Regulatory Interactions between the Amino Terminus of G-protein βγ Subunits and the Catalytic Domain of Phospholipase Cβ2

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We previously identified a 10-amino acid region from the Y domain of phospholipase Cβ2 (PLCβ2) that associates with G-protein βγ subunits (Sankaran, B., Osterhout, J., Wu, D., and Smrcka, A. V. (1998) J. Biol. Chem. 273, 7148–7154). We mapped the site for cross-linking of a synthetic peptide (N20K) corresponding to this Y domain region to Cys25 within the amino-terminal coiled-coil domain of Gβγ (Yoshikawa, D. M., Bresciano, K., Hatwar, M., and Smrcka, A. V. (2001) J. Biol. Chem. 276, 11246–11251). Here, further experiments with a series of variable length cross-linking agents refined the site of N20K binding to within 4.4–6.7 Å of Cys25. A mutant within the amino terminus of the Gβ subunit, Gβγ(23–27)γγ, activated PLCβ2 more effectively than wild type, with no significant change in the EC50, indicating that this region is directly involved in the catalytic regulation of PLCβ2. This mutant was deficient in cross-linking to N20K, suggesting that a binding site for the peptide had been eliminated. Surprisingly, N20K could still inhibit Gβγ(23–27)γγ-dependent activation of PLC, suggesting a second N20K binding site. Competition analysis with a peptide that binds to the Ga subunit switch II binding surface of Gβγ indicates a second N20K binding site at this surface. Furthermore, mutations to the N20K region within the Y domain of full-length PLCβ2 inhibited Gβγ-dependent regulation of the enzyme, providing further evidence for a Gβγ binding site within the catalytic domain of PLCβ2. The data support a model with two modes of PLC binding to Gβγ through the catalytic domain, where interactions with the amino-terminal coiled-coil domain are inhibitory, and interactions with the Ga subunit switch II binding surface are stimulatory.

Activation of G-protein-coupled receptors releases the Gβγ subunit from Ga-GTP, leaving Gβγ free to interact with effector molecules such as enzymes or ion channels (1–3). A particular group of enzymes regulated by Gβγ belong to the phosphoinositide-specific phospholipase Cβ (PLCβ1) class. Once activated, PLCβ cleaves phosphatidylinositol 4,5-bisphosphate (PIP2) into the two second-messengers, diacylglycerol and inositol 1,4,5-trisphosphate. All PLCβ isoforms have an N-terminal pleckstrin homology (PH) domain, four sets of EF-hand domains, X and Y domains comprising the catalytic center, and a C2 domain followed by a C-terminal extension (4). There are four isoforms of PLCβ, β1–4; however, only PLCβ2 and PLCβ3 are regulated by βγ subunits (5).

G-protein β subunits have two distinct domains, an N-terminal α helix, which forms a coiled-coil interaction with the γ subunit, and a seven-blade β-propeller structure composed of seven WD repeating motifs (6, 7). Amino acids within blades one, two, and three make extensive contacts with α-GDP (7, 8). Since α subunits block Gβγ-dependent regulation of all effectors, effector-binding sites on Gβγ were predicted to overlap with the α subunit binding site. Indeed, mutagenesis of particular amino acids on Gβγ important for α subunit binding blocked activation of PLCβ2 (9, 10). Furthermore, mutations to the outer strands of blades 2, 6, and 7, which do not make direct contact with Ga, rendered Gβγ unable to effectively activate PLCβ2 (11).

The mechanism for regulation of PLCβ2 by Gβγ subunits is not fully understood. Approaches for elucidating the activation mechanism have been to characterize the effects of a variety of mutants, peptides, and fusion proteins on regulation of PLCβ2 by Gβγ. Two major regions on PLCβ2 have been implicated as Gβγ-binding sites, the N-terminal PH domain and a portion of the catalytic Y domain. A large body of evidence supports a role for interactions between the catalytic domain of PLCβ2 and Gβγ subunits. Overexpression of the Y domain in COS-7 cells blocked receptor-mediated Gβγ-dependent, but not Gaα-dependent activation of PLC. A purified glutathione S-transferase fusion protein comprising amino acids 526–641 of the Y-domain bound directly to purified Gβγ subunits (12). Overlapping peptides representing a portion of the Y-domain directly cross-linked to both Gβ and Gγ subunits with the heterobifunctional cross-linker SMCC, and this was blocked by purified PLCβ2 but not at α. The peptides also inhibited Gβγ regulation of PLCβ2 with an EC50 of 30–50 μM (13). The crystal structure of PLCδ reveals that the region implicated by the peptide studies corresponds to the α helix on the surface of the catalytic domain and therefore is accessible to interact with Gβγ subunits (14). We hypothesized that direct binding of this helix within the catalytic domain to Gβγ is involved in Gβγ-dependent PLCβ2 activation.

To define the site cross-linking of one of the catalytic domain peptides, N20K (amino acids 565–574 in PLCβ2), to the Gβ subunit and identify a potential Y-domain interaction site on Gβ, we used a systematic cysteine mutagenesis approach where each cysteine on Gβ was individually mutated to ala nine. The cysteine residue critical for the majority of peptide cross-linking was cysteine 25 within the amino-terminal coiled-coil region of Gβ (15). This region is outside the Ga subunit-
binding site, complementing data demonstrating that Go11 could not block cross-linking of N20K to Gβγ (13, 15). Previous studies have not implicated the amino terminus of Gβγ as an important effector signaling site in mammals, although it has been implicated to be important in the yeast pheromone pathway (16, 17).

These data suggest that the amino-terminal coiled-coil of Gβγ directly binds to the αG helix of the Y-domain to regulate PLC activity. The goals of this study were 3-fold: 1) to further delineate the specific region within the amino terminus of Gβγ subunits that interact with PLCβ; 2) to demonstrate a functional role for the amino terminus of Gβ in mammalian effector regulation; and 3) to demonstrate the functional importance of the N20K region within full-length PLC for Gβγ regulation. These results would be the first demonstrating a specific region on PLC interacting with a specific region on Gβγ, leading to a better understanding of how Gβγ subunits regulate PLC.

**EXPERIMENTAL PROCEDURES**

**Materials**—Peptides (N20K, Ac-NSSYVVSSFTLAKYDLLSK; SIGK, Ac-SSVYVVSSFTLAKYDLD; LDG, Ac-SSVYVVSSFTLAKYDNYD) were purchased from Alpha Diagnostic International (San Antonio, TX) and had purity greater than 90% based on HPLC chromatography analysis. The masses of the peptides were confirmed by mass spectrometry. All cross-linking reagents used in this study were from Pierce. Ni2⁺-nitrilotriacetic acid resin was from Qiagen. [3H]PiP, and [3H]NAD were from PerkinElmer Life Sciences.

Pertussis toxin was purchased from List Biological Laboratories. All molecular biology reagents were from Invitrogen, unless otherwise indicated. Baculoviruses were generated as per the manufacturer’s instructions (Invitrogen).

**Construction of Gβ Subunit and PLCβ Mutants**—Mutants in rat Gβ2, and in human PLCβ2 were constructed by overlap-extension PCR with Pfu polymerase (Stratagene) using standard protocols. Final constructs were sequenced to confirm the presence of the mutations before use. Procedures were as per the manufacturer’s instructions.

**Purification of βγ Subunits—** Gβγ subunits were purified from 2 liters of SF-9 cells triply infected with His6-Gβγ, wild type or mutant Gβ2 subunits, and Gγγ subunits essentially as described (18) with the following changes. SF-9 cell membranes were prepared as described (18) and then extracted in buffer A containing 50 mM HEPES, pH 8.0, 0.3 mM MgCl2, 10 mM β-mercaptoethanol, 10 mM GTP, 50 mM NaCl, 1% polyoxyethylene 10 lauryl ether (C10E9), and a protease inhibitor mixture for 2 h at 4 °C. Detergent-extracted proteins were diluted 5-fold with buffer B, 20 mM HEPES, pH 8.0, 1 mM MgCl2, 10 mM β-mercaptoethanol, 10 μM GDP, 100 mM NaCl, 0.5% C10E9, and a protease-inhibitor mixture and loaded onto a 4-ml Ni2⁺ resin. 

**RESULTS**

**Delineation of the N20K Binding Site on Gβγ**—Previous studies in our laboratory demonstrated two overlapping peptides from the catalytic domain of PLCβ2, N20K, and E20K (amino acids 564–584 and 574–594, respectively) could be covalently cross-linked to Gβ subunits (13, 15). Based on this, and other studies, we hypothesized the 10-amino acid region of overlap between these peptides, ELKAYDLLSK (amino acids 574–584), representing an α helix on the surface of PLCβ2, directly bound to Gβγ subunits. To define this potential PLCβ2 interaction site on Gβγ, we mapped a cross-linking site for the N20K peptide using a cysteine to alanine mutagenesis approach (15). From this data, it was determined the sulfhydryl group of cysteine 25 is within reach of the peptide-binding site. However, since the cross-linker, SMCC, had a spacer arm length of 12 Å, we could only conclude the peptide was binding within a 15-Å radius of cysteine 25 (the length of the spacer arm in the SMCC cross-linker plus the length of the lysine side chain). To further refine the nature of the N20K interaction within Gβγ, we determined which residue(s) in N20K was/were directly bound to Gβγ. We also determined that the sulfhydryl group of cysteine 25 is within reach of the peptide-binding site. However, since the cross-linker, SMCC, had a spacer arm length of 12 Å, we could only conclude the peptide was binding within a 15-Å radius of cysteine 25 (the length of the spacer arm in the SMCC cross-linker plus the length of the lysine side chain). To further refine the nature of the N20K interaction within Gβγ, we determined which residue(s) in N20K was/were directly bound to Gβγ. We also determined that the sulfhydryl group of cysteine 25 is within reach of the peptide-binding site.
higher molecular weight immunoreactive $\beta$ subunit band only in the presence of peptide and cross-linker, although neither was as effective as N20K. Cross-linking of both of these peptides to $\beta_1$ subunits where Cys25 was mutated to alanine ($\beta_1(C25A)$) was substantially inhibited.

To further restrict the radius of binding of these two lysine residues, cross-links with various spacer arms (12–15 Å) were used to cross-link R1 and R2 to Gβγ. Both R1 and R2 effectively cross-linked to Gβγ with N-[γ-maleimidobutyryloxy]-succinimide ester (6.7 Å) but not N-[α-maleimidodiacetoxy]-succinimide ester (4.4 Å) (Fig. 1C), demonstrating both that lysine residues are approximately equidistant from Cys25 and that the site of binding of N20K to Gβγ is within 4.4–6.7 Å of Cys25.

**Purification of Amino-terminal Site-directed Mutants of $\beta$γ Subunits near Cys25—**The cross-linking data suggest a PLC binding site in the amino terminus of Gβγ within 7 Å of cysteine 25. Therefore, surface amino acids in the coiled-coil region of Gβγ within 10 Å of cysteine 25 were chosen for site-directed mutagenesis to confirm a functional role for the GβγN terminus in PLC regulation. Six amino acids on $\beta_2$ and six amino acids on $\gamma$ neighboring cysteine 25 were mutated three amino acids at a time. The mutations were named Gβγ1,2(23–27) based on the stretches of amino acids where the mutations were located (Fig. 2A). Similar mutations made in Gγγ failed to dimerize with Gβγ and were not analyzed further. Wild type and mutant Gβγ dimers were purified simultaneously and in parallel to near homogeneity in one step based on binding to His$_6$-Go_{i} and elution by activation of the Go subunit with AlF$_4^-$ (Fig. 2B). This purification strategy insured that the mutants were properly assembled, folded, and functional. Proper assembly with the Gγ subunit was confirmed by immunoblotting for the presence of Gγ subunits in the purified mutant preparations (Fig. 2C). The proteins were >90% pure based on Coomasie Blue staining. No detectable endogenous Sf-9 Gβγ (which runs at a higher molecular weight than overexpressed mammalian Gβ1) was detected on either a Coomasie Blue-stained gel or immunoblots with an antibody that recognizes both endogenous Sf-9 and overexpressed Gβ1.

**Activation of PLCβ by Wild Type and Mutated Gβγ Subunits**—To demonstrate a functional role for the amino-terminal amino acids of Gβγ in mammalian PLCβ2 regulation, the purified mutated dimers were tested for activation of PLCβ2. Surprisingly, Gβ1(23–27)$\gamma$ activated PLCβ2 2–4-fold more effectively than wild type with no significant change in the EC$_{50}$ (Fig. 3A and Table I). There was no significant difference in either the -fold activation or the EC$_{50}$ for activation by Gβ1(17–21) (Fig. 3A and Table I). Activation of PLCβ2 was abolished by boiling the mutant prior to performing the assay, indicating that the enhancement was not due to a non-protein contaminant within the buffer (data not shown). The addition of GDP-loaded Go, blocked activation of PLCβ2 by Gβ1(23–27)$\gamma$, ensuring that the enhancement was due to the addition of Gβγ and not a contaminating protein (Fig. 3B). The addition of purified PLC was required to observe accumulation of inositol 1,4,5-trisphosphate, indicating that no endogenous SF-9 phosphatases were co-purified with the mutant Gγγ (data not shown). These results were repeated with three different preparations of Gβ1(23–27)$\gamma$, and two different preparations of wild type Gβ1γγ, demonstrating the reproducibility of the results with different batches of protein. As a final confirmation that both proteins were properly folded and functional postpurification, the ability of both Gβ1γγ and Gβ1(23–27)$\gamma$ to support pertussis toxin-mediated ADP-ribosylation of Go$_i$ was tested. Both proteins supported ADP-ribosylation of Go$_i$, with no significant difference in either potency or efficacy (Fig. 3C). These data confirm that...
Fig. 3. Activation of PLC by wild type and mutated βγ subunits. A, wild type and mutated Gβγ subunits were incubated with 2 ng of PLCβ2 and sonicated lipid vesicles containing 25 μM PIP2 and 100 μM liver phosphatidylethanolamine. All reactions contained 0.06% octyl glucoside. Reactions were initiated by the addition of 100 nM free Ca²⁺ and then incubated for 10 min at 30 °C water bath for 7 min before quenching by the addition of ice-cold 10% trichloroacetic acid. Data shown are mean ± S.E. of duplicate determinations from one of three (Gβγ1(17–21)) or seven (Gβγ2(23–27)) independent experiments. B, 100 nM Gαq-GTP blocked the activation of PLCβ2 by 100 nM Gβγ2(23–27). Data are from one of two independent experiments. C, Gβγ2(23–27)γ2 supported ADP-riboseylation of Gαq, 15 pmol of purified Gαq was incubated with various amounts of wild type or mutated Gβγ subunits in the presence of pertussis toxin and [35S]P[NAD. Data shown are mean ± S.E. of duplicate determinations. This experiment was repeated three times with similar results. D, wild type and mutated Gβγ subunits were incubated with 5 ng of PLCβ2 as in A, except reactions were at 50 °C for 10 min. The data are mean ± S.E. of duplicate determinations from a representative experiment. This experiment was repeated three times with similar results. All curves were fit with a single site binding hyperbolic function using Graph Pad Prism Software. IP₃, inositol 1,4,5-trisphosphate.

Table I

| PLCβ2 activationᵃ | N20K inhibition of PLCβ2 activationᵇ | PLCβ γ activationᵇ |
|-------------------|-------------------------------------|---------------------|
| EC₅₀ | Maximal activation | IC₅₀ | Maximal inhibition | EC₅₀ | Maximal activation |
|-----------------|-----------------|------|------------------|------|------------------|
| WT | 56 ± 5.8 | 6.4 ± 0.06 | 18 ± 0.05 | 85 ± 3.0 | 37 ± 9.5 | 8.1 ± 2.2 |
| Gγ1(17–21)γ₂ | 50 ± 8.5 | 6.3 ± 0.56 | 27 ± 0.04 | 77 ± 3.8 | ND | ND |
| Gγ1(23–27)γ₂ | 68 ± 14 | 17 ± 2.2 | 20 ± 0.08 | 53 ± 3.7 | 15 ± 1.7 | 9.6 ± 3.5 |

ᵃ Average ± S.D. of three (Gγ1(17–21)γ₂) or six (Gγ2(23–27)γ₂) independent experiments.
ᵇ Average ± S.D. of three independent experiments.
ᶜ Significantly different as compared with wild type (p < 0.05).
ᵈ ND, not determined.

Both proteins are properly folded and functional and that there are no errors in the protein concentrations.

To determine whether this enhancement of activity was specific for PLCβ2, activation of PLCβ3 by mutant and wild type Gβγ was tested. In this case, both WT and Gβγ1(23–27)γ₂ activated PLCβ3 to a similar extent, but Gβγ2(23–27)γ₂ was nearly 2-fold more potent than wild type (Fig. 3D and Table I). These data strongly support a function of the amino terminus in the regulation of both PLCβ2 and PLCβ3. In both cases, the role of the amino terminus appears to be inhibitory, although the mechanisms are different. Others have reported differential activation of PLCβ2 and PLCβ3 by Gγ subunits (10, 11, 22).

Interaction between N20K and Mutated βγ Dimers—The amino acids chosen for mutation were amino acids that, due to their close proximity to Cys₂⁵, were likely candidates to directly interact with the N20K peptide. To determine whether the Gβ subunit mutations eliminated N20K peptide binding, we tested whether N20K could still cross-link to the amino-terminal mutants. Gβγ1(17–21)γ₂ did cross-link to N20K, although higher concentrations of peptide were required for cross-linking compared with wild type, whereas Gβγ2(23–27)γ₂ substantially lost its ability to cross-link to N20K (Fig. 4A). Normally, N20K cross-linking to Gβγ generates two higher molecular weight bands. N20K cross-linking to Gβγ2(23–27)γ₂ produces only one faint higher molecular weight band. We predicted that if the functional effects of N20K on activation of PLCβ by Gβγ subunits were through binding at the amino terminus, then elimination of this binding site would eliminate N20K inhibition of Gβγ-dependent activation of PLCβ2. Unexpectedly, N20K still inhibited Gβγ2(23–27)γ₂ activation of PLCβ2 with a similar potency as wild type. Gβγ2(23–27)γ₂-dependent activation of PLC was inhibited by 50%, whereas wild type Gβγ-dependent activation was inhibited 85%. The inability of N20K to fully inhibit PLC activation by Gβγ2(23–27)γ₂ may be due to the increased efficacy of Gβγ2(23–27)γ₂. Taken together, the cross-linking data and the peptide inhibition data suggest that there may be two binding sites for N20K on Gβγ, one within the amino terminus of Gβγ eliminated by the Gβγ1(23–27)γ₂ mutation and one remaining intact. The ability of N20K to inhibit Gβγ1(23–27)γ₂ activation of PLC would be through binding to this second site. From these experiments, we hypothesize that Y-domain binding to the amino terminus of Gβγ inhibits PLC activity, whereas Y-domain binding to this second site increases PLC activity.
βγ-Dependent Activation of PLC

Defining a Second Site for N20K Interaction—Cross-linking data presented here and in our previous publications support the notion of two N20K binding sites. Cross-linking of N20K to Gβγ produces two higher molecular weight bands (Figs. 1B and 4A). The first higher molecular weight band is Gβ γ cross-linked to one N20K peptide. The nature of the second band has been unclear, but its appearance was prevented by preincubation of one N20K peptide. The nature of the second band had been molecular weight band (15). We propose that this second higher molecular weight band is Gγ (15). To determine the second higher molecular weight band is Gβ cross-linked to two N20K peptides, one at the amino terminus and one elsewhere. We believe the residual cross-linking of peptide to the Gβγ[23–27] mutant and Gβγ(C25A) is through binding at the second site.

In order to define the second N20K binding site, we utilized the Gβγ(C25A) mutant. Our previous data showed that N20K cross-linked weakly to Gβγ(C25A)γγ. Since there are no cysteine residues near Cys25 capable of interacting with the cross-linker, G81(C25A) should have abolished all cross-linking if the N terminus was the sole site of binding. This finding indicates that there is a second N20K binding site outside of the amino terminus, and binding to this second site results in the residual cross-linking to Gβγ(C25A)γγ. N20K has sequence similarity to Gβγ binding peptides identified in a random peptide phage display screen conducted in our laboratory (23). This sequence similarity led us to hypothesize that these peptides were binding to a similar site on Gβγ as the Y-domain of PLC (23). Recently, a binding site for one of these peptides was clearly defined in the x-ray crystal structure of one of these peptides, SIGKAF-

KILGYPDYD (SIGK), bound to Gβγ(252). SIGK was identified in a dodge mutagenesis screen to identify derivatives of one of the original peptides obtained in the phage display screen, SIRKALNILGYPDYD (SIRK). SIGK binds to Gβγ with higher affinity than SIRK but retains most of the characteristics of SIRK. The SIGK peptide bound to the top surface of Gβγ overlapping with the switch II binding site of Go. To determine whether this site was the second N20K binding site, we tested the ability of SIGK to compete away cross-linking of N20K to Gβγ (SIGK itself does not cross-link to Gβγ under these conditions). Fig. 5A shows that replacement of Cys25 with alanine greatly reduces, but does not completely eliminate, cross-linking of N20K, confirming our previous studies (due to poor resolution of the second higher molecular weight band, we are focusing only on the lower band, which is a combination of Gβ cross-linked to N20K at either one of the two sites). The addition of SIGK at concentrations above the Kd (1–5 μM) partially reduces N20K cross-linking to wild type Gβγ, suggesting that binding to one of the two sites has been blocked by SIGK, but one remains intact. However, incubation with SIGK completely eliminated cross-linking of N20K to βγ(C25A)γγ (Fig. 5, A and B). The addition of a control peptide that does not bind βγ, L9A (23), has no effect on N20K cross-linking (Fig. 5A).

2 T. L. Davis, T. M. Bonacci, A. V. Smrcka, and S. R. Sprang, submitted for publication.

3 T. M. Bonacci, M. Ghosh, S. Malik, and A. V. Smrcka, unpublished data.
These data demonstrate that N20K has two distinct binding sites on Gβγ, one within the amino terminus near Cys25 and one at the Go interface, at the SIGK binding site.

We hypothesized that N20K blocked Gβγ2(23–27)γ2-dependent activation of PLCβ2 by interacting with the site outside of the amino-terminal binding site. Our cross-linking studies presented in Fig. 5, A and B, show that this second site overlaps with the SIGK peptide binding site; therefore, SIGK should also block PLCβ activation by both wild type Gβγ and Gβγ2(23–27)γ2 subunits. Fig. 5C demonstrates that the SIGK peptide blocked Gβγ-dependent activation of PLCβ2 by both wild type and Gβγ2(23–27)γ2 with no significant change in either the maximal inhibition or the IC50. From this finding, it is reasonable to conclude that the ability of N20K to block activation of PLCβ2 by Gβγ2(23–27)γ2 was through binding to this site.

**Regulation of Catalytic Domain Mutants of PLCβ2 by Gβγ Subunits**—This study and previous data suggesting that this peptide from the Y-domain of PLC binds to two sites are taken as evidence that the α5 helix on PLC represented by this peptide can bind to these two sites. If N20K is truly a model of the Y-domain α5 helix in full-length PLC, then mutations of the region corresponding to N20K within full-length PLC should have altered Gβγ-dependent activation. We constructed three triple alanine mutants (Fig. 6A) corresponding to the last 10 amino acids of N20K and the α5 helix in full-length PLCβ2. The basal Ca2+-dependent activities of all three PLCβ2 mutants were similar or slightly less than wild type (Fig. 6B), indicating that they were properly folded and active. The three PLCβ2 mutants were then tested for their ability to be activated by saturating concentrations of Gβγ2, PLCβ2(ELK/AAA) had a significant decrease in its Gβγ-dependent activation as compared with wild type. PLCβ2(YDL/AAA) was also significantly deficient, whereas PLCβ2(LSK/AAA) was not significantly different from wild type. Full titration curves for Gβγ-dependent activation of PLCβ2(ELK/AAA) indicate that this mutant was activated very poorly at all concentrations tested (Fig. 6D). To confirm that the Gβγ-dependent activation resulted from specific alteration of Gβγ-dependent PLC activation, activation by Goi was tested. Previous studies have shown that the regions in PLCβ necessary for Goi-dependent activation are different from those required for Gβγ25. The data in Fig. 6E show that PLCβ2(ELK/AAA) was activated to a greater extent than wild type PLCβ2, indicating that this mutant has no global deficiency in the catalytic machinery or in the ability to be activated in general. The nature of the enhanced activation by Goi is unknown and will be the subject of further investigation. Overall, these data demonstrate that there is a functional interaction between Gβγ subunits and the α5 helix of PLCβ2.

**DISCUSSION**

In this work, we have reinforced and extended previous data suggesting that the catalytic domain of PLCβ2 can bind to the amino-terminal coiled-coil region of Gβγ subunits near cysteine 25. Importantly, the mutagenic analysis demonstrates a functional role for interactions between the catalytic domain of PLCβ2 and both the N terminus and propeller regions of Gβγ. The amino-terminal region we identified in mammalian Gβγ is the same region previously identified as an effector binding site in yeast Gβγ subunits involved in the pheromone response pathway (17, 26). Another study with single point mutations in the N terminus of mammalian β failed to produce a mutant deficient in Gβγ-dependent effector activation when transfected into COS-7 cells, but interestingly, one mutant, K20A, showed a slight gain of function with respect to c-Jun N-termi-
nal kinase activation but not activation of PLCβ2 (22). This study did not include mutations to Lys$^{23}$ or Ala$^{24}$, which were mutated in our analysis. Other mutagenesis studies of Gβ subunits did not test for effects of mutations in the amino-terminal coiled-coil region, focusing on regions at the Gα subunit interface and other amino acids within the propeller region (9–11, 27).

Despite apparently eliminating a binding site for N20K in the Gβ$_2$(23–27) mutant, N20K still inhibited the ability of this mutant to activate PLCβ2 with a similar potency as wild type (Table I, Fig. 4B), indicating that N20K must still be associating with the mutant. To explain this result, we hypothesize that there are two binding sites for N20K on the Gβ subunit, one eliminated by the Gβ$_2$(23–27)$^{γ_2}$ mutation and one still intact. This hypothesis is supported by our cross-linking studies in which both Gβ(C25A)$^{γ_2}$ and Gβ$_2$(23–27)$^{γ_2}$ still displayed some cross-linking to N20K (Figs. 5A and 4A, respectively). Any cross-linking to Gβ(C25A) must be through binding of the peptide to a second binding site on Gβγ. Since the cross-linking of Gβ$_2$(23–27)$^{γ_2}$ is similar to Gβ(C25A), we suspected that this residual cross-linking is due to N20K binding at the site outside of the amino terminus. Our previous work (13, 15) and Fig. 1B support this idea, showing that cross-linking of wild type Gβγ to N20K leads to two cross-linked species on a Western blot. The lowermost band is uncross-linked Gβ. We believe that the first cross-linking band corresponds to Gβ cross-linked to one N20K peptide at one of two binding sites, and the upper band is Gβ cross-linked to two N20K peptides. This band is not Gβ cross-linked to Gy due to the nature of the cross-linking protocol and confirmation by immunoblotting (data not shown; see Ref. 13).

We recently identified a family of peptides in a random peptide phage display screen that have homology to N20K. The binding site for a peptide derived from this screen, SIGK, has recently been defined at the Gα subunit-switch II binding interface on the β-propeller. Since SIGK competes for the residual cross-linking of N20K to Gβ$_2$(C25A), the second N20K binding site must overlap with the SIGK binding site. This surface on βγ subunits has been shown to be important for PLC regulation (9, 10). There are two surface cysteine residues in proximity to this site, Cys$^{204}$ and Cys$^{271}$, which could be participating in the cross-linking reaction. This interface is ~30 Å from Cys$^{25}$. With SMCC as a cross-linker with a 12-Å spacer arm, it is not possible for the peptide to be binding at the amino terminus and use either of these two cysteine residues for cross-linking; nor is it possible for the peptide to be solely binding at this interface and use cysteine 25 for cross-linking. Binding of SIGK to this surface is able to block Gβγ-dependent activation of PLC, implying that it is a functional site for PLC binding.

Previous data suggesting that the Y-domain of PLCβ2 interacts with Gβγ subunits has been inferred through indirect experiments from peptide and small domain binding and inhibition assays. Other data have suggested involvement of the PH domain in βγ-dependent regulation of PLCβ (28). To conclusively demonstrate that the Y-domain is indeed important for Gβγ regulation of PLCβ2, we made mutations in the $α_5$ helix within the Y-domain on the surface of PLCβ corresponding to the last 10 amino acids N20K. PLCβ2(ELK/AAA) was significantly impaired with respect to Gβγ-dependent activation relative to wild type PLCβ2 (Fig. 6, C and D). However, none of the three mutations tested completely blocked Gβγ-dependent activation. Previous work by Illenberger et al. (29) demonstrated that replacement of the PH domain of PLCβ2 with the PH domain of PLCβ1 (a phospholipase poorly regulated by Gβγ subunits) blocked some but not all of the activation by Gβγ subunits, leading the authors to believe that interactions between Gβγ subunits and the catalytic domain of PLCβ2 were both necessary and sufficient for Gβγ-dependent activation. Thus, data suggesting the PH domain is important for PLC regulation by Gβγ subunits, taken together with the data presented here and previously, suggest that both the PH domain and the Y-domain of PLCβ2 play important roles in regulation of the enzyme by Gβγ subunits. We also found that PLC(ELK/AAA) was activated to a greater extent by Gα$_4$ relative to wild type PLCβ2. This is an interesting observation that needs to be investigated in further detail but clearly demonstrates that these mutants are selectively impaired with respect to Gβγ-dependent activation of PLC.

The results of this study suggest that the Y-domain of PLCβ2 can bind two distinct regions on the Gβγ subunit, the N-terminal α helix and the Gα subunit switch II binding interface. Fig. 7 outlines a simple model for PLC regulation through binding at these two distinct sites. In the model, binding of the Y-domain of PLCβ2 (shown as a triangle, with the N20K region of the Y-domain highlighted) to the amino terminus of Gβγ is inhibitory. This complex is in equilibrium with a Gβγ-PLC complex where the Y-domain is bound to the propeller region. This contact is stimulatory and necessary for enzymatic activation of PLC. During interactions between wild type PLC and wild type Gβγ, these two processes are in equilibrium, with the balance resulting in a net activation of the enzyme. For the Gβ$_2$(23–27) mutant, disruption of the interaction between the amino terminus of Gβγ and PLC relieved this inhibitory constraint, resulting in the enhanced activation. The N20K peptide was still able to block this mutant’s activation of PLC, because the stimulatory binding contact between the Y-domain and the blade region remained intact. Mutations to the N20K region within full-length PLCβ2 would be expected to reduce the ability of the enzyme to be activated by Gβγ, since binding to both the stimulatory and the inhibitory region would be equally affected. This was confirmed with the PLCβ2(ELK/AAA) mutant.

There are two simple mechanisms by which binding to the amino terminus of Gβγ could result in an inhibition in the activation of the enzyme. First, binding of the Y-domain to this
region would sequester it from binding to the propeller region, which is necessary to stimulate PLC activation, as shown by our N20K and SIGK competition data. Second, the N20K region within the Y-domain of PLCβ is within close proximity to the substrate binding site of the enzyme (14). Recent low resolution structures of Gβγ bound to membrane tubules place the N-terminal region of Gβγ far from the membrane surface (30). Therefore, if the Y-domain of PLCβ is bound to the N terminus, it would be sequestered from its substrate.

Some possible physiological implications for an inhibitory binding constraint within the amino terminus of Gβγ for PLCβ2 can be imagined. First, inhibitory binding to the amino terminus could be a mechanism for regulating the activation of the enzyme. There are data to suggest that G-proteins can negatively regulate PLC activity. It had previously been demonstrated that the addition of a nonhydrolyzable version of GTP, Gpp(NH)p, to solubilized membranes from cortical neurons, decreased inositol phosphate production at low concentrations, whereas it stimulated production at high concentrations (31, 32). Moreover, activation of Goα/ο-coupled receptors, such as the D2 dopamine receptor and the A1 adenosine receptors, could antagonize inositol phosphate production resulting from activation of the Goα-linked thyrotropin-releasing hormone receptor (24, 33, 34). These experiments suggest Gβγ could negatively regulate PLC activation in vivo. Alternatively, the amino terminus could be functioning as a scaffold, anchoring the PLC near its activator and its substrate, while the G-protein is in its inactive heterotrimer and the propeller region is blocked by Go. Upon activation of the G-protein signaling cascade and release of α-GTP, the Y-domain could dissociate from the amino terminus, and both the Y-domain and the PH domain of PLC could now make contact with the stimulatory areas within the propeller region, leading to enzyme activation.

Three novel conclusions have evolved from the course of this study. First, we clearly demonstrate that the amino-terminal coiled-coil region of Gβγ is directly involved in effector regulation in mammalian isoforms of Gβγ subunits, the first such data to map a mammalian effector binding site outside of the propeller region. Second, we show that mutations within the catalytic domain of PLCβ2 had reduced activation by Gβγ subunits, supporting previous data with synthetic peptides and GST fusion proteins suggesting that the Y-domain of PLC was important for regulation by Gβγ subunits. Finally, we show evidence suggesting that the N20K peptide may bind two distinct regions on the Y-domain subunit, leading to the possibility the Y-domain of full-length PLC may also bind Gβγ at two distinct sites as well. What is becoming clearly apparent with Gβγ regulation of effectors is that no single binding interaction defines all of the contact interfaces. Thus, the mechanisms for Gβγ binding and effector activation appear to be significantly more complex than had been previously imagined.

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