Role of Dynamic Interactions in Effective Signal Transfer for Gβ Stimulation of Phospholipase C-β2*

Received for publication, June 5, 2002, and in revised form, September 23, 2002 Published, JBC Papers in Press, October 17, 2002, DOI 10.1074/jbc.M205553200

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Heterotrimeric G protein subunits regulate their effectors by protein-protein interactions. The regions involved in these direct interactions have either signal transfer or general binding functions (Buck, E., Li, J., Chen, Y., Weng, G., Scarlata, S., and Iyengar, R. (1999) Science 283, 1332–1335). Although key determinants of signal transfer regions for G protein subunits have been identified, the mechanisms of signal transfer are not fully understood. We have used a combinatorial peptide approach to analyze one Gβ region, Gβ66–105, involved in signal transfer to the effector phospholipase C (PLC)-β2 to gain a more mechanistic understanding of Gβ/PLC-β2 signaling. Binding and functional studies with the combinatorial peptides on interaction with and stimulation/inhibition of phospholipase Cβ2 indicate that binding affinity can be resolved from EC50 values. Although more potent, these peptides display a much lower extent of maximal stimulation. These peptides synergize with Gβγ or peptides encoding the second Gββ2–54 signal transfer region in maximally stimulating phospholipase C-β2. Other combinatorial peptides from the Gβ66–105 region that bind to PLC-β2 by themselves submaximally stimulate and extensively inhibit Gβγ stimulation of PLC-β2. The intrinsic stimulation function can be attributed to Arg-96 and Ser-97, the synergy function to Trp-99, and the binding affinity to Thr-87, Val-90, Pro-94, Arg-96, Ser-97, and Val-100. These results indicate that, even within signal transfer regions, residues involved in binding can be resolved from those involved in signal transfer and that signal transfer is likely to be achieved through dynamic rather than steady-state interactions.

Protein-protein interactions represent a major mode by which information is propagated along cell signaling pathways. The heterotrimeric guanine nucleotide binding protein (G protein) regulates the activity of a multitude of different effectors within the cell by direct protein-protein interactions (1). Both the Gs subunit and the Gβγ complex of the G protein can interact with effectors (2). Effectors for Gα include adenyl cyclases and phospholipase C-β (PLC-β) isofoms. Effectors for Gβγ subunits include G protein inwardly rectifying K+ channels, Ca2+ channels, and PLC-β isoforms (3).

We have found that regions of G protein subunits important for signal transfer can be resolved from regions important for binding alone (4, 5). For Gβγ stimulation of the effector PLC-β2, one Gβ region, Gβ66–105, functions directly in signal transmission. A peptide derived from this region can regulate PLC-β2 activity on its own in the absence of Gβγ subunits. Another region of Gβ, 115–135, is involved in binding but does not transmit signals, because it does not affect PLC-β2 activity by itself but inhibits Gβγ stimulation. Therefore, it is possible to separate general binding domains from signal transfer regions for a protein-protein interaction within intracellular signal flow. Further analysis has shown that Gβ relies on modular collections of these signal transfer and general binding units (6).

We had previously used substituted and truncated peptides to determine the amino acid characteristics of one of the Gβ signal transfer regions, the Gβ 86–105 region, that render it capable of PLC-β2 stimulation (4). Residues Lys-89 and Arg-96 are important for its potency (4, 7). The six-amino acid region Gβ 96–101 represents a core signal transfer region, and all contacts contributing to signal transfer for the Gβ 86–105 signal transfer region likely lie within the Gβ 96–101 six-amino acid region. However, the Gβ 96–101 region displays significantly lower EC50 for PLC-β2 stimulation, indicating that there may exist important binding contacts within Gβ 86–105 but outside of Gβ 96–101.

How the architecture of the Gβ 86–105 signal transfer region supports effector regulation is still unclear. Would the mechanism for signal transfer be driven largely by complementarity of the interactions between preformed surfaces, including those between charged residues, or by dynamic processes wherein residues on Gβ involved in signal transfer to PLC-β2 retain conformational flexibility to induce change in activity in PLC-β2 by transient interactions? To address these issues, herein, we have used a combinatorial peptide library approach to study both binding and functional regulation. Our data suggest that the mechanism of signal transfer to PLC-β2 is likely to rely on dynamic contacts with this effector that are distinct from those contacts involved in general binding affinity.

*This work was supported in part by National Institutes of Health Grants DK-38761 (to R.I.) and GM53132 (to S. S.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡A predoctoral trainee supported by Molecular Endocrinology Training Grant DK-07135.

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¶The abbreviations used are: G protein, guanine nucleotide binding protein; PLC, phospholipase C; PLD, phospholipase D; PBS, phosphate-buffered saline; DTT, dithiothreitol; BSA, bovine serum albumin; PIP2, phosphatidylinositol 4,5-bisphosphate; FRET, fluorescence resonance energy transfer; MBP, myelin basic protein; ELISA, enzyme-linked immunosorbent assay.
EXPERIMENTAL PROCEDURES

Materials—Library vectors and electrocompetent cells were a gift from Affymax, Palo Alto, CA. All oligonucleotides were from Genescript, New Brunswick, NJ. All peptides were purchased from the Tufts University core facility. The 96-well plates were from Dynatech. Sources of other reagents have been previously described (4, 6). For other experiments, all reagents used were of the highest quality available.

Expression of PLC-β2—Human PLC-β2 was expressed in H15 insect cells by infection with recombinant baculovirus. H15 cells were grown in a 10-liter suspension culture in 50 ml of sf900 media (Invitrogen) with shaking until the cell density reached 0.5 × 10^6 cells/ml. The cells were then infected with 20 ml of PLC-β2 recombinant baculovirus supernatant. About 3 days post infection the cells were collected. The cells were spun at 10,000 rpm for 10 min to produce a pellet. The media was decanted, and the cell pellet was resuspended into 20 ml phosphate-buffered saline (PBS). The cells were again spun at 1000 rpm for 10 min. The wash was decanted, and the pellet was resuspended into 20 ml of ice-cold PBS supplemented with protease inhibitors and DTT (10 μg/ml aprotinin, 1 μg/ml leupeptin, 200 μM phenylmethylsulfonyl fluoride, and 1 mM DTT). The cells were lysed by decompression in a Par bomb after equilibration at 600 p.s.i. for 30 min at 4 °C. The lysate was then ultracentrifuged at 35,000 rpm for 1 h at 4 °C. The supernatant was removed and distributed into 50-μl aliquots. These aliquots were frozen on dry ice and ethylene glycol and then stored at −70 °C.

Purification of PLC-β2—Human PLC-β2 was expressed in H15 insect cells, harvested, and lysed in 25 ml of lysis buffer, as described previously. Following lysis using the Par bomb, NaCl was added to a final concentration of 500 mM. The lysis mix was allowed to rotate at 4 °C for about 1 h. The supernatant was then centrifuged at 35,000 rpm for 45 min at 4 °C. The supernatant was reserved (25 ml) and added to 8.25 ml of 50% nickel-nitriolactritriacetic acid bead slurry (Qiagen) that had been equilibrated with lysis buffer. The slurry mix was allowed to rotate for about 2 h at 4 °C. The slurry mix was then poured into a Kontex column at 4 °C. The column was first washed with 70 ml (about 10 column volumes) of low salt wash buffer (10 mM sodium Hepes, pH 8.0, 0.1 mM EDTA, 0.1 mM EGTA, 800 mM NaCl, 0.5% C12E10, 15 mM imidazole) supplemented with protease inhibitors and DTT, as before. PLC-β2 was then eluted by washing with the column with six successive 4-ml elutions of elution buffer (10 mM sodium Hepes, pH 8.0, 0.1 mM EDTA, 0.1 mM EGTA, 50 mM NaCl, 125 mM imidazole) supplemented with protease inhibitors and DTT, as before. 25-μl aliquots of each of the six fractions were run on an SDS-polyacrylamide gel along with BSA standards to determine purity and the presence of PLC-β2. Usually, the second and third fractions that contained highly (>95%) purified PLC-β2 were aliquoted, frozen on dry ice and ethanol, and stored at −70 °C.

Peptide Synthesis—All peptides were purchased from the Tufts University Core Facility. Peptides were high performance liquid chromatography-purified, and their identity was verified by mass spectrometry. When needed, peptides were dissolved in HED buffer (10 mM Hepes (pH 7.0) 1 mM EDTA (pH 8.0), and 1 mM DTT).

Expression of Gβδ—Gβδ was purified from bovine brain as previously described (8) and was a kind gift of Dr. John Hildebrandt. Two Gβδ pools (pools 1 and 2) were isolated as described (9). About 10^5 peptide was used in the experiments shown in Fig. 3 the EC50 for PLC-β2 stimulation was 20 nM for PLC-β2 stimulation.

Subcloning into the MBP Vector—pELM3 was digested with Age1 (New England BioLabs) followed by SalI. The digest was run on a 1% agarose gel and electroeluted. Digests were then ligated to the MBP vector pMAL-c2X (New England BioLabs) to give pELM3-MBP. All the constructs were transformed into ARI814 electrocompetent cells, the pM14 vector was transformed into Peter Schatz at Affymax Corp., Palo Alto, CA. The use of these reagents to construct combinatorial libraries has been previously described (11). The degenerate library oligonucleotide (5′-GA GGT GGT ... NNN ... TAA CTA AGT AAA GC), where NNN denotes the nucleotides encoding the 20-amino acid degenerate library, was chemically synthesized, gel-purified, and 5′-phosphorylated. Here, N denotes a probability of 70% wild type base and 10% each of the other three bases. This distribution leads to an approximate 50% probability that the wild type residue at each of the 20-amino acid positions will be mutated to another amino acid. The two-linker oligonucleotides, ON-829 and ON-830, were synthesized and 5′-phosphorylated. All oligonucleotides were from Genescript, Syraucuse, NY.

Library Construction—A 2× 10^6 combinatorial peptide library based on the peptides on plasmids method was constructed and expressed as described in detail previously (11, 12). Briefly, the library oligonucleotide was annealed with the two linker oligonucleotides and ligated into the pJS142 peptide on plasmid vector. The ligation was electroporated into AR1814 electrocompetent cells, amplified, and frozen in aliquots at −70 °C. A portion of the library was removed prior to amplification to determine the number of individual library clones. The size of the Gβδ 86–105 combinatorial peptide library was greater than 10^10.

Panning—The methods used for the panning protocol have been described in detail elsewhere (11). For round 1 of panning 1% BSA in HEK buffer (35 mM Hepes, 0.1 mM EDTA, 50 mM KCl, 1 mM DTT) was added to the wells of a 96-well microtiter plate (Dynatech) and allowed to shake gently at 4 °C for 1 h. This allowed PLC-β2 to adhere to the wells of the plates. The wells with PLC-β2 were designated as (+) PLC wells. For (−) PLC control wells, 100 μl of HEK buffer was added. All the wells were then non-specific binding. The plates were washed with 200 μl of a blocking agent. For round 1 of panning 1% BSA in HEK buffer (35 mM Hepes, 0.1 mM EDTA, 50 mM KCl, 0.2 M α-lactate, 1 mM DTT (adjust pH to 7.5 with KOH)) was used to block the wells. For rounds two and three 1% nonfat dry milk in HEK buffer was used as the blocking agent. After 1 h of blocking agent the plate was allowed to shake gently for 1 h at 4 °C. The wells were then washed four times with HEK blocking agent. After washing, wash buffer, 200 μl of washing buffer containing 100 μl of blocking agent, and 200 μl of wash buffer was added to the wells, and the plate was allowed to shake gently at 4 °C for 1 h. For the panning procedure, see Ref. 11.

During one set of round 3 panning the native Gβδ 86–105 peptide was added at this step at a final concentration of 40 μM to compete with the library peptide clones for binding. The wells were then washed four times with 200 μl of HEK blocking agent. After the third wash the wells were blocked with 200 μl of blocking agent, and 200 μl of wash buffer was added to the wells, and the plate was allowed to shake gently at 4 °C for 1 h. The panning procedure, see Ref. 11.

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has previously described. Typica1 experiments are shown. Were repeated at least three times with qualitatively similar results.

The procedure for the MBP ELISA has been previously described. 0.25 µg of purified PLC-β2 was added to the wells of a microtiter 96-well plate (Dy1atech) and allowed to shake gently at 4 °C for 1 h. In no-PLC control wells 100 µl of HEK buffer was added. All wells were then blocked by adding 100 µl of 2% BSA in HEK with 1 mM DTT. Blocking was carried out shaking at 4 °C for 1 h. The MBP lysates were then thawed and diluted 1:1000 in HEK with 1 mM DTT. Following blocking, the wells of the plate were washed 4× with HEK, 1 mM DTT. 100 µl of the diluted MBP lysates was then added to the wells, and the plate was allowed to shake for 1 h at 4 °C. The plate was washed, and the plate was washed 4× with PBS/0.05% Tween. 100 µl of the diluted primary antibody was added to each well, and the plate was allowed to shake for 30 min at 4 °C. The plate was washed 4× with PBS/0.05% Tween. The secondary antibody, goat anti-rabbit conjugated to horseradish peroxidase (Roche Molecular Biochemicals), was diluted 1/1000 in PBS. The primary antibody, rabbit anti-MBP (New England BioLabs), was diluted 1/1000 in PBS. 100 µl of the diluted secondary antibody was added to each well, and the plate was allowed to shake for 30 min at 4 °C. The plate was washed 4× with PBS/0.05% Tween. The secondary antibody, goat anti-rabbit monovalently as a chimera with the maltose binding protein (MBP), thus enabling us to score the selected library peptides individually for binding in an MBP ELISA assay. We scored a peptide as a positive binder if it generated an ELISA signal that was greater than two standard deviations above the background signal. 25 peptides tested positively in the ELISA, and we sequenced the DNA that encoded each of these peptide clones.

The sequences of the ELISA positive clones for round 3 of panning in the absence of and in presence of the wild type peptide are shown in Tables I and II, respectively. An amino acid position was considered as “selected for” if, in the pool of selected peptides, the homology at that position was greater than 70%. The amino acid positions in each group that we considered to be part of the consensus sequence are highlighted. Wild type amino acids Lys-89 (86% homology), Val-90 (93% homology), Ser-97 (71% homology), and Val-100 (75% homology) are selected for in binding interactions with PLC.

For one group of round 3 panning we used the wild type Gβ86–105 peptide to compete away any peptide sequences that bound with affinities less than that of the wild type. Following panning, the DNA encoding captured peptides was subcloned into the pELM3 vector so that peptides could be expressed monovalently as a chimera with the maltose binding protein (MBP), thus enabling us to score the selected library peptides individually for binding in an MBP ELISA assay. We scored a peptide as a positive binder if it generated an ELISA signal that was greater than two standard deviations above the background signal. 25 peptides tested positively in the ELISA, and we sequenced the DNA that encoded each of these peptide clones.

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Combinatorial Analysis of G\textsubscript{86–105}

**TABLE II**

Sequences of ELISA-positive clones from round three of panning in the presence of 40 \(\mu\text{M} \) of the G\textsubscript{86–105} wild type peptide

| Peptides | wt |
|----------|----|
| TTTT | T |
| TTNT | H |
| TTHR | R |
| THRL | L |
| HRLG | G |
| RLVN | V |
| NNPQ | N |
| ALPV | P |
| SLRS | S |
| VYRS | Y |
| SCR | C |
| R2 |
| TTKR | K |
| KVRG | V |
| RLPQ | R |
| QPAF | A |
| FYTC | Y |
| CTFR | F |
| RFLA | L |
| T4 |
| TTTK | T |
| VKHQ | K |
| IAPR | I |
| LRFH | A |
| SPTK | P |
| FCTC | T |
| SCLA | C |
| T8 |
| TTVD | V |
| KDIQ | D |
| ANPL | N |
| LRFH | R |
| SPTK | T |
| FCTC | T |
| SCLA | C |
| T9 |
| TTTV | T |
| KVQT | V |
| TMPK | M |
| LKSC | L |
| AFAQ | A |
| CFLA | C |
| T11 |
| TTNL | T |
| DLHS | L |
| PRSP | R |
| RYSL | Y |
| FLEN | E |
| PLNL | P |
| T13 |
| STNK | S |
| LAMN | A |
| PRKL | R |
| SLRS | L |
| VINS | N |
| MLIC | L |
| T14 |
| AIRN | I |
| KVMH | K |
| ASLR | S |
| RRSP | R |
| MPFR | P |
| DRYM | D |
| T15 |
| RTIK | R |
| LPAI | P |
| PLKS | L |
| SSWN | S |
| RFYY | F |
| T17 |

Those amino acid positions for which the homology at that position is greater than 70% are highlighted.

G\textsubscript{86–105}, the region we had previously found to be the core signal transfer region for G\textsubscript{86–105}. A number of truncated peptides were selected, suggesting that the last one or two amino acid positions of this region, G\textsubscript{86–104} and G\textsubscript{86–105}, probably do not substantially contribute to binding affinity for PLC-\beta. We did not find any strong consensus, i.e. greater than 70% homology, for a switch of one amino acid for another at any position within G\textsubscript{86–105}. However, we did select some peptides where the lack of homology at a consensus position is accompanied by a switch at another amino acid position. An example is seen with the T14 peptide. This peptide lacks homology at consensus position 96. It has the substitution R96C. However, this mutation is accompanied by a mutation at position 99, W99R. This second mutation might compensate for the lack of homology at consensus position 96. It has the substitution R96C. Few selected peptides lack consensus at more than one amino acid position. Among the two groups of peptides, i.e. 25 total sequences, a non-conservative mutation of the basic amino acids at positions 89 and 96 is selected for only once.

Our previous analysis of substituted peptides from the G\textsubscript{86–105} region led us to predict that Lys-89, Arg-96, Ser-97, and Met-101 would likely to be important for binding and signal transfer for PLC-\( \gamma \) (4). For round 3 of panning, when the wild type peptide was allowed to compete with the binding of the library peptides, we found consensus at positions Arg-96 and Ser-97 but failed to find good consensus at position 89 and very little consensus at position 101. Interestingly, the homology at position 89 dropped from 86% to 64% when the wild type peptide was used to compete during panning. Our interpretation of this result is that the contribution of position Lys-89 to binding affinity is less than that of Arg-96 and Ser-97. In selecting Arg-96 and Ser-97 for binding, changes at position Lys-89 might be tolerated. In our substituted peptide studies the peptide G\textsubscript{86–105} M101N showed no measurable binding or activity. We did not find much of a consensus at position 101 in the library, but, as expected, we did not find any selected peptides with the M101N substitution.

**Combinatorial Peptides That Display Synergism**—During the library screening we selected peptides that bound PLC-\( \beta \) 2 with affinities greater than or equal to the wild type G\textsubscript{86–105} region, and we tested whether the binding would translate into stimulation of PLC-\( \beta \) 2 activity. To test if amino acid residues that make contacts important for tight binding also make contacts that directly generate signal transmission, we synthesized representative peptides that display the consensus sequence and measured the effect of these peptides on the activity of basal and G\textsubscript{86–105} wild type peptide. We selected peptides from both panning groups on the basis of how well conserved they were at the six consensus positions for round 3 of panning in the presence of the wild type peptide. Our criterion was that they would have consensus at 5 or more of these 6 positions. Most of the peptides that fit the cutoff criteria were from round 3 of panning in the presence of the wild type peptide (Table II). Of these 11 clones 8 showed consensus at 5 or more of the consensus positions. However, 5 of the 14 clones from round 3 of panning in the absence of the wild type peptide had consensus at 5 or more of the 6 sites. We, therefore, also included these peptides in our activity measurements.

The P9 peptide variant displays 5 of the 6 elements of the consensus sequence. The effect of the P9 peptide on PLC-\( \beta \) 2 basal activity is shown in Fig. 1A. This peptide stimulates PLC-\( \beta \) 2 with a much better EC\textsubscript{50} value than the wild type peptide, about 3 \( \mu\text{M} \) as compared with 45 \( \mu\text{M} \), but with a maximal stimulation much lower than that of the wild type G\textsubscript{86–105} sequence, about 1.3-fold as compared with greater than 3-fold. A comparison of the relative EC\textsubscript{50} extents and extents of stimulation is shown in Fig. 1B.

To determine if the decreased EC\textsubscript{50} value for the P9 peptide is accompanied by a decrease in the K\textsubscript{D} value for PLC-\( \beta \) 2, we measured the binding affinity of the P9 peptide to PLC-\( \beta \) 2 by FRET analysis and compared it to the binding affinity of the G\textsubscript{86–105} wild type peptide. The binding affinity of the P9 variant is roughly the same as that of the wild type peptide (Fig. 1C). It is possible that the P9 peptide may have a lower affinity, because it does not appear to become fully saturating at 10\textsuperscript{–7} \( \mu\text{M} \) peptide concentration. To test for binding specificity, we measured the binding of two control peptides, G\textsubscript{86–105} M, but with a K\textsubscript{D} of 300–313 and G\textsubscript{64–84}. These peptides have no effect on PLC-\( \beta \) 2 activity, and our data indicate that they are from regions of G\textsubscript{86} that are important structurally but do not play a direct role in protein-protein interactions with PLC-\( \beta \) 2. We found that they show no measurable binding to PLC-\( \beta \) 2 (Fig. 1D).

These results indicate that the better EC\textsubscript{50} value for the P9 peptide is not accompanied by a decrease in the K\textsubscript{D} value,
suggesting that there are distinct determinants within the Gβ 86–105 signal transfer region contributing to its EC50 and Kd values for PLC-β2. We wondered what molecular characteristic of the P9 peptide rendered it with a decrease in maximal stimulation even though it had a significantly better EC50 value and no change in Keq value as compared with the wild type Gβ 86–105 peptide. Comparison of the amino acid sequence of the P9 peptide with the sequence of the wild type peptide shows it to differ in homology at only two amino acid positions within the core Gβ 96–101 region, the six-amino acid core signal transfer region. The two amino acid changes are Ser-98P and M101F. Because we have previously found that contacts within Gβ 86–105 involved in signal transfer to PLC lie within the Gβ 96–101 region, it is possible that one or both of these amino acids are directly involved in signal transfer. The changes at positions Ser-98 and/or Met-101 may result in a decrease in maximal observed stimulation even though there is no change in binding affinity. To determine which of these amino acids, or both of them, are responsible for the better EC50 value and decreased maximal stimulation, we tested another library clone, the P3 peptide. The P3 peptide also has the amino acid change S98P but has the wild type residue Met at position 101. The effect of the P3 peptide clone on PLC-β2 basal activity is shown in Fig. 2A. Like the P9 peptide, P3 also has a decreased EC50 value and decreased maximal stimulation as compared with the wild type region peptide. The binding affinity of the P3 peptide, as measured by FRET, is about 0.5 μM, not significantly different from that of the P9 peptide variant or the Gβ 86–105 wild type peptide (Fig. 2B). Because the P9 and P3 peptides behave very similarly in binding and stimulating PLC-β2, it is likely that the common amino acid sequence shared by these two peptides, RSPW, Gβ 96–99 S98P, renders the P3 and P9 peptide clones with a decreased maximal stimulation and better EC50 values even though there is not a significant change in binding affinity from the wild type peptide. This sequence motif is highlighted in Fig. 2C.

Given the low extent of stimulation, we wondered if the P9 peptide would act as a partial antagonist to Gβγ stimulation; therefore, we measured the effect of the P9 peptide in the presence of subsaturating stimulation by Gβγ subunits. The result was surprising. The presence of subsaturating concentrations of Gβγ subunits increased the observed maximal stimulation by the P9 peptide, while not having much effect on the EC50 value of the P9 peptide (Fig. 3A). P9 stimulates PLC-β2 to a maximal extent of greater than 3-fold in the presence of Gβγ, whereas it only modestly stimulates about 1.3-fold by itself. The effect of varying concentrations of Gβγ on PLC-β2 activity in the presence and absence of maximal stimulation by the P9 peptide is shown in Fig. 3B, and this data indicate that the effect of P9 is synergistic with the effect of Gβγ.

One explanation for these results is that the P9 peptide induces structural changes in PLC so that other signal transfer regions on Gβ can form more productive interactions with PLC, resulting in a greater extent of stimulation. We have previously identified another signal transfer region on Gβ for stimulation of PLC-β2, the Gβ 42–54 signal transfer region. We wondered if the P9 peptide, by presumably inducing key structural changes in PLC-β2, would enable the Gβ 42–54 signal transfer region to be a better stimulator of PLC-β2 activity, and so we tested the effect of varying concentrations of the Gβ 42–54 peptide on PLC-β2 activity in the presence of close to saturating concentrations of the P9 peptide, 8 μM. The Gβ 42–54 peptide stimulates basal activity by about 20% by itself, however, in the presence of 8 μM P9 peptide its maximal extent of stimulation increases by ~50% (Fig. 3C). The inverse of this experiment yields similar results (Fig. 3D). Here we measured the effect of varying concentrations of the P9 peptide in the presence of subsaturating concentrations of the Gβ 42–54 peptide. The P9 peptide stimulates basal activity by about 20% by itself; however, in the presence of 1 μM Gβ 42–54 its maximum extent of stimulation increases by greater than 2-fold, to about 50% (Fig. 3D). These data would suggest that the effects of the P9 signal transfer region variant peptides are likely more than additive with the Gβ 42–54 signal transfer region peptide. The cooperative effect of the P9 peptide with Gβ 42–54 is not as pronounced as that observed with Gβγ subunits. This might indicate there are other regions of Gβ, not yet characterized, that function in signal transfer or the general binding domains within Gβ contribute to the synergy and that binding determinants of Gβ 42–54 are not sufficiently strong by themselves to allow for effective signal transfer. These alternatives will have to be experimentally resolved and will require combinatorial analysis of the Gβ 42–54 region as well.
We tested whether the G\(\beta\)86–105 wild type peptide had the same effect as the P9 variant on G\(\beta\)Y and G\(\beta\)42–54 stimulation of PLC-\(\beta\)2. G\(\beta\)86–105 stimulates PLC-\(\beta\)2 to a higher extent, greater than 3-fold, than the P9 variant. We next measured the effect of 5 \(\mu\)M G\(\beta\)42–54 peptide on G\(\beta\)86–105 stimulation. Subsaturating concentrations of G\(\beta\)42–54 increase the maximal observed stimulation attained by the G\(\beta\)86–105 signal transfer region (Fig. 3F). These data indicate that the G\(\beta\)86–105 and G\(\beta\)42–54 signal transfer regions have the capability to act in a synergistic manner to stimulate PLC-\(\beta\)2.

**Combinatorial Peptides That Behave as Antagonists/Partial Agonists**—Two other peptides from the library, T7 and T8, display all six elements of the consensus sequence but differ within the G\(\beta\)96–101 region from both the wild type sequence and the P9/P3 sequences. We measured the effect of these variant peptides on PLC-\(\beta\)2 activity. The T8 peptide has an EC\(_{50}\) value of about 50 \(\mu\)M, in the same range as the G\(\beta\)86–105 wild type peptide (Fig. 4A). However, it only stimulates PLC activity about 60%. However, in contrast to the P9 and P3 peptides, the T8 peptide is capable of inhibiting most of G\(\beta\)86–105 stimulation (Fig. 4B). Thus, the T8 variant of the G\(\beta\)86–105 signal transfer region is an antagonist as well as a partial agonist. Measurement of the binding affinity of the T8 peptide for PLC-\(\beta\)2 by FRET shows that the \(K_d\) value in the 1–2 \(\mu\)M range is similar to the wild type G\(\beta\)86–105 peptide. We tested the binding affinity of the T8 peptide for PLC-\(\beta\)2 by FRET shows that the \(K_d\) value in the 1–2 \(\mu\)M range is similar to the wild type G\(\beta\)86–105 peptide.
PLC-β2. Substituting these residues within Gβ 86–105 may render this signal transfer region with a lower maximal stimulation even though it has EC_{50} and K_d values that are similar to those of the wild type. We have already found that positions 98 and 99 are likely to be directly involved in contacts for signal transfer to PLC-β2, and the Gβ 99–99 sequence motif RSPW likely renders a higher potency but lower maximal stimulation of PLC-β2 compared with the wild type sequence of RSSW. The RSPW sequence motif also appears to be responsible for synergizing with other signal transfer regions on Gβ to stimulate PLC-β2. To better understand how the changes of the T8 peptide result in a decrease in maximal effect on PLC-β2 and loss of synergism with Gβ subunits, we tested the effect of another library peptide, the T7 peptide, on PLC-β2 activity. The T7 peptide is very similar in sequence to the T8 peptide in the Gβ 99–101 region. It has the same sequence as the wild type sequence peptide and also only minimally stimulates PLC-β2, around 1.5-fold. The T7 peptide clone affects Gβγ stimulation of PLC-β2 in a manner similar to that of the T8 peptide (Fig. 4D) and has a K_d value for PLC-β2 that is similar to the wild type sequence (data not shown). Thus, the T7 peptide is also a partial agonist and antagonist of PLC-β2. We noted, however, that the T7 peptide fully inhibits Gβγ stimulation of PLC-β2, suggesting that in the presence of Gβγ it is unable to stimulate PLC-β2.

These results indicate that it is likely the shared amino acid sequence between these two peptides in the Gβ 96–101 region, highlighted in Fig. 4E, that renders them as antagonists/partial agonists. Specifically, these residues are Arg-96, Arg-99, and Arg-101. It is interesting that the substitutions W99R and V100F affect the maximal extent of stimulation for this signal transfer region peptide but not its EC_{50} value. This indicates for residues 99 and 100 of Gβ that the EC_{50} and K_d values are independent of the maximal extent of stimulation.

**DISCUSSION**

Through a combinatorial screen we have identified variant peptides of the Gβ86–105 signal transfer region that have binding affinities for PLC-β2, are very similar to that of the wild type Gβ86–105 peptide, but have very different signal transfer properties. The range of signaling behaviors we observe provides clues toward initial understanding of the underlying mechanisms of signal transfer. From these analyses we have identified two key features. First, for the wild type Gβ86–105 peptide we find that the EC_{50} for the stimulation of PLC-β2 is over 20-fold to the right of the binding affinity and that the position of the EC_{50} is inversely related to the extent of stimulation. Second, intrinsic stimulation as evidenced by partial agonist activity could co-exist with two very opposite behaviors: synergistic stimulation with Gβγ or antagonist activity in the presence of Gβγ. Both these features provide mechanistic insights into how signal transfer might occur. The residues involved in the distinct signaling functions are summarized in Table III.

**Relationship between Binding Affinity, EC_{50} and Maximal Stimulation**—The FRET experiments showed that all of the variant peptides had binding affinities similar to that of the wild type peptide. Our lack of success in identifying any variant of Gβ86–105 region that had a significant increase in binding affinity suggests that the Gβ86–105 region may have been engineered for relatively modest binding affinity. A number of positively charged residues were identified in the library screen as important for binding affinity, and it is likely that electrostatics contribute to at least part of the interaction affinity of the Gβ 86–105 region for PLC-β2. We have also found this to be true for the other signal transfer region Gβ 42–54. One reason we did not find any higher affinity peptide is that it might be energetically more costly to reengineer a protein-protein interaction for higher affinity when most of the binding affinity is contributed by electrostatics rather than hydrophobic interactions.

The Gβ 86–105 region of Gβ has been shown to interact with a number of other Gβγ effectors, including adenylyl cyclases. One requirement for this diverse interaction capability might be flexibility that results in an inherently low affinity interaction surface. A plausible model for signal transfer from Gβγ to PLC-β2 from inherently low affinity binding regions, such as the Gβ 86–105 region, would involve initial interactions driven by electrostatic forces using induced fit mechanisms for additional dynamic contacts to affect activity changes in the effector (PLC-β2). These signal transfer regions might be interspersed with higher affinity binding regions that utilize hydrophobic forces and are more selective for specific effectors. Such a model
would also explain the role of Gγ subunits in stimulation of effectors (14, 15).

Of the library peptide variants selected and tested, all show both conserved and variant residues within the Gβ 86–101 region, the core signal transfer region. Residues Arg-96 and Ser-97 are part of the consensus sequence for selected library clones. These residues likely contribute to binding affinity. Other residues within the Gβ 96–101 sequence are somewhat varied, suggesting that amino acid substitutions in this region might be tolerated with no significant changes in binding affinity. These changes, however, affect signal transfer as evidenced by the change in maximal stimulation. As indicated in Table III, these data suggest that contacts made by positions 98–101 of Gβ that are not involved in binding affinity are involved in signal transfer. An example is observed with the T8 peptide variant. Here the amino acid substitutions W99R, V100F, and M101L render this peptide with a better EC$_{50}$ but decreased maximal stimulation even though there is no significant change in overall binding affinity. Contacts made by one or all of these positions in the wild type peptide might be important for signal transmission but not for binding affinity.

None of the variants of Gβ 86–105 that we identified in this screen had a maximal stimulation as great as that of the wild type sequence. For each variant peptide tested, its maximal stimulation was less than 2-fold, whereas the maximal stimulation of the wild type Gβ 86–105 peptide is generally greater than 3-fold. Thus, the Gβ 86–105 signal transfer region appears to be optimized in terms of the efficacy with which it can regulate PLC-β2 basal activity. This is to be true, at least, for variants of Gβ 86–105 with binding affinities equal to or greater than the wild type sequence. It is possible that there might be variants of Gβ 86–105 that have weaker binding affinities for PLC-β2 but are more efficacious in stimulating PLC-β2 activity. An example of this scenario was seen with a substituted peptide from the Gβ 42–54 region. The substituted peptide Gβ 42–54 R48A displayed a weaker EC$_{50}$ that was accompanied by an increase in maximal stimulation (6). That none of the peptide variants could transfer signals to the same extent as the wild type region also indicates that positions within the core signaling region Gβ 96–101 that are not important for binding affinity can be important for signaling; therefore, the roles of binding and signal transfer for this region can be resolved at an amino acid level.

**Synergism and Antagonism**—The P9 peptide was found to act in a synergistic fashion with Gβγ and the second Gβ signal transfer region, Gβ 42–54 and displayed no antagonist properties, whereas the other set of library peptides, T7 and T8, acted as antagonists to Gβγ simulation. However, by themselves, all the peptides stimulated PLC-β2 only 30–60%. These data would suggest that the set of contacts leading to the small amount of stimulation is distinct from those interactions that generate synergism with other signal transfer regions on Gβ, including the Gβ 42–54 region to yield extensive (severalfold) stimulation. As summarized in Table III, amino acids Arg-96 and Ser-97 of Gβ, residues common to both sets of peptides are important for binding and for generating low efficacy signal transmission. A distinct contact from the amino acid at position 99 appears to be required for generating additional efficacy for signal transmission by synergizing with other signal transfer regions on Gβ such as the Gβ 42–54 region. The synergistic effect of the wild type Gβ86–105 peptide with the Gβ 42–54 signal transfer region is not as pronounced as that observed with Gβγ subunit. This observation suggests that there might be additional regions in Gβ that play a role in synergistic signal transfer. Whether these are additional signal transfer regions or general binding domains need to be experimentally determined.

Overall we propose that synergism occurs because the binding of one signal transfer region may enhance the efficiency of signal transfer from the second region, because it appears that there are multiple domains in PLC-β2 that are capable of receiving signals (16, 17). Such a model would be based on the idea that interaction of the Gβ 86–105 region with PLC-β2 may induce several discrete conformational changes in this effector. These conformational changes can be functionally resolved from one another, and this is illustrated by the T7 and T8 variants where the small extent of activation is not accompanied by synergism with Gβγ subunits. Both of these peptides have the amino acid substitution W99R. This substitution, although not affecting contacts involved in binding affinity or contacts for generating a low efficacy signal transmission, might prevent contacts with PLC-β2 important for synergism with other signal transfer regions. Thus the resultant functional effect is antagonism in the presence of Gβγ subunits.

Although parts of our model are speculative, our data clearly indicate that binding does not directly translate into signal transmission for the Gβ86–105 signal transfer region. Signal transfer from this Gβ region to PLC-β2 must involve at least a partially non-overlapping set of protein-protein contacts as compared with binding. This conclusion is supported by two sets of observations: first, individual amino acids important for binding can be resolved from those important for signaling, and second, some of these signaling residues appear to play little or no direct role in contributing to the overall binding affinity. There have been similar findings for the resolution of binding from signal transfer in other signaling systems as well. Hamm and coworkers (18) have shown that at least two distinct regions are involved in interactions between Gαs and Gαi subunits of cGMP phosphodiesterase and that only one of these appears to be important for stimulation of cGMP phosphodiesterase. Similarly for the Rho family member Cdc42, interactions with its effector phospholysis D1 (PLD1), a region from Cdc42 that is important for stimulating PLD1 activity is not involved in binding affinity (19). The data from Gtα/phosphodiesterase γ (18), Cdc42/PLD (19), Gαi/adenyl cyclase (5), and the Gβγ/PLC-β2 systems indicate that the design of separating signal transfer regions from general binding domains may be widely used for signal transfer in G protein systems.

In summary, the studies presented here provide an initial mechanistic model for signal transfer where initial contact within signal transfer regions and possibly concurrent interactions between general binding domains induces the effector to attain a state of high receptivity such that signals from multiple signal transfer regions can be effectively transmitted. Such a model predicts a coordinated set of induced fit interactions between the signal transfer regions on the G protein subunits and signal-receiving regions of the effectors. Future experiments that provide direct information about dynamics of the interacting structures involved in signal transfer from Gβ to PLC-β2 will be needed to test this prediction.

**Acknowledgments**—We thank Drs. John Hildebrandt and Jane Dingus for purified Gβγ subunits, Drs. Heidi Hamm and Annette Gilchrist for useful advice, and Dr. Yihang Chen and Burney Yoo for a critical reading of the manuscript.

**REFERENCES**

1. Gilman, A. G. (1987) Annu. Rev. Biochem. 56, 615–649
2. Logothetis, D. E., Kurachi, Y., Galper, J., Neer, E. J., and Clapham, D. E. (1987) Nature 325, 321–326
3. Clapham, D. E., and Neer, E. J. (1997) Annu. Rev. Pharmacol. Toxicol. 37, 167–203
4. Buck, E., Li, J., Chen, Y., Weng, G., Scarlata, S., and Iyengar, R. (1999) Science 283, 1322–1325
5. Chen, Y., Yao, R., Lee, J. B., Weng, G., and Iyengar, R. (2001) J. Biol. Chem. 276, 45751–45754
6. Buck, E., and Iyengar, R. (2001) J. Biol. Chem. 276, 36014–36019
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7. Buck, E., and Iyengar, R. (2001) *Methods Enzymol.* 344, 513–521
8. Dingus, J., Wilcox, M. D., Kohnken, R., and Hildebrandt, J. B. (1994) *Methods Enzymol.* 237, 457–471
9. De Vivo, M. (1994) *Methods Enzymol.* 238, 131–140
10. Runnels, L. W., Jenco, J., Morris, A., and Scarlata, S. (1996) *Biochemistry* 35, 16824–16832
11. Schatz, P. J., Cull, M. G., Martin, E. L., and Gates, C. M. (1996) *Methods Enzymol.* 267, 171–191
12. Gilchrist, A., Li, A., and Hamm, H. E. (2000) *Methods Enzymol.* 315, 388–404
13. Ford, C. E., Skiba, N. P., Bae, H., Duaka, Y., Reuveny, E., Shketler, L. R., Rosal, R., Weng, G., Yang, C. S., Iyengar, R., Miller, R. J., Jan, L. Y., Lefkowitz, R. J., and Hamm, H. E. (1998) *Science* 280, 1271–1274
14. Myung, C. S., Yasuda, H., Liu, W. W., Harden, T. K., and Garrison, J. C. (1999) *J. Biol. Chem.* 274, 16595–16603
15. Akgoz, M., Azpiazu, I., Kalyanaraman, V., and Gautam, N. (2002) *J. Biol. Chem.* 277, 18573–18578
16. Wang, T., Dowal, L., El-Maghrabi, M. R., Rebecchi, M., and Scarlata, S. (2000) *J. Biol. Chem.* 275, 7466–7469
17. Wang, T., Pentayal, S., Elliot, J. T., Gupta, E., Rebecchi, M. J., and Scarlata, S. (1999) *Proc. Natl. Acad. Sci. U. S. A.* 96, 7843–7846
18. Artemyev, N. O., Rarick, H. M., Mills, J. S., Skiba, N. P., and Hamm, H. E. (1992) *J. Biol. Chem.* 267, 25067–25072
19. Walker, S. J., Wu, W.-J., Cerione, R. A., and Brown, H. A. (2000) *J. Biol. Chem.* 275, 15665–15668