The G protein Gβγ subunit complex stimulates effectors by direct interactions utilizing extensive Gβ regions over the surface of its propeller structure that faces the Gα subunit. Our previous experiments have shown the resolved functions of signal transfer and general binding for Gβ regions involved in stimulation of the effector phospholipase C-β2, PLC-β2, within the region Gβ-(86–135), which comprises three β strands arranged in a structurally contiguous fashion (Buck, E., Li, J., Chen, Y., Weng, G., Sacarlata, S., and Iyengar, R. (1999) Science 283, 1332–1335). This raises an important question as to why mutagenesis studies indicate that an extensive set of sites all over the Gβ propeller structure and outside the 86–135 region are involved in Gβ regulation of PLC-β2. Using peptides to define functions of these Gβ regions, we find that Gβ signaling to PLC-β2 relies on a collection of modular signal transfer and general binding units, each with lower apparent affinity relative to Gβγ-PLC interactions. Gβ-(42–54) functions as a signal transfer region, Gβ-(228–249) and Gβ-(321–340) function in general binding, and Gβ-(64–84) and Gβ-(300–313) seem to play a structural role rather than a direct contact with the effector. A substitution within the Gβ-(42–54) signal transfer region that increases the $K_{act}$ of this peptide for PLC-β2 is accompanied by an increase in the observed maximal extent of signal transfer. We conclude that the lower $K_{act}$ for individual signal transfer regions may result in a decrease in the maximal effect of signal transfer. The spatial resolution of the signal transfer and general binding regions over a wide surface of Gβ allow geometrical constraints to achieve specificity even with relatively low affinity interactions.

Many receptors transmit their signals through heterotrimeric guanine nucleotide-binding proteins, G proteins. Both the α and βγ subunits of the G protein are capable of regulating effectors. Effectors for Gβγ include classical second messenger-producing enzymes such as adenyl cyclase and phospholipase C-β2, PLC-β2.7 Signal transfer from the G protein subunits to these effectors occurs through direct protein-protein interactions (1). A noteworthy feature of Gβγ in regulation of effectors is that it occurs with a much lower affinity (2) compared with the Gα subunit (3). Nevertheless, the interaction between Gβγ and effectors is quite specific. For instance, it stimulates PLC-β2, but not PLC-β4 (4). These observations lead to a central question in G protein signaling of how specificity is achieved even when interactions are of low affinity. This study addresses this question for Gβγ regulation of PLC-β2.

Molecular biological techniques, such as yeast two-hybrid screening and site-directed mutagenesis have been extensively used to map the regions of Gβ involved in interactions with effectors including PLC-β2 (5–9). The sites mapped by these approaches cover a large surface of Gβ on the side that interacts with Gα. These interaction sites are spread through all seven blades in the structure of the Gβ subunit.

As identified by site-directed mutagenesis, regions of Gβ that mediate regulation of PLC-β2 could serve three distinct functions. Some regions may make direct physical contact with the effector protein and transfer signals to it. Other regions, which form direct contact sites, might contribute to overall binding affinity but not be involved directly in signal transfer. Still other regions may be important from a structural standpoint for Gβ while not making any direct physical contacts with this effector protein. Such a region could be responsible for the global folding of the protein or could serve to stabilize local secondary structure or side chain alignment important for mediating an interaction with PLC-β2, even though the region by itself makes no direct contact with this effector. Mutagenesis studies are not useful for ascribing such functions to various regions. However, peptides that encode these various regions may be used for this purpose. In previous work we demonstrated that the functions of two sites in a spatially contiguous region on Gβ (Gβ-(86–135)), important for regulation of PLC-β2, could be resolved and separately identified as signal transfer and general binding regions (10). This finding leads to the question of why the mechanism of Gβγ stimulation of PLC-β2 relies extensively on sites outside the Gβ-(86–105) region to coordinate stimulation of PLC-β2. Here we use the peptide approach to define the functions of the various regions of Gβ outside Gβ-(86–135) that mutagenesis studies indicate are involved in PLC-β2 function. We find that Gβ utilizes a modular design of multiple signal transfer and general binding domains to allow for stimulation of PLC-β2. These findings suggest a mechanism that ensures that PLC-β2 regulation is both specific and reversible.

### Experimental Procedures

**Matteria**—Amino acids and reagents for peptide synthesis were from Bachem. lHphosphatidylinositol-4,5-bisphosphate (lHIP IP$_2$) was from PerkinElmer Life Sciences. Lipids were obtained from Sigma. All other reagents used were the best analytical grade that was commercially available.

**Peptide Synthesis**—Peptides Gβ-(64–84), Gβ-(300–313), Gβ-(321–340), Gβ-(312–340)-F355A, Gβ-(228–249), and Gβ-(228–249)-D226R,
D246S were synthesized on an Applied Biosystems peptide synthesizer (Model 431A). Peptides were lyophilized and stored at −20 °C. Peptides Gβ(42–54), Gβ(42–54)R48A, Gβ(42–54)R49A, and Gβ(46–54) were synthesized at the Tufts University Core Facility. These peptides were all purified by HPLC. When needed, peptides were dissolved in HED buffer (10 mM Hepes (pH 7.0), 1 mM EDTA (pH 8.0), 1 mM dithiothreitol) at a stock concentration of 3 mM. All peptides were prepared fresh at the time of the experiment. The pH of each peptide in solution was tested to ensure that the pH of the peptide stocks was pH 7.0. The identity and purity of peptides were verified by mass spectrometry and HPLC. We ensure that the pH of the peptide stocks was pH 7.0. The identity and purity of peptides were verified by mass spectrometry and HPLC. We ensure accurate identification of the peptides, their effect was tested within 1 week of mass spectrometry analysis. For controls we used substituted peptides. Substitutions were made at the same residues that had been shown by mutagenesis to affect Gβγ regulation of PLC-β2. This allowed for direct comparison of our experiments with the mutagenesis studies.

**Gβγ Expression**—Recombinant Gβ1γ2-His6 was expressed in High 5 insect cells by co-infection of recombinant baculovirus. Three to 4 days postinfection, the cells were lysed by decompression in a Parr bomb after equilibration at 600 p.s.i. The lysate was ultracentrifuged for 1 hour at 35,000 rpm at 4 °C. The supernatant was decanted and stored in aliquots at −70 °C.

**Measurement of PLC-β2 Activity**—10 μg of protein from the cytosolic fraction of H5 cells expressing PLC-β2 was used/100 μl of reaction volume. Phospholipid substrate is a mixture of [3H]PIP2 and unlabeled phospholipids. Unlabeled phospholipids (Sigma-P-6023) were crude lipids from bovine brain. The total diphosphoinositide and triphosphoinositol content was 20–40%. The remainder was a mixture of phosphatidylinositol and phosphatidylserine. Phospholipids were stored in chloroform stocks. When needed an aliquot of phospholipids was dried under nitrogen and then resuspended and sonicated in 10 mM Hepes (pH 7.0) to form lipid micelles. A total of 0.01 μCi of [3H]PIP2, corresponding to 3000–7000 cpm, and 5 μg of unlabeled mixed phospholipids was used per reaction. The PLC assay was done as previously described (11). Briefly, substrate, PLC-β2 (10 μg of protein cytosolic fraction), peptide, and Gβγ subunits (400 nM recombinant Gβ1γ2) were mixed on ice in 100 μl of buffer containing 10 mM NaCl, 2 mM EGTA, 1 mM EDTA, and 1 mM MgCl2. Reactions were started by the addition of 25 μl of 2.5 mM CaCl2 in 10 mM Hepes (pH 7.0) and incubated at 32 °C for 15 min. Reactions were stopped by the addition of 1 ml CMH (chloroform:methanol:HCl mixed 100:1:1 by volume) and 250 μl of 10 mM EDTA. After extraction, the aqueous phase (400 μl) was counted on a Beckman scintillation counter to indicate PLC activity. All dose response curves have been fitted with the curve for a sigmoidal dose response curve using the equation y = minimum + (maximum − minimum)/(1 + 10^(logEC50 − X)), where X is the logarithm of concentration of peptide and Y is the activity of PLC. The PLC activity is expressed as pmol inositol 1,4,5-trisphosphate/min/μg total protein. Over the course of this study two different batches of PLC-β2 were used. Basal activities between the batches varied by about 2-fold. All experiments were repeated a minimum of three times, and typical results for each set of experiments are shown.
Blade 7 in the structure of the Gβ propeller has been implicated by a number of studies to be important for the stabilization of the Gβ structure and for effector regulation (9, 12, 13). The crystal structure of Gβ shows that blade 7 is the only blade of Gβ that is not composed of a contiguous amino acid sequence (13). The outermost β strand (the d-strand) of this blade is formed from a sequence near the amino terminus of Gβ, Gβ-(47–54), while the remaining β strands (a, b, and c) are formed by the carboxyl terminus of Gβ (Fig. 1A). Here, amino-terminal residues in the d-strand make contact with carboxyl-terminal residues in the c-strand of this blade to form a so-called snap to close the propeller structure. In addition to this structural role some data suggest that residues within the d-strand of blade 7 may play a direct role in Gβ effector interactions (7–9). Yeast two-hybrid data show the first 100 amino acids of Gβ to bind to a number of effectors including PLC-β2 (8). Site-directed mutagenesis data also suggest this region to be involved in Gβγ regulation of PLC-β2 as select mutations within this region decrease the observed maximal effect for Gβγ stimulation of PLC-β2 activity by up to 85% (7). To ascertain if direct contact with PLC-β2 occurs with this region of Gβ and what function this contact might serve in regulation of PLC-β2, we tested a peptide that includes this region of Gβ, Gβ-(42–54), on PLC-β2 activity. This peptide, highlighted in magenta in Fig. 1A, has the ability to modestly stimulate PLC-β2 activity on its own, with a $K_{\text{act}}$ value of $\sim 7$ µM. This indicates that direct contact with PLC-β2 may occur within this region of Gβ and that this contact is capable of stimulating PLC activity (Fig. 1B). We also tested a truncated peptide from this region, Gβ-(46–54) (Fig. 1B). The Gβ-(46–54) region composes just the outermost d strand of blade 7. This peptide has a 3-fold higher $K_{\text{act}}$ value than Gβ-(42–54); however, the observed maximal effect of this truncated peptide for stimulating PLC-β2 is the same as that for Gβ-(42–54). These data indicate that the amino acids necessary for signal transfer within the Gβ-(42–54) region reside in the blade 7 d strand.

We substituted two of the residues within the Gβ-(42–54) region, Arg-48 and Arg-49, which were indicated by the mutagenesis studies to be important (7) to see how these specific amino acids are involved in stimulating PLC-β2 within this signal transfer region. Fig. 2A shows the positions of Arg-48 and Arg-49 within blade 7 of Gβ. Fig. 2B, upper panel, shows the effect of the Gβ-(42–54)-R48A-substituted peptide as compared with wild-type peptide sequence. This substituted peptide displayed very interesting behavior. The R48A substitution leads to a nearly 8-fold increase in $K_{\text{act}}$; however, this increase in $K_{\text{act}}$ is accompanied by a 2-fold increase in the maximal observed stimulation of PLC-β2. This observation suggests Arg-48 to be important for binding to PLC-β2 as well as for contributing to signal transfer capabilities. On the other hand, the Gβ-(42–54)-R49A-substituted peptide, while also displaying an increase in the $K_{\text{act}}$ value, does not show an increase in the maximal observed effect seen with the Gβ-(42–54)-R48A-substituted peptide (Fig. 2B, middle panel). Even though the Gβ-(42–54)-R48A-substituted peptide has a higher $K_{\text{act}}$, it is able to evoke an enhanced enzymatic response from PLC-β2. This observation may indicate for residue Arg-48 of Gβ the subtle balance between the potential ability to tightly bind to PLC-β2 and the ability to initiate the presumed allosteric change in PLC-β2 required for stimulation of enzyme activity.

**RESULTS**

FIG. 2. A, ribbon diagram showing regions of Gβ involved in the formation of blade 7 of the Gβ propeller structure. Amino-terminal residues 47–54 (dark blue) form the outermost d β strand of this blade. Carboxyl-terminal residues 311–340 (light blue) form the remaining a, b, and c β strands. Residues Arg-48 (magenta) and Arg-49 (red) are highlighted. The ribbon diagram was generated with MidasPlus. B,
FIG. 3. A, effects of 300 μM of Gβ(321–340) peptide on basal and Gβγ (400 nM recombinant Gβγγ) stimulated activity of PLC-β2. B, upper panel, effects of varying concentrations of the Gβ(321–340) peptide on Gβγ (400 nM recombinant Gβγγ) stimulated activity. Lower panel, effects of varying concentrations of the Gβ(321–340) and Gβ(321–340)-F335A-substituted peptide on Gβγ (400 nM recombinant Gβγγ) stimulation of PLC-β2.

Modular Design of Gβ

Substituting Ala for Arg at amino acid position 48 causes two major changes in the side chain properties of the amino acid at this position. First, the size of the side chain is a much smaller one. Second, there is a loss of positive charge and replacement with a hydrophobic side chain. We wondered which property of Arg-48 was important for its binding and signal transfer capabilities, and so we tested another substituted peptide from the Gβ(228–340) sequence, are also likely candidates to function in Gβγ-mediated stimulation of PLC-β2. Mutation of the conserved Asp residue in this blade, Asp-333, shows the most dramatic loss in the rate of Gβγ complex formation as compared with analogous Asp mutations in other blades (12). Trypsin digestion shows that Gβγ complexes, which do form, seem at least on a global scale to fold properly; however, local structural changes at the level of this individual blade might disrupt Gβγ signaling even if the gross structure of the Gβ propeller remains largely intact. When Trp-332, a Gγ contact point, is mutated to Ala, the mutant Gβ is much less effective at modulating a number of effectors including PLC-β2 (6). A chimeric Gβ constructed by substituting the last 20 amino acids of Gβγ with those of Dictyostelium Gβ, an isoform of Gβ that poorly regulates the effector PLC-β2, resulted in a Gβ that had lost nearly all ability to regulate PLC-β2 activity but was still fairly effective at Gβ modulation of some other pathways, such as the MAPK pathway (9). Truncation of six amino acids from the carboxyl terminus of STE4, a yeast homolog of Gβ, rendered Gβ incapable of interaction with downstream effectors, although it could still associate with STE18, the yeast homologue of Gγ (14). These data suggest that while the carboxyl-terminal 20 amino acids of Gβ, β strands b and c of blade 7, are important from a gross structural standpoint, they may also play roles in direct protein-protein interactions. To test whether these last 20 residues of Gβ form direct interactions with PLC we tested a peptide derived from these 20 residues on basal and Gβγ-stimulated PLC activity. This peptide is very effective at blocking nearly all Gβγ stimulation, whereas basal activity is largely unaffected (Fig. 3A). This effect, at varying concentrations of the Gβ(321–340) peptide, is shown in Fig. 3B, upper panel. The results show that Gβγ(321–340) has roles beyond participating in the folding of the Gβ propeller structure or in maintaining a local conformation important for forming interactions with a
Gβ effector protein. Specifically, our data indicate that this region is part of a general binding domain and not a signal transfer region because the 20-mer peptide was incapable of regulating PLC-β2 on its own. The last six amino acids within this carboxy-terminal region of Gβ, Gβ 335–340, appear to play dual roles. By forming the c β strand within blade 7 they seem to form a snap through interactions with the d β strand within this blade to structurally close the Gβ propeller. Also, this region seems to make direct binding contact with PLC-β2. We tested a peptide from the Gβ-(321–340) region that was substituted at position 335, F335A. Phe-335 is a residue implicated by the homologous scanning mutagenesis experiments to be important in the ability of Gβγ to stimulate PLC-β2 (9) on Gβγ stimulation of PLC-β2. This substitution greatly reduced the ability of the peptide to inhibit Gβγ stimulation as compared with the wild type peptide. This observation highlights Phe-335 of Gβ as an important binding contact within this general binding domain (Fig. 3B, lower panel).

The region Gβ-228–249, β strands a and b of blade 5, has been shown to be important for the folding of Gβ into its propeller structure and the formation of the Gβγ dimer. When the conserved Asp residue in this blade is mutated, dimer formation is reduced (12). Mutation of residues within the region Gβ-228–249 leads to a decrease in Gβγ stimulation of PLC-β2 (7). To determine whether this reduction in effector regulation is the result of a structural change, either affecting the global folding of the propeller or altering the conformation of another PLC-β2 contact site, or if Gβ-228–249 is a direct contact region for PLC-β2, we tested the effect of the Gβ-(228–249) peptide on basal and Gβγ stimulation of PLC-β2. Gβ-228–249 had no measurable effect on PLC-β2 basal activity, but it could inhibit close to 100% the stimulation by Gβγ with an apparent Kd of about 200 μM (Fig. 4, upper panel). A Gβ-228–249 peptide that was substituted at two positions, D228R and D246S, was ineffective at modulating Gβγ stimulation of PLC-β2 (Fig. 4, lower panel). This is consistent with the mutagenesis studies in which these same substitutions led to a nearly complete loss of the effect of Gβγ on PLC-β2 (5). This region, thus, seems to be part of a general binding domain that makes direct physical contact with PLC-β2 but plays no role in signal transfer. Asp residues at positions 228 and 246 are important in this binding interaction.

Mutagenesis studies have predicted two additional regions of Gβ to be potentially important in forming protein–protein interactions with PLC-β2. These are the regions Gβ-(300–313) and Gβ-64–84 (6–8). When chimeras are made with analogous Dictostelium sequence both showed a reduction in Gβγ stimulation of PLC-β2 (9). For one of these regions, Gβ-64–84, mutation of amino acids at positions Ser-72, Asp-76, and Trp-82 renders Gβγ with a loss of function for modulating PLC activity (8). However, the Gβ-64–84 region appears to be important from a structural standpoint. Amino acids Ser-72, Asp-76, and Trp-82 form part of a hydrogen bonding network that is conserved in all WD repeats, and so mutations at these positions might disrupt Gβγ regulation of PLC-β2 by causing a structural change that prevents proper folding of this blade (13). Also, residues within the Gβ-64–84 region make intramolecular contacts with the Gβ 86–105 region, a region of Gβ that we have previously found to be a signal transfer region for PLC stimulation (10). Mutations in the Gβ-64–84 region could alter Gβγ regulation of PLC indirectly by changing the structure of a signal transfer region on Gβ. We tested a peptide encoding the Gβ-64–84 region and found it to have no measurable effect on either basal or Gβγ stimulation of PLC (Fig. 5A). This experiment suggests that this region might not be important for making a direct contact with PLC and supports its structural role by either maintaining local secondary structure or the structure of another signal transfer region.

Mutagenesis experiments highlight specific amino acids in the Gβ-(300–313) region as critical in Gβγ regulation of PLC-β2 (7). For example the L300A mutation can affect regulation of PLC-β2. However, Leu-300 is also a direct Gγ subunit contact. The L300A mutation could affect the structure of the Gβγ dimer. We tested a peptide from the Gβ-(300–313) region and found it to have no observed effect on either basal or Gβγ stimulation of PLC (Fig. 5B). Thus, it seems that this region also does not make contact with PLC-β2. The Gβ-(300–313) region may be important in Gβγ stimulation of PLC-β2 because it functions to stabilize a certain structure whether it be through interactions with Gγ or through local interactions within the blade, which is necessary for effective signal transfer to PLC-β2.

**DISCUSSION**

The experiments in this study show that Gβγ interactions with PLC have evolved in modular domains so that multiple sites of interaction can regulate binding affinity and signal transfer. The roles of the various sites are summarized in Fig. 6. The multiplicity of both signal transfer and general binding domains raises questions about the advantage of such a configuration.

It is rather surprising that we have identified two signal transfer regions of Gβ. Each appears sufficient to transfer signals because each can independently activate PLC-β2. Why have two such regions? One explanation is that multiplicity of signal transfer regions increases the probability of signal transfer even when interaction affinities are low. This same reasoning can explain why signal transfer regions are not contiguous with general binding domains. It is likely that these functions have been distributed to different effector contact sites because the affinity required to enable these two proteins to interact fruitfully with specificity might not allow the flexibility necessary to induce local allosteric changes in the effector protein required for change in activity. Protein interaction regions must compromise between tight specific binding and efficacious
signal transfer. This idea is supported by the analysis of Arg-48 substitutions within the Gβ(42–54) peptide. Here it seems that while the R48A substitution renders the Gβ(42–54) peptide with a higher $K_{\text{act}}$ for stimulating PLC-β2, it increases the observed maximal extent of this stimulation. Specifically, eliminating the positive charge from the amino acid side chain at this position might prevent this region of Gβ from interacting tightly with PLC. This weaker binding configuration might allow more effective signal transfer from this region of Gβ to the effector and hence increase the extent of stimulation. Here nature, it seems, has sacrificed this enhanced extent of stimulation to gain a tighter, more specific interaction with this signal transfer region.

Multiple general binding domains may exist to perform a variety of functions. Some domains might function in adhesion and act as the "velcro" to hold the two protein partners together. Here, multiple dispersed and lower affinity interactions may be important to permit reversibility while utilizing spatial geometry to achieve specificity. Other general binding domains may function in orientation, perhaps to align a signal transfer region into the proper configuration. The possibility that some may function in alignment, perhaps to align a signal transfer geometry to achieve specificity. Other general binding domains may be important to permit reversibility while utilizing spatial orientation interactions between signal transfer regions and the effector cannot be ruled out. Such a counterintuitive interaction would be useful in obtaining reversible interactions with the effector while still retaining fine-tuned specificity.

The modular design of Gβ for regulation of PLC may have evolved to allow for reversibility of interaction and effective signal transfer while maintaining specificity of interaction with PLC-β2. The geometrical constraint imposed by spatially separated sites can confer a unique level of specificity for low affinity interactions between Gβ and its effector. Such mechanism will easily promote the transient and reversible regulation of specific effector enzymes, achieving an essential requirement for signal flow within the cell.

Our studies highlight the flexibility with which nature has designed protein-protein interactions to achieve specific and transient signal flow within signaling pathways. Such information is important not only to define the fundamental principles of the molecular mechanisms by which these two signaling proteins communicate but also to serve as a foundation for the design of small molecules that may mimic or inhibit such signal flow.

Acknowledgements—We thank Drs. Gezhi Weng and George Brown for useful advice, Dr. Jinrong Li for help with peptide synthesis, and Dr. Maria Diverse and Dedrick Jordan for critical reading of the manuscript.

REFERENCES

1. Hamm, H. E. (1998) J. Biol. Chem. 273, 669–672
2. Ueda, N., Iniguez-Lluhi, J., Lee, E., Smreka, A. V., Robishaw, J. D., Gilman, A. G. (1994) J. Biol. Chem. 269, 4388–4395
3. Birnbaumer, L. (1992) Cell 71, 1069–1072
4. Jiang, H., Wu, D., Simon, M. I. (1994) J. Biol. Chem. 269, 7593–7596
5. Li, Y., Sternweis, P. M., Charnecki, S., Smith, T. F., Gilman, A. G., Neer, E. J., Kozasa, T. (1998) J. Biol. Chem. 273, 16285–16292
6. Ford, C. E., Skiba, N. P., Bae, H., Daaka, Y., Reuveny, E., Shechter, L. R., Rosal, R., Weng, G., Yang, C., Iyengar, R., Miller, R. J., Jan, L. Y., Lefkowitz, R. J., Hamm, H. E. (1998) Science 280, 1271–1274
7. Panchenko, M. P., Saxena, K., Li, Y., Charnecki, S., Sternweis, P. M., Smith, T. F., Gilman, A. G., Kozasa, T., Neer, E. J. (1999) J. Biol. Chem. 274, 28298–28304
8. Yan, K., Gautam, N. (1997) J. Biol. Chem. 272, 2056–2059
9. Zhang, S., Cose, O. A., Collins, R., Gutkind, J. S., Simonds, W. F. (1999) J. Biol. Chem. 274, 1332–1335
10. Buck, E. Li, J., Chen, Y., Weng, G., Scarlata, S., and Iyengar, R. (1999) Science 283, 1332–1335
11. DeVivo, M. (1994) Methods Enzymol. 233, 131–140
12. Garcia-Higuera, I., Gaitatzes, C., Smith, T. F., Neer, E. J. (1998) J. Biol. Chem. 273, 9041–9048
13. Sondek, J., Bohm, A., Lambricht, D. G., Hamm, H. E., Sigler, P. B. (1996) Nature 379, 369–374
14. Cona, R., Savvinin-Tejeda, A., Birnbaumer, L. (1995) FEBS Lett. 367, 122–126