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G Protein βγ Subunits Synthesized in Sf9 Cells

FUNCTIONAL CHARACTERIZATION AND THE SIGNIFICANCE OF PRENYLATION OF γ*

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Heterotrimeric guanine nucleotide-binding regulatory proteins (G proteins) consist of a nucleotide-binding α subunit and a high-affinity complex of β and γ subunits. There is molecular heterogeneity of β and γ, but the significance of this diversity is poorly understood. Different G protein β and γ subunits have been expressed both singly and in combinations in Sf9 cells. Although expression of individual subunits is achieved in all cases, βγ subunit activity (support of pertussis toxin-catalyzed ADP-ribosylation of $rG_{i\alpha1}$) is detected only when β and γ are expressed concurrently. Of the six combinations of βγ tested (β_1 or β_2 with γ_1, γ_2, or γ_3), only one, $\beta_2\gamma_1$, failed to generate a functional complex. Each of the other five complexes has been purified by subunit exchange chromatography using $G_{o\alpha}$-agarose as the chromatographic matrix. We have detected differences in the abilities of the purified proteins to support ADP-ribosylation of $G_{i\alpha1}$; these differences are attributable to the γ component of the complex. When assayed for their ability to inhibit calmodulin-stimulated type-I adenylylcyclase activity or to potentiate $G_{s\alpha}$-stimulated type-II adenylylcyclase, recombinant $\beta_1\gamma_1$ and transducin βγ are approximately 10 and 20 times less potent, respectively, than the other complexes examined. Prenylation and/or further carboxyl-terminal processing of γ are not required for assembly of the βγ subunit complex but are indispensable for high affinity interactions of βγ with either G protein α subunits or adenylylcyclases.

Guanine nucleotide-binding regulatory proteins (G proteins)[1] transmit signals that are generated by a large number of plasma membrane-bound receptors to intracellular effector enzymes and ion channels (reviewed in Refs. 1–3). G proteins are heterotrimers, composed of a guanine nucleotide-binding α subunit (molecular mass of 39–46 kDa) and a tight complex of β (37 kDa) and γ (8–10 kDa) subunits. The signaling process involves the sequential formation and dissociation of complexes between G protein α and βγ subunits and between G protein subunits and both upstream (receptor) and downstream (effector) elements. This process is driven by the binding of agonist ligands to receptors and by the binding and hydrolysis of GTP by the G protein α subunit (1–4). Receptors catalyze exchange of GDP for GTP on G protein α subunits; activated GTP-bound α then dissociates from the receptor and βγ, and both appear capable of regulating downstream effector molecules. This active state is transient, however, and decays because of the intrinsic GTPase activity of the α subunit. GDP-bound α has a high affinity for βγ, reassociates to form a heterotrimer, and is thus available for further stimulation by receptors.

Most interest in G proteins has been focused on their α subunits, since these proteins bind and hydrolyze GTP and most obviously regulate the activity of the best-studied effectors (cyclic GMP phosphodiesterase and adenylylcyclase). Much less is known about the structure and function of βγ. The βγ subunit complex is essential for functional interactions of G protein α subunits with receptors (5–7). Recent data also suggest that the interaction of βγ with receptors is important for agonist-dependent receptor phosphorylation, suggesting an important role for βγ in desensitization (8). βγ subunits can also regulate effector molecules. Genetic studies of *Saccharomyces cerevisiae* indicate that βγ carries the signal from pheromone receptors to an as yet unidentified downstream effector (9–11). βγ can also regulate adenylylcyclase activity, and the effects of the protein complex differ for individual forms of the enzyme. Whereas βγ inhibits type-I adenylylcyclase when stimulated by either $G_{s\alpha}$ or calmodulin, βγ greatly potentiates the stimulatory effect of $G_{s\alpha}$ on either type-II or type-IV adenylylcyclase (12, 13). Although controversial, βγ may also be involved in the direct or indirect regulation of ion channels (14–16).

There is substantial molecular diversity in both β and γ. Sequences of four distinct mammalian β (17–21) and five γ (22–25) subunits have been reported to date. Although βγ subunit complexes are, to at least a certain extent, interchangeable among different α subunits, the molecular diversity of β and γ demands examination of functional significance. However, it has been very difficult to search for such differences, since preparations of βγ are often heterogeneous and their composition is poorly defined. Nevertheless, some distinctions have been made with purified proteins (26–29). In addition, little is known about the functional importance of the fact that γ subunits are prenylated and processed at

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[1] The abbreviations used are: G protein, guanine nucleotide-binding regulatory protein; $rG_{s\alpha\text{-}s}$, the short form of $G_{s\alpha}$ expressed in *E. coli*; $rG_{i\alpha1}$, the α subunit of G_i expressed in *E. coli*; $G_{o\alpha}$, the α subunit of the major 39-kDa G protein from brain; DTT, dithiothreitol; GTPγS, guanosine 5′-(3-*O*-thio)triphosphate; PAGE, polyacrylamide gel electrophoresis; kb, kilobase pair(s).

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their carboxyl termini in a manner analogous to that described in particular for $p21^{ras}$ (30, 31). To approach these questions, we have synthesized β and γ subunits singly and in combinations in Sf9 cells using recombinant baculovirus. Individual βγ subunit complexes have been purified from this source, and we have initiated their characterization.

MATERIALS AND METHODS

Plasmid Construction—cDNAs encoding G protein β_1, β_2, γ_1, γ_2, γ_2C68S, and γ_3 subunits were transferred to a baculovirus expression vector (pVL1392 or pVL1393) as follows. For the β_1 subunit (18), a 3.0-kb cDNA ligated at the *Eco*RI site of pBluescript (+) was digested with *Asp*700 and *Nco*I. The resulting 1.1-kb fragment containing the β_1 coding sequence was isolated after filling the 3′ recessed terminus generated by *Nco*I. This fragment was transferred to pVL1393 at the *Sma*I site. For the β_2 subunit, the 1.06-kb *Apa*I fragment from the Okayama and Berg vector containing the β_2 cDNA (32) was ligated at the *Apa*I site of pGEM-11Zf(−). The resulting plasmid was digested with *Bam*HI and *Not*I. The 1.08-kb fragment containing the entire coding sequence was then transferred to pVL1393 that had been digested with the same enzymes. For the γ_1 subunit (24), the 0.26-kb *Ava*I/*Asp*700 fragment containing the entire γ_1 sequence was generated from pNG-2-1. This fragment was transferred to pVL1392 that had been digested with *Sma*I and *Not*I. The termini generated by *Ava*I and *Not*I were made complementary by partially filling the *Ava*I site with dTTP and dCTP and the *Not*I site with dGTP. For the γ_2 subunit (25), a 0.425-kb *Bgl*II/*Eco*RI fragment containing the entire coding sequence was isolated from a pBluescript based vector. This fragment was transferred to pVL1392 that had been digested with the same enzymes. The γ_2C68S sequence, in which cysteine 68 has been replaced by serine (33), was subcloned into pVL1392 as was γ_2. The coding region of the bovine γ_3 cDNA (23) was prepared by polymerase chain reaction amplification of reverse transcribed mRNA with the introduction of *Pst*I and *Xba*I sites at the 5′ and 3′ ends, respectively. This fragment was cloned into pVL1392.

Cell Culture and Cloning of Recombinant Baculoviruses—Fall armyworm ovary (Sf9) cells were propagated in IPL-41 medium supplemented with 10% fetal calf serum as described (34). Recombinant baculoviruses were generated by cotransfection of Sf9 cells with the expression vectors described above and with linearized AcRP23-*lacZ* viral DNA by the lipofection method (35). Positive viral clones were isolated by plaque assay and were identified by their ability to direct the expression of the appropriate proteins as revealed by immunoblotting.

Gel Electrophoresis and Immunoblotting—To resolve the γ subunits, SDS-PAGE was carried out in linear gradient gels (10–20% acrylamide; acrylamide:bisacrylamide 26:1) containing 4 M urea. Samples were reduced and alkylated with *N*-ethylmaleimide as described (36). In some cases samples were precipitated with 15% trichloroacetic acid and the pellets washed with cold acetone before reduction and alkylation. Gels were either stained with silver (37) or processed for immunoblotting as described (38). The following antisera were used at the indicated dilutions: β-specific, U-49 1:10,000 (38) and affinity-purified K-521 1:400 (32); γ-specific, affinity-purified NG-1 (23), affinity-purified X-263 (33), and B-53, all at 1:500. B-53 antiserum was generated using a peptide corresponding to the amino-terminal 17 residues of the bovine γ_3 sequence. Detection was achieved using donkey anti-rabbit IgG F(ab′)$_2$ conjugated to horseradish peroxidase and the enhanced chemiluminescence detection system according to the manufacturer's instructions (Amersham Corp.).

Expression of βγ Subunits in Sf9 Cells and Cell Fractionation—Typically, Sf9 cells (100–250 ml at 2×10^6 cells/ml) were infected at a multiplicity of infection of one for each of the indicated viruses. Cells were collected by centrifugation 60 h after infection and were resuspended in a solution containing 20 mM NaHepes (pH 8.0), 2 mM $MgCl_2$, 1 mM EDTA, 2 mM DTT, and protease inhibitors[2] at a density of 25×10^6 cells/ml. After 5 min on ice, cells were homogenized by forcing the cell suspension (six times) through a 25-gauge needle attached to a disposable syringe. The lysate was then centrifuged in a TL-100.3 rotor (Beckman) at 200,000 × *g* for 15 min at 4 °C. The supernatant or cytosolic fraction was removed, and the crude particulate fraction was resuspended in 0.25–0.5 ml of the same buffer. Detergent extracts were made by diluting particulate fractions to a final protein concentration of 5 mg/ml and addition of 10% Lubrol PX or 20% sodium cholate to final concentrations of 0.6 or 1%, respectively. The mixtures were then homogenized by forcing them through a 25-gauge needle (six times). The detergent extracts were collected after centrifugation at 200,000 × *g* for 15 min at 4 °C in a TL-100.3 rotor. Solubilization of total protein approximated 20% with Lubrol PX and 50% with sodium cholate.

Purification of βγ—Sf9 cells (1 liter) were grown to a density of 1×10^6 cells/ml in IPL 41 medium supplemented with 1% fetal calf serum and lipid concentrate (GIBCO). Cultures were coinfected with the appropriate recombinant baculoviruses at a multiplicity of infection of 1 for each virus, and cells were collected 60 h after infection. Unless otherwise indicated all subsequent manipulations were carried out at 4 °C. Preparation of Sf9 cell membranes was carried out essentially as described (34). The cells were lysed in 20 mM NaHepes (pH 8.0), 1 mM EDTA, 1 mM EGTA, 5 mM $MgCl_2$, 2 mM DTT, 150 mM NaCl, and protease inhibitors, and the membrane pellets were washed and resuspended in 20 mM NaHepes (pH 8.0), 2 mM $MgCl_2$, 1 mM EDTA, 2 mM DTT, and protease inhibitors. Membranes (typically 30 mg) were solubilized in the same buffer containing 1% sodium cholate at a protein concentration of 5 mg/ml. Functional βγ complexes were purified by affinity chromatography using a 10-ml column of bovine brain $G_{o\alpha}$-agarose. Purified bovine brain $G_{o\alpha}$ (20 mg) was coupled to 10 ml of ω-aminobutyl-agarose by the method of Sternweis *et al.* (30). The concentration of βγ binding sites on the resulting resin was approximately 0.6 μM. (We have observed, however, that the binding capacity of the column is lower when crude detergent extracts are loaded.) The resin was packed in a Pharmacia XK-26–20 column fitted with flow adapters and was used in a fast protein liquid chromatography system. Approximately 6 nmol of βγ activity (determined by support of ADP-ribosylation (see below), using purified bovine brain βγ as a standard) was diluted to 10 ml with 20 mM NaHepes (pH 8.0), 400 mM NaCl, 1 mM EDTA, 2 mM DTT, 5 μM GDP (buffer A), and 0.5% Lubrol PX and applied at a rate of 0.3 ml/min to the $G_{o\alpha}$-agarose, which had been equilibrated in the same buffer. After washing the column with 20 ml of buffer A containing 0.5% Lubrol PX, the flow was increased to 3 ml/min and the concentration of Lubrol PX in the wash buffer was reduced to 0.05% over 20 ml. The resin was washed with 200 ml of buffer A containing 0.05% Lubrol PX. Elution was then achieved with 20 mM NaHepes (pH 8.0), 400 mM NaCl, 2 mM DTT, 5 μM GDP, 30 μM $AlCl_3$, 20 mM $MgCl_2$, 10 mM NaF (buffer B), and 0.05% Lubrol PX. Fractions (10 ml) were collected at a rate of 0.3 ml/min. To increase the rate of dissociation of βγ during the elution, the resin was warmed to approximately 15 °C with a water jacket. Fractions containing βγ (judged by SDS-PAGE and silver stain) from two or three chromatographic runs were pooled (100–200 ml) and concentrated in a PM-10 Amicon ultrafiltration device to a volume of 10–20 ml. This material was loaded onto a 1-ml hydroxylapatite column previously equilibrated with 20 mM NaHepes (pH 8.0), 100 mM NaCl, 2 mM DTT, and 0.05% Lubrol PX. The column was then washed with 5 ml of the same buffer and βγ was eluted in 1-ml fractions with 5 ml of the same buffer containing 50 mM potassium phosphate. Fractions containing βγ were pooled and subjected again to chromatography on $G_{o\alpha}$-agarose. The pooled fractions were concentrated and subsequently adsorbed to hydroxylapatite and eluted as described above. The final preparations were diluted appropriately to achieve a βγ concentration of 0.5 μM (based on an Amido Black protein assay (39) using bovine serum albumin as standard) and a Lubrol PX concentration of 0.025%. The preparations were stored at −80 °C. The final yields varied from 50 to 200 μg of βγ, depending on the particular complex that was synthesized. Endogenous βγ from Sf9 cells was purified as described above from membranes of cells infected with control (Lac-Z) virus encoding β-galactosidase. Bovine brain βγ was purified as described by Sternweis *et al.* (36) and corresponds to a pool from the second heptylamine-Sepharose column in the presence of Al^{3+}, Mg^{2+}, and F^-. Transducin βγ was a generous gift of Dr. Susanne Mumby. Both bovine brain and transducin βγ were subjected to $G_{o\alpha}$-agarose chromatography, followed by hydroxylapatite chromatography, as described.

Partial Purification of $\beta_1\gamma_2$C68S—A cytosolic fraction (10 ml, 2 mg/ml) from Sf9 cells infected with recombinant baculoviruses encoding β_1 and γ_2C68S was loaded onto a Pharmacia Mono-Q HR 5/5 column equilibrated with 50 mM Tris-HCl (pH 8.0), 1 mM EDTA, and 2 mM DTT. After washing the column with 5 ml of the same buffer, proteins were eluted with a linear gradient of NaCl (0–350

[2] 16 μg/ml each 1-1-tosylamido-2-phenylethyl chloromethyl ketone, 1-chloro-3-tosylamido-7-amino-2-heptanone, and phenylmethylsulfonyl fluoride; 3.2 μg/ml each leupeptin and soybean trypsin inhibitor; and 1 μg/ml aprotinin.

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mM). Fractions containing $\beta_1\gamma_2$C68S (eluting at approximately 150 mM NaCl) were pooled and concentrated. The amount of βγ in the pool was estimated by quantitative immunoblotting, using purified $\beta_1\gamma_2$ as a standard. The expressed protein constituted approximately 20% of the total protein in the pool.

Support of ADP-ribosylation and Adenylylcyclase Assays—The capacity of βγ to support the ADP-ribosylation of $rG_{i\alpha1}$ was assessed as described, with some modifications (40). The final lipid concentration was increased to 1 mM, and purified $rG_{i\alpha1}$ (20 pmol/assay) was used as a substrate. The use of a high concentration of substrate devoid of any endogenous βγ allowed the reliable quantitation of 50 fmol of βγ activity (1.2 nM final concentration in the assay), even in crude detergent extracts. To assay column fractions containing Al^{3+}, Mg^{2+}, and F^-, the $MgCl_2$ in the reaction mixture was replaced with EDTA to yield a final concentration of EDTA that was at least 1 mM in excess of the concentration of $MgCl_2$ contributed by the sample. This change has essentially no effect on the kinetics of the reaction (26). For adenylylcyclase assays, membranes from Sf9 cells infected with recombinant baculoviruses encoding type-I or type-II adenylylcyclase were prepared as described (12). Purified recombinant *Drosophila* calmodulin and $rG_{s\alpha\text{-}s}$ were provided by Dr. Wei-Jen Tang. $rG_{s\alpha\text{-}s}$ was activated with GTPγS for 30 min at 30 °C in 20 mM NaHepes (pH 8.0), 1 mM EDTA, 1 mM DTT, 10 mM $MgSO_4$, and 100 μM GTPγS. Free nucleotide was removed by gel filtration. Membranes containing type-I or type-II adenylylcyclase were incubated with Ca^{2+}/calmodulin or GTPγS-$rG_{s\alpha\text{-}s}$, respectively, for 10 min at 30 °C. Aliquots (10 μl) were then distributed to tubes containing the indicated amounts of purified βγ (10 μl). The reactions were initiated by the addition of 80 μl of the remaining reaction components (12). After 10 min at 30 °C, the reactions were terminated and cyclic AMP was quantified by the method of Salomon (41). The final concentrations of activators were 50 μM $CaCl_2$ and 100 nM calmodulin for type-I adenylylcyclase and 50 nM GTPγS-$rG_{s\alpha\text{-}s}$ for type-II adenylylcyclase.

Gel Filtration Chromatography—Cytosolic fractions and cholate extracts of membranes were prepared as described above from Sf9 cells independently expressing β_1 or γ_2C68S as well as from cells expressing both subunits. Before chromatography, cytosolic fractions were adjusted to 1% sodium cholate. Aliquots (200 μl; 0.5–1 mg of protein) of samples containing β_1 (cholate extract), γ_2C68S (cytosol), or $\beta_1\gamma_2$C68S (cytosol) were injected onto a Pharmacia Superose 12 HR 10/30 gel filtration column that had been equilibrated with 20 mM NaHepes (pH 8.0), 2 mM $MgCl_2$, 150 mM NaCl, 1 mM EDTA, 2 mM DTT, and 1% sodium cholate. Proteins were eluted at a rate of 0.3 ml/min, and 0.5-ml fractions were collected (starting 2 ml after injection of the sample). Aliquots (100 μl) of selected fractions were precipitated with 15% trichloroacetic acid, and 25% of each sample was subjected to SDS-PAGE and immunoblotting as described above. β and γ subunits were identified with antisera U-49 and X-263, respectively. The intensities of bands were quantified with a laser densitometer.

RESULTS

Expression of G Protein βγ Subunits in Sf9 Cells—Concurrent infection of Sf9 cells with recombinant baculoviruses encoding the G protein β_2 and γ_2 subunits leads to a substantial, time-dependent increase in βγ subunit activity as assessed by the capacity of detergent extracts of cell membranes to support the ADP-ribosylation of $rG_{i\alpha1}$ (Fig. 1). No such activity is detected when either subunit is expressed alone or when the cells are infected with a control virus encoding β-galactosidase (Lac-Z). The lack of activity when each subunit is expressed independently is not due to a failure to synthesize the polypeptides, since expression of the subunits was confirmed by SDS-PAGE and immunoblotting (Fig. 1, *inset*). It thus appears that this characteristic βγ activity is dependent on both subunits. We have not been successful in attempts to reconstitute activity by mixing detergent extracts from cells expressing each subunit independently (data not shown).

Little is known about the selectivity of interactions between different β and γ subunits. However, by coinfecting Sf9 cells with β_1- or β_2-encoding viruses and either γ_1, γ_2, or γ_3 viruses we have tested the ability of these six combinations to yield a complex capable of supporting the ADP-ribosylation of

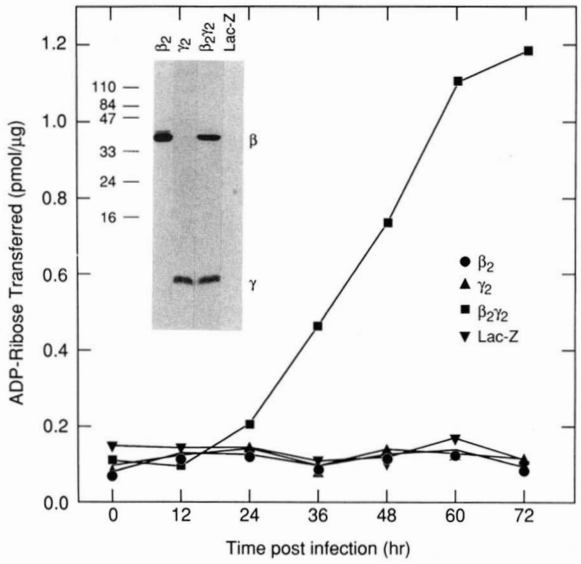

FIG. 1. **Expression of β and γ subunits in Sf9 cells.** Cultures (100 ml) of Sf9 cells (1×10^6 cells/ml) were infected at a multiplicity of infection of 1 with control (Lac-Z) virus (▼), with recombinant baculoviruses encoding G protein β_2 (●) or γ_2 (▲) subunits, or with both β_2 and γ_2 viruses (■). At the indicated times after infection, 10-ml aliquots were withdrawn and 0.6% Lubrol PX extracts of crude particulate fractions were obtained and assayed (0.5 μg of protein) for support of ADP-ribosylation activity as described under "Materials and Methods." *Inset*, 20 μg of crude particulate fractions from cells collected 72 h after infection were subjected to SDS-PAGE and immunoblotting as described under "Materials and Methods." The *upper* and *lower* sections of the blot were separated and probed with affinity-purified K-521 and X-263 antisera, respectively. The migrations of molecular weight standards and of the β and γ subunits are indicated at the *left* and *right*, respectively.

$rG_{i\alpha1}$. All combinations tested led to the accumulation of such activity except that of the retinal γ_1 subunit with the nonretinal β_2 subunit. As shown in Fig. 2*A*, ADP-ribosylation activity is present in detergent extracts of Sf9 cells when γ_1 is expressed with the retinal β_1 subunit. However, no activity is detected when γ_1 is expressed with β_2. Immunoblot analysis of crude particulate fractions and of detergent extracts from cells infected with $\beta_1\gamma_1$ and $\beta_2\gamma_1$ (Fig. 2*B*) reveals that all subunits are synthesized. For cells expressing β_1 and γ_1, the detergent extract contains immunoreactivity for both β and γ. In contrast, little or no β_2 immunoreactivity is detected in extracts from cells expressing β_2 and γ_1, although γ can be solubilized efficiently. This extraction behavior is similar to that observed when the subunits are expressed alone (data not shown). The results suggest that γ_1 fails to form a functional complex with β_2 under these conditions, although it can do so with β_1. For the β and γ combinations that yield functional complexes, we estimate that active βγ comprises from 0.5 to 2% of the protein present in the cholate extract, depending on the particular combination.

Purification of βγ Complexes—To perform careful comparisons of the properties of different βγ subunit complexes, it is necessary to purify each of these in a functional form. To achieve this we have adapted a powerful affinity purification scheme based on the immobilized bovine brain $G_{o\alpha}$-agarose matrix originally described by Sternweis *et al.* (30). This method allows the selective purification of βγ complexes that can associate stably but reversibly with $G_{o\alpha}$. To minimize copurification of endogenous Sf9 cell βγ species, extracts were prepared under conditions that do not favor dissociation of the endogenous heterotrimeric G proteins. An example of preparation of $\beta_1\gamma_2$ by $G_{o\alpha}$-agarose chromatography is shown

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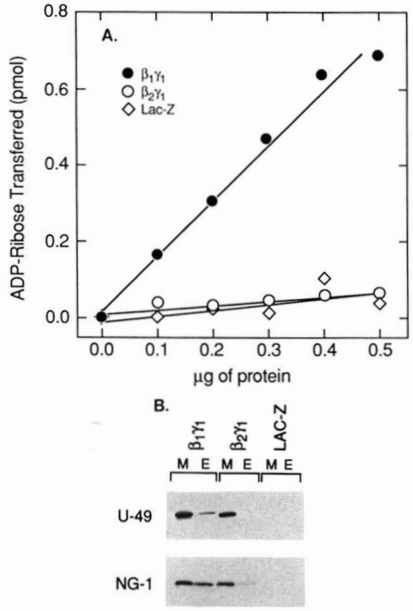

FIG. 2. **Concurrent expression of γ_1 with β_1 or β_2 subunits.** *A*, the indicated amounts of cholate extract protein derived from membranes of Sf9 cells were assayed for support of ADP-ribosylation activity as described under "Materials and Methods." Cells were infected with control (Lac-Z) virus (◇) or were infected concurrently with β_1 and γ_1 viruses (●) or with β_2 and γ_1 viruses (○). *B*, membranes (*M*) (1 μg, *upper panel*; 10 μg, *lower panel*) and cholate extracts (*E*) (5 μg, *both panels*) of membranes from Sf9 cells infected with the indicated viruses were subjected to SDS-PAGE and immunoblotting as described under "Materials and Methods." The *upper* and *lower panels* were probed with affinity-purified K-521 and NG-1 antisera, respectively. Only the regions of the blot showing immunoreactivity are shown. The amount of membranes used in the *upper panel* was reduced because of the large amount of β immunoreactivity.

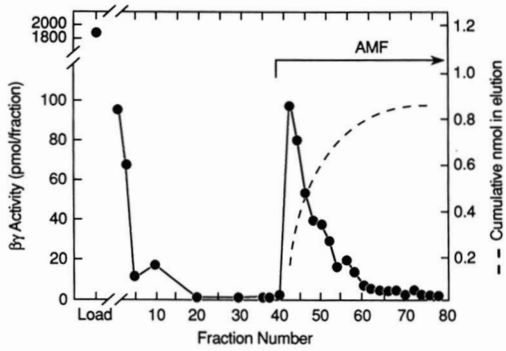

FIG. 3. **Purification of $\beta_1\gamma_2$ by $G_{o\alpha}$-agarose chromatography.** An aliquot (1.5 ml, 1.3 mg/ml) of a 1%-cholate extract of membranes from Sf9 cells infected with β_1 and γ_2 viruses was diluted to 5 ml with buffer A containing 0.5% Lubrol PX and was added to 5 ml of $G_{o\alpha}$-agarose. After a 1-h incubation at 4 °C with gentle mixing, the resin was packed into a column and drained. The resulting flowthrough was reapplied to the column and collected as *fraction 1*. The column was then washed with 50 ml of buffer A containing 0.5% Lubrol PX (*fractions 2–11*), followed by 50 ml of buffer A containing 0.1% Lubrol PX (*fractions 12–21*) and by 65 ml of buffer A containing 0.05% Lubrol PX (*fractions 22–35*). βγ was eluted at room temperature with buffer B containing 0.05% Lubrol PX, and 1.5-ml fractions were collected. The fractions were assayed for support of ADP-ribosylation activity as described under "Materials and Methods." A standard curve (using purified bovine brain βγ) was used to estimate the amount of βγ in each fraction. The *dashed line* indicates the cumulative amount of βγ activity recovered in the elution. In a similar experiment using an equivalent amount of cholate extract from cells infected with control (Lac-Z) virus, the total yield of βγ activity in the elution was 45 pmol.

in Fig. 3. Typical recoveries of βγ activity were in the range of 50–60%. Although substantial purification is achieved by $G_{o\alpha}$-agarose chromatography followed by concentration on a hydroxylapatite column, more highly purified preparations were obtained when the material was subjected to $G_{o\alpha}$-agarose chromatography a second time. Largely purified preparations of βγ from bovine brain and bovine rod outer segments were also purified further by this method to select for functionally active material. We have also purified small amounts of βγ from Sf9 cells infected with a control virus (Lac-Z).

The different preparations of βγ have been compared by SDS-PAGE and silver staining and by immunoblot analysis using various selective antisera (Fig. 4). Endogenous Sf9 cell βγ can be distinguished by the faster migration of the insect γ subunit (Fig. 4*A*); this band is not detected in the other preparations purified from Sf9 cells. The mobilities of γ subunits are anomalous in this SDS-PAGE system. Based on the predicted molecular weights of the fully processed γ_1, γ_2, and γ_3 subunits of 8500, 7900, and 8300, respectively, the γ_1 subunit should have the slowest mobility; by contrast, it has the fastest. As expected, the γ subunit of transducin comigrates with the γ subunit of the $\beta_1\gamma_1$ complex, and they are both poorly stained with silver. The immunoblotting results shown in Fig. 4*B* indicate that the pattern of immunoreactivity of β subunits is consistent with the reactivity of U-49 (β_1-specific) and affinity-purified K-521 (β_1- and β_2-reactive) anti-

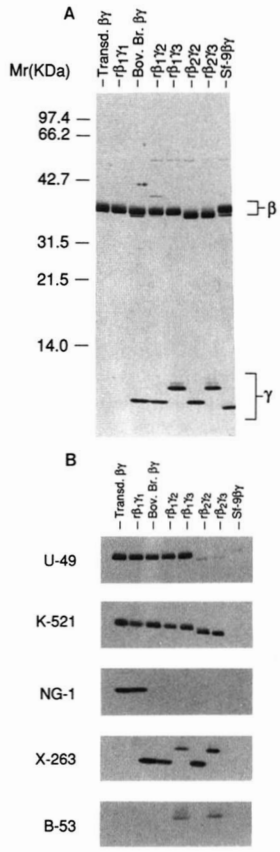

FIG. 4. **Silver stains and immunoblots of purified βγ preparations.** Each of the purified βγ preparations (2.5 pmol) was subjected to SDS-PAGE as described under "Materials and Methods." One of the gels was stained with silver (*panel A*). The migrations of molecular weight standards and of the β and γ subunits are indicated at the *left* and *right*, respectively. γ_1 Subunits migrate slightly faster than do γ_2 and stain poorly with silver. Identical gels were transferred to nitrocellulose and probed with β-specific antisera U-49 and K-521 or with γ-specific antisera NG-1, X-263, and B-53, as described under "Materials and Methods." The areas of immunoreactivity for each antiserum are shown in *panel B*.

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sera. The Sf9 cell βγ preparation reacts only weakly with both antisera. The affinity-purified NG-1 antiserum raised against a peptide sequence unique to γ_1 recognizes only the γ subunits of transducin and $\beta_1\gamma_1$. As anticipated, based on the amino acid sequence of the peptide used as the antigen (30), affinity-purified antiserum X-263 reacts with both γ_2 and γ_3 subunits. Antiserum B-53 recognizes only γ_3. None of the antibodies recognize the Sf9 cell γ polypeptide. Based on silver staining and immunoblotting, the bovine brain βγ preparation is most probably composed largely of β_1 and γ_2 subunits.

Support of ADP-ribosylation by Purified βγ Preparations—ADP-ribosylation of G protein α subunits by pertussis toxin is a sensitive means to monitor interactions between α and βγ, since the heterotrimeric G protein is the preferred substrate for the toxin. As an initial screen to evaluate potential functional differences between individual βγ subunits, we have determined the ability of the purified βγ preparations to support catalytically the ADP-ribosylation of $rG_{i\alpha1}$ and bovine brain $G_{o\alpha}$. When $rG_{i\alpha1}$ is used as substrate, the rate of ADP-ribosylation is substantially different for the different βγ complexes. Irrespective of the β subunit, the highest rates are observed for the complexes containing γ_2, while the lowest rates are observed with γ_1 (including transducin βγ). Intermediate rates are observed for $\beta_1\gamma_3$ and $\beta_2\gamma_3$. The largest difference observed was approximately 6-fold. When bovine brain $G_{o\alpha}$ is used as substrate (Fig. 5*B*), the pattern is different, and the largest difference in rates is only 2-fold. ADP-ribosylation is most rapid with transducin βγ, and the lowest rate is observed with $\beta_2\gamma_3$. There is no clear grouping based on γ subunit composition. It thus appears that G protein α subunits can discriminate between different βγ complexes. However, we have not yet addressed whether the differences observed are due to intrinsic differences between the two α subunits or to the fact that the recombinant protein ($G_{i\alpha1}$) is not myristoylated. In addition, although this assay is simple technically and very sensitive, it is complex mechanistically and does not allow a straightforward assessment of the relative affinities of α for βγ (see below).

Effect of Different βγ Complexes on Type-I and Type-II Adenylylcyclase Activity—Type-I adenylylcyclase is activated by either Ca^{2+}/calmodulin or $G_{s\alpha}$; such activation can be partially reversed by βγ in a noncompetitive fashion (12). By contrast, when type-II adenylylcyclase is stimulated by $G_{s\alpha}$, further addition of βγ causes a large potentiation of $G_{s\alpha}$-stimulated activity (12). We examined the ability of the different βγ complexes to inhibit Ca^{2+}/calmodulin-stimulated type-I adenylylcyclase (Fig. 6). All preparations can inhibit type-I adenylylcyclase activity. However, $\beta_1\gamma_1$ and transducin βγ are approximately 10 and 20 times, respectively, less potent than the other βγ preparations. The difference is thus attributable to the γ subunit. The bovine brain preparation behaves essentially as does its recombinant counterpart, $\beta_1\gamma_2$. The pattern was very similar when we examined the effect of the different βγ preparations on $rG_{s\alpha\text{-}s}$-stimulated type-II adenylylcyclase (Fig. 7); again, transducin βγ and $\beta_1\gamma_1$ are approximately 10 and 20 times less potent than the other complexes, and $\beta_1\gamma_2$ is very similar to bovine brain βγ.

Effects of Prenylation on βγ Function—We have compared the properties of the wild type γ_2 subunit with those of a mutant γ_2 in which the cysteine four amino acid residues from the carboxyl terminus has been changed to serine (γ_2C68S). This cysteine residue is the presumed site of prenylation of γ, and this mutant protein cannot be prenylated *in vivo* (33, 42). When both wild type and mutant γ_2 subunits are coexpressed with β_1, ADP-ribosylation activity is detected only in detergent extracts of Sf9 cells infected with wild type γ_2 (Fig. 8*A*). In a mammalian cell expression system, coexpression of γ_2C68S with β_1 causes distribution of β to the cytosolic fraction. SDS-PAGE and immunoblot analysis of Sf9 cell fractions (Fig. 8*C*) indicates that substantial γ_2 immunoreactivity is found in the cholate extract of membranes, with little or no reactivity in the cytosolic fraction. By contrast, little or no γ_2C68S is found in the cholate extract, whereas substantial immunoreactivity is present in the cytosol. Furthermore, coinfection of γ_2C68S with β_1 leads to a

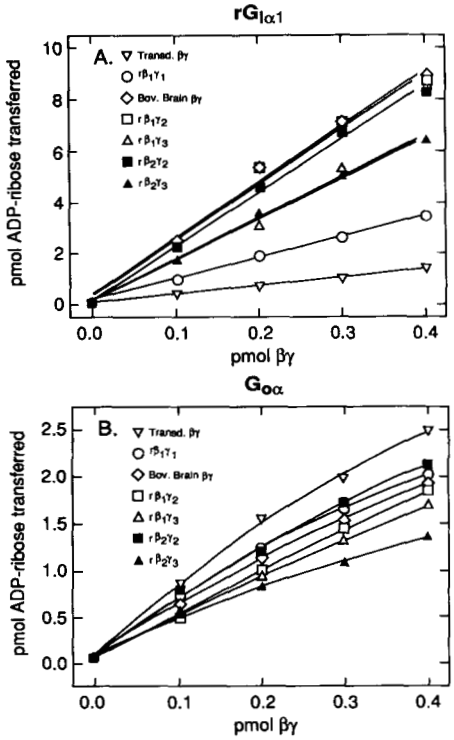

FIG. 5. **Support of ADP-ribosylation of $rG_{i\alpha1}$ and bovine brain $G_{o\alpha}$ by purified βγ preparations.** The indicated amounts of βγ were assayed for support of ADP-ribosylation activity as described under "Materials and Methods" using 20 pmol of $rG_{i\alpha1}$ (*panel A*) or bovine brain $G_{o\alpha}$ (*panel B*) as substrates.

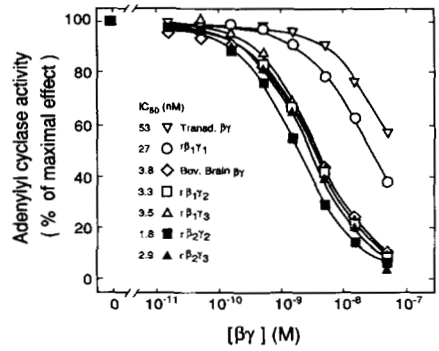

FIG. 6. **Inhibition of Ca^{2+}/calmodulin-stimulated type-I adenylylcyclase by purified βγ preparations.** Aliquots (10 μg) of Sf9 cell membranes containing type-I adenylylcyclase were assayed for 10 min in the presence of 100 nM calmodulin, 50 μM $CaCl_2$, and the indicated concentrations of the purified preparations of βγ. Values are expressed as a percent of the maximal calmodulin-stimulated activity in the absence of βγ. Data from duplicate determinations of two to four independent experiments are included. The average basal and calmodulin-stimulated activities were 0.4 and 5.1 nmol of cyclic AMP × min^{-1} × mg^{-1}, respectively. The *curves* shown are the nonlinear least-squares fits to the four parameter logistic equation $Y = (A/(1 + (C/X)^B)) + D$, and the derived IC_{50} values are indicated in the *inset*.

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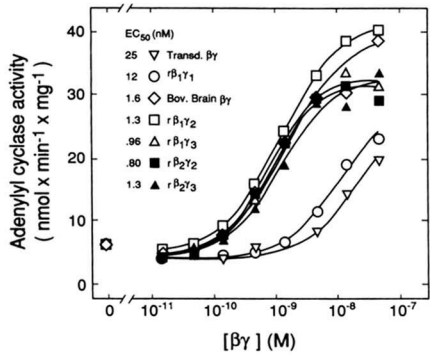

FIG. 7. **Potentiation of $rG_{s\alpha\text{-}s}$-stimulated type-II adenylylcyclase activity by purified βγ preparations.** Aliquots (1 μg) of Sf9 cell membranes containing type-II adenylylcyclase were assayed for 10 min in the presence of 50 nM GTPγS-$rG_{s\alpha\text{-}s}$ and the indicated concentrations of the different purified βγ preparations. Data shown are the averages of duplicate determinations from a single experiment, which is representative of three such experiments. The *curves* shown are the nonlinear least-squares fits to the four parameter logistic equation $Y = (A/(1 + (C/X)^B)) + D$, and the derived EC_{50} values are indicated in the *inset*.

shift in the distribution of β from the cholate extract to the cytosolic fraction. This pattern is in agreement with the results obtained in the mammalian system (33). Since most of the γ_2C68S is located in the Sf9 cell cytosol, we tested this fraction for support of ADP-ribosylation (Fig. 8*B*). There is no cytosolic activity from cells expressing either wild type γ_2 or γ_2C68S. Distribution of β to the cytosol when expressed with γ_2C68S suggests that unprenylated γ_2 is still associated with β. To examine this possibility, we have analyzed the behavior of β and γ_2C68S subunits during gel filtration chromatography. When a cytosolic fraction from cells infected with β_1 and γ_2C68S viruses is fractionated on a Superose 12 gel filtration column, β_1 and γ_2C68S coelute at a position consistent with the formation of a complex (Fig. 9). Furthermore, $\beta_1\gamma_2$C68S elutes before γ_2C68S, as determined by gel filtration of a cytosolic fraction from cells expressing γ_2C68S alone (Fig. 9). β_1 and γ_2C68S also copurify during anion exchange chromatography of a cytosolic fraction from cells expressing both subunits, again consistent with the existence of a complex between the subunits (data not shown). Taken together, these data indicate that prenylation and subsequent carboxyl-terminal processing are not required for interaction between β and γ.

The failure of $\beta_1\gamma_2$C68S to support ADP-ribosylation of $rG_{i\alpha1}$ could be due to a very low affinity of this complex for G protein α subunits or to the formation of a complex that is not a substrate for ADP-ribosylation by pertussis toxin. We thus subjected a cytosolic fraction from cells coexpressing β_1 and γ_2C68S and a cholate extract from cells expressing the wild type complex to $G_{o\alpha}$-agarose chromatography (Fig. 10). The wild type complex binds stably to immobilized $G_{o\alpha}$ and is eluted when the α subunit is activated with Al^{3+}, Mg^{2+}, and F^-. In contrast, the mutant complex flows through the column. These data indicate that $\beta_1\gamma_2$C68S has a much lower affinity for the GDP-bound form of $G_{o\alpha}$ than does its wild type counterpart. This result also indicates that $G_{o\alpha}$-agarose chromatography selects for βγ complexes that have been properly prenylated.

Although γ subunit prenylation appears to be essential for interaction of βγ with α, this mutant complex might still interact productively with effectors such as adenylylcyclase. We have therefore examined the ability of partially purified $\beta_1\gamma_2$C68S to regulate adenylylcyclase activity (Fig. 11). High concentrations of $\beta_1\gamma_2$C68S do not inhibit calmodulin-stimulated type-I adenylylcyclase activity, nor do they potentiate $G_{s\alpha}$-stimulated type-II adenylylcyclase activity. Furthermore, the mutant complex fails to diminish the effects of wild type βγ when the mutant is in 100-fold excess. Thus, it appears that γ subunit prenylation and/or additional carboxyl-terminal processing of the protein are not required for interaction between β and γ; however, at least certain of these modifications are essential for other important protein-protein interactions: those between βγ and α and between βγ and adenylylcyclase.

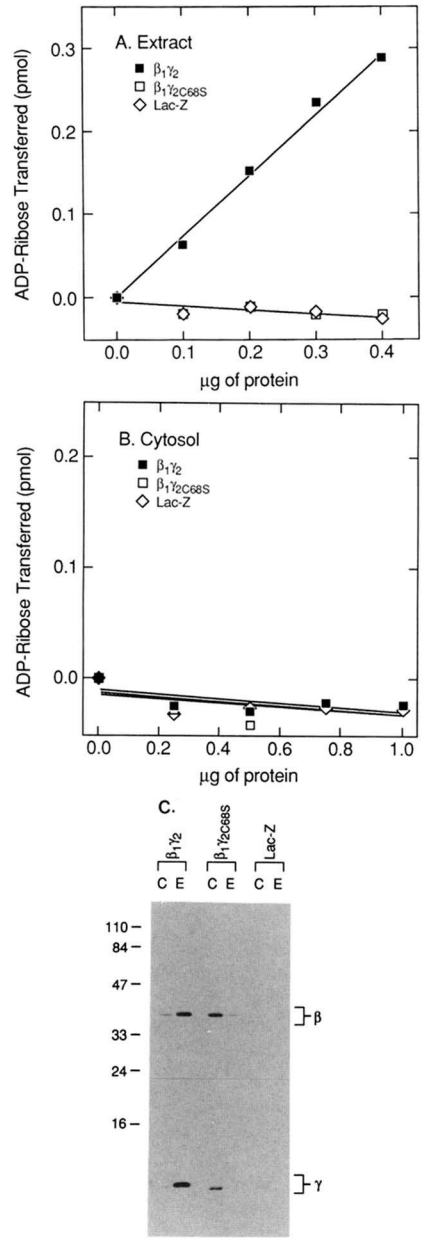

FIG. 8. **Support of ADP-ribosylation by $\beta_1\gamma_2$ and $\beta_1\gamma_2$C68S.** Cytosolic fractions as well as 1%-cholate extracts from crude particulate fractions were obtained from Sf9 cells infected with control (Lac-Z) virus (◇) or from cells coinfected with β_1 and γ_2 viruses (■) or with β and γ_2C68S viruses (□). The indicated amounts of cholate extract (*panel A*) or cytosolic protein (*panel B*) were assayed for support of ADP-ribosylation activity as described under "Materials and Methods." *C*, for each infection, cytosolic protein (30 μg, *C*) and cholate-extract protein (20 μg, *E*) derived from an equal number of cells were subjected to SDS-PAGE and immunoblotting as described under "Materials and Methods." The *upper* and *lower* sections of the blot were probed with U-49 and affinity-purified X-263 antisera, respectively.

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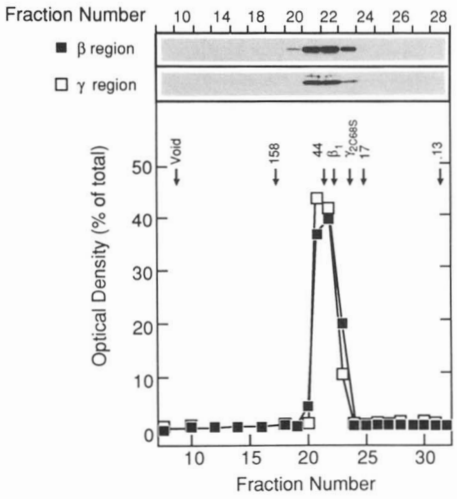

FIG. 9. **Superose 12 gel filtration chromatography of $\beta_1\gamma_2$C68S.** A cytosolic fraction from Sf9 cells expressing $\beta_1\gamma_2$C68S was adjusted to contain 1% sodium cholate, and 200 μl (580 μg) of this fraction was subjected to gel filtration chromatography as described under "Materials and Methods." Aliquots (100 μl) of the indicated fractions were precipitated with trichloroacetic acid, and 25% of each sample was subjected to SDS-PAGE and immunoblotting as described under "Materials and Methods." Blots were probed with antiserum U-49 (β region) and affinity-purified antiserum X-263 (γ region). Quantitation of band intensity was performed with a laser densitometer, and data are expressed as percent of the total immunoreactivity. The *arrows* show the elution positions for molecular size standards of the indicated molecular masses (kDa), as well as the elution position of β_1 and γ_2C68S when a 1%-cholate extract (β_1) and a cytosolic fraction (γ_2C68S) of Sf9 cells expressing these subunits individually were run under identical conditions.

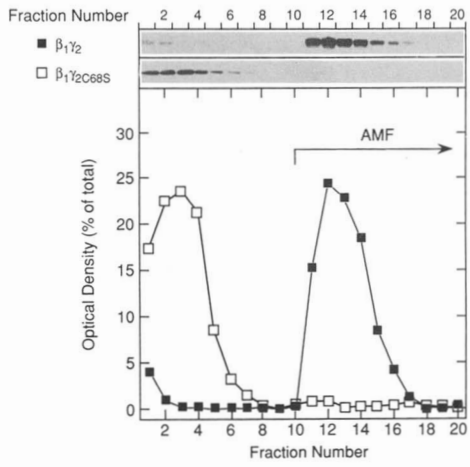

FIG. 10. **$G_{o\alpha}$-agarose chromatography of $\beta_1\gamma_2$ and $\beta_1\gamma_2$C68S.** An aliquot (300 μl, 3 mg/ml) of a 1%-cholate extract of a crude particulate fraction from cells expressing $\beta_1\gamma_2$ (■) or 300 μl (7.3 mg/ml) of a cytosolic fraction (adjusted to contain 1% sodium cholate) from cells expressing $\beta_1\gamma_2$C68S (□) were diluted to 1 ml with buffer A and mixed with separate 1-ml aliquots of $G_{o\alpha}$-agarose. After a 1-h incubation at 4 °C with gentle mixing, the resins were packed into columns. The resulting flowthroughs were reapplied to the columns and collected as *fraction 1*. The columns were then washed with 4 ml of buffer A containing 0.25% Lubrol PX (*fractions 2–5*), followed by 5 ml of buffer A containing 0.05% Lubrol PX (*fractions 6–10*). Elution was initiated by stopping the column and resuspending the resin in 1 ml of buffer B. After a 30-min incubation at room temperature with gentle mixing, the columns were allowed to drain (*fraction 11*) and were subsequently washed with 9 ml of buffer B (*fractions 12–20*). Aliquots (250 μl) of the indicated fractions were precipitated with trichloroacetic acid, and 25% of each sample was subjected to SDS-PAGE and immunoblotting as described under "Materials and Methods." β_1 immunoreactivity was detected with antiserum U-49, and data were quantified as described in the legend to Fig. 9. Similar results were obtained when γ_2 immunoreactivity was monitored with affinity-purified antiserum X-263.

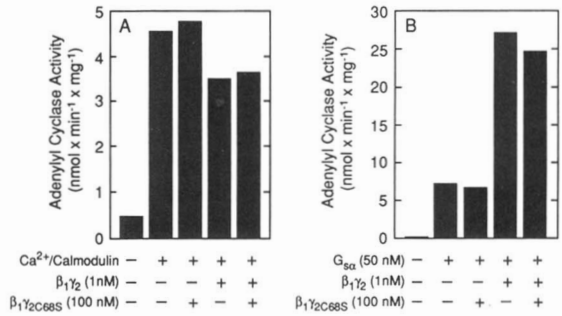

FIG. 11. **$\beta_1\gamma_2$C68S fails to regulate adenylylcyclase activity.** Sf9 cell membranes containing type-I (*panel A*) or type-II (*panel B*) adenylylcyclase were assayed as described in the legends of Figs. 6 and 7 in the absence or presence of activators (50 μM $CaCl_2$, 100 nM calmodulin for type I; 50 nM GTPγS-r$G_{s\alpha\text{-}s}$ for type II) and βγ (1 nM $\beta_1\gamma_2$; 100 nM $\beta_1\gamma_2$C68S), as indicated.

DISCUSSION

G protein β and γ subunits function as a tightly bound complex that is disrupted only by denaturants. The results presented here indicate that coexpression of both subunits is required to detect the activity of the complex. This suggests that the functional properties of the complex are dependent on contributions from both proteins. Schmidt and Neer (43) noted that most β subunit protein becomes aggregated when translated *in vitro*, although a monomeric fraction of this protein is capable of forming a complex when mixed with γ (also translated *in vitro*). We have not been able to reconstitute βγ activity by mixing detergent extracts from cells expressing β and γ independently. Expression of β by itself in Sf9 cells also appears to lead to aggregation of the protein, since very little immunoreactivity can be extracted from such membranes. It is possible that β and γ adopt an active conformation only when they are mutually involved in a complex.

The ability to coinfect Sf9 cells with baculoviruses encoding different β and γ subunits has allowed us to examine the selectivity of interaction between these proteins. Of the six βγ combinations tested, only one appears not to be allowed. The observation that γ_1 will associate readily with β_1 but not with β_2 is somewhat surprising, since β_1 and β_2 are 90% identical in their amino acid sequence; most of the changes are conservative. However, the fact that γ_1 and β_2 yield functional complexes with other partners argues against most trivial explanations for the phenomenon. It is interesting, however, that this particular combination is not likely to occur in nature, since expression of γ_1 appears restricted to photoreceptors, cells in which expression of β_2 has not been reported and in which β_1 is by far the predominant form. Although the amino-terminal region of β_1 (sequences surrounding Cys^{25}) has been proposed to interact with γ_1 (44), the amino acid sequence surrounding this residue is nearly identical in β_1 and β_2. Very subtle differences in primary sequence or as yet unidentified post-translational modifications may be responsible for such subunit selectivity. It will be interesting to extend these observations to other more divergent forms of β and γ to determine how much of the potential combinatorial diversity is actually available.

The substantial levels of expression of functional βγ complexes in this system have provided us with a source of material of defined composition. Furthermore, purification of βγ complexes by subunit-exchange chromatography on a ma-

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trix of $G_{o\alpha}$-agarose permits valid comparison between the different complexes, since the material purified in this fashion is functional, at least in its ability to interact with $G_{o\alpha}$.

Analysis of the rates of βγ-dependent ADP-ribosylation of two different α subunits indicates that a single α is capable of distinguishing between different species of βγ. This is most notable in the case of $rG_{i\alpha1}$. When this α subunit is used as substrate, preference for individual forms of βγ appears attributable to γ. Previous studies have failed to reveal differences in the capacity of different βγ complexes to support ADP-ribosylation of G protein α subunits (26, 29). G_i and G_o α subunits are myristoylated at their amino termini, and this modification increases affinity for βγ (45). The lack of this modification of the *Escherichia coli*-derived $rG_{i\alpha1}$ subunit could accentuate differences in the interaction of this α subunit with individual forms of βγ. The overall rate of the ADP-ribosylation reaction is determined in this assay, and it is not possible to derive estimates of affinity of α for βγ based solely on these data. βγ acts catalytically in this reaction and is present in substoichiometric amounts. Thus, the rate of ADP-ribosylation reflects the ability of βγ to recycle between G protein α subunits. It is therefore possible that a high rate of ADP-ribosylation might reflect a higher rate of dissociation of βγ from α, allowing it to recycle more rapidly. Direct estimates of the affinity of α for βγ can be made by determination of the concentration of βγ required to slow dissociation of GDP from α at appropriately low concentrations of the protein reactants.

We have also compared the ability of the different βγ complexes to modulate adenylylcyclase activity, where the effect of βγ is dependent on the type of enzyme utilized (12). There are quantitative differences in the ability of different βγ subunits to regulate adenylylcyclases; $\beta_1\gamma_1$ is approximately 10-fold less potent than the other complexes tested. Our results are consistent with previous observations that transducin βγ is less potent than brain βγ in inhibiting type-I adenylylcyclase (12) or brain adenylylcyclase activity in a reconstituted system (27). The differences observed are attributable to γ. One major difference between γ_1 and the other γ subunits tested is the isoprenoid used to modify the carboxyl terminus; γ_1 is farnesylated, whereas γ_2 and γ_3 are predicted to be geranylgeranylated. Although we have not yet analyzed the type of modification carried out by Sf9 cells on G protein γ subunits, these cells do modify $p21^{ras}$ appropriately with a farnesyl group (46). It is possible, then, that the type of isoprenoid might be an important determinant of the potency of a βγ complex in these assays. It must be noted, however, that the amino acid sequence of γ_1 is substantially different from those of the more closely related γ_2 and γ_3 polypeptides. Further experimentation will be necessary to delineate the relative contributions of the polypeptides and the type of isoprenoid to the properties of these complexes.

In the assays described here, most of the functional differences can be attributed to the γ subunits. It seems likely, however, that functional differences due to β may be revealed when interactions of βγ with other components of the signaling system (*e.g.* receptors or other effectors) are examined or when a more extensive group of β subunits is examined. In this respect, Law *et al.* (47) have suggested that brain somatostatin receptors associate preferentially with complexes containing β_1. It will also be of interest to examine the effects of individual βγ complexes on phosphoinositide-specific phospholipases. Complex effects of βγ have been observed on receptor- and guanine nucleotide-sensitive phosphoinositide hydrolysis in turkey erythrocyte membranes (48).

The carboxyl-terminal posttranslational modifications of G protein γ subunits are important determinants of the subcellular location of βγ (33, 42). The functional consequences of prenylation and carboxymethylation are now being addressed. Carboxymethylation of γ occurs after prenylation and proteolytic removal of the last 3 amino acid residues (31). The mutation examined here (C68S) presumably blocks all of these steps. Blockade of carboxyl-terminal processing of γ does not appear to impair its ability to associate with β. However, the resulting complex is inactive in all other assays. The inability of this mutant complex to compete with the wild type analog further indicates that the COOH-terminal modifications are essential for high affinity interactions of βγ with both α subunits and adenylylcyclases. The data presented here are consistent with reports indicating that both prenylation and carboxymethylation enhance the ability of transducin βγ to facilitate rhodopsin-catalyzed binding of guanine nucleotides to transducin α (49, 50) and extend the observations to include effectors like adenylylcyclase. Together, these observations support the hypothesis that lipid modifications of proteins are important determinants of protein-protein interactions, and these interactions may well underlie the effects of lipid modification on subcellular localization.

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