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

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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.

PH[3] 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 syntrophin 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-syntrophin 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-syntrophin.

Phospholipase C (PLC) hydrolyzes phosphatidylinositol 4,5-biphosphate to produce the second messengers inositol 1,4,5-triphosphate 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 ζ (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 isozymes 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. 1*A*). 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 Ca^{2+} entry via the TRPC3

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The atomic coordinates and structure factors (code 2FJL) have been deposited in the Protein Data Bank, Research Collaboratory for Structural Bioinformatics, Rutgers University, New Brunswick, NJ (http://www.rcsb.org/).

[S] The on-line version of this article (available at http://www.jbc.org) contains supplemental Figs. 1–3.

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[3] The abbreviations used are: PH, pleckstrin homology; PLC, phospholipase C; NOE, nuclear Overhauser effect; NOESY, nuclear Overhauser effect spectroscopy; GST, glutathione *S*-transferase; TOCSY, total correlation spectroscopy; HSQC, heteronuclear single quantum coherence; SH, Src homology.

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 (PLCγ1-PH_C) can directly associate with TRPC3. Very recently, the same research group showed that PLCγ1-PH_C 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 PLCγ1-PH_C-binding segment of TRPC3 represents a complementary partial PH domain "hidden" in the ion channel. They demonstrated that binding of the two partial PH domain fragments from PLC-γ1 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 PLCγ1 can fold into a canonical PH domain structure; whether PLCγ1-PH_C 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 PLCγ1 and TRPC3 (or PLCγ1 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 PLC-γ1. 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 PLCγ1-PH_C with the hypothetical hidden PH_N fragment from TRPC3.

MATERIALS AND METHODS

Protein Expression and Purification—The joined PH_N-PH_C domain (residues 489–547 and 851–933), PH_C fragment (residues 851–933), and the PH_N-SH2SH2SH3-PH_C (residues 489–933) of rat PLC-γ1 were cloned into a modified version of the pET32a vector (21). The joined PH_N-PH_C 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 PH_N fragment (residues 489–547) were cloned into the pET32a vector. GST-fused PH_N-PH_C, the PH_C fragment, and the SH3-PH_C fragment (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 isopropyl β-D-thiogalactoside at 16 °C overnight. Uniformly ^{15}N- and $^{15}N/^{13}C$-labeled proteins were prepared by growing bacteria in M9 medium containing $^{15}NH_4Cl$ with or without $^{13}C_6$-glucose. The His-tagged fusion proteins were purified under native conditions using an Ni^{2+}-nitrilotriacetic acid-agarose (Qiagen) affinity chromatography. The remaining small amount of contaminant proteins was removed by size-exclusion chromatography. The GST-fused proteins were purified using GSH-Sepharose affinity chromatography followed by size-exclusion chromatography.

Lipid Binding Assay—Liposomes consisting of total bovine brain lipids were prepared by resuspending brain lipid extracts (Folch fraction I, Sigma B1502, which contains ~10% phosphatidylinositol lipids) at 2 mg/ml in a buffer containing 20 mM HEPES, pH 7.4, 150 mM NaCl, 1 mM dithiothreitol. The protein sample (5–10 μM) was incubated with 0.6 mg/ml liposomes in 40 μl of buffer for 15 min at room temperature and then spun at 65,000 × *g* for 15 min at 4 °C in a Beckman TLA100.1 rotor. The supernatants were removed for determination of proteins not bound to liposomes. The pellets were washed twice with the same buffer and brought up to the same volume as the supernatant. The supernatant and the pellet proteins were subjected to SDS-PAGE and visualized by Coomassie Blue staining.

Pull-down Experiments—Purified GST-PH_C, GST-PH_N-PH_C, or GST-SH3-PH_C (10 μg) was mixed with purified Trx-TRPC3-(1–52) (100 μg) with or without the presence of 0.5 mg/ml of brain liposome. Then the complexes were pelleted with 30 μl of fresh GSH-Sepharose beads (Amersham Biosciences). The pelleted beads were washed extensively with phosphate-buffered saline buffer and subsequently boiled with 2× SDS-PAGE sample buffer. The proteins were resolved by SDS-PAGE and visualized by Coomassie Blue staining.

NMR Spectroscopy—NMR samples contained ~1.0 mM of the PH_N-PH_C tandem in 50 mM potassium phosphate, pH 6.5, in 90% H_2O, 10% D_2O or 99.9% D_2O. 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-TOCSY experiments. The side chains of aromatics were assigned by standard ^1H two-dimensional TOCSY/NOESY experiments.

Structure Calculations—Approximate interproton distance restraints were derived from the NOESY spectra (a ^1H two-dimensional homonuclear NOESY, a ^{15}N-separated NOESY, and a ^{13}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), 1.8–3.3 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 (φ and ψ angles) 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 ^1H-^{15}N HSQC spectra of ^{15}N-labeled protein samples (~0.2 mM) with or without addition of their respective binding partners at natural abundance. The N-terminal SH2 domain-binding phosphotyrosine peptide (D{pY}IIPLPDP) 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 PH_N and PH_C) 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 PH_N-SH2SH2SH3-PH_C supramodule, resulting in a fusion protein with the two halves of the split PH domain connected directly (*i.e.* PH_N-PH_C). The recombinant PH_N-PH_C was eluted at a molecular mass indicative of a stable monomer when analyzed by analytical gel filtration chromatography (data not shown). The well dispersed ^1H, ^{15}N HSQC spectrum indicates that the joined PH_N-PH_C is well folded (Fig. 1*B*, *black dots*). It is possible that the covalent linkage of PH_N and PH_C may artificially induce folding of the linked protein. To address this possibility, in the middle of the linking sequence of PH_N-PH_C, we inserted an 8-residue peptide fragment that can be cleaved by protease 3C. Digestion of PH_N-

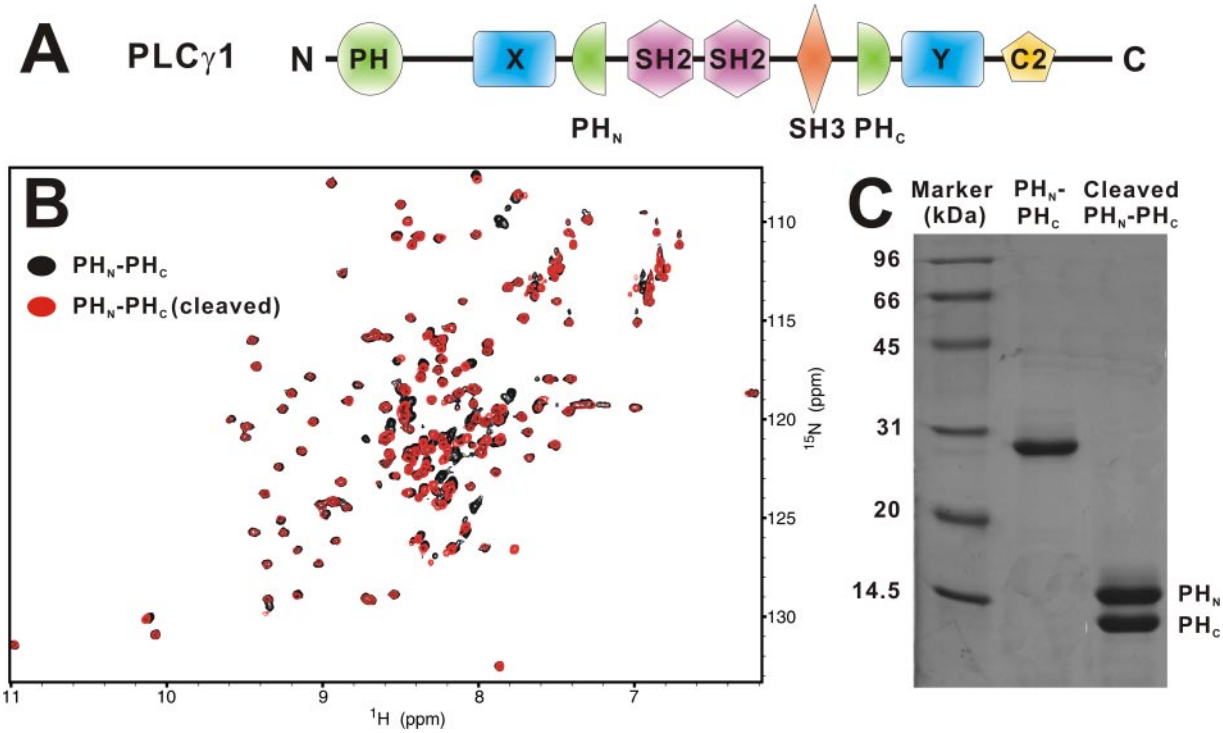

FIGURE 1. **Folding and interaction of the two halves of the split PH domain from PLC-γ1.** *A,* schematic diagram showing the domain organization of PLC-γ1. *B,* overlay plot of the ^{1}H, ^{15}N HSQC spectra of the joined PH$_N$-PH$_C$ (*black dots*) and its protease 3C cleaved form (*red dots*). *C,* SDS-PAGE showing the purification of the joined PH$_N$-PH$_C$ domain and protease 3C cleavage of the domain into PH$_N$ and PH$_C$ fragments.

PH$_C$ with protease 3C produces two fragments with molecular masses corresponding to PH$_N$ and PH$_C$, respectively (Fig. 1*C*). The NMR spectrum of the protease 3C-cleaved PH$_N$-PH$_C$ is essentially identical to that of the uncleaved protein (Fig. 1*B*), indicating that the covalent linkage between PH$_N$ and PH$_C$ is dispensable to the folding of the split PH domain in PLC-γ1. Furthermore, both the joined and cleaved PH$_N$-PH$_C$ 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 PH$_N$-PH$_C$ by NMR spectroscopy (Fig. 2 and Table 1). The PH$_N$ and PH$_C$ 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 PH$_N$ half is composed of three β-strands (β1–β3), and the PH$_C$ 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 ^{1}H, ^{15}N 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 PLCγ1 are highly conserved throughout evolution (Fig. 3*A*). When compared with a number of PH domains that bind to phosphoinositide head groups with high affinities (29–33), the split PH domain of PLCγ1 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_{μ}(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. 3*A*) (32–35). These conserved basic

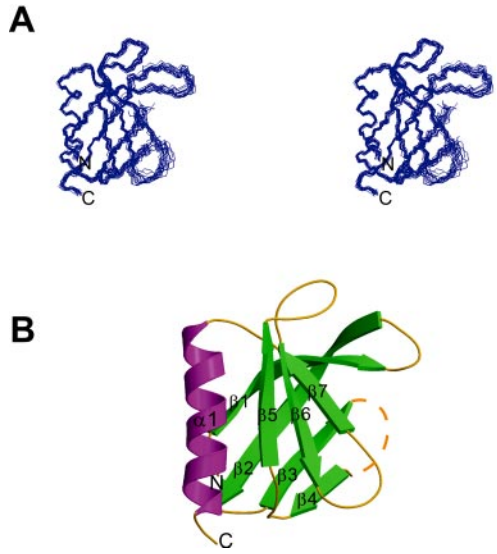

FIGURE 2. **Structure of the joined PH$_N$-PH$_C$ domain.** *A,* stereo view showing the backbones of 15 superimposed NMR-derived structures of the joint PH$_N$-PH$_C$ domain. In this drawing, the flexible β3/β4-loop is shown in *gray*. *B,* ribbon diagram of a representative structure of the PH domain. The flexible β3/β4-loop is omitted and indicated by a *dashed line*.

residues play critical roles in binding to negatively charged phosphate groups from the head groups of phosphoinositides (see Fig. 3*B* 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 phosphoinositol 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

TABLE 1

Structural statistics for the family of 15 structures of the joined PH_N-PH_C domain

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.

Distance restraints	
Intraresidue ($i - j = 0$)	837
Sequential ($\|i - j\| = 1$)	501
Medium range ($2 \leq \|i - j\| \leq 4$)	220
Long range ($\|i - j\| > 5$)	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^{-1})	
E_{NOE}[a]	10.26 ± 0.44
E_{cdih}[a]	0.00 ± 0.00
$E_{L\text{-}J}$	−368 ± 19
Ramachandran plot[b]	
Residues 1–33 and 90–150	
% residues in the most favorable regions	74.6
Additional allowed regions	20.0
Generously allowed regions	4.6
Disallowed regions	0.8
Atomic r.m.s. difference (Å)[c]	
Residues 1–11, 16–31, 91–118, and 125–147	
Backbone heavy atoms (N, Cα, and C′)	0.45
Heavy atoms	0.93

[a] The final values of the square-well NOE and dihedral angle potentials were calculated with force constants of 50 kcal mol^{-1} Å^{-2} and 200 kcal mol^{-1} rad^{-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.

module. To test this hypothesis, we assayed the binding of the joined PH_N-PH_C domain of PLCγ1 to liposomes prepared from total bovine brain lipids. As predicted, the split PH domain of PLC-γ1 showed no detectable binding to brain liposomes (Fig. 3*C*). We further demonstrated that the PH_N-SH2SH2SH3-PH_C supramodule of PLCγ1 does not bind to brain liposomes either, indicating that the SH2SH2SH3 insertion does not alter the lipid binding property of the split PH domain (Fig. 3*C*).

The SH2SH2SH3 insertion is known to play important roles in regulating the enzyme activities of PLC-γ1. It has been suggested, based on indirect experimental evidence, that the insertion may change the structure of the split PH domain, thereby influencing the assembly of the catalytic X and Y boxes (10, 11). We used NMR spectroscopy to investigate potential structural changes that the SH2SH2SH3 insertion might exert on the split PH domain. As shown in Fig. 4*A* and supplemental Fig. 3, the HSQC spectrum of the joined PH_N-PH_C domain overlaps well with a subset of peaks from the HSQC spectrum of the PH_N-SH2SH2SH3-PH_C supramodule, indicating that the insertion of the SH2SH2SH3 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 SH2SH2SH3 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_N-SH2SH2SH3-PH_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-γ1 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_N-SH2SH2SH3-PH_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. 4*C*). Residue-specific chemical shift assignments of the PH_N-SH2SH2SH3-PH_C supramodule are required for correlating the peptide-induced shift changes to the individual residues within the SH2SH2SH3 domains.

Characterization of the Interaction between the Split PH Domain of PLC-γ1 and TRPC3—Having characterized the structure of the split PH domain of PLC-γ1 in detail, we went on to study the earlier reported interaction between PLC-γ1 and TRPC3 (17, 18), hoping to lay a foundation for structural characterization of the PLCγ1-TRPC3 complex. We were able to obtain large quantities of recombinant proteins encompassing the N-terminal 52 residues of TRPC3 (Fig. 5*A*), 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-γ1-binding sequence reported by van Rossum *et al.* (18). We tested potential direct interaction between PH_N-PH_C of PLC-γ1 and TRPC3 by titrating the unlabeled TRPC3 fragment to the ^{15}N-labeled PH_N-PH_C at a concentration of ~0.1 mM. No chemical shift changes to PH_N-PH_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-γ1. Consistent with the above NMR-based assay, no binding between PH_N-PH_C and TRPC3 was detected when we used purified GST-fused TRPC3 to "pull-down" PH_N-PH_C (or GST-fused PH_N-PH_C to pull-down the TRPC3 fragment) (supplemental Fig. 2*A*).

It is possible that TRPC3 only binds to PLC-γ1 when the PH_C half is somehow dissociated from the PH_N half. We set out to test this hypothesis. Sufficient amounts of pure, recombinant PH_C could be obtained, and this half of the PH domain remained soluble at concentrations up to 0.5 mM (Fig. 5, *A* and *B*). The backbone amide resonances seen in the ^{1}H, ^{15}N HSQC spectrum of PH_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. 5*B*). Circular dichroism spectrum also confirmed that PH_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 ^{15}N-labeled PH_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_C (Fig. 5, *B* and *C*), indicating that even the PH_C half of PLC-γ1 alone does not interact with TRPC3 (or under this assay condition, the dissociation constant of the TRPC3/PH_C mixture is higher than 10^{-2} M). We also used several biochemical methods to test potential interactions between TRPC3 and PH_C of PLCγ1. No binding could be detected when we tried to use GST-fused PH_C to "pull down" TRPC3 (supplemental Fig. 2*B*). No interaction was detected when the purified TRPC3 fragment and PH_C were co-injected into an analytical gel filtration column (data not shown). Finally, we tested the possibility that the interaction between TRPC3 and PH_C of PLC-γ1 may be dependent on the presence of phosphoinositol lipids. We incubated GST-fused TRPC3 with PH_C in the

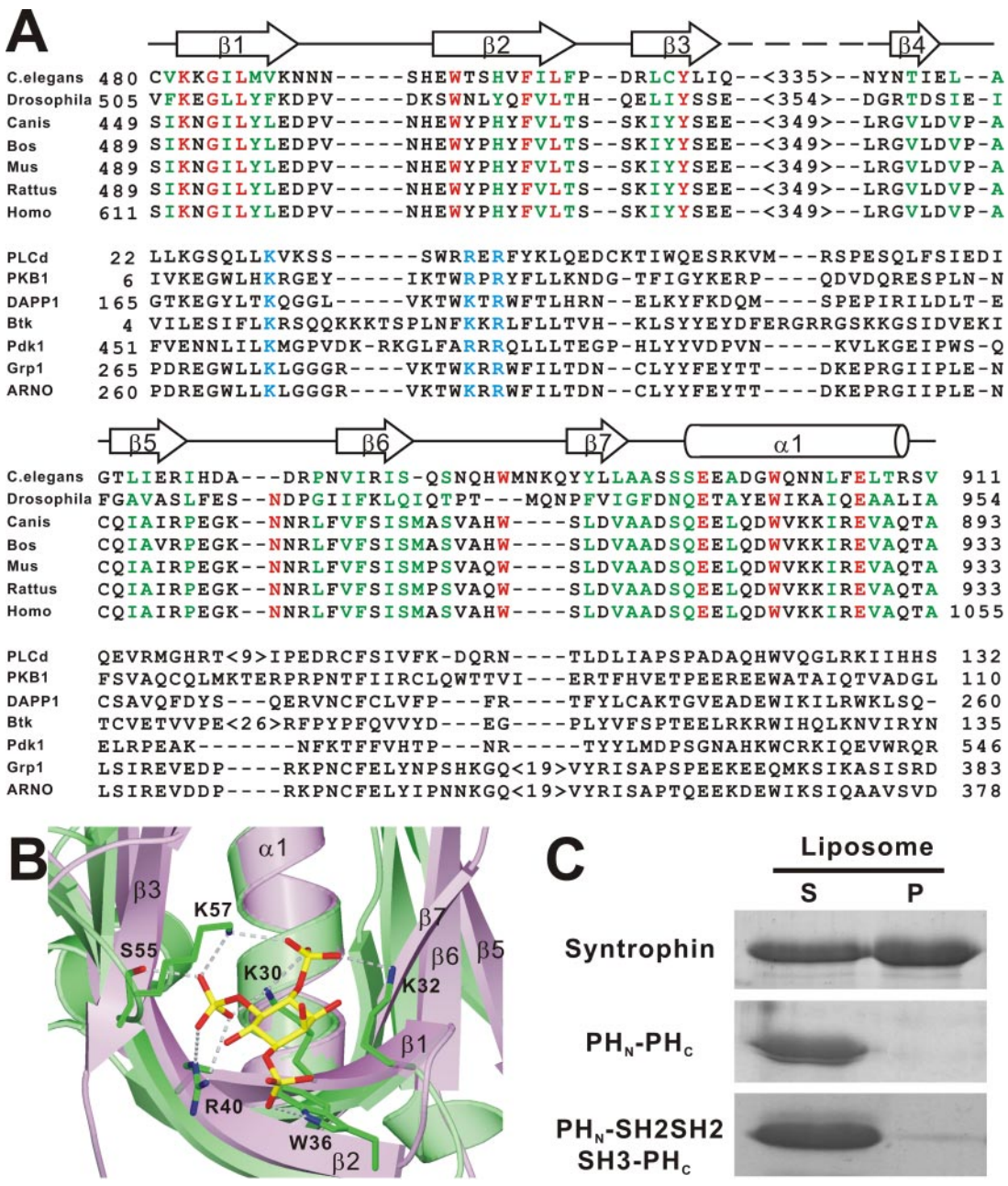

FIGURE 3. **Structural features of the split PH domain of PLC-γ1.** *A,* amino acid sequence alignment of the split PH domains of PLC-γ1 family proteins (*upper panel*) and structure-based sequence alignment of some PH domains known to bind to lipids specifically (*lower panel*). In the sequence alignment of PH domains from PLC-γ1, the absolutely conserved amino acids are shown in *red*, the highly conserved residues in *green*, and the variable residues in *black*. The residues from the phosphoinositide-binding signature motifs of other PH domains are highlighted in *cyan*. The secondary structure of the PLC-γ1 PH$_N$-PH$_C$ domain is also included at the top of the figure. *B,* comparison of the phosphatidylinositol phosphate lipid head binding pocket of the PLC-δ1 PH domain (*green*) with the same region of the PLC-γ1 PH$_N$-PH$_C$ domain. The residues from the PLC-δ1 PH domain responsible for binding to for inositol 1,4,5-trisphosphate are drawn using explicit atom representation. *C,* brain liposome-based lipid binding assay of the joined PH$_N$-PH$_C$ domain and the PH$_N$-SH2SH2SH3-PH$_C$ supramodule. "*S*" and "*P*" denote proteins recovered in the supernatants and pellets, respectively, in the centrifugation-based liposome binding assays. The PH$_N$-PDZ-PH$_C$ supramodule of α-syntrophin is used as a positive control.

presence of brain liposomes or *in vitro* reconstituted PC/PS liposome containing 10% phosphatidylinositol 4,5-P_2 (8). Again, no interaction could be detected between TRPC3 and PH$_C$ of PLC-γ1 (Fig. 5*E*). To rule out the possibility that the negative binding between TRPC3 and PH$_C$ might result from an unanticipated alteration of PH$_C$, we studied the interaction between two complementary halves of the PH domain (*i.e.* PH$_N$ and PH$_C$). When thioredoxin-fused PH$_N$ and ^{15}N-labeled PH$_C$ 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 ^{15}N-labeled PH$_C$ mixture overlap very well with a subset of peaks corresponding to the PH$_C$ portion of the PH$_N$-PH$_C$ fusion protein (Fig. 5*D*, *red dots*), indicating that the PH$_C$ 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-γ1 could interact intermolecularly to form a PH domain structure indistinguishable from that of the halves covalently connected together.

Because the SH3 domain of PLCγ1 had been suggested to play critical roles in mediated lipase-independent activities of PLCγ1 (14–17), we tested whether a longer fragment of PLC-γ1 containing both the SH3 domain and the PH$_C$ portion might interact with TRPC3. Again, we

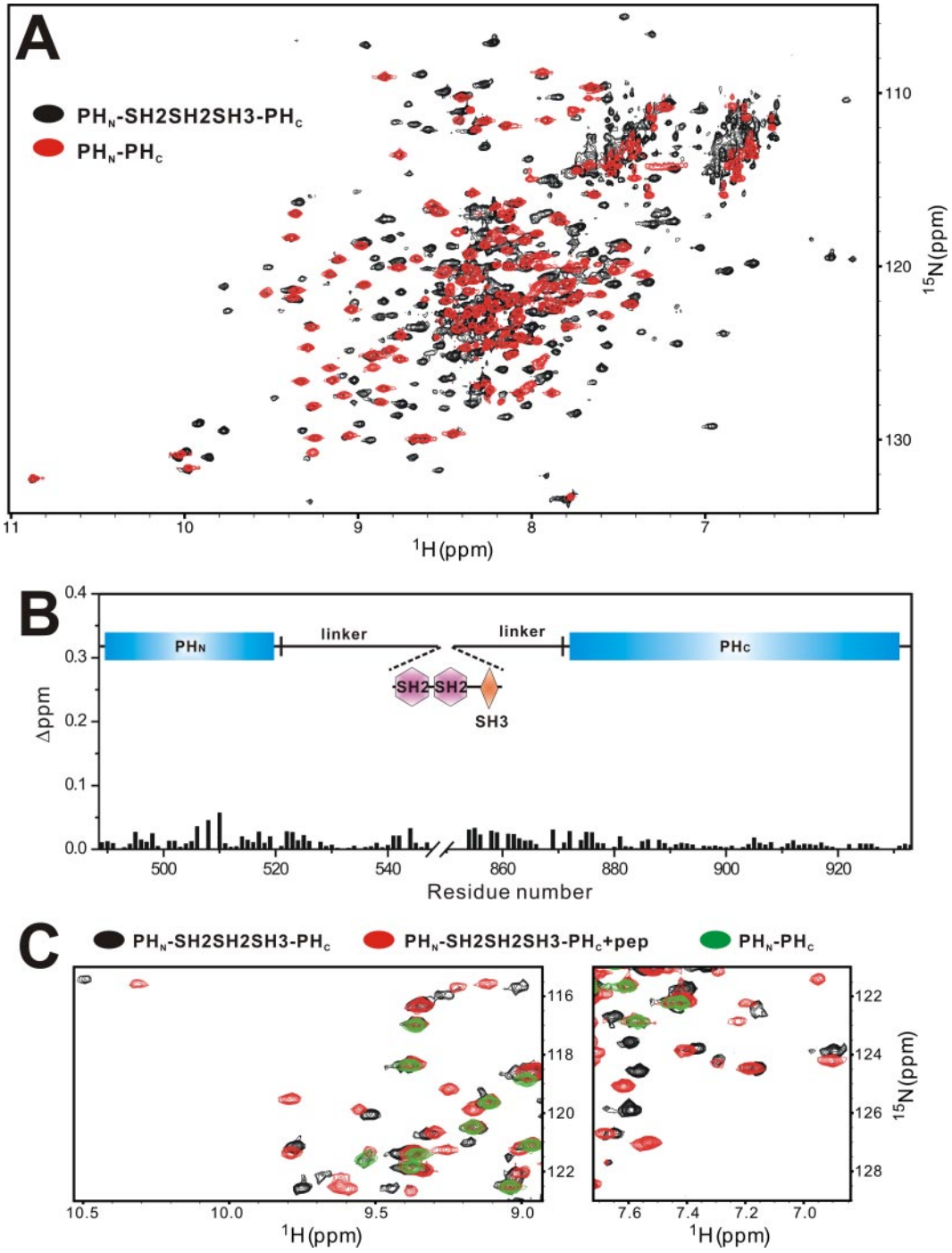

FIGURE 4. **Structural comparison of the PLC-γ1 PH domain in the form of the joined PH$_N$-PH$_C$ domain and in the PH$_N$-SH2SH2SH3-PH$_C$ supramodule.** *A,* superposition plots of ^{1}H, ^{15}N HSQC spectra of the PH$_N$-SH2SH2SH3-PH$_C$ tandem (*black dots*) and the joined PH$_N$-PH$_C$ domain (*red dots*). *B,* plot of the backbone amide chemical shift differences as a function of the residue number of the split PH domains in the PH$_N$-SH2SH2SH3-PH$_C$ supramodule with and without the addition of the "D{pY}IIPLPDP" peptide. The combined ^{1}H and ^{15}N chemical shift changes are defined as follows: $\Delta_{ppm} = ((\Delta\delta_{HN})^2 + (\Delta\delta_N \times \alpha_N)^2)^{1/2}$, where $\Delta\delta_{HN}$ and $\Delta\delta_N$ represent the chemical shift differences of amide proton and nitrogen chemical shifts of each residue. The scaling factor (α_N) used to normalize the ^{1}H and ^{15}N chemical shifts is 0.17. The domain organization of PH$_N$-SH2SH2SH3-PH$_C$ is indicated at the *top* of the plot. *C,* superposition plot of ^{1}H, ^{15}N HSQC spectra of the joined PH$_N$-PH$_C$ domain (*green*), the free PH$_N$-SH2SH2SH3-PH$_C$ supramodule (*black dots*), and the D{pY}IIPLPDP peptide-bound form of the supramodule (*red dots*). For clarity, only two selected regions of each spectrum are shown.

were not able to detect any interaction between this longer fragment of PLC-γ1 and TRPC3 (Fig. 5*F*).

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 PH$_N$-PH$_C$ 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

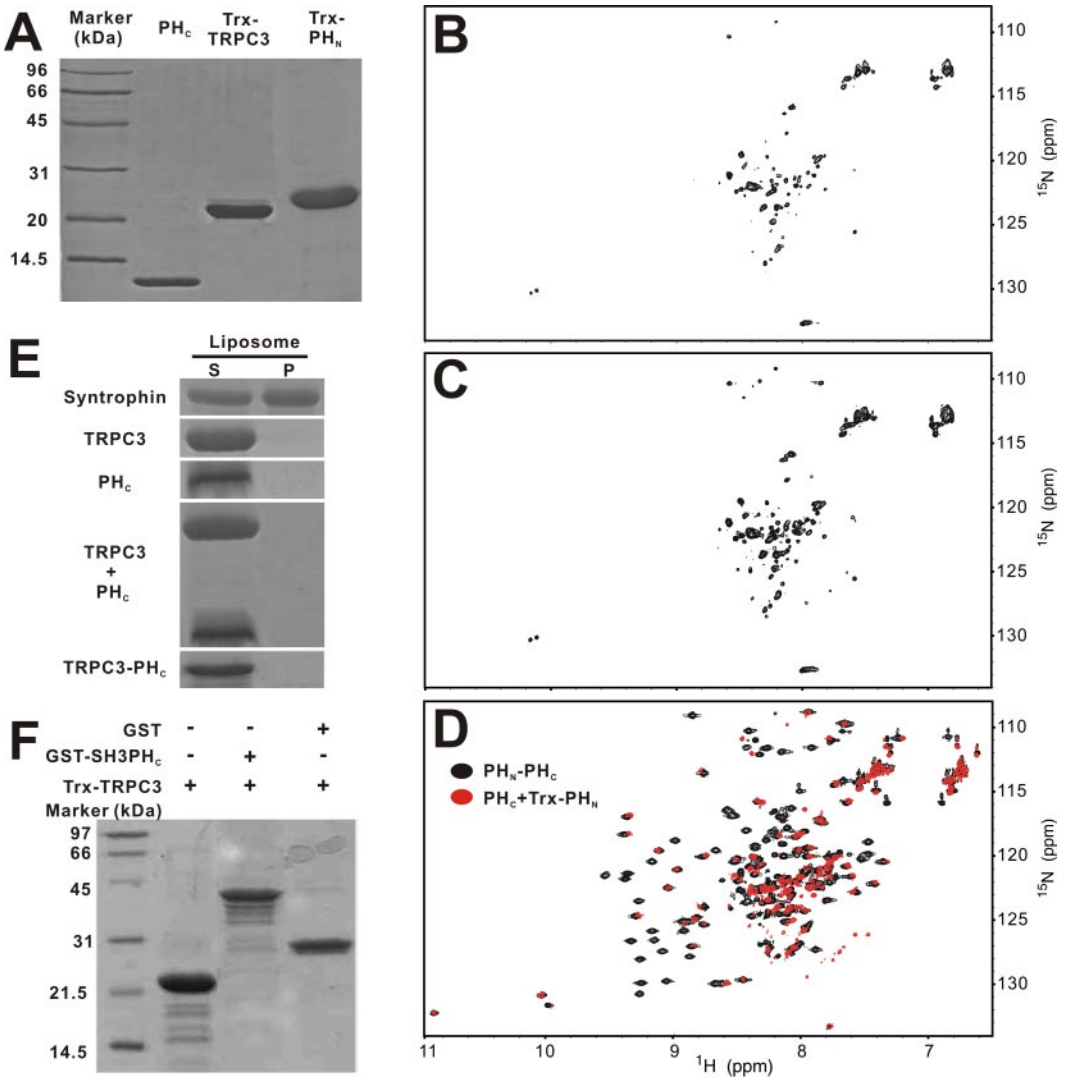

FIGURE 5. **Biochemical characterization of the interaction between the split PH domain of PLC-γ1 and TRPC3.** *A,* SDS-PAGE showing the purification of ^{15}N-labeled PH_C of PLC-γ1, unlabeled Trx-TRPC3, and unlabeled Trx-PH_N. *B* and *C,* ^{1}H, ^{15}N HSQC spectra corresponding to the isolated PH_C fragment and the same protein mixed with two molar ratio amounts of unlabeled Trx-TRPC3, respectively. *D,* overlay plot of the ^{1}H, ^{15}N HSQC spectra of the joined PH_N-PH_C (*black dots*), and the 1:1 molar ratio mixture of ^{15}N-labeled PH_C and unlabeled Trx-PH_N (*red dots*). *E,* brain liposome-based lipid binding assay of binding of various protein mixtures. In this panel, "Trx-TRPC3-PH_C" denotes a fusion protein with the N-terminal 52-residue TRPC3 fragment fused with the PH_C half of PLC-γ1. "Trx-TRPC3 + PH_C" represents an equal molar mixture of the purified N-terminal TRPC3 fragment with PH_C of PLC-γ1. *S* and *P* denote proteins recovered in the supernatants and pellets, respectively, in the liposome binding assays. Again, PH_N-PDZ-PH_C of α-syntrophin serves as the positive control. *F,* pull-down assay of potential interactions between the N-terminal 52-residue fragment of TRPC3 and the SH3-PH_C tandem of PLC-γ1. In this assay, the SH3-PH_C tandem is in the GST-fused form.

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. 2*A*). In contrast, the corresponding loops in the split PH domain of syntrophin are highly flexible. We further showed that the SH2SH2SH3 insertion does not alter the structure of the split PH domain. The 300-residue SH2SH2SH3 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 $KX_n(K/R)XR$ 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 SH2SH2SH3 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 SH2SH2SH3 insertion does not show detectable phospholipid binding in our liposome-based lipid binding assay (Fig. 3). This observation is perhaps not surprising, as the N-terminal PH domain 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 PH_N-SH2SH2SH3-PH_C supramodule (Fig. 1*A*). 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 PH_N-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 PH_C of the enzymes with the TRPC3 calcium channel (17, 18), as this interaction allows direct coupling of PLCγ with 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 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 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 was 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 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 PH_N and 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 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 PH_C half of PLCγ1 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 PLCγ1 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 PH_C half of PLCγ1 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-PH_C and the PH_N-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.

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