen from calf skin (Sigma).

2.2. Transfection

1. Distilled water.
2. 2.5 *M* $CaCl_2$.
3. 2X HEPES-balanced salt solution (HeBS): 16.4 g NaCl, 0.38 *M* final; 11.9 g HEPES, 0.05 *M* final; 0.21 g Na_2HPO_4, 1.5 m*M* final; 800 mL H_2O. Titrate to exactly pH 7.05 with 5 *N* NaOH and add H_2O to 1 L. Store at –20°C in 50-mL aliquots.
4. Falcon 2063 tubes.
5. 1-mL Sterile plastic serological pipettes.
6. β-Arrestin2-eGFP plasmid cDNA (available on request).

2.3. Microscopy

1. LSM-510 laser scanning confocal microscope (Zeiss) or equivalent instrument.
2. Zeiss 63 × 1.4 numerical aperture oil immersion lens.
3. Laser excitation at 488 nm and emission 515–540-nm emission filter set.

2.4. Data Analysis

1. Zeiss LSM-510 physiological software package.
2. Adobe Photoshop.

3. Methods

3.1. Cell Culture (see Note 1)

1. Day 1: Aspirate the media from stock cultures of HEK293 cells grown to 95% confluency in T175 cell culture flasks (*see* **Note 2**). Wash the cells twice with PBS, then treat with 4 mL 0.25% trypsin-EDTA for 1 min and gently resuspend in at least one equal volume of MEM containing 10% FBS and 50 μg/μL gentamicin. Count the cells using a hemocytometer and reseed at a density of 2×10^6 HEK 293 cells per 100-mm tissue culture dishes in a final volume of 10 mL MEM containing 10% FBS and 50 μg/mL gentamicin.

2. Day 2: Transfect HEK 293 cells cultured in 100-mm dishes by calcium phosphate precipitation as outlined in **Subheading 3.3.**, then maintain overnight at 37°C in a humidified atmosphere of 95% air, 5% CO_2.
3. Day 3: 15–18 h after transfection, exchange the culture media for 10 mL fresh MEM containing 10% FBS and 50 µg/mL gentamicin. Six to eight hours after changing the media, wash the 100-mm cell culture dishes twice with 5 mL PBS and treat with 2 ml of 0.05% trypsin for 1 min, then gently resuspend in 10 mL MEM containing 10% FBS and 50 µg/mL gentamicin. Reseed 2 mL of the suspension in 35-mm glass coverslip-bottomed culture dishes that are pretreated for 5 min with 1 mL collagen solution, then wash twice with 2 mL PBS (*see* **Note 3**). Allow the cells to recover overnight. This should result in cultures that are 40–50% confluent (*see* **Note 4**).
4. Day 4: Exchange the cell culture media in the 35-mm culture dishes for 1 mL HBSS (37°C) prior to experimentation.

3.3. Transfection (see *Notes 5–7*)

1. Pipet 1 µg pEGFP-C1 β-arrestin2 cDNA (β-arrestin2-GFP) along with 5–10 µg plasmid cDNA encoding the receptor protein into the bottom of a Falcon 2063 tube.
2. Add 450 µL sterile distilled water to the plasmid cDNA.
3. Add 50 µL 2.5 *M* $CaCl_2$ and mix the solution by vortexing for 5 s.
4. Add 500 µL 2X HeBS drop by drop using a 1-ml serological pipet and mix thoroughly using the pipet.
5. Carefully administer drop by drop the mixture containing the plasmid cDNA across the surface of the 100-mm cell culture dish. Apply the drops 1–1.5 in. above the surface of the liquid.
6. Culture the cells overnight at 37°C in a humidified atmosphere of 95% air, 5% CO_2 as described in **Subheading 3.2.**

3.4. Microscopy (see *Note 8*)

1. Place 35-mm glass coverslip-bottomed culture dishes containing HEK 293 cells transfected with receptor and β-arrestin2-GFP on the microscope stage.
2. Image the cells using a Zeiss 63X 1.4 numerical aperture oil immersion lens and focus the cells using transmitted light.
3. It is desirable to obtain a field of β-arrestin2-GFP transfected cells using the fluorescent microscope prior to switching to laser-scanning mode. Healthy HEK 293 cells take on a flat irregular shape, β-arrestin2-GFP should be localized to the cytoplasm of the cell and excluded from the nucleus. A cell with a defined plasma membrane and large cytoplasm should be chosen for imaging. The most desirable image plane is through the center of the cell because this will provide the best definition of the plasma membrane and cytosolic compartments of the cell.
4. The LSM-510 laser can be used to zoom in on the field of cells. A zoom function of no more than 4X should be employed. The pinhole should be set to unity. The image quality can be increased both by reducing the pixel scan rate of the laser and by increasing the number of image frames that are integrated to provide an

image. A pixel scan rate of 2.24–4.48 µs combined with the integration of eight image frames was found to provide a desirable confocal image every 15–30 s. The laser excitation should be set at 488 nm and the emission filter set to 515–540 nm. Laser power is an issue because GFP is readily bleached. Therefore, the lowest possible laser power should be employed, which is usually 1–2%.

5. Acquire images every 15–30 s. Obtain a time series of images using the time-series software available in the LSM-510 software package. Obtain one or two control images of the cell prior to the addition of agonist. Apply agonist to the cell culture dish in a 100–µL volume at a final concentration that will maximally activate the cell surface GPCRs (*see* **Note 9**).

3.5. Data Analysis

1. Analyze data using the LSM-510 physiological software package or other publicly or commercially available software packages. Export images into Adobe Photoshop for data presentation.

4. Notes

1. All liquid media should be filter-sterilized using 0.2-µm bottle-top filters and all tissue culture glassware must be autoclaved before use. Sterile techniques should be followed at all times.
2. Laboratory stocks of HEK 293 cells seeded in T175 tissue culture flasks containing 15 mL MEM containing 10% FBS and 50 µg/mL gentamicin are maintained at 37°C in a humidified atmosphere of 95% air, 5% CO_2 (*see* **Note 2**).
3. Stock cultures should be seeded with 2×10^6 HEK 293 cells. The media should be replaced every 3–4 d and cultures split and reseeded in new flasks once a week.
4. The treatment of the 35-mm glass coverslip-bottomed culture dishes with collagen facilitates the attachment of cells to the glass coverslip and avoids detachment of cells during experimentation.
5. It is useful that cells be evenly distributed at a confluence of no more than 40–50% for β-arrestin2-GFP translocation experiments.
6. Good receptor expression is essential for effective β-arrestin2-GFP translocation. Expression levels of greater than 1 pmol/mg protein are desirable. However, for many receptors, translocation can be detected at much lower expression levels. β-Arrestin2-GFP expression should be low.
7. In the case of some receptors, it may be desirable to cotransfect 1–2 µg plasmid cDNA-encoding GRK2. The endogenous complement of GRK2 expressed in HEK 293 cells may not be sufficiently high enough to promote phosphorylation of some receptors. This is particularly the case for chemokine receptors ***(10)***. Moreover, if the receptor being studied internalizes as a complex with β-arrestin2-GFP, this can be avoided (if desired) by cotransfecting the cells with 3 µg dynamin I-K44A plasmid cDNA ***(11)***.
8. Alternative methods of cell transfection like lipofectamine can also be utilized. However, in our experience, the calcium phosphate precipitation method for cell

transfection provides a simple cost-effective method for transfecting HEK 293 cells with a cotransfection efficiency that ranges between 50% and 80%.

9. A microscope equipped with a heated stage, Zeiss environmental control chamber or perfusion chamber, is desirable. The heated stage and environmental control chamber allow cell cultures to be maintained at optimal temperatures. Cell cultures do not need to be maintained at 37°C for these experiments, and under some conditions, it may be desirable to lower temperatures to slow the translocation process. A cell perfusion chamber should be used if either agonist–dose response curves are desired or the experiment requires changes in drug treatment (e.g., agonist recovery or antagonist treatment).

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11

Detection of G Protein-Coupled Receptors by Immunofluorescence Microscopy

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Summary

G protein-coupled receptors (GPCRs) are activated by a wide array of signals, which include light, neurotransmitters, hormones, cytokines, and drugs. Knowledge of the subcellular distribution of GPCRs is required in many experimental situations. Most GPCR signaling occurs in response to activators that interact with receptors localized on the cell surface. GPCRs must move from their site of synthesis to the plasma membrane, often undergoing redistribution in response to ligand binding. Endocytosis and recycling of receptors are important for desensitization and resensitization. Furthermore, mutations in GPCRs can alter receptor trafficking and prevent normal receptor function. This chapter describes a widely applicable method for visualizing a GPCR by indirect immunofluorescence microscopy.

Key Words: G protein-coupled receptor; epitope tag; antibody; tissue culture; fluorescence microscopy; immunocytochemistry.

1. Introduction

This chapter describes a generally applicable method for visualizing the subcellular distribution of a G protein-coupled receptor (GPCR). Like other intrinsic membrane proteins, GPCRs are synthesized in the rough endoplasmic reticulum, processed in the Golgi apparatus, and trafficked to the plasma membrane. Many GPCRs are internalized in response to agonist binding, moving from the plasma membrane through clathrin-coated pits to endosomes before undergoing lysosomal degradation or recycling to the plasma membrane ***(1)***. Mutations that interfere with normal GPCR trafficking can lead to disease; for example, mutant rhodopsins unable to travel to the plasma membrane are responsible for some forms of retinitis pigmentosa ***(2)***, and mutant V2 vaso-

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pressin receptors that are constitutively internalized give rise to nephrogenic diabetes insipidus ***(3)***.

There are several methods for studying the subcellular location of a GPCR. The most direct is to tag the GPCR with an intrinsically fluorescent protein like green fluorescent protein (GFP) and observe its behavior in live cells ***(4,5)***. This is a powerful technique, but it entails the addition of a 27-kD protein to the receptor, which may perturb normal trafficking and signaling; the method is limited to genetically engineered receptors that must be introduced into cells and expressed at supraphysiological concentrations. A more widely applicable approach is to visualize receptors immunocytochemically using fluorescence microscopy. If a suitable antibody is available, immunolocalization is applicable to cells expressing endogenous receptors. However, unless the epitope is in the extracellular domain of the receptor, immunocytochemistry requires fixation and permeabilization. Receptor distribution can also be inferred from the binding of radioactive ***(6)*** or fluorescent-labeled ***(7,8)*** ligands, but binding studies can only detect functional unoccupied receptors accessible to the labeled probe.

Because most GPCRs are expressed at low concentrations, immunolocalization requires both a high-affinity, high-titer antibody and a sensitive detection technique (e.g., immunofluorescence). Antibodies against native receptors offer a major advantage, but obtaining good antibodies against GPCRs can be challenging. Antisera against some GPCRs are commercially available, and many laboratories have successfully raised useful antireceptor antibodies after immunizing with peptides from receptor sequences, fusion proteins, or purified receptors ***(9,10)***. Antibodies to native GPCRs can be directed against the extracellular N-terminal domain, intracellular loops, or most commonly, the cytoplasmic C-terminal region.

A different strategy for localizing a GPCR is to engineer a receptor with an epitope tag fused to either the amino- or carboxy-terminus. An epitope tag is a sequence of 8–12 amino acids, which is recognized by a well-characterized, high-affinity antibody. Excellent monoclonal and polyclonal antibodies against frequently used epitopes, such as myc, hemagglutinin (HA), and Flag, are commercially available. The addition of two or three copies of the epitope sequence increases sensitivity. The choice of where to add an epitope tag depends on the receptor as well as the goals of the experiment. Placement of an epitope tag at the amino-terminus of a GPCR may interfere with receptor processing or necessitate the addition of a signal peptide, and nearby N-linked carbohydrate groups can interfere with antibody recognition. On the other hand, addition of an epitope to the C-terminus is generally well tolerated, but has the potential to interfere with protein–protein interactions involving this region of the receptor. A plasmid encoding the epitope-tagged GPCR is introduced into cells by transfection or other means. If receptor expression is driven by a typical strong constitutive promoter, the GPCR may be expressed at abnormally high levels.

Despite these potential drawbacks, much insight into GPCR trafficking has come from studies in which epitope-tagged receptors have been expressed in model cell lines (e.g., COS, CHO, and HEK293).

This chapter describes in detail how to use standard immunological reagents to localize GPCRs in tissue culture cells. The specific example describes the visualization of a GPCR-tagged HA epitope, YPYDVPDYA, but the methods are basically the same for the identification of an endogenous GPCR with a polyclonal antibody. Procedures can be readily adapted for the identification of GPCRs in tissue sections.

2. Materials

2.1. Preparation of Mounting Solution

1. Mowiol (Calbiochem).
2. Glycerol.
3. 0.2 *M* Tris-HCl, pH 8.5.
4. 1,4-diazobicyclo-[2.2.2]-octane (DABCO; Aldrich).

2.2. Cell Culture

1. Cell line expressing receptor (e.g., HEK293 cells expressing HA-tagged receptor).
2. Control cell line not expressing receptor, if available (e.g., HEK293 cells).
3. Sterile 25-mm round glass coverslips, which can be placed in a glass Petri dish and autoclaved.
4. Adherent such as CellTac, ECL, collagen, or poly-L-lysine, if necessary.
5. Growth media (e.g., Dulbecco's modified Eagle's medium [DMEM] + 5% fetal bovine serum [FBS]).

2.3. Fixation and Immunocytochemistry

1. Paraformaldehyde (Sigma).
2. Primary antibody against receptor (e.g., HA11 mouse anti-HA antibody from Covance). Store antibody undiluted or at 1:10 dilution in blocking buffer in the refrigerator or freezer as recommended by the manufacturer.
3. Control antibody. If the primary antibody is monoclonal, an appropriate control is a similar preparation (purified IgG or ascites fluid) of a control monoclonal antibody of the same class, i.e., one that does not recognize any cellular protein. If the primary antibody is polyclonal, the appropriate control is preimmune serum or, if this is unavailable, normal rabbit serum. Control antiserum should be used at the same concentration as primary antiserum.
4. Immunizing peptide. If the primary antibody has been raised against a peptide, antibody that has been absorbed with an excess of the peptide can serve as an additional control.
5. Fluorescently labeled secondary antibody (e.g., rhodamine-labeled goat anti-mouse IgG conjugate from Molecular Probes or American Qualex). Store as recommended by the manufacturer.

6. PBS: 135 m*M* NaCl, 4.5 m*M* Na_2HPO_4, 1.5 m*M* KH_2PO_4, pH 7.3.
7. Blocking buffer: 5% goat serum, 0.2% NP40 (Calbiochem or BRL) in phosphate-buffered saline (PBS). Store at 0–4°C (*see* **Note 1**).

2.4. Fluorescence Microscopy

Fluorescence microscope with appropriate detection filters (e.g., TRITC cube for detection of rhodamine, available from Chroma or Omega) and oil objective with high numerical aperture (40–100x for most applications; *see* **Note 2**).

3. Methods

3.1. Preparation of Mounting Solution (11)

1. Add 2.4 g Mowiol to 6 g glycerol in a beaker and stir to mix.
2. Add 6 mL distilled water and leave at room temperature for several hours.
3. Add 12 mL 0.2 *M* Tris-HCl, pH 8.5, and heat to 50°C with occasional mixing until dissolved for approx 10 min.
4. Transfer to conical tubes, centrifuge at top speed in a clinical desktop centrifuge for 20 min, and retain supernatant fluid.
5. Add 0.6 g DABCO to reduce fading.
6. Aliquot in microfuge tubes and store at –20°C. Tubes can be stored at least 1 yr but should be discarded if the solution becomes cloudy.

3.2. Cell Culture

1. Place individual sterile coverslips in 35-mm culture dishes. It is easy to lift and transfer coverslips by suction using a sterile glass Pasteur pipet or sterile plastic pipet tip attached to a vacuum tubing.
2. If necessary for good adhesion, coat coverslips with an adherent according to the manufacturer's instructions. Most cells do not adhere to glass coverslips as well as to plastic culture dishes.
3. Plate cells onto coverslips in usual growth medium following normal procedures for subculture and allow to adhere at least overnight. Prepare enough coverslips for both experimental and control staining (*see* **Subheading 3.3., step 6**).
4. Grow to desired cell density and treat cells as appropriate.

3.3. Fixation (see Note 3) and Immunocytochemistry (see Note 4)

1. Prepare 4% paraformaldehyde fixer fresh daily before use. Add 0.2 g paraformaldehyde to 5 mL boiling PBS in a flask on a heated stir plate in a chemical fume hood and heat until paraformaldehyde is dissolved (*see* **Note 5**). Cool to room temperature.
2. Aspirate culture medium and wash cells twice with 2 mL PBS. Remove PBS.
3. Add 1 mL 4% paraformaldehyde fixer and place dishes on a shaker at 75 rpm at room temperature for 10 min (*see* **Note 6**).
4. Aspirate fixer and rinse dishes three times with 2 mL PBS (*see* **Note 7**).

5. Add 1 mL blocking buffer and incubate at room temperature for 30 min on shaking platform.
6. Dilute receptor-specific antibody as appropriate in blocking buffer on the day of use. For HA11 antibody, dilute 1:1000.
   a. If feasible, stain two cell lines with the antireceptor antibody, one line expressing receptor and a control cell line not expressing the receptor. This is a stringent control that helps distinguish between real and artifactual staining when antibodies with marginal affinity or specificity must be used.
   b. Include a nonspecific antibody control, in which the experimental cell line is stained with an equivalent concentration of control antibody (*see* **Subheading 2.3., step 3**).
   c. If antipeptide antibodies are used, antibody that has been absorbed with the peptide can serve as an additional control. Preincubate the antiserum with peptide for at least 1 h before adding to cells. Peptides can usually be used at 10 μg/mL, although higher concentrations may be required.
   d. Include one autofluorescence control in initial experiments using no primary or secondary antibody.
7. Cover cells with 0.5 mL antibody solution (*see* **Note 8**) and incubate at room temperature for 2 h (*see* **Note 9**) on a shaker set at 75 rpm.
8. Remove antibody solution and rinse the dish three times with 2 mL PBS for 5 min each wash, placing dishes on shaker at room temperature.
9. Dilute fluorescently labeled secondary antibody in blocking buffer and wrap tube in aluminum foil. Use rhodamine-labeled anti-mouse IgG at 1:500 (*see* **Note 10**).
10. Add 0.5 mL fluorescent-labeled secondary antibody to each dish, cover dishes with aluminum foil, and incubate on shaker at room temperature for 30 min. After cells have been incubated with the secondary antibody, minimize exposure to light.
11. Aspirate secondary antibody and wash cells three times with PBS as described in **step 8**.
12. Add 2 mL PBS to each dish and cover with aluminum foil.

3.4. Attachment of Glass Coverslips to Microscope Slides

1. Prelabel microscope slides and add 15 μL Mowiol to the center of the microscope slide. Prepare slides one at a time because the mounting medium dries quickly.
2. Using fine tweezers, pick up the glass coverslip and carefully touch the edge to a Kimiwipe to remove liquid.
3. Carefully invert the glass coverslip on the drop of Mowiol on the microscope slide cell side down.
4. Store microscope slides covered with foil or in a dark slide box at least overnight before observing.

3.5. Fluorescence Microscopy

1. Visualize cells on a fluorescence microscope using filters appropriate for the fluorescent label under an oil ×40, ×63 or ×100 objective with high numerical aper-

ture. For cells stained with rhodamine-labeled goat anti-mouse antiserum, use a standard TRITC filter set.
2. To minimize bleaching, locate cells in brightfield, then observe under fluorescence. Capture images of control and experimental cells under identical conditions.
3. Evaluate background staining by comparing control with experimental coverslips (*see* **Note 11**).
4. Store slides in a dark box in a cold room for maximum shelf life. The quality of the slides should not deteriorate for at least 1 mo.

4. Notes

1. Blocking buffer should contain 5% serum from the same species as the fluorescently labeled secondary antibody, usually goat or sheep.
2. If a confocal microscope is available, it will give better resolution.
3. As described here, paraformaldehyde fixation usually works well for GPCRs. An alternative is to fix with ice-cold 1:1 methanol:acetone for 1 min. We have found that methanol:acetone fixation works better for staining certain endoplasmic reticulum or Golgi apparatus markers, and methanol:acetone fixation may be preferable if such proteins are being colocalized.
4. The directions here are written for staining with a single monoclonal antibody. If cells are to be stained simultaneously for two proteins, the fixed coverslip should be incubated with two primary antibodies from different species, often a mouse monoclonal antibody and a rabbit polyclonal antibody. Two secondary antibodies labeled with different fluorescent tags, such as FITC-labeled anti-mouse IgG and TRITC- or Texas red-labeled anti-rabbit IgG, are then used to identify the mouse and rabbit antibodies. The Molecular Probes website has excellent information on choices of fluorescent labels. For double labeling, the microscope must have filter sets appropriate for observing each fluorochrome individually. Controls should be run with each of the labeled secondary antibodies alone, and bleedthrough should be assessed by viewing these control slides at each wavelength. If a choice is available, stain the less abundant protein with a monoclonal antibody. Use the longer wavelength secondary antibody with the protein that is more difficult to stain well.
5. Formaldehyde fumes are dangerous. This step should be performed in a well-ventilated chemical fume hood.
6. Fixation conditions should be optimized to obtain the best possible morphology and receptor staining. Paraformaldehyde concentrations may range between 2% and 5% and fixation times between 5 and 30 min.
7. Some protocols call for a quenching step in which cells are incubated for 1 min with 0.5% *M* sodium borohydride after fixation. In our experience with tissue culture cells, this step has not altered background detectably.
8. This method is convenient and reliable if the primary antibody is inexpensive and readily available. If antibody is precious, aspirate buffer from the dish thoroughly and pipet 200 µL of the antibody on the coverslip. The solution should be held by surface tension; in this case, the dish should not be moved. Antibody use

can be further reduced by inverting the coverslip on a drop of antibody and incubating without agitation. With experience, dilute solutions of some antibodies can be saved and reused.

9. Incubation times can usually be reduced to 1 h at room temperature or 37°C or extended to overnight in the cold room, if more convenient.
10. The optimal dilution of secondary antibody should be determined experimentally because this will depend on the supplier, preparation, and sensitivity of the microscope and camera. Begin with the concentration recommended by the supplier.
11. Signal-to-noise ratios will depend on the density of receptors on the cells and the antibody. If high background staining is encountered, the controls previously outlined should distinguish between autofluorescence and background because of the antibody. The following steps can be taken to reduce nonspecific staining.
    a. If autofluorescence is high, try different fixation conditions. Because autofluorescence will be lower at longer wavelengths, switching from FITC to TRITC or Texas red will reduce background fluorescence.
    b. If autofluorescence is low, but background staining is high in antibody-stained cultures, run additional controls in which either the primary or the secondary antibody is omitted. If high background is the result of to the secondary antibody, use a new source of fluorescently labeled antiserum.
    c. High background is usually caused by nonspecific staining by the primary antibody. In general, background staining will be higher with rabbit antibodies than with monoclonal antibodies and higher if antibodies must be used at high concentrations (>1:200). If possible, lower the antibody concentration. Try adding one or two additional washes and vary the fixation conditions to make sure that immunoreactivity is maximized and background minimized. Including NP40 in wash solutions may help. If these steps are insufficient, it may be necessary to purify the IgG fraction of a crude antiserum, to affinity purify the primary antiserum or to switch to a different antibody preparation.

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12

Assay of G Protein-Coupled Receptor Activation of G Proteins in Native Cell Membranes Using [^{35}S]GTPγS Binding

Jean M. Bidlack and Amy L. Parkhill

Summary

The interaction of a G protein-coupled receptor (GPCR) with the G protein is the first step in the transduction of receptor binding to the activation of second-messenger systems mediated by G proteins. Binding of the poorly hydroylzable GTP analog guanosine-5'-*O*-(3-[^{35}S]thio)triphosphate ([^{35}S]-GTPγS) to the α subunit of a G protein has been used as a biochemical assay to measure the efficacy of a compound. The maximal percent stimulation of [^{35}S]GTPγS binding, induced by an agonist, correlates well with the efficacy of an agonist in an in vivo system. The concentration of agonist necessary to achieve 50% of the maximal stimulation is indicative of the affinity of the agonist for the receptor, under the assay conditions. The [^{35}S]GTPγS binding assay, which uses membranes from either myeloma cells or endogenous tissues, is a biochemical assay to determine the efficacies of agonists for receptors that are expressed endogenously. Novel compounds that have been shown to have high affinity in radioligand receptor-binding assays are screened in the [^{35}S]GTPγS binding assay to determine if they are agonists, antagonists, or partial agonists at a particular receptor.

Key Words: [^{35}S]GTPγS; neuroblastoma; opioid; G protein.

1. Introduction

G proteins undergo an activation/inactivation cycle (for reviews, *see* **refs. *1*** and ***2***). At resting state, the guanine nucleotide-binding site of the α subunits of G proteins is occupied by guanosine diphosphate (GDP). When agonists activate the G protein-coupled receptor (GPCR), guanosine triphosphate (GTP)

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displaces GDP bound to the α subunit. Binding of GTP causes dissociation of G proteins into α and β/γ subunits. GTP-bound α subunit and β/γ subunits activate downstream effectors. Agonist effects are terminated by hydrolysis of GTP to GDP by a low K_m GTPase that is intrinsic to the α subunit ***(3–5)***. The GDP-bound α subunit then reassociates with β/γ subunits to re-enter the cycle. When the poorly hydrolyzed GTP analog [^{35}S]GTPγS is used instead of GTP, the half-lives of GTPγS-bound α subunits are prolonged, and α subunits are persistently activated ***(6)***.

Agonist-stimulated binding of [^{35}S]GTPγS to G proteins has been used as a sensitive assay of activation of many GPCRs. It was first used in a reconstitution system of purified G proteins and purified β adrenergic receptors ***(7)***. Subsequently, this method has been applied to many GPCRs in cell membranes, including muscarinic ***(8,9)***, A1 adenosine ***(10)***, α_2 adrenergic ***(11)***, and μ opioid receptors ***(12)***. By in vitro autoradiography, [^{35}S]GTPγS binding has been used to localize G proteins activated by agonists for μ opioid, cannabinoid, and γ-aminobutyric $acid_B$ receptors in rat brain sections ***(13)***.

The [^{35}S]GTPγS binding assay has been used with myeloma cell lines ***(12,14–19)***, transfected cells ***(9,20,21)***, tissue plasma membranes ***(4,8,10)***, or brain slices ***(12)***. An advantage of using myeloma cell lines instead of transfected cells is that the number of receptor and G proteins expressed more closely parallels the number seen in tissues. However, transfected cell lines have been used because it is possible to express only one type of receptor, whereas many neuroblastoma cell lines contain multiple GPCRs from the same family. Differences in the coupling of receptors to G proteins have been observed in this assay, depending on whether membranes from transfected cells or membranes from a cell line were used ***(20)***. Because [^{35}S]GTPγS will not penetrate the plasma membrane, this assay cannot be used with whole cells, but it was used with cells treated with the detergent digitonin to permeabilize the plasma membrane ***(21)***.

The [^{35}S]GTPγS binding assay is also used to determine if a novel compound is an agonist, antagonist, partial agonist, or inverse agonist at a receptor. The efficacy of agonists can be determined by this assay. By rank ordering the efficacy of agonists in stimulating [^{35}S]GTPγS binding, it is possible to determine which compounds are the least and most efficacious. There is a good correlation between the in vitro [^{35}S]GTPγS binding assay results, and the ability of opioid agonists to produce antinociception ***(12)***. If a compound does not have agonistic properties, it may be an antagonist. To determine if a compound is an antagonist, the [^{35}S]GTPγS binding assay is performed in the presence of a concentration of an agonist that is known to stimulate [^{35}S]GTPγS binding, mediated by the receptor of interest. If the compound has no intrinsic activity, but inhibits agonist-stimulated [^{35}S]GTPγS binding,

then the compound is an antagonist in this assay. Likewise, this assay can be used to determine if a compound is a partial agonist or an inverse agonist. When a compound is a partial agonist, it will produce some stimulation of [35S]GTPγS binding. However, it will also antagonize [35S]GTPγS binding that was stimulated by another agonist acting at the same receptor. An inverse agonist is a compound that reduces the basal amount of [35S]GTPγS binding to the G proteins. In this assay, the ability of a compound to inhibit basal-specific binding would be determined.

In summary, the [35S]GTPγS binding assay can be used with membranes from tissues, myeloma cell lines, and transfected cells. The pharmacological properties of compounds can be determined with this assay. The efficacies of agonists ***(6)***, as well as if a compound is a partial ***(22)*** or an inverse agonist ***(15)***, can be determined. This relatively simple assay has the ability to produce significant pharmacological information about a compound. Also, the [35S]GTPγS binding assay can be used to measure desensitization of a receptor ***(17)***.

2. Materials

2.1. Neuroblastoma Cell Lines (see Note 1)

Many neuroblastoma cell lines, derived from different species, including human, are available from the American Type Culture Collection (ATCC) (Rockville, MD).

2.2. Reagents and Equipment for the Neuroblastoma Membrane Preparation and [35S]GTPγS Binding Assay

1. 10X Phosphate-buffered saline (PBS), pH 7.2, without $CaCl_2$ and $MgCl_2$ (GIBCO, Grand Island, NY).
2. [35S]GTPγS (~1250 Ci/mmol) can be obtained from New England Nuclear (Boston, MA) and is stored at –20°C.
3. Unlabeled GTPγS stored at –80°C.
4. GDP stored at –80°C.
5. Opioid peptides and alkaloids stored at –80°C.
6. Schleicher & Schuell no. 32 glass fiber filters (Keene, NH).
7. Brandel 48-well cell harvester (Brandel, Gaithersburg, MD).
8. Ecoscint A scintillation fluid (National Diagnostics, Atlanta, GA).

2.3. Stock Solutions

1. PBS, pH 7.2.
2. Assay buffer: 50 m*M* Tris-HCl, pH 7.4, 3 m*M* $MgCl_2$, 100 m*M* NaCl, 0.2 m*M* EGTA.
3. 100 μ*M* GDP.
4. 10 μ*M* GTPγS.
5. 1 mg/mL Bovine serum albumin (BSA).

3. Methods

Neuroblastoma membranes are prepared from cells that are at approx 70% confluence. After preparing the membranes, the protein concentration of the membrane preparation is determined by the Bradford method ***(23)***. If possible, the membranes are used in the [^{35}S]GTPγS binding assay on the same day that they are prepared. Otherwise, they are stored at –80°C at a protein concentration of ≥3 mg/mL (*see* **Note 2**). Because [^{35}S]GTPγS does not penetrate membranes, this assay cannot be performed with whole cells.

3.1. Preparation of Neuroblastoma Cell Membranes

1. Culture neuroblastoma cells in 162-cm^2 tissue culture flasks in 75 mL media as recommended by the supplier of the cells. At the time of preparing membranes, neuroblastoma cells should be at approx 70–90% confluence. The following procedure is for one flask of cells.
2. Add sterile 15 mL PBS to cover the cells after the media has been decanted.
3. Incubate the PBS-covered cells at 4°C for 10 min (*see* **Note 3**).
4. Remove the cells from the flask by repetitive pipeting or gently tapping the side of a culture flask to dislodge the cells, which are then transferred into 50-mL disposable centrifuge tubes.
5. Centrifuge the cells at 200*g* for 10 min at 4°C.
6. Aspirate the supernatant from the cells.
7. Resuspend the cell pellet in 10 mL assay buffer for each 50-mL centrifuge tube.
8. Homogenize the cells with a Dounce homogenizer.
9. Transfer the homogenized cells to one 40-mL Beckman centrifuge tube. Bring the volume up to 40 mL with assay buffer.
10. Centrifuge the homogenate at 37,000*g* for 30 min at 4°C.
11. Resuspend the pellet in 10 mL assay buffer with a Dounce homogenizer.
12. Bring the membrane suspension to 20 mL by adding 10 mL assay buffer.
13. Centrifuge the homogenate at 37,000*g* for 30 min at 4°C.
14. Resuspend the membrane pellet in 2 mL assay buffer with the use of a Polytron homogenizer at a setting of 2 for 20 s. Determine the concentration of protein in the sample by the Bradford method ***(23)*** protein assay with BSA as the standard.

3.2. [^{35}S]GTPγS Binding Assay

The [^{35}S]GTPγS binding assay is useful in determining if a compound is an agonist, antagonist or partial agonist at a GPCR. In addition, the efficacies of compounds in stimulating [^{35}S]GTPγS binding strongly correlate with their in vivo efficacies. Before characterizing a new compound, the [^{35}S]GTPγS binding assay must be optimized with regard to the necessary amount of protein used in the assay and the necessary concentration of GDP that should be included, which will vary among cell lines.

3.2.1. Assay Optimization

1. To determine the amount of protein needed to produce optimal stimulation of [35S]GTPγS binding with membranes from a particular cell line or tissue, varying amounts of protein, usually ranging from 2 to 50 µg, are used in a final volume of 500 µL assay buffer.
2. To measure nonspecific [35S]GTPγS binding, 10 µ*M* GTPγS is included.
3. For each amount of protein, a total binding, nonspecific binding, and an agonist-stimulated binding sample are performed. The agonist used in the protein titration experiment will have high affinity for the receptor of interest and should be used under saturating conditions. For the protein titration experiment, the agonist should be used at a high concentration (e.g., 0.1–10 µ*M*). For an initial experiment, it is suggested that GDP be used at a concentration of 3 µ*M*. In the protein titration experiment, the protein amount that gives the best ratio of specific-to-nonspecific binding and shows a large stimulation of agonist-induced [35S]GTPγS binding should be used in future experiments.
4. To determine the optimal GDP concentration to use in the experiments, GDP should be titrated from 1 to 30 µ*M*. Like the protein titration experiment, an agonist should be used at a high concentration where appropriate. Each set of samples in a final volume of 500 µL will contain a sample that measures total binding, one that measures nonspecific binding, and a sample that contains an agonist for the receptor of interest. The concentration of GDP will be constant for the total, nonspecific, and agonist-stimulated tubes.

3.2.2. Titration of Agonist-Stimulated [35S]GTPγS Binding (see ***Notes 4*** *and* ***5****)*

Once the optimal protein and GDP concentrations have been determined, it is possible to titrate an agonist to determine its EC_{50} value, a measure of affinity, and its E_{max} value, a measure of efficacy. The E_{max} value is the maximal stimulation obtained that does not significantly change over three concentrations of the agonist. The EC_{50} value is the concentration of agonist needed to obtain half maximal stimulation of [35S]GTPγS binding. **Table 1** outlines a typical experiment for titrating the stimulation of [35S]GTPγS binding by the µ opioid peptide, [D-Ala^2,(Me)-Phe^4,Gly(ol)^5]enkephalin (DAMGO), using human SH-SY5Y neuroblastoma cell membranes. Usually two basal-binding and two nonspecific-binding samples are performed with each experiment, which is performed in triplicate.

1. Add the components of the assay to 12 × 75-tubes in the order listed in a final volume of 0.5 mL as in **Table 1**.
2. Add the appropriate volume of assay buffer to each assay tube calculated based on the considerations shown in **Table 1**.
3. Dilute the opioid peptide, DAMGO, from a 10^{-3} *M* frozen stock to the appropriate concentration (*see* **Table 1**) in assay buffer and added to the tubes.

Table 1
Titration of GTPγS Binding to Membranes with DAMGO

Condition	Assay buffer (μL)	100 m*M* GTPγS (μL)	10^{-8} *M* DAMGO (μL)	10^{-7} *M* DAMGO (μL)	10^{-6} *M* DAMGO (μL)	10^{-5} *M* DAMGO (μL)	10^{-4} *M* DAMGO (μL)	30 m*M* GDP (μL)	0.8 n*M* [^{35}S]GTPγS (μL)	Membranes (μL)
Basal	300	—	—	—	—	—	—	50	50	100
Nonspecific	250	50	—	—	—	—	—	50	50	100
2 n*M* DAMGO	200	—	100	—	—	—	—	50	50	100
4.8 n*M* DAMGO	276	—	—	24	—	—	—	50	50	100
11.6 n*M* DAMGO	242	—	—	58	—	—	—	50	50	100
27.6 n*M* DAMGO	162	—	—	138	—	—	—	50	50	100
66 n*M* DAMGO	267	—	—	—	33	—	—	50	50	100
160 n*M* DAMGO	220	—	—	—	80	—	—	50	50	100
380 n*M* DAMGO	110	—	—	—	190	—	—	50	50	100
920 n*M* DAMGO	254	—	—	—	—	46	—	50	50	100
2.2 m*M* DAMGO	190	—	—	—	—	110	—	50	50	100
5.2 m*M* DAMGO	274	—	—	—	—	—	26	50	50	100
12.6 m*M* DAMGO	237	—	—	—	—	—	63	50	50	100
30 m*M* DAMGO	150	—	—	—	—	—	150	50	50	100
Basal	300	—	—	—	—	—	—	50	50	100
Nonspecific	250	50	—	—	—	—	—	50	50	100

4. For the nonspecific binding tubes only, add 50 µL 100 µ*M* GTPγS.
5. Add 50 µL GDP to give the final concentration of GDP determined during the assay optimization described in **Subheading 3.2.1.** For the SH-SY5Y neuroblastoma cell membranes, a final concentration of 3 µ*M* GDP was found to be optimal.
6. [35S]GTPγS (1250 Ci/mmol; 250 µCi/250 µL): 0.8 µL stock [35S]GTPγS is added to 10 mL assay buffer, and 50 µL is added to each assay tube, giving a final [35S]GTPγS concentration of 0.08 n*M*.
7. Mix samples before the addition of membranes.
8. Dilute membranes to give the optimal protein amount, determined by performing a protein titration as described in **Subheading 3.2.1.**, and added in 100-µL volumes. With the SH-SY5Y neuroblastoma cells, 50 µg of membrane protein has been shown to be optimal.
9. Incubate the samples for 60 min at 30°C.
10. Filter samples through Schleicher & Schuell no. 32 glass fiber filters using a Brandel cell harvester.
11. Wash the filters three times rapidly with cold 50 m*M* Tris-HCl, pH 7.5.
12. Count the filters in 2 mL Ecoscint A scintillation fluid after incubating in the dark for at least 12 h.

Nonspecific binding is subtracted from basal binding, to give specific [35S]GTPγS binding that is not stimulated by agonist. Also, nonspecific binding is subtracted from the samples containing agonist. The basal-specific binding is set at "0" percent stimulation. The percent stimulation of [35S]GTPγS binding by an agonist is calculated and plotted on a semi-log graph.

4. Notes

1. The cells should be cultured in the medium recommended by ATCC. Cells should not be kept in culture for an extended period of time (e.g., >1 mo), and the cells should not be overgrown at any time. When cultured for extended periods of time or stressed, cells will often reduce the synthesis of certain proteins (possibly the receptors and G proteins that are of interest).
2. Some membranes from neuroblastoma cells can be frozen at –80°C, and the [35S]GTPγS assay can be performed on a separate day. However, for some neuroblastoma cell lines, optimal [35S]GTPγS binding is obtained only when freshly prepared membranes are used. Whether it is feasible to freeze membranes and perform the [35S]GTPγS binding assay on a different day will have to be determined empirically for each cell line.
3. If necessary, use trypsin in PBS to remove the cells from the culture dish or flask. Avoidance in using trypsin is the preference because it may digest the receptor or G protein of interest, however, trypsin is sometimes needed to detach the cells.
4. To determine if a compound is an antagonist, the compound is titrated in the presence of a known agonist. For example, at the µ opioid receptor, the compound would be titrated in the presence of a constant concentration of the µ agonist (DAMGO). A concentration of the agonist is chosen so that it gives approx 70% of the maximal stimulation. The compound is added at varying concentra-

tions. Controls consist of determining the control-specific binding and the percent stimulation of [^{35}S]GTPγS binding in the presence of the agonist alone. If the unknown compound is an antagonist, it will reduce the amount of [^{35}S]GTPγS binding in the presence of the agonist.

5. If a compound gives low-percentage stimulation of [^{35}S]GTPγS binding when tested alone, it may be a partial agonist. After generating a concentration-dependent curve for the compound alone, it is tested to determine if it will inhibit [^{35}S]GTPγS binding stimulated by a known agonist. This experiment is performed exactly like the experiment described in **Note 4** to determine if the compound is an antagonist.

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13

Analysis of the Coupling of $G_{12/13}$ to G Protein-Coupled Receptors Using a Luciferase Reporter Assay

Bo Liu and Dianqing Wu

Summary

G protein-coupled receptors (GPCRs) transduce signals from cell surface to intracellular effectors through various G proteins. In this chapter, a luciferase reporter gene assay system is described for the study of the coupling of GPCRs to $G\alpha_{12/13}$. This assay system, in which ligand-stimulated production of the reporter luciferase under the control of a modified serum-responsive element (SRE) in a cell line derived from mice lacking $G\alpha_{q/11}$ that are transfected with a GPCR of interest, allows for easy, fast, and valid determination of the coupling of the GPCR to $G\alpha_{12/13}$.

Key Words: G_{12}; G_{13}; G proteins; GPCR; SRE; luciferase.

1. Introduction

Hormones, neurotransmitters, chemoattractants, and many other biologically active molecules exert their effects on cells through a large family of serpentine G protein-coupled receptors (GPCRs) ***(1,2)***. Ligand-induced receptor activation leads to guanine nucleotide exchange on specific G proteins and the dissociation of G protein heterotrimers, allowing signals to be transduced to downstream effectors. G proteins are usually classified into four classes (G_S, G_i, G_q, and G_{12}) based on their amino acid sequence similarity and function of the $G\alpha$ subunits ***(3)***. The G_s and G_i proteins regulate adenylyl cyclases (ACs), and the G_q proteins activate phospholipase C (PLC). More recently, the G_{12} family, consisting of G_{12} and G_{13} was found to regulate RhoA ***(4,5)***. Although one of the mechanisms by which G_{13} regulates RhoA is via a direct interaction between $G\alpha_{13}$ and a RhoA-specific exchange factor GEF115 ***(6–8)***, the mechanism by which G_{12} regulates RhoA is not completely understood ***(9)***. We previously showed that both G_{12} and G_{13} could activate RhoA via the Tec/Btk

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family of nonreceptor tyrosine kinases ***(10)***. As the activities of many G protein effectors can be assayed in cultured cells, methods have been developed to use the activities of the effectors as read-outs for the coupling of GPCRs to G proteins. This chapter describes a cell-based assay system for testing the coupling of GPCRs to $G_{12/13}$.

Because both $G\alpha_{12}$ and $G\alpha_{13}$ can activate serum response factor (SRF) through RhoA ***(9)***, changes in the activity of $G\alpha_{12}$ or $G\alpha_{13}$ may be monitored by examining the levels of SRF-dependent transcriptional activity using a SRF-specific reporter gene assay. In our system, a SRE.L luciferase reporter gene is used. Derived from *c-fos* serum response element (SRE), SRE.L binds to only SRF, but not tertiary complex factors (TCFs) ***(11)***. Therefore, the luciferase activity of this reporter gene corresponds only to SRF activation. Although the SRE.L-based reporter system also responds to the activation of Rac and Cdc42, the effect of Rac and Cdc42 can be blocked by coexpression of C3 toxin, which inactivates Rac and Cdc42, but not RhoA ***(11)***. To eliminate interference from G_q proteins, which also activate RhoA ***(9)***, a cell line that was derived from mouse embryos lacking both $G\alpha_q$ and $G\alpha_{11}$ ***(12)*** is used. Our study has demonstrated that this $G\alpha_{q/11}$-deficient cell line does not contain other members of the $G\alpha_q$ family, including $G\alpha_{14}$ and $G\alpha_{15}$ ***(9)***. Thus, GPCR-mediated activation of SRF in this cell line is mediated by G_{12} and/or G_{13}.

This chapter uses the endothelin receptors (ETRs) as a practical example. ETRs is a potent vasoactive peptide that can induce a wide range of pharmacological responses ***(13)***. In mammalian cells, there are at least two ETR subtypes: ET_A and ET_B. They both couple to PLC via the G_q proteins ***(14)***. In addition, both receptors regulate AC activity through G_s (ET_A receptor) or G_i (ET_B receptor) ***(15,16)***. To test if ETRs can couple to $G_{12/13}$, ETR is expressed together with the SRE.L luciferase reporter system in the cells lacking $G\alpha_{q/11}$. ET-induced luciferase activity is determined and normalized against coexpressed green fluorescence protein (GFP). To confirm that ET acts through RhoA rather than Rac or Cdc42, C3 toxin is coexpressed. As shown previously, both ETRs act primarily through RhoA as C3 blocked ET-induced activity ***(9)***. However, caution should be used when C3 is used because it is extremely cytotoxic. Optimal amounts of the C3 expression plasmid should be used, at which there should be minimal reduction in GFP expression or inhibition of Cdc42 or Rac. Therefore, the normalization procedure and proper controls are essential for this type of assay, especially when inhibitory effects are observed.

2. Materials

2.1. Cell Culture and Transfection

1. NIH3T3 cells (ATCC, Rockville, MD) and cells lacking $G\alpha_{q/11}$ (designated as Fq/11; kindly provided by Melvin Simon, Caltech, Pasadena, CA).

2. 1X Dulbecco's modified Eagle's medium (Life Technologies, Rockville, MD): 4.5 g/L D-glucose, L-glutamine, 25 m*M* HEPES buffer, and pyridoxine hydrochloride, but no sodium pyruvate.
3. Fetal bovine serum (FBS) (Hyclone Laboratories Inc., Logan, UT).
4. 24-Well cell culture plates (Corning, Corning, NY).
5. LipofectAMINE and PLUS reagent kit (Life Technologies, Rockville, MD).
6. SRE.L luciferase reporter gene plasmid.
7. GFP expression plasmid (Clontech, Palo Alto, CA).
8. LacZ expression plasmid.
9. GPCR construct plasmids.

2.2. Ligand Treatment and Luciferase Assay

1. ET-1 (Sigma, St. Louis, MO).
2. 1X Phosphate-buffered saline (PBS) buffer: 0.137 *M* NaCl, 2.7 m*M* KCl, 1.4 m*M* KH_2PO_4, 0.01 *M* Na_2HPO_4.
3. Distilled water.
4. Luciferase reporter gene assay kit (Roche Biochemical, Indianapolis, IN).
5. 96-Well white fluorescence plates (VWR, West Chester, PA).

3. Methods (see Note 1)

3.1. Cell Culture and Transfection

1. Maintain NIH3T3 and Fq/11 cells in DMEM containing 10% FBS at 37°C under 5% CO_2.
2. The day before transfection, seed the cells at 5×10^4 cells/well into 24-well cell culture plate.
3. Perform transfection using LipofectAMINE Plus reagent in 250 µL serum-free DMEM for each well (*see* **Note 2**). The total amount of DNA transfected in each well is 0.5 µg for NIH3T3 cells and 0.25 µg for Fq/11 cells, respectively. The SRE.L luciferase reporter gene is usually used at 0.1 µg/well. In addition, cotransfect 0.1 µg/well GFP expression plasmid with receptors for the purpose of normalization. Use LacZ expression plasmid to make up the total DNA amount as desired.
4. Add all transfection components into each well, then put the cell culture plates back into the incubator at 37°C under 5% CO_2 for 3 h (NIH3T3) or 5 h (Fq/11 cells), respectively.
5. Then stop transfection by removing transfection mix from each well. Add immediately 500 µL/well of cell culture medium containing 0.5% FBS (*see* **Note 3**).
6. Culture cells for another 24 h for NIH3T3 and 48 h for Fq/11, respectively, before the luciferase assay.

3.2. Ligand Treatment and Luciferase Assay (see Note 4)

1. Add ligand ET-1 to culture media at desired concentrations for 6 h.
2. Remove the media and wash the cells with 200 µL/well PBS.

3. Add 150 µL/well 1X cell lysis buffer provided by the luciferase assay kit.
4. Put the cell plates on a shaker at room temperature for 5 min.
5. Measure the background of the 96-well fluorescence plates used in the assay. Add 40 µL/well distilled water into plates and count under the fluorescence mode in the Victor2 counter (Wallac, Gaithersburg, MD).
6. Discard the water. Transfer the cell lysate 40 µL/well into the above plates.
7. Measure the GFP expression level under the fluorescence mode.
8. Add 40 µL/well luciferase substrate provided by the kit into the same plates, and measure the luminescence intensity in the counter using the luminescence mode.
9. Normalize the results (*see* **Note 5**). The relative luciferase activity in each well is obtained by dividing the luminescence intensity by the corresponding, background corrected fluorescence intensity.

4. Notes

1. SRE.L-mediated transcription can be activated by RhoA in a number of cell lines, Including NIH3T3 mouse fibroblast cells, human embryonic kidney (A293) cells, COS7 monkey kidney cells, and various lymphocytes. Transient transfection is preferred because the chromosome-integrated reporter gene appears to be regulated less efficiently ***(17)***.
2. The transfection protocol, including the amounts of plasmid DNA and LipofectAMINE reagents, needs to be optimized for different cell lines. The transfection efficiency could be affected by a number of factors, such as the cell condition and density, the quality of prepared plasmids, and the transfection duration.
3. Because serum activates SRF, the cells need to be maintained in the DMEM containing 0.5% FBS after transfection, rather than the growth medium containing 10% FBS, in order to minimize the basal level of luciferase activity.
4. The luciferase assay is usually performed at 24 h following transfection. However, the protein expression duration should also be adjusted according to cell types and the strength of the promoters in the plasmids. For Fq/11 cells, we use 48-h expression.
5. The normalization of assay results is helpful for comparing data from different experiments. Coexpression of β-galactosidase or alkaline phosphatase has also been widely utilized for this purpose. The use of GFP for the normalization process has more advantages: high sensitivity, wider linear dynamic range, less time consuming, and more importantly, reduced variations caused by sample handling as both fluorescence and luminescence are measured in the same plates.

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14

Selective Inhibition of G Protein-Mediated Pathways Using RGS Domains

Tohru Kozasa and Richard D. Ye

Summary

Regulators of G protein signaling (RGS) proteins are recently identified regulators of G protein-mediated pathways. These proteins recognize specific members of G protein α subunits and, in most cases, act as GTPase activating proteins (GAPs). RGS proteins interact with activated forms of Gα subunits through RGS domain of about 120 amino acids. Thus, the overexpression of the RGS domain in cells can specifically block the signalling pathways that are mediated by the interacting G proteins. The method is particularly useful to differentiate between G_q-mediated and $G_{12/13}$-mediated pathways.

Key Words: G protein; RGS protein; p115RhoGEF; G protein receptor kinase (GRK).

1. Introduction

The α subunits of heterotrimeric G proteins are commonly classified into four subfamilies based on amino acid sequence homology and function (**Table 1**) ***(1,2)***. Members of the G_s and G_q subfamilies are activators of adenylyl cyclases (AC) and phospholipase C (PLC)-βs, respectively. Members of the G_i subfamily are involved in inhibition of certain subtypes of AC or stimulation of a retinal cGMP phosphodiesterase. Members of G_{12} subfamily are involved in activation of Rho GTPase. In addition to these effector molecules, novel effectors or interacting proteins for heterotrimeric G proteins have recently been identified and evidence is accumulating to suggest that G protein-mediated pathways are involved in a broad range of signaling systems ***(3)***.

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Table 1
Classification of Gα Subunit

Subfamily	Gα subunits	Effector regulation
G_s	$G_s\alpha$, $G_{olf}\alpha$	Activation of AC
G_i	$G_i\alpha$, $G_o\alpha$, $G_z\alpha$ $G_t\alpha$	Inhibition of AC, etc.
G_q	$G_q\alpha$, $G_{11}\alpha$, $G_{14}\alpha$, $G_{16}\alpha$	Activation of PLCβs
G_{12}	$G_{12}\alpha$, $G_{13}\alpha$	Activation of RhoA

To understand the mechanism and regulation of signal transduction pathways, identification of G proteins or G protein subfamilies that couple to particular G protein-coupled receptor (GPCR)-mediated signaling events is critically important. There are several different methods to identify the G proteins in individual signaling pathways. The most direct method is to measure the agonist induced change of second messenger level in cells, such as cAMP for AC or inositol-1,4,5-triphosphate (IP_3) for PLC-β. However, these biochemical assays are not always possible. In addition, various G protein-mediated effects that do not involve these second messengers have been reported. Another approach is to utilize specific inhibitors like pertussis toxin. Pertussis toxin has been most extensively used to determine whether G proteins of G_i subfamily are involved in the signaling. Pertussis toxin specifically adenosine 5'-diphosphate (ADP)-ribosylates α subunits of G_i subfamily (except for $G\alpha_z$) at a cysteine residue four amino acids from the C-terminus. The C-terminal region of G protein α subunits is involved in the coupling with receptors and G_i or $G_o\alpha$ subunits can no longer couple to receptors if they are ADP ribosylated by pertussis toxin. Since this modification is quite specific for Gα subunits of $G_{i/o}$ subfamily and does not affect other Gα subunits, it can be concluded that responses that are blocked by pertussis toxin treatment involve G_i or G_o. No other specific reagents are well established to differentiate among other G protein subfamilies. Particularly, it has not been possible to differentiate between G_q and $G_{12/13}$ involvement in receptor-mediated responses. GPCRs that couple to $G_{12/13}$ often couple to G_q. Since the $G_{12/13}$ pathway is involved in Rho GTPase activation and does not change soluble second-messenger levels, it can be fairly difficult to conclude that observed cellular effects are mediated through $G_{12/13}$. Here we describe a method that uses regulator of G protein signaling (RGS) domains with different specificity for Gα subunits to identify G proteins involved in particular pathways. We focus on a specific method to differentiate between G_q- and $G_{12/13}$-mediated pathways.

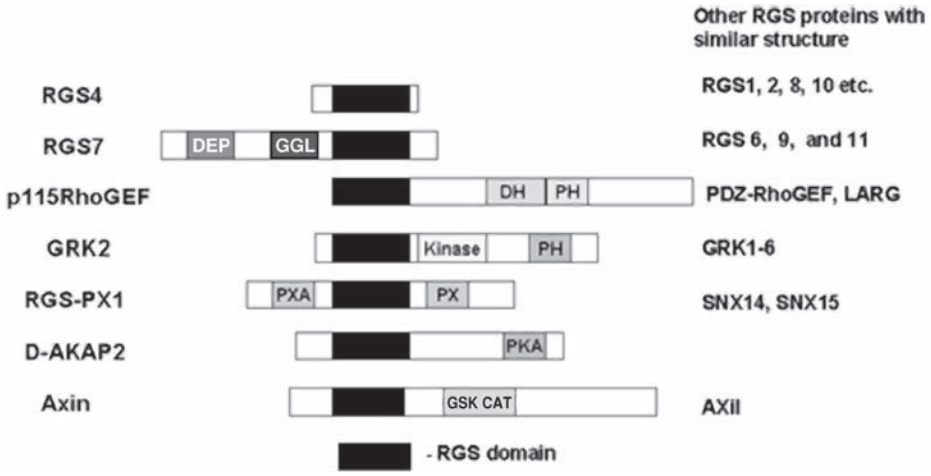

Fig. 1. Domain structures of RGS proteins. Domain structures of representative RGS proteins are shown. Black box represents RGS domain: DEP, Disheveled/EGL10/ Pleckstrin domain; GGL, Gγ-like domain; DH, Dbl homology domain; PH, Pleckstrin homology domain; PKA, protein kinase A binding domain; kinase, receptor kinase domain; GSK CAT, GSK3β and β catenin-binding domain; PX, phosphatidyl inositol binding domain; PXA, PX-associated domain.

RGS proteins were initially identified as negative regulators of G protein signaling in yeast and *C. elegans* through genetic screening ***(5)***. Subsequently, RGS proteins were found in mammalian cells. To date, more than 20 mammalian RGS proteins have been identified to contain homologous core domains (RGS domains) of approx 120 amino acids. RGS proteins interact with activated Gα subunits through their RGS domains. Biochemical studies revealed that several RGS proteins act as GTPase-activating proteins (GAPs) to facilitate GTPase activity of Gα subunits. Besides the common RGS domain, RGS proteins contain various protein motifs involved in cell signaling (**Fig. 1**). Recent studies indicate that RGS proteins have additional functions besides GAP activities and they participate in various physiological functions in cells ***(4)***.

RGS domains selectively recognize the active form of specific Gα subunits and do not interact with inactive GDP-bound Gα subunit. The three-dimensional crystal structure of a complex of $G\alpha_i$ with the RGS domain of RGS4 revealed that the RGS domain interacts with three switch regions of $G\alpha_i$ ***(5)***. These switch regions change conformation between GTP-bound and GDP-bound states, being critically involved in GTP hydrolysis. Thus, this crystal structure shows that RGS domains recognize and stabilize the transition state of GTPase reaction and act as a GAP for Gα subunits ***(6)***. Using nonhydrolyz-

Table 2
Gα Subunit Specificity of RGS Proteins

RGS	Interacting Gα
RGS4	$G_i\alpha$, $G_q\alpha$
RGS7	$G_o\alpha$
p115RhoGEF	$G_{12}\alpha$, $G_{13}\alpha$
GRK2	$G_q\alpha$
RGS-PX1	$G_s\alpha$
D-AKAP2	?
Axin	?

able analog of GTP, GTPγS, it was also found that RGS proteins can function as an effector antagonists by sequestering the active form of Gα subunits. For example, RGS4 inhibited PLCβ activation by GTPγS activated $G_q\alpha$ in vitro *(7)*. Thus, RGS proteins negatively regulate G protein-mediated pathways by two different mechanisms: by accelerating Gα deactivation by its GAP activity and by sequestering activated form of Gα as an effector antagonist. From these results, it was expected that RGS proteins could be used as specific blockers for G-signaling pathways to differentiate coupling G proteins.

RGS proteins can be classified into several subfamilies based on domain structure *(8)*. As shown in **Table 2**, members of each subfamily demonstrate different specificity for Gα subunits. Most RGS proteins in the RGS4 subfamily have a molecular weight of 20–25 kDa and interact with Gα subunits in G_i and G_q subfamily. They do not interact with Gα subunits of G_s or G_{12} subfamily *(6,9)*. Members of RGS7 subfamily are large and contain disheveled, egl-10, pleckstrin (DEP) domain and Gγ-like domain (G protein γ subunit like [GGL]) domain in the amino-terminal region. These RGS proteins selectively complex with G protein β5 subunits through their GGL domains and interact with Gα subunit of Gi subfamily, especially with $G_\alpha o$ *(10)*. It is well established that six isoforms of GPCR kinases (GRKs) are involved in receptor-mediated desensitization by phosphorylation of agonist-bound GPCRs. They all have RGS domains at the amino-terminus. Recently, the RGS domain of GRK2 was found to selectively interact with activated $G\alpha_q$ *(11)*. Unlike members of the RGS4 family, it does not interact with $G\alpha_i$. This suggested that sequestration of active $G\alpha_q$ through the RGS domain may be responsible for phosphorylation-independent desensitization of G_q-mediated PLC activation. RGS domains of RhoGEFs (p115RhoGEF, PDZ-RhoGEF, and LARG) selectively interact with $G\alpha_{12}$ or $G\alpha_{13}$ and RGS domains of p115RhoGEF or LARG are efficient GAPs

for these Gα subunits ***(12,13)***. These RGS domains do not interact with Gα subunits from G_s, G_i, or G_q subfamily, and RhoGEFs proteins are RGS proteins that have effector activity in addition to RGS activity. Recently, RGS-PX1 was identified as an RGS protein-specific for $G\alpha_s$ ***(14)***. Gα subunits partners for RGS domains of Axin or D-AKAP2 have not been identified.

In summary, there are RGS proteins specific for each subfamily of Gα subunit, and these RGS domains can be used as specific blockers for each G protein in cellular signaling pathways. In particular, $G_{12/13}$-mediated signaling can be specifically blocked by overexpression of the RGS domain of p115. Overexpression of this RGS domain will not affect $G\alpha_q$-mediated pathways. On the other hand, G_q-mediated pathways can be blocked by RGS domains from the RGS4 subfamily (RGS2, 3, or 4) or from GRK2. These RGS domains will not affect the $G\alpha_{12/13}$-mediated pathway. Several examples of experiments that dissect G_q- or $G_{12/13}$-mediated pathways using these RGS domains are presented in the following sections, then a specific application of the method will be described in detail in **Subheading 2.**

1.1. M1 Receptor-Mediated Pyk2 Activation and Serum Response Element (SRE) Activation

It is well known that the M1 muscarinic acetylcholine receptor couples to G_q subfamily members to activate PLCβs. However, it is not clearly understood whether the M1 receptor couples to the G_{12} subfamily. As shown in **Fig. 2**, in HeLa cells expressing the M1 receptor, carbachol induced Rho-dependent SRE activation, and this response was blocked by exogenously expressing the RGS domain of p115RhoGEF ***(15)***. The result indicated that carbachol activated SRF activity through $G_{12/13}$ in these cells. It was further demonstrated that this M1 receptor-mediated SRE activation involved Pyk2 tyrosine kinase activation. This Pyk2 activation was also blocked by RGS domain of p115 (**Fig. 3**). On the other hand, Pyk2 activation induced by constitutive active Gαq mutant was not inhibited by p115RGS. These results indicate that in HeLa cells, M1 receptor couples to $G_{12/13}$ to induce Pyk2 activation and SRE activation.

1.2. Endothelin-1 (ET-1)-Mediated MAP Kinase Activation

ET-1-ET_A receptor pathway couples to the G_q subfamily to activate PLC and increase intracellular Ca^{2+}. It has been reported that the ET_A receptor also couples to $G_{12/13}$. In CHO cells expressing ET_A receptor, RGS3 blocked $G\alpha_q$-mediated Ca^{2+} responses ***(16)***. ET-1 also activates MAP kinase in these cells and overexpression of RGS3, but not p115RGS blocked this effect (**Fig. 4**). The results indicate that ET-1 induces MAP kinase activation through G_q, but not thorough $G_{12/13}$ in CHO cells.

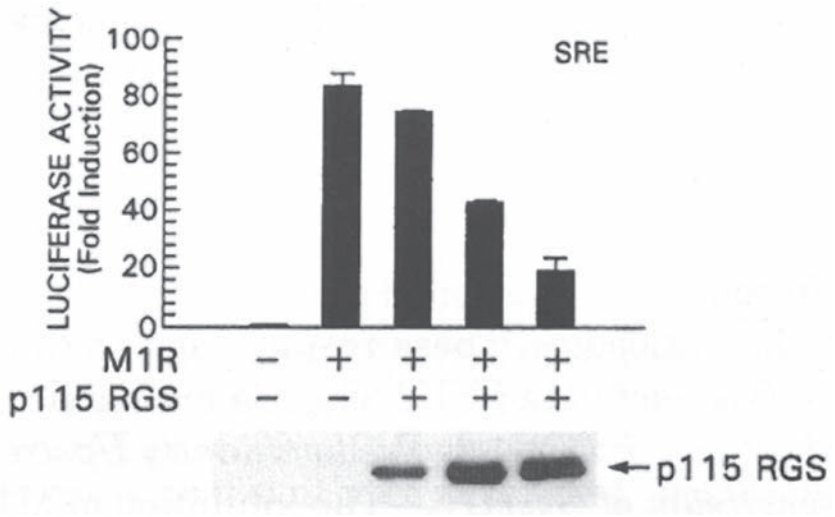

Fig. 2. Inhibition of carbachol induced SRE activation by p115RGS.SRE-luciferase activity was assayed in HeLa cells expressing M1 receptor and p115RGS (0.5, 1.0, or 1.5 μg). Cells were exposed to 100 μ*M* carbachol and SRE activity of cell lysates were assayed. (Reproduced from **ref. *15*** with permission.)

1.3. KSHV-GPCR-Mediated NF-κB Activation

The Kaposi's sarcoma-associated herpes virus encodes a GPCR (KSHV-GPCR) that is homologous to interleukin-8 (IL-8) receptors. Overexpression of KSHV-GPCR induces cell transformation and focus formation without agonist stimulation, suggesting constitutive activation of this receptor. It was demonstrated that overexpression of KSVH-GPCR activated NF-κB and this response was partially blocked by expressing p115RGS ***(17)***. Thus, KSHV-GPCR constitutively activates G_{13} to stimulate NF-κB activity (**Fig. 5**). The details of these specific methods will be presented in the experimental section of this chapter.

1.4. α1 Adrenergic Receptor-Induced Cardiac Hypertrophy

In neonatal cardiomyocytes, α_1-adrenergic receptor activation induces cardiac hypertrophy through JNK activation. This α_1-mediated JNK activation was not blocked by pertussis toxin treatment, suggesting the involvement of G_q and/or G_{12} subfamilies of G proteins. Here, overexpression of p115RGS or the RGS domain of GRK2 or RGS4 in these cells using recombinant adenovirus inhibited α_1-mediated JNK activation ***(18)***. The results suggest that both $G_{12/13}$ and $G_{q/11}$ mediate α_1 adrenergic agonist-induced cardiac hypertrophy.

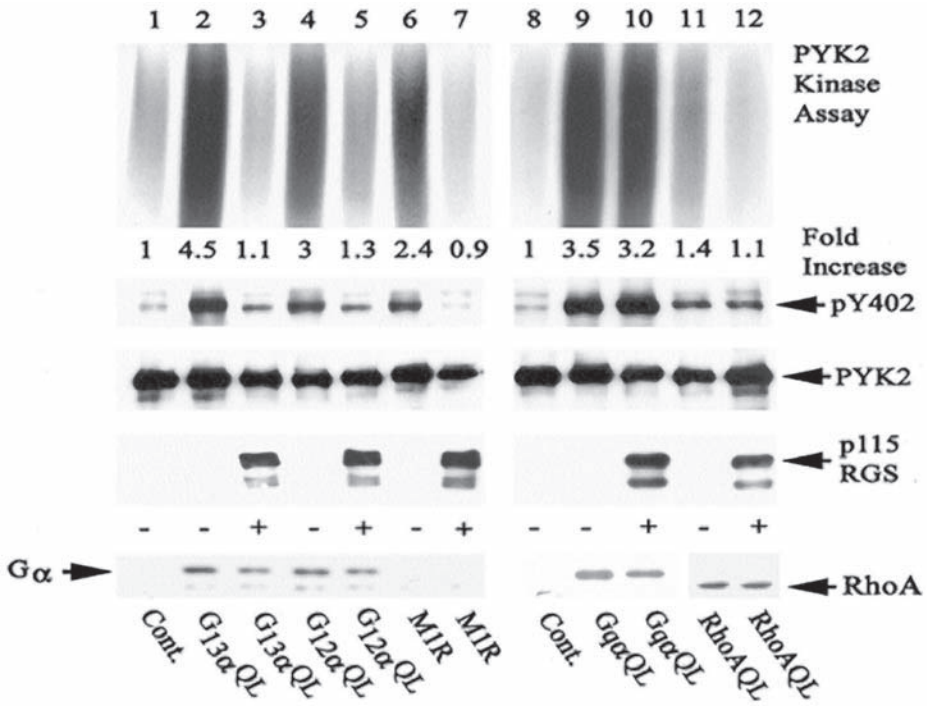

Fig. 3. Effect of p115RGS on activation of PYK2 by constitutive active Gα and M1R signaling. Lysates from HeLa cells expressing PYK2 and either $G_{13}\alpha_{QL}$, $G_{12}\alpha_{QL}$, $G_q\alpha_{QL}$, RhoAQL, or M1R in the presence and absence of p115RGS were analyzed for PYK2 kinase activity. The fold increase in kinase activity when compared with basal is indicated. The cells expressing M1R were exposed 100 μ*M* carbachol for 15 min prior to lysis. (Reproduced from **ref. *15*** with permission.)

Currently, we have RGS proteins that are specific for each of four G protein subfamilies. As stated previously, the RGS domain of p115 RhoGEF is a powerful and convenient tool to analyze the involvement of $G_{12/13}$ in the signaling event. In order to block G_q-mediated pathways, RGS domains of RGS2, 3, 4 or GRK2 have been useful ***(11,16,19)***. For G_i-mediated pathways, pertussis toxin treatment is more efficient than overexpression of RGS domains. Application of the $G\alpha_s$ specific RGS protein RGS-PX1 to analyze G_s-mediated signal transduction pathways has not been reported.

The method described is most useful to determine the subclass of G protein family. However, it cannot differentiate Gα subunits within the same subfamily. For example, the apparent affinity of p115RGS for $G\alpha_{12}$ or $G\alpha_{13}$ is similar, and no RGS domain has been identified that is specific for either $G\alpha_{12}$ or $G\alpha_{13}$.

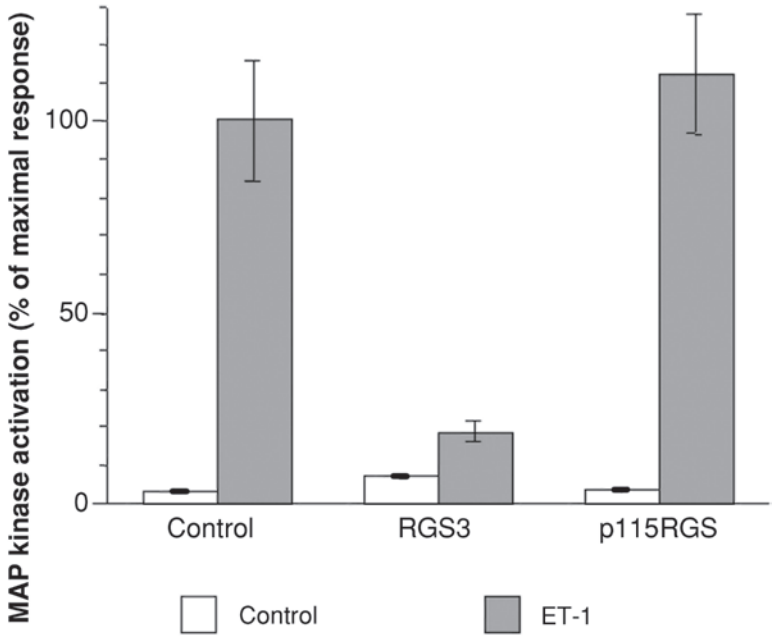

Fig. 4. RGS3, but not p115RGS, inhibits MAP kinase activation by ET-1. CHO cells were transfected with ET_A receptor with or without RGS3 or p115RGS. Cells were stimulated by ET-1 and MAP kinase activity of cell lysate were assayed (N. Dulin, University of Chicago, unpublished).

Also, the RGS method cannot differentiate members within the G_q subfamily or G_i subfamily. In order to identify Gα subunit within the same subfamily, the method using carboxyl-terminal region of each G protein α subunit may be useful ***(20)***. The combination of these two methods was successfully applied to the ET-1 induced response in cardiomyocytes ***(21)***.

2. Materials

2.1. Expression Constructs

1. *Myc*-tagged p115 RGS construct. Amino-acid residues from 1 to 252 of p115RhoGEF were subcloned into pCMV5 vector with N-terminal *myc* tag.
2. G_{13}(Q226L) construct. Glutamine 226 of $G\alpha_{13}$ was mutated to leucine. This mutation reduces GTPase activity of $G\alpha_{13}$ and makes it constitutively active. The mutant cDNA was subcloned into pCMV5 vector.
3. 3X κB luciferase reporter construct. This construct was generated by ligation of 3 consensus NF-κB site (5'-AGGGGACTTTCCCA-3') in tandem into the pGL2-Luc vector (Promega), upstream from the firefly luciferase reporter ***(22)***. A simi-

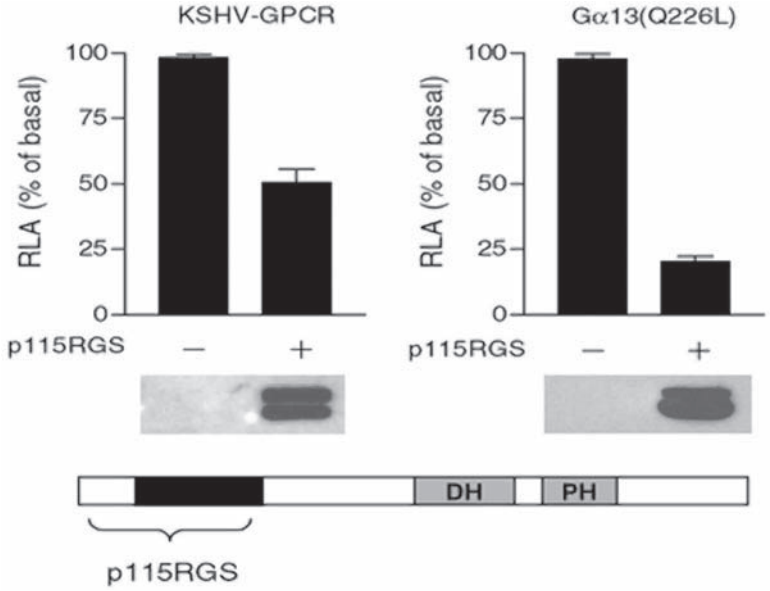

Fig. 5. Negative regulation of KSHV-GPCR induced NF-κB activation by p115RGS. HeLa cells were transfected with KSHV-GPCR or $G_{13}\alpha_{QL}$ with and without p115RGS. Inhibition of the κB luciferase activity is expressed as percent of the maximal response induced by KSHV-GPCR (9.5-fold increase over baseline). (Reproduced from **ref.** ***17*** with permission.)

lar reporter construct, with 5X κB sites, is available from Stratagene and can be used in place of the 3X κB reporter.

4. AU5-tagged KSHV-GPCR construct. This expression construct was made by RT-PCR, using a forward oligonucleotide primer coding for the AU5 sequence (TDFYLK) that was placed N-terminal to the KSHV-GPCR sequence. The initiation codon of KSHV-GPCR was removed. Consensus translational initiation sequence that included an initiation codon, CCACCATG, was placed in front of the AU5 sequence.
5. A β-galactosidase expression construct directed by a constitutive promoter, such as pSV-β-galactosidase control vector (Promega).

2.2. Tissue Culture Cells and Reagents

1. HeLa cells: these and other suitable cell lines are available from American Type Culture Collection.
2. Six-well cell tissue culture plates.
3. Dulbecco's modified Eagle's medium (DMEM) and Opti-MEM (Invitrogen/Life Technologies).
4. Heat-inactivated fetal bovine serum (FBS) (Invitrogen/Life Technologies).

5. 2 m*M* Glutamine (Invitrogen/Life Technologies)
6. Penicillin/streptomycin (Invitrogen/Life Technologies).

2.3. Transfection and Assay Reagents

1. Reporter lysis buffer and luciferase assay reagent (Promega).
2. Luminescence β-galactosidase detection reagent (Clontech).
3. Luminometer (e.g., a Femtomaster FB12 luminometer, Zylux Corporation).
4. Transfection reagent. Transfection of adherent cells can be achieved by using calcium phosphate precipitation, dextran sulfate-mediated transfection, or liposome-based transfection. LipofectAMINE reagents (Invitrogen/Life Technologies) works well with HeLa and COS7 cells.

2.4. Western Blotting Reagents

1. Anti-*myc* monoclonal antibody (9E10 from Covance Research Products), 1 mg/mL stock solution for purified MAb, or use ascites with 1:500 dilution.
2. Goat anti-mouse horse radishperoxidase-linked antibody (Sigma).
3. Supersignal West Pico Chemiluminescent reagent (Pierce).

2.5. Flow Cytometry Reagents

1. Anti-AU5 monoclonal antibody (Covance Research Products).
2. Fluorescein isothiocyanate-labeled goat anti-mouse antibody (Sigma).
3. Flow cytometer.

3. Methods

In this example, the question being asked is what G proteins couple to a transfected GPCR, the KSHV-GPCR, to result in activation of NF-κB signaling. As indicated by the various examples that have been presented this system is generally applicable to studying various transfected GPCRs (provided the appropriate expression construct is available) as well GPCRs already present in a cell line of interest. Critical to the success of such experimentation is the appropriate endpoint assay. In this example, a plasmid containing an NF-κB responsive promoter is used to monitor the activity of the NF-κB pathway. *See* **Notes 1** and **2**.

3.1. Cell Culture and Transfection

1. HeLa cells (a cervical carcinoma-derived adherent cell line) are grown in cell culture dishes with complete media: DMEM supplemented with 2 m*M* glutamine, 10% heat-inactivated FBS, 100 IU/mL penicillin, and 50 µg/mL streptomycin at 37°C in a tissue culture incubator with 5% CO_2 (*see* **Note 3**).
2. One day before transfection, cells are seeded in 6-well plates so that they reach 40–50% confluency by the time of transfection.
3. 200 ng each of the constructs are mixed with the transfection reagents according to **Table 3** such that the total DNA added to each well equals 1 µg.

Table 3
Transfection of Adherent Cells

3X κB reporter construct	200 ng
PSV-β-gal expression vector	200 ng
GPCR expression vector	200 ng
± p115 RGS	200 ng
Empty vector (e.g., pRK5)	200 ng (or bring total to 1000 ng)
Add Opti-MEM to above mixture	Bring to 0.1 mL (A)
LipofectAMINE reagent (2–5 μL)	Bring to 0.1 mL with Opti-MEM (B)
Mix (A) and (B)	Incubate at room temperature (~23°C), 30 min

Per well for 6-well plate.

4. Remove the media and wash the cells with Opti-MEM or serum-free DMEM.
5. Add 0.6 mL Opti-MEM to the transfection mix (**Table 3**; 0.2 mL for each well of a 6-well plate), such that the total volume is 0.8 mL. Mix gently and add to the well. Incubate cells for 5 h at 37°C in a humidified CO_2 incubator.
6. After incubation, add 1 mL complete media and allow cells to incubate for 24–28 h.
7. After this incubation period, remove the media and replace with media lacking FBS and incubate overnight (16–18 h) to serum starve the cells.

3.2. Agonist Addition and Reporter Assay

For this example, no agonist is added and basal activity of the KSHV-GPCR is monitored. In general, however, agonists should be added at concentrations and for durations specific to each receptor (*see* **Note 4**).

1. After overnight incubation quickly remove the cells from the incubator, wash the cells twice with PBS at 37°C and add 1 mL PBS to each well. If no agonist is to be added, skip this step and go directly to **step 3**.
2. Add agonist to the final concentration in PBS at 37°C, and return the cells to the incubator for the incubation time desired (usually 4–5 h).
3. Remove the cells from the incubator, place on ice, and wash each well twice with 2 mL ice cold PBS.
4. Add 0.25 mL 1X reporter lysis buffer, made fresh from 5X stock with H_2O, to each well. Harvest cells using a cell scraper (Sarstedt).
5. Collect cell lysate into a 1.5-mL microcentrifuge tube. Vortex briefly, and centrifuge at 12,000*g* for 15 s.
6. Transfer supernatant to a clean tube for assay or storage at –80°C.
7. For measurement of the induced luciferase activity, take 20 μL supernatant, then mix with 100 μL luciferase assay reagent in a clear microcentrifuge tube. Vortex briefly and measure luminescence for 6–10 s.

3.3. β-Galactosidase Assay

1. For measurement of the constitutively expressed β-galactosidase, first prepare reaction buffer mix. For each reaction, add 2 µL reaction substrate to 98 µL reaction buffer. Prepare a master reaction buffer mix depending on the number of assays to be performed.
2. In a clear microcentrifuge tube, mix 2–5 µL supernatant recovered from cell lysate with 100 µL reaction buffer mix. Gently mix and incubate at room temperature (20–25°C) for 60 min.
3. At the end of incubation, measure luminescence.
4. Normalize for transfection efficiency: divide the luciferase activity reading by the β-galactosidase activity reading from the same sample:

$$\text{Normalized NF-}\kappa\text{B liciferase activity} = \frac{\text{Observed luciferase activity}}{\text{Observed } \beta\text{-galactosidase activity}}$$

3.4. Western Blotting to Confirm Expression of Constructs

It is critical to confirm that the RGS domain of interest is indeed expressed in your experiments.

1. Take 50–100 µL reporter lysate and make a gel sample for polyacrylamide gel electrophoresis.
2. For *c-myc*-p115RhoGEF a 10% mini-gel can be used.
3. Electrophorese the samples at 30 mA.
4. Transfer the proteins to a nitrocellulose membrane at 100 V for 1 h at 4°C. It is best to use fresh transfer buffer at this step.
5. After transfer pretreat the membrane with 5% nonfat dry milk in TTBS (20 m*M* Tris-HCl, pH 7.5, 120 m*M* NaCl, 0.05% Tween-20) for 1–2 h at room temperature.
6. Remove the milk solution and wash twice with TTBS for 5 min each.
7. Incubate the membrane with 1:1000 *c-myc* antibody in TTBS plus 5% bovine serum albumin (BSA) overnight (16 h) at 4°C.
8. Wash the membrane with TTBS 3X 10 min each and incubate with goat anti-mouse secondary antibody coupled to horseradish peroxidase (1:5000) for 1 h.
9. Wash three times 10 min each with TTBS.
10. Develop using supersignal chemiluminescence reagents and exposure to film for 30 s to 5 min.

3.5. Flow Cytometry to Monitor Receptor Surface Expression in HeLa Cells (see Note 5)

1. Approximately 2–4 × 10^5 of transfected HeLa cells are prepared in suspension by dissociation of cells from culture dish with enzyme-free cell dissociation buffer (Invitrogen/Life Technologies). After dissociation, wash cell pellet with cold PBS twice, and resuspend in 250 µL phosphate-buffered

saline (PBS) with 1% BSA. The AU5 MAb is added to 1:500, and cells are incubated for 1 h on ice.
2. After washing and centrifugation of the cells three times with PBS to remove the anti-AU5 MAb, resuspend cells to 250 µL PBS with 1% BSA. Add fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse antibody (1:200) and incubate on ice for another hour.
3. After washing cells for three times with ice-cold PBS, resuspend cells in 400 µL and proceed to flow cytometry analysis. Use as a negative control untransfected or mock-transfected (with empty vector) HeLa cells, incubated similarly with the above antibodies.

4. Notes

1. The combination of specific constructs to be used is dependent on the nature of the system being studied. In general there are multiple RGS domain constructs that can be employed (**Fig. 1**). In this example, a 3X κB luciferase reporter plasmid is used. The specific reporter plasmid is dependent on the particular pathway of interest. A large number of reporter plasmids for different signal transduction pathways are available from Stratagene.
2. Critical to the success of the transfection approach is the transfection efficiency of the cells with the RGS construct. Depending on the cells of interest the transfection efficiency may range from as low as 10% of the cells transfected to 90% of the cells transfected. If you are looking for inhibition of a pathway with a transfected RGS construct, only those cells in a population that are transfected with the RGS construct have any chance having the pathway inhibited. If this is only 10–20% of the cell population, this inhibition may not be observed if a global cellular assay is used. For this, the decision to use either a reporter plasmid or a transfected receptor is critical. The use of transfected reporter plasmids allows the ability to monitor signal transduction events that are only occurring in the transfected cells thus circumventing this problem. If the cells are transfected with 80% efficiency, an endogenous signaling pathway can be monitored directly.
3. Again, the cell type used is specific to the question being asked as can be seen with the various examples given in the **Introduction**. HeLa cells have the advantage that they are readily transfectable and have a robust NF-κB pathway.
4. In this example, the constitutive activity of the KSHV-GPCR is being assayed. Generally, receptors thought to be coupled to a protein require agonist stimulation.
5. The receptor is tagged with an AU5 epitope tag to monitor surface expression, but other methods, such as ligand binding, can also be used to monitor receptor expression.

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15

Ribozymes as Tools for Suppression of G Protein γ Subunits

Janet D. Robishaw, Zheng-ping Guo, and Qin Wang

Summary

RNA suppression approaches provide a rapid survey of gene function. Of these approaches (i.e., antisense oligonucleotides, ribozymes, and RNA interference), ribozymes offer significant advantages by operating as strigent site-specific ribonucleases. The purpose of this chapter is to provide some guidelines and protcols for the use of ribozymes to identify the functions of closely related members of the G protein γ subunit family. Improvements in their design and delivery will be discussed, including the use of "second generation" ribozymes targeted against multiple cleavage sites.

Key Words: Signal transduction; heterotrimeric G proteins; RNA suppression; ribozymes.

1. Introduction

The large number of α, β, and γ subunits creates the potential to generate several hundred distinct G protein trimers. The current challenge is to identify which G protein trimers actually exist in vivo; and to establish their roles in particular signaling pathways in a native system. Increasingly, RNA-based approaches are being used to address these questions. Particularly advantageous, these strategies provide a rapid survey of functions for the study of large gene families. Three basic strategies are available for this purpose: antisense oligonucleotides ***(1**,**2)***, RNA interference ***(3**,**4)***, and ribozymes ***(5**,**6)***.

Antisense oligos act by binding to the target mRNA and suppressing the translation of the target protein. Although early reports were promising ***(7)***, their use has often been plagued by poor specificity. This appears to relate to the secondary action of RNase H, which degrades not only the target mRNA,

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but also "nontarget" mRNAs sharing as little as a 5-bp match ***(1,8)***. A recent strategy showing promise in blocking RNase H action is to modify the oligonucleotide backbone producing morpholino antisense oligos ***(9,10)***. Currently, the effectiveness of this strategy for γ subunit suppression is being tested in our laboratory. Interference RNAs act by triggering the assembly of a nuclease complex that directs the target mRNA for degradation. Although this approach has evolved into a powerful tool for analyzing the functions of the estimated 19,000 genes in *C. elegans* ***(11)***, initial reports suggest that this strategy may have more limited utility in mammals, which appear to lack some of the enzymatic machinery required for this process ***(3,4)***. Uniquely different from the previous approaches, ribozymes possess an inherent cleavage activity that degrades the target mRNA ***(5,6)***. Because their action is dependent on both sequence complementarity and the presence of a specific cleavage site, ribozymes bind more stringently to the target mRNA than antisense oligos ***(12,13)***. Moreover, a control for ribozyme activity can be made by substituting a single nucleotide in the catalytic domain to produce an inactive form of the enzyme. Collectively, this combination of properties makes ribozymes the most highly efficacious tools for suppressing gene expression known to date.

Recently, we used the ribozyme strategy for the first time to suppress specific G protein γ subunit genes in cells, and to identify their functional roles in particular signaling pathways ***(14,15)***. In the process, we optimized the design, delivery, and efficacy of synthetic ribozymes targeted against the γ subunits ***(16)***. Although establishing proof-of-concept, synthetic ribozymes are restricted to certain cell types that are efficiently transfected with lipofectamine. Moreover, their efficacy is quite variable. To address both of these issues, we recently modified the ribozyme strategy to use virally expressed ribozymes targeted against multiple cleavage sites within the γ subunit. This chapter focuses on the theory underlying the design and delivery of these second-generation ribozymes.

2. Materials

2.1. Ribozyme Design

1. Tools for data mining (National Center for Biotechnology Information; www.ncbi.nlm.nih.gov).
2. RNA folding programs for predicting accessibility of ribozyme cleavage sites ***(17,18)***.

2.2. Ribozyme Construction

1. pLEGFP-C1 vector for constructing retroviral delivery system (Clontech).

2.3. Assembly of the Ribozymes in Tandem Configuration

1. QuickChange site-directed mutagenesis kit (Stratagene).

2.4. Delivery of the Ribozymes into Cells

1. T67 cells for packaging recombinant retroviruses (Clontech).
2. MM19 cells for studying consequences of γ_{11} suppression (ATCC).
3. DME medium (Invitrogen/Life Technologies).
4. Fetal bovine serum (FBS) (Invitrogen/Life Technologies).
5. LipoFECTAMINE PLUS (Invitrogen/Life Technologies).
6. Geneticin disulfate (G418) for selecting stably transduced cells (Invitrogen/Life Technologies).

2.5. Assess the Suppression of γ mRNA/Protein

1. RNeasy kit for isolating total RNA (Qiagen).
2. Diethyl pyrocarbonate (DEPC; Sigma).
3. Ethanol (AAPER Alcohol and Chemical Company).
4. EZ rTth RNA PCR kit (Perkin-Elmer).
5. Thermocycler (MJ Research).
6. Agarose (Invitrogen/Life Technologies).
7. Ethidium bromide (Sigma).
8. Low-salt buffer for lysing cells: 2 m*M* magnesium chloride, 1 m*M* EDTA, 20 m*M* HEPES, pH 7.0, 10 m*M* DTT, 1 m*M* aminoethylbenzenesulfonyl fluoride, 1 µg/mL pepstatin A, and 1 m*M* benzamidine.
9. 25-gauge Needle for lysing cells.
10. 1% Sodium cholate for solubilizing G proteins (Cal BioChem).
11. 15% Polyacrylamide-sodium dodecyl sulfate (SDS) gel for resolving G protein γ subtypes (29:1; Bio-Rad).
12. 0.45-mm Pore size nitro-plus nitrocellulose for immunoblotting (Osmonics).
13. High-detergent blotto buffer for immunoblotting: 50 m*M* Tris-HCl, pH 8.0, 80 m*M* NaCl, 2 m*M* $CaCl_2$, 5% nonfat powdered milk, 1% Tween-20, and 0.2% SDS.
14. Low-detergent blotto buffer for immunoblotting: 50 m*M* Tris-HCl, pH 8.0, 80 m*M* NaCl, 2 m*M* $CaCl_2$, 5% nonfat powdered milk, 0.1% Tween-20.
15. Buffer A for immunoblotting: 50 m*M* Tris-HCl, pH 8.0, 80 m*M* sodium chloride and 2 m*M* calcium chloride.
16. Anti-G protein γ antibodies (Cytosignal; Robishaw laboratory).
17. Anti-rabbit Ig, horseradish peroxidase-linked $F(ab')_2$ fragment from donkey (Amersham).
18. Enhanced chemiluminescence (ECL) substrate (SuperSignal, Pierce).
19. Biomax MS film (Kodak).
20. PhosphorImager SI analysis (Molecular Dynamics).

3. Methods

Figure 1 shows a schematic of the design process for a multitarget ribozyme targeted against several cleavage sites in the γ_{11} mRNA. This is based on our published experience in developing synthetic ribozymes against the γ_7 subtype ***(14–16)***, as well as more recent work in designing virally expressed ribozymes against the γ_{11} subtype.

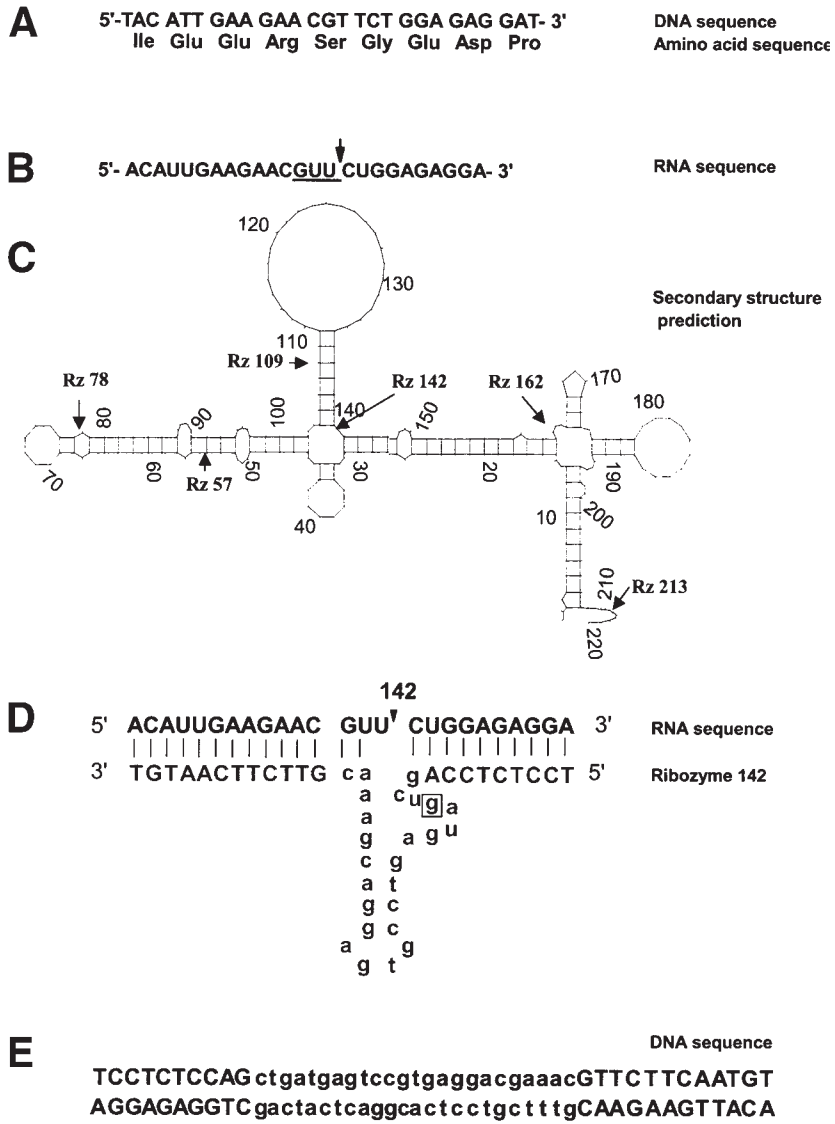

Fig. 1. Design of multitarget ribozyme. This shows a schematic of the design process for a multitarget ribozyme targeted against several cleavage sites in the γ_{11} mRNA. **(A)** DNA sequence and predicted protein sequence for a portion of γ_{11} subunit. **(B)** Corresponding RNA sequence with cleavage site at position 142 underlined. **(C)** Secondary structure prediction of γ_{11} mRNA, with cleavage sites at multiple positions marked. **(D)** Aligned ribozyme 142 sequence. **(E)** Double-stranded DNA sequence-encoding ribozyme 142 ready for synthesis and cloning into expression vector with catalytic core indicated by lower case letters and flanking arms designated by upper case letters.

3.1. Design and Construction of Ribozymes

1. Choose the target γ subtype and obtain the nucleotide sequence. Although this should be obvious, it is very important that the cells, target mRNA sequence, and ribozyme sequence are derived from the same species because even a single base mismatch can disrupt binding.
2. Identify cleavage sites (*see* **Note 1**). Search the nucleotide sequence of the targeted γ subunit for all possible cleavage sites. In the case of hammerhead ribozymes, the preferred cleavage site is GUH, where H = C, A, U ***(19)***. In the example, a portion of the γ_{11} nucleotide sequence and predicted protein sequence is shown with the potential cleavage site underlined (**Fig. 1A,B**).
3. Prioritize cleavage sites (*see* **Note 2**). Select several cleavage sites, which are located in those regions of the γ subunit showing the most sequence divergence and the least secondary structure. In our experience, cleavage sites in the region surrounding the translation start site meet both of these criteria, and ribozymes targeted against this region exhibit consistently high activities. However, more often than not, cleavage sites fall outside this region. In those cases, computer predictions and experimental verification are needed. In the example, a computer prediction of the secondary structure of the γ_{11} mRNA shows the cleavage sites at positions 78, 142, 162, and 213 are located at the junction of stem-loop structures, predicting their accessibility (**Fig. 1C**).
4. Design the ribozyme (*see* **Note 3**). The typical hammerhead ribozyme contains two types of domains: (1) two flanking arms that confer specificity of binding of the ribozyme to the target mRNA; and (2) a catalytic domain that mediates cleavage 3' to the GUH site. In general, the length of each flanking domain should be ≤12 nucleotides (nt). This provides the requisite specificity, and at the same time, allows the rapid association and dissociation of the ribozyme from the target mRNA that is required for catalytic cleavage. In the example, the ribozyme is designed with flanking arms of 12 and 10 nt (**Fig. 1D**; designated by capital letters). The catalytic core contains the nucleotide sequence required for cleavage activity. The ribozyme is designed with this invariant sequence (**Fig. 1D**; designated by lower case letters). Importantly, a substitution of the boxed residue within the catalytic core has been shown to abolish cleavage activity, providing an important control to test the specificity of ribozyme action.
5. Construct the ribozyme. Depending on the application, the ribozyme can be chemically synthesized, or recombinantly expressed. Because we previously published the procedure for the former ***(16)***, this description will focus on the latter. The minimal ribozyme expression unit contains: (1) the double-stranded DNA sequence encoding the ribozyme, which must be preceded by a 5' start site; and followed by a 3' termination signal; (2) a promoter to drive transcription of the ribozyme; and (3) a marker to permit stable selection in eucaryotic cells. In the example, the double-stranded DNA sequence encoding one unit of the ribozyme is shown ready for synthesis and cloning into the pLEGFP-C1 expression vector (**Fig. 1E**). Briefly, the deoxyribonucleotides are chemically synthesized, purified, and inserted into the multiple cloning site of the vector

using the QuickChange site-directed mutagenesis kit. An ATG start codon is placed 5' to the ribozyme construct, and a stop codon is present 3' in the vector.

6. Assemble the ribozymes in tandem configuration (*see* **Note 4**). The greatest obstacle to the use of ribozymes is predicting the accessibility of the cleavage sites within the target mRNA. Because these sites may be masked by RNA folding or protein binding, there is no alternative but to empirically test several different ribozymes to determine their relative efficacies in the intact cell setting. This process is costly and time-consuming. To speed it up, ribozymes targeted against different sites in the target RNA can be assembled in a tandem configuration to simultaneously evaluate their usage of specific sites, and their relative efficiencies. Notably, such multitarget ribozymes have been shown to be functional with no apparent interference between the cleavage sites ***(20)***. In the example, the DNA sequences coding for two, four, or six ribozyme units targeted against the indicated cleavage sites within the γ_{11} mRNA are cloned into the pLEGFP-C1 vector (**Fig. 2A**). Short linkers with the sequence GCATC are introduced to connect the ribozymes in a tandem configuration. DNA sequencing is used to confirm the correct insertion of the ribozymes between the upstream promoter and the downstream termination signal. As will be described, the multitarget ribozymes are introduced into cells, where their effects to suppress the γ_{11} mRNA and protein are assessed.

3.2. Delivery of Ribozymes into Cells (see Note 5)

Virtually any cell line that expresses the γ mRNA and protein of interest can be used, yet some knowledge of the types of G protein signaling pathways that are present in the cell line can be useful. In the example, a mouse lung tumor cell line (MM19) that expresses the γ11 mRNA/protein is used.

Retroviral vectors display distinct advantages, including high transduction efficiency, stable integration, and high level expression of the ribozyme in the same intracellular compartment as the target mRNA ***(6,21)***. The retroviral vector pLEGFP-C1 includes a 5' viral LTR that directs the expression of the *neomycin* resistance gene in eucaryotic cells; and a human *cytomegalovirus* (*CMV*) immediate early promoter that directs the expression of the linked ribozyme and *EGFP* genes. In the example, the vector is engineered to express two, four, or six ribozymes. The vector lacking any ribozymes is used as a control.

1. To generate cell lines producing recombinant retroviruses, PT67 packaging cells are transfected with each of the linearized control, two, four, or six ribozyme constructs in the presence of LipofectaminePlus reagent. For this purpose, the PT67 cells are grown in 60-mm dishes in Delbecoo's modified Eagle's medium (DMEM) supplemented with 10% FBS.
2. Prior to transfection, the cells are preincubated with serum-free DMEM for 1 h. Then, the cells are incubated with each of the linearized ribozyme constructs premixed with 15 µg/mL LipofectaminePLUS reagent.

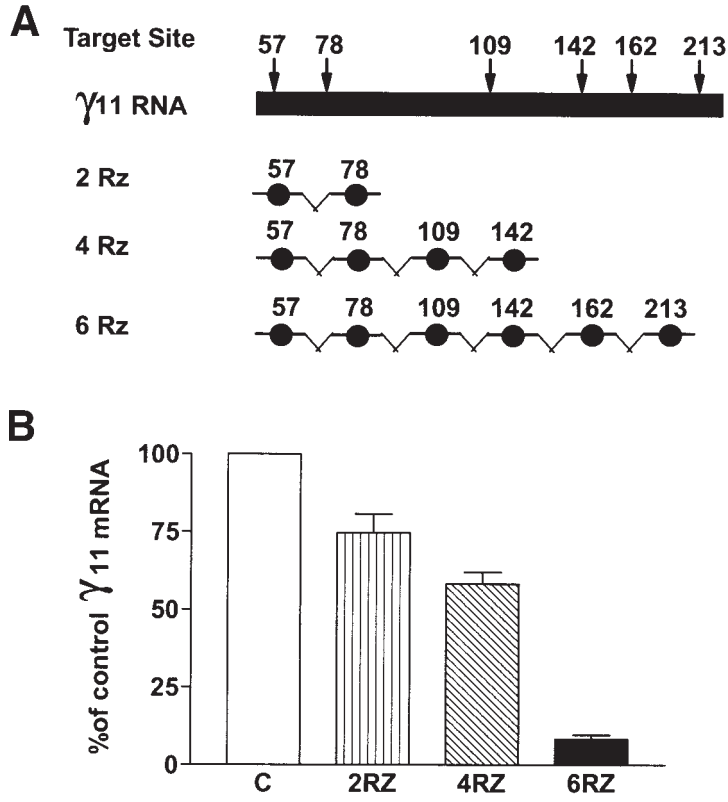

Fig. 2. Efficacy of multitarget ribozyme. **(A)** This shows a schematic of three multitarget ribozymes targeted against various cleavage sites in the γ_{11} mRNA with numbers indicating the number of the 3' residue in each cleavage site relative to the ATG initiation site. **(B)** Dose-dependent suppression of the γ_{11} mRNA, comparing two, four, or six ribozyme constructs to a control construct with no ribozyme.

3. At 5 h posttransfection, cells are supplemented with heat-inactivated FBS at a final concentration of 6%.
4. At 48 h posttransfection, cells are continually cultured with fresh medium containing 1.0 mg/mL G418 until >95% of the cells show fluorescence.
5. This procedure generates PT67 cell lines expressing recombinant retroviruses for the various ribozyme constructs.
6. When the PT67 cell lines reach approx 80% confluence, the media containing the ribozyme viruses are collected, and placed on ice.
7. After addition of polybrene to a final concentration of 4 µg/mL, the ribozyme viruses are collected by filtration with an 0.45-µm filter. Their titers are deter-

mined by serially diluting the virus, infecting HEK 293 cells, and counting the number of fluorescent cells with a microscope.
8. The ribozyme viruses can then be used to stably transduce a wide variety of cells. In the example, mouse lung tumor cell lines expressing zero, two, four, or six ribozyme units are grown in media containing 1.0 mg/mL G418 until more than 95% of the cells show fluorescence.

3.3. Assess Suppression of γ Subunit mRNA (see Note 6)

1. To confirm suppression, total RNA is prepared from stably transduced cells with the RNeasy kit (Qiagen); precipitated with ethanol, and redissolved in DEPC-treated water.
2. The relative levels of γ mRNA are then determined by reverse transcription-polymerase chain reaction (RT-PCR) or Northern slot blot *(16)* analysis. In the example, the relative amounts of γ_{11} mRNA are determined using the EZ rTth RNA PCR kit according to the manufacturer's instructions (Perkin-Elmer). The primers for the γ_{11} mRNA were derived from the mouse cDNA (sense primer: 5'-CACATCGAGGATCTGCCAGA-3'; antisense primer: 5'-AGAATTTCCCT CCCCCAGAG-3').
3. Total RNA (0.5 μg) is added to a tube containing 20 pmol of the primers along with other listed kit components in a final volume of 50 μL.
4. The reverse transcriptase reaction is performed at 60°C for 30 min.
5. After denaturation at 94°C for 60 s, the PCR amplification step is performed from 25 to 40 cycles (94°C for 15 s; 60°C for 30 s); and terminated at 60°C for 7 min.
6. The resulting PCR products are resolved by electrophoresis on a 1.8% agarose gel, visualized by ethidium bromide staining, and quantified within the linear range of amplification. In the example, multitarget ribozymes suppress the level of the γ_{11} mRNA in a dose-dependent fashion with the six ribozyme construct showing the greatest suppression (**Fig. 2B**). The specific action of the six ribozyme construct is demonstrated if the γ_{11} mRNA is significantly reduced relative to control mRNAs. Although not shown, the levels of elongation factor and other γ mRNAs are comparable between cells lacking or expressing the six ribozyme construct. Presumably, the higher efficacy of the six ribozyme construct results from the cumulative action of the individual ribozymes.

3.4. Assess Suppression of γ Subunit Protein (see Note 6)

1. It is important to confirm that ribozyme mediated loss of the γ mRNA is paralleled by suppression of the γ protein. For this purpose, cholate-solubilized membranes from control and stably transduced cells are evaluated by Western blot analysis. Briefly, equal amounts of proteins from these cells (50 μg) are resolved on a 15% polyacrylamide-SDS gel, and transferred to Nitro-plus nitrocellulose using a high-temperature transfer procedure *(22)*.
2. Next, the nitrocellulose blot is blocked for 1 h in high-detergent blotto buffer.
3. Then, the blot is incubated for 1 h in low-detergent blotto buffer containing the γ_{11} subtype specific antibody at a 1:100 dilution *(23)*.

4. After three successive washes with low-detergent blotto buffer, the blot is incubated for 1 h in low-detergent blotto buffer containing anti-rabbit Ig, horseradish peroxidase linked $F(ab')_2$.
5. After three successive washes with low-detergent blotto and two rinses with buffer A, the blot is incubated with ECL substrate and exposed to X-ray film.
6. The intensities of the immunodetectable bands are quantified, and the relative amounts of γ protein in ribozyme-expressing cells are expressed as percentages of their levels in control cells. The specific action of the ribozyme is demonstrated if the target γ protein is significantly reduced relative to control proteins including other related γ proteins.

3.5. Determine the Functional Consequences

The final step in the process is to determine how the ribozyme mediated loss of the γ subunit affects the G protein-mediated signaling pathways. Assays for the more common G protein-mediated signaling pathways are published elsewhere in this handbook (*see* Chapters 6–8, 13, 14). The specific role of a γ subunit is demonstrated when one or more these signaling pathways is altered by the active ribozyme, but not the catalytically inactive form.

4. Notes

1. Most naturally occurring hammerhead ribozymes utilize the GUC cleavage site, although kinetic studies reveal that other sites are also subject to cleavage. These are, in order of decreasing preference: GUC>GUA>GUU>AUH>CUH~UUH ***(19)***.
2. Because each γ mRNA has multiple cleavage sites from which to choose, these sites are prioritized based on the following guidelines: (1) specificity and (2) accessibility. Regarding the specificity issue, the obvious preference is to utilize those sites whose sequences show the least conservation to other related γ subunit mRNAs, in particular, and other unrelated mRNAs, in general. This can be accomplished by searching the databases. Regarding the accessibility issue, the challenge is even greater. Because the γ mRNAs form higher order structures within the cell, prediction of accessible cleavage sites is difficult. One approach is to use computer programs to predict the secondary structure of the γ mRNAs. At least two RNA folding programs are available for this purpose ***(17,18)***. From this analysis, regions with loops or stem-loop structures are generally considered to be most accessible to the ribozyme. Although progress is being made, computer simulations of secondary structure are often inaccurate. Therefore, the other approach is to construct a multitarget ribozyme targeted against several different cleavage sites, thereby mitigating the problem of accessibility as well as increasing the issue of specificity.
3. Once a suitable cleavage site has been identified, the ribozyme is engineered to hybridize to the nucleotide sequences flanking the GUH triplet. The catalytic domain is invariant and is not discussed further. By contrast, the flanking domains are critical to the successful design of the ribozyme. Of the issues to be

considered, the most important are the uniqueness and length of the flanking domains, because these determine the specificity and activity of the ribozyme, respectively. Particularly in the case of a multigene family, it is important to select those flanking regions that show the greatest sequence divergence, in particular, between the γ family members and, in general, other sequences in the database. Though having a high probability of being unique to the human genome ***(1)***, any sequence of 17 nt or longer would also have a high probability of stably binding to the target mRNA, which would decrease the efficiency of ribozyme action and increase the likelihood of RNase H-dependent action that accounts for most of the nonspecific antisense effects in cells. To circumvent both of these problems, sequences of 12 nt or less should be used for the flanking arms with the requirement for their juxtaposition around the cleavage site providing the requisite specificity.

4. Multitarget ribozymes are connected in a tandem configuration by short pieces of random sequence to produce a multimeric ribozyme ***(20)***. When compared to monoribozymes, these multitarget ribozymes not only maintain the target specificities of the individual ribozymes, but also significantly boost the overall cleavage efficacy ***(13,20)***. Although cleavage can be assessed in the test tube ***(16)***, such in vitro assays cannot predict the ability of the multitarget ribozyme to function in cells. Therefore, it is necessary to introduce the ribozyme into cells, where its effect to suppress the targeted γ mRNA and protein can be assessed.
5. There are two strategies for ribozyme delivery. For the exogenous delivery system, the ribozyme is chemically synthesized, then delivered to cells by transient transfection with cationic liposomes. Though this strategy is suitable for certain cell types, one of the problems associated with this mode of delivery is trapping of the ribozyme in the endosome at a site distinct from its target mRNA ***(24)***. For the endogenous delivery system, the ribozyme is introduced into cells, where it is expressed most commonly with a viral vector. Ideally, the vector should express high levels of ribozyme RNA in the same intracellular compartment as its target RNA. Generally, these criteria are met by a retroviral vector ***(6,21)***.
6. Ribozyme are considered to be specific when cell viability is maintained and the levels of target mRNA and protein fall much more than those of control mRNAs and proteins. In this regard, several issues warrant consideration. The active ribozyme should inhibit the expression in a dose-dependent fashion. The failure of the inactive ribozyme containing a point mutation in the catalytic domain to cause these changes is particularly convincing. Moreover, the active ribozyme should not inhibit the expression of other gene products, including closely related members of the γ subunit family as well as unrelated gene products. Because there is a limit on the number of gene products that can be examined, a homology search of the nucleic acids database should be carried out to rule out other possible mRNAs that may bind to ribozyme as a result of sequence similarity.

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16

In Vivo Adenoviral-Mediated Gene Transfer of the βARKct to Study the Role of Gβγ in Arterial Restenosis

Guido Iaccarino and Walter J. Koch

Summary

A large body of in vitro studies has helped to elucidate intracellular pathways that lead to mitogenic signaling in vascular smooth muscle (VSM) cells. However, a limitation of these studies is that they fail to test the in vivo physiological significance especially because VSM proliferation, in the forms of intimal hyperplasia and restenosis, is an important clinical problem. The recent advent of adenoviral gene transfer technology has made possible to test the in vivo effects of specific molecular modulations of intracellular signal transduction pathways on physiological responses. For example, in VSM, adenoviruses can be delivered to the vessel wall to determine a gene/protein's role in proliferative responses to vascular injury. This technology, once standardized and rendered safe for human applications, will be the basis of gene therapy and molecular medicine. Several exemplary applications have now been generated in the vascular system, including the use of an adenovirus containing the carboxyl-terminus of the β-adrenergic receptor kinase (βARKct), which binds to the βγ-subunits of activated heterotrimeric G proteins (Gβγ), to study the in vivo role of Gβγ in VSM intimal hyperplasia after vascular injury that leads to restenosis.

Key Words: G Proteins; βγ subunit; pleckstrin homology domain; adenovirus; gene transfer; cardiovascular system; heart; vascular smooth muscle cells; restenosis; intimal hyperplasia; signal transduction; angioplasty.

1. Introduction

Intracellular signal transduction pathways allow the fine-tuned regulation of cellular responses. These pathways are activated by a large number of receptors that are expressed on the cell surface, which transmit signals to the cytoplasm

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and often to the nucleus. The G protein-coupled receptors (GPCRs) are the largest class of these receptors and, over the past decade, information has flourished regarding the mechanisms by which GPCRs respond to a large array of molecules, from neurohormones to peptides to lipids to odorants, and even photons ***(1)***. In general, GPCR signaling is represented by three units: the receptor, which detects the ligand in the extracellular milieu; the heterotrimeric G protein, composed of an α subunit (Gα) and a βγ subunit (Gβγ); and the effector, that following the interaction with Gα or Gβγ, mediate the intracellular events activated by the ligand binding ***(1,2)***. Classically, Gα subunits have been considered the primary signal transducing unit, whereas to Gβγ function was attributed only structural and membrane localization properties. However, research over the last few years has led to the appreciation of Gβγ being critical-transducing components themselves as they can interact with and regulate several effector molecules such as adenylyl cyclase (AC), phospholipase C (PLC) and ion channels ***(3,4)***.

Of special interest to this chapter is the fact that Gβγ has been shown to be capable of transmitting mitogenic signals ***(5,6)***. Our laboratory was the first to directly demonstrate that Gβγ released from G_i-coupled receptor activation could activate mitogen-activated protein (MAP) kinases via a $p21^{Ras}$ (Ras)-dependent mechanism ***(7)***. Other laboratories have also shown that Gβγ, primarily through G_i proteins, can activate MAP kinases through specific intracellular signaling pathways independent of classical effector systems, such as AC and PLC ***(8)***. Most of these studies (including ours) diagnosed the role of Gβγ by employing cellular expression of Gβγ-sequestering peptides which specifically eliminate Gβγ signaling ***(7–9)***. We have always used the carboxyl-terminus of the β-adrenergic receptor kinase (βARK1), which contains a pleckstrin homology (PH) domain that binds to Gβγ ***(5,10)***. This peptide, comprised of the last 194 amino acids of bovine βARK1, has been termed the βARKct ***(10)***. The Gβγ-mediated translocation of βARK1 is required for the activation of this kinase for the phosphorylation and desensitization of activated GPCRs ***(5,10)***. Expression of the βARKct in cells, by the use of plasmids or adenoviral vectors, results in effective inhibition of not only βARK1 activity ***(10,11)***, but also other Gβγ-mediated signaling pathways through a variety of GPCRs including PLC-β activation ***(12)*** and importantly, MAP kinase signaling ***(7,9,13)***.

The βARKct was originally used to directly demonstrate that G_i-coupled receptors could activate the MAP kinase-signaling cascade via Gβγ-mediated Ras activation ***(7)***. This was shown in cellular studies in HEK-293 cells as well as fibroblasts, primarily involving signaling via lysophosphatidic acid (LPA), a major serum mitogen. The βARKct was also utilized to demonstrate, for the first time, that the insulin-like growth factor (IGF-1) receptor, which is a classical tyrosine kinase receptor, could activate MAP kinase in a Gβγ-dependent

(via G_i) manner ***(9)***. This was a significant finding because it demonstrated that Gβγ could be the trigger for several important mitogenic agents that signal through GPCRs and also via tyrosine kinase receptors ***(6)***.

Specifically concerning vascular smooth muscle (VSM) mitogenic signaling and proliferation, we have recently tested the hypothesis in vitro that agents present in serum, such as LPA or other growth factors that trigger mitogenesis via the activation of GPCRs, may play critical roles in uncontrolled smooth muscle proliferation triggered by Gβγ-dependent Ras-MAP kinase signaling. In primary-cultured rat aorta VSM cells, LPA is able to activate MAP kinase through a Gβγ sensitive pathway ***(13)***. In fact, when the βARKct is expressed in these VSM cells following adenovirus infection, the activation of the extracellular-regulated kinase (ERK) family of MAP kinases in response to LPA is attenuated by 75% ***(13)***. Importantly, we also found that proliferative signaling through the epidermal growth factor (EGF), which activates the Ras-MAP kinase cascade through means of the tyrosine kinase receptor pathway, was not affected by the βARKct, demonstrating the specificity of this PH domain-containing peptide for Gβγ ***(13)***.

In these in vitro experiments, serum can mimic, at least partially, the complex signaling pathways that stimulate in vivo VSM proliferation. Importantly, we have shown that serum activation of ERK in VSM cells in culture is largely inhibited by the βARKct ***(13)***. Moreover, serum-induced proliferation, as measured by [^{3}H]-thymidine incorporation into VSM cells, is also significantly inhibited by the presence of the βARKct and thus, it appears that factors present in serum that direct mitogenesis do so via GPCRs and, more specifically, via Gβγ ***(13)***.

These in vitro results in VSM cells led to our hypothesis that Gβγ may be critically involved in VSM proliferation in vivo, which is often associated with significant pathology. This pathological process is known as intimal hyperplasia; limiting its progression is an important therapeutic target. Major clinical problems caused by unchecked VSM proliferation and migration (intimal hyperplasia) can occur following surgical arterial bypass, leading to vein-graft failure or restenosis following arterial angioplasty because of injury-dependent stimulation of VSM growth ***(14,15)***. Accordingly, the results reviewed previously suggest that targeting Gβγ inhibition by use of in vivo βARKct expression may be a novel therapeutic approach to limiting in vivo VSM intimal hyperplasia.

Importantly, gene delivery to the vessel wall can be accomplished by either plasmid DNA or, more effectively, by adenoviral vectors and represent powerful tools for dissecting in vivo signal transduction pathways important for disease processes. The use of βARKct plasmids, and more recently a βARKct adenovirus (Adeno-βARKct) has led to the direct testing of the role of Gβγ in

pathological VSM proliferation in vivo in both vein-graft and restenosis models ***(13,16,17)***. Interestingly, expression of the βARKct has led to the significant attenuation of intimal hyperplasia associated with both vein-graft failure ***(16,17)*** and arterial restenosis after angioplasty ***(13)***. This chapter specifically describes the surgical model of Adeno-βARKct delivery to the balloon-catheter-injured rat carotid artery as a model to utilize a PH-domain peptide to diagnose the in vivo role of Gβγ in VSM intimal hyperplasia associated with restenosis following angioplasty.

2. Materials

2.1. Propagation and Purification of Adenoviral Vectors Containing the βARKct (Adeno-βARKct) or Control Transgenes

1. HEK293 cells (*see* **Note 1**).
2. Dulbecco's modified Eagle medium (DMEM) (Gibco, cat. no. 12387-015).
3. F-12 nutrient mixture (HAM) (Gibco, cat. no. 12396-016).
4. Fetal bovine serum (FBS).
5. Phosphate-buffered saline (PBS).
6. Liquid N_2.
7. RQDNase (17 microI/8 mL cells, Promega, cat. no. M610A).
8. CsCl.
9. 112 Trichloro-trifluoro-ethane 99.8% pure (Freon; Sigma, cat. no. 200-36-1).
10. 17-mL Centrifuge tube (polyallomer).
11. Sucrose.
12. Cryotubes.
13. Dialysis cassettes.
14. Ultracentrifuge.
15. Vortexer.
16. 37°C Water bath.
17. DNAs, RNAse-free bidistilled H_2O.
18. Seed stocks or aliquots of adenoviral vectors of interest (i.e., Adeno-βARKct).

2.2. Rat Carotid Balloon-Catheter Injury and Vessel Collection

2.2.1. Surgery and Medical Supplies

1. 280–350 g Wistar rats (Charles River).
2. 10 mg/mL Ketamine (Sigma, cat. no. K2753).
3. 10 mg/mL Xylazine (Sigma, cat. no. X1251).
4. Enrofloxacin.
5. Pentobarbital.
6. 2 French Fogarty balloon catheter (Baxter).
7. Microsurgery instruments: scalpel handle, blades, dissecting scissor, Dissecting holder (0.1–0.5 mm tip), 1 Cockers, 3 Klemmers, 1 microdivaricator, 1 fine point (0.05–0.07 mm) Dumont no. 5 straight and 1 curved, blunted tip holder, Vannas

Wolf scissor, 6-0 silk brides, and 5-0 silk curved needle, Olsen-Hegar needle holder, 2 curved tip, microvascular clips, 1 clip applicator (Fine Science Tools).
8. Dissection microscope (World Precision Instruments).
9. Fiber optic illuminator (World Precision Instruments).
10. Sterile pads.
11. 1-, 2-, 5-, and 5-mL Syringes.
12. 1.7-mL Snap-cap microcentrifuge tubes.
13. Liquid N_2.
14. Liquid N_2 container.
15. Rat restrainer.
16. Formalin.
17. Silastic tube, 0.63/0.30 mm, OD/ID, 10 cm (SFM3-1050, World Precision Instruments).
18. Miscellaneous supplies: tape, betadine, ethanol, 28G blunt needle, heparin.

2.3 Rat Carotid Reverse Transcription-Polymerase Chain Reaction (RT-PCR) for Detection of the βARKct Transgene

2.3.1. RNA Preparation

1. Stainless steel tissue smasher.
2. Guanidinium-based RNA extraction kit (RNAzol, Ambion).
3. 1.7 mL RNAse-DNAse free microcentrifuge tubes.

2.3.2. Reverse Transcriptase (RT)

1. Mouse Maloney Murine Leukemia Virus-Reverse Transcriptase (MMLV-RT) (50 U/µL) and MMLV-RT buffer.
2. 100 m*M* Mixed dNTPs.
3. 100 ng/µL Random primers.
4. First-strand buffer.
5. RNAse block inhibitor.

2.3.3. PCR

1. 2 U/µL Taq polymerase.
2. 25 m*M* Mixed dNTPs.
3. Primers: forward 5'-GAATTCGCCGCCACCATGGG-3'; reverse: 5'-GGAACA AAGGAACCTTTAATAG-3' (*see* **Note 2**).
4. 50 m*M* $MgCl_2$.
5. H_2O.
6. Thermocycler.

2.3.4. Agarose Gel

1. TAE buffer: 45 m*M* Tris, 45 m*M* acetic acid, 0.5 m*M* EDTA.
2. Low EEO agarose: 1% in TAE buffer.
3. 100-bp Molecular weight markers (Sigma).
4. Ethidium bromide: 10 mg/mL stock solution in distilled water.
5. 6X Loading dye buffer: 0.25% bromophenol blue, 15% glycerol.

3. Methods

3.1. Adenovirus Expansion and Purification in HEK293 Cells (see Note 1)

1. Grow cells to 60–75% confluence in DMEM/F-12 (50/50) (+10% FBS) in large 150-mm dishes (*see* **Note 3**).
2. Remove medium and add 5 mL above media without serum containing 1.5×10^8 total viral particles (tvp) to achieve a rough titer of 100 tvp/cell considering 1.5 million cells. Let incubate for 15 min at 37°C.
3. Add 10 mL DMEM/F-12 (+15%FBS).
4. Let grow cells for approx 2 d or until a majority of the cells have been lifted off of the plate.
5. Wash cells off plates by pipetting or scraping (should come off easily).
6. Spin cells at 650*g* for 10 min. Remove media and resuspend cells in 5–10 mL PBS.
7. Freeze in liquid N_2 and thaw at 37°C.
8. Repeat three times (total of four cycles) and make sure they are thoroughly frozen and thawed each time (if necessary can be frozen at –80°C after first liquid N_2 freeze).
9. Once the last thaw is brought to 37°C, digest with RQDNase at 37°C for 30 min (17 µL/8 mL cells, Promega, cat. no. M610A).
10. Add an equal volume of freon and vortex well.
11. Spin at 650*g* for 10 minutes.
12. While cells are spinning, prepare CsCl gradient: in a 17-mL polyallomer centrifuge tube, add approx 6 mL 1.3 *M* CsCl, then carefully layer underneath of this 6 mL 1.4 *M* CsCl. To prepare 1.3 *M* CsCl, calculate 0.39 g CsCl/mL of H_2O. For 1.4 *M* CsCl, use 0.53 g/mL.
13. Layer the top layer of the freon spin (avoid interface) to the 4–5 mL CsCl column. The centrifuge tubes should have at least 16 mL liquid. Do not load <6 mL CsCls and add equal volumes (*see* **Note 4**).
14. Spin CsCl gradients at 160,000*g* for 3 h at 4°C.
15. Extract (pull) the virus band and load onto another CsCl column (*see* **Note 5**).
16. Spin overnight at 160,000*g* at 4°C (*see* **Note 6**).
17. Extract virus band with a minimum volume of 0.5–2.0 mL total CsCl.
18. Add sucrose to the virus CsCl solution to a final concentration of 1%.
19. Dialyze the sample against PBS/1% sucrose (2-L buffer change every 2 h, minimum 4 h/maximum overnight). Remove sample from dialysis.
20. Add 5 µL virus stock to 95 µL PBS/1% sucrose and read OD_{260} (aim for absorbance at OD_{260} of ≥0.5). Use PBS 1% sucrose as a blank. An OD260 of 0.5 is approx 1×10^{12} tvp. Dilute adenoviral stock with PBS/1% sucrose to a final concentration of 1×10^{12} tvp/mL.
21. Freeze in 100-µL aliquots (1×10^{11} tvp) at –80°C using prechilled cryotubes.

3.2. Rat Surgery

3.2.1. Performing the Balloon Injury

1. Prepare the anesthetic by mixing together 10 mL ketamine and 1 mL xylazine. Load a 1-mL syringe with 0.5 mL mixture, put a 24-gauge needle on top of the syringe.
2. Pull the animal from the cage, and allow the rat to run in to the tunnel restrainer. Secure the tunnel with the back door and gently pull the tail of the rat.
3. Inject the anesthetic mixture in the lower hindlimb.
4. Take the animal out of the tunnel restrainer and put it back in the cage.
5. Once the animal is adequately anesthetized (*see* **Note 7**), put on the nonsterile side of the surgical table, pour some ethanol on the fur below the chin, and shave with a surgical blade from the chin down to half the way of the thorax and the origin of the upper hindlimb.
6. With a sterile gauze, clean the shaved skin and the limiting fur with Betadine.
7. Place the animal on the sterile drape with the head toward you. Secure the animal to the table using five pieces of tape, four for the legs, and one for the tail (*see* **Note 8**).
8. With a new sterile blade, cut the skin along a midline from the chin to the sternum handle.
9. With the holder in the left hand, peel up the left end of the wound and with the dissecting scissors in the right hand, spread apart (*see* **Note 9**) the connective tissue underneath the skin. Repeat the same maneuver for the other side of the wound.
10. With the Cocker, pick one end of the wound on the skin to open the surgery field as much as possible. Repeat the maneuver of the other side.
11. Use the holder to peel up the connective tissue that is above the muscular plan and along the midline use the scissor to spread apart this covering linen from the muscle underneath, moving toward the right.
12. Isolate the right sternocleidomastoideus along the internal border from the surrounding muscles.
13. Engage the microdissector with one end along the trachea, the other end along the medial border of the right sternocleidomastoideus.
14. Place the rat underneath the dissecting microscope and adjust the optical fibers to light up the surgical field (*see* **Note 10**).
15. Clean the carotid free from the connective tissue around, from its origin in the neck up to the bifurcation into internal and external carotids. Follow the external carotid up to the iodine bone.
16. With a 6-0 silk suture, make a loop around the first bifurcation of the external carotid (the tyrode artery) and tie it up. Cut the suture close to the tie so that there are no sutures in the surgical field.
17. With another 5-cm long 6-0 silk suture, place a loop around the external carotid and tie it up as cranially as possible. Pull the external carotid up by putting the

two ends of the suture together and clip them together with a klemmer. Lie the klemmer by the side of the rat head.
18. Place one vascular clip on the origin of the internal carotid (*see* **Note 11**) and another one on the origin of the common carotid in the neck (*see* **Note 12**).
19. Having interrupted the blood flow in the carotid axle, use the Vannas microscissors to make an incision on the external carotid right below the tie.
20. Pick up the Fogarty catheter tip with the curved microholder in your left hand, making sure that the blades are not placed on the balloon (*see* **Note 13**).
21. Hold the upper side of the cut with the micro holder in your left hand and move the tip of the catheter into the external carotid (*see* **Note 14**).
22. Once the balloon is inside the catheter, release the left holder and place it around the external carotid to prevent the catheter to be pushed out. Keep pushing the catheter in the carotid using the curved holder in the right hand (*see* **Note 15**). Place the catheter in the common carotid as far as possible. At this point, connect the catheter with a 1-cc syringe and inflate air up to a pressure of approx 2 atmospheres (ATM) (*see* **Note 16**). Move the balloon up and down three times with the right hand, securing the vessel with the microholder in the left hand.
23. Deflate the catheter and remove it.
24. Place a loose 6-0 loop around the external carotid and put the ends of the suture on the thorax.
25. Connect the sylastic catheter to a blunted 28-gauge needle (*see* **Note 17**). Cut the tube to approx 10 cm, making an oblique cut.
26. Connect the tube to a 1-cc syringe containing 100 µL virus solution (*see* **Note 18**). Fill up the tube.
27. Through the cut in the external carotid, advance the tubing down to common carotid. Start injecting the virus in the common carotid. As soon as the liquid starts to come out from the cut on the external carotid, tighten up the loose loop around the external carotid. While keeping the common carotid distended by pushing more virus in, tie securely the suture around the external carotid, and let the virus incubate for 15 min.
28. Aspirate the virus back in the syringe.
29. Briefly release the clip on the common carotid and let some blood to flow back in the tube. Put the clip back on.
30. Loosen the tie around the external carotid and the tubing. Remove the tubing.
31. Tie the external carotid as close as possible to the origin from the bifurcation.
32. Release the clips on the internal and the common carotid. Check for bleeding.
33. Close the wound in layers.
34. Administer antibiotics to the rat (*see* **Note 19**).
35. Make sure the rat is fully awake and recumbant before putting it back in the cage.

3.2.2. Carotid Collection

1. On the day of sacrifice, inject the animal with 5000 U heparin as indicated in **Subheading 3.2.1., steps 2** and **3**.
2. Anesthetize the rat as previously indicated.

3. Expose the treated carotid as indicated.
4. Tie up the common carotid as close to the thorax as possible.
5. Tie up the internal carotid.
6. Cut out the common carotid, wash it with 0.9% NaCl, and place it in an empty 1.7-mL screw-cap cryotube, snap-freeze in liquid N_2; store at –80°C.
7. Inject the rat with 5 cc pentobarbital intraperitoneally (*see* **Note 20**).
8. Properly dispose of the corpse (*see* **Note 21**).

3.2.3. RT-PCR

1. Immerge the Inox mortar in liquid nitrogen until it is completely frozen.
2. Place the frozen carotid in the mortar and smash it with an hammer three times
3. Place (scoop) the smashed carotid into a 13-mL polypropylene centrifuge tube, add 1 mL RNAzol, and homogenize the sample with a tissue homogenator.
4. Follow the instructions of the manufacturer for the RNA extraction (*see* **Note 22**).
5. Resuspend the total RNA in 38 µL diethyl pyrocarbonate (DEPC)-treated water.
6. Add 3 µL random primers (100 ng/µL).
7. Incubate at 65°C for 5 min.
8. Let the reaction cool down to room temperature and add in the following order: 5 µL 10X MMLV-RT buffer (*see* **Note 23**); 1 µL RNASE block inhibitor (40 U/mL), 2 µL 100 m*M* dNTPs; 1 µL MMLV-RT (60 U/mL).
9. Incubate at 37°C for 1 h.
10. Prepare the PCR mix, using 5 µL cDNA, using 2.5 µL each primer, 2.5 µL MgCl 10 m*M*, 2.5 µL 10 m*M* dNTP's mix, 2.5 µL 10X Taq buffer, and DEPC H_2O to a final volume of 25 µL.
11. Amplify for 30 cycles using 54°C as annealing temperature.
12. While amplifying, prepare a 1.5% agarose gel in TAE.
13. Load the entire volume of each reaction (25 µL + 6 µL dye) into the appropriate well of the gel. The βARKct product is a 670-bp fragment.

4. Notes

1. HEK293 cells are used because they contain the adenoviral E1 gene that is deleted in replication-deficient adenoviruses and thus, these cells supply this needed gene in trans to support vector propagation ***(18)***.
2. These primers specifically amplify only the βARKct DNA as the original βARKct construct was designed to contain a 3'-segment of the untranslated β-globin sequence for mRNA stability ***(7)***. Therefore, this strategy amplifies only the βARKct transgene, not endogenous βARK1.
3. Typically for the large-scale purification of the βARKct adenovirus, we use 75 large 150-mm plates of HEK293 cells ***(11)***.
4. It is very important that the tubes are balanced.
5. The adenovirus band is generally in the middle of the gradient. Use a 20-gauge needle to extract the virus band. Sometimes there can be more than one band, but the proper band is always the largest.
6. Centrifugation should always be for a minimum of 3 h. The overnight spin is typically done for convenience.

7. To verify that the animal is fully anesthetized, check the pain reflex by pinching the pad of the upper limb with a nonsterile klemmer.
8. Placing the upper limbs open will make it easier to secure the upper limbs to the table and will help to keep the wound open during surgery.
9. Using the scissors in this way will help to avoid cutting small vessels in the connective tissue. There should be no bleeding during the whole surgical procedure.
10. It is pivotal that you adjust the focus of the microscope before starting to operate. It may be best to focus one eye at a time.
11. Soon after the bifurcation there is another smaller vessel that has an origin from the external carotid and going in the same direction of the internal carotid, i.e., medially and deeper in the muscular plans. Use clips with blades long enough to close both vessels. If the two vessels are too far apart to be closed by one clip (it's an anatomic anomaly that occurs in approx 2–5% of wistar rats), use a silk loop to temporarily close that vessel.
12. Make sure not to put the tie around the nerve, as it will create respiratory problems to the rat. Also, if the blades are not correctly placed, there will be blood flowing in the carotid. To avoid these inconveniences, you should place the clips in a manner that allow you to see the tip of the clip.
13. To make the catheter go into the carotid smoothly, dip the tip of the catheter in NaCl 0.9% solution.
14. Make sure that you made a cut large enough for the catheter to get into the external carotid. Drops of Xylocaine can be used to induce vasorelaxation of the vessel.
15. Do not try to push it with your hand, it will be too large of a movement to be seen under the microscope.
16. You should be able to measure the ATM generated in the balloon using a three-way luer lock and a pressure transducer. Put the luer locker between the 1-cc syringe and the catheter, connect the third opening to a pressure transducer, and inflate up until the pressure has risen to 2 ATM. At that point, take note of what volume of air you have inflated so that by using that volume, you will not need to measure the ATM each time. Alternatively, you can get an Indeflator (a special syringe with a dial transducer on the tip) from a Cardiac Cath laboratory that will allow you to measure the pressure each time you apply to the balloon.
17. A blunted needle can be made by using a commercial cable peeler. Once you have advanced the catheter on the needle, secure it by making a couple of ties around the tube on the needle with a 5-0 suture.
18. The virus should be kept frozen in liquid N_2, then thawed on ice right before the application. The viral solution should contain at least 10^{11} tvp, so that at least 5×10^{10} tvp will be injected into the common carotid, whereas the other half stays in the tube.
19. Treat with 1 mg intramuscular daily for 7 d.
20. Just stick the needle in the belly of the animal and inject.
21. Follow this step according to your institutional guidelines.
22. The whole procedure is performed on ice.
23. This buffer is supplied with the MMLV-RT.

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17

Analysis of RGS Protein Palmitoylation

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Summary

Palmitoylation refers to the covalent attachment of a 16-carbon fatty acid to cysteine residues of proteins. This modification occurs on many intracellular signaling proteins including regulators of G protein signaling proteins (RGS). Palmitoylation mediates the interaction of proteins with membranes and other proteins and can control the biological activity of a protein. Palmitate attachment occurs through a labile thioester bond and is readily reversible in cells, thus providing a particularly important means for protein regulation. This chapter presents protocols for investigating RGS protein palmitoylation in mammalian cells. The RGS protein of interest is heterologously expressed in HEK293 cells, and cells are metabolically labeled with [^{3}H]palmitate. The RGS protein is isolated from fractionated cells by immunoprecipitation and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and fluorography to determine if [^{3}H] has been incorporated.

To confirm that the radiolabeled fatty acid is linked to the protein through a thioester bond, labeled proteins are treated with neutral hydroxylamine. Oxyester-linked palmitate, which is occasionally found on serine and threonine residues, is insensitive to this treatment, whereas thioesters are sensitive. To verify that incorporated radiolabel is palmitate, the protein is treated with base, which also cleaves thioester bonds. The resulting lipids are extracted from the sample, then analyzed by chromatography.

Key Words: Palmitoylation; fatty acylation; thioacylation; lipid modifications; RGS protein.

1. Introduction

Many eukaryotic signaling proteins are modified by the covalent attachment of lipid molecules. Three classes of lipids are commonly found on intracellular

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proteins ***(1)***. Prenylation is the thioether attachment of an isoprenoid moiety to cysteine residues near the C-terminus of a protein. Myristoylation, the cotranslational amide linkage of a 14-carbon saturated fatty acid, occurs at N-terminal glycine residues. Palmitoylation, the attachment of a 16-carbon unsaturated fatty acid to cysteine residues, occurs through a thioester bond. In addition to palmitate, other fatty acids are found thioester-linked to proteins. Although both prenylation and myristoylation are considered to be stable modifications, palmitoylation is a reversible process, which is believed to be controlled by two different classes of enzymes in cells. There has been no consensus sequence identified for palmitoylation, thus any cysteine residue is a putative site for palmitoylation. These properties suggest a potentially unique role for palmitoylation in the regulation of a protein's activity. Palmitoylation has been studied extensively in G protein α subunits, small G proteins such as Ras, nonreceptor tyrosine kinases, and G protein-coupled receptors (GPCR). Although it has been difficult to define the precise role that this lipid modification serves, palmitoylation has demonstrated effects on membrane affinity, subcellular localization, and activity of these proteins. For a comprehensive review of palmitoylation of signaling proteins, *see* **ref. *2***.

Members of the recently discovered family of regulators of G protein signaling (RGS) proteins are potent inhibitors of heterotrimeric G protein activity. Although they were first identified as GTPase-activating proteins for Gα subunits, RGS proteins are now known to be multifunctional regulators of heterotrimeric G protein signaling pathways. Because RGS proteins are such effective G protein-signaling inhibitors, they are likely to be carefully regulated in cells. Palmitoylation is one potential means of controlling RGS activity spatially and temporally. A number of RGS proteins are reportedly modified by palmitate, including RGS3 ***(3)***, RGS4 ***(4)***, RGS7 ***(5)***, RGS10 ***(6)***, RGS16 ***(7)***, and GAIP ***(8)***. Of these, the B/R4 subfamily members RGS4, RGS16, and RGS10 have been the best characterized. RGS4 and its close relative RGS16 are both palmitoylated at conserved cysteine residues 2 and 12 within their N-terminal ***(4)***. In addition, RGS4 is palmitoylated at a cysteine within its RGS domain; RGS10 is palmitoylated at the corresponding internal site ***(6)***. The contribution of palmitoylation toward activity of these RGS proteins is complex, and results have varied depending on the system.

The protocols presented in this chapter outline how to assess if an RGS protein is palmitoylated in vivo. The general approach is to incubate cells expressing the protein of interest with [^{3}H]palmitate, isolate the protein from the cells and determine whether it incorporated radioactive palmitate using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and fluorography. In some cases, palmitate may be incorporated into serine or threonine residues through an oxyester linkage, thus it is necessary to verify that a labeled protein is

a result of authentic thioester-linked lipid. This is accomplished by treatment of the gel containing the labeled proteins with neutral hydroxylamine, which hydrolyzes only thioester-linked lipids. Identity of the attached fatty acid is confirmed by cleaving thioester bonds with base, extracting released fatty acids from the hydrolysate and analyzing by high-performance liquid chromatography (HPLC) or thin-layer chromatography (TLC). Once one establishes that an RGS protein is authentically palmitoylated, the site(s) for palmitate attachment can be determined by mutation of cysteines to alanine or serine and the mutant proteins compared to the wild-type. Proteins that incorporate palmitate at more than one site may contain a primary cysteine, where palmitoylation is required prior to incorporation at other sites within the protein. For this reason, it is important to design both individual and multiple cysteine mutants to properly map the sites of palmitate incorporation.

To successfully perform this analysis, several general considerations should be made. First, specific antibodies are required in order to immunoprecipitate the expressed protein. Because it is often difficult to produce antibodies against the RGS protein directly, epitope-tagged versions of the protein can be engineered. We designed RGS4 fused at the C-terminus to three sequential *myc* tags for our studies. Other convenient epitopes can be used instead.

Second, large quantities of protein need to be expressed in order to produce a [^{3}H] labeled signal. Sf9 insect cells have provided an excellent expression system for palmitoylation studies, particularly for heterotrimeric G protein α subunits (Gα) ***(9)***. The primary advantage of insect cells is that infection with recombinant Baculovirus produces relatively high levels of heterologous protein expression. Because they are eukaryotic cells, Sf9 cells contain the cellular machinery to modify expressed proteins. For detailed protocols of radiolabeling Sf9 cells and analysis of lipid modifications, *see* **ref. *10***.

Although Sf9 cells have proven to be a good model system for palmitoylation studies of Gα, some mammalian proteins may be processed or regulated inappropriately in an insect expression system. These subtle processing differences can have significant effects on palmitate incorporation into the protein. Although it is desirable to analyze palmitoylation of RGS proteins in a native system, most RGS proteins are difficult to detect in their endogenous cells or tissues. This chapter describes a method for studying palmitoylation of RGS proteins heterologously expressed in a mammalian cell line. We used HEK293 cells for our studies, although COS7, CHO, or other readily transfectable adherent cell line may be substituted (*see* **Note 1**). Most of the methods described are the same or similar to those for Sf9 cells ***(10)***. The differences between them are in the introduction of the construct into the cells and the method of cell lysis. Because many laboratories are equipped for mammalian cell culture, these protocols are likely to be straightforward to adopt.

2. Materials

2.1. Reagents for Transfection and Metabolic Labeling of RGS Protein in Mammalian Cells

1. HEK293 cells (American Type Culture Collection, Manassas, VA, cat. no. CRL-1573).
2. Transfection-quality RGS4-*myc* pcDNA3.1 plasmid DNA (*see* **Note 2**).
3. Lipofectamine reagent (Invitrogen, Frederick, MD).
4. OPTI-MEM reduced serum media (Invitrogen).
5. Complete HEK293 medium: Dulbecco's modified Eagle medium (DMEM) (Invitrogen), 10% fetal bovine serum (FBS), pencillin/streptomycin.
6. [^{35}S]methionine wash media: 95% methionine-free DMEM, 5% normal media, 2.5% dialyzed FBS.
7. [^{35}S]methionine labeling media: 95% methionine-free DMEM, 5% normal DMEM, 2.5% dialyzed FBS, sodium pyruvate, 100 µCi/mL [^{35}S]methionine (>1000 Ci/mmol) (Amersham Biosciences, Piscataway, NJ). Aliquot the [^{35}S] methionine and store at –80°C. Make media fresh before use.
8. [^{3}H]palmitate labeling media: 1 mCi/mL [^{3}H]palmitate (30–60 Ci/mmol, DuPont New England Nuclear, Boston, MA), 1% dimethylsulfoxide (DMSO), 2.5% FBS, sodium pyruvate, nonessential amino acids (*see* **Note 3**). Make fresh before use.
9. Nitrogen gas.

2.2. Subcellular Fractionation Reagents

1. Ball-bearing cell homogenizer (cell-cracker), 0.1566 in. bore size, 0.1554 in. ball bearing (or equivalent 0.0012 in. clearance) (H&Y Enterprises, Redwood City, CA).
2. Phosphate-buffered saline (PBS).
3. Hypotonic lysis buffer: 20 m*M* Tris-HCl, pH 7.4, 1 m*M* EDTA, 1 m*M* dithiothreitol (DTT), protease inhibitors (0.1 m*M* phenylmethylsulfonyl fluoride [PMSF], 21 µg/mL *N*-p-tosyl-L-phenylalanine chloromethyl ketone [TPCK], 21 µg/mL *N*-r-tosyl-L-lysine chloromethyl ketone [TLCK], 3.2 µg/mL leupeptin, 3.2 µg/mL lima bean trypsin inhibitor). Add protease inhibitors just before use.
4. Microfuge tubes for high-speed centrifugation (Beckman polyallomer, cat. no. 357448).
5. 1X RIPA buffer: PBS, 1% Nonidet P-40, 0.5% deoxycholic acid, 0.1% sodium dodecyl sulfate, protease inhibitors (0.1 m*M* phenylmethylsulfonyl fluoride [PMSF], 21 µg/mL *N*-p-tosyl-L-phenylalanine chloromethyl ketone [TPCK], 21 µg/mL *N*-r-tosyl-L-lysine chloromethyl ketone [TLCK], 3.2 µg/mL leupeptin, 3.2 µg/mL lima bean trypsin inhibitor). Add protease inhibitors fresh just before use.

2.3. Immunoprecipitation of Labeled Protein, SDS-PAGE, and Fluorography

1. *c-Myc* monoclonal antibody (9E10 epitope).

2. *Staphylococcus aureus* (Calbiochem, Pansorbin, cat. no. 507858; *see* **Note 4**).
3. Coomassie blue stain: 30% isopropanol, 10% acetic acid, 0.25% Coomassie Brilliant blue R-250.
4. Destain solution: 30% methanol and 10% acetic acid.
5. Amplify Fluorgraphic Reagent (Amersham Biosciences, cat. no. NAMP100; *see* **Note 5**).
6. 2X Sample buffer: 100 m*M* Tris-HCl, pH 6.8, 0.5% SDS, 20% glycerol, 0.5% β-mercaptoethanol, 0.004% bromphenol blue, 20 m*M* DTT. Add DTT fresh before use.

2.4. Hydroxylamine Sensitivity

1. Hydroxylamine solution: 1 *M* hydroxylamine, 50% isopropanol, pH 7.0. Use concentrated HCl to pH the solution. Note that it takes a lot of acid to bring up the pH.
2. 1 *M* Tris-HCl, pH 7.0, in 50% isopropanol.

2.5. Identification of Fatty Acids on Labeled Protein

1. 13 × 100-mm glass tubes with Teflon-lined caps (Corning, cat. no. 9826-13).
2. HPLC-grade methanol, choloroform, acetonitrile, acetic acid.
3. [^{3}H] fatty acid standards: [^{3}H]palmitate, [^{3}H]myristate, [^{3}H]stearate (DuPont New England Nuclear [NEN]).
4. N_2 gas.
5. NaOH.
6. 6 *N* HCl, high-grade (Pierce Chemicals, cat. no. 24309).
7. Palmitic acid (Sigma, cat. no. P5585).
8. Trifluoroacetic acid.
9. C:18 reverse-phase TLC plates (Whatman, cat. no. 4800-620).
10. En3Hance spray (DuPont NEN).

3. Methods

3.1. Measuring Palmitate Incorporation into RGS-myc Protein in Mammalian Cells

3.1.1. Transfection and Metabolic Labeling of RGS Protein in Mammalian Cells

1. Plate 2–10-cm dishes of HEK293 cells so that they will be 50–80% confluent the next day.
2. Transfect HEK293 cells with RGS-*myc* (e.g., RGS4-myc pcDNA3.1) construct using lipofectamine reagent according to manufacturer's instructions (*see* **Note 6**). Incubate 5 h, 37°C, 5% CO_2/95% air.
3. Replace transfection media with complete media and allow cells to recover in 37°C incubator overnight (*see* **Note 7**).
4. At approx 36 h following the transfection, label one plate with [^{35}S]methionine: wash once in [^{35}S]methionine wash media, then add labeling media and incubate overnight in the 37°C incubator.

5. The following day, label the second plate with [^{3}H] palmitate: wash cells once with prewarmed DMEM, then incubate for 1–2 h in [^{3}H]palmitate-labeling medium.
6. Harvest each plate 48 h post-transfection: wash plates once with PBS, add 1 mL PBS, then scrape cells with rubber policeman into separate conical tubes.
7. Collect cells by centrifugation at 500*g*, 4°C, for 10 min.

3.1.2. Subcellular Fractionation (see ***Note 8***)

1. Resuspend each cell pellet in 1 mL hypotonic lysis buffer.
2. Pass cells through ball-bearing homogenizer 20 times (0.0012 in. clearance) to lyse cells (*see* **Note 9**).
3. Centrifuge the lysate at 500*g*, 10 min, 4°C to collect nuclei, unbroken cells, and plasma membrane sheets.
4. Transfer the supernatants to Beckman polyallomer microfuge tubes. Spin in TLA100.3 with adaptors, 100,000*g*, 30 min at 4°C.
5. Remove the supernatant from the high-speed spin (cytosol) to Eppendorf tubes and add 1 mL 2X RIPA buffer. Resuspend the pellet (membranes) in 500 µL 1X RIPA buffer in the polyallomer tubes (*see* **Note 10**).
6. Solublize the membranes by incubating at 4°C, 1 h, on an end-over-end rocker.
7. Pellet the insoluble material by centrifuging 100,000*g*, 30 min, 4°C.
8. Remove the supernatants (membrane extract) to new microfuge tubes (*see* **Note 11**).

3.1.3. Immunoprecipitation of Labeled Protein, SDS-PAGE, and Fluorography (see ***Note 12***)

1. Incubate the membrane extracts and cytosol with 5 µL *c-myc* antibody. Incubate at least 3 h or overnight if convenient at 4°C, rocking end-over-end.
2. To prewash the Pansorbin Staph A, remove 10 µL/sample of prepared Pansorbin suspension. Spin in microcentrifuge 30 s, remove supernatant, and resuspend pellet in 1 mL 1X RIPA. Spin 30 s, remove supernatant, and resuspend pellet in the original volume of 1X RIPA.
3. Add 10 µL prewashed Pansorbin to each sample. Incubate at 4°C, 30 min, rocking end-over-end.
4. Collect immunoprecipitated complex by centifugation, 2 min in a microcentrifuge. Wash once with 1 mL 1X RIPA, then once with 500 µL PBS.
5. Suspend the pellets in 25 µL 2X SDS-PAGE sample buffer.
6. Boil samples 1 min, then spin 1 min in microcentrifuge. Load the supernatant samples on SDS-PAGE gels and run (*see* **Note 13**).
7. Stain gels with Coomassie blue dye, rocking, 30 min.
8. Incubate in several changes of destain solution, rocking, until proteins are visualized on the gel (*see* **Note 14**).
9. Soak gels in Amplify solution, 30 min, rocking.
10. Dry down the gels on a vacuum dryer, 60°C.
11. Expose the gels to film in a cassette. Store the cassette at –80°C for at least 3 d to visualize the [^{35}S]-labeled bands. Replace with new film and expose for 1–4 wk to visualize [^{3}H]labeled bands (*see* **Note 15**).

3.2. Hydroxylamine Sensitivity of [^{3}H]Palmitate-Labeled Proteins (see *Note 16)*

1. Transfect cells, label, and fractionate cells as in **Subheadings 3.1.1.** and **3.1.2.** (*see* **Note 17**).
2. Immunoprecipitate RGS protein from cells as outlined in **Subheading 3.1.3., steps 1–5**.
3. Boil samples 1 min, then pellet for 1 min in microcentrifuge. Split each sample in half and run duplicate gels of each.
4. Fix gels by incubating in destain solution for 30 min, rocking.
5. Soak one gel in fresh hydroxylamine solution for 12–18 h overnight. As a control, soak the other gel in 1 M Tris, pH 7.0, in 50% isopropanol. Replace the solutions at least once after approx 1 h. The gels will shrink as they are dehydrated by the isopropanol.
6. Wash each gel four times in 50% isopropanol for a total of 48 h.
7. Soak gels in Coomassie stain, then destain solutions to visualize precipitated proteins. The gels will rehydrate back to their original size during this step.
8. Soak gels in Amplify solution, 30 min, rocking.
9. Dry down the gels on a vacuum dryer, 60°C and expose to film at –80°C.

3.3. Identification of Fatty Acids on Labeled Protein

3.3.1. Base Hydrolysis and Extraction of Thioester-Linked Fatty Acids

1. Transfect cells, label, and fractionate cells as in **Subheadings 3.1.1.** and **3.1.2.**
2. Immunoprecipitate RGS protein from cells as outlined in **Subheading 3.1.3., steps 1–5**.
3. Boil samples 1 min, then pellet for 1 min in microcentrifuge. Split each sample in half and run duplicate SDS-PAGE gels.
4. Stain gels with Coomassie blue solution, rocking, 30 min.
5. Incubate in several changes of destain solution until proteins are visualized on the gel.
6. Excise the immunoprecipitated protein bands from membranes and cytosol with a razor blade or scalpel, keeping gel slices equal in size. Also excise three gel slices from unused regions of the gel to run with standards.
7. Place gel slices in to air-tight, screw-cap 13 × 100-mm glass tubes. Wash gel slices overnight with five changes of 50% methanol.
8. Remove methanol with Pasteur pipet. To the blank gel slices, add [^{3}H]myristate, [^{3}H]stearate, or [^{3}H]palmitate standards, 25,000–50,000 dpm per sample. Dry gel slices under a nitrogen stream.
9. To hydrolyze thioester-linked lipids, resuspend gel slices in 0.7 mL 1.5 *M* NaOH. Flush tubes with nitrogen and cap. Incubate 3 h, 37°C, mixing periodically.
10. Acidify the solutions to pH 1.0–2.0 by adding high-grade 6 *N* HCl. This typically requires 100–200 µL acid.
11. To extract the released fatty acids, add 3.75 vol chloroform:methanol (1:2 v:v) in a fume hood. This results in chloroform:methanol:aqueous 1.25:2.5:1 v:v:v. Incubate 10 min at room temperature with occasional mixing.

12. To separate the fatty acids from water-soluble components and gel slices, add 1.25 vol of original aqueous volume of chloroform and 1.25 vol water.
13. Vortex tubes, then centrifuge at 1000*g* at room temperature. This will separate the mixture into an upper aqueous phase and a lower organic phase, with the gel slice in between.
14. Remove the bottom phase containing fatty acids by bubbling a Pasteur pipet through the aqueous phase to the bottom of the tube. Transfer to new screw-cap tubes.
15. To extract remaining fatty acids, add the same amount of chloroform as in **step 12**. Incubate 5 min, room temperature with occasional mixing. Centrifuge 1000*g* and remove bottom phase as before. Combine with first extracts.
16. Dry down extracts under nitrogen.
17. Add 0.5 mL chloroform and 40 µg cold palmitic acid as a carrier. Flush tubes with nitrogen and store at –20°C.

3.3.2. TLC Analysis of Hydrolyzed Lipids

1. Count a small volume of each 50 µL sample in scintillation vials to determine how much sample to load. Let the chloroform dry before counting or it will quench.
2. Spot equivalent amounts of each sample (25,000–50,000 dpm) on a reverse-phase C:18 TLC plate. Also spot equivalent standards.
3. Run samples in 90% acetonitrile/10% acetic acid in a chromatography tank. This should take 20–30 min.
4. After the solvent has migrated to the top, remove plates, and allow them to dry thoroughly in the hood.
5. Spray plates with En3Hance. Let plates dry slightly and expose to film at –80°C for 5–10 h. Reexpose for 24–48 h if signal is undetectable.

4. Notes

1. The protocols for heterologous protein expression, cell lysis and fractionation (**Subheadings 3.1.1.** and **3.1.2.**) are described here specifically for mammalian cells. The remainder of the methods are performed the same independently of cell type used.
2. We use Midi- or Maxi-prep kits from Qiagen to produce DNA for transfection. Follow the manufacturer's protocols.
3. [^{3}H]palmitate is supplied as a solution in ethanol and cannot be added directly to media. First, measure the appropriate amount into a glass tube and evaporate the ethanol under a nitrogen stream. When the palmitate is dry, it will appear as a light film on the bottom of the tube. Resuspend the palmitate first in DMSO, then add the FBS. Add the remaining reagents to the dissolved [^{3}H]palmitate.
4. We use the following protocol to prepare Pansorbin Staph A for immunoprecipitations: First, centrifuge the suspension for 3 min in a microcentrifuge. Resuspend the pellet in 3% SDS, 10% β-mercaptoethanol, 50 m*M* Tris, pH 7.5, 150 m*M* NaCl. Incubate in a boiling water bath for 5 min, then cool to room temperature. Repeat once more, then centrifuge. Wash the pellet twice in 50 m*M* Tris, pH 7.5,

2 m*M* EDTA, 150 m*M* NaCl. Resuspend it in the same buffer, 900 µL/mL of original volume. Store the prepared Pansorbin at 4°C. These can be prepared as a large batch and stored for at least 1 yr. Note that the manufacturer does not specify this wash step.

5. We have successfully used a homemade version of fluorographic reagent: 1 *M* sodium salicylate in 15% methanol. Store in a dark bottle.
6. Detailed instruction for optimizing transfection conditions with lipofectamine reagent are provided by the manufacturer. The amount of DNA and lipofectamine used per dish can vary depending on the protein. For RGS4-*myc* we used 10 µg DNA and 40 µL of lipofectamine.
7. The optimal time to harvest transfected cells for peak protein expression may vary depending on the protein. In this protocol, cells are collected 48 h posttransfection. For shorter posttransfection incubations, labeling should be started at the appropriate time so that [^{35}S]methionine-labeling media is incubated overnight and [^{3}H]palmitate media is incubated for 1–2 h.
8. All solutions should be kept cold at 4°C, and all samples should be kept on ice during the following steps in order to minimize protein degradation.
9. When using the ball-bearing homogenizer for both [^{35}S]- and [^{3}H]-labeled cells during an experiment, pass the [^{3}H] samples first to avoid accidental transfer of the higher energy [^{35}S] signal into the [^{3}H] samples. Wash out the homogenizer thoroughly with ethanol to remove trace [^{3}H] residue. Alternatively, cells may be lysed by another convenient method (e.g., dounce, freeze-thaw).
10. Other buffers may be successfully used to solubilize the protein. We found this RIPA buffer formulation to work well for the *myc* antibody. If a different antibody is used, the buffer may need to be adjusted.
11. At this step, the membrane extracts and cytosol samples may be snap-frozen in liquid nitrogen and stored at –80°C until later use, as limited by the half-life of the [^{35}S] signal. Frozen samples should be thawed rapidly and kept on ice.
12. Immunoprecipitation protocols vary widely. For a review and guide to optimization of immunoprecipitation using different types of antibodies and solid support matrices, *see* **ref. *11***. Other protocols may be easily substituted.
13. Avoid loading Pansorbin onto the SDS-PAGE gel. Thioester-linked fatty acid is susceptible to prolonged heating and exposure to reducing agents. We have found that 20 m*M* DTT in the sample buffer and heating for 1 min is sufficient to obtain good resolution of RGS proteins on gels without a substantial loss of radioactive palmitate on the protein.
14. Gel can be left in destain solution overnight if desired.
15. Remove plastic wrap from the gel before exposure or it will block the [^{3}H] signal. Storage of the cassette at –80°C is essential for the fluorography reagent to work. Do not used a screen. The [^{35}S]-labeled samples may be visualized within a few days. The [^{3}H] labeled bands may be detected after 1-wk exposure, but it can take 1 mo or longer, depending on the amount of protein on the gel.
16. If the membrane-bound RGS protein incorporates [^{3}H]palmitate, further analysis should be done to confirm authentic thioester palmitate attachment. Treatment of

the gel containing the [^{3}H]-labeled sample with neutral hydroxylamine should result in the reduction or disappearance of the labeled band. As a control, a duplicate gel is soaked in Tris buffer at the same pH. The [^{3}H]-labeled sample should be visible in this gel. In addition, [^{35}S]methionine-labeled proteins should be run in parallel to control for the loss of protein from the hydroxylamine-treated and Tris-treated gels during processing.

17. The number of cells required for these experiments may vary depending on protein expression and stoichiometry of palmitoylation. Adjust the size and/or number of dishes transfected based on the autoradiograph in **Subheading 3.1.**

Acknowledgments

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18

Methods for Measuring RGS Protein Phosphorylation by G Protein-Regulated Kinases

Susanne Hollinger and John R. Hepler

Summary

Little is known about cellular regulation of the regulators of G protein signaling (RGS) proteins, principal players in G protein signaling. These proteins are known for their capacity to negatively regulate G protein signals, however, their chief cellular functions may expand beyond this limited role. Comprehensive understanding of cellular roles of RGS proteins requires knowledge of their regulation by short latency and inducible signals, such as kinase activation by G proteins. A number of RGS proteins are phosphorylated in cells, with varied effects on their function and localization. These studies focus on RGS14, which contains recognition motifs for several G protein-regulated kinases. Procedures used in our laboratory to study the phosphorylation of RGS14 are outlined, and the method used to purify RGS14 is described with notes on complications that may be encountered. Standard protocols used to investigate the recognition of RGS proteins by 3-5-cyclic adenosine monophosphate (cAMP)-dependent protein kinase (PKA), extracellular signal-regulated kinase (ERK), and protein kinase C (PKC) are described, followed by strategies used to establish the specific amino acids modified by these kinases. Although this chapter focuses on investigations into RGS14, the protocols described are readily modified for other RGS proteins.

Key Words: RGS14; RGS proteins; phosphorylation; PKA; PKC; ERK.

1. Introduction

Regulators of G protein signaling (RGS), are now recognized as key players in intracellular signaling. Initially identified as negative regulators of G protein signals in lower eukaryotes, recent discoveries make it clear that RGS proteins also link G proteins to diverse signals, such as Ca^{++} oscillations, neu-

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rotransmitter release, and activation of monomeric GTPases ***(1–3)***. A comprehensive understanding of the capacity of RGS proteins to modulate signals requires the study of mechanisms by which RGS proteins are regulated within the cell. This chapter describes methods that have been employed to test the capacity of a selection of G protein-regulated kinases to phosphorylate specific RGS family members.

RGS proteins were originally classified because of their shared RGS domains, through which these proteins act as GTPase activating proteins (GAP). This protein superfamily now includes more than 30 distinct proteins, grouped into 6 subfamilies (A/RZ, B/R4, C/R7, D/R12, E/RA, and F/RL), which share little in common other than their RGS domains (for recent reviews, *see* **refs. *2*** and ***3***). The RGS domain itself is a protein-binding module that can be found alone or in combination with a variety of other motifs. These include G gamma-like (GGL), PDZ, Rho exchange factor (DH), and phosphotyrosine-binding (PTB) domains, among many others ***(2,3)***. In several instances, RGS proteins directly link multiple-signaling cascades through these domains. RGS14, one focus of our current research, is a member of the D/R12 family of RGS proteins. It contains several protein-binding domains, including the RGS domain, a Rap-binding domain (RBD), and a GoLoco (GL) motif ***(4–7)***. Currently, not much is known of the mechanisms by which RGS proteins are activated or what controls their interactions with diverse-binding partners. However, recent studies indicate that posttranslational modifications, particularly protein phosphorylation, are an important cellular mechanism for this regulation.

Activation of kinase cascades is a central component of virtually all cellular signals. The most immediate effects of phosphorylation occur at the level of protein–protein interactions or enzymatic activity, however, phosphorylation can also regulate protein stability as well as trafficking through intracellular compartments. Phosphoprotein regulation is particularly important because of the rapid and reversible nature of the modification. The possible role of phosphorylation of RGS proteins as a mechanism for feedback regulation of G proteins is especially exciting because G protein-coupled signals initiate a number of kinase cascades. Three of the most familiar of these are those linked to protein kinase A (PKA), protein kinase C (PKC), and the extracellular signal-regulated kinases (ERK1 and ERK2).

Recent studies have shown that a number of RGS proteins are phosphorylated in cells with varying effects on their function ***(8–14)***. RGS2, for example, is phosphorylated by PKC, which significantly reduces its GAP activity, initiating a feed-forward loop that leads to increased $G\alpha_q$ activation of inositol lipid-signaling ***(13)***. On the other hand, ERK2 phosphorylation of RGS-GAIP enhances GAP activity and reduces $G\alpha_i$-linked signals, including ERK activa-

tion ***(15)***. Phosphorylation can also dictate the subcellular localization and availability of RGS proteins. For example, PKA-mediated phosphorylation of RGS10 causes the protein to shuttle to the nucleus, thereby reducing its availability at the membrane and possibly allowing it to participate in non-GPCR-related pathways.

Studying posttranslational modifications of native or recombinantly expressed RGS proteins in mammalian host cells has often proven difficult, both because proteins are frequently found at low levels and because protein expression (in some cases) can be toxic. Moreover, the methods available to identify phosphorylation sites on proteins from cell lysates (i.e., mass spectrometry ***[16]***) are costly and technically limited. Research into the effects of phosphorylation on RGS-mediated cell signals has also proven difficult. The effect that different sets of RGS proteins may have on cell signals or how the expression of varied binding partners can affect these is relatively unknown. Furthermore, most RGS proteins are likely regulated by multiple kinases. Therefore, the effect of phosphorylation on the catalytic or binding properties of these proteins is more readily identified in vitro using recombinant proteins. This information can then be used to design further studies to correlate the modification of specific sites with in vivo signal regulation.

The assays described were used by our laboratory to study the capacity of different kinases to phosphorylate RGS family members. Although this article focuses on RGS14, the protocols discussed are readily adapted to other RGS proteins. The assays fall into three categories: preparation of purified recombinant protein, phosphorylation of the wild-type protein by the individual kinases, and analysis of the specific sites of phosphorylation. In studying in vitro phosphorylation of any protein, several issues should be considered. First, the relative ease with which the RGS protein can be prepared. Many RGS proteins are readily purified from *E. coli* using standard-affinity fusion tags, such as hexahistidine or glutathione-S-transferase. However, some RGS proteins require special conditions, which must be taken into account when designing kinase protocols (*see* **Note 1**). Second, the specificity of the kinase of interest should be taken into account. Some kinases, such as ERK, recognize well-defined motifs whereas others, such as PKC, interact with less stringency at a number of recognition sites. Because in vitro reaction conditions for phosphorylation are optimized, they can encourage nonspecific kinase activity. Mutational analysis can aid in demonstrating the degree of nonspecific kinase activity. Additionally, although many assays are more readily approached using recombinant protein, we recommend always correlating in vitro experimental results with parallel studies using recombinant or native protein in host cells.

2. Materials

2.1. Protein Purification

2.1.1. Reagents

1. Isopropyl-β-D-thiogalactoside (IPTG; Fisher, cat. no. BP1755-10).
2. Nickel-nitrilotriacetic acid (Ni-NTA) agarose (Qiagen, cat. no. 1000632).
3. BL21DE3 bacterial strain (Stratagene, cat. no. 200131).
4. RGS protein in bacterial expression vector. While the following protocols are given for RGS14 in pET32b, which was a gift of Dr. D. P. Siderovski (University of North Carolina, Chapel Hill), we have also successfully employed these protocols for a variety of RGS proteins, including RGS2, RGS4, RGS7, and RGS16.
5. French-pressure cell press (SIM Aminco or equivalent).
6. Superdex-75 or Superdex-200 HR 10/30 column (Pharmacia Biotech).
7. Fast-protein liquid chromatography (FPLC; Pharmacia P-500 pump and controller or equivalent).

2.1.2. Reagents

1. Lysis buffer: 50 m*M* HEPES, 500 m*M* sodium chloride (NaCl), 1 m*M* ethylenediaminetetracetic acid (EDTA), supplemented with protease inhibitors (phenylmethylsulfonyl fluoride, aprotinin, leupeptin).
2. Running buffer: 50 m*M* HEPES, 100 m*M* NaCl, 1 m*M* β-mercaptoethanol (BME).
3. Wash buffer: 50 m*M* HEPES, 100 m*M* NaCl, 10 m*M* BME, 10 m*M* imidazole.
4. Elution buffer: 50 m*M* HEPES, 100 m*M* NaCl, 10 m*M* BME, 200 m*M* imidazole.

2.2. In Vitro Phosphorylation

2.2.1. Reagents

1. [^{32}P] Adenosine 5-triphosphate (ATP) (Perkin Elmer Life Sciences, "Easytides," cat. no. Blu/NEG/502A).
2. 4X Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer: 200 m*M* Tris-HCl, pH 6.8, 4% SDS, 40% glycerol, 600 m*M* BME, bromophenol blue, 20 m*M* dithiothreitol (DTT), added fresh.
3. cAMP-dependent PKA phosphorylation.
4. Catalytic subunit PKA (PKAcat; Calbiochem, cat. no. 539486).
5. 5X PKA reaction buffer: 250 m*M* Tris-HCl, pH 7.5, 50 m*M* $MgCl_2$, 500 m*M* ATP.
6. PKA dilution buffer: 50 mM Tris-HCl, pH 7.5, 10 m*M* $MgCl_2$, and 100 μ*M* DTT.

2.2.2. ERK Phosphorylation

1. Activated ERK2(Calbiochem, cat. no. 454855).
2. 5X ERK reaction buffer: 125 m*M* HEPES, pH 7.5, 125 m*M* $MgCl_2$, 25 m*M* EDTA, 250 μ*M* ATP.

2.2.3. PKC Phosphorylation

1. Sodium salt phosphatidylserine (PS; Avanti Polar Lipids, cat. no. 840032).
2. 1,2-Dioleolyl Glycerol (DOG; Avanti Polar Lipids, cat. no. 800811).

3. PKC, 2 mU/25 µL (Boehringer/Roche, cat. no. 145965).
4. CHAPS buffer: 50 µL 10 m*M* CHAPS, 10 m*M* Tris-HCl, pH 7.4.
5. 5X PKC reaction buffer: 72.5 mM Tris-HCl, pH 7.5, 50 m*M* $MgCl_2$, 5 m*M* $CaCl_2$, 750 µ*M* ATP.
6. PKC activation buffer: 20 m*M* Tris-HCl, pH 7.5, 0.5 m*M* EDTA, 0.5 m*M* EGTA.

3. Methods

3.1. Protein Purification

Because the family of RGS proteins is very diverse in their biochemical properties, purification techniques should be modified depending on the protein of interest. The following section describes the methods employed to purify RGS14 from *E. coli*. Several groups, including ours, have found RGS14 a particularly difficult protein to purify. It is highly susceptible to degradation and is often found in inclusion bodies when expressed in *E. coli*. To overcome this instability, we utilized a fusion protein consisting of RGS14 and thioredoxin, which is made from RGS14 cDNA cloned into the pET32b expression vector (Novagen, *see* **Materials**). Several other constructs were prepared but proved less soluble and prone to degradation (*see* **Notes 2** and **3**).

1. Seed RGS14 grown in BL21DE3 or other strain permissive for protein production.
2. Grow culture overnight.
3. Using the overnight culture, seed 1 L LB+ 50 µg/mL carbenicillin and grow to OD = 0.5 – 0.6.
4. Add IPTG to final concentration of 0.5 m*M*.
5. Grow 2 h at 37°C, shaking at 250 rpm.
6. Spin in a Beckman J6 centrifuge at 3000 rpm for 30 min.
7. Resuspend the pellet in cold 10 mL lysis buffer with 1 m*M* DTT.
8. Lyse cells using the French press method (*see* **Note 4**).
9. Add 60 mL cold lysis buffer with 1 m*M* BME.
10. Spin at 75,000*g* for 1 h.
11. While lysate is spinning, wash 1 mL bed-volume, gravity packed Ni-NTA with at least 50 mL dH_2O in a column at 4°C.
12. Wash S-200 sephadex column (Pharmacia) with 40 mL running buffer at 0.7 mL/min (*see* **Note 5**) using an FPLC controller. Make sure the column is precooled to 4°C.
13. After spin, remove supernatant and discard pellet (*see* **Note 6**).
14. Load supernatant onto washed Ni-NTA at 4°C, saving the flow-through.
15. Reload flow-through onto Ni-NTA, making sure that column does not run dry.
16. Repeat **step 15**.
17. Wash the Ni-NTA column three times with 3 mL cold wash buffer.
18. Add 1.5 mL cold elution buffer onto Ni-NTA column and incubate at 4°C for 10 min (*see* **Note 7**).
19. Repeat three times, labeling each elution "E1," "E2," and so forth (*see* **Note 8**).
20. Load 250 µL E2 (see **Note 8**) onto the washed Sephadex sizing column using a 200 µL "load loop" (*see* **Note 9**).

21. Using FPLC, run the protein through the sizing column with running buffer at 0.33 mL/min, taking 20-drop fractions. After the program is complete, take aliquots of fractions and test the protein concentration. We find that full-length RGS14 is usually concentrated in fraction 21, however each column varies slightly and several fractions should always be tested.
22. Aliquot the purified protein into 25-µL aliquots (see **Note 10**).
23. Aliquot elutions 2 and 3 from the Ni-NTA column into 0.25-mL aliquots.
24. Snap-freeze all aliquots in liquid nitrogen and store at –80°C.

3.2. In Vitro Phosphorylation (Fig. 1)

3.2.1. cAMP-Dependent PKA

Our laboratory uses the purified PKAcat for these assays because it is constitutively active without requiring cAMP. To ensure that our assays are consistent, we make multiple single-use aliquots of stock enzyme (50 U/tube) immediately on arrival, which are snap-frozen in liquid nitrogen and stored at –80°C. Because we use 5 U/reaction, this allows for 10 reactions/aliquot.

1. Thaw purified recombinant RGS protein. For most standard applications, we use 2 µg purified protein/reaction. However, this amount can be adjusted based on availability of protein and the level of phosphorylation necessary for detection (*see* **Note 11**).
2. Make 5X PKA reaction buffer with enough volume for 4 µL/reaction. We carry out our final reaction in 50 m*M* Tris-HCl, pH 7.5, 10 m*M* $MgCl_2$ (or $MgSO_4$), and 100 µ*M* ATP.
3. Take 2 µL [^{32}P]ATP stock, dilute this to 20 µL and count 2 µL in 2 mL scintillation fluid. Based on the cpm value, calculate the cpm/µL of your [^{32}P]ATP dilution. For these assays, we use 2000 cpm/pmol ATP in the reaction mix, however this amount can be adjusted as long as internal controls are used. Each 20 µL assay uses 4×10^6 cpm [^{32}P]ATP.
4. In a microfuge tube on ice dilute your protein of interest, 4×10^6cpm of [^{32}P]ATP, and 4 µL 5X PKA reaction buffer to a final volume of 18 µL with dH_2O.
5. Thaw enough aliquots of PKAcat to make 5 U/reaction. Dilute the PKAcat to a final concentration of 1–5 U/µL (we use 2.5 U/µL) in PKA dilution buffer.
6. To start the assay, add 5 U of diluted PKA to each reaction tube, mix, and transfer to 30°C (*see* **Note 12**).
7. Incubate the reaction for 20 min at 30°C (*see* **Note 13**).
8. To stop the reaction, move the tubes to ice and add 15 µL Lammeli's sample buffer. Boil the samples for 10 min and subject them to SDS-PAGE.
9. After separation on SDS-PAGE, stain the proteins in the gel using coomassie blue (*see* **Note 14**).

3.2.2. Mitogen-Activated Protein Kinase (MAPK, ERK)

ERK1 and ERK2 activation is essential in cell-cycle progression and a major component of G protein regulation of transcription factors. A unique feature of

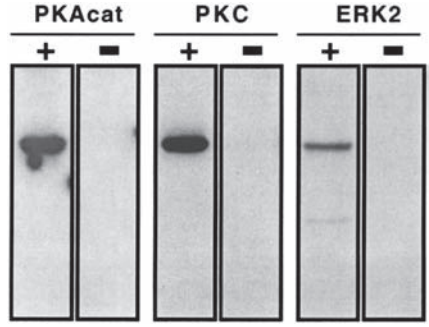

Fig. 1. RGS14 is phosphorylated by PKA, PKCα, and ERK2. Using the protocols described, we showed phosphorylation of full-length RGS14 by three G protein-linked kinases. 2 µg RGS14 were incubated for 30 min with PKAcat, PKC, or ERK2. Proteins were separated by SDS-PAGE and exposed to film. Left panels are in the presence kinase, right panels show kinase-free controls.

ERKs is that they require activation by phosphorylation at both threonine and tyrosine residues by their upstream-binding partner, MEK. To save time and improve consistency, our laboratory uses ERK2 that has been preactivated with MEK. The kinase is unstable, so we make single-use aliquots on arrival, snap-freeze them in liquid nitrogen and store these at –80°C. This protocol is essentially the same as that for PKAcat (*see* above), varying only in the buffer conditions used. Therefore we include an abbreviated version and refer readers to the PKA protocol for more detailed explanations.

1. Thaw 2 µg purified recombinant RGS protein.
2. Make enough 5X ERK reaction buffer for 4 µL/reaction.
3. Calculate the volume needed to achieve 2×10^6 cpm [^{32}P]ATP (2000 cpm/pmol).
4. In a microfuge tube on ice, dilute your protein of interest, 2×10^6 cpm of [^{32}P]ATP, and 4 µL 5X ERK reaction buffer to a final volume of 18 µL with dH_2O.
5. Thaw enough ERK2 to allow 10 U/reaction. Dilute the stock ERK2 to a final concentration of 5 U/µL in 25 m*M* HEPES, 25 m*M* $MgCl_2$, and 5 m*M* EDTA.
6. To start the assay, add 2 µL diluted ERK2 to each reaction tube, mix, and transfer to 30°C.
7. Follow **steps 7–9** in **Subheading 2.2.1** to complete reaction and visualize phosphorylation.

3.2.3. PKC

The protocol for PKC phosphorylation is similar to that for PKA and ERK, however, the standard protocol is more involved because lipids are used. An alternative protocol (*see* **Note 15**) utilizes the purified catalytic subunit of PKCα.

1. Combine 5 µL each of PS and DOG (*see* **Materials**) in a glass vial. Dry lipids under N_2 gas in fume hood and emulsify by sonication in a bath sonicator in 50 µL CHAPS buffer (*see* **Note 16**).
2. Thaw 2 µg of purified recombinant RGS protein.
3. Make enough 5X PKC reaction buffer for 4 µL/reaction.
4. Activate the PKC by diluting the stock (80 µU/µL) 1:8 with PKC activation buffer, supplemented with 5 m*M* DTT, added immediately before PKC.
5. Calculate volume needed to achieve 6×10^6 cpm [^{32}P]ATP (2000 cpm/pmol).
6. In a microfuge tube on ice dilute your protein of interest, 6×10^6 cpm of [^{32}P]ATP, 1 µL reconstituted lipids, and 4 µL 5X PKA reaction buffer to a final volume of 18 µL with dH_2O.
7. To start the assay, add 2 µL activated PKC to each reaction tube, mix, and transfer to 30°C.
8. Incubate the reaction for 30 min at 30°C.
9. Follow **steps 8** and **9** in **Subheading 2.2.1.** to complete reaction and visualize phosphorylation.

3.3. Quantifying Phosphorylation

The efficiency of phosphorylation can be quantified by analyzing the molar amount of ATP incorporated/mole of protein. Although multiple methods are available to test this, many involve precipitating all the proteins in the sample to quantify the incorporation. We have compared the method described below with an alternate approach which employs trichloroacetic acid (TCA) to precipitate protein samples. The TCA precipitation returns approx 20% higher counts, however this may be due to minor protein contaminants in the sample. Because RGS14 is prone to degradation, we use a method in which we measure the [^{32}P]-bound only to the protein of interest.

1. Perform in vitro phosphorylation as described in **Subheading 2.2.** However, to quantify phosphorylation efficiency, we perform kinase assays using a time course of incubation of 0, 5, 10, 15, 30, and 60 min at 30°C.
2. After completing visualization of [^{32}P]-labeling by autoradiography or Phosphorimager analysis, excise the band of interest from the dried gel.
3. Place excised band in scintillation vial and add 2 mL scintillation fluid. Cap vial and count the [^{32}P] recovered.
4. To calculate the stoichiometry:
   a. Calculate the specific activity of [^{32}P]-ATP added to the reaction (cpm/pmol total ATP).
   b. Divide the cpm recovered by the specific activity to determine the pmol of phosphate recovered.
   c. Divide the pmol of phosphate recovered by the pmol of protein in the reaction.
   d. If this number is less than 1, there is <100% stoichiometry. Numbers significantly above 1 indicate multiple phosphorylation sites.

3.4. Mutagenesis

Several available techniques test the in vitro specificity of a kinase. For kinases such as ERK and PKA, where recognition sites are well identified, the most straightforward method is to mutate the sites of interest and test the activity of the kinase on the mutant proteins. This strategy can only be used if the number of potential sites is low. For kinases such as PKC, for which the recognition motif is less well defined, the most straightforward methods include phosphopeptide mapping and phosphoamino acid analysis ***(17,18)***. A full review of all methods available is beyond the scope of this chapter. We briefly review the steps involved in mutating residues to test in vitro phosphorylation by PKA and ERK. We use the "Quickchange" site-directed mutagenesis kit (Stratagene) for this process. Primer design is the most difficult aspect of this process.

1. Sense and antisense primers are designed, making as few substitutions as possible to alter the amino acid(s) of interest (*see* **Note 17**).
2. In a 0.2 mL PCR tube mix 5 µL 10X reaction mix, 10–20 ng of template DNA, 125 ng of each primer, 1 µL each dNTP, and dH_2O to a final volume of 49 µL.
3. Using a thermocycler with heated lid, heat reaction to 95°C. After 60 s, add 1 µL *Pfu* DNA polymerase and start the PCR cycle. The cycle should be as follows:
   a. Melting: 95°C for 20–30 s.
   b. Annealing: 55°C for 1 min.
   c. Extension: 68°C (or adjusted extension temperature) for 2 min/kb plasmid.
   d. Repeat this cycle 16 times for single amino-acid changes.
4. After completion of the cycle, cool the reaction to 37°C. Add 1 µL *Dpn*I restriction enzyme at 37°C for 1 h (*see* **Note 18**).
5. Remove supercompetent *E. coli* (SoloPack Gold, Stratagene) from –80°C and place on ice to thaw.
6. Add 1 µL DNA to one tube of cells on ice (*see* **Note 19**).
7. Swirl and incubate on ice for 30 min.
8. Heat-shock *E. coli* at 54°C for 60 s without swirling tubes.
9. Cool on ice for 2 min.
10. Add 150 µL Luria-Bertani (LB) or NZY media and let cells recover for 1 h at 37°C.
11. Plate onto LB plate supplemented with antibiotic, grow at 37°C overnight.
12. Sequence plasmid DNA from one or two colonies to confirm mutation.
13. Following the standard purification procedure, purify mutant protein and test phosphorylation by kinases (**Fig. 2**).

4. Notes

1. In our experience, several of the larger, more complex RGS proteins were difficult to purify in experimentally useful quantities. As described, we were only able to purify RGS14 after addition of a thioredoxin molecule. RGS7 also requires modi-

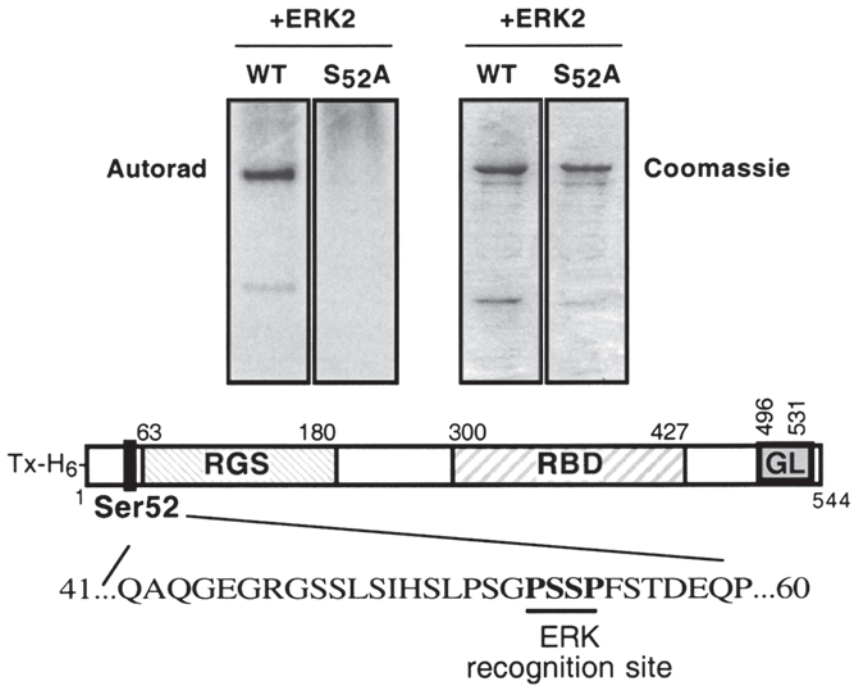

Fig. 2. RGS14 ERK phosphorylation site is identified by site-directed mutagenesis. RGS14 is not phosphorylated after mutation of Serine 52 to Alanine. Serine 52 was identified as the best ERK recognition site in the RGS14 sequence, and the suppression of protein phosphorylation indicates that ERK specifically modifies this residue. Top, left panel: autoradiogram of ERK2 phosphorylation of RGS14 and RGS14(Ser52Ala); right panel: Coomassie-stained gel showing protein bands. Bottom: diagram of RGS14, highlighting the ERK recognition site near the RGS domain.

fied conditions, most notably coexpression with its binding partner $G\beta_5$, and is not readily purified from *E. coli* in any condition. "Simple" RGS proteins can also require customized conditions for purification that may necessitate adjustment of the kinase protocols. The most obvious example of this is RGS2, which requires 500 m*M* NaCl for solubility.

2. RGS14 is particularly prone to degradation and remains largely insoluble in *E. coli* and Sf9 insect cells. We found that neither amino-, nor carboxy-terminal hexahistidine or glutathione-*S*-transferase tags alleviate degradation or enhance the solubility. The Trx-Tag™ (Novagen) in the pET32b vector codes for a modified, 109 amino-acid thioredoxin protein that attaches to the amino-terminal of RGS14, increasing protein solubility and stability. Because thioredoxin reduces disulfide bonds, several host strains of *E. coli* are available that allow sulfide bonds in proteins that require these to properly fold ***(19)***.

3. Because pET32b attaches a thioredoxin molecule and a hexahistidine tag to the amino-terminus of the protein, our recombinant RGS14 runs at approx 80 kD (the RGS14 sequence codes for a 60 kD protein). The vector includes a thrombin cleavage site that the allows removal of the large amino-terminal tag, which we were able to efficiently cleave from RGS14. However, we were subsequently unable to purify the nontagged RGS14 for use in further experiments because the protein precipitated during the purification steps in the absence of thioredoxin.
4. If a French-pressure cell is not available, cells can also be lysed on ice using 0.2 mg/mL lysozyme for 30 min. After lysis, cells must be treated with DNAse to reduce viscosity of the lysate. As an alternative to DNAse treatment, 1 mg/mL Streptomycin sulfate can be added to the lysate to bind and precipitate DNA.
5. Many hexahistidine-tagged proteins can be adequately purified using only a Ni-NTA column. Unfortunately, because RGS14 is partially cleaved in every condition we have tested, we found that the best method to achieve pure, full-length protein was to include an additional step of separating the smaller cleavage products from the full-length protein by size-exclusion chromatography. If an FPLC apparatus is unavailable, purity can also be achieved by conventional gravity flow.
6. We have not encountered difficulties recovering acceptable amounts of thioredoxin-tagged RGS14 from *E. coli*, although it is good practice to save a small amount of pellet in the initial attempts at purification to test for expression in case recoveries are particularly low. Several RGS proteins are insoluble when produced in *E. coli* and accumulate in inclusion bodies in the pellet. In this case, the rate of induction can be changed, and if this fails, alternate protein-production strategies (e.g., production in insect cells) should be considered.
7. To increase the yield of protein in the early elutions off the Ni-NTA beads, allow 1 mL initial 1.5 mL elution buffer to run into the column before pausing the buffer flow. This allows the imidazole to come into contact with the Ni-NTA beads for the first 10-min incubation.
8. We find that the majority of our full-length protein elutes from the Ni-NTA column in E2 and E3. Therefore, these samples are used for subsequent steps and aliquots. However, small variations in technique can cause proteins to come off the column slightly earlier or later. Initial experiments should be carried out to determine in which elutions the protein appears.
9. The elutions from the Ni-NTA column are added into the loading loop using a syringe. The volume added should not exceed 1% of the bed-volume of the column (in our case, 24 mL) for proper size exclusion. Do not remove the syringe after injecting the sample. Removing the syringe allows air into the loop and subsequently onto the superdex column, which disturbs the resin bed and leads to distorted results.
10. Avoid freeze-thaw. Each freeze-thaw cycle will cause approx 50% of purified thioredoxin-RGS14 to precipitate out of solution. We have reduced this problem by adding 10% glycerol into the running buffer, however, this step may affect subsequent assays. We usually aliquot purified thioredoxin tagged RGS14 into single-use volumes, snap freeze in liquid nitrogen, and store these at –80°C.

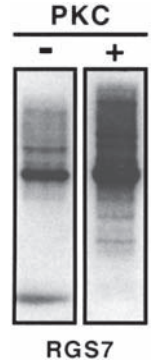

Fig. 3. Nonspecific kinase activity in proteins purified from insect cells. Hexahistidine-tagged RGS7 was purified using standard protocols from Sf9 cells. Left panels: kinase-free control; right panels: incubated with PKCα. These results highlight the importance of including kinase-free controls.

11. Many proteins purify more readily from insect cells than from *E. coli*. However, when we attempted to investigate the capacity of PKC and ERK2 to phosphorylate several proteins that we purified from eukaryotes, we found that unidentified, nonspecific kinases copurified with out proteins and were active under these reaction conditions. We have included **Fig. 3** as an example of this nonspecific phosphorylation. Histidine-tagged RGS7 was purified by affinity chromatography from Sf9 insect cells. Note the high-background phosphorylation in the absence of PKC, although addition of the enzyme clearly increases phosphorylation.
12. All assays should be performed in parallel either without kinase or using heat-inactivated kinase. To reduce the total cost, we recommend kinase-free controls.
13. Depending on the protein, longer reaction times can lead to some nonspecific phosphorylation, so reactions should be kept short to enhance the specificity of phosphorylation. However, longer reaction times may help increase the stoichiometry of phosphorylation (**Subheading 2.3.**) and should be tested in parallel with phosphorylation-deficient mutants (**Subheading 2.4.**) to determine the level of nonspecific phosphorylation.
14. Coomassie blue staining: Soak gels in Coomassie brilliant blue dissolved in 30% isopropanol, 10% acetic acid for 20 min, destain in 30% methanol, 10% acetic acid until protein bands are easily visible, and fix in 1% glycerol, 10% acetic acid for 15 min. After fixing, gels are dried under vacuum.
15. An alternative protocol utilizes the purified PKCcat. This alternate protocol is less time-consuming and less complicated, however, the higher cost of the catalytic subunit could prove prohibitive when large numbers of experiments are proposed.
    a. Make a PKC reaction buffer: 50 m*M* HEPES, 12.5 m*M* $MgCl_2$, 1.25 m*M* EDTA, 125 μ*M* ATP, 9×10^6cpm/reaction, and a phosphatase inhibitor (i.e., microcystin) in a final volume of 43 μL/reaction.

b. Add your protein of interest to a final volume of 48 µL.
c. To start the reaction, add 2 µL undiluted PKCcat and transfer reaction tube to 30°C.
d. Incubate the reaction for 30 min at 30°C.
e. Follow **steps 8** and **9** in **Subheading 2.2.1.** to complete reaction and visualize phosphorylation.

16. Emulsified lipids should appear cloudy. If lipids appear to aggregate, the stocks may have expired and fresh lipids should be purchased.
17. Primers should be between 25 and 45 bp long, with a melting temperature (T_m) of approx 78°C. We have had success with primers at several T_m, ranging from 68–82°C. When working with primers that have a T_m close to 68°C, we have reduced the extension temperature several degrees. The mutation desired should be at the center of the primer, with 10–15 bases of correct sequence on either side and a GC content of at least 40%. We order custom primers through Operon and have found that their standard purification procedures work well for the mutagenesis reaction.
18. *Dpn*I is a restriction enzyme that selectively acts on methylated DNA. In virtually all strains of *E. coli*, DNA is methylated during plasmid replication by several enzymes number, including *dam* and *dcm* methylases. On the other hand, DNA that is polymerized by polymerase chain reaction (PCR) is not modified. Therefore, only the template DNA will be degraded when incubated with *Dpn*I, leaving the nicked DNA available for bacterial transformation. This strategy allows easy detection of mutants with low-background colonies. To control for *Dpn*I activity, use template DNA that has not undergone PCR and digest this in parallel to the PCR reaction. After transformation and overnight growth on selection media, there should be little or no colony growth on this plate. If there are a number of colonies on this plate, the enzyme should be replaced and the digestion carried out again.
19. Our laboratory typically uses Solopack-gold supercompetent *E. coli* from Statagene, although almost any supercompetent cells will work well for this purpose. The Solopack cells do not need to be transferred to polypropylene tubes. If several mutants are being produced at once, half the number of cells can be used to conserve cost. Stratagene also suggests adding BME to the cells for 10 min before incubation with DNA, although we have found little benefit from this.

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19

The Use of Green Fluorescent Proteins to View Association Between Phospholipase Cβ and G Protein Subunits in Cells

Suzanne Scarlata and Louisa Dowal

Summary

A major advance in biology is the ability to attach either green fluorescence protein (GFP) or one of its variants to a target protein and follow its cellular localization and interaction with other partners by fluorescence microscopy. Our laboratory has previously developed fluorescence energy-transfer methods to measure the kinetics and affinities of the lateral association between phospholipase C (PLC) and G protein subunits on membrane surfaces. We are currently developing methods to view these associations in living cells using fluorescence resonance energy transfer (FRET) between GFP-based chimeras. Because the improvements and variations of these GFP-based FRET techniques has continued on a rapid pace, we focus only on the basic principles behind these measurements and the methods used, which may continue to be applicable as improvements become available.

Key Words: Phospholipase Cβ; G proteins; fluorescence resonance energy transfer; fluorescence microscopy; green fluorescent protein; cell culture.

1. Introduction

1.1. Green Fluorescent Protein

Green fluorescent protein (GFP) from the jellyfish *Aequorea victoria* was reported nearley 40 yr ago (for recent reviews, *see* **refs. *1*** and ***2***). The isolated protein forms a dimer (Kd ~100 μ*M*), whose monomers consist of a tightly compact barrel of 11 β sheets. Within the hydrophobic core is an axial α helix containing a tripeptide that has the ability to cyclize, then oxidize to produce a

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fluorescent species ***(1,2)***. Altering the residues in the local environment of the tripeptide has allowed for the production of proteins with higher fluorescent yields and improved photostability and shifts in spectrum to wavelengths with less overlap of cellular components. Presently, there are approx 10 types of GFPs that vary in their extinction coefficient and excitation and emission spectrum. The detection limit has been estimated to be 10^5 GFPs/cell ***(1)***, but this value will likely decrease as improved optics and GFP design develops. Specific information about the photophysics of these constructs can be found in the review by Tsien ***(1)***, and their applicability to the G protein–phospholipase C (PLC) system is given in **Note 1**.

1.2. Bird's Eye View of Fluorescence Resonance Energy Transfer (FRET)

Fluorescence is the emission of light energy from an excited state of a chromophore after it absorbs a certain amount of light, where the amount of absorbed light is given by the extinction coefficient (for complete background, *see* **ref. *3***). After a probe absorbs light, it spends a certain period of time in the excited state before the light is released. This period of time, termed the fluorescence lifetime (τ), is determined by the ability of the local environment to quench or promote relaxation to the ground state by nonradiative mechanisms. For biological probes, the fluorescent lifetime is generally in the nanosecond range. Agents that promote these nonradiative processes include the presence of oxygen, most metal ions, and other ionic groups. Many of these groups quench dynamically (i.e., while the probe is in the excited state) and reduce the fluorescent lifetime. Alternately, some quenching agents quench statically form complexes with the probe, which reduces the amount of fluorescence without changing the lifetime. Fortunately, the barrel structure of GFP insulates the internal fluorophore well; therefore, quenching by most agents is usually not problem (*see* **Notes 2** and **3**).

Many years ago, Förster described a process that would compete with the normal rate of fluorescence emission. This would occur in the presence of a second molecule (i.e., acceptor), whose absorption spectrum overlaps the emission spectrum of the fluorophore. In this process, the excited energy of the probe can be transferred to the ground state acceptor and cause it to be excited. Thus, the resulting emission will occur from the acceptor, giving the characteristic emission spectrum of the acceptor. Note that this process, termed Förster resonance energy transfer (FRET), is distinct from trivial reabsorption, where a donor emits light that is subsequently absorbed by the acceptor, which then fluoresces. The distinguishing feature of FRET is its excited-state resonance transfer that is critically dependent on the distance between the donor and acceptor. If it is assumed that the interaction between the donor and acceptor does not result in any structural alterations, then the rate of energy transfer (k_t) is given by:

$$k_t = 1/\tau_D \, (R_o/R_{DA})^6$$

where τ_D is the fluorescence lifetime of the donor, R_{DA} is the distance between the donor and the acceptor, and R_o is the critical transfer distance or the distance at which 50% of the donor fluorescence is lost to transfer. R*o* can be calculated from the quantum yield of the donor (Q_D), which refers to the number of photons emitted over those absorbed), the index of refraction of the solvent between the donor and acceptor (n), the overlap integral between the donor emission and the acceptor absorption spectra, and the orientation between the donor and acceptor dipoles (κ^2) where

$$R_o = (8.8 \times 10^{25} \, \kappa^2 Q_D J/n^4)^{1/6} \text{ cm}$$

$$\text{where } J = \int \varepsilon_a(\lambda) \, f_D(\lambda) \, \lambda^4 d\lambda$$

where ε_a is the extinction coefficient of the acceptor at wavelength λ, and $f_D(\lambda)$ is the emission intensity of the donor normalized so that the integral of $f_D(\lambda)$ over its emission band equals QD.

$$\kappa^2 = (\sin\zeta_D \sin\zeta_A \cdot cos\theta - 2 \, cos\zeta_D \cos\zeta_A)^2$$

where ζ_D and ζ_A are the angles between the vector of R_{DA} and the donor and acceptor dipole directions, and θ is the azimuthal angle between the donor and acceptor dipoles.

We present these equations to show that when choosing energy-transfer probes, several features besides spectral overlap should be kept in mind. Among them is the ability of the donor to absorb the light transferred to the acceptor; thus, a donor with a high-extinction coefficient and high-quantum yield is desired. Similarly, the acceptor should have a high-extinction coefficient to absorb the excitation energy from the donor. A very difficult parameter to assess is the orientation factor or κ^2. The simple reason for its importance is that transfer can only occur when some population of the dipoles are similarly aligned. If the dipoles are perpendicular, transfer will not occur. Fortunately, in solution, the donor and acceptor molecules can rotate and sample a large number of orientations during the excited state of the donor. Under conditions of free rotation, κ^2 is 2/3. In most circumstances, free rotation is assumed, and the extent of rotational motion can always be checked by fluorescence anisotropy (*see* below). Unless one is using energy transfer to determine intermolecular distances, then the 2/3 assumption is reasonable.

Values for the extinction coefficient and the quantum yield of GPF can be found in the literature ***(1,2)***. Presently, the best energy transfer pair among athe GFPs uses cyan fluorescent protein (CFP) as a donor and yellow fluorescent protein (YFP) as an acceptor. The R_o for this pair is 49–52 Å. Other pairs that use blue fluorescent protein (BFP) are less desirable because of photoinstability and low extinction and absorption coefficients (*see* **Note 4**).

The requisite dependence on distance (i.e., R^6) is the key feature of FRET that sets it apart from other techniques that detect protein interactions. For a probe pair whose R_o is 50 Å, the transfer will be reduced to 33% at 60 Å and 8.8% at 75 Å. Thus, the observation of FRET essentially only occurs when protein association takes place.

A special case of FRET is the transfer among identical probes, which will occur when there is good overlap between the absorption and emission spectra. Probes like fluorescein are particularly good at self-transfer. GFP can also transfer energy to itself, and we have used homotransfer to view the dimerization of a GFP-labeled receptor tyrosine kinase on activation in a fluorometer (Runnels and Scarlata, unpublished results). Because homotransfer produces little or no change in the emission spectrum of a probe, the best method to view homotransfer is by fluorescence anisotropy (A) or polarization. Fluorescence anisotropy is experimentally measured by exciting a probe with polarized light, which can easily be obtained by inserting a polarization filter in the incident beam, and measuring the polarization of the emitted light. The anisotropy is calculated from:

$$A = (I_v - gI_H)/(I_v + 2gI_H) \text{ and } P = (I_v - gI_H)/(I_v + gI_H)$$

where I_v refers to vertically polarized light, I_H to horizontally polarized light, and g is a instrument parameter that must be used when the emission monochromater does not identically pass vertical and horizontal components. Most commercial instruments will automatically calculate the g factor by determining the ratio of the horizontal and vertical emission when exciting with horizontally polarized light (i.e., $g = [I_v/I_H]$ $ex = H$).

Anisotropy measures the depolarization of the emitted light and is therefore used to assess whether a probe is rotationally restricted, where high-anisotropy values (i.e., those approaching 0.35) have little freedom of movement, whereas small freely rotating probes have anisotropy values close to zero. Because the full rotation of most proteins cannot be realized in the nanosecond lifetime of the probes, their solution anisotropy values usually fall in the range of 0.1–0.25.

When a probe transfers its energy in the excited state, the polarization of the transferred light decreases and will decrease further with each transfer. Thus, when homotransfer occurs, the anisotropy decreases and shows a systematic decrease with each transfer. Homotransfer, like Förster energy transfer, has an R^6 distance dependence. We have previously used this method to estimate the number of subunits in a membrane protein oligomer ***(4)***. Although homotransfer has not yet been adapted to fluorescence microscopy, in principle, it should be easy to apply experimentally and may prove useful to view higher order protein complexes.

2. Materials

2.1. Plasmids and Cells

1. Plasmids encoding the fusion proteins, $G\alpha_q$-EGFP, $G\alpha_q$-ECFP, $G\alpha_q$-RC-ECFP, and ECFP, have been cloned into the vector pcDNAI/Amp from Invitrogen and were the generous gifts of Dr. Catherine Berolot (Yale, New Haven, CT).
2. A $PLC\beta_1$-EYFP fusion protein was cloned into a pcDNA6-V5HisB vector from Invitrogen and was the generous gift of Dr. Loren Runnels (Rutgers University, Piscataway, NJ).
3. Rat pheochromocytoma (PC12) cells (American Type Culture Collection [ATCC], cat. no. CRL-1721). Other cell types such as HEK 293 cells could be used.

2.2. Labware

1. LabTek Chambers (Nalg Nunc International): 2-well chamber 1–2 mL volume and each equal to 4.4 cm^2.
2. 1.5 × 2-in. Quick-Seal polyallomer tube (Beckman).
3. Cyan/Topaz energy transfer filter set (Chroma Tech, cat. no. 31052).

2.3. Solutions

2.3.1. DNA Preparation

1. STE: 100 m*M* NaCl, 10 m*M* Tris-HCl, pH 8.0, 1 m*M* EDTA.
2. GTE: 25 m*M* Tris-HCl, pH 8.0, 10 m*M* EDTA, 20% glucose.
3. LB/ampicillin: 200 µg/mL Ampicillin in Lubria-Broth (LB; Invitrogen, cat. no. 12795-084).
4. TE: 10 m*M* Tris-HCl, pH 8.0, 1 m*M* EDTA.
5. 0.2 *M* NaOH/1% sodium dodecyl sulfate (SDS): make fresh each time.
6. 1 g/mL CsCl/TE.
7. 10 mg/mL Ethidium bromide (EtBr).
8. 5 mg/mL stock Lysozyme.

2.3.2. Cell Culture

1. PC12 media: Dulbeco's modified Eagle's medium (DMEM) supplemented with 10% equine serum, 5% fetal calf serum (FCS), and 1 m*M* sodium pyruvate.
2. PC12 microscopy media: Opti-MEM (Invitrogen) 10% equine serum, 5% FCS, 15 m*M* HEPES, and 250 µg/mL $CaCl_2$.
3. Nerve growth factor (NGF; Sigma, St. Louis, MO) (100 ng/mL DMEM).
4. Trypsin/EDTA: 0.05% trypsin, 0.53 m*M* EDTA-$4Na^+$.
5. ICEB: 125 m*M* KOH, 4 m*M* NaOH, 73 m*M* PIPES, 34 m*M* myo-inositol, 10 m*M* $NaHCO_3$, 5 m*M* K_2HPO_3, 5 m*M* KH_2PO_3, 5 m*M* D-glucose, 4 m*M* $MgCl_2$, 1 m*M* $MgSO_4$, 1 µ*M* $CsCl_2$, pH 7.0, and filter sterilized.
6. 50 µg/mL Fibronectin.
7. 1X Dulbecco's phosphate-buffered saline (PBS) without $CaCl_2$ and without $MgCl_2$ (GIBCO, cat. no. 12377-016).
8. Alexafluor594 phalloidin (Molecular Probes, Eugene, OR).

2.2. Instrumentation

Our laboratory uses an ISS photon-counting spectrofluorometer (Champaign, IL). Although the particular type of fluorometer is not important, having photon-counting ability increases the sensitivity 10 fold, which is important for these types of measurements. Because cells tend to produce a lot of scattered light that must be eliminated for accurate measurements, it is also very useful to have cut-off filters that eliminate the contribution of scattered excitation light. For these measurements we usually place a Corion 470 nm cut-off filter after the sample. This filter prevents of scattered 440-nm excitation light from reaching the monochromater. If the fluorometer is equipped with polarizers, then crossing the polarizers (i.e., placing the excitation polarizer in the parallel position and the emission polarizer in the perpendicular position) works because scattered light is vertically polarized. However, crossing the polarizers will greatly reduce the amount of detected emission by 70%, so if one has the choice, using cut-off filters is preferred.

We have imaged the cells on a Nikon IMT-2 epifluoresense microscope equipped with an Olympix Astra Cam camera and filter packages from Chroma Tech., including the Cyan/Topaz energy transfer filter set (cat. no. 31052). Cells have also been viewed on the confocal apparatus in our university microscopy facility.

3. Methods

3.1. Plasmid Purification

1. All plasmids are purified from XL-Blue competent cells using the Cesium Chloride Ultra Centrifugation Method modified from Sambroook and colleagues ***(5)***.
2. In brief, inoculate 500 mL LB/ampicillin is inoculated with a single colony and grow overnight with shaking at 37°C.
3. The following day, harvest cells in a JA-10 rotor at 5000*g* for 15 min at 4°C.
4. Resuspend cells in 50 mL STE and recentrifuge.
5. Resuspend the cell pellet in GTE containing 25 mg/mL lysozyme and incubate at room temperature for 5 min.
6. Add 2 vol 0.2 *M* NaOH/1% SDS to the lysate, mix, then 7.5 mL of 5 *M* K/acetate, and incubate the lysate on ice for 5 min.
7. Centrifuge lysed cells are then centrifuged at 35,000*g* in a JA-20 rotor for 30 min at 4°C.
8. Add 0.6 vol of isopropanol to the supernatant, and incubate for 10 min at room temperature.
9. Centrifuge the supernatant at 5031*g* for 15 min. Wash the resulting pellet with 70% ethanol and allowed to dry.
10. When the pellet is dry, suspend it in 4 mL CsCl/TE containing EtBr and load into a Quick-Seal polyallomer tube and heat seal.
11. Centrifuge the tube at 20°C in a Beckman TLA 100.4 rotor for 18 h at 204,000*g*.

12. To retrieve the plasmid DNA, clamp the tube in front of a UV light source, and puncture the top with a syringe needle. Just below the lower band (intact DNA), insert a syringe needle, and draw the band into the syringe.
13. Extract the DNA with water-saturated isobutanol until the pink-colored EtBr is removed.
14. Dialyze plasmid DNA against 1 L TE at 4°C for 1 h.
15. Determined DNA concentration by reading the absorbance at 260/280 nm and calculated by multiplying the A_{260} by the dilution then by 50 to give μgDNA/mL.

3.2. Cell Culture

We have done most of our studies using PC12 cells. These cells are cultured in PC12 medium and incubated at 37°C with 5% CO_2. When viewing cells under the microscope, cultures are kept in. Opti-MEM. PC12 differentiation was induced with NGF (Sigma, St. Louis, MO) at a concentration of 100 ng/mL for 3 d.

1. Transfect cells with fusion protein plasmids by electroporation.
2. Wash a 70–80% confluent T-25 flask with PBS and lift cells with a short 1-min incubation in Trypsin/EDTA.
3. Bring trypsinized cells up in DMEM and spin down at for 5 min at 490*g*.
4. Bring cells up in Opti-MEM, centrifuge as above, and suspend the cell pellet in ICEB (*see* below).
5. Separated the resuspended cells into four, 15-mL conical tubes and centrifuge again.
6. Bring the resulting pellets up in 500 μL ICEB containing 10–30 μg plasmid DNA.
7. Transferred the cell/Intracellular electroporation buffer (ICEB) mixtures to 0.4-cm electroporation cuvets (Bio-Rad), and pulsed once at 200 V with a 960 μF capacitance.
8. To the pulsed samples, add 1 mL of Opti-MEM and removed cells from cuvets into 15-mL conical tubes containing 3 mL DMEM.
9. Centrifuge cells for 5 min at 1500 rpm, bring up in 1.5 mL DMEM and plated onto LabTek Chambers coated with 50 μg/mL fibronectin.
10. Allow cells to attach to the substrate for 3–4 h, at which time wash the wells gently with PBS, and add fresh DMEM containing 100 ng/mL NGF. Cells undergo differentiation during incubation for 3 d, after that they are viewed and imaged.

3.3. Cell Fixation and Staining

Transfected cells in LabTek Chambers are stained with Phalloidin to determine fusion protein colocalization with actin.

1. Wash chamber wells once with Opti-MEM and fixed in 500 μL of 4% formaldehyde in PBS for 20 min at room temperature.
2. Then wash wells twice with PBS for 3 min each, and permeabilized cells with 400 mL 0.2% Triton X-100 in PBS for 5 min at room temperature.

3. The wash wells three times for 3 min each followed by staining with 500 µL 1.75 ng/mL Alexafluor/phalloidin in PBS for 30 min at room temperature in the dark.
4. After staining, wells are washed three times with PBS for 7 min each. Add 1 mL PBS to each well, and store chambers protected from light at 4°C.

3.5. FRET Determinations

View FRET by one of two methods: the loss of donor fluorescence in the presence of acceptor or the increase in fluorescence in the presence of acceptor, termed sensitized emission. Because many factors influence the fluorescence intensity of these species, it is often difficult to be convinced that FRET is actually occurring. A current problem in viewing FRET between GFP pairs is the contribution of the donor emission to the acceptor spectrum (i.e., bleed-through) and alternately, the contribution of acceptor emission to the donor spectrum. Bleed-through problems can greatly reduce the sensitivity of the system to transfer. Although corrections can be made, the ideal situation would be to increase the separation between the emission spectra of the donor and acceptor. Very recently, Clontech has offered a more red-shifted GFP acceptor which may reduce bleed-through. This very red GFP may even offer the possibility of viewing transfer of two distinct sets of probes or between ternary species.

Fortunately, FRET can be assessed in a ratiometric measurement that helps to more carefully control the system. Because contributions from light scattering can be minimized using a spectrofluometer, it is sometimes beneficial to evaluate the amount of energy transfer on this instrument before using a microscope. Also, measurements on a spectrofluorometer allows the assess to overall intensity. Either membrane preparations or whole cells can be placed in a cuvette and the emission of the probes followed.

For these measurements, we excite the system at the absorption maximum of the CFP donor (440 nm) and measure the emission intensities at the wavelength maxima for CFP and YFP at the two wavelengths where the GFPs have the greatest difference (i.e., 490 and 525 nm; *see* **Fig. 1**). We then compare the ratios of the fluorescence intensities at the two wavelengths to the values obtained when one of the GFP proteins is expressed with a vector of the protein partner without a GFP tag. A good positive control is to express both GFPs linked together. For the G protein heterotrimer, this latter control is not critical. The percent FRET is simply calculated as:

$$\%\ \text{FRET} = [Ratio(\text{CFP}) - Ratio(sample)]/[Ratio(CFP) - Ratio\ (YFP)]$$

The CFP filter cube set we use has an excitation filter of 440/21 nm with a dichroic beamsplitter of 455 nm and emission filter of 480/30 nm. The YFP filter cube set uses an 500/25 nm excitation, 525 nm beamsplitter and 545/35

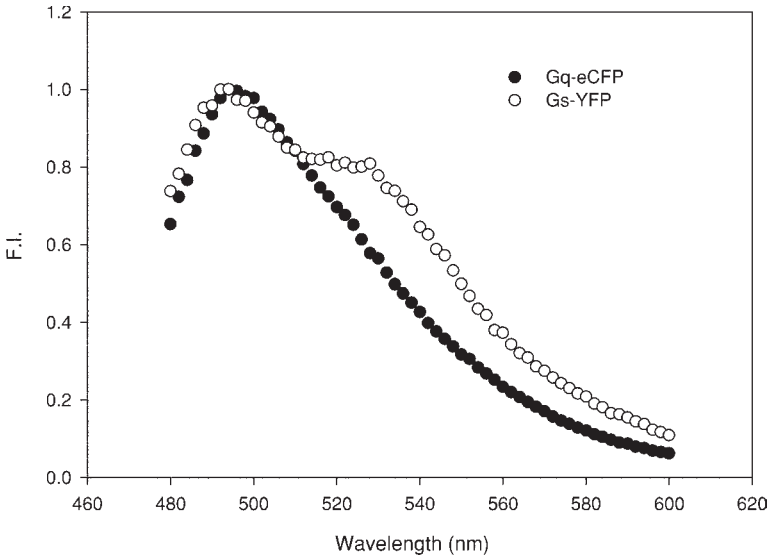

Fig. 1. Comparison of Rau emission spectra of HEK cell membranes that have been transfected with G_q-eCFP(●) or G_s-YFP (○).

emission filters. The FRET filter cube set uses a 440/21 nm excitation, 455 nm beamsplitter, and 535/26 nm emission filter.

When using a fluorescent microscope, a similar methodology is used, although the optical properties of the filter cubes and the software play critical roles ***(9)***. Chroma and other companies now make filter cubes that have been optimized for CFP–YFP fluorescence, and many microscope software often have FRET packages. A software package is necessary that will integrate the pixel intensity of a given image and take images using filter sets for CFP, YFP, and FRET. Data can be analyzed by a similar ratiometric procedure.

4. Notes

1. One concern about GFP tags is that they are not small proteins and may influence the properties of their host proteins. Regarding the PLCβ-G protein signaling system, we and others have made constructs which do not appear to perturb their cellular properties. For example, placement of GFP on the N-terminus of PLCβ still results in normal signal transduction under carbachol stimulation in transfected HEK cells (Loren Runnles, personnal communication). Catherine Berlot (Dept. of Physiology, Yale University) has placed GFP analogs on loops in G_q ***(6)*** and Gβγ (Berlot, personal communication) and these proteins all form heterotrimers and behave normally in transfected HEK cells. A more biophysical approach to determine the integrity of GFP protein chimeras was to separately transfect cells with the DNA of eGFP-PLC and/or eGFP-G protein subunits, pre-

pare membrane fraction, and measure the energy transfer from the GFP proteins to purified proteins tagged with a fluorescence acceptor, as the latter is added into the membrane preparation. We find the resulting interaction energies to be on the same order of those using purified proteins.
2. Because oxygen tends to permeate through a protein matrix, its quenching ability under the particular experimental circumstances should be checked by blanketing the sample with an inert gas, such as argon or nitrogen, when applicable.
3. An important quenching agent in the phospholipase C–G protein system is aluminum fluoride. However, under standard G_q activation conditions, we do not detect significant quenching.
4. When using YFP it is important to keep in mind that the Q69M type, known as citrene, is better expressed at 37°C, is less pH-sensitive and less susceptible to nonradiative emission pathways when compared to the other YFP types *(7,8)*.

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Cellular Localization of GFP-Tagged α Subunits

Thomas R. Hynes, Thomas E. Hughes, and Catherine H. Berlot

Summary

Heterotrimeric G proteins transmit signals from a wide range of cell surface G protein-coupled receptors (GPCRs) to mediate multiple cellular events. Within the plasma membrane, G proteins interact with GPCRs and effector proteins such as adenylyl cyclase (AC) and phospholipase C (PLC). Plasma membrane subdomains (e.g., lipid rafts and caveolae) may organize and regulate these interactions. G protein subunits have been reported to be in additional cellular regions, such as the Golgi apparatus and the cytoskeleton, and G protein α subunits may move within the cell during the activation cycle. Changes in the cellular localization of α subunits could be important for interactions with effectors that are not in the plasma membrane and/or could be a means for terminating G protein signaling. However, until recently, the topic of G protein α subunit localization under basal and activated conditions has been controversial, partly because of spatial and temporal limitations inherent to procedures like cell fractionation and immunohistochemistry. Green fluorescent protein (GFP)-tagging is a useful way to enable real-time visualization of proteins in living cells. This chapter describes how to produce and visualize functional GFP-tagged α subunits and to investigate whether activation affects their subcellular localization.

Key Words: G protein α subunit; green fluorescent protein; fluorescence microscopy; subcellular localization; plasma membrane targeting; transfection; HEK-293 cells; G protein-coupled receptor; Triton X-100, aluminum fluoride.

1. Introduction

Heterotrimeric G proteins transmit signals from G protein-coupled receptors (GPCRs) to intracellular effectors and regulate many physiological processes. They are associated with the plasma membrane, where they can interact

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with GPCRs, but there are also various and sometimes conflicting reports about localization to other cellular regions under basal and/or activated conditions. Given the large number of cellular processes that G proteins can regulate, the many potential proteins with which they may interact, and differences in specificity that have been observed between in vivo conditions and in vitro reconstitution systems, it is very likely that subcellular localization plays an important role in regulating the specificity of G protein signaling. However, until recently, attempts to elucidate the connections between localization and signaling specificity have been limited to conventional procedures, such as cell fractionation and immunohistochemistry, which provide only a restricted view of the process and are subject to artifacts. Green fluorescent protein (GFP)-tagging now makes it possible to measure the localization of G protein subunits in living cells with a spatial and temporal resolution that has been unattainable previously. This chapter describes how to produce functional GFP-tagged α subunits and how to determine their localization patterns under basal and activated conditions.

Designing functional fluorescent G protein α subunits requires careful consideration of the site of GFP insertion. GFP must be inserted at an internal site of the G protein α subunit, because the amino- and carboxyl-termini of α subunits are involved in interactions with receptors, effectors, and βγ, as well as membrane attachment ***(1–3)***. GFP insertion sites that have maintained the functional integrity of an α subunit are shown in **Fig. 1**. Each of these sites is located near the surface of the α subunit helical domain, a region that does not appear to specify interactions with receptors or effector proteins ***(4–7)***. For α_q, insertion of GFP in the αB/αC loop of the helical domain (site 1, **Fig. 1**) maintains functional interactions with receptors, phospholipase C (PLC), and βγ ***(8)***. This insertion site corresponds to a region in GPA1, a G protein α subunit in *Saccharomyces cerevisae*, in which there is an insertion of approx 100 residues relative to other α subunits ***(9,10)***. Including linkers with the sequence S-G-G-G-G-S at both the amino- and carboxyl-termini of GFP was critical for maintaining α_q function. For the *Dictyostelium discoideum* Gα2 subunit, stable expression of a fusion protein in which cyan fluorescent protein (CFP) is inserted in the αA/αB loop (site 2, **Fig. 1**) rescues chemotactic and developmental defects seen in g$\alpha 2^-$ cells ***(11)***. For α_s, interactions with receptors and adenylyl cyclase (AC) are maintained when GFP is inserted in the α1/αA loop (site 3, **Fig. 1**), which is a site of alternative splicing (***12***; our unpublished results) and within the amino-terminal portion of the αA helix (site 4, **Fig. 1**; ***13***). Another recent strategy that produced CFP-tagged α subunits that could signal between receptors and effectors was to fuse an amino-terminal membrane-targeting segment from GAP43 to CFP, which was then fused to the amino termini of α_i or α_o subunits lacking their native myristoylation and palmitoylation sites ***(14)***. However, a potential concern with

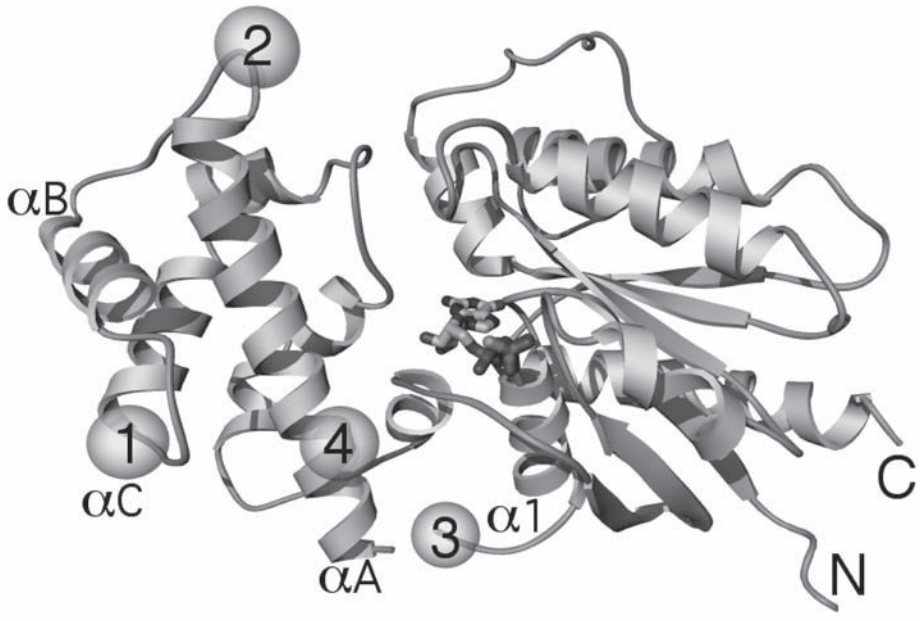

Fig. 1. Model of G protein α subunit indicating GFP insertion sites. The α subunit structure is that of α_s·GTPγS (28). Insertion sites for GFP that resulted in functional α subunits are indicated as numbered spheres. Site 1, F124-S-G-G-G-G-S-[GFP]-S-G-G-G-G-S-E125, was used for α_q-GFP ***(8)***. Numbered residues refer to α subunit residues. The GFP sequence is indicated by [GFP]. Linker residues between the α subunit and GFP sequences are shown (without numbers). Site 2, G90-T-[CFP]-S-M91, was used for *Dictyostelium discoideum* Gα2-CFP ***(11)***. Site 3, E71-[GFP]-S82 ***(12)***, or G72-S-G-G-G-G-S-[GFP]-S-G-G-G-G-S-D85 (our unpublished results), was used for α_s-GFP. Site 4, V92-Q93-D94-L-S-L-I-H-I-[GFP]-G-G-G-P-G-L-D-V-Y-K-R-Q-V92- Q93-D94, is another α_s-GFP fusion site generated by an in vitro transposition reaction ***(13)***. Note that this site is within an α helix (αA). This surprising location demonstrates the usefulness of a random-insertion approach.

this latter approach is that membrane localization and regulation of localization may reflect that of GAP43, rather than the α subunit.

Before imaging a GFP-tagged α subunit, it is important to demonstrate that the construct is functional. The first level of analysis should be to check that the expression level and amount of membrane association of the fusion protein is comparable to that of the α subunit from which it is derived. Including an internal epitope in both the α subunit and the GFP-tagged α subunit makes it possible to compare them directly without interference from endogenous α subunits. Methods for epitope-tagging α subunits ***(15,16)*** and membrane prepa-

rations ***(15)*** as well as cell fractionation ***(8)*** from transiently transfected cells have been described. Fusion proteins that are expressed appropriately should then be tested for functionality. Simple assays to test the functionality of α subunits that regulate adenosine 3',5'-cyclic monophosphate (cAMP) ***(15)*** or inositol phosphate ***(5)*** production have been described.

The method described here involves imaging GFP-tagged α subunits in transiently transfected cells. An advantage of transient expression is the ability to image numerous types of cells expressing different constructs within day(s) of transfection. Transient transfection results in a wide range of expression levels, which makes it possible to determine whether expression level affects the subcellular localization pattern. Also, expression level can be regulated by varying the amount of plasmid used in the transfections. If a more homogeneous population of transfected cells is desired, stable lines can be produced.

Association of G protein α subunits with the plasma membrane results from amino-terminal myristoylation and/or palmitoylation and association with the G protein βγ subunits ***(17)***. Some α subunits (e.g., α_i and α_o) are both myristoylated and palmitoylated, whereas others such as α_q and α_s are palmitoylated, but not myristoylated. α_q has two palmitoylation sites; whereas α_s has only one. The degree and amount of α subunit modification will affect its affinity for the plasma membrane and its dependence on βγ for plasma membrane localization. Depending on the α subunit, it may be necessary to co-transfect with βγ-expressing plasmids to obtain plasma membrane localization. For instance, we have found that under transfection conditions in which α_q-GFP exhibits clear plasma membrane localization, α_s-GFP requires co-expression with βγ. Therefore, when investigating the localization pattern of a novel GFP-tagged α subunit, it is important to look at a range of expression levels and to determine whether co-expression with βγ affects localization.

Detection of plasma membrane-associated GFP-tagged α subunits requires that the signal associated with the plasma membrane-exceed that of any signal in the cytosol. If the signals are equal, the membrane-associated GFP-tagged α subunits will be masked. To test for plasma membrane-associated signal, cells can be treated with Triton X-100, which releases the cytosolic signal, leaving the plasma membrane. This procedure can be useful for quantifying the relative amount of plasma membrane association of different GFP-tagged α subunits or of mutant versions of the same α subunit.

Methods are described to test the effects of activation on targeting and localization of GFP-tagged α subunits. To determine whether the activation state of a G protein α subunit affects its ability to be targeted to the plasma membrane after synthesis, the effects of mutations that cause activation can be tested. In the case of α_q-GFP, mutational activation disrupts plasma membrane association ***(8)***. To determine whether α subunits associated with the plasma mem-

brane change their localization during the activation cycle, the effect of stimulating cells with receptor agonists or with aluminum fluoride (AlF_4^-) can be tested. In the case of α_q-GFP, stimulation with either receptor agonists or AlF_4^- does not affect localization *(8)*.

2. Materials

2.1. Producing cDNAs Encoding GFP-Tagged α Subunits

1. Vector: pcDNAI/Amp or pcDNA3 (Invitrogen).
2. cDNAs encoding fluorescent proteins: EGFP, EYFP, ECFP, DsRed2 (BD Biosciences Clontech).
3. Muta-Gene T7 Enzyme kit (Bio-Rad, cat. no. 170-3581).
4. Polymerase chain reaction (PCR) machine

2.2. Transient Expression of GFP-Tagged α Subunits

1. HEK-293 cells (ATCC, CRL-1573).
2. Minimal essential medium (MEM) with Earle's salts with L-glutamine (Invitrogen/Life Technologies, cat. no. 11095-080).
3. Fetal bovine serum (FBS; Hyclone, cat. no. A-1115-L; *see* **Note 1**).
4. Trypsin-EDTA solution: 0.05% trypsin, 0.53 m*M* EDTA (Invitrogen/Life Technologies, cat. no. 25300-054).
5. Lipofectamine 2000 reagent (Invitrogen/Life Technologies, cat. no. 11668).
6. Opti-MEM I reduced serum medium (Invitrogen/Life Technologies, cat. no. 31985).
7. 35-mm Tissue culture dishes containing a glass coverslip (glass bottom no. 1.5, P35G-1.5-14-C, MatTek Corporation, Ashland, MA).

2.3. Imaging of GFP-Tagged α Subunits in Living Cells

1. MEM powder with Earle's salts and L-glutamine, without sodium bicarbonate (Invitrogen/Life Technologies, cat. no. 61100-061). To prepare HEPES-buffered MEM, add HEPES to 20 m*M* and pH to 7.4, then sterilize by filtration.

2.4. Testing for Effects of Activating Mutations on Targeting of GFP-Tagged α Subunits in Living Cells

1. Muta-Gene T7 Enzyme kit (Bio-Rad, cat. no. 170-3581).

2.5. Imaging of GFP-Tagged α Subunits after Extraction with Triton X-100

1. 1% Triton X-100 (Sigma) in Dulbecco's phosphate-buffered saline (PBS; Invitrogen/Life Technologies, cat. no. 14040).

2.6. Testing for Effects of Agonists on the Localization of GFP-Tagged α Subunits in Living Cells

1. Stock solution of UK-14,304, α_2-adrenergic agonist (Research Biochemicals International/Sigma Aldrich, U-104). Make 20 m*M* stock in dimethylsulfoxide (DMSO). Aliquots are stored at –20°C.

2. 4X Stimulating solution of UK-14,304. On the day of experiment, prepare 40 μ*M* UK-14,304 in PBS (Invitrogen/Life Technologies, cat. no. 14040).

2.7. Testing for Effects of AlF_4^- on the Localization of GFP-Tagged α Subunits in Living Cells

1. 1 *M* NaF (Sigma) in PBS (Invitrogen/Life Technologies, cat. no. 14040; *see* **Note 2**).
2. 10 m*M* $AlCl_3$ in H_2O (*see* **Note 3**).
3. 4X AlF_4^- solution: 120 μ*M* $AlCl_3$, 40 m*M* NaF. Prepare fresh from 1 *M* NaF and 10 m*M* $AlCl_3$ stock solutions.

3. Methods

3.1. Producing cDNAs Encoding GFP-Tagged α Subunits

1. Select location for GFP insertion (*see* **Note 4**).
2. Introduce unique restriction endonuclease site for insertion of GFP (*see* **Note 5**). **Figure 2A** shows the oligonucleotide used to introduce a *Bam*HI site at the alternative splice site in α_s. This can be done using oligonucleotide-directed in vitro mutagenesis ***(18)*** using the Bio-Rad Muta-Gene kit or by using PCR to produce DNA fragments with overlapping ends that are combined subsequently in a fusion polymerase chain reaction ***(19)***.
3. Amplify GFP cDNA bracketed by linker and restriction sites using PCR. **Figure 2B** shows the sequences of oligonucleotides that can be used to amplify GFP and append *Bam*HI sites and S-G-G-G-G-S linkers at each end.
4. Subclone the PCR product into unique restriction endonuclease site in α subunit cDNA. Confirm subcloning and mutagenesis procedures by restriction enzyme analysis and DNA sequencing.

3.2. Transient Expression of GFP-Tagged α Subunits

1. For each transfection, plate out 0.5×10^6 HEK-293 cells in 2.5 mL MEM containing 10% FBS on a 35-mm tissue culture dish containing a glass coverslip (*see* **Note 6**). Incubate the cells at 37°C, 5% CO_2.
2. Transfect the cells with α-GFP-expressing plasmid 24 h later. Transfect with a range of plasmid amounts (i.e., 0.01, 0.03, 0.09, 0.27, 0.81, and 2.43 μg) (*see* **Notes 7** and **8**). For each transfection, dispense plasmid into a sterile 1.5-mL microcentrifuge tube. In sterile hood, add 250 μL Opti-MEM I to each tube.
3. In a separate microcentrifuge tube, add 5 μL Lipofectamine 2000 reagent to 250 μL Opti-MEM I. Mix well by inverting the tube several times.
4. After 5 min, add the Lipofectamine 2000 mixture to the plasmid mixture.
5. After 20 min, add the 500 μL plasmid-Lipofectamine 2000 mixture to the cells by dripping gently all over the plate (*see* **Note 9**). Incubate the cells at 37°C, 5% CO_2.

3.3. Imaging of GFP-Tagged α Subunits in Living Cells

1. Cells can be imaged over a range of times after transfection. Generally, 24–48 h is optimal (*see* **Note 10**).

A Strategy to introduce unique Bam HI site for inserting GFP into α_s.

Wild type sequence (α_s residues 67–89)
Location where new Bam HI site will be placed is indicated in bold type.
Residues/bases to be changed or deleted are underlined or crossed out, respectively.

```
Gly Phe Asn Gly Glu Gly Gly Glu Glu Asp Pro Gln Ala Ala Arg Ser Asn Ser Asp Gly Glu Lys Ala
 GG TTT AAC GGA GAG GGC GGC GAA GAG GAC CCG CAG GCT GCA AGG AGC AAC AGC GAT GGT GAG AAG GC
```

Sequence of Oligo 1 (New Bam HI site is indicated in bold type)

```
Gly Phe Asn Gly Glu Gly Ser Asp Gly Glu Lys Ala
 GG TTT AAC GGA GAG GGA TCC GAT GGT GAG AAG GC
```

B PCR primers for GFP that include Bam HI site and S-G-G-G-G-S linker.

Sequence of Oligo 2 5′ coding oligo

```
        Gly Ser Gly Gly Gly Gly Ser Met Val Ser Lys Gly Glu Glu
GGA GAG GGA TCC GGT GGG GGC GGG AGC ATG GTG AGC AAG GGC GAG GAG
```

Design of Oligo 3 3′ Noncoding oligo

```
Met Asp Glu Leu Tyr Lys Ser Gly Gly Gly Gly Ser
  G GAC GAG CTG TAC AAG TCC GGA GGT GGA GGA TCC TAC ACG
  C CTG CTC GAC ATG TTC AGG CCT CCA CCT CCT AGG ATG TGC
```

Sequence of Oligo 3

```
CGT GTA GGA TCC TCC ACC TCC GGA CTT GTA CAG CTC GTC C
```

Fig. 2. Oligonucleotides used to produce α_s-GFP. **(A)** Oligonucleotide used to introduce a *Bam*HI site by oligonucleotide-directed in vitro mutagenesis ***(18)*** for fusing GFP at the alternative splice site in α_s. **(B)** PCR primers used to amplify GFP and add *Bam*HI sites and S-G-G-G-G-S linkers at each end.

2. At least 1 h before imaging, replace the bicarbonate-buffered medium with HEPES-buffered MEM (*see* **Note 11**).
3. Cells can be imaged using an inverted fluorescence microscope or a confocal microscope. Computer-controlled filter wheels facilitate rapid sequential imaging of different fluorescent proteins. This minimizes movement of the cells and/or the fluorescent proteins between images. Filter sets for imaging GFP, CFP, YFP, and DsRed are available from Chroma Technology (Brattelboro, VT) and Omega Optical (Brattelboro, VT). For confocal microscopy, it is important to be sure that the microscope can produce the laser lines necessary to excite the fluorescent proteins to be imaged.

3.4. Testing for Effects of Activating Mutations on Targeting of GFP-Tagged α Subunits in Living Cells

1. Introduce a mutation in either of two conserved α subunit residues to produce constitutive activation by inhibiting GTP hydrolysis. One of these residues is an arginine, corresponding to R201 in α_s ***(20)***, R179 in α_{i2} ***(21)***, and R183 in α_q ***(22)***, and the other is a glutamine, corresponding to Q227 in α_s ***(23)***, Q205 in α_{i2} ***(21)***, and Q209 in α_q ***(24)***.
2. Transfect and image as previously described in **Subheading 3.2.** and **3.3. Figure 3** shows that replacing R183 in α_q with cysteine (to produce α_qRC-GFP) greatly

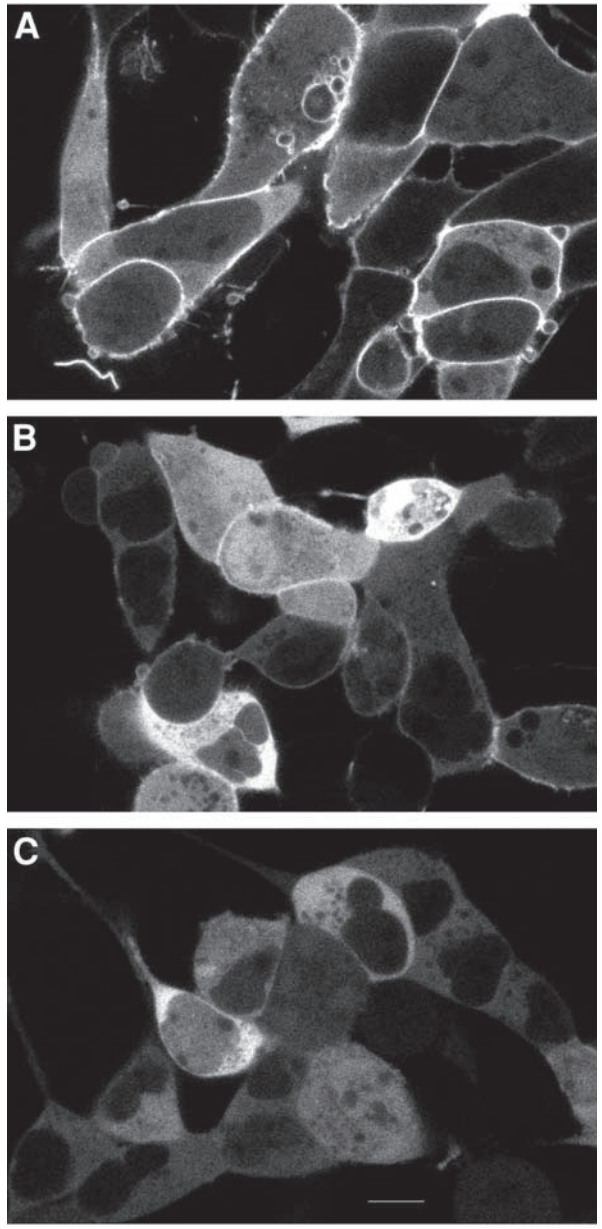

Fig. 3. Confocal imaging of α_q-GFP and α_q-GFP mutants in living cells. HEK-293 cells were transiently transfected with (**A**) α_q-GFP, (**B**) α_qRC-GFP, or (**C**) α_qC9S/C10S-GFP. The cells were imaged with a 100× lens, numerical aperture of 1.2. α_q-GFP exhibits distinct signal in the plasma membrane, as well as in the cytoplasm and the nucleus. The RC mutation greatly reduces the relative amount of signal in the plasma membrane compared to that in the cytoplasm, whereas the C9S/C10S mutations eliminate all detectable plasma membrane signal. Bar = 10 μm. (Reproduced from **ref. *8*** with permission.)

reduces the relative amount of signal in the plasma membrane when compared to the cytoplasm. For comparison, substituting serines for the two palmitoylated cysteines in α_q, C9 and C10, to produce α_qC9S/C10S-GFP, eliminates all detectable plasma membrane signal.

3.5. Imaging of GFP-Tagged α Subunits After Extraction with Triton X-100

1. Using a 20× lens, find a field of transfected cells (*see* **Note 12**).
2. Carefully remove the medium from the plate and replace with 3 mL ice-cold 1% Triton X-100. Wait 2.5 min and image again (*see* **Note 13**). **Figure 4** shows how this method enables a clear distinction to be made between the localization patterns of α_qRC-GFP and α_qC9S/C10S-GFP.

3.6. Testing for Effects of Hormones on the Localization of GFP-Tagged α Subunits in Living Cells (see Note 14)

1. Prepare a plate of cells transfected with a GFP-tagged α subunit, a GPCR that activates the α subunit, and, if possible, an indicator for its activation, such as protein kinase C (PKC)-γ-DsRed1 (BD Biosciences Clontech). For example, for α_q-GFP, transfect with 2 µg α_q-GFP in pcDNAI/Amp, 2 µg α_{2a}-adrenergic receptor in pCMV4 ***(25)***, and 2 µg pPKC-γ-DsRed1.
2. Place the plate of cells on a stage heated to at least 30°C (*see* **Note 15**). Carefully remove 1.5 mL media, leaving 1.5 mL. Start collecting images at 2-s intervals. Stimulate with 0.5 mL 40 µ*M* UK-14,304 for a final concentration of 10 µ*M*, with 0.05% DMSO (*see* **Notes 16** and **17**). Continue to image at 2-s intervals for at least 10 min or as determined by the timing of activation of a downstream indicator (*see* **Note 18**).

3.7. Testing for Effects of AlF_4^- on the Localization of GFP-Tagged α Subunits in Living Cells (see Note 19)

1. Prepare a plate of cells co-transfected with a GFP-tagged α subunit and, if possible, an indicator for its activation (e.g., PKC-γ-DsRed1).
2. Carefully remove the medium and replace with 2.25 mL pre-warmed HEPES-buffered MEM. Allow cells to equilibrate to 37°C on heated stage for 60 min (*see* **Note 20**).
3. Start imaging the cells at 1-min intervals and stimulate with 0.75 mL 4X AlF_4^-. The final concentration is 30 µ*M* $AlCl_3$, 10 m*M* NaF. Continue to collect images every 1 min for 20 min. Then collect another 200 images using 10-s intervals (*see* **Note 21**).

4. Notes

1. If other brands of serum are used, the viability of the cells after transfection may be decreased.
2. Make fresh. NaF tends to fall out of solution over time.
3. Add H_2O carefully to $AlCl_3$ in a fume hood. $AlCl_3$ can react explosively when hydrated. This solution can be stored at 4°C.

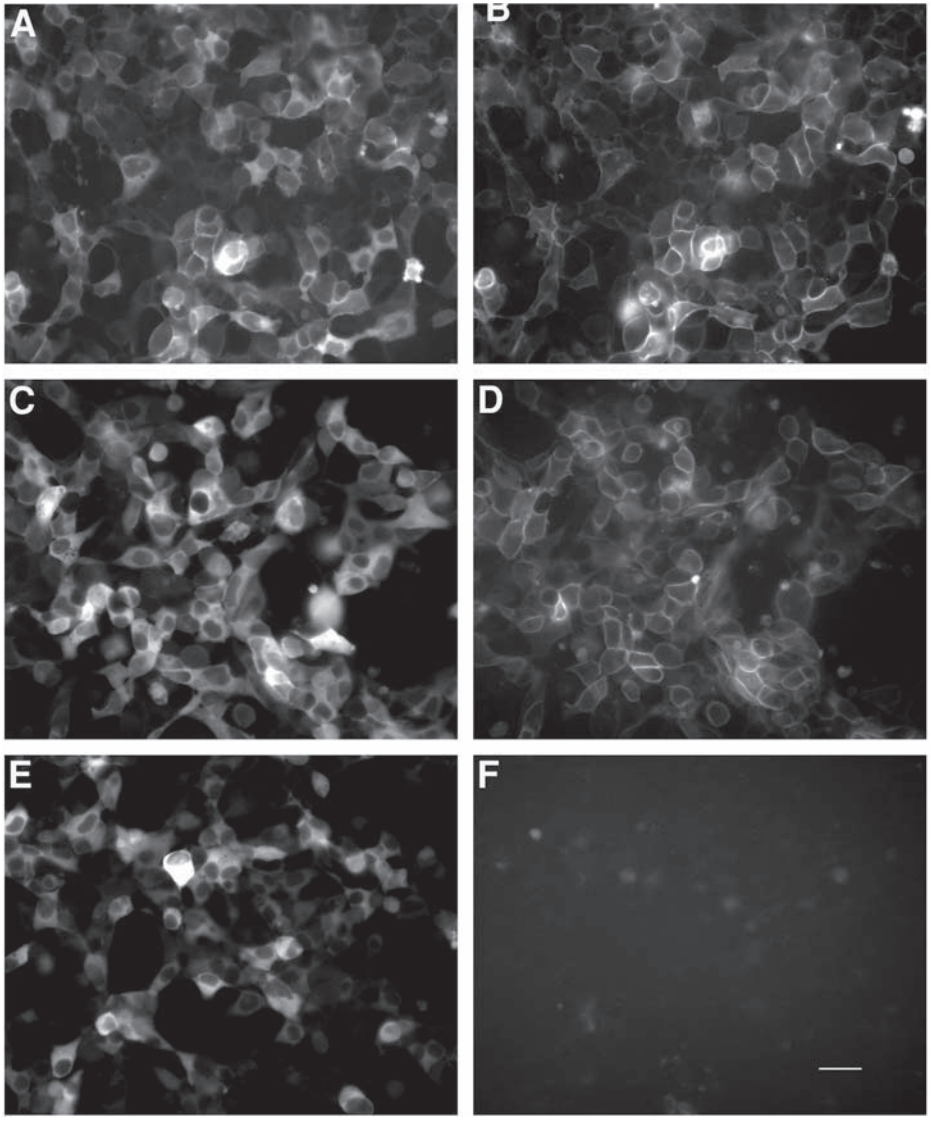

Fig. 4. Imaging of α_q-GFP constructs in living cells and after extraction with 1% Triton X-100. Transiently transfected HEK-293 cells were imaged on an inverted Zeiss microscope before and after the addition of ice-cold 1% Triton X-100. The cells were transfected with plasmid encoding α_q-GFP (**A** and **B**), α_qRC-GFP (**C** and **D**), or α_qC9S/C10S-GFP (**E** and **F**). A, C, and E are images of the living cells, while B, D, and F are images derived from the same cells 2.5 min after the addition of detergent. Although there is much less detectable plasma membrane signal due to α_qRC-GFP than to α_q-GFP in living cells, the signals in the Triton-treated cells are similar. However, although the signals due to α_qRC-GFP and α_qC9S/C10S-GFP are quite similar in living cells, there is no detectable signal after Triton treatment of cells transfected with α_qC9S/C10S-GFP. Two-second acquisition times were used for all

4. An alternative approach is to randomly insert GFP using an in vitro transposition reaction ***(13)***. This method will rapidly create a set of constructs in which GFP is fused within the sequence of the α subunit and is recommended if the GFP insertion sites described in the **Introduction** do not result in a functional α subunit.
5. Bracketing GFP with a unique restriction endonuclease site is desirable so that different fluorescent proteins (i.e., CFP, YFP, DsRed) can be swapped in later. It may be necessary, as was the case with α_s, to eliminate other restriction sites within the α subunit cDNA so that the one used for the GFP insertion is unique.
6. For best results, use cells that are less than passage number 45. At higher passage numbers, viability after transfection may decrease.
7. It is important to optimize the amount of GFP-tagged α subunit plasmid to be transfected and to consider co-transfecting with equal amounts of plasmids encoding β and γ subunits that the α subunit is known to interact with. In the case of α_q-GFP, clear staining of the plasma membrane is seen with up to 2 μg transfected plasmid. However, for α_s-GFP, plasma membrane staining is seen when 0.01–0.1 μg plasmid is used, but when 0.3 μg or more of plasmid is transfected, α_s-GFP is distributed throughout the cytoplasm. The absence of plasma membrane localization at higher doses of α_s-GFP plasmid appears to be the result of insufficient amounts of endogenous βγ, because co-expression of equal amounts of plasmids encoding the G protein subunits β_1 and γ_7, which interact with α_s in HEK-293 cells ***(26,27)***, restores localization to the plasma membrane. A possible explanation for this observation is that α_s is more dependent on βγ than α_q is for plasma membrane localization, because α_q has two palmitoylation sites, whereas α_s has only one.
8. Expression level can be regulated by varying the amount of plasmid used for the transfection. We have found that using 5 μL Lipofectamine 2000 and increasing the amount of α_s-GFP-expressing plasmid between 0.01 and 1 μg results in a higher percentage of transfected cells as well as, on average, a higher level of expression in individual cells.
9. Do not dispense the plasmid-Lipofectamine 2000 mixture all in one place, because this will be toxic to the cells.
10. The optimal imaging time will need to be determined for the particular GFP-tagged α subunit. Forty-eight hours was optimal for α_q-GFP. If the cells are imaged too early, there may not be sufficient time for the GFP-tagged α subunits to reach the plasma membrane. If the cells are imaged too late, the GFP-tagged α subunits may reach excessively high expression levels and aggregate into very bright intracellular structures.
11. It is important to replace bicarbonate-buffered medium with HEPES-buffered medium to keep the pH constant when viewing cells in the room environment

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Fig. 4. *(continued)* images, but considerable variation in signal strength required adjusting the brightness/contrast of the final images by plotting restricted ranges of the pixel values as follows: (A) 400–2000; (B) 400–1000; (C) 400–3000; (D) 400–1500; (E) 300–2500; (F) 300–1250. Bar = 30 μm. (Reproduced from **ref. *8*** with permission.)

because changes in pH can alter localization patterns. Alternatively, the dish can be kept in a 5% CO_2 atmosphere while imaging.

12. Low magnification is preferred because it is easier to stay in focus after removing medium and adding the Triton X-100 solution. It is also easier to do this using a wide-field fluorescence microscope rather than a confocal microscope.
13. We used the same exposure time for image acquisition before and after Triton treatment to make it possible to compare the intensities of the images directly. Most likely, the signal will be reduced after Triton treatment. To enable visualization of all images, restricted ranges of the pixel values can be plotted.
14. It is important to have a positive control to demonstrate activation of the GFP-tagged α subunit on a cell-by-cell basis. In the case of α subunits that activate PLC like α_q, stimulus-dependent translocation of PKC can be monitored *(8)*. PKC can be tagged with a distinguishable fluorescent protein (e.g., DsRed) and monitored in the same cells as the GFP-tagged α subunit. This control is important, because not every cell shows a PKC response.
15. When using an oil immersion objective, it is best to use an objective heater to assure that the sample is maintained at the desired temperature.
16. It is important to be sure that the carrier does not have an effect on its own. For instance, DMSO can cause PKC translocation responses at a concentration of 1%.
17. It is also important to control for hormone-induced cell shape changes if they occur. This can be done by co-transfecting cells with a distinguishable fluorescent protein to control for cell volume changes or with a membrane marker (pECFP-Mem or pEYFP-Mem, BD Biosciences Clontech).
18. In HEK-293 cells transfected with the α_{2a}-adrenergic receptor, α_q-GFP, and PKC-γ-DsRed1, the PKC translocation response to 10 μ*M* UK-14,304 generally peaks at 2–3 min at 30°C. Because the PKC response is downstream of α_q activation, any activation-dependent changes in α_q-GFP localization should be over by the time of the PKC-γ-DsRed1 translocation response.
19. AlF_4^- irreversibly activates α subunits by binding to the GDP-bound form and mimicking the γ phosphate. It activates all heterotrimeric G proteins and can also inhibit phosphatases. However, in HEK-293 cells, translocation of PKC-γ-DsRed1 in response to aluminum fluoride is generally dependent on co-expression of α_q-GFP.
20. Incubating at 37°C is essential. The PKC translocation response to AlF_4^- is not observed at 30°C.
21. The basis for this choice of imaging times is that there is a delay after adding AlF_4^- before PKC responses are seen. The delay usually varies between 10–20 min. Then PKC oscillates in and out of the plasma membrane for at least 50 min. Extended incubations eventually result in cell death.

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