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Structure of the High Affinity Complex of Inositol Trisphosphate with a Phospholipase C Pleckstrin Homology Domain

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Summary

The X-ray crystal structure of the high affinity complex between the pleckstrin homology (PH) domain from rat phospholipase C-δ_1 (PLC-δ_1) and inositol-(1,4,5)-trisphosphate (Ins(1,4,5)P_3) has been refined to 1.9 Å resolution. The domain fold is similar to others of known structure. Ins(1,4,5)P_3 binds on the positively charged face of the electrostatically polarized domain, interacting predominantly with the $\beta1/\beta2$ and $\beta3/\beta4$ loops. The 4- and 5-phosphate groups of Ins(1,4,5)P_3 interact much more extensively than the 1-phosphate. Two amino acids in the PLC-δ_1 PH domain that contact Ins(1,4,5)P_3 have counterparts in the Bruton's tyrosine kinase (Btk) PH domain, where mutational changes cause inherited agammaglobulinemia, suggesting a mechanism for loss of function in Btk mutants. Using electrostatics and varying levels of head-group specificity, PH domains may localize and orient signaling proteins, providing a general membrane targeting and regulatory function.

Introduction

Pleckstrin homology (PH) domains are structural modules of around 120 amino acids that appear to be important for membrane association of proteins involved in intracellular signaling and the cytoskeleton (Gibson et al., 1994). Structural studies have shown that they share a common architecture, consisting of a β sandwich closed off at one end by a C-terminal α helix (Ferguson et al., 1995). Each domain is also electrostatically polarized, with a positively charged region that coincides with the most variable face of the domain (Ferguson et al., 1994). A number of lines of evidence suggest that PH domains bind to phosphoinositides and their head groups. Harlan et al. (1994) showed that the N-terminal PH domain from pleckstrin binds to detergent-solubilized phosphatidylinositol-(4,5)-bisphosphate (PtdIns(4,5)P_2). This interaction occurs with low affinity ($K_d \approx 30$ μM), and little stereo specificity is evident. Hyvönen et al. (1995) have also reported weak binding of the β-spectrin PH domain to inositol-(1,4,5)-trisphosphate (Ins(1,4,5)P_3). The K_d for this interaction was >40 μM, and only limited specificity was demonstrated. In both cases, structural studies showed that the phosphoinositide interacts with the positively charged face of the PH domain (Harlan et al., 1994; Hyvönen et al., 1995). In neither of these cases, however, is there any evidence for the physiological relevance of the interaction studied.

Studies aimed at identifying cytoplasmic Ins(1,4,5)P_3-binding molecules led Kanematsu et al. (1992) to clone phospholipase C-δ_1 (PLC-δ_1). Like other PLC isoforms, PLC-δ_1 has a PH domain at its N-terminus (Parker et al., 1994), and this region has been implicated in the stereo-specific binding of both Ins(1,4,5)P_3 and PtdIns(4,5)P_2 to PLC-δ_1 (Cifuentes et al., 1993, 1994; Yagisawa et al., 1994). The isolated PLC-δ_1 PH domain (PLCδPH) has since been shown to bind Ins(1,4,5)P_3 with high affinity (K_d = 210 nM) in a stereo-specific manner (Lemmon et al., 1995). It also binds to PtdIns(4,5)P_2 in phospholipid vesicles (K_d = 1.7 μM). It has been proposed that the PH domain of PLC-δ_1 serves to anchor the enzyme to PtdIns(4,5)P_2-rich membranes, permitting processive or scooting mode hydrolysis of substrate (Lemmon et al., 1995; Rebecchi et al., 1992; Cifuentes et al., 1993; Ramirez and Jain, 1991). Ins(1,4,5)P_3 binding to the PH domain competes with PtdIns(4,5)P_2 binding (Lemmon et al., 1995) and inhibits the activity of PLC-δ_1 (Kanematsu et al., 1992; Cifuentes et al., 1994). Studies of other PH domains, including those from β-adrenergic receptor kinase (Pitcher et al., 1995), Bruton's tyrosine kinase (Btk) (Li et al., 1995), insulin receptor substrate 1 (IRS-1) (Myers et al., 1995; Voliovitch et al., 1995), and the guanine nucleotide–releasing factor Sos (for Son of sevenless; McCollam et al., 1995) all indicate that a membrane anchoring function may represent a general role. The present challenge is to determine whether such anchoring can arise from weak, relatively unspecific interactions such as those seen for the PH domains from pleckstrin and spectrin, or requires high affinity, stereo-specific binding such as that seen for PLCδPH.

In our efforts to understand the basis for the specificity and high affinity of PLCδPH for Ins(1,4,5)P_3, we have determined the X-ray crystal structure of the PLCδPH–Ins(1,4,5)P_3 complex at 1.9 Å resolution. This is the most clearly physiologically relevant and most stable complex reported for a PH domain. PLCδPH has the same architecture and electrostatic polarization as other PH domains, and Ins(1,4,5)P_3 is bound to the positively charged surface of the domain. Two residues that contact Ins(1,4,5)P_3 have counterparts in the Btk PH domain at which mutational changes cause inherited agammaglobulinemia (Rawlings et al., 1993; Thomas et al., 1993; Vihinen et al., 1995). This suggests that elements of the PH domain–phosphoinositol interactions seen in this study may have broad implications for the function of PH domains in general.

Results and Discussion

PLCδPH, corresponding to residues 11–140 of rat PLC-δ_1, was produced in Escherichia coli and purified as described (Lemmon et al., 1995). Crystals of the unliganded PH do-

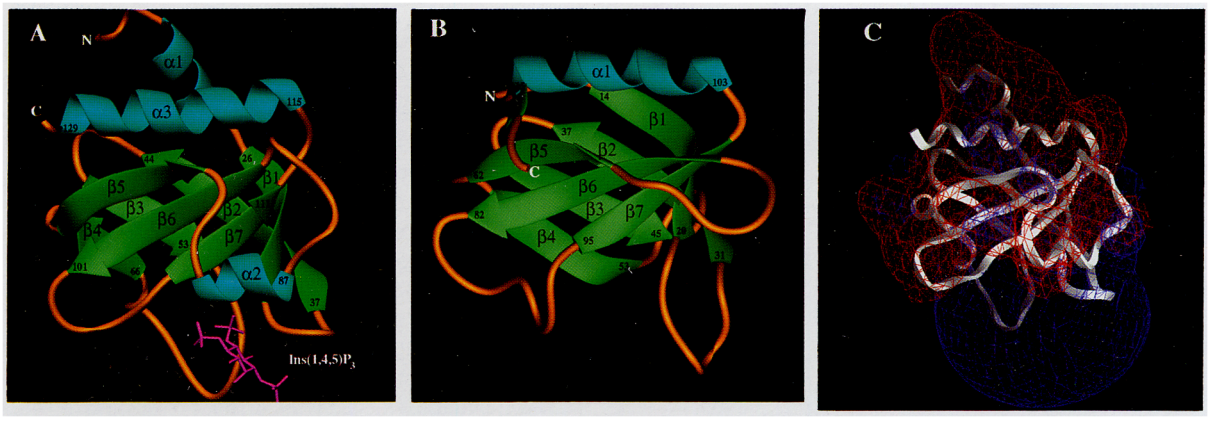

Figure 1. Overall Fold and Electrostatic Polarization of the PLCδPH–Ins(1,4,5)P_3 Complex

(A) A RIBBONS (Carson, 1991) representation of amino acids 12–130 of PLCδPH in complex with Ins(1,4,5)P_3, viewed perpendicular to the two β sheets. Ins(1,4,5)P_3 is shown in magenta in a ball-and-stick representation.

(B) A RIBBONS (Carson, 1991) representation of the PH domain from human dynamin (DynPH) (Ferguson et al., 1994), in the same orientation as that seen for PLCδPH in (A).

(C) Polarized electrostatic potential of PLCδPH calculated without the bound Ins(1,4,5)P_3, contoured at +2 kT (red) and −2 kT (blue). The orientation of PLCδPH is as for (A). This polarization of the electrostatic potential is conserved in other PH domains of known structure (Ferguson et al., 1995).

main could not be obtained, whereas tetragonal crystals ($P4_32_12$; a = 59.2 Å, c = 80.7 Å) could be grown from hanging drops of a PLCδPH:Ins(1,4,5)P_3 (1.0:1.2) mixture with 12% PEG 20,000 as the precipitant. Crystals grew to a typical size of 0.06 × 0.06 × 0.8 mm in 3 days at 18°C and diffracted X-rays to better than 1.9 Å at the National Synchrotron Light Source (NSLS) X25 beamline. To solve the structure, phases were determined by multiple isomorphous replacement plus anomalous scattering using a mercury derivative and crystals of selenomethionine (Se-Met)-containing protein (see Experimental Procedures; Table 1). The position of the bound Ins(1,4,5)P_3 was clearly interpretable in initial experimental electron density maps. Solvent flattening, histogram matching, and partial model combination were employed to improve phases, permitting clear interpretation of amino acids 12–130 of PLC-δ_1 as well as the Ins(1,4,5)P_3 in the electron density map. The resulting model was refined to a final R factor of 20.5% (>2σ data) with 110 water molecules included. A Ramachandran plot showed no violators of accepted backbone torsion angles, and the model has good geometry (Table 1).

Structure of PLCδPH

The overall architecture of PLCδPH is similar to that of other PH domains (Saraste and Hyvönen, 1995). Figure 1 shows a view of the PLCδPH–Ins(1,4,5)P_3 complex (Figure 1A) alongside a view of the dynamin PH domain (Figure 1B) (Ferguson et al., 1994) for comparison. Each PH domain is a β sandwich closed off at one of its splayed corners (Chothia, 1984) by a C-terminal α helix. PLCδPH contains two additional short α helices not found in other PH domains: one is at the N-terminus, and the other is in the β5/β6 loop, and neither directly contacts the bound Ins(1,4,5)P_3. The spectrin PH domain also contains an additional α helix, in the β3/β4 loop (Macias et al., 1994). In view of the very weak sequence identity, the structural similarity with other PH domains is remarkable. PLCδPH shares only around 10% sequence identity with the other domains of known structure.

Like other PH domains (Macias et al., 1994; Ferguson et al., 1994, 1995), PLCδPH shows marked electrostatic sidedness (Figure 1C). The positively charged faces of the PH domains from dynamin and spectrin were previously invoked as a likely surface for interaction with negatively charged membrane surfaces (Ferguson et al., 1994; Gibson et al., 1994). This surface coincides with the segments of PH domains that exhibit the greatest sequence variability, supporting the suggestion that the positive face also determines the specificity. As described below, the binding site for Ins(1,4,5)P_3 is in the center of this positively charged region on the surface of PLCδPH, at the splayed corner of the β sandwich opposite the C-terminal α helix (Figures 1A and 1C).

Ins(1,4,5)P_3 Binding to PLCδPH

The amino acids that interact with Ins(1,4,5)P_3 are mostly in the β1/β2 and β3/β4 loops of PLCδPH (Figures 2 and 3A). Direct hydrogen bonds are seen between the bound Ins(1,4,5)P_3 and seven amino acids, as seen in Figure 3. Two lysine side chains, K30 and K57, appear to clamp the 4- and 5-phosphate groups of Ins(1,4,5)P_3 in the binding pocket, each hydrogen bonding with both phosphates (Figures 3B and 3C). These dual contacts suggest a stereochemical cooperativity of interactions that enhances specificity. Through additional interactions with the side chains of R40, S55, and the backbone NH of R56, plus a water-mediated hydrogen bond with the backbone carbonyl of E54, the 5-phosphate of Ins(1,4,5)P_3 is completely buried. The 4-phosphate forms hydrogen bonds directly to the side chains of K30, K32, and K57, and via a water molecule to the backbone carbonyl of T107. Although nearly all of the Ins(1,4,5)P_3–PH domain interactions involve the 4- and 5-phosphate groups, our previous binding

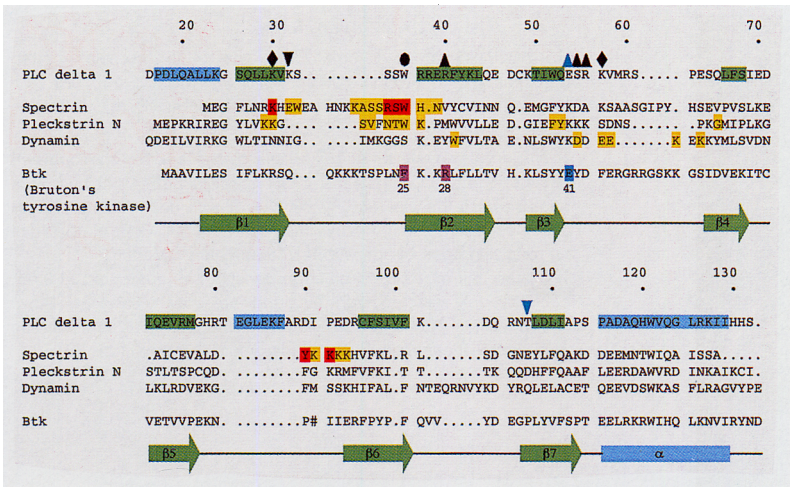

Figure 2. Sequence Alignment and Comparison with Other PH Domains

Structure-based sequence alignment of PLCδPH with the three other PH domains of known structure: human N-terminal pleckstrin PH domain (1–103), mouse brain spectrin PH domain (2199–2304), and human dynamin PH domain (506–629). An alignment of the Bruton's tyrosine kinase (Btk) PH domain (1–136) is also shown. The elements of secondary structure of PLCδPH are shaded green for β strands and blue for α helices. The secondary structure elements that are conserved among PH domains are shown under the sequences. Amino acids in PLCδPH that contact Ins(1,4,5)P_3 are indicated with the following symbols above the sequence (black for direct contacts, blue for water-mediated interactions): inverted triangle, contacts the 4-phosphate; triangle, contacts the 5-phosphate; diamond, contacts both the 4- and 5-phosphate; circle, contacts the 1-phosphate. Amino acids boxed in yellow in the spectrin, pleckstrin N-terminal, and dynamin PH domain sequences correspond to those positions implicated by NMR analysis in binding to Ins(1,4,5)P_3 (Hyvönen et al., 1995), PtdIns(4,5)P_2 (Harlan et al., 1994), and 1-(α-glycerophosphoryl)-Ins(4,5)P_2 (Zheng et al., 1996), respectively. Those boxed red in the spectrin PH domain sequence were seen to contact Ins(1,4,5)P_3 in the crystal structure (Hyvönen et al., 1995). The two positions of XLA mutations in BtkPH (Vihinen et al., 1995) that correlate with Ins(1,4,5)P_3 binding in PLCδPH are boxed in magenta, while the position of the activating mutation (Li et al., 1995) is shaded blue.

analyses (Lemmon et al., 1995) showed that at least one additional phosphate is required. The structure suggests that additional phosphates could be accommodated at either the 1 or the 2 position. However, Ins(1,4,5)P_3 binds 23-fold more tightly to PLCδPH than does Ins(2,4,5)P_3 (Lemmon et al., 1995), indicating that specific interactions involving the 1-phosphate are more important for high affinity binding than nonspecific electrostatic attraction. A direct hydrogen bond is seen between the 1-phosphate of Ins(1,4,5)P_3 and the indole nitrogen of the W36 side chain, which is in van der Waals contact with part of the inositol ring (Figure 3B). Here again, local cooperativity may enhance specificity; i.e., this inositol ring contact buttresses Ins(1,4,5)P_3 for optimal interaction of its 4- and 5-phosphates with the protein, and vice versa. In agreement with our previous studies, the structure shows that phosphates at the 3 or 6 positions would sterically interfere with inositol phosphate binding.

The Binding Site for Ins(1,4,5)P_3 Suggests Explanations for XLA Mutations

A number of mutations in the PH domain of Bruton's tyrosine kinase (BtkPH) have been shown to cause X-linked agammaglobulinemia (XLA) (Thomas et al., 1993; Rawlings et al., 1993; Vihinen et al., 1995). Although the physiological ligand for this PH domain is not yet known, the locations of several such natural mutations suggest that the functional surface of BtkPH is close to the Ins(1,4,5)P_3-binding site on PLCδPH. XLA may therefore result from an impaired interaction of BtkPH with a ligand such as an inositol phosphate or similar compound. Two amino acids so implicated are analogous in sequence position and side chain type to those in PLCδPH that interact directly with Ins(1,4,5)P_3. Substitution in BtkPH of F25 with serine or R28 with histidine results in XLA. These amino acids correspond to W36 and R40 in PLCδPH (see Figure 2), which form hydrogen bonds to the 1- and 5-phosphates of Ins(1,4,5)P_3, respectively, and van der Waals contact with the inositol (W36). Changes analogous to the XLA mutations at either position in PLCδPH would be likely to reduce affinity for Ins(1,4,5)P_3. A gain-of-function mutation has also been observed in BtkPH (E41K) (Li et al., 1995) that can cause transformation of NIH 3T3 cells and results in enhanced membrane localization of whole Btk. E41 in BtkPH is the counterpart of E54 in PLCδPH, which is in the β3/β4 loop. The backbone carbonyl of E54 in PLCδPH contacts the 5-phosphate of Ins(1,4,5)P_3 through a water-mediated hydrogen bond. Substitution with a basic amino acid at this position could enhance binding to a phosphoinositide or similar ligand and result in the observed gain of function.

Comparison with Low Affinity Binding of Phosphoinositides to Other PH Domains

Ins(1,4,5)P_3 binding to the PH domain from spectrin (Hyvönen et al., 1995) is at least 200-fold weaker ($K_d \geq 40$ μM) than its binding to PLCδPH ($K_d = 210$ nM). Similarly, PtdIns(4,5)P_2 binds to the N-terminal pleckstrin PH domain with a K_d of approximately 30 μM (Harlan et al., 1994), while 1-(α-glycerophosphoryl)–Ins(4,5)P_2 binds weakly to the dynamin PH domain ($K_d \approx 4.3$ mM) (Zheng et al., 1996). In each case, nuclear magnetic resonance (NMR) studies have identified amino acids in the PH domains that are involved in these interactions. In addition, Hyvönen et al. (1995) have described the crystal structure of the Ins(1,4,5)P_3–spectrin PH domain complex.

In an effort to understand the stereochemical basis for the higher stability of the PLCδPH–Ins(1,4,5)P_3 complex that we describe, we compare the interactions involved in phosphoinositide binding to each of the three PH domains. The amino acids implicated in each case are highlighted in Figure 2. Broadly, phosphoinositides and inositol phos-

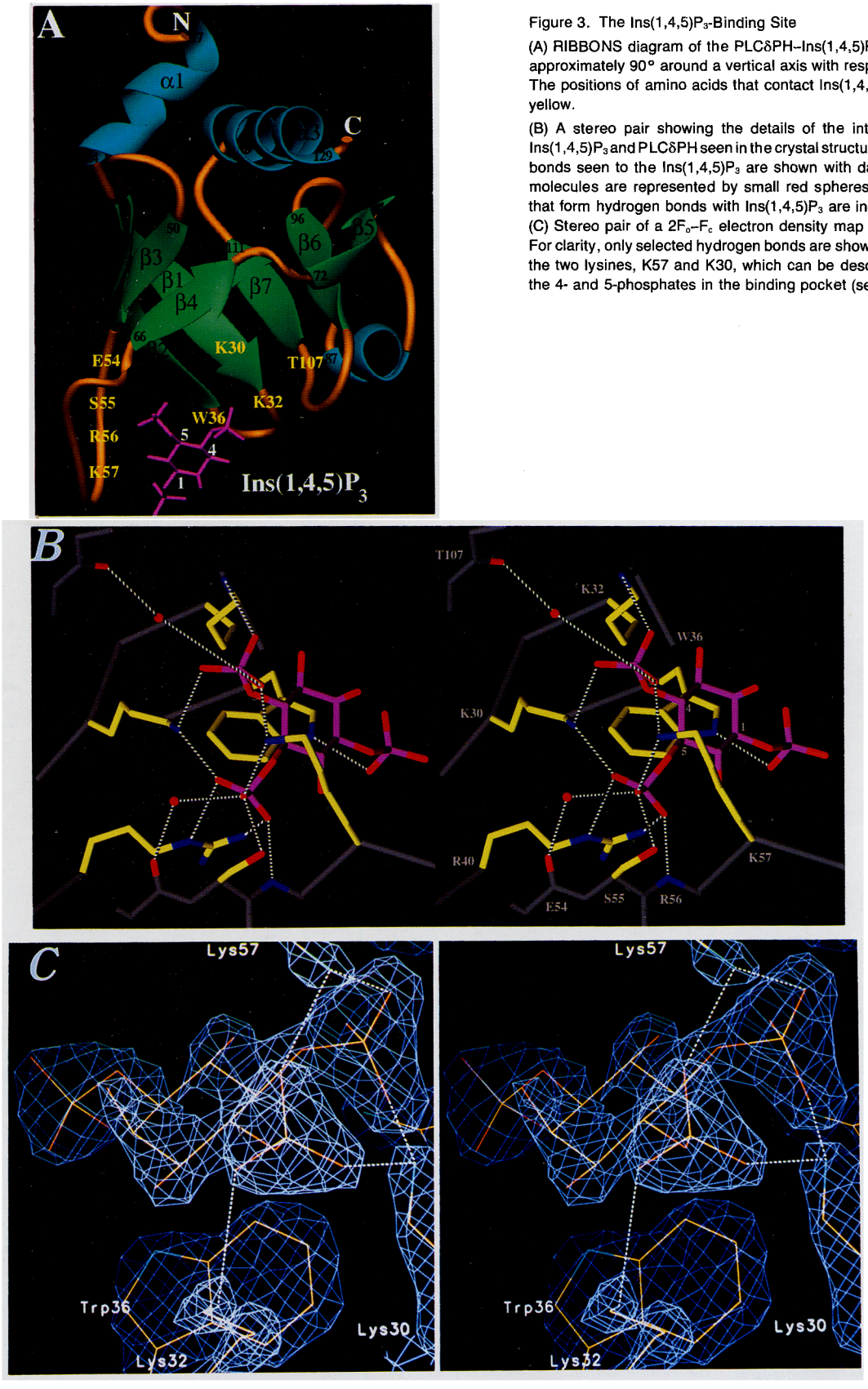

Figure 3. The Ins(1,4,5)P_3-Binding Site

(A) RIBBONS diagram of the PLCδPH–Ins(1,4,5)P_3 complex rotated approximately 90° around a vertical axis with respect to Figure 1A. The positions of amino acids that contact Ins(1,4,5)P_3 are labeled in yellow.

(B) A stereo pair showing the details of the interactions between Ins(1,4,5)P_3 and PLCδPH seen in the crystal structure. All the hydrogen bonds seen to the Ins(1,4,5)P_3 are shown with dashed lines. Water molecules are represented by small red spheres. Only side chains that form hydrogen bonds with Ins(1,4,5)P_3 are included.

(C) Stereo pair of a $2F_o–F_c$ electron density map contoured at 2.0σ. For clarity, only selected hydrogen bonds are shown. This view shows the two lysines, K57 and K30, which can be described as clamping the 4- and 5-phosphates in the binding pocket (see Figure 3B).

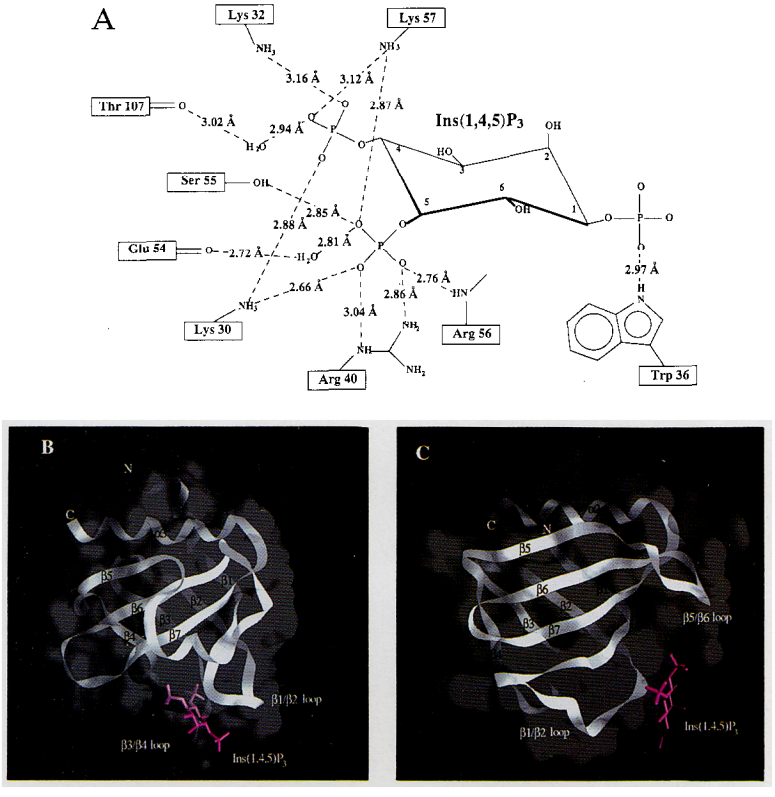

Figure 4. Comparison of Ins(1,4,5)P_3 Binding to PLCδPH and the Spectrin PH Domain

(A) A schematic representation of hydrogen bonds formed between Ins(1,4,5)P_3 and PLCδPH (see also Figure 3B).

(B) Representation of the relationship of Ins(1,4,5)P_3 to the surface of PLCδPH, showing how portions of Ins(1,4,5)P_3 project into a cavity. A solvent-accessible surface is shown around the protein. The 4- and 5-phosphates can be seen behind the partially transparent representation of the protein surface. The orientation shown is the same as that in Figure 1A.

(C) A representation of Ins(1,4,5)P_3 bound to the surface of the spectrin PH domain, shown in an orientation similar to that in (B). Ins(1,4,5)P_3 is seen to bind in a different location from its binding site on PLCδPH (see text), with a different orientation and little penetration into any cavity on the protein surface. Coordinates for the spectrin PH domain–Ins(1,4,5)P_3 complex were provided by Mathias Wilmanns of EMBL and were used, with permission, to generate (C) (Hyvönen et al., 1995).

phates bind to the same side of each PH domain, in the region of the β1/β2 loop. The binding site is in the middle of the positively charged face of each electrostatically polarized PH domain (Ferguson et al., 1995). The proposed phosphoinositide-binding sites on the pleckstrin and dynamin PH domains coincide approximately with the site of Ins(1,4,5)P_3 binding to PLCδPH (Figure 3A). Amino acids implicated in binding are in the region of the β1/β2 and β3/β4 loops in each case (Harlan et al., 1994; Zheng et al., 1996). The Ins(1,4,5)P_3-binding site on the spectrin PH domain, by contrast, occurs on the opposite side of the β1/β2 loop, and additional interactions are contributed largely by the β5/β6 loop (Hyvönen et al., 1995). This displacement of the binding site on the spectrin PH domain correlates with the altered position of its positively charged face (Ferguson et al., 1995).

A detailed comparison of the interactions with Ins(1,4,5)P_3 is only possible with the spectrin PH domain, for which the crystal structure of the complex is known (Hyvönen et al., 1995). There are large differences in the number and arrangement of interactions that are likely to account for the 200-fold higher stability of the complex formed between Ins(1,4,5)P_3 and PLCδPH. Seven hydrogen bonds, involving five amino acids, form between Ins(1,4,5)P_3 and the spectrin PH domain, while there are 12 hydrogen bonds involving nine amino acids between Ins(1,4,5)P_3 and PLCδPH (Figure 4A). The starkest contrast is seen for the 5-phosphate of Ins(1,4,5)P_3, which is involved in three hydrogen bonds to two amino acids in the spectrin PH complex, but participates in seven hydrogen bonds to six amino acids in the PLCδPH complex. The number of interactions between the 5-phosphate and PLCδPH is thus greater than that seen between the entire Ins(1,4,5)P_3 molecule and the spectrin PH domain. Further differences between the modes of Ins(1,4,5)P_3 binding to the two PH domains can be seen in interactions involving the inositol ring. One side of the inositol ring of Ins(1,4,5)P_3 bound to PLCδPH is in van der Waals contact with the aliphatic portion of the K32 side chain and the side chain of W36, which is in turn is held in position by its interaction with the 1-phosphate of Ins(1,4,5)P_3. By contrast, in binding to the spectrin PH domain, the inositol ring of Ins(1,4,5)P_3 is not in close contact with the protein. Indeed, as can be seen in a comparison of Figures 4B and 4C, Ins(1,4,5)P_3 is significantly more buried within a binding pocket in the PLCδPH–Ins(1,4,5)P_3 complex (Figure 4B) than in the spectrin PH domain–Ins(1,4,5)P_3 complex. Ins(1,4,5)P_3 appears to lie on the surface of the spectrin PH domain, its phosphate groups interacting with surface-located side chains (Figure 4C).

A unique feature of PLCδPH that could contribute further to its high affinity for Ins(1,4,5)P_3 is the position of the β3/β4 loop, which is intimately involved in ligand binding (see Figure 3A). In the dynamin PH domain (Ferguson et al., 1994; Fushman et al., 1995), and others of known structure (Macias et al., 1994; Yoon et al., 1994), this loop projects away from the plane of strands β1 to β4 (Figure 5B). By contrast, in the PLCδPH–Ins(1,4,5)P_3 complex, the β3/β4 loop remains in this plane and extends toward the ligand (Figure 5A). This difference in the position of a loop that makes significant contact with the ligand may help explain the much higher affinity shown by PLCδPH

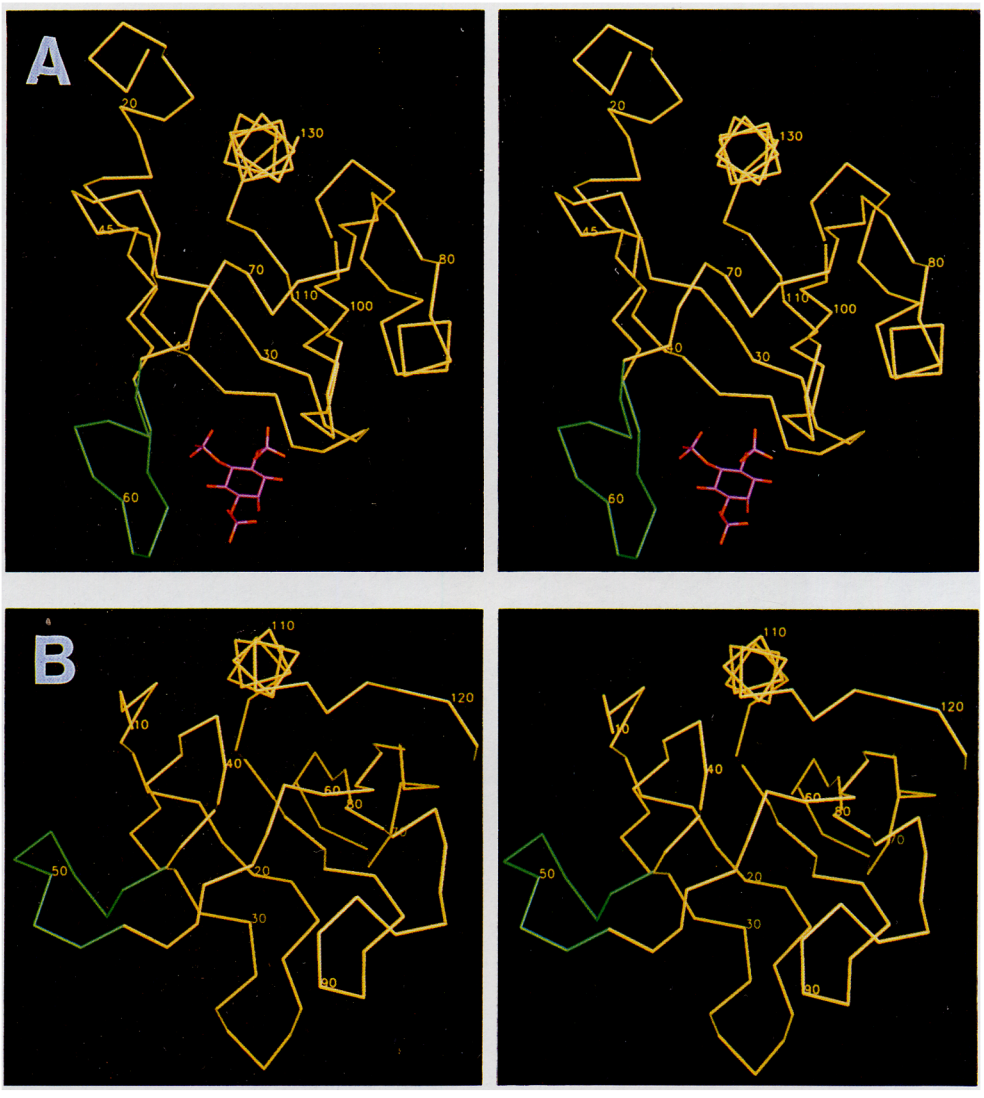

Figure 5. A Possible Conformational Change upon Ligand Binding

Cα-trace, viewed down the long axis of the C-terminal α helix, of PLCδPH (A) and DynPH (B). The β3/β4 loop, which is in a different orientation in the PLCδPH–Ins(1,4,5)P_3 complex compared with other unliganded PH domains (see text), is colored green. The root-mean-square deviation between the backbone of the core elements of secondary structure of PLCδPH and DynPH is 1.75 Å (amino acids aligned between PLCδPH and DynPH are the following: 25–32 and 15–22, 37–44 and 30–37, 50–54 and 41–45, 65–69 and 55–59, 73–77 and 62–66, 95–101 and 76–82, and 109–129 and 96–116).

for Ins(1,4,5)P_3. Without the unliganded PLCδPH structure for comparison, however, we can only speculate on the origin of this difference. It is possible that the difference in position of the β3/β4 loop reflects a conformational change in PLCδPH upon ligand binding, one reminiscent of the change seen in streptavidin upon biotin binding (Weber et al., 1989). No such conformational changes are seen upon binding of Ins(1,4,5)P_3 to the spectrin PH domain (Hyvönen et al., 1995). Their occurrence in PLCδPH could contribute to the higher binding affinity.

Implications for PH Domain Ligand Recognition

The major challenge in studies of PH domains is to establish the physiological relevance of the ligand-binding interactions that have been reported. PLC-δ_1 was cloned as a result of experiments aimed at identifying Ins(1,4,5)P_3 receptors in rat brain cytosol (Kanematsu et al., 1992). Ins(1,4,5)P_3 is the head group of the preferred substrate for PLC-δ_1 and was clearly found to be the preferred ligand for PLCδPH in our previous studies (Lemmon et al., 1995). Ins(1,4,5)P_3 binding to PLCδPH is just 5-fold weaker than its binding to the purified endoplasmic reticulum Ins(1,4,5)P_3 receptor (K_d = 40 nM) (Ferris et al., 1989). Furthermore, Ins(1,4,5)P_3 has been shown to inhibit PLC-δ_1 activity in vitro by reducing the affinity of the enzyme for substrate-rich membranes (Cifuentes et al., 1994; Yagisawa et al., 1994). Proteolytic removal of a portion of the PH domain from PLC-δ_1 abolishes these effects and itself reduces the affinity of the enzyme for PtdIns(4,5)P_2-containing membranes (Cifuentes et al.,

1993, 1994; Yagisawa et al., 1994). It is therefore likely that D-myo-Ins(1,4,5)P_3 represents a physiologically relevant ligand for PLCδPH. The stereospecific character of this interaction is indicated in our previous studies of specificity of ligand binding, which showed that any change in the arrangement or number of phosphate groups leads to at least a 15-fold reduction in the affinity of the inositol phosphate for PLCδPH (Lemmon et al., 1995). The PLCδPH–Ins(1,4,5)P_3 interface seen in the crystal structure provides a stereochemical explanation for this.

It is possible that the binding sites for phosphoinositides that have been located by NMR and crystallographic studies of the PH domains from spectrin, pleckstrin, and dynamin reflect limited interactions with noncognate ligands (Harlan et al., 1994; Hyvönen et al., 1995; Zheng et al., 1996). The strongest of these interactions has a K_d of 30 μM (Harlan et al., 1994), while all inositol polyphosphates tested (with three or more phosphates) bind to PLCδPH with K_d values of 13 μM or less (Lemmon et al., 1995). Therefore, the interactions of PLCδPH with inositol phosphates against which it discriminates are still stronger than those seen in studies of any other PH domain. This fact, together with a lack of functional relevance, argues that the physiological importance of phosphoinositide or inositol phosphate binding to the PH domains of spectrin, pleckstrin, and dynamin must be questioned. The physiologically relevant ligands for these other PH domains, if such ligands exist, may be molecules other than inositol polyphosphates that contain phosphate groups or other negatively charged moieties.

It is also possible that the other PH domains do not have highly specific strongly interacting ligands, and that PLCδPH is a special case. The positively charged face of PH domains is likely to be important for their binding to anionic membrane surfaces (Ferguson et al., 1994, 1995; Hyvönen et al., 1995). This could involve delocalized electrostatic attraction between the protein and the membrane surface, as shown for secretory phospholipase A_2 molecules (Scott et al., 1994). Binding to PtdIns(4,5)P_2 or other phosphoinositides could serve to augment this electrostatic interaction, while also imposing some stereospecificity. PLCδPH binds to PtdIns(4,5)P_2 in vesicles 8-fold more weakly (K_d = 1.7 μM) than it does to its soluble head-group Ins(1,4,5)P_3. This may result from a reduction in the degrees of freedom or accessibility (or both) of the head group in the lipid bilayer (Figure 6). The diminished affinity for the phospholipid head group under these conditions may be partly compensated for by delocalized electrostatic attraction between the protein and the membrane surface. This suggestion is strengthened by the fact that binding of whole PLC-δ_1 to PtdIns(4,5)P_2-containing membranes is enhanced by the presence of other anionic lipids (Rebecchi et al., 1992). Furthermore, PLCδPH binds to PtdIns(4)P in negatively charged lipid vesicles with a K_d of approximately 15 μM, while it does not interact with the soluble head group of this phospholipid, Ins(1,4)P_2 (Lemmon et al., 1995).

Following these arguments, binding of the pleckstrin PH domain to PtdIns(4,5)P_2-containing vesicles (Harlan et al., 1994) could result primarily from the electrostatic interaction between the membrane surface and the positively charged face of the PH domain. We have been unable to detect significant interactions between the pleckstrin PH domain and Ins(1,4,5)P_3 (Lemmon et al., 1995), which supports this suggestion. The spectrin PH domain binds with high affinity to stripped bovine brain membranes (Davis and Bennett, 1994; Lombardo et al., 1994). The binding to Ins(1,4,5)P_3 reported by Hyvönen et al. (1995) is not sufficiently tight to explain this observation, but could augment and provide some stereo specificity to a delocalized electrostatic interaction with the membrane surface.

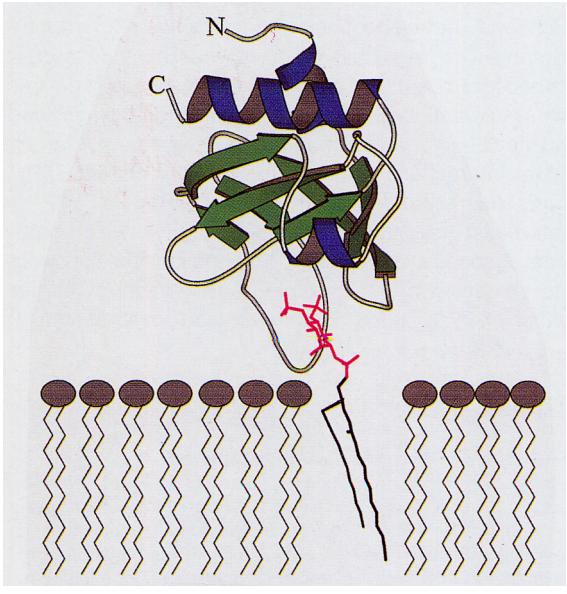

Figure 6. Schematic Representation of PLCδPH Binding to PtdIns(4,5)P_2 in a Membrane

PLCδPH is shown in a ribbon representation in the same orientation as in Figure 1A and Figure 2. Ins(1,4,5)P_3 is shown bound to PLCδPH as seen in the crystal structure. Diacylglycerol has been attached to the Ins(1,4,5)P_3 to give an impression of the way PtdIns(4,5)P_2 might associate with PLCδPH. The PtdIns(4,5)P_2 molecule is shown in a highly schematized bilayer. The positive face of the PLCδPH (Figure 1C) faces the membrane. This figure was generated with MOLSCRIPT (Kraulis, 1991).

Functional Implications

By anchoring PLC-δ_1 to substrate-containing membranes, interaction of PLCδPH with PtdIns(4,5)P_2 (Lemmon et al., 1995) may allow processive substrate hydrolysis by the enzyme (Cifuentes et al., 1993, 1994; Kanematsu et al., 1992). The product of this hydrolysis, Ins(1,4,5)P_3, competes with PtdIns(4,5)P_2 for binding to PLCδPH and reduces the affinity of PLC-δ_1 for PtdIns(4,5)P_2-containing membranes (Cifuentes et al., 1994). This inhibits the enzyme (Kanematsu et al., 1992), suggesting that product inhibition could be important in PLC-δ_1 regulation, and that the PH domain is required for this effect. Most PH domains are found in proteins that require membrane association for their function, such as the β-adrenergic receptor kinase (Pitcher et al., 1995), guanine nucleotide–releasing factors, and protein kinases (Gibson et al., 1994). All PH do-

mains may have phosphoinositide-like ligands and may be subject to regulated membrane association as described for PLC-δ_1. The physiological relevance of the low affinity interactions of PH domains with phosphoinositides and their head groups remains to be determined. Analysis of the interactions between Ins(1,4,5)P_3 and PLCδPH shows a correspondence between amino acids in PLCδPH that contact Ins(1,4,5)P_3 and those in BtkPH at which mutational changes alter function. This suggests that the structure presented here can serve as a valuable model for further analysis of PH domain structures and functional interactions with their ligands.

Experimental Procedures

Preparation and Crystallization of PLCδPH

PLCδPH, corresponding to residues 11–140 of rat PLC-δ_1, was produced in Escherichia coli by using a T7 expression system and was purified by cation exchange and size-exclusion chromatography as previously described (Lemmon et al., 1995). Following gel filtration, the protein was concentrated and buffer exchanged for a further hydroxyapatite purification step. Protein was loaded on to a 2 ml column of Macro-Prep ceramic hydroxyapatite (Bio-Rad Laboratories, Incorporated) equilibrated with 10 mM Na/K phosphate, 10% glycerol, 0.5 mM $CaCl_2$ (pH 7.0) and was eluted by running a Na/K phosphate concentration gradient from 10 to 200 mM. The resulting material was at least 99% pure as assessed on overloaded silver-stained SDS gels. D-myo-Ins(1,4,5)P_3 was purchased from Sigma and used without further purification. PLCδPH was concentrated by ultrafiltration and buffer exchanged with 25 mM NaCl, 10% glycerol, 10 mM morpholineethanesulfonic acid (MES) (pH 6.5). Protein concentration was determined by measurement of OD_{278}, using the calculated extinction coefficient. PLCδPH was then mixed with Ins(1,4,5)P_3 in a 1.0:1.2 molar ratio (PLCδPH:Ins(1,4,5)P_3) at a final protein concentration of 1.2 mM. Hanging drops were formed by mixing 3 μl of this complex with 3 μl of 12% PEG 20,000, 10% glycerol, 50 mM Na-citrate (pH 5.0) and were equilibrated by vapor diffusion against a reservoir of this solution. No crystals could be obtained under these conditions (or any other conditions tested) in the absence of Ins(1,4,5)P_3. Tetragonal crystals of the PLCδPH–Ins(1,4,5)P_3 complex ($P4_32_12$; a = 59.4 Å, c = 80.7 Å) grew in three days at 18°C to a typical size of 0.06 × 0.06 × 0.8 mm. Se-Met-containing protein was produced as described previously (Ferguson et al., 1994). Crystals were stabilized in 20% PEG 20,000, 10% glycerol, 50 mM Na-citrate (pH 5.0), which also served as a cryosolvent. A single mercury derivative was obtained by soaking crystals in 1 mM $HgCl_2$ in this stabilizing solution for 12 hr.

Data Collection and Structure Solution

Data were collected at 100K on crystals (stabilized in the cryosolvent) that had been flash-frozen in liquid nitrogen-cooled liquid propane. The native and $HgCl_2$-derivative data were collected on an R-Axis II image plate detector and a Siemens Xentronics multiwire area detector, respectively, in both cases using double mirror-monochromated/focused Cu K_α X-rays from a 0.2 mm focus of a Rigaku generator operating at 3 kW (50 kV × 60 mA). Data for crystals of Se-Met-containing protein were collected at the X25 beamline (NSLS) tuned to the Se absorption edge as determined for this crystal (a wavelength of 0.9 Å), by use of a MAR image plate detector. R-Axis and X25 data were processed with DENZO (Otwinowski, 1993), while Xentronics data were processed with the program XDS (Kabsch, 1988a, 1988b). All data were scaled with SCALEPACK (Otwinowski, 1993). Two Se sites were located in the anomalous difference Patterson map calculated from the Se-Met data set. The two major Hg sites in the $HgCl_2$ derivative were found in isomorphous difference Fourier maps from the Se-Met-derived phases. A further three minor sites were found in the $HgCl_2$ derivative from isomorphous difference Fourier maps calculated with phase information from both derivatives. The resulting heavy

Table 1. Summary of Crystallographic Data

Data Collection Statistics

	Source	Resolution Limit (Å)	R_{sym}[a] (%)	Completeness (%)	Number of Heavy Atom Sites
Native	R-Axis II	2.4	9.2	96.5	–
Se-Met	NSLS X25	1.9	6.4	99.6	2
$HgCl_2$	Xentronics	2.8	8.0	89.1	2 major, 3 minor

Phasing Statistics									
Resolution (Å)	15–9.06	6.49	5.05	4.14	3.50	3.04	2.68	2.4	Overall
Phasing power[b]									
$HgCl_2$ isomorphous	2.92	2.87	2.94	2.08	1.51	1.54	1.70	–	1.91
$HgCl_2$ anomalous	0.63	0.53	0.53	0.50	0.41	0.31	0.27	–	0.40
Se-Met isomorphous	1.01	0.93	0.78	0.56	0.37	0.34	0.38	0.29	0.41
Se-Met anomalous	0.34	0.43	0.52	0.45	0.45	0.40	0.41	0.41	0.43
Mean figure of merit[c]	0.62	0.70	0.67	0.57	0.45	0.34	0.30	0.20	0.37

Refinement Statistics									
Resolution (Å)	8.00–3.69	2.98	2.62	2.38	2.22	2.09	1.99	1.90	Overall
Unique reflections	1554	1491	1476	1441	1449	1441	1423	1420	11695
$\langle I/\sigma \rangle$	26.5	24.8	18.9	16.3	13.8	12.3	6.8	5.1	20.3
R factor, all data[d]	17.2	17.7	22.9	24.5	24.5	25.4	28.3	30.7	21.3 (30.2)
R factor, >2.0σ data[d]	17.1	17.5	22.1	23.4	23.3	23.9	26.1	27.3	20.5 (29.1)
Rms deviations									
Bond length (Å)	0.008								
Bond angle (degrees)	1.48								

[a] $R_{sym} = \Sigma_{h'}|I_h - I_{h'}|/\Sigma_{h'}I_{h'}$, where $|I_h - I_{h'}|$ is the absolute deviation of a reflection $I_{h'}$ from the average I_h of its symmetry and Friedel equivalents (Friedel pairs were not merged for the Se-Met or $HgCl_2$ data sets).

[b] Phasing power = $\Sigma|F_h^c|/\Sigma||F_p^o| \exp(i\Phi_c)+F_h^c|-|F_{ph}^o||$, where $|F_h^c|$ = heavy-atom structure factor, and $|F_p^o|$ and $|F_{ph}^o|$ are observed amplitudes for the protein and heavy-atom derivatives respectively, and Φ_c is the experimental phase.

[c] Figure of merit = $\int P(\Phi)\exp(i\Phi)d\Phi / \int P(\Phi)d\Phi$ where P is the probability distribution of Φ, the phase angle.

[d] Number in parentheses is the free R value (Brünger, 1992).

atom sites were refined, and phases were calculated with MLPHARE (Otwinowski, 1991). Solvent flattening, histogram matching, and partial model combination were employed to improve phases by use of DPHASE (G. Van Duyne). The program O (Jones et al., 1991) was used for all model building and adjustments, and the structure was refined with XPLOR (Brünger, 1993). The final refined model includes amino acids 12–130 of PLC-δ_1, plus Ins(1,4,5)P_3 and 110 water molecules. A Ramachandran plot shows no violators of accepted backbone torsion angles. The crystallographic data are presented in Table 1. Figures 1C, 4B, and 4C were generated with the program GRASP (Nicholls and Honig, 1991).

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