The pleckstrin homology (PH) domain of phosphatidylinositol-specific phospholipase C-δ1 (PLC-δ1) binds to both d-myoinositol 1,4,5-trisphosphate (Ins(1,4,5)P₃) and phosphatidylinositol 4,5-bisphosphate (PtdIns(4,5)P₂) with high affinities. We have previously identified a region rich in basic amino acids within the PH domain critical for ligand binding (Yagisawa, H., Hirata, M., Kanematsu, T., Watanabe, Y., Ozaki, S., Sakuma, K., Tanaka, H., Yabuta, N., Kamata, H., Hirata, H., and Noyjima, H. (1994) J. Biol. Chem. 269, 20179–20188; Hirata, M., Kanematsu, T., Sakuma, K., Koga, T., Watanabe, Y., Ozaki, S., and Yagisawa, H. (1994) Biochem. Biophys. Res. Commun. 205, 1563–1571). To investigate the role of these basic residues, we have performed site-directed mutagenesis replacing each of the basic amino acid in the N-terminal 60 residues of PLC-δ1 (Lys²⁴, Lys²⁶, Lys²⁷, Arg²⁸, Arg³⁰, Arg³³, Arg³⁵, Arg³⁶, Lys³⁷, and Arg³⁹) with a neutral or an acidic amino acid. The effects of these mutations on the PH domain ligand binding properties and their consequence for substrate hydrolysis and membrane interactions of PLC-δ1 were analyzed using several assay systems. Analysis of [³⁵S]-Ins(1,4,5)P₃ binding, measurement of the binding affinities, and measurements of phospholipase activity using PtdIns(4,5)P₂-containing phospholipid vesicles, demonstrated that residues Lys³⁷, Lys³⁷, Arg³⁵, Arg³⁶, Arg³⁹, and Lys³⁷ were required for these PLC-δ1 functions. Further, other mutations resulted in a moderate reduction. A subset of selected mutations was further analyzed for the enzyme activity toward substrate present in cellular membranes of permeabilized cells and for interaction with the plasma membrane after microinjection. These experiments demonstrated that mutations affecting ligand binding and PtdIns(4,5)P₂ hydrolysis in phospholipid vesicles also resulted in reduction in the hydrolysis of cellular polyphosphoinositides and loss of membrane attachment. All residues (with the exception of the K43E substitution) found to be critical for the analyzed PLC-δ1 functions are present at the surface of the PH domain shown to contain the Ins(1,4,5)P₃ binding pocket.

The pleckstrin homology (PH) domain has been initially identified as a region of sequence similarity of about 120 amino acid residues (3, 4). At the last count, more than 100 proteins have been reported to have this sequence motif; many of these proteins are involved in cellular signaling and cytoskeletal functions (5–8). Studies of several PH domains using x-ray crystallography or NMR (9–12) revealed a conserved structural motif containing a seven-stranded β-sheet and an α-helix. The loops between the β-strands, particularly the β1/β2, β3/β4, and β6/β7, differ greatly in length and sequence. Each PH domain is electrostatically polarized, and the most variable loops coincide with the positively charged face.

By analogy with other conserved structural modules (e.g. SH2 and SH3 domains), it has been proposed that the PH domain could be involved in signaling by mediating intermolecular interactions. Consequently, a great effort has been made to identify ligand(s) for this domain. Although there are examples of PH domains involved in protein-protein interactions (e.g. binding of Gβγ by β-adrenergic receptor kinase PH domain (13) or recognition of phosphotyrosine by Sch PH/PTB domain (14)) there is an increasing evidence that many PH domains interact with different inositol lipids and inositol phosphates (15, 16). In this respect, the PH domain of phospholipase C-δ1 (PLC-δ1) has been studied most extensively. Determination of association constants for different inositol lipids and their head groups (1, 2, 17), and relative abundance of these phospholipids in the cell identified PtdIns(4,5)P₂ as a potentially important physiological ligand (18, 19). Ins(1,4,5)P₃ can bind to the same binding pocket as the head group of cellular polyphosphoinositides and loss of membrane attachment. All residues (with the exception of the K43E substitution) found to be critical for the analyzed PLC-δ1 functions are present at the surface of the PH domain shown to contain the Ins(1,4,5)P₃ binding pocket.
Mutational Analysis of the PH Domain of Phospholipase C-β1

PtdIns(4,5)P₂ with even higher affinity (1, 20). Studies of PH domain interactions also identified inositol lipids other than PtdIns(4,5)P₂ as possible ligands for several PH domains. Findings that some of the phosphoinositides generated by activation of phosphatidylinositol 3-kinase (PtdIns(3)P₂, PtdIns(3,4,5)P₃, and PtdIns(3,4,5)P₃) bind to the PH domains of the serine-threonine kinase PKR/RAC/Akt (21–24), protein GAP1 (25), and protein GRP1/ARNO/cytohesin-1 (22, 26, 27) suggested that these proteins could be signaling targets of phosphatidylinositol 3-kinase. Broton's tyrosine kinase (Btk) PH domain has also been reported to bind PtdIns(3,4,5)P₃ (28) and several inositol polyphosphates (1,3,4,5-tetrakisphosphate, 1,3,4,6-pentakisphosphate, and 1,2,3,4,5,6-hexakisphosphate) (29). Other examples of high affinity binding of different inositol phosphates to PH domains include binding of inositol 1,4,5,6-tetrakisphosphate to p130 inositol phosphate-binding protein (30).

In this article, we focus on the PLC-β1 PH domain located at the N terminus of the molecule. Although it is not clear how the PH domain interacts with the three-domain core structure of PLC-β1 (containing the EF-hand, catalytic, and C2 domain), a flexible, surface-exposed link has been suggested (31). Previous studies indicating that the PH domain could play a role in the regulation and catalysis of PLC-β1 demonstrated its requirement for a high rate of substrate hydrolysis (32) and membrane interactions (33). The structure of the PLC-β1 PH domain has been obtained in a complex with Ins(1,4,5)P₃, revealing not only structural elements but also identified residues within the Ins(1,4,5)P₃ binding pocket (12). Based on detailed structural information, availability of different functional assays, and our previous studies, we performed extensive structure/function analysis. It was demonstrated that single basic amino acid replacements in the N-terminal part of the PH domain, when microinjected into cells, could cause a significant reduction in the Ins(1,4,5)P₃/PtdIns(4,5)P₂ binding, the catalytic activity, and the interaction with the plasma membrane.

**EXPERIMENTAL PROCEDURES**

**Materials**—[1H]Ins(1,4,5)P₃ (specific radioactivity: 777 GBq/mmol) was obtained from NEN Life Science Products. Non-radioactive Ins(1,4,5)P₃ and (inositol-2-13C]PtdIns(4,5)P₂ (specific activity: 37 GBq/mmol) was from Amersham Life Science. Monoclonal antibodies against PLC-β1 were obtained from Upstate Biotechnology, Inc. 

**Generation and Site-directed Mutagenesis**—The cDNA encoding rat aortic PLC-β1 (34) was subcloned into a pGEX-2T-based bacterial expression vector (pGEX-2T from Pharmacia Biotech Inc.), designated as PGST3 (1). Individual point mutations were introduced to the plasmid pGST3/PLC-β1 encoding the wild-type enzyme by a T4 DNA polymerase-based mutagenesis using a Transformer™ site-directed mutagenesis kit (CLONTECH) with a selection primer (5'-CTCGTGGCGTGAGGAACGCTTCTA-3').

**Assay of [1H]Ins(1,4,5)P₃ Binding**—[1H]Ins(1,4,5)P₃ binding assays were carried out as described elsewhere (35, 36). In brief, the assay mixture (0.45 ml) contained 50 mM Tris-HCl buffer (pH 8.3), 1 mM EDTA, 1.07 nM [1H]Ins(1,4,5)P₃, and a protein sample. After incubation of the mixture on ice for 15 min, the [1H]Ins(1,4,5)P₃-protein complex was precipitated with 0.5 ml of polyethylene glycol 6000 (30%, w/v) together with 50 μl of bovine γ-globulin (10 mg/ml) as a carrier protein. The pellet after centrifugation was dissolved in 0.3 ml of 0.1 N NaOH and then subjected to liquid scintillation counting. The specific binding was obtained after subtracting the radioactivity of samples in the presence of 1 μM non-radiolabeled Ins(1,4,5)P₃.

**Measurement of Binding Constants Using an Optical Evanescent Resonance Biosensor**—An optical evanescent resonance mirror cuvette system (Lays™, Affinity Sensors), whose principle is based on a quantum mechanical phenomenon (37) similar to that of a surface plasmon resonance biosensor (38, 39), was used to measure interaction kinetics between an immobilized Ins(1,4,5)P₃ analog and PLC-β1 mutants.

Briefly, the hydrolgel containing carboxymethylxanthran on the cuvette surface was activated by a mixture of N-ethyl-N'-3-dimethylaminopropyl-N'-carboxyoxysuccinimide as described in the coupling kit (Affinity Sensors). Streptavidin (Vector Laboratories Inc., 0.2 mg/ml) in 10 mM sodium acetate buffer (pH 4.5) was then covalently attached to the activated cuvette at pH 4.5. Remaining reactive esters on the surface were blocked by a subsequent washing of the cuvette with 1 M ethanolamine-HCl (pH 8.5). Then, 0.2 mg/ml of a biotinylated Ins(1,4,5)P₃ analog, 2-O-[4-15N]O-4-azidoazobenzoyl-N'-biotinyl-L-lysine amide, and 1,2-hydroxy-2-monoazobenzoyl-1,4,5-triphosphoserine, previously designated as 204BTN3 (40, 41), in 10 mM PBS plus 0.05% Tween 20 (pH 7.4) was added to the cuvette. After washing the cuvette with the binding buffer (10 mM HEPES/NaOH (pH 8.5), 0.1 mM NaCl, 2 mM EDTA, 10 mM mercaptoethanol), affinity-purified GST/PLC-β1 or mutant fusion proteins dissolved in the binding buffer (1 mg/ml) was added. The association process was monitored for about 10 min, after which the solution in the cuvette was replaced with the free binding buffer to see dissociation (for about 5 min). Regeneration of the ligand was performed by washing the cuvette with 200 μl of the binding buffer containing 2 mM NaCl. The binding experiments were carried out at room temperature.

The traces of the association and dissociation process were analyzed using FASTFIT™ kinetics analysis software supplied with the instrument. Experimentally derived association and dissociation data at various concentrations of analytes were fitted to equations containing a single exponential function (43, 42). The pseudo first-order rate constant, kₐ, with units of s⁻¹, was obtained for each concentration of the analyte protein. The association rate constant, kₐ, with units of M⁻¹·s⁻¹, was derived from the gradient of the plot of kₐ, against analyte concentration. The dissociation rate constant, kₐ, with units of M⁻¹·s⁻¹, was derived directly from the time course of dissociation (43). The affinity constant, Kₒ, is equivalent to kₐ/kₐ. The values were examined for self-consistency of the data as described (44).

**Assay of PLC Activity**—PLC activity was measured using PtdIns(4,5)P₂ as a substrate. The assay mixture (50 μl) contained the protein sample, 2.5 μg of PtdIns(4,5)P₂, 25 μg of phosphatidylinethanol-
amino containing 370 Bq (0.1 µCi) of [3H]PtdIns(4,5)P2, 20 mM Hepes/NaOH (pH 7.2), 0.83 mM MgCl2, 1 mM dithiothreitol, 2 mM EGTA, 0.2 mM EDTA, 30 mM KCl, and 2 mM CaCl2. Incubation was carried out for 1 h at room temperature. Labeled preparations were mounted under coverglass and examined with a Bio-Rad MRC 600 confocal microscope.

Microinjection and Immunofluorescence Analysis—Subcellular localization of PLC-δ1 was analyzed according the method described previously (33). MDCK cells grown to confluence were subjected to microinjection on a Zeiss microinjection workstation (Carl Zeiss). Samples of purified proteins (2 mg/ml) were mixed with 10% fetal calf serum and injected into MDCK cells by 10% fetal calf serum for 15 min. Incubation with a pool of mouse anti-PLC-δ1 antibodies (primary antibody) diluted 1:150 was followed by 10% fetal calf serum for 15 min followed by 10% fetal calf serum for 15 min. Incubation with a pool of mouse anti-PLC-δ1 antibodies (primary antibody) diluted 1:150 was followed by 10% fetal calf serum for 15 min followed by 10% fetal calf serum for 15 min. Incubation with a pool of mouse anti-PLC-δ1 antibodies (primary antibody) diluted 1:150 was followed by 10% fetal calf serum for 15 min followed by 10% fetal calf serum for 15 min. 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We changed all the amino acids with basic side chains between residues 24 and 60 of PLC-$\delta_1$ individually to a typical neutral amino acid, which brings about minimal structural change or to a charge-reversed amino acid. A charge-reversed double substitution with Asp and Glu was made for residues Arg$^{37}$ and Arg$^{38}$, respectively, noted as R37D/R38E. Pure proteins could be isolated as GST fusion proteins, and the levels of expression were comparable for most of the mutants with some exceptions: K43L and R60A, whose yields as soluble protein were slightly reduced, and K30L, which showed marked reduction (data not shown).

The ligand binding properties and activity of PLC-$\delta_1$ proteins with mutations within the PH domain were subjected to analysis by several different methods. In most cases, purified proteins were used (the binding kinetics to an immobilized Ins(1,4,5)P$_3$ analog, the substrate hydrolysis, and the ability in targeting the plasma membrane after microinjection into cells). For some measurements, however, crude preparations of the mutants, standardized for their expression, were sufficient.

**Determination of Basic Amino Residues Critical for $[^{3}H]$Ins(1,4,5)P$_3$ Binding**—Analysis of GST/PLC-$\delta_1$ fusion proteins using $[^{3}H]$Ins(1,4,5)P$_3$ binding demonstrated its stereospecific, high affinity ($K_{d}$ $\approx$ 0.01 $\mu$m and $K_{d}$ $\approx$ 0.1 $\mu$m, respectively) binding to PLC-$\delta_1$ or to its PH domain (1, 50). The specific $[^{3}H]$Ins(1,4,5)P$_3$ binding activity of the PH domain mutants was compared with that of the wild-type using preparations normalized for their expression levels. Results of this analysis are shown in Fig. 2. Substitutions at Lys$^{30}$, Lys$^{32}$, Arg$^{37}$, Arg$^{38}$, Arg$^{40}$, and Lys$^{57}$ resulted in the complete loss of the binding or a large reduction, with activity remaining below 10% of the wild-type PLC-$\delta_1$ binding activity. In addition to residues implicated in Ins(1,4,5)P$_3$ binding by structural studies (Lys$^{30}$, Lys$^{32}$, Arg$^{40}$, and Lys$^{57}$) residues Arg$^{37}$ and Arg$^{38}$ are also essential for binding. The only double mutant analyzed in this study with substitutions by negative charges, R37D/R38E, was completely inactive in ligand binding.

Another group of mutants with substitutions at residues Lys$^{24}$, Lys$^{43}$, Lys$^{49}$, and Arg$^{60}$ could clearly bind Ins(1,4,5)P$_3$, but the binding was reduced to 10–50% of the wild-type PLC-$\delta_1$ binding. In the case of Lys$^{43}$, the mutant with a neutral substitution (K43L) showed reduced but still substantial (34% of control) binding, but the mutant with a charge-reversed substitution (K43E) resulted in the loss of binding.

The only mutation that did not reduce Ins(1,4,5)P$_3$ binding was at residue Arg$^{46}$. Structural studies implicated this residue in direct interactions with the phosphoryl group at position 5 of the inositol ring. This interaction, therefore, does not seem to be critical for Ins(1,4,5)P$_3$ binding when other residues that coordinate the 5-phosphate are in place.

Generally, amino acid substitution of each of the basic amino acids in the N-terminal 60 residues of PLC-$\delta_1$ resulted in attenuation of the Ins(1,4,5)P$_3$ binding. The reduction was most prominent when substitutions were made in residues directly implicated in the ligand binding, with the exception of Arg$^{56}$. In addition, substitution of two basic residues in their vicinity with either neutral or negatively charged residues (Arg$^{51}$ and Arg$^{59}$) resulted in loss of binding.

**Determination of Kinetic Parameters for the Ins(1,4,5)P$_3$ Binding Using an Evanescent Resonance Biosensor**—Simple binding experiments using $[^{3}H]$Ins(1,4,5)P$_3$ as a ligand provides equilibrium rate constants, but do not usually give kinetic constants such as the association rate constant ($k_{a}$) and the dissociation rate constant ($k_{d}$). To estimate these constants and the dissociation constant for the Ins(1,4,5)P$_3$ binding of each site-directed mutant, we have introduced a real-time kinetic assay using an optical evanescent wave biosensor, IAsys$^\text{TM}$.

An Ins(1,4,5)P$_3$ analog, 204BTN3, was immobilized to the cuvette surface of the biosensor. Addition of GST alone to the cuvette did not show a response, whereas the wild-type GST/PLC-$\delta_1$ and all mutants brought about significant responses (data not shown). Kinetic constants were obtained from both the association and dissociation data of these responses, and the values of the kinetic equilibrium constant ($K_{d}$) were compared. The average $K_{d}$ value for wild-type enzyme thus measured was about 5.2 $\times$ 10$^{-7}$ M, about 2 orders of magnitude larger than that obtained from $[^{3}H]$Ins(1,4,5)P$_3$ binding experiments (1). At the moment, we do not have a rationale for this difference, but it could be due to modifications introduced into the Ins(1,4,5)P$_3$ analog and/or to changes in its presentation caused by immobilization to the matrix. Nonetheless, the wild type had the smallest $K_{d}$ value whereas each neutral mutation showed a 2–10-fold decrease in the affinity (data not shown). Charge-reversed mutants, K30E and K32E, and a deletion mutant, Δ1–223, which lacks the entire PH domain showed further increase (about 60–70%, and 50-fold, respectively) in $K_{d}$ values as compared with that for the wild-type (data not shown).

**Substrate Hydrolyzing Activities of the Site-directed Mutants**—To examine the effect of point mutations within the PLC-$\delta_1$ PH domain on substrate hydrolysis by the enzyme, several assay systems have been used. When substrate was presented as 0.5% sodium cholate/PtdIns(4,5)P$_2$ mixed micelles, wild-type PLC-$\delta_1$ and all the mutations examined had similar specific activities (about 800–1000 units/mg). This is consistent with our previous observation (51) that deletion of the PH domain did not reduce the specific activity of the enzyme in this assay. In assays with reduced concentrations of

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**Figure 2. Specific $[^{3}H]$Ins(1,4,5)P$_3$ binding activity of site-directed mutants.** The specific Ins(1,4,5)P$_3$ binding (B) of the site-directed mutants was normalized to that of the wild-type GST/PLC-$\delta_1$, taking the expression level of the GST (G) of the mutants into account. The specific $[^{3}H]$Ins(1,4,5)P$_3$ binding of the mutant (%$B = B \times 100$) of the mutant/B of the wild-type/G of the mutant/G of the wild-type) × 100. Fifty μg of soluble proteins from transformed E. coli were used for Ins(1,4,5)P$_3$ binding assay and GST assay, respectively. Nonspecific binding was measured in the presence of 1 μM non-radioactive Ins(1,4,5)P$_3$. Figure shows means ± S.D. of three separate experiments carried out in duplicates.
the absence of detergent is shown. Assay methods were described under “Experimental Procedures.” These are the average ± S.D. of three independent experiments carried out in duplicates.

sodium cholate (0.08%), some differences between the mutants and the wild-type enzyme could be seen (data not shown). The differences, however, could be more clearly detected and measured when phospholipid vesicles containing PtdIns(4,5)P2 were used as a substrate instead of mixed micelles (Fig. 3). For these measurements, crude preparations of the mutants (normalized for their expression) were used. The activity was greatly reduced in mutants K30L, K30E, K32L, K32E, R37D, R38E, R37D/R38E, R40A, R40D, K43L, K57A. Although the charge-reversed mutation almost completely abolished the activity, the proteins with neutral substitutions R37L, R38V, and, in particular, K43L retained some activity. Decreased but still significant activities were shown for mutants K24A, K49A, R56A, and R60A.

It has been shown previously that exogenous phospholipase C preparations can hydrolyze inositol lipids present in membranes of permeabilized HL-60 cells (52). Addition of a GST/PLC-δ1 fusion protein to the permeabilized cells resulted in the production of inositol phosphates at calcium concentrations 1–10 μM. Deletion of the PH domain resulted in a significant reduction (10–20-fold) of substrate hydrolysis compared with the wild-type enzyme could be seen (data not shown). The data presented in Table I shows that hydrolysis of inositol lipids present in permeabilized cells was much more reduced for mutants R37D, R37L, R38E, R40A, and R40D (9–24% of wild-type activity) than for neutral substitutions at positions Lys43, Lys49, Lys56, and Arg60 (51–73% of wild-type activity).

Subcellular Localization of Site-directed Mutants—We have demonstrated previously (33) that a portion of PLC-δ1 associates with the plasma membrane after microinjection of purified protein into MDCK cells; the protein was visualized by immunofluorescence using a pool of anti-PLC-δ1 monoclonal antibodies. It has been shown also (33) that deletion of the PH domain (or portions of this domain) resulted in a loss of membrane association. The data from this type of analysis cannot be easily quantified and only substantial reduction in membrane association can be detected as absence of membrane staining.

Using the same assay, we analyzed the ability of the PLC-δ1 point mutants to interact with the plasma membrane. The wild-type PLC-δ1 and the PH domain mutants were prepared as purified GST fusion proteins. The presence of the GST portion in the fusion protein did not affect subcellular localization of PLC-δ1 when compared with PLC-δ1 molecule without any tag. The panel of PLC-δ1 PH domain mutants included those that are clearly different in their ability to bind Ins(1,4,5)P3 or hydrolyze PLC substrate: K24A, R37D, R37L, R38E, R40A, R40D, K43L, K49A, R56A, and R60A. Results of these experiments are shown in Fig. 4. PLC-δ1 mutants R37L, R37D, R38E, R40A, and R40D lost their ability to interact with the plasma membrane and staining was only visible in the cytoplasm. For other mutants, membrane staining with the PLC-δ1 antibody was detected and in most cases was comparable with that of the wild-type protein.

**DISCUSSION**

In this article, we describe the structure/function relationship of PLC-δ1 PH domain. Results presented here demonstrate that single amino acid replacements within the PH domain, affecting binding of the ligands for this domain, can result in reduction of enzyme activity and loss of PLC-δ1 interaction with the plasma membrane.

Our previous studies (1) and studies of others (20, 50) demonstated that PLC-δ1 PH domain, present at the N terminus of the molecule, binds Ins(1,4,5)P3 and PtdIns(4,5)P2 with high affinity and specificity. Estimated Kd values are within the range of 3–200 nM for Ins(1,4,5)P3 and 0.1–10 μM for PtdIns(4,5)P2. These interactions are sufficiently strong to mediate the PH domain binding to these molecules in vitro at their estimated physiological concentrations (18, 19). Ligand binding studies in vitro also demonstrated that the binding of Ins(1,4,5)P3 to a great extent reflects binding of PtdIns(4,5)P2. It has been shown that the recognition of the head group of this phospholipid is critical for its binding and interaction with PtdIns(4,5)P2-containing vesicles, and that this can be readily inhibited by Ins(1,4,5)P3. The presence of the lipid moiety seems to restrict rather than facilitate the binding (50). For comparison of the PH domain ligand binding properties of the wild-type and mutant PLC-δ1 molecules in this study (Figs. 1

![Graph showing inositol lipid hydrolysis in permeabilized HL-60 cells](image-url)
and 2), binding of [3H]Ins(1,4,5)P3 was measured. The binding of [3H]Ins(1,4,5)P3 to PLC-δ1 molecules with mutations K30L, K30E, K32L, K32E, R37D, R37V, R38E, R37D/R38E, R40A, R40D, K43E, and R57A was abolished or greatly reduced, whereas K24A, K43L, K49A, R56A, and R60A resulted only in a moderate reduction or had no effect.

The enzyme activity of PLC-δ1 and other phospholipases has been studied in different assay systems in vitro using substrate presented as detergent mixed micelles, phospholipid vesicles or monolayers (45, 53–55). Studies of the mechanism of PtdIns(4,5)P2 hydrolysis and binding to PtdIns(4,5)P2-containing phospholipid vesicles suggested that the substrate is hydrolyzed in a processive mode of interfacial catalysis (56, 57). This would involve interaction with the phospholipid surface via site(s) distinct from the active site followed by several cycles of PtdIns(4,5)P2 hydrolysis in the active site. Since the PH domain is required for the processive mode of catalysis and binding to the vesicles, it has been suggested that this domain represents a major non-catalytic membrane interaction site (33, 57). Comparison of the full-length and PH domain deletion mutant in the sodium cholate/PtdIns(4,5)P2 mixed micelle assay, where processivity cannot be observed, demonstrated similar substrate hydrolysis (33). Data presented here, demonstrating that point mutations that abolish Ins(1,4,5)P3 binding also resulted in a loss of membrane attachment, further support the proposed role of PtdIns(4,5)P2 as a membrane component involved in interaction with the PLC-δ1 PH domain. The data are also consistent with the possibility that Ins(1,4,5)P3, the product of the enzyme reaction, could interfere with the membrane attachment and consequently affect PLC-δ1 function.

All mutations analyzed in this study are present within the N-terminal portion of PLC-δ1 PH domain, a region previously identified as essential for the ligand binding (1, 56). Determination of the crystal structure of the PLC-δ1 PH domain in a complex with Ins(1,4,5)P3 provided an insight into the overall structure of the domain and identified residues that could interact directly with the ligand (Fig. 1) (12). Positions of residues selected for mutagenesis within the PH domain structure and the impact of these mutations on analyzed functions of PLC-δ1 are summarized in Fig. 5. Residues where replacements have a greater effect on PLC-δ1 functions (with the exception of acidic substitution at position 43) are present at the surface containing Ins(1,4,5)P3 binding pocket. They include residues directly involved in interactions with 4- and/or 5-phosphoryl groups: Lys30 and Lys57 (both position 4 and 5), Lys32 (position 4), and Arg46 (position 5). Results from another study also demonstrated inhibition of PLC-δ1 by K30G, K32G, and R37G mutations (55). In addition, it has been shown that the mutation of Trp36, a residue interacting with phosphoryl group at position 1, has an inhibitory effect (55). Together, these data suggest that the recognition of all phosphoryl groups of Ins(1,4,5)P3 is important for the binding. Residues exhibiting
strong inhibition of PLC-δ1 functions also include residues Arg<sup>195</sup> and Arg<sup>248</sup> that have not been directly implicated in Ins<sup>(1,4,5)P<sub>3</sub></sup> binding but are present in their vicinity; several mutations of these residues have been analyzed (R37L, R37D, R37G, R38V, R38E, and R37D/R38E) (this article and Lomasney et al. (55)). Substitution of two other residues present at the same part of the molecules, Arg<sup>160</sup> and arginine residue involved in Ins<sup>(1,4,5)P<sub>3</sub></sup> binding (Arg<sup>256</sup>), had only moderate effect. However, other mutations showing moderate inhibition, Lys<sup>α11</sup>, Lys<sup>α13</sup> and Lys<sup>α14</sup> (this article and Lomasney et al. (55)), are present at the opposite end to the Ins<sup>(1,4,5)P<sub>3</sub></sup>/PtdIns<sup>(4,5)P<sub>2</sub></sup> binding pocket and, as visualized by rotating the model of the PH domain structure (Fig. 5), are roughly in the same vicinity as the binding pocket. Their influence on Ins<sup>(1,4,5)P<sub>3</sub></sup>/PtdIns<sup>(4,5)P<sub>2</sub></sup> binding may imply a secondary low affinity site of interaction. This secondary site may be involved in interaction with any anionic phospholipids on the membrane surface and could indirectly influence the binding of inositol head group to the binding pocket. Involvement of acidic phospholipids in membrane interactions has been suggested by the observation that the presence of acidic phospholipids in phospholipid vesicles increased binding of the isolated PH domain about 10-fold (57) and that the PLC-δ1 activity was stimulated by interaction with phosphatidic acid (58). It is also intriguing that an acidic-to-basic substitution (E54K) of the PLC-δ1 PH domain enhances the enzymatic activity (59).

Studies of PH domains have demonstrated that this conserved structural module can bind a number of different ligands and, potentially, perform a variety of functions (14, 15). It is therefore difficult to predict whether basic residues of other PH domains present at similar positions as those analyzed in this study would affect their function. Nonetheless, the observation that single point mutations within the PH domain can have a dramatic effect on the function of the whole molecule seem to be more general. For example, a point mutation within the PH domain of the serine-threonine kinase PKB/Akt (R25C) abolished binding of PtdIns<sup>(3,4)P<sub>2</sub></sup> activation by this phospholipid <em>in vitro</em>, and activation by PISK kinase <em>in vitro</em> (60). It has also been demonstrated that each of the point mutations identified in the PH domain of Btk and linked to human agammaglobulinemia and murine immunodeficiency, can cause loss of inositol tetrakisphosphate binding <em>in vitro</em> (29).

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