The Pleckstrin Homology Domain of Phospholipase Cβ Transmits Enzymatic Activation through Modulation of the Membrane−Domain Orientation†

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ABSTRACT: Phospholipase Cβ (PLCβ) enzymes are activated by Gαq and G$\beta\gamma$ subunits and catalyze the hydrolysis of the minor membrane lipid phosphatidylinositol 4,5-bisphosphate [PI(4,5)P_2]. Activation of PLCβ_2 by G$\beta\gamma$ subunits has been shown to be conferred through its N-terminal pleckstrin homology (PH) domain, although the underlying mechanism is unclear. Also unclear are observations that the extent of G$\beta\gamma$ activation differs on different membrane surfaces. In this study, we have identified a unique region of the PH domain of the PLCβ_2 domain (residues 71−88) which, when added to the enzyme as a peptide, causes enzyme activation similar to that with G$\beta\gamma$ subunits. This PH domain segment interacts strongly with membranes composed of lipid mixtures but not those containing lipids with electrically neutral zwitterionic headgroups. Also, addition of this segment perturbs interaction of the catalytic domain, but not the PH domain, with membrane surfaces. We monitored the orientation of the PH and catalytic domains of PLC by intermolecular fluorescence resonance energy transfer (FRET) using the G$\beta\gamma$ activatable mutant, PLCβ2/δ1(C193S). We find an increase in the level of FRET with binding to membranes with mixed lipids but not to those containing only lipids with electrically neutral headgroups. These results suggest that enzymatic activation can be conferred through optimal association of the PHβ71−88 region to specific membrane surfaces. These studies allow us to understand the basis of variations of G$\beta\gamma$ activation on different membrane surfaces.

Phospholipase C enzymes are intracellular enzymes that catalyze the hydrolysis of lipid phosphatidylinositol 4,5-bisphosphate [PI(4,5)P_2][1] to give 1,4,5-inositol trisphosphate (IP_3) and diacylglycerol (DAG) which in turn promote the release of Ca^{2+} from intracellular stores and the activation of protein kinase C. There are several families of mammalian PLCs whose tissue distribution and cellular regulation differ. The PLCβ family is regulated by heterotrimeric G proteins. All four known subtypes of PLCβ are activated by Gαq subunits, while two (PLCβ_2 and PLCβ_3) are additionally activated by G$\beta\gamma$ subunits (*1*, *2*).

PLCβs are multidomain enzymes consisting of an N-terminal PH domain, four EF hands, a catalytic X/Y domain, a C2 domain, and a C-terminal tail. While the only domain of PLCβ whose structure has been determined is the C-terminal tail (*3*), high-resolution structures of the PH (*4*) and catalytic-C2 domains (*5*) of the closely related enzyme PLCδ_1 are available. The relatively high degree of sequence homology between PLCβ_2 and PLCδ_1 has allowed the construction of theoretical models of the PH and catalytic domains of PLCβ_2 (*6*−*8*).

The ability of PLC ancillary domains to regulate the activity of the catalytic core has been studied. The C2 domain and C-terminal tail play key roles in regulating binding and activation of Gαq subunits (*9*, *10*). The N-terminal PH domain of PLCβ_2 serves a dual role of anchoring the enzyme to the membrane surface and securing binding and activation by G$\beta\gamma$ subunits (*11*, *12*). In parallel, the PH domain of PLCδ_1 binds strongly and specifically to PI(4,5)P_2 (*13*), resulting in activation (*8*, *14*). The importance of the PH domain in conferring activation can be clearly seen through studies showing that swapping the PH domain of PLCβ_2 into PLCδ_1 results in a PLCβ2/δ1 chimera possessing the same G$\beta\gamma$ and membrane binding properties as PLCβ_2, and which can be identically activated by G$\beta\gamma$ subunits (*15*). Similarly, swapping the PH domain of PLCδ_1 into PLCβ_2 results in a PLCβ2/δ1 chimera that can no longer be activated by G$\beta\gamma$ but instead can be activated by PI(4,5)P_2 in a manner similar to that of PLCδ_1 (*8*). The observation that activation of PLCβ_2 and PLCδ_1 can be conferred through binding of the PH domain to a membrane activator leads to the suggestion that the orientation of the enzyme with respect to the membrane surface and surface-associated proteins may be

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[1] Abbreviations: acrylodan, 6-acryloyl-2-(dimethylamino)naphthalene; CM-SE, 7-(dimethylamino)coumarin-4-acetic acid succinimidyl ester; CPM, 7-(diethylamino)-3-(4′-maleimidylphenyl)-4-methylcoumarin; DABCYL-SE, 4-{[4-(dimethylamino)phenyl]azo}benzoic acid succinimidyl ester; IP_3, inositol 1,4,5-trisphosphate; Laurdan, 6-lauroyl-2-(dimethylamino)naphthalene; LUVs, large unilamellar vesicles; PLC, mammalian phosphosinositide-specific phospholipase C; PH, pleckstrin homology; PI(4,5)P_2, phosphatidylinositol 4,5-bisphosphate; POPE, 1-palmitoyl-2-oleoylphosphatidylethanolamine; POPS, 1-palmitoyl-2-oleoylphosphatidylserine; POPC, 1-palmitoyl-2-oleoylphosphatidylcholine.

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critical in regulating catalytic activity.

Since PLC works on lipid surfaces, it is not surprising that enzyme activity and activation depend strongly on the physical properties of the membrane surface. It has been shown that the activity of PLCβ_2 and the activity of PLCδ_1 depend on the matrix in which their PI(4,5)P_2 substrate is immersed. For example, while sphingomyelin lipids inhibit PLCδ_1 (*16*), fatty acids promote its activity (*17*). PLCβ_1 has a higher activity in the presence of an excess of PA lipids (*18*), while the binding of PLCβ_2 is somewhat promoted by the inclusion of PE lipids (*11*). It is generally found that inclusion of PE in assays improves the ability of PLCβ_2 to be activated by G$\beta\gamma$ subunits (*19*), but the mechanism for this is unclear. It is possible that activation may be promoted by the expansion of the membrane surface due to the reduced size and polarity of PE headgroups which may allow better membrane penetration into the surface to access substrate (see ref *20*).

In this study, we continued our previous work aimed at understanding the role of the PH domain of PLCβ_2 in conferring G$\beta\gamma$ activation. We began with the observation that PHβ_2 contains a unique sequence (PH71−88) which can interact with membranes. Surprisingly, addition of a peptide having this sequence to PLCβ_2 simulates its activity to an extent similar to that with G$\beta\gamma$. Combining these two observations, we find that this peptide can affect the membrane orientation of the enzyme. These studies lead to a model in which activation of PLCβ_2 by G$\beta\gamma$ involves critical alignment of the catalytic core with the membrane surface which is mediated by PH71−88.

MATERIALS AND METHODS

Protein Expression and Purification. His6-PLCβ2/δ1 and mutants, obtained by using the site-directed mutagenesis kit from Stratagene, were expressed for 20 h at room temperature in *Escherichia coli* BL21D3 growing in Superbroth medium after an induction at an OD of 0.6 with 1 mM IPTG. The bacteria were harvested by centrifugation at 6000*g*. The pellet was resuspended in lysis buffer [50 mM NaH_2PO_4 (pH 8), 300 mM NaCl, and 10 mM imidazole] containing 1 mM PMSF, 2.5 mM benzamidine, and 1 mM DTT and supplemented with a protease inhibitor cocktail (Sigma), and the bacteria were broken. The lysate was treated for 30 min with DNAse I in the presence of Mg^{2+} and with RNAse A. The cell material was centrifuged for 90 min at 140000*g* and 4 °C, and the supernatant was loaded three times on a Ni^{2+}−NTA column (Qiagen). After the column had been washed twice with buffer containing 20 mM imidazole, the protein was eluted with 250 mM imidazole. Fractions showing the highest PLC activity were pooled and diluted 10 times in 25 mM Tris (pH 7.4) buffer with 1 mM DTT and brought to a final NaCl concentration of 30 mM. The material was loaded onto a 15Q column and eluted with a NaCl gradient from 0 to 500 mM.

His6-PLCβ_2 was expressed using a *Sf*9 expression system and purified as described with minor modifications (*21*). G$\beta_1\gamma_2$ subunits were prepared by coexpression of Gαq, Gβ_1, and His6-Gγ_2 in *Sf*9 cells and purified on a Ni^{2+}−NTA column (*22*). This construct activates PLCβ_2 in a manner identical to that for the non-His-tagged counterpart (*15*).

The purity of proteins was assessed by electrophoresis on a SDS−PAGE gel, and concentrations were determined with a Bradford assay (Bio-Rad) or on a SDS−PAGE gel with known concentrations of BSA for reference.

Peptides. PHβ71−88 (NH_2-KFAKMPKSQKLRDVFNMD-$CONH_2$), sPHβ71−88 (NH_2-VSKQDLKMFNKMRDAPFK-$CONH_2$), and PHβ120−135 (NH_2-ENVGKAWAEDVLA-LVK-$CONH_2$) were purchased from the American Peptide Co. (Sunnyvale, CA) with a purity of >90% as confirmed by analytical HPLC and mass spectrometry and were used as received. The peptides Gβ86−105 and Gβ86−105-(M101N) are gifts from R. Iyengar (Department of Pharmacology, Mt. Sinai School of Medicine, New York, NY). The concentrations of PHβ71−88 and sPHβ71−88 were estimated by a BCA assay (Pierce), whereas the concentration of other peptides is estimated by absorption at 280 nm.

Lipids. 1-Palmitoyl-2-oleoylphosphatidylethanolamine (POPE), 1-palmitoyl-2-oleoylphosphatidylserine (POPS), 1-palmitoyl-2-oleoylphosphatidylcholine (POPC), and phosphatidylinositol 4,5-bisphosphate [PI(4,5)P_2] in chloroform were purchased from Avanti Polar Lipids, Inc. (Alabaster, AL), whereas [^{3}H]PI(4,5)P_2 was from New England Nuclear, Inc.

Enzyme Activity Studies. Measurements of PI(4,5)P_2 hydrolysis by PLC enzymes were carried out using small, unilamellar vesicles composed of POPE, POPS, and PI(4,5)-P_2 at a 66:32:2 molar ratio and doped with enough [^{3}H]PI-(4,5)P_2 to obtain approximately 10 000 dpm/assay. The purified enzymes were diluted to 1−3 nM in 15 μL of Ca^{2+}-free MAIN buffer [50 mM Hepes (pH 7.2), 160 mM KCl, 3 mM EGTA, and 1 mM DTT]. G$\beta\gamma$ subunits were dialyzed three times in a Slide-ALyzer (Pierce) with a molecular mass cutoff of 3500 Da, for 30 min at 4 °C against 50 mM Hepes (pH 7.4), 150 mM NaCl, and 1 mM DTT buffer. When appropriate, enzymes were premixed with G$\beta\gamma$ subunits or with an identical volume of G$\beta\gamma$ stock buffer dialyzed as mentioned above.

To measure PLC activity, the proteins (15 μL) were placed on ice and mixed with 10 μL of 3 mM freshly sonicated lipids and 5 μL of MAIN buffer containing 12 mM $CaCl_2$ to give a final Ca^{2+} concentration of 1000 nM (*11*). The reaction was initiated by placing the samples in a water bath at 37 °C for 30 s to 5 min (depending on the phospholipase) and terminated by the addition of 0.2 mL of ice-cold 10% trichloroacetic acid, followed by 0.1 mL of cold 1% BSA. The aqueous and organic phases were separated by centrifugation (5 min at 7200*g*), and 0.27 mL of the upper aqueous phase, mixed with 2 mL of scintillation liquid, was counted by liquid scintillation. All experiments were done in triplicate and the samples compared to control samples containing all the reaction components but carried out on ice for the same incubation time.

Fluorescence Labeling and Measurements. All probes were purchased from Molecular Probes Inc. (Eugene, OR). Enzymes, stored at −80 °C in 10% glycerol, were thawed and dialyzed for 1 h against 500 mL of 20 mM Hepes (pH 7.4) and 150 mM NaCl buffer. Afterward, the proteins were labeled on ice with the thiol-reactive probe 7-diethylamino-3-(4′-maleimidylphenyl)-4-methylcoumarin (CPM) at a probe:protein ratio of 2:1, or the thiol-reactive probe acrylodan at a probe:protein ratio of 4:1. We have found that these ratios labeled at a 1:1 probe:enzyme molar ratio as determined by absorption for the probe concentration and BCA analysis for the protein concentration. After 1 h, the reaction was stopped

by adding 10 mM β-mercaptoethanol. The labeled proteins were separated from the free excess probe by applying the mixture to a 1 mL PD6 column followed by elution with 1 mL of 20 mM Hepes (pH 7.4), 150 mM NaCl, and 1 mM DTT buffer, or by extensive dialysis with the same buffer. In the studies described here, we used also DABCYL-SE or CM-SE, each of which covalently attaches to free amine groups. Labeling by these probes was performed at pH 7 to favor specific labeling of the N-terminus, and labeling was verified by elimination of the susceptibility of the protein to Edman degradation carried out at the Promeotics Center of the State University of New York (Stony Brook, NY).

Fluorescence measurements were performed on an ISS (Champaign, IL) spectrofluorometer using 3 mm quartz cuvettes. Peptide and protein stocks were diluted into 20 mM Hepes (pH 7.2), 160 mM NaCl, and 1 mM DTT. The emission spectrum of the CPM-labeled protein was measured from 415 to 530 nm ($\lambda_{ex} = 384$ nm), whereas the emission of the acrylodan-labeled protein was measured from 400 to 580 nm ($\lambda_{ex} = 360$ nm). The background spectra of unlabeled protein or peptide were subtracted from each spectrum along the titration curve. All of the spectra were corrected for the 10−12% dilution that occurred during the titration.

Intramolecular FRET was assessed by the decrease in CPM or acrylodan fluorescence when DABCYL, a nonfluorescent energy transfer acceptor, was attached to the N-terminus (see above). For these studies, proteins labeled with the thiol-reactive probe were either subsequently reacted with the FRET acceptor or saved as a control but taken through the same dialysis steps. The ratios of the doubly labeled to singly labeled intensities are reported.

Membrane Binding Studies. Membrane binding was carried out by titrating large, unilamellar vesicles (prepared by manual extrusion through a 0.1 μm polycarbonate filter) into a 100 nM solution of labeled protein and by measuring the change in the integrated area of the probe spectra after correcting for background and dilution using the settings described above. Membrane binding of unlabeled PLCβ2/δ1 was carried out by monitoring the change in the intrinsic fluorescence intensity ($\lambda_{ex} = 280$ nm, scanning from 300 to 450 nm). Membrane binding of PHβ71−88 and sPHβ71−88 was assessed by using LUVs labeled with 0.1% Laurdan (see ref *23*) and scanning the emission from 400 to 580 nm with a λ_{ex} of 360 nm. After correction for dilution and background, the change in fluorescence intensity was plotted as a function of lipid concentration and fit to a hyperbolic curve using SigmaPlot (Jandel, Inc.) to obtain the apparent partition coefficient (K_p) which corresponds to the lipid concentration at which 50% of the protein or peptide is bound.

Homology Modeling. We built a structural model of the PH domain of human PLCβ_2 by using the first 135 residues (SwissProt accession number Q00722). Two distinct on-line modeling servers were used [@TOME (*24*) and ROBETTA (*25*)], and both identified the PH domain of PLCδ_1 (PDB entry 1MAI) as the best template for homology modeling. Since the servers use different methods for the alignment and building steps, the two resulting structures are different (rmsd of 4.86 Å), especially at the N-terminus (residues 1−12) and the loops of residues 48−59 and 71−88. The models were minimized in a water box (10 Å × 10 Å × 10 Å) by using AMBER version 7.00 (*26*) and then evaluated by using PROSA II (*27*), Verify3D (*28*), ProQ (LGscore/MaxSub) (*29*), What If Fine Packing quality, and the Ramachandran plot (by using the MolProbity server). The best model was created by ROBETTA with the following evaluation scores: Verify3D = 0.238; PROSA II = −0.199; ProQ (LGscore/MaxSub) = 2.220/0.232; Ramachandran (percent of residues in favorable/allowed region) = 97.7/100; WhatIF (Av/*Z*-score) = 0.405/−2.70 [compared to those calculated for the PHδ_1 structure (0.415, −1.671, 4.526/0.551, 96.58/99.15, and 0.279/1.79, respectively)]. A major advantage of this homology modeling method is to build the additional residues in PHβ_2 which are not present in the template.

Membrane Insertion Simulation. The orientation and insertion of the PHβ_2 domain into a phospholipid membrane were simulated by using the method described by Ducarme et al. (*30*, *31*). We note that specific lipids are not used in the simulations but rather a restraint force field to mimic the membrane, and the lipid−water interface is described as a continuum medium by an empirical function, $C(z)$, which varies along the z-axis perpendicular to the plane of the membrane (the origin of the z-axis is the center of the bilayer).

$$C(z) = 0.5\frac{1}{1 + e^{\alpha(|z|-z_0)}}$$

where $\alpha = 2.042275$ and $z_0 = 15.75$ as indicated previously (*31*).

The protein is described by its full atomic coordinates. A standard Monte Carlo procedure is applied to explore the configuration of the protein in the membrane. Each run was performed twice at 298 K for 10^5 steps. Translations along the z-axis (maximum of a 1 Å step) and rotations (maximum of a 5° step) are randomly generated. The metropolis procedure is used to accept or reject the new energetic configuration based on the following energy calculation:

$$E_{tot} = E_{int} + E_{lip}$$

with

$$E_{int} = -\sum_{i=1}^{N} S(i)E_{tr}(i)C(Z_i)$$

and

$$E_{lip} = \alpha_{lip}\sum_{i=1}^{N} S(i)C(Z_i)$$

E_{int} increases when accessible hydrophilic atoms penetrate into the membrane and decreases in cases of hydrophobic atoms (favorable configuration), whereas E_{lip} accounts for the perturbation of the lipid bilayer due to the protein insertion and increases with the surface of the protein in contact with lipids. Energy calculation is based on the accessible surface S of each atom i of the protein. Seven atomic types are defined and associated with an empirical transfer energy E_{tr}. The accessible surface area was calculated using NSC (*32*) with the atomic radii from ref *33*. α_{lip} is an empirical factor fixed to −0.018 (*30*).

FIGURE 1: (A) Structural model of the PH domain of PLCβ_2 with residues 71−88 represented in CPK. (B) Structural alignment of PHβ_2 and PHδ_1 highlighting the loop between β5 and β6 in PH−PLCβ_2 not found in PH−PLCδ_1. The residues in lowercase letters are those of PHδ_1 not included in the structure (*4*). Residues 71−88 are colored blue and define the β5−β6 loop in PLCβ_2. Asterisks and periods indicate identical and homologous residues, respectively.

The electrostatic potential of PHβ_2 models was evaluated by using APBS [Adaptive Poisson_Boltzmann Solver (*25*)]. The atomic radius and charges were AMBER ones. The concentration of mobile ion species ([positive ions] = [negative ions]) was 0.150 M during the calculation. VMD (Visual Molecular Dynamics) software (*34*) was then used to visualize the electrostatic potential with the 1 kT/e potential isocontour as a blue transparent surface and the −1 kT/e isocontour as a red one.

RESULTS

Identification of the PHβ2 Region that Produces Activation. The PH domain of PLCβ_2 has been shown to bind to G$\beta\gamma$ and mediate its ability to activate the catalytic core (*15*), while the PH domain of PLCδ_1 binds specifically to PI(4,5)-P_2 to confer activation (*8*, *14*). The structure of the PHδ_1−Ins(1,4,5)P_3 complex has been determined (*4*) and has been used as a basis for theoretical models of PHβ_2 (*6*, *7*). However, these models give little insight into the reason PHβ_2 binds strongly to model membranes with little specificity, whereas PHδ_1 binds specifically to membranes that contain PI(4,5)P_2 (*11*, *12*). PH domains share little sequence identity despite their conserved fold (*35*), and homology profiles show that the sequence of PHβ_2 is most homologous to PHδ_1 with 11% of the sequence being identical. When building a model of the PH domain of PLCβ_2 based on the high-resolution structure of PHδ_1 (Figure 1A), we noted this former contains a unique insertion of nine amino acids between β-strands 5 and 6 (residues 75−83). The structural alignment of PHβ_2 and PHδ_1 highlights this observation (Figure 1B), as well as indicating that PHβ_2 contains a more apolar N-terminal region than PHδ_1. Neither of the reported models of PHβ_2 has included this latter region (*6*, *7*). The 71−88 region of PHβ_2 (i.e., the β5−β6 loop), which was included in the study of Singh and Murray (*6*), is predicted to be outside the core of the domain. We tested the possibility that it may be involved in G$\beta\gamma$ activation.

We synthesized a peptide corresponding to the PLCβ_2 71−88 segment (PHβ71−88) and a peptide with a scrambled sequence to use as a control (sPHβ71−88). We then tested the ability of these peptides to affect the activity of PLCβ_2 and the activity of a chimeric enzyme consisting of the PH domain of PLCβ_2 and the remaining domains of PLCδ_1 (PLCβ2/δ1). PLCβ2/δ1 [previously called PLC$\beta2^{PH-L}$/δ1 (*15*)] is activated to the same extent as PLCβ_2 by G$\beta\gamma$ subunits. The rationale for using this protein is that it lacks the PLCβ_2 C-terminal tail which may result in enzyme dimerization (*36*, *37*) and it can be expressed in bacteria, unlike PLCβ_2, allowing for easier preparation of point mutations. We note that key studies were repeated using wild-type PLCβ_2.

In Figure 2A, we show the effect of PHβ71−88 on PLCβ2/δ1 activity and its activation by G$\beta\gamma$ subunits using substrate dispersed in bilayers composed of POPE, POPS, and PI(4,5)P_2 (66:32:2). Unexpectedly, we find that the peptide increases the activity of PLCβ2/δ1 4-fold as compared to the control in the absence of G$\beta\gamma$ subunits. In addition, at >0.66 μM peptide, we find that PLCβ2/δ1 is no longer activated by G$\beta\gamma$ subunits. Parallel studies using the scrambled peptide, sPHβ71−88, show a reduced effect on activity and allow for the G$\beta\gamma$ activation at the highest peptide concentration that was tested. As a control, we tested the ability of the peptide to activate wild-type PLCβ_2. In Figure 2B, we show that the addition of 6.6 μM PHβ71−88 but not sPHβ71−88 to PLCβ_2 increases its activity and that the complex cannot be further activated by G$\beta\gamma$ subunits. We note that the extent of activation of PLCβ_2 by G$\beta\gamma$ subunits in our assays varies 2−5-fold depending on the age of the protein preparations and the nature and concentration of detergent used to solubilize G$\beta\gamma$ (see ref *18*). However, the extent of G$\beta\gamma$ activation of PLCβ_2 and PLCβ2/δ1 has been shown to be identical (*15*), and we find that the extent of activation produced by PHβ71−88 is similar to that with G$\beta\gamma$ for both types of enzymes.

In an effort to understand the mechanism underlying the enhancement of PLCβ2/δ1 activity by PHβ71−88, we noted that this region contains five positively charged residues and two negatively charged residues. Previous studies have reported that cationic peptides including polylysine can activate PLCβ_2 (*38*), although the amount of peptide required is much higher than we find for PHβ71−88 but within the range seen for the scramble peptide, suggesting that activation by PHβ71−88 is specific. Therefore, we produced two mutants of PLCβ2/δ1 in which each has two cationic side chains replaced with anionic ones, PLCβ2/δ1(K74E/K77E) and PLCβ2/δ1(K80E/R82E). Both mutants, and in particular PLCβ2/δ1(K80E/R82E), were not as strongly activated by G$\beta\gamma$ subunits as the wild-type chimera, although their binding to POPC and POPC/POPS (2:1) bilayers was the same within error as that of the wild-type chimera. The

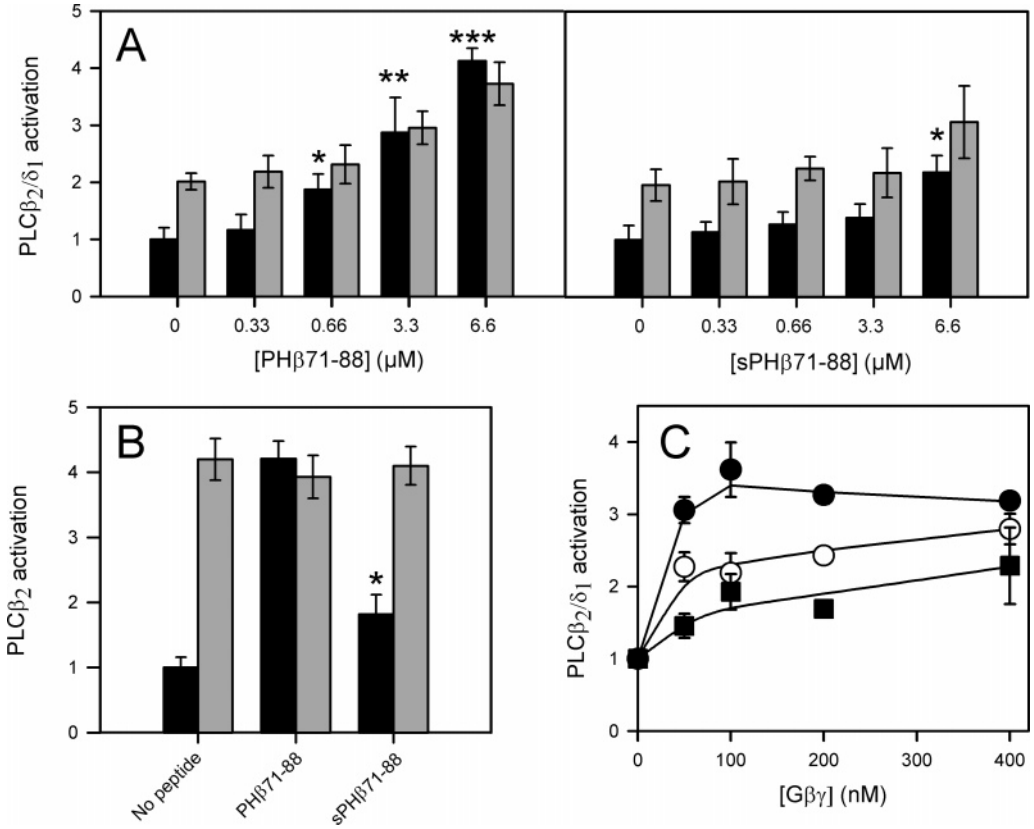

FIGURE 2: (A) Activation of the PLC$\beta 2/\delta 1$ chimera in the presence of increasing concentrations of PHβ71−88 or scrambled sPHβ71−88 in the absence (black bars) or presence (gray bars) of 50 nM G$\beta\gamma$. The activity was normalized to the basal activity (5−7.5 μmol min^{-1} mg^{-1}). Each bar is the mean of five separate experiments, each performed in triplicate, and the error bars indicate the standard deviation. (B) Activation of PLCβ_2 by 6.6 μM peptide PHβ71−88 or sPHβ71−88 in the absence (black bars) or presence (gray bars) of 50 nM G$\beta\gamma$. The activity was normalized to the basal activity (8.2 μmol min^{-1} mg^{-1}). Each bar is the mean of six separate experiments with the error bars indicating the standard deviation. In these studies, PLC$\beta 2/\delta 1$ and PLCβ_2 were incubated for 30 s and 3 min, respectively, at 37 °C in Ca^{2+}-containing MAIN buffer with 1 mM POPE/POPS/PI(4,5)P_2 (66:32:2) vesicles, freshly sonicated, and doped with [^{3}H]PIP(4,5)P_2 as described in Materials and Methods. The statistical significance of difference between the activity of phospholipase in the absence and presence of peptide is indicated (ANOVA, $*p < 0.1$; $**p < 0.01$; $***p < 0.001$). (C) Activation of PLC$\beta 2/\delta 1$ (●), PLC$\beta 2/\delta 1$(K74E/K77E) (○), and PLC$\beta 2/\delta 1$(K80E/R82E) (■) with increasing concentrations of G$\beta\gamma$ with 0.6 mM vesicles. Each value is the mean of two independent experiments performed in duplicate with error bars corresponding to the standard deviation. The activity was normalized to the basal activity which is 6.6 μmol min^{-1} mg^{-1} for PLC$\beta 2/\delta 1$, 6.2 μmol min^{-1} mg^{-1} for PLC$\beta 2/\delta 1$(K74E/K77E), and 16.6 μmol min^{-1} mg^{-1} for PLC$\beta 2/\delta 1$(K80E/R82E).

reduced activity of these mutants supports the idea that residues 71−88 play a role in enzyme regulation (Figure 2C). Interestingly, as noted in the legend of Figure 2C, PLC$\beta 2/\delta 1$(K80E/R82E) has a basal activity 2-fold higher than that of PLC$\beta 2/\delta 1$, suggesting that in the wild-type enzyme, residues 71−88 may have an inhibitory effect. These results indicate that other features of the PH71−88 region, besides electrical charge, play a role in PLC$\beta 2/\delta 1$ activation.

PHβ71−88 Interacts with Membrane Surfaces. We undertook a series of studies to understand the basis for PLC$\beta 2/\delta 1$ activation by PHβ71−88. Since the PH domains of PLCβ enzymes bind strongly and nonspecifically to neutral and charged membrane surfaces (*11*, *12*), we determined the most probable orientation of PHβ_2 in an electrically neutral membrane by using a continuous restraint force field (*31*). As shown in Figure 3A, the simulation identified a single minimum with the PHβ_2 mass center at $z = 28.3$ Å (center of membrane $z = 0$ Å) with a total energy of −17.7 kcal/mol. This minimum is associated with a configuration of PHβ_2 where its nonpolar N-terminus is inserted deeply into the membrane and where residues 71−88 lie close to the membrane surface. Electrostatic potential calculations indicate that this region is part of the positively charged side of the PH domain in contact with the membrane interface, whereas the negatively charged side of the domain is oriented toward the solvent (Figure 3B).

If residues 71−88 of PHβ_2 interact with membranes, it is possible that it may change enzyme activation by modulating its orientation on the membrane surface. To test this idea, we directly assessed the membrane binding of PHβ71−88 and sPHβ71−88 to negatively charged or electrically neutral surfaces. This study was carried out by doping large, unilamellar vesicles with a lipophillic fluorescence reporter probe, Laurdan (*12*, *23*), at 0.1 mol % and measuring the change in its fluorescence spectrum properties as peptides are added. Systematic changes in Laurdan fluorescence are observed when negatively charged lipid bilayers were added but not when electrically neutral bilayers were used (Figure 4). This lack of change suggests little or weak interaction with neutral membranes. In accord with this idea, PHβ71−88 exhibited binding affinities similar to those of POPC/POPS (2:1) and POPC/POPS/POPE (1:1:1) bilayers, indicating that binding is primarily through electrostatic interactions. Interestingly, the scrambled sequence, sPHβ71−88, had a much weaker affinity for POPC/POPE/POPS bilayers than PHβ71−88 (Figure 4), even though it has an

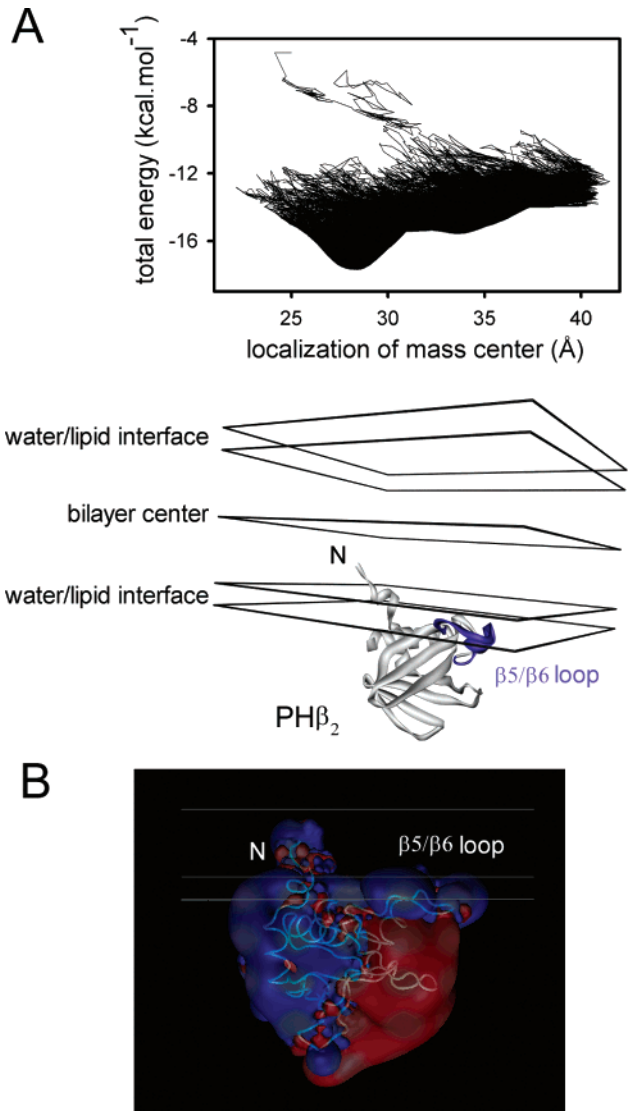

FIGURE 3: (A) Simulated energy profile of PHβ_2 embedded in an electrically neutral lipid bilayer. Each point corresponds to the total energy of an accepted configuration during the Monte Carlo run as a function of the position of the center of mass of the protein along the z-axis. The protein is represented as a ribbon with residues 71−88 (β5−β6 loop) colored blue and is inserted into the membrane with the configuration corresponding to the lowest total energy (−17.7 kcal/mol). (B) Mapping of the electrostatic potential on the optimized configuration with the 1 kT/e potential isocontour as a blue transparent surface and the −1 kT/e isocontour as a red one.

identical electrical charge suggesting that the distribution of charge and/or the helix-forming propensity contributes to PHβ71−88 membrane binding.

We then tested whether PHβ71−88 altered PLCβ2/δ1 membrane interactions by measuring the capacity of the enzyme to bind to membranes in the absence or presence of 10 μM peptide. In an initial series of studies, we monitored the binding of PLCβ2/δ1 to POPC/POPS/POPE large, unilamellar vesicles through changes in intrinsic fluorescence. We have previously found that the emission intensity of PLCβ_2 significantly decreases by ~35% upon membrane binding most likely due to the quenching of interfacial Trp residues by the ionic lipid headgroups (*11*). We find that this quenching also occurs upon the association of PLCβ2/δ1 with these membranes, giving a membrane partition coefficient (K_p) of 11 ± 5 μM. This identical value was

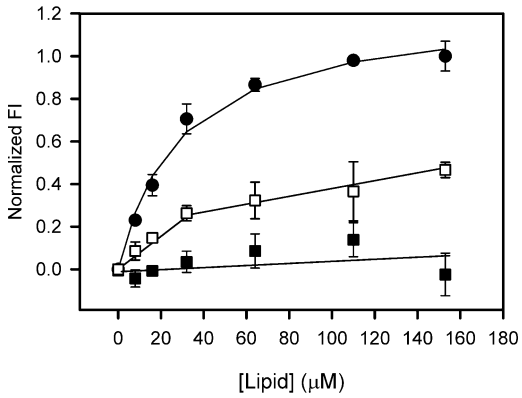

FIGURE 4: Membrane binding of 1 μM PHβ71−88 peptide to POPC/POPS/POPE (1:1:1) (●) or POPC (■) LUVs labeled with 0.1% Laurdan. Also shown is the binding of 10 μM sPHβ71−88 to POPC/POPS/POPE (1:1:1) LUVs (□). Labeling was carried out by adding a small amount of probe from a concentrated stock in DMF to preformed membranes and by incubating them in a bath sonicator for 5 min at room temperature. Emission spectra were recorded from 380 to 580 nm with a λ_{ex} of 350 nm. The integrated areas were corrected for dilution background controls in which buffer was substituted for peptide to give the change in fluorescence intensity (FI). These values were normalized to the maximum change in signal which was 14.2%.

obtained in the presence of 10 μM scrambled peptide sPHβ71−88. However, in the presence of 10 μM PHβ71−88, the level of binding was reduced ~10-fold, indicating that PHβ71−88 interferes with membrane binding. This result prompted us to carry out a more comprehensive membrane binding study.

PHβ71−88 Affects Membrane Interactions of the Catalytic Domain. In analyzing our data, we noted that our previous studies showed that the intrinsic fluorescence of PHβ2 does not change upon membrane binding (*12*). Therefore, the changes in PLCβ2/δ1 intrinsic fluorescence must be due to Trp/Tyr residues in the EF-catalytic domains. Thus, the results described above imply that PHβ71−88 is affecting membrane interactions at regions other than the PH domain.

To better understand which domains may be affected by PHβ71−88, we undertook a series of studies to determine whether membrane binding of the PH and catalytic domains is linked. We note that we could distinguish membrane binding of the PH domain by labeling with an amine-reactive probe under conditions where attachment to the N-terminus is preferred (see Materials and Methods), although we cannot discount the possibility of covalent labeling of Lys side chains in other regions of the protein. We also note that PLCβ2/δ1 contains 10 Cys residues which all lie in the catalytic domain except for one located in the EF hand domain (i.e., Cys193). Therefore, mutating Cys193 to Ser would allow us to follow membrane interactions of the catalytic domain through labeling with a thiol-reactive fluorescent probe. The corresponding PLCβ2/δ1(C193S) mutant is correctly activated by G$\beta\gamma$ (data not shown).

We labeled PLCβ2/δ1 with amine-reactive probe coumarin SE (CM-SE) under conditions that favor N-terminal labeling and monitored its change in fluorescence upon membrane binding. Addition of POPC/POPS/POPE bilayers to CM-labeled PLCβ2/δ1 resulted in a 3-fold increase in coumarin fluorescence after background subtraction with little change in the center of spectral mass and yielded a K_p of 16 ± 3 μM. Addition of 10 μM PHβ71−88 did not significantly

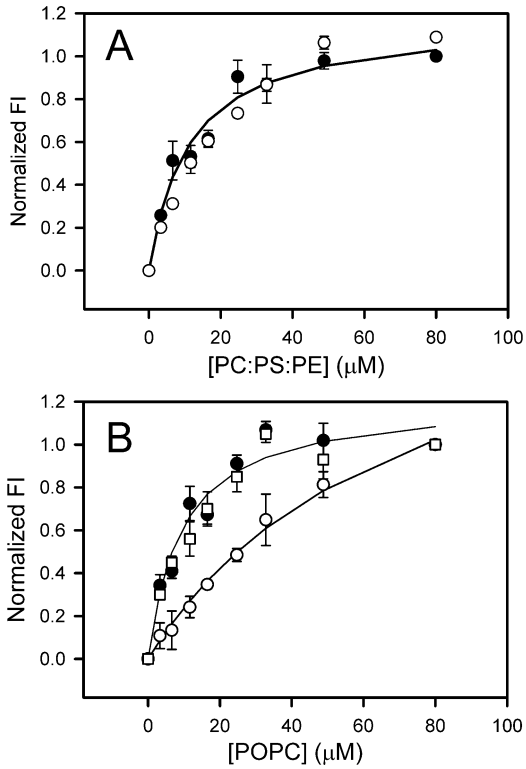

FIGURE 5: (A) Binding to POPC/POPE/POPS (1:1:1) LUVs in the absence (●) or presence (○) of 10 μM PHβ71−88. We note that an identical binding behavior was seen in the presence of 10 μM sPHβ71−88 and after substitution of POPC LUVs for POPC/POPE/POPS (1:1:1) LUVs. (B) Effect of 10 μM PHβ71−88 on the binding of acrylodan-labeled PLCβ2/δ1 to POPC bilayers (○) as compared to binding in the absence of peptide (●). We also found that the inclusion of 10 μM sPHβ71−88 did not affect the binding membrane interactions of CM-PLCβ2/δ1 (□).

change the membrane binding of the enzyme (Figure 5A), nor did addition of 10 μM scrambled peptide. We note that identical data were obtained using the PLCβ2/δ1(C193S) mutant. These data show that PHβ71−88 does not interfere with the membrane interaction of the N-terminal region of PLCβ2/δ1.

To follow membrane interactions of the catalytic domain of PLCβ2/δ1, we labeled the PLCβ2/δ1(C193S) mutant with the thiol-reactive probe acrylodan and followed membrane binding. We found that binding of the enzyme to POPC/POPS/POPE membranes was ~3-fold stronger in the absence of 10 μM PHβ71−88 ($K_p = 11 \pm 2$ μM) than in the presence of peptide ($K_p = 36 \pm 4$ μM) or in the presence of 10 μM scrambled peptide (data not shown). Interestingly, the effect of peptide was more pronounced when membranes with electrically neutral headgroups were used (i.e., $K_p = 10 \pm 2$ vs 71 ± 10 μM; see Figure 5B). These results show that the presence of the peptide affects membrane interactions as monitored by a probe attached to the catalytic domain.

The results described above suggest that changes in the distance and/or orientation of the PH domain relative to the catalytic domains occur upon membrane binding. To determine whether this is the case, we used intramolecular fluorescence resonance energy transfer (FRET). Since the amount of energy transfer from a donor to an acceptor follows an R^6 distance dependence, where R is the distance between the probes, then appropriately placed probes should be sensitive to changes in the distance between the domains. We carried out FRET studies by first labeling the enzyme in the catalytic domain with a fluorescent thiol-reactive probe to act as a FRET donor (CPM) and then further labeled a portion of this material on the N-terminus with a nonfluorescent FRET acceptor (DABCYL SE). If the two probes are positioned close enough for interdomain FRET to occur [~25 Å (*39*)], then the emission intensity of the doubly labeled probe should be significantly lower than that of its singly labeled counterpart under identical conditions. Therefore, we assessed the presence of FRET by comparing the ratio of the DABYLC/CPM-labeled protein versus that of the CPM-labeled one.

We carried out these studies by labeling PLCβ2/δ1(C193S) with CPM (see Materials and Methods). On the basis of the crystal structure of the catalytic domain (*5*), there are two fully exposed Cys residues (C334 and C367) that are in the proximity of each other and three (C188, C393, and C546) that are partially exposed. All other Cys residues are buried. Since the enzyme consistently and rapidly labels with thiol-reactive probes at a 1:1 probe:protein molar ratio, we postulate that one of the two Cys residues is preferentially labeled. We took half of PLCβ2/δ1(C193S)-CPM and labeled it under conditions favoring attachment to the N-terminus with a non-fluorescence energy transfer acceptor DABCYL SE; labeling was verified by Edman degradation, and the molar labeling ratio of probe to protein was ~0.8, as estimated by absorption of the probe and BCA analysis.

We stress that we are only viewing the changes in FRET upon membrane binding due to interdomain movement and not trying to assess the absolute changes in distance. Thus, precise knowledge of the exact location of the probe sites is not required. We note also we cannot discount the possibility that a small percentage of the DABCYL label is linked to the catalytic domain. If this were the case though, this population would be nonfluorescent since the probes would be in the proximity of each other and not contribute to changes seen upon lipid binding since it is extremely unlikely that the catalytic domain would unfold upon membrane binding. The following studies were designed to qualitatively assess differences in movement when binding to lipids occurred.

As shown in Figure 6, we found a significant amount of fluorescence energy transfer in the absence of lipids (80%). Addition of POPC/POPS/POPE LUVs results in a small but significant increase in the amount of transfer. In contrast, addition of POPC reduces the amount of transfer. These data suggest that the N-terminus moves closer to the catalytic domain upon binding to POPC/POPS/POPE LUVs but not POPC. Thus, changes in FRET differ with the nature of the membrane surface.

Although we could not selectively label the individual domains of wild-type PLCβ_2, we determined whether PHβ71−88 could alter its membrane binding properties. We labeled the protein with a thiol-reactive probe at a 1:1 stoichiometry but note that there are accessible Cys residues in the C-terminal region as well as the catalytic domain and the EF hands. We found that the presence of 10 μM PHβ71−88 did not significantly affect the binding affinity for POPC/POPS/POPE bilayers ($K_p = 8 \pm 0.5$ μM). However, when the peptide concentration was increased to 30 μM, a decrease in the binding affinity was seen ($K_p = 13 \pm 1$ μM). These same affinities were obtained for DOPC as well as POPC,

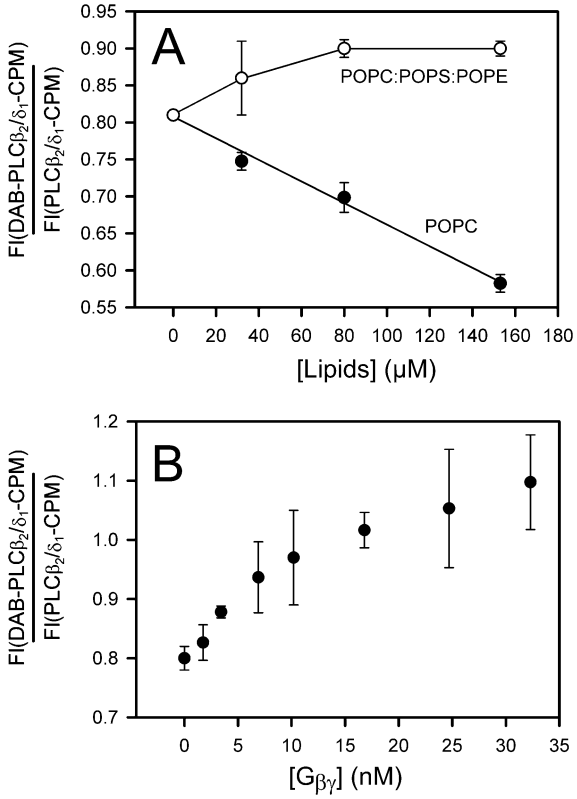

FIGURE 6: Qualitative assessment of the change in interdomain distance using FRET. PLCβ2/δ1(C193S) was labeled in the catalytic domain with the thiol-reactive probe CPM. Half of this material was further reacted with DABCYL-SE under conditions that favor covalent modification of the N-terminus. (A) Effect of the addition of POPC/POPE/POPS (1:1:1) (○) or POPC (●) LUVs on the FRET signal. The ratio of the fluorescence intensity of the doubly labeled to singly labeled protein was then monitored as a function of lipid concentration. (B) Effect of the increasing amount of G$\beta\gamma$ on the FRET signal. These measurements were carried out in the absence of added lipid and detergent by dialyzing purified G$\beta\gamma$ against a 10000-fold excess of buffer [20 mM Hepes (pH 7.5), 160 mM NaCl, and 1 mM DTT] for 30 min to remove excess storage detergent (0.1% cholate).

showing that the nature of the hydrocarbon region is not affecting enzyme binding. Since the ~400-residue C-terminus of PLCβ_2 contributes significantly to membrane binding (*8*), it is likely that the PLCβ_2−PHβ71−88 complex (see below) interacts with membranes in a manner similar to that of the uncomplexed enzyme, and the observed decrease in the level of binding of PLCβ_2 to POPC/POPS/POPE LUVs in the presence of 30 μM peptide is due to direct competition for the membrane surface by the peptide.

Activation of PLCβ2/δ1 Is Dependent on Membrane Curvature. Our membrane binding studies suggest that residues 71−88 will confer responsiveness to G$\beta\gamma$ more readily on membrane surfaces to which it is bound more strongly (i.e., to POPC/POPS/POPE vesicles rather than POPC vesicles). Previous studies have also noted that PE lipids promote membrane binding of PLCβ_2 and activation by G$\beta\gamma$ subunits (*19*). To better test this idea, we monitored the activity of PLCβ2/δ1 employing a peptide derived from the Gβ subunit, Gβ86−105, which activates PLCβ_2 (*40*). The rationale for using this peptide activator is that it does not bind to membranes as indicated by Trp fluorescence experiments (data not shown), and the orientation of G$\beta\gamma$ subunits may be sensitive to the physical properties of the membrane surface (see ref *41*). Thus, this peptide will allow us to isolate membrane effects on PLCβ2/δ1.

We first verified that the Gβ86−105 peptide, but not the Gβ86−105(M101N) mutant, affects the activity of PLCβ2/δ1 in a manner similar to that of PLCβ_2 (Figure 7A). Interestingly, we found that this peptide also activated PLCδ_1, thereby showing that activation by this peptide does not depend on its binding to the PHβ_2 domain. This observation supports the idea that PH domains of PLCβ_2 and PLCδ_1 bind and orient the activator [i.e., G$\beta\gamma$ or PI(4,5P)$_2$] to a conserved site in the catalytic domain to achieve full activation (*8*, *42*). Also, as shown in Figure 7B, we found that PLCβ2/δ1, when completely activated by Gβ86−105, is not activated by G$\beta\gamma$, which is similar to the behavior reported for PLCβ_2 (*40*).

In Figure 7C, we show a direct comparison of Gβ86−105 activation of PLCβ2/δ1 on three types of membrane surfaces. We find that PE lipids are required for activation. We propose that this PE dependence is linked to better association of the β5−β6 loop with the membrane surface.

The β3 and β4 Strands Play a Role in G$\beta\gamma$ Interaction. If the β5−β6 loop of PHβ_2 modulates membrane association, then other regions of the PH domain must interact with G$\beta\gamma$ subunits. Recently, the structure of the GRK$_2$ (G-receptor kinase 2) PH domain in complex with G$\beta\gamma$ has been determined (*43*). From a structural alignment of PHβ_2 with PH-GRK2, we determined whether a FζDK motif (ζ corresponding to a hydrophobic residue), essential for the binding of PH-GRK2 to the Gβ subunit, is also present in PLCβ_2. The Phe residue in this motif (F147) would be the equivalent of Met664 of PH-GRK$_2$ interacting with Trp99 and Met101 within the Gβ subunit. When superposing our model of the PHβ_2 with the structure of the PH domain of GRK2 (PDB entry 1OMW), we found the same motif in terms of sequence alignment (data not shown). However, we found that the activation of PLCβ2/δ1(F147E) by G$\beta\gamma$ remains similar to that of PLCβ2/δ1. In contrast, we observed that the level of activation by G$\beta\gamma$ of PLCβ2/δ1(Y48A), PLCβ2/δ1(Y48R), and the double mutant PLCβ2/δ1(Y48R/F147E) is systematically reduced compared to that of the native chimera (Figure 8A,B).

The crystal structure of the GRK2−G$\beta\gamma$ complex also suggests that the C-terminal helix of the PH domain served as a binding site for G$\beta\gamma$ subunits. Additionally, we tried to define whether the segment of residues 120−135 of the PHβ_2 domain, which is strongly predicted to be an α-helix, is implicated in the PLCβ_2−G$\beta\gamma$ interaction. We find that the presence of 5 μM PHβ120−135 shows a slight inhibition of activation of PHβ_2/δ_1 by G$\beta\gamma$ (Figure 8C,D). However, this peptide did not inhibit the G$\beta\gamma$-mediated activation of PLCβ_2.

DISCUSSION

The PH domains of PLCβ_2 and PLCδ_1 have been shown to confer activation to their catalytic core when binding to their particular activators [i.e., G$\beta\gamma$ and PI(4,5)P$_2$, respectively], and these activators can be interchanged by swapping PH domains (*8*, *15*). In this study, we have identified a region unique to the PHβ_2 domain, residues 71−88. As discussed below and shown in Figure 9, we propose that this region will play a role in inhibitory interactions between the PH and catalytic domains. Relief of inhibition will be achieved

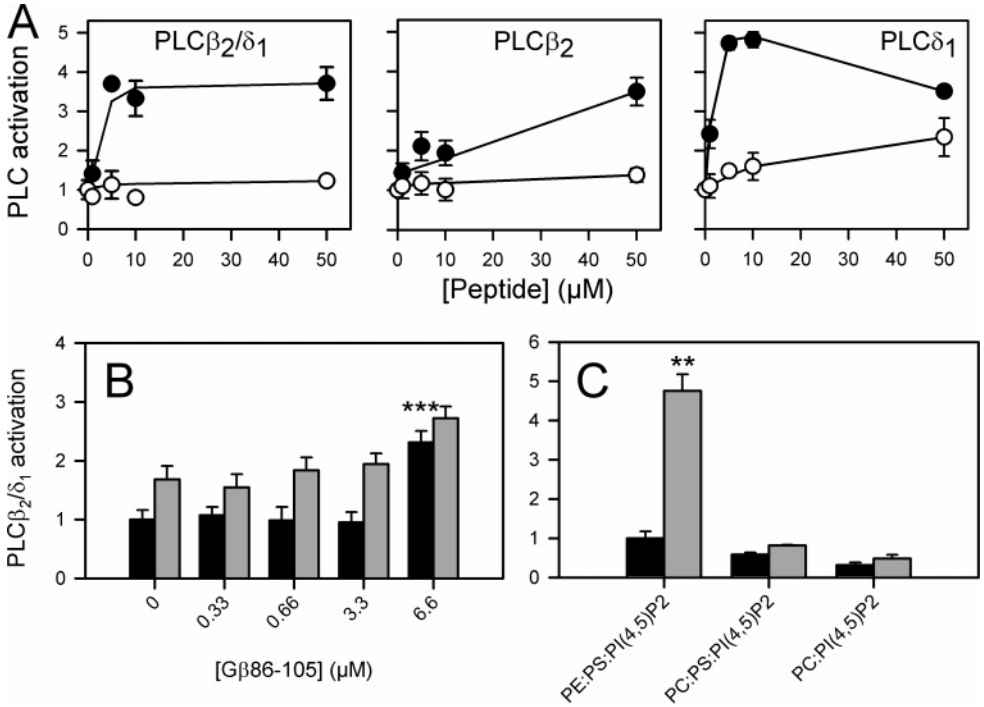

FIGURE 7: (A) Effect of the Gβ86−105 or Gβ86−105(M101N) peptide on the rate of PI(4,5)P_2 hydrolysis by 1 nM PLCβ2/δ1, PLCβ_2, or PLCδ_1. PLCβ2/δ1 and PLCδ_1 were incubated for 30 s, whereas PLCβ_2 was incubated for 2 min at 37 °C in Ca^{2+}-containing MAIN buffer with 1 mM POPE/POPS/PI(4,5)P_2 (66:32:2) vesicles doped with [^{3}H]PIP(4,5)P_2. The catalytic activity (normalized to the basal activity) was examined in the presence of various concentrations of Gβ86−105 (●) or Gβ86−105(M101N) (○). Each point is the mean of five independent experiments, with the error bars indicating the standard deviation. (B) Activation of PLCβ2/δ1 by increasing concentrations of peptide Gβ86−105 in the absence (black bars) or presence of 75 nM G$\beta\gamma$ (gray bars). Each bar is the mean of five separate experiments, with the error bars indicating the standard deviation. The difference in activity of PLCβ2/δ1 in the absence or presence of the peptide is statistically significant when indicated (*$p < 0.1$). (C) Activity of PLCβ2/δ1 in the absence (black bars) or presence (gray bars) of 10 μM Gβ86−105 peptide with vesicles with different compositions. The chimera was incubated for 30 s at 37 °C with 1 mM POPE/POPS/PIP-(4,5)P2 (66:32:2), POPC/POPS/PIP(4,5)P_2 (66:32:2), or POPC/PIP(4,5)P_2 (98:2) LUVs. Each bar is the mean of two separate experiments performed in triplicate, with the error bars indicating the standard deviation. The difference in activity of PLCβ2/δ1 in the absence or presence of the peptide is statistically significant when indicated (**$p < 0.01$).

through binding of G$\beta\gamma$ subunits or by addition of an appropriate peptide that competes for this inhibitory interaction. Importantly, relief of this inhibition comes about when the enzyme is on an appropriate surface, such as membranes containing PE lipids. This model also suggests that the low level of activation seen at high concentrations of other cationic peptides (*38*), including the scrambled peptides used here, occurs through a similar mechanism. Disrupting the inhibitory interactions through mutation can increase the basal activity and thereby reduce the net amount of activation by G$\beta\gamma$ subunits, as seen in the data for the K80E mutant. As discussed below, we propose that activation by PHβ71−88 is not through direct interaction with the catalytic core but through modulation of the orientation of the active site on the membrane surface.

In trying to understand the basis for enzyme activation by this peptide, we noted that the segment of residues 71−88 of PHβ_2 (i.e., the β5−β6 loop) has five positively charged residues that could participate in the regulation of PLCβ_2 and the PLCβ2/δ1 chimera. Mutation of these residues did not affect membrane binding of PLCβ2/δ1 but modified the activation by G$\beta\gamma$, as exemplified by the Lys80/Arg82 mutant. We note also that this mutant displayed a higher basal level, suggesting that the region of residues 71−88 may inhibit the catalytic domain, allowing for a higher extent of activation by G$\beta\gamma$ subunits. Likewise, the observation that apparent activation of PLCβ2/δ1 and PLCβ_2 by G$\beta\gamma$ is eliminated when they are incubated with PHβ71−88 suggests a common mechanism between these activators. Taken together, these results suggest that the loop of residues 71−88 of PHβ2 plays a role in regulating the catalytic domain rather than being directly involved in G$\beta\gamma$ subunit interactions.

To assess whether the PHβ5/β6 loop may play a similar role in other PLCβ isozymes, we note that while all PLCβ isoforms are activated by Gαq subunits, only PLCβ_2 and PLCβ_3 are also activated by G$\beta\gamma$. The lack of G$\beta\gamma$ activation of PLCβ_1 correlates well with its inability to bind G$\beta\gamma$ subunits strongly, even though its isolated PH domain binds to G$\beta\gamma$ with high affinity (*12*). Multiple-sequence alignments with rat and human PLCβ1−4 show a pair of positively charged residues corresponding to the K74/K77 pair in PLCβ_2. Additionally, PLCβ_1 and PLCβ_3 contain positively charged residues corresponding to K80/R82. Therefore, while PLCβ_1 has the potential to be activated by G$\beta\gamma$ through PH71−88, its lack of association prevents this mechanism from occurring. Studies aimed at improving the understanding of the underlying mechanism for this lack of activation are underway.

Our studies show that PHβ71−88 has the ability to bind to negatively charged membranes, and we propose that this interaction may serve to stabilize the position of the catalytic core in an orientation favorable for G$\beta\gamma$ activation. Several pieces of data support this idea. First, we find that PH71−88 affects the fluorescence properties of Laurdan embedded in POPC/POPS (2:1) and POPC/POPS/POPE (1:1:1) bilay-

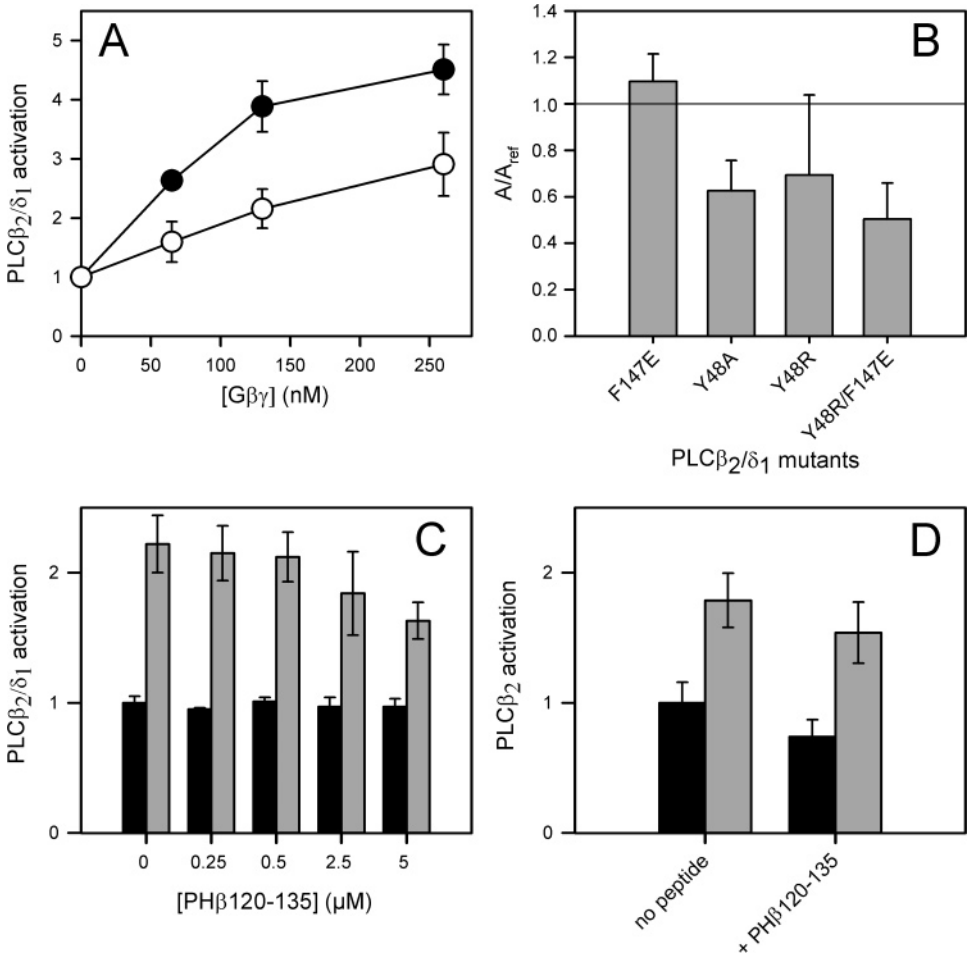

FIGURE 8: (A) Activation of PLCβ2/δ1 and PLCβ2/δ1(Y48A) with increasing amounts of G$\beta\gamma$ at 0.6 mM POPE/POPS/PI(4,5)P_2 (66:32:2) vesicles doped with [^{3}H]PI(4,5)P_2. Each value is the mean of three independent measures with error bars corresponding to the standard deviation and representative of two independent experiments. The activity is normalized to the basal activity in the absence of G$\beta\gamma$. (B) Relative G$\beta\gamma$ activation of mutants PLCβ2/δ1(F147E), PLCβ2/δ1(Y48A), PLCβ2/δ1(Y48R), and PLCβ2/δ1(Y48R/F147E) at 200 nM G$\beta\gamma$. (C) Effect of an increasing level of PHβ120−135 on the activity of PLCβ2/δ1 in the absence (black bars) or presence (gray bars) of 75 nM G$\beta\gamma$. Each bar is the mean of three separate experiments ($n = 5-7$) with error bars indicating the standard deviation. (D) Activation of PLCβ_2 by 5 μM PHβ120−135 in the absence (black bars) or presence (gray bars) of 200 nM G$\beta\gamma$. Each bar is the mean of five separate experiments performed in triplicate, with the error bars indicating the standard deviation.

ers, but not in POPC, suggesting very weak or superficial PC interactions. Thus, if PH71−88 played a role in enzymatic regulation through membrane interactions, we would expect inhibition of PLCβ_2 activity and G$\beta\gamma$ activation on electrically neutral surfaces, and this is what is observed. Also, if PH71−88 played a role in membrane orientation, then we would not expect productive G$\beta\gamma$ activation on surfaces where optimization of orientation cannot occur, such as on micelles, and it has been shown that PLCβ_2 is not activated by G$\beta\gamma$ on micellar surfaces (*19*). By this argument, the nature of the membrane surfaces is the critical determinant for activation rather than the hydrocarbon interior, and this idea is under investigation. Thus, the lipid specificity of PLCβ2/δ1−G$\beta\gamma$ activation can be rationalized by the membrane binding specificity of PHβ71−88 even though the entire PH domain binds to membranes with little specificity (*12*).

It is important to note that the PH domains of PLCδ_1 and PLCβ_2 serve to anchor the protein to the membrane to allow the catalytic domain to processively catalyze substrate. This mechanism stems from the very strong membrane interactions of the PH domain versus the ~1000-fold weaker membrane interactions of the catalytic domain (see refs *13*, *19*, and *44*). The lack of an effect of PHβ71−88 on membrane binding as seen by the N-terminal region containing the PH domain is due to the strong binding of this region, which overshadows the weaker membrane interactions of the catalytic domain. This idea is further supported by the similarities of the membrane binding affinities between the isolated PH domains and the whole proteins (*12*, *44*). Thus, the overall membrane binding of the enzyme is dictated by the PH domain, while membrane interactions of the weakly binding catalytic domain are susceptible to the weakening and changing of the domain orientation in the presence of PHβ71−88. Our results support a model in which the two domains have some independence of motion even though they are in some way linked.

Support for the idea that PHβ7 1−88 modulates the position of the catalytic core comes from fluorescence studies with the PLCβ2/δ1 chimera. We find that PHβ71−88 alters the membrane binding affinity of PLCβ2/δ1 as measured by Trp fluorescence, presumably in the catalytic domain. This idea was confirmed using a probe located in the catalytic domain. However, the presence of PHβ71−88 did not affect

the binding of a probe that targets the N-terminus. These data show that PHβ71−88 preferentially affects the membrane interactions of the catalytic domain.

These studies suggest that movement of the PH and catalytic domains of PLCβ2/δ1 may be independent. This idea was tested by intramolecular FRET studies between probes in the catalytic domain and N-terminal regions. In general, an increased amount of transfer corresponds to a reduced distance between two probes. We find that enzyme binding to POPC/POPS/POPE bilayers results in an increase in the level of FRET, suggesting that PE surfaces stabilize PH catalytic domain orientation that allows for more efficient transfer. In contrast, a reduction in the level of FRET is observed when the enzyme binds to POPC membranes, suggesting an increase in the interdomain distance. Taken together, these studies support the idea that different membrane surfaces promote differing distances and/or orientations between the PH and catalytic domains. This finding correlates well with the observation that the PH domain of PLCδ_1 is unresolved in crystals which suggests multiple domain orientations (*5*). We also note that the independence of the PH versus catalytic domains in membrane orientation may explain some of the different membrane binding to POPC seen in previous studies (*45*, *46*) versus our own (*11*).

While the PHβ71−88 peptide also stimulated the activity of wild-type PLCβ_2, it did not greatly affect its membrane binding. The simplest explanation is that the membrane interaction sites in the C-terminal region are masking any effect of the peptide either directly or indirectly by promoting protein dimerization. Selective labeling of the catalytic domain to determine interdomain movement is not straightforward and is the subject of current study.

Our results show that activation of the enzyme by Gβ86−105 depends on the PE content of vesicles, as in studies using G$\beta\gamma$ (*19*). Even though membrane charge is important in PHβ71−88−membrane interactions, activation is optimal on an expanded PE surfaces. This result, in combination with the membrane binding studies, allows us to speculate that the increased curvature and hydrophobicity of PE-containing vesicles allow residues 71−88 to orient the PH domain with the catalytic domain. It is possible that this orientation allows G$\beta\gamma$ subunits, the Gβ86−105 peptide, or PHβ71−88 to bind to the enzyme allowing activation.

The studies here employed model membranes that allowed us to isolate the effects of an expanded membrane surface. It is interesting to speculate about the situations that may arise on cellular membranes whose surfaces are variable. Our results suggest that activation would be inhibited on the compacted surfaces of lipid rafts but may be promoted on membranes with a high PE content. Importantly, membrane proteins may assist in modulating PHβ71−88−membrane interactions. More studies are needed to determine whether different cellular membrane have the ability to modulate PLCβ_2 activity and its ability to be activated by G$\beta\gamma$ subunits.

Many reports have identified potential sites of interaction on G$\beta\gamma$ for PLCβ_2 (*40*, *47*−*50*) and potential sites of G$\beta\gamma$ binding on PLCβ_2 (*9*, *12*, *44*). We have found here that the Gβ86−105 peptide, which corresponds to a segment of the Gβ subunit, not only activates PLCβ_2 as previously demonstrated (*40*) but also stimulates the PLCβ2/δ1 chimera and PLCδ_1. This finding suggests that the segment interacts with

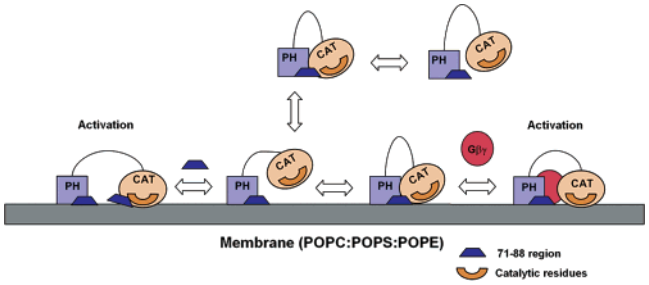

FIGURE 9: Model of G$\beta\gamma$ activation of PLCβ_2. In solution, the PH domain of PLCβ2/δ1 can be docked to the catalytic domain in several interchangeable orientations. Binding to membranes can stabilize particular contacts between the PH domain with the catalytic region which are inhibitory. Addition of cationic peptides (i.e., PHβ71−88) competes with these contacts, allowing the catalytic domain to have productive interactions with the membrane surface containing substrate. Inhibitory contacts are also overcome by addition of G$\beta\gamma$ subunits. We note that optimal interactions between the catalytic domain and the membrane surface comprise an expanded surface as produced by PE lipids.

PLCβ_2 in a manner that is independent of the PH domain. Since the catalytic domains of PLCβ_2 and PLCδ_1 are very homologous, it is conceivable that they share a binding site for the region of residues 86−105 of G$\beta\gamma$. We note that structural studies of the GRK2−G$\beta\gamma$ complex (*43*) show that the primary interaction site is between Gβ86−105 and the C-terminal region of the GRK2 PH domain. However, our studies show that the C-terminal region of PHβ_2 does not play a significant role in G$\beta\gamma$ activation (Figure 8).

So far, the mechanism through which PHβ2 binds to membranes is unknown, in contrast to that of PHδ_1 whose membrane interactions have been clearly identified (*4*, *50*). A structural model of PHβ_2 and subsequent simulation of its insertion into an electrically neutral membrane allow us to propose a dominant membrane orientation in which the region of residues 71−88 resides on the membrane surface (Figure 3). It has been suggested that the accessible hydrophobic patch in the β3−β4 region, not present in PHδ_1 (see the alignment in Figure 1), may play a role in the membrane binding of PHβ_2 (*6*). Interestingly, our simulations show that when the orientation of the 10 first residues is modified by a trans-to-cis conversion of the Pro11−Lys12 amide bond, a second and additional local minimum is found in which the β3−β4 loop lies close to the membrane (data not shown). Indeed, this study highlights the possibility that the orientation of the PHβ_2 domain with respect to the catalytic domain and the membrane surfaces is dynamic. In any case, the β3−β4 hydrophobic patch is accessible to the solvent in the configuration that is thought to be necessary for the G$\beta\gamma$ activation. We note that in our model the C-terminal helix of the PH domain lies parallel to the membrane surface correlating with our observation that the C-terminus of PHβ_2 does not play a significant role in G$\beta\gamma$ activation.

On the basis of our results, we propose a model of G$\beta\gamma$ activation (Figure 9) in which in solution, the PH domain of PLCβ2/δ1 can be docked to the catalytic domain in several interchangeable orientations. Binding to membranes containing PE lipids can stabilize a particular membrane orientation of the PH domain due to the penetration of the β5−β6 loop into PE surfaces, most likely due to the higher degree of curvature or more expanded surface caused by these lipids.

This orientation of the PH domain stabilizes an inhibited form of the enzyme but promotes G$\beta\gamma$ binding and subsequent activation of the catalytic core.

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REFERENCES

1. Rebecchi, M., and Pentylana, S. (2000) Structure, function and control of phosphoinositide-specific phospholipase C, *Physiol. Rev. 80*, 1291−1335.
2. Rhee, S. G. (2001) Regulation of phosphoinositide-specific phospholipase C, *Annu. Rev. Biochem. 70*, 281−312.
3. Singh, S., and Murray, D. (2002) Molecular modeling studies of phospholipase C PH domains, *Biophys. J. 82*, 168a.
4. Ferguson, K. M., Lemmon, M. A., Schlessinger, J., and Sigler, P. B. (1995) Structure of the high affinity complex of inositol trisphosphate with a phospholipase C pleckstrin homology domain, *Cell 83*, 1037−1046.
5. Essen, L. O., Perisic, O., Cheung, R., Katan, M., and Williams, R. L. (1996) Crystal structure of a mammalian phosphoinositide-specific phospholipase C-δ, *Nature 380*, 595−602.
6. Singh, S. M., and Murray, D. (2003) Molecular modeling of the membrane targeting of phospholipase C pleckstrin homology domains, *Protein Sci. 12*, 1934−1953.
7. Philip, F., Guo, Y., and Scarlata, S. (2002) Multiple roles of the pleckstrin homology domains in phospholipase Cβ function, *FEBS Lett. 531*, 28−32.
8. Guo, Y., Philip, F., and Scarlata, S. (2003) The pleckstrin homology domains of phospholipases C-β and -δ confer activation through a common site, *J. Biol. Chem. 278*, 29995−30004.
9. Wang, T., Pentyala, S., Elliott, J., Dowal, L., Gupta, E., Rebecchi, M., and Scarlata, S. (1999) Selective interaction of the C2 domains of phospholipase C-β_1 and -β_2 with activated Gαq subunits: An alternative function for C2-signaling modules, *Proc. Natl. Acad. Sci. U.S.A. 96*, 7843−7846.
10. Lee, S. B., Shin, S. H., Hepler, J. R., Gilman, A. G., and Rhee, S. G. (1993) Activation of phospholipase C-β2 mutants by G protein αq and $\beta\gamma$ subunits, *J. Biol. Chem. 268*, 25952−25957.
11. Runnels, L. W., Jenco, J., Morris, A., and Scarlata, S. (1996) Membrane binding of phospholipases C-β_1 and C-β_2 is independent of phosphatidylinositol 4,5-bisphosphate and the α and $\beta\gamma$ subunits of G proteins, *Biochemistry 35*, 16824−16832.
12. Wang, T., Pentyala, S., Rebecchi, M., and Scarlata, S. (1999) Differential association of the pleckstrin homology domains of phospholipases C-β1, C-β2 and C-δ1 with lipid bilayers and the $\beta\gamma$ subunits of heterotrimeric G proteins, *Biochemistry 38*, 1517−1527.
13. Cifuentes, M. E., Delaney, T., and Rebecchi, M. J. (1994) D-myo-Inositol 1,4,5-trisphosphate inhibits binding of phospholipase C-δ1 to bilayer membranes, *J. Biol. Chem. 269*, 1945−1948.
14. Lomasney, J. W., Cheng, H. F., Wang, L. P., Kuan, Y., Liu, S., Fesik, S. W., and King, K. (1996) Phosphatidylinositol 4,5-bisphosphate binding to the pleckstrin homology domain of phospholipase C-δ1 enhances enzyme activity, *J. Biol. Chem. 271*, 25316−25326.
15. Wang, T., Dowal, L., Rebecchi, M., and Scarlata, S. (2000) The pleckstrin homology domain of phospholipase C-β_2 links the binding of G$\beta\gamma$ to the catalytic core, *J. Biol. Chem. 275*, 7466−7469.
16. Scarlata, S., Gupta, R., Garcia, P., Keach, H., Shah, S., Kasireddy, C. R., Bittman, R., and Rebecchi, M. J. (1996) Inhibition of phospholipase C-δ1 catalytic activity by sphingomyelin, *Biochemistry 35*, 14882−14888.
17. Kobayashi, M., Mutharasan, R. K., Feng, J., Roberts, M. F., and Lomasney, J. W. (2004) Identification of hydrophobic interactions between proteins and lipids: Free fatty acids activate phospholipase Cδ1 via allosterism, *Biochemistry 43*, 7522−7533.
18. Litosch, I. (2000) Regulation of Phospholipase Cβ_1 Activity by Phosphatidic Acid, *Biochemistry 39*, 7736−7743.
19. Feng, J., Roberts, M., Drin, G., and Scarlata, S. (2005) Dissection of the steps of phospholipase Cβ_2 activity that are enhanced by G$\beta\gamma$ subunits, *Biochemistry 44*, 2577−2584.
20. Gennis, R. B. (1989) *Biomembranes: Molecular Structure and Function*, Springer-Verlag, New York.
21. Ghosh, M., Wang, H., Kelley, G. G., and Smrcka, A. V. (2004) Purification of phospholipase Cβ and phospholipase Cϵ from Sf9 cells, *Methods Mol. Biol. 237*, 55−64.
22. Kozasa, T. (2004) *Methods Mol. Biol. 237*, 21−38.
23. Scarlata, S. (2002) Determination of the strength and specificity of membrane-bound protein association using fluorescence spectroscopy: Application to the G protein−phospholipase C system, in *Methods in Enzymology* (Hildrebrandt, I., Ed.) Vol. 345C, pp 306−327, Academic Press, New York.
24. Douguet, D., and Labesse, G. (2001) Easier threading through web-based comparison and cross-validations, *Bioinformatics 17*, 752−753.
25. Kim, D. E., Chivian, D., and Baker, D. (2004) Protein structure prediction and analysis using the Robetta server, *Nucleic Acids Res. 32* (Suppl. 2), W526−W531.
26. Pearlman, D. A., Case, D., Caldwell, J. W., Ross, W. R., Cheatham, T. E. I., DeBolt, S., Ferguson, D., Siebel, G., and Kollman, P. (1995) AMBER, a computer program for applying molecular mechanics, normal mode analysis, molecular dynamics and free energy calculations to elucidate the structures and energies of molecules, *Comput. Phys. Commun. 91*, 1−41.
27. Sippl, M. J. (1993) Recognition of errors in three-dimensional structures of proteins, *Proteins 17*, 355−362.
28. Eisenberg, D., Luthy, R., and Bowie, J. U. (1997) VERIFY3D: Assessment of protein models with three-dimensional profiles, *Methods Enzymol. 277*, 396−404.
29. Wallner, B., and Elofsson, A. (2003) Can correct protein models be identified? *Protein Sci. 12*, 1073−1086.
30. Vogt, B., Ducarme, P., Schinzel, S., Brasseur, R., and Bechinger, B. (2000) The topology of lysine-containing amphipathic peptides in bilayers by circular dichroism, solid-state NMR, and molecular modeling, *Biophys. J. 79*, 2644−2656.
31. Ducarme, P., Rahman, M., and Brasseur, R. (1998) IMPALA: A simple restraint field to simulate the biological membrane in molecular structure studies, *Proteins 30*, 357−371.
32. Eisenhaber, F., Persson, B., and Argos, P. (1995) Protein structure prediction: Recognition of primary, secondary, and tertiary structural features from amino acid sequence, *Crit. Rev. Biochem. Mol. Biol. 30*, 1−94.
33. Bondi, A. (1964) van der Waals volumes and radii, *J. Phys. Chem. 68*, 441−451.
34. Humphrey, W., Dalke, A., and Schulten, K. (1996) VMD: Visual molecular dynamics, *J. Mol. Graphics 14*, 27−38.
35. Rebecchi, M. J., and Scarlata, S. (1998) Pleckstrin homology domains: A common fold with diverse functions, *Annu. Rev. Biophys. Biomol. Struct. 27*, 503−528.
36. Ilkaeva, O., Kinch, L., Paulssen, R., and Ross, E. M. (2002) Mutations in the carboxyl-terminal domain of phospholipase C-β1 delineate the dimer interface and a potential Gαq interaction site, *J. Biol. Chem. 277*, 4294−4230.
37. Singer, A., Waldo, G., Harden, T. K., and Sondek, J. (2002) A unique fold of phospholipase C-β mediates dimerization and interaction with GαQ, *Nat. Struct. Biol. 9*, 32−36.
38. Simoes, A. P., Schnabel, P., Pipkom, R., Camps, M., and Gierschik, P. (1993) A peptide corresponding to a potential polyphosphoinositide binding site of phospholipase C-β2 enhances its catalytic activity, *FEBS Lett. 331*, 248−251.
39. van der Meer, W., Coker, G., and Chen, S. S.-Y. (1994) *Resonance Energy Transfer, Theory and Data*, VCH Publishers, Inc., New York.
40. Buck, E., Li, J., Chen, Y., Weng, G., Scarlata, S., and Iyengar, R. (1999) Resolution of a signal transfer region from a general binding domain in G-β for stimulation of phospholipase C-β_2, *Science 283*, 1332−1335.
41. Scarlata, S. (2002) Regulation of the lateral association of phospholipase C-β_2 and G protein subunits by lipid rafts, *Biochemistry 41*, 7092−7099.

42. Sankaran, B., Osterhout, J., Wu, D., and Smrcka, A. (1998) Identification of a structural element in phospholipase Cβ2 that interacts with G protein $\beta\gamma$ subunits, *J. Biol. Chem. 273*, 7148−7154.
43. Lodowski, D. T., Pitcher, J. A., Capel, W. D., Lefkowitz, R. J., and Tesmer, J. J. (2003) Keeping G proteins at bay: A complex between G protein-coupled receptor kinase 2 and G$\beta\gamma$, *Science 300*, 1256−1262.
44. Garcia, P., Gupta, R., Shah, S., Morris, A. J., Rudge, S. A., Scarlata, S., Petrova, V., McLaughlin, S., and Rebecchi, M. J. (1995) The pleckstrin homology domain of phospholipase C-δ1 binds with high affinity to phosphatidylinositol 4,5-bisphosphate in bilayer membranes, *Biochemistry 34*, 16228−16234.
45. Romoser, V., Ball, R., and Smrcka, A. V. (1996) Phospholipase C β_2 Association with Phospholipid Interfaces Assessed by Fluorescence Resonance Energy Transfer. G Protein $\beta\gamma$ subunit-mediated translocation is not required for enzyme activation, *J. Biol. Chem. 271*, 25071−25078.
46. Jenco, J. M., Becker, K. P., and Morris, A. J. (1997) Membrane-binding properties of phospholipase C-β_1 and phospholipase C-β_2: Role of the C-terminus and effects of polyphosphoinositides, G-proteins and Ca^{2+}, *Biochem. J. 327*, 431−437.
47. Ford, C. E., Skiba, N. P., Bae, H., Daaka, Y., Reuveny, E., Shekter, 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) Molecular basis for interactions of G protein $\beta\gamma$ subunits with effectors, *Science 280*, 1271−1274.
48. Fogg, V., Azpiazu, I., Linder, M. E., Smrcka, A., Scarlata, S., and Gautam, N. (2001) Role of the γ subunit prenyl moiety in G protein $\beta\gamma$ complex interaction with phospholipase Cβ, *J. Biol. Chem. 276*, 41787−41802.
49. Bonacci, T. M., Ghosh, M., Malik, S., and Smrcka, A. V. (2005) Regulatory interactions between the amino terminus of G-protein $\beta\gamma$ subunits and the catalytic domain of phospholipase Cβ2, *J. Biol. Chem. 280*, 10174−10181.
50. Tuzi, S., Uekama, N., Okada, M., Yamaguchi, S., Saito, H., and Yagisawa, H. (2003) Structure and dynamics of the phospholipase C-δ1 pleckstrin homology domain located at the lipid bilayer surface, *J. Biol. Chem. 278*, 28019−28025.

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