Sites for Gα Binding on the G Protein β Subunit Overlap with Sites for Regulation of Phospholipase Cβ and Adenylyl Cyclase*

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Heterotrimeric G proteins, composed of α and βγ subunits, forward signals from transmembrane receptors to intracellular effector enzymes and ion channels. Free βγ activates downstream targets, but its action is terminated by association with GDP-liganded α subunits. Because α can inhibit activation of many effectors by βγ, it is likely that the α subunit binding surfaces on βγ overlap the surfaces necessary for effector activation. To test this hypothesis, we mutated residues on β shown to contact α in the recently published crystal structures of the αβγ heterotrimer (Wall, M. A., Coleman, D. E., Lee, E., Iniguez-Lluhi, J. A., Posner, B. A., Gilman, A. G., and Sprang, S. R. (1995) Cell 83, 1047–1058; Lambright, D. G., Sondek, J., Bohm, A., Skiba, N. P., Hamm, H. E., and Sigler, P. B. (1996) Nature 379, 311–319.). The α subunit binds to the flat, top surface of the toroidal β subunit and also extends a helix along the side of the β subunit at blade 1. We mutated four residues on the top surface of β (Hβ[1,L117A], Hβ[2,D228R], Hβ[3,D246S], and Hβ[5,W332A]) and two residues on the side of β that contacts α (Hβ[3,N88A/K89A]). Each of the mutant proteins was able to form βγ dimers, but they differed in their ability to bind α and to activate phospholipase C β2 (PLCβ2), PLCβ3, and adenylyl cyclase II. Mutation of residues along the side of the torus at blade 1 diminishes affinity for α but do not prevent activation of any of the effectors. Mutations on the α binding site differentially affected PLCβ2, PLCβ3, and adenylyl cyclase II. Residues that affect PLCβ and adenylyl cyclase II activity are found on opposite sides of the central tunnel, suggesting that PLC and adenylyl cyclase, like the α subunit, make many contacts on the top surface. None of the mutations affected the ability of βγ to inhibit adenylyl cyclase I. We conclude that α, PLCβ2, PLCβ3, and adenylyl cyclase II share an interaction on the top surface of β. The importance of individual residues is different for α binding and for effector activation and differs even between closely related isoforms of the same effector.

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adenyl cyclase. The function of βγ subunits containing these mutations were analyzed in three different expression systems: in vitro translation, transient expression in COS-7 cells, and in vitro reconstitution with proteins purified from baculovirus-infected Sf9 cells. None of the mutations interfered with the ability of β subunits to form βγ dimers. As expected, some, but not all, mutations affected the ability of mutant βγ dimers to interact with α. Most importantly, the results show that the α contact surface on the flat, narrow end of the propeller is important for effector activation. Moreover, the mutations did not have equal consequences for the effectors tested, nor even between closely related subtypes of the same effector.

**EXPERIMENTAL PROCEDURES**

**Cell Culture and Transfection—**COS-7 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. Transfection was done with LipofectAMINE according to the manufacturer's protocol using 6–7 μg of LipofectAMINE/ml of culture medium (Opti-MEM, Life Technologies, Inc.) for 5 h or overnight. The medium containing LipofectAMINE was removed, and cells were washed twice with Dulbecco's modified Eagle's medium/5% fetal bovine serum.

**Mutagenesis and Plasmid Construction—**Site-directed mutagenesis was performed in the pAlter vector (Promega) according to the manufacturer's instructions. The cDNA of the βγ subunit was cloned into pAlter vector as described (8, 9). The single-stranded βγ DNA was synthesized and used as a template for mutagenesis. To facilitate the transfer of mutants among different vectors, a silent mutation corresponding to amino acids 144 and 145 was introduced into the HindIII site of the HindIII site and the EcoRI site from the pALTER vector. The amino acid sequence added to the amino terminus with hemagglutinin epitope) was described in Ref. 10.

**Cells—**Cells were starved for 24 h in methionine- and cysteine-deficient RPMI 1640 medium containing 15% (vol/vol) of LipofectAMINE/ml of culture medium supplemented with 10% fetal bovine serum in a humidified 10% CO2/90% air atmosphere. A cell line of 0.5–2 × 10^6/well in six-well plates were transfected with 10 μg of plasmid DNA and 10 μg of LipofectAMINE. The extracts were combined, neutralized to pH 7.5 with 30 mM ammonium hydroxide, and loaded on 0.5 m AG1-X8 anion exchange columns. Prior to use, the columns were washed with 2 ml of 1 M NaOH and 2 ml of 1 M formic acid and equilibrated with water to neutrality. The columns were washed with 10 bed volumes of water and 10 bed volumes of 5 mM borax and 60 mM sodium formate. The inositol phosphates were eluted with 10 bed volumes of 1 mM ammonium formate.

**In Vitro Translation, Immunoprecipitation, and Cross-linking—**All subunits were transcribed and translated using the TNT-coupled reticulocyte lysate system (Promega). Typically, 1 μg of plasmid DNA and 20 μCi of [3H]methionine were used in a 50-μl reaction. In all cases, transcription was directed by the TT promoter. Synthesis of the desired product was routinely verified by running 5 μl of the translation mixture in a small 11 or 13% polyacrylamide gel (13), followed by autoradiography with overnight exposure. Independently translated β and γ subunits were mixed together and incubated at 37 °C for 90 min to allow dimer formation. Because γ translation was usually more efficient, 10–15 μl of γ translation mixture was typically added to 50 μl of β translation mixture. Fifty μl of the βγ mixture was passed over an 8 ml Aca34 column (Sepracor) equilibrated with HMSE plus 0.05% Lubrol PX at 4 °C in order to remove DTT and to separate the βγ dimers from unimerized β. The fractions containing βγ were concentrated 5–10-fold using a Centricron-30 concentrator (Amicon).

**For cross-linking, 30 μl of this sample was mixed with 10 μl of α1 (2–5 μg/μl) (Protein A-Sepharose beads) in HMSE or 10 μl of HMSE buffer alone, and the reaction was initiated by the addition of 1.6 μl of freshly prepared 50 mM bismaleimidyl hexane (BMH) (Pierce) in Me2SO (8, 9). In control un-cross-linked samples, 20 μM DTT was added prior to BMH. After 40 min at 4 °C, DTT (20 μM) and/or Laemmli sample buffer containing 15% β-mercaptoethanol was added, and the samples were boiled and resolved by SDS-PAGE on 5% polyacrylamide gels (13). Densitometric scans were taken in Excel and then used for autoradiography. The radioactive bands could be visualized after 2–7 days of exposure at −70 °C.

**Sf9 Cell Culture and Construction of Recombinant Baculovirus—**Sf9 cells were cultured in suspension in IPL-14 medium containing 1% Pluronic F68, 10% heat-inactivated fetal bovine serum, and 50 μg/ml gentamicin at 27 °C with constant shaking (125 rpm).

To generate recombinant βγ4 mutant viruses, the mutated βγ cDNAs (K98A, L117A, D228R, D246S, and W332A) were subcloned into pVL1392 transfer vector, and the resulting plasmids were cotransfected into Sf9 cells with BacPac6 viral DNA linearized with Bsu361 (CLONTECH) using Lipofectin (Life Technologies, Inc.). Two viruses, one with and one without Hisα,tag, were generated for [L117A], [D228R], or [W323A], and the one that gave higher protein expression was used for subsequent studies. The βγ, [D228R] and βγ, [D246S] mutants were used with a hexahistidiny (His12) tag at the amino terminus; the other mutants were used without a tag. Recombinant viruses were plaque-purified and amplified as described (15).

Recombinant baculoviruses encoding βγ, [γ2, and Hisγ4γ3 have been described previously (16, 17). Recombinant βγ1 and Other Protein–Deficient Sf9 Cells (1 liter; 1.5 × 10^9 cells/ml) were cotransfected with recombinant baculoviruses encoding β1, γ2, and His6γ3. Cells were harvested after 48–66 h, and membranes were prepared as described (17). Sodium cholate was added to a final concentration of 1%, and the mixture was stirred on ice for 1 h, followed by centrifugation at 100,000 × g for 40 min. The supernatant was diluted 3-fold with Buffer A (20 mM HEPES, pH 7.6, 6 mM MgCl2, 75 mM sucrose, 1 mM EDTA, 1 mM DTT, 1% Triton X-100, and 3 mM benzamidine; HMSE, 50 mM NaH2P2, pH 7.5, 6 mM MgCl2, 75 mM sucrose, and 1 mM EDTA).
NaHEPES, pH 8.0, 100 mM NaCl, 1 mM MgCl₂, 10 mM β-mercaptoethanol, 0.5% polyoxethylene 10-lauryl ether) and loaded onto a Ni-NTA (Qiagen) column (0.5 ml) that had been equilibrated with Buffer A. The column was washed with 5 ml of Buffer A containing 400 mM NaCl and 10 mM imidazole and 5 ml of Buffer B (20 mM NaHEPES, pH 6.0, 100 mM NaCl, 1 mM MgCl₂, 10 mM β-mercaptoethanol, 0.2% octyl β-D-glucopyranoside, 10 mM imidazole). Recombinant βγ was eluted with 2 ml of Buffer B containing 1% octyl β-D-glucopyranoside and 150 mM imidazole. The eluate was concentrated and exchanged into 20 ml NaHEPES, pH 8.0, 1 mM EDTA, 1 mM DTT, and 1% octyl β-D-glucopyranoside with a Centricon-30 (Amicon). Because of low expression, the eluate from the Ni-NTA column containing βγ (W332A) was further purified over Mono Q 5/5 by fast protein liquid chromatography (Amersham Pharmacia Biotech) in the presence of 1% octyl β-D-glucopyranoside with a gradient (0–400 mM) of NaCl. The peak fractions were combined and processed as above. The capacity to support ADP-ribosylation of α₁₂ by pertussis toxin was performed as described (18).

Recombinant α₁ and wild-type β₁γ were purified from SF9 cells as described (17). Myristoylated α₁ was purified from Escherichia coli as described (19). PLCβ₂ was purified from SF9 cells and kindly provided by Dr. Paul C. Sternweis (University of Texas Southwestern Medical Center). Protein was measured as described in Ref. 20.

**In Vitro Assays for Phospholipase C and Adenyl Cyclase Activity—**Phospholipase C activity was measured using sonicated micelles containing 50 μM phosphatidylinositol 4,5-bisphosphate, 500 μM phosphatidylethanolamine, and inositol-[2-3H] phosphatidylinositol 4,5-bisphosphate (NEN Life Science Products) (2,500 cpm/assay) in a solution containing 50 mM NaHEPES, pH 7.5, 0.42 mM EDTA, 3 mM EGTA, 2 mM MgCl₂, 1.7 mM CaCl₂, 42 mM NaCl, 47 mM KCl, 4 mM GDP, 0.125 mg/ml bovine serum albumin, 1 mM DTT, and 0.375% octyl β-D-glucopyranoside with 0.1 mM PLC-β2 and the indicated amount of βγ. The mixture (60 μl) was incubated at 30 °C for 8 min, and the amount of IP₃ generated was quantitated as described (21).

To measure adenyl cyclase activity, purified βγ mutants were reconstituted with 10 μg of membranes from SF9 cells expressing type I or type II adenyl cyclase for 3 min at 30 °C in a final volume of 20 μl. Assays were then performed as described (22) for 7 min at 30 °C in a total volume of 50 μl containing 4 mM MgCl₂ and 0.2% octyl β-D-glucopyranoside. The presence of the hexahistidine tag at the amino terminus of either β₁ or γ₂ did not affect any of the enzymatic assays (data not shown).

**RESULTS**

**Formation of βγ Dimers by Mutant Hβ₂ and Their Association with α₁—**To evaluate the relationship between surfaces of β that bind α and that activate PLCβ, we mutated four residues on the flat, top surface of β₁ and two residues on the side of blade I (see Fig. 1). The mutations were introduced into the background of rat β₁ tagged at the amino terminus with six additional histidine residues. Addition of the hexahistidine tag was extremely important for assays in COS-7 cells because the size difference between Hβ₁ and wild-type β₁ allowed us to discriminate transfected, mutated β subunits from endogenous β₁ subunits. We could detect no differences in these assays between the hexahistidine β₁ (Hβ₁) and wild-type β₁ (see below). Before we could assess the ability of mutated β subunits to activate PLC, it was essential to establish that they could form dimers. We expected that some, but not all, mutations would also affect the ability of βγ to form heterotrimers with α. To evaluate these two issues, transfected COS-7 cells were labeled with Trans[35S]-Label and Hβ₁ or the mutant proteins were immunoprecipitated through cotransfected HAγ₂ (10). Because we immunoprecipitated through one subunit (HAγ₂) but measured the other, this assay measured only the amount of βγ dimers that accumulate and not the total synthesis of β. When HAγ₂ was transfected, it dimerizes with both wild-type endogenous β and from the transfected Hβ₂ subunits. Therefore, both types of β subunits were immunoprecipitated with the anti-HAγ₂ antibody, but the two can be readily distinguished by their mobility in SDS-PAGE (Fig. 2A).

We also used immunoprecipitation to measure the ability of mutant Hβ₁γ₂ dimers to associate with transfected α₁₂. Co-transfection of α₁₂ had no reproducible effect on the amount of

βγ dimer formed. Because antibody to HAγ₂ precipitates both endogenous and transfected βγ, it was essential to be able to subtract the amount of α₁₂ coprecipitating with a dimer containing endogenous β and HAγ₂, from the amount coprecipitating with a dimer of transfected Hβ₂ mutants and HAγ₂. The amount of α₁₂ coprecipitated with endogenous β was determined from the lysates of cells transfected with HAγ₂, α₁₂, and vector but no additional β. From the relative density of the α and β bands (taking into account the number of methionine and cysteine residues), we calculated that 0.6–0.8 mol of α were precipitated per mol of endogenous β₂.

To determine whether the α₁₂ immunoprecipitating with Hβ₂HAγ₂ dimers was associating correctly, we measured the ability of coimmunoprecipitated α to be [32P]ADP-ribosylated by pertussis toxin. Although only the α subunit is ADP-ribosylated, the substrate for the toxin is the αβγ heterotrimer (14). Alternate lanes in the top panel of Fig. 2 show [35S] label only or [35S] + [32P]. The bottom panel shows [32P] only. These experiments are quantitated and summarized in Fig. 2B and Table I. The results show that mutation Hβ₂[W332A] has little effect on the ability of βγ to interact with α, but each of the other mutations diminishes coimmunoprecipitation of α through βγ. For each mutant, the results were the same whether we measured the amount of α by [35S] or by ADP-ribosylation. This correlation suggests that there are no dramatic differences in the ability of mutant βγ to support ADP-ribosylation of α once a complex has formed.

Another way to assess the interaction of mutant βγ dimers with α is chemically to cross-link mutant βγ dimers to α using the cysteine-specific reagent, BMH. We have previously shown that this reagent specifically cross-links cysteine 215 of α₀.

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2 In principle, it would be possible to immunoprecipitate α through HA-tagged β assembled with untagged γ₂. However, one would then immunoprecipitate both dimerized and undimerized βγ, so the results would be very difficult to interpret.
either to cysteine 204 or cysteine 271 of β, giving two cross-linked products (8, 9). In the wild-type βγ, the two cross-linked products are formed approximately equally (Fig. 3). From the crystal structure, we know that the distance between the residues on αα and on β is very close to that of the fully extended cross-linking reagent. The ability of the reagent to reach to one or the other of the cysteines on αβ depends on a correct orientation of α with respect to β. The βγ formed from one of the mutants on the top surface of β (Hβ1[D246S]) gave the same two cross-linked products as wild-type in the same ratio. In βγ containing each of three other mutants, the ratio of cross-linked products was different. The upper band produced by cross-linking Hβ1[N88A/K89A] greatly diminished the affinity of βγ for α. Indeed, βγ containing this mutation produced barely detectable cross-linked products, although both bands were faintly visible. The cross-linking reaction is an irreversible reaction and is therefore able to reveal even low affinity interactions between the subunits. We explain the altered cross-linking pattern of Hβ1[W332A] and Hβ1[L171A] in Hβ1[D228R], the lower band (produced by cross-linking α Cys215 to β Cys271) was missing. Mutating the two residues that contact the aminoterminal α helix of α (Hβ1[N88A/K89A]) greatly diminished the affinity of βγ for α. Indeed, βγ containing this mutation produced barely detectable cross-linked products, although both bands were faintly visible. The cross-linking reaction is an irreversible reaction and is therefore able to reveal even low affinity interactions between the subunits. We explain the altered cross-linking pattern of Hβ1[W332A], Hβ2[L171A] and Hβ1[D228R] by suggesting that the α subunit is still able to interact with the mutated β, but that it is tilted on its binding site. It is unlikely the changes in the cross-linking pattern are due to local effects of the mutations themselves, because no mutated residue is adjacent to the cysteine whose cross-linking it affects.

**Activation of Phospholipase C β isoforms by βγ**

PLCβ2 and PLCβ3 are two isoforms of PLC that are activated by the βγ dimer (11, 23, 24). We used two methods to compare the ability of mutant βγ proteins to interact with α and to activate the two isoforms of PLC. First, we cotransfected COS-7 cells expressing the mutants with various amounts of HA35S proteins in the presence of BMH to activate the PLCβ isoforms. The activated PLCβ isoforms were immunoprecipitated using monoclonal antibodies against the HA epitope. The amount of immunoprecipitated PLCβ was quantitated by densitometry of the autoradiogram. The relative phospholipase C activation was calculated as follows.

\[
\text{Relative activation} = \frac{\text{cpm PLCβ} + \text{Hβγ}}{\text{cpm PLCβ alone}} \times 100.
\]

**Table 1**

| Mutation          | Immunoprecipitation of αγ | % of Hβγα | Activation of PLCβ2 | Activation of PLCβ3 |
|-------------------|---------------------------|-----------|---------------------|---------------------|
| N88A/K89A         | 3 ± 2                     | 72 ± 19   | 88 ± 8              |                     |
| L117A             | 19 ± 9                    | 102 ± 11  | 14 ± 15             |                     |
| D228R             | 8 ± 4                     | 27 ± 12   | 2 ± 4               |                     |
| D246S             | 31 ± 3                    | 37 ± 16   | 80 ± 10             |                     |
| W332A             | 71 ± 1                    | 22 ± 8    | -13 ± 8             |                     |

The values given were the mean ± S.D. for at least three independent experiments for each mutant.

The amount of αγ immunoprecipitated was quantitated by densitometry of the 35S autoradiogram for separate experiments. The amount of immunoprecipitated αγ accounted for by the endogenous βHAγ was subtracted from the total value. The relative phospholipase C activation was calculated as follows.

\[
\text{Relative activation} = \frac{\text{cpm PLCβ + Hβγ} - \text{cpm PLCβ alone}}{\text{cpm PLCβ} + \text{Hβγ} - \text{cpm PLCβ alone}} \times 100.
\]
cells with wild-type and mutant H12 subunits, HAγ2 and PLCβ2, or PLCβ3, and measured the increase in inositol phosphate production. Second, we synthesized the proteins in Sf9 cells, purified them, and measured activation of PLCβ2 in vitro. Transfection of βγ into COS-7 cells did not significantly affect basal PLC activity (probably because the βγ level is elevated only in the fraction of the cells that took up the cDNA, whereas all cells contribute to the basal activity) (data not shown). Transfection of PLCβ2 together with β and γ caused a 3-fold increase in inositol phosphate production compared with transfection of PLCβ alone (Fig. 4A). Neither β nor γ alone increased the activity of transfected PLCβ (data not shown). Addition of the hexahistidine tag had no effect on the activity of β, and HAYγ2 was as effective as γ2. As was previously shown by Katz et al. (11), we found that βγ dimers that contain a mutant γ that cannot be prenylated at the carboxyl terminus do not activate PLCβ in the COS-7 cells (data not shown). Finally, activation of PLCβ by βγ was blocked by cotransfection of α2 (see below). Taken together, these controls, together with published in vivo and in vitro studies (11, 25–29), support the interpretation that the elevation of inositol phosphates that we measured reflects activation of PLCβ by βγ. This interpretation is further strengthened by agreement of the data obtained in transfected cells with those obtained with purified proteins.

As shown in Table I, mutation in residues on the side of the β torus (Hβ1[N88A/K89A]) had little effect on the ability of the β subunit to activate PLCβ or PLCβα, despite a profound effect on the affinity for α2 as measured in solution. In contrast, three of the four mutations on the top surface of β markedly diminished the ability of the mutant βγ to activate phospholipase Cβ2. The ability of Hβ1[L117A] to activate PLCβ2 was equal to that of the wild-type. Another mutation in a known αβ contact point (Hβ1[W332A]) had little effect on binding of α to βγ but diminished stimulation of PLCβ2. Mutations that affect the ability of the βγ subunit to activate PLCβ2 do not always have similar effects on the ability to activate PLCβα. For example, Hβ1[D246S] activated PLCβ2 almost as well as wild-type, but was blunted in its ability to activate PLCβα. In contrast, Hβ1[L117A] was fully active with respect to PLCβ2 but inactive with respect to PLCβα. These results are consistent with a model in which the interaction interfaces of βγ with α or βγ differ between overexpressed, but the importance of specific residues for each function is different.

Cotransfection of α2 blocks PLCβ2 activation by βγ (Fig. 4A), even when the βγ has a diminished affinity for α in solution (for example, Hβ1[L117A] or Hβ1[D246S]). Of the mutations we made, changes in residues on the side of the β torus (Hβ1[N88A/K89A]) had the most profound effect on the affinity for α2 in solution, as measured by immunoprecipitation. Nevertheless, in cells, expression of α2 blocked activation of PLCβ2 by Hβ1[N88A/K89A] with a dose-response curve similar to its inhibition of wild-type βγ (Fig. 4B). Analysis of α2 expression by Western blot at each cDNA concentration showed that the α2 levels rose approximately equally in cotransfections with PLCβ2 and wild-type or mutant β (data not shown).

To confirm the results in COS-7 cells, recombinant βγ mutant proteins were synthesized in Sf9 cells and purified; SDS-PAGE analysis of these samples is shown in Fig. 5. All five β1 mutants were purified as complexes with the γ2 subunit. In these studies, we used a single β1[K89A] rather than a double Hβ1[N88A/K89A] mutant at the side of the β1 torus. All of the complexes supported ADP-ribosylation of α1 by pertussis toxin, although the potency of Hβ1[K89A]γ2 was about half that of the wild-type protein, presumably reflecting the lower affinity of this mutant for α1, consistent with the properties of the double mutant (data not shown).

Activation of PLCβ2 by purified wild-type and mutant βγ complexes is shown in Fig. 6. Consistent with results in COS-7 cells, Hβ1[K89A]γ2 and Hβ1[L117A]γ2 were approximately equal to wild-type βγ in activating PLCβ2, but the other three mutations on the top surface of β1 were severely blunted in their ability to activate PLCβ2. Although there are quantitative differences in the degree of impairment of D228R, D246S, and W332A in the two experimental systems, the conclusion that mutating each of the three residues diminishes PLCβ2 activation is consistent in both. In analyzing a large number of mutations at various sites in β, we have sometimes observed differences in the ability of mutant proteins to fold correctly,
depending on the expression system, with the most native state achieved when the protein is made in mammalian cells (28). It is possible that the final conformation of the mutant \( \beta \) subunit is slightly different when they are made in mammalian COS-7 cells as opposed to insect cells.

Activation and Inhibition of Adenylyl Cyclase by \( \beta \gamma \) Mutants—The effects of mutant \( \beta \gamma \) complexes on adenylyl cyclase activities are shown in Fig. 7. \( \beta \gamma \) activates type II adenylyl cyclase in the presence of \( \alpha \), but it inhibits type I adenylyl cyclase (29). The apparent affinities of \( \beta \gamma_{[D246S]} \) and \( \beta \gamma_{[W332A]} \) for type II adenylyl cyclase are clearly diminished; we were unable to assess unequivocally their maximal capacities to activate the enzyme because of our inability to achieve higher concentrations of these proteins in the assay (Fig. 7B). Within a similar range of concentrations, \( \beta \gamma_{[D228R]} \) did not activate type II adenylyl cyclase. The \( \beta \gamma_{[K89A]} \) and \( \beta \gamma_{[L117A]} \) mutants were indistinguishable from wild-type complex. In contrast, all five mutant \( \beta \gamma \) complexes inhibited type I adenylyl cyclase (Fig. 7A). These inhibitory activities were lost after inactivation of the proteins at 95 °C for 5 min (data not shown). The observation that \( \beta \gamma_{[D228R]} \) is able to inhibit type I adenylyl cyclase (albeit with the lowest apparent potency of the group tested), whereas it is inactive in PLC\( \gamma \), PLC\( \delta \), and type II adenylyl cyclase assays, confirms the conclusion, based on communoprecipitation and cross-linking studies, that the protein is not grossly misfolded (Figs. 2 and 3).

The inability of any of the mutations on the top surface of \( \beta \) to interfere with the interaction of type I adenylyl cyclase raises the possibility that \( \beta \gamma \) inhibition of type I adenylyl cyclase would not require the \( \alpha \) binding surface and would be an exception to the rule that association with \( \alpha \) blocks interaction of \( \beta \gamma \) with all effectors. However, incubation of wild-type \( \beta \gamma \) with GDP-\( \alpha \) interfered with both activation of type II adenylyl cyclase (Fig. 6).
cyclohexane and inhibition of type I adenylyl cyclase $\beta\gamma$ (Fig. 8), suggesting overlap of $\alpha$ with these interacting surfaces. The interface between $\beta\gamma$ and the two adenylyl cyclases must require different parts of the $\beta$ top surface.

**DISCUSSION**

The interpretation of the functional consequences of mutation introduced into a protein structure depends on demonstrating, as far as possible, that the mutation produces only a local change and not a global one. We have mutated some of the residues in $\beta$ known to contact $\alpha$ (2, 3) in order to test the hypothesis that $\alpha$ and effectors share a common surface. We have analyzed the properties of the mutant $\beta$ subunits in three kinds of expression systems, which allows us to evaluate different aspects of their function. None of the mutant proteins reported in this paper appeared to have global effects on $\beta$ structure or its ability to assemble with $\beta\gamma$. Indeed, in one assay (inhibition of type I adenylyl cyclase), all were fully active. When the same property was evaluated in more than one system (e.g. activation of PLC$\beta_2$ in COS-7 cells or reconstituted with pure proteins in vitro), we found general agreement in the results.

Our results show that some residues that are important for binding of $\alpha$ by $\beta\gamma$ are also important for activation of PLC$\beta_2$, PLC$\beta_3$, and adenylyl cyclase II. Mutating the residues on the side of blade 1 (Asn$^{266}$ and Lys$^{295}$) has little effect on activation of either PLC isofor or regulation of adenylyl cyclase I and II. These results suggest that the side of the torus at blade 1 is not involved in activation of these effectors, although it is very important for $\alpha$ binding. In contrast, mutations on the flat top surface of $\beta$ affect activation of both PLC isoforms and adenylyl cyclase II. The $\alpha$ subunit, PLC$\beta_2$, PLC$\beta_3$, and adenylyl cyclase II appear to share an overlapping region on the top surface of $\beta\gamma$, but the relative importance of particular residues is different among them. Changing Trp$^{332}$ to Ala severely inhibits the ability to activate adenylyl cyclase II. The amount of $\alpha$ subunit, PLC$\beta_2$, PLC$\beta_3$, and adenylyl cyclase II poorly. Nevertheless, $\beta\gamma$ containing the purified H$\beta_1[D228R]$ protein is able fully to inhibit adenylyl cyclase I, confirming our studies in vitro and in COS-7 cells that this mutation does not prevent folding of the mutant $\beta$ into $\beta\gamma$.

The observation that $\beta\gamma$ dimers containing each of the mutated $\beta$ subunits were able to inhibit type I adenylyl cyclase was surprising. Therefore, we wanted to be sure that $\beta\gamma$ inhibition of type I adenylyl cyclase could, indeed, be inhibited by $\alpha$. As shown in Fig. 8, activation of type II adenylyl cyclase and inhibition of type I adenylyl cyclase follow the rule that $\alpha$ blocks all effector regulation by $\beta\gamma$. We propose that type I adenylyl cyclase interacts with a different part of the top surface from PLC$\beta_2$, PLC$\beta_3$, and type II adenylyl cyclase. The footprint of $\alpha$ on the top surface of $\beta$ is quite large. Steric hindrance could prevent interactions over a much greater portion of the surface than that immediately forming contact sites for $\alpha$ on $\beta$.

Residues that affect PLC$\beta$ and adenylyl cyclase II activity are found on opposite sides of the central tunnel (see Fig. 1), suggesting that PLC$\beta$ and adenylyl cyclase II, like $\alpha$, makes many contacts with the top surface. Overlap of the footprint of $\alpha$, PLC or adenylyl cyclase II on $\beta$ explains why binding of $\alpha$ to $\beta\gamma$ prevents activation of these effectors by $\beta\gamma$. Analysis of conserved residues on $\beta$ subunits from several species also suggested that the $\alpha$ binding surface is important for effector activation (31).

The amount of $\alpha$ coimmunoprecipitated with wild-type or mutant $\beta\gamma$ gives information about the relative importance of some residues in $\beta$ for binding to $\alpha$ in solution. However, for molecules to coimmunoprecipitate requires that they have a fairly high affinity for each other. Another assay for the ability of mutant $\beta\gamma$ subunits to interact with $\alpha$ is chemical cross-linking with BMH, an irreversible reaction. In addition to affecting the apparent affinity, some mutations of the $\alpha$ binding surface H$\beta_1[L117A], H\beta_2[D228R], H\beta_3[W332A]$ appear to cause the proteins to interact with $\alpha$ abnormally. They give cross-linking patterns that differ from wild-type, suggesting that $\alpha$ is not docking properly on the $\beta\gamma$ surface. The irreversible cross-linking reaction traps even transient associations, so that even those proteins that are poorly immunoprecipitated can still be cross-linked (H$\beta_1[D228R]$ and H$\beta_3[W332A]$). They can also interact with $\alpha$ in cells as shown by the ability of cotransfected $\alpha_{12}$ to block PLC$\beta$ activation by all the mutants (Fig. 4A). Even the mutant $\beta\gamma$ most severely affected with respect to $\alpha$ binding (H$\beta_3[N88A/K89A]$) can be inhibited by cotransfected $\alpha_{12}$ with a cDNA dose-response curve similar to...
reversal of PLCβ2 activation by wild-type βγ (Fig. 4B). We suggest that when α and the mutant βγ proteins are correctly positioned in the membrane, the local concentration is high enough to allow productive interactions.

Besides α, two other molecules that virtually universally block the action of βγ have been described. The first is the carboxyl-terminal region of the β-adrenergic receptor kinase (32). This region has been shown to bind βγ. When expressed in COS-7 cells, the βARK fragment is able to inhibit activation of adenylyl cyclase and PLC by βγ. The βARK fragment does not bind to the αβγ heterotrimer. We presume, therefore, that like PLC and type II adenylyl cyclase, it binds to the Gβ on the surface that faces α. The second is a 12-amino acid peptide derived from the putative βγ binding site on adenylyl cyclase that blocks βγ activation of adenylyl cyclase, the K⁺ channel, and PLC and may bind to the top surface of βγ (33, 34).

The region of βγ that we have identified as important for interaction with PLCβ and type II adenylyl cyclase is unlikely to be the only surface of βγ that interacts with these enzymes, although it is probably the one crucial for regulation by α. Yan et al. (35) recently described interaction in the yeast two hybrid system of the first 100 residues of Gβ (including the amino-terminal coiled coil plus blade 1) with the amino terminus of the muscarinic K⁺ channel and a portion of adenylyl cyclase II. The interactions have not yet been verified by any other means, but the data are consistent with the genetic findings in S. cerevisiae that the amino-terminal region may contain binding sites for some effectors. In addition to the crystal structure of the αβγ dimer (2, 3), the structure of βγ in a complex with phosducin, a potential regulator of βγ function, has recently been solved (36). Like α, phosducin binds to the top surface of β as well as to the side of the torus. It is likely that PLC and adenylyl cyclases also have important contacts along the side of the Gβ torus. Future studies will determine whether such contacts exist and where they are.

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REFERENCES
1. Neer, E. J. (1995) Cell 86, 249–257
2. Wall, M. A., Coleman, D. E., Lee, E., Iniguez-Lluhi, J. A., Posner, B. A., Gilman, A. G., and Sprang, S. R. (1995) Cell 83, 1047–1058
3. Lambright, D. G., Sondek, J., Bohn, A., Skiba, N. P., Hamm, H. E., and Sigler, P. B. (1996) Nature 379, 311–319
4. Sondek, J., Bohm, A., Lambright, D. G., Hamm, H. E., and Sigler, P. B. (1996) Nature 379, 369–374
5. Fung, B. K.-K., and Nash, C. R. (1983) J. Biol. Chem. 258, 10503–10510
6. Winslow, J. W., Van Amsterdam, J. K. R., and Neer, E. J. (1986) J. Biol. Chem. 261, 7571–7579
7. Denker, B. M., Neer, E. J., and Schmidt, C. J. (1992) J. Biol. Chem. 267, 6727–6727
8. Thomas, T. C., Schmidt, C. J., and Neer, E. J. (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 10295–10299
9. Garcia-Higuera, I., Thomas, T. C., Yi, F., and Neer, E. J. (1996) J. Biol. Chem. 271, 528–535
10. Mende, U., Schmidt, C. J., Yi, F., Spring, D. J., and Neer, E. J. (1995) J. Biol. Chem. 270, 15892–15898
11. Katz, A., Wu, D., and Simon, M. I. (1992) Nature 360, 686–689
12. Berridge, M. J., Dawson, R. M. C., Downes, C. P., Heslop, J. P., and Irvine, R. F. (1983) Biochem. J. 212, 473–482
13. Lasemmler, U. K. (1970) Nature 227, 680–685
14. Neer, E. J., Lok, J. M., and Wolf, L. L. G. (1984) J. Biol. Chem. 259, 14222–14229
15. Summers, M. D., and Smith, G. E. (1987) A Manual of Methods for Bacterial Vectors and Insect Cell Culture Procedures, Texas Agricultural Experiment Station, Bulletin 1555, College Station, TX.
16. Iniguez-Lluhi, J. A., Simon, M. I., Robishaw, J. D., and Gilman, A. G. (1992) J. Biol. Chem. 267, 23409–23417
17. Kozasa, T., and Gilman, A. G. (1995) J. Biol. Chem. 270, 1734–1741
18. Casey, P. J., Pang, I.-H., and Gilman, A. G. (1991) Methods Enzymol. 195, 315–321
19. Lee, E., Linder, M. E., and Gilman, A. G. (1994) Methods Enzymol. 237, 146–164
20. Schaffner, W., and Weisman, C. (1973) Anal. Biochem. 56, 502–514
21. Hepler, J. R., Kozasa, T., Smrcka, A. V., Simon, M. I., Rhee, S. G., Sternweis, P. C., and Gilman, A. G. (1993) J. Biol. Chem. 268, 14367–14375
22. Smigel, M. D. (1986) J. Biol. Chem. 261, 1976–1982
23. Camps, M., Carozzi, A., Schnabel, P., Scheer, A., Parker, P. J., and Gierschik, P. (1992) Nature 360, 684–688
24. Smrcka, A. V., and Sternweis, P. C. (1993) J. Biol. Chem. 268, 9667–9674
25. Kueang, Y., Wu, Y., Smrcka, A., Jiang, H., and Wu, D. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 2964–2968
26. Conklin, B. R., Chabre, O., Wong, Y. H., Federman, A. D., and Bourne, H. R. (1992) J. Biol. Chem. 267, 31–34
27. Conklin, B. R., Farfel, Z., Lustig, K. D., Julius, D., and Bourne, H. R. (1993) Nature 363, 274–276
28. Garcia-Higuera, I., Gaitatzes, C., Smith, T. F., and Neer, E. J. (1998) J. Biol. Chem. 273, 9041–9049
29. Tang, W. J., and Gilman, A. G. (1991) Science 254, 1500–1503
30. Blank, J. L., Shaw, K., Ross, A. H., and Exton, J. H. (1993) J. Biol. Chem. 268, 25184–25191
31. Lichtarge, O., Bourne, H. R., and Cohen, F. E. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 7507–7511
32. Pitcher, J. A., Inglese, J., Higgins, J. B., Arriza, J. L., Casey, P. J., Kim, C., Benovic, J. L., Kwatra, M. M., Caron, M. G., and Lefkowitz, R. J. (1992) Science 257, 1264–1267
33. Chen, J., DeVivo, M., Dingus, J., Li, A. H. J., Sui, J., Carty, D. J., Bla, J. L., Exton, J. H., Stoffel, R. H., Inglese, J., Lefkowitz, R. J., Logothetis, D. E., Hildebrandt, J. D., and Iyengar, R. (1995) Science 268, 1166–1169
34. Chen, Y., Weng, G., Li, J., Harry, A., Fierion, J., Dingus, J., Hildebrandt, J. D., Guarnieri, F., Weinstein, H., and Iyengar, R. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 2711–2714
35. Yan, K., and Gautam, N. (1996) J. Biol. Chem. 271, 17597–17600
36. Gaudet, R., Bohn, A., and Sigler, P. B. (1996) Cell 87, 577–588