Structural Characterization of the Split Pleckstrin Homology Domain in Phospholipase C-γ1 and Its Interaction with TRPC3

Received for publication, January 12, 2006, and in revised form, February 17, 2006 Published, JBC Papers in Press, February 24, 2006, DOI 10.1074/jbc.M600336200

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Phospholipase C (PLC)-γ is unique among the PLC enzymes because each PLC-γ isozyme contains a split pleckstrin homology (PH) domain with an SH2SH2SH3 tandem repeat insertion (where SH indicates Src homology domain) in the middle of its sequence. Split PH domains exist in a number of other proteins that play crucial signaling roles. However, little is known about the structure and function of split PH domains. The C-terminal half of the PLC-γ-split PH domain has been implicated to interact directly with the TRPC3 calcium channel, thereby providing a direct coupling mechanism between PLC-γ and agonist-induced calcium entry. However, this interaction has not been proved by direct biochemical or structural studies. Here we determined the three-dimensional structure of the split PH domain of PLC-γ1, and we found that the split PH domain of the enzyme folds into a canonical PH domain fold with high thermostability. The SH2SH2SH3 insertion between the β3 and β4 strands does not change the structure of the split PH domain. In contrast to the majority of phospholipid-binding PH domains, the PLC-γ1 split PH domain lacks the signature lipid-binding motif located between the β1 and β2 strands. Consistent with this structural feature, the split PH domain of PLC-γ1 does not bind to phospholipids. Multiple biochemical and biophysical experiments have argued against a direct interaction between TRPC3 and the C-terminal half of the PLC-γ1 split PH domain. Our data pointed to the existence of a yet to be elucidated interaction mechanism between TRPC3 and PLC-γ1.

PH1 domains are abundant protein modules that play critical roles in cellular signaling and cytoskeletal organization (1). All PH domains with known structures contain a conserved core structure composed of a partially open, two-sheeted β-barrel with one end of the barrel capped with a C-terminal α-helix (1–5). The best characterized function of PH domains is binding to inositol phospholipids (1). Only a minority of PH domains are capable of binding to lipids with high affinity and specificity. Some PH domains are known to be weak, nonspecific membrane phosphoinositide binders (6), whereas others interact with proteins (e.g. the PH domain of the β-adrenergic receptor) (7). However, the functions of the majority of PH domains are unknown (6).

Split PH domains represent a unique subclass of PH domains that are characterized by insertions of one or several autonomously folded protein modules in the middle of PH domain sequences. Split PH domains are also found in various proteins, including the second messenger generating enzymes phospholipase C-γ (PLC-γ), the synaptophin scaffold proteins (8), the Rock1 family Ser/Thr kinases, and the actin filament-based molecular motor myosin X (9). Recent biochemical and structural studies showed that the split PH domain of α-synaptophin folds into a canonical PH domain fold with or without the PDZ domain insertion. It was further demonstrated that the PDZ domain insertion functions synergistically with the split PH domain in binding to phosphoinositol lipids (8). Little is known about the structural and biochemical properties of the split PH domains other than what has been demonstrated in α-synaptophin.

Phospholipase C (PLC) hydrolyzes phosphatidylinositol 4,5-bisphosphate to produce the second messengers inositol 1,4,5-trisphosphate and diacylglycerol. There are as many as 12 different PLC gene products found in mammalian cells that can be grouped into five subfamilies: β (β1–β4), γ (γ1 and γ2), δ (δ1–δ4), ε, and η (10–12). All PLC isoforms are modular proteins invariably containing from their N-terminal to C-terminal ends a PH domain, catalytic X and Y domains, and a C2 domain. Among the various isoforms of PLC, members of the γ subfamily are structurally distinct in that the catalytic X and Y domains are separated by an ~450-residue insertion. The center of the insertion sequences in each PLC-γ isozyme consists of two Src homology 2 (SH2) domains and an SH3 domain, and the two ends of the SH2SH2SH3 supramodule are flanked by the split halves of a PH domain (Fig. 1A). Extensive studies in the past demonstrated that in addition to docking the enzymes to various receptors and adaptor proteins, the SH2 and SH3 domains also directly regulate the catalytic activities of PLC-γ (11–13). An emerging feature of the PLC-γ family isozyme is that many PLC-γ cellular functions are not dependent on lipase activity. For example, the mitogenic activity of PLC-γ1 was not affected by a lipase-inactive mutation (14) but could be inhibited by the SH3 domain of the enzyme (15). The SH3 domain of PLC-γ1 was found to contain guanine nucleotide exchange factor activity specifically for the phosphatidylinositol 3-kinase enhancer small GTPase PIKE (16), and it was suggested that the guanine nucleotide exchange factor activity of the SH3 domain may be associated with the lipase-independent mitogenic activity of the enzyme. Another example of lipase-independent activity of PLC-γ is the regulation of agonist-induced Ca2+ entry via the TRPC3.
calcium channel (17). In that study, the authors showed that the lipase-inactive mutant of PLC-γ1 functions as effectively as the wild-type enzyme in augmenting agonist-induced Ca\(^{2+}\) entry in PC12 cells. It was further found that a fragment of the enzyme containing the SH3 domain and the C-terminal half of the split PH domain of PLC-γ1 (PLCy1–PHC) can directly associate with TRPC3. Very recently, the same research group showed that PLCy1-PHC is solely responsible for direct binding to a short fragment of TRPC3 located at its N-terminal end (18). More significantly, the authors suggested that the PLCy1-PHC-binding segment of TRPC3 represents a complementary partial PH domain “hiding” in the ion channel. They demonstrated that binding of the two partial PH domain fragments from PLCy1 and TRPC3 forms a functional “PH domain” capable of binding to specific lipids and regulating the surface expression of the TRPC3 ion channel. Given the potentially wide distribution of split PH domains in diverse proteins and enzymes, the work presented by van Rossum et al. (18) suggests a novel mode of function of many PH domains (19). To advance this important hypothesis, it is critical to know whether the two halves of the split PH domain in PLCy1 can fold into a canonical PH domain structure; whether PLCy1-PHC alone can stably exist in solution for binding to a complementing partial PH domain from another protein such as TRPC3; and whether the complex formed by two fragments from PLCy1 and TRPC3 (or PLCy1 and translational elongation factor 1α (20)) or from other proteins with split PH domains can indeed assume a PH domain-like fold.

In this study, we determined the solution structure of the split PH domain of PLCy1. We further showed that the insertion of the SH2SH2SH3 domain does not affect the structure of the split PH domain of PLC-γ1. Finally, we characterized potential interactions of PLCy1-PHC with the hypothetical hidden PHN fragment from TRPC3.

**MATERIALS AND METHODS**

**Protein Expression and Purification**—The joined PHN1-PHC domain (residues 489–547 and 851–933), PHC fragment (residues 851–933), and the PHN1-SH2SH2SH3-PHC domain (residues 489–933) of rat PLCy1 were cloned into a modified version of the pET32a vector (21). The joined PHN1-PHC domain contains an 8-residue protease 3C recognition sequence (“LEVLFQGP”) at the joint site of the two halves of the PH domain. The human TRPC3 fragment (residues 1–52) and the PLC-γ1 (residues 794–933) were cloned into pGEX4T-1 plasmid (Amersham Biosciences). Bacterial cells harboring each fusion protein expression plasmid were grown at 37 °C, and protein expression was induced by 1.0 mM of the PHN1-PHC tandem in 50 mM potassium phosphate, pH 6.5, in 90% H\(_2\)O, 10% D\(_2\)O or 99.9% D\(_2\)O. NMR spectra were acquired at 35 °C on Varian Inova 500- and 750-MHz spectrometers each equipped with an actively z-gradient shielded triple resonance probe. Backbone and side chain resonance assignments of the protein were obtained by standard heteronuclear correlation experiments (22, 23). Nonaromatic, nonexchangeable side chain resonances were assigned using HCCH-TOSY experiments. The side chains of aromatics were assigned by standard \(^1\)H two-dimensional TOCSY/NOESY experiments.

**Structure Calculations**—Approximate interproton distance restraints were derived from the NOESY spectra (a \(^1\)H two-dimensional homonuclear NOESY, a \(^1\)H\(^-\)\(^1\)N-separated NOESY, and a \(^1\)H\(^-\)\(^1\)C-separated NOESY). The NOEs were grouped into three distance ranges as follows: 1.8–2.7 Å (1.8–2.9 Å for NOEs involving NH protons), 1.8–3.5 Å (1.8–3.5 Å for NOEs involving NH protons), and 1.8–5.0 Å, corresponding to strong, medium, and weak NOEs, respectively. Hydrogen bonding restraints were generated from the standard secondary structure of the protein based on the NOE patterns and backbone secondary chemical shifts. The backbone dihedral angle restraints were derived from the chemical shift analysis program TALOS (24). Structures were calculated using the program CNS (25). Figures were generated using MOLMOL (26), MOLSCRIPT (27), and Raster3D (28).

**NMR Titration**—NMR-based interaction studies were performed by recording \(^1\)H-\(^1\)H HSQC spectra of \(^1\)H\(^-\)\(^1\)N-labeled protein samples (\(\sim 0.2 \text{mM}\)) with or without addition of their respective binding partners at natural abundance. The N-terminal SH2 domain-binding phosphotyrosine peptide (Dip\(\gamma\)YIPILPD) was commercially synthesized (GenScript Corp., Piscataway, NJ)). The buffer condition was identical to that used in the samples for the structural determination of the split PH proteins.

**RESULTS**

The Split PH Domain of PLC-γ1 Adopts a Stable Fold—Several approaches were used to assess whether the two split halves of the PH domain of PLC-γ1 (referred to as PHN and PHC) can directly interact with each other to form a stable structure. First, we deleted the SH2SH2SH3 insert (residues 548–850 in rat PLC-γ1) from the PHN1-SH2SH2SH3-PHC supramodule, resulting in a fusion protein with the two halves of the split PH domain connected directly (i.e., PHN1-PHC). The recombinant PHN1-PHC was eluted at a molecular mass indicative of a stable monomer when analyzed by analytical gel filtration chromatography (data not shown). The well dispersed \(^1\)H, \(^1\)N HSQC spectrum indicates that the joined PHN1-PHC is well folded (Fig. 1B, black dots). It is possible that the covalent linkage of PHN1 and PHC may artificially induce folding of the linked protein. To address this possibility, in the middle of the linking sequence of PHN1-PHC, we inserted an 8-residue peptide fragment that can be cleaved by protease 3C. Digestion of PHN1-PHC...
PHC with protease 3C produces two fragments with molecular masses corresponding to PHN and PHC, respectively (Fig. 1C). The NMR spectrum of the protease 3C-cleaved PHN-PHC is essentially identical to that of the uncleaved protein (Fig. 1B), indicating that the covalent linkage between PHN and PHC is dispensable to the folding of the split PH domain in PLC-γ1. Furthermore, both the joined and cleaved PHN-PHC showed excellent thermostability, as the proteins remained well folded in the NMR tubes at temperature as high as 50 °C (supplemental Fig. 1).

Structure of the Split PH Domain of PLC-γ1 — To determine whether the split PH domain of PLC-γ1 folds into a canonical PH domain structure, we solved the three-dimensional structures of the joined PHN-PHC by NMR spectroscopy (Fig. 2 and Table 1). The PHN and PHC fragments fold together to form a canonical PH domain structure containing seven β-strands and one C-terminal α-helix. As in the split PH domain of α-syntrophin (8), the PHN half is composed of three β-strands (β1–β3), and the PHC half contains the remaining four β-strands (β4–β7) and the C-terminal α-helix. Inserted at the β3/β4-loop of the PH domain is a 56-residue flexible linker. The flexibility of this 56-residue linker is confirmed by a lack of any detectable medium, long range NOEs and negative backbone amide 1H, 15N NOE values (data not shown). In the native PLC-γ1, the β3/β4-loop of the split PH domain also contains a 300-residue SH2SH2SH3 tandem insertion in the middle of the loop.

Sequence alignment analysis showed that the split PH domains of PLC-γ1 are highly conserved throughout evolution (Fig. 3A). When compared with a number of PH domains that bind to phosphoinositide head groups with high affinities (29–33), the split PH domain of PLC-γ1 lacks a number of critical residues necessary for binding to phosphoinositides. For example, the phosphoinositide-binding PH domains share a signature motif with conserved positively charged amino acid residues, "KX_3(K/R)XR," where the first Lys locates at the penultimate position of the β1 strand, and the (K/R)XR sequence corresponds to residues 2–4 of the β2 strand (Fig. 3A) (32–35). These conserved basic residues play critical roles in binding to negatively charged phosphate groups from the head groups of phosphoinositides (see Fig. 3B for an example). In contrast, the penultimate residue in the β1 strand is a Leu instead of a Lys, and the second and the fourth residues in the β2 strand are Tyr and His, respectively, in the split PH domains of PLC-γ1. Because all three positively charged residues in the otherwise phosphoinositidol lipid-binding signature motif are absent, we predicted that the split PH domain of PLC-γ1 is not likely to function as a lipid binding
module. To test this hypothesis, we assayed the binding of the joined PH\textsubscript{N}-PH\textsubscript{C} domain of PLC\textsubscript{y1} to liposomes prepared from total bovine brain lipids. As predicted, the split PH domain of PLC\textsubscript{y1} (Fig. 3, the HSQC spectrum of the joined PH\textsubscript{N}-PH\textsubscript{C} domain overlaps well with a subset of peaks from the HSQC spectrum of the PH\textsubscript{N}-SH2SH2H3-PH\textsubscript{C} supramodule, indicating that the insertion of the SH2SH2H3 tandem domains in the β3/β4-loop does not alter the structure of the split PH domain. We further tested whether ligand binding to the SH2SH2H3 insertion might result in structural changes of the split PH domain. We chose a peptide ligand that is specific to the C-terminal SH2 domain to test potential ligand binding-induced structural changes to the split PH domain, as the two SH2 domains have been shown to play critical roles in enzyme activity regulation (11, 12). Binding of a phospho-Tyr-containing peptide encompassing the Tyr(P)-1021 site of platelet-derived growth factor receptor (36) to the PH\textsubscript{N}-SH2SH2H3-PH\textsubscript{C} supramodule induced minimal chemical shift changes in the entire split PH domain (Fig. 4, B and C), indicating that the binding of the C-terminal SH2 ligand to PLC-\textsubscript{y1} does not change the conformation and therefore the assembly of the split PH domain. As expected, binding of the C-terminal SH2 ligand peptide to the PH\textsubscript{N}-SH2SH2H3-PH\textsubscript{C} supramodule induced significant chemical shift changes in a number of residues other than those from the split PH domain, and these residues presumably belong to the ligand-binding SH2 domain (Fig. 4C). Residue-specific chemical shift assignments of the PH\textsubscript{N}-SH2SH2H3-PH\textsubscript{C} supramodule are required for correlating the peptide-induced shift changes to the individual residues within the SH2SH2H3 domains.

Characterization of the Interaction between the Split PH Domain of PLC-\textsubscript{y1} and TRPC3—Having characterized the structure of the split PH domain of PLC-\textsubscript{y1} in detail, we went on to study the earlier reported interaction between PLC-\textsubscript{y1} and TRPC3 (17, 18), hoping to lay a foundation for structural characterization of the PLC\textsubscript{y1}-TRPC3 complex. We were able to obtain large quantities of recombinant proteins encompassing the N-terminal 52 residues of TRPC3 (Fig. 5A), as well as a fragment containing the N-terminal 48 residues (data not shown). The authenticity of the TRPC3 fragments was verified using mass spectrometry. Both TRPC3 fragments contained the necessary PLC-\textsubscript{y1}-binding sequence reported by van Rossum et al. (18). We tested potential direct interaction between PH\textsubscript{N}-PH\textsubscript{C} of PLC-\textsubscript{y1} and TRPC3 by titrating the unlabeled TRPC3 fragment to the 15N-labeled PH\textsubscript{N}-PH\textsubscript{C} at a concentration of ∼0.1 mM. No chemical shift changes to PH\textsubscript{N}-PH\textsubscript{C} were observed upon addition of excess molar ratio amounts (up to 10 eq molar ratio) of the 52-residue TRPC3 fragment (data not shown), indicating that TRPC3 does not bind to the folded split PH domain of PLC-\textsubscript{y1}. Consistent with the above NMR-based assay, no binding between PH\textsubscript{N}-PH\textsubscript{C} and TRPC3 was detected when we used purified GST-fused TRPC3 to “pull-down” PH\textsubscript{N}-PH\textsubscript{C} (or GST-fused PH\textsubscript{N}-PH\textsubscript{C} to pull-down the TRPC3 fragment) (supplemental Fig. 2A).

It is possible that TRPC3 only binds to PLC-\textsubscript{y1} when the PH\textsubscript{C} half is somehow dissociated from the PH\textsubscript{N} half. We set out to test this hypothesis. Sufficient amounts of pure, recombinant PH\textsubscript{C} could be obtained, and this half of the PH domain remained soluble at concentrations up to 0.5 mM (Fig. 5, B and C). The backbone amide resonances seen in the 1H, 13N HSQC spectrum of PH\textsubscript{C} are clustered within a narrow chemical shift window (between 7.8 and 8.8 ppm), indicating that this half of the PH domain is largely unfolded (Fig. 5B). Circular dichroism spectrum also confirmed that PH\textsubscript{C} does not contain a significant level of secondary structures (data not shown). Again, we titrated an unlabeled N-terminal 52-residue fragment of TRPC3 to this 13N-labeled PH\textsubscript{C} (∼0.1 mM). To our surprise, addition of an excess amount (up to 10-fold) of TRPC3 induced no observable chemical shift changes to PH\textsubscript{C} (Fig. 5, B and C), indicating that even the PH\textsubscript{C} half of PLC-\textsubscript{y1} alone does not interact with TRPC3 (or under this assay condition, the dissociation constant of the TRPC3/PH\textsubscript{C} mixture is higher than 10\textsuperscript{−7} M). We also used several biochemical methods to test potential interactions between TRPC3 and PH\textsubscript{C} of PLC-\textsubscript{y1}. No binding could be detected when we tried to use GST-fused PH\textsubscript{C} to “pull down” TRPC3 (supplemental Fig. 2B).

Table 1

<table>
<thead>
<tr>
<th>Structure statistics for the family of 15 structures of the joined PH\textsubscript{N}-PH\textsubscript{C} domain</th>
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<tbody>
<tr>
<td>None of the structures exhibits distance violations greater than 0.3 Å or dihedral angle violations greater than 4°. r.m.s. indicates root mean square.</td>
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**Distance restraints**

- Intraresidue (|i−j| = 0) 837
- Sequential (|i−j| = 1) 501
- Medium range (2 ≤ |i−j| ≤ 4) 220
- Long range (5 ≤ |i−j| ≤ 7) 465
- Hydrogen bonds 62
- Total 2085

**Dihedral angle restraints**

| φ | 31 |
|Ψ | 29 |
|Total | 60 |

**Mean r.m.s. deviations from the experimental restraints**

- Distance (Å) 0.008 ± 0.000
- Dihedral angle (°) 0.003 ± 0.005

**Mean r.m.s. deviations from idealized covalent geometry**

- Bond (Å) 0.001 ± 0.000
- Angle (°) 0.291 ± 0.004
- Improper (°) 0.125 ± 0.004

**Mean energies (kcal mol\textsuperscript{−1})**

- E\textsubscript{vdw} 10.26 ± 0.44
- E\textsubscript{elas} 0.45
- E\textsubscript{elec} −368 ± 19

**Ramachandran plot**

- Residues 1–33 and 90–150: 74.6
- Additional allowed regions: 20.0
- Generously allowed regions: 4.6
- Disallowed regions: 0.8

**Atomic r.m.s. difference (Å)**

- Residues 1–11, 16–31, 91–118, and 125–147: 0.45
- Backbone heavy atoms (N, C\textsubscript{α}, and C\textsubscript{β}): 0.93
- Heavy atoms:

   - (A) The final values of the square–well NOE and dihedral angle potentials were calculated with force constants of 50 kcal mol\textsuperscript{−1} Å\textsuperscript{−2} and 200 kcal mol\textsuperscript{−1} rad\textsuperscript{−2}, respectively.
   - (B) The program Procheck (40) was used to assess the overall quality of the structures.
   - (C) The precision of the atomic coordinates is defined as the average r.m.s. difference between 15 final structures and the mean coordinates of the protein.
presence of brain liposomes or in vitro reconstituted PC/PS lipid containing 10% phosphatidylinositol 4,5-P2 (8). Again, no interaction could be detected between TRPC3 and PHC of PLC·/H9253 (Fig. 5E). To rule out the possibility that the negative binding between TRPC3 and PHC might result from an unanticipated alteration of PHC, we studied the interaction between two complementary halves of the PH domain (i.e. PHN and PHC). When thioredoxin-fused PHN and 15N-labeled PHC were mixed together, the interaction of the two halves of the PH domain induced de novo folding of both fragments. Furthermore, the NMR spectrum showed that the peaks from the 15N-labeled PHC mixture overlap very well with a subset of peaks corresponding to the PHC portion of the PHN-PHC fusion protein (Fig. 5D, red dots), indicating that the PHC fragment used in the TRPC3 binding assay contains all necessary structural features for the C-terminal half of the split PH domain. Our NMR data further demonstrated that the two cognate halves, probably only two cognate halves, of the split PH domain of PLC·/H9253 could interact intermolecularly to form a PH domain structure indistinguishable from that of the halves covalently connected together. Because the SH3 domain of PLC·/H9251 had been suggested to play critical roles in mediated lipase-independent activities of PLC·/H9251 (14–17), we tested whether a longer fragment of PLC·/H9251 containing both the SH3 domain and the PHC portion might interact with TRPC3. Again, we...
were not able to detect any interaction between this longer fragment of PLC-γ1 and TRPC3 (Fig. 5F).

DISCUSSION

The second PH domain of PLC-γ is unique among ~200 PH domains found in each mammalian genome because the domain is split into two halves by insertion of tandem repeats of SH2SH2SH3 domains. It was not known prior to this study whether the split PH domain of PLC-γ is folded. We wondered if the split PH domain folds into a canonical PH domain structure and what the consequence of the SH2SH2SH3 insertion is to the structure of the split PH domain. The solution structure of the joined PHc-PHe domain of PLC-γ1 determined in this study is the second three-dimensional structure solved for split PH domains. Similar to the structure of the split PH domain of α-syntrophin (8), the split PH
domain of PLC-γ1 folds into a canonical PH domain structure. Detailed comparison does reveal significant differences between the two split PH domains with known structures. The most significant differences are the flexibilities in the loop regions connecting β1/β2 and β5/β6. The conformations of the β1/β2 and β5/β6 loops of the split PH domain of PLC-γ1 are partially defined (Fig. 2A). In contrast, the corresponding loops in the split PH domain of syntrophin are highly flexible. We further showed that the SH2H2H3 insertion does not alter the structure of the split PH domain. The 300-residue SH2H2H3 tandem domains are inserted between the β3 and β4 strands of the split PH domain (Fig. 2). Curiously, the position of the PDZ domain insertion also falls between the β3 and β4 strands of the α-syntrophin split PH domain (8).

Amino acid sequence analysis of the split PH domain of myosin X also predicts that the domain is split into two halves by insertion of another intact PH domain between the β3 and β4 strands. We do not know whether this is just a coincidence or if insertion between the β3 and β4 strands is a requirement for proper folding of a split PH domain.

PH domains that are known to bind to phospholipids with medium to high affinities typically contain the KXn(K/R)XR signature motif with three highly conserved basic residues located at the C and N termini of the β1 and β2 strands, respectively (29–35). In contrast, the PH domain of PLC-γ1 lacks these three basic residues within this so-called signature motif. Additionally, residues from the β3/β4 loop of the PH domains are also known to be intimately involved in binding to head groups of phosphatidylinositol phosphate lipids (29–35). The insertion of bulky SH2H2H3 domains in the β3/β4 loop of the split PH domain of PLC-γ1 would interfere with its lipid-binding capacity. Consistent with this observation, the split PH domain either alone or together with the SH3-PHC tandem of PLC-γ1 is known to bind to phosphatidylinositol phosphate lipids with high affinity (37). A tempting hypothesis is that the split PH domain of PLC-γ1 plays structural roles in regulating the activities of the enzyme. It is
interesting to note that the catalytic X- and Y-boxes of PLC-γ1 represent another example of a split domain organization, and the two halves of the catalytic domain are split by the \( \text{PH}_1\)-SH2SH2SH3-PH\(_C\) supramodule (Fig. 1A). The structure of the split PH domain indicates that the two halves of the split PH domain bring X- and Y-boxes of PLC-γ1 into close proximity. By the same token, any conformational changes to the split PH domain could alter the assembly of the X and Y boxes, thereby influencing the catalytic activity of the enzyme. However, we found that the split PH domains of PLC-γ1 in the \( \text{PH}_1\)-SH2SH2SH3-PH\(_C\) supramodule, as well as that formed by the two separate halves of the domain, are highly stable. It is unlikely that the two halves of the split PH domain will spontaneously dissociate or undergo major conformational changes. If this indeed exists, the split PH domain-mediated enzyme activity regulation requires regulated conformational changes of the split PH domain. Such conformational changes can be induced either by direct alteration of the split PH domain structure (e.g. by binding to certain factors) or by allosteric changes induced by binding of ligand(s) to the SH2 and/or SH3 domains splitting the PH domain. As a first step, we used a peptide ligand specific to the C-terminal SH2 domain to test this hypothesis. We found that ligand binding to the C-terminal SH2 domain does not alter the conformation of the split PH domain. However, further work is required to validate the above hypothesis, as the SH2 and SH3 domains of PLC-γ are known to interact with numerous proteins (11).

A number of lipase-independent functions of PLC-γ have been shown to associate directly with the split PH domain (14–18). Perhaps the most important function of the split PH domain of PLC-γ1 reported to date is the direct interaction between \( \text{PH}_1\) of the enzymes with the TRPC3 calcium channel (17, 18), as this interaction allows direct coupling of PLC-γ1 to agonist-induced calcium entry. Using yeast two-hybrid assays, van Rossum et al. (18) showed that a 7-residue fragment from 40 to 46 in the N-terminal intracellular domain of TRPC3 is necessary and sufficient for direct binding to \( \text{PH}_C\) (residues 861–920) of PLC-γ1. The authors further suggested that the N-terminal fragment (residue 1–46) of TRPC3 represents a hidden partial PH domain that is complementary to \( \text{PH}_C\) of PLCγ1. In the same study, it was demonstrated that the PH domain formed by the intermolecular two halves of the PH domain from TRPC3 and PLC-γ1 was shown to be able to bind to phosphatidylinositol 4,5-P\(_2\) phospholipids. To advance this important hypothesis, detailed biochemical and structural studies of the interaction between TRPC3 and PLC-γ1 were urgently needed and thus motivated this study. The TRPC3 fragment used for detailed analysis in this study contained the N-terminal 52 residues, which contains the 7-residue PLC-γ1-binding sequence reported by van Rossum et al. (18). Additionally, domain structure analysis using SMART (38) of a merged sequence containing this 52-residue TRPC3 fragment followed by PH\(_C\) of PLC-γ1 (residues 851–933) indeed suggested the existence of a PH domain-like fold. However, detailed biochemical studies using several different approaches described in this study argued against a direct interaction between the N-terminal TRPC3 fragment and the \( \text{PH}_C\) half of PLC-γ1. The most direct and convincing experiment was the NMR-based binding assay, the results of which are shown in Fig. 5. Simple mixing of isolated \( \text{PH}_1\) and \( \text{PH}_C\) of PLC-γ1 resulted in direct binding and de novo folding of the two halves of the PH domain. In contrast, mixing of the N-terminal TRPC3 fragment with the same \( \text{PH}_C\) half of PLC-γ1 showed no detectable interaction between the two fragments. We further showed that phospholipids do not promote the interaction between the two fragments from TRPC3 and PLC-γ1. We note that the \( \text{PH}_C\) half of PLC-γ1 used in the study by van Rossum et al. (18) lacked a large part (~10 residues) of the C-terminal α-helix. The compact structures of the split PH domain of PLC-γ1 (Fig. 2) and those of other PH domains predict that removal of the C-terminal α-helix would compromise the folding of PH domains. Additionally, the 7-residue fragment of TRPC3 that was shown to be sufficient for binding to PLC-γ1 would be much too short to encompass the β1 to β3 strands of a PH domain. We conclude, based on our biochemical studies and amino acid sequence analysis, that the N-terminal TRPC3 fragment and the \( \text{PH}_C\) half of PLC-γ1 are unlikely to interact with each other to form an inter-molecular PH domain fold. Additionally, we did not observe any direct interactions of the N-terminal TRPC3 fragment with several longer forms of PLCγ1, including SH3-\( \text{PH}_C\) and the \( \text{PH}_1\)-SH2SH2SH3-PH\(_C\) supramodule. Given the strong evidence supporting direct coupling between PLC-γ1 and the TRPC3 calcium channel and the lipase-independent role of PLC-γ1 in this coupling (17, 18), our study points to an alternative interaction mechanism between these two important signaling molecules other than direct intermolecular PH domain complementation. For example, the C2 domain located at the carboxyl tail of PLC-γ1 was shown to bind directly to another calcium channel TRPM7 (39). Alternatively, the interaction between TRPC3 and PLC-γ1 could be indirect and mediated by an unknown factor. Further work is required to elucidate the exact interaction mechanism between PLC-γ1 and TRPC3.

Acknowledgments—We thank Dr. G. Carpenter for the rat PLC-γ1 cDNA clone and Drs. J. H. Wang and V. Unkefer for critical reading and comments on the manuscript. The NMR spectrometer used in this work was purchased with funds donated to the Biotechnology Research Institute by the Hong Kong Jockey Club.

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Split PH Domain of PLC-γ1