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†

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ABSTRACT: Pleckstrin homology (PH) domains are recognized in more than 100 different proteins, including mammalian phosphoinositide-specific phospholipase C (PLC) isozymes (isotypes β, γ, and δ). These structural motifs are thought to function as tethering devices linking their host proteins to membranes containing phosphoinositides or $\beta\gamma$ subunits of heterotrimeric GTP binding (G) proteins. Although the PH domains of PLC-δ and PLC-γ have been studied, the comparable domains of the β isotypes have not. Here, we have measured the affinities of the isolated PH domains of PLC-β_1 and -β_2 (PH-β_1 and PH-β_2, respectively) for lipid bilayers and G-$\beta\gamma$ subunits. Like the intact enzymes, these PH domains bind to membrane surfaces composed of zwitterionic phosphatidylcholine with moderate affinity. Inclusion of the anionic lipid phosphatidylserine or phosphatidylinositol 4,5-bisphosphate [PI(4,5)P_2] and inclusion of G-$\beta\gamma$ subunits had little affect on their membrane affinity. In contrast, binding of PLC-δ_1 or its PH domain was highly dependent on PI(4,5)P_2. We also determined whether these domains laterally associate with G-$\beta\gamma$ subunits bound to membrane surfaces using fluorescence resonance energy transfer. Affinities for G-$\beta\gamma$ were in the following order: PH-$\beta_2 \geq$ PH-$\beta_1 >$ PH-δ_1; the affinities of the native enzyme were as follows: PLC-$\beta_2 \gg$ PLC-$\delta_1 >$ PLC-β_1. Thus, the PH domain of PLC-β_1 interacts with G-$\beta\gamma$ in isolation, but not in the context of the native enzyme. By contrast, docking of the PH domain of PLC-β_2 with G-$\beta\gamma$ is comparable to that of the full-length protein and may play a key role in G-$\beta\gamma$ recognition.

Phosphatidylinositide-specific phospholipase C (PLC)[1] isozymes catalyze hydrolysis of phosphatidylinositol 4,5-bisphosphate [PI(4,5)P_2] to generate two intracellular second messengers diacylglycerol and inositol 1,4,5-triphosphate [Ins(1,4,5)P_3] (for reviews, see refs *1*−*3*). Three isotypes, PLC-β, -γ, and -δ, comprise the family of intracellular PLCs. Each isotype has several subtypes and a distinct mode of cellular regulation. The PLC-β subtypes are regulated by heterotrimeric GTP binding (G) proteins. All PLC-βs are stimulated by the α subunits of the G_q family, while two subtypes, PLC-β_2 and -β_3, are activated by G-$\beta\gamma$ subunits as well. PLC-γ isozymes are activated by protein tyrosine kinases and the PI-3 kinase product, PI(3,4,5)P_3 (*4*). Regulation of the PLC-δ family has not been established, although intracellular Ca^{2+} levels (*5*), α_h subunits of heterotrimeric G proteins (*6*), and RhoGAP (*7*) have been implicated in the control of the δ_1 subtype.

The eukaryotic PLCs have a modular domain organization (*1*−*3*). With the exception of a few spliced variants, all eukaryotic PLCs contain an amino terminal pleckstrin homology (PH) domain followed by four EF hand motifs, an X/Y motif constituting a catalytic α/β barrel, and a single C-2 domain. Structures of the PH domain of PLC-δ_1 and a truncated form lacking this domain have been separately solved (*8*, *9*). Whether the PH domain is complexed with other regions of the protein or tethered by a flexible linker is uncertain.

Approximately 110 residues in length, PH domains are found in more than 100 different proteins (for review, see ref *11*). Despite very low sequence similarities, all PH domains consist of a seven-stranded β sandwich with one edge sealed by a long C-terminal α helix. These domains are polarized with a lobe of positive charge at the open end of the β sandwich. This positive lobe has been suggested to bind anionic ligands.

The function of the PH domains has only been determined for a few proteins, including PLC-δ_1 and -γ_1. In the case of PLC-δ_1, the PH domain forms numerous specific contacts, allowing this protein to bind the PI(4,5)P_2 polar headgroup with high affinity (*11*). This high-affinity binding directs PLC-δ_1 to membranes enriched in this lipid (*11*, *12*), allowing it to processively hydrolyze substrate (*13*, *14*). The amino-

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[1] Abbreviations: PH, pleckstrin homology; PLC, mammalian phosphoinositide-specific phospholipase C; PI(4, 5)P_2, phosphatidylinositol 4,5-bisphosphate; Ins(1,4,5)P_3, D-myoinositol 1,4,5-trisphosphate; Laurdan, 6-lauroyl-2-(dimethylamino)naphthalene; Dabcyl SE, 4-(dimethylamino)phenylazophenyl-4-sulfonyl chloride succinyl ester; C, 7-(diethylamino)-3-(4′-maleimidylphenyl)-4-methylcoumarin; CB, cascade blue; POPC, 1-palmitoyl-2-oleoylphosphatidylcholine; POPS, 1-palmitoyl-2-oleoylphosphatidylserine; acrylodan, 6-acryloyl-2-(dimethylamino)naphthalene.

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terminal PH domain of PLC-γ_1 has a comparable function, binding strongly and specifically to PI(3,4,5)P_3, linking this isozyme to the membrane surface and the PI-3 kinase pathway. In contrast, the PH domains of the PLC-β isozymes and their roles in membrane binding and processive catalysis have not been directly examined.

PH domains have been implicated as binding sites for G-$\beta\gamma$ subunits. Studies of the β-adrenergic receptor kinase (also known as βARK or GRK2) have shown that the C-terminal region of its PH domain and the 20 residues that follow are responsible for strong interactions with G-$\beta\gamma$ (*15*, *16*). It has been suggested that this binding reflects a general interaction between PH domains and proteins containing the WD-40 motif found in the G-β subunit and a host of other proteins. High-affinity binding to G-$\beta\gamma$ subunits, however, is not a general property of the other PH domains examined thus far (*10*). Nevertheless, this possibility is intriguing since a number of proteins containing these domains are regulated by G-$\beta\gamma$ subunits, including PLC-β_2 and -β_3. Furthermore, PH domains bound to the membrane surface may mediate the lateral association with membrane-resident G-$\beta\gamma$ subunits, thereby contributing to the formation of specific signaling complexes. This aspect of PH domain function has received little attention.

Here, we have investigated the binding of the PH domains of PLC-β_1 and -β_2 (PH-β_1 and PH-β_2, respectively) to phospholipids and to G-$\beta\gamma$ subunits, and compared their binding behavior to that of PH-δ_1. Our results demonstrate that the PH domains of PLC-β_1 and -β_2 strongly associate with phospholipid bilayers, independent of phosphoinositide or G-$\beta\gamma$ subunits. Our results also provide evidence that the PH domain of PLC-β_2 contributes to specificity in the lateral association of this isozyme with membrane-resident G-$\beta\gamma$ subunits, leading to formation of an activated complex.

MATERIALS AND METHODS

Preparation of PLC Isozymes and G-$\beta\gamma$. Rat PLC-β_1 and -β_2 were expressed in Sf9 cells and purified as previously described (*18*). The expression of human recombinant PLC-δ_1 and its PH domain in *Escherichia coli* and their purification have been described elsewhere (*11*, *18*). G-$\beta\gamma$ was purified from bovine brain using the procedure of Sternweis and Robishaw (*19*) with modifications (*17*). Purified G-$\beta\gamma$ was solubilized in 0.7% CHAPS, flash-frozen in liquid N_2, and stored at −80 °C until it was used.

Construction of Prokaryotic Expression Vectors and Isolation of Recombinant PH Domains. The coding sequences of PH domains of PLC-β_1 and -β_2 were amplified by PCR using corresponding PLC cDNAs. PH-β_1 and PH-β_2 consisted of residues 1−188 and 1−171, respectively, of the native enzymes which includes residues of the first EF hand (143−188 for PH-β_1 and 139−171 for PH-β_2). The calculated molecular weights, including the His tag sequences, are 22 291 for PH-β_1 and 20 708 for PH-β_2.

The amplified products were digested with *Nde*I and *Hin*dIII and inserted into the corresponding sites in the bacterial expression vector pET-15b (Novagen) to generate the constructs pET-PH-β_1 and pET-PH-β_2. *E. coli* BL-21-(DE3) cells, transformed with pET-PHs expression vectors, were grown in Super broth containing ampicillin (50 μg/mL). PH domain expression was induced with isopropyl β-D-thiogalactopyranoside (1 mM) at 18 °C, and the bacteria were harvested 24 h later.

PH-β_1 and PH-δ_1 were extracted from the soluble fraction of the bacterial lysates and purified using His-Bind resin (Novagen) as described previously (*11*). PH-β_2 was recovered from inclusion bodies by extracting in 8 M urea, 150 mM NaCl, and 10 mM Tris-HCl (pH 8) containing 10 mM imidazole and incubating for 4 h at 4 °C. The extract was centrifuged for 30 min at 20000*g*, and the supernatant was passed through His-Bind resin. The protein was eluted with increasing concentrations of imidazole (0.1 to 0.4 M) in the presence of 8 M urea. The purified denatured protein was refolded by stepwise reduction of the urea concentration (6, 5, 4, 2, 1, 0.5, and 0 M urea). Protein products were verified by amino acid sequencing and by immunoblotting with anti-His tag antibody (BabCo). All PH domains were stored at 4 °C in 20 mM Hepes and 150 mM NaCl (pH 7.2).

Circular dichroic spectra of PH-β_1 and PH-β_2 in 20 mM PO_4 were obtained using the apparatus at the National Synchrotron Light Source at Brookhaven National Laboratories (Upton, NY) and on an Aviv 62A DS spectrometer. CD spectra of the domains are shown in Figure 1, and the corresponding structural elements as calculated by Chang (*20*) are given below. The secondary structural elements are consistent with the known structures of PH domains.

	% α helix	% β sheet	% β turn	% random coil
PH-β_1	21	25	3	26
PH-β_2	18	19	15	33
PH-δ_1	11	21	6	26

Removal of the His Tag. To verify that the N-terminal His tag on the proteins did not affect the measured affinities, several key studies, as noted in the text, were performed with proteins where the His tag was removed. The His tag sequence was cleaved using the Thrombin Cleavage Capture Kit (Novagen Inc.). Briefly, the hexa-His-tagged proteins were incubated with biotinylated thrombin at 1:2000 (w:w) in cleavage buffer [20 mM Tris-HCl (pH 8.4), 150 mM NaCl, and 2.5 mM $CaCl_2$] for 16 h at 20 °C. The cleavage reaction was stopped by adding PMSF (1 mM). Biotinylated thrombin was removed by adding streptavidin−agarose (in a ratio of 10 μL of settled resin per unit of enzyme) and incubating for 30 min at room temperature while the mixture was being gently shaken. The whole reaction mixture was transferred to a spin filter cup and centrifuged at 1000*g* for 5 min to remove the agarose beads. The supernatant, containing the cleaved protein free of the His tag and thrombin, was dialyzed against 20 mM Hepes and 150 mM NaCl.

Preparation of Lipid Bilayers. Large unilamellar vesicles (LUVs), 100 nm in diameter, were prepared by extrusion (*21*). Sucrose-loaded LUVs were prepared as described previously (*22*).

Protein Labeling and Reconstitution. All probes were purchased from Molecular Probes, Inc. (Eugene, OR). G protein subunits were labeled with thiol-reactive probes which do not affect their ability to activate PLC-β_2 or -β_3 (*23*). Prior to labeling with the thiol-reactive reagents, G-$\beta\gamma$ and the PH domains were dialyzed briefly (∼2 h) with two changes of a 5000-fold excess volume of buffer [20 mM Hepes and 160 mM KCl (pH 7.0)] under N_2 to remove DTT

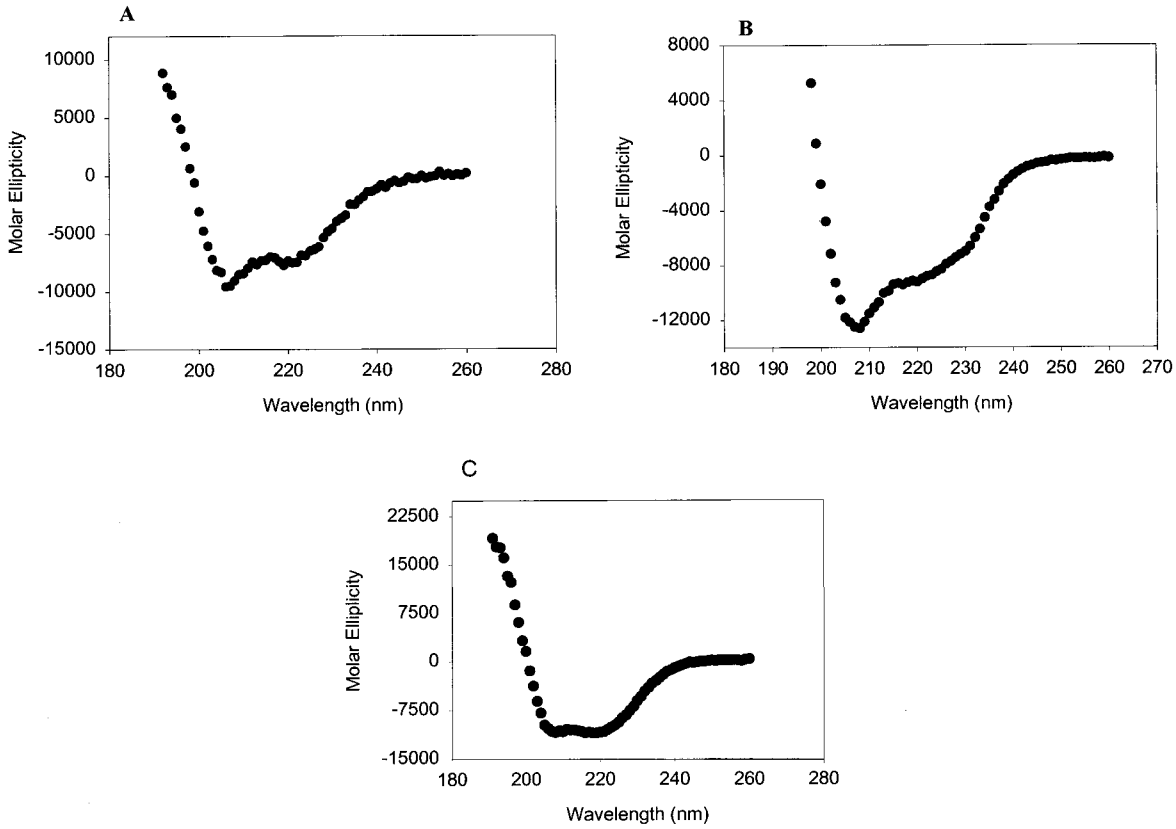

FIGURE 1: Circular dichroic spectra of (A) PH-β_1, (B) PH-β_2, and (C) PH-δ_1 solubilized in 20 mM phosphate buffer (pH 7). Spectra were fit to secondary structural elements that are listed in Materials and Methods.

at 5 °C. Proteins were labeled on ice for 30−120 min by a 4-fold molar excess of probe, and dialyzed for at least 18 h against several changes of a 1000-fold excess of buffer. PH domains were also labeled with the amine-reactive probe Dabcyl SE. In this reaction mixture, the pH of the solution was increased to pH 7.5 to favor modification of the N-terminus. The DTT concentration was 1 mM. Otherwise, all labeling conditions were the same. The protein:probe labeling ratio was determined by absorption spectroscopy using the calculated extinction coefficients for the protein and the coefficients provided by the probe manufacturer and was ~1:1 (mole:mole) for all preparations.

Labeled G-$\beta\gamma$ was reconstituted into lipid vesicles by adding the detergent-solubilized protein to a large excess of preformed extruded vesicles. This resulted in capture of the G-$\beta\gamma$ by the membranes without affecting bilayer stability (*23*).

Fluorescence Measurements and Data Analysis. Fluorescence measurements were performed on an ISS spectrofluorometer (Champaign, IL) using a 3 mm cuvette with a magnetic stirrer. Coumarin-labeled proteins were excited at 380 nm and scanned from 420 to 560 nm. Laurodan probes were excited at 350 nm and scanned from 380 to 560 nm. The level of coumarin−Dabcyl SE resonance energy transfer was determined by the loss of coumarin fluorescence caused by addition of the nonfluorescent Dabcyl-labeled acceptor protein. Signals were corrected for dilution and compared to loss of fluorescence caused by addition of buffer alone.

To determine the affinities of the PH domains G-$\beta\gamma$, the energy transfer data were analyzed as binding isotherms assuming that the maximum loss of fluorescence represents the maximum extent of complex formation and that the stoichiometry of this complex is 1:1 (mole:mole). From this analysis, apparent K_d's with units of moles per liter were derived. Because the PH domain−G-$\beta\gamma$ interaction is a lateral association on a membrane surface, and thus occurs in two dimensions, the apparent K_d depends on the concentration of the proteins on the membrane surface, and thus the lipid concentration or total available membrane surface area. We have previously translated the apparent K_d that we experimentally observe, which depends on the concentration of lipid (*23*; but see refs *24* and *25*), to the corresponding dissociation constant that would be observed if the proteins were not bound to membranes, but instead distributed homogeneously within a surface phase volume (υ) that was 100 nm thick. The concentration of the protein in this bulk phase [P_b] is related to the concentration bound at the membrane surface [P_m] by

$$[P_m] = (V_b/\upsilon)[P_b]$$

where V_b is the bulk volume. The relationship between K_b, the dissociation constant for lateral association of the proteins, and K_{d-app}, the apparent dissociation constant that is dependent on lipid concentration, is

$$K_b = (V_b/\upsilon)K_{d-app}$$

The ratio V_b/υ is calculated from the amount of lipid used and equal to 0.0443/[lipid] assuming that the average area of a lipid headgroup is 0.75 nm^2 (*23*).

RESULTS

Membrane Binding. Binding of β_1, β_2, and δ_1 PH domains to lipid bilayers was assessed with two independent assays

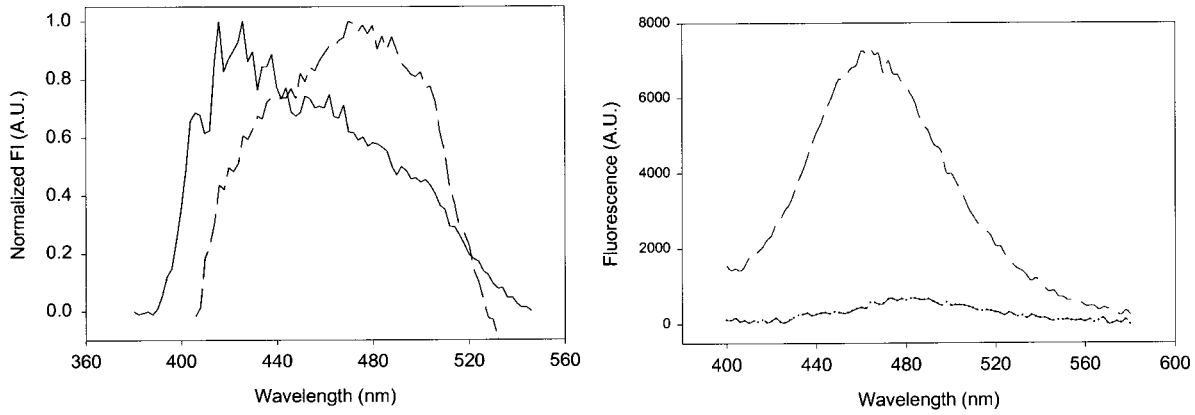

FIGURE 2: Examples of the two fluorometric methods used to monitor PH domain − membrane binding. (A) Shift in the emission spectra of 100 μM (solid line) and 10 μM POPC (dashed line) labeled with 0.1 mol % Laurodan in a dilute (100 nM) solution of unlabeled PH-β_2. (B) Increase in emission intensity of CM-PH-β_2 in the absence (dashed line) and presence (dotted-dashed line) of saturating (100 μM) POPC bilayers.

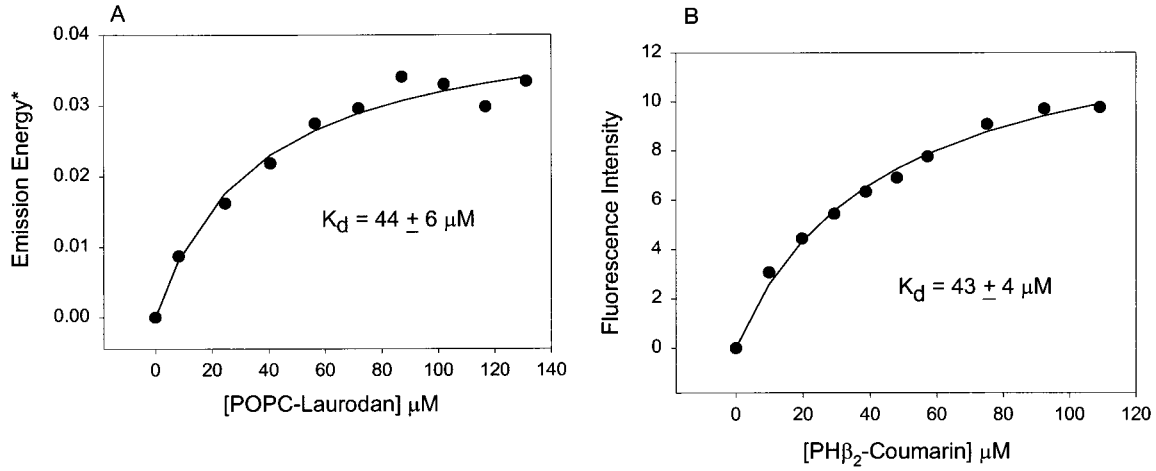

FIGURE 3: Titration curves of PH-β_2 to POPC bilayers obtained using the two fluorescence assays displayed in Figure 2. (A) Change in Emission Energy* of Laurdan-labeled POPC/PI(4,5)P_2 (98:2) when added to a dilute solution (100 nM) of unlabeld PH-β_2 where Emission Energy* is defined as (1 − EE)/EE_{max} and EE is the weighted emission maximum. (B) Increase in the integrated fluorescence intensity of CM-PH-β_2 as unlabeled POPC bilayers are added.

using a fluorophore either incorporated into the membrane (Laurdan) or attached to the protein (coumarin succinyl ester or CSE). In the former assay, Laurdan was incorporated into bilayer vesicles at 0.1 mol %. The polar headgroup of Laurdan is highly sensitive to the polarity of its environment. As proteins bind the membrane surface and displace water, the emission intensity increases and the center of spectral mass shifts to higher energies (shorter wavelengths). In the second type of binding measurement, the PH domains were labeled with coumarin. The emission intensity of this probe increases when the labeled protein binds to the membrane surface. Examples of spectra obtained with these two approaches are shown (Figure 2A,B).

The affinities of PH-β_1, -β_2, and -δ_1 for bilayers were determined from titration curves based on the spectral changes such as those seen in Figure 2. These titrations were carried out by adding lipid to a dilute solution of protein (~200 nM), and examples are shown in Figure 3A,B. Results of the binding of the PH domains and their corresponding full-length enzymes to different types of membranes using both types of assays are summarized in Table 1.

Binding of the PH domains to bilayers composed of electrically neutral phospholipids (POPC) and binding to ones containing 2% PI(4,5)P_2 (Table 1) were compared. As

Table 1: Apparent Membrane Binding Affinities (K_p in Micromolar) of the PH Domains and Their Corresponding Proteins[a]

	PH-δ_1	PLC-δ_1	PH-β_1	PLC-β_1	PH-β_2	PLC-β_2
POPC	>1000	>1000	28 ± 4	200 ± 41	44 ± 7	92 ± 8
POPC/PIP_2	33 ± 4	25 ± 9	39 ± 1	83 ± 21	55 ± 4	72 ± 9
PC/PS (2:1)	>1000	>1000	26 ± 1	ND	35 ± 1	57 ± 7

[a] Membrane binding affinities are the lipid concentrations at which 50% of the protein is membrane-bound. Data, averaged from two to four binding curves (see Figures 2 and 3) from both the Laurdan-lipid-based assay and the coumarin-PH domain assay, were fit to a hyperbola, and the midpoint of this fit and its associated error are reported. ND means not determined. The binding constants for PLC-δ_1 were from ref *23*, and those for PLC-β_1 and -β_2 were from ref *18*.

previously reported, PH-δ_1 does not adsorb to pure POPC bilayers but binds avidly to POPC membranes containing 2% PI(4,5)P_2 (*11*, *12*). In contrast, both PH-β domains bind to pure POPC in the absence of PI(4,5)P_2. Inclusion of PI(4,5)P_2 results in a small change for PH-β_1 and no significant change for PH-β_2 (Table 1). Likewise, the extent of binding of PH-β_1 and -β_2 domains to POPC membranes did not increase substantially when the anionic lipid PS was included. This binding behavior mirrors that of the native proteins measured by similar fluorescence techniques (*17*). In Figure 4, we compare the binding of PH-β_2 and its corresponding

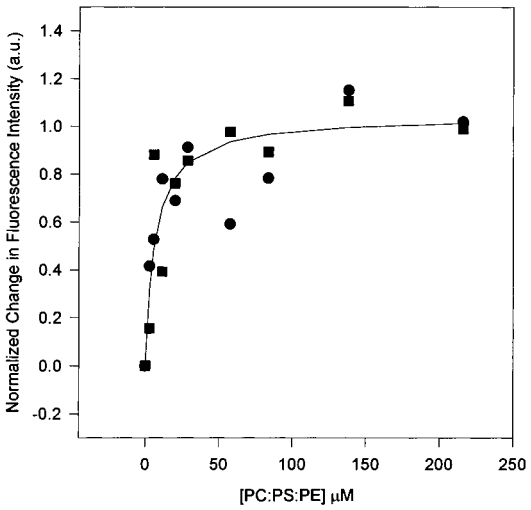

FIGURE 4: Comparison of the membrane binding affinity of PH-β_2 binding to its intact enzyme. Binding curves for PLC-β_2 (●) and PH-β_2 (■) were determined by monitoring the increase in emission intensity of Laurdan-labeled PC/PS/PE (1:1:1).

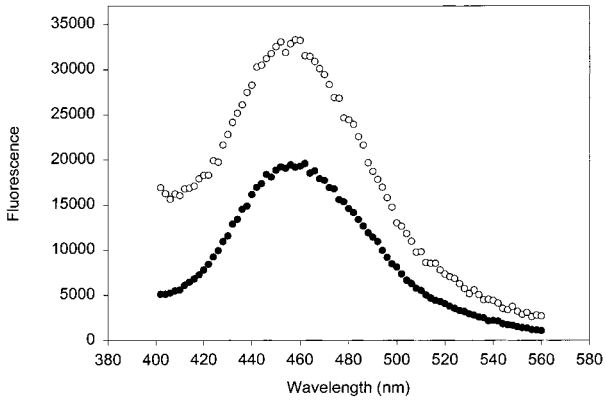

FIGURE 5: Example of the emission decrease due to FRET as PH domain associates with G-$\beta\gamma$ subunits. Emission spectra of coumarin-labeled G-$\beta\gamma$, reconstituted into POPC/PIP_2 (98:2) bilayers (300 μM), in the absence (●) and presence (○) of saturating amounts of Dabcyl SE-labeled PH-β_2. The decrease in intensity is due to energy transfer from the coumarin label to the Dabcyl as the labeled proteins move into close proximity (~20 Å) of each other.

full-length enzyme to PC/PS/PE vesicles. We found that the membrane affinities of these two proteins are identical within experimental error.

In the Laurdan-based binding measurements, we found that the PH-β proteins cause a greater shift in the emission energy than PH-δ_1 (450 as opposed to 160 cm^{-1}). It is possible that the smaller emission shift of Laurdan observed for PH-δ_1 results from reduced coverage of the membrane surface caused either by the reduced area of binding or by a significant nonbinding population (the fluorescence assays are only sensitive to changes in emission produced by the proteins that bind). Previous studies by our groups of binding of PH-δ_1 to POPC/PIP_2 bilayers in which a sedimentation-based assay was used show that all of the protein completely binds (*11*). Thus, the greater shift in emission energy of Laurdan from PH-β_2 binding as opposed to PH-δ_1 is due either to differences in the area covered by the two proteins or to the electrical character of the protein−lipid interface.

Association of PH Domains with G-$\beta\gamma$ Subunits. G-$\beta\gamma$ subunits reconstituted on PC/PIP_2 bilayers at ~20:1 to 500:1 lipid:protein ratios did not promote the binding of either PH-β_1, -β_2, or -δ_1 when the same assays described above was used (data not shown). This agrees with our previous findings which showed that the presence of G-$\beta\gamma$ does not measurably increase the affinity of intact PLC-β_1 or -β_2 for membrane bilayers (*17*). Thus, the affinities of these domains for G-$\beta\gamma$ subunits must be considerably weaker than their association with the zwitterionic lipid, PC. Nevertheless, lateral interactions between these membrane-bound domains and G-$\beta\gamma$ could contribute to formation of a specific activation complex. This would be most relevant to PLC-β_2 whose catalytic activity is strongly stimulated by the G protein subunits (*1*−*3*).

Because membrane binding of these domains is strong, we could work under conditions where all of the proteins are membrane-bound and monitor their lateral association with G-$\beta\gamma$. In the case of PH-δ_1, membranes contained sufficient $PI(4,5)P_2$ and other acidic lipids to ensure that all of the proteins were membrane-bound.

Two types of fluorescence assays were used to measure lateral association. In the simplest approach, G-$\beta\gamma$ subunits were labeled with dansyl chloride or acrylodan and reconstituted into lipid bilayers. Addition of unlabeled PH domain to the membrane surface systematically decreased the dansyl-G-$\beta\gamma$ emission, and shifted the acrylodan emission to shorter wavelengths. In the second type of assay, we measured FRET between a coumarin probe covalently attached to G-$\beta\gamma$ (C-G-$\beta\gamma$) and Dabcyl SE, an acceptor covalently attached to the PH domain. The Dabcyl group is a nonfluorescent energy acceptor, so FRET is observed as a loss in coumarin fluorescence as the two proteins interact laterally (Figure 5).

A number of control studies demonstrate that the observed quenching is due to fluorescence energy transfer. No comparable changes in fluorescence were observed when unlabeled PH domains were added or when PLC-β_1, which binds to G-$\beta\gamma$ with a low affinity, was substituted for PLC-β_2 which binds with a high affinity (*23*). No changes in emission intensity were detected when C-G-$\beta\gamma$ was replaced with C-α_q(GTPγS) in FRET measurements with Dabcyl (data not shown). Additionally, we conducted these studies at protein concentrations far below those where nonspecific changes in fluorescence due to membrane crowding would occur (*23*).

Figures 6−8 show the fluorescence results of the lateral association of the PH domains with G-$\beta\gamma$ subunits. The apparent binding constants obtained from the FRET studies are summarized in Table 2, and identical values, within error, were obtained using the dansyl-G-$\beta\gamma$ assay.

Figure 8 shows that as the initial concentration of C-G-$\beta\gamma$ decreased, the steepness of the titration increased and the apparent dissociation constant for these curves were the same within experimental error. Hence, the data in Figures 6 and 7 reflect the free energy of complex formation since the apparent dissociation constant did not change when the initial amount of C-G-$\beta\gamma$ on the membrane surface differed.

The results summarized in Table 2 demonstrate that the PH domains of PLC-β_2 and -β_1 interact much more strongly with G-$\beta\gamma$ than with PH-δ_1 or full-length PLC-δ_1. PLC-β_2 and its PH domain associated with G-$\beta\gamma$ with comparable affinities (*23*); binding of the native enzyme was 3-fold stronger. By contrast, PLC-β_1, which is not strongly activated by G-$\beta\gamma$ subunits, interacted very weakly, suggesting that

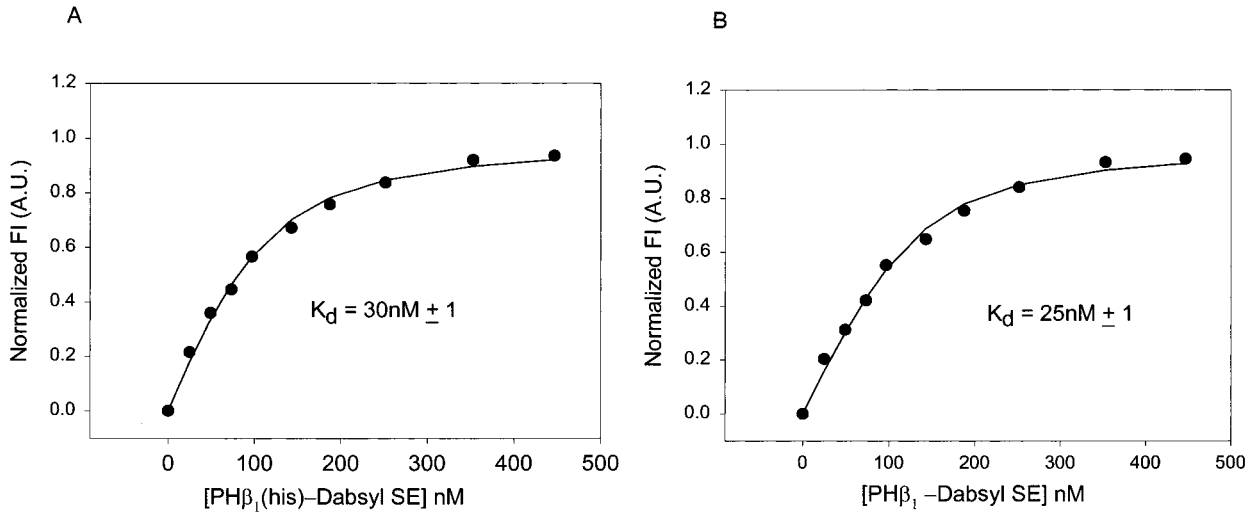

FIGURE 6: Lateral association of PH-β_1 and G-$\beta\gamma$ on membrane surfaces. PH-β_1, labeled with the fluorescence energy transfer acceptor, Dabcyl SE, was titrated into a solution containing 75 nM coumarin-labeled G-$\beta\gamma$ reconstituted on 350 μM POPC/PI(4,5)P_2 (98:2) bilayers. The degree of association was derived from the loss in coumarin fluorescence due to energy transfer to the Dabcyl SE probe assuming the proteins bind in a 1:1 stoichiometry. Panels A and B present data obtained using PH-β_1 with and without the His tag (see Materials and Methods), respectively.

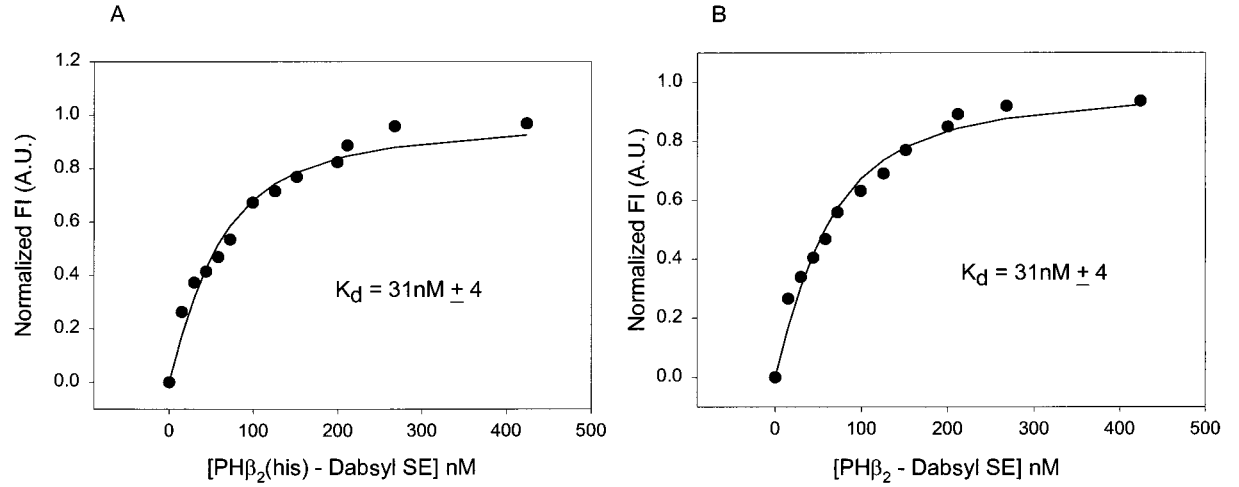

FIGURE 7: Lateral association of PH-β_2 and G-$\beta\gamma$ on membrane surfaces. PH-β_2, labeled with the fluorescence energy transfer acceptor, Dabcyl SE, was titrated into a solution containing 75 nM CM-$\beta\gamma$ reconstituted on 350 μM POPC/PIP_2 bilayers. The degree of association was derived from the loss in coumarin fluorescence due to energy transfer to the Dabcyl SE probe assuming the proteins bind in a 1:1 stoichiometry. Panels A and B present data obtained with and without the His tag (see Materials and Methods), respectively.

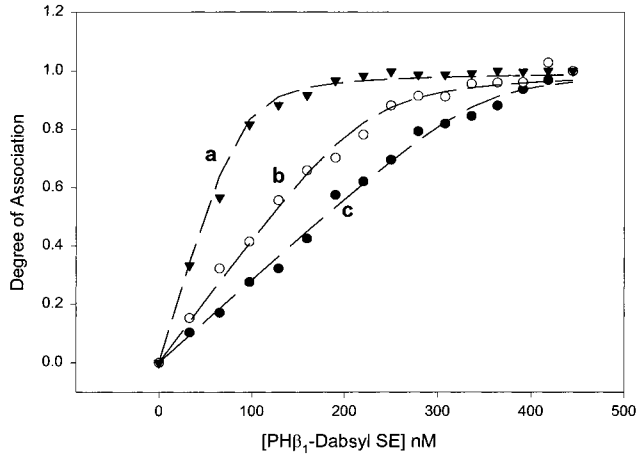

FIGURE 8: Concentration dependence of PH-β_1−G-$\beta\gamma$ lateral association. Dabcyl SE-PH-β_1 was titrated into a solution containing 200 μM POPC reconstituted with (a) 20, (b) 100, and (c) 200 nM C-G-$\beta\gamma$. All three curves give identical values of K_{d-app} within experimental error.

Table 2: Affinities of PH Domains and PLCs for G-$\beta\gamma$ Subunits on Membrane Surfaces[a]

	K_{d-app} (nM) (μM lipid)	K_b (μM)
PH-δ	98 ± 35 (255)	17 ± 6
PLC-δ	174 ± 16 (255)	30 ± 3
PH-β_1	31 ± 2 (400)	6.2 ± 3.6
PLC-β_1	>300 (400)	>50
PH-β_2	36 ± 2 (400)	4.0 ± 1.6
PLC-β_2	10 ± 5 (350)	1.3 ± 6.8

[a] K_{d-app} is the experimentally observed apparent dissociation constant at the lipid concentration given in parentheses, and K_b is the dissociation constant that would be obtained if the proteins associated in a three-dimensional or bulk phase (see Materials and Methods). The binding constants are averages of two to five independent measurements. These data were taken from FRET measurements as described in the text, but identical values (within error) were obtained by titrating unlabeled PH domains into membrane-bound dansyl-G-$\beta\gamma$.

in the context of the full-length protein G-$\beta\gamma$ is unable to form a similar complex with this PH domain.

The apparent K_d values that were obtained for the association of membrane-bound proteins are highly dependent on the lipid concentration; increasing the amount of lipid decreases the concentration of the proteins on the membrane surface, thereby reducing the apparent K_d. In Table 2, we present values for the observed apparent dissociation constant for the membrane-bound proteins that have been translated to a bulk or three-dimensional value, K_b (see Materials and Methods and ref *23*). Note that K_b values indicate a much weaker association than when the proteins are concentrated on a membrane surface.

Synergistic activation of β-ARK by PI(4,5)P_2 and G-$\beta\gamma$ has been reported (*26*, *27*). We examined the possibility that the presence of PI(4,5)P_2 on the membrane surface would enhance the affinity of the PH domains for G-$\beta\gamma$ subunits by comparing the affinity of PH-β_2 for G-$\beta\gamma$ in the presence and absence of PI(4,5)P_2. No differences in affinities could be detected (data not shown).

DISCUSSION

In this study, we determined the affinities of the isolated PH domains of PLC-β_1, -β_2, and -δ_1 for lipid bilayers and G-$\beta\gamma$ subunits. PH domains have been identified in a large number of diverse proteins, but their functions are still unclear (*28*). The current ideas that these domains are used to tether their host proteins to inositol lipids or G-$\beta\gamma$ subunits are based only on a few well-studied examples. Our results show that the PH domains of PLC-β_1 and PLC-β_2 bind strongly to membranes regardless of the presence of PI(4,5)-P_2 or other anionic lipids, suggesting that these domains are nonspecific membrane-binding anchors.

The lack of PI(4,5)P_2 specificity for PH-β_1 and -β_2 is not surprising since membrane binding of PLC-β_1 and -β_2 is independent of PI(4,5)P_2 as well (*17*, *29*). With the exception of SOS-1 and β-spectrin, other PH domain proteins, like pleckstrin and dynamin, show far weaker affinities for PI-(4,5)P_2 than PLC-δ_1 (*30*, *31*). The PLC-β isozymes may fall into this latter group. In a previous discussion of the membrane binding behavior of these two PLC-βs, we rationalized its lack of PI(4,5)P_2 specificity by comparing the sequence of its PH domains to those of PH-δ (*17*). Most of the specific contacts between PH-δ_1 and the PI(4,5)P_2 polar headgroup involve hydrogen bonds to the 4 and 5 position phosphomonoesters contributed by nine amino acids that are nonconservatively replaced in the PLC-β proteins. Also, many of these residues are replaced in PLC-δ_2 and -δ_4. Thus, even among mammalian PLC-δ subtypes, high-affinity PI(4,5)P_2 binding may be restricted to PLC-δ_1 and the related noncatalytic InsP_3 binding protein (*23*), suggesting a highly specialized role for this function.

On the other hand, some PH domain proteins recognize alternative inositol lipids. The PH domains of PLC-γ_1, cytohesin, and the related protein GRP1 bind to PI(3,4,5)P_3 or 3-phosphorylated inositol polyphosphates with high affinity and specificity (*33*, *34*). In the case of PLC-γ_1, binding to PI(3,4,5)P_3 stimulates catalytic activity. Binding of the PLC-β PH domains to products of the PI-3 kinase pathway has not yet been investigated.

Our lipid binding studies suggest that PH-β_1 and -β_2 have a polar character that is different from that of PH-δ_1. PH-δ_1 has a large positively charged lobe surrounding the IP_3 binding site (*9*). This positive lobe is presumably responsible for enhanced binding of PH-δ_1 when the mole fraction of acidic lipid is increased. Unlike PH-δ_1, PH-β_1 and -β_2 bind strongly to surfaces composed entirely of the electrically neutral lipid, PC. Neutral lipids such as phosphatidylethanolamine enhance binding of the intact enzymes (*17*). The inability of negatively charged lipids to enhance binding significantly suggests that these domains are not as electrically polarized. Further support for this idea comes from the observation that the binding of PH-β_1 and -β_2 causes a much larger shift in Laurodan emission than that of PH-δ_1. The larger shift to a higher emission energy is consistent with a lower polarity of the membrane binding surfaces of PH-β_1 and -β_2. We speculate that hydrophobic rich regions participate in membrane interactions. In addition, polar uncharged residues located on the binding surfaces may be attracted by the high dipole potential of the PC membrane contributing to the apparent binding constant.

PH-β_2 and -β_1 bind weakly to G-$\beta\gamma$ compared to their affinities for PC membranes. When lateral interactions were examined using FRET techniques, the affinities of PH-β_2 and -β_1 for G-$\beta\gamma$ were found to be comparable and stronger than those of PH-δ_1, which is weakly activated by G-$\beta\gamma$.

Although the interactions between the PH domains of PLC and G-$\beta\gamma$ are weak compared to those of the extended PH domain of βARK, they are in the same range as other PH domains which have been tested (*35*). While the low bulk phase affinities do not support a specific interaction between the PH domains and G-$\beta\gamma$ in solution, the relevant affinities, i.e., those that occur on the membrane surface, reveal a specific interaction based on the rank order of binding measured here.

Our comparisons of the PLC isozymes show that the lateral association of $\beta\gamma$ subunits with PLC is a function of their surface concentrations (moles of protein per unit area of membrane). Like the activation experiments, the order of affinities are as follows: PLC-$\beta_2 \gg$ PLC-β_1 and -δ_1 (Table 2; *23*). FRET measurements of PH-β_2 and -δ_1 domain interactions with G-$\beta\gamma$ are in close agreement with this order. Interestingly, PH-β_1 associates almost as strongly as PH-β_2. One plausible explanation is that the PH domain of this isozyme is unavailable for docking in the native enzyme.

While a strong correlation exists between the extent of PLC activation and the affinities of PLC for G-$\beta\gamma$, the extent of activation by G protein subunits is not necessarily related to their affinities for the intact enzyme or its PH domain. Regions located in the catalytic core, especially the Y region, may provide additional sites for docking of G-$\beta\gamma$ that lead to activation (*36*, *37*). We suggest that the initial lateral association of PLC with G-$\beta\gamma$ occurs through the PH domain which positions G-$\beta\gamma$ for interaction with another site located in the catalytic core leading to activation.

In summary, we find that the PH domains of PLC-β_1 and -β_2 may function as a general membrane binding motif. Once bound to the membrane surface, these domains are capable of docking with G-$\beta\gamma$ subunits. In the case of PLC-β_2, this lateral association leads to its activation. Experiments in which mutant PLC-β_2 is used to explicitly test the role of the PH domain are currently underway.

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