The Pleckstrin Homology Domain of Phosphoinositide-specific Phospholipase Cδ₄ Is Not a Critical Determinant of the Membrane Localization of the Enzyme*

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The inositol lipid and phosphate binding properties and the cellular localization of phospholipase Cδ₄ (PLCδ₄) and its isolated pleckstrin homology (PH) domain were analyzed in comparison with the similar features of the PLCδ₁ protein. The isolated PH domains of both proteins showed plasma membrane localization when expressed in the form of a green fluorescent protein fusion construct in various cells, although a significantly lower proportion of the PLCδ₁ PH domain was membrane-bound than in the case of PLCδ₄-PH-GFP. Both PH domains selectively recognized phosphatidylinositol 4,5-bisphosphate (PI(4,5)P₂), but a lower binding of PLCδ₁-PH to lipid vesicles containing PI(4,5)P₂ was observed. Also, higher concentrations of inositol 1,4,5-trisphosphate (Ins(1,4,5)P₃) were required to displace the PLCδ₁-PH from the lipid vesicles, and a lower Ins(1,4,5)P₃ affinity of PLCδ₁-PH was found in direct binding assays. In sharp contrast to the localization of its PH domain, the full-length PLCδ₁ protein localized primarily to intracellular membranes mostly to the endoplasmic reticulum (ER). This ER localization was in striking contrast to the well-documented PH domain-dependent plasma membrane localization of PLCδ₁. A truncated PLCδ₁ protein lacking the entire PH domain still showed the same ER localization as the full-length protein, indicating that the PH domain is not a critical determinant of the localization of this protein. Most important, the full-length PLCδ₁ enzyme still showed binding to PI(4,5)P₂-containing micelles, but Ins(1,4,5)P₃ was significantly less potent in displacing the enzyme from the lipid than with the PLCδ₁ protein. These data suggest that although structurally related, PLCδ₁ and PLCδ₄ are probably differentially regulated in distinct cellular compartments by PI(4,5)P₂ and that the PH domain of PLCδ₄ does not act as a localization signal.

Phosphatidylinositol 4,5-bisphosphate (PI(4,5)P₂) is a minor phospholipid component of the plasma membrane and a key regulator of several cellular processes. PI(4,5)P₂ is a precursor of important second messengers, such as the water-soluble InsP₃, which regulates Ca²⁺ release from intracellular Ca²⁺ stores, and the hydrophobic diacylglycerol, a potent activator of protein kinase C (1, 2). PI(4,5)P₂ has been shown to regulate proteins, by interacting with their lipid recognition domains, and to participate in membrane remodeling events, including the fusion of secretory vesicles with the plasma membrane (3), and at several steps along the endocytic pathway (4–6).

The best-known regulators of PI(4,5)P₂ levels are the phospholipase C (PLC) enzymes that hydrolyze the phosphodiesters between the diacylglycerol backbone and the phosphate group linking the inositol ring within the PI(4,5)P₂ molecule. Several isoforms of PLC have been described that can be classified into five major groups, PLCβ, PLCγ, PLCδ, PLCε, and PLCζ (7, 8). PLCβ isotypes are regulated by the α- and βγ-subunits of heterotrimeric G proteins, whereas the two PLCγ isotypes are activated by receptor tyrosine kinases as well as by the lipid products of PI 3-kinases. PLCε is a recently identified enzyme that is associated with the small GTP-binding protein, Ras (9, 10), and PLCζ is a novel sperm-specific PLC isoform that is responsible for the initiation of Ca²⁺ oscillations following fertilization (11). PLCζ is the isoform that evolutionarily is the most conserved, its homologue already appearing in yeast, yet the regulation of this enzyme is the least understood. As with all other PLCs, PLCδ is activated by Ca²⁺ ions, and the cytosolic Ca²⁺ increase is believed to be the primary means by which this enzyme is regulated. One important and distinctive feature of PLCδ has been its high affinity binding to InsP₃ and the ability of InsP₃ to inhibit the catalytic activity of the enzyme (12, 13). The part of the molecule responsible for InsP₃ binding is the pleckstrin homology (PH) domain (12), a conserved motif first described in pleckstrin (14), and one which is also present in all β, γ, and δ PLC isozymes (8), as well as in a number of other regulatory molecules (15). The isolated PH domain of PLCδ has been shown to specifically bind PI(4,5)P₂ both in vitro (10) and in vivo (17, 18), and its crystal structure was solved with a bound Ins(1,4,5)P₃ molecule (19). Although the PH domains found in the other PLCs are also capable of binding certain isomers of inositol lipids, they do not display a...
Membrane Localization of PLCδ3 PH Domains

Similarly high affinity to InsP$_3$. Most intriguing, a PLCδ3 homologue with similar InsP$_3$ binding properties but without PLC enzymatic activity, termed p130, has been isolated and characterized (20).

Additional genes encoding isoforms of PLCδ have been described recently (21–24). Among these, PLCδ6 was found to be critical in sperm to induce the acrosome reaction in the zona pellucida (25). PLCδ3 is also unique in that it has three splice variants, one of which has been reported recently to be a potent negative regulator of the PLCδ enzymes (24). This latter study also showed that the PH domain of PLCδ3 binds InsP$_3$ very poorly but still binds the phospholipid, PI(4,5)P$_2$, a feature crucial to the inhibitory effect of the unique splice variant. The finding of a PH domain with the ability to bind PI(4,5)P$_2$ and not being influenced by Ins(1,4,5)P$_3$ would greatly aid studies in which such PH domains are fused to GFP in order to report PI(4,5)P$_2$ regulation to the protein.

As shown in the previous study (18), the PH domain of PLCδ3, and -δ in vitro and to study the localization and dynamics of these domains when expressed in cells as GFP fusion proteins in vivo. Our data suggest that the PH domain of PLCδ3 has a lower affinity to both InsP$_3$ and PI(4,5)P$_2$ than the similar domain of PLCδ and, therefore, shows less prominent plasma membrane localization in intact cells than the PLCδ8 PH domain. Nevertheless, the PLCδ3 PH domain is still capable of reporting PLC activation. Most surprising, the localization of the full-length PLCδ3 protein is significantly different from that of its PH domain, and both the full-length protein and its truncated form missing the PH domain show primarily ER localization. These findings prompted us to compare the inositol phosphate and inositol lipid binding properties of the PH domains of PLCδ3 and -δ in vitro and to study the localization and dynamics of these domains when expressed in cells as GFP fusion proteins in vivo.

Our data suggest that the PH domain of PLCδ3 has a lower affinity to both InsP$_3$ and PI(4,5)P$_2$ than the similar domain of PLCδ and, therefore, shows less prominent plasma membrane localization in intact cells than the PLCδ8 PH domain. Nevertheless, the PLCδ3 PH domain is still capable of reporting PLC activation. Most surprising, the localization of the full-length PLCδ3 protein is significantly different from that of its PH domain, and both the full-length protein and its truncated form missing the PH domain show primarily ER localization. These findings prompted us to compare the inositol phosphate and inositol lipid binding properties of the PH domains of PLCδ3 and -δ in vitro and to study the localization and dynamics of these domains when expressed in cells as GFP fusion proteins in vivo.

EXPERIMENTAL PROCEDURES

Reagents—Angiotensin II (human) was purchased from Peninsula Laboratories. Ionomycin, 1,2-bis(2-aminophosphonooxyethyl)-N,N,N',N'-tetraacetic acid, InsP$_3$, and InsP$_4$ were obtained from Calbiochem. Phosphatidylinositol, phosphatidylserine, and phosphatidyethanolamine were all from Sigma. myo-[3H]Inositol (68 Ci/mmol) and [3H]InsP$_4$ (48 Ci/mmol) were from Amersham Biosciences. The anti-HA monoclonal antibody (HA.11) was from Covance, and the fluorescent secondary antibody (1:1000) for staining was from Molecular Probes. All other chemicals were of high performance liquid chromatography or analytical grade.

DNA Constructs—The PH domain of human PLCδ3 (NM_006225) (residues 1–170) and its mutant R40L has been described previously (18). The PH domain of the PLCδ3 (NM_080688) (residues 1–163) was amplified from the cDNA clone described previously (22), using primers with KpnI/SmaI restriction sites. After digestion with the restriction enzymes, the PCR product was ligated into the pEGFP-N1 plasmid (Clontech) cut with the same two restriction enzymes. A shorter variant of this domain, containing residues 1–127, was also constructed, which showed very similar results in localization studies. The full-length PLCδ3 as well as the truncated version (Δ1–125) were cloned into a pCDNA3.1 plasmid with an HA epitope placed at the C termini of the proteins. All constructs were sequenced with dideoxy sequencing. The integrity and expression levels of the fusion proteins were assessed by Western blot analysis from cells lysates prepared from COS-7 or NIH 3T3 cells transfected with the constructs, using a polyclonal antibody against GST (Clontech). The same HA-tagged constructs were subcloned into the pET23b bacterial expression vector (Novagen) using the NdeI/EcoRI restriction sites. The PLCδ3 PH was also subcloned into the pGEX6P3 expression plasmid (Amersham Biosciences) for its expression as a GST fusion protein. The resulting plasmids were used to transform the BL-21-DE3 strain of E. coli (Novagen). Bacterial cells were grown to an OD$_{600}$ as follows: 0.6 at 37 °C and induced with 300 μM isopropyl-1-thio-β-D-galactopyranoside at 18–20 °C for 7 h. Bacterial lysates were prepared by sonication in lysis buffer (20 mM NaCl and 20 mM Tris, pH 8.0) followed by centrifugation at 10,000 × g for 30 min at 4 °C. The supernatant was incubated with Ni$^{2+}$–nitritotriacetic acidagarose beads (Qiagen) in the presence of 5 mM imidazole for 1 h at 4 °C. The beads were washed three times with lysis buffer and the recombinant proteins were eluted with the same buffer containing 1 M imidazole. GST fusion proteins were isolated from bacterial lysates on glutathione-Sepharose columns (Amersham Biosciences) following standard procedures. Protein samples were concentrated and stored in PBS containing 5 mM dithiothreitol and 20% glycerol at −20 °C.

For bacterial expression of PLCδ3 and its truncated form lacking the PH domain, the C-terminally HA-tagged proteins were expressed from the pET23b plasmid and purified using an anti-HA antibody and protein A/G plus-Sepharose (Calbiochem). Induction of the expression was achieved by overnight incubation in the presence of 100 μM isopropyl-1-thio-β-D-galactopyranoside at 12 °C, because induction at higher temperatures (see above) results in the expression of mostly insoluble protein. The concentration of recombinant proteins was assessed by quantifying the bands of Coomassie Blue-stained SDS gel containing the recombinant proteins and bovine serum albumin as a standard.

The incubation buffer of the in vitro binding assay contained 50 mM Na-Hepes, pH 7.4, 50 mM KCl, 0.5 mM MgCl$_2$, and 0.01 mM CaCl$_2$. A total of 5 μg of soluble recombinant proteins were used in each reaction. The reaction mixture was incubated at 37 °C for 10 min. The reaction was stopped by adding 20 μl of 2% SDS, and the radioactivity was counted in a liquid scintillation counter.

Measurements of Binding to PI(4,5)P$_2$-containing Lipid Vesicles—Phospholipid binding was performed with mixed lipid vesicles as described recently (29). Briefly, 110 μg of PL(4,5)P$_2$ (Roche Applied...
**A.** Amino acid sequence alignment between the PH domains of PLCβ1 (human) and PLCβ4 (rat). The positions of the β-sheets and α-helices are indicated above the sequences. Conserved residues are labeled as dots and are highlighted with a dark background, and hyphens indicate gaps. Residues participating in Ins(1,4,5)P3 binding in PLCβ1PH (19) are marked with black dots.

**B.** Lipid binding specificity of the PLCβ4 PH domain expressed as a GST fusion protein based on binding to PIP strips (31). PE, phosphatidylethanolamine; PC, phosphatidylcholine; PA, phosphatidic acid.

**C.** Graph showing binding to lipid vesicles (% of control) versus Ins(1,4,5)P3 (μM) with and without PK4,8P2. The graph illustrates the concentration-dependent binding of PLCβ4 PH to lipid vesicles in the presence and absence of the phosphatidylinositol 4,8-bisphosphate.

**D.**
Binding Properties of Isolated PH Domain-GFP Fusion Proteins—It has been reported recently (24) that the isolated PH domain of PLCδ3 is unable to bind InsP3, despite its ability to bind the lipid PI(4,5)P2. Because inositol lipids and their watersoluble inositol phosphate counterparts usually compete for the same binding pocket within the PH domain, we wanted to explore further this unique difference between the binding of lipids versus inositol phosphates by comparing the PH domains of PLCδ3 and PLCδ4, which show very significant sequence homology within this domain (Fig. 1A). Both PH domains were created as GFP fusion proteins so that their in vitro binding properties could be compared with the cellular distribution of a similar protein construct expressed in mammalian cells. As shown in Fig. 1B, the PLCδ3 PH showed the same selectivity for binding only PI(4,5)P2 as described for the PLCδ3 PH (e.g. Ref. 29), and both recombinant PH-GFPs showed PI(4,5)P2-dependent association with lipid vesicles. A larger fraction of the PLCδ3 PH domain was associated with lipids than of the PLCδ4 PH domain (87 ± 2.9 versus 72 ± 5.7, mean ± S.E., n = 6) (Fig. 1C). When InsP3 was added to displace the protein from the lipids, higher concentrations were needed to displace the PH domain of PLCδ3 (IC50, 8 μM) compared with that of PLCδ4 (IC50, 1.2 μM) (Fig. 1D). These data showed that the PLCδ3 PH domain displays high specificity for PI(4,5)P2, but it binds both the lipid and the soluble Ins(1,4,5)P3 with lower affinity than the PLCδ4 PH domain.

Next, the ability of the domains to bind the water-soluble Ins(1,4,5)P3 was investigated using [3H]Ins(1,4,5)P3 as a tracer. In these assays, there was a clearly measurable specific binding of [3H]Ins(1,4,5)P3 to the PLCδ3 PH domain, although this was only about 50–60% of that bound to equal amounts of the PLCδ4 PH-GFP protein. Displacement curves showed a significantly lower affinity of the PLCδ3 PH domain to the soluble Ins(1,4,5)P3 (IC50, 50 and 15 mM for PLCδ3 and PLCδ4 PH domains, respectively) (Fig. 2A), and this difference was also observed when Ins(1,3,4,5)P4 was used as a displacer (IC50, 500

![Fig. 2. Binding of Ins(1,4,5)P3 to recombinant PLCδ3-PH-GFP and PLCδ4-PH-GFP proteins. Bacterially expressed and purified proteins were incubated with increasing concentrations of unlabeled Ins(1,4,5)P3 (A) or Ins(1,3,4,5)P4 (B) for 10 min on ice. Proteins were precipitated by the addition of ice-cold 4% γ-globulin and polyethylene glycol 6000 and centrifuged at 10,000 × g for 10 min as described under the “Experimental Procedures.” The pellet was dissolved in 2% SDS, and its 3H activity was determined by scintillation spectrometry. Binding was expressed as percent of binding observed (Bb) without the unlabeled inositol phosphates. Means ± S.E., n = 3 (A) and means ± range, n = 2 (B). PLCδ3-PH-GFP, closed symbols; PLCδ4-PH-GFP, open symbols.](image)

acid; PS, phosphatidylserine. C, binding of recombinant PLCδ3-PH-GFP and PLCδ4-PH-GFP proteins to lipid vesicles in the presence of increasing concentrations of Ins(1,4,5)P3. Lipid vesicles containing PI(4,5)P2 and phosphatidylethanolamine (or only the latter) were made by sonication and incubated with the recombinant proteins as described under “Experimental Procedures.” The fraction of proteins bound to the lipid vesicles was separated by ultracentrifugation, and both the pellets (P) and the supernatants (S) were dissolved in Laemmli buffer and subjected to PAGE analysis. Wet gels were analyzed and quantitated by a PhosphorImager, using the blue laser for excitation of the GFP molecule. D, displacement of the PH domains from the lipid vesicles by Ins(1,4,5)P3. The combined results from six experiments are shown where the initial localization (87 ± 2.9 and 72 ± 5.7, percent (mean ± S.E.) of the total for PLCδ3-PH-GFP and PLCδ4-PH-GFP, respectively) was taken as 100%. PLCδ3-PH-GFP, filled circles; PLCδ4-PH-GFP, open circles. The faint band migrating below the PLCδ3-PH-GFP protein is a minor proteolytic fragment that is invariably present in this preparation.
after removal of Ca\(^{2+}\) mobilizing AT\(_1\)-angiotensin II receptor. The distribution of the Ins(1,4,5)P\(_3\), but did not indicate a large degree of discrepancy plasma membrane localization, the plasma membrane localiza-

"focal microscopy."

...transfected with plasmid DNA containing the indicated constructs, and after 36 h, live cells were examined by confocal microscopy. A shows the localization of the PLC\(_{\delta_4}\)-PH-GFP protein in the plasma membrane as well as in the nucleus. Note the bright spots (usually four dots per nucleolus) within the nucleoli (A, middle panel). After addition of the Ca\(^{2+}\) ionophore, ionomycin (to activate endoge-

...nous PLCs), the fluorescence falls off the plasma membrane and appears in the cytosol within minutes (B, middle panels). After removal of Ca\(^{2+}\) by the addition of the Ca\(^{2+}\) chelator, 1,2-bis(2-aminophenoxy)-ethane-N,N,N',N'-tetraacetic acid (BAPTA), the fluorescence returns to the plasma membrane, reflecting the resynthesis of the PI(4,5)P\(_2\) pools (B, right panels). The translocation of the PLC\(_{\delta_4}\)-PH-GFP is very similar to that of PLC\(_{\alpha_1}\)-PH-GFP (B, lower panels). Note that the nuclear and nucleolar localization of PLC\(_{\delta_4}\)-PH-GFP does not change in re-

...response to the Ca\(^{2+}\) increase or removal (A, right panel).

To compare the dynamic properties of the two constructs within the cells, FRAP analysis was performed in N1E-115 neuroblastoma cells where such properties of the PLC\(_{\delta_4}\)-PH-GFP had been characterized previously (27). These experiments showed a significantly faster recovery of the fluorescence in the bleached area in the case of the PLC\(_{\delta_4}\)-PH-GFP than with the PLC\(_{\alpha_1}\)-PH-GFP construct (Fig. 4). Because FRAP of PLC\(_{\delta_4}\)-PH-GFP is already much faster than that of a membrane-delimited fluorescent construct (27), these data indi-

...that the weak membrane localization of PLC\(_{\delta_4}\)-PH-GFP is a result of a dynamic equilibrium in which the PH domain rapidly dissociates and re-associates with the membrane PI(4,5)P\(_2\). Most interesting, FRAP was very slow (\(\tau >100\) s) in the nuclear spots, suggesting that the association of PLC\(_{\delta_4}\)-PH-GFP with nuclear structures is of a completely different nature than that with the plasma membrane.

Next, the kinetics of changes of PH domain translocation during more physiological conditions was studied. HEK 293 cells stably expressing the AT\(_1\) receptor were transfected with the PH domain GFP constructs, and the distribution of the fluorescent protein between the plasma membrane and the cytosol was followed after agonist addition. Similar experiments were performed in N1E-115 neuroblastoma cells stimu-

...via endogenous bradykinin receptors. As shown in Fig. 5, angiotensin II stimulation caused a rapid and full translocation of both PH domains from the membrane to the cytosol in HEK 293 cells. When the fluorescence ratios (\(I_{\text{membrane}}/I_{\text{cytosol}}\)) were used as an index of localization, the translocation responses
could be plotted as a function of time following stimulation (Fig. 6). These data showed that although the translocation response of PLCδ4PH-GFP was somewhat more transient than that of PLCδ1PH-GFP in angiotensin II-stimulated HEK cells, the opposite was true in N1E-115 neuroblastoma cells. These small kinetic differences were not statistically significant, but in both cases, the amplitudes of changes were smaller in the case of the PLCδ4 PH domain, simply because of its weaker association with the plasma membrane (Fig. 6, A and C). These data showed that, despite its lower lipid affinity, the PLCδ4 PH domain is still able to detect PI(4,5)P₂ changes after stimulation with a Ca²⁺-mobilizing agonist with no significant difference in the kinetics compared with PLCδ1 PH.

Studies on the Full-length PLCδ4 Protein—Whereas the interaction of the PH domains of PLCδ4 and PLCδ1 with the inositol lipid, PI(4,5)P₂, showed many similarities and only quantitative differences, we wanted to determine whether the PH domain of PLCδ4 has a similarly important role in the localization of the enzyme as has been widely documented in the case of PLCδ1 (32). For this, the full-length enzyme was HA epitope-tagged in its C terminus, and its cellular localization was determined by immunocytochemistry after transfection.

**Fig. 4. FRAP of PLCδ4PH-GFP and PLCδ1PH-GFP in N1E-115 cells.** Cells grown on coverslips were transfected with the respective plasmid DNAs containing the indicated constructs and transferred to the confocal microscope after 24 h. Small spots (~1.3 μm) in the basal membrane were bleached, and fluorescence recovery was detected as detailed under the “Experimental Procedures” (A). The relatively noisier trace of PLCδ4PH-GFP is because of the lower membrane association of this construct. Recovery time constants (Tₐₑ) for these constructs were determined by fitting single exponentials to the curves and are depicted as mean ± S.E. of 15 experiments each (B).

**Fig. 5. Redistribution of the PLCδ4PH-GFP protein after angiotensin II stimulation in HEK 293 cells stably expressing AT₁a-angiotensin receptors.** Cells were transfected with plasmid DNA containing the indicated constructs, and after 24 h, live cells were examined by confocal microscopy. Angiotensin II (100 nM) was added at time 0, and pictures were taken at every 15 s. Both the PLCδ4PH-GFP (A) and the PLCδ1PH-GFP (B) show a rapid, complete, and transient translocation from the membrane to the cytosol.
into COS-7 cells. As shown in Fig. 7, the enzyme was mostly localized to intracellular membranes, with prominent staining in the nuclear membrane, the pericentriolar area, and the endoplasmic reticulum. There was some signal detectable also over the plasma membrane, but clearly, most of the signal was over the ER structures. Because the localization in fixed cells is often slightly different from that observed in live cells, we determined the localization of the PLCδ1 protein as well as both isolated PH domains as GFP fusion proteins under similar conditions in the fixed cells for a comparison. As shown in Fig. 7, PLCδ1 and both isolated PH domains showed the prominent plasma membrane localization, and only a small signal was present in the pericentriolar area. These data already indicated that the PLCδ4 PH domain does not make an important contribution to the localization of the enzyme. To test this conclusion further, we created a truncated PLCδ4 protein that lacked the entire PH domain, and the localization of this truncated version was found indistinguishable from that of the full-length protein (Fig. 7). Moreover, increasing the cytoplasmic Ca\textsuperscript{2+} concentration by ionomycin treatment did not make a significant change in the distribution of the protein other than what was because of the fragmentation of the ER (not shown).

Next we determined whether the PH domains still can display their lipid and Ins\textsubscript{(1,4,5)P}\textsubscript{3} binding within the full-length enzymes. Therefore, we examined the lipid associations of the full-length proteins and their displacement with Ins\textsubscript{(1,4,5)P}\textsubscript{3} in a similar manner as with their isolated PH domains. As shown in Fig. 8, although both enzymes were able to bind to the lipid vesicles, higher concentrations of Ins\textsubscript{(1,4,5)P}\textsubscript{3} were required to displace the full-length proteins from the lipid vesicles than their respective isolated PH domains (IC\textsubscript{50}, 10 and 210 \textmu m for PLCδ1 and PLCδ4, respectively). Moreover, a bigger difference was observed between the Ins\textsubscript{(1,4,5)P}\textsubscript{3} sensitivities of the full-length δ1 and δ4 enzymes (∼20-fold) than between their PH domains (∼6-fold) (Fig. 8). It is important to note that neither the full-length nor the PH domain-deleted PLCδ4 enzyme showed measurable association with PI(4,5)P\textsubscript{2} in the PIP strip assays, and instead, both proteins showed a weak association with the PI(3)P, PI(5)P, and PI(4)P but not with PI(4)P of animal origin (not shown). No specific binding to PI(4,5)P\textsubscript{2}-containing lipid vesicles was demonstrable with the PLCδ4 truncated protein (not shown).

DISCUSSION

The PH domain is an important determinant of the mode of operation of PLCδ enzymes. In the case of PLCδ1, it has been well documented that its relatively high affinity to PI(4,5)P\textsubscript{2} allows the enzyme to be tethered to the plasma membrane so that it can effectively catalyze the hydrolysis of many PI(4,5)P\textsubscript{2} molecules without being released from the membrane. In this model of processive catalysis (33), one of the reaction products, Ins(1,4,5)P\textsubscript{3}, serves as a negative regulator by binding to the PH domain and competing for PI(4,5)P\textsubscript{2} binding and hence disrupting localization of the enzyme. Although all PLC isoforms contain a PH domain, their binding specificity differs from that of PLCδ1, and they do not show the high affinity binding and inhibitory effect of Ins(1,4,5)P\textsubscript{3}, described above (8).
Recent studies performed with PLCγ4 already indicated that this enzyme may not be regulated in the same manner as PLCγ1, because it was shown that the γ4 isoform does not show a great sensitivity to inhibition by Ins(1,4,5)P3 (24). Our studies with the isolated PLCγ4 PH domain indicate that although it also binds PI(4,5)P2, it does so with a lower affinity than the PLCγ1 PH domain. The lower lipid affinity is also paralleled with a comparably lower affinity to Ins(1,4,5)P3, although, contrary to the conclusion of a previous report (24), we found that this domain is still capable of binding Ins(1,4,5)P3. We cannot explain the difference between our results and those of Nagano et al. (24), but these authors used the PLCγ4 PH domain as a GST fusion protein, whereas our studies utilized the PH domain in its natural N-terminal position fused to GFP. Most

![Cellular localization of PLCγ4 and PLCγ1 as well as their isolated PH domains in fixed COS-7 cells.](image-url)
intriguing, in a recent study (34) molecular modeling of the PH domains of various PLC proteins predicted that the PI(4,5)P$_2$ binding affinity of PLC$_\delta$PH would be lower than that of PLC$_\delta$PH.

In agreement with the in vitro binding data, the PLC$_\delta$PH-GFP construct showed only moderate plasma membrane recruitment when expressed in mammalian cells. The faster recovery of PLC$_\delta$PH-GFP than PLC$_\delta$PH-GFP after photo-bleaching is also consistent with a larger fraction of the protein being cytosolic, causing a faster association rate, resembling that observed with the PLC$_\delta$PH-GFP construct after a partial PLC activation (27). Even with its limited plasma membrane binding, the PLC$_\delta$ PH domain shows translocation from the membrane to the cytosol when phospholipase C is activated by a Ca$^{2+}$ ionophore or by a Ca$^{2+}$-mobilizing agonist, indicating that it can also detect the PI(4,5)P$_2$ changes within the cell. Comparison of the kinetics of translocation obtained by the two PH domains after stimulation with the Ca$^{2+}$-mobilizing agonist, indicating that high levels of PI(4,5)P$_2$ (39). Unfortunately, in vitro PLC assays cannot answer these questions, because under in vitro conditions PLC enzymes can usually hydrolyze PI, PI(4)P, and PI(4,5)P$_2$ (8).

In the present study, the concentrations of Ins(1,4,5)P$_3$ required to inhibit the binding of full-length PLC$_\delta_1$ or -$\delta_4$ to lipid vesicles have been one order of magnitude higher than those found with the isolated PH domains. Such a difference has also been documented in the literature based on the reported affinity values of Ins(1,4,5)P$_3$ binding to isolated PH domains (16, 26, 29) and the inhibitory concentrations of Ins(1,4,5)P$_3$ on lipid binding (40, 41) or PLC activity (40, 41) of the full-length molecule. One explanation for this difference could be simply that the high affinity binding site of the PH domain is not fully exposed in the full-length protein. It is not unreasonable to assume that the PH domain interacts with the rest of the molecule, and Ins(1,4,5)P$_3$ binding to the PH domain would alter the intramolecular interaction so that the access or affinity of the catalytic site to the substrate lipid is changed. The potent PH domain-mediated inhibitory effect of the PLC$_\delta_4$ splice variant, AltIII, on PLC$\delta$ (but not -$\beta$ or -$\gamma$) catalytic activity (24) could be also interpreted as an indication of an inhibitory interaction between the PH domain and the rest of the molecules affecting catalysis. It has been suggested recently (42) that the PH domain could serve as an allosteric

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**Fig. 8. Binding of full-length PLC$_\delta_1$ and PLC$_\delta_4$ proteins to lipid vesicles in the presence of increasing concentrations of Ins(1,4,5)P$_3$.** For experimental details, see the legend to Fig. 1.
modulator of the catalytic activity of PLCδ1 via an intramolecular interaction. Although there is no structural information available to evaluate this hypothesis (the PH domain and the rest of PLCδ1 have been crystallized separately), several functional data reported in the literature and some of our present findings could be consistent with such a cooperative interaction between the PH domain and other parts of the molecule.

In summary, the present study shows that the PH domains of PLCδ1 and PLCδ4 show major differences in their Ins(1,4,5)P3 and P(4,5)P2 affinities but still report very similar Ca2+ binding specificities. Consistent with its lower PI(4,5)P2 affinity, despite its lipid binding, the PH domain of PLCδ4, which is primarily associated with the ER. Therefore, the PH domain of PLCδ4 may act as a regulator of the enzyme rather than a localization signal via possible intramolecular interactions. More studies are needed to explore the intriguing question of whether the PH domains could serve as lipid-mediated allosteric regulators of the catalytic activity of the PLCδ enzymes.

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