Role of the Prenyl Group on the G Protein γ Subunit in Coupling Trimeric G Proteins to A1 Adenosine Receptors

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The coupling of receptors to heterotrimeric G proteins is determined by interactions between the receptor and the G protein α subunits and by the composition of the βγ dimers. To determine the role of the γ subunit prenyl modification in this interaction, the CaaX motifs in the γ1 and γ2 subunits were altered to direct modification with different prenyl groups, recombinant βγ dimers expressed in the baculovirus/Sf9 insect cell system, and the dimers purified. The activity of the βγ γ subunit was compared in two assays: formation of the high affinity agonist binding conformation of the A1 adenosine receptor and receptor-catalyzed exchange of GDP for GTP on the α subunit. The β1γ1 dimer (modified with farnesyl) was significantly less active than β1γ2 (modified with geranylgeranyl) in either assay. The β1γ2-S74L dimer (modified with geranylgeranyl) was nearly as effective as β1γ2 in either assay. The β1γ2-L71S dimer (modified with farnesyl) was significantly less active than β1γ2. Using [3H]labeled βγ subunits, it was determined that native and altered βγ dimers reconstituted equally well into Sf9 membranes containing A1 adenosine receptors. These data suggest that the prenyl group on the γ subunit is an important determinant of the interaction between receptors and G protein γ subunits.

The membranes of all cells contain multiple receptors and transmembrane signaling systems that regulate cell function (1–5). One major unsolved problem in cell signaling is understanding how a cell selects its response to a hormone or growth factor given the possibilities available. The signaling mechanism used by receptors coupled to the heterotrimeric G proteins provides an excellent example of the complexities. Current evidence suggests that specificity is determined at many levels in this pathway. In addition to the selectivity provided by the receptor itself, the interaction between the intracellular loops of the receptors and the αβγ subunits in the heterotrimer is a major determinant of specificity (6, 7). Interestingly, some receptors couple selectively to certain G protein α subunits. For example, the β-adrenergic receptor couples primarily to members of the Gs family (1, 6) and rhodopsin to the Ga1ββ subunit (2). Other receptors, such as the angiotensin AT1 or muscarinic receptors couple to multiple α subunits including members of the Ga and Gq class of α subunits (6, 8), leading to activation of multiple signaling networks by a given ligand (5).

Although the receptor and the α subunit provide one determinant of selectivity, the βγ subunit is clearly required for the receptor to couple to the α subunit (9, 10). Thus, a third level of selectivity may be provided by the type of βγ subunit used to form the receptor-αβγ complex. In addition, since the βγ dimer can activate effectors directly (11–13), the diversity of these proteins may contribute to the specificity of signaling. Two lines of experimental evidence support this conclusion. Using recombinant βγ subunits purified from Sf9 insect cells, some investigators have demonstrated differences in βγ activity using in vitro assays. For example, the β1γ1 dimer is less potent in activating the muscarinic K2 channel (14), type I1 adenylyl cyclase (15, 16), and phospholipase C-β2 (16) compared with the β1γ2 dimer. In contrast, the β1γ1 dimer is far more effective at coupling rhodopsin to the Gα1 subunit than β1γ2 (17). Using an antisense RNA approach to study G protein-dependent regulation of the voltage-dependent Ca2+ channel in GH3 cells, Kleuss et al. (18–20) suggested that the Gαoβγ complex heterotrimer coupled preferentially to the muscarinic receptor and that the Gαoβγγ2 combination coupled to the somatostatin receptor. Thus, the structural diversity of the γ subunit may be an important determinant of the selectivity of coupling of receptors to G proteins and of the βγ dimer to effectors.

The 11 known γ subunits are modified by the addition of isoprenoid lipids to an invariant cysteine residue in a CaaX motif at their carboxyl terminus (21). These lipids have been assumed to be responsible for attaching proteins to membranes (22–24), but growing evidence suggests the prenyl groups may also play a major role in determining activity and interaction with other proteins (24, 25). For example, prenylation of the γ2 subunit is necessary for the formation of an active transducin αβγ complex (26, 27), for ADP-ribosylation of α subunits by pertussis toxin (15), and for the stimulation of phospholipase C-β by the βγγ2 dimer (28). Moreover, studies with synthetic peptides indicate that the carboxyl-terminal region of the γ subunit is an important domain for the interaction among the receptor, the α subunit, and the βγ dimer. For example, peptides made from the carboxyl terminus of the γ subunit are most effective at stabilizing the rhodopsin Gα subunit interaction if they contain the farnesyl group (29); peptides with a geranyl or geranylgeranyl group have much lower potency in the stabilization assay (30). These results strongly suggest that
in addition to the primary amino acid sequence of the \( \gamma \) subunit, the prenyl group is important in establishing receptor- \( \beta\gamma \) coupling. To examine this possibility, we have purified recombinant \( \beta \gamma \) dimers from Sf9 cells containing \( \gamma \) subunits with altered prenyl groups (31) and examined their activity in two assays of receptor coupling which are dependent on \( \beta\gamma \) activity. Membranes from Sf9 cells expressing recombinant A1 adenosine receptors were reconstituted with purified G, \( \alpha \) subunits and various types of \( \beta\gamma \) dimers and assayed for the ability to establish the high affinity agonist binding conformation of the receptor and the ability of the receptor to activate the exchange of GDP for GTP on the \( \alpha \) subunit. The data indicate that the A1 adenosine receptor markedly prefers \( \gamma \) subunits containing the geranylgeranyl group.

**EXPERIMENTAL PROCEDURES**

Construction of Recombinant Baculoviruses—The virus encoding the bovine A1 adenosine receptor was prepared as described. Briefly, the cDNA for the bovine A1 adenosine receptor (33) was altered to minimize the length of the construct to 5' to the start codon using polymerase chain reaction amplification, and the resulting insert was subcloned into the multiple cloning polylinker of the baculovirus transfer vector, pVL1393 (Invitrogen), using the 5'Smal and 3'XbaI sites. To assure fidelity, the completed construct was sequenced in the forward and reverse directions. The recombinant baculoviruses coding for the \( \alpha \) subunits of the G proteins and the \( \beta\gamma \) subunits for the G proteins were prepared as described (34, 35). The baculoviruses coding for G proteins \( \gamma \), \( \gamma \), and \( \gamma \) subunits with altered prenylation sequences in the Caa\( \text{X} \) motif were prepared as described in the accompanying manuscript (31). All recombinant baculoviruses were produced by standard techniques in Sf9 insect cells and isolated by four sequential rounds of plaque purification (36). After the fourth round of purification, the viral titers were calculated by the end point dilution method (36). High titer stocks were prepared for each virus and stored at 4°C.

Cell Culture Expression, and Purification of Recombinant G Protein \( \alpha \) and \( \beta\gamma \) Subunits—Sf9 cell culture conditions were as described previously (35, 37). Recombinant G protein \( \alpha \) subunits were expressed and purified as described previously (35). The \( \beta\gamma \) subunits were extracted with 0.1% Genapol C-100 from frozen cell pellets and purified on a DEAE column followed by affinity chromatography on a G,\( \alpha \)-agarose column as described (31, 36). To remove the GDP and AIF- used to elute the \( \beta\gamma \) dimers from the \( \alpha \) subunit affinity column, they were exchanged into storage buffer (20 mM dithiothreitol, 200 mM NaCl, 0.6% CHAPS, pH 8.0) by centrifuging through a 1 x 3-cm polypropylene column (Quik-Sep, Isolab Inc.) containing 2 ml of Bio-Gel P-6 DG gel filtration medium (Bio-Rad) equilibrated in storage buffer. About 200 \( \mu \)l of \( \beta\gamma \) dimer in elution buffer was loaded onto a spin column that had been prepared previously by centrifuging at 2,000 x g for 10 min. The loaded column was again centrifuged at 2,000 x g for 10 min, and the pass-through containing the \( \beta\gamma \)-dimer was collected. In a control experiment, this procedure separated 99.9% of the \[^{35}\text{S}]\text{GTP}^\text{S} \) added to a sample of \( \beta\gamma \).

Quantification of the Yield of \( \beta\gamma \) Dimer by Silver Staining—The \( \beta\gamma \) dimers in storage buffer were loaded onto 12% acrylamide gels (0.75 ml of Bio-Gel P-6 DG gel filtration medium (Bio-Rad) equilibrated in storage buffer (20 mM Hepes, 1 mM EDTA, 1 mM dithiothreitol, 500 mM GDP, and 0.04% CHAPS (w/v), pH 7.4)). G protein subunits were mixed and diluted in reconstitution buffer containing 0.1 ng/ml bovine serum albumin such that the desired quantity was contained in a small volume. Typically, a 5-\( \mu \)l volume containing the desired quantity of G protein \( \alpha \) and \( \beta\gamma \) subunits was added to a 27.5-\( \mu \)l volume of membrane suspension containing ~70 fmol of recombinant A1 adenosine receptors (measured as [\[^{3}\text{H}\text{]cyclopentyl-xanthine binding sites]).

Prenylation of G Protein Subunits—Membranes from Sf9 cell cultures expressing recombinant A1 adenosine receptors with various amounts of reconstituted G protein \( \alpha \) and/or \( \beta\gamma \) subunits were measured in binding assay buffer containing 5 units/adenosine deaminase present in the vehicle of various additional components in a final volume of 100 \( \mu \)l. The binding isotherm shown in Fig. 1A was performed with concentrations of \[^{125}\text{I}]\text{ABA}- 

\[^{35}\text{S}]\text{GTP}^\text{S} \) added to a 27.5-\( \mu \)l volume of membranesuspension containing 70 fmol of adenosine receptor agonist R-PIA was added to a final concentration of 20 fmol of A1 adenosine receptor was contained in a 50-\( \mu \)l volume.

Measurement of Radioligand Binding—The \[^{125}\text{I}]\text{ABA}- was prepared by iodination of the parent compound to theoretical specific activity of 100 Ci/mol (DuPont NEN) as described. The binding of [\[^{35}\text{S}]\text{GTP}^\text{S} \) to recombinant A1 adenosine receptors in membranes containing the desired quantity of G protein \( \alpha \) and/or \( \beta\gamma \) subunits was measured in binding assay buffer containing 5 units/adenosine deaminase present in the presence of various additional components in a final volume of 100 \( \mu \)l. The binding isotherm shown in Fig. 1A was performed with concentrations of \[^{35}\text{S}]\text{GTP}^\text{S} \) ranging from 0.03 to 10 nM and 10 \( \mu \)M unlabeled ligand was added, and the concentration of CHAPS varied from 0.005 to 0.04% (w/v). The results were expressed as binding to membranes containing 5 units of membranes, recombinant G proteins, and various compounds in binding assay buffer. Binding assays were incubated for 3 h at 25°C. The binding assays were terminated by filtration over Whatman GF/C glass fiber filters. The filters were rinsed three times with 2 ml of ice-cold 10 mM Hepes pH 7.4, 1 mM EDTA, 100 \( \mu \)M MgCl\(_2\). The filters (about 4 x 10\(^{6}\) cpm/tube) were counted in a Beckman 4000 series gamma counter at an efficiency of 75%. For each binding assay, specific binding in the presence of 10 \( \mu \)M GDP, and non-specific binding in the presence of 10 \( \mu \)M R-PIA was assayed in triplicate. Non-specific binding of \[^{35}\text{S}]\text{GTP}^\text{S} \) to membranes was minimal, ranging from 2% to 0.03 nmol to 6% to 10 nmol ligand. The appropriate value was subtracted from all data before presentation.

Measurement of Receptor-stimulated \[^{35}\text{S}]\text{GTP}^\text{S} \) Binding to \( \alpha \) Subunits—Sf9 cell membranes expressing recombinant bovine A1 adenosine receptors (~8 pmol/mg of membrane protein) were reconstituted with G protein \( \alpha \) and \( \beta\gamma \) subunits as described above. Prior to reconstitution, membranes containing the receptor were incubated with 1 mM AMP-PNP for 1 h to occupy all binding sites with GTP. This treatment greatly reduced the background of \[^{35}\text{S}]\text{GTP}^\text{S} \) binding. Membranes were then reconstituted with the G protein \( \alpha \) subunit (containing 5 \( \mu \)M GDP) and the indicated \( \beta\gamma \) dimer by incubation for 30 min on ice in a buffer containing 25 mM Hepes, 100 mM NaCl, 5 mM MgCl\(_2\), 1 mM EDTA, 1 mM dithiothreitol, pH 7.4. The final GDP concentration in the assay (provided in part by the \( \alpha \) subunit) was kept constant at 0.005% (w/v). In these experiments, the \( \alpha \) subunit was G\(_{\text{a}}\), and the effects of various \( \beta\gamma \) dimers were examined. The final concentrations of G\(_{\text{a}}, \beta\gamma \), and GTP\(_{\text{S}}\) were 6, 6, and 6 nm, respectively, and the receptor to G protein ratio was approximately 1:6. Following reconstitution, about 270 \( \mu \)l of the membrane suspension was transferred to a 25°C water bath and the assay begun by the addition of 30 \( \mu \)l of carrier-free \[^{35}\text{S}]\text{GTP}^\text{S} \) (about 4 x 10\(^{6}\) cpm/tube). The adenosine receptor agonist R-PIA was added to a final concentration of 2. R. A. Figler, S. G. Graber, M. A. Lindorfer, H. Yasuda, J. Linden, and J. C. Garrison, submitted for publication.
100 nm 8 min after addition of [35S]GTP-γ-S and 30-µl aliquots containing membranes (~30 fmol of receptor) were removed for filtration through nitrocellulose filters (Millipore, HAWP-025) every 30 s.

Preparation of [125I]-Labeled βγ Subunits—Pure, recombinant βγ dimers were iodinated with IODO-GEN iodination reagent (Pierce) according to the method of Frazer and Speck (41). Approximately 100 µl of purified βγ dimers in elution buffer was introduced into an IODO-GEN-coated vial. Carrier-free [125I]NaI, diluted in borate saline buffer, pH 8.0, was added (10 µCi) and the reaction allowed to proceed for 20 min at 4°C. The reaction was stopped by removal of the reaction mix from the IODO-GEN-coated vial and immediate loading of the mix onto a Bio-Gel P-6 DG column (1 × 3 cm) equilibrated with reconstitution buffer plus 0.1 mg/ml bovine serum albumin (fraction V, Sigma). The βγ dimers were eluted by gravity flow and 0.2-ml fractions collected. The [125I]-labeled protein eluting at the void was pooled, aliquoted, and stored at −80°C. The specific activity of the iodinated βγ dimers was calculated by determining the amount of βγ subunit by scanning densitometry of silver-stained 12% acrylamide minigels (0.75 mm thickness) as described above and by counting the labeled protein in a Beckman 4000 gamma counter at a calibrated efficiency of 75%.

Calculations and Expression of Results—Most data presented under “Results” are from representative experiments repeated 3–10 times. The number of repetitions for each experiment is provided in the figure legends. Error bars represent the mean ± the S.E. Statistical differences were determined for the data in Fig. 2A using the paired t test. The rates of GTP-γ-S binding were determined by linear regression analysis of the amount of nucleotide bound at each time point taken between 8 and 12 min (see Fig. 6 for an example).

Materials—All reagents used in the culture of Sf9 cells and for the expression and purification of G protein α and βγ subunits have been described in detail (31, 35, 37). Genapol C-100 and GTP-γ-S were purchased from Calbiochem, CHAPS was from Boehringer Mannheim, and AMP-PNP was from Sigma. The radioisotopes, carrier-free [125I]NaI, and [35S]GTP-γ-S were purchased from DuPont NEN. All other reagents were of the highest purity available.

RESULTS

To examine the ability of the various βγ dimers to support the establishment of the high affinity agonist binding conforma-
tion of a receptor, bovine A1 adenosine receptors were expressed in the membranes of Sf9 insect cells using recombinant baculoviruses. Previous studies have shown this procedure to produce membranes containing high levels of receptors that are not coupled to G proteins and that the receptors can be effectively recoupled by reconstitution of the G or Gα subunits in the presence of the βγ dimer.2 The experiments shown in Fig. 1A were performed to establish the binding parameters for the membranes used in the experiments presented in this report using the G12 α subunit. The membranes expressing the bovine receptor contained ~4 pmol of receptor/mg of membrane protein, as measured using binding isotherms with the agonist ligand [3H]cyclopentylxanthine. When measured using the agonist ligand [125I]-ABA, recombinant bovine A1 adenosine receptors bound ligand with low affinity (Fig. 1A, –G protein). As expected, addition of increasing concentrations of purified recombinant G12 α subunit and β1γ2 dimer to the Sf9 cell membrane expressing recombinant bovine A1 adenosine receptors resulted in the reestablishment of high affinity [125I]-ABA binding (Fig. 1A, + G protein). Note that increasing the G protein concentration in the reconstitution mix from 3 to 40 nM produces a progressively larger fraction of adenosine receptors in the high affinity conformation. These curves demonstrate the very high affinity expected of the bovine receptor for the [125I]-ABA ligand (33). Based on the data in Fig. 1A, an [125I]-ABA concentration of 0.3–0.5 nM and a G protein concentration of 6 nM were chosen for subsequent experiments as a reasonable compromise between the size of the response, the basal level of binding, and the amount of G protein and ligand used in each experiment.

The ability of the reconstituted G protein α subunit to increase the high affinity state of the receptor absolutely requires the βγ dimer (9, 10). Thus, this assay is a sensitive measure of the ability of different βγ dimers to support the first step in the formation of the receptor-αβγ complex. The β1γ2 dimer has been found to be less potent than other βγ combinations in its ability to activate effectors (13–16, 42) and to reestablish high affinity agonist binding.2 To examine the concentrations of βγ needed for the assay, we compared the ability of various concentrations of β1γ2, or β1γ2 dimers in combination with the G12 α subunit to reconstitute high affinity [125I]-ABA binding (Fig. 1B). As shown in Fig. 1A, increasing the amount of either βγ subunit increased the levels of [125I]-ABA binding, but the β1γ2 subunit was less effective at all concentrations compared with the β1γ2 dimer. At 10 nM βγ, the Bmax values were about 880 fmol/mg of membrane protein for β1γ2 and 462 fmol/mg of protein for β1γ2. There was no apparent difference in EC50 value for the two βγ dimers (EC50 = 0.1 nM). Based on the data shown in Fig. 1B, concentrations of βγ dimers between 3 and 10 nM were used in most subsequent experiments.

To characterize the role of the prenyl group of the γ subunits in establishing the high affinity binding conformation
of the receptor, we prepared γ1 and γ2 subunits with altered prenyl groups and examined their ability to support high affinity agonist binding. The mutant γ1-S74L has a geranylgeranyl lipid in place of the wild type γ1 farnesyl group, and the mutant γ2-L71S has a farnesyl group in place of the wild type γ2 geranylgeranyl group (31). The ability of these different βγ pairs to induce high affinity binding of bovine A1 adenosine receptors using 3 nM G12 α subunit and a 3 nM concentration of various βγ dimers is shown in Fig. 2A. The β1γ1-S74L mutant (which contains the geranylgeranyl group) produced an increment in binding about three times greater than the wild type β1γ1 (which contains the farnesyl group). The wild type β1γ1 dimer produced a 2-fold increase in high affinity binding. Interestingly, the β2γ2-L71S mutant containing the farnesyl group was much less active than wild type β1γ2 and was approximately equal to the wild type β1γ2 in generating the high affinity state of the receptor. The responses of all of the βγ dimers were statistically significant (see the legend of Fig. 2A). Experiments analogous to those shown in Fig. 2A were performed with the G11 α subunit (Fig. 2B). The same order of effectiveness at

![Graph](image)

**Fig. 2.** Comparison of the ability of native and altered βγ dimers to support reconstitution of the high affinity agonist binding state of the bovine A1 adenosine receptor. Panel A, Sf9 cell membranes expressing recombinant bovine A1 adenosine receptors were reconstituted with 3 nM G12 α subunit and a 3 nM concentration of the indicated βγ dimers, and the amount of high affinity 125I-ABA binding was measured as described under “Experimental Procedures.” Each tube in the binding assay contained 20 fmol of receptor and 0.3 nM 125I-ABA. The ratio of receptor to α to βγ was approximately 1:10:10 on a mol:mol:mol basis. Shaded bars represent binding in the presence of 100 μM GTPγS. Data are the mean ± S.D. of triplicate determinations compiled from five independent experiments. The ability of each βγ combination to support high affinity binding was significantly different from that of the α subunit alone (p < 0.03). The effect of the β1γ1 dimer was significantly different from both the α subunit alone and the β1γ1-S74L dimer (p < 0.03). The effect of the β1γ2 dimer was significantly different from both the α subunit alone and the β1γ2-L71S dimer (p < 0.03). Panel B, analogous experiments performed with 3 nM G11 α subunit and a 3 nM concentration of the indicated βγ dimers. Data are the mean ± S.D. of two independent experiments, each performed in triplicate.

![Graph](image)

**Fig. 3.** Comparison of the efficacy of wild type and altered βγ subunits to support reconstitution of high affinity 125I-ABA binding. Panel A, Sf9 cell membranes expressing recombinant A1 adenosine receptors were reconstituted with 6 nM G12 α subunit and the indicated concentrations of β1γ1 or β1γ1-S74L as described under “Experimental Procedures.” Each tube in the binding assay contained 20 fmol of receptor and 0.3 nM 125I-ABA. The ratio of receptor to α to βγ was approximately 1:20:0–33. The open circles represent incubations in which 100 μM GTPγS was included in the binding assay from the beginning of the incubation. Panel B, analogous experiments performed using Sf9 cell membranes expressing recombinant bovine A1 adenosine receptors reconstituted with the G11 α subunit and β1γ2 or β1γ2-L71S. The results are representative of six similar experiments performed in triplicate.

establishing high affinity agonist binding was observed using the G11 α subunit as with the G12 α subunit, i.e. β1γ2 or β1γ1-S74L. Note that alteration of the γ2 prenyl group from farnesyl to geranylgeranyl leads to a significant increase in agonist binding at all concentrations of βγ dimer reconstituted into the membrane. The increase in binding is sensitive to GTPγS (open circles), suggesting that the increase in binding is due to receptors coupled to the αβγ heterotrimer. Fig. 3B shows an analogous experiment performed with the native β1γ1 dimer and the prenyl mutant β1γ1-L71S. Clearly, changing the prenyl group from geranylgeranyl to farnesyl results in a significant decrease in binding at the relatively low concentrations of βγ dimer tested. It is important to note that mass spectral analysis of the structure of the four γ subunits used in these experiments showed that the primary amino acid sequences are correct and that all γ proteins are fully modified with the expected prenyl group (31). These data suggest that the type of prenyl group on the γ subunit plays an important role in the reconstitution of the receptor-αβγ complex and the...
The resulting formation of the high affinity agonist binding state of the receptor. As most of the response to the $\beta\gamma$ dimers occurs over a narrow concentration range (1–10 nM), the differences in activity observed could be due to differences in the ability of the proteins to incorporate into the Sf9 cell membrane. Thus, it was important to demonstrate that $\gamma$ subunits with different prenyl groups (farnesyl versus geranylgeranyl) reconstitute into the membrane equally. To this end, we labeled the four different recombinant $\beta\gamma$ dimers with $^{125}$I and examined the ability of the proteins to partition between the buffer phase and the Sf9 cell membrane under the conditions used to measure high affinity agonist binding. As shown in Table I, all four of the $\beta\gamma$ dimers tested reconstitute into the Sf9 cell membrane equally well. About 25% of the G protein $\beta\gamma$ subunits added to the reconstitution mixture are incorporated into the membrane under these conditions. Thus, as judged by this experimental approach, the length of the prenyl chain does not significantly affect the concentration of the $\beta\gamma$ dimer reconstituted into the Sf9 cell membrane.

To determine if the $\alpha_1$ subunit used in the assay affected the results, the ability of wild type and altered $\gamma$ subunits to reestablish high affinity agonist binding was investigated using three different recombinant $\alpha_1$ subunits. As shown in the left panels of Fig. 4 (A–C), the mutant $\beta_1\gamma_1$-S74L dimer was more effective than the native $\beta_1\gamma_1$ dimer regardless of the $\alpha_1$ subunit used in the assay. The increase in high affinity binding induced by the $\beta_1\gamma_1$-S74L dimer was about 1.5-2 times larger than that produced by the $\beta_1\gamma_1$ subunit. Similarly, the right panels of Fig. 4 (D–F) demonstrate that the altered $\beta_1\gamma_2$-L71S dimer was less effective than the native $\beta_1\gamma_2$ when paired with any of the three $\alpha_1$ subunits. Again, the increase in high affinity binding induced by the native $\beta_1\gamma_2$ subunit was about 1.5-1.8 times larger than the mutant $\beta_1\gamma_2$-L71S dimer.

TABLE I

| $\beta\gamma$ subunit | Added dimer | Dimer incorporated into membrane | Reconstituted |
|-----------------------|-------------|---------------------------------|--------------|
| $\beta_1\gamma_1$     | 990         | 287                             | 29           |
| $\beta_1\gamma_1$-S74L| 986         | 203                             | 21           |
| $\beta_1\gamma_2$     | 1,199       | 324                             | 27           |
| $\beta_1\gamma_2$-L71S| 999         | 260                             | 26           |

The $\beta\gamma$ subunits were iodinated and their specific activities quantified as described under "Experimental Procedures." The $^{125}$I-$\beta\gamma$s were reconstituted into Sf9 cell membranes as described under "Experimental Procedures" and incubated at 4 °C for 30 min. The membranes were separated from the buffer phase by centrifugation at 14,000 × g at 4 °C for 10 min and the supernatants and pellets counted in a Beckman 4000 gamma counter. These data are representative of six separate experiments performed in duplicate.

FIG. 4. Comparison of the abilities of three recombinant $\alpha_1$ subunits and different concentrations of $\beta\gamma$ dimers to reconstitute high affinity $^{125}$I-ABA binding in membranes from Sf9 cells expressing recombinant A1 adenosine receptors. Panels A–C, membranes were reconstituted with a 6 nM concentration of the indicated $\alpha_1$ subunit and the indicated concentration of the $\beta_1\gamma_1$ or $\beta_1\gamma_1$-S74L dimers and formation of the high affinity binding state was measured as described under "Experimental Procedures." Each tube in the binding assay contained 20 fmol of receptor and 0.4 nM $^{125}$I-ABA.

The ratio of receptor to $\beta\gamma$ was approximately 1:200–33. Panels D–F, membranes were reconstituted with a 6 nM concentration of the indicated $\alpha_1$ subunit and the indicated concentration of the $\beta_1\gamma_2$ or $\beta_1\gamma_2$-L71S dimers. Formation of the high affinity binding state was measured as described above, except $^{125}$I-ABA was 0.55 nM. The results are representative of three similar experiments performed in triplicate.
mutant γ subunits with altered prenyl groups on the rate of receptor-catalyzed GDP/GTP exchange by comparing the activity of the native β1γ1 subunit alone for 30 min at 0 °C as described under "Experimental Procedures." The reaction tube was brought to 25 °C, and [35S]GTP-γ-S was added to a concentration of 6 nM and incubated for 8 min before adding 100 nM R-PIA. Samples were taken for filtration through glass fiber filters at 30-s intervals after adding the agonist. Panel B, an analogous experiment in which both α and γ subunits were reconstituted into the Sf9 cell membrane as described above. The results are representative of 10 similar experiments.

![FIG. 5. Receptor-catalyzed guanine nucleotide exchange in Sf9 cell membranes expressing A1 adenosine receptors and reconstituted with recombinant G protein α and βγ subunits. Panel A, control experiments showing that neither the Gi2α-subunit alone nor the β1γ2 subunit alone is sufficient to catalyze [35S]GTP-γ-S binding. Sf9 cell membranes (30 fmol of receptor/time point) were reconstituted with either 6 nM Gi2α subunit or 6 nM β1γ2 subunit alone for 30 min at 0 °C as described under "Experimental Procedures." The reaction tube was brought to 25 °C, and [35S]GTP-γ-S was added to a concentration of 6 nM and incubated for 8 min before adding 100 nM R-PIA. Samples were taken for filtration through glass fiber filters at 30-s intervals after adding the agonist. Panel B, an analogous experiment in which both α and βγ subunits were reconstituted into the Sf9 cell membrane as described above. The results are representative of 10 similar experiments.

![FIG. 6. Comparison of the ability of β1γ1 and β1γ2 to support receptor-catalyzed guanine nucleotide exchange on the G protein α subunit. Sf9 membranes expressing the A1 adenosine receptor (40 fmol of receptor/time point) were reconstituted with 6 nM Gi2α subunit and a 6 nM concentration of the indicated βγ subunit as described under "Experimental Procedures" and in the legend to Fig. 5. The results are representative of two similar experiments.

![FIG. 7. Effect of the γ subunit prenyl group on the rate of receptor-catalyzed guanine nucleotide exchange on the G protein α subunit. Panel A, Sf9 membranes containing 30 fmol of receptor/time point were reconstituted with 6 nM Gi2α subunit and a 6 nM concentration of the β1γ1 or the β1γ1-S74L dimers and the assay conducted as described in the legend to Fig. 5. The [35S]GTP-γ-S concentration was 6.6 nM. Panel B, an analogous experiment in which the membranes were reconstituted with 6 nM Gi2α subunit and a 6 nM concentration of the β1γ2 or the β1γ2-L71S dimers. The [35S]GTP-γ-S concentration was 8.6 nM. The results shown are representative of five similar experiments.

increase in the rate of nucleotide exchange, from 65 fmol/min/mg of protein for the β1γ1 subunit (about a 2.3-fold increase over the basal rate of 22.6 fmol/min/mg of protein) to 139 fmol/min/mg of protein (about a 6.3-fold increase over basal) for the β1γ1-S74L dimer. Likewise, changing the prenyl group from geranylgeranyl to farnesyl on the γ2 subunit caused a significant decrease in the rate of nucleotide exchange, from 272 fmol/min/mg of protein (about 4.8-fold over the basal rate of 57 fmol/min/mg of protein) to 123 fmol/min/mg of protein (about 2.1-fold over the basal rate) for the β1γ2-L71S dimer (Fig. 7B).

Based on these data, it appears that the bovine A1 adenosine receptor prefers the geranylgeranyl group on either γ subunit to catalyze a rapid rate of guanine nucleotide exchange on the Giα subunit. This result is consistent with the effects of exchanging the γ subunit prenyl groups observed for high affinity agonist binding in Figs. 2–4 given the kinetic differences between these two assays.

**DISCUSSION**

A major increase in the understanding of the signaling networks activated by the heterotrimeric G proteins has come from the realization that both the α and the βγ subunits have important roles in the generation of intracellular messengers (5, 11–13). The basal state of the G protein is an abg complex capable of interacting with receptors. In the presence of an agonist-bound receptor, the appropriate α subunit is activated by binding GTP and releases the βγ dimer (44). In the GTP-ligated state, the activated α subunit stimulates different effectors such as adenylyl cyclase or phospholipase C-β (44, 45).

The βγ subunits form a functional complex and appear to have...
at least three roles; one to stabilize the GDP-bound form of the α subunit, a second as a required member of the receptor-αβγ complex, and a third as a direct activator of certain effectors (5, 13–16, 38). Although the mechanisms that lead to specific interaction between receptors and the different α subunits are becoming clear (6, 7), much less is known about the factors that determine the specificity of the actions of the βγ dimer (13, 46). The major focus of the present study was to examine the role of the γ subunit in determining signaling specificity.

There are two major differences among the 11 known γ subunits: the marked differences in their primary amino acid sequences and the different prenyl groups attached to their carboxyl terminus (26, 47–51). There are three γ subunits (γ1, 8, and 11) which code for the farnesyl group, and eight other γ subunits are predicted to incorporate the geranylgeranyl group (26, 47–53). The current evidence suggesting that βγ dimers containing the farnesylated γ1 subunit are much less potent in certain assays of βγ function compared with dimers containing γ subunits modified with the geranylgeranyl lipid (14–16, 54) provides a compelling reason to examine functional differences between the two types of γ subunits. Therefore, to dissect the functional differences between the amino acid sequences and the different prenyl groups of the γ1 and γ2 proteins, we expressed recombinant γ subunits incorporating the two different prenyl groups and tested their function in assays of receptor-G protein coupling.

The data in this report provide strong evidence that the A1 adenosine receptor prefers G protein αβγ complexes containing the geranylgeranyl lipid attached to either the γ1 or the γ2 subunit. The experimental system in which recombinant adenosine receptors were expressed in Sf9 insect cell membranes and reconstituted with recombinant α and βγ subunits of defined composition provides a sensitive and specific vehicle for examining the ability of the βγ dimer to interact with the α subunit and the receptor. Among the four βγ dimers tested, the two containing a γ subunit with the geranylgeranyl moiety (native β1γ1 and the β1γ1S74L mutant) were far more effective at coupling to the receptor than the dimers with γ subunits containing the farnesyl group (native β1γ2 and the β1γ2L715 mutant). This result could arise because the different prenyl groups influence the affinity of the βγ dimer for the α subunit; the affinity of the αβγ complex for the receptor; the affinity of the βγ dimer for the receptor; or some combination of these possibilities. Our current data suggest that differences in the affinity of the βγ dimers for the α subunit is the least likely reason as no major differences were found in the ability of the four βγ dimers to bind to or elute from the G1, α-agarose column used in the purification of the proteins (31). Since the carboxyl-terminal 21 amino acids of the γ1 and γ2 proteins are only 48% identical (61% similar), both the peptide sequence and the prenyl group at the carboxyl terminus must play a major role in the receptor-αβγ coupling (29, 46). It is important to realize that the newly discovered γ11 subunit has a carboxyl-terminal amino acid sequence nearly identical to the γ1 protein, is farnesylated, and has a wide tissue distribution (50). Thus, there may be a number of receptor-G protein complexes that prefer interaction with the farnesyl group on the carboxyl terminus of the γ subunit. Finally, since the β1γ1 dimer is less potent in activating effectors compared with β1γ2 (13–16, 54), it is interesting to speculate that the γ subunit’s prenyl group may also play a role in the interaction between the βγ dimer and effectors.

The data obtained in this study provide an interesting counterpoint to similar experiments done using the receptors and G proteins of the visual transduction system. Using rhodopsin as the receptor and the G proteins purified from bovine rod outer segments (the G1, α subunit and the β1γ1 dimer), it has been observed that the β1γ1 dimer is far more effective in coupling the G1, α subunit to rhodopsin than other βγ dimers. For example, using rhodopsin to catalyze GDP/GTP exchange on the G1, α subunit, the β1γ1 dimer supported a much faster rate of exchange than did β1γ2 or β1γ3 (17). Moreover, peptides corresponding to the 11 amino acids of the carboxyl-terminal end of the γ1 subunit stabilize the formation of the active form of rhodopsin much more effectively if they contain the farnesyl group (29) than peptides containing the geranyl or geranylglyceranyl group (30). Other investigators using ADP-ribosylation with pertussis toxin to measure the effect of the prenyl group on the direct interaction of the G1, α and β1γ1 subunits found that farnesylated peptides blocked subunit association and that nonprenylated peptides had no effect (55). Overall these results suggest that both the carboxyl-terminal region of the γ subunit and its prenyl group in the heterotrimer are important for interaction with the receptor. Moreover, it is possible to speculate that the receptor itself may prefer interacting with different prenyl groups. Thus, rhodopsin may be an example of receptors that prefer the farnesyl group on the γ subunit and the A1 adenosine receptor an example of those that prefer the geranylgeranyl group. Interestingly, the 5-hydroxytryptamine 1A receptor, which couples to G1, α subunits, also prefers βγ dimers modified with the geranylgeranyl group (56).

The recently solved x-ray crystal structure of the G protein αβγ heterotrimer provides critical information about the spatial orientation of the α and βγ subunits in the complex (57, 58). Interestingly, the contacts holding the α subunit and the βγ dimer together occur primarily between the α and β subunits, whereas the γ subunit lies across a face of the β subunit without direct contact with the α subunit. However, the carboxyl terminus of the γ protein does lie within 18–19 Å of the amino terminus of the α subunit and is also relatively near the α subunit’s carboxyl terminus. As the amino terminus of the α subunit and the carboxyl terminus of the γ subunit group are the two domains in the heterotrimer known to contain post-translational lipid modifications and the lipid groups on the α and γ subunits are thought to anchor the heterotrimer to the membrane (24), it is presumed that the amino terminus of the α and the carboxyl terminus of the γ subunit may lie near the inner surface of the plasma membrane (57, 58). Thus, these two domains and the nearby carboxyl terminus of the α subunit would be in a position to interact with the intracellular loops of the receptor. This interpretation of the heterotrimer crystal structure is in agreement with a large amount of biochemical and genetic data that point to the carboxyl terminus of the α subunit as one important site of receptor interaction with the α subunit (59). The data in this report argue that the γ subunit’s prenyl group is also important in the receptor-αβγ interaction and are consistent with the orientation of this face of the heterotrimer toward the receptor in the plasma membrane.

Overall, the data presented in this report agree with the developing picture of receptor-G protein interaction. Clearly the prenyl group on the γ subunit is one important determinant of the receptor-αβγ interaction. Moreover, the data suggest that the prenyl group has a role in determining protein/protein interactions in addition to the role of anchoring the βγ dimer to the membrane. Interestingly, prenyl groups have been suggested to play a role in protein/protein interactions in a number of other systems (24, 25), and a number of models have been proposed in which lipid groups insert into hydrophobic pockets of proteins or interface with a hydrophobic pocket and the membrane (24, 32, 60, 61). Further work will be necessary to
determine if the G protein-coupled receptors have such lipid
binding domains.

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