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The Pleckstrin Homology Domain of Phospholipase C β Transmits Enzymatic Activation through Modulation of Membrane - Domain Orientation[§]

Guillaume Drin¹, Dominique Douguet², and Suzanne Scarlata^{*}

¹Department of Physiology and Biophysics, Stony Brook University, Stony Brook, NY 11794-8661, USA

²Centre de Biochimie Structurale, 29 rue de Navacelles, 34090 Montpellier, France

^{*}Department of Physiology and Biophysics, Stony Brook University, Stony Brook, NY 11794-8661, USA

Abstract

Phospholipase C-beta (PLC β) enzymes are activated by G α_q and G $\beta\gamma$ subunits and catalyze the hydrolysis of the minor membrane lipid phosphatidylinositol 4,5 biphosphate (PI(4,5)P₂). Activation of PLC β_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 PLC β_2 domain (residues 71-88) which, when added to the enzyme as a peptide, causes enzyme activation similar to 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 head groups. Moreover, 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, PLC $\beta_2/\delta 1$ (C193S). We find an increase in FRET with binding to membranes with mixed lipids but not to those containing only lipids with electrically neutral head groups. These results suggest that enzymatic activation can be conferred through optimal association of the PH β 71-88 region to specific membrane surfaces. These studies allow us to understand the basis of variations of G $\beta\gamma$ activation on different membrane surfaces.

Phospholipases C are intracellular enzymes that catalyze the hydrolysis of lipid phosphatidylinositol 4,5 biphosphate (PI(4,5)P₂) to give 1,4,5 inositol trisphosphate (IP₃) and diacylglycerol (DAG) which in turn promote the release of Ca²⁺ 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 PLC β family is regulated by heterotrimeric G proteins. All four known subtypes of PLC β are activated by G α_q subunits while two (PLC β_2 and PLC β_3) are additionally activated by G $\beta\gamma$ subunits[1,2].

PLC β s are multidomain enzymes consisting of an N-terminal PH domain, 4 EF hands, a catalytic X/Y domain, a C2 domain and a C-terminal tail. While the only domain of PLC β whose structure has been solved is the C-terminal tail [3], high-resolution structures of the PH [4] and catalytic-C2 domains [5] of the closely related enzyme PLC δ_1 are available. The

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Address correspondence to: Suzanne Scarlata, Department of Physiology and Biophysics, Basic Science Tower, Stony Brook University, Stony Brook, NY 11794-8661, USA, Tel: 631 444 3071; Fax: 631 444 3432; [email: Suzanne.Scarlata@stonybrook.edu](mailto:Suzanne.Scarlata@stonybrook.edu).

relatively high sequence homology between PLC β_2 and PLC δ_1 has allowed the construction of theoretical models of the PH and catalytic domains of PLC β_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 G α_q subunits [9]9(9)(9)(9)(9)(9)(9)(9)(9)(9)(9)(9)[10]. The N-terminal PH domain of PLC β_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 PLC δ_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 PLC β_2 into PLC δ_1 results in a chimera PLC β_2/δ_1 possessing the same G $\beta\gamma$ and membrane binding properties as PLC β_2 , and which can be identically activated by G $\beta\gamma$ subunits [15]. Similarly, swapping the PH domain of PLC δ_1 into PLC β_2 results in a chimera PLC β_2/δ_1 that can no longer be activated by G $\beta\gamma$ but instead can be activated by PI(4,5)P $_2$ similar to PLC δ_1 [8]. The observation that activation of PLC β_2 and PLC δ_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 critical in regulating catalytic activity.

Since PLC works on lipid surfaces, it is not surprising that enzyme activity and activation depends strongly on the physical properties of the membrane surface. It has been shown that the activity of both PLC β_2 and PLC δ_1 depend on the matrix in which their PI(4,5)P $_2$ substrate is immersed. For example, while sphingomyelin lipids inhibit PLC δ_1 [16], fatty acids promote its activity [17]. PLC β_1 has a higher activity in the presence of an excess of PA lipids [18], while the binding of PLC β_2 is somewhat promoted by the inclusion of PE lipids [11]. It is generally found that inclusion of PE in assays increases the ability of PLC β_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 head groups which may allow better membrane penetration into the surface to access substrate (see [20]).

In this study, we continued our previous work in understanding the role of the PH domain of PLC β_2 in conferring G $\beta\gamma$ activation. We began with the observation that PH β_2 contains a unique sequence (PH71-88) which can interact with membranes. Surprisingly, addition of a peptide having this sequence to PLC β_2 simulates its activity to an extent similar to 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 PLC β_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- PLC β_2/δ_1 and mutants, obtained by using the site-directed mutagenesis kit from Stratagene, were expressed for 20 hours at room temperature in BL21D3 *E. coli* growing in Superbroth medium after an induction at OD of 0.6 with 1 mM IPTG. The bacteria were harvested by centrifugation at 6000 x g. The pellet was resuspended in lysis buffer (50 mM NaH $_2$ PO $_4$ (pH 8), 300 mM NaCl, 10 mM imidazole) containing 1mM PMSF, 2.5 mM benzamidine, 1mM DTT and supplemented with a protease inhibitor cocktail (Sigma) and the bacteria were broken. The lysate was treated for 30 minutes with DNaseI in the presence of Mg $^{2+}$ and with RNase A. The cell material was centrifuged for 90 minutes at 140000 g at 4° C and the supernatant was loaded 3 times on a Ni $^{2+}$ -NTA column (Qiagen). After washing the column 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 1mM DTT, and brought to a final NaCl concentration of 30 mM. The material was loaded onto a 1 5Q column and eluted with a NaCl gradient from 0 to 500 mM.

His6-PLC β_2 was expressed using a *Sf9* expression system and purified as described with minor modifications [21]. G $\beta_{1\gamma_2}$ subunits were prepared by co-expression of G α_q , G β_1 and His6-G γ_2 in *Sf9* cells and purified on a Ni²⁺-NTA column [22]. This construct activates PLC β_2 identically to the non-His tagged counterpart [15].

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

Peptides

PH β 71-88 (NH₂-KFAKMPKSQKL RDVFNM D-CONH₂), sPH β 71-88 (NH₂-VSKQDLKMFNKMRDAPFK-CONH₂) and PH β 120-135 (NH₂-ENVGKAWAEDVLALVK-CONH₂) were purchased from the American Peptide Company (Sunnyvale, CA) with a purity >90 % as confirmed by analytical HPLC and mass spectrometry and were used as received. The peptide G β 86-105 and G β 86-105(M101N) are gifts from Dr. R.Iyengar (Dept. of Pharmacology, Mt. Sinai School of Medicine). The concentration of PH β 71-88 and sPH β 71-88 were estimated by BCA assay (Pierce) whereas the concentration of other peptides is estimated by absorption at 280 nm.

Lipids

1-palmitoyl-2-oleoyl phosphatidylethanolamine (POPE), 1-palmitoyl-2-oleoylphosphatidylserine (POPS), 1-palmitoyl-2-oleoylphosphatidylcholine (POPC) and phosphatidylinositol 4,5 bisphosphate (PI(4,5)P₂) in chloroform were purchased from Avanti Polar Lipids, Inc (Alabaster, AL) whereas the [³H]PI(4,5)P₂ was from New England Nuclear, Inc.

Enzyme Activity Studies

Measurements of PI(4,5)P₂ hydrolysis by PLC enzymes were carried out using small, unilamellar vesicles composed of POPE:POPS:PI(4,5)P₂ at a 66:32:2 molar ratio and doped with enough [³H]PI(4,5)P₂ to obtain approximately 10000 dpm/assay. The purified enzymes were diluted to 1-3 nM in 15 μ l Ca²⁺ free-MAIN buffer (50 mM Hepes pH 7.2, 160 mM KCl, 3 mM EGTA, 1 mM DTT). G $\beta\gamma$ subunits were dialyzed three times in a Slide-A-Lyzer (Pierce) with a molecular weight cut-off of 3500 Da, for 30 minutes at 4° C against 50 mM Hepes (pH 7.4), 150 mM NaCl, 1mM DTT buffer. When appropriate, enzymes were premixed with G $\beta\gamma$ subunits or with an identical volume of G $\beta\gamma$ -stock buffer dialysed as mentioned above.

To measure PLC activity, the proteins (15 μ l) were placed on ice and mixed with 10 μ l of 3mM freshly sonicated lipids and 5 μ l of MAIN buffer containing 12mM CaCl₂ to give a final concentration of 1000 nM Ca²⁺ [11]. The reaction was initiated by placing the samples in a water bath at 37 °C for 30s-5 minutes (depending on the phospholipase) and terminated by the addition of 0.2 ml 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 x 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 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 hour against 500 ml of 20 mM Hepes (pH 7.4), 150 mM NaCl buffer. After, 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 yields proteins labeled at a 1:1 probe:enzyme molar ratio was determined by absorption to determine the probe concentration and BCA analysis to determine the protein concentration. After one hour, 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 1ml PD6 column followed by elution with 1 ml of 20 mM Hepes (pH 7.4), 150 mM NaCl, 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, which covalently attaches to free amine groups. Labeling by these probes was performed at pH 7 to favor a 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 SUNY Stony Brook.

Fluorescence measurements were performed on an ISS spectrofluorometer (Champaign, IL) using 3 mm quartz cuvettes. Peptide and protein stocks were diluted into 20 mM Hepes (pH 7.2), 160 mM NaCl, 1 mM DTT. The emission spectrum of CPM-labeled protein was measured from 415 to 530 nm ($\lambda_{\text{ex}}=384$ nm) whereas the emission of acrylodan-labeled protein was measured from 400 to 580 nm ($\lambda_{\text{ex}}=360$ 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 non-fluorescent energy transfer acceptor, is 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 ratio of the doubly labeled to singly labeled intensities are reported.

Membrane binding studies

Membrane binding was carried out either by titrating large, unilamellar vesicles, (prepared by manual extrusion through 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 PLC β_2/δ_1 was carried out by monitoring the change in the intrinsic fluorescence intensity ($\lambda_{\text{ex}}=280$ nm, scanning from 300-450 nm). Membrane binding of PH β_71 -88 and sPH β_71 -88 was determined by using LUVs labeled with 0.1% Laurdan (see[23]) and scanning the emission from 400 to 580 nm with $\lambda_{\text{ex}}=360$ 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 the human PLC β_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 PLC δ_1 (PDB code: 1MAI) as the best template for a homology modeling. Since the servers use different methods for the alignment and building steps, the two resulting structures are different (RMS = 4.86 Å), especially at the N-terminus (residues 1-12) and loops 48-59 and 71-88 segments.

The models were minimized in a water box ($10 \times 10 \times 10$ Angstroms) by using the program AMBER v7.00 [26]26(26)(26)(26)(26)(26)(26)(26)(26)(26)(26)(26)[26] and then evaluated by using programs 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; PROSAII = -0.199; ProQ (LGscore/ MaxSub) = 2.220 / 0.232; Ramachandran (% residue in favorable/allowed region) = 97.7 / 100; WhatIF (Av/Z-score) = 0.405 / -2.70 compared to those calculated for PH δ_1 structure (0.415; -1.671; 4.526 / 0.551; 96.58 / 99.15; 0.279/1.79). A major advantage of this homology modeling method is to build the additional residues in PH β_2 which are not present in the template.

Membrane insertion simulation

The orientation and insertion of the PH β_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 z-axis is the center of the bilayer).

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

with $\alpha = 2.042275$ 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 1 Å step) and rotations (maximum of 5° step) moves 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} . Accessible surface area was calculated using the program NSC [32] with the atomic radii from A. Bondi [33]. α_{lip} is an empirical factor fixed to - 0.018 [30].

The electrostatic potential of PH β_2 models was evaluated by using the program APBS (Adaptive Poisson-Boltzmann Solver) [25]. Atomic radius and charges were AMBER ones. The concentration of mobile ion species [positives] = [negatives] were 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 PH β 2 region that produces activation

The PH domain of PLC β 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 PLC δ 1 binds specifically to PI(4,5)P₂ to confer activation [8,14]. The structure of the PH δ 1-Ins(1,4,5)P₃ has been solved [4] and has been used as a basis for theoretical models of PH β 2 [6,7]. However, these models give little insight into the reason why PH β 2 binds strongly to model membranes with little specificity whereas PH δ 1 binds specifically to membranes that contain PI(4,5)P₂ [11,12]. PH domains share little sequence identity despite their conserved fold [35] and homology profiles show that the sequence of PH β 2 has highest homology to PH δ 1 with 11% of sequence identity. When building a model of the PH domain of PLC β 2 based on the high-resolution structure of PH δ 1 (Figure 1A), we noted this former contains a unique insertion of 9 amino-acids between β strands 5 and 6 (segment 75-83). The structural alignment of PH β 2 and PH δ 1 highlights this observation (Figure 1B), as well as indicating that PH β 2 contains a more apolar N-terminal region compared to PH δ 1. Neither of the reported models of PH β 2 has included this latter region [6,7]. The 71-88 region of PH β 2 (i.e. the β 5/ β 6 loop), which was included in the study of Singh & 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 PLC β 2 71-88 segment (PH β 71-88) and a peptide with a scrambled sequence to use as a control (sPH β 71-88). We then tested the ability of these peptides to affect the activity of PLC β 2 and the activity of a chimeric enzyme consisting of the PH domain of PLC β 2 and the remaining domains of PLC δ 1 (PLC β 2/ δ 1). PLC β 2/ δ 1, (previously described as PLC β 2^{PH-L}/ δ 1 [15]), is activated to the same extent as PLC β 2 by G $\beta\gamma$ subunits. The rationale for using this protein is that it lacks the PLC β 2 C-terminal tail which may result in enzyme dimerization [36,37] and it can be expressed in bacteria, unlike PLC β 2, allowing for easier preparation of point mutations. We note that key studies were repeated using wild type PLC β 2.

In Figure 2A, we show the effect of PH β 71-88 on PLC β 2/ δ 1 activity and its activation by G $\beta\gamma$ subunits using substrate dispersed in bilayers composed of POPE:POPS :PI(4,5)P₂ (66:32:2). Unexpectedly, we find that the peptide increases the activity of PLC β 2/ δ 1 4-fold as compared to control in the absence of G $\beta\gamma$ subunits. In addition, above a peptide concentration of 0.66 μ M, we find that PLC β 2/ δ 1 is no longer activated by G $\beta\gamma$ subunits. Parallel studies using the scrambled peptide, sPH β 71-88, show a reduced effect on activity and allow for the G $\beta\gamma$ activation at the highest peptide concentration tested. As a control, we tested the ability of the peptide to activate wild type PLC β 2. In Figure 2B we show that the addition of 6.6 μ M of PH β 71-88 but not sPH β 71-88 to PLC β 2 increases its activity and that the complex can not be further activated by G $\beta\gamma$ subunits. We note that the extent of activation of PLC β 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 [18]). However, the extent of G $\beta\gamma$ activation of PLC β 2 and PLC β 2/ δ 1 has been shown to be identical [15] and we find that the extent of activation produced by PH β 71-88 is similar to G $\beta\gamma$ for both types of enzymes.

In an effort to understand the mechanism underlying the enhancement of PLC β 2/ δ 1 activity by PH β 71-88, we noted that this region contains 5 positively charged residues and 2 negatively charged residues. Previous studies have reported that cationic peptides including polylysine can activate PLC β 2 [38] although the amount of peptide required is much higher than we find for PH β 71-88 but within the range seen for the scramble peptide suggesting that activation by

PH β 71-88 is specific. Therefore, we produced two mutants of PLC β 2/ δ 1 in which each has 2 cationic side chains replaced with anionic ones, PLC β 2/ δ 1(K74E/K77E) and PLC β 2/ δ 1(K80E/R82E). Both mutants, and in particular PLC β 2/ δ 1 (K80E/R82E) were not as strongly activated by G $\beta\gamma$ subunits as compared to the wild type chimera although their binding to POPC and POPC:POPS (2:1) bilayers were the same within error as the wild type chimera. The reduced activity of these mutants support the idea that the 71-88 region plays a role in enzyme regulation (Figure 2C). Interestingly, as noted in the legend of Figure 2C, PLC β 2/ δ 1 (K80E/R82E) has a basal activity two-fold higher than PLC β 2/ δ 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, plays a role in PLC β 2/ δ 1 activation.

PH β 71-88 interacts with membrane surfaces

We undertook a series of studies to understand the basis for PLC β 2/ δ 1 activation by PH β 71-88. Since the PH domains of PLC β enzymes bind strongly and non-specifically to neutral and charged membrane surfaces [11,12], we determined the most probable orientation of PH β 2 into an electrically neutral membrane by using a continuous restrain force-field [31]. As shown in the Figure 3A, the simulation identified a single minima with the PH β 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 PH β 2 where its non-polar N-terminus is inserted deeply into the membrane and where the 71-88 segment lies 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 the 71-88 region of PH β 2 interacts 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 measured the membrane binding of PH β 71-88 and sPH β 71-88 to negatively charged or electrically neutral surfaces. This study was carried out by doping large, unilamellar vesicles with 0.1 mol% of a lipophilic fluorescence reporter probe, Laurdan [12,23] and measuring its change in 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, PH β 71-88 showed similar binding affinities to POPC:POPS (2:1) as POPC:POPS:POPE (1:1:1) indicating that binding is primarily through electrostatic interactions. Interestingly, the scrambled sequence, sPH β 71-88, had a much weaker affinity to POPC:POPE:POPS bilayers compared to PH β 71-88 (Figure 4) even though it has an identical electrical charge suggesting that the distribution of charge and/or the helix-forming propensity contributes to PH β 71-88 membrane binding.

We then tested whether PH β 71-88 altered PLC β 2/ δ 1 membrane interactions by measuring the capacity of the enzyme to bind to membranes in the absence or the presence of 10 μ M peptide. In an initial series of studies, we monitored the binding of PLC β 2/ δ 1 to POPC:POPS:POPE large, unilamellar vesicles through changes in intrinsic fluorescence. We have previously found that the emission intensity of PLC β 2 significantly decreases by ~35% upon membrane binding most likely due to the quenching of interfacial Trp residues by the ionic lipid head groups [11]. We find that this quenching also occurs upon the association of PLC β 2/ δ 1 to these membranes giving a membrane partition coefficient (K_p) of 11 ± 5 μ M. This identical value was obtained in the presence of 10 μ M scrambled peptide sPH β 71-88. However, in the presence of 10 μ M PH β 71-88, binding was reduced ~10 fold indicating that PH β 71-88 interferes with membrane binding. This result prompted us to carry out a more comprehensive membrane binding study.

PH β 71-88 affects membrane interactions of the catalytic domain

In analyzing our data, we noted that our previous studies showed that the intrinsic fluorescence of PH β 2 does not change upon membrane binding [12]. Therefore, the changes in PLC β 2/ δ 1 intrinsic fluorescence must be due to Trp/Tyr residues in the EF-catalytic domains. Thus, the above results imply that PH β 71-88 is affecting membrane interactions at regions other than the PH domain.

To better understand which domains may be affected by PH β 71-88, we undertook a series of studies to determine whether membrane binding of the PH and catalytic domains are linked. We note that we could discriminate membrane binding of the PH domain by labeling with an amine reactive probe under conditions where attachment to the N-terminus is preferred (see methods) although we cannot discount the possibility of covalent labeling of Lys side chains in other regions of the protein. We also note that PLC β 2/ δ 1 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 mutant PLC β 2/ δ 1(C 193S) is correctly activated by G β γ (*data not shown*).

We labeled PLC β 2/ δ 1 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-PLC β 2/ δ 1 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 $16 \pm 3 \mu\text{M}$. Addition of $10 \mu\text{M}$ PH β 71-88 did not significantly change the membrane binding of the enzyme (Figure 5A), nor did addition of $10 \mu\text{M}$ scrambled peptide. We note that identical data were obtained using the PLC β 2/ δ 1(C193S) mutant. These data show that PH β 71-88 does not interfere with the membrane interaction of N-terminal region of PLC β 2/ δ 1.

To follow membrane interactions of the catalytic domain of PLC β 2/ δ 1, we labeled the PLC β 2/ δ 1(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 $10 \mu\text{M}$ PH β 71-88 ($K_p = 11 \pm 2 \mu\text{M}$) than in the presence of peptide ($K_p = 36 \pm 4 \mu\text{M}$) or in the presence of $10 \mu\text{M}$ scrambled peptide (*data not shown*). Interestingly, the effect of peptide was more pronounced when membranes with electrically neutral head groups were used (i.e. $K_p = 10 \pm 2$ versus $71 \pm 10 \mu\text{M}$, see Figure 5B). These results show that the presence of 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 non-fluorescent FRET acceptor (DABCYL SE). If the two probes are positioned close enough for interdomain FRET to occur (~ 25 Angstroms, [39]), then the emission intensity of the double-labeled probe should be significantly lower than its single labeled counterpart under identical conditions. Therefore, we assessed the presence of FRET by comparing the ratio of the DABYLC/CPM labeled protein versus the CPM labeled one.

We carried out these studies by labeling PLC β 2/ δ 1 (C193S) with CPM (see methods). Based on the crystal structure of the catalytic domain [5], there are two fully exposed Cys (C334 and C367) that are in close proximity 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 are preferentially labeled. We took half of PLC β 2/ δ 1 (C193S) - CPM and labeled it under conditions favoring attachment to the N-terminus with a non-fluorescence energy transfer acceptor DABCYL SE and labeling was verified by Edman degradation and the molar labeling ratio was $\sim$ 0.8 probe:protein 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 non-fluorescent since the probes would be in close proximity 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 movement when binding to lipids.

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 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 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 PLC β 2, we determined whether PH71-88 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 μ M PH β 71-88 did not significantly affect the binding affinity for POPC:POPS:POPE bilayers ($K_p = 8 \pm 0.5 \mu$ M). However, when the peptide concentration was increased to 30 μ M, a decrease in the binding affinity was seen ($K_p = 13 \pm 1 \mu$ M). These same affinities were obtained for DOPC as well as POPC showing that the nature of the hydrocarbon region is not affecting enzyme binding. Since the $\sim$ 400 residue C-terminus of PLC β 2 contributes significantly to membrane binding [8], then it is likely that the PLC β 2 - PH71-88 complex (see below) interacts with membranes similarly to the uncomplexed enzyme, and the observed decrease in PLC β 2 binding to POP:POPS:POPE in the presence of 30 μ M peptide is due to direct competition for the membrane surface by the peptide.

Activation of PLC β 2/ δ 1 is dependent on membrane curvature

Our membrane binding studies suggest that the 71-88 region will confer responsiveness to G $\beta\gamma$ more readily on membrane surfaces to where 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 PLC β 2 and activation by G $\beta\gamma$ subunits [19]. To better test this idea, we monitored the activity of PLC β 2/ δ 1 employing a peptide derived from the G β subunit, G β 86-105, which activates PLC β 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 [41]). Thus, this peptide will allow us to isolate membrane effects on PLC β 2/ δ 1.

We first verified that G β 86-105 peptide, but not the mutant G β 86-105(M101N), affects the activity of PLC β 2/ δ 1, similar to PLC β 2 (Figure 7A). Interestingly, we found that this peptide also activated PLC δ 1 thereby showing that activation by this peptide does not depend on its binding to the PH β 2 domain. This observation supports the idea that PH domains of PLC β 2 and PLC δ 1 binds and orients 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 PLC β 2/ δ 1, when completely activated by G β 86-105 peptide, is not activated by G $\beta\gamma$ similar to the behavior reported for PLC β 2 [40].

In Figure 7C we show a direct comparison of G β 86-105 activation of PLC β 2/ δ 1 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 β 5/ β 6 loop with the membrane surface.

The β 3/ β 4 strands plays a role in G $\beta\gamma$ interaction

If the β 5/ β 6 loop of PH β 2 modulates membrane association, then other regions of the PH domain must interact with G $\beta\gamma$ subunits. Recently, the structure of the GRK2 (G-receptor kinase 2) PH domain in complex with G $\beta\gamma$ has been solved [43]. From structural alignment of PH β 2 with PH-GRK2, we determined whether a motif F ζ DK (ζ corresponding to a hydrophobic residue), essential for the binding of PH-GRK2 to G β subunit, is also present in PLC- β 2. The Phe residue in this motif (F147) would be the equivalent of the Met664 of PH-GRK2 interacting with Trp99 and Met101 within G β subunit. When superposing our model of the PH β 2 with the structure of the PH domain of GRK2 (PDB code:1OMW) we found the same motif in terms of sequence alignment (*data not shown*). However, we found that the activation of PLC β 2/ δ 1 (F147E) by G $\beta\gamma$ remains similar to that of PLC β 2/ δ 1. In contrast, we observed that the activation by G $\beta\gamma$ of mutant PLC β 2/ δ 1(Y48A), PLC β 2/ δ 1(Y48R) and the double-mutant PLC β 2/ δ 1 (Y48R/F147E) is systematically reduced compared to the native chimera. (Figure 8 A and B).

The crystal structure of the GRK2 - G $\beta\gamma$ 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 120-135 of the PH β 2 domain, which is strongly predicted to be an α -helix, is implicated in the PLC β 2/G $\beta\gamma$ interaction. We find that the presence of 5 μ M the PH β 120-135 shows a slight inhibition of activation of PH β 2/ γ 1 by G $\beta\gamma$ (Figure 8 C and D). However, this peptide did not inhibit of the G $\beta\gamma$ -mediated activation of PLC β 2.

DISCUSSION

The PH domains of PLC β 2 and PLC δ 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 PH β 2 domain, residues 71-88. As discussed below and shown in Figure 9, we propose that this region will plays a role in inhibitory interactions between the PH and catalytic domains. Relief of inhibition will be achieved through binding of G $\beta\gamma$ subunits, or by adding 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, occur 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 by the data for the K80E mutant. As discussed below, we propose that activation by PH β 71-88 is not through direct interaction with the catalytic core but through modulating 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 71-88 segment of PH β_2 (i.e. the β_5/β_6 loop) has 5 positively charged residues that could participate in the regulation of PLC β_2 and the chimera, PLC β_2/δ_1 . Mutation of these residues did not affect membrane binding of PLC β_2/δ_1 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 suggesting that the region 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 PLC β_2/δ_1 and PLC β_2 by G $\beta\gamma$ is eliminated when incubated with PH β_2 71-88 suggests a common mechanism between these activators. Taken together these results suggest that the 71-88 loop of PH β_2 plays a role in regulating the catalytic domain rather than being directly involved in G $\beta\gamma$ subunit interactions.

To assess whether the PH β_5/β_6 loop may play a similar role in other PLC β isozymes, we note that while all PLC β isoforms are activated by G α_q subunits, only PLC β_2 and PLC β_3 are also activated by G $\beta\gamma$. The lack of G $\beta\gamma$ activation of PLC β_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 PLC β_1 -4 show a pair of positively charged residues corresponding to the K74/K77 pair in PLC β_2 . Additionally, PLC $\beta_{1,3}$ contain positively charged residues corresponding to K80/R82. Therefore, while PLC β_1 has the potential to be activated by G $\beta\gamma$ through PH71-88, its lack of association prevents this mechanism from occurring. Studies are underway to better understand the underlying mechanism for this lack of activation.

Our studies show that PH β_2 71-88 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), POPC:POPS:POPE (1:1:1) bilayers, 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 PLC β_2 activity and G $\beta\gamma$ activation on electrically neutral surfaces, and this is what is observed. Moreover, 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 PLC β_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 PLC β_2/δ_1 - G $\beta\gamma$ activation can be rationalized by the membrane binding specificity of PH β_2 71-88 even though the entire PH domain binds to membranes with little specificity [12].

It is important to note that the PH domains of PLC γ_1 and PLC β_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 [13,19,44]). The lack of effect of PH β_2 71-88 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 through the PH domain while membrane interactions of the weakly binding catalytic domain are susceptible to weakening and changing the domain orientation in the presence of PH β_2 71-88. 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 PH β 71-88 modulates the position of the catalytic core comes from fluorescence studies with the PLC β 2/ δ 1 chimera. We find that PH β 71-88 alters the membrane binding affinity of PLC β 2/ δ 1 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 PH β 71-88 did not affect the binding of a probe that targets the N-terminus. These data show that PH β 71-88 preferentially affects the membrane interactions of the catalytic domain.

The above studies suggest that movement of the PH and catalytic domains of PLC β 2/ δ 1 may be independent. This idea was tested by intramolecular FRET studies between probes in the catalytic domain and N-terminal regions. In general, increased transfer corresponds to a reduced distance between two probes. We find that enzyme binding to POPC:POPS:POPE bilayers results in an increase in FRET suggesting that PE surfaces stabilizes PH-catalytic domain orientation that allows for more efficient transfer. In contrast, a reduction in FRET is observed when the enzyme binds to POPC membranes suggesting an increase in interdomain distance. Taken together, these studies support the idea that different membrane surfaces promote differing distances/orientations between the PH and catalytic domains. This finding correlates well with the observation that the PH domain of PLC δ 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 PH β 71-88 peptide also stimulated the activity of wild type PLC β 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 G β 86-105 depends on the PE content of vesicles similar to studies using G β γ [19]. Even though membrane charge is important in PH β 71-88 - membrane interactions, activation is optimal on an expanded PE surfaces. This result, in combination to the membrane binding studies, allows us to speculate that the increased curvature and hydrophobicity of PE-containing vesicles allows residues 71-88 to orient the PH domain with the catalytic domain. It is possible that this orientation allows G β γ subunits, the G β 86-105 peptide or PH β 71-88 to bind to 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 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 PH β 71-88 membrane interactions. More studies are needed to determine whether different cellular membrane have the ability to modulate PLC β 2 activity and its ability to be activated by G β γ subunits.

Many reports have identified potential sites of interaction on G β γ for PLC β 2 [40,47-50] and potential sites of G β γ binding on PLC β 2 [9,12,44]. We have found here that the G β 86-105 peptide, which corresponds to a segment of G β subunit, not only activates PLC β 2 as previously demonstrated [40], but also stimulates the PLC β 2/ δ 1 chimera and PLC δ 1. This finding suggests that the segment interacts with PLC β 2 independently of the PH domain. Since the catalytic domains of the PLC β 2 and PLC δ 1 are very homologous, it is conceivable that they share a binding site for the region 86-105 of G β . We note that structural studies of the GRK2-G β γ complex [43] show that the primary interaction site is between G β 86-105 and the C-terminal

region of the GRK2 PH domain. However, our studies show that the C-terminal region of PH β_2 does not play a significant role in G $\beta\gamma$ activation (Figure 8).

So far, the mechanism through which PH β_2 binds to membranes is unknown in contrast to PH δ_1 whose membrane interactions have been clearly identified [4,50]. A structural model of PH β_2 and subsequent simulation of its insertion into an electrically neutral membrane allows us to propose a dominant membrane orientation where the 71-88 region resides on the membrane surface (Figure 3). It has been suggested that the accessible hydrophobic patch in the β_3/β_4 region, not present in PH δ_1 (see alignment in Figure 1) may play a role in the membrane binding of PH β_2 [6]. Interestingly, our simulations show that when the orientation of the ten first residues is modified by a *trans*-to-*cis* conversion of the Pro11-Lys12 amide bond, a second and additional local minima is found in which β_3/β_4 lies close to the membrane (*data not shown*). Indeed, this study highlights the possibility that the orientation of the PH β_2 domain with respect to the catalytic domain and the membrane surfaces is dynamic. In any case, the β_3/β_4 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 PH β_2 does not play a significant role in G $\beta\gamma$ activation.

Based on our results, we propose a model of G $\beta\gamma$ activation (Figure 9) in which in solution, the PH domain of PLC β_2/δ_1 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 β_5/β_6 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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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 phosphoinositide-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

REFERENCES

1. Rebecchi M, Pentylana S. Structure, function and control of phosphoinositide-specific phospholipase C. *Physiological Reviews* 2000;80:1291–1335. [PubMed: 11015615]

2. Rhee SG. Regulation of phosphoinositide-specific phospholipase C. *Ann.Rev.Biochem* 2001;70:281–312. [PubMed: 11395409]
3. Singh S, Murray D. Molecular modeling studies of phospholipase C PH domains. *Biophys.J* 2002;82:168a.
4. Ferguson KM, Lemmon MA, Schlessinger J, Sigler PB. Structure of the high affinity complex of inositol trisphosphate with a phospholipase C pleckstrin homology domain. *Cell* 1995;83:1037–1046. [PubMed: 8521504]
5. Essen LO, Perisic O, Cheung R, Katan M, Williams RL. Crystal structure of a mammalian phosphoinositide-specific phospholipase C- δ . *Nature* 1996;380:595–602. [PubMed: 8602259]
6. Singh SM, Murray D. Molecular modeling of the membrane targeting of phospholipase C pleckstrin homology domains. *Protein Sci* 2003;12:1934–1953. [PubMed: 12930993]
7. Philip F, Guo Y, Scarlata S. Multiple roles of the pleckstrin homology domains in phospholipase C β function. *FEBS Lett* 2002;531:28–32. [PubMed: 12401198]
8. Guo Y, Philip F, Scarlata S. The pleckstrin homology domains of phospholipases C- β and - δ confer activation through a common site. *J.Biol.Chem* 2003;278:29995–30004. [PubMed: 12761218]
9. Wang T, Pentylala S, Elliott J, Dowal L, Gupta E, Rebecchi M, Scarlata S. Selective interaction of the C2 domains of phospholipase C- β_1 and - β_2 with activated G α_q subunits: An alternative function for C2 - signaling modules. *Proc. Natl. Acad. Sci. USA* 1999;96:7843–7846. [PubMed: 10393909]
10. Lee SB, Shin SH, Hepler JR, Gilman AG, Rhee SG. Activation of phospholipase C- β_2 mutants by G protein α_q and $\beta\gamma$ subunits. *Journal of Biological Chemistry* 1993;268:25952–25957. [PubMed: 8245028]
11. Runnels LW, Jenco J, Morris A, Scarlata S. 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* 1996;35:16824–16832. [PubMed: 8988021]
12. Wang T, Pentylala S, Rebecchi M, Scarlata S. Differential association of the pleckstrin homology domains of phospholipases C- β_1 , C- β_2 and C- δ_1 with lipid bilayers and the $\beta\gamma$ subunits of heterotrimeric G proteins. *Biochemistry* 1999;38:1517–1527. [PubMed: 9931017]
13. Cifuentes ME, Delaney T, Rebecchi MJ. D-myo-inositol 1,4,5-trisphosphate inhibits binding of phospholipase C-delta 1 to bilayer membranes. *Journal of Biological Chemistry* 1994;269:1945–1948. [PubMed: 8294445]
14. Lomasney JW, Cheng HF, Wang LP, Kuan Y, Liu S, Fesik SW, King K. Phosphatidylinositol 4,5-bisphosphate binding to the pleckstrin homology domain of phospholipase C-delta1 enhances enzyme activity. *Journal of Biological Chemistry* 1996;271:25316–25326. [PubMed: 8810295]
15. Wang T, Dowal L, Rebecchi M, Scarlata S. The pleckstrin homology domain of phospholipase C- β_2 links the binding of G $\beta\gamma$ to the catalytic core. *Journal of Biological Chemistry* 2000;275:7466–7469. [PubMed: 10713048]
16. Scarlata S, Gupta R, Garcia P, Keach H, Shah S, Kasireddy CR, Bittman R, Rebecchi MJ. Inhibition of phospholipase C-delta 1 catalytic activity by sphingomyelin. *Biochemistry* 1996;35:14882–14888. [PubMed: 8942652]
17. Kobayashi M, Mutharasan RK, Feng J, Roberts MF, Lomasney JW. Identification of hydrophobic interactions between proteins and lipids: free fatty acids activate phospholipase C delta 1 via allosterism. *Biochemistry* 2004;43:7522–7533. [PubMed: 15182194]
18. Litosch I. Regulation of Phospholipase C β_1 Activity by Phosphatidic Acid. *Biochem* 2000;39:7736–7743. [PubMed: 10869178]
19. Feng J, Roberts M, Drin G, Scarlata S. Dissection of the steps of phospholipase C β_2 activity that are enhanced by G $\beta\gamma$ subunits. *Biochem* 2005;44:in press
20. Gennis, RB. *Biomembranes: Molecular Structure and Function*. Springer-Verlag; New York: 1989.
21. Ghosh M, Wang H, Kelley GG, Smrcka AV. Purification of phospholipase C beta and phospholipase C epsilon from Sf9 cells. *Methods Mol Biol* 2004;237:55–64. [PubMed: 14501038]
22. Kozasa T. *Meth.Molec.Biol* 2004;237:21–38.
23. Scarlata, S. Determination of the strength and specificity of membrane-bound protein association using fluorescence spectroscopy: Application to the G protein - phospholipase C system. In: Hildrebrandt, I.a., editor. *Methods in Enzymology*. 345C. Academic Press; New York: 2002. p. 306-327.

24. Douguet D, Labesse G. Easier threading through web-based comparison and cross-validations. *Bioinformatics* 2001;17:752–753. [PubMed: 11524382]
25. Kim DE, Chivian D, Baker D. Protein structure prediction and analysis using the Robetta server. *Nucl.Acids.Res* 2004;32(Suppl2):W526–531. [PubMed: 15215442]
26. Pearlman DA, Case D, Caldwell JW, Ross WR, Cheatham TEI, DeBolt S, Ferguson D, Siebel G, Kollman P. 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. *Comp. Phys.Commun* 1995;91:1–41.
27. Sippl MJ. Recognition of errors in three-dimensional structures of proteins. *Proteins* 1993;17:355–362. [PubMed: 8108378]
28. Eisenberg D, Luthy R, Bowie JU. VERIFY3D: assessment of protein models with three-dimensional profiles. *Methods Enzymol* 1997;277:396–404. [PubMed: 9379925]
29. Wallner B, Elofsson A. Can correct protein models be identified? *Protein Sci* 2003;12:1073–1086. [PubMed: 12717029]
30. Vogt B, Ducarme P, Schinzel S, Brasseur R, Bechinger B. The topology of lysine-containing amphipathic peptides in bilayers by circular dichroism, solid-state NMR, and molecular modeling. *Biophys J* 2000;79:2644–2656. [PubMed: 11053137]
31. Ducarme P, Rahman M, Brasseur R. IMPALA: a simple restraint field to simulate the biological membrane in molecular structure studies. *Proteins* 1998;30:357–371. [PubMed: 9533620]
32. Eisenhaber F, Persson B, Argos P. Protein structure prediction: recognition of primary, secondary, and tertiary structural features from amino acid sequence. *Crit Rev Biochem Mol Biol* 1995;30:1–94. [PubMed: 7587278]
33. Bondi A. *J.Phys.Chm* 1964;68:441–451.
34. Humphrey W, Dalke A, Schulten K. VMD: visual molecular dynamics. *J Mol Graph* 1996;14:33–38. 27–38. [PubMed: 8744570]
35. Rebecchi MJ, Scarlata S. Pleckstrin homology domains: A common fold with diverse functions. *Annu.Rev.Biophys.Biomolec.Struc* 1998;27:503–528.
36. Ilkaeva O, Kinch L, Paulssen R, Ross EM. Mutations in the carboxyl-terminal domain of phospholipase C-beta1 delineate the dimer interface and a potential G(alpha)q interaction site. *J.Biol.Chem* 2002;277:4294–4230. [PubMed: 11729196]
37. Singer A, Waldo G, Harden TK, Sondek J. A unique fold of phospholipase C-beta mediates dimerization and interaction with GalphaQ. *Nature Struc Biol* 2002;9:32–36.
38. Simoes AP, Schnabel P, Pipkom R, Camps M, Gierschik P. A peptide corresponding to a potential polyphosphoinositide binding site of phospholipase C-[beta]2 enhances its catalytic activity. *FEBS Lett* 1993;331:248–251. [PubMed: 8397113]
39. van der Meer, W.; Coker, G.; Chen, SS-Y. *Resonance Energy Transfer, Theory and Data*. VCH Publishers, Inc.; New York: 1994.
40. Buck E, Li J, Chen Y, Weng G, Scarlata S, Iyengar R. Resolution of a signal transfer region from a general binding domain in G-β for stimulation of phospholipase C-β2. *Science* 1999;283:1332–1335. [PubMed: 10037604]
41. Scarlata S. Regulation of the lateral association of phospholipase C-β2 and G protein subunits by lipid rafts. *Biochem* 2002;41:7092–7099. [PubMed: 12033943]
42. Sankaran B, Osterhout J, Wu D, Smrcka A. Identification of a structural element in phospholipase C beta 2 that interacts with G protein beta gamma subunits. *Journal of Biological Chemistry* 1998;273:7148–7154. [PubMed: 9507029]
43. Lodowski DT, Pitcher JA, Capel WD, Lefkowitz RJ, Tesmer JJ. Keeping G proteins at bay: a complex between G protein-coupled receptor kinase 2 and Gβγ. *Science* 2003;300:1256–1262. [PubMed: 12764189]
44. Garcia P, Gupta R, Shah S, Morris AJ, Rudge SA, Scarlata S, Petrova V, McLaughlin S, Rebecchi MJ. The pleckstrin homology domain of phospholipase C-δ1 binds with high affinity to phosphatidylinositol 4,5-bisphosphate in bilayer membranes. *Biochemistry* 1995;34:16228–16234. [PubMed: 8519781]
45. Romoser V, Ball R, Smrcka AV. Phospholipase C β2 Association with Phospholipid Interfaces Assessed by Fluorescence Resonance Energy Transfer. G Protein βγ subunit-mediated translocation

- is not required for enzyme activation. *Journal of Biological Chemistry* 1996;271:25071–25078. [PubMed: 8810260]
46. Jenco JM, Becker KP, Morris AJ. 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+} . *Biochemical Journal* 1997;327:431–437. [PubMed: 9359412]
47. Ford CE, Skiba NP, Bae H, Daaka Y, Reuveny E, Shekter LR, Rosal R, Weng G, Yang CS, Iyengar R, Miller RJ, Jan LY, Lefkowitz RJ, Hamm HE. Molecular basis for interactions of G protein betagamma subunits with effectors. *Science* 1998;280:1271–1274. [PubMed: 9596582]
48. Fogg V, Azpiazu I, Linder ME, Smrcka A, Scarlata S, Gautam N. Role of the γ subunit prenyl moiety in G protein $\beta\gamma$ complex interaction with phospholipase C β . *J.Biol.Chem* 2001;276:41787–41802.
49. Bonacci TM, Ghosh M, Malik S, Smrcka AV. Regulatory interactions between the amino terminus of G-protein betagamma subunits and the catalytic domain of phospholipase C β_2 . *J Biol Chem* 2005;280:10174–10181. [PubMed: 15611108]
50. Tuzi S, Uekama N, Okada M, Yamaguchi S, Saito H, Yagisawa H. Structure and dynamics of the phospholipase C-delta1 pleckstrin homology domain located at the lipid bilayer surface. *J Biol Chem* 2003;278:28019–28025. [PubMed: 12736268]

Figure 1.

(A) Structural model of the PH domain of PLC β_2 with residues 71-88 represented in CPK.

(B) Structural alignment of PH β_2 and PH δ_1 highlighting the loop between β_5 and β_6 PH-PLC β_2 not found in PH-PLC δ_1 . The residues in lowercase are those of PH δ_1 not included in the structure [4]. The residues 71-88 are colored in blue and define the β_5/β_6 loop in PLC β_2 . Asterisks and dots indicate respectively the identical and homologous residues.

Figure 2.

(A) Activation of the PLCβ2/δ1 in presence of increasing concentration of PHβ71-88 or scrambled sPHβ71-88 in the absence (black bars) or presence (gray bars) of 50 nM Gβγ. 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 the PLCβ2 by 6.6 μM of peptide PHβ71-88 or sPHβ71-88 in the absence (black bars) or the presence (gray bars) of 50 nM Gβγ. 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, PLCβ2/δ1 and PLCβ were incubated for 30 s or 3 minutes respectively at 37°C in Ca²⁺-containing MAIN buffer with 1 mM POPE:POPS:PI(4,5)P₂ (66:32:2) vesicles, freshly sonicated, doped with [³H]PIP (4,5)P₂ as described in the methods section. The statistical significance of difference between the activity of phospholipase in absence and presence of peptide is indicated (ANOVA, *p<0.1; ** p<0.01, *** p<0.001). (C) Activation of PLCβ2/δ1 (●), PLCβ2/δ1(K74E/K77E) (○) and PLCβ2/δ1(K80E/R82E) (■) with increasing concentration of Gβγ with 0.6 mM of vesicles. Each value is the mean of two independent experiments performed in duplicate with errors bars corresponding to the standard deviation. The activity was normalized to the basal activity which is 6.6 μmol.min⁻¹.mg⁻¹ for PLCβ2/δ1 6.2 μmol.min⁻¹.mg⁻¹, for PLCβ2/δ1 (K74E/K77E) and 16.6 μmol.min⁻¹.mg⁻¹ for PLCβ2/δ1 (K80E/R82E).

Figure 3.

(A) Simulated energy profile of PHβ₂ 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 in ribbon with the 71-88 region (β5/β6 loop) colored in blue and is inserted into the membrane with the configuration corresponding to the lowest total energy (-17.7 kcal.mol⁻¹). (B) Mapping of the electrostatic potential on the optimized configuration with + 1 kT/e potential isocontour as a blue transparent surface and the -1 kT/e isocontour as a red one.

Membrane Binding of PH β 71-88

Figure 4.

Membrane binding of 1 μ M PH β 71-88 peptide to POPC:POPS:POPE (1:1:1) (●) or POPC (■) LUVs labeled with 0.1% Laurdan. Also shown is the binding of 10 μ M sPH β 71-88 to POPC:POPS:POPE (1:1:1) (□). 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 minutes at room temperature. Emission spectra were recorded from 380 to 580 nm with λ_{ex} =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%.

Figure 5.

(A) Binding of to POPC:POPE:POPS (1:1:1) LUVs in the absence (●) or in the presence (○) of 10 μM PHβ7 1-88. We note that an identical binding behavior was seen in the presence of 10 μM sPHβ71-88, and substituting POPC for POPC:POPE:POPS (1:1:1). (B) The 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 (□).

FRET studies of Interdomain Movement of PLC β_2/δ_1

Figure 6.

Qualitative assessment of the change in interdomain distance using FRET. PLC β_2/δ_1 (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 FRET signal. The ratio of the fluorescence intensity of the doubly labeled versus singly labeled protein was then monitored as a function of lipid concentration. **(B)** Effect of 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 (20mM Hepes pH=7.5, 160 mM NaCl, 1mM DTT) for 30 minutes to remove excess storage detergent (0.1% cholate).

Figure 7.

(A) Effect of the peptide Gβ86-105 or Gβ86-105(M101N) on the rate of PI(4,5)P₂ hydrolysis by 1nM PLCβ₂/δ₁, PLCβ₂ or PLCδ₁. PLCβ₂/δ₁ and PLCδ₁ were incubated for 30 s whereas PLCβ₂ was incubated for 2 min at 37°C in Ca²⁺-containing MAIN buffer with 1 mM POPE:POPS:PI(4,5)P₂ (66:32:2) vesicles doped with [³H]PIP(4,5)P₂. The catalytic activity (normalized to the basal activity) was examined in the presence of various concentration of Gβ86-105 (●) or Gβ86-105(M101N) (○). Each point is the mean of five independent experiments, the error bars indicating the standard deviation. (B) Activation of PLCβ₂/δ₁ by increasing concentration of peptide Gβ86-105 in the absence (black bars) or presence of 75 nM Gβγ (gray bars). Each bar is the mean of 5 separate experiments, the error bars indicating the standard deviation. The difference in activity of PLCβ₂/δ₁ in the absence or in the presence of the peptide is statistically significant when indicated (*p<0. 1). (C) Activity of PLCβ₂/δ₁ in the absence (black bars) or in the presence (gray bars) of 10 μM Gβ86-105 peptide with vesicles with different composition. The chimera was incubated for 30s at 37°C with 1mM of POPE:POPS:PIP(4,5)P₂ (66:32:2), POPC:POPS:PIP(4,5)P₂ (66:32:2) or POPC:PIP(4,5)P₂ (98:2) Each bar is the mean of 2 separate experiments performed in triplicate, the error bars indicating the standard deviation. The difference in activity of PLCβ₂/δ₁ in the absence or in the presence of the peptide is statistically significant when indicated (** p<0.01).

Figure 8.

(A) Activation of PLCβ₂/δ₁ and PLCβ₂/δ₁(Y48A) with increasing amounts of Gβγ at 0.6 mM of POPE:POPS:PI(4,5)P₂ (66:32:2) vesicles doped with [³H]PI(4,5)P₂. Each value is the mean of 3 independent measures with errors bars corresponding to standard deviation and representative of two independent experiments. The activity is normalized to the basal activity in the absence of Gβγ. (B) Relative Gβγ activation of mutants PLCβ₂/δ₁(F147E), PLCβ₂/δ₁(Y48A), PLCβ₂/δ₁(Y48R), PLCβ₂/δ₁(Y48R/F147E) at 200 nM Gβγ. (C) Effect on increasing PHβ120-135 on the activity of the PLCβ₂/δ₁ in the absence (black bars) or the presence (gray bars) of 75 nM Gβγ. Each bar is the mean of three separate experiments (n=5-7) with errors bars indicating the standard deviation. (D) Activation of the PLCβ₂ by 5 μM

PH β 120-135 in the absence (black bars) or the presence (gray bars) of 200 nM G $\beta\gamma$. Each bar is the mean of five separate experiments performed in triplicate, the error bars indicating the standard deviation.

Figure 9. Model of G $\beta\gamma$ activation of PLC β_2

In solution, the PH domain of PLC β_2/δ_1 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. PH β 71-88) compete 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.