G Protein βγ Subunits Synthesized in Sf9 Cells

FUNCTIONAL CHARACTERIZATION AND THE SIGNIFICANCE OF PRENYLATION OF γ*

(Received for publication, June 25, 1992)

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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 monolayer 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 rGαi1) is detected only when β and γ are expressed concurrently. Of the six combinations of βγ tested (β′ or βγ with γ1, γ2, or γ3), only one, βγγ1, failed to generate a functional complex. Each of the other five complexes has been purified by subunit exchange chromatography using Gαγ-agarose as the chromatographic matrix. We have detected differences in the abilities of the purified proteins to support ADP-ribosylation of Gα; 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 Gαγ-stimulated type-II adenylylcyclase, recombinant β1,γ1 and transducin βγ are approximately 10 and 20 times less potent, respectively, than the other complex 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) transmit signals that are generated by a large number of plasma membrane-bound receptors to intracellular effectors 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 Gαγ or calmodulin, βγ greatly potentiates the stimulatory effect of Gαγ 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

Vol. 267, No. 32, Issue of November 15, pp. 23400–23417, 1992
Printed in U.S.A.
their carboxyl termini in a manner analogous to that described in particular for p21 (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 β1, β2, γ1, γ2, γ3, and γS is, and γ1, subunits were transferred to a baculovirus expression vector (pVL1392 or pVL1393) as follows. For the β1 subunit (18), a 3-kb cDNA ligated at the EcoRI site of pBHR5 (plus) (+) was digested with ApaI and NefI. The resulting 1.1-kb fragment containing the β1 coding sequence was isolated after filling the 3' recessed terminus generated by NefI. This fragment was transferred to pVL1393 at the Smal site. For the β2 subunit, the 1.06-kb Apal fragment from the Okayama and Berg vector containing the β2 cDNA (32) was ligated at the Apal site of pGEM-12R(−). The resulting plasmid was digested with BamHI and NefI. The 1.08-kb fragment containing the entire coding sequence was then transferred to pVL1392 that had been digested with the same enzymes. For the γ1 subunit (24), the 0.26-kb Apal/ApaI fragment containing the entire γ1 sequence was generated from pNG-2-1. This fragment was transferred to pVL1392 that had been digested with Smal and NefI. The termini generated by Apal and NefI were complementary filling the ends at the NefI site with dTTP and dGTP and the Smal site with dGTP. For the γ2 subunit (25), a 0.425-kb BglII/EcoRI fragment containing the entire coding sequence was isolated from a pBluescript vector based construct. This fragment was transferred to pVL1392 that had been digested with the same enzymes. The γ3S sequence, in which cysteine 68 has been changed to serine (33), was subcloned into pVL1392 as γ3S. The coding region of the bovine γ2 cDNA (23) was prepared by polymerase chain reaction amplification of reverse transcribed mRNA with the introduction of PstI and XhoI sites at the 5' and 3' ends, respectively. This fragment was cloned into pVL1392.

**Cell Culture and Infecting of Recombinant Baculoviruses**—Fall armyworm ovary (Sf9) cells were propagated 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 membrane was carried out essentially as described (34). The cells were lysed in 20 mM NaHepes (pH 8.0), 1 mM EDTA, 1 mM MgCl2, 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 MgCl2, 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-m column of bovine brain Gs, aga-rose. Purified bovine brain Gs (20 mg) was coupled to 10 ml of α-aminoethyl-butyrate by the method of Sternweis et al. (30). The concentration of Gs binding sites on the sepharose resin was approximately 0.6 μM. (9 μmol/ml). Purification of 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 Gs,-agarose, which had been equilibrated in the same buffer. After the column was washed and resuspended in buffer 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 0.5% Lubrol PX. Elution was then achieved with 20 mM NaHepes (pH 8.0), 400 mM NaCl, 2 mM DTT, 5 μM GDP, 30 μM AlCl3, 20 mM MgCl2, 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 (containing 40 nmol) were pooled and subjected again to chromatography on Gs, agarose. The column was then washed and resuspended in 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 purified as described above 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 Gs,-agarose. The pooled fractions were concentrated and subsequently adsorbed to hydroxyapatite and eluted as described above. The final preparations were dilute to 25 μmol of βγ activity (determined by support of ADP-ribosylation (see below), 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 a pool of the second heptylamine-Sepharose column in the presence of protein A, Mg2+, and F−. Transducin βγ was a generous gift of Dr. Armenian K. Dumb. The pool of the second heptylamine-Sepharose column in the presence of protein A, Mg2+, and F−. Transducin βγ was a generous gift of Dr. Armenian K. Dumby. The pool of the second heptylamine-Sepharose column in the presence of protein A, Mg2+, and F−. Transducin βγ was a generous gift of Dr. Armenian K. Dumby. The pool of the second heptylamine-Sepharose column in the presence of protein A, Mg2+, and F−. Transducin βγ was a generous gift of Dr. Armenian K. Dumby. The pool of the second heptylamine-Sepharose column in the presence of protein A, Mg2+, and F−. Transducin βγ was a generous gift of Dr. Armenian K. Dumby. The pool of the second heptylamine-Sepharose column in the presence of protein A, Mg2+, and F−. Transducin βγ was a generous gift of Dr. Armenian K. Dumby. The pool of the second heptylamine-Sepharose column in the presence of protein A, Mg2+, and F−. Transducin βγ was a generous gift of Dr. Armenian K. Dumby. The pool of the second heptylamine-Sepharose column in the presence of protein A, Mg2+, and F−. Transducin βγ was a generous gift of Dr. Armenian K. Dumb.
Fractionation of any endogenous detergent extracts. To assay column fractions containing Al**+, Me, as a substrate. The use of a high concentration of substrate devoid of mM). Fractions containing 8~2C68S (eluting at approximately 150 were prepared as described (12). Purified recombinant Drosophila calmodulin and rGan.*  were provided by Dr. Wei-Jen Tang. rGIR.*  was activated with GTPyS for 30 min at 30 °C in 20 mM NaHepes (pH 8.0, 1 mM EDTA, 10 mM MgSO4, and 100 μM GTPyS. 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 rGan were prepared for the ADP-ribosylation of rGan, as described with modifications (40). The final lipid concentration was increased to 1 mM, and purified rGan, (30 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**+, Me, and F-, the MgCl2 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 MgCl2 contributed by the sample. This change has essentially no effect on the kinetics of the reaction (20).

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panels A, membranes of Sf9 cells were assayed for support of ADP-ribosylation activity as described under "Materials and Methods." The indicated viruses were subjected to SDS-PAGE and immunoblotting. One of the gels was stained with silver (panel A). An aliquot (1.5 ml, 1.3 mg/ml) of a 1% cholate extract of membranes from Sf9 cells infected with β and γ viruses was diluted to 5 ml with buffer A containing 0.5% Lubrol PX and was added to 5 ml of Gγ-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.

FIG. 3. Purification of βγ by Gγ-agarose chromatography. An aliquot (1.5 ml, 1.3 mg/ml) of a 1% cholate extract of membranes from Sf9 cells infected with β, and γ viruses was diluted to 5 ml with buffer A containing 0.5% Lubrol PX and was added to 5 ml of Gγ-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.

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 Subunits migrate slightly faster than do γγ 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.
The Sf9 cell βγ preparation reacts only weakly with both anti sera. The affinity-purified NG-1 antisera raised against a peptide sequence unique to γ, recognizes only the γ subunits of transducin and βγ. As anticipated, based on the amino acid sequence of the peptide used as the antigen (30), affinity-purified antisera X-263 reacts with both γ2 and γ subunits. Antiserum B-53 recognizes only γ. 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 β1 and γ 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 rGiml and bovine brain Go-a. When rGiml 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, while the lowest rates are observed with (including transducin βγ). Intermediate rates are observed for βγ and βγ. The largest difference observed was approximately 6-fold. When bovine brain Go-a is used as substrate (Fig. 5B), 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 βγ. 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 (Go-a) 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 Adenylyl cyclase Activity—Type-I adenylyl cyclase is activated by either Ca⁺⁺/calmodulin or Go-a; such activation can be partially reversed by βγ in a noncompetitive fashion (12). By contrast, when type-II adenylyl cyclase is stimulated by Go-a, further addition of βγ causes a large potentiation of Go-a-stimulated activity (12). We examined the ability of the different βγ complexes to inhibit Ca⁺⁺/calmodulin-stimulated type-I adenylyl cyclase (Fig. 6). All preparations can inhibit type-I adenylyl cyclase activity. However, βγ 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, βγ. The pattern was very similar when we examined the effect of the different βγ preparations on rGo-a-stimulated type-II adenylyl cyclase (Fig. 7); again, transducin βγ and βγ are approximately 10 and 20 times less potent than the other complexes, and βγ is very similar to bovine brain βγ.

Effects of Prenylation on βγ Function—We have compared the properties of the wild type γ subunit with those of a mutant γ in which the cysteine four amino acid residues from the carboxyl terminus has been changed to serine (γ-C68S). 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 subunits are coexpressed with β1, ADP-ribosylation activity is detected only in detergent extracts of Sf9 cells infected with wild type γ (Fig. 8A). In a mammalian cell expression system, coexpression of γ-C68S with β1 causes distribution of β to the cytosolic fraction. SDS-PAGE and immunoblot analysis of Sf9 cell fractions (Fig. 8C) indicates that substantial γ immunoactivity is found in the cholate extract of membranes, with little or no reactivity in the cytosolic fraction. By contrast, little or no γ-C68S is found in the cholate extract, whereas substantial immunoactivity is present in the cytosol. Furthermore, coinfection of γ-C68S with β1 leads to a
concentrations of the different purified cyclase activity by purified are Sf9 cell membranes containing type-I1 adenylylcyclase were assayed equation cytosolic fraction. This pattern is in agreement with the results obtained in the mammalian system (33). Since most of the γC68S is located in the Sf9 cell cytosol, we tested this fraction for support of ADP-ribosylation (Fig. 8B). There is no cytosolic activity from cells expressing either wild type γ or γC68S. Distribution of β to the cytosol when expressed with γC68S suggests that unprenylated γ is still associated with β. To examine this possibility, we have analyzed the behavior of β and γC68S subunits during gel filtration chromatography. When a cytosolic fraction from cells infected with β and γC68S viruses is fractionated on a Superose 12 gel filtration column, β1 and γC68S coelute at a position consistent with the formation of a complex (Fig. 9). Furthermore, βγγC68S elutes before γC68S, as determined by gel filtration of a cytosolic fraction from cells expressing γC68S alone (Fig. 9). β1 and γC68S 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 βγγC68S to support ADP-ribosylation of rG111 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 β1 and γC68S and a cholate extract from cells expressing the wild type complex to Gao-agarose chromatography (Fig. 10). The wild type complex binds stably to immobilized Gao 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 βγγC68S has a much lower affinity for the GDP-bound form of Gao than does its wild type counterpart. This result also indicates that Gao-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 βγγC68S to regulate adenylylcyclase activity (Fig. 11). High concentrations of βγγC68S do not inhibit calmodulin-stimu-
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 γ1 will associate readily with β1 but not with β2 is somewhat surprising, since β1 and β2 are 90% identical in their amino acid sequence; most of the changes are conservative. However, the fact that γ1 and β2 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 γ1 appears restricted to photoreceptors, cells in which expression of β2 has not been reported and in which β1 is by far the predominant form. Although the amino-terminal region of β1 (sequences surrounding Cys36) has been proposed to interact with γ1 (44), the amino acid sequence surrounding this residue is nearly identical in β1 and β2. 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 Gα,-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 Gα.

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 rGαi. 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). G1 and G2 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 rGα11 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 concentrations 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 adenylyl cyclase 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 adenylyl cyclases; β1γ1 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 adenylyl cyclase (12) or brain adenylyl cyclase activity in a reconstituted system (27). The differences observed are attributable to γ. One major difference between γ1 and the other γ subunits tested is the isoprenoid used to modify the carboxyl terminus; γ1 is farnesylated, whereas γ2 and γ3 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 p21Gα11 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 γ1 is substantially different from those of the more closely related γ2 and γ3 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 receptor α associates preferentially with complexes containing β1. 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. Carboxy terminal of γ occurs after prenylation and proteolytic removal of the last 3 amino acid residues (31). The mutation examined here (G68S) 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 both βγ subunits and adenylyl cyclases. 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 adenylyl cyclase. Together, these observations support the hypothesis that lipid modifications of proteins are important determinants of protein-protein interactions, and these interactions may well underline the effects of lipid modification on subcellular localization.

Acknowledgments—We thank Dr. Randall Reed (Johns Hopkins University) and Dr. Boning Gao (University of Texas Southwestern) for β1 and β2 CDNAs, respectively, Dr. N. Gautam (California Institute of Technology) for γ1 and γ2 constructs and NG-1 antiserum, Dr. Suzanne Munilly (University of Texas Southwestern) for the γμGαS construct and for purified transducin βγ. Dr. Wei-Jen Tang (University of Texas Southwestern) for purified rGα11, recombinant Droshophila calmodulin, and adenylyl cyclase baculoviruses, Dr. Maurine Linder (University of Texas Southwestern) for rGα11, and Ethan Lee (University of Texas Southwestern) for bovine brain Gα11. We also thank Dr. Paul Sternweis for many helpful suggestions and Vivian Kolman for excellent technical assistance.

REFERENCES

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