Intrinsic Pleckstrin Homology (PH) Domain Motion in Phospholipase C-β Exposes a Gβγ Protein Binding Site*

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Mammalian phospholipase C-β (PLC-β) isoforms are stimulated by heterotrimeric G protein subunits and members of the Rho GTPase family of small G proteins. Although recent structural studies showed how Goq and Rac1 bind PLC-β, there is a lack of consensus regarding the Gβγ binding site in PLC-β. Using FRET between cerulean fluorescent protein-labeled Gβγ and the Alexa Fluor 594-labeled PLC-β pleckstrin homology (PH) domain, we demonstrate that the PH domain is the minimal Gβγ binding region in PLC-β3. We show that the isolated PH domain can compete with full-length PLC-β3 for binding Gβγ but not Goq. Using sequence conservation, structural analyses, and mutagenesis, we identify a hydrophobic face of the PLC-β PH domain as the Gβγ binding interface. This PH domain surface is not solvent-exposed in crystal structures of PLC-β, necessitating conformational rearrangement to allow Gβγ binding. Blocking PH domain motion in PLC-β by cross-linking it to the EF hand domain inhibits stimulation by Gβγ without altering basal activity or Goq response. The fraction of PLC-β cross-linked is proportional to the fractional loss of Gβγ response. Cross-linked PLC-β does not bind Gβγ in a FRET-based Gβγ-PLC-β binding assay. We propose that unliganded PLC-β exists in equilibrium between a closed conformation observed in crystal structures and an open conformation where the PH domain moves away from the EF hands. Therefore, intrinsic movement of the PH domain in PLC-β modulates Gβγ access to its binding site.

Phospholipase C (PLC) isoforms integrate signaling inputs downstream of diverse receptors to catalyze the hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP2) in the inner leaflet of the plasma membrane. This reaction generates two products, inositol 1,4,5-trisphosphate and diacylglycerol, important second messengers and second messenger precursors that regulate multiple cellular processes (1). The mammalian genome encodes six families of PLCs (β, δ, γ, ε, η, and ζ) that share a conserved core architecture composed of a pleckstrin homology (PH) domain, four EF hands, a split TIM barrel, and a C2 domain. The TIM barrel contains the active site, and its two halves are separated by the so-called X-Y linker that is thought to occlude the active site and whose motion is central to the regulation of activity. In the PLC-β isoforms, the linker is highly cationic, flanked by a highly anionic region, and disordered. Sondek and co-workers (2) speculated that PLC recruitment to the plasma membrane by protein activators causes electrostatic repulsion of the linker by negatively charged phospholipids that moves it away from the active site to relieve autoinhibition. Proteolysis or genetic removal of the linker elevates basal enzyme activity across many PLC isoforms (3, 4), supporting the generality of this mechanism. However, mutant PLC-βs that lack this linker are activated further by their physiological ligands (2), suggesting the existence of additional regulatory mechanisms that are yet to be discovered.

The four mammalian PLC-β isoforms are stimulated to different extents by Goq subunits of the Gq family, Gβγ subunits, and members of the small Rho GTPase family. All PLC-β isoforms are stimulated by GTP-bound Goq, but Gβγ activates only PLC-β1, PLC-β2, and PLC-β3 (5). PLC-β2 is the most responsive to Gβγ stimulation and is also the only isoform that is significantly sensitive to the small G protein Rac1 (6). PLC-β3 responds less than 2-fold to Rac1 (7) but is stimulated more than 100-fold by both Goq and Gβγ (8). In previous work, we showed that PLC-β3 and, to a lesser extent, PLC-β2 respond synergistically to Goq and Gβγ (8). Such synergism indicates that the binding sites for these activators are independent and do not overlap but is consistent with a two-state allosteric model that requires only one mechanism of activation (8). The PLC-β isoforms are also GTPase-activating proteins for Goq (9), accelerating the hydrolysis of Goq-bound GTP to modulate the amplitude, duration, and response kinetics of signaling from Goq-coupled receptors (10).

Although the physiology of PLC-β-regulated signaling pathways is well studied, we have only begun to understand the structural basis of PLC-β activation. Crystal structures of PLC-β-bound to Goq (11) or Rac1 (12) established the independence of the binding sites for these activators. However, the overall conformation of PLC-β in these structures is remarkably similar to the conformation in the absence of bound activator. This similarity suggests the absence of any large-scale conformational changes during activation, except perhaps at the autoinhibitory X-Y linker, which is not visible and presumably disordered in all available structures. Recent studies identi-
tified a helix-turn-helix extension of the C2 domain that is bound to the TIM barrel in the unliganded structure but is refolded and bound to Goq upon complex formation (13), supporting the notion that PLC-β activation requires more than simple recruitment to the membrane. Mutational analysis suggested that this helix-turn-helix extension may stabilize the X-Y linker in its inhibitory conformation. Unlike the other PLC families, PLC-β isosforms possess an ~400-amino acid helical bundle C-terminal to this extension and are separated from it by a protease-sensitive and probably disordered linker. A PLC-β truncation mutant that lacks this helical domain binds Goq with about the same affinity as wild-type PLC but displays markedly reduced sensitivity to stimulation by Goq (14). Multiple single amino acid mutations in the helical domain also inhibit stimulation by Goq (15). However, the helical bundle is dispensable for stimulation by Gβγ or Rac1 (16).

In the absence of a co-crystal structure of the GβγPLC-β complex, the mechanism of PLC-β regulation by Gβγ is the least well understood. Gβγ stimulates the phospholipase activity of PLC-β but inhibits its GTPase-activating protein activity (17). There is no consensus on where Gβγ binds or how it coordinates the dual regulation of PLC-β activities. Two binding sites for Gβγ on PLC-β have been proposed. The N-terminal PH domain is required for stimulation by Gβγ. Removal of the PH domain abolishes Gβγ stimulation without blocking either Gβγ inhibition of GTPase-activating protein activity or stimulation of PLC activity by Goq (18). PLC-δ, an isoform not stimulated by Gβγ, gains sensitivity to Gβγ when its PH domain is replaced by the PLC-β PH domain and the first EF hand (19). Gβγ binds the PH domains of many proteins, including some with low nanomolar affinity (20), suggesting that Gβγ may bind to a conserved surface in PH domains. The second proposed site is a conserved helix in the C-terminal half of the TIM barrel. Smrcka and co-workers (21) showed that a peptide corresponding to this region inhibits Gβγ stimulation of PLC-β and that the peptide can be chemically cross-linked to Gβγ. Point mutations in this region also diminished the response to Gβγ (21). These two sites, the PH domain and this region of the TIM barrel surface, are not adjacent in the tertiary structure of PLC-β, and it is hard to envision how both could be involved in Gβγ binding unless there is substantial domain movement.

In this study, we developed a FRET-based assay for the binding of Gβγ to PLC-β and to its isolated PH domain, establishing the PH domain as the minimal Gβγ binding site in PLC-β. We also identify a Gβγ binding surface on the PH domain that is consistent with other known Gβγ-PH domain interfaces. This Gβγ binding surface is buried in the available crystal structures of PLC-β, and movement of the PH domain is therefore required for Gβγ-PLC-β binding and activation. We confirm this prediction by showing that anchoring the PH domain by cross-linking it to the EF hand domain blocks PLC-β activation by Gβγ. Based on these data, we propose a new model for PLC-β interaction with Gβγ in which the PH domain is in equilibrium between distinct positions and Gβγ binds and stabilizes a conformation needed for enzyme activation.

**Experimental Procedures**

**Proteins**—cDNA encoding PLC-β3 (human) with an N-terminal His6 tag was cloned into pQE60 for expression in Escherichia coli. Cysteines at positions 193, 516, 614, 892, 1005, 1176, and 1207 were predicted to be solvent-exposed and mutated to serine (1176 to valine) by multiple rounds of QuikChange mutagenesis. The activity and regulation of this modified PLC-β3 purified from E. coli was virtually indistinguishable from wild-type PLC-β3 expressed in S9 cells, and we refer to this mutant as wild-type throughout. Point mutations to introduce cysteine residues for cross-linking or fluorescence labeling were made in this background by QuikChange mutagenesis. To quantitate the fraction of intramolecular cross-linking in the Cys60,Cys164 construct, we introduced a TEV protease recognition sequence after Gly94 by overlap PCR (22) (G4Gsenlyfgagaaag95); the inserted sequence is in lowercase, and the numbers at the ends indicate native PLC-β3 residue numbers. We refer to this construct as PLC-TEV. All mutants were verified by sequencing over the entire PLC-β3 open reading frame. Wild-type PLC-β3 and variants were purified from transformed BL21DE3/p REP4 cells grown in T7 medium (2% Trp-tone, 1% yeast extract, 0.2% glycerol, 0.5% NaCl, 50 mM potassium P0, pH 7.2) and induced with 60–100 μM isopropyl-1-thio-β-D-galactopyranoside for 9 h at 25 °C. Frozen cell pellets were lysed with 0.5 mg/ml lysozyme in buffer A (20 mM NaHepes, 50 mM NaCl, and 10 μM PMSF) plus 500 mM NaCl. After sonication, the suspension was centrifuged for 30 min at 100,000 × g, and the supernatant was mixed with Ni2+-nitrotriacetic acid resin (Qiagen) for 2 h. The resin was washed sequentially with buffer A plus 500 mM NaCl and 5 mM imidazole and buffer A plus 100 mM NaCl and 5 mM imidazole until A280 reached baseline. PLC was eluted with buffer A plus 100 mM NaCl and 120 mM imidazole. The eluate was diluted into buffer B (20 mM NaMES (pH 6.0), 0.1 mM EDTA, 1 mM DTT, 0.2 mM PMSF, and 10% glycerol) and applied to a 1-ml SourceS column that was equilibrated with buffer B. After washing the column with 200 mM NaCl in buffer B, protein was eluted by a gradient of 200–500 mM NaCl in buffer B. Peak fractions were pooled, exchanged into buffer C (20 mM NaHepes, 100 mM NaCl (pH 7.5), 0.1 mM DTT, and 10% glycerol) and flash-frozen to −80 °C for storage. N-terminally His6-tagged PLC-β2 (mouse) was purified from baculovirus-infected insect cells as described earlier (8).

The PH domain (residues 2–147) of human PLC-β3 was amplified by PCR and cloned into a pET28 vector with an N-terminal MBP tag and a TEV protease recognition sequence (a gift from Neal Alto, University of Texas Southwestern Medical Center) (23). The resulting fusion protein had the sequence His6-MBP-His6-ENLYFQSG followed by the PLC-β3 PH domain. TEV protease cleaves after the Gln residue in ENLY-FQSG. Point mutations were made in this background by QuikChange mutagenesis. Proteins were expressed at 16 °C in BL21DE3 grown in T7 medium for 16–20 h after induction with 0.5 mM isopropyl-1-thio-β-D-galactopyranoside. Cells were lysed in buffer A plus 500 mM NaCl. After sonication, the lystate was centrifuged for 30 min at 100,000 × g. The superna-
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tant was bound to nickel-nitrotriacetic acid resin (Qiagen) for 2 h. The resin was washed sequentially with buffer A plus 500 mM NaCl and buffer A plus 100 mM NaCl. Bound protein was eluted with buffer A plus 100 mM NaCl and 150 mM imidazole.

To remove the MBP tag, proteins were incubated with 1% (w/w) TEV protease for ~16 h at 4 °C, followed by removal of the cleaved His6-MBP by passage over amylose resin (New England Biolabs). Proteins were further purified by size exclusion chromatography on a Superdex 75 10/30 column in 20 mM NaCl, 100 mM NaCl, and 1 mM DTT. Eluted protein was concentrated to and eluted from Gb1g2-CFP with 1% (w/w) TEV protease for 2 h. The resin was washed sequentially with buffer A plus 500 mM NaCl and 150 mM imidazole.

For quantification of the fraction of PLC cross-linked, the protein was buffer exchanged into 20 mM HEPES (pH 7.5), 100 mM NaCl to remove dithiothreitol. The protein was reacted with a 10-fold molar excess of BMOE before use. For competitive binding and competition data, the fraction of PLC cross-linked, the protein was buffer exchanged into 20 mM HEPES (pH 7.5), 100 mM NaCl to remove dithiothreitol. The fraction of PLC cross-linked was determined by iteratively simulating the experimental data using the total concentrations of each protein and the previously determined Kd of 550 nM for GBγ-CFP binding to PH-97Alx.
Results

**Gβγ Binds the PH Domain of PLC-β3**—Deletion of the PH domain in PLC-β abolishes regulation by Gβγ (19), suggesting that the PH domain is required for Gβγ interaction with the intact enzyme. To determine whether the PLC-β PH domain is a Gβγ binding site, we developed a FRET-based binding assay. We used CFP fused to Gβγ as the donor and covalently labeled the isolated PLC-β3 PH domain that has a single reactive cysteine (PH-A97C) with an Alexa Fluor 594 acceptor (PH-97Alx). As shown in Fig. 1, PH-97Alx quenches the fluorescence of Gβγ-CFP, accompanied by simultaneous enhancement of Alexa Fluor 594 acceptor fluorescence. We detected no quenching of CFP fluorescence when we added unlabeled PH domain or free dye to Gβγ-CFP or when the labeled PH domain was unfolded by heat denaturation prior to addition. By this assay, Gβγ binds the PH domain with a $K_d$ of 550 nM (Fig. 1B) in detergent solution.

Termination of Gβγ signaling in cells occurs through sequestration by Gao-GDP after GTP hydrolysis, and Gao-GDP blocks Gβγ stimulation of PLC-β3 (8). As predicted, Gao-GDP competes with PH-97Alx for Gβγ binding, as evidenced by the recovery of CFP donor fluorescence (Fig. 1C). The $IC_{50}$ for Gao-GDP was 30 nM, and correction for the affinity with which Gβγ binds PH-97Alx (Fig. 1B) (see “Experimental Procedures”) indicates that the $K_d$ for Gao-GDP-Gβγ binding is ~3 nM (range, 2–5 nM based on standard error of fit shown in Fig. 1B), consistent with the subunits existing as a constitutive heterotrimer when Gao is in the GDP-bound inactive state. Phosducin, another regulator of Gβγ signaling that shares the same interface on Gβγ as Gao-GDP (31), also competitively displaced PH-97Alx from the Gβγ-PH complex with an $IC_{50}$ of ~70 nM. From these data, the $K_i$ for Gβγ-phosducin binding is 30 nM, in good agreement with previous reports (29). These observations suggest that the assay reports on specific Gβγ-PH domain binding. PH-A97C also displaced PH-97Alx to bind Gβγ, although with a much higher $K_i$ (Fig. 1D) (see below).

To determine whether PH domain binding is necessary for Gβγ interaction with intact PLC-β, we asked whether the PH domain can inhibit Gβγ-stimulated PLC-β3 activity. As shown in Fig. 2, the addition of isolated PH domain inhibited Gβγ stimulation of PLC-β3 with an $IC_{50}$ of 18 μM and inhibition of 65% at the highest concentration tested. The PH domain did not alter the basal or Gq-GTPγS-stimulated activity of PLC-β3 at any concentration, indicating that the inhibition was an effect on Gβγ stimulation and not on the catalytic activity of PLC-β itself. These data indicate that the isolated PH domain
competes with the PH domain in intact PLC-β to specifically block the Gβγ-PLC-β3 interaction. Thus, we propose that the PH domain defines the minimal Gβγ binding region in PLC-β.

The data in Figs. 1–3 measure the affinity of Gβγ binding to the isolated PLC-β PH domain using several different experimental approaches: FRET-based binding measurement, the effect on stimulation of PLC-β catalytic activity, and competition in both of these assays. The tightest interaction is that of the direct binding of Gβγ-CFP to PH-97Alx, with $K_d \sim 0.5 \mu M$, and the weakest interactions are for competition by unlabeled PH domain for binding or activation in the presence of phospholipid vesicles. Disagreement among these values appears to arise both from the use of phospholipids rather than cholate in the various assay buffers and from the ability of Alexa Fluor labeling to increase affinity of binding. FRET-based binding and competition assays conducted in medium containing phospholipid vesicles report a lower-affinity interaction than when measured in cholate buffer, in agreement with the low-potency inhibition of PLC-β stimulation in vesicles. More significant is the observation that Alexa Fluor labeling increases affinity by 15- to 20-fold (Fig. 3B). Consistent with these observations, we observed only 10% inhibition of Gβγ-CFP-PH-97Alx binding by $\sim 80 \mu M$ PH-A97C in phospholipid-containing buffer. Although we do not understand the mechanism of these effects in any detail, the consistency of results from two assays, with full-length PLC and isolated PH domain, and under different conditions indicates that the variations do not undercut the interpretability of the binding interactions.

Rac1 and Gβγ Bind Distinct Surfaces on PLC-β—PLC-β2 and PLC-β3 are directly activated by the small GTPase Rac1. GTPγS-loaded Rac1 binds intact PLC-β with a $K_d$ of $\sim 25 \mu M$, similar to that for the isolated PH domain (32). Structural studies later confirmed the PH domain as the Rac1 binding site (12). We therefore asked whether Rac1 competes with Gβγ for stimulation of PLC-β activity using PLC-β2, the most Rac1-sensitive isoform. As observed previously (7), activation by Rac1 and Gβγ was essentially additive even at near-saturating concentrations of either ligand (Fig. 4). Further, earlier studies showed that point mutations within the PH domain diminish Rac1-stimulated activity without altering Gβγ-stimulated activity (12). These results indicate that both Rac1 and Gβγ interact with the PLC-β PH domain through distinct binding sites that do not overlap.

**Prediction of the PH Domain-Gβγ Binding Interface by Structural Homology—Because Gβγ binds PH domains in many proteins, it is likely that it recognizes a common PH domain surface. The GRK2 PH domain-Gβγ interface has been extensively characterized biochemically (33) and structurally (34). The top face of the Gβ propeller makes extensive contact with strands β3 and β4 of the GRK2 PH domain core and with an unstructured, basic C-terminal extension past the PH domain, burying $\sim 2200 \AA^2$ of surface area (34). The PH domain in PLC-β adopts a fold similar to the GRK2 PH domain (root mean square deviation 3.8 Å for equivalent Cα atoms) (Fig. 5A), although it does not possess an analogous extension and is immediately followed by the EF hand domain. However, the β strands of the PH domain structurally align with the equivalent GRK2 regions even though the Gβγ contact residues are not strictly conserved (Fig. 5B). Furthermore, we noted that PLC-β1, PLC-β2, and PLC-β3 display a stronger conservation of residues in this region in comparison with PLC-β4, the only isoform that is not detectably stimulated by Gβγ (5).

To test whether the PH domains of GRK2 and PLC-β3 bind Gβγ in a similar manner, we examined the role of strands β3 and β4 in the PLC-β PH domain by mutational analyses. The hydrophobic character of this region suggested that Gβγ-PH domain binding is driven by non-polar interactions. To increase the likelihood that a single amino acid substitution at this interface would result in significant perturbation, we chose to introduce the large polar amino acid glutamine. We found that replacement of a conserved hydrophobic residue with glutamine (F50Q) was sufficient to completely abolish binding to Gβγ (Fig. 5C). Consistent with our results, a GRK2 mutant harboring a substitution at the same position in the PH domain (GRK2 R587Q) was insensitive to stimulation by Gβγ (33). Because PLC-β4 is not activated by Gβγ, we predicted that replacement of PLC-β3 residues in this region with the corresponding PLC-β4 residues would diminish Gβγ binding affinity. We picked three positions, Tyr35, Thr35, and Asp62, which were conserved across Gγ-responsive PLC-β isoforms but not in PLC-β4 (Asp62 is replaced by Glu in PLC-β1 and PLC-β2). Mutations at two of these residues, T35R and D62Q, displayed weaker binding to Gβγ with affinities 1.5- and 3-fold weaker than the wild-type protein. These results suggest that residues from strands β3 and β4 in the PH domain are important and specific binding determinants for Gβγ.

Next, we asked what effect these mutations have on the ability of the PH domain to compete with intact PLC-β3 for Gβγ binding. If a mutation weakened PH domain-Gβγ binding, then it would be predicted not to impede Gβγ stimulation of PLC-β3 catalytic activity. Addition of wild-type PH domain inhibited Gβγ stimulation of PLC-β3, as observed earlier. The PH (F50Q) mutant, which does not bind Gβγ, inhibited Gβγ-stim-
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**FIGURE 3. Effect of vesicles and labeling on Gβγ interactions.** A, equilibrium binding of Gβγ to the PH domain in the presence of phospholipid vesicles was measured by FRET. Data show the percent of CFP fluorescence that is quenched. Data are the average and range of measurements from two separate experiments, with measurements done once in each experiment. The solid line shows the fit to a single-site competition model with a $K_d$ of 7.6 μM and maximal quenching of 57%. Note the difference in concentration of PH-97Alx from Fig. 1B. B, competition by PH-A97C (○) or PH-97Alx (●) for stimulation of PLC-β3 by 10 nM Gβγ. Error bars show the range for duplicate samples in one of two separate experiments. The solid lines show fits to single-site competition models. The IC$_{50}$ values in the two experiments were 0.7 and 1.4 μM for PH-97Alx and 10.5 and 16.3 μM for PH-A97C.

**FIGURE 4. Rac1 and Gβγ bind distinct regions on PLC-β.** A, stimulation of PLC-β2 by Gβγ was measured in the presence (●) or absence (○) of 15 nM Rac1. Error bars show the range from duplicate measurements in a single experiment. The solid lines show fits to saturation functions with EC$_{50}$ values of 13 and 17 nM, respectively. A separate experiment with a different batch of proteins gave similar results, with EC$_{50}$ values of 9.5 and 12 nM. B, Rac1-stimulated PLC-β2 activity was assayed in the presence (●) and absence (○) of 15 nM Gβγ. Data show average and range from two independent measurements done in duplicate. The solid lines are fits to saturation functions with EC$_{50}$ values of 6.7 and 5.5 nM.

![Graph A](image1.png)  
![Graph B](image2.png)

ulated activity by less than 10% so that PLC-β3 activity was within error of the control sample (Fig. 5D). The T55R mutation in the PH domain also reduced its ability to inhibit stimulation by Gβγ, although loss of inhibition was only partial. PH (T55R) inhibited Gβγ-stimulated activity by 45% at 30 μM, the highest concentration tested, compared with 65% for the wild-type PH domain. This loss is consistent with the 1.5-fold reduction in its affinity for Gβγ. The PH (D62Q) mutant, which had 3-fold lower affinity for Gβγ, was similarly compromised in its ability to compete with PLC-β3 for Gβγ binding. At 10 μM, inhibition of Gβγ-stimulated PLC-β3 activity by PH(D62Q) was insignificant, whereas maximal inhibition was only 20% at the highest concentration tested.

Collectively, these data support our hypothesis that strands β3 and β4 in the PH domain form the Gβγ binding face. These results also suggest that the presence of polar residues in PLC-β4 in this region interferes with productive Gβγ interaction, rendering it insensitive to activation by Gβγ.

The Predicted Gβγ Binding Interface of PLC-β Is Not Surface-exposed—To understand how Gβγ interacts with PLC-β, we examined the predicted Gβγ binding residues of the PH domain in PLC-β crystal structures. The interface formed by these residues is distinct from the footprint of Rac1 on the PH domain of PLC-β2 (Fig. 6A), consistent with the lack of competition between Rac1 and Gβγ for binding PLC-β (Fig. 4). Although the Rac1 binding surface is completely surface-exposed, the predicted Gβγ binding face of the PH domain forms an intramolecular interface with the EF hands that buries close to 1200 Å$^2$ of total accessible surface area. Close inspection of the PLC-β structure reveals that this interface is composed of only a few hydrogen bonds and van der Waals contacts, suggesting that the interaction between the two surfaces is weak (Fig. 6B). The hydrogen bond interactions between the PH domain and EF hand residues involve residues whose side chain donor and acceptor atoms are 3–4 Å apart, significantly weaker in energy than the average hydrogen bond lengths observed in proteins (~1.5–2 Å) (35). Taken together with the absence of electrostatic interactions or water bridges at this interface, we hypothesized that the PH domain is mobile in solution, existing in equilibrium between the conformation observed in crystal structures and an “open” conformation where the PH domain moves so that the residues required for Gβγ binding become accessible.

If the PH domain of PLC-β has to swing away from the EF hand domain to bind Gβγ, then preventing mobility of the PH domain should obstruct Gβγ access to its binding site. We used intramolecular cross-linking to restrict PH domain motion by introducing cysteines in the PH domain and EF hands to give PLC-β3-Cys$^{60}$,Cys$^{164}$. These residues lie at the solvent-exposed edge of the PH domain-EF hand interface (Fig. 7A) so that they can be cross-linked using BMOE, an 8-Å cysteine-reactive cross-linker. Irreversible cross-linking of these residues should
block the motion of the PH domain and stabilize it in the conformation observed in PLC-β crystal structures. As predicted, cross-linking PLC-β-Cys\(^{60},\)Cys\(^{164}\) inhibited Gβγ-stimulated PLC-β activity (Fig. 7B) without blocking stimulation by Gα\(_q\)-GTPγS (Fig. 7C). Alkylation of PLC-β-Cys\(^{60},\)Cys\(^{164}\) with a monovalent maleimide or alkylation with BMOE of single-cysteine mutants, PLC-β-Cys\(^{60}\) or PLC-β-Cys\(^{164}\), had no effect. These data indicate that intramolecular cross-linking at the PH domain-EF hand interface interferes with Gβγ-PLC-β interaction uniquely without global effects on PLC-β structure or catalytic activity. BMOE cross-linking of PLC-β-Cys\(^{60},\)Cys\(^{164}\) decreased the maximal Gβγ-stimulated activity of cross-linked PLC-β only partially under optimized conditions, but the EC\(_{50}\) for Gβγ was unchanged (~20–30 nM, Fig. 7D). If cross-linking partially blocked a Gβγ binding site on PLC-β, we would predict an increase in EC\(_{50}\) for Gβγ activation because of decreased affinity rather than a decrease in maximal response.

The data in Fig. 7D suggest that BMOE cross-linking is incomplete, with the cross-linked PLC insensitive to Gβγ and the rest retaining full sensitivity to Gβγ. Initial attempts to quantify cross-linking failed because the electrophoretic mobility of cross-linked PLC-β was indistinguishable from that of the control, and mass spectrometry gave poor peptide coverage in the relevant regions. We therefore designed a mutant PLC-β to quantify the fraction of cross-linked species. We introduced a TEV protease cleavage site in an exposed loop of the PH domain of PLC-β-Cys\(^{60},\)Cys\(^{164}\) to give a construct we refer to as PLC-TEV. Proteolytic cleavage of PLC-TEV should generate two separate polypeptides but would produce a single species in the enzyme that had been correctly cross-linked between Cys\(^{60}\) and Cys\(^{164}\) (Fig. 8A). The relative sizes of cross-linked and non-cross-linked species after proteolysis are such that they can be separated by gel electrophoresis. Treatment of PLC-TEV with protease yielded a species that migrated faster on a SDS-PAGE gel because of loss of the ~10-kDa N-terminal fragment (Fig. 8A). Treatment with the cross-linker prior to proteolysis abrogated this shift as expected, confirming that this strategy can be used to quantitate the fraction of PLC-β cross-linked. To test whether cross-linking proceeds to completion, we incubated PLC-TEV with the cross-linker for varying times and measured the fraction of cross-linked species. Cross-linking occurred rapidly, on the order of minutes, but plateaued with only ~70% of total PLC-TEV cross-linked after 30 min (Fig. 8B). Changing the pH or temperature or including a mild reducing agent, tris(2-carboxyethyl)phosphine, in the cross-linking buffer did not increase fractional cross-linking. Incubating a fixed concentration of protein with increasing amounts of BMOE gave similar results. We then analyzed the Gβγ response of these species and observed a negative correlation between the fraction of cross-linked PLC-β and its stimulation by Gβγ (Fig. 8C). This result indicates that the residual activity observed in Fig. 7D was from PLC-β that had not been cross-linked. Importantly, stimulation by Gα\(_q\)-GTPγS was not dependent on the cross-linked status. Therefore, our observations are consistent with the idea that Gβγ is unable to stimulate PLC-β when the PH domain is immobilized by tethering to the first EF hand.

Restraining PH Domain Motion Blocks Gβγ Binding—Our results suggested that cross-linking PLC-β leads to reduced Gβγ sensitivity by prohibiting its binding to the G protein. We
developed a FRET-based assay for Gβγ interaction with PLC-β3 to directly test whether cross-linking blocks Gβγ-PLC-β binding. We labeled PLC-β3 at Cys97 in the PH domain with Alexa Fluor 594 (PLC-97Alx), as we had done for the isolated PH domain, and asked whether PLC-97Alx is a FRET acceptor for Gβγ-CFP. PLC-97Alx quenched Gβγ-CFP fluorescence as predicted, similar to PH-97Alx. The Kd for Gβγ-PLC-β3 binding was 220 nM (Fig. 9A). Unlabeled PLC-β3 inhibited binding between Gβγ-CFP and PLC-97Alx with an IC50 of 370 nM (Fig. 9B), equivalent to a Ki of 170 nM, which is approximately equal to the directly determined Kd for labeled PLC-β3. These data indicate that the assay reports on specific binding between Gβγ and PLC-β3.

We then used this assay to ask whether cross-linked PLC-β binds Gβγ. Because BMOE and Alexa Fluor 594 both react with the sulphydryl group of cysteines, we could not label cross-linked PLC-β with Alexa Fluor 594. Rather, we used competition to test the binding of cross-linked PLC-β3 to Gβγ. We found that PLC-TEV treated with DMSO competes with PLC-97Alx for Gβγ binding with an IC50 of ~360 nM or a Ki of ~150 nM. (Fig. 9C), which agrees with the Ki for wild-type PLC-β3 (Fig. 9B). BMOE-treated PLC-TEV also competed for binding (Fig. 9C, inset), but PLC-TEV treated with BMOE is not completely cross-linked and competition apparently reflects only the residual non-cross-linked PLC (see above). The TEV proteolysis assay indicated that 35% of BMOE-treated PLC-TEV used in the experiment in Fig. 9C was not cross-linked, and correction for fractional cross-linking indicated that only the non-cross-linked fraction accounts for the observed inhibition (Fig. 9C). Collectively, these results indicate that PLC-β does not bind Gβγ when cross-linked.

Discussion

Stimulation of PLC-β isoforms by Gβγ is well established, but the identity of the binding site for Gβγ has eluded consensus. In this study, we show that the N-terminal PH domain is the minimal

FIGURE 6. The predicted Gβγ binding face of the PH domain is not surface-exposed. A, top panel, schematic of PLC-β. The C-terminal helical bundle is not shown. Bottom panel, crystal structure of PLC-β3 with the PH domain depicted as a surface (PDB code 2ZKM). Residues predicted to be involved in Gβγ binding based on homology with the GRK2-Gβγ structure (PDB code 1OMW) are shown in red. This surface is not solvent-accessible and does not overlap with the region that binds Rac1 (green). B, details of interactions between the PH domain and EF hands in intact PLC-β3. Residues are colored to match the schematic in A. The numbers indicate the distance between donor and acceptor atoms for residues involved in hydrogen bonds.
Identification of the Gβγ-binding surface led to a new structural model for Gβγ binding that involves substantial movement of the PH domain that may help anchor and orient PLC-β at the bilayer surface for PIP2 hydrolysis. This work also describes a reliable and quantitative assay for binding of Gb1g2 to PLC-β3 that should be applicable to any PLC-β and Gβγ dimer.
The FRET-based binding assays indicate that Gβγ binds similarly to both the isolated PH domain of PLC-β3 and to intact PLC-β. The free PH domain also inhibited PLC-β activation by Gβγ, but not Gαi (Fig. 2), by competing with the intact enzyme for binding Gβγ. Binding of Gβγ to the PH domain was inhibited by Gαi-GDP, consistent with the idea that Gαi sequesters Gβγ in the Gi heterotrimer until Gi is activated by GTP.

We defined the Gβγ binding surface of the PLC-β PH domain starting with analogy to the crystal structure of Gβγ in complex with GRK2. PH domains in multiple proteins are implicated in Gβγ binding (20), and, of these, Gβγ binding to the PH domain of GRK2 is the best studied (34). The crystal structure of Gβγ in complex with GRK2 shows that Gβγ recognizes a hydrophobic face of the GRK2 PH domain, and this surface was readily mapped to homologous residues of the PLC-β PH domain (Fig. 5). The effect of mutating residues on this face of the PH domain confirms the importance of this hydrophobic surface for regulation and binding by Gβγ. Mutation of the conserved Phe30 at this surface to a polar residue abolished Gβγ binding and inhibition of Gβγ-stimulated PLC-β3 activity, and milder mutations at other residues diminished binding to a lesser extent. Previous studies showed that point mutations at Ile80, Trp99, Met101, Leu117, and Trp332 in the opposing surface of Gβ reduced stimulation of PLC-β (36, 37). Taken together, these data suggest that non-polar rather than electrostatic interactions contribute most of the free energy of binding between Gβγ and the PH domain.

In the GRK2-Gβγ complex, a cluster of Lys/Arg residues in an extension past the GRK2 PH domain also interacts with the Gβγ surface. Strikingly, the first EF hand domain (EF-1) immediately C-terminal to the PH domain in all four PLC-β isoforms is similarly rich in Lys/Arg residues, and Barr et al. (38) showed that a quadruple charge-reversal mutation in EF-1 is less responsive to Gβγ. They also showed that a GST fusion construct of the PH domain plus EF-1 bound Gβγ in pulldown experiments but that the charge reversal mutation reduced binding. In that same study, Gβγ also bound a fusion construct of GST and the PH domain alone, in agreement with our observation that the PH domain is the minimal Gβγ binding region, but just the PH domain was less efficient in pulldowns than the
PH plus EF-1 construct. These data argue that Gβγ minimally binds the PH domain and that interactions of basic residues in EF-1 may enhance affinity.

The Gβγ-interacting, hydrophobic face of the PH domain we identified is surprisingly buried in an intramolecular interface with the EF hands in crystal structures of PLC-β (Fig. 6) so that a substantial conformational change would be required to allow Gβγ binding. We used intramolecular cross-linking of the PH domain to the EF hands to confine the PH domain in the conformation observed in PLC-β crystal structures. Consistent with our proposal, immobilizing the PH domain makes PLC-β refractory to stimulation by Gβγ (Figs. 7 and 8). Furthermore, we showed that Gβγ failed to bind PLC-β in which the PH domain was immobilized (Fig. 9). The Gαq response did not change when PLC-β was cross-linked, indicating that the impaired interaction of cross-linked PLC-β was Gβγ-specific. However, cross-linking-mediated immobilization also likely blocked access to the PH domain surface that faces the TIM barrel. We tested various mutants in this region and found no change in PH domain inhibition of Gβγ-stimulated PLC-β activity, suggesting that this surface is not involved in Gβγ binding.

**Gβγ-PLC-β Binding Requires Movement of the PH Domain**

If our identification of the Gβγ binding interface in PLC-β is correct, then there must be significant movement to expose it because it is mostly occluded in the resting PLC-β structure by juxtaposition to the EF hand domain (Fig. 6). Furthermore, much of the exposed surface of the PH domain is needed for the Rac1 binding site, and we showed that Rac1 and Gβγ can bind PLC-β simultaneously (Fig. 4). To allow regulatory Gβγ binding, we propose that PLC-β exhibits intrinsic flexibility between closed and open conformational states of the PH domain (Fig. 10). The closed state, in which the PH domain faces the EF hands, is favored under crystallization conditions and, presumably, in solution in the absence of activators. PLC-β can, however, transiently sample an open state in which the PH domain moves away from the EF hands to expose the hydrophobic Gβγ binding face and, by a still not understood mechanism, stimulate catalytic activity. The need for such motion is supported by the observation that locking PLC-β3 in the closed state by cross-linking the PH domain to EF-1 blocks both Gβγ binding and the ability of Gβγ to activate (Figs. 8 and 9).

Crystallography of PLC-δ1, the PH domain and EF-1 were both invisible, suggesting that they undergo significant motion even in crystals (39, 40). Truncation of the PH domain alone stabilized PLC-δ so that its structure could be determined. Further, Drin et al. (41) showed that Gβγ-PLC-β binding reduced intramolecular FRET between the PH domain and the TIM barrel, suggesting that movement of the PH domain away from the TIM barrel to an open state is coupled to Gβγ binding (41). The transition from the closed to the open conformation in PLC-β may also involve loss of EF-1 structure. Unfolding EF-1 would achieve twin objectives: higher affinity Gβγ binding from interaction with the EF-1 basic residues (38), as discussed above, and a longer tether to allow whatever PH domain movement is needed for Gβγ to bind in an appropriately activating orientation.

The requirement for PH domain motion in PLC-β activation by Gβγ does not produce a detailed model for the PLC activation process, but it is consistent with a general model for PLC activation proposed by Sondek and co-workers (2). PLC-β contains an anionic autoinhibitory strand that occludes the active site and that must be moved to allow activation (1). Sondek and co-workers proposed (2) that simply holding the PLC molecule tightly against the bilayer surface shoves the autoinhibitory X-Y linker away from the active site by electrostatic and/or steric repulsion by negatively charged lipids (1). Although we do not know precisely how the Gβγ-PLH domain complex is oriented with respect to the bilayer, anchoring PLC-β at the membrane surface is an important part of the Gβγ activation mechanism. Gβγ with a non-prenylated Gγ does not bind to membranes and does not activate PLC-β (42). Less direct support for the role of bilayer anchorage comes from studies of a PLC chimera with a PIP2-binding PH domain (43).

A mobile PH domain can allow binding of PLC-β to Gβγ and consequent activation, but this mechanism by itself does not constrain PLC-β orientation. Such a simple mechanism might, however, explain why Gβγ is a less efficacious activator of PLC-β than Gαq. Gαq binds PLC-β by contacting regions in the EF hand domain, the C2 domain, and a helical extension immediately C-terminal to the C2 domain (11) and may thereby help orient PLC-β with its active site facing the bilayer to facilitate access to the PIP2 substrate. Gαq binding has also been proposed to relieve autoinhibition by moving the helical extension away from the autoinhibitory strand to allow it to move away from the active site (13). There are no data to suggest that Gβγ
performs either function and may therefore be a weaker activator.

Mobility of the PH domain in binding Gβγ may also reconcile PH-Gβγ binding with the suggestion by Smrcka and coworkers (21) that Gβγ has a second binding site on PLC-β on the TIM barrel domain. This site is essentially on the side of PLC-β opposite from that of the PH domain in crystal structures. If the PH domain exhibits significant motion, it is possible that Gβγ binds simultaneously to the PH and TIM barrel domains, although this proposal awaits rigorous investigation.

Our work suggests the presence of a previously unappreciated PLC-β conformation critical to Gβγ binding and regulation of phospholipase activity. PLC-β is also synergistically activated by Goq and Gβγ (8), and the Gq GTPase-activating protein activity of PLC-β is inhibited by Gβγ (17). How the physical and functional interactions of Gβγ with PLC-β and Goq direct the dynamics of complex assembly/disassembly in this three-protein system is uncertain. Studies focused on visualizing these interactions will shed light on the mechanisms coordinating Gβγ regulation of PLC-β activities.

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