Mouse α1-Syntrophin Binding to Grb2: Further Evidence of a Role for Syntrophin in Cell Signaling†

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ABSTRACT: Syntrophins have been proposed to serve as adapter proteins. Syntrophins are found in the dystrophin glycoprotein complex (DGC); defects in the constituents of this complex are linked to various muscular dystrophies. Blot overlay experiments demonstrate that α-dystroglycan, *β*-dystroglycan, and syntrophins all bind Grb2, the growth factor receptor bound adapter protein. Mouse α1-syntrophin sequences were produced as chimeric fusion proteins in bacteria and found to also bind Grb2 in a Ca^{2+}-independent manner. This binding was localized to the proline rich sequences adjacent to and overlapping with the N-terminal pleckstrin homology domain (PH1). Grb2 bound syntrophin with an apparent K_D of 563 ± 15 nM. Grb2-C-SH3 domain bound syntrophin with slightly higher affinity than Grb2-N-SH3 domain. Crk-L, an SH2/SH3 protein of similar domain structure but different specificity, does not bind these syntrophin sequences.

Syntrophins are a group of peripheral membrane proteins first identified in *Torpedo* postsynaptic membranes (*1*). Syntrophins have been found to be closely associated with dystrophin (*2*), the protein product of the Duchenne muscular dystrophy gene locus (*2*, *3*). In skeletal muscle, dystrophin and syntrophins are found in a complex with other proteins and glycoproteins, the dystrophin glycoprotein complex or DGC[1] (*4*, *5*) whose defects cause Duchenne, Becker, various limb girdle, and other muscular dystrophies. Syntrophins are a multigene family of homologous proteins. Three syntrophin isoforms, α1, *β*1, and *β*2, are products of different genes (*6*−*9*). The α1-syntrophin is primarily expressed in striated muscles and brain, while the *β*-syntrophins are ubiquitous in mammalian tissues (*10*). Each of the syntrophins contains two pleckstrin homology (PH) domains, an N-terminal PH1 domain and a PH2 domain (see Figure 1). The PH1 domain of α1-syntrophin has been reported to bind phosphatidylinositol 4,5-bisphosphate (PtdIns4,5P_2) (*11*). Recently, this domain has also been shown to be involved in the oligomerization of syntrophin in vitro in a Ca^{2+}-dependent manner. Calmodulin inhibits oligomerization in a Ca^{2+}-independent manner (*12*). The amino-terminal PH domain is interrupted by an inserted PDZ domain. The PDZ domain is 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. Syntrophin's PDZ domain has been shown to bind neuronal nitric oxide synthetase (*8*, *13*), muscle and nerve voltage-gated Na^+ channels (*14*), and the MAP kinase, SAPK3 (*15*). Syntrophins also bind calmodulin (*16*). A domain unique to syntrophins, the SU domain, has been shown to bind Ca^{2+}-calmodulin (*17*), and the SU domain in addition to other sequences in the C-terminal of the protein binds to dystrophin. Ca^{2+}-calmodulin binding inhibits the syntrophin−dystrophin interaction (*17*). The N-terminal of the PH1 domain and the N-terminal of the PDZ domain have been reported to bind calmodulin (*18*) in a Ca^{2+}-independent manner (*12*, *17*). Thus, syntrophins act as adapters, between the dystrophin complex of proteins and components of the cell signaling apparatus.

Grb2 (growth factor receptor bound 2), the human homologue of the nematode *Caenorhabditis elegans* protein Sem-5, is an adapter protein consisting of one *src* homology (SH) 2 domain flanked by two SH3 domains. The N-terminal SH3 domain stretches from amino acids 5 to 55, the SH2 domain from 60 to 158, and the C-terminal SH3 domain from 164 to 214. This structure suggests that Grb2 is capable of binding phosphotyrosine proteins through its SH2 domains, while its SH3 domain interacts with proteins possessing PXXP motifs. While investigating the known interaction (*19*) between Grb2 and *β*-dystroglycan, we discovered that syntrophin was also a Grb2 binding protein. The N-terminus

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[1] Abbreviations: PH, Pleckstrin homology; MBP, maltose binding protein; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; PtdIns4,5P_2, phosphatidylinositol 4,5-bisphosphate; NBT, nitroblue tetrazolium; BCIP, 5-bromo-4-chloro-3-indolyl phosphate; NTA, nitrilotriacetic acid; SAPK3, stress-activated protein kinase 3; BSA, bovine serum albumin; DGC, dystrophin glycoprotein complex; Grb2, growth factor receptor bound 2; GST, glutathione-*S*-transferase.

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of syntrophin contains two proline-rich regions that could interact with SH3 domains of signaling proteins, namely:

PADGPGPEPEPAQLNGAAEPGAAPPQLPEAL
(α-syntrophin 44−75)

WASPPASPLQRQPSSPGPQPRNLSEAKHVSLKMA-
YVSRRCTPTDPEPRY (181−229)

These are located in the intervening sequences between the PH1a and PDZ domain sequences and between the PDZ and PH1b domains and overlapping the N-terminus of the latter.

In this paper we have characterized the interaction of the mouse α1-syntrophin with the adapter protein, Grb2.

EXPERIMENTAL PROCEDURES

Materials. Antibodies against recombinant Grb2 (anti-Grb2), GST (anti-GST), and α-syntrophin were produced in rabbit and purified by affinity chromatography. Mouse monoclonal Grb2 antibody was from Transduction Laboratories. Crk-L rabbit polyclonal antibody was from Santa Cruz Biotechnology. S-protein−alkaline phosphatase conjugate was from Novagen. Goat anti-rabbit IgG (H+L)−alkaline phosphatase conjugate, goat anti-mouse IgG (H+L)−alkaline phosphatase conjugate, and goat anti-rabbit IgG (H+L)−horseradish peroxidase conjugate were from BioRad. Ni^{2+}-NTA−agarose was from Qiagen. Cyanogen bromide preactivated Sepharose was from Sigma. All other chemicals were of the highest purity available commercially.

Dystrophin glycoprotein complex (DGC) was partially purified from digitonin-solubilized rabbit skeletal muscle using the procedure of Ervasti et al. (*20*) up through the succinylated wheat germ agglutinin (sWGA) chromatography. Aliquots were stored frozen at −85 °C. DGC was further fractionated by electrophoresis on 4−15% gradient sodium dodecyl sulfate−polyacrylamide gels and blotted to nitrocellulose as previously described (*21*). The gel blots were then overlaid with Grb2 as described under Overlay Experiments below. Gel blots were also stained with specific antibodies for α-syntrophin, α- and β-dystroglycan, and α-sarcoglycan to confirm the identity of bands interacting with Grb2.

Fusion Proteins. The syntrophin fusion proteins $[His]_6$-Syn and $[His]_6$-Syn A and maltose binding protein (MBP) fusions MBP-Syn B, MBP-Syn H, and MBP-Syn I were prepared as described previously (*17*). pET32 plasmid constructs for PH1, PH2, and the PDZ domain were the 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 as $[His]_6$-PH1, -PH2, and -PDZ (*14*). The His-Tag fusion proteins were purified by using Ni^{2+}-NTA−agarose from Qiagen as described earlier (*17*). The MBP fusion proteins were purified using the batch method described previously by Jarrett and Foster on amylose resin (*22*). GST-Grb2, GST-Grb2-N-SH3 (amino acids 1−54), and GST-Grb2-C-SH3 (amino acids 163-217) domains (kindly provided by Dr. O. Segatto, Instituto Regina Elena, Roma, Italy) and GST-βC1 (rabbit β-dystroglycan coding amino acids 787−895) (*23*) were all expressed in *E. coli* BL21 strain and purified by affinity chromatography on glutathione−agarose beads (Amersham Pharmacia Biotech) as described elsewhere (*23*, *24*). The purity of the proteins was determined by 12% SDS−polyacrylamide gel electrophoresis using the method of Laemmli (*25*). The major bands of the fusion proteins were of the expected size and relatively high purity (data not shown). The Bradford assay (*26*) was used to determine the protein concentrations using bovine serum albumin as the standard.

Overlay Experiments. The fusion proteins Syn, Syn A, Syn B, Syn H, PH1, MBP-LacZα, and $[His]_6$-green fluorescent protein (GFP) were applied to electrophoresis on a 12% SDS−PAGE gel, and electroblotted onto nitrocellulose paper. The paper was then blocked with 10 mg/mL BSA in TTBS (20 mM Tris-HCl, pH 7.5, 0.5 M NaCl, and 0.2% Tween-20). After washing extensively with 1 mM $CaCl_2$ in BSA/TTBS (1 mg/mL BSA in TTBS), the blot was overlaid with 0.1 mg/mL GST-Grb2 for 2 h in the presence of 1 mM Ca^{2+}. The blot was then washed extensively with Ca^{2+}/BSA/TTBS, and incubated with affinity-purified anti-GST (1:5000 dilution). Goat anti-rabbit IgG (H+L)−alkaline phosphatase conjugate (1:1000 dilution, BioRad) was used, following the primary antibody. The blot was developed using 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 $NaHCO_3$, 1 mM $MgCl_2$, pH 9.8). Alternatively, after primary antibody and washing as above, blots were probed with 1:3000 diluted goat anti-rabbit IgG (H+L)−horseradish peroxidase conjugate, washed as above, and developed for chemiluminescence by incubating the blot in a mixture of 15 mL of solution A (90 mM coumaric acid and 250 mM Luminol, 0.1 M Tris-HCl, pH 8.5) and 15 mL of solution B (7.2 μL of H_2O_2 in 0.1 M Tris-HCl, pH 8.5) in the dark room and then exposing it to Kodak Scientific Imaging film. MBP-LacZα and $(His)_6$-GFP were used as controls for maltose and His-Tag fusion proteins, respectively. A similar experiment was performed to test binding of Grb2 to syntrophin from partially purified dystrophin glycoprotein complex. Bovine serum albumin was used as a control for this experiment.

Immunoprecipitation. Samples in phosphate-buffered saline (0.13 M NaCl, 2.68 mM KCl, 5.37 mM Na_2HPO_4, 1.76 mM KH_2PO_4, pH 7.4) containing 1% Triton X-100 were prepared by incubation with protein A−Sepharose at 4 °C for 1 h. The supernatants were incubated with 100 μL of fresh protein A−Sepharose with anti-syntrophin or anti-MBP polyclonal antibodies at room temperature for 1 h and then on ice for 30 min. The beads were washed 3 times with phosphate-buffered saline containing 1% Triton X-100. The bound proteins were eluted by boiling in SDS−PAGE sample buffer and separated on a 4−15% gradient sodium dodecyl sulfate−polyacrylamide gel (BioRad) and blotted onto a nitrocellulose membrane as described previously (*27*). The blots were then analyzed by using different antibodies and were developed using the enhanced chemiluminescence method.

Solid-Phase Binding Assays. To 0.9 mg of GST-Grb2 (0.9 mg/mL) was coupled 1 g of cyanogen bromide-activated Sepharose (Sigma) using procedures recommended by the manufacturer (Pharmacia). The support was then washed with the coupling buffer (0.1 M $NaHCO_3$, pH 8.3, 0.5 M NaCl) and blocked for 2 h with 0.1 M Tris-HCl, pH 8, 0.5 M NaCl.

The amount of protein coupled (0.115 mg of Grb2/mL of 50% slurry Sepharose) 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 was used to which either no protein or glutathione-*S*-transferase (GST) was coupled.

Two hundred microliters of a 50% slurry of Grb2-Sepharose containing 23 μg of Grb2 was equilibrated with buffer A (50 mM Tris, pH 7.5, 100 mM KCl) containing 0.1 mM EGTA and then incubated with different fusion proteins (25 μg) or rabbit skeletal muscle extract prepared in a manner similar to the immunoprecipitation experiment. Incubation was 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 or Sepharose with glutathione-*S*-transferase coupled to it was used. After the incubation, Grb2-Sepharose was washed 3 times with 0.5 mL of 1 mg/mL BSA/TTBS. The protein was eluted using 60 μL of 2× Laemmli sample buffer (*25*). 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 a 12% SDS−PAGE gel (*25*), and electroblotted onto nitrocellulose paper (*27*). The paper was then blocked with 10 mg/mL BSA in TTBS. After washing extensively with 1 mg/mL BSA/TTBS, the blot was incubated with affinity-purified anti-MBP (1:1000 dilution) for MBP fusion proteins or with 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) were used for the rabbit and mouse primary antibodies, respectively. For fusion proteins containing S-Tag, namely, PH1, PH2, and PDZ, S-protein−alkaline phosphatase conjugate (1:5000, Novagen) was used.

Surface Plasmon Resonance. SPR assays were performed using a BIAcore instrument (BIAcore X) equipped with a two flow cell sensor chip, using purified Grb2 or Grb2-C-SH3 or Grb2-N-SH3 domain GST fusion proteins. Proteins were immobilized by covalent coupling to CM-5 sensor chips (research grade) (BIAcoreAB) after activation of the carboxymethylated dextran surface by a mixture of 0.05 M *N*-hydroxysuccinimide and 0.2 M *N*-ethyl-*N*′-3-(dimethylaminopropyl)carbodiimide hydrochloride. The reaction was performed by injecting 45 μg/mL Grb2 in 10 mM acetate buffer, pH 4.8, at a flow rate of 10 μL/min at 25 °C for 7 min. Residual activated groups were blocked with 1 M ethanolamine hydrochloride, pH 8.5. Immobilization resulted in about 4000 resonance units (RU) of Grb2 protein. Binding assays were performed in HBS (10 mM HEPES, 0.15 M NaCl, 3 mM EDTA, 0.5% surfactant P20, pH 7.4) with a flow rate of 2, 5, 10, 15, and 20 μL/min. The soluble ligand (Syn) was applied in the range of concentration of 15.5 nM−82.7 μM. At the end of the sample plug, HBS buffer was allowed to flow past the sensor surface to allow dissociation. The sensor surface was regenerated for the next Syn sample using a 30−120 s pulse of regenerating buffer (50 mM NaOH) at a flow rate of 20 μL/min. Nonspecific binding on the sensor surfaces and mass-transport problems were avoided by flowing the same solution past the two sensor surfaces in sequence and using different flow rates. The response was monitored as a function of time (sensogram) at 25 °C. The integrity of immobilized proteins was tested with anti-Grb2 antibody.

The data evaluation was carried out using BIAevaluation software version 3.0, following the manufacturers' instructions. The kinetic constants were calculated by nonlinear regression of data using the pseudo-first-order rate equation as described in detail by Herzog et al. (*28*).

Solid-Phase Immunoassay. To measure the binding of GST-Grb2 to Syn A, a microtiter assay was used. A microtiter plate (Dynatech Immulon 1) was coated by incubation of 100 μL/well of 0.05 mg/mL His-Tag Syn or His-Tag Syn A for 4 h at 4 °C. The plate was then blocked by replacing the fusion proteins with 300 μL/well of 30 mg/mL BSA in AC7.5 buffer (50 mM Tris-HCl, pH 7.5, 100 mM KCl, 3 mM $MgCl_2$, 1 mM $CaCl_2$). All subsequent operations were at room temperature and 100 μL/well. After washing the plate 3 times (5 min per wash) with AC7.5T/BSA (buffer AC7.5, 0.1% Tween-20, 1 mg/mL BSA), serial dilutions of GST-Grb2, GST-Grb2-C-SH3, GST-Grb2-N-SH3, or GST were added. The plate was incubated for 2 h. The plate was washed 3 times as described above and then incubated with anti-GST antibody for 1 h. The plate was washed 3 more times as described and incubated with goat anti-rabbit IgG (H+L)−alkaline phosphatase conjugate (1:1000 dilution, BioRad) for 1 h. The plate was then washed once with 1 mg/mL AC7.5T/BSA followed by two immediate washes of diethanolamine/Mg (10 mM diethanolamine, 0.5 mM $MgCl_2$). The plate was developed by incubation with 100 μL/well of 1 mg/mL *p*-nitrophenyl phosphate in diethanolamine/Mg. The absorption at 405 nm was determined versus time. The concentration giving half-maximal response was calculated and is denoted here as K_d.

For the experiments performed in EGTA, $CaCl_2$ was replaced by EGTA in all the buffers up to the stage at which the anti-GST antibody was added. After that stage, the experiment was continued as specified above using the $CaCl_2$ containing AC7.5T/BSA.

RESULTS

The schematic representation of mouse α1-syntrophin and Grb2 sequences expressed as fusion proteins is shown 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. Syn, Syn A, PH1, PH2, and PDZ were produced as His-Tag fusion proteins while Syn B, Syn H, and Syn I were produced as maltose binding fusion proteins. Glutathione-*S*-transferase (GST) fusions of Grb2 and selected regions of it were also produced. Each fusion protein was affinity-purified.

Gel blots of partially purified dystrophin glycoprotein complex binding Grb2 are shown in Figure 2. Coomassie blue staining of the purified dystrophin glycoprotein complex is shown in the figure. Clearly, the dystrophin glycoprotein complex is only partially purified and other proteins are present, but it is sufficient to determine Grb2 binding. In the Coomassie stain, the syntrophins appear as two bands, but the upper, more intense band is itself a doublet of the two β-syntrophins. The lower, less intense band is α-syntrophin. The three asterisks show the position, in descending

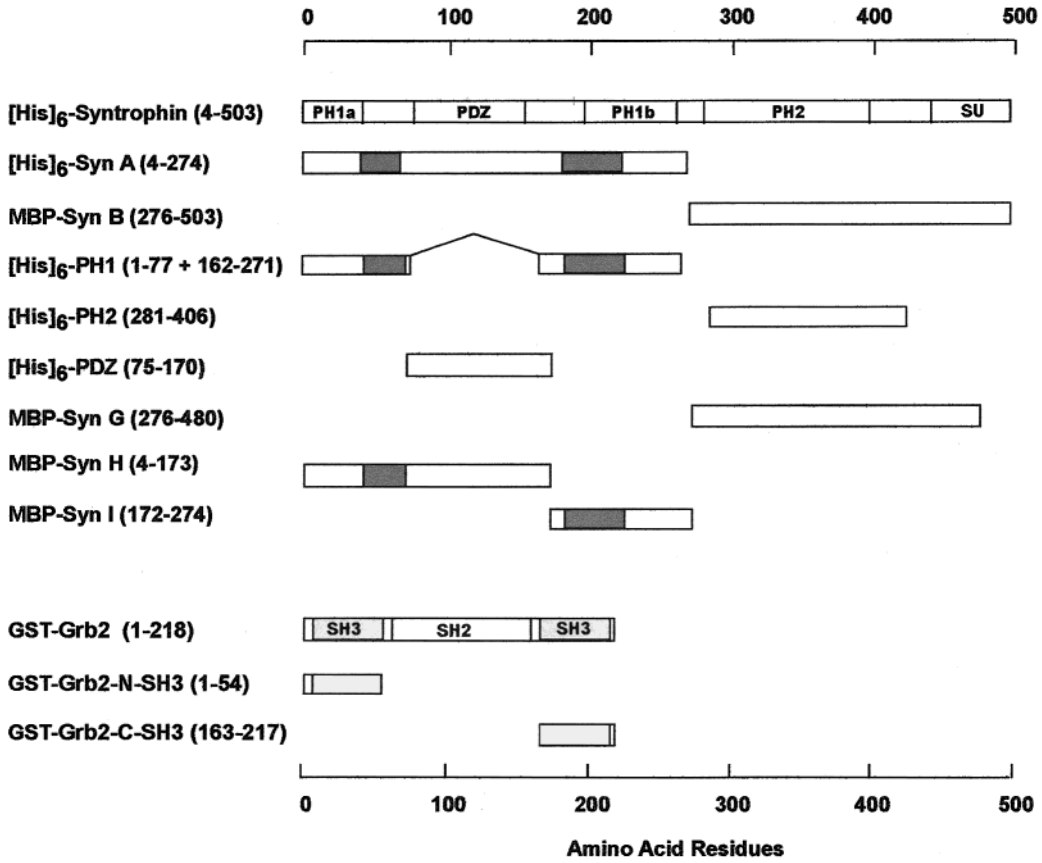

FIGURE 1: Diagrammatic representation of fusion proteins containing α1-syntrophin and Grb2 sequences. The darkly shaded portion shows the location of proline-rich motifs in syntrophin. The lighter shading highlights the SH3 domains of Grb2. Numbers in parentheses represent the amino acid sequences present in each fusion protein.

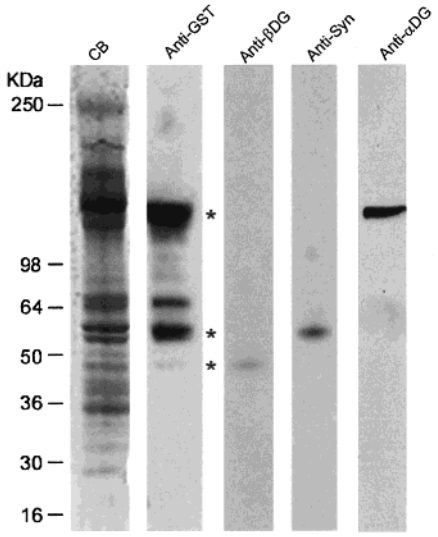

FIGURE 2: Grb2 binds syntrophin in the partially purified dystrophin glycoprotein complex (DGC) from rabbit skeletal muscle. Partially purified DGC was applied to electrophoresis on a 4−15% gradient sodium dodecyl sulfate−polyacrylamide gel and electroblotted onto nitrocellulose paper. The blot was then overlaid with 0.1 mg/mL GST-Grb2 for 2 h in the presence of 1 mM $CaCl_2$. The binding was detected with anti-GST antibody. Three asterisks (*) show the positions of α-dystroglycan, the syntrophins, and β-dystroglycan in descending order. Anti-βDG, Anti-Syn, and Anti-αDG show the bands detected by the β-dystroglycan, α-syntrophin, and α-dystroglycan antibodies, respectively. To the left are shown molecular mass markers.

order, of the α-dystroglycan, the syntrophins, and β-dystroglycan; all bind Grb2. Specific staining with α-syntrophin, β-dystroglycan, and α-dystroglycan antibodies confirms these identities. The DGC was also probed with dystrophin antibody (NCL-DYS2) from Novacastra (data not shown). The DGC was also overlaid with GST alone and did not show any binding (data not shown). β-Dystroglycan (lowermost asterisk) had been previously shown to bind Grb2 (*29*), but this is actually the least intense of the staining observed. α-Sarcoglycan showed binding to Grb2 in some of the dystrophin glycoprotein complex preparations we tested (data not shown) though it did not bind Grb2 well in this particular preparation. Close inspection of its sequence demonstrated that the only PXXP motifs present are PYNP from amino acids 83−86 and PEGP from amino acids 128−131, regions of the protein believed to be on the exterior face of the sarcolemma (*30*), and are of questionable relevance to in vivo binding. α-Dystroglycan (uppermost asterisk) shows the strongest binding to Grb2. Though its sequence contains PXXP motifs such as PSEP from amino acids 29−32, PTLP from amino acids 302−305, PTSP from amino acids 340−343, PIQP from amino acids 382−385, PATP from amino acids 432−435, and PRTP from amino acids 451−454, this protein is believed to be on the exterior face of the sarcolemma and, therefore, is also of questionable relevance to in vivo intracellular binding. Therefore, the remainder of this paper will focus on this newly discovered (Figure 2) interaction with α-syntrophin (middle asterisk), an intracellular peripheral membrane protein.

In other experiments, the partially purified dystrophin glycoprotein complex was probed with other antibodies (data not shown). The uppermost band seen in the Coomassie-

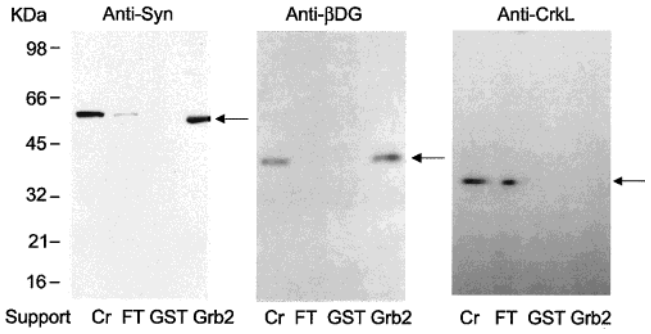

FIGURE 3: Grb2 binds syntrophin and β-dystroglycan from rabbit skeletal muscle membrane. GST-Grb2-Sepharose or GST-Sepharose was incubated with the rabbit skeletal muscle extract containing 1% Triton X-100 as described under Experimental Procedures. Cr represents crude extract, FT is the fraction not bound by the Grb2-Sepharose, GST represents GST-Sepharose that was used as negative control, and Grb2 represents the GST-Grb2-Sepharose-bound fraction. The top arrow shows the band detected by α-syntrophin antibody (Anti-Syn), the middle arrow shows the band detected by β-dystroglycan antibody (Anti-βDG), and the bottom arrow shows the band detected by the Crk-L antibody (Anti-Crk-L). Molecular mass markers are shown to the left.

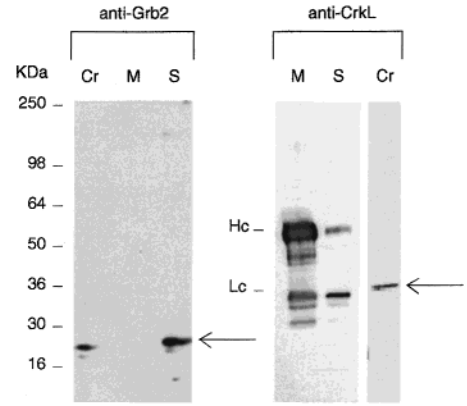

FIGURE 4: Immunoprecipitation of Grb2 from rabbit skeletal muscle extract. Rabbit skeletal muscle extract containing 1% Triton X-100 was incubated with Protein A Sepharose and α-syntrophin antibody (S) or anti-MBP antibody (M), a negative control. After extensive washing, bound proteins were eluted with SDS−PAGE sample buffer. Samples, after electrophoresis and electroblotting, were probed with antibodies for Grb2 (mouse monoclonal, anti-Grb2) and Crk (rabbit polyclonal, anti-Crk-L). The positions of Grb2 (lower arrow) and Crk (higher arrow) are indicated. Heavy and light chains of IgG are indicated in the middle as Hc and Lc, respectively. The crude extract (Cr) was also applied to the same gel.

stained gel in Figure 2 is dystrophin and is detected with a dystrophin antibody (NCL-Dys2 from Novacastra). It comigrates with the 250 kDa marker in this gel system although its true molecular mass is much larger. Dystrophin did not bind Grb2 in any of our experiments and neither did the other (β, δ, and γ) sarcoglycans. There is a band at about 70 kDa which does bind Grb2 (Figure 2, lane 2), which is near the molecular mass of one of the shortened dystrophin (DP71) forms, but it did not bind dystrophin C-terminal antibody and so it is not this protein. We made no other attempts to identify it.

To confirm the observed interaction of syntrophin with Grb2, GST-Grb2-Sepharose was incubated with rabbit skeletal muscle extract. GST-Sepharose was used as a control. Although a small amount of syntrophin is present in the flow through, most of the syntrophin binds to Grb2 (top arrow, Anti-Syn) as can be seen in Figure 3. β-Dystroglycan has been shown to bind Grb2 by Yang et al. (*29*) and is used as a positive control (middle arrow, Anti-βDG). Anti-Crk-L was used as an unrelated antibody which served as a negative control. Grb2 did not show any binding to Crk-L, and all of the Crk-L was present in the flow through.

To investigate if a complex of syntrophin and Grb2 is present in skeletal muscle and if this interaction is specific, syntrophin was immunoprecipitated ("S" in Figure 4) from rabbit skeletal muscle extract, and results were analyzed by immunoblotting. For comparison, the irrelevant anti-MBP antibody was also used ("M" in Figure 4). The gel blot of the bound proteins was probed with two antibodies against SH3 domain proteins: the mouse monoclonal anti-Grb2 antibody (Trasduction Laboratories) and the anti-Crk-L antibody (Santa Cruz). It can be seen from Figure 4 that Grb2 binds syntrophin (Figure 4, anti-Grb2) while Crk does not (Figure 4, anti-Crk-L). The antibody light-chain band in the immunoblot (Figure 4, anti-Crk-L, lane S) is near the expected size for Crk but migrates faster on the gel than expected for Crk and faster than the Crk band detected in the muscle extract used (Figure 4, anti-Crk-L, lane Cr). Similar results were also obtained using an anti-Grb2 polyclonal antibody (data not shown); Grb2 bound syntrophin while Crk did not although the muscle extract contained both proteins (data not shown). Thus, Grb2 binds, but not Crk. Syntrophin contains a PQLP sequence (69−72) which is similar to a motif known to bind Crk (*31*). Thus, this experiment was designed to test if a reasonable alternative SH3 domain protein bound instead of Grb2. Apparently though, Grb2 binds while Crk does not. The reverse experiment using mouse monoclonal anti-Grb2 and anti-Crk-L antibody was also performed to immunoprecipitate syntrophin from rabbit skeletal muscle extract and showed also that Grb2 but not Crk binds syntrophin (data not shown). This was also confirmed using muscle extract and syntrophin-Sepharose; Grb2 bound while Crk-L did not (data not shown).

Figure 5 summarizes the Grb2 interaction observed for all the syntrophin fusion proteins tested, namely, Syn, Syn A, Syn B, Syn H, Syn I, PH1, PH2, and PDZ. A typical experiment is shown in Figure 5A. Syn, Syn A, Syn B, PH1, and PDZ were briefly incubated with Grb2-Sepharose in the presence of 1 mM EGTA, since Syn, Syn A, and PH1 have been shown to oligomerize in the presence of Ca^{2+} (*12*). Syn, Syn A, and PH1 showed binding to Grb2-Sepharose while Syn B and PDZ did not bind (Figure 5A). CNBr-activated Sepharose-4B without any protein coupled to it was used as a negative control. This assay also demonstrated that Syn H and Syn I also bound Grb2 in other experiments (data not shown).

To confirm the region involved in this interaction, an overlay experiment was carried out where gel blots of syntrophin fusion proteins were overlaid with Grb2 in the presence of 1 mM Ca^{2+}. Figure 5B confirms the localization of the Grb2−syntrophin interaction. $[His]_6$-green fluorescent protein (GFP) and MBP-LacZα were used as controls for His-Tag and maltose binding fusion proteins, respectively, and as expected, neither shows any binding to Grb2. Syn, Syn A, Syn H, Syn I, and PH1 all bind Grb2 while Syn B, PH2, and PDZ do not. Notice that the two proline-rich regions are separately expressed in Syn H and Syn I (see

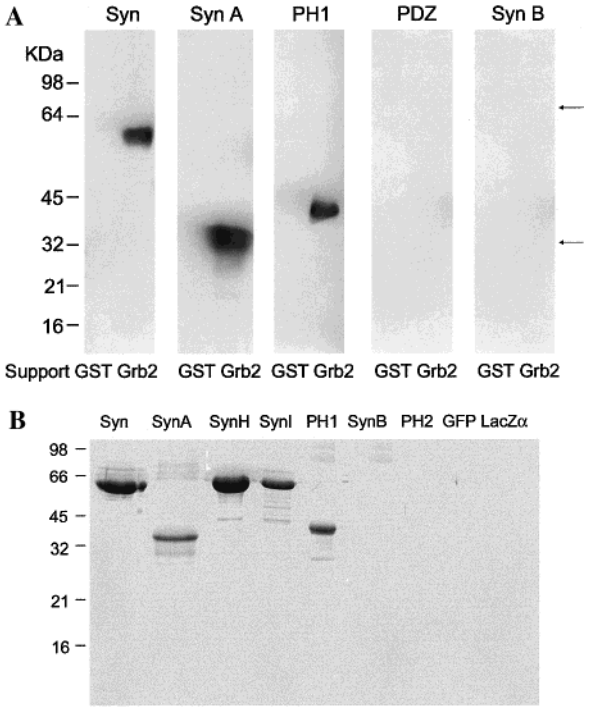

FIGURE 5: Syntrophin's Grb2 binding region is localized to the proline-rich motifs adjacent to and overlapping the PH1 domain. To the left are shown molecular mass markers. (A) Grb2-Sepharose was incubated with the Syn fusion protein in the presence of 1 mM EGTA. GST represents GST-Sepharose; Grb2 represents Grb2-Sepharose. To the right are two arrows; the upper arrow shows the molecular mass of Syn B, and the lower arrow shows the molecular mass of PDZ which do not bind to Grb2-Sepharose. (B) A gel blot overlay experiment was performed as described under Experimental Procedures in the presence of 1 mM $CaCl_2$. Syn fusion proteins were separated by electrophoresis, blotted to nitrocellulose, and overlaid with GST-Grb2, and the binding was detected with anti-GST antibody. $[His]_6$-green fluorescent protein (GFP) and MBP-LacZα were used as controls for His-Tag and maltose binding fusion proteins, respectively.

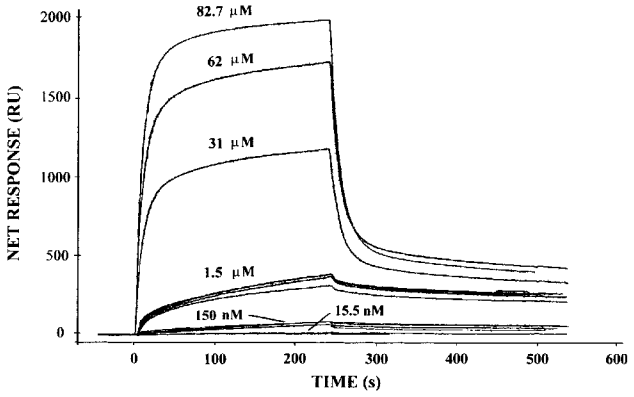

FIGURE 6: Surface plasmon resonance profiles of the binding of soluble Syn to Grb2 immobilized to the sensor chip. Individual curves from bottom to top were obtained with Syn concentrations ranging from 15.5 nM to 82.7 μM. Association and dissociation phases were started at 0 s and at 240 s, respectively.

Figure 1) and that both bind full-length Grb2 (Figure 5B). Binding occurs to the same proteins in the absence (Figure 5A) or presence (Figure 5B) of Ca^{2+}. The binding affinity of Syn for Grb2 was determined by surface plasmon resonance.

Sensograms for the binding are shown in Figure 6. Similar experiments were also done for Syn/Grb2-C-SH3 and Syn/Grb2-N-SH3 interactions (data not shown). The initial part of each curve corresponds to the signal of the buffer flowing on the sensor surface. The rising part of the curve (association) results from syntrophin binding to the Grb2 on the surface of the sensor chip. Finally, buffer alone flows over the chip, and the decreasing signal results as bound syntrophin dissociates. Control experiments demonstrate that the syntrophin association occurs only when the chip surface contains Grb2 (data not shown). Increasing concentrations of Syn ranging from 15.5 nM to 15.5 μM were allowed to flow over the sensor surface. The observed association rate constant, k_{on} (7.89×10^3 M^{-1} s^{-1}), and the dissociation rate constant, k_{off} (4.44×10^{-3} s^{-1}), were calculated from the association and dissociation sensorgrams, respectively, using a simple bimolecular association model (*28*), and the apparent dissociation constant, K_d, was calculated from the ratio of the rate constants (i.e., k_{off}/k_{on}). For the binding of full-length syntrophin to full-length Grb2 (Figure 6), $K_d = 563 \pm 15$ nM. For the binding of full-length syntrophin to the two separate SH3 domains of Grb2, $K_d = 351 \pm 4$ and 438 ± 4 nM for Grb2-N-SH3 and Grb2-C-SH3, respectively (data not shown).

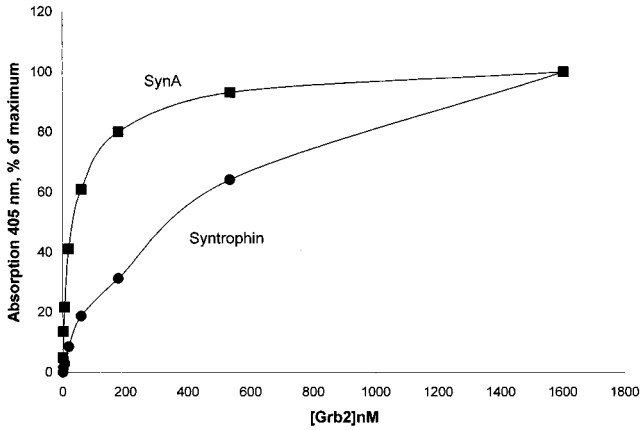

FIGURE 7: Grb2 binds to Syn A with higher affinity than to full-length syntrophin. Solid-phase immunoassay was performed on Syn or Syn A coated microtiter plates as described under Experimental Procedures. Binding of GST-Grb2 was detected by using anti-GST antibody. Absorption 405 nm measures the amount of GST-Grb2 bound by syntrophin (circles) and Syn A (squares). The data were scaled by subtracting blank values (obtained using GST-coated plate wells) and defining the maximum signal for each data set as 100%.

To confirm the binding affinity of syntrophin for Grb2, $[His]_6$-Syn or -Syn A was used to coat the wells of a microtiter plate, and the binding of GST-Grb2 was assessed. The results in Figure 7 demonstrate an interesting phenomenon—that Syn A binds Grb2 with considerably higher affinity than does the full-length Syn. For Syn, the affinity was 355 ± 25 nM, in reasonably good agreement with the value (563 nM) measured in the surface plasmon resonance experiment (Figure 6). However, Syn A bound with an apparent affinity of 35 ± 10 nM, an order of magnitude higher affinity (both are the average $\pm$ standard deviation for three separate determinations). Syn A was also mixed with Syn B to reconstitute the complete sequence in two parts, and this mixture was assayed for binding to Grb2. The affinity was not significantly different from that observed for Syn A alone (data not shown).

To determine if Grb2 was binding preferentially to either Syn H or Syn I, GST−Grb2 or GST alone was used to coat

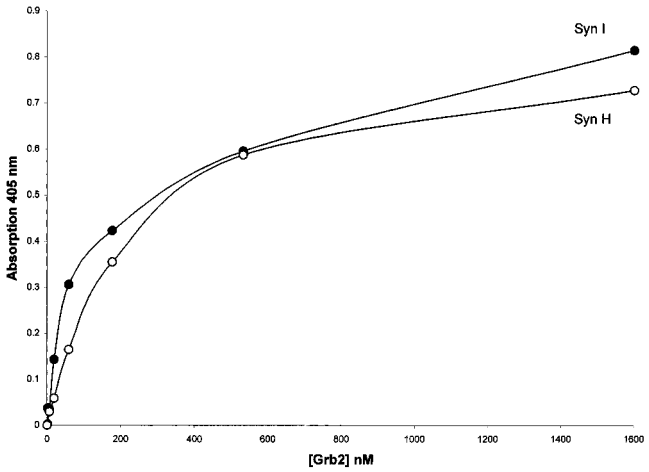

FIGURE 8: Grb2 binds to Syn H and Syn I with similar affinities. Solid-phase immunoassay was performed on GST-Grb2-coated microtiter plates as described under Experimental Procedures. Binding of MBP-Syn H (open circles) or MBP-Syn I (closed circles) was detected by using anti-MBP antibody. Absorption 405 nm measures the amount of MBP-Syn H or MBP-Syn I bound by Grb2. The data were scaled by subtracting blank values (obtained using GST-coated plate wells).

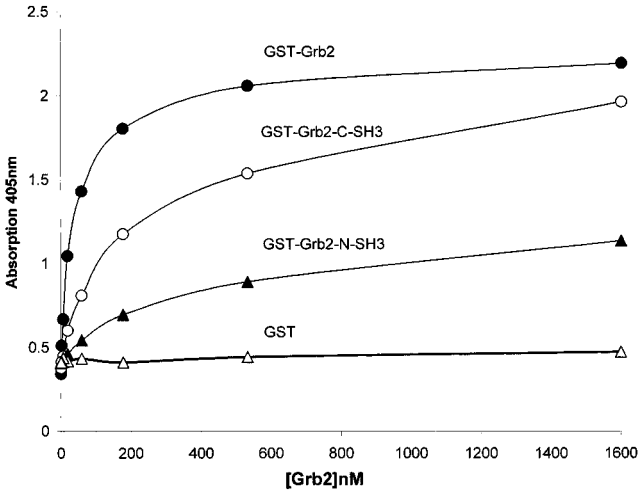

FIGURE 9: Syn A binds best to Grb2's C-terminal SH3 domain. Solid-phase immunoassay was performed as described under Experimental Procedures using $[His]_6$-Syn A-coated microtiter plates. Binding of GST-Grb2 fusion proteins was detected by using anti-GST antibody. Absorption 405 nm measures the amount of GST-Grb2 (closed circles), GST-Grb2-C-SH3 (open circles), GST-Grb2-N-SH3 (closed triangles), or GST (open triangles) bound.

the wells of a microtiter plate, and overlaid with MBP-Syn H or MBP-Syn I. The binding was detected with anti-MBP antibody. The results in Figure 8 demonstrate that Syn H and Syn I bind Grb2 with a K_d of 182 and 171 nM, respectively.

The issue of whether the Grb2-C-SH3 or Grb2-N-SH3 domains bound preferentially to either Syn H or Syn I was addressed in a similar experiment (data not shown but submitted for review). The result was that Grb2-C-SH3 is bound to higher levels by both Syn H and Syn I but both Grb2 SH3 domains bind to both regions of syntrophin and the affinities are quite similar. Thus, both syntrophin sequences show some preference for Grb2-C-SH3, but the differences are not large.

A peptide containing polyproline sequences in Syn H, EPGAAPPQLPEALLLQ (63−78), was synthesized and used in an ELISA experiment. It did not inhibit the interaction between Syn A and Grb2 (data not shown), supporting the results observed in Figure 8 that the polyproline sites present in both Syn H and Syn I are required for the high-affinity Grb2 binding.

To further localize the Grb2−syntrophin interaction, $[His]_6$-Syn A was used to coat the wells of a microtiter plate, and the binding of GST-Grb2, GST-Grb2-C-SH3, and GST-Grb2-N-SH3 was assessed in the presence of 1 mM EGTA. Binding was detected using the anti-GST antibody. A typical experiment is presented in Figure 9. Averaging the data from three different experiments gave half-maximal binding at 35 ± 10 nM for Grb2, 80 ± 10 nM for Grb2-N-SH3, and 60 ± 10 nM for Grb2-C-SH3. This assay was also used to confirm that other fusion proteins, including Syn I and Syn H, bound Grb2. Furthermore, experiments in Ca^{2+} or EGTA gave similar binding affinities, showing that this interaction is independent of Ca^{2+} (data not shown).

Syntrophin has been shown to bind dystrophin, but this binding has no effect on the syntrophin−Grb2 interaction, since we find that the DysS9 fusion protein, containing the C-terminus of dystrophin that binds syntrophin (*32*), does not interfere with Grb2 binding (data not shown). The N-terminal of the PH1 domain and the N-terminal of the PDZ domain have been reported (*18*) to also bind calmodulin. Binding of calmodulin to syntrophin in the presence or absence of Ca^{2+} also does not influence the Grb2 interactions (data not shown). Phosphatidylinositol 4,5-bisphosphate binds syntrophin's PH1 domain (*11*), but it does not influence the syntrophin−Grb2 interaction (data not shown). Binding this lipid probably serves the role of localizing syntrophin to the membrane surface.

DISCUSSION

Syntrophins have been suggested to function as adapters, linking cellular proteins to the DGC (*33*). It is now clear that this adapter protein binds another adapter, the SH2/SH3 domain protein Grb2. Syntrophin contains polyproline sequences that bind Grb2. This binding is Ca^{2+}-independent (Figures 5, 8, and 9). This Ca^{2+} independence is significant since Ca^{2+} binding by syntrophin results in large syntrophin oligomers and oligomerization occurs utilizing PH1 sequences (*12*). Ca^{2+} binding or oligomerization could block Grb2 binding, but this is apparently not the case.

Binding does not occur with fusion proteins lacking the polyproline sequences (Figure 5A,B). The Grb2-C-SH3 and Grb2-N-SH3 domains bind with similar affinity (Figure 9), and both bind to both of syntrophins' polyproline regions (in Syn H and I, data not shown). The affinities measured here are quite high for SH3 interactions which typically occur in the 1−10 μM range (*34*), while here we observed affinities ranging from 35 nM (for Syn A, Figure 9) to 563 nM (for full-length Syn, Figure 6). Thus, syntrophin binding to Grb2 represents one of the highest affinity interactions known for either protein, which argues strongly for its physiological relevance. Both the proline-rich sequences at syntrophin 44−75 (present in Syn H) and the one at 181−229 (present in Syn I) bind Grb2 (Figure 5B and Figure 8). The K_ds of Syn H and Syn I are 182 and 171 nM, respectively, which are intermediate between those of Syn (563 nM) and Syn A (35

nM), suggesting that binding to both sequences is required for highest affinity Grb2 binding. Since Grb2 has two SH3 domains that bind to syntrophin with similar affinity (Figure 9), presumably the two domains of Grb2 bind the two sequence regions of syntrophin. In their study to design peptides which bound with high affinity to the SH2/SH3 adapter protein Crk, Posern et al. (*34*) found that affinities as high as 96 nM (for Crk-N-SH3) or 11 μM (for Grb2-N-SH3) could be achieved with peptides only 11 amino acids long. Sparks et al. (*31*) have used 16 amino acid long combinatorial libraries to characterize the PXXP SH3 binding motifs. Thus, 10−16 amino acids should be sufficient to define the binding sites on syntrophin. There are four PXXP motifs in Syn A, which fall into two groups: group 1 containing one PXXP motif

EPGAAPPQLPEALLLQ (63−78)

present in Syn H and group 2 containing three PXXP motifs:

VGWASPPASPLQRQPS (179−194)

SPLQRQPSSPGPQPRN (187−202)

VSRRCTPTDPEPRYLE (216−231)

present in Syn I.

Both groups bind Grb2 (Figure 5B and Figure 8). Some Grb2 interactions, such as the Grb2−dynamin or Grb2−SOS interaction, are essentially mediated by the single N-terminal SH3 domain of Grb2 (*35*, *36*); however, our data strongly suggest that both SH3 domains of Grb2 bind syntrophin well, and both PXXP regions of syntrophin are bound by both SH3 domains of Grb2. The interaction is specific for Grb2; muscle Grb2 binds, while muscle Crk-L is not bound. SH3 domains are thus necessary but not sufficient for this specific interaction (*31*, *37*). As can be seen from Figure 8, Grb2 does not bind preferentially to either one of the PXXP regions. The difference in the binding affinities for the Syn−Grb2 interaction and that of the Syn A−Grb2 interaction is interesting. The binding affinities for the Syn−Grb2 interaction (563 nM) and that of the Syn A−Grb2 interaction (35 nM) differ by an order of magnitude (Figure 7), but either one is still a quite high affinity. This is possible if the polyproline sites that are involved in the Grb2 interaction are less accessible in the full-length syntrophin, and by removing the C-terminal part of syntrophin (Syn B), these sites are made more accessible in Syn A. When the PXXP motifs are separated and are more accessible in Syn H and Syn I, the apparent dissociation constants are 182 and 171 nM, respectively, which are intermediate between that of Syn (563 nM) and that of Syn A (35 nM). Both Grb2 SH3 domains bind to both regions of syntrophin (data not shown but submitted for review), and the affinities are quite similar. Our model to account for the affinities is that the two motif groups are less accessible in Syn and become more so in Syn A. The two SH3 domains of Grb2 can bind to each motif group, resulting in many possible ways to bind the multiple sites and high-affinity binding. When the motifs are separated in Syn I and Syn H, binding is restricted to a simple, single interaction of lower apparent affinity.

Simply mixing the Syn A and Syn B sequences did not reconstitute the lower affinity, suggesting that the intact, covalent structure is required. The binding affinity for the Syn−Grb2 interaction would probably be of more significance to the in vivo situation.

Syntrophins are PH/PDZ domain adapter proteins that bind Na^+-channels (*14*), NO synthetase (*8*, *13*), dystrophin, α- and γ-sarcolglycans (*21*), and SAPK3 (*15*) and to themselves (*12*). This self-association could bring together cellular signaling components and ion channels. That syntrophin binds the SH2/SH3 adapter protein, Grb2, brings together a large number of motifs important to cellular signaling in one place within the cell. SH2/SH3 adapters are necessary for MAP kinase pathway activation and tyrosine kinase recruitment in many different cell processes. Grb2 has also been shown to recruit focal adhesion kinase, FAK^{125}, to the DGC (*38*, *39*). Phosphorylated tyrosines on FAK^{125} act as docking sites for molecules such as Grb2 that participate in multiple signal transduction pathways (*40*). The Grb2−SOS complex also binds to small G proteins such as Ras or Rac1, leading to activation of signaling pathways to initiate cell growth and differentiation. Thus, the association of Grb2 with syntrophin (this report) and β-dystroglycan (*19*) could have important roles in muscle regulation and growth.

Syntrophin also binds calmodulin (*16*−*18*), and while Ca^{2+}-calmodulin inhibits the syntrophin−dystrophin interaction (*17*), it does not affect syntrophin/Grb2 binding. Syntrophins are also found associated near the acetylcholine receptor at the neuromuscular junction (*31*), and Grb2 is also bound by the tyrosine-phosphorylated acetylcholine receptor δ-subunit (*41*). Grb2 additionally binds the C-terminus of β-dystroglycan (*19*), through its N-terminal SH3 domain. Grb2 has been reported to be present in DGC preparations from bovine brain synaptosomes (*38*). The presence of Grb2 and syntrophin in the dystrophin glycoprotein complex preparation (*38*) and our ability to trap Grb2 (but not Crk) directly from muscle extracts with syntrophin−Sepharose strongly support the hypothesis that the interaction between them observed in vitro also occurs in vivo (*38*, *39*). Indeed, the numerous signal transduction proteins now known to have association with the DGC make it almost certain that this complex is involved somehow in cell signaling as we originally suggested in 1992 (*16*). Within this complex, the dystroglycan proteins bind the muscle laminin, merosin (*42*). This may be the receptor function of the complex, much as the binding of fibronectin and other RGD sequence proteins initiates integrin signaling. In fact, there is great similarity between what is known of the signal transduction proteins associated with the DGC and what is known of integrin signaling, as has been pointed out by Yoshida et al. (*43*). It may be that signaling cellular attachment to laminin is the long sought function of this complex.

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