The Pleckstrin Homology Domain of Phospholipase C-δ_1 Binds with High Affinity to Phosphatidylinositol 4,5-Bisphosphate in Bilayer Membranes†

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ABSTRACT: The pleckstrin homology (PH) domain of phospholipase C-δ_1 (PLC-δ_1) binds to phosphatidylinositol 4,5-bisphosphate (PI(4,5)P_2) in phospholipid membranes with an affinity ($K_a \sim 10^6\ M^{-1}$) and specificity comparable to those of the native enzyme. PLC-δ_1 and its PH domain also bind inositol 1,4,5-trisphosphate, the polar head group of PI(4,5)P_2, with comparable affinity and approximately 1:1 stoichiometry. A peptide corresponding to amino acids 30−43 of the PLC-δ_1 PH domain contains several basic residues predicted to bind PI(4,5)P_2, but binds weakly and with little specificity for PI(4,5)P_2; hence the tertiary structure of the isolated PH domain is required for high affinity PI(4,5)P_2 binding. Our PI-(4,5)P_2 binding results support the hypothesis that the intact PH domain, serving as a specific tether, directs PLC-δ_1 to membranes enriched in PI(4,5)P_2 and permits the active site, located elsewhere in the protein, to hydrolyze multiple substrate molecules before this enzyme dissociates from the membrane surface.

The phosphoinositide-specific phospholipase C (PLC)[1] isozymes hydrolyze phosphatidylinositol 4,5-bisphosphate (PI(4,5)P_2), generating two second messengers, inositol 1,4,5-trisphosphate (Ins(1,4,5)P_3) and diacylglycerol (DAG) (Berridge, 1993). The isozymes can be divided into three groups (β, γ, δ) that exhibit unique patterns of tissue expression and control (for reviews, see Lee & Rhee, 1995; Exton, 1994; Majerus, 1992). Members of the β group are activated by G_q α or $\beta\gamma$ subunits derived from heterotrimeric GTP-binding (G) proteins, whereas members of the γ group are stimulated by receptor and nonreceptor protein tyrosine kinases. By contrast, the factors that control PLC-δ activity are unknown.

The PLC isozymes are peripheral membrane proteins that operate at a phospholipid/solution interface; hence components of the membrane that link these enzymes to the interface are likely to be critical factors in their regulation. PLC-δ_1 (Rebecchi et al., 1992; Pawelczyk & Lowenstein, 1993) and PLC-β, isolated from turkey erythrocytes (James et al., 1995), bind strongly to PI(4,5)P_2 through a domain that may not be catalytic. This idea has been tested. Deletion of the amino terminal 60 amino acids of PLC-δ_1 inactivates high affinity PI(4,5)P_2 and Ins(1,4,5)P_3 binding, although the catalytic core (X and Y region), which contains essential active site residues (Ellis & Katan, 1995; Cheng et al., 1995), remains functional (Cifuentes et al., 1993, 1994; Yagisawa et al., 1994). These results are consistent with the suggestion that the membrane anchoring region is distinct from the catalytic domain. The 60 amino acid region deleted in these experiments is now recognized as part of a domain with pleckstrin homology (PH).

Pleckstrin, the major protein kinase C substrate in thrombin-stimulated platelets (Haslam et al., 1993), contains N-terminal and C-terminal repeats of approximately 100 amino acids. Motifs similar to these repeats have been found, with various extensions and insertions, in over 70 proteins, including guanine nucleotide exchange factors, GTPase activating proteins, tyrosine or serine/threonine protein kinases, kinase substrates, structural and regulatory elements of the cytoskeleton, and all mammalian phospholipase C isoforms (for recent reviews, see Gibson et al., 1994; Cohen et al., 1995; Pawson, 1995). All PH domains exhibit a core structure consisting of a pair of nearly orthogonal antiparallel β-sheets with a long C-terminal α-helix that ties together one end of a collapsed β-barrel. The lengths and sequences of the loops connecting the 7 β-strands are variable. Although the biological role of pleckstrin itself remains unknown, PH

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[1] Abbreviations: PH domain, pleckstrin homology domain; PI(4,5)-P_2, phosphatidylinositol 4,5-bisphosphate; PLC, phosphoinositide-specific phospholipase C; PC, phosphatidylcholine; PI(4)P, phosphatidylinositol 4-monophosphate; PI, phosphatidylinositol; PA, phosphatidic acid; PS, phosphatidylserine; PG, phosphatidylglycerol; Ins(1,4,5)P_3, D-*myo*-inositol 1,4,5-trisphosphate; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; Tris, tris(hydroxymethyl)aminomethane; MES, 2-(*N*-morpholino)ethanesulfonic acid; MOPS, 3-(*N*-morpholino)-propanesulfonic acid; IPTG, isopropyl β-D-thiogalactopyranoside; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′,-tetraacetic acid; EDTA, ethylenediaminetetraacetic acid; BCA, bicinchoninic acid; BSA, bovine serum albumin; ECL, enhanced chemiluminescence; DTT, dithiothreitol; LUVs, large unilamellar vesicles; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; CD, circular dichroism; β-ark, β-adrenergic receptor kinase; G-proteins, GTP-binding proteins.

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domains are believed to function as modular binding units akin to SH2 and SH3 domains. Despite their weak sequence homologies, the structures of pleckstrin N-terminal (Yoon et al., 1994), β-spectrin (Macias et al., 1994), and dynamin PH domains (Ferguson et al., 1994; Fushman et al., 1995) demonstrate a remarkable conservation of tertiary structure.

Recent work by Harlan et al. (1994) demonstrated that the PH domains of pleckstrin (N-terminal and C-terminal), T-cell specific tyrosine kinase, ras GTPase activating protein, and the β-adrenergic receptor kinase (β-ark) bind to phospholipid vesicles that contain either PI(4,5)P_2 or PI(4)P. Heteronuclear NMR experiments indicate that binding to PI(4,5)P_2 perturbs several residues located in the loop region between the first two β-strands of pleckstrin (Harlan et al., 1994). Not all PH domain proteins, however, bind polyphosphoinositides (Ferguson et al., 1994), which implies that there are additional functions for these motifs. For example, the PH domains found in β-ark, PLC-γ_1 (Touhara et al., 1994), Sos 1, and Dbl (Mahadevan et al., 1995) bind $\beta\gamma$ subunits of heterotrimeric G-proteins. Recent work demonstrating that $\beta\gamma$ G-protein subunits and PI(4,5)P_2 act synergistically to stimulate β-ark activity (Pitcher et al., 1995) implies that both regulatory factors bind the PH domain of this protein simultaneously.

In this study, we demonstrate that the isolated PLC-δ_1 PH domain binds polyphosphoinositides and inositol phosphates with an affinity and specificity comparable to that of the native protein. Our results are consistent with the idea that the PLC-δ_1 PH domain facilitates processive hydrolysis of PI(4,5)P_2 by tethering the catalytic core of this enzyme to the membrane surface.

EXPERIMENTAL PROCEDURES

Lipids. Phosphatidic acid (PA) of egg origin was purchased from Sigma. The synthetic lipids, phosphatidylcholine (PC), phosphatidylserine (PS), and phosphatidylglycerol (PG), which contain palmitic and oleic acid esterified at the 1 and 2 positions of the *sn*-glycerol backbone, were purchased from (Avanti). PI(3,4,5)P_3 and PI(3,4)P_2 were prepared enzymatically from PI(4,5)P_2 and PI(4)P using the recombinantly expressed p85α/p110β isoform of phosphoinositide 3-kinase and purified by anion-exchange high-pressure liquid chromatography (Morris et al., 1995). PI(4,5)P_2 and PI(4)P were prepared from Folch fraction I of bovine brain (Sigma) according to the method of Low (1990).

Construction of Human PLC-δ_1 PH Domain Expression Plasmid. Polymerase chain reaction (PCR) was used to generate the cDNA encoding a region containing the conserved PH domain, amino acids 1−155 (bases 1−465) of human PLC-δ_1. The 465 base pair fragment was purified by agarose gel electrophoresis and ligated into pET-19b (Novagen) digested with *Nde*I and *Eco*RI. Part of the pET19b expression plasmid, the $(His)_{10}$-Tag, was engineered into the coding sequence at the amino terminus of the PH domain to aid in purification of the soluble protein. The calculated molecular mass of the expressed PH domain protein was 22.6 kDa. For the remainder of this paper, we refer to this protein with the attached $(His)_{10}$-Tag as the PH domain. The insert and flanking regions were sequenced on an automated 370A DNA sequenator (Applied Biosystems).

Expression of Human PLC-δ_1 PH Domain in Escherichia coli Strain BL21(DE3). *E. coli* strain BL21(DE3) was transformed with the expression plasmid containing the PH domain coding sequence. A single, transformed colony was selected for growth inhibition by isopropyl β-D-thiogalactopyranoside (IPTG), as described previously (Studier, 1990), and used to inoculate 1 L of superbroth (32 g/L Bacto-tryptone, 20 g/L Bacto-yeast, 5 g/L NaCl, pH 7.5) containing 150 μg/mL ampicillin. Cells were grown at 37 °C until mid-log phase. The culture was cooled to 20 °C, and protein expression was induced by addition of IPTG (90 mg/L). An additional dose of ampicillin was added at this time. The cells were induced for 18−24 h at 20 °C, harvested by centrifugation at 5000 rpm in a Beckman JA-10 rotor for 10 min at 4 °C, and resuspended in 100 mL of harvesting buffer (500 mM NaCl, 20 mM Tris-HCl, pH 7.9, 5 mM imidazole, 2 mM phenylmethanesulfonyl fluoride, 5 mM benzamidine, 10 μg/mL leupeptin, 5 μg/mL pepstatin, 10 μg/mL aprotinin, 50 μg/mL of lysozyme).

Cells were disrupted by flash freezing in liquid nitrogen and thawing in cold water and then sonicated using a 1 cm Biosonix IV sonicator probe (VWR) at high power with four 20 s bursts. The lysate was centrifuged at 35000*g* (17 000 rpm) in a Beckman JA-20 rotor for 30 min at 4 °C. The clear supernatant fluid was loaded onto 12 mL of His-Bind resin (Novagen) at a rate of 60 mL/h. The column was washed with buffers according to the manufacturer's instructions and eluted with 30 mL of 0.5 M NaCl, 20 mM Tris-HCl, and 0.4 M imidazole, pH 7.9, and 36 mL of 0.5 M NaCl, 20 mM Tris-HCl, and 1 M imidazole, pH 7.9. Most of the PLC-δ_1 PH domain eluted with the 0.4 M imidazole buffer.

The pH of the eluted protein solution was adjusted to pH 6.5 by dropwise addition of 0.5 M MES buffer, pH 2, with constant stirring. This was important to prevent precipitation of the protein, which was eluted from the column near its isoelectric point. The protein solution was dialyzed for 18 h at 4 °C against 4 L of 0.5 M NaCl, 20 mM MES, pH 6.2, and 0.5 mM DTT and stored at 4 °C. Protein concentration was estimated from the calculated molar extinction coefficient (25 000 M^{-1} cm^{-1}); approximately 60 mg of pure PH domain was yielded per liter of *E. coli* culture. The soluble fraction of *E. coli* lysate and fractions from the His-Bind affinity column were analyzed by sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE) and immunoblot. Protein bands were identified on immunoblots using a peptide-specific polyclonal antiserum that recognizes the sequence 3−16 in the PH domain of bovine and human PLC-δ_1 as described previously (Cifuentes et al., 1993). Antibody binding was detected by goat anti-rabbit IgG linked to horseradish peroxidase and ECL reagents (Amersham). Details of the expression and purification of recombinant human PLC-δ_1 in *E. coli* will be published elsewhere.

Binding of PLC-δ_1 PH Domain and Native PLC-δ_1 to Membrane Bilayers. Binding of PLC-δ_1 PH domain to sucrose-loaded large unilamellar vesicles (LUVs) was measured using a modification of the method previously described (Rebecchi et al., 1992). The experiments were performed as follows: PLC-δ_1 PH domain (8−12 μg) and LUVs were mixed in 250 μL of solution containing 100 mM NaCl, 0.5 mM DTT, 0.5 mM EDTA, and 25 mM HEPES, pH 7.2, at room temperature. The LUVs were then sedimented by centrifugation at 100 000*g* for 40 min at 25 °C. $[PH\ domain]_{free}$ and $[PH\ domain]_{bound}$ were determined by measuring protein concentration in the supernatant fluid and

pellet, respectively, using the BCA protein assay (Pierce). Vesicles without protein were used to determine the reactivity of the lipid components in the protein assay. This value, which was less than 15% of the readings in the presence of protein, was subtracted from each measurement of the bound fraction. Binding of native recombinant PLC-δ_1 was determined as described previously (Rebecchi et al., 1992).

The binding of the PH domain, the PLC-δ_1 (30−43) peptide, or the native enzyme to phospholipid vesicles is expressed as a molar partition coefficient, K (M^{-1}), which makes no assumption as to lipid:protein stoichiometry. As defined in Peitzch and McLaughlin (1993), K is the proportionality constant between the mole ratio of protein bound to the outer monolayer of the vesicle, $[\text{protein}]_{\text{bound}}/[\text{lipid}]$, and the concentration of protein in the bulk aqueous phase, [protein]. Thus, $[\text{protein}]_{\text{bound}} = K[\text{protein}][\text{lipid}]$. [Lipid] is the concentration of lipid present in the outer monolayer of the LUVs (equal to half the total lipid concentration). Under our conditions, $[\text{protein}]_{\text{bound}} \ll [\text{lipid}]$. It follows that

$$\% \text{ protein bound} = K[\text{lipid}](100)/(1 + K[\text{lipid}]) \quad (1)$$

Equation 1 was fit to the binding data by a nonlinear least squares method to determine values of K.

Binding of Peptides to Membrane Bilayers. Binding of PLC-δ_1 (30−43) peptide, KVKSSSWRRERFYK (net charge = 5+), to LUVs was measured by mixing the peptide (1 μM) and LUVs in 900 μL of solution containing 100 mM KCl and 1 mM MOPS, pH 7, and separating free peptide from bound by sedimentation of the sample at 100 000g for 1 h at 25 °C. $[\text{Peptide}]_{\text{free}}$ and $[\text{peptide}]_{\text{bound}}$ were determined by measuring the peptide concentration in the supernatant fluid and pellet, respectively, using a fluorescamine assay modified slightly from Weigele et al. (1972) as described by Buser et al. (1994). Vesicles contained PG rather than PS, which reacts with fluorescamine.

Binding of PLC-δ_1 PH Domain and Native PLC-δ_1 to Ins(1,4,5)P_3. One hundred microliters of PH domain or native PLC-δ_1 (0.8 nmol) in buffer (100 mM NaCl, 0.5 mM DTT, 0.5 mM EDTA, 25 mM HEPES, pH 7.2) was mixed with 100 μL of [^{3}H]Ins(1,4,5)P_3 (3×10^{-3} Ci/mmol, DuPont New England Nuclear) at room temperature. Similarly, 100 μL of bovine serum albumin (BSA) solution (the same concentration, w/v) was mixed with a 100 μL portion of [^{3}H]Ins(1,4,5)P_3 solution. This served as the control (null) binding reaction. The samples were transferred to Ultrafree-MC (Millipore) filters (10 K nominal MW cutoff) and subjected to centrifugation in a microfuge at 4000g for 10 min. Approximately 30 μL of fluid was filtered in this time. Ten microliter portions of the filtrates were transferred to vials for liquid scintillation counting. The difference between the cpm in the BSA (control) filtrate and the cpm in the filtrate of the test protein was used to calculate the concentration of [^{3}H]Ins(1,4,5)P_3 bound to protein. BSA alone did not significantly reduce the cpm in the filtrate compared to buffer without protein. For the competition experiments, unlabeled Ins(1,4,5)P_3 (LC Laboratories) was added to the binding reaction and the concentration of [^{3}H]Ins(1,4,5)P_3 bound to protein was determined.

Fluorescence Emission and Circular Dichroism Spectra. Fluorescence emission measurements were performed on an I.S.S. K2 spectrofluorometer (I.S.S. Champaign, IL). Spectra were taken without polarizers. Samples of the PLC-δ_1 PH domain were diluted to 1 μM in buffer: 100 mM NaCl, 0.5 mM DTT, 0.5 mM EDTA, 25 mM HEPES, pH 7.2, and 1.2 mL of protein solution transferred to a quartz cuvette in the spectrofluorometer. The chamber of the spectrofluorometer was continuously flushed with N_2, and the sample was magnetically stirred at room temperature. Ins(1,4,5)P_3 was titrated from 0 to 30 μM. At each titration step, the sample was excited at 280 nm and the emission spectra were scanned from 310 to 420 nm. Emission spectra were corrected for dilution and background, which was determined with a solution of PH domain ultrafiltrate. CD spectra were acquired with a JASCO J-20 spectropolarimeter, scanned from 260 to 195 nm, and at the National Synchrotron Light Source at Brookhaven National Laboratory, scanned from 300 to 185 nm. Samples of the PH domain protein solution (1 mg/mL) were dialyzed overnight against 1 L of 150 mM NaCl and 50 mM sodium phosphate, pH 6.7, before the CD spectra were obtained.

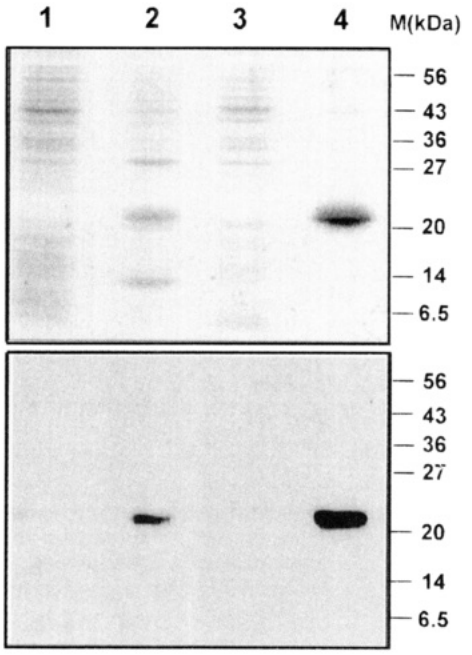

FIGURE 1: Purification of PLC-δ_1 PH domain expressed in *E. coli* strain BL21(DE3). Portions of the soluble fractions of *E. coli* lysates were subjected to SDS−PAGE. Fractions were obtained: prior to IPTG induction (lane 1), after 18 h of induction (lane 2), from the flow-through fraction of the His-Bind affinity column (lane 3), and the fraction eluted from this column with buffer containing 0.4 M imidazole (lane 4). The 15% polyacrylamide gels were stained with Coomassie Brilliant Blue R-250 dye (top) or electroblotted onto nitrocellulose and probed with antibody raised against the peptide sequence (3−16) of the PLC-δ_1 PH domain. Binding of the peptide-specific antibody was detected with a goat anti-rabbit IgG linked to horseradish peroxidase.

RESULTS

Expression of the PLC-δ_1 PH Domain. We expressed the putative membrane anchor of human PLC-δ_1, which includes the entire PH domain sequence (amino acids 1−155). Synthesis of the PH domain was induced in *E. coli* transformed with the expression plasmid. The predicted molecular mass with the attached $(\text{His})_{10}$-Tag sequence was 22.6 kDa. After 18 h, a major protein band with a relative molecular mass of approximately 22 kDa appeared in the soluble fraction (Figure 1, top, lanes 1 and 2). The 22 kDa

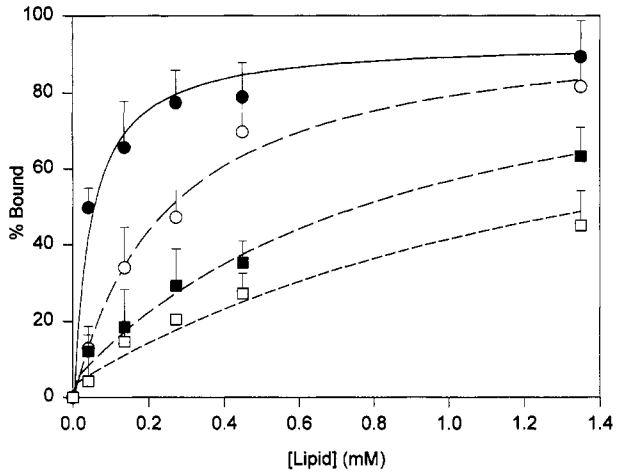

FIGURE 2: Binding of PLC-δ_1 PH domain to membrane bilayers. Binding of the PLC-δ_1 PH domain to LUVs formed from mixtures of PC:PS (2:1, mol:mol) (open squares), PC:PS:PI (2:1:0.09) (filled squares), PC:PS:PI(4)P (2:1:0.09) (open circles), or PC:PS:PI(4,5)-P_2 (2:1:0.09) (filled circles) were compared. The ordinate is the percentage of protein bound and the abscissa is the concentration of lipid in the outer monolayer of the LUVs. Maximum binding was never greater than 95%. Each symbol is the average of at least 3 experiments, each determined in duplicate. Bars indicate standard deviations of the mean. The curves represent the best fits of eq 1 to the data. The molar partition coefficents deduced from these and similar experiments are summarized in Table 1.

protein completely adsorbed to His-Bind resin and specifically eluted with buffer containing 0.4 M imidazole (lane 4). An antibody against the peptide corresponding to amino acids 3−16 of the PLC-δ_1 PH motif (Cifuentes et al., 1993) recognized the same 22 kDa bands in the soluble fraction and the 0.4 M elution step (Figure 1, bottom, lanes 2 and 4).

Binding of PLC-δ_1 PH Domain to Membrane Bilayers. The ability of the PLC-δ_1 PH domain to serve as a PI(4,5)-P_2-specific membrane anchor was assessed by measuring the binding of the PH domain to sucrose-loaded LUVs containing various phosphoinositides (Figure 2 and Table 1).[2] To provide a rigorous test of binding specificity, a 2:1 mixture of PC and PS was used to maintain a nearly constant membrane surface potential (about −50 mV) with different concentrations and types of test lipid (McLaughlin, 1989). Figure 2 shows that the PLC-δ_1 PH domain binds strongly to bilayers composed of PC:PS (2:1) and 3 mol % PI(4,5)-P_2, less strongly to vesicles containing 3 mol % PI(4)P, and weakly to vesicles containing 3 mol % PI or no phosphoinositide (see also Table 1, lines 3, 6 and 8). Inclusion of 3 mol % PI(4,5)P_2 in the PC:PS (2:1) membrane increased the binding affinity (molar partition coefficient) nearly 40-fold (Figure 2; Table 1, lines 3 and 10). We observed no significant binding of the control protein, BSA (data not shown). A 10-fold molar excess of Ins(1,4,5)P_3 suppressed most of the binding of PLC-δ_1 PH domain to LUVs containing PI(4,5)P_2 (data not shown).

The molecular determinants of membrane binding were examined further by comparing binding of the PH domain to other phosphoinositides, including PI(3,4,5)P_3 and PI(3,4)-P_2 (Table 1). Phosphoinositides with phosphate linked to the 3 position of the inositol ring are produced in living cells in response to various stimuli (Stephens et al., 1991; Cantley, 1991) and could serve as recognition sites for binding PH domain proteins to membrane surfaces. The PLC-δ_1 PH domain binds less strongly to PI(3,4)P_2 compared to PI(4,5)-P_2 (Table 1, lines 2 and 4), indicating that the phosphomonoester group at position 3 of the inositol ring is not a strong determinant of binding. The PH domain binds more strongly, however, to vesicles composed of PC, PS, and PI-(3,4,5)P_3 than to vesicles containing PI(4,5)P_2 (lines 1 and 2).[3] Binding of PLC-δ_1 PH domain to PC:PS vesicles containing 1 mol % PI or PA was much weaker than to vesicles formed with 1 mol % polyphosphoinositide (lines 2, 7 and 9). Eliminating the acidic monovalent lipid, PS, from the bilayer lowers the magnitude of the surface potential and decreases the K for binding approximately 10-fold to vesicles containing 1 mol % PI(4,5)P_2 (lines 2 and 11). Binding of PLC-δ_1 PH domain to vesicles formed from the zwitterionic lipid, PC, was nearly undetectable (line 14).

Table 1: Binding of the PH Domain of PLC-δ_1 to Membrane Bilayers

composition	ratio (mol:mol)	K (M^{-1})[a] ± SD[b]	n[c]
PC:PS:PI(3,4,5)P_3	2:1:0.03	2.2 (0.9) × 10^4	2
PC:PS:PI(4,5)P_2	2:1:0.03	7.7 (1.5) × 10^3	4
PC:PS:PI(4,5)P_2	2:1:0.09	2.1 (0.6) × 10^4	4
PC:PS:PI(3,4)P_2	2:1:0.03	4.8 (2.5) × 10^3	2
PC:PS:PI(4)P	2:1:0.03	1.6 (0.2) × 10^3	2
PC:PS:PI(4)P	2:1:0.09	4.0 (0.8) × 10^3	4
PC:PS:PI	2:1:0.03	1.4 (0.2) × 10^3	3
PC:PS:PI	2:1:0.09	1.0 (0.2) × 10^3	4
PC:PS:PA	2:1:0.03	3.8 (0.1) × 10^2	2
PC:PS	2:1	6.2 (1.1) × 10^2	4
PC:PI(4,5)P_2	99:1	7.1 (0.2) × 10^2	2
PC:PI(4)P	98:2	2.3 (0.1) × 10^2	2
PC:PI	99:1	$<10^2$	2
PC	100	$<10^2$	2

[a] Results are expressed as molar partition coefficients (K). To calculate the apparent K_a for a 1:1 protein:PI(4,5)P_2 complex in PC:PS membranes containing 1 mol % PI(4,5)P_2, K is divided by the PI(4,5)P_2 mole fraction (0.01) ($K_a = 7.7 \times 10^5$ M^{-1}). [b] SD is standard deviation for determination of the parameter, K, estimated by fitting eq 1 to the binding data. [c] n is the number of independent binding experiments.

Binding of PLC-δ_1 (30−43) Peptide to Membrane Bilayers. Yagisawa et al. (1994) and Hirata et al. (1994) claimed that a peptide corresponding to amino acids 30−43 of PLC-δ_1 binds to PI(4,5)P_2 with high affinity. This sequence contains the conserved residues found in the loop connecting the first two β-strands of pleckstrin's amino terminal domain (K13, K14, W21, K22), which are known to interact with PI(4,5)P_2 (Harlan et al., 1994, 1995). Unlike the native

The apparent association constants (K_a) for binding of the PLC-δ_1 PH domain to PI(4,5)P_2 or PI(3,4,5)P_3 (calculated from Table 1 assuming a 1:1 polyphosphoinositide:protein stoichiometry) are similar to those obtained for recombinant human PLC-δ_1, about 10^6 M^{-1} (data not shown). These binding constants, as well as the K_a for binding Ins(1,4,5)P_3 (see below), are at least 10-fold greater than the values reported for other PH domain proteins (Harlan et al., 1994, 1995; Hyvonen, 1995).

[2] Harlan and co-workers (1994) demonstrated that control proteins containing a His-Tag sequence, such as the Shc SH2 domain, do not bind to vesicles formed from PC and PI(4,5)P_2.

[3] The somewhat stronger binding of the PLC-δ_1 PH domain to PI-(3,4,5)P_3 does not mean that PLC-δ_1 is bound predominantly to this lipid in living cells. Even in stimulated cells, the level of PI(3,4,5)P_3 is less than $^1/_{10}$ the level of PI(4,5)P_2 (Stephens et al., 1991; Cantley et al., 1991).

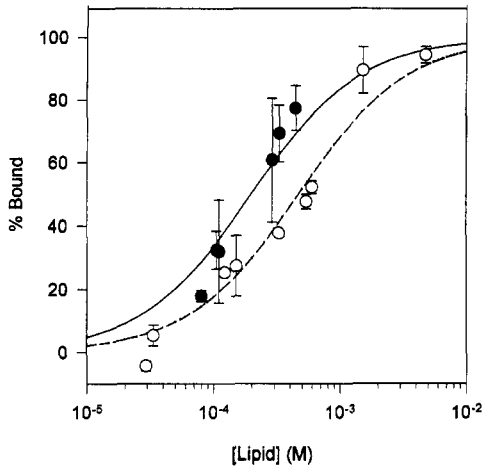

FIGURE 3: Binding of PLC-δ_1 (30−43) peptide to membrane bilayers. Binding of PLC-δ_1 (30−43) peptide to LUVs formed from PC:PG (2:1) (open circles) and PC:PG (2:1) vesicles containing 2 mol % PI(4,5)P_2 (filled circles) were compared. The ordinate is the percentage of peptide bound, and the abscissa the concentration of lipid in the outer monolayer of the LUVs. Each symbol is the average of 2 measurements, and bars indicate standard deviations of the mean. The curves represent the best fits of the data to eq 1.

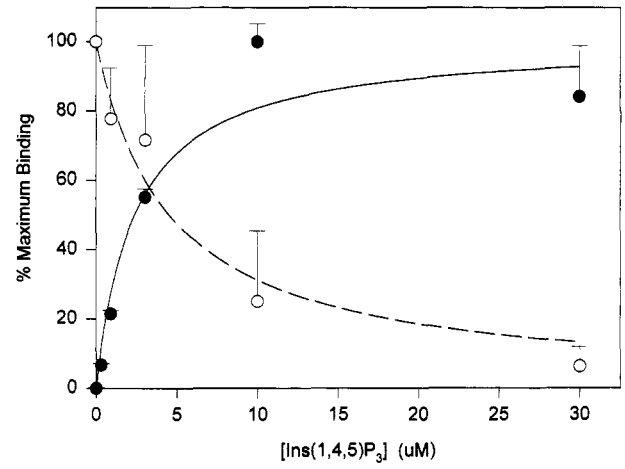

FIGURE 4: Binding of PLC-δ_1PH domain to Ins(1,4,5)P3. Binding of [^{3}H]Ins(1,4,5)P_3 to PLC-δ_1 PH domain (filled circles) was measured by equilibrium ultrafiltration. The ordinate is the percentage of maximum binding achieved when approximately 80% of the protein was bound to Ins(1,4,5)P_3. [^{3}H]Ins(1,4,5)P_3 binding was suppressed by unlabeled Ins(1,4,5)P_3 (open circles). The concentrations of [^{3}H]Ins(1,4,5)P_3 in the competition experiments were 3 μM. Each symbol is the average of 2 experiments, each determined in duplicate. Bars indicate standard deviations of the mean. The curve (solid line) represents the best fit of eq 1 to the data. The curve (dashed line) represents the best fit to the data of an equation describing a simple competitive inhibition system (Segel, 1975).

enzyme or PH domain protein, this peptide demonstrated marginal specificity for binding to PI(4,5)P_2 in our experiments (Figure 3). K was 5×10^3 M^{-1} for binding PC:PG:PI(4,5)P_2 (2:1:0.06) LUVs compared to 2×10^3 M^{-1} for PC:PG (2:1) vesicles. Recall that incorporation of 3 mol % PI(4,5)P_2 into LUVs containing 33% monovalent acidic lipid increased the binding of native PLC-δ_1 (Rebecchi et al., 1992) or recombinant enzyme (data not shown) 100-fold and the isolated PH domain of PLC-δ_1 40-fold (Table 1). The value of K for the PLC-δ_1 (30−43) peptide binding to PC:PG vesicles containing PI(4,5)P_2 is similar to that observed for pentalysine or another 5+ valent peptide corresponding to amino acids 430−442 located in the X box of PLC-δ_1 (Rebecchi et al., 1992).

Binding of Ins(1,4,5)P_3 to the PLC-δ_1 PH Domain. We also measured binding of the PLC-δ_1 PH domain to the polar head group of PI(4,5)P_2 in the absence of membrane vesicles. [^{3}H]Ins(1,4,5)P_3 bound specifically and with high affinity to PLC-δ_1 PH domain as assessed by equilibrium filtration experiments (Figure 4): binding stoichiometry was nearly 1:1 with an apparent association constant of 4×10^5 M^{-1}. Addition of a 10-fold excess of unlabeled Ins(1,4,5)P_3 abolished nearly all the radioligand binding. The apparent association constant of Ins(1,4,5)P_3 binding to recombinant human PLC-δ_1 was similar (9×10^5 M^{-1}) (data not shown).[4]

Structural Studies. We attempted to follow binding of inositol phosphates to PLC-δ_1 PH domain, which contains four tryptophan residues, by monitoring the intrinsic fluorescence emission spectrum, but we were unable to detect any significant changes in response to binding Ins(1,4,5)P_3. Likewise, we were unable to detect significant changes in the CD spectrum. These results are consistent with the idea that the PLC-δ_1 PH domain forms a stable modular structure that does not undergo major conformational changes upon binding to Ins(1,4,5)P_3.

DISCUSSION

We have demonstrated that the amino terminal region of PLC-δ_1 (amino acids 1−155), which we refer to as the PH domain, binds to PI(4,5)P_2 with high affinity and specificity. Our measurements of Ins(1,4,5)P_3 binding suggest that the PH domain recognizes the polar head group of PI(4,5)P_2 and binds with a 1:1 stoichiometry. The apparent association constant (K_a) for binding of the PLC-δ_1 PH domain to PI(4,5)P_2 is similar to those reported for PLC-δ_1 purified from brain, $K_a \sim 10^6$ M^{-1} (Rebecchi et al., 1992), and liver cytosol, $K_a \sim 10^5-10^6$ M^{-1} (Pawelczyk & Lowenstein, 1993). Likewise, the relative binding affinities for PI(4,5)P_2, PI(4)P, and PI, and the effects of monovalent acidic lipids such as PS on phosphoinositide binding, are the same for the PH domain and native PLC-δ_1 proteins (Rebecchi et al., 1992; Pawelczyk & Lowenstein, 1993).

Structural studies of the PH domains isolated from different proteins suggest a molecular explanation for the selective, high affinity PI(4,5)P_2 binding we observe. The PI(4,5)P_2 binding site of pleckstrin's amino terminal domain has been identified (Harlan et al., 1994, 1995). Heteronuclear NMR experiments indicate that binding of PI(4,5)P_2 perturbs residues K13, K14, W21, and K22, located in the loop that links the first two β-strands of this PH domain. The three-dimensional structure of the β-spectrin PH domain, complexed with the PI(4,5)P_2 polar head group, reveals that residues located in the corresponding loop (K8, R21, and W23) make contact with the position 4 and 5 phosphomonoester groups (Hyvonen et al., 1995). A similar binding site does not exist in the PH domain of dynamin (Ferguson et al., 1994; Fushman et al., 1995). If the sequences of PLC-δ_1, pleckstrin, and β-spectrin PH domains are compared by alignment and secondary structure analysis (Hyvonen et al.,

[4] The specific binding of Ins(1,4,5)P_3 to the PLC-δ_1 PH domain has no clear biological function. Ins(1,4,5)P_3 could in principle compete with PI(4,5)P_2 for the PH domain, but the concentrations of Ins(1,4,5)P_3 in living cells are unlikely to ever reach those required to displace a significant fraction of PLC-δ_1 (>5 μM).

1995), amino acids K30, K32, W36, and R38 are predicted to be in this loop region, which suggests these residues form part of the PI(4,5)P_2 binding site of PLC-δ_1.

Hirata et al. (1994) synthesized a peptide of 14 amino acids corresponding to residues 30−43 of PLC-δ_1. This peptide was capable of binding to Ins(1,4,5)P_3 and PI(4,5)P_2, although it did not demonstrate the selectivity of the native protein. Our results (Figure 3) demonstrate that this simple peptide, like others of similar charge, fails to specifically bind PI(4,5)P_2 with an affinity comparable to that of the native enzyme or its PH domain (Figure 2). Thus, strong, selective binding of PI(4,5)P_2 appears to require a three-dimensional binding pocket that exists in the native PLC-δ_1 protein or isolated PH domain, but not in these simple peptides.

The PH domain of PLC-δ_1 may be attracted by electrostatic forces to membrane bilayers formed with a high content of monovalent acidic lipid. This prediction is consistent with our observation that increasing the mole fraction of monovalent acidic lipid enhances the binding of the PH domain to membranes containing PI(4,5)P_2 10-fold (Table 1, compare lines 2 and 11). Structures of the pleckstrin, β-spectrin, and dynamin PH domains reveal that they are electrically polarized: clusters of basic residues surround the PI(4,5)P_2 binding pocket, and clusters of acidic residues are located on the opposite surface (Yoon et al., 1994; Macias et al., 1994; Ferguson et al., 1994; Harlan et al., 1994; Fushman et al., 1995; Hyvonen et al., 1995).

Does the affinity of the PLC-δ_1 PH domain for PI(4,5)P_2 mean that a significant fraction of PLC-δ_1 is bound to the minute amounts of PI(4,5)P_2 found in eukaryotic cells? If we assume that PI(4,5)P_2 is confined mainly to the plasma membrane and comprises 1% of the lipid in this compartment, the effective concentration of PI(4,5)P_2 in a spherical cell of radius 10 μm is about 10^{-5} M. As the K_a for PI(4,5)P_2 $\sim 10^6$ M^{-1}, the PH domain could tether 90% of the PLC-δ_1, which suggests the PH domain is capable of directing PLC to membranes enriched in this lipid.

The function of the PH domain as a membrane tether fits readily into a model of processive catalysis originally proposed for extracellular lipases (Deems & Dennis, 1975; Hendrickson & Dennis, 1984; Jain & Berg, 1989). In this model, the enzyme associates with the membrane via a distinct anchoring site and remains bound to the interface throughout multiple cycles of catalysis before dissociating back into the bulk solution. Similar kinetic behavior has been described for the PLC isozymes: PLC-β from turkey erythrocytes (James et al., 1995), γ_1 (Wahl et al., 1992), and δ_1 (Cifuentes et al., 1993) exhibit a dependence of initial reaction velocity on substrate mole fraction that is characteristic of processive catalysis. In the case of the δ_1 isozyme, an intact PH domain is required for this behavior (Cifuentes et al., 1993).

Results of this study support the hypothesis that PLC-δ_1 hydrolyzes PI(4,5)P_2 processively. In our simple model, the PH domain tethers the active site to membranes enriched in PI(4,5)P_2. Such an arrangement permits the catalytic core of the enzyme to hydrolyze multiple substrate molecules before dissociating from the membrane surface. It is reasonable to extend this model to other δ subtypes (δ_2 and δ_3) and some members of the PLC-β group. PLC-δ_2 and -δ_3 contain PH motifs with sequences that are nearly identical to the PH domain of δ_1 (Parker et al., 1994). Although the PH domain sequence of PLC-β from turkey erythrocyte differs considerably from those of the δ isozymes, the turkey erythrocyte form (James et al., 1995) (but not the PLC-β_2 isozyme, unpublished) binds PI(4,5)P_2 with comparable affinity, consistent with the presence of a PI(4,5)P_2-specific membrane anchoring site in this protein.

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