

Structure of the split PH domain and distinct lipid-binding properties of the PH–PDZ supramodule of α -syntrophin

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Pleckstrin homology (PH) domains play diverse roles in cytoskeletal dynamics and signal transduction. Split PH domains represent a unique subclass of PH domains that have been implicated in interactions with complementary partial PH domains 'hidden' in many proteins. Whether partial PH domains exist as independent structural units alone and whether two halves of a split PH domain can fold together to form an intact PH domain are not known. Here, we solved the structure of the PH_N–PDZ–PH_C tandem of α -syntrophin. The split PH domain of α -syntrophin adopts a canonical PH domain fold. The isolated partial PH domains of α -syntrophin, although completely unfolded, remain soluble in solution. Mixing of the two isolated domains induces *de novo* folding and yields a stable PH domain. Our results demonstrate that two complementary partial PH domains are capable of binding to each other to form an intact PH domain. We further showed that the PH_N–PDZ–PH_C tandem forms a functionally distinct supramodule, in which the split PH domain and the PDZ domain function synergistically in binding to inositol phospholipids.

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Introduction

Syntrophins are a family of multi-domain scaffold proteins that associate with dystrophin and the dystrophin-related proteins, utrophin and dystrobrevin (Adams *et al.*, 1993; Ahn *et al.*, 1994; Yang *et al.*, 1994). All five members (α , β 1, β 2, γ 1, and γ 2) of the syntrophin family share the same domain organization: an N-terminal split PH domain containing an embedded PDZ domain, a central PH domain, and a C-terminal syntrophin unique domain (SU) (Figure 1A; Adams

et al., 1993; Ahn *et al.*, 1994; Yang *et al.*, 1994; Piluso *et al.*, 2000). The second PH domain and the SU domain of syntrophins are responsible for binding to dystrophin, utrophin, and dystrobrevin (Kramarcy *et al.*, 1994; Ahn and Kunkel, 1995; Suzuki *et al.*, 1995; Kachinsky *et al.*, 1999). A large number of signaling proteins that bind to the PDZ domain of syntrophins have been identified. These proteins include neuronal nitric oxide synthase (nNOS) (Brenman *et al.*, 1996; Adams *et al.*, 2001), aquaporin-4 (Adams *et al.*, 2001; Neely *et al.*, 2001), voltage-gated sodium channels (Gee *et al.*, 1998; Ou *et al.*, 2003), potassium channels (Connors *et al.*, 2004; Leonoudakis *et al.*, 2004), serine/threonine protein kinases (Hasegawa *et al.*, 1999; Lumeng *et al.*, 1999), the ATP-binding cassette transporter A1 (Buechler *et al.*, 2002), and others. The first PH domain of syntrophin has been implicated in phospholipid binding (Chockalingam *et al.*, 1999). Since none of the defined domains contain intrinsic enzyme activity, syntrophins are regarded as adaptors that function by targeting PDZ-binding partners to the membrane via the dystrophin complex (Albrecht and Froehner, 2002).

PH domains are abundant protein modules that play critical roles in cellular signaling and cytoskeletal organization (Lemmon and Ferguson, 2000). Despite their low amino-acid sequence identity, 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 (Ferguson *et al.*, 1994; Macias *et al.*, 1994; Yoon *et al.*, 1994; Fushman *et al.*, 1995; Lemmon and Ferguson, 2000). The first β -sheet is composed of four anti-parallel β -strands (β 1– β 4), and the second β -sheet contains three strands (β 5– β 7). The best characterized function of PH domains is binding to inositol phospholipids (Lemmon and Ferguson, 2000). Only a minority of PH domains (e.g., the PH domains from phospholipase C δ , Bruton's tyrosine kinase, and protein kinase B; Fukuda *et al.*, 1996; Frech *et al.*, 1997; Rameh *et al.*, 1997; Kavran *et al.*, 1998; Yu *et al.*, 2004) are capable of binding to lipids with high affinity and specificity. Some PH domains are known to be weak, nonspecific membrane phosphoinositide binders (Yu *et al.*, 2004), while others interact with proteins (e.g., the PH domain of β -adrenergic receptor; Touhara *et al.*, 1994). However, the function of the majority of PH domains is unknown (Yu *et al.*, 2004).

The PH domain organization of syntrophins is unique. Based on the amino-acid sequence analysis, the N-terminal PH domain is split into two halves by insertion of the PDZ domain (Figure 1A). The insertion of the PDZ domain in the middle of the N-terminal PH domain is a highly conserved molecular characteristic of all isoforms of syntrophins, suggesting the potential functional significance of this arrangement. However, it is not known whether the split PH domains of syntrophins can fold properly into a canonical PH domain structure. Additionally, the functional significance (if any) of the PDZ domain-mediated PH domain splitting has not been

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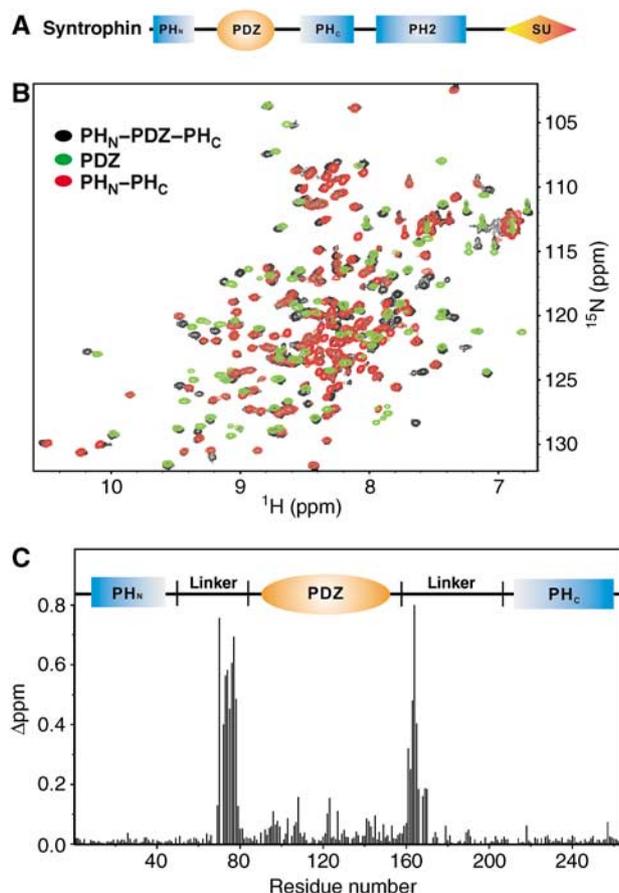


Figure 1 Comparison of the split PH and PDZ domains in the PH_N-PDZ-PH_C tandem and in their respective isolated state. (A) Schematic diagram showing the domain organization of syntrophins. (B) Superposition plots of ¹H, ¹⁵N HSQC spectra of the PH_N-PDZ-PH_C tandem (black), the isolated PDZ domain (green), and the joint PH_N-PH_C domain (red). (C) Plot of chemical shift changes as a function of residue number of the split PH and PDZ domains in the PH_N-PDZ-PH_C tandem and in their respective isolated forms. The combined ¹H and ¹⁵N chemical shift changes are defined as:

$$\Delta_{\text{ppm}} = [(\Delta\delta_{\text{HN}})^2 + (\Delta\delta_{\text{N}} \times \alpha_{\text{N}})^2]^{1/2}$$

$\Delta\delta_{\text{HN}}$ and $\Delta\delta_{\text{N}}$ represent chemical shift differences of amide proton and nitrogen chemical shifts between the PH_N-PDZ-PH_C tandem and the isolated PH and PDZ domains, respectively. The scaling factor (α_{N}) used to normalize the ¹H and ¹⁵N chemical shifts is 0.17. The domain organization of the PH_N-PDZ-PH_C tandem is indicated at the top of the plot.

addressed. Another example of PH domain splitting by insertion of other protein-protein interaction domains is phospholipase C γ 1. The C-terminal PH domain of phospholipase C γ 1 contains an insert of a tandem array of SH2-SH2-SH3 domains (Chang *et al*, 2002). It was reported that the split halves of the phospholipase C γ 1 PH domain may function independently in binding to target proteins (Chang *et al*, 2002, 2005). Very recently, van Rossum *et al* (2005) reported that the C-terminal half of the phospholipase C γ 1 PH domain (PLC γ 1-PH_C) can interact with a short fragment of peptide sequence in the TRPC3 ion channel. The authors suggested that the PLC γ 1-PH_C-binding segment of TRPC3 represents a complementary partial PH domain 'hidden' in the ion channel. Binding of the two partial PH domain fragments from PLC γ 1 and TRPC3, respectively, forms a functional PH

domain capable of binding to specific lipids and regulating surface expression of the TRPC3 ion channel (van Rossum *et al*, 2005). Given the potentially wide distribution of split PH domains in diverse proteins and enzymes, the work presented by van Rossum *et al* (2005) suggests a novel mode of function of many PH domains. To advance this important hypothesis, it is critical to know whether the complex formed by two fragments from PLC γ 1 and TRPC3 (or PLC γ 1 and translational elongation factor 1 α (Chang *et al*, 2002)) or from other proteins with split PH domains can indeed assume a PH domain-like fold.

In this work, we solved the three-dimensional structure of the PH_N-PDZ-PH_C supramodule of α 1-syntrophin by NMR spectroscopy. We show, for the first time, that the two halves of the split PH domain associate with each other, forming a canonical, intramolecular PH domain fold. The insertion of the PDZ domain has negligible impact on the conformation of the PH domain. We found, however, that the split PH_N-PDZ-PH_C supramodule has distinct phosphoinositide lipid-binding properties when compared to an isolated PH domain formed by simply connecting the PH_N and the PH_C with a linker sequence or the PDZ domain alone.

Results

The split PH domain of the PH_N-PDZ-PH_C tandem from α -syntrophin adopts the same fold as the two halves linked together

We chose the PH_N-PDZ-PH_C tandem of α -syntrophin for detailed structural analysis as its biological function is best known within the syntrophin family (Kameya *et al*, 1999; Adams *et al*, 2000, 2001; Neely *et al*, 2001; Hosaka *et al*, 2002). The recombinant PH_N-PDZ-PH_C tandem of α -syntrophin (residues 2-264, referred to hereafter as PH_N-PDZ-PH_C) was eluted at molecular mass indicative of a stable monomer when analyzed by analytical gel filtration chromatography (data not shown). The well-dispersed ¹H, ¹⁵N HSQC spectrum of PH_N-PDZ-PH_C indicates that both the split PH domain and the PDZ domain are folded (Figure 1B, black peaks). Next, we deleted the PDZ domain from the PH_N-PDZ-PH_C, and purified a covalently linked form of PH domain composed of PH_N-PH_C. Analytical gel filtration analysis of the joined PH_N-PH_C showed that the protein also exists as a stable monomer in solution (data not shown). The ¹H, ¹⁵N HSQC spectrum of PH_N-PH_C (Figure 1B and C, red peaks) overlaps very well with a subset of peaks which originate from the two split halves of the PH domain in PH_N-PDZ-PH_C. The HSQC peaks of the isolated PDZ domain (green) overlap less well with the peaks from the PDZ domain in the PH_N-PDZ-PH_C tandem (Figure 1B). After assigning the chemical shifts of the PH_N-PDZ-PH_C tandem as well as the isolated PH and PDZ domains, we plotted the chemical shift differences of backbone amides as a function of residue number between the PH_N-PDZ-PH_C tandem and the isolated PH and PDZ domains (Figure 1C). The largest chemical shift differences are located in the linker regions that connect the two halves of the PH domain with the PDZ domain. Such large chemical shift changes are expected when the PDZ domain is removed from the PH_N-PDZ-PH_C tandem. The two halves of the PH domain showed minimal shift differences whether they are in the PH_N-PDZ-PH_C tandem form or in the joined PH_N-PH_C form, indicating that the two halves of the PH domain of

α -syntrophin can fold into the same stable structure with or without the PDZ insertion. The residues that show small but significant differences in the two forms of PDZ domain are concentrated within an area in the vicinity of the two termini of the domain (Supplementary Figure 1). Taken together, our NMR and gel filtration chromatography studies demonstrate that the split PH domain of α -syntrophin can adopt a stable, intramolecular structure.

Structure of the PH_N -PDZ- PH_C supramodule

To determine if the split PH domain of α -syntrophin folds into a canonical PH domain structure, we solved the three dimen-

sional structures of the PH_N -PDZ- PH_C tandem as well as the joined PH_N - PH_C domain and the isolated PDZ domain by NMR spectroscopy (Figure 2 and Table I, Supplementary Figures 2 and 3, and Supplementary Table 1). Consistent with the data shown in Figure 1, the PH_N -PDZ- PH_C tandem contains two separate domains. 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 (Figure 2A-C). The PH_N half is composed of three β -strands ($\beta 1$ - $\beta 3$), and the PH_C half contains the remaining four β -strands ($\beta 4$ - $\beta 7$) and the C-terminal α -helix. Inserted at the $\beta 3/\beta 4$ -loop of the PH domain is a well-folded PDZ domain and two long

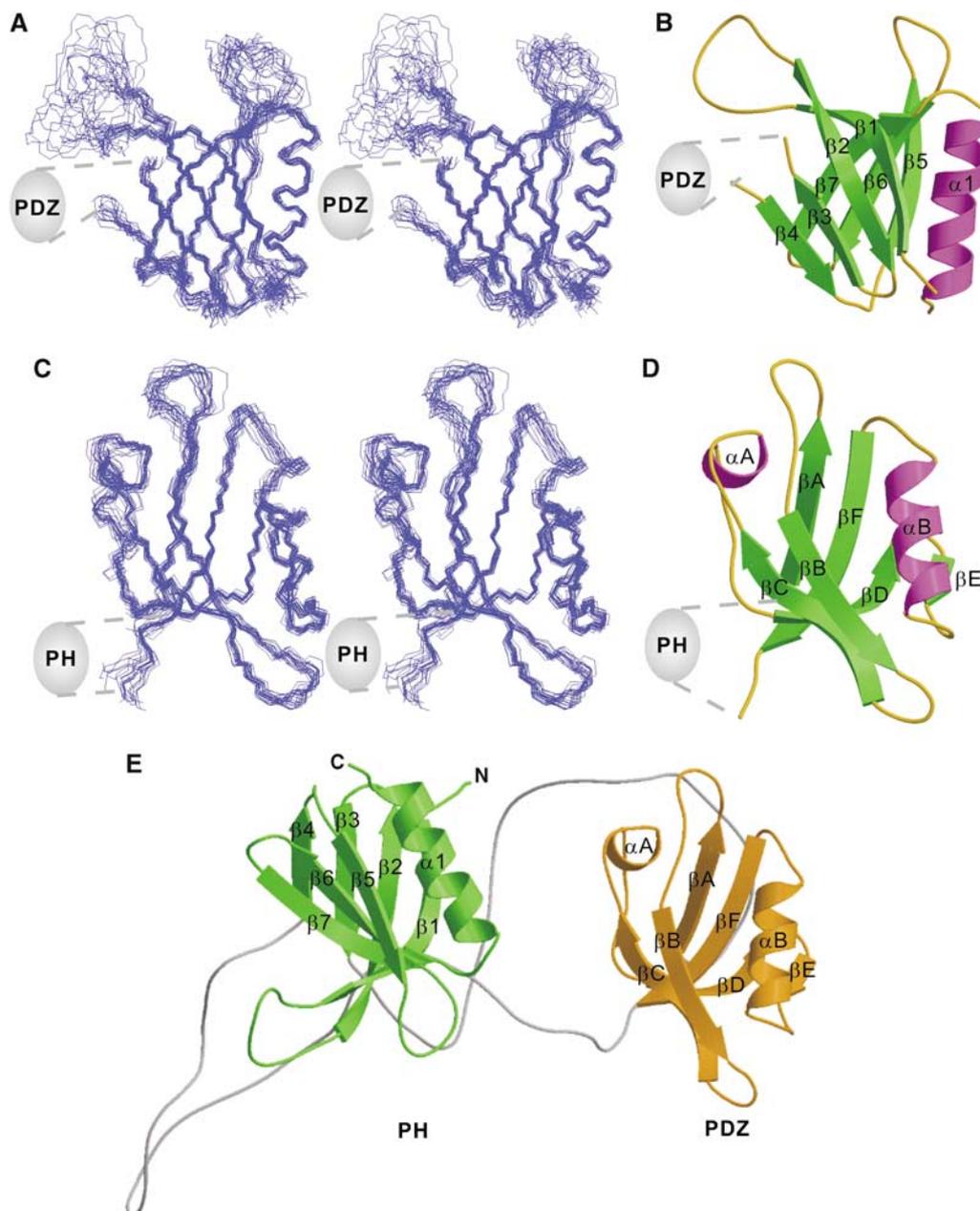


Figure 2 Structure of the PH_N -PDZ- PH_C tandem. (A) Stereo view showing the backbones of 20 superimposed NMR-derived structures of the split PH domain from the PH_N -PDZ- PH_C tandem. Since the orientation between the split PH domain and the PDZ domain in the PH_N -PDZ- PH_C tandem is not fixed, we chose to present the structures of each domain separately. (B) Ribbon diagram of a representative NMR structure of the split PH domain. The insertion of the PDZ domain in the $\beta 3/\beta 4$ -loop of the split PH domain is indicated. (C) Stereo view plot of 20 superimposed NMR structures of the PDZ domain from the PH_N -PDZ- PH_C tandem. (D) Ribbon diagram drawing of the PDZ domain structure. The split PH domain connected to the βA and βF of the domain is indicated. (E) Ribbon diagram drawing of a representative NMR structure of the PH_N -PDZ- PH_C tandem. In this drawing, the split PH domain is in green, and the PDZ domain is in gold.

Table 1 Structural statistics for the final 20 structures of the PH_N-PDZ-PH_C tandem^a

<i>Distance restraints</i>	
Intraresidue ($i-j=0$)	1160
Sequential ($ i-j =1$)	627
Medium range ($2 \leq i-j \leq 4$)	206
Long range ($ i-j > 5$)	607
Hydrogen bonds	140
Total	2740
<i>Dihedral angle restraints</i>	
Φ	94
Ψ	90
Total	184
<i>Mean r.m.s. deviations from the experimental restraints</i>	
Distance (Å)	0.012 ± 0.000
Dihedral angle (deg)	0.102 ± 0.015
<i>Mean r.m.s. deviations from idealized covalent geometry</i>	
Bond (Å)	0.002 ± 0.000
Angle (deg)	0.306 ± 0.007
Improper (deg)	0.149 ± 0.010
<i>Mean energies (kcal mol⁻¹)</i>	
E _{NOE^b}	27.75 ± 0.71
E _{cdih^b}	0.12 ± 0.03
E _{L-J}	-488.03 ± 31.34
<i>Ramachandran plot^c</i>	
(Residues 2-45, 79-163, 205-264)	
residues in the most favorable regions	69.3
Additional allowed regions	26.1
Generously allowed regions	3.0
Disallowed regions	1.6
<i>Atomic r.m.s. difference (Å)^d</i>	
(residues 9-15, 29-44, 206-219, 229-262 in PH)	
Backbone heavy atoms (N, C α , and C')	0.54
Heavy atoms	1.14
(residues 80-85, 93-162 in PDZ)	
Backbone heavy atoms (N, C α , and C')	0.51
Heavy atoms	1.09

^aNone of the structures exhibits distance violations greater than 0.3 Å or dihedral angle violations greater than 4°.

^bThe 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.

^cThe program Procheck (Laskowski *et al*, 1996) was used to assess the overall quality of the structures.

^dThe precision of the atomic coordinates is defined as the average r.m.s. difference between 20 final structures and the mean coordinates of the protein.

linkers (Figure 2C-E). No interdomain NOEs between the split PH domain and the PDZ domain were observed; therefore, the orientations of the two domains were not defined. Potential long-range interactions between the split PH domain and the PDZ domain were further investigated by NMR-based approaches. First, we measured the residual dipolar coupling constants of the backbone amides of the PH_N-PDZ-PH_C tandem by aligning the protein in a 4.5% positively charged copolymer containing 50% of (3-acrylamidopropyl)-trimethylammonium chloride and 50% of acrylamide (Cierpicki and Bushweller, 2004). The alignment tensors of the split PH domain and the PDZ domain in this alignment medium were very different (data not shown), indicating that the orientations of the two domains are not fixed in the PH_N-PDZ-PH_C tandem. We further probed the orientation of the split PH domain and the PDZ domain in the PH_N-PDZ-PH_C

tandem by tagging the split PH domain with S-cysteaminy-EDTA, an EDTA derivative used to detect long-range interactions in proteins using paramagnetic pseudo-contact shifts (Ikegami *et al*, 2004; Pintacuda *et al*, 2004). Addition of Co²⁺ to the S-cysteaminy-EDTA-tagged PH_N-PDZ-PH_C induced chemical shift changes limited to the split PH domain (Supplementary Figure 4). This paramagnetic pseudo-contact shift perturbation data further indicated that the orientation of the split PH domain and the PDZ domain in the PH_N-PDZ-PH_C tandem is not fixed.

Consistent with the nearly complete overlap of the peaks in the NMR spectra (Figure 1B), the structure of the joined PH_N-PH_C is essentially the same as the split PH domain structure in the PH_N-PDZ-PH_C tandem (a root-mean-square difference of 1.0 Å for the backbones of the two structures; Supplementary Figure 2). The structure of the isolated PDZ domain is also essentially the same as the domain in the PH_N-PDZ-PH_C tandem (Supplementary Figure 3; also see Schultz *et al*, 1998a).

Since split PH domains have been observed in several proteins in addition to syntrophins, it is critical to understand whether the inserted sequences between the two halves of a split PH are required for the domain to fold. This issue becomes particularly relevant as the C-terminal half of the PLC γ 1 split PH domain (PLC γ 1-PH_C) was suggested to bind to a partial PH fragment hidden in TRPC3 instead of the PH_N within PLC γ 1 (van Rossum *et al*, 2005). To address this question, we replaced the PDZ domain of PH_N-PDZ-PH_C with an eight-residue peptide fragment that can be cleaved by protease 3C (referred to as PH_N-C'-PH_C; Figure 3A). The NMR spectra indicated that the purified PH_N-C'-PH_C folds into a structure indistinguishable from that of the joined PH_N-PH_C domain (Figures 1B and 3B). Digestion of PH_N-C'-PH_C with protease 3C produces two fragments with molecular masses corresponding to PH_N and PH_C, respectively (Figure 3A). The NMR spectrum of the protease 3C-cleaved PH_N-C'-PH_C is essentially identical to that of the uncleaved protein (Figure 3B), indicating that the covalent linkage between PH_N and PH_C (and hence the PDZ domain) is dispensable for the folding of the split PH domain in syntrophins.

To investigate whether two partial but complementary PH domains can indeed interact with each other intermolecularly, we studied the structure of the two isolated PH_N and PH_C fragments of α -syntrophin, as well as a 1:1 mixture of the two fragments. The cleaved His₆-tagged PH_N was separated from PH_C under denatured condition by passing the protease-cleaved mixture through a Ni²⁺-NTA affinity column. The denaturant (urea) was subsequently dialyzed away from each half of the purified PH domain (Figure 3A). The NMR spectra of the two isolated fragments showed that each domain alone is completely unfolded, but remains soluble and stable in solution, indicating that partial PH domains can exist as independent structural units (Figure 3C and D). When mixed together, the interaction of the two partial PH domains induces *de novo* folding of both fragments. Further, the NMR data showed that the two partial PH domains fold into an intact PH domain structure indistinguishable from that of the split PH domain in the PH_N-PDZ-PH_C tandem (Figure 3E). The PH domain formed by the two isolated halves remains stably folded for a number of days under our NMR measurement conditions.

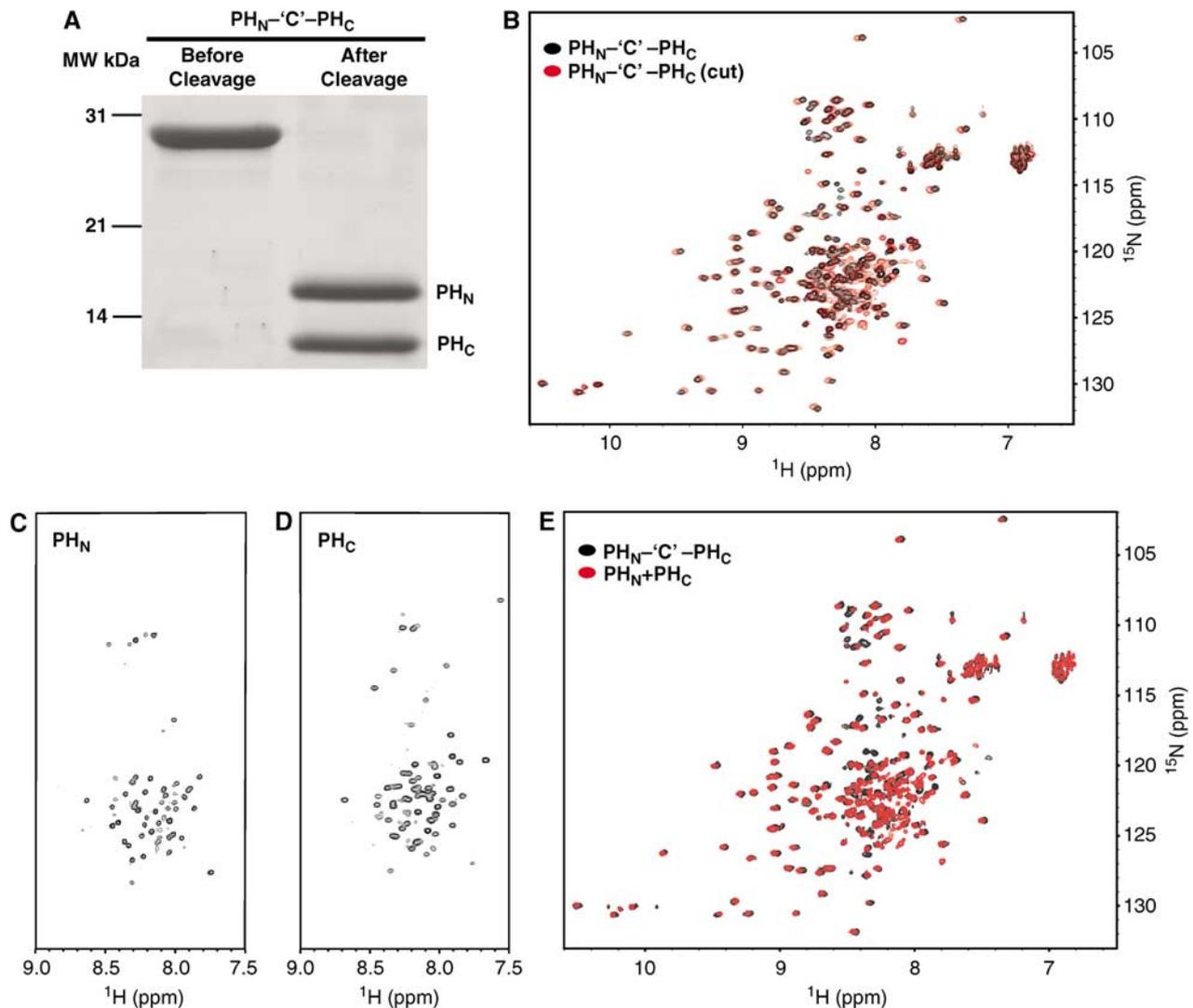


Figure 3 Folding and interaction of the two isolated, partial PH domain fragments. (A) SDS-PAGE showing the purification of the joined PH_N-C'-PH_C domain and cleavage of the domain into PH_N and PH_C fragments. (B) Overlay plot of the ¹H, ¹⁵N HSQC spectra of the joined PH_N-C'-PH_C (black) and its protease cleaved form (red). ¹H, ¹⁵N HSQC spectra of the isolated PH_N (C) and PH_C (D) fragments. (E) Overlay plot of the ¹H, ¹⁵N HSQC spectra of the joined PH_N-C'-PH_C (black) and the 1:1 mixture of the two halves of the split PH domain (red).

The α -syntrophin PH_N-PDZ-PH_C tandem functions as a supramodule with distinct inositol phospholipid-binding properties

Since the split PH domain of PH_N-PDZ-PH_C can fold into a canonical PH domain structure, we assayed the binding of the PH_N-PDZ-PH_C tandem to liposomes prepared from total bovine brain lipids (Figure 4). The PH_N-PDZ-PH_C tandem was found to bind efficiently to these liposomes (Figure 4B, top panel). The interaction between the PH_N-PDZ-PH_C tandem and the brain liposome is dose-dependent, with an estimated *K*_d value of ~5 μM (Figure 4C). To our surprise, binding of the joined PH_N-PH_C domain to liposomes is much weaker than the PH_N-PDZ-PH_C tandem, even though the PH_N-PH_C domain folds into the same structure as the split PH domain in the PH_N-PDZ-PH_C tandem (Figure 4B, second panel). Since PDZ domains have been shown to bind directly to inositol phospholipids (Zimmermann *et al*, 2002; Mortier *et al*, 2005), we tested whether the PDZ domain of α -syntrophin could also bind to lipids, using the same brain liposome-binding assay. The α -syntrophin PDZ domain dis-

played some binding to lipids, albeit with a much weaker binding capacity when compared to the PH_N-PDZ-PH_C tandem (Figure 4B, third panel). Further, the enhanced lipid-binding avidity of α -syntrophin requires that the split PH domain and the PDZ domain be covalently linked, as a mixture containing the isolated PH_N-PH_C domain and the PDZ domain binds to brain liposomes only weakly (Figure 4B, bottom panel). To ensure that the altered lipid-binding properties of the mutants shown in Figure 4 did not result from mutation-induced protein stability changes, we compared the stabilities of these mutants (PH_N-PH_C and isolated PDZ domain) with that of the wild-type PH_N-PDZ-PH_C tandem. Both the temperature- and urea-dependent denaturation profiles of the mutants and the PH_N-PDZ-PH_C tandem are highly similar (Supplementary Figure 5), indicating that the decreased lipid-binding avidities of the mutants are not the result of their compromised stability.

It is conceptually easy to rationalize that, when covalently connected, two relatively weak lipid-binding domains can function synergistically to increase the lipid-binding avidity

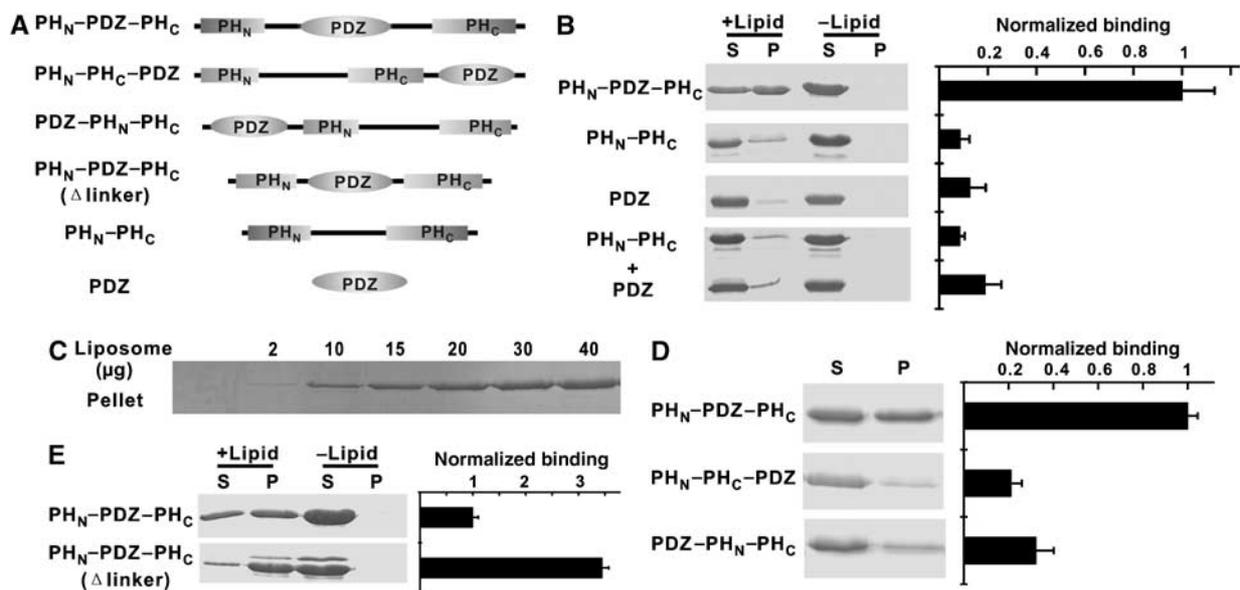


Figure 4 The PH_N-PDZ-PH_C supramodule binds to brain liposomes with enhanced avidity. **(A)** Schematic diagrams showing the various forms of proteins purified for liposome-binding assays. **(B)** Brain liposome-binding assays of the PH_N-PDZ-PH_C tandem and its fragments. ‘S’ and ‘P’ denote proteins recovered in the supernatants and pellets, respectively, in the centrifugation-based liposome-binding assays. **(C)** Dose-dependent binding between the PH_N-PDZ-PH_C tandem and the brain liposome. In this assay, the amount of the PH_N-PDZ-PH_C tandem is fixed at 10 μM, and the concentration of liposome varies. The liposome-bound PH_N-PDZ-PH_C tandem is recovered in the pellet in the binding assay. **(D)** Lipid binding of the two mutants of the PH_N-PDZ-PH_C tandem. In these two mutants, the PDZ domain was placed either at the front or after the split PH domain. In these mutants, the PH and the PDZ domains were connected by a flexible ‘Gly-Ser-Gly-Gly-Ser-Gly-Gly-Ser-Gly-Ser’ linker. **(E)** Lipid binding of a PH_N-PDZ-PH_C tandem mutant with the two connecting linkers shortened to six residues. Amino-terminal His₆-tagged proteins were used in all of the brain liposome-binding assays.

of the PH_N-PDZ-PH_C tandem. However, it is intriguing that the PH domain of every member of the syntrophin family contains a PDZ domain inserted in the middle of its primary sequence; yet, the folding and structure of the PH domains are not affected by this insertion. We hypothesized that the position-specific insertion of the weak lipid-binding PDZ domain in the middle of the PH domain may somehow specifically modify the lipid-binding properties of the PH_N-PDZ-PH_C tandem. To test this hypothesis, we created two mutants of the PH_N-PDZ-PH_C tandem by placing the PDZ domain either in front of or after the joined PH_N-PH_C domain. To avoid artificial conformational restraints, a 10-residue flexible linker (‘Gly-Ser-Gly-Gly-Ser-Gly-Gly-Ser-Gly-Ser’) was inserted between the PDZ domain and the joined PH_N-PH_C domain in both mutants. Both mutants displayed weaker lipid binding compared to the PH_N-PDZ-PH_C tandem (Figure 4D). In the protein stability test, both PDZ-PH_N-PH_C and PH_N-PH_C-PDZ showed similar stability to the PH_N-PDZ-PH_C tandem (Supplementary Figure 5). Our data indicate that splitting the PH domain by insertion of the PDZ domain creates a PH_N-PDZ-PH_C supramodule with unique lipid-binding properties.

If the linkers between the split PH domain and the PDZ domain in the PH_N-PDZ-PH_C tandem are entirely flexible, we should not observe the obvious differences in lipid-binding avidity between the wild-type PH_N-PDZ-PH_C and the two mutants shown in Figure 4D. To address this, we analyzed the structural feature of the two linkers connecting the two halves of the split PH domain and the PDZ domain in detail. The secondary structure-induced ¹³C_α and ¹³C_β chemical shifts of the split PH domain in the PH_N-PDZ-PH_C tandem showed that the two linkers are partially structured

(Supplementary Figure 6). Specifically, the linker connecting the PH_N and PDZ contains a β-strand-like structure immediately following the β3 of PH_N and an α-helix-like segment N-terminal to the PDZ domain. The linker connecting the PH_C and PDZ contains two partial β-strands N-terminal to the β4 strand of PH_C. Presumably, the two partially structured linkers restrict the degree of motion between the split PH domain and the PDZ domain to a certain extent, thereby promoting binding of the PH_N-PDZ-PH_C tandem to liposomes. The small but significant chemical shift differences between the isolated PDZ domain and the domain in the PH_N-PDZ-PH_C tandem (Supplementary Figure 1) are consistent with the notion that the linkers may influence the motion of the PDZ domain. To experimentally test if the interdomain motion between the split PH domain and the PDZ domain is correlated with the lipid-binding avidity of the protein, we shortened both linkers of the PH_N-PDZ-PH_C tandem to six residues, and found that the resulting mutant binds to liposomes with a significantly higher avidity than the wild-type protein (Figure 4E).

The binding specificity of the PH_N-PDZ-PH_C tandem towards different lipids was assayed using membrane-immobilized lipid strips. Of the 15 lipids tested, the PH_N-PDZ-PH_C tandem displayed strong binding to several phosphoinositides including PI(3,5)P₂ and PI(5)P, modest binding to PI(3,4)P₂ and PI(4,5)P₂, and weak binding to other inositol phospholipids (Figure 5A). No binding could be detected between the PH_N-PDZ-PH_C tandem and two major components of mammalian membrane lipids, phosphatidylcholine and phosphatidylethanolamine, suggesting that the interaction between the PH_N-PDZ-PH_C tandem and phosphoinositides is relatively specific. However, the stereo-specificity

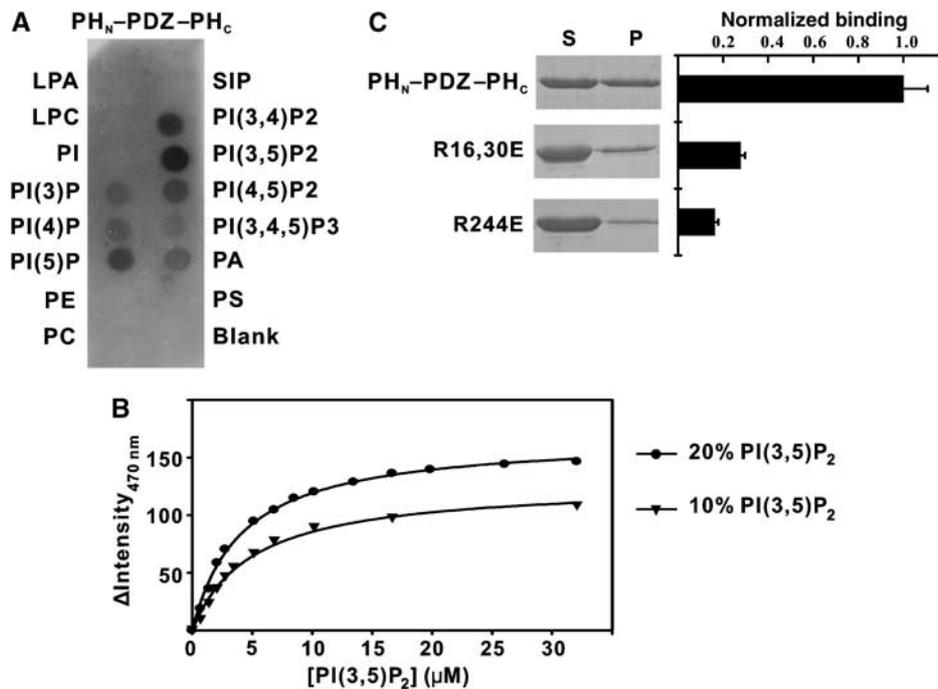


Figure 5 Lipid-binding specificity of the PH_N-PDZ-PH_C supramodule using phospholipid strip overlay assay. (A) Binding of the PH_N-PDZ-PH_C tandem to the phospholipids spotted on a cellulose membrane. The amount of lipid per spot was 100 pmol. Abbreviations for the lipids: SIP: sphingosine-1-phosphate; LPA: lysophosphatidic acid; LPC: lysophosphocholine; PE: phosphatidylethanolamine; PS: phosphatidylserine; PA: phosphatidic acid; PC: phosphatidylcholine; PtdIns: phosphatidylinositol. The amount of protein used in the assay is 0.5 μg/ml. (B) Binding isotherms of PH_N-PDZ-PH_C with PC/PS liposomes containing either 10 or 20% PI(3,5)P₂. (C) Identification of the residues in the split PH domain of the PH_N-PDZ-PH_C tandem that are involved in the lipid binding.

among different phosphoinositides is not very high. Consistent with the data derived from the brain liposome assay, the isolated PH_N-PH_C domain, the isolated PDZ domain, and the mutants with the PDZ domain position swapped all displayed very low lipid-binding affinity in the lipid strip-binding assay (data not shown). Since lipid strip-based assay is very qualitative, we used a fluorescence perturbation assay to quantitatively measure affinity between the PH_N-PDZ-PH_C tandem and PI(3,5)P₂. Binding was monitored via fluorescence change of a 7-diethylamino-3-(4'-maleimidylphenyl)-4-methylcoumarin (CPM) fluorophore-labeled protein (Papayannopoulos *et al*, 2005) upon addition of vesicles (phosphatidyl choline (PC), phosphatidyl serine (PS), and (PI(3,5)P₂) at ratios of 70:20:10 and 60:20:20, respectively). No detectable binding of the PH_N-PDZ-PH_C tandem is observed to vesicles containing only PC/PS (80:20). Strong binding is observed to vesicles containing 10 and 20% PI(3,5)P₂ (apparent $K_d \sim 5.1$ and $3.9 \mu\text{M}$, respectively) (Figure 5B), indicating that the interaction between the PH_N-PDZ-PH_C tandem and phosphoinositides is specific.

To identify the phosphoinositide-binding site in the PH domain, we mutated several positively charged residues in the $\beta 1/\beta 2$ -loop (Arg16 and Arg30) and in the $\beta 7$ -strand (Arg244) of the split PH domain. These positively charged residues are clustered at one end of the split PH domain (data not shown), and are expected to be involved in lipid binding (Ferguson *et al*, 1995; Lemmon and Ferguson, 2000; Lietzke *et al*, 2000; Thomas *et al*, 2002). Substitutions of Arg16 and Arg30 or Arg244 by Glu significantly reduced the lipid-binding capacity of the PH_N-PDZ-PH_C tandem, indicating that these positively charged residues are indeed required for lipid

binding (Figure 5C). We tried to map the lipid-binding site of the split PH domain by titrating the joined PH_N-PH_C domain or the PH_N-PDZ-PH_C tandem (both uniformly labeled with ¹⁵N) with water-soluble head groups of phosphoinositides that display significant binding to the PH_N-PDZ-PH_C tandem. However, we were not able to detect specific interactions between the head groups of these phosphoinositides with the split PH domains. In agreement with a very recent study (Mortier *et al*, 2005), we were not able to detect binding of the isolated PDZ domain to water-soluble head groups of phosphoinositides.

Next, we studied the potential impact of the PDZ ligands on the lipid binding of the PH_N-PDZ-PH_C tandem. We found that addition of an excess amount of α -syntrophin PDZ ligand (a synthetic peptide containing a consensus α -syntrophin PDZ-binding motif 'S-X-V*', or the nNOS PDZ domain) did not significantly affect the lipid-binding property of the PH_N-PDZ-PH_C tandem. Thus, α -syntrophin PDZ ligands do not interfere with the lipid-binding capacity of the PH_N-PDZ-PH_C supramodule (Figure 6A and data not shown). Our data also imply that the lipid-binding site and the PDZ ligand-binding site on the α -syntrophin PDZ domain do not overlap with each other. Consistent with this observation, we found that mutations of the two positively charged residues in the carboxyl-binding loop of the target recognition groove of the PDZ domain (Arg85 and Arg86 to Ser) had no impact on lipid binding of the PH_N-PDZ-PH_C tandem (Figure 6B). In a reverse experiment, we showed that the binding of lipids to the PH_N-PDZ-PH_C tandem does not affect the binding of α -syntrophin PDZ domain to the nNOS PDZ domain (Figure 6C).

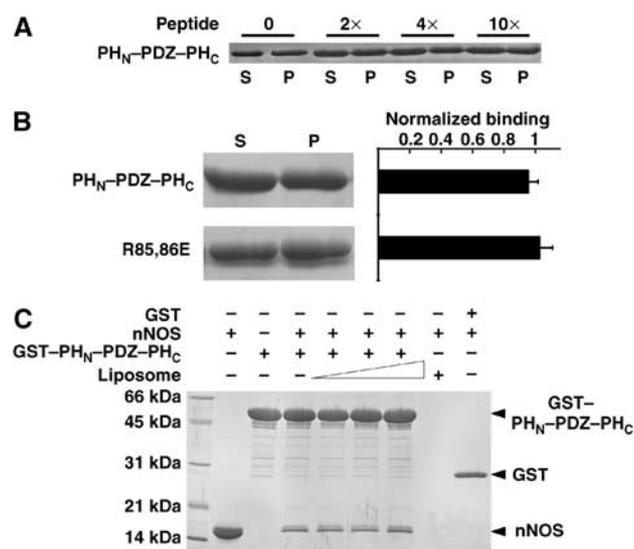


Figure 6 The PDZ ligands do not affect lipid binding of the PH_N-PDZ-PH_C tandem. (A) Addition of excess amounts (up to ~10 equivalents) of a PDZ-binding peptide to the liposome-binding assay buffer did not alter the lipid-binding capacity of the PH_N-PDZ-PH_C tandem. The amino-acid sequence of the PDZ ligand peptide is: KLSSIESDV. (B) Mutation of the two positively charged residues (Arg85 and Arg86) in the carboxyl group-binding loop of the target recognition groove of the PDZ domain did not change lipid-binding capacity of the PH_N-PDZ-PH_C tandem. (C) Binding of lipids to the PH_N-PDZ-PH_C tandem does not change the binding of PDZ domain to its ligand, the nNOS PDZ domain. Three different liposome concentrations (0.4, 0.6, and 0.8 mg/ml) were tested in this assay.

Discussion

As one of the most abundant protein modules in the mammalian genome, PH domains have been extensively studied both structurally and functionally (Schultz *et al*, 1998b; Lemmon and Ferguson, 2000). PH domains are generally regarded as lipid-binding modules capable of targeting PH domain proteins to the cell membrane or detecting lipid signals generated in cellular signaling processes. A recent genome-wide analysis of all yeast PH domains suggested that only a minor population of PH domains binds to lipids specifically. Thus, the functions of the majority of PH domains are unknown (Yu *et al*, 2004). Protein domain searching using the SMART program (Schultz *et al*, 1998b) identifies several proteins, for example, syntrophins, PLC γ 1, myosin X (Berg *et al*, 2000), and Rho kinases, that contain a split PH domain (i.e. the PH domain contains one or more other intact protein modules in the middle of its primary sequence). The significance of split PH domains has not been studied until a very recent report by van Rossum *et al* (2005), suggesting that the C-terminal half of the PLC γ 1 PH domain may be complemented by a hidden partial PH domain-like segment in TRPC3. It was suggested that partial PH domains exist in many proteins (van Rossum *et al*, 2005). Detailed structural studies of split PH domains are pressingly needed to understand the novel functions of this unique subgroup of PH domain proteins.

As one of the major findings in this study, we found that the split PH domain of α -syntrophin adopts an intramolecular, canonical PH fold. We also showed that the PDZ domain insertion does not have significant impact on the structure of

the split PH domain. We further showed that the two halves of the split PH domain, even in the absence of covalent linkage, can autonomously fold into a native-like PH domain structure. In contrast, the isolated partial fragments of the split PH domain of α -syntrophin are both unfolded, but soluble in solution. Our data indicate that partial PH domains may stably exist in proteins, and such partial PH domains easily escape detection by conventional computer algorithms. Interaction of the two complementary halves of a PH domain induces *de novo* folding of the domain. Thus, our study provides a structural basis for potential interactions of two hidden partial PH domains within one protein or between two different proteins. Our study also suggests that the intramolecular, two half-PH domain complementation dominates the assembly of the split PH domain of α -syntrophin. If this is also the case for the split PH domain of PLC γ 1, the interaction between PLC γ 1-PH_C and the hypothetical partial PH domain of TRPC3 suggested by van Rossum *et al* (2005) requires a prior dissociation of PLC γ 1-PH_C from PLC γ 1-PH_N. If such intermolecular PH domain assembly indeed occurs, a key question is whether and how such a process is regulated.

Another key finding of this work is that the PH_N-PDZ-PH_C tandem functions as a supramodule with distinctly higher avidity in binding to lipids when compared to the isolated PH_N-PH_C and PDZ domains. The simplest explanation for the high avidity of the PH_N-PDZ-PH_C supramodule is the synergism afforded by the two weak lipid-binding domains (the split PH domain and the PDZ domain) covalently connected to each other. It is important to note that the binding between inositol phospholipids and the PH_N-PDZ-PH_C tandem requires the membrane bilayer and the hydrophobic tails of the lipids, as soluble head groups showed no detectable binding to the PH_N-PDZ-PH_C tandem. Presumably, anchoring of phosphoinositides onto the membrane bilayer is entropically favorable for the lipid molecules binding to the bidentate PH_N-PDZ-PH_C tandem. The interaction between PDZ domains and lipids is an emerging concept. The molecular basis of lipid/PDZ interaction is largely unknown, and this is an important area in the future research. It is interesting to note that the lipid-binding affinity of the PH_N-PDZ-PH_C tandem also depends on the position of the PDZ domain insertion. Furthermore, the canonical protein/peptide target-binding site of the PDZ domain does not overlap with the lipid-binding site of the domain. Many of the PDZ domain-binding proteins are membrane proteins (Sheng and Sala, 2001; Zhang and Wang, 2003). One can envision that the coordinated actions of lipid binding by the PH_N-PDZ-PH_C tandem and the PDZ domain-mediated interactions with membrane proteins can target syntrophins very efficiently to specialized membrane domains, such as neuromuscular junctions and astroglial endfeet. In a broader sense, the lipid- (and potentially protein-) binding properties of many PH domains could be influenced by other protein domains both intra- and intermolecularly. It is perhaps not surprising that many PH domains do not bind to lipids in their isolated forms.

Materials and methods

Protein expression and purification

The PH_N-PDZ-PH_C tandem (residues 2–264), the joint PH_N-PH_C domain (residues 2–79 and 165–264), and the PDZ domain

(residues 80–164) of mouse α -syntrophin were cloned into a modified version of pET32a vector, respectively (Feng *et al*, 2002). The protease-cleavable form of the PH_N-PH_C domain was constructed by insertion of a protease 3C recognition sequence at the joint site of the two halves of the PH domain. Point mutations of the PH_N-PDZ-PH_C tandem (R16,30E, R244E, and R85,86S) were created by PCR-based mutagenesis. The PDZ-PH_N-PH_C and PH_N-PH_C-PDZ mutants were constructed, respectively, by connecting the PDZ domain and joint PH_N-PH_C domain with a 10-residue 'Gly-Ser-Gly-Gly-Ser-Gly-Gly-Ser-Gly-Ser' linker. 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 ¹⁵N- and ¹⁵N/¹³C-labeled proteins were prepared by growing bacteria in M9 medium containing ¹⁵NH₄Cl with or without ¹³C₆-glucose. The His-tagged fusion proteins were purified under native conditions using a Ni-NTA agarose (Qiagen) affinity chromatography. The remaining small amount of contaminant proteins was removed by size-exclusion chromatography.

NMR structure determination

NMR samples contained ~1.0 mM of the PH_N-PDZ-PH_C tandem in 100 mM potassium phosphate, pH 7.0, in 90% H₂O/10% D₂O or 99.9% D₂O. NMR spectra were acquired at 30 °C on a Varian Inova 750 MHz spectrometer 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 (Bax and Grzesiek, 1993; Kay and Gardner, 1997). The stereo-specific assignments of the Val and Leu methyl groups were obtained using a 10% ¹³C-labeled sample (Neri *et al*, 1989). Approximate interproton distance restraints were derived from NOESY spectra (a ¹H 2D homonuclear NOESY, a ¹⁵N-separated NOESY, and a ¹³C-separated NOESY). NOEs were grouped into three distance ranges: 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. Backbone dihedral angle restraints (ϕ and ψ angles) were derived from the chemical shift analysis program TALOS (Cornilescu *et al*, 1999). Structures were calculated using the program CNS (Brunger *et al*, 1998). Figures were generated using MOLMOL (Koradi *et al*, 1996), MOLSCRIPT (Kraulis, 1991), and Raster3D (Merritt and Murphy, 1994).

Lipid-binding assay

Brain lipid extracts (Folch fraction I, Sigma B1502) were resuspended at 2 mg/ml in a buffer containing 20 mM HEPES, pH 7.4, 150 mM NaCl, and 1 mM DTT. The protein sample (5 μ M) was incubated with 0.6 mg/ml liposomes in 40 μ l 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.

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Phosphatidylcholine, phosphatidylserine, and PI(3,5)P₂ were purchased from Avanti Lipids (>99% pure). Large unilamellar vesicles (LUV) were generated by mixing di(dibromostearoyl) phosphatidylcholine and dipalmitoyl phosphatidyl-L-serine dissolved in chloroform in a 4:1 molar ratio. Addition of PI (3,5)P₂ was counteracted by reduction of equal moles of PC. Mixed lipids were freeze dried, and resuspended in 50 mM Tris (pH 7.4) and 100 mM NaCl at 4.0 mM. Hydrated lipids were subjected to at least 10 cycles of freeze–thawing in liquid nitrogen, followed by 1 min bath sonication and extrusion through a 0.1 μ m filter with a lipid extruder (Avanti). Purified PH_N-PDZ-PH_C was labeled with CPM (Molecular Probes) containing single Cys (Cys232) at the end of β 6 of the split PH domain (Cys17 and Cys219 were substituted with Ser). PH_N-PDZ-PH_C was labeled with 10-fold molar excess of CPM in 50 mM Tris (pH 7.4) and 100 mM NaCl at 4°C. The reaction mixture was cleared by centrifugation, the excess CPM was removed by PD-10 column (Amersham Pharmacia). For constant-density PIP₂ titrations, lipid vesicles were titrated into a 0.6 ml solution containing 0.3 μ M CPM-labeled PH_N-PDZ-PH_C in 50 mM Tris (pH 7.4), 100 mM NaCl. The sample was excited at 386 nm, and emission was detected at 470 nm. Data from binding experiments were fit by nonlinear least-squares methods using Prism 4 from GraphPad.

Lipid strip assay

The PIP strips (Echelon, P-6001) were blocked by 3% fatty acid-free BSA in TBS-T buffer (50 mM Tris, pH 8.0, 150 mM NaCl, 0.1% Tween-20) at room temperature for 1 h. The strips were then incubated with 0.5 μ g/ml of PH_N-PDZ-PH_C at 4°C overnight, washed five times in TBS-T buffer and subsequently incubated for 1 h with the pan-specific syntrophin antibody SYN1351 (1:8000) (Froehner *et al*, 1987) at room temperature. The signals were generated using the enhanced chemiluminescence detection kit (Perkin-Elmer).

Coordinates

The atomic coordinates have been deposited in the Protein Data Bank under access numbers of 1Z87, 2ADZ, and 1Z86 corresponding to the PH_N-PDZ-PH_C tandem, the joined PH_N-PH_C domain, and the isolated PDZ domain, respectively. The chemical shift assignments of the three proteins have been deposited in the BioMagResBank.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online.

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