Pleckstrin Homology Domain 1 of Mouse α1-Syntrophin Binds Phosphatidylinositol 4,5-Bisphosphate†

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ABSTRACT: Mouse α1-syntrophin sequences were produced as chimeric fusion proteins in bacteria and found to bind phosphatidylinositol 4,5-bisphosphate (PtdIns4,5P_2). Half-maximal binding occurred at 1.9 μM PtdIns4,5P_2 and when 1.2 PtdIns4,5P_2 were added per syntrophin. Binding was specific for PtdIns4,5P_2 and did not occur with six other tested lipids including the similar phosphatidylinositol 4-phosphate. Binding was localized to the N-terminal pleckstrin homology domain (PH1); the second, C-terminal PH2 domain did not bind lipids. Key residues in PtdIns4,5P_2 binding to a PH domain were found to be conserved in α-syntrophins' PH1 domains and absent in PH2 domains, suggesting a molecular basis for binding.

Pleckstrin homology (PH)[1] domains derive their name from pleckstrin, the major protein kinase C substrate of platelets. Pleckstrin contains 2 copies of this domain of about 100 amino acyl residues (*1*). PH domains frequently possess two biological activities: they bind phosphatidylinositol 4,5-bisphosphate (PtdIns4,5P_2) and the heterotrimeric GTPase protein $\beta\gamma$-subunits ($G_{\beta\gamma}$) (*2*−*4*).

Other than association with PtdIns4,5P_2 and $G_{\beta\gamma}$, PH domains are often implicated in signal transduction events in other ways. Small G-protein GDP releasing factors and GTPase activating proteins, protein tyrosine kinases, Ser/Thr kinases, phospholipase C isoforms, and several oncogenes contain PH domains (*5*, *6*). These observations suggest a role for PH domains in cellular signaling. PH domains are also frequently found in cytoskeletal proteins, such as nonerythroid β-spectrin and, recently, syntrophin (*5*).

Syntrophins are a group of homologous proteins originally identified in *Torpedo* postsynaptic membranes (*7*). More recently, syntrophins have been found to be constituents of the dystrophin glycoprotein complex (*8, 9*), a complex of proteins whose defects cause Duchenne, Becker, various limb-girdle, and other muscular dystrophies (*10*). Mouse, rabbit, and human syntrophin cDNAs (*8, 9, 11, 12*) revealed three syntrophin isoforms, α1, β1, and β2, which are products of different genes. Each contains two pleckstrin homology (PH) domains, one PDZ domain, and a domain unique to syntrophins, the SU domain. This modular domain structure suggests that syntrophins may function as adapters, linking cellular proteins to the DGC (*13*).

The amino-terminal PH domain of syntrophin is interrupted by another domain inserted in roughly the middle. This other domain, the PDZ domain, is also found in membrane proteins and was named for the first three proteins in which this ∼90 amino acid motif was identified: the Postsynaptic density protein, PSD-95, the *Drosophila disks-large* protein, Dlg, and the Zona Occludens 1 (ZO-1) protein (*14*). Syntrophin's PDZ domain has recently been shown to bind to neuronal nitric oxide synthetase (*15*) and muscle and nerve voltage-gated Na^+-channels (*16*, *17*). The crystal structure of a PDZ domain from PSD-95 shows that the N- and C-termini are close to one another in space (*18*); if this is also true for syntrophin, this PDZ domain insertion in syntrophin's PH domain may be of only minor consequence to its structure.

Syntrophin also binds calmodulin (*19*). A Ca^{2+}-calmodulin binding site comprises the C-terminal 24 residues of syntrophin, in the syntrophin unique domain. Other binding was found in the N-terminal 174 residues (*20*). Recently, the location of this binding has been localized to two sites, one at the N-terminal end of the PH1 domain and the other at the N-terminal end of the PDZ domain (*21*). Thus, calmodulin and Ca^{2+} may affect the activity of syntrophin's domains. Furthermore, syntrophin has been shown to bind Ca^{2+} (*20*) and Ca^{2+} binding is a property of PH domains in other proteins (*22*).

The function of syntrophin's PH domains is currently unknown. Here, we show that one of the two domains binds PtdIns4,5P_2 specifically and in low stoichiometry.

MATERIALS AND METHODS

Materials. Phosphatidylinositol 4,5-bisphosphate disodium salt from bovine brain (PtdIns4,5P_2)[1] from both Sigma and Fluka was used for the experiments. PC, 40% from soybean,

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[1] Abbreviations: PH, pleckstrin homology; PtdIns4,5P_2, phosphatidylinositol 4,5-biphosphate; $G\beta\gamma$, heterotrimeric GTPase β- and γ-subunit complex; PC, phosphatidylcholine; PA, phosphatidic acid; PE, phosphatidylethanolamine; PtdIns, phosphatidylinositol; PS, phosphatidylserine; SM, sphingomyelin; PtdIns4P, phoshatidylinositol 4-monophosphate; MBP, maltose binding protein; SDS, sodium dodecyl sulfate; PBS, phosphate-buffered saline.

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PA, PE, PS, SM, PtdIns, and PtdIns4P were all obtained from Sigma. Endoproteinase Xa (from bovine plasma) was from New England Biolabs. All other chemicals were of the highest purity obtainable commercially.

Fusion Proteins. The syntrophin fusion proteins $(His)_6$-Syn, $(His)_6$-Syn A, $(His)_6$-Syn I, MBP-Syn B, MBP-Syn G, and MBP-Syn H were prepared as described earlier (*20*). pET32 plasmids encoding $(His)_6$-thioredoxin-PH1, -PH2, and -PDZ were used to express the $(His)_6$-PH1, -PH2, and -PDZ proteins as previously described (*16*). The His-Tag fusions were purified on Ni−NTA−agarose following the procedure given earlier (*20*). The MBP fusions were purified using the batch method described by Jarrett and Foster (*23*) on amylose resin. The proteins were checked for their purity on a 10% SDS−polyacrylamide gel and the bands stained with Coomassie brilliant blue (*24*). The protein concentrations were determined using the Bradford assay (*25*) with bovine serum albumin as the standard.

Preparation of Lipid Vesicles. Lipids were dissolved (at 10 mg/mL for PC and 1 mg/mL for all the others) in chloroform under nitrogen and mixed to give solutions that were (i) 100% (w/w) PC; (ii) 95% (w/w) PC and 5% (w/w) PtdIns4,5P_2, PA, PE, PI, PS, SM or PtdIns4P; and (iii) 10.0, 5.0, 1.25, 0.63, 0.31, 0.16, 0.08, or 0.04% (w/w) PtdIns4,5P_2 with the remainder as PC (w/w) resulting in 100% lipid. Mixtures were dried under a stream of nitrogen and rehydrated with 10 mM Tris-HCl (pH 7.2), 100 mM NaCl to a final lipid concentration of 3.4 mg/mL.

The resuspended lipids were vortexed under nitrogen followed by two cycles of ultrasonication on ice with a microprobe for 15 s with 45 s cooling intervals using a probe-type sonicator (VirSonic 50, The Virtis Co. Inc.) at power setting 12. Samples were then bubbled with nitrogen and stored at room temperature. Prepared vesicles were used within 24 h. Unused vesicles could be stored frozen at −20 °C and were used only after repeating the sonication. The samples stored and used for the second time were then discarded.

Binding of the Proteins to Lipid Vesicles. The lipid binding assay is a modification of that described by Touhara et al. (*26*).The proteins were dialyzed in PBS (8 g of NaCl, 0.2 g of KCl, 1.44 g of Na_2HPO_4, 0.24 g of KH_2PO_4 in 1 L, pH adjusted to 7.4) overnight at 4 °C and centrifuged at a speed of 100 000 rpm (Beckman TL-100 Ultracentrifuge, TLA-100 rotor, 440000g_{max}) for 15 min at 4 °C in polycarbonate tubes (7 × 20 mm; Beckman). The proteins in the supernatant were used for the lipid binding assay.

The lipid vesicles (20 μL) were added to the tubes containing protein (2 μg), and the reaction mixture was diluted to a final volume of 40 μL with PBS. The final lipid concentration in the mixture was 1.7 mg/mL in all the assays. Ten microliters of the mixture was saved as total protein in the reaction mixture (T). After incubation at room temperature for 10 min and on ice for 5 min, the tubes were centrifuged at 100 000 rpm for 15 min at 4 °C. The supernatant (S) was saved and the pellet rinsed once with PBS. The pellet was resuspended in 60 μL of Laemmli sample buffer (*24*). Then 20 μL of this was loaded on a gel to check the precipitated or lipid-bound protein (P). The 'T' and 'S' samples, 10 μL, were mixed with 10 μL of twice-concentration (2×) Laemmli sample buffer and loaded on the same gel. The gels were stained with Coomassie brilliant blue, and the amounts of protein in 'T', 'S', and 'P' were compared using the Alpha Innotech camera system (Alpha Innotech Corp.) and AlphaImager 2000 3.3b software. The protein concentrations of the test (e.g., 95% PC and 5% PtdIns4,5P_2 or other lipids) were compared with that of protein not exposed to lipid [blank with 10 mM Tris-HCl (pH 7.2), 100 mM NaCl in the place of lipid]; 100% PC was also used as another control to correct for any precipitation due to PC or due to ultracentrifugation itself.

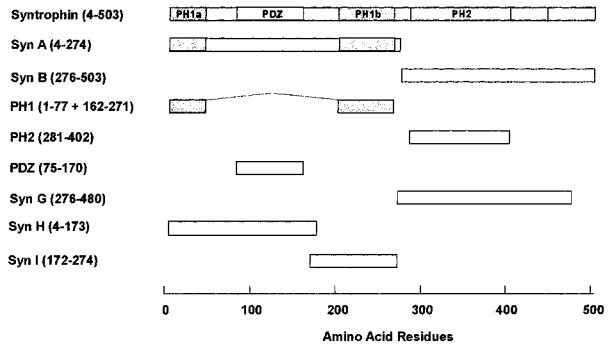

FIGURE 1: Diagrammatic representation of the mouse α1-syntrophin fusion proteins. The shaded portion shows the location of the PH1 domain.

His-Tag Digestion. Fifty microliters of Syn A (0.8 mg/mL) was digested with 1 μL of endoproteinase Xa (1 mg/mL) by dialyzing the mixture overnight at 4 °C against digestion buffer (50 mM Tris, pH 8.0, 100 mM NaCl, 2 mM $CaCl_2$, and 1 mM NaN_3). The protein was checked on the gel, and the molecular weights were compared with that of the undigested control. The digested sample was dialyzed in PBS buffer before assaying for lipid binding.

RESULTS

The sequences of mouse α1-syntrophin expressed as fusion proteins are represented diagrammatically in Figure 1. In parentheses are given the regions of mouse α1-syntrophin's 503 amino acid sequence contained in each construct. The PH1 domain sequences are shaded in those fusion proteins which contain them since these were found to bind PtdIns4,5P_2 as demonstrated below.

The proteins were produced as His-Tag fusion proteins in the case of Syn, Syn A, Syn I, PH1, PH2, and PDZ, and maltose-binding protein fusions in the case of Syn B, Syn G, and Syn H. Additionally, the vector used for PH1, PH2, and PDZ (pET32) adds thioredoxin sequences immediately following the His-Tag at the amino terminus. These different expression systems were found necessary to obtain adequate amounts of full-length proteins. Each fusion protein was expressed and purified using either Ni^{2+}−NTA-agarose (for His-Tag binding fusions) or amylose resin (for maltose binding ones). Figure 2 shows the purified proteins on a 10% SDS−polyacrylamide gel. The proteins were relatively pure and full-length in most cases, with a few showing partial proteolysis. This partial proteolysis has been investigated and discussed previously (*20, 23*). The obvious exception in Figure 2 is the PH2 protein which is degraded to a greater extent than the other proteins. This was variable from preparation to preparation, with some giving predominantly the intact protein. Also, the PDZ domain protein migrates as a dimer in Figure 2 regardless of being boiled for 2 min

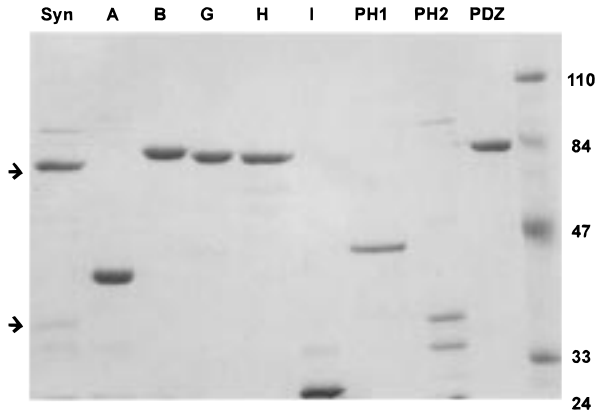

FIGURE 2: Purity of the proteins used. Purified fusion proteins (1 μg) were electrophoresed on a 10% sodium dodecyl sulfate−polyacrylamide gel and stained with Coomassie brilliant blue. The molecular masses of the markers, in kilodaltons, are shown to the right. To the left are two arrows: the upper is at the position of full-length syntrophin, the lower at full-length PH2. The proteins, indicated across the top of the gel, are (from left to right) syntrophin, syntrophin A, B, G, H, and I (see Figure 1 for sequence regions), and the isolated syntrophin PH1, PH2, and PDZ domain fusion proteins.

in the presence of SDS and 2-mercaptoethanol. Boiling for extended periods is necessary to observe predominantly the monomer molecular weight (unpublished data). The experiments were all repeated with several different preparations of each protein and were reproducible with all preparations tested.

The dialyzed proteins were centrifuged at 100 000 rpm before the lipid binding assay in order to remove any insoluble protein. To assess lipid binding, a centrifugal liposome-based binding assay was used. Liposomes pellet in the ultracentrifuge under these conditions and carry with them those proteins which bind. As a control for protein precipitation or nonspecific lipid binding, protein solutions were also centrifuged without added liposomes or with liposomes containing only PC. These controls are especially important for syntrophin since we (*20*, *27*) and others (*28*) have reported aggregation or oligomerization of syntrophin which can also affect its behavior in the ultracentrifuge. PtdIns4,5P_2 binding is a property of those proteins which pellet or pellet to a greater extent, only when PtdIns4,5P_2 is present in the liposomes.

Figure 3 shows the results of the lipid binding assay for Syn A on a 10% SDS−polyacrylamide gel. For Syn A and each of the other fusion proteins, precipitation in the ultracentrifuge was examined under three conditions: no lipid, 100% PC, or 5% PtdIns4,5P_2 + 95% PC. While 30−35% of this preparation of SynA pelleted in the absence of PtdIns4,5P_2, in its presence precipitation was nearly complete. Thus, while a portion of SynA aggregates under these conditions, the majority must also bind PtdIns4,5P_2.

Figure 4 summarizes the binding observed for Syn, Syn A, and PH1. In each case, it was observed that despite prior centrifugation, some portion of each protein pelleted during the assay even when PtdIns4,5P_2 was absent. This is seen in the two controls. The precipitation in the controls appears to be due to aggregation of the syntrophin rather than PC binding since it is also observed in the control in which lipid is absent. However, liposomes containing PtdIns4,5P_2 always bound and pelleted much greater amounts of these three

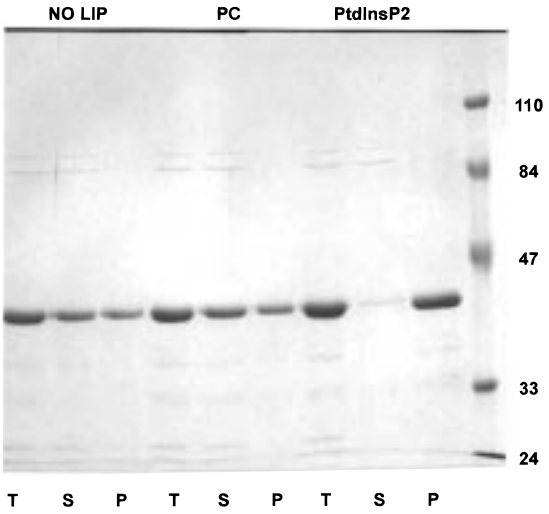

FIGURE 3: PtdIns4,5P_2 binding to Syn A as compared to controls with no PtdIns4,5P_2. Binding is shown on a 10% sodium dodecyl sulfate−polyacrylamide gel stained with Coomassie brilliant blue. The molecular masses of the markers, in kilodaltons, are to the right. T, total protein in the reaction mixture; S, protein in the supernatant after centrifugation; P, protein in the pellet. No lipid (lanes 1−3), 10 mM Tris-HCl (pH 7.2), 100 mM NaCl in the place of lipid; PC (lanes 4−6), 100% (w/w) PC; PtdIns4,5P_2 (lanes 7−9), 5% (w/w) PtdIns4,5P_2 and 95% (w/w) PC.

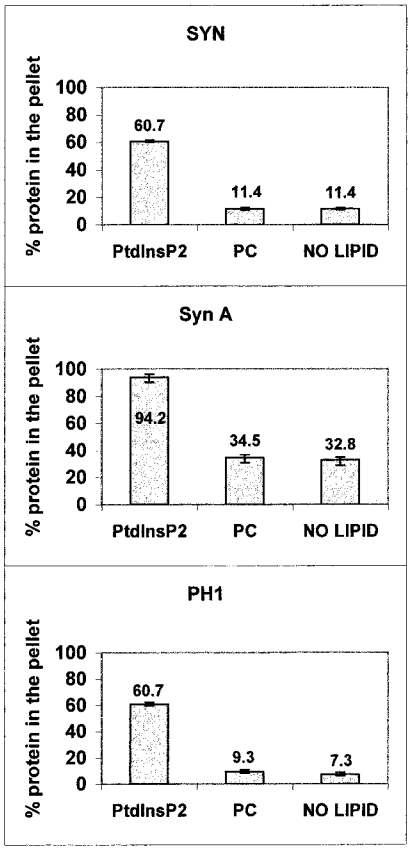

FIGURE 4: Extent of PtdIns4,5P_2 binding to Syn, Syn A, and PH1. This is presented as a percentage of the total protein (2 μg) pelleted along with PtdIns4,5P2, and the binding level compared to that of two different controls. The labels are as defined in Figure 3. The range of deviation from the mean (of triplicates) is given as error bars in each graph.

fusion proteins than could be accounted for in the controls, presumably because of a specific interaction between PtdIns4,5P_2 and the PH1 domain present in all three proteins.

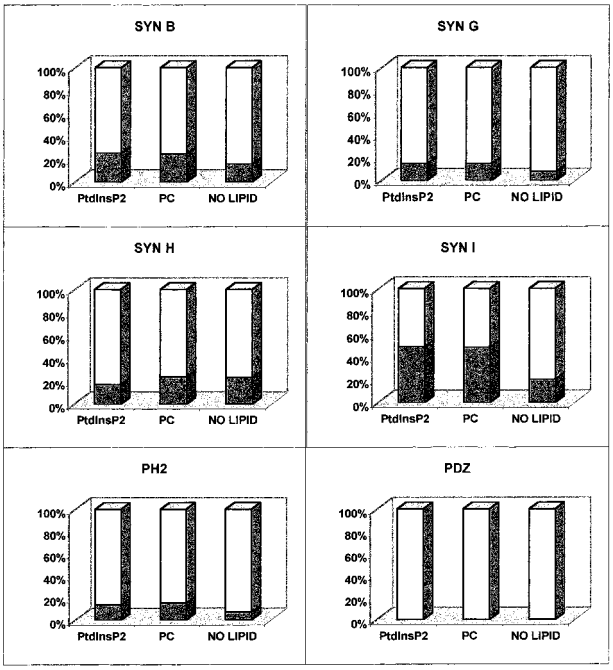

FIGURE 5: PtdIns4,5P_2 does not bind to Syn B, Syn G, Syn H, Syn I, PH2, and PDZ. The percentage of total protein (2 μg, full bar length) precipitated along with PtdIns4,5P_2-containing liposomes is shown compared to two different controls. Labels are as defined in Figure 4. Open bars (white), percent protein in the supernatant; closed bars (black), percent protein in the pellet; full bar length represents protein in the supernatant + protein in the pellet. The deviation from the mean (of duplicates) ranged from −1.5 to +1.5 for all the experiments.

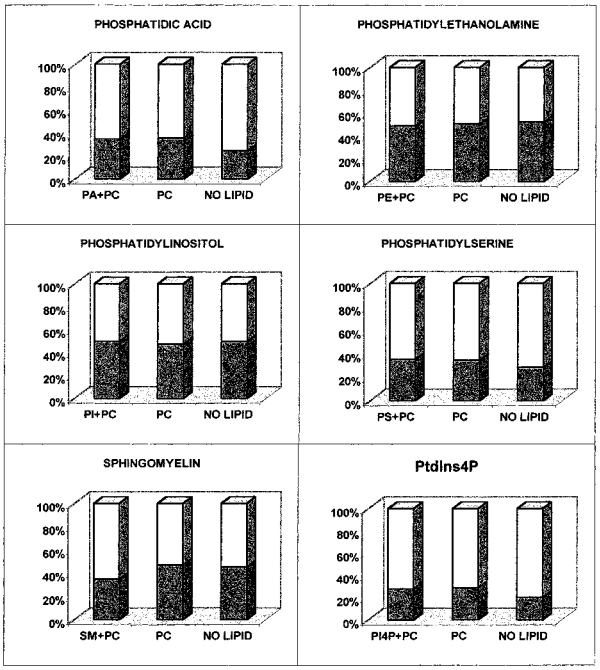

FIGURE 6: Specificity of lipid that can bind Syn A. This is given as the percentage of total protein (2 μg, full bar length) precipitated due to various lipids by comparing the binding level to that of two different controls. Lipid+PC, 5% (w/w) of the corresponding lipid and 95% (w/w) PC; other labels and the bar graph representation are described in Figure 5. The deviation from the mean (of duplicates) ranged from −3.8 to +2.3 for all the experiments.

Other proteins tested, namely, Syn B, Syn G, Syn H, PH2, and PDZ, did not show any significant lipid binding. Figure 5 summarizes the results with these proteins and Syn I. Even though there is some binding in the case of Syn I with PtdIns4,5P_2, it is not due to PtdIns4,5P_2 since the same amount of precipitation is seen in the control containing 100% PC. This lipid binding may result from the PH1 domain C-terminal sequences (i.e., PH1b) present in Syn I (see Figure 1), but the binding in this case is not specific to PtdIns4,5P_2. The combined results shown in Figures 4 and 5 show that those fusion proteins containing an intact PH1 domain bind PtdIns4,5P_2 while those lacking these sequences do not. Interestingly, while these results strongly suggest that PH1 binds PtdIns4,5P_2, they also reveal that PH2 is not functional. This will be discussed further.

The specificity of the lipid that can bind syntrophin was tested. Liposomes containing 5% PA, PE, PI, PS, SM, PtdIns4P, or PtdIns4,5P_2 mixed with 95% PC were used for lipid binding assays. The results for Syn A are shown in Figure 6. Syn A did not bind to any of the other lipids (Figure 6) in contrast to the binding of 5% PtdIns4,5P_2 already demonstrated. Thus, the lipid binding appears to be very specific to PtdIns4,5P_2 and does not occur even with the very similar lipid, PtdIns4P, which lacks only the 5-phosphate.

Figure 7 shows the binding of different concentrations of PtdIns4,5P_2 to Syn A. The binding saturates above 2.5% PtdIns4,5P_2 with no additional binding at higher concentrations. The concentrations of Syn A in these assays (0.9 and 1.8 μM for 1 and 2 μg, respectively) may well be near or above the binding affinity for PtdIns4,5P_2. Thus, it is uncertain whether the concentration of PtdIns4,5P_2 which

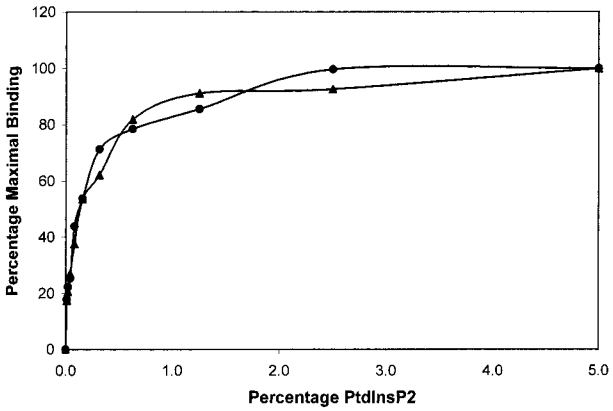

FIGURE 7: Binding of different concentrations of PtdIns4,5P_2 to two different concentrations of Syn A. (●) represents experiments with 2 μg of total protein, and (▲) represents experiments with 1 μg of total protein in the reaction mixture. The percent of total protein in the pellet in the case of liposomes containing only PC (control) is less than the ones containing PtdIns4,5P_2 (0.01−5%), and was subtracted from all the percent protein precipitated to correct for nonspecific binding by PC. This gives the amount of protein bound and pelleted by PtdIns4,5P_2. The maximal binding observed for 5% PtdIns4,5P_2 (nearly 54% of the total protein) was plotted as 100% binding in this case in order to show the concentration of PtdIns4,5P_2 at which there is half-maximal binding. Half-maximal binding occurs at 0.12−0.14% PtdIns4,5P_2.

gives 50% binding represents a binding affinity or a measure of stoichiometry, though we suspect both contribute. Half-maximal binding occurs at 1.8−1.9 μM (~0.12%) PtdIns4,5P_2 (for 2 and 1 μg of Syn A, respectively). From the 2 μg of Syn A data, an upper limit of the stoichiometry of binding can be calculated. Half-maximal binding occurs at a stoichiometry of 1 PtdIns4,5P_2/Syn. Thus, maximal binding must occur at about 2 PtdIns4,5P_2 or less. A value of 1 would be

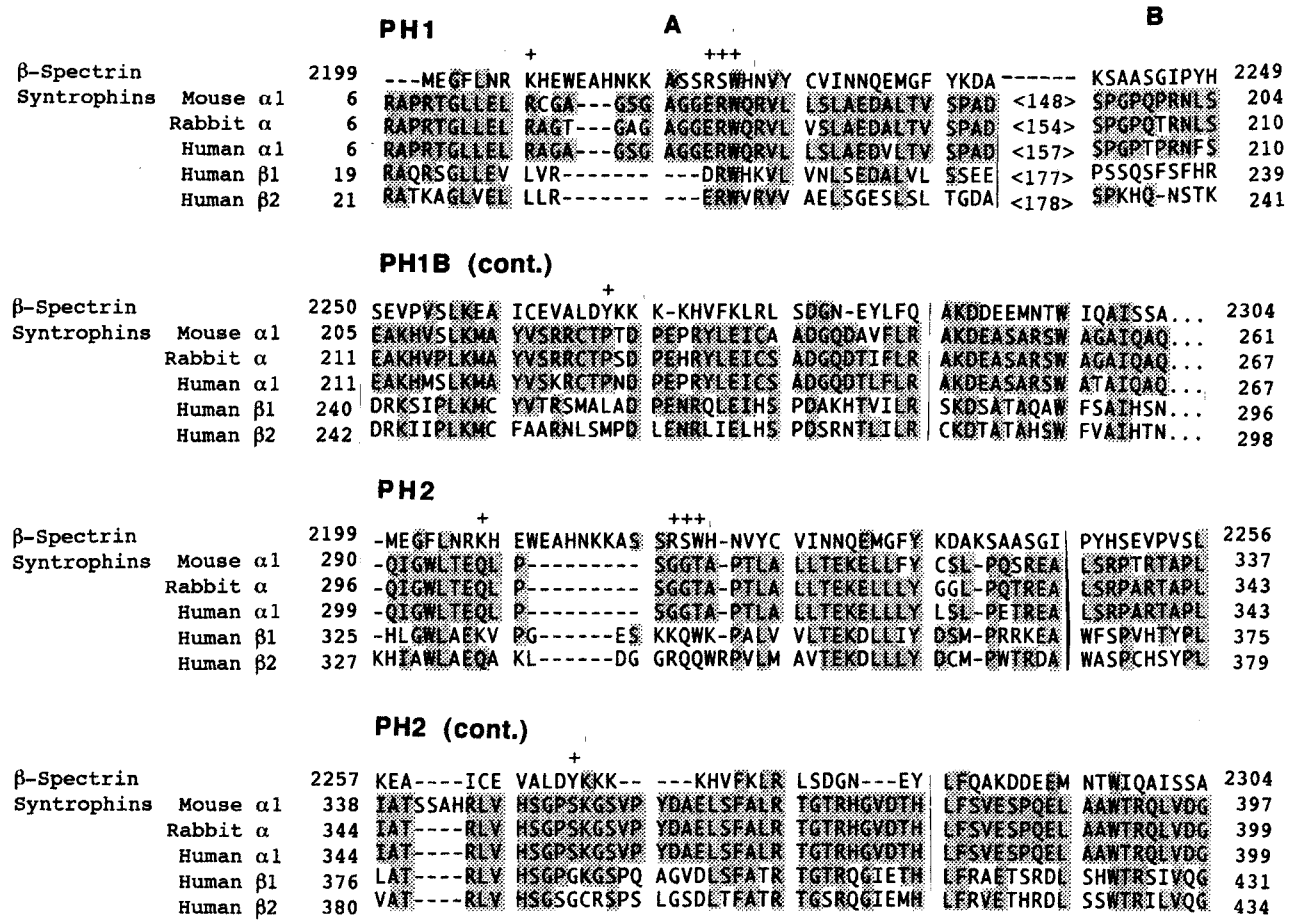

FIGURE 8: Comparison of syntrophin and spectrin PH domains. The PH domains of the syntrophins shown were aligned using the CLUSTAL4 algorithm included in the DNASIS software package. These aligned sequences were then aligned with mouse brain β-spectrin following the alignment given by Gibson et al. (*5*) for rabbit α- and human β2-syntrophin.

expected for this stoichiometry, and this result may suggest either that the lipid is impure or that the dissociation constant lying within the micromolar range is affecting this estimate of stoichiometry. No further experiments were performed to address this issue.

Since Syn, Syn A, and PH1 are all His-Tag fusion proteins, we also removed these fused sequences to prove they were not responsible for the observed behavior. The fused sequences were removed from Syn A with endoproteinase Xa. The lipid assay was performed using the digested sample and compared with that of the undigested sample. The digested and undigested samples were bound and pelleted to a similar extent by PtdIns4,5P_2 (data not shown). That PtdIns4,5P_2 binding is not due to His-Tag is also shown by the PH2 and PDZ proteins; both are also His-Tag binding fusions, but they did not exhibit any PtdIns4,5P_2 binding (Figure 5).

Since PH domains in proteins such as the oncogene *dbl* product bind Ca^{2+} (*22*) and we have shown that syntrophin binds Ca^{2+} (*20*), we also assayed syntrophin fusion proteins in 0.1 mM EGTA or in 0.1 mM $CaCl_2$ and found no differences in PtdIns4,5P_2 binding. Furthermore, the presence of a 5-fold molar excess of calmodulin over syntrophin also did not affect its PtdIns4,5P_2 binding (data not shown). Other experiments showed that replacing NaCl with KCl, or the phosphate buffer with HEPES were also without effect (data not shown).

Since both dystrophin (*32*) and syntrophin (this report) bind PtdIns4,5P_2, we also assayed whether the binding of dystrophin to syntrophin was disrupted by PtdIns4,5P_2. Syntrophin interacts with the COOH-terminal domain of dystrophin which we have expressed as a fusion protein called DysS9 (*20*). This protein still binds to syntrophin with approximately the same affinity whether PtdIns4,5P_2 is present. Furthermore, when mixed together, syntrophin and DysS9 pellet together with PtdIns4,5P_2-containing liposomes but not with liposomes containing only phosphatidylcholine (data not shown). Thus, PtdIns4,5P_2 does not appear to affect this interaction of syntrophin with dystrophin, an interaction we have previously shown is inhibited by Ca^{2+}-calmodulin (20).

DISCUSSION

The PH domains of syntrophin were known only by homology, and no biological activity of these domains had been demonstrated. Here, we have shown that the PH1 domain of mouse α1-syntrophin binds PtdIns4,5P_2 (Figures 3 and 4). This activity is not present in other syntrophin fusion proteins which lack the PH1 domain sequences (Figure 5). Interestingly, fusion proteins containing the PH2 domain do not appear to function in lipid binding. This is not due to the partial proteolysis of PH2 (Figure 2). Preparations of the PH2 fusion protein showing less proteolysis also do not bind, and other fusion proteins containing the PH2 domain (i.e., Syn B and Syn G) also do not bind PtdIns4,5P_2.

The lipid binding to PH1 is quite specific for PtdIns4,5P_2 and does not occur with other lipids tested, including other phosphatidylinositides (i.e., PtdIns4P) or with other acidic lipids (Figure 6). This agrees with the observation by Harlan et al. (*4*) that the N-terminal PH domain of pleckstrin binds PtdIns4P with about 23-fold lower affinity than it binds PtdIns4,5P_2. Half-maximal binding occurs at 1.9 μM PtdIns4,5P_2. In comparison, the N-terminal PH domain of pleckstrin gives half-maximal binding at 51 μM under similar conditions; thus, syntrophin is a high-affinity PtdIns4,5P_2 binding protein. Our binding data allow an upper limit to be established at 2.4 PtdIns4,5P_2 bound per syntrophin (Figure 7).

PH domains are present in other proteins which, like syntrophin, are membrane-associated. The nonerythroid β-spectrin PH domain is particularly well characterized (*29*). The structure of the complex has been studied using mutation, ORD/CD, NMR, and diffraction techniques and reveals that major interactions are salt bridges between the PtdIns4,5P_2 phosphates and K8 and R21, and hydrogen bonds with S22, W23, and Y69 (this is domain numbering where M2199 is position 1). These positions are denoted by a "+" in Figure 8 where β-spectrin is aligned with mammalian syntrophins. Of these interactions, K8 was considered to be a key residue (*29*). In mouse α1-syntrophin's PH1 domain, the homologous residue is R16 which should also be capable of forming a salt bridge. W23 of spectrin is conserved as W28 of syntrophin. While the other positions do not appear to be well conserved, a similar amino acid is usually found nearby which may serve the same role. For example, R21 of spectrin aligns with E26 of syntrophin, but nearby is syntrophin R27 (and R30) which may actually serve the homologous role. Focusing upon these key spectrin residues, none is conserved in α-syntrophin's PH2 domain, perhaps accounting for why this domain is nonfunctional for PtdIns4,5P_2 binding.

The β-syntrophins were not a part of our study, but Figure 8 does reveal some interesting differences. The PH1 domain is even less well conserved in terms of these important residues than it was in α-syntrophins. This may suggest that the β-syntrophin PH1 domain is less capable of PtdIns4,5P_2 binding. Interestingly, in human β1-syntrophin, the PH2 domain shows the greatest similarity to spectrin, and this domain may be a functional lipid binding domain.

Binding to PtdIns4,5P_2 may account for syntrophin's membrane localization. PtdIns4,5P_2 binding appears to account reasonably well for the membrane localization of other PH domain proteins such as the β-adrenergic receptor kinase (*2*, *30*, *31*). However, with all of the known interactions syntrophin has with other membrane proteins, the precise interactions anchoring syntrophin to the membrane may be quite complex.

A recent report has shown that PtdIns4,5P_2 binding to highly purified dystrophin inhibits its interaction with actin. Half-maximal inhibition occurred at less than 10 μM PtdIns4,5P_2 (*32*). Thus, PtdIns4,5P_2 binds to at least two constituents of the dystrophin−glycoprotein complex.

PtdIns4,5P_2 also serves an important role in cell signaling. Its hydrolysis by phospholipase C results in two important second messengers: diacylglycerol and inositol 1,4,5-trisphosphate. This hydrolysis, by altering the amount of PtdIns4,5P_2 in the membrane, may also affect the cellular localization of syntrophins. Conversely, syntrophin may make PtdIns4,5P_2 in its immediate vicinity unavailable for signal transduction. Furthermore, syntrophin binding to dystrophin (*27*, *28*, *33*−*35*) or other DGC proteins (*27*), Na^+-channels (*16*, *17*), and nitric oxide synthetase (*15*) may be affected by PtdIns4,5P_2 binding. While syntrophin binding to dystrophin's C-terminal domain was not affected in our experiments, it remains to be seen how PtdIns4,5P_2 functions in the intact DGC.

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