Preface

Since the initial discovery of the G protein-coupled receptor system that regulates cyclicAMP production, the G protein field has rapidly expanded. Cell surface receptors that couple to heterotrimeric G proteins, the G protein-coupled receptors (GPCRs), number in the hundreds and bind to a wide diversity of ligands including, biogenic amines (e.g., adrenaline), lipid derivatives (e.g., lysophosphatidic acid), peptides (e.g., opioid peptides), proteins (e.g., thyroid-stimulating hormone), and odorants to name a few. The GPCR system is found throughout biology in such simple organisms as yeast and in such more complex organisms as *Dictyostelium discoideum* (slime mold), *Caenorhabditis elegans* (nematode worm), and of course in humans. GPCRs and their associated G protein systems are the subject of intense academic research and because of their involvement in a human biology and disease, the pharmaceutical industry has large research initiatives dedicated to the study of GPCRs. By some estimates, more than 50% of the pharmaceuticals on the market are targeted at GPCRs.

The G protein/G protein-coupled receptor system consists of a receptor (GPCR), a heterotrimeric G protein consisting of α, β, and γ subunits, and an effector. G protein effector molecules, such as enzymes or ion channels, respond to activation by the G protein to generate second messengers or changes in membrane potential that lead to alterations in cell physiology. Superimposed on this classical G protein framework are the recently discovered regulators of G protein signaling (RGS proteins) that provide an additional level of G protein regulation, but whose physiological functions remain undefined. The heterotrimeric G proteins themselves form a diverse family of subunits with more than 20 α subunits, 6 β subunits and 13 γ subunits. There are many effectors, each of which may be represented by multiple isoforms. Analysis of such a complex system requires methods that can tackle each of these molecules and the interactions between them in biochemical and cell biological contexts. The variety of strategies that are employed range from studies with purified components to studies in cell biological and whole animal systems. In this volume we have gathered together many of the methodologies that are used to study mechanisms of G protein and G protein coupled receptor function and the roles of G protein subunits in cell biology and disease. Many of the chapters cover topics that use a variety of G protein methodologies, so within single chapters multiple specific methodologies are described in detail. In some cases not only is the method itself described, but the principles that underlie the experimental design are also out-

lined in ways that are not described in other publications. And of course in each chapter there is a Notes section dealing with critical details that are often not included in formal publications owing to space limitations and other issues.

G Protein Signaling: Methods and Protocols has been divided into seven parts somewhat arbitrarily, but based on the general types of systems being used. The first part deals with purification of G proteins and effector enzymes from heterologous expressions systems. To study G proteins and their interactions with receptors and targets it is often necessary to purify the proteins prior to analysis. The second part of the volume has protocols for assays of the interactions between these purified G proteins and effector enzymes. Alternate strategies outlined in Part III are for the study of G protein interactions with effectors in intact cells, either with the endogenous components or with expressed components. Each of these approaches has advantages and disadvantages with regard to data interpretation and analysis. The fourth part is concerned with various assays of G protein coupled receptor structure, function, and localization. The fifth part has protocols for studying the physiological roles for endogenous G proteins, either in cell culture systems or in whole animals, using approaches that inhibit these endogenous systems. Regulators of G protein signaling (RGS) proteins are a novel class of proteins that regulate the activity of heterotrimeric G proteins. Part VI describes methods for studying lipid and phosphate modifications of these proteins. Finally, green fluorescent proteins and their derivatives have been used to study the localization and interactions of many proteins in cells. In Part VII, the specific application of such green fluorescent proteins to G protein signaling systems are described.

The methods outlined in *G Protein Signaling: Methods and Protocols* should be of interest to scientists studying the physiological roles of G protein systems, the signal transduction systems associated with these G protein systems, as well as the molecular nature of the G proteins themselves. The volume contains complementary biochemical, molecular biological, and cell biological approaches to addressing specific questions so that they can be studied from multiple perspectives and adopted by different types of laboratories.

Alan V. Smrcka

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.

From: *Methods in Molecular Biology, vol. 237: G Protein Signaling: Methods and Protocols*
Edited by: A. V. Smrcka © Humana Press Inc., Totowa, NJ

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 $1.5–2 \times 10^6$ 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**).

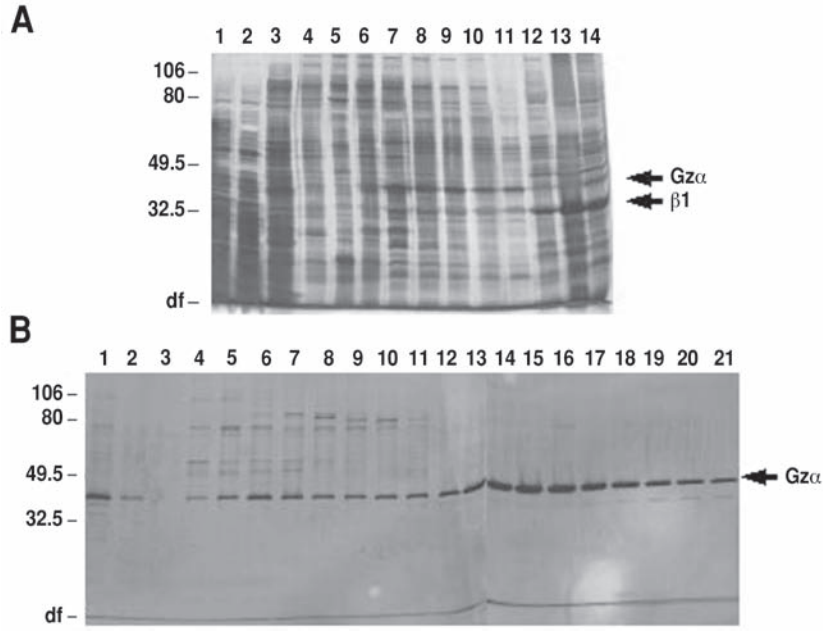

Fig. 1. Purification of $G_z\alpha$. **(A)** Ni-NTA column for purification of $G_z\alpha$. Fractions of 4 µL were subjected to SDS-PAGE and stained by silver nitrate. Lane 1, load; lane 2, flow-through; lanes 3–5, wash with high salt and low imidazole; lanes 6–11, elution with AMF; lanes 12–14, elution 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 Sf9$G_i\alpha$-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**).

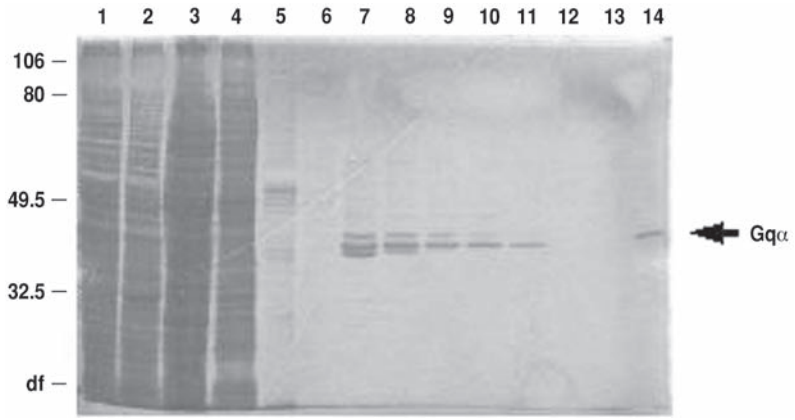

Fig. 2. Purification of $G_q\alpha$. Ni-NTA column for purification of $G_q\alpha$. Fractions of 4 µL were subjected to SDS-PAGE and stained by silver nitrate. In this purification, $G_q\alpha$ was eluted with two steps; first with 1% cholate containing buffer, then with AMF containing buffer. Lane 1, 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.

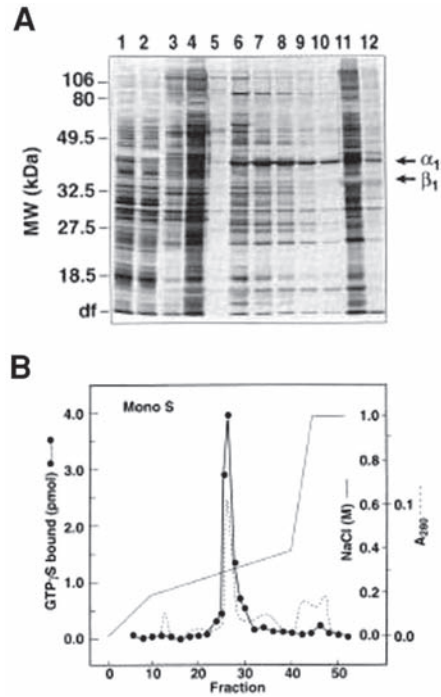

Fig. 3. Purification of $G_{12}\alpha$. **(A)** Ni-NTA column for purification of $G_{12}\alpha$. Fractions of 4 µL were subjected to SDS-PAGE and stained by silver nitrate. Lane 1, load; lane 2, flow-through; lanes 3–5, wash with low imidazole; lanes 6–10, elution with AMF; lanes 11 and 12, elution 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 βγ 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.

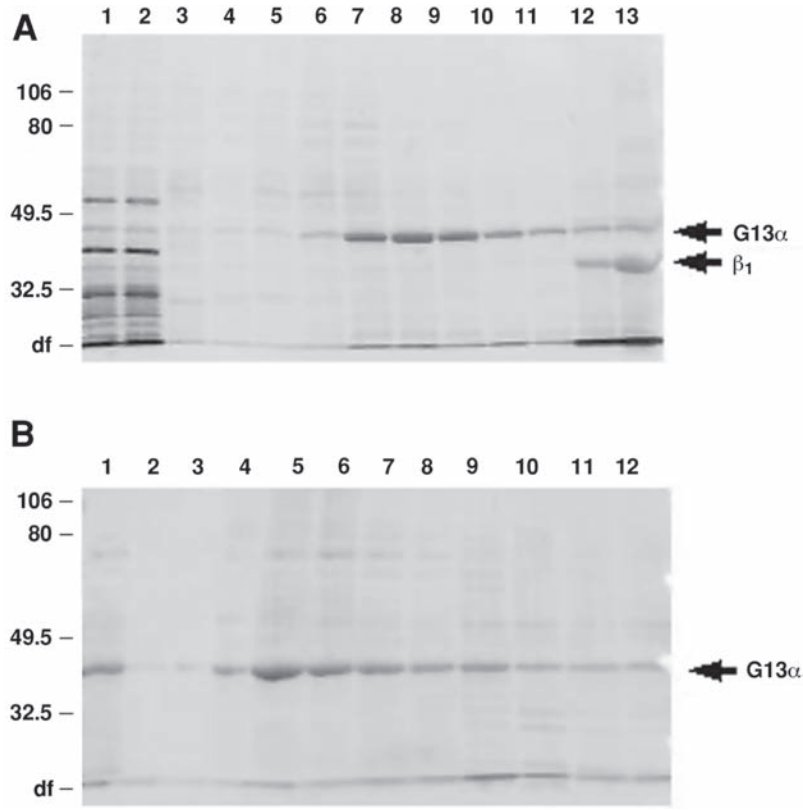

Fig. 4. Purification of $G_{13}\alpha$. **(A)** Ni-NTA column for purification of $G_{13}\alpha$. Fractions of 4 µL were subjected to SDS-PAGE and stained by silver nitrate. Lane 1, load; lane 2, flow-through; lanes 3–5, wash with low imidazole; lanes 6–11, elution with AMF; lanes 12 and 13, elution 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*

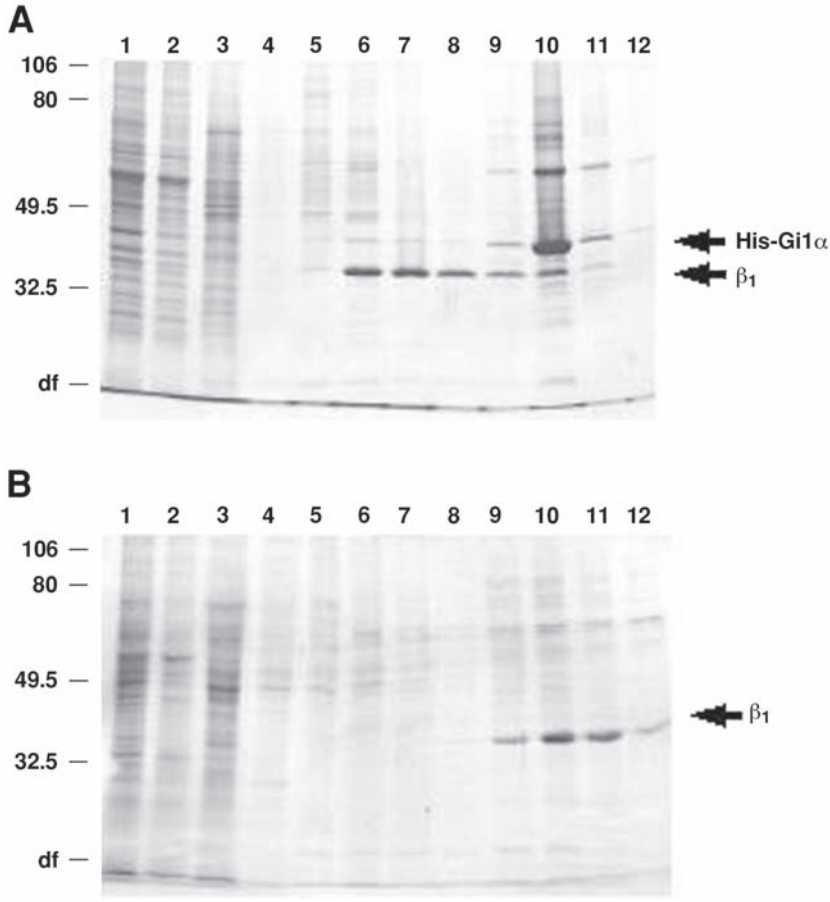

Fig. 5. Purification of βγ subunit.Ni-NTA column for purification of wild-type $\beta_1\gamma_2$ **(A)** or 6-His-$\beta_1\gamma_2$ **(B)** Fractions of 4 µL were subjected to SDS-PAGE and stained by silver nitrate. Lane 1, load; lane 2, flowthrough; lanes 3–5, wash with high salt and low imidazole; 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 Gα, GTP-bound form of Gα 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 βγ 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 βγ 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 βγ 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 βγ subunits that have less affinity for Gα subunits can be purified with this method. Wild-type, nontagged βγ subunit is recommended for receptor reconstitution assays. The imidazole bump fractions from Ni-NTA column of Gα 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.

References

1. Gilman, A. G. (1987) G proteins: transducers of receptor-generated signals. *Annu. Rev. Biochem.* **56,** 615–649.
2. Kaziro, Y., Itoh, H., Kozasa, T., Nakafuku, M., and Satoh, T. (1991) Structures and function of signal-transducing GTP-binding proteins. *Annu . Rev. Biochem.* **60,** 349–400.
3. Neer, E. J. (1995) Heterotrimeric G proteins: organizers of transmembrane signals. *Cell* **80,** 249–257.
4. Hepler, J. R. and Gilman, A. G. (1992) G proteins. *Trends Biochem. Sci.* **17,** 383–387.
5. Hart, M. J., Jiang, X., Kozasa, T., Roscoe, W., Singer, W. D., Gilman, A. G., et al. (1998) Direct stimulation of the guanine nucleotide exchange activity of p115 RhoGEF. *Science* **280,** 2112–2114.
6. Kozasa, T., Jiang, X., Hart, M. J., Sternweis, P. M., Singer, W. D., Gilman, A. G., et al. (1998) p115 RhoGEF is a GTPase activating protein for $G\alpha_{12}$ and $G\alpha_{13}$. *Science* **280,** 2109–2111.
7. Clapham, D. E. and Neer, E. J. (1997) G protein βγ subunits. *Annu. Rev. Pharmacol. Toxicol.* **37,** 167–203.
8. Mumby, S. M. and Linder, M. E. (1994) Myristoylation of G protein α subunits. *Meth. Enzymol.* **237,** 254–268.
9. Iniguez-Lluhi, J. A., Simon, M. I., Robishaw, J. D., and Gilman, A. G. (1992) G protein βγ subunits synthesized in Sf9 cells: Functional characterization and the significance of prenylation of γ. *J. Biol. Chem.* **267,** 23,409–23,417.
10. Hepler, J. R., Kozasa, T., Smrcka, A. V., Simon, M. I., Rhee, S. G., Sternweis, P. C., et al. (1993) Purification from Sf9 cells and characterization of recombinant $G_q\alpha$ and $G_{11}\alpha$: Activation of purified phospholipase C isozymes by Gα subunits. *J.Biol. Chem.* **268,** 14,367–14,375.
11. Kozasa, T., Hepler, J. R., Smrcka, A. V., et al. (1993) Purification and characterization of recombinant $G_{16}\alpha$ from Sf9 cells: activation of purified phospholipase C isozymes by G protein α subunits. *Proc. Natl. Acad. Sci. USA* **90,** 9176–9180.
12. Kozasa, T. and Gilman, A. G. (1995) Purification of recombinant G proteins from Sf9 cells by hexa-histidine tagging of associated subunits: Characterization of α_{12} and inhibition of adenylyl cyclase by α_z. *J. Biol. Chem.* **270,** 1734–1741.
13. Singer, W. D., Miller, T. R., and Sternweis, P. C. (1994) Purification and characterization of the α subunit of G_{13}. *J. Biol. Chem.* **269,** 19,796–19,802.
14. Ueda, N., Iniguez-Lluhi, J. A., Lee, E., Smrcka, A. V., Robishaw, J. D., and Gilman, A. G. (1994) G protein βγ subunits: Simplified purification and properties of novel isoforms. *J. Biol. Chem.* **269,** 4388–4395.
15. Summers, M. D. and Smith, G. E. (1987) *A manual of methods for baculovirus vectors and insect cell culture procedures*, College Station, TX: Texas Agricultural Experiment Station, TX, Bulletin no. 1555.
16. O'Reilly, D. R., Miller, L. K., and Luckow, V. A. (1993) *Baculovirus Expression Vectors. A Laboratory Manual*. W. H. Freeman & Co., New York.

17. Pang, I.-H. and Sternweis, P. C. (1990) Preparation of G proteins and their subunits, in *Receptor-Effector Coupling. A Practical Approach* (Hulme, E. J., ed.), Oxford University Press, Oxford.
18. Gutowski, S., Smrcka, A. V., Nowak, L., Wu, D., Simon, M. I., and Sternweis, P. C. (1991) Antibodies to the α_q subfamily of guanine nucleotide-binding regulatory protein a subunits attenuates activation of polyphosphoinositide 4, 5-bisphosphate hydrolysis by hormones. *J. Biol. Chem.* **266,** 20,519–20,524.
19. Li, Y., Sternweis, P. M., Charnecki, S., Smith, T. F., Gilman, A.G., Neer, E.J., and Kozasa, T. (1998) Sites for Gα binding on the G protein β subunit overlap with sites for regulation of phospholipase Cβ and adenylyl cyclase. *J. Biol. Chem.* **273,** 16,265–16,272.
20. Panchenko, M. P., Saxena, K., Li, Y., Charnecki, S., Sternweis, P., Smith , T. F., et al. (1998) The sides of the G protein βγ subunit propeller structure contain regions important for phospholipase C β2 activation. *J. Biol. Chem.* **273,** 28,298–28,304.
21. Hepler, J. R., Berman, D. M., Gilman, A. G., and Kozasa, T. (1997) RGS4 and GAIP are GTPase-activating proteins for Gqα and block activation of phospholipase Cα by γ-thio-GTP-Gqα. *Proc. Natl. Acad. Sci. USA* **94,** 428–432.
22. Chidiac, P. and Ross, E. M. (1999) Phospholipase C-β1 directly accelerates GTP hydrolysis by Gαq and acceleration is inhibited by Gβγ subunits. *J. Biol. Chem.* **274,** 19,639–19,643.
23. Chidiac, P., Gadd, M. E., and Hepler, J. R. (2002) Measuring RGS protein interactions with Gqα. *Methods Enzymol.* **344,** 686–702.
24. Lindorfer, M. A., Myung, C. S., Savino, Y., Yasuda, H., Khazan, R., and Garrison, J. C. (1998) Differential activity of the G protein β5γ2 subunit at receptors and effectors. *J. Biol.Chem.* **273,** 34,429–34,436.
25. Yoshikawa, D. M., Hatwar, M., and Smrcka, A. V. (2000) G protein β_5 subunit interaction with a subunits and effectors. *Biochemistry* **39,** 11,340–11,347.
26. Posner, B. A., Gilman, A. G., and Harris, B. A. (1999) Regulators of G protein signaling 6 and 7. Purification of complexes with Gβ5 and assessment of their effects on G protein-mediated signaling pathways. *J. Biol. Chem.* **274**, 31,087–31,093.