Role of C-terminal domains of the G protein β subunit in the activation of effectors

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Edited by Robert J. Lefkowitz, Duke University Medical Center, Durham, NC, and approved June 5, 2000 (received for review March 13, 2000)

The prenyl group on the G protein γ subunit is an important determinant of protein–protein interactions between the $\beta\gamma$ dimer and its targets, such as α subunits, receptors, and effectors. In an effort to identify domains of the β subunit important for the activation of effectors, we have prepared two types of mutants, one set in the domain suggested to form a hydrophobic prenyl-binding pocket for the γ subunit's prenyl group (prenyl pocket mutants) and the other set in a domain between Gly^{306} and Gly^{319} in the β propeller, which undergoes a conformational change when the dimer binds to phosducin (conformational change mutants). Recombinant baculoviruses for each set of mutants were prepared, and the nine mutant β subunits were overexpressed with either the γ_2 subunit (modified with geranylgeranyl) or the $\gamma_{2\text{-L71S}}$ subunit (γ_2 with altered *CAAX* sequence and modified with farnesyl). The purified dimers were tested for their ability to couple $G\alpha_{i1}$ to the A1 adenosine receptor and to activate phospholipase C-β or type II adenylyl cyclase. All dimers containing mutant β subunits were indistinguishable from wild-type $\beta_1\gamma_2$ or $\beta_1\gamma_{2\text{-L71S}}$ in coupling the receptor to $G\alpha_{i1}$. The prenyl pocket mutants expressed with γ_2 were 10-fold less potent in activating phospholipase C-β and adenylyl cyclase than $\beta_1\gamma_2$ and had similar activities to $\beta_1\gamma_{2\text{-L71S}}$. The conformational change mutants caused a 15- to 23-fold decrease in EC_{50} values for activation of these two effectors. Overall, the results suggest that the sites in Gβ identified by these mutants are very important in the activation of effectors. Furthermore, the nature of the prenyl group on Gγ may play an important role in the conformational change leading to the activity of G$\beta\gamma$ on effectors.

The $\beta\gamma$ subunit of heterotrimeric G proteins is a fundamental part of the signaling system used by G protein-coupled receptors (1). G$\beta\gamma$ is essential for the proper interaction of Gα with the receptor to form the high-affinity agonist-binding conformation of the receptor (2, 3) and to initiate GDP/GTP exchange on Gα (3, 4). Once released from Gα, G$\beta\gamma$ regulates over 12 effectors, including PLC-β, adenylyl cyclases, and ion channels (1, 5). Thus, understanding the domains in G$\beta\gamma$ that interact with these multiple effectors is necessary for understanding the function of this signaling molecule within the cell.

The activity of Gα is closely regulated by multiple mechanisms, including the receptor itself, G$\beta\gamma$, and RGS proteins(1, 6). However, G$\beta\gamma$ has been assumed to be fully active once released from Gα (1, 5, 7), perhaps because structural studies initially suggested that the conformation of free G$\beta\gamma$ was the same as that in the α-$\beta\gamma$ heterotrimer (8–10). Interestingly, one x-ray structure of G$\beta\gamma$ suggests that Gβ does undergo a conformational change in three regions when bound to at least one protein, phosducin (11). A more recent x-ray structure of the phosducin–$\beta_1\gamma_1$ complex solved with an intact Gγ containing the farnesyl group shows the farnesyl group inserts into a hydrophobic pocket in the β subunit, and a conformational change occurs in the amino acids between Gly^{306} and Gly^{319} near the C terminus (12).

All Gγ subunits are subject to posttranslational modification by the addition of the 15-carbon farnesyl group or the 20-carbon geranylgeranyl group to an invariant cysteine residue in the *CAAX* motif at their C terminus (13, 14). This lipid modification is important for anchoring G$\beta\gamma$ to the membrane and for determining functional interaction with other proteins (14, 15). We have found that switching the prenyl group on $\beta_1\gamma_2$ from geranylgeranyl to farnesyl causes a significant decrement in its ability to couple $G\alpha_{i1}$ to A1 adenosine receptors (3) and to activate either PLC-β or adenylyl cyclase (16). These observations clearly indicate that the prenyl group on Gγ is an important determinant of interactions between $\beta\gamma$ dimer and its targets.

To examine further the role of Gγ's prenyl group and the predicted conformational changes in Gβ on the ability of G$\beta\gamma$ to activate effectors, we investigated two types of $G\beta_1$ mutants, one set in the hydrophobic prenyl-binding pocket (termed *prenyl pocket mutants*) and the other to minimize the potential effect of conformational changes in certain amino acids between Gly^{306} and Gly^{319} (termed *conformational change mutants*). The nine mutated β_1 subunits were coexpressed with either the γ_2 (modified with geranylgeranyl) or $\gamma_{2\text{-L71S}}$ (γ_2 subunit modified with farnesyl) subunit and the purified dimers tested for their ability to support coupling of the A1 adenosine receptor to $G\alpha_{i1}$ subunits or to activate PLC-β and adenylyl cyclase. Their activity was compared with that of wild-type $\beta_1\gamma_2$ and $\beta_1\gamma_{2\text{-L71S}}$. The results demonstrate that none of the dimers containing mutant β_1 subunits affect the interaction between the A1 adenosine receptor and the α subunit. However, both the prenyl pocket mutants and the conformational change mutants decrease the activity of G$\beta\gamma$ on PLC-β and type II adenylyl cyclase, suggesting that these sites in Gβ are very important for activation of effectors.

Materials and Methods

Mutagenesis and Construction of Recombinant Baculoviruses. The prenyl pocket mutants were made in the six amino acids predicted to interact with Gγ's prenyl group as observed in the phosducin-$\beta_1\gamma_1$ structure, Val^{315}, Thr^{329}, Ser^{331}, Phe^{335}, Lys^{337}, and Trp^{339} (12). Each of these amino acids was mutated individually to Ala with the exception of Thr^{329}, which was mutated to Lys. The point mutations were made in the cDNA encoding $G\beta_1$ by using PCR-based site-directed mutagenesis. As an example of making the Val^{315} mutation (V315A), two independent PCRs were performed to switch GTC (Val) to GCC (Ala) at the position of 315 on $G\beta_1$ by using the primers: sense I 5′-CCGCTCCAGAATTCAAGATGAGTG-3′ and antisense I 5′-AGGCAGCTGGCGCGGTTGTCA-3′ for one reaction and sense II 5′-TGACAACCGCGCCAGCTGCCT-3′ and antisense II 5′-CCAGGAAAGGATCCGCGTTAGTTC-3′ for the other reaction. The final PCR was performed by using the sense I primer and the antisense II primer to give an *Eco*RI restriction site at the 5′ position and a *Bam*HI site at the 3′ position. The PCR product was subcloned into the pCNTR shuttle vector (5 Prime → 3 Prime), digested with *Xba*I, and the *Xba*I fragment was ligated into the pVL1393 baculovirus transfer vector (Invitrogen) (16). The five other mutations (T329K, S331A,

This paper was submitted directly (Track II) to the PNAS office.

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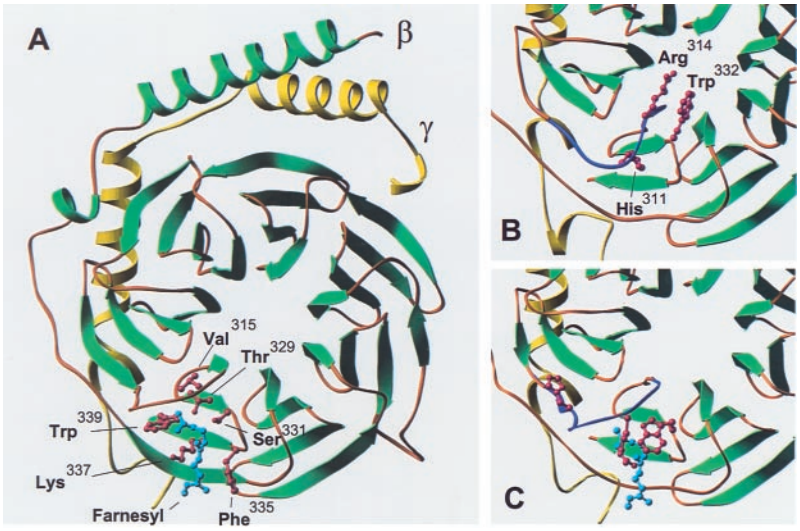

Fig. 1. Important features of the C-terminal region of Gβ. (*A*) The structure of G$\beta\gamma$ containing the farnesyl lipid derived from the $\beta_1\gamma_1$–phosducin complex as determined by Loew *et al.* (12). Gβ is shown in green and Gγ in yellow. The farnesyl group at the C terminus of Gγ is shown in cyan. The six amino acids predicted to interact with the farnesyl group based on the x-ray structure of the phosducin–$\beta_1\gamma_1$ complex are shown in red as ball-and-stick models. (*B*) The structure of free $\beta_1\gamma_1$ as determined by Sondek *et al.* (9). The molecule was placed in an identical orientation to the $\beta\gamma$ structure shown in *A* by using the program O. Only the lower left corner of G$\beta\gamma$ is shown. The region of Gβ (Gly^{306}–Gly^{319}) that undergoes a conformational change when the dimer interacts with phosducin is shown in purple. Three amino acids that undergo dramatic conformational changes on formation of the phosducin–$\beta_1\gamma_1$ complex are shown as red ball-and-stick models. (*C*) The region of G$\beta\gamma$ shown in *B* from the $\beta\gamma$–phosducin complex determined by Loew *et al.* (12). The amino acids between Gly^{306} and Gly^{319} are shown in purple. The dramatic conformational changes in the side chains of His^{311}, Arg^{314}, and Trp^{332} are indicated by the red ball-and-stick models. The illustrations of G$\beta\gamma$ were generated with the program RIBBONS.

F335A, K337A, and W339A) were generated by using the same method.

Two conformational change mutations were made at His^{311} and Arg^{314} as the side chains of these residues were observed to undergo a dramatic conformational change when free G$\beta\gamma$ (9) binds to phosducin (11, 12). These two amino acids were mutated to Ala (H311A and R314A). The point mutations were made in Gβ_1 by using PCR as described above. A β subunit containing a mutant at Trp^{332} was also studied because this amino acid undergoes a significant conformational change in the complex between phosducin and $\beta_1\gamma_1$ (11). The recombinant baculovirus encoding the alanine mutation of Trp^{332} (W332A) was a kind gift from T. Kozasa (University of Illinois, Chicago). The pVL1393 transfer vectors encoding the eight mutant β_1 subunits were sequenced to ensure fidelity. The recombinant baculoviruses encoding each mutant Gβ_1, α_s, α_{i1}, γ_2, and $\gamma_{2\text{-L71S}}$ subunits were produced as described (17–19).

Expression and Purification of Recombinant Gα and G$\beta\gamma$. Gα and G$\beta\gamma$ subunits were overexpressed in Sf9 insect cells and purified as described (17–19). The $\beta\gamma$ dimers containing wild-type or mutated Gβ_1 subunits with either γ_2 (modified with geranylgeranyl) or $\gamma_{2\text{-L71S}}$ (farnesyl modification) subunits were purified on a DEAE column followed by affinity chromatography on a G_{i1}-α-agarose column (19). The purified $\beta\gamma$ dimers were resolved on 12% polyacrylamide gels, stained with silver, and the concentration of the purified $\beta\gamma$ dimers estimated by using ovalbumin standards (16, 17). The experiments shown in *Results and Discussion* were performed by using two different preparations of each $\beta\gamma$ dimer.

Mass Spectrometrical Analysis of the Posttranslational Processing of Gγ. Because analysis of many previous batches of G$\beta\gamma$ by using electrospray mass spectrometry has shown that the γ_2 and $\gamma_{2\text{-L71S}}$ subunits are fully modified with the appropriate prenyl group (16, 20, 21), this set of 20 $\beta\gamma$ dimers was analyzed by using matrix-assisted laser desorption ionization mass spectrometry. The deconvoluted mass spectra of all $\beta\gamma$ dimers indicate that the γ_2 and $\gamma_{2\text{-L71S}}$ proteins in the sample were composed of one molecular weight species with molecular masses of 7,751 Da or 7,683 Da, respectively. These results are consistent with full processing of $G\gamma_2$ by removal of three C-terminal amino acids (-AIL for γ_2; -AIS for $\gamma_{2\text{-L71S}}$), addition of either a geranylgeranyl for γ_2 or a farnesyl for $\gamma_{2\text{-L71S}}$ and a carboxylmethyl group to the C-terminal cysteine, and the removal of the N-terminal methionine and acetylation of the resulting N-terminal alanine.

Expression of A1 Adenosine Receptors, PLC-β, and Type II Adenylyl Cyclase. Sf9 cells were infected with a recombinant baculovirus encoding bovine A1 adenosine receptors, and membranes containing these receptors were prepared (2). Recombinant turkey PLC-β was purified as described (22). Sf9 insect cell membranes overexpressing recombinant, rat type II adenylyl cyclase were prepared as described (17). The experiments shown in *Results and Discussion* were performed by using a single preparation of each type of membrane and recombinant turkey PLC-β.

Measurement of the Activities of Wild-Type and Mutant $\beta\gamma$ Dimers. To measure the ability of each particular G$\beta\gamma$ to couple to receptors, Sf9 insect cell membranes expressing recombinant A1 adenosine receptors were reconstituted with G protein α and $\beta\gamma$ subunits on ice for 30 min. Each reaction tube contained 20 fmol of receptor, 6 nM $G\alpha_{i1}$, 0–30 nM of a G$\beta\gamma$, and 50 nM GDP. After the reconstitution, the high-affinity agonist-binding conformation of the receptor was measured by using 0.3 nM of the agonist ligand [^{125}I]-N^6-(aminobenzyl)adenosine (3). The ability of each G$\beta\gamma$ dimer to stimulate PLC-β and type II adenylyl cyclase was measured as described (23). To determine whether the lower activity observed with the $\beta_1\gamma_{2\text{-L71S}}$ dimer containing farnesyl could be increased by including geranylgeranyl in the assay medium, the synthetic lipid vesicles (23) used in the PLC-β assay were extruded in the presence of 10–50 μM geranylgeranyl or farnesyl alcohol (American Radiolabeled Chemicals, St. Louis). The vesicles were then passed over an AcA-34 column to remove unincorporated lipids (24). The vesicles enriched with geranylgeranyl or farnesyl were reconstituted with $\beta_1\gamma_2$, $\beta_1\gamma_{2\text{-L71S}}$, $\beta_{1\text{-S331A}}\gamma_2$, or $\beta_{1\text{-K337A}}\gamma_2$ and PLC assays performed as described (23).

Calculation and Data Expression. Experiments presented under *Results and Discussion* are the average of three or more similar experiments. Data expressed as concentration-response curves were fit to sigmoid curves by using the fitting routines in the GRAPHPAD PRISM software (GraphPad Software, San Diego). Statistical differences between the fitted curves were determined by using all of the individual data points from multiple experiments to calculate the F statistic (25).

Results and Discussion

To examine the possibility that the prenyl group of Gγ participates in the activation of effectors by G$\beta\gamma$, we have studied two types of Gβ mutants. On the basis of a crystal structure of the phosducin–$\beta_1\gamma_1$ complex that contained the farnesyl group, Loew *et al.* argued that the isoprenoid group folds into a hydrophobic pocket formed by blades 6 and 7 of the β propeller (12). Fig. 1*A* shows the structure of G$\beta\gamma$ from the $\beta\gamma$–phosducin complex reported by Loew *et al.* (12). The six amino acids forming contacts with the farnesyl group are highlighted in red. Therefore, our first strategy was to mutate these six amino acids to determine whether modifying this region had an effect on the function of the $\beta\gamma$ dimer. Val^{315} appears to be capping the pocket-binding farnesyl (shown in cyan) and was mutated to Ala in an attempt to disrupt hydrogen bonding and remove a barrier at the end of the pocket. Thr^{329} makes a hydrogen bond with Trp^{339} and helps hold the alignment of the cavity. Thr^{329} was mutated to the larger Lys residue to force a bulky group to protrude into the prenyl-binding cavity and disrupt the hydrogen bond alignment with Trp^{339}. This mutation was expected to force the prenyl group out of the pocket. Ser^{331}, Phe^{335}, Lys^{337}, and Trp^{339} form the sides of the pocket and were mutated to Ala. These changes should make the cavity bigger and decrease the interactions holding the prenyl group in the pocket.

The structure of free G$\beta\gamma$ is nearly identical to that observed in the crystal structure of the heterotrimer (8, 10). Structures of the phosducin–$\beta\gamma$ complex (11, 12) indicate that amino acids near the C-terminal end of Gβ undergo conformational changes when G$\beta\gamma$ binds to phosducin as compared with the structure of free G$\beta\gamma$ (9). One study of the phosducin–$\beta_1\gamma_1$ complex identified three local conformational changes in Gβ between residues 287–295, residues 308–318, and residues 329–338 (11). A second study highlighted the conformational changes in the region between Gly^{306} and Gly^{319} and identified the hydrophobic pocket that binds the farnesyl group (12). The conformational change between Gly^{306} and Gly^{319} is shown in purple in Fig. 1 *B* and *C*, respectively. Therefore, our second experimental strategy was to study certain amino acids that underwent dramatic conformational changes in the $\beta_1\gamma_1$–phosducin complex. Two amino acids in this region on $G\beta_1$, His^{311}, and Arg^{314} were chosen, and the conformational change mutants were made by mutating to Ala. Although not in this region, Trp^{332} [first studied as an α-$\beta\gamma$ contact site (26, 27)] also undergoes a significant conformational change in the complex of phosducin–$\beta_1\gamma_1$ (11). Therefore, in addition to H311A and R314A, we also studied the alanine mutation of Trp^{332} (W332A) as a conformational change mutant. The positions of these three amino acids in free G$\beta\gamma$ are indicated as red ball-and-stick models in Fig. 1*B*, and the conformational changes induced by formation of the phosducin–$\beta\gamma$ complex are shown in Fig. 1*C*.

The mutant $G\beta_1$ were expressed with either the γ_2 or the $\gamma_{2\text{-L71S}}$ subunit in baculovirus-infected Sf9 insect cells, and the resulting $\beta\gamma$ dimers were purified by $G\alpha_{i1}$-agarose affinity chromatography (19). Silver-stained SDS gels of the 20 $\beta\gamma$ dimers used in this study showed the proteins to be highly pure; only two bands at about 36 and 7 kDa were visible, as described in our previous studies (16, 20).

Neither Type of Mutant Affects Receptor Coupling. All 20 $\beta\gamma$ dimers were tested for their ability to reestablish the high-affinity agonist-binding conformation of recombinant bovine A1 adenosine receptor expressed in Sf9 cell membranes. The data in Fig. 2*A* present the effect of $\beta_{1\text{-K337A}}\gamma_2$ as representative of the activity of the prenyl pocket mutants. Each dimer containing a mutant β subunit was able to support high-affinity agonist binding at the bovine A1 adenosine receptor with a potency and efficacy equal to $\beta_1\gamma_2$ (see Fig. 2*A* and Table 1). As expected, the $\beta_1\gamma_{2\text{-L71S}}$ dimer, in which the γ_2 subunit was modified with farnesyl, was measurably less active than $\beta_1\gamma_2$ (3). Interestingly, the activity of $\beta_{1\text{-K337A}}\gamma_{2\text{-L71S}}$ was the same as that of $\beta_1\gamma_{2\text{-L71S}}$. All other $\beta\gamma$ dimers containing mutant $G\beta_1$ expressed with the $\gamma_{2\text{-L71S}}$ subunit were also equal in receptor coupling to the wild-type $\beta_1\gamma_{2\text{-L71S}}$ (see Table 1). The data in Fig. 2*B* indicate that the activity of dimers containing any of the three conformational change mutants was equal to wild-type $\beta_1\gamma_2$. Therefore, neither type of mutant affects the ability of G$\beta\gamma$ to bind to the $G\alpha_{i1}$ and support formation of the high-affinity ligand-binding state of A1 adenosine receptor. In addition, all of the nine $\beta\gamma$ dimers containing altered β_1 subunits were able to support the ability of an agonist-stimulated A1 adenosine receptor to initiate GDP/GTP exchange on $G\alpha_{i1}$ (data not shown). These results are consistent with structural data showing that the C-terminal region of Gβ has no direct interaction with Gα (8, 10). Overall, these data suggest that when G$\beta\gamma$ interacts with Gα and the receptor, the prenyl group may extend away from Gβ and insert into the membrane. This possibility is consistent with data suggesting that the C-terminal end of Gγ and its prenyl group are important for the interaction with the receptor and the Gα subunit (3, 14).

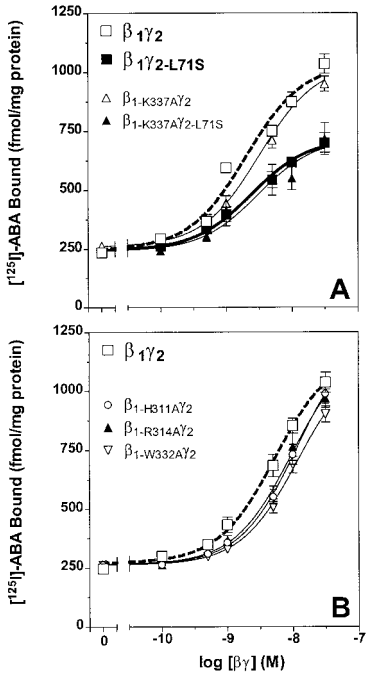

Fig. 2. Comparison of the ability of wild-type $\beta\gamma$ dimers and those containing prenyl pocket mutants or conformational change mutants to support the high-affinity agonist-binding state of the A1 adenosine receptor. (*A*) The activity of $\beta_1\gamma_2$ (open squares, thick dotted line) and $\beta_1\gamma_{2\text{-L71S}}$ (closed squares, thick line) is compared with two representative prenyl pocket mutants, $\beta_{1\text{-K337A}}\gamma_2$ and $\beta_{1\text{-K337A}}\gamma_{2\text{-L71S}}$. (*B*) An analogous experiment performed with $\beta_{1\text{-H311A}}\gamma_2$, $\beta_{1\text{-R314A}}\gamma_2$, and $\beta_{1\text{-W332A}}\gamma_2$ and compared to wild-type $\beta_1\gamma_2$ (open squares, thick dotted line). Overall, the EC_{50} values of wild-type and mutant $\beta\gamma$ dimers containing the same γ subunit are not significantly different. Each data point is an average of three similar experiments performed in triplicate.

Activity of Prenyl Pocket Mutants on Effectors. The full set of mutated $\beta\gamma$ dimers was also tested for their ability to activate effectors. The data in Fig. 3*A* illustrate the ability of four representative $\beta\gamma$ dimers to activate recombinant turkey PLC-β.

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Table 1. Comparison of the ability of the wild-type $\beta\gamma$ dimers and those containing prenyl pocket mutants to support the high-affinity agonist-binding state of the A1 adenosine receptor and the activation of PLC-β or type II adenylyl cyclase

		Receptor coupling		PLC-β		AC-II	
Type of $\beta\gamma$ dimer	Designation	K_{act}, nM	B_{max}, fmol/mg of protein	EC_{50}, nM	V_{max}, μmol/mg of PLC/min	EC_{50}, nM	V_{max}, nmol/mg of protein/min
Wild type	$\beta_1\gamma_2$	2.0	1,116.0 ± 43.0	3.0	3.26 ± 0.05	4.1	22.19 ± 0.65
Prenyl pocket mutants with γ_2 subunit modified with geranylgeranyl	$\beta_{1\text{-V315A}}\gamma_2$	4.8	1,157.0 ± 91.3	31.0*	2.81 ± 0.15†	34.2*	12.11 ± 1.96†
	$\beta_{1\text{-T329K}}\gamma_2$	4.2	1,120.9 ± 85.3	26.1*	3.12 ± 0.10	35.2*	12.39 ± 1.80†
	$\beta_{1\text{-S331A}}\gamma_2$	4.7	1,108.4 ± 70.9	26.3*	3.24 ± 0.09	46.2*	22.01 ± 0.74
	$\beta_{1\text{-F335A}}\gamma_2$	4.2	1,030.9 ± 35.1	26.2*	3.10 ± 0.15	33.4*	16.50 ± 3.89†
	$\beta_{1\text{-K337A}}\gamma_2$	3.2	1,036.7 ± 36.6	28.1*	3.12 ± 0.08	35.9*	21.02 ± 0.94
	$\beta_{1\text{-W339A}}\gamma_2$	4.5	1,059.9 ± 62.9	27.5*	2.71 ± 0.20†	35.2*	16.48 ± 2.41†
Wild type	$\beta_1\gamma_{2\text{-L71S}}$	5.4	718.4 ± 30.7	30.3	3.08 ± 0.10	42.8	23.51 ± 0.88
Prenyl pocket mutants with $\gamma_{2\text{-L71S}}$ subunit modified with farnesyl	$\beta_{1\text{-V315A}}\gamma_{2\text{-L71S}}$	5.6	741.2 ± 43.1	42.2‡	1.81 ± 0.22‡	40.2	9.05 ± 1.13‡
	$\beta_{1\text{-T329K}}\gamma_{2\text{-L71S}}$	9.4	834.9 ± 82.0	47.3‡	2.12 ± 0.18‡	45.3	7.22 ± 0.65‡
	$\beta_{1\text{-S331A}}\gamma_{2\text{-L71S}}$	8.2	646.6 ± 40.4	49.5‡	2.01 ± 0.18‡	47.0‡	10.43 ± 3.04‡
	$\beta_{1\text{-F335A}}\gamma_{2\text{-L71S}}$	5.3	685.6 ± 54.4	43.2‡	1.53 ± 0.11‡	52.4‡	14.28 ± 3.12‡
	$\beta_{1\text{-K337A}}\gamma_{2\text{-L71S}}$	5.0	718.5 ± 57.7	39.5‡	1.81 ± 0.14‡	40.3	9.15 ± 0.61‡
	$\beta_{1\text{-W339A}}\gamma_{2\text{-L71S}}$	6.3	692.7 ± 68.6	40.2‡	1.71 ± 0.20‡	41.9	12.98 ± 1.90‡

The K_{act}, EC_{50}, B_{max}, or V_{max} values of wild-type and mutant $\beta\gamma$ dimers were determined by fitting each data set to sigmoid curves as described in *Materials and Methods*.

*Significant differences in responses to prenyl pocket mutants in comparison with corresponding wild-type $\beta_1\gamma_2$; $P < 0.001$.

†Protein concentration of these mutants is too low to construct full concentration-response curves, so it is not possible for V_{max} to be estimated with full accuracy.

‡Significant differences in responses to prenyl pocket mutants in comparison with corresponding wild-type $\beta_1\gamma_{2\text{-L71S}}$; $P < 0.001$.

As expected, $\beta_1\gamma_2$ activated PLC-β about 10-fold with an estimated EC_{50} value of 2.6 nM and was about 10-fold more potent at activating PLC-β than $\beta_1\gamma_{2\text{-L71S}}$ (16). Note that $\beta\gamma$ dimers containing $\beta_{1\text{-S331A}}$ or $\beta_{1\text{-K337A}}$ are significantly less potent than wild-type $\beta_1\gamma_2$. Interestingly, the EC_{50}s of these mutants are not significantly different from that of $\beta_1\gamma_{2\text{-L71S}}$ (see Table 1). Analogous experiments were performed with the other four dimers containing mutant β subunits and combined with either the γ_2 or the $\gamma_{2\text{-L71S}}$ subunit. An analysis of the data from these experiments is presented in Table 1. Note especially that the potency of dimers containing any of six prenyl pocket mutants is similar to that of $\beta_1\gamma_{2\text{-L71S}}$ and about 10-fold lower than that of $\beta_1\gamma_2$. Even if the Gγ subunit in the dimers was modified with geranylgeranyl, their ability to stimulate PLC-β was similar to that of $\beta_1\gamma_{2\text{-L71S}}$ (Fig. 3*A* and Table 1). To determine whether the lower activity observed with the $\beta_1\gamma_{2\text{-L71S}}$ dimer containing farnesyl could be increased by including geranylgeranyl in the assay medium, the vesicles (23) used in the PLC-β assay were extruded in the presence of 10–50 μM geranylgeranyl or farnesyl alcohol. These vesicles were reconstituted with 0, 2, 5, or 10 nM $\beta_1\gamma_2$, $\beta_1\gamma_{2\text{-L71S}}$, $\beta_{1\text{-S331A}}\gamma_2$, or $\beta_{1\text{-K337A}}\gamma_2$ and the activity of PLC-β measured. In the geranylgeranyl-enriched vesicles, the activity of the $\beta_1\gamma_2$ dimer is about the same as in the control vesicles; however, in this concentration range, the activity of the $\beta_1\gamma_{2\text{-L71S}}$ dimer is increased about 2-fold to become equal to that of the $\beta_1\gamma_2$ dimer. Moreover, the activity of neither prenyl pocket mutant is increased in the geranylgeranyl-enriched vesicles, and the activity of the $\beta_1\gamma_{2\text{-L71S}}$ dimer is not increased in farnesyl-enriched vesicles ($n = 3$; data not shown). Overall, these results and those in Table 1 strongly suggest that the geranylgeranyl lipid can bind in the prenyl-binding pocket of the β subunit and cause the most favorable conformation for activating effectors.

The data in Fig. 3*B* illustrate the effects of these four dimers on the activity of type II adenylyl cyclase. Note that the $\beta_{1\text{-S331A}}\gamma_2$ and $\beta_{1\text{-K337A}}\gamma_2$ dimers were about 10-fold less potent than wild-type $\beta_1\gamma_2$. Similar results were found with the four other mutant Gβ_1 expressed and purified with γ_2 (see Table 1). The six mutant β subunits expressed with $\gamma_{2\text{-L71S}}$ had even lower EC_{50}s and estimated V_{max} values in the cyclase assay (see Table 1).

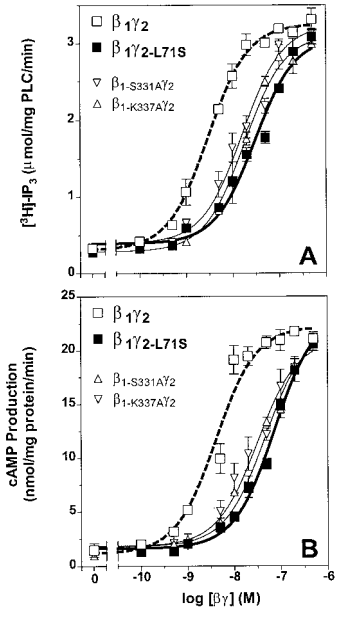

Fig. 3. Comparison of the ability of wild-type $\beta\gamma$ dimers and those containing prenyl pocket mutants to activate PLC-β or type II adenylyl cyclase. (*A*) The activity of $\beta_1\gamma_2$ (open squares, thick dotted line) and two representative prenyl pocket mutants, $\beta_{1\text{-S331A}}\gamma_2$ and $\beta_{1\text{-K337A}}\gamma_2$, compared with that of $\beta_1\gamma_{2\text{-L71S}}$ (closed squares, thick line). The indicated concentrations of the two representative prenyl pocket mutants were reconstituted with recombinant, turkey PLC-β in phospholipid vesicles containing [^{3}H]phosphatidylinositol 4,5-bisphosphate, and PLC activity was measured as described in *Materials and Methods*. The difference between the effect of wild-type $\beta_1\gamma_2$ and the prenyl pocket mutants was statistically significant ($P < 0.001$; see Table 1). (*B*) The ability of the two representative prenyl pocket mutants, $\beta_{1\text{-S331A}}\gamma_2$ and $\beta_{1\text{-K337A}}\gamma_2$, to activate type II adenylyl cyclase as compared with the effect of wild-type $\beta_1\gamma_2$ (open squares, thick dotted line) and $\beta_1\gamma_{2\text{-L71S}}$ (closed squares, thick line). The cyclase reaction was performed with the indicated concentrations of $\beta\gamma$ dimers as described in *Materials and Methods*. The difference between the effect of wild-type $\beta_1\gamma_2$ and prenyl pocket mutant $\beta\gamma$ dimers was statistically significant ($P < 0.0001$; see Table 1). Each data point is an average of three independent experiments, each performed in duplicate.

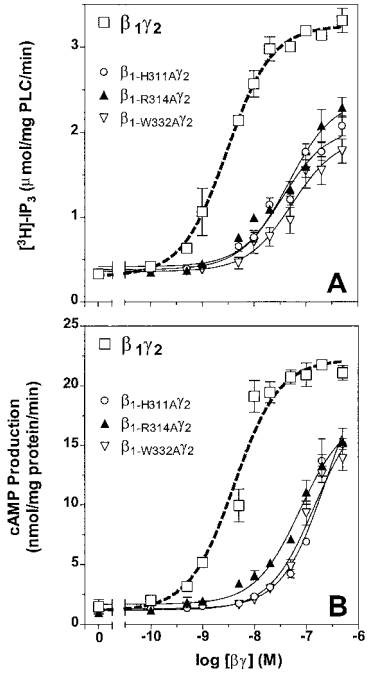

Fig. 4. Comparison of the ability of the wild-type $\beta_1\gamma_2$ (open squares, dotted lines) and the conformational change mutants, $\beta_{1\text{-H311A}}\gamma_2$, $\beta_{1\text{-R314A}}\gamma_2$ and $\beta_{1\text{-W332A}}\gamma_2$, to stimulate PLC-$\beta$ (*A*) or type II adenylyl cyclase (*B*). In both assays, the difference between the effect of wild-type $\beta_1\gamma_2$ and the three conformational change mutants was significant ($P < 0.0001$; see Table 2). Each data point is an average of three independent experiments, each performed in duplicate.

Overall, the mutation of any of the six amino acids forming the postulated hydrophobic binding pocket for the prenyl group in Gβ decreases the potency of the dimer in both effector assays. Their maximal efficacies are not greatly changed. Interestingly, the loss of activity is very similar to that observed when the prenyl group on Gγ_2 is switched from geranylgeranyl to farnesyl ($\gamma_{2\text{-L71S}}$), and the lower activity of $\beta_1\gamma_{2\text{-L71S}}$ can be increased by adding exogenous geranylgeranyl lipid in the PLC-β assay. Given that our previous experiments showed that the prenyl group on Gγ does not affect the partitioning of G$\beta\gamma$ into phospholipid vesicles (16, 24) or into Sf9 cell membranes (3), these results indicate that the nature of the prenyl group can affect the affinity of the interaction of the dimer with PLC-β or type II adenylyl cyclase. Thus, $\beta\gamma$ dimers containing the geranylgeranyl group may assume somewhat different conformations when complexed with effectors as compared with $\beta\gamma$ dimers containing the farnesyl group. This suggests the intriguing possibility that the interaction between the γ subunit's prenyl group and the β subunit may be important for inducing the active form of G$\beta\gamma$.

Activity of Conformational Change Mutants on Effectors. The data in Fig. 4*A* show that the ability of this set of mutant $\beta\gamma$ dimers to activate PLC-β is much less than that of $\beta_1\gamma_2$. Analysis of the data shows that the EC_{50} for the activation of PLC-β is increased from 3.0 nM to about 50.0 nM, and the estimated V_{max} is reduced about 40% (see Table 2). When tested in the type II adenylyl cyclase assay, the potency of these three mutant $\beta\gamma$ dimers was about 25-fold less than $\beta_1\gamma_2$ (Fig. 4*B*). The estimated V_{max} values were also slightly reduced (see Table 2). In both cases, the change is much larger than changes observed with the prenyl pocket mutants. Interestingly, any of the three mutants expressed with $\gamma_{2\text{-L71S}}$ is much less active at stimulating PLC-β or type II adenylyl cyclase than wild-type $\beta_1\gamma_{2\text{-L71S}}$ (see Table 2). Therefore, alanine mutations of three amino acids whose conformation changes dramatically on binding phosducin result in a $\beta\gamma$ dimer which has significantly reduced activity on either PLC-β or type II adenylyl cyclase. This observation indicates that rearrangement of the domain in Gβ between Gly^{306} and Gly^{319} (shown in purple in Fig. 1*C*) and Trp^{332} may be critical for forming the active conformation of G$\beta\gamma$ at these effectors. These findings provide a biochemical correlate to the observation that the structure of free G$\beta\gamma$ (9) is the same as that observed in the structure of the heterotrimer (8, 10) and the conformation changes when G$\beta\gamma$ is complexed with phosducin (11, 12). Overall, these results argue that Gβ undergoes a conformational change when it binds to effectors such as PLC-β and type II adenylyl cyclase, much as it does when it binds to phosducin. Thus, the domain of Gβ between Gly^{306} and Gly^{319} may be very important for the interaction with certain effectors.

A number of studies have identified the C-terminal region of Gβ as a critical domain for the activation of effectors. Replacement of only four mammalian residues (Val^{327}, Ala^{328}, Phe^{335}, and Asn^{340}) in Gβ_1 with those from *Dictyostelium* produced $\beta\gamma$ dimers that were severely impaired in their ability to activate PLC-β2 (28). These residues overlap with the region of the hydrophobic prenyl-binding pocket in Gβ. In addition, mutant β subunits lacking the C-terminal two amino acids (Trp^{339} and Asn^{340}) did not activate mitogen-activated protein kinase but were able to stimulate c-Jun N-terminal kinase/stress-activated protein kinase (29). Taylor *et al.* found that a photoaffinity-

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Table 2. Comparison of the ability of wild-type $\beta\gamma$ dimers and those containing conformational change mutants to support the high-affinity agonist-binding state of the A1 adenosine receptor and the activation of PLC-β or type II adenylyl cyclase

		Receptor coupling		PLC-β		AC-II	
Type of $\beta\gamma$ dimer	Designation	K_{act}, nM	B_{max}, fmol/mg of protein	EC_{50}, nM	V_{max}, μmol/mg of PLC/min	EC_{50}, nM	V_{max}, nmol/mg of protein/min
Wild type	$\beta_1\gamma_2$	2.0	1,116.0 ± 43.0	3.0	3.26 ± 0.05	4.1	22.19 ± 0.65
Conformational change mutants with	$\beta_{1\text{-H311A}}\gamma_2$	4.3	1,149.1 ± 75.7	45.3*	2.06 ± 0.11*	95.5*	23.48 ± 1.89
γ_2 subunits modified with geranylgeranyl	$\beta_{1\text{-R314A}}\gamma_2$	5.2	1,180.4 ± 96.3	47.0*	2.40 ± 0.12*	76.8*	17.56 ± 0.99*
	$\beta_{1\text{-W332A}}\gamma_2$	4.6	1,142.9 ± 67.3	54.2*	1.95 ± 0.15*	95.3*	18.55 ± 1.16*
Wild type	$\beta_1\gamma_{2\text{-L71S}}$	5.4	718.4 ± 30.7	30.3	3.08 ± 0.10	42.8	23.51 ± 0.88
Conformational change mutants with	$\beta_{1\text{-H311A}}\gamma_{2\text{-L71S}}$	5.7	710.3 ± 27.0	49.7†	0.91 ± 0.06†	94.1†	19.03 ± 6.15
$\gamma_{2\text{-L71S}}$ subunits modified with farnesyl	$\beta_{1\text{-R314A}}\gamma_{2\text{-L71S}}$	7.8	671.8 ± 35.6	41.3†	1.08 ± 0.09†	81.4†	12.04 ± 1.89†
	$\beta_{1\text{-W332A}}\gamma_{2\text{-L71S}}$	7.6	728.3 ± 44.7	51.1†	1.08 ± 0.12†	98.3†	7.65 ± 0.92†

The K_{act}, EC_{50}, B_{max}, or V_{max} values of wild-type and mutant $\beta\gamma$ dimers were determined by fitting each data set to sigmoid curves as described in *Materials and Methods.*

*Significant differences in responses to conformational change mutants in comparison with corresponding wild-type $\beta_1\gamma_2$; $P < 0.0001$.

†Significant differences in responses to conformational change mutants in comparison with corresponding wild-type $\beta_1\gamma_{2\text{-L71S}}$; $P < 0.0001$.

labeled peptide derived from the third intracellular loop of the α_2-adrenergic receptor crosslinked to the C-terminal 60 amino acids of Gβ (4). Taken together, the results of these studies strongly indicate that the C-terminal region of Gβ is important for interaction with multiple effectors.

The crystal structure of the G protein heterotrimer has provided critical information for three recent mutagenesis studies. To examine the possibility that the $\beta\gamma$ dimer uses a common surface to interact with both the α subunit and its downstream effectors, Ford *et al.* mutated 15 amino acids in the β_1 subunit to alanine (26). These amino acids were located in the switch interfaces between the α and β subunits (Lys^{57}, Tyr^{59}, Ser^{98}, Trp^{99}, Met^{101}, Leu^{117}, Asn^{119}, Thr^{143}, Asp^{186}, Asp^{228}, and Trp^{332}) and in the N-terminal interface (Leu^{55}, Lys^{78}, Ile^{80}, and Lys^{89}). The mutants were expressed with either the γ_1 or the γ_2 subunit and tested for their ability to interact with the G_t α subunit or to activate downstream targets such as the β-adrenergic receptor kinase, PLC-β2, type II adenylyl cyclase, or K^+ and Ca^{2+} channels. The results of this study show that the regions of the $\beta\gamma$ dimer that interact with effectors are in the domain covered by the α subunit in the heterotrimer. However, the different effectors were found to interact with distinct but partially overlapping domains on the β subunit. These results provide two important concepts for $\beta\gamma$ signaling. First, they indicate that different domains of the $\beta\gamma$ dimer may be used for activation of different effectors. Second, apparently all of these domains are covered by the α subunit in the heterotrimer. Thus, formation of the heterotrimer is able to regulate the activity of the $\beta\gamma$ dimer on multiple downstream effectors.

Another study showed that four point mutations in the top surface of Gβ inhibited the ability of the dimers to activate PLC-β2, PLC-β3, and type II adenylyl cyclase (27). Interestingly, none of the mutations affected the ability of G$\beta\gamma$ to inhibit type I adenylyl cyclase. The two point mutations on the side of Gβ in blade 1 diminished the affinity for Gα but did not inhibit activation of effectors (27). In a more extensive study of the importance of the side surfaces of Gβ in activating effectors, Panchenko *et al.* mutated multiple amino acids in each of the seven blades of the β propeller and found that mutations in the amino acids of blades 2, 6, and 7 greatly inhibited the ability of G$\beta\gamma$ to activate PLC-β2 but did not alter the activity at type I or type II adenylyl cyclase (30). Moreover, Buck *et al.* showed that a peptide derived from amino acids 86–105 of Gβ_1 could activate PLC-β2, and a peptide derived from 115–135 could block G$\beta\gamma$ activation of PLC-β2 (31). The latter region is on the outer surface of blade 2 of Gβ and overlaps one of the domains identified as important for activation of PLC-β2 by Panchenko *et al.* (30). Overall, the results of these studies suggest that the amino acids in both the top surface and the edges of the β torus are important for activating PLC-β, but that activation of type II adenylyl cyclase depends more on interaction with the top surface of Gβ, indicating that multiple regions of G$\beta\gamma$ are involved in the interaction with its targets.

Clearly, the region between Gly^{306} and Gly^{319} identified in the present work is another important region in G$\beta\gamma$ activity. One interesting aspect of the Ala mutations in the region between amino acids 306 and 319 in Gβ_1 in this study is that they markedly inhibit activation of both PLC-β and type II adenylyl cyclase. These three conformational change mutants are located on the top surface of the β torus; thus, our results are in keeping with the general observations that the top surface of the β propeller is important for interaction with effectors. It is important to note that the amino acids represented by both the prenyl pocket mutants and the conformational change mutants are strictly conserved in 11 β subunits from a variety of species. The three amino acids that undergo a conformational change (His^{311}, Arg^{314}, and Trp^{332}) are absolutely conserved in all five mammalian β subunits, yeast, *Dictyostelium*, *Caenorhabditis elegans*, *Drosophila*, and squid. Moreover, the glycines at positions 306 and 319 that provide the putative hinges for the conformational change in the structure are also strictly conserved across species (9). Thus, this domain in Gβ may be very important for the interaction of the dimer with multiple effectors. In addition, the residues that form the hydrophobic prenyl pocket are about 95% conserved in these six species. Therefore, both regions of Gβ studied in these experiments may be generally important for the activity of G$\beta\gamma$ at downstream effectors in multiple species.

We thank Drs. Ravi Iyengar (Mt. Sinai School of Medicine, New York) and Tohru Kozasa for the baculoviruses encoding type II adenylyl cyclase and the β_1 subunit containing the W332A mutation, respectively. We thank Dr. T. K. Harden (University of North Carolina, Chapel Hill, NC) for purified recombinant turkey phospholipase C-β, the University of Virginia Diabetes Core Facility for cAMP Assays, and the Biomolecular Research Facility for DNA sequencing and mass spectrometric analysis. We also acknowledge Warren J. Clingan, III for excellent technical assistance and Qiang Zhao for help with the RIBBONS program. This work was supported by a fellowship from the Virginia Affiliate of the American Heart Association (to C.-S. M.) and by National Institutes of Health Grants CA40042 and DK-19952.

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