Oligomerization of Mouse α1-Syntrophin and Self-Association of Its Pleckstrin Homology Domain 1 Containing Sequences†

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ABSTRACT: Syntrophins are known to self-associate to form oligomers. Mouse α1-syntrophin sequences were produced as chimeric fusion proteins in bacteria and were found to also oligomerize and in a micromolar Ca^{2+}-dependent manner. The oligomerization was localized to the N-terminal pleckstrin homology domain (PH1) or adjacent sequences; the second, C-terminal PH2 domain did not show oligomerization. PH1 was found to self-associate, and calmodulin or Ca^{2+}-chelating agents such as ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA) could effectively prevent this oligomerization. A single calmodulin bound per syntrophin to cause inhibition of the precipitation. Since calmodulin inhibited syntrophin oligomerization in the presence or absence of Ca^{2+}, Ca^{2+} binding to syntrophin is responsible for the inhibition by EGTA of syntrophin oligomerization.

Syntrophin, a peripheral membrane protein, was first identified in the postsynaptic membrane of *Torpedo* (*1*). Syntrophins were found to be associated with the dystrophin glycoprotein complex (*2*) whose defects cause Duchenne, Becker, various limb girdle, and other muscular dystrophies (*3*). Syntrophins are a multigene family of homologous proteins, namely, α1, β1, and β2. α1-Syntrophin is predominantly expressed in striated muscles and brain, whereas β-syntrophins are ubiquitous in mammalian tissues (*4*). All syntrophins are known to contain two pleckstrin homology (PH)[1] domains, an N-terminal PH1 domain and a PH2 domain. This domain structure is shown diagrammatically in Figure 1. The PH1 domain is interrupted by an inserted PDZ domain, a homologous domain found in the postsynaptic density 95 kDa protein, neuronal nitric oxide synthetase, and other membrane proteins (*5*, *6*). Syntrophins also have a unique domain at the C-terminus, the syntrophin unique (SU) domain. Recently, the PH1 domain of α1-syntrophin has been reported to bind phosphatidylinositol 4,5-bisphosphate (PtdIns4,5P_2) (*7*). Syntrophins also bind calmodulin (*8*). The SU domain of syntrophin has also been shown to bind Ca^{2+}−calmodulin (*9*), a ubiquitous calcium binding protein. The N-termini of the PH1 domain and the PDZ domain have been reported (*10*) to also bind calmodulin. Whether this N-terminal calmodulin binding is Ca^{2+}-dependent (*10*) or independent (*9*) is controversial (*11*). α1-Syntrophin has also been shown to bind calcium (*9*). Thus, Ca^{2+}, calmodulin, and PtdIns4,5P_2 may affect the activities of syntrophins. Syntrophins may also play a role as an adapter that links different cellular proteins to the dystrophin glycoprotein complex (*12*). PH domains are also known to bind heterotrimeric G protein, $G_{\beta\gamma}$, and syntrophin's PDZ domain binds voltage-gated Na^+-channels in muscles and nerves (*13*) and the MAP kinase SAPK3 (*14*). The SU domain binds Ca^{2+}−calmodulin and the SU domain plus other sequences in the C-terminus of the protein bind to dystrophin, the protein product of the Duchenne muscular dystrophy gene. Ca^{2+}−calmodulin binding inhibits the syntrophin−dystrophin interaction (*9*). α1-Syntrophins are known to self-associate and associate with other syntrophins (*15*, *9*, *4*, *16*). This oligomerization is currently only poorly characterized. Oligomerization of syntrophins is also likely to be physiologically relevant since the dystrophin glycoprotein complex contains one copy of most constituents but two syntrophins, suggesting self-association to at least a dimer in vivo (*17*). In this paper, we have studied oligomerization of the mouse α1-syntrophin. We show here that the PH1 domain of α1-syntrophin is involved in the oligomerization of syntrophin in vitro and that Ca^{2+} regulates it.

MATERIALS AND METHODS

Materials. Endoproteinase Xa (from bovine plasma) was from New England Biolabs. T7 monoclonal antibody for His_6-tag fusion proteins was from Novagen. Goat anti-rabbit IgG (H + L)−alkaline phosphatase conjugate and goat anti-mouse IgG (H + L)−alkaline phosphatase conjugate were from Bio-Rad. Ni^{2+}−NTA−agarose was from Qiagen. Cyanogen bromide-preactivated Sepharose was from Sigma. All other chemicals were of the highest purity available commercially.

Fusion Proteins. The syntrophin fusion proteins His_6−Syn and His_6−Syn A and the maltose binding protein (MBP) fusions MBP−Syn B and MBP−Syn H were prepared as

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[1] Abbreviations: PH, pleckstrin homology; $G_{\beta\gamma}$, heterotrimeric guanosine triphosphatase β- and γ-subunit complex; MBP, maltose binding protein; SDS−PAGE, sodium dodecylsulfate−polyacrylamide gel electrophoresis; PtdIns4,5P_2, phosphatidylinositol 4,5-bisphosphate; NBT, nitroblue tetrazolium; BCIP, 5-bromo-4-chloro-3-indolylphosphate; NTA, nitrilotriacetic acid; SAPK3, stress-activated protein kinase 3; BSA, bovine serum albumin; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid.

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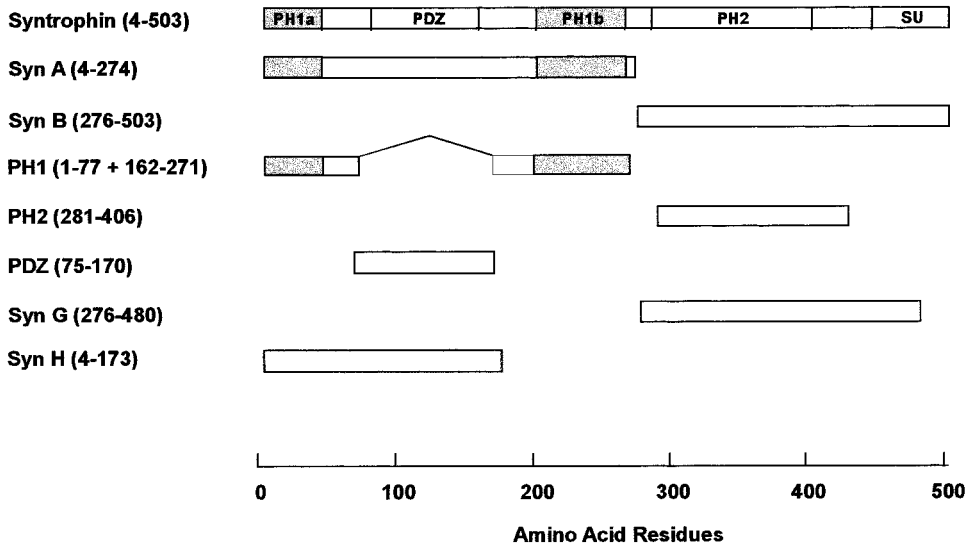

FIGURE 1: Diagrammatic representation of fusion proteins containing α1 syntrophin sequences. The shaded portion shows the location of PH1 domain. Numbers in the parentheses represent the syntrophin amino acid sequences present in each fusion protein.

described previously (*9*). pET32 plasmid constructs for PH1, PH2, and the PDZ domain were a generous gift from Drs. Steven Gee and Stan Froehner, (Department of Physiology, University of North Carolina, Chapel Hill). pET32 plasmids encoding His_6-thioredoxin-PH1, −PH2, and −PDZ were used to express proteins referred to here as His_6−PH1, −PH2, and −PDZ (*13*). The His-tag fusion proteins were purified by use of Ni^{2+}−NTA−agarose from Qiagen as described earlier (*9*). The MBP fusion proteins were purified by amylose affinity chromatography as described previously (*18*). The purity of the proteins was determined by SDS−12% polyacrylamide gel electrophoresis by the method of Laemmli (*19*). The major bands of the fusion proteins were of expected size and relatively high purity. The Bradford assay (*20*) was used to determine the protein concentrations with bovine serum albumin as the standard.

Ultracentrifuge Oligomerization Assay. All proteins were centrifuged at 100 000 rpm (Beckman TLA-100 rotor) before dialysis in order to remove any insoluble protein. Fusion protein (50 μL) was dialyzed in buffer A (50 mM Tris, pH 7.5, and 100 mM KCl) containing 100 μM Ca^{2+} or 100 μM EGTA overnight at 4 °C. The next day 10 μL of the dialyzed sample was saved as the total T. To this was added 10 μL of 2× Laemmli buffer. Twenty μL of the remaining sample was subjected to centrifugation at 100 000 rpm (Beckman TL-100 ultracentrifuge, TLA-100 rotor, 440 000g_{max}) for 15 min at 4 °C in polycarbonate tubes (7 × 20 mm; Beckman). Then 10 μL of the supernatant (S) was taken and 10 μL of 2× Laemmli buffer (1× is 62.5 mM Tris-HCl, pH 6.8, 0.001% bromophenol blue, 6.25% glycerol, 2% sodium dodecyl sulfate, and 0.7 M 2-mercaptoethanol) was added to it. After removal of the remaining supernatant, the pellet (P) was dissolved in 40 μL of 1× Laemmli buffer with vigorous vortexing and a bath-type sonicator. The samples were heated for 5 min at 95 °C, applied to an SDS−12% polyacrylamide gel (*19*), electrophoresed, and stained with Coomassie brilliant blue. The amount of protein in the total, supernatant, and pellet was compared by use of Alpha Innotech camera system (The Alpha Innotech Corp.) and AlphaImager 2000 3.3b software. The amount of the protein in T was treated as 100%, and the percentage of the protein in S and P was calculated accordingly.

Solid-Phase Binding Assays. Syn (1.6 mg, 0.8 mg/mL) and 1.8 mg of PH1 (1.5 mg/mL) were coupled to 1 g of cyanogen bromide-activated Sepharose (Sigma) by procedures recommended by the manufacturer (Pharmacia). The support was then washed with the coupling buffer (0.1 M $NaHCO_3$, pH 8.3, and 0.5 M NaCl) and blocked for 2 h with 0.1 M Tris-HCl, pH 8. The amount of the protein coupled (0.2 mg/g of Sepharose for syntrophin and 1.67 mg/g of Sepharose for PH1) was determined by the difference in the ultraviolet absorption of the added protein and that recovered from coupling in the wash fractions. For negative controls, CNBr-activated Sepharose to which no protein was coupled was used.

A 50% slurry of Syn-Sepharose (200 μL) containing 3 μg of Syn was equilibrated with buffer A containing 100 μM Ca^{2+} and then incubated with different fusion proteins (25 μg) for 1 h at room temperature and for 30 min on ice with gentle mixing in a final volume of 200 μL. For control, Sepharose without any protein coupled to it was used. After the incubation, Syn-Sepharose was washed three times with 0.5 mL of BSA/TTBS (1 mg/mL BSA in 20 mM Tris-HCl, pH 7.5, 0.5 M NaCl, and 0.05%Tween-20). The protein was eluted with 60 μL of 2× Laemmli buffer. Samples were heated for 5 min at 95 °C. The samples were then centrifuged for 5 min at room temperature to remove the resin, applied to electrophoresis on an SDS−12% polyacrylamide gel (*19*), and electroblotted onto nitrocellulose paper (*21*). The paper was then blocked with 10 mg/mL BSA in TTBS. After extensive washing with 1 mg/mL BSA/TTBS, the blot was incubated with affinity-purified anti-MBP (1:1000 dilution) for MBP fusion proteins or T7 monoclonal antibody (Novagen, 1:10 000 dilution) for His-tag fusion proteins. Goat anti-rabbit IgG (H + L)−alkaline phosphatase conjugate and goat anti-mouse IgG (H + L)−alkaline phosphatase conjugate (both 1:1000 dilution, Bio-Rad) were used, respectively, following the primary antibodies. The blot was developed with a 1:100 dilution each of 30 mg/mL NBT (in 70% dimethylformamide) and 15 mg/mL BCIP (in 100% dimethylformamide)

in buffer AP (0.1 M $NaHCO_3$ and 1 mM $MgCl_2$, pH 9.8). A similar experiment was carried out with PH1−Sepharose.

His Tag Digestion. Syn A (50 μL) was digested with 1 μL of endoproteinase Xa (1 mg/mL) by dialyzing for 12 h at 4 °C in digestion buffer (50 mM Tris, pH 7.5, 100 mM NaCl, 2 mM $CaCl_2$, and 1 mM NaN_3). Cleavage of the fused sequences was confirmed on SDS−12% PAGE. The ultracentrifugation experiment was performed with the digested protein and compared with the results for the undigested protein.

Size-Exclusion Chromatography. Syn A (0.8 mg/mL) was dialyzed in MC (10 mM Mops, pH 7.0, 90 mM KCl, and 1 mM $CaCl_2$) or ME buffer (10 mM Mops, pH 7.0, 90 mM KCl, and 1 mM EGTA) for 12 h at 4 °C. The dialyzed protein (300 μL) was loaded on a Sepharose-4B column (1.4 cm × 42.5 cm). Mobile phase (MC or ME buffer, 80 mL) was passed through the column at a flow rate of 0.33 mL/min, and 1.5 mL fractions were collected. Three equivalents of calmodulin was added to Syn A in the experiments involving calmodulin. A microtiter plate (Dynatech Immulon 1) was coated by incubation of 100 μL/well of each fraction for 4 h at 4 °C. The plate was then blocked by replacing the fractions in each well with 300 μL of 30 mg/mL BSA in AC7.5 buffer (50 mM Tris-HCl, pH 7.5, 100 mM KCl, 3 mM $MgCl_2$, and 1 mM $CaCl_2$). All subsequent operations were at room temperature and 100 μL/well. After extensive washing with AC7.5T/BSA (buffer AC7.5 plus 0.1% Tween-20 and 1 mg/mL BSA), the plate was incubated with T7 monoclonal antibody (Novagen, 1:10 000 dilution). Goat anti-mouse IgG (H + L)−alkaline phosphatase conjugate (1:1000 dilution, Bio-Rad) was used following the primary antibody. The plate was then washed once with 1 mg/mL AC7.5T/BSA followed by two immediate washes of diethanolamine buffer (10 mM diethanolamine and 0.5 mM $MgCl_2$). The plate was then developed by incubation with 100 μL/well of 1 mg/mL *p*-nitrophenyl phosphate in diethanolamine buffer. The absorption at 405 nm was determined.

RESULTS

Mouse α1-syntrophin sequences expressed as fusion proteins are diagrammatically represented in Figure 1. The regions of mouse α1-syntrophin's amino acid sequence in each construct are given in parentheses. The boundaries of the PH1 domain are not well defined. Our PH1 construct contains some additional sequences as shown in Figure 1. PH1 domain sequences are shaded in the fusion proteins containing them, as these are involved in oligomerization (see below). Syn, Syn A, PH1, PH2, and PDZ were produced as His-tag fusion proteins, while Syn B and Syn H were produced as maltose binding fusion proteins. Each fusion protein was expressed and affinity-purified by use of Ni^{2+}−NTA−agarose for His-tag fusion proteins and amylose−agarose for maltose binding fusion proteins.

Figure 2 shows the purity of the proteins used for the experiments. The purified proteins are mostly full-length but some show partial proteolysis. This partial proteolysis has been investigated and discussed previously (*9*, *18*). All the proteins were boiled for 5 min in the presence of SDS and 2-mercaptoethanol. This was necessary to observe predominantly the monomer molecular weight of PDZ domain protein, which otherwise migrates as a dimer (*7*). The

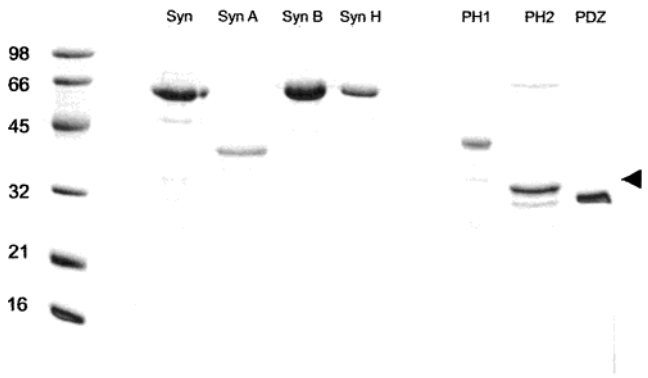

FIGURE 2: Purity of the fusion proteins used. Two micrograms of each fusion protein was subjected to SDS−PAGE and stained with Coomassie brilliant blue. The molecular mass of the markers, in kilodaltons, is shown to the left. The arrowhead to the right shows the position of full-length PH2.

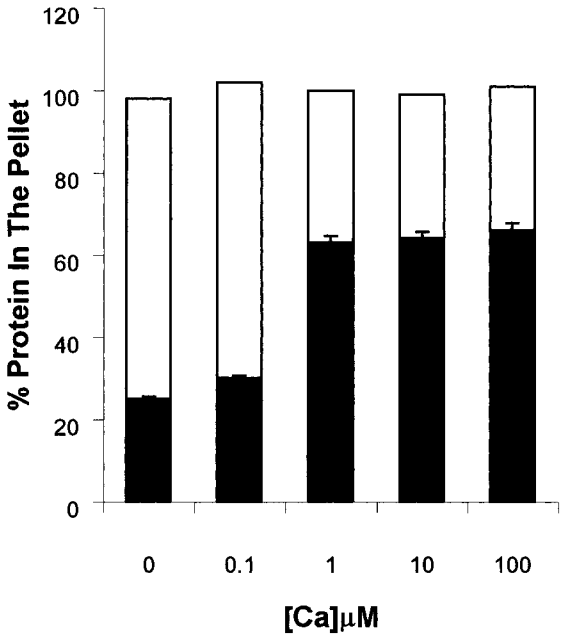

FIGURE 3: Syn A precipitates in a Ca^{2+}-dependent manner and the Ca^{2+}-chelating reagent EGTA inhibits it. The ultracentrifuge oligomerization assay was performed as described under Materials and Methods. Full bar length represents total protein. Open portion of bar, percent protein in the supernatant; solid portion of bar, percent protein in the pellet. The standard error of the mean (of duplicates) is given as an error bar for each sample. The amount of Ca^{2+} given here is the calculated, free concentration after addition of Ca^{2+} to 0.1 mM EGTA in the buffer.

experiments were repeated with different preparations of each protein and were reproducible with all of the preparations tested.

Syntrophins self-associate (*15*, *9*, *4*, *16*) and the larger oligomers can be collected by centrifugation. Oligomerization and precipitation in the centrifuge for Syn A in different Ca^{2+} concentrations or in EGTA is shown in Figure 3. Though approximately 20% of the protein precipitated in the presence of EGTA, the majority of the protein precipitated at Ca^{2+} concentrations at or above micromolar. This difference is statistically significant.

Figure 4 summarizes the precipitation observed for all the proteins tested—namely, Syn, Syn A, Syn B, Syn H, PH1, PH2, and PDZ—in the presence of 100 μM Ca^{2+} and 100 μM EGTA. His_6−green fluorescent protein (GFP) (*22*) and

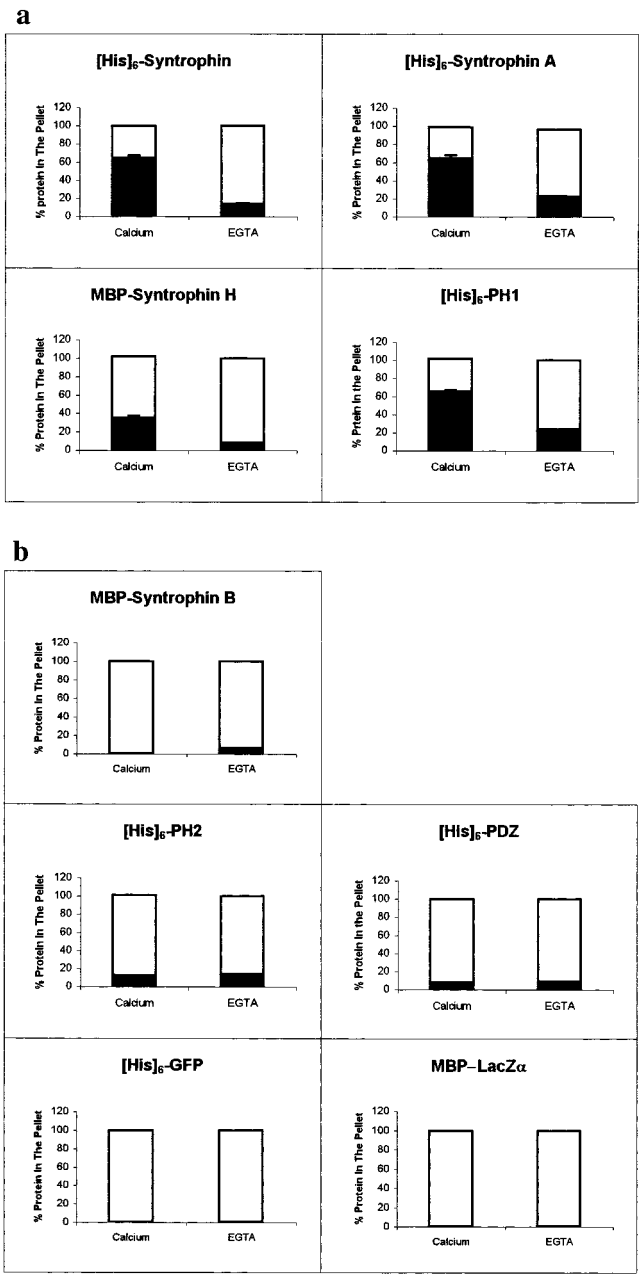

FIGURE 4: (a) Extent of precipitation of Syn, Syn A, Syn H, and PH1 in a Ca^{2+}-dependent manner. (b) Syn B, PH2, PDZ, and the controls used for His-Tag and MBP fusion proteins, GFP and MBP−LacZα, do not show significant precipitation. The labels are as defined in Figure 3. The standard error of the mean (of duplicates) is given as error bars in each graph.

MBP−LacZα (*18*) were used as controls for His-tag and maltose binding fusion proteins, respectively, and as expected, neither shows appreciable aggregation. Figure 4a shows that Syn, Syn A, Syn H, and PH1 aggregate in Ca^{2+}, while other proteins, namely, Syn B, PH2, and PDZ, which did not show any significant precipitation, are shown in Figure 4b. Thus, fusion proteins containing the PH1 domain precipitate in a Ca^{2+}-dependent manner. Though Syn H shows less precipitation as compared to other PH1 domain-containing fusion proteins, it should be noted (see Figure 1) that Syn H has only the N-terminal portion of the PH1 domain (PH1a) and lacks C-terminal sequences (PH1b).

To show that these results were not artifacts caused by prolonged dialysis or centrifugation, freshly dialyzed protein

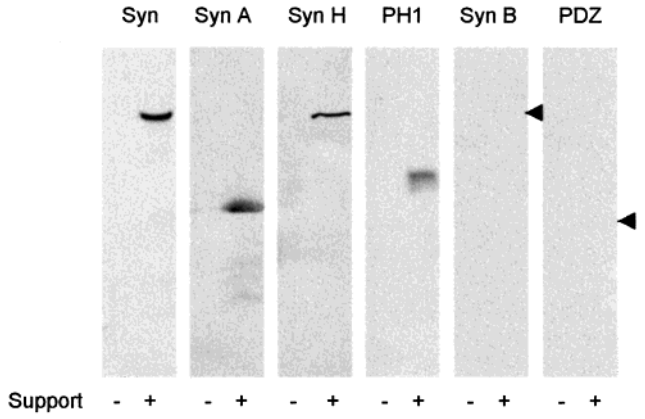

FIGURE 5: Localization of the region involved in the oligomerization of syntrophin. Syn, Syn A, Syn H, and PH1 show binding to Syn-Sepharose. (−) represents control Sepharose (no protein coupled); (+) represents Syn-Sepharose. To the right are two arrowheads; the upper shows the position of Syn B and the lower shows the position of PDZ, which do not show binding to Syn-Sepharose.

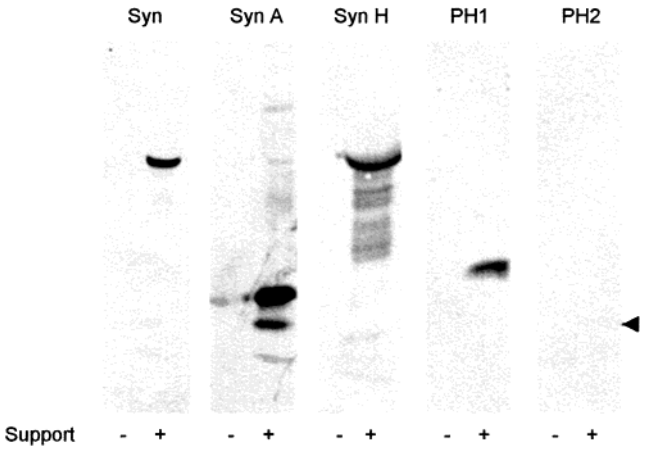

FIGURE 6: Syntrophin's self-association is localized to the PH1 domain-containing sequences. Syn, Syn A, Syn H, and PH1 show binding to PH1−Sepharose. (−) represents control Sepharose, (+) represents PH1−Sepharose. The arrowhead to the right shows the position of PH2, which does not bind to PH1−Sepharose.

was centrifuged to remove any aggregate and this was briefly incubated with Syn-Sepharose. Figure 5 confirms the localization of the syntrophin-syntrophin interaction as shown previously in Figure 4. Syn, Syn A, Syn H, and PH1 show binding to Syn-Sepharose, while Syn B and PDZ did not show any binding. CNBr-activated Sepharose-4B without any protein coupled to it was used as a negative control. Other fusion proteins such as PH2 also did not show any binding (data not shown).

The syntrophin−syntrophin interaction is further localized in Figure 6. Syn, Syn A, Syn H, and PH1 show binding to PH1−Sepharose, while PH2 did not show any binding. None of the fusion proteins bound significantly to the control Sepharose (lacking PH1). The combined results of Figures 5 and 6 show that the sequences present in the PH1 fusion protein are necessary and sufficient for syntrophin−syntrophin interaction. These also demonstrate that PH2 and PDZ domains do not play an obvious role in syntrophin−syntrophin interactions.

Ca^{2+}−calmodulin has been shown to bind the N-terminal sequences of the PH1 domain (*10*), which are also involved

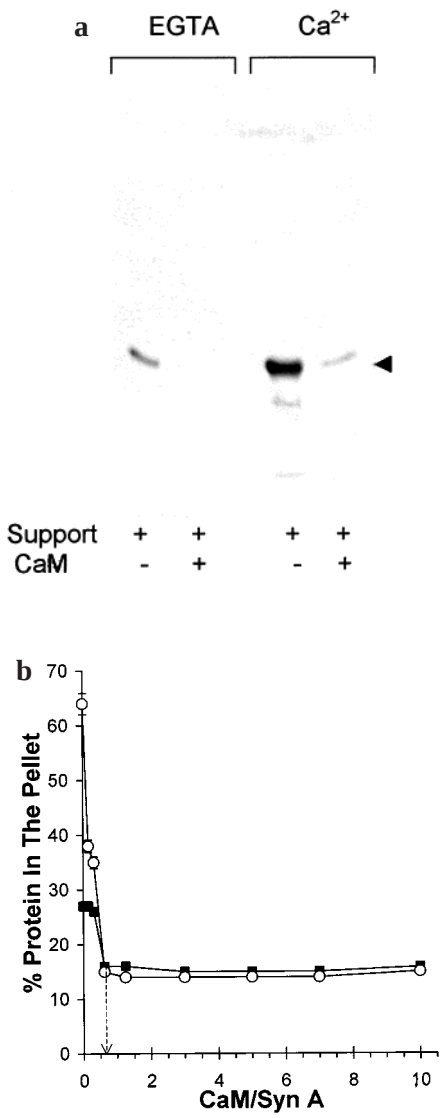

FIGURE 7: Calmodulin inhibits syntrophin self-association in the presence or absence of Ca^{2+}. (a) PH1−Sepharose was incubated with the PH1 fusion protein in the presence (+) and absence (−) of calmodulin in the presence of 100 μM Ca^{2+} or 100 μM EGTA. (b) Precipitation of Syn A (by the ultracentrifuge oligomerization assay) at different concentrations of calmodulin in the presence of 100 μM Ca^{2+} (○) or 100 μM EGTA (■). The standard error of the mean is given as error bars. The dotted line shows that the breakpoint in the titration occurs near 1 calmodulin/syntrophin.

in oligomerization. The effect of calmodulin on the syntrophin−syntrophin interaction was also tested. PH1 was incubated with PH1−Sepharose in the presence or absence of calmodulin in the presence of 100 μM Ca^{2+} or 100 μM EGTA. Inhibition of the PH1−PH1 interaction by calmodulin is shown in Figure 7a. While Ca^{2+} enhances the PH1−PH1 interaction, calmodulin inhibits it in Ca^{2+} or EGTA. Figure 7b shows the precipitation of Syn A at different concentrations of calmodulin in the presence of 100 μM Ca^{2+} or 100 μM EGTA. In Ca^{2+}, calmodulin reduces the precipitation of Syn A from 64% to 14% in a dose-dependent manner. In addition, Figure 7b shows that calmodulin also inhibits precipitation of Syn A in EGTA over a similar concentration range. For both curves, a sharp break occurs at 0.65 mol of calmodulin/mol of syntrophin, suggesting that a single calmodulin binds to cause inhibition of the precipitation.

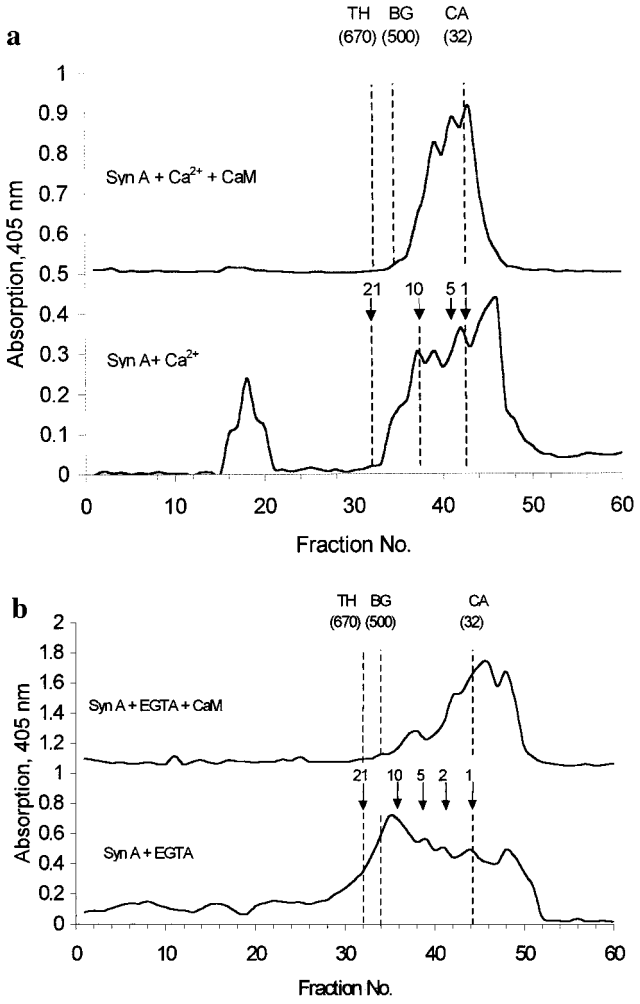

FIGURE 8: Size-exclusion chromatography of Syn A in the presence of Ca^{2+} or EGTA. The column was run as described under Materials and Methods. The dashed lines show the position of molecular mass standards thyroglobulin (TH), β-galactosidase (BG), and carbonic anhydrase (CA) on the chromatogram for reference. The numbers in the parentheses indicate molecular mass of the standards in kilodaltons. The arrows indicate the size of different oligomers of Syn A on the chromatogram, also for reference. (a) In the presence of Ca^{2+}; (b) in the presence of EGTA.

Nickel has been reported to induce oligomerization of proteins containing the His_6−tag, and removal of this affinity tag by treatment with endoproteinase Xa abolished the ability of the proteins to form oligomers (*23*). Since Syn, Syn A, and PH1 are all His-tag fusion proteins, to show that the observed precipitation was not due to the affinity tag, these fused sequences were digested with endoproteinase Xa to test whether they contributed to the precipitation. The experiment was performed with the digested protein and compared with the undigested protein. Both the digested and undigested proteins precipitated to a similar extent in a Ca^{2+}-dependent manner (data not shown). The fact that PH2, PDZ, and GFP (also His-tag fusion proteins; see Figure 4) do not precipitate confirms that precipitation is not due to His-tag sequences.

Syntrophin oligomerizes in Ca^{2+} and, to a lesser extent, in EGTA; to determine the size of Syn in the presence of Ca^{2+} or EGTA, His-tag Syn A was subjected to size-exclusion chromatography on Sepharose-4B. Figure 8a (lower panel) shows that, in the presence of Ca^{2+}, Syn A forms oligomers of several different sizes but 17% of the

protein is larger than a 20 mer (>670 kDa). When calmodulin was added to Syn A (upper trace) in the presence of Ca^{2+}, it effectively inhibited oligomerization of Syn A, resulting in protein that was predominantly present as monomeric to octameric forms.

When a similar experiment was repeated in the presence of EGTA, protein was predominantly present as monomeric to decameric forms even in the absence of calmodulin as shown in Figure 8b. The small peak at fraction number 16, which represents protein larger than a 20-mer, could account for the small amount of protein that precipitates in the ultracentrifugation experiments described earlier. When calmodulin was added to Syn A in the presence of EGTA, it prevented oligomerization and most of the protein was present at sizes consistent with a monomer.

The PH1 domain of syntrophin has been reported to bind PtdIns4,5P_2 (*7*). Other experiments showed that the presence of PtdIns4,5P_2 did not have any effect on oligomerization of Syn (data not shown), suggesting that lipid binding does not alter syntrophin's oligomeric state.

DISCUSSION

α-Syntrophins were known to self-associate and associate with other syntrophins (*15*, *9*, *4*, *16*). A consistent level of aggregation of the syntrophins in the immunoprecipitation experiments was observed by Ahn et al. (*4*). In vitro-translated α1-syntrophin has been shown to bind to all the three components of the syntrophin triplet in the overlay experiment by Yang et al. (*16*). The binding of α1-syntrophin with both *β*1 and *β*2 syntrophins has been previously seen in the overlay experiments by Madhavan and Jarrett (*15*). Peters et al. (*24*) have shown that pairs of different syntrophins occur in vivo. This oligomerization was poorly characterized. Recently, it has been shown that the PH1 domain of mouse α1-syntrophin binds PtdIns4,5P_2 (*7*). Syntrophin's PDZ domain is known to form a dimer (*7*) and thus was thought to play a role in the oligomerization of syntrophin. The syntrophin sequence has a leucine approximately every seventh residue at its N-terminus from amino acid 107 to 137, similar to the leucine zipper motif (where leucine is present at exactly every seventh residue). Leucine zippers are known to mediate protein−protein interaction, raising the possibility that this leucine zipper could function as an oligomerization motif of syntrophin. But in none of the experiments was His_6−PDZ that contains the leucine-rich sequence found to bind syntrophin or aggregate in the ultracentrifugation experiments. It may, however, account for the PDZ dimerization reported by others (*7*).

Here, we have shown that the PH1 domain-containing sequence of α1-syntrophin is required for the oligomerization of syntrophin, which occurs in a Ca^{2+}-dependent manner (Figures 4 and 5), and that micromolar concentrations of Ca^{2+} increase oligomerization (Figure 3). Conversely, Ca^{2+}-chelating agents such as EGTA inhibit oligomerization (Figure 3). Since syntrophin alone, in the absence of calmodulin, responds to Ca^{2+}, Ca^{2+} must bind directly to syntrophin to cause this effect. Since Ca^{2+} affects even constructs as small as PH1, Ca^{2+} binding must occur somewhere within these sequences. The PH domain of Dbl has been shown to bind Ca^{2+} with a K_d of 10 μM (*25*). It is curious to note that Ca^{2+} also regulates the oligomerization of other proteins such as actin (*26*) and tubulin (*27*).

Oligomerization does not occur with fusion proteins lacking the PH1 domain (Figure 4b). Fusion proteins containing an intact PH1 domain precipitate to a larger extent than does Syn H, which lacks C-terminal sequences (PH1b) of the PH1 domain (Figure 4a). This may suggest that that this oligomerization is primarily a property of the N-terminal sequences of the PH1 domain (PH1a) or adjacent sequences. Interestingly, fusion proteins containing the PH2 domain do not seem to play a role in the oligomerization of syntrophin (Figures 4 and 6). Non-PH1 domain sequences do not oligomerize (Figures 3 and 5).

We have also shown that the PH1 domain containing sequence alone is capable of self-association (Figure 6). Calmodulin inhibits the PH1−PH1 interaction (Figure 7a) and inhibition does not require Ca^{2+} (Figure 7). This agrees with the observation by Iwata et al. (*10*) that calmodulin binds to N-terminal sequences of PH1 domain. However, Iwata et al. (*10*) found that calmodulin binding to this region was Ca^{2+}-dependent, while we had previously found it to be Ca^{2+}-independent. These results confirm that calmodulin binding to this region is Ca^{2+}-independent. Figure 7b shows that calmodulin inhibits oligomerization in the presence or absence of Ca^{2+}. Thus, Ca^{2+}-calmodulin or apocalmodulin (Ca^{2+}-free) can prevent self-association of the PH1 domain-containing sequences and hence oligomerization of syntrophin. A single calmodulin apparently binds per syntrophin to inhibit syntrophin self-association (Figure 7b).

We have also shown that when syntrophin oligomerizes in the presence of Ca^{2+}, much of the protein is larger than a 20-mer (>670 kDa). It also forms several species of syntrophin oligomers such as 5-mer, 10-mer, etc. The presence of calmodulin effectively prevents this oligomerization, converting the protein from 20-mer and larger to predominantly monomer and hence shifting the peaks to the right on the chromatogram (Figure 8a). The peaks actually shift in both directions. When calmodulin is added to Syn A, Syn A can no longer form large aggregates and hence the peak is shifted to the right on the chromatogram, but because calmodulin binds to it, the molecular masses of the complexes increase, shifting the peaks slightly to the left on the chromatogram.

In the presence of EGTA, protein is predominantly present as monomeric to decameric forms (Figure 8b). When calmodulin is added to Syn A in the presence of EGTA, it prevents oligomerization, converting the protein to predominantly monomers.

The oligomerization of syntrophin is complex, and a model depicting this oligomerization process is presented in Figure 9. This model suggests that the oligomerization of syntrophin, denoted by $(Syn^*)_p$, where P represents polymeric forms of syntrophin occurs by binding of Ca^{2+}−syntrophin (denoted by an asterisk), and this process is regulated by Ca^{2+} (Figures 3−5) and calmodulin (Figure 7). Calmodulin inhibits the oligomerization in a Ca^{2+}-independent manner (Figure 7b). Syn in this model is not meant to strictly refer to monomeric syntrophin but to include this and simpler oligomers. The somewhat larger oligomers (Figure 8b) (up to decamer) that occur in the absence of Ca^{2+} are shown as $(Syn)_O$. In our previous report (*9*), another form of syntrophin is shown, indicated as Syn^X, which occurs at high concentrations in

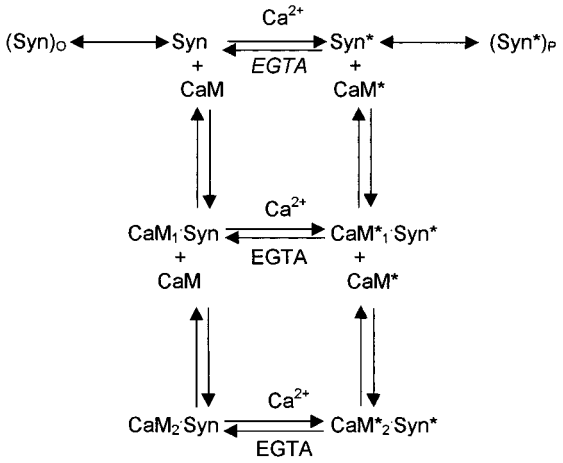

FIGURE 9: Model depicting oligomerization of syntrophin. In this model calcium-bound forms of syntrophin and calmodulin (CaM) are denoted with an asterisk. The polymeric forms of Ca^{2+}-syntrophin are denoted as $(Syn^*)_p$. The smaller oligomeric forms of syntrophin in the absence of Ca^{2+} are shown as $(Syn)_O$. CaM_1·Syn and CaM_2·Syn represent calmodulin binding to one or both of the two sites known (*10*) at the N-terminus of syntrophin.

the absence of Ca^{2+} and does not bind dansyl-calmodulin. This form is not shown in this scheme because it was not investigated in the experiments presented and is currently less well-defined, but it may represent a predominance of $(Syn)_O$ at high protein concentrations.

From these in vitro results, the state of syntrophin in vivo may be inferred. Calmodulin is present in many tissues at concentrations as high as micromolar and is probably in sufficient concentration to inhibit or limit the oligomerization of syntrophin. In the resting muscle, Ca^{2+} would be submicromolar in concentration and this would also limit aggregation. The calmodulin bound would be the Ca^{2+}-free, apocalmodulin conformer (*11*). As intracellular Ca^{2+} increases, preceding and during muscle contraction, this Ca^{2+} could bind to syntrophin and favor syntrophin aggregation. Additionally, as Ca^{2+} binds calmodulin, calmodulin would be able to bind to other more numerous cell proteins, which bind only the Ca^{2+}−calmodulin conformer. Thus, Ca^{2+}−calmodulin could be bound elsewhere, allowing syntrophin's oligomerization. Thus, one can envision syntrophin as monomeric in the resting cell and oligomeric in the contracting muscle. The oligomeric state of syntrophin may be important to maintaining the clusters of ion channels at the neuromuscular junction during contraction. Alternatively, syntrophins may respond to Ca^{2+} during contraction in a way that makes the protein complexes that contain it more resistant to the contractile forces. Indeed, it is now apparent that the dystrophin−actin (*18*), syntrophin−dystrophin (*15*), and syntrophin−syntrophin interactions all are responsive to Ca^{2+} and calmodulin, suggesting that during muscle contraction the protein−protein interactions within the dystrophin glycoprotein complex are altered and probably rearrange.

Phosphatidylinositol-4,5-bisphosphate binds PH1 domain (*7*) but it does not influence oligomerization. This would not be predicted from other PH domains, particularly of both dynamin isoforms, that require oligomerization for high-affinity phosphoinositide binding (*28*). Since PtdIns4,5P_2 did not affect oligomerization, it is clear that it does not regulate this syntrophin activity. What role(s) it plays remains to be elucidated. Binding this lipid probably serves the role of localizing syntrophin to the membrane surface.

Syntrophins bind Na^+ channels (*13*), NO synthetase (*5*, *6*), dystrophin, α- and γ-sarcolglycans (*15*), SAPK3 (*14*), and to themselves. This self-association could bring together cellular signaling components and ion channels. Calmodulin can thus inhibit syntrophin oligomerization in a Ca^{2+}-independent manner (this report) and inhibit syntrophin's interaction with dystrophin in a Ca^{2+}-dependent manner (*9*). Furthermore, since Ca^{2+} promotes aggregation of syntrophin even in the absence of calmodulin, it must be able to bind to syntrophin itself and alter its chemical and physical properties. This agrees with our previous observation of Ca^{2+} binding by syntrophin.

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REFERENCES

1. Froehner, S. C. (1984) Peripheral proteins of postsynaptic membranes from Torpedo electric organ identified with monoclonal antibodies, *J. Cell Biol. 99*, 88−96.
2. Campbell, K. P., and Kahl, S. D. (1989) Association of dystrophin and an integral membrane glycoprotein, *Nature 338* (6212), 259−62.
3. Straub, V., and Campbell, K. P. (1997) Muscular dystrophies and the dystrophin−glycoprotein complex, *Curr. Opin. Neurol. 10*, 168−175.
4. Ahn, A. H., et al. (1996) The three human syntrophin genes are expressed in diverse tissues, have distinct chromosome locations, and each bind dystrophin and its relatives, *J. Biol. Chem. 271*, 2724−2730.
5. Gibson, T. J., et al. (1994) PH domain: the first anniversary, *Trends Biochem. Sci. 19*, 349−353.
6. Adams, M. E., et al. (1995) Mouse α1- and β2-syntrophin gene structure, chromosome localization, and homology with a disc's large domain, *J. Biol. Chem. 270*, 25859−25865.
7. Chockalingam, P. S., Gee, S. H., and Jarrett, H. W. (1999) Pleckstrin Homology Domain 1 of Mouse α1-Syntrophin Binds Phosphatidylinositol-4,5-bisphosphate, *Biochemistry 38*, 5596−5602.
8. Madhavan, R., Massom, L. R., and Jarrett, H. W. (1992) Calmodulin specifically binds three proteins of the dystrophin−glycoprotein complex, *Biochem. Biophys. Res. Commun. 185*, 753−759.
9. Newbell, B. J., Anderson, J. T., and Jarrett, H. W. (1997) Ca^{2+}−Calmodulin Binding to Mouse α1 Syntrophin: Syntrophin Is also a Ca^{2+}-Binding Protein, *Biochemistry 36*, 1295−1305.
10. Iwata, Y., et al. (1998) α1-Syntrophin has distinct binding sites for actin and calmodulin, *FEBS Lett. 423*, 173−177.
11. Jurado, L. A., Chockalingam, P. S., and Jarrett, H. W. (1999) Apocalmodulin, *Physiol. Rev. 79*, 661−682.
12. Froehner, S. C., et al. (1997) in *Cytoskeletal Regulation of Membrane Function* (Froehner, S. C., and Bennett, V., Eds.) pp 197−207, The Rockefeller University Press, New York.
13. Gee, S. H., et al. (1998) Interactions of muscle and brain sodium channels with multiple members of the syntrophin family of dystrophin-associated proteins, *J. Neurosci. 18*, 128−137.

14. Hasegawa, M., et al. (1999) Stress-activated protein kinase 3 interacts with the PDZ domain of α1-syntrophin. A mechanism for specific substrate recognition, *J. Biol. Chem. 274*, 12626−12631.
15. Madhavan, R., and Jarrett, H. W. (1995) Interactions between dystrophin glycoprotein complex proteins, *Biochemistry 34* (38), 12204−9.
16. Yang, B., et al. (1995) Identification of α-syntrophin binding to syntrophin triplet, dystrophin, and utrophin, *J. Biol. Chem. 270* (10), 4975−8.
17. Ervasti, J. M., and Campbell, K. P. (1991) Membrane organization of the dystrophin−glycoprotein complex, *Cell 66*, 1121−1131.
18. Jarrett, H. W., and Foster, J. L. (1995) Alternate binding of actin and calmodulin to multiple sites on dystrophin, *J. Biol. Chem. 270* (10), 5578−86.
19. Laemmli, U. K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4, *Nature 227*, 680−685.
20. Bradford, M. M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein−dye binding, *Anal. Biochem. 72*, 248−254.
21. Towbin, H., Staehelin, T., and Gordon, J. (1979) Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications, *Proc. Natl. Acad. Sci. U.S.A. 76* (9), 4350−4354.
22. Jarrett, H. W., and Taylor, W. L. (1998) Transcription factor-green fluorescent protein chimeric fusion proteins and their use in studies of DNA affinity chromatography, *J. Chromatogr. A 803*, 131−139.
23. Hom, L. G., and Volkman, L. E. (1998) *Nickel-induced oligomerization of proteins containing 10-Histidine tags*, *BioTechniques 25* (1), 20−22.
24. Peters, M. F., Adams, M. E., and Froehner, S. C. (1997) Differential association of syntrophin pairs with the dystrophin complex, *J. Cell Biol. 138* (1), 81−93.
25. Mahadevan, D., et al. (1995) Structural studies on the PH domains of Dbl, Sos 1, IRS-1, and βARK1 and their differential binding to G$\beta\gamma$ subunits, *Biochemistry 34*, 9111−9117.
26. Wang, L. L., and Spudich, J. A. (1984) A 45,000-mol-wt protein from unfertilized sea urchin eggs severs actin filaments in a calcium-dependent manner and increases the steady-state concentration of nonfilamentous actin, *J. Cell Biol. 99* (3), 844−51.
27. Soto, C., Rodriguez, P. H., and Monasterio, O. (1996) Calcium and gadolinium ions stimulate the GTPase activity of purified chicken brain tubulin through a conformational change, *Biochemistry 35* (20), 6337−44.
28. Klein, D. E., et al. (1998) The pleckstrin homology domains of dynamin isoforms require oligomerization for high affinity phosphoinositide binding, *J. Biol. Chem. 273* (42), 27725−33.

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