The \( \beta \gamma \) subunits of the heterotrimeric GTP-binding proteins (G proteins) that couple heptahelical, plasma membrane-bound receptors to intracellular effector enzymes or ion channels directly regulate several types of effectors, including phospholipase C\( \beta \) and adenylyl cyclase. The \( \beta \) subunit is made up of two structurally different regions: an N-terminal \( \alpha \) helix followed by a toroidal structure made up of 7 blades, each of which is a twisted \( \beta \) sheet composed of four anti-parallel \( \beta \) strands (Wall, M. A., Coleman, D. E., Lee, E., Íñiguez-Lluluí, J. A., Posner, B. A., Gilman, A. G., and Sprang, S. R. (1995) Cell 83, 1047–1058; Lambricht, D. G., Sondek, J., Bohm, A., Skiba, N. P., Hamm, H. E., and Sigler, P. B. (1996) Nature 379, 311–319). We have previously shown that sites for activation of PLC\( \beta_2 \), PLC\( \beta_3 \), and adenylyl cyclase II overlap on the “top” surface of the propeller, where Ga also binds (Li, Y., Sternweis, P. M., Charnecki, S., Smith, T. F., Gilman, A. G., Neer, E. J., and Kozasa, T. (1998) J. Biol. Chem. 273, 16265–16272). The present study was undertaken to identify the regions on the side of the torus that might be important for effector interactions. We made mutations in each of the outer \( \beta \) strands of the G protein \( \beta \) propeller, as well as mutations in the loops that connect the outer strands to the adjacent \( \beta \) strands. Our results suggest that activation of PLC\( \beta_2 \) involves residues in the outer strands of blades 2, 6, and 7 of the propeller. We tested three of the mutations that most severely affected PLC\( \beta_2 \) activity against two forms of adenylyl cyclase (ACI and ACII). Both inhibition of ACI and activation of ACII were unaffected by these mutations, suggesting that if ACI and ACII contact the outer strands, the sites of contact are different from those for PLC\( \beta_2 \). We propose that distinct sets of contacts along the sides of the propeller will define the specificity of the interaction of \( \beta \gamma \) with effectors.

The transmembrane machinery that transmits information from outside of the cell to enzymes and ion channels inside the cell is made up of a receptor (usually one that spans the membrane seven times), a heterotrimeric GTP binding protein (G protein) made up of \( \alpha \), \( \beta \), and \( \gamma \) subunits, and an effector enzyme or ion channel. Both \( \alpha \) and \( \beta \gamma \) subunits of the G protein regulate the activity of their target effectors, and both are active only when they are dissociated from each other. Hydrolysis of GTP to GDP at the active site of \( \alpha \) leads to reassociation of the \( \alpha \) and \( \beta \gamma \) subunits and reformation of the inactive heterotrimer (reviewed in Ref. 1).

The \( \beta \) subunit is made up of two structurally different regions: an N-terminal \( \alpha \) helix followed by a toroidal structure made up of seven repeating units. The repeating units make a structure called a \( \beta \) propeller with seven blades, each of which is a twisted \( \beta \) sheet composed of four anti-parallel \( \beta \) strands (Fig. 1) (2, 3). The repetitive three-dimensional structure reflects a repeating amino acid sequence made up of WD repeats (reviewed in Ref. 4). The \( \beta \) propeller motif occurs 4 – 16 times in over 100 proteins with many different cellular functions. It can be divided into two regions: a region of variable length that includes the outermost \( \beta \) strand of each blade, followed by a conserved core that encodes the inner three \( \beta \) strands of the propeller structure (4).

The toroidal, WD-repeat portion of the \( \beta \) subunit is made up of four surfaces. The narrow “top” surface interacts with the \( \alpha \) subunit (2, 3) (see Fig. 1). The same surface also provides sites for interaction with phosducin, a protein that may regulate \( \beta \gamma \) function (5). The wider “bottom” surface of the torus is the dominant site for interaction with \( \gamma \) (2, 6). The outer surface of the torus is largely made up of the outer \( \beta \) strands of the blades that are encoded in the variable region of the WD repeat. Finally, a surface of unknown function lines the central tunnel of the torus. In addition to contacting the top surface of the torus, both \( \alpha \) and phosducin make contacts with the outer \( \beta \) strands of the propeller blades, but each protein interacts with a different subset of the blades.

The \( \beta \gamma \) subunit directly regulates several effectors, including subtypes of phospholipase C\( \beta \) (PLC\( \beta \)1) and adenylyl cyclase (7–12). We (13) and others (26) have recently shown that the top surface of the torus is an interaction site for these effectors. This common binding site for \( \alpha \) and effectors explains why the \( \beta \gamma \) subunit can interact with \( \alpha \) or effectors but not with both simultaneously. The present study was undertaken to identify the regions on the sides of the torus that might be important for effector interaction with the \( \beta \gamma \) subunit. It is our hypothesis that all effector proteins will interact with \( \beta \) on its top surface.

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The abbreviations used are: PLC\( \beta \), phospholipase C; H\( \alpha \), hexahistidine-tagged \( \beta \gamma \); HA\( \gamma \), hemagglutinin-tagged \( \gamma \); PCR, polymerase chain reaction; PBS, phosphate-buffered saline; PAGE, polyacrylamide gel electrophoresis.

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but that each effector will have a characteristic footprint along the sides of the molecule. To test the hypothesis, we have made mutations in each of the outer β strands of the G protein β₁ propeller, as well as mutations in the loops that connect the outer strands to the adjacent β strands. We tested the ability of the mutant proteins to activate phospholipase Cβ₂ (PLCβ₂) and to regulate two forms of adenylyl cyclase (AC₁ and ACII). Our results suggest that activation of PLCβ₂ involves residues in the outer strands of blades 2, 6, and 7 of the propeller. None of the mutations substantially affects activation or inhibition of adenylyl cyclase, indicating that these enzymes and PLCβ interact with different domains of βγ.

**EXPERIMENTAL PROCEDURES**

cDNA Constructs—The cDNAs encoding rat β₁ and α₁ subunits were generously provided by R. Reed (HHMI, Johns Hopkins School of Medicine, Baltimore). The cDNA for γ₁ was a gift of N. Gautam (University School of Medicine, St. Louis, MO). They were subcloned into pcDNA3 vector (Invitrogen). Human PLCβ₂ (in pMT2 vector) was a gift of M. Simon (CIT, Pasadena, CA). The N-terminally hemagglutinin (HA) epitope-tagged γ₁, encoding cDNA and the N-terminally (His)₆-tagged β₁ (H₆β₁) encoding cDNA with a silent mutation (to create a unique KpnI site), corresponding to amino acids 144 and 145, were constructed in pcDNA3 vector as described previously (13, 14). To identify the possible surfaces of the β subunits that interact with effectors, we made a series of localized alanine-scanning mutations on various blades. A restriction site (XhoI in blades 1, 3, 4, 5, 6, 7 and SpeI in blade 2) was introduced by site-directed mutagenesis in the center of the region in which the amino acid residues were to be replaced by alanines. These mutations are called 1A–7A in Table I. Site-directed mutagenesis in β₁ cDNA was performed using the Altered Sites in vitro mutagenesis system (Promega). Then, PCR primers for alanine mutation were made containing 17–19 base pairs of wild-type sequence followed by the alanine-scanning region and the new restriction site. The cDNA of the H₆β₁ subunit in pcDNA3 was used as a template, and the PCR reactions were carried out with the mutagenic primers and the SP6 or T7 primer, depending on the orientation of the mutagenic primer. The PCR products were digested with the appropriate restriction enzymes and cloned in the cDNA of H₆β₁ subunit in pcDNA3 with HindIII-XhoI, XbaI-KpnI, XbaI-HindIII, XbaI-SpeI, SpeI-BamHI, depending on the location and the orientation of the mutagenic primer. The HindIII site following the H₆ tag is located at the 5′ end of the β₁ cDNA, the BamHI at the 3′ end of the β₁ cDNA, and the KpnI site corresponds to amino acids 144 and 145. The part of the final construct derived from PCR was sequenced. The mutations were confirmed from both strands by direct nucleotide chain termination sequencing method using Sequenase Version 2.0 (US Biological).

**Culture and Transfection of Cells—**COS-7 cells maintained in Dulbecco's modification of Eagle's medium (MediTech) containing 4.5 g/liter glucose, 2 mM l-glutamine, and supplemented with 10% heat-inactivated (56 °C, 1 h) fetal bovine serum, 50 units/ml penicillin, and 50 µg/ml streptomycin at 37 °C in a humidified 5% CO₂ atmosphere. Cells were harvested and reestablished in complete growth medium 24 h before transfection. Cells at 70–80% confluence in 12-well dishes or in 60-mm culture dishes were starved at 37 °C for 2 h in 1 or 2 ml, respectively, of serum-depleted Opti-MEM I (Life Technologies, Inc.). The medium was replaced with 2 ml (60-mm dish) or 1 ml (12-well plate) of Opti-MEM I, containing preformed DNA/LipofectAMINE (Life Technologies, Inc.) complex. The total amount of DNA and LipofectAMINE was 4 µg and 32 µg per 60-mm dish, and 1 µg and 8 µg per well in a 12-well plate. All cDNAs constructed used for transfection of COS-7 cells were purified with a plasmid mini-prep kit from Qiagen. After 5 h, transfection was stopped by adding 4 ml (60-mm dish) or 2 ml (12-well plate) of complete growth medium, and the cells were maintained overnight. The next morning, the medium was replaced with complete growth medium. The efficiency of transfection was consistently 30–40%, as determined by transfection of the cells with CMV-β-galactosidase vector, and 48 h later, staining them with X-Gal.

**35S Metabolic Labeling and Immunoprecipitation—**48 h after transfection, cells in 60-mm culture dishes were starved for 2 h at 37 °C in 2 ml of RPMI 1640 media (ICN) supplemented with 10% dialyzed, heat-inactivated (56 °C, 1 h) fetal bovine serum and 2 mM l-glutamine (Sigma Cell Culture). The medium was then replaced with 2 ml of the same one containing 300 µCi of [35S]-Express Protein Labeling Mix (NEN Life Science Products). 5 h later, cells were rinsed with room temperature PBS and lysed with gentle rocking at 4 °C for 30 min in 1 ml of ice-cold buffer containing 50 mM HEPES-Na (pH 7.5), 6 mM MgCl₂, 1 mM EDTA, 75 mM sucrose, 3 mM benzamidine, and 1% (v/v) Triton X-100. Lysates were precleared by mixing with 50 µl of protein A-Sepharose (Sigma) slurry (50% v/v in PBS) and 48 h later, washing them with X-Gal.

**Western Blot Analysis—**For Western blot analysis, media in 12-well plates were changed to 1 ml of inositol-free PBS and lysed with gentle rocking at 4 °C for 4 h at 15,000 × g. Supernatants were incubated with 4 µl of monoclonal anti-HA-epitope antibody (Berkeley Antibody Co.) at 4 °C for 12–14 h with gentle rocking. The samples were centrifuged at 4 °C for 30 min at 15,000 × g. One-half of the clear supernatants were mixed with 50 µl of protein A-Sepharose slurry (50% v/v in PBS), incubated and washed as described above, and then heated for 15 min with 1 ml of ice-cold lysis buffer, also containing 150 mM NaCl, and once for 15 min with 1 ml of room temperature PBS. SDS-PAGE sample buffer (20 µl) was added to the final pellets; the samples were heat-denatured at 100 °C for 10 min and centrifuged, and 10 µl of the supernatant was loaded onto 4%–12% SDS-PAGE gel. Electrophoresis was performed at constant current (20 mA/0.75-mm thick gel). The gels were dried, and autoradiography was performed for 6–24 h with two intensifying screens at ~80 °C.

**Analysis of Inositol Phosphates in COS-7 Cells—**After transfection, media in 12-well plates were changed to 1 ml of inositol-free Dulbecco’s modified Eagle’s medium supplemented with 5% heat-inactivated fetal bovine serum and 1 mM sodium pyruvate. After a 3-h incubation at 37 °C, the medium was changed to 1 ml of the same one to which 2 µCi of myo-[2-3H]inositol (Amersham) was added. 15 min later, 1 µCi of LiCl was added to a final concentration of 10 mM. After 14–16

![Fig. 1. Structure of the βγ subunit viewed from the α surface.](image-url)
PLCβ2 Activation Sites on the Sides of the Gβγ Propeller

...h of labeling at 37 °C, the medium was aspirated, and cells were extracted on ice twice for 0.5 h each with 0.5 ml of 20 mM formic acid. The extracts were combined, neutralized to pH 7.5 with a solution containing 7.5 mM HEPES and 150 mM KOH, and centrifuged at room temperature for 2 min at 15,000 x g; the clear supernatants were loaded onto 0.5-ml Dowex AG-1-X8 anion exchange columns (Bio-Rad), equilibrated with 2 ml of 1 M NaOH, 2 ml of 1 M formic acid, and 5 x 5 ml of H2O. After sample application, the columns were washed with 5 ml of water and 5 ml of 5 mM borax and 60 mM sodium formate. The inositol phosphates were eluted with 3 ml of 0.9 M ammonium formate, 0.1 mM formic acid and counted in a scintillation counter (7, 16).

...of six histidines residues (termed H6, cdNAS were subcloned into pAcSG2 baculovirus transfer vector (PharMingen) using existing restriction enzyme sites, and the resulting plasmids were cotransfected into Sf9 cells according to the manufacturer’s protocol. Recombinant virions were amplified as described (17). Recombinant baculoviruses encoding β1, γC, and His6-γC have been described previously (18, 19).

...Of Mutant βγ and Other Proteins from Sf9 Cells—Sf9 cells were cultured in suspension in IPL-41 medium containing 1% Pluronic F68, 10% heat-inactivated fetal bovine serum, and 50 μg/ml gentamicin at 27 °C with constant shaking (125 rpm). Sf9 cells (1 liter; 1.5 x 10^6 cells/ml) were infected with amplified recombinant baculovirus at an MOI of 10 and cultured after 48–66 h. Microsomes were prepared as described (13, 19).

...Recombinant βγ and wild-type β1, γC were purified from Sf9 cells as described (19). Myristoylated αi1 was purified from Erwinichia coli as described (20). The capacity of mutant βγ to support ADP-ribosylation of αi2 by pertussis toxin was measured as described (21). 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 by Schaffner and Weisman (22).

...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-biphosphate (NEF 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, 47 mM KCl, 4 mM GDP, 0.125 mg/ml bovine serum albumin, 1 mM dithiothreitol, 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 37 °C for 8 min, and the amount of IP3 generated was quantitated as described (23).

...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 (24) 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 N terminus of either β1 or γC did not affect any of the enzymatic assays (data not shown).

...RESULTS

To test the hypothesis that regions on the side surface of the βγ torus are important for activation of PLCβ2, we mutated residues in the outer β strand one or two at a time or replaced some residues in the outer β strands with alanines. The location and nature of each mutation is shown in Table I. Each mutation is numbered according to the blade in which it is placed and given a letter to designate the specific mutation. This notation is used throughout. For these mutants to be useful in testing our hypothesis, each had to form βγ dimers with an efficiency similar to wild type. All but two of our mutations were made in a background of β1 tagged at the N terminus with six histidine residues (termed H6β1). We have previously shown (13) and now show in controls for these experiments that addition of six histidines at the N terminus has no effect on the ability of βγ to activate PLCβ2. However, it has an extremely important advantage for experiments in COS-7 cells because the terminally tagged β subunit is larger than the endogenous β subunit and is easily distinguished from it by SDS-PAGE.

In interpreting the results of PLCβ2 activation in COS-7 cells, it is important to know how much β protein is properly assembled into a βγ subunit, not simply how much β is synthesized. We used three criteria to assess the ability of the mutant β subunits to form functional βγ subunits in COS-7 cells. First, transfected mutated β1 subunits were immunoprecipitated through hemagglutinin-tagged γ2 subunits, indicating that βγ dimers were formed. Second, the ability of the transfected, mutant β subunits to associate with α was measured by assessing the ability of the mutant βγ subunit to coimmunoprecipitate αi2. Third, the functional interaction between the mutant β subunit and α was tested by measuring the ability of the immunoprecipitated βγ to support ADP-ribosylation of the coimmunoprecipitated αi2 by pertussis toxin. Fig. 2 shows the results of immunoprecipitation of 32P-labeled proteins from COS-7 cells. None of the mutations in blade 1 (Fig. 2A) prevented formation of βγ dimers. Although mutation 1A seems to form slightly less βγ in the example shown, this was within the range of variation given by the value in Table I. βγ subunits with mutations 1B, 1C, and 1D were each impaired in their ability to coimmunoprecipitate α subunits. These observations are consistent with the crystal structure of the αβγ heterotrimer, which shows that the N-terminal α helix of α runs down the side of blade 1. We have previously shown that mutations of residues 88 and 89 to alanine completely prevent formation of αγβ in solution (13). None of the other mutations in any of the blades had a significant effect on the ability of the βγ dimers to coimmunoprecipitate α subunits (see Table I). Note that the value in Table I is the relative amount of α that could be coimmunoprecipitated by the mutated βγ present in the sample. If βγ formation was slightly diminished, for example as in mutant 2C, the absolute amount of α that was coimmunoprecipitated was smaller, but the ratio of α to Hβγ was no different from wild type. In all cases, the amount of immunoprecipitated α that could be accounted for by the endogenous βγ was subtracted. The crystal structure indicates that the residues altered do not perturb the N-terminal helix of α directly. We assume that substitution of SR for HA into the outer β strand of blade 1 distorts it sufficiently to diminish the affinity of α for βγ. The ability of the mutant Hβγ or βγ to support ADP-ribosylation of the coimmunoprecipitated α was tested as described under “Experimental Procedures.” The incorporation of ADP-ribose was exactly proportional to the amount of α precipitated in every case (data not shown). Therefore, we conclude that the interaction of α with βγ was functionally intact.

The ability of each of the mutants to activate phospholipase Cβ2 was assayed in COS-7 cells transfected with the mutant β subunit, γ2, and PLCβ2 with and without αi2. Fig. 3 shows a number of experiments that were done to validate the assays in COS-7 cells. Expression of βγ or Hβγ in COS-7 cells had no effect on the endogenous PLC activity (lanes 1–3). Expression of PLCβ2 resulted in increased basal concentration of inositol phosphates (lane 4). Coexpression of αi2 with PLCβ2 lowered the activity, presumably by inhibiting stimulation of the enzyme by endogenous βγ (lane 5). Neither β alone nor γ alone significantly increased the activity of transfected PLCβ2, but transfection of the two together caused an approximately 3-fold stimulation of PLCβ2-dependent accumulation of inositol phosphates (lanes 6, 8, and 10). There was only a slight difference in the ability of histidine-tagged Hβ2 to activate PLCβ2 compared with wild type (lanes 10 and 12). Coexpressed αi2 entirely blocked the stimulatory effect of transfected βγ on PLCβ2 activity. Transfection of PLCβ2 led to the appearance of an approximately 145-kDa protein recognized by anti-PLCβ2 antibodies (Fig. 3B). The amount of immunoreactive PLCβ2 did not change when G protein subunits were cotransfected. In addition, as will be described below, some mutant proteins were synthesized in baculovirus-infected Sf9 cells, purified, and reconstituted with purified PLCβ2. There was good agreement...
were transfected that interacted poorly with a variation in the PLC the other components that were also present. The intra-assay cotransfected was quite constant in a given assay, regardless of results to those obtained with purified proteins (13). The amount of immunoprecipitated 15% of the mean. This was true even when mutant 1C was blocked by a mutant 1C that assays in COS-7 cells give similar between these assays. We had previously shown with another set of mutants in Hβ1 that assays in COS-7 cells give similar results to those obtained with purified proteins (13).

As illustrated in Fig. 3A, the PLCβ2 activity when αβ2 was cotransfected was quite constant in a given assay, regardless of the other components that were also present. The intra-assay variation in the PLCβ2 activity with αβ2 was ordinarily ±10–15% of the mean. This was true even when mutant β subunits were transfected that interacted poorly with α in the immunoprecipitation assay (for example, mutants 1B, 1C, and 1D). We had previously shown that the activation of PLCβ2 and PLCβ3 by mutant 1C was blocked by αβ2 with the same dose-response curve as was activation by βγ (13). We concluded that differences in affinity that are sufficient to affect an immunoprecipitation assay are not sufficient to prevent interaction of α with β when the subunits are confined to the plasma membrane. Not only may the local concentration be higher, but the subunits are probably oriented with respect to one another in a way that is likely to enhance their ability to interact.

Each of the mutant β subunits was assayed for its ability to activate PLCβ2 with and without cotransfected αβ2. The results for all 26 mutations are given in Table I. Fig. 4 shows a DNA dose-response curve for wild-type Hβ1 and five of the more informative mutants. The βγ-dependent stimulation of trans-

### Table I

| Blade | WT and mutated sequence | βγ formation | αβγ formation | PLCβ2 activation |
|-------|------------------------|--------------|--------------|-----------------|
| 1     | 84                     | 94           |              |                 |
| WT    | SYTTNVHAIP             |              |              |                 |
| 1A    | --SR------             | 91 ± 4 (11)  | 106 ± 7 (10) | 98 ± 4 (4)      |
| 1B    | --SRAA----             | 114 ± 5 (6)  | 0 (4)        | 71 ± 9 (3)      |
| 1C    | -----AA-------         | 109 ± 6 (4)  | 0 (3)        | 79 ± 13 (2)     |
| 1D    | ---------SR----------  | 93 ± 5 (5)   | 14 ± 4 (4)   | 111 ± 9 (2)     |
| 2     | 126                    | 140          |              |                 |
| WT    | LKTREGNVRVREL         |              |              |                 |
| 2A    | -----TS--------        | 96 ± 5 (11)  | 111 ± 5 (8)  | 107 ± 10 (5)    |
| 2B    | AAAAAATS----          | 108 ± 7 (9)  | 84 ± 6 (6)   | 73 ± 4 (6)      |
| 2C    | -----TSAAAAAA-------- | 108 ± 5 (8)  | 108 ± 5 (8)  | 51 ± 2 (12)     |
| 2D    | -------YR------------  | 87 ± 6 (3)   | 111 ± 10 (3) | 97 ± 12 (4)     |
| 2E    | ---------G-----------  | 91 ± 7 (2)   | 95 ± 5 (2)   | 86 ± 16 (2)     |
| 3     | 171                    | 181          |              |                 |
| WT    | IETGQTTTFT             |              |              |                 |
| 3A    | ----SR------           | 71 ± 8 (10)  | 104 ± 16 (6) | 95 ± 10 (5)     |
| 3B    | AAAAAAAR              | 0 (4)        | 0 (3)        | ND              |
| 3C    | ------SRAAA           | 0 (4)        | 0 (3)        | ND              |
| 4     | 213                    | 223          |              |                 |
| WT    | VREGMCQFT              |              |              |                 |
| 4A    | -----S--------         | 94 ± 8 (6)   | 101 ± 4 (5)  | 85 ± 3 (3)      |
| 4B    | AAAAAA----             | 0 (4)        | 0 (3)        | ND              |
| 4C    | ------SAAAA            | 0 (4)        | 0 (3)        | ND              |
| 5     | 255                    | 264          |              |                 |
| WT    | LRADQELMTY             |              |              |                 |
| 5A    | ----SR------           | 89 ± 9 (7)   | 104 ± 8 (6)  | 51 ± 2 (6)      |
| 5B    | AA-ASR-----            | 0 (4)        | 0 (3)        | ND              |
| 5C    | ------SRAAA           | 0 (4)        | 0 (3)        | ND              |
| 6     | 299                    | 309          |              |                 |
| WT    | ALKADRAYLA             |              |              |                 |
| 6A    | -----S--------         | 103 ± 11 (13)| 95 ± 6 (9)   | 117 ± 7 (11)    |
| 6B    | --AA-S----             | 51 ± 3 (5)   | 82 ± 9 (3)   | 21 ± 5 (6)      |
| 6C    | ----S--AAA            | 79 ± 9 (13)  | 82 ± 6 (7)   | 14 ± 2 (11)     |
| 6D    | --ACS-------           | 75 ± 10 (5)  | 87 ± 3 (4)   | 57 ± 6 (8)      |
| 6E    | -----S-TC--           | 78 ± 12 (9)  | 113 ± 11 (6) | 101 ± 14 (10)   |
| 6F    | --ACS-TC--            | 81 ± 15 (9)  | 111 ± 6 (6)  | 42 ± 5 (10)     |
| 7     | 42                     | 52           |              |                 |
| WT    | RIQMTTRTFLR           |              |              |                 |
| 7A    | ---------SR--         | 96 ± 13 (12) | 88 ± 15 (7)  | 38 ± 4 (5)      |
| 7B    | ------T-CSR---------- | 94 ± 8 (11)  | 87 ± 8 (8)   | 16 ± 5 (10)     |

a The top sequence in each set is the wild type (WT). The mutated residues are indicated. Dashes mean the same residue as WT. The mutated β1 subunits are named according to the blade containing the mutated residues with a letter designating the specific mutant.

b The formation of βγ dimers was calculated as a % of WT, either histidine-tagged or not as appropriate. All mutations were in the Hβ1 background, except 2E and 3A. The comparison was based on densitometry of radioautographs of 35S-labeled immunoprecipitates (see Fig. 2 and experimental procedures).

c The formation of αβγ complexes was calculated from experiments like that shown in Fig. 2. The amount of immunoprecipitated α was quantitated by densitometry of the 35S radioautogram for the number of experiments shown in parentheses. The amount of immunoprecipitated α accounted for by endogenous βHAγ2 was subtracted from the total value from α. The percentage was then calculated as follows:

\[
\frac{a/β\text{ mutant}}{a/β\text{ wild type}} \times 100
\]

d The relative phospholipase C activation was calculated as follows:

\[
\frac{(cpm \text{ PLC}β2 + mutant βγ2) - (cpm \text{ PLC}β2 + mutant βγ2 + αβ2)}{(cpm \text{ PLC}β2 + WTβγ2) - (cpm \text{ PLC}β2 + WTβγ2 + αβ2)} \times 100
\]

e Not determined.
PLC\(\beta_2\) Activation Sites on the Sides of the G\(\beta\gamma\) Propeller

Fig. 2. Immunoprecipitation of wild-type and mutant \(\beta_1\) subunits and \(\alpha\) subunits through HA\(\gamma\). The subunits were cotransfected, metabolically labeled, and immunoprecipitated as described under “Experimental Procedures.” The autoradiograms shown are representative of experiments that were repeated at least three times. The positions of wild-type \(\beta_1\) or mutant H\(\beta_1\) subunits, as well as the endogenous \(\beta\) and the immunoprecipitated \(\alpha\) subunits, are indicated on the figure. The mutations in each blade are defined in Table I. A, mutants in blade 1. B, mutants in blade 2. Note that mutant 2E is made in \(\beta_1\) that is not tagged with six histidine residues and therefore is not resolved from wild-type \(\beta_1\). C, mutants in blades 3, 4, and 5. Note that mutant 3A is made in \(\beta_1\), which is not tagged with six histidine residues. D, mutants in blades 6 and 7.

Fig. 3. Activation of PLC\(\beta_2\) in COS-7 cells. A, PLC\(\beta_2\) activity was measured as described under “Experimental Procedures.” One representative experiment analyzed in duplicate is shown. The entire experiment was repeated three times. Portions of it (lanes 4, 5, 12, and 13) were included as controls in all experiments reported in Table I. The error bars indicate the range of duplicate determinations. Where no error is shown, it was too small to graph. B, coexpression of G protein subunits does not change the level of expression of cotransfected PLC\(\beta_2\). PLC\(\beta_2\) was transfected into COS-7 cells in 60-mm dishes, together with the indicated subunits. The figure shows Western blots of cell lysates. Expression of endogenous PLC\(\beta_2\) could not be detected in these lysates.

Table \(\alpha_{12}\). Although PLC\(\beta_2\) activation is saturated at 40–100 ng DNA/100,000 cells, the amount of \(\beta\gamma\) expressed continues to increase. The observation confirms that the level of activity measured is not limited by the amount of \(\beta\gamma\), even for a mutant such as H\(\beta_1\)2C, whose yield of \(\beta\gamma\) dimers is somewhat lower than the others. The first two lanes in Fig. 4B are controls. When no HA\(\gamma_2\) is transfected, no proteins are immunoprecipitated. When no \(\beta\) is transfected, anti-HA\(\gamma_2\) antibody brings down only endogenous \(\beta\) and the \(\alpha\) associated with it. The antibody was always in excess in these experiments so that the apparent plateau in the protein is not due to an inadequate amount of antibody. Fig. 4C shows that cotransfection of G protein subunits does not change the amount of PLC\(\beta_2\) detected by Western blot.

Fig. 5 shows the activation of purified PLC\(\beta_2\) by purified mutant \(\beta\gamma\) subunits. The rank order of activation by the five mutants tested is the same as that found in the COS-7 cell assay. H\(\beta_6\)6A was the most active, followed by H\(\beta_6\)2B. Both H\(\beta_6\)6C and H\(\beta_6\)2C were inactive in both assays, whereas H\(\beta_1\)7A had an intermediate activity. The purified proteins...
were also tested for their ability to inhibit ACI or activate ACII synergistically with \( \alpha \) (Table II). All three of the mutants that decrease or prevent activation of PLC\( \beta_2 \) in vitro or in COS-7 cells were active in the adenylyl cyclase assays.

**DISCUSSION**

Mutations in three blades, 2, 6, and 7, greatly reduce the ability of \( \beta \) to activate PLC\( \beta_2 \). Replacement of most of the blade 2 “d” strand with alanine residues reduces the ability of the mutant protein to activate PLC\( \beta_2 \) to 15% of wild type. In contrast, an equal number of alanines introduced into the loop between the “c” and “d” strands interferes little, if at all, with the ability of the mutant \( \beta \) subunit to activate PLC\( \beta_2 \). Smaller mutations in the “d” strand do not affect PLC\( \beta_2 \) activation.

The valine to glycine mutation at residue 135 in blade 2 is equivalent to the V173G mutation in the *Saccharomyces cerevisiae* \( \beta \) subunit, Ste4 (25). The mutation confers a dominant negative phenotype on Ste4, suggesting that the mutant mol-

**FIG. 5.** Activation of PLC\( \beta_2 \) by purified mutant \( \beta \) subunits in vitro. The indicated amount of each \( \beta \) mutant was reconstituted with 0.1 nM PLC\( \beta_2 \), and the synthesis of IP\( _3 \) was measured over 8 min at 30 °C as described under “Experimental Procedures.” The data shown are the average of duplicate determinations from a single representative experiment. 2B, 2C, 2D, 6A, 6B, 6C, 7A, 7B; wild-type \( \beta_1 \).

were also tested for their ability to inhibit ACI or activate ACII synergistically with \( \alpha \) (Table II). All three of the mutants that decrease or prevent activation of PLC\( \beta_2 \) in vitro or in COS-7 cells were active in the adenylyl cyclase assays.

Mutations in three blades, 2, 6, and 7, greatly reduce the ability of \( \beta \) to activate PLC\( \beta_2 \). Replacement of most of the blade 2 “d” strand with alanine residues reduces the ability of the mutant protein to activate PLC\( \beta_2 \) to 15% of wild type. In contrast, an equal number of alanines introduced into the loop between the “c” and “d” strands interferes little, if at all, with the ability of the mutant \( \beta \) subunit to activate PLC\( \beta_2 \). Smaller mutations in the “d” strand do not affect PLC\( \beta_2 \) activation.

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12-well plates were cotransfected with plasmids encoding wild-type or mutated \( \beta \) subunits (4–200 ng of DNA/well), PLC\( \beta_2 \) (200 ng of DNA/well), and either \( \alpha_5 \) (100 ng of DNA/well) or the same amount of vector DNA. Each well contained 10⁵ cells. PLC\( \beta_2 \) activity was measured as described under “Experimental Procedures.” B, DNA concentration-dependent expression of \( \beta \) subunits, \( \beta_1 \), \( \beta_2 \), and \( \beta_3 \) in 60-mm dishes (top), \( \beta \) subunits (bottom). COS-7 cells in 60-mm dishes were transfected with the same cDNA expression constructs described above. For ease of comparison with Fig. 4A, the amount of DNA used is given in ng/10⁵ cells. The 60-mm dishes actually contained 10⁶ cells and were transfected with a total of 5 µg of DNA/dish. Empty vector DNA was added as needed to compensate for the variable amount of \( \beta_1 \) DNA. The cells were metabolically labeled, and the heterotrimers were immunoprecipitated as described under “Experimental Procedures.” Positions of expressed wild-type or mutated \( \beta_1 \) subunits, endogenous \( \beta \), HA\( \gamma_2 \), and expressed \( \alpha_5 \) are indicated by arrows. The first two lanes on the left are controls. The first lane shows that without transfected HA\( \gamma_2 \), nothing is precipitated by anti-HA antibody. The second lane shows that with transfected \( \beta_1 \), HA\( \gamma_2 \) assembles with endogenous \( \beta \) and forms heterotrimers with transfected \( \alpha_5 \). C, expression of \( \beta \) does not affect expression of transfected PLC\( \beta_2 \). Transfection and Western blot analysis were carried out as described under “Experimental Procedures.” The position of PLC\( \beta_2 \) immunoreactivity is shown by the arrow. The antibody did not detect endogenous PLC. The experiment was done exactly as described in B and shows the results with mutants H\( \beta_1 \), H\( \beta_2 \), H\( \beta_3 \), H\( \beta_5 \), H\( \beta_6 \), H\( \beta_7 \), and H\( \beta_8 \).
other neighboring mutations severely impaired the ability of blades that make extensive contacts with the activity was measured as described under “Experimental Procedures.” The activities of no βγ for type I and type II adenylyl cyclase are 1.22 nmol of cAMP/min/mg and 7.7 nmol of cAMP/min/mg, respectively.

| Type IAIC | Type IIAIC |
|-----------|------------|
| No βγ     | 100%       |
| 2β        | 35 ± 2     |
| 7A        | 52 ± 6     |
| Wild type | 36 ± 1     |

ecule binds to, but cannot activate, the normal target of Ste4. That direct target is still not known, but if it is a PLC, it is, not unexpectedly, different from mammalian PLCβ in its contact sites with the G protein β subunit. Two mutations in blade 6 severely impair the ability of the mutant molecules to activate PLCβ2 (Hβ5B, Hβ6C) (Figs. 4A and 5, Table I), whereas two other mutations (Hβ6D, Hβ6F) partially impair the ability to activate PLCβ2 (Table I). The locations of the mutations suggest that leucine 300, valine 307, and leucine 308 may be important residues for activation of PLCβ2. Two mutations that affect the “d” β strand of blade 7 also inhibit PLCβ2 activation (Table I, Figs. 4A and 5).

One mutation in blade 5 (Hβ5A) showed a partial loss of activation of PLCβ2. This result is difficult to interpret because other neighboring mutations severely impaired the ability of βγ to fold. The partial inhibition may be due to mutation of a direct contact for PLCβ2 or this blade may be particularly sensitive to any mutation. Since we have only one testable mutant in blade 5, we reserve judgment of its significance. Blades 3, 4, and 5 are blades that make extensive contacts with the γ subunit and may, for that reason, be more sensitive to mutation.

We have previously shown that mutation of residues on the top surface of βγ (the surface that contacts the α subunit) affects the ability of the βγ subunit to activate PLCβ2, PLCβ3, and type II adenylyl cyclase. We now show for PLCβ2 that several regions on the side surface of the torus are also important, both when the mutant subunits are expressed in COS-7 cells and when the pure proteins are reconstituted in vitro.

Three mutations (2C, 6C, 7A) that reduce or prevent activation of PLCβ2 both in vitro and in COS-7 cells do not block the ability of βγ to activate ACII or inhibit ACI. These results are useful because they confirm in vitro that the decreased activity of 2C, 6C, and 7A is not due to gross misfolding of the proteins. Two of these mutants, 2C and 7A, do have modest blunting of the inhibition of ACI and the activation of ACII. However, further analysis will be necessary to map the contact sites of β for adenylyl cyclase and for other effectors.

The G protein β subunit belongs to a large family of proteins containing a highly related repeating motif, the WD repeat. We have identified over 140 unique proteins containing this repeat and have analyzed over 900 unique repeats within these proteins (4). The conserved core of the repeat extends from a highly conserved dipeptide GH to the signature WD residues. In βγ, this sequence comprises the inner three strands of each propeller blade. The outer “d” strand is contained in a region that is highly variable, not only in length but in composition among all the members of the family. This degree of variability in itself suggests that the sequences in the outer strand and the two loops connecting to it may contain regions that are important for the specific function for each of the WD-repeat proteins. We know from two crystal structures, those of the αβγ heterotrimer and the complex of βγ with the regulatory protein, phosducin, that both of these proteins make contacts not only with the “top” surface of the βγ subunit, but they also make important contacts along the sides of the torus. We propose that each effector binds not only to the top surface but also makes contacts along the sides of the propeller. PLCβ may bind like a clamp over βγ extending part of its structure down the sides. These extensions need not be large. For example, in the α subunit, a single α helix makes contact with the side of β.

Since there are more proteins known to interact with βγ than there are blades, it is clear that there will not be a simple 1:1 relationship between blades and effectors. Some effectors may make very circumscribed contact with the side of the propeller (similar to the α subunit, whose N-terminal α helix contacts only a part of the outer “d” β strand of blade 1), whereas others (like PLCβ2) may have a larger footprint. It is possible that an overlapping subset of contacts along the sides of the propeller will define the specificity of interaction of βγ with any effector. By extension, from our results with PLCβ2 and from the crystal structures of the complexes of α or phosducin with βγ, we propose that the regions equivalent to the outer strands of many WD proteins are important sites for contact with their partners. It would, therefore, be reasonable to target site-directed mutagenesis to those regions to define the requirements of other WD proteins for interaction with their targets.

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