Mechanism of Membrane Binding of the Phospholipase
D1 PX Domain*

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Mammalian phospholipases D (PLD), which catalyze the hydrolysis of phosphatidylcholine to phosphatic acid (PA), have been implicated in various cell signaling and vesicle trafficking processes. Mammalian PLD1 contains two different membrane-targeting domains, pleckstrin homology and Phox homology (PX) domains, but the precise roles of these domains in the membrane binding and activation of PLD1 are still unclear. To elucidate the role of the PX domain in PLD1 activation, we constructed a structural model of the PX domain by homology modeling and measured the membrane binding of this domain and selected mutants by surface plasmon resonance analysis. The PLD1 PX domain was found to have high phosphoinositide specificity, i.e. phosphatidylinositol 3,4,5-trisphosphate (PtdIns(3,4,5)P3) → phosphatidylinositol 3-phosphate → phosphatidylinositol 5-phosphate → other phosphoinositides. The PtdIns(3,4,5)P3 binding was facilitated by the cationic residues (Lys119, Lys121, and Arg179) in the putative binding pocket. Consistent with the model structure that suggests the presence of a second lipid-binding pocket, vesicle binding studies indicated that the PLD1 PX domain could also bind with moderate affinity to PA, phosphatidylserine, and other anionic lipids, which were mediated by a cluster of cationic residues in the secondary binding site. Simultaneous occupancy of both binding pockets synergistically increases membrane affinity of the PX domain. Electrostatic potential calculations suggest that a highly positive potential near the secondary binding site may facilitate the initial adsorption of the domain to the anionic membrane, which is followed by the binding of PtdIns(3,4,5)P3 to its binding pocket. Collectively, our results suggest that the interaction of the PLD1 PX domain with PtdIns(3,4,5)P3 and/or PA (or phosphatidylserine) may be an important factor in the spatiotemporal regulation and activation of PLD1.

Mammalian phospholipase D (PLD) catalyzes the hydrolysis of phosphatidylcholine to generate phosphatic acid (PA) and choline (1, 2). PA may act as a lipid mediator for various proteins involved in cell signaling and vesicle trafficking (3, 4) and may also regulate the physical property of the cellular membranes (5, 6). Two isoforms of mammalian PLDs, PLD1 and PLD2, have been implicated in numerous cellular processes, including vesicle trafficking, cytoskeletal rearrangement, and proliferation (1, 3, 4, 7, 8). PLDs are activated in many cell types in response to growth factors, hormones, and neurotransmitters (9). It has been reported that PLD activities are regulated through interactions with a wide variety of molecules, including small GTP-binding proteins, such as ADP-riboseylation factor (Arf), Rho, Rac, and Cdc42, and protein kinase C isoforms (10–16).

In most mammalian cells, PLD activities have been found associated with the membrane fraction but PLDs show complex membrane localization patterns depending on cell types. While PLD2 is mainly found at the plasma membrane (17), PLD1 shows dynamic localization between the plasma membrane and the intracellular membranes of endocytic and secretory origin, including Golgi apparatus, endoplasmic reticulum, early and late endosomes, and multivesicular bodies (18–22). The mechanisms underlying the subcellular localization and activation of PLDs are not fully understood. In particular, the role of lipid-protein interactions in the processes is not well defined. It has been shown that phosphatidylinositol 4,5-bisphosphate (PtdIns(4,5)P2) and phosphatidylinositol 3,4,5-trisphosphate (PtdIns(3,4,5)P3) can activate PLDs in vitro (14, 23, 24). All mammalian PLDs contain two membrane targeting domains, a Phox homology (PX) and a pleckstrin homology (PH) domain, in the amino-terminal region and a putative PtdIns(4,5)P2-binding polybasic motif in the catalytic domain. Although PH and PX domains from other proteins have been shown to bind various phosphoinositides (PIs) (25–30), direct binding of any PI to neither isolated PH domain nor isolated PX domain of PLD1 has been unambiguously demonstrated. As such, the exact roles of PH and PX domains in the membrane binding and the regulation of mammalian PLDs remain controversial.

The PX domain, which is composed of 100–140 amino acids, was first found in two cytosolic subunits (p47phox and p40phox) of NADPH oxidase (31) and has been found since in a variety of proteins involved in cell signaling (i.e. PI 3-kinases, cytokine-independent survival kinase, and FISH) and membrane traf-
ficking (i.e. Mvp1p, Vps5p, Bem1p, Grd19p, and sorting nexin proteins) (25–32). Sequence comparisons of PX domains have shown that the domain contains several conserved regions, including a proline-rich stretch (PXXP) and a number of basic residues. Recently, PX domains have been shown to interact with different PIs via conserved basic residues and target the host proteins to specific subcellular locations (25, 32–34). Sequence comparisons of PX domains (see Fig. 1) show that the domain contains several conserved residues, i.e. Arg58, specifically forms hydrogen bonds with the side chain of the 3-phosphate of PtdIns(3)P. The PX domains lacking this Arg, such as the PX domains from Class II PI 3-kinase-C2 (40) and p47\(^{phox}\) (41), prefer longer chains of the anionic lipid with varying affinities (39). The recent x-ray crystal structure of the PX domain of p40\(^{Mvp2}\) (28) illustrated how the domain achieves the stereospecific recognition of PtdIns(3)P (26). The structure revealed that a basic residue, Arg\(^{58}\), specifically forms hydrogen bonds with the 3-phosphate of PtdIns(3)P. The PX domains lacking this Arg, such as the PX domains from Class II PI 3-kinase-C2a (40) and the yeast protein Bem1p (25), bind PtdIns(4,5)\(_2\) and phosphatidylinositol 4-phosphate (PtdIns(4)P), respectively. Furthermore, the p47\(^{phox}\) PX domain has been reported to preferentially interact with phosphatidylinositol 3,4-bisphosphate (PtdIns(3,4)\(_2\)) (29, 37).

Sequence alignment of PX domains (Fig. 1) shows that PLD1 contains a Lys (Lys\(^{119}\)) instead of Arg in the putative 4-phosphate-binding site. Also, PLD1 harbors an Arg (Arg\(^{179}\)) in the putative 4-phosphate interaction site. However, still unresolved is the phospholipid binding capability of the PLD1 PX domain. This study was undertaken to determine the membrane binding properties of the PLD1 PX domain, with an emphasis on elucidating the PI and/or anionic lipid binding specificity. Results from in vitro membrane binding measurements by surface plasmon resonance (SPR) analysis as well as structural modeling indicate that PLD1 PX specifically binds PtdIns(3,4,5)\(_3\) and also have a secondary binding site for anionic phospholipids, including PA and phosphatidylserine (PS). This information provides new insight into the membrane targeting and activation mechanism of PLD1.

**EXPERIMENTAL PROCEDURES**

**Materials**—1-Palmitoyl-2-oleoyl-sn-glycerol-3-phosphatidic acid (POPC), 1-palmitoyl-2-oleoyl-sn-glycerol-3-phosphocholine (POPC), 1-palmitoyl-2-oleoyl-sn-glycerol-3-phosphoethanolamine (POPE), 1-palmitoyl-2-oleoyl-sn-glycerol-3-phosphoserine (POPS) were purchased from Avanti Polar Lipids, Inc. (Alabaster, AL) and used without further purification. 1,2-Dipalmitoyl derivatives of PtdIns(3)P, PtdIns(3,4)P, phosphatidylinositol 5-phosphate (PtdIns(5)P), PtdIns(3,4,5)\(_3\), PtdIns(4,5)\(_2\), and PtdIns(3,4,5,6)\(_4\) were synthesized as described elsewhere (41). Phospholipid concentrations were determined by a modified Bartlett analysis (42). The Liposofast microextruder and a number of fractions using 0.5 ml of 50 mM Tris-HCl, pH 7.4, containing 0.1 M NaCl, 1 mM phenylmethylsulfonyl fluoride, 0.4% Triton X-100, and 0.4% sodium deoxycholate. The mixture was incubated on ice with gentle shaking for 1 h. After this time, the mixture was poured onto a column, which was washed with 20 ml of 50 mM Tris-HCl, pH 8.0, containing 0.1 M KCl and 10 mM imidazole. Subsequently, the protein was eluted from the column in six fractions using 0.5 ml of 50 mM Tris-HCl, pH 8.0, containing 0.1 M KCl and 10 mM imidazole.

**SPR Analysis**—All SPR measurements were performed at 23 °C. A detailed protocol for coating the L1 sensor chip has been described elsewhere (44, 45). Briefly, after washing the sensor chip surface, 90 l of vesicles containing various phospholipids (see Table I) were injected in 5 s to give a response of 5000 resonance units (RU). Similarly, a control surface was coated with vesicles, typically without the PI of interest, to give the same resonance unit response as the active binding surface. Under our experimental conditions, no binding was detected to this control surface beyond the refractive index change for the PI surface. Our detailed protocol for coating the L1 sensor chip has been described elsewhere (44, 45). Briefly, after washing the sensor chip surface, 90 l of vesicles containing various phospholipids (see Table I) were injected in 5 s to give a response of 5000 resonance units (RU). Similarly, a control surface was coated with vesicles, typically without the PI of interest, to give the same resonance unit response as the active binding surface. Under our experimental conditions, no binding was detected to this control surface beyond the refractive index change for the PI surface. Under our experimental conditions, no binding was detected to this control surface beyond the refractive index change for the PI surface. Under our experimental conditions, no binding was detected to this control surface beyond the refractive index change for the PI surface.

**Circular Dichroism Measurements**—CD spectra of PX domains were recorded in 1-1 mm quartz cuvettes in a Jasco J810 Spectropolarimeter. Protein concentration for all measurements was 0.3 mg/ml. Following the association of sample to final concentration in a 10 mM sodium phosphate buffer solution, pH 7.0, four scans were taken from 190 to 280 nm and averaged.

**Mutagenesis and Protein Expression**—CDNA for rat PLD1 was kindly provided by Dr. Sungho Ryu of Pohang University. Constructs of PLD1-PX containing residues 76–214 were obtained by the overlap extension polymerase chain reaction method (43). Each construct was ligated into the pET28a (44) and human p47\(^{phox}\) PX domains were shown. Residues involved in PI binding are highlighted in *bold-faced* characters; see “Results” for description.
protein concentrations (C), and the $K_d$ value was determined by a nonlinear least squares analysis of the binding isotherm using an equation, $R_e = R_{	ext{monolayer}}(1 + K_d/C)$. Mass transport (48, 49) was not a limiting factor in our experiments, since change in flow rate (from 10 to 60 μl/min) did not affect kinetics of association and dissociation. After curve fitting, residual plots and $\chi^2$ values were checked to verify the validity of the binding model. Each data set was repeated three times to calculate a standard deviation value.

**Membrane Binding of PLD1 PX Domain**

The structures of the p40 phox PX domain and the p47 phox PX domain (Protein Data Bank code 1H6H) (26) were constructed as described previously (51). Five to ten μl of phospholipid solution in ethanol/hexane (1.9 v/v%) was spread onto 10 ml of subphase (20 mM Tris-HCl, pH 7.4, containing 0.16 M KCl) to form a monolayer with a given initial surface pressure ($\pi_0$). The subphase was continuously stirred at 60 rpm with a magnetic stir bar. Once the surface pressure reading of monolayer had been stabilized (after ~10 min), the protein solution (typically 100 μl) was injected into the subphase through a small hole drilled at an angle through the wall of the trough and the change in surface pressure ($\Delta \pi$) was measured as a function of time. Typically, the $\Delta \pi$ value reached a maximum after 30 min. It has been shown empirically that $\Delta \pi$ caused by a protein is mainly due to the penetration of the protein into the lipid monolayer (50). The maximal $\Delta \pi$ value at a given $\pi_0$ depended on the protein concentration and reached a saturation value. Protein concentrations in the subphase were therefore maintained above such values to ensure that the observed $\Delta \pi$ represented a maximal value. The critical surface pressure ($\pi_c$) was determined by extrapolating the $\Delta \pi$ versus $\pi_0$, plot to the x axis (51).

**Homology Modeling and Electrostatic Potential Computation—**Models for the PLD1 PX domain were constructed as described previously for the p47 phox domain (52). Fold recognition programs (53, 54) identified the structures of the p40 phox PX domain (Protein Data Bank code 1H6H) (26) and the p47 phox PX domain (Protein Data Bank code 1O7K) (29) as high confidence structural representations of the PLD1 and p47 phox PX domain sequences and were, therefore, used as structural templates in modeling. The sequence alignments between the PLD PX domains and its structural templates were manually edited and constructed by combining (a) the results of alignment algorithms (55, 56), (b) threading analysis (53, 54), and (c) alignment of predicted (57–61) and known (62) secondary structure elements of the target and template, respectively. Homology models were constructed by overlaying the target sequence on the template structure according to the optimized sequence alignment using the program Nest (63). Alignments were further manually edited to produce models that maximized the fitness scores obtained in the structure evaluation program, Verify 3D (64).

**RESULTS**

**Structural Modeling of the PLD1 PX Domain—**Structural analyses of the PX domains of p40 phox, p47 phox, cytokine-independent survival kinase, and Vam7p have shown that these PX domains have similar tertiary structures (26, 29, 66, 67). Thus, we constructed a model structure of the PLD1 PX domain by means of the homology modeling to these PX domains, to gain better insight into its potential membrane binding properties. Fold recognition programs (53, 54) identified the structures of the p40 phox PX domain (Protein Data Bank code 1H6H) (26) and the p47 phox PX domain (Protein Data Bank code 1O7K) (29) as high confidence structural representations of the PLD1 PX domain sequence and were, therefore, used as structural templates in modeling. Homology models were constructed by overlaying the target sequence on the template structure according to the optimized sequence alignment using the program Nest (63). Alignments were further manually edited to produce models that maximized the fitness scores obtained in the structure evaluation program, Verify 3D (64).

The resulting model (see Fig. 2) predicts the presence of two basic pockets as potential lipid-binding sites: a primary pocket similar to the binding sites of other PX domains and a shallower secondary site. A predicted PI-binding pocket contains three cationic residues, Lys119, Lys121, and Arg179. As shown in Fig. 1, Lys119 of the PLD1 PX domain aligns to Arg58 of the p40 phox PX domain and Arg43 of the p47 phox PX domain, respectively, both of which are involved in 3-phosphate binding (26, 29). On the other hand, Arg179 of the PLD1 PX domain aligns to Arg350 of the p40 phox PX domain and Arg350 of the p47 phox PX domain, respectively. The former interacts with the 4- and 5-hydroxyl groups of PtdIns(3)P bound to the domain (26), whereas the latter may be involved in interaction with the 4-phosphate (29). Thus, these cationic residues in the putative PI-binding pocket are good candidates for binding to PIs containing 3- and 4-phosphate (and possibly 5-phosphate). However, Lys119 in place of Arg may attenuate the specificity and/or affinity for 3-phosphorylated PIs, since Lys cannot provide the bidentate interaction of Arg observed in the crystal structure of the p40 phox PX-PtdIns(3)P complex.

The location of a secondary lipid-binding pocket was predicted for the PLD1 PX domain (Fig. 2), based upon the crystal structure of the p47 phox PX domain with a secondary lipid-binding site (29). In this case, however, the exact residues involved in forming the second site could not be predicted with confidence, since our modeling procedure could not reliably predict the orientation of loops. Nevertheless, the model suggests that a large cluster of cationic residues in this region, including Lys132, Lys144, Arg145, Arg149, Arg150, and Arg159, might be involved in interaction with an (or more) anionic phospholipid.

Our model structure also positions two hydrophobic residues Ile171 and Phe148 near the PI-binding pocket and the secondary binding site, respectively. Hydrophobic residues surrounding the PI-binding pocket of the PX domains of p40 phox and p47 phox have been shown to participate in partial membrane penetration (47), while corresponding residues in Vam7p PX domain have been shown to undergo chemical shifts in NMR in the presence of phospholipids (35). A hydrophobic residue near the secondary lipid-binding site of p47 phox PX has also been shown to penetrate the membrane (47). Thus, Ile171 and Phe148 might...
be involved in partial membrane penetration and hydrophobic interactions with the membrane.

To validate our model structure for the PLD1 PX domain, we bacterially expressed the domain and measured its CD spectrum. In general, the bacterial expression and purification of the PLD1 PX domain and its mutants gave a lower yield (<0.5 mg from 1 liter of growth medium) than other PX domains previously characterized, due to their tendency to aggregate. As shown in Fig. 3, the CD spectrum of the PLD1 PX domain is similar to that of the p47phox PX, which was used as a template for the homology modeling of the PLD1 PX domain. This indicates that the bacterially expressed PLD1 PX domain is functionally folded and its folded conformation is similar to that of the p47phox PX, as predicted by our homology modeling.

Phosphoinositide Specificity of the PLD1 PX Domain—To see if the two putative lipid-binding sites actually interact with phospholipids, we measured the binding of the PLD1 PX domain to vesicles with different lipid compositions. We first measured by kinetic SPR analysis the binding of wild type PLD1 PX domain to various PI-containing lipid vesicles immobilized to the sensor surface. Representative sensograms are shown in Fig. 4A for binding of the wild type to POPC/POPE/PtdIns(3,4,5)P3 (77:20:3) vesicles. To validate the Kd value determined from the kinetic SPR analysis, we also determined Kd by equilibrium SPR analysis. The Kd value (20 ± 0.4 nM) calculated from the equilibrium binding isotherm (Fig. 4B) agreed well with Kd determined from the kinetic analysis (Kd = 18 ± 4 nM).

In the absence of PI, the PLD1 PX domain showed extremely low affinity for POPC/POPE (80:20) vesicles (Kd > 30 μM). The addition of PI to the vesicles enhanced the vesicle affinity of the PLD1 PX domain in a concentration-dependent manner (data not shown), but the extent varied widely among PIs. When compared at 3 mol % PI (see Table I), the PLD1 PX domain shows the highest affinity for PtdIns(3,4,5)P3-containing vesicles, modest affinity for PtdIns(3)P- and PtdIns(5)P-containing vesicles, and much lower affinity for vesicles with other PIs. Specifically, its affinity for PtdIns(3,4,5)P3 is 8-fold higher than that for PtdIns(3)P and >200-fold higher than that for PtdIns(3,4)P2, demonstrating specificity for PtdIns(3,4,5)P3. It should be noted that the PLD1 PX domain binds mono-phosphorylated species, PtdIns(3)P or PtdIns(5)P, much better than bis-phosphorylated species, PtdIns(3,4)P2 or PtdIns(3,5)P2. This precludes the possibility that the PtdIns(3,4,5)P3-specificity of the PLD1 PX domain is simply due to a nonspecific electrostatic effect caused by highly negative PtdIns(3,4,5)P3.

In the case of the p47phox PX domain, the secondary lipid-binding pocket was shown to bind with moderate affinity an anionic phospholipid with a smaller headgroup, such as PA and PS, but not PIs (29). Also, simultaneous occupation of the two sites by PI and PA (or PS), respectively, synergistically enhanced the membrane affinity of the p47phox PX domain. To see if the PLD1 PX domain shows similar behaviors, we measured its binding to vesicles containing varying concentrations of PA or PS. The PLD1 PX domain showed no detectable binding to POPC/POPE vesicles containing 3 mol % POPA or POPS (i.e. POPC/POPE/POPA = 77:20:3); however, its affinity gradually increased with the increase in POPA (or POPS) concentration. In fact, the PLD1 PX domain showed higher affinity for POPC/POPE/POPA (47:20:30) vesicles than for POPC/POPE/PtdIns(3,4,5)P3 (77:20:3) vesicles. This suggests that the primary PI-binding site or the secondary site has moderate affinity for PA (or PS). To see if PA (or PS) binding synergizes with PtdIns(3,4,5)P3 binding, we measured the binding to POPC/POPE/PtdIns(3,4,5)P3/POPA (or POPS) (74:20:3:3) vesicles. Although 3 mol % POPS or POPA alone did not increase the affinity of the PLD1 PX domain for POPC/POPE (80:20) vesicles, the addition of 3 mol % POPS or POPA to POPC/POPE/PtdIns(3,4,5)P3 (77:20:3) vesicles resulted in a 4–8-fold increase in affinity. Thus, it appears that PA (or PS) binding to the secondary site (see below) and PtdIns(3,4,5)P3 binding to the primary PI-binding site synergistically enhance the membrane affinity of the PLD1 PX domain. Interestingly, the addition of 3 mol % PtdIns(3,4)P3, which has extremely low affinity for the primary PI-binding site, to POPC/POPE/PtdIns(3,4,5)P3 (77:20:3) vesicles had a similar enhancing effect. This indicates that unlike the secondary lipid-binding site of the p47phox PX domain, the secondary site of the PLD1 PX domain can also interact with some PIs, presumably due to its strongly cationic nature and conformational flexibility.

Specificities of the Two Lipid-Binding Sites—The lipid specificities of the two phospholipid-binding pockets were further clarified by measuring the binding of a series of site-specific mutants to vesicles containing PtdIns(3,4,5)P3 and different anionic lipids. All putative lipid-binding residues except Lys119 were mutated to Ala; for Lys119, the K119Q mutation was made due to instability of the K119A mutant. We first measured the binding of mutants to POPC/POPE/PtdIns(3,4,5)P3 (77:20:3) vesicles by SPR analysis. Results are summarized in Table I. Clearly, mutation of Arg179 in the putative PI-binding pocket abrogated binding to PtdIns(3,4,5)P3-containing vesicles, demonstrating its critical role in PtdIns(3,4,5)P3 binding. This was not due to deleterious structural changes caused by the mutation, as evidenced by similar CD spectra of wild type and R179A (see Fig. 3). Mutation of Lys119 and Lys121, which are 7-fold reduction, respectively, in vesicle affinity. This suggests that Lys119 and Lys121 are also involved in PtdIns(3,4,5)P3 binding. In contrast to mutations in the PI-binding pocket, mutations of cationic residues located in the putative secondary site (K144A/R145A, R149A/R150A, and R159A) resulted in little decrease in affinity for POPC/POPE/PtdIns(3,4,5)P3 vesicles, indicating that PtdIns(3,4,5)P3 does not interact with the secondary anion-binding pocket with high affinity.

We then measured the binding of mutants to POPC/POPE/POPA (50:20:30) vesicles in the absence of

**FIG. 3.** CD spectra of the PLD1 PX domain and mutant. The CD spectra of PLD1 PX domain (○), R179A (●), and the p47phox PX domain (△) were measured in 10 mM sodium phosphate buffer, pH 7.0. Protein concentrations were 0.3 mg/ml. Curves represent an average of four scans.
Phospholipid headgroup selectivity of the PLD1PX domain determined by SPR analysis

Values represent the mean and standard deviation from three determinations. All measurements were performed in 10 mM HEPES, pH 7.4, containing 0.16 M KCl.

| Lipids \(^a\) | \(k_a\) | \(k_d\) | \(K_d\) | Fold increase in \(K_d\) \(^b\) |
|------------|---------|---------|---------|-----------------|
| PtdIns(3,4,5)P\(_3\) | \((9.1 \pm 0.8) \times 10^4\) | \((1.6 \pm 0.3) \times 10^{-3}\) | \((1.8 \pm 0.4) \times 10^{-8}\) | 1 |
| PtdIns(3)P | \((2.3 \pm 0.4) \times 10^4\) | \((3.2 \pm 0.6) \times 10^{-3}\) | \((4.4 \pm 0.4) \times 10^{-7}\) | 8 |
| PtdIns(5)P | \((1.7 \pm 0.3) \times 10^4\) | \((3.7 \pm 0.5) \times 10^{-3}\) | \((2.2 \pm 0.5) \times 10^{-7}\) | 12 |
| PtdIns(4,5)P\(_2\) | \((9.1 \pm 0.8) \times 10^3\) | \((9.5 \pm 0.6) \times 10^{-3}\) | \((1.0 \pm 0.1) \times 10^{-6}\) | 56 |
| PtdIns(4)P | \((6.6 \pm 0.7) \times 10^3\) | \((1.2 \pm 0.3) \times 10^{-3}\) | \((1.8 \pm 0.5) \times 10^{-6}\) | 99 |
| PtdIns(3,5)P\(_2\) | \((3.6 \pm 0.4) \times 10^3\) | \((1.2 \pm 0.3) \times 10^{-3}\) | \((3.2 \pm 0.9) \times 10^{-6}\) | 178 |
| PtdIns(3,4,5)P\(_3\) | \((2.9 \pm 0.5) \times 10^3\) | \((1.4 \pm 0.4) \times 10^{-2}\) | \((5.0 \pm 1.7) \times 10^{-6}\) | 267 |
| 30% POPS | \((2.6 \pm 0.4) \times 10^5\) | \((1.6 \pm 0.3) \times 10^{-3}\) | \((6.1 \pm 1.1) \times 10^{-9}\) | 0.34 |
| 30% POPA | \((2.1 \pm 0.3) \times 10^5\) | \((1.1 \pm 0.3) \times 10^{-3}\) | \((5.2 \pm 1.6) \times 10^{-9}\) | 0.28 |
| PtdIns(3,4,5)P\(_3\) + POPS | \((1.9 \pm 0.4) \times 10^5\) | \((5.5 \pm 0.7) \times 10^{-4}\) | \((4.6 \pm 1.1) \times 10^{-10}\) | 0.26 |
| PtdIns(3,4,5)P\(_3\) + POPA | \((1.9 \pm 0.3) \times 10^5\) | \((4.5 \pm 0.5) \times 10^{-4}\) | \((2.4 \pm 0.5) \times 10^{-9}\) | 0.13 |
| PtdIns(3,4,5)P\(_3\) + PtdIns(3,4,5)P\(_3\) | \((9.8 \pm 0.9) \times 10^4\) | \((4.9 \pm 0.5) \times 10^{-4}\) | \((5.0 \pm 0.7) \times 10^{-9}\) | 0.27 |

\(^a\) POPC/POPE/PI vesicles (77:20:3).

\(^b\) Increase in \(K_d\) compared to \(K_d\) for PtdIns(3,4,5)P\(_3\).

\(^c\) POPC/POPE/PtdIns(3,4,5)P\(_3\)/POPA (or others) vesicles (74:20:3:3).

PtdIns(3,4,5)P\(_3\) to assess the roles of the two binding pockets in PA or PS binding. As shown in Table II, mutations in the PI-binding pocket (K119Q, K121A, or R179A) did not significantly affect the binding to POPA or POPS-containing vesicles, demonstrating the specificity of this site. However, the mutations in the secondary lipid-binding pocket (K144A/R145A and R149A/R150A) caused a 4–5-fold decrease in PA or PS affinity, indicating that the secondary site is solely responsible for binding to PA or PS.

We also measured the binding of wild type and two representative mutants, R179A and R149A/R150A, for POPC/POPE/PtdIns(3,4,5)P\(_3\)/POPA (74:20:3:3) vesicles. Notice that the R179A mutation abrogated binding to POPC/POPE/PtdIns(3,4,5)P\(_3\) (77:20:3) vesicles. Interestingly, R179A exhibited less pronounced 100-fold reduction in affinity for POPC/POPE/PtdIns(3,4,5)P\(_3\)/POPA (74:20:3:3) vesicles. Thus, the binding of POPA or POPS to the secondary anion site lessens the deleterious effect of the mutation in the PI-binding site, underscoring the complementarity nature of two of the binding sites. On the other hand, R149A/R150A, which has similar affinity to wild type for POPC/POPE/PtdIns(3,4,5)P\(_3\) (77:20:3) vesicles, has 50% reduction in affinity for POPC/POPE/PtdIns(3,4,5)P\(_3\)/POPA (74:20:3:3) vesicles. This data corroborates that binding of POPA or POPS to the secondary lipid-binding site considerably implements the PI-mediated membrane binding of the PLD1 PX domain.

**Roles of Hydrophobic Residues in Membrane Penetration**—It has been shown that hydrophobic residues near the PI-binding pocket (K119Q, K121A, or R179A) did not significantly affect the binding to POPA or PS binding. As shown in Table II, respectively. Conversely, I171A and F148A had 1.5- and 4-fold lower affinity than the wild type (Table II), respectively. Conversely, I171A and F148A had 1.5- and 1.6-fold lower affinity than the wild type for POPC/POPE/PI vesicles (77:20:3). These results indicate that Ile\(^{171}\) and Phe\(^{48}\) near the PI-binding pocket and the secondary binding site, respectively, in membrane binding of the PLD1 PX domain, we measured the binding of I171A and F148A mutants to two different types of vesicles. When POPC/POPE/PtdIns(3,4,5)P\(_3\) (77:20:3) vesicles were used, I171A and F148A had 4- and 1.6-fold lower affinity than the wild type (Table II), respectively.
Membrane Binding of PLD1 PX Domain

Table II

Membrane binding parameters for the PLD1 PX domain and mutants

| Protein                          | $K_d$ | $K_a$ | $K_{d0}$ | Fold increase in $K_d$ |
|----------------------------------|-------|-------|----------|------------------------|
| POPC/POPE/PtdIns(3,4,5)P3 (77:20:3) |       |       |          |                        |
| Wild type                        | 9.1 ± 0.8 × 10^4 | 1.6 ± 0.3 × 10^3 | 1.8 ± 0.4 × 10^-8 | 1                      |
| K119Q                            | 3.7 ± 0.6 × 10^4 | 8.4 ± 0.8 × 10^3 | 2.3 ± 0.4 × 10^-7 | 13                     |
| K212A                            | 4.4 ± 0.5 × 10^4 | 5.0 ± 0.6 × 10^3 | 1.2 ± 0.2 × 10^-7 | 6.7                    |
| F148A                            | 8.5 ± 0.8 × 10^4 | 2.5 ± 0.4 × 10^3 | 2.9 ± 0.6 × 10^-8 | 1.6                    |
| K144AR145A                       | 8.3 ± 0.8 × 10^4 | 1.5 ± 0.4 × 10^3 | 1.8 ± 0.5 × 10^-8 | 1.0                    |
| R149AR150A                       | 8.5 ± 0.7 × 10^4 | 1.9 ± 0.4 × 10^3 | 2.2 ± 0.5 × 10^-8 | 1.2                    |
| R159A                            | 8.4 ± 0.8 × 10^4 | 1.7 ± 0.5 × 10^3 | 2.0 ± 0.5 × 10^-8 | 1.1                    |
| H171A                            | 9.3 ± 0.7 × 10^4 | 7.1 ± 0.6 × 10^3 | 7.6 ± 1.2 × 10^-8 | 4.2                    |
| R179A                            | ND$^b$ | ND$^b$ | ND$^b$   | 100                    |
| POPC/POPE/POPS (77:20:30)        |       |       |          |                        |
| Wild type                        | 2.6 ± 0.4 × 10^5 | 1.6 ± 0.3 × 10^3 | 6.1 ± 1.5 × 10^-9 | 1                      |
| K119Q                            | 1.8 ± 0.3 × 10^5 | 1.9 ± 0.4 × 10^3 | 1.1 ± 0.3 × 10^-8 | 1.8                    |
| K212A                            | 2.0 ± 0.4 × 10^5 | 1.7 ± 0.5 × 10^3 | 8.4 ± 3.0 × 10^-8 | 1.4                    |
| F148A                            | 2.3 ± 0.2 × 10^5 | 3.5 ± 0.5 × 10^3 | 1.5 ± 0.3 × 10^-8 | 2.5                    |
| K144AR145A                       | 1.1 ± 0.2 × 10^5 | 2.9 ± 0.5 × 10^3 | 2.6 ± 0.2 × 10^-8 | 4.3                    |
| R149AR150A                       | 9.8 ± 1.1 × 10^4 | 3.2 ± 0.4 × 10^3 | 3.3 ± 0.6 × 10^-8 | 5.4                    |
| R159A                            | 1.5 ± 0.2 × 10^5 | 2.7 ± 0.3 × 10^3 | 1.8 ± 0.2 × 10^-8 | 3.0                    |
| H171A                            | 2.3 ± 0.4 × 10^5 | 2.1 ± 0.3 × 10^3 | 9.1 ± 2.0 × 10^-9 | 1.5                    |
| R179A                            | 2.1 ± 0.3 × 10^5 | 1.8 ± 0.2 × 10^3 | 8.6 ± 1.6 × 10^-9 | 1.4                    |
| POPC/POPE/PtdIns(3,4,5)P3/POPA (74:20:3:3) | 1.9 ± 0.4 × 10^5 | 5.5 ± 0.7 × 10^4 | 2.9 ± 0.7 × 10^-9 | 1                      |
| Wild type                        | 1.6 ± 0.5 × 10^5 | 6.9 ± 0.8 × 10^4 | 4.3 ± 1.4 × 10^-9 | 1.5                    |
| R149AR150A                       | 3.4 ± 0.6 × 10^4 | 9.8 ± 0.9 × 10^3 | 2.9 ± 0.6 × 10^-7 | 100                   |

$^a$ Increase in $K_d$ compared to $K_d$ of wild type under each condition.
$^b$ ND, not detectable.

The PLD1 PX domain may not significantly penetrate the membrane.

To test this notion, we measured the interactions of the PX domain with lipid monolayers of different lipid compositions. We first measured the effects of PtdIns(3,4,5)P3 and other anionic lipids on the monolayer penetration of the PLD1 PX domain (see Fig. 5). When the penetration of the PLD1 PX domain into the POPC/POPE (80:20) monolayer of varying $\pi_c$ was measured, the PLD1 PX domain showed low penetrating power with a $\pi_c$ value of 26 dyne/cm, implying that PLD1 PX has low intrinsic membrane penetrating capability. Incorporation of 3 mol % PtdIns(3,4,5)P3 into the monolayer (i.e. POPC/POPE/PtdIns(3,4,5)P3 (77:20:3)) modestly elevated the monolayer penetrating capability of the domain, with the $\pi_c$ value near 30 dyne/cm. However, this monolayer penetration was significantly less than that of the p47$^{phox}$ PX domain that has the $\pi_c$ value above 35 dyne/cm with the POPC/POPE/PtdIns(3,4,5)P3 (77:20:3) monolayer. Furthermore, 3 mol % POPA, POPS, PtdIns(3)P, PtdIns(5)P, or PtdIns(4,5)P2 exhibited essentially the same effect on the monolayer penetration as PtdIns(3,4,5)P3, indicating that this enhancing effect is due not to the specific PI-induced membrane penetration reported for the p47$^{phox}$ PX domain (47) but to nonspecific electrostatic attraction.

**Electrostatic Potential Calculations**—The above studies indicate that although the PLD1 PX domain and the p47$^{phox}$ PX domain are similar with respect to the tertiary structure and dual lipid binding, they also have noticeably different membrane binding properties, particularly with respect to PI-mediated membrane penetration. To understand the basis of these differences, we calculated the electrostatic potentials of the PLD1 PX domain model structure in the presence and absence of PtdIns(3,4,5)P3 and PS, respectively. Our previous electrostatic potential calculations of p47$^{phox}$ PX showed that binding of PtdIns(3,4,5)P3 and PS reduces the electrostatic potentials surrounding their respective binding sites, which in turn allows favorable partitioning of hydrophobic residues in these regions into the membrane due to the reduced desolvation penalty associated with the process (29, 47).

The electrostatic potential calculation of the PLD1 PX domain showed a highly cationic surface in the vicinity of the secondary binding site, due to the presence of a large number of surface cationic residues, whereas the PI-binding site has a less pronounced positive electrostatic potential (see Fig. 6A). This suggests that the secondary site may play a role of initially bringing the domain to the anionic membrane through nonspecific electrostatic interactions. The binding of PtdIns(3,4,5)P3 was measured as a function of $\pi_c$ for PLD1 PX wild type with POPC/ POPE (80:20) (○), POPC/POPE/PtdIns(3,4,5)P3 (77:20:3) (●), and POPC/POPE/POPS (77:20:3) (□) monolayers. 3 mol % PtdIns(3)P, PtdIns(5)P, or PtdIns(4,5)P2 showed essentially the same effect as 3 mol % PtdIns(3,4,5)P3 (data not shown). The subphase was 10 mM HEPES buffer, pH 7.4, containing 0.16 M KCl for all experiments.

![Change in Surface Pressure vs Initial Surface Pressure](http://www.jbc.org/)

**FIG. 5.** Monolayer penetration analysis of PLD1 PX domain. $\Delta \pi$ was measured as a function of $\pi_c$ for PLD1 PX wild type with POPC/POPE (80:20) (○), POPC/POPE/PtdIns(3,4,5)P3 (77:20:3) (●), and POPC/POPE/POPS (77:20:3) (□) monolayers. 3 mol % PtdIns(3)P, PtdIns(5)P, or PtdIns(4,5)P2 showed essentially the same effect as 3 mol % PtdIns(3,4,5)P3 (data not shown). The subphase was 10 mM HEPES buffer, pH 7.4, containing 0.16 M KCl for all experiments.
to the putative PI-binding site caused a dramatic change in electrostatic potential (i.e., electrostatic switch) surrounding the binding site (Fig. 6B). However, the negative potential surrounding Ile	extsuperscript{171} remains relatively unchanged after PtdIns(3,4,5)P	extsubscript{3} binding. Thus, it is not expected that PtdIns(3,4,5)P	extsubscript{3} binding will facilitate the membrane penetration of Ile	extsuperscript{171} by reducing the desolvation penalty associated with the membrane insertion. This is consistent with the finding that PtdIns(3,4,5)P	extsubscript{3} had a relatively modest effect on the monolayer penetration of the PLD1 PX domain (see Fig. 5). Fig. 6C illustrates that the docking of PS to the secondary pocket does not significantly reduce the electrostatic potential surrounding this site and Phe	extsuperscript{148}. This again explains the modest effect of the F148A mutation on the membrane affinity for POPC/POPE/POPS (57:20:30) vesicles. Last, we calculated the change in electrostatic potential of PLD1 PX in the presence of both PtdIns(3,4,5)P	extsubscript{3} and PS. As shown in Fig. 6 (B and D), the docking of PS does not significantly affect the potential of the PtdIns(3,4,5)P	extsubscript{3}-bound PLD1 PX domain. Collectively, our electrostatic calculations on the PLD1 PX domain suggest that neither PtdIns(3,4,5)P	extsubscript{3} binding to the PI-binding site nor PS (or PA) binding to the secondary site has an electrostatic unmasking effect on the neighboring surface hydrophobic residues, which is consistent with its lower degree of monolayer penetration when compared with the p47	extsuperscript{phox} PX domain (see Fig. 5). The exceptionally positive electrostatic potential of this PX domain suggests that its membrane binding be driven primarily by electrostatic forces.

**DISCUSSION**

PLD1 has at least three structural parts that might play a role in its membrane targeting: PX domain, PH domain, and the polybasic motif in the catalytic domain. It has been reported that PtdIns(4,5)P	extsubscript{2} and PtdIns(3,4,5)P	extsubscript{3} stimulate PLD activities, whereas other PIs, including PtdIns(3,4)P	extsubscript{2} and PtdIns(4)P, are ineffective (14, 23, 24). Previous studies have indicated that both the PH domain (24) and the polybasic motif (69) interact with PtdIns(4,5)P	extsubscript{2}, albeit with different affinities, and are therefore involved in PtdIns(4,5)P	extsubscript{2}-mediated membrane targeting and activation of PLD1. A recent study (19) suggested that the PLD1 PX domain might bind PtdIns(5)P and mediate targeting of the protein to endocytic vesicles. The present study clearly shows that the PX domain of PLD1 has high affinity and specificity for PtdIns(3,4,5)P	extsubscript{3}, due to the presence of three cationic residues in the PI-binding pocket that might be able to interact with the 3'-, 4'-, and 5'-phosphate groups in PtdIns(3,4,5)P	extsubscript{3}. High affinity and specificity of the PLD1 PX domain for PtdIns(3,4,5)P	extsubscript{3} suggest that it can mediate the reported action of PtdIns(3,4,5)P	extsubscript{3} and allow PLD1 to function as a downstream effector of PtdIns(3,4,5)P	extsubscript{3}. This notion is supported by findings that the inhibition of PI 3-kinase results in the down-regulation of PLD activity (70–72) and that PLD is involved in insulin-like growth factor-I-induced activation of extracellular signal-regulated kinase (ERK) in Chinese hamster ovary cells (73). The study also indicates that anionic lipids, such as PS or PA, in the membrane can synergistically enhance the PtdIns(3,4,5)P	extsubscript{3}-mediated membrane binding of the PLD PX domain.

Our previous studies on ENTH, FYVE, and PX domains have shown that these domains initially bind the anionic membrane containing their cognate PIs through nonspecific electrostatic interaction, which is followed by the PI binding and concomitant (or subsequent) PI-induced membrane penetration of surface hydrophobic residues (46, 47, 68). PIs induce the membrane penetration of their binding domains by causing local conformational changes of proteins and by neutralizing the positive electrostatic potential surrounding the hydrophobic loop residues that restricts the membrane penetration of the domains. For the p47	extsuperscript{phox} PX domain, binding of PA (or PS) to

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**FIG. 6. Effects of anionic lipids on the electrostatic potential of the PLD1 PX domain.** The electrostatic potentials for the PLD1 PX domain were calculated and visualized in the program GRASP by two-dimensional equipotential contours in 0.1 M KCl. The red and blue contours represent −25 mV and +25 mV, respectively. The structure is shown in the same orientation as in Fig. 2, and Ile	extsuperscript{171} (left) and Phe	extsuperscript{148} (right) are shown in green space-filling representation. A, in the absence of lipids; B, in the presence of PtdIns(3,4,5)P	extsubscript{3} (pink); C, in the presence of PS (yellow); D, in the presence of both PtdIns(3,4,5)P	extsubscript{3} (pink) and PS (yellow).
the secondary lipid-binding site synergistically enhances the PI-mediated membrane penetration (29, 47). Our vesicle binding, monolayer penetration, and electrostatic calculation studies indicate that the PLD1 PX domain has a different membrane binding mechanism. It is not clear from our studies whether the secondary binding site binds a single anionic lipid molecule in the pocket or interacts nonspecifically with anionic membrane surface. In either case, however, the strongly cationic region surrounding the secondary lipid-binding site would seem to make initial contact with the anionic membrane during the membrane binding of the PLD1 PX domain, which is followed by the binding of PtdIns(3,4,5)P_3 to its pocket. Unlike the p47phox PX domain, neither PI binding nor PS (or PA) binding has a large effect on the membrane penetration of the PLD1 PX domain. Instead, the membrane interactions of the PLD1 PX domain appear to be primarily electrostatic in nature, as is the case with many PH domains.

The affinity (K_d = 18 nM) of the PLD1 PX domain for POPC/POPE/PtdIns(3,4,5)P_3 (77:20:3) vesicles is comparable with that of p47phox PX domain (K_d = 38 nM) for its preferred vesicles (i.e. POPC/POPE/PtdIns(3,4,5)P_2 (77:20:3)) (29). Since the isolated p47phox PX domain is not localized at cell membranes (47), the PLD1 PX domain may not be able to autonomously translocate to cell membranes unless the localization of anionic lipids to the secondary site. The membrane interaction of the PLD1 PX domain will significantly enhance the affinity of PLD1 for cell membranes under appropriate conditions and may also play a role of activating the membrane-bound enzyme. This study opens up a new avenue to study the role of the PX domain in subcellular targeting and activation of mammalian PLDs.

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Mechanism of Membrane Binding of the Phospholipase D1 PX Domain
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