Specific Isoprenyl Group Linked to Transducin γ-Subunit Is a Determinant of Its Unique Signaling Properties among G-Proteins†

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ABSTRACT: Among 11 subtypes of heterotrimeric G-protein γ-subunit, γ_1 (rod), γ_8 (cone) and γ_{11} are modified with farnesyl while the others are modified with geranylgeranyl at the C-terminus. To understand the role of specific isoprenylation (farnesylation) of retinal transducin, we examined how and to what extent the type of isoprenyl group affects transducin-$\beta\gamma$ ($\beta_1\gamma_1$) functions such as interactions with membranes, Gα/receptor, and effectors. To this end, the C-terminal farnesylation signal sequence (CVIS) of γ_1 was replaced by a geranylgeranylation signal (CVIL), and the resultant mutant (S74L) or wild-type (WT) γ_1 was coexpressed with β_1 in the baculovirus-Tn5 insect cell system. Both γ_1WT and γ_1S74L expressed as a $\beta\gamma$ complex were mixtures modified with farnesyl and geranylgeranyl groups. The ratio of farnesyl to geranylgeranyl in preparations of $\beta_1\gamma_1$WT and $\beta_1\gamma_1$S74L purified from the Tn5 cell membrane fraction was about 1:2 and 1:6, respectively. These two forms of recombinant $\beta_1\gamma_1$ and retinal $\beta_1\gamma_1$ were different in their abilities to associate with rod outer segment membranes with the following rank order: $\beta_1\gamma_1$S74L > $\beta_1\gamma_1$WT > retinal $\beta_1\gamma_1$. Functionally, $\beta_1\gamma_1$S74L was the most potent to promote pertussis toxin-catalyzed ADP ribosylation of transducin-α (Tα), to stimulate metarhodopsin II-catalyzed GTPγS-binding reaction to Tα and to modulate adenylyl cyclase and phospholipase C activities. All of the $\beta_1\gamma_1$ functions absolutely required the isoprenylation of the γ-subunit. As for the interaction with Goα and adenylyl cyclase, predominantly geranylgeranylated $\beta_1\gamma_1$S74L was less effective than geranylgeranylated $\beta_1\gamma_2$ purified from bovine brain. These results demonstrate that the properties of G$\beta\gamma$ are strongly affected by the type of functionally indispensable isoprenylation in addition to the amino acid sequence of Gγ. The relative contribution of the two factors depends on proteins with which G$\beta\gamma$ interacts.

The heterotrimeric guanine nucleotide-binding regulatory proteins (G-proteins[1]) play a central role in transducing extracellular signals from cell surface receptors to diverse intracellular effectors (*1*, *2*). G-Proteins are composed of three subunits termed α, β, and γ. The α subunit (Gα) possesses a guanine nucleotide-binding site and GTPase activity, while the β (Gβ) and γ (Gγ) subunits form a tight $\beta\gamma$ complex which is disrupted only by denaturation.

To date, 5 subtypes of Gβ and 11 subtypes of Gγ have been identified in mammalian cells (*3*, *4*). Such a diversity is believed to contribute to selective coupling between receptors and effectors (*5*, *6*). Comparison of the properties among several $\beta\gamma$ complexes in vitro, however, revealed substantially no difference except for retinal transducin $\beta_1\gamma_1$. Among G$\beta\gamma$'s, only $\beta_1\gamma_1$ showed considerably low affinity for Gα in GDP-bound form (*7*) and little ability to regulate activities of adenylyl cyclase (*7*, *8*), phospholipase C (*9*), receptor kinase (*10*), and K^+ channel (*11*). A more clear difference is that $\beta_1\gamma_1$ can be easily extracted from cell membranes by using hypotonic buffer, while the others require detergents to be extracted. These unique properties of $\beta_1\gamma_1$ have been attributed to γ_1 because most $\beta\gamma$ complexes used in those experiments share a common β_1 subunit.

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[1] Abbreviations: CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; DTT, dithiothreitol; FPLC, fast protein liquid chromatography; G-protein, guanine nucleotide-binding regulatory protein; GTPγS, guanosine 5′-*O*-(thiotriphosphate); MOPS, 3-(*N*-morpholino)propanesulfonic acid; PIP_2, phosphatidylinositol 4,5-bisphosphate; ROS, rod outer segment; Tα, α-subunit of retinal transducin; T$\beta\gamma$, $\beta\gamma$ complex of retinal transducin. For clarity, this term "T$\beta\gamma$" was used for specifying $\beta_1\gamma_1$ purified from bovine retinas to distinguish it from recombinant $\beta_1\gamma_1$.

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Gγ's are 68−75 amino acid polypeptides containing a consensus C-terminal sequence designated CAAX motif (C, cysteine; A, aliphatic amino acid; X, any amino acid). The cysteine residue in the CAAX motif is modified with C15 farnesyl or C20 geranylgeranyl, followed by a removal of AAX, and a newly exposed carboxyl group of the cysteine residue is methyl-esterified (*12*). The X residue in the CAAX motif is a major determinant of the isoprenyl group, C15 or C20 (*12*). When X is serine, methionine, glutamine, or alanine, the proteins are recognized by farnesyltransferase, whereas a leucine residue at this position leads to the modification by geranylgeranyltransferase-I. For example, γ_1, γ_8(cone) and γ_{11} with serine at the X position are modified with farnesyl (*13*−*16*), while the other γ-subunits with leucine are modified with geranylgeranyl (*4*, *17*−*19*). These posttranslational modifications are required for membrane attachment of $\beta\gamma$ complexes (*20*, *21*). Functionally, the modifications facilitate interactions of $\beta\gamma$ with its cognate Gα (*8*, *21*−*26*) and the effectors such as adenylyl cyclase (*8*), phospholipase C (*27*, *28*), and phosphoinositide 3-kinase (*29*). Thus, selective farnesylation of transducin $\beta\gamma$ is one of the candidates for a determinant of the unique signaling properties of $\beta_1\gamma_1$ among a variety of G-protein $\beta\gamma$ complexes (*30*).

In this report, by using native $\beta_1\gamma_1$ (farnesylated), native $\beta_1\gamma_2$ (geranylgeranylated), and recombinant $\beta_1\gamma_1$ (farnesylated/geranylgeranylated), we examined to what extent the characteristic properties of $\beta_1\gamma_1$ are attributed to "selective isoprenylation" and "amino acid sequence" of γ_1. The results demonstrate that the replacement of farnesyl linked to γ_1 by geranylgeranyl remarkably altered the affinity of $\beta\gamma$ complex for Gα and effectors as well as cell membranes, but the functions of $\beta_1\gamma_1$ were not fully converted to those of the other geranylgeranylated G$\beta\gamma$ by a simple substitution of the isoprenyl group, indicating important contributions of both the type of isoprenylation and the amino acid sequence of γ-subunits.

EXPERIMENTAL PROCEDURES

Construction of Recombinant Baculoviruses. The cDNAs encoding β_1 and γ_1 were generated by polymerase chain reactions with bovine retinal cDNA as the template and with primers corresponding to the 5′ and 3′ ends of the coding regions (plus 5′ restriction site for *Eco*RI and 3′ restriction site for *Not* I). The amplified cDNAs were ligated into baculovirus expression vector pVL1393. Site-directed mutagenesis (S74L) of the serine (CAT) at position 74 of γ_1 into leucine (TAT) was performed by PCR using the following primers: 5′-CTAGAATTCC ACCATGCCAGTGATC-3′ and 5′-GCAGCGGCCGCTTATAAAATCACACAG-3′. The amplified product was digested with *Eco*RI and *Not* I and ligated into pVL1393. After nucleotide sequences were verified by DNA sequencing, the vectors were cotransfected into *Spodoptera frugiperda* (Sf9) cells with BaculoGold viral DNA (Pharmingen). Recombinant baculoviruses thus obtained were plaque-purified and amplified.

Expression of Recombinant $\beta_1\gamma_1$ Complex. For production of recombinant $\beta_1\gamma_1$ complex, *Trichoplusia ni* (Tn5) cells were grown to a density of 4×10^6 cells/mL at 27 °C in a serum-free SF900 II medium (Life Technologies). Cells (6×10^8 cells) were coinfected with β- and γ-recombinant baculoviruses at a multiplicity of infection of 5 for each virus and harvested 72 h after the infection. The cells were washed twice with PBS (10 mM Na−Pi, 140 mM NaCl, 1 mM $MgCl_2$; pH 7.4), suspended in 60 mL of buffer A (20 mM Tris-HCl, 2 mM $MgCl_2$, 1 mM DTT, 0.1 mM phenylmethanesulfonyl fluoride, 4 μg/mL aprotinin, 4 μg/mL leupeptin; pH 8.0), and homogenized using a Teflon homogenizer (10 strokes), followed by drawing/ejecting the suspension 10 times through a 25-gauge needle. The homogenate was centrifuged at 100000*g* for 30 min at 4 °C to isolate "cytosol" and "membrane" fractions. The membrane fraction was homogenized with 60 mL of buffer B (buffer A containing 1% CHAPS) for solubilization, and then centrifuged at 100000*g* for 30 min at 4 °C to isolate "CHAPS extract" and "insoluble" fractions.

Protein Purification. All the purification procedures were carried out at 4 °C unless otherwise stated.

Recombinant $\beta_1\gamma_1$ complex was purified from both cytosol and CHAPS extract fractions. For purification of $\beta_1\gamma_1$ complex from CHAPS extract fraction, the final extract (60 mL) was applied to a Blue-Sepharose (Pharmacia) column (diameter 14 × 60 mm), and the flow-through was applied to a DEAE-Toyopearl 650S (Toso, Japan) column (diameter 10 × 60 mm) preequilibrated with buffer B. After application of the sample, the column was washed with 50 mL of buffer B and proteins were eluted with a linear gradient of NaCl (0−500 mM) in buffer B (30 mL) at a flow rate of 22 mL/h. Fractions containing $\beta_1\gamma_1$ complex were pooled (5 mL) and applied to a gel filtration TSKgel-G3000SW column (diameter 25 × 600 mm, Toso, Japan), from which $\beta_1\gamma_1$ complex was eluted with buffer B containing 100 mM NaCl at a flow rate of 3 mL/min using a FPLC system (Pharmacia). The $\beta_1\gamma_1$ complex-rich fractions were pooled (15 mL), applied to a MonoQ HR 5/5 (Pharmacia) equilibrated with buffer C (20 mM Tris-HCl, 5 mM $MgCl_2$, 1mM DTT, 1% CHAPS; pH 7.7), and eluted with a linear gradient of NaCl (0−500 mM) in buffer C (50 mL) at a flow rate of 1 mL/min using a FPLC system. Similarly, $\beta_1\gamma_1$ complex in cytosol fraction was purified using the buffers without CHAPS.

Transducin was purified from bovine retinas as described previously (*21*). Farnesylated $\beta_1\gamma_1$ was separated into carboxylmethylated and nonmethylated forms (*21*). Goα and $\beta_1\gamma_2$ were purified from bovine brain as described (*7*, *31*). Transducin-depleted rod outer segment membranes were prepared as described (*24*). Purified proteins were divided into aliquots and stored at −80 °C.

Electrophoresis and Immunoblotting. SDS−PAGE was carried out as described by Laemmli (*32*). For better separation of γ-subunit bands, Tricine/SDS−PAGE was performed (*33*). For immunoblotting, proteins resolved by SDS−PAGE were transferred to Immobilon P (Millipore) and probed with a mixture of mouse antisera AS$\beta\gamma$-3 raised against bovine $\beta_1\gamma_1$ complex (*34*) and anti-γ_1 against purified bovine γ_1 at final dilutions of 1:2000 (AS$\beta\gamma$-3) and 1:100 (anti-γ_1). The two antisera, AS$\beta\gamma$-3 and anti-γ_1, stained no specific bands of endogenous proteins (e.g., Gβ and Gγ) in insect cells. Bound antibodies were detected by goat anti-mouse IgG conjugated to horseradish peroxidase and visualized by using Renaissance (DuPont).

Analyses of γ-Subunits by Reversed-Phase HPLC. To analyze the C-terminal structure (lipid modifications) of

recombinant γ_1, purified $\beta\gamma$ complexes were directly injected onto a reversed-phase column (Cosmosil $5C_{18}$-P300, diameter 4.6 × 150 mm; Nacalai Tesque, Japan) equipped with an HPLC system (model 600E; Waters). The γ-subunits were eluted from the column with a linear gradient (1%/min) of acetonitrile (30−80%) in 0.1% trifluoroacetic acid at a flow rate of 1 mL/min. Eluted peak fractions were collected and characterized by electrospray mass spectrometry as described (*24*).

Quantification of $\beta\gamma$ Complexes Associated with ROS Membranes. The amounts of $\beta\gamma$ complexes associated with transducin-depleted ROS membranes were estimated as described previously (*21*) with slight modifications. Briefly, purified $\beta\gamma$ (1.5 μM) in 30 μL of buffer B containing 200 mM NaCl was mixed with the transducin-depleted ROS membranes (25 μM rhodopsin) in 120 μL of buffer D (10 mM MOPS−NaOH, 2 mM $MgCl_2$, 1 mM DTT, 0.1 mM phenylmethanesulfonyl fluoride, 4 μg/mL aprotinin, 4 μg/mL leupeptin; pH 7.5). The mixture was centrifuged at 18000*g* for 30 min at 4 °C to sediment the membranes with bound $\beta\gamma$. The resultant clear supernatant (120 μL) containing unbound subunits was subjected to SDS−PAGE and transferred to the PVDF membrane. The amounts of β_1 and γ_1 were estimated by densitometric scanning of their bands detected by immunoblotting.

Miscellaneous Procedures. ADP-ribosylation of Tα catalyzed by pertussis toxin and the binding of GTPγS to Tα catalyzed by metarhodopsin II were measured as described (*21*). The rate of GDP release from Goα and calmodulin-stimulated adenylyl cyclase activity in rat retinal membranes were measured as described (*7*). Phospholipase C activity in the cytosol fraction of HL-60 cells was assayed as described (*35*).

Protein concentrations were determined using bovine serum albumin as a standard (*36*). Rhodopsin concentration was calculated from the difference in absorbance at 498 nm before and after complete bleaching of rhodopsin in the presence of 100 mM NH_2OH.

RESULTS

Expression of $\beta_1\gamma_1$ Complex in Tn5 Insect Cells. To examine to what extent the functional characteristics of transducin $\beta\gamma$ are ascribed to its specific isoprenyl group linked to γ_1, the farnesylation signal sequence (CVIS) at the C-terminus of γ_1 was mutated into a geranylgeranylation signal sequence (CVIL). This mutant γ_1 (S74L) or wild-type γ_1 (WT) was coexpressed with β_1 in insect cells using a baculovirus expression system. For this purpose, we used Tn5 cells derived from the cabbage looper instead of widely used fall armyworm Sf9 cells because of the higher level of expressed proteins in Tn5 cells. As shown in Figure 1A, γ_1WT and γ_1S74L were recovered in the "cytosol (Cyt)" or "CHAPS extract (Ext)" fraction but not in the "insoluble (Ins)" fraction. On the other hand, most of expressed β_1 was found in the insoluble fraction (Ins; Figure 1A), indicating low efficiency of complex formation between β_1 and γ_1 expressed in Tn5 cells. The cytosol and CHAPS extract fractions, however, contained a small amount of β_1 immunoreactivity, which was not detected in the cells infected with β_1 virus alone, and thus it appeared to represent $\beta_1\gamma_1$ complex.

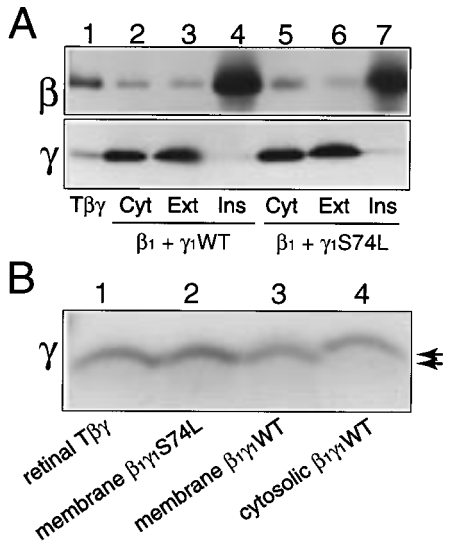

FIGURE 1: Electrophoretic analysis of β_1 and γ_1 expressed in Tn5 insect cells. (Panel A) Western blot analysis of β_1 and γ_1 in fractions of Tn5 cells infected with a combination of β_1 and γ_1WT (lanes 2−4) or β_1 and γ_1S74L (lanes 5−7) viruses. The cells were recovered 72 h after infection and fractionated into the cytosol (Cyt; lanes 2 and 5), CHAPS extract (Ext; lanes 3 and 6), and insoluble (Ins; lanes 4 and 7) fractions. Proteins in these fractions and T$\beta\gamma$ purified from bovine retinas (lane 1) were electrophoresed in an SDS-polyacrylamide (13%) gel, transferred to Immobilon P membrane, and then probed with antibodies to β_1 and γ_1. Parts of the membrane sheet with β_1 and γ_1 bands are shown. (Panel B) Tricine/SDS−PAGE analysis of purified preparations of retinal T$\beta\gamma$ (lane 1), CHAPS-extracted $\beta_1\gamma_1$S74L (lane 2), CHAPS-extracted $\beta_1\gamma_1$WT (lane 3), and cytosolic $\beta_1\gamma_1$WT (lane 4). The gel was stained with Coomassie Blue, and only the γ_1 bands are shown. Note that the mobility of cytosolic γ_1WT (lane 4) is slightly different from those of the other γ-subunits (lanes 1−3).

We purified $\beta_1\gamma_1$WT complex from these two fractions independently and also purified $\beta_1\gamma_1$S74L from CHAPS extract fraction (see Experimental Procedures). The final yields of $\beta_1\gamma_1$ complexes purified from 600-mL cell cultures were approximately 700, 350, and 350 μg for cytosolic $\beta_1\gamma_1$WT, CHAPS-extracted $\beta_1\gamma_1$WT, and CHAPS-extracted $\beta_1\gamma_1$S74L, respectively. Hereafter, the $\beta_1\gamma_1$ complex purified from the CHAPS extract fraction is termed "membrane $\beta_1\gamma_1$ (WT or S74L)".

Structural Analysis of γ_1 in Recombinant $\beta_1\gamma_1$ Complex. The three preparations of purified $\beta\gamma$ complexes (cytosolic $\beta_1\gamma_1$WT, membrane $\beta_1\gamma_1$WT, membrane $\beta_1\gamma_1$S74L) were subjected to Tricine/SDS−PAGE for analysis of G-protein γ-subunits (*19*). As shown in Figure 1B, the mobilities of γ-subunits in membrane $\beta_1\gamma_1$S74L (lane 2) and membrane $\beta_1\gamma_1$WT (lane 3) were identical to that of farnesylated Tγ purified from bovine retinas (lane 1), whereas γ-subunit in cytosolic $\beta_1\gamma_1$WT (lane 4) showed slightly slower mobility. This suggested that both membrane γ_1WT and membrane γ_1S74L are isoprenylated while cytosolic γ_1WT is not, because the C-terminal isoprenylation is known to increase the electrophoretic mobility of proteins including γ_1 (*22*, *37*).

To determine the C-terminal structures of the recombinant γ_1's, we analyzed their chromatographic behaviors by using a reversed-phase C_{18} column (Cosmosil $5C_{18}$-P300) under denaturing conditions (*24*, *30*). When retinal T$\beta\gamma$ was injected onto the column (Figure 2, trace a), two peaks corresponding to the methylated and nonmethylated forms of farnesylated γ_1 were detected as reported previously (*21*,

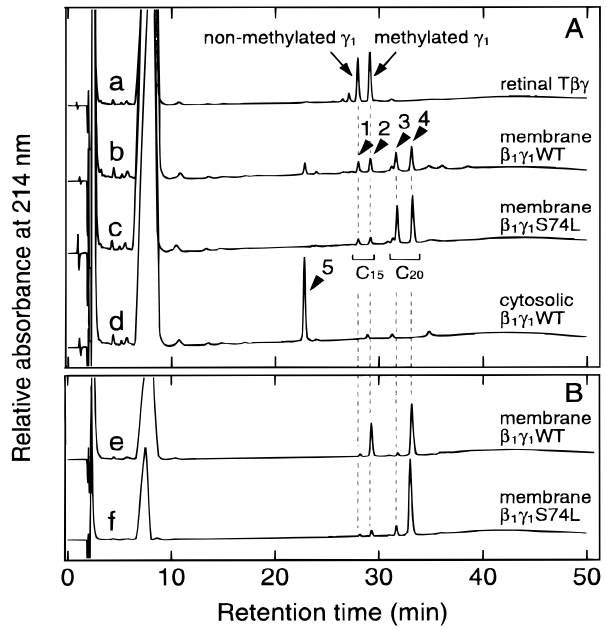

FIGURE 2: Reversed-phase HPLC analysis of γ-subunits. (Panel A) Each of purified $\beta_1\gamma_1$ complexes was injected onto a reversed-phase column (Cosmosil $5C_{18}$-P300, diameter 4.6 × 150 mm) equipped with an HPLC system (model 600E; Waters). The γ-subunits were eluted with a linear gradient of acetonitrile (30−80%, 1%/min) in 0.1% trifluoroacetic acid at a flow rate of 1 mL/min, and the absorbance at 214 nm of the eluate was continuously monitored. (a) Injection of retinal T$\beta\gamma$ gave two γ_1 peaks corresponding to carboxylmethylated and nonmethylated forms of farnesylated γ_1. (b) Injection of membrane $\beta_1\gamma_1$WT gave four γ_1 peaks designated 1−4. Electrospray-ionization mass spectrometry revealed that these correspond to farnesylated/nonmethylated (peak 1), farnesylated/methylated (peak 2), geranylgeranylated/nonmethylated (peak 3), and geranylgeranylated/methylated γ_1 (peak 4). A peak eluted at 22.8 min was ascribed to a contaminated protein. (c) The γ_1 subunit of membrane $\beta_1\gamma_1$S74L eluted into the same four peak fractions as in (b) with a different relative composition. (d) The γ_1 subunit of cytosolic $\beta_1\gamma_1$WT eluted much faster in peak 5 (= unprocessed γ_1). (Panel B) another preparation of membrane $\beta_1\gamma_1$WT (e) or membrane $\beta_1\gamma_1$S74L (f) was analyzed similarly.

24). In addition to these two peaks, another pair of peaks was detected when membrane $\beta_1\gamma_1$WT was injected onto the column (Figure 2, trace b). The four peak fractions (peaks 1−4) contained electrophoretically indistinguishable γ_1 (not shown), but electrospray-ionization mass spectrometry revealed a difference in mass: peak 1, 8318.5; peak 2, 8332.0; peak 3, 8385.0; peak 4, 8400.0. These values respectively agreed well with the calculated masses of farnesylated (8315.7), farnesylated/methylated (8329.7), geranylgeranylated (8383.8), and geranylgeranylated/methylated (8397.8) forms of γ_1 (Pro^2−Cys^{71}). Such a heterogeneous modification of γ_1 in Tn5 cells is consistent with a previous report that γ_1 expressed in Sf9 cells incorporates not only farnesyl but also geranylgeranyl (*38*). The ratio in area intensity of peaks 1−4 was 14:19:30:37 (Figure 2, trace b), and the ratio of farnesylated γ_1WT to geranylgeranylated γ_1WT was (14 + 19):(30 + 37) ≈ 1:2.

Similarly, membrane γ_1S74L was a mixture of the four components (Figure 2, trace c) with a ratio in peak area intensity of 6:8:39:47. In this case, the ratio of farnesylated γ_1S74L to geranylgeranylated γ_1S74L was (6 + 8):(39 + 47) ≈ 1:6. That is, γ_1S74L incorporated a small amount of farnesyl despite its geranylgeranylation signal sequence. The degree of γ_1 methylation varied from preparation to preparation (compare panel A with B in Figure 2), representing an artificial demethylation during the purification procedures (*21*). In contrast with this, the ratio of farnesylated form to geranylgeranylated form was almost constant: approximately 1:2 in wild type and 1:6 in S74L mutant, respectively.

On the other hand, when cytosolic $\beta_1\gamma_1$WT was injected onto the column, the γ-subunit was eluted much faster (Figure 2, trace d). An electrospray-ionization mass spectrum of this peak fraction (peak 5) gave a molecular mass of 8409.9, which is in good agreement with the calculated mass (8410.7) of Pro^2−Ser^{74} of γ_1. Together with its fast elution from the C_{18} column, this indicates that cytosolic γ_1-WT is not modified posttranslationally at the C-terminus. Overexpression of the γ-subunit in insect cells would result in an accumulation of unmodified $\beta\gamma$ in the cytosol, and it is concluded that the modification of γ_1 is not required for formation of $\beta_1\gamma_1$ complex.

Effect of the Prenyl Groups on Membrane Association of $\beta\gamma$. We examined the effects of the prenyl groups in detail by using the four preparations of $\beta_1\gamma_1$ as follows:

(i) retinal T$\beta\gamma$ (pure farnesyl)
(ii) membrane $\beta_1\gamma_1$WT (farnesyl:geranylgeranyl = 1:2)
(iii) membrane $\beta_1\gamma_1$S74L (farnesyl:geranylgeranyl = 1:6)
(iv) cytosolic $\beta_1\gamma_1$WT (unmodified).

We have previously shown that the carboxyl methylation of γ_1 affects the function of the $\beta_1\gamma_1$ complex (*21*). Therefore, we selected a set of the $\beta_1\gamma_1$ preparations (i−iii), in which the degree of methylation of γ_1 is nearly identical to each other, to make clear the contribution of the prenyl groups. That is, the ratio of nonmethylated to methylated form was 45:55 (as shown in Figure 2A) in experiments of Figures 3−5 or 5:95 (as shown in Figure 2B) in experiments of Figures 6−8.

To see how the chain length of isoprenoid affects the interaction between $\beta_1\gamma_1$ complex and lipid membranes, we compared the binding of $\beta_1\gamma_1$ complexes (i−iv) to transducin-depleted ROS membranes (Figure 3). The amount of $\beta_1\gamma_1$ bound to the membrane was estimated by quantifying unbound β_1 and γ_1 in supernatant after centrifugation. As depicted in Figure 3, cytosolic $\beta_1\gamma_1$WT showed almost no affinity for the membranes, indicating an essential role of the C-terminal modification in the membrane association of $\beta\gamma$. On the other hand, three preparations of isoprenylated $\beta_1\gamma_1$ showed distinct membrane affinity which increased in parallel with the increase in relative content of geranylgeranylated γ_1 (membrane $\beta_1\gamma_1$S74L > membrane $\beta_1\gamma_1$WT > retinal T$\beta\gamma$). It is concluded that geranylgeranylated $\beta\gamma$ has much higher affinity for the membrane than farnesylated one.

Interaction of Recombinant $\beta\gamma$ *Complexes with* Gα *and Rhodopsin*. To examine whether the type of the prenyl group affects the interaction of $\beta_1\gamma_1$ complex with α-subunit, we first compared the ability of the $\beta_1\gamma_1$ complex to support pertussis toxin-catalyzed ADP-ribosylation of Tα in the absence of lipid membranes. Since pertussis toxin ADP-ribosylates Tα-GDP, which is associated with $\beta_1\gamma_1$, quantitative analysis of the reaction rate is one of the sensitive methods to detect Tα−$\beta_1\gamma_1$ association (*39*). As shown in Figure 4, the initial rate of the reaction was elevated by the addition of isoprenylated $\beta\gamma$ preparations in a dose-dependent manner, but cytosolic $\beta_1\gamma_1$WT with unmodified γ_1 induced no enhancement of the reaction rate. This observation is consistent with the reports showing an essential role of the

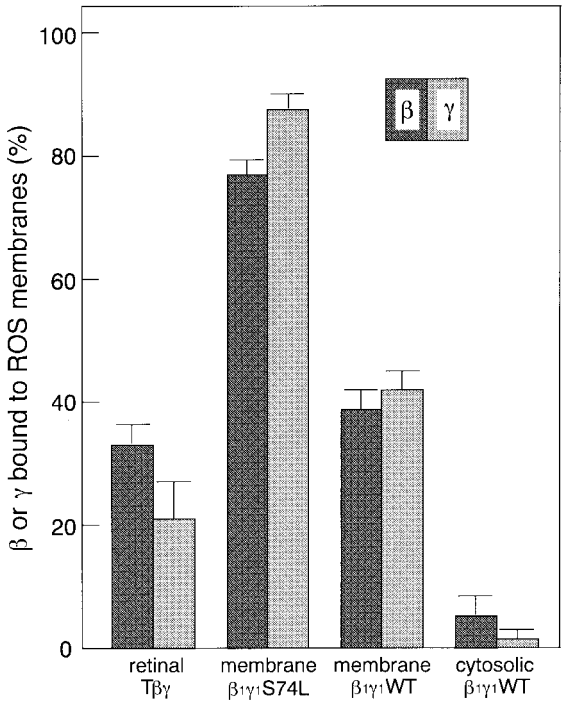

FIGURE 3: Effect of isoprenylation of $\beta_1\gamma_1$ complex on membrane association. Each $\beta\gamma$ complex (0.3 μM) was mixed with nonirradiated stripped ROS membranes (20 μM rhodopsin) in the dark and centrifuged to obtain a clear supernatant containing unbound $\beta\gamma$ complex. This supernatant was subjected to SDS−PAGE/immunoblot analysis (see Experimental Procedures), and the amounts of β_1 and γ_1 were quantified by densitometric scanning of an X-ray film. The relative amounts of membrane-bound β_1 and γ_1 were calculated by subtracting the amount of unbound one from the total added to the tube, and they were expressed as percent of total amount. Data are the average ± range of results from two independent experiments.

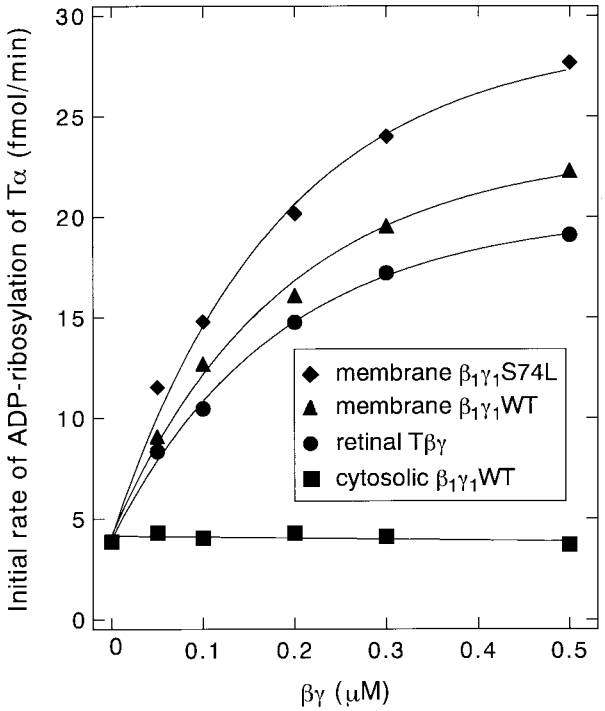

FIGURE 4: Effect of $\beta_1\gamma_1$ complex on pertussis toxin-catalyzed ADP-ribosylation of Tα. Prior to experiments, pertussis toxin (Kaken-Seiyaku Co.) was preactivated at 30 °C for 15 min in buffer E (72 mM HEPES−NaOH, pH 6.6, 180 mM NaCl, 18 mM EDTA, 10mM DTT, 3.6 mM ATP and 0.7 mM GDP). The ADP ribosylation reactions were carried out at 30 °C in reaction mixtures (150 μL:120 μL of buffer D containing 100 mM NaCl plus 30 μL of buffer E) containing various concentrations (0.05, 0.1, 0.2, 0.3, or 0.5 μM) of $\beta\gamma$ complex, 0.5 μM Tα, 10 μg/mL preactivated pertussis toxin, 2.5 mg/mL ovalbumin (Sigma), 0.17% CHAPS, and 10 μM [^{32}P]NAD (1 Ci/mmol). At 15-min intervals of incubation time (≤60 min), 20-μL aliquots of the reaction mixture were withdrawn and mixed with 200 μL of ice-cold buffer F (100 mM Tris-HCl and 1 mM $MgCl_2$) containing 15% (w/v) trichloroacetic acid for terminating the reaction. [^{32}P]ADP-ribosylated Tα was isolated from free [^{32}P]NAD by filtrating the samples over 0.45-μm cellulose membranes (type HATF; Millipore) fitted with MultiScreen Assay System (Millipore). After filtration, membranes were washed four times with 0.3 mL of buffer F. The radioactivities of the membranes were determined by liquid scintillation counting. The initial rate of the reaction was calculated from the slope of the linear fitting of the data and plotted against the concentration of $\beta\gamma$. Data represent a typical set of three independent experiments with similar results.

isoprenylation in the functional interaction between Gα and G$\beta\gamma$ (*8*, *21*−*23*, *25*, *26*, *30*). Previously we showed that totally inactive T$\beta\gamma$ has a truncated form of Tγ which lacks the C-terminal CVIS tetrapeptide including farnesyl (*13*, *22*). Therefore, it is reasonable to conclude that the low activity of unmodified $\beta_1\gamma_1$ produced in the current study is due to the absence of the prenylation, though a possibility is not completely excluded that the VIS tripeptide present in the unmodified $\beta_1\gamma_1$ might also contribute to the low activity.

Among the isoprenylated $\beta\gamma$ preparations, the ability to enhance the ADP-ribosylation reaction ranked with the same order as the membrane binding; membrane $\beta_1\gamma_1$S74L > membrane $\beta_1\gamma_1$WT > retinal T$\beta\gamma$ (Figure 4). Clearly geranylgeranylated $\beta_1\gamma_1$ is more potent to stimulate the ADP-ribosylation reaction than farnesylated one, suggesting that Tα interacts with geranylgeranylated $\beta_1\gamma_1$ more efficiently than with farnesylated one.

We next compared the abilities of these $\beta_1\gamma_1$ complexes to promote the binding of GTPγS to Tα in the presence of the activated receptor, metarhodopsin II, reconstituted in phospholipid vesicles. As shown in Figure 5, the rate constant of the reaction was progressively elevated with increasing concentrations of membrane $\beta_1\gamma_1$S74L, membranne $\beta_1\gamma_1$WT, and retinal T$\beta\gamma$, while cytosolic $\beta_1\gamma_1$WT showed no catalytic activity. The activities of modified $\beta_1\gamma_1$ complexes were again proportional to the relative content of geranylgeranylated γ_1 in the complex. We concluded that the C-terminal isoprenylation of γ_1 is essential for the light signal transduction and that the types of the isoprenyl groups heavily affect the signaling efficiency from rhodopsin to transducin.

The results of Figures 3−5 demonstrate that some unique properties of $\beta_1\gamma_1$ among $\beta\gamma$ complexes are at least partly ascribed to the farnesylation of γ_1 despite the divergence of the primary structures of the γ-subunits. Then we asked whether the functions of $\beta_1\gamma_1$ can be completely converted to those of the other $\beta\gamma$ complexes simply by substitution of the isoprenyl group. To answer this question, we compared the affinity for Goα of the $\beta_1\gamma_1$ complexes expressed in Tn5 cells with that of geranylgeranylated $\beta_1\gamma_2$ purified from bovine brain. The interaction of Goα with each $\beta\gamma$ complex in the absence of membranes was assessed by measuring the rate of GDP release from Goα, which is slowed by α−$\beta\gamma$ trimer complex formation (*7*). Figure 6 shows that all the $\beta\gamma$ complexes except for cytosolic $\beta_1\gamma_1$WT slowed the dissociation rate of GDP from Goα,

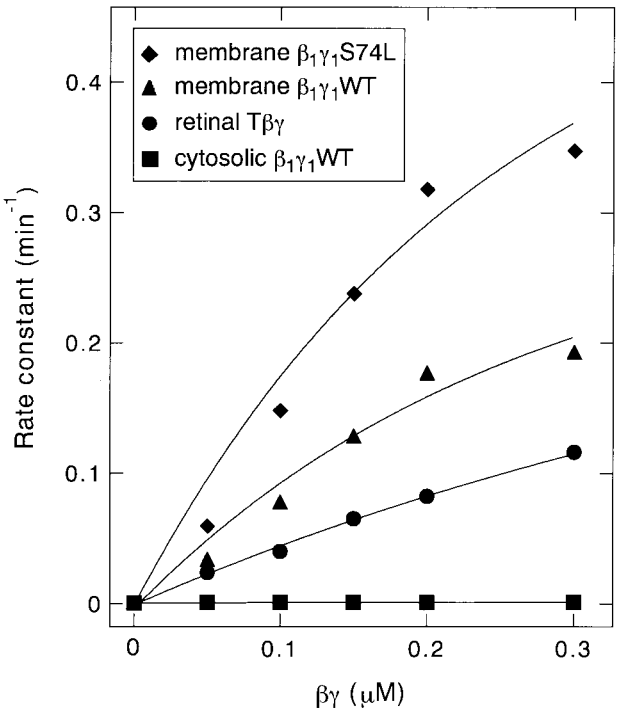

FIGURE 5: Effect of $\beta_1\gamma_1$ complex on metarhodopsin II-catalyzed GTPγS binding to Tα. Prior to experiments, purified bovine rhodopsin reconstituted in phosphatidylcholine liposomes was light-activated with the orange light (>540 nm) for 1 min at 4 °C. The time course of the GTPγS binding to Tα catalyzed by metarhodopsin II was measured at 4 °C in a reaction mixture (150 μL of buffer D containing 100 mM NaCl) composed of various concentrations (0.05, 0.1, 0.15, 0.2, or 0.3 μM) of $\beta\gamma$ complex, 1.0 μM Tα, 30 nM metarhodopsin II in liposomes, 2.5 mg/mL ovalbumin, 0.1% CHAPS, and 10 μM [^{35}S]GTPγS (3 Ci/mmol). The reaction was started by the addition of [^{35}S]GTPγS and terminated by diluting 10-μL aliquots, which were withdrawn at 2.5-min intervals of incubation time ($\leq$15 min) and mixed with 200 μL of ice-cold buffer F containing 2 mM GTP. Tα-bound [^{35}S]GTPγS was isolated from free [^{35}S]GTPγS by membrane filtration as described in the legend to Figure 5. The data points were best fitted to a single-exponential equation, $B(t) = B_m(1 - \exp(-kt))$, where $B(t)$ is the amount of Tα-bound GTPγS at time t, B_m is the maximum binding at infinite time, and k is the rate constant. The rate constants calculated from the kinetic experiments were plotted against the concentration of $\beta\gamma$ in the reaction mixtures. Data represent a typical set of five independent experiments with similar results.

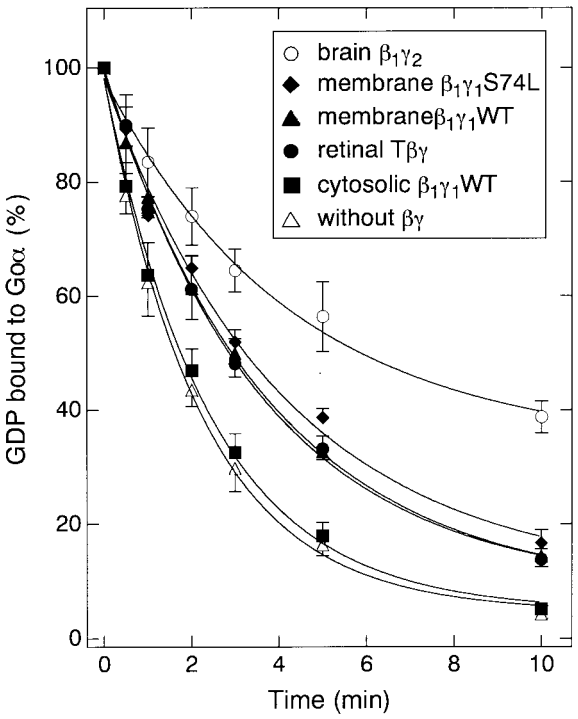

FIGURE 6: Effect of $\beta\gamma$ complex on inhibition of GDP release from Goα. Each $\beta\gamma$ complex was mixed with equimolar amount of Goα (final concentration of 100 nM) and incubated at 0 °C for 10 min. This mixture or Goα alone was then supplemented with [^{3}H]GDP at 300 nM (final concentration). After incubation at 20 °C, unlabeled GTP (final concentration of 500 μM) was added to the mixture (at time 0), followed by incubation at 20 °C. At appropriate time of incubation (0.5, 1, 2, 3, 5, and 10 min), aliquots were withdrawn from the mixture for measurement of [^{3}H]GDP bound to Goα. Data are the average $\pm$ SD of results from four independent experiments.

indicating functional interaction of Goα only with the modified $\beta\gamma$ complex. Here, it should be noted that the slowdown effect of $\beta_1\gamma_2$ was much stronger than that of membrane $\beta_1\gamma_1$S74L, membrane $\beta_1\gamma_1$WT, or retinal T$\beta\gamma$, the latter three of which showed an effect similar to each other. These results indicate that the major determinant of Goα−$\beta\gamma$ interaction is the amino acid sequence of the γ-subunit rather than the type of isoprenyl group, though the modification is indispensable for the interaction.

Interaction of Recombinant $\beta\gamma$ Complexes with Effectors. The isoprenyl group also plays an important role in the interaction of $\beta\gamma$ with its effectors (*8*, *27*−*29*). In the present study, the four forms of $\beta_1\gamma_1$ and bovine brain $\beta_1\gamma_2$ were compared with each other in ability to regulate two kinds of effectors, i.e., inhibition of calmodulin-dependent adenylyl cyclase and activation of phospholipase C.

In the former experiment, we used an enzyme source from rat retinal membranes, from which endogenous transducin and calmodulin had been washed out. Adenylyl cyclase activity in the membranes was stimulated about 6-fold in the presence of calmodulin at high (10 μM) Ca^{2+} concentration (Figure 7). This Ca^{2+}/calmodulin-stimulated activity was suppressed by the addition of increasing amounts of the modified $\beta\gamma$ preparations but not by cytosolic $\beta_1\gamma_1$WT (Figure 7), indicating clearly that the isoprenylation of γ is required for the inhibition of calmodulin-dependent adenylyl cyclase activity. The rank order of the inhibitory effect (Figure 7) shows that geranylgeranylated $\beta_1\gamma_1$ is a more potent inhibitor than farnesylated one (membrane $\beta_1\gamma_1$S74L $\geq$ membrane $\beta_1\gamma_1$WT > retinal T$\beta\gamma$). Also, it is suggested that $\beta_1\gamma_2$ is more potent to inhibit the activity than $\beta_1\gamma_1$ when both are geranylgeranylated (brain $\beta_1\gamma_2$ > membrane $\beta_1\gamma_1$S74L).

Now adenylyl cyclase is a membrane-bound enzyme; it is not clear whether the effect of the isoprenyl group observed in Figure 7 is ascribed to a change in protein−protein interaction or to different membrane affinities among the $\beta\gamma$ complexes. As a test for a soluble effector system, we investigated the stimulatory effect of $\beta\gamma$ complex on the phospholipase C activity present in the cytosolic fraction of HL-60 cells. This enzyme activity has been found to be activated by $\beta\gamma$ complexes in the absence of membranes (*35*). As shown in Figure 8, every $\beta\gamma$ preparation except for cytosolic $\beta_1\gamma_1$WT stimulated the PIP_2 hydrolysis in a dose-dependent manner, indicating a requirement of the isoprenyl group for the effector regulation through a protein−protein interaction at least in this $\beta\gamma$−effector system (*27*, *28*). And

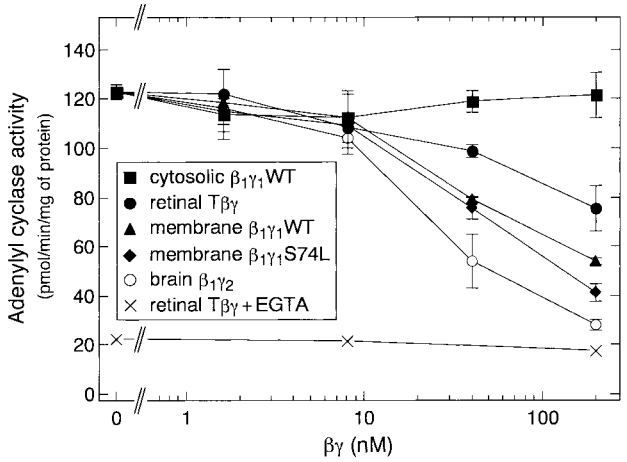

FIGURE 7: Effect of isoprenylation of $\beta\gamma$ complex on calmodulin-stimulated adenylyl cyclase activity. Rat retinal membranes were washed with buffer containing 1 mM EGTA and 100 μM GTP to remove endogenous calmodulin and transducin (*7*). The membranes were preincubated with 200 nM calmodulin and each $\beta\gamma$ complex at various concentrations (1.6, 8, 40, or 200 nM) in the presence of high (10 μM $CaCl_2$) or low (100 mM EGTA) Ca^{2+} concentration at 0 °C for 15 min. Adenylyl cyclase activity of the mixture was measured by incubating with 250 μM ATP at 30 °C for 15 min. Data are the average ± SD of results from three independent experiments.

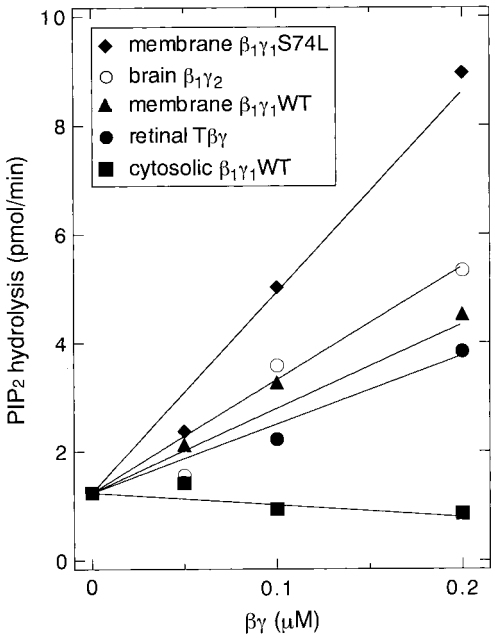

FIGURE 8: Effect of $\beta\gamma$ complex on activation of phospholipase C activity in HL-60 cytosol. Cytosolic fraction of HL-60 cells (*35*) was mixed with each $\beta\gamma$ complex at various concentrations (0.05, 0.1, or 0.2 μM) and phospholipid vesicles containing [^{3}H]PIP_2. The reaction was terminated by the addition of chloroform/methanol-HCl, followed by measurement of inositol phosphate formed. Data represent a typical set of five independent experiments with similar results.

again, the rank order of the activation ability of the complexes correlates with the relative content of the geranylgeranylated species in $\beta_1\gamma_1$ complex. It is evident that a replacement of farnesyl by the geranylgeranyl group in $\beta_1\gamma_1$ leads to a stronger activation of phospholipase C activity. In contrast with the effects on bovine brain Goα (Figure 6) and on rat retinal adenylyl cyclase (Figure 7), it seems likely that the phospholipase C is preferentially activated by the sequence of γ_1 over that of γ_2 under our experimental conditions (membrane $\beta_1\gamma_1$S74L > brain $\beta_1\gamma_2$).

DISCUSSION

Isoprenylation of γ_1 in Insect Cells. There are two variant reports on the isoprenylation of γ_1 expressed in Sf9 insect cells (*38*, *40*). Kalman et al. (*38*) demonstrated that both farnesyl and geranylgeranyl are incorporated into γ_1 expressed in Sf9 cells, while Lindorfer et al. (*40*) showed that most of the expressed γ_1 is farnesylated. In the present study, we determined the structures of γ_1WT and γ_1S74L expressed in different insect cells' Tn5 and showed the occurrence of both farnesylated and geranylgeranylated γ_1. Our observation agrees with the former report (*38*), but the ratio of farnesyl to geranylgeranyl found in the present study (33:67) diverges from it (ref *38*, approximately 66:34). Lindorfer et al. (*40*) demonstrated that about 50% of the expressed β-subunit was recovered in the detergent extract, while in the present study, the bulk of expressed β could not be extracted, and it was found predominantly in the insoluble fraction. The expression levels of β_1 and γ_1 in insect cells and/or purification procedures of $\beta\gamma$ complex may affect the ratio of farnesyl to geranylgeranyl in the final preparation. Recently, it was reported that rhodopsin kinase with the farnesylation signal sequence is modified by a mixture of C5, C10, C15, and C20 isoprenyl groups when it is expressed in Sf21 insect cells (*41*). The protein sequence and/or the insect cell type may also affect the composition of incorporated isoprenoids.

Relative Importance of the Type of Prenylation and Primary Structure of γ. The wild-type and mutant (S74L) γ_1 expressed in insect cells enabled us to conclude that functional properties of $\beta_1\gamma_1$ are remarkably altered when C15 farnesyl is replaced by C20 geranylgeranyl. The results presented in this study would explain the previous observations that $\beta_1\gamma_1$ is less potent than the other $\beta\gamma$ complexes to interact with Goα (*7*) and with effectors such as adenylyl cyclase (*7*, *8*), phospholipase C (*9*), and the K^+ channel (*11*). Our results, however, argue against the idea that rhodopsin might selectively recognize the farnesyl moiety attached to the Gγ (*42*, *43*), because the rate of metarhodopsin II-catalyzed GTPγS binding to Tα was significantly enhanced by the recombinant $\beta_1\gamma_1$ in which farnesyl was replaced by geranylgeranyl (Figure 5).

The importance of the type of the prenyl group in $\beta\gamma$ function is also supported by a recent report showing that the lower efficiency of $\beta_1\gamma_1$ to couple with the A1 adenosine receptor becomes nearly equal to that of $\beta_1\gamma_2$ by replacement of farnesyl with geranylgeranyl (*44*). As shown in Figures 6−8, however, the properties of predominantly geranylgeranylated $\beta_1\gamma_1$ are not identical to those of geranylgeranylated $\beta_1\gamma_2$. These results suggest an important contribution of the primary structure of the γ-subunit toward determining the $\beta\gamma$ function as a signal transducer. Among 11 subtypes of γ-subunits, the amino acid composition of γ_1 shows a noticeably acidic net charge (*30*), which may also contribute to the unique features of $\beta_1\gamma_1$. A weak membrane affinity of $\beta_1\gamma_1$ could be also ascribed to both less hydrophobic farnesyl and acidic net charge of γ_1. Taken together, it is likely that the relative importance of the type of prenylation and the primary structure of γ depends on targets with which $\beta\gamma$ complex interacts.

Protein−Protein Interaction via Isoprenoid. In the present study, we demonstrated that (i) isoprenylation is essential for the interaction between Gα and G$\beta\gamma$ and (ii) the affinity between Gα and G$\beta\gamma$ heavily depends on the type of prenylation. These results are consistent with a model that the isoprenyl moiety of γ_1 directly interacts with Tα (*30*). It is also reported that γ_2 free of Gβ interacts with Goα in a GTP- and an isoprenylation-dependent manner (*26*). Although the crystal structure of G-protein α$\beta\gamma$ trimer (*45*, *46*) indicates no direct contact between Gα and Gγ, the crystallized Gγ lacks several amino acid residues at the C-terminus, which seems to reach forward to the N-terminal region of Tα (*45*, *46*). We speculate that the farnesyl group may interact with Tα at the N-terminal region including covalently linked myristate or structurally related fatty acids (*47*) via lipid−lipid association (*48*). Alternatively, the structure of lipid-modified α$\beta\gamma$ trimer may be different from that of the unmodified one. That is, the farnesyl of γ_1 may act as an allosteric regulator which induces a conformational change of $\beta_1\gamma_1$ leading to an effective interaction with Tα or effectors. The three-dimensional structure of the lipid-modified form of G-proteins will help us to understand the roles of lipid modifications in more detail.

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