Communication

Identification of *α*-Syntrophin Binding to Syntrophin Triplet, Dystrophin, and Utrophin*

**Bin Yang‡§¶, Daniel Jung‡§, Jill A. Rafael∥,
Jeffrey S. Chamberlain∥**, and
Kevin P. Campbell‡§‡‡

*From the ‡Howard Hughes Medical Institute and
§Department of Physiology and Biophysics, University
of Iowa College of Medicine, Iowa City, Iowa 52242 and
the ∥Department of Human Genetics and **Human
Genome Center, University of Michigan Medical School,
Ann Arbor, Michigan 48109*

Syntrophin represents three cytoplasmic components of the dystrophin-glycoprotein complex that links the cytoskeleton to the extracellular matrix in skeletal muscle. α-Syntrophin has now been translated *in vitro* and shown to associate directly with all three components of the syntrophin triplet and with dystrophin. The *in vitro* translated 71-kDa non-muscle dystrophin isoform, containing the cysteine-rich/C-terminal domain, can also interact with the syntrophin triplet. The syntrophin binding motif in dystrophin was localized to exons 73 and 74 including amino acids 3447-3481 by comparing the interactions of α-syntrophin and seven overlapping human dystrophin fusion proteins. More than one syntrophin interaction site in this binding motif was suggested. α-Syntrophin also interacts directly with a C-tenninal utrophin fusion protein. α-Syntrophin is localized to the muscle sarcolemma as well as to the neuromuscular junction in control mouse muscle. However, similar to utrophin, α-syntrophin is only present at the neuromuscular junction in *mdx* mouse muscle in which dystrophin is absent. Our data suggest that α-syntrophin binds all syntrophin isoforms, and syntrophin directly interacts with dystrophin through more than one binding site in dystrophin exons 73 and 74 including amino acids 3447-3481.

The dystrophin-glycoprotein complex (DGC)[1] is composed of five sarcolemmal glycoproteins (α-dystroglycan, 50-DAG, β-dystroglycan, 43-DAG and 35-DAG), a 25-kDa transmembrane protein (25-DAP), and two intracellular proteins (59-DAP and dystrophin) (1-6). The α-dystroglycan (156-DAG) binds laminin and dystrophin binds to actin filaments, indicating that one function of the dystrophin-glycoprotein complex is to provide a link between the extracellular matrix and the actin cytoskeleton (5, 6). 59-DAP (now named syntrophin) is a 59-kDa cytoplasmic protein triplet in the purified dystrophin-glycoprotein complex (1-6). Together with dystrophin and the 87-kDa protein homologous to dystrophin, syntrophin is also highly enriched in AChR-rich Torpedo postsynaptic membrane (7-9). cDNA cloning and peptide sequencing further demonstrate that the syntrophin triplet contains a family of homologous but distinct syntrophin proteins encoded by several separate genes (10-12). The greatly reduced expression of dystrophin-glycoprotein complex in *mdx* mice and Duchenne muscular dystrophy (DMD) patients suggests that syntrophin-containing dystrophin-glycoprotein complex is important in maintaining the normal structure and function of muscle sarcolemma (8, 11, 13, 14).

Several lines of biochemical evidence suggest that dystrophin and syntrophin may directly interact with each other. *Torpedo* dystrophin can bind either *Torpedo* 52/58-kDa protein or the corresponding mouse protein in protein overlay assays (15). The 59-kDa protein band corresponding to β-syntrophin in the purified DGC separated on SDS-PAGE can be overlaid by dystrophin fusion protein (16). Dystrophin, utrophin, 87-kDa postsynaptic dystrophin homologous protein, and DP71 were able to be immunoprecipitated by an anti-syntrophin antibody (17). Transgenic *mdx* mice, which express a 71-kDa non-muscle dystrophin isoform in skeletal muscle, were able to rescue all components of the dystrophin-glycoprotein complex including syntrophin (18, 19). The biochemical evidence above suggests that some syntrophins, if not all, directly interact with dystrophin. However, the protein interactions among dystrophin and all protein components in the syntrophin triplet are still elusive.

Here, α-syntrophin has been translated *in vitro* and shown to directly bind dystrophin as well as all the components of the syntrophin triplet. *In vitro* translated 71-kDa non-muscle dystrophin isoform, which contains the cysteine-rich/C-terminal domain, can also associate with the syntrophin triplet. More than one syntrophin binding site in dystrophin has been identified in dystrophin exons 73 and 74 including amino acids 3447-3481. The data suggest that syntrophin isoforms may associate directly with dystrophin *in vivo* as a complex through multiple interaction sites.

[1] The abbreviations used are: DGC, dystrophin-glycoprotein complex; DMD, Duchenne muscular dystrophy; AChR, acetylcholine receptor; PCR, polymerase chain reaction; GST, glutathione *S*-transferase; MBP, maltose-binding protein; FP, fusion protein; DAG, dystrophin-associated glycoprotein.

EXPERIMENTAL PROCEDURES

In Vitro Transcription and Translation of ³⁵S-Labeled α-Syntrophin and the Non-muscle 71-kDa Dystrophin Isoform—The full-length open reading frame of rabbit α-syntrophin (GenBank accession no. U01243) was amplified by polymerase chain reaction (PCR) with the following primers: sense (5'-CCCATGGCCGTCCGGCAGGCG-3') and antisense (5'-TTCTAGACCAGCAGCCCCAGG-3'). The resulting product was digested with *Nco*I and *Xba*I and then subcloned into pGEM3 vector containing a 50-nucleotide alfalfa mosaic virus consensus initiation site (20). This construct was used to synthesize a ³⁵S-labeled probe by coupled *in vitro* transcription and translation with the TNT system as described (20). The DNA template was incubated at 30 °C for 2 h with TNT rabbit reticulocyte lysate with T7 RNA polymerase and an amino acid mixture containing [³⁵S]methionine (Amersham Corp.). The ³⁵S-labeled α-syntrophin was examined on SDS-PAGE. The non-muscle 71-kDa dystrophin isoform corresponding to the sequence of DP71 was constructed as described elsewhere (21). ³⁵S-Labeled non-muscle 71-

kDa dystrophin isoform was synthesized as described above.

Protein Overlay Assay—Rabbit skeletal muscle surface membrane and dystrophin-glycoprotein complex were purified as described previously (2, 3, 6), resolved by SDS-PAGE, and transferred onto nitrocellulose membranes. The membrane was blocked for 2 h at 4 °C in blocking buffer (0.1% gelatin, 5% bovine serum albumin, and 0.1% Tween 20 in phosphate-buffered saline, pH 7.5) and then incubated with *in vitro* translated ^{35}S-labeled α-syntrophin or non-muscle 71-kDa dystrophin isoform (10 μl·ml^{-1}) in overlay buffer (150 mM NaCl, 20 mM Hepes, 2 mM MgCl$_2$, 1 mM dithiothreitol, and 5% bovine serum albumin, pH 7.5). After extensive washing with overlay buffer, the membrane was exposed to film (X-Omat AR, Eastman Kodak Co.).

Generation of GST and Maltose-binding Protein (MBP) Fusion Proteins (FPs)—The overlapping human dystrophin (GenBank accession no. M18533) C-terminal domains were amplified by PCR with corresponding primers: for FP (3054-3685), sense (5'-TGAATTCCACGTCT-GTCCAGGG-3') and antisense (5'-AGAATTCCTACATTGTGTCCTC-3'); for FP (3054-3446), sense (5'-TGAATTCCACGTCTGTCCAGGG-3') and antisense (5'-AGAATTCTGCTACCTGCTAGCATAATGTTC-3'); for FP (3435-3685), sense (5'-TGAATTCACGCATTGAACATTATGC-3') and antisense (5'-AGAATTCCTACATTGTGTCCTC-3'); for FP (3435-3482), sense (5'-TGAATTCACGCATTGAACATTATGC-3') and antisense (5'-GAATTCCTGGTTCAAACTTTGGCAGTAATG-3'); for FP (3481-3685), sense (5'-GAATTCAGGACTCCCCCCTGAGCCAGCC-3') and antisense (5'-AGAATTCCTACATTGTGTCCTC-3'); for FP (3054-3463), sense (5'-TGAATTCCACGTCTGTCCAGGG-3') and antisense (5'-GGAATTCCTCTCATTAGGAGAGATGCTATC-3'); for FP (3464-3685), sense (5'-CGGATCCAGCATAGATGATGAACATTTG-3') and antisense (5'-AGAATTCCTACATTGTGTCCTC-3') (22). These PCR products were subcloned into pGEX vectors and introduced into *Escherichia coli* DH5α cells. Overnight cultures were diluted 1:10, incubated for 1 h, and induced for 3 h with 1 mM isopropyl-1-thio-β-D-galactopyranoside. Each induced bacterial pellet was resuspended in phosphate-buffered saline, separated on SDS-PAGE, and transferred onto nitrocellulose membranes. The murine utrophin (EMBL accession no. X69086) C-terminal domain corresponding to dystrophin exons 70 through the beginning of 79 was amplified by PCR and the resulting product was subcloned into the pMAL-p2 vector from New England Biolabs to make MBP fusion protein (18).

Immunofluorescence—Cryosections (7 μm) of quadriceps muscle of adult control mouse and adult *mdx* mouse were stained with anti-α-syntrophin FP2 antibody and detected with a fluorescein isothiocyanate-conjugated secondary antibody (11). Neuromuscular junctions were identified by double staining with rhodamine-labeled α-bungarotoxin as described previously (23).

Immunoblotting—Rabbit skeletal muscle total membranes, surface membranes or DGC were prepared as described previously (2, 3, 6). All membranes (200 μg of protein/lane) and purified DGC (8 μg of protein/lane) were separated on 3-12% gradient SDS-polyacrylamide gels and transferred onto nitrocellulose. Immunoblot staining was performed either with affinity-purified antibody against dystrophin C-terminal 15 amino acids or with affinity-purified anti-syntrophin antibodies from sheep anti-DGC polyclonal antisera (1-3, 13).

RESULTS AND DISCUSSION

To test the hypothesis that syntrophin may directly interact with dystrophin, the full-length open reading frame of α-syntrophin was amplified by PCR and subcloned into pGEM3 vector containing the 50-nucleotide alfalfa mosaic virus consensus initiation site as described (20). This construct was used to synthesize a [^{35}S]methionine-labeled probe by coupled in vitro transcription and translation in the TNT system (Promega). An overlay assay was carried out to test for syntrophin-binding proteins in skeletal muscle surface membrane and purified DGC. Fig. 1 shows that α-syntrophin binds to all three components of the syntrophin triplet as well as dystrophin. An 87-kDa protein, which is not enriched in purified DGC, also bound α-syntrophin. This protein could be related to the 87-kDa postsynaptic membrane protein, which has been demonstrated to colocalize with the AChR and associate with *Torpedo* dystrophin and syntrophin (8, 9). Our data demonstrate that α-syntrophin binds directly to dystrophin in skeletal muscle. It also provides the first evidence that syntrophins may form a syntrophin complex *in vivo*.
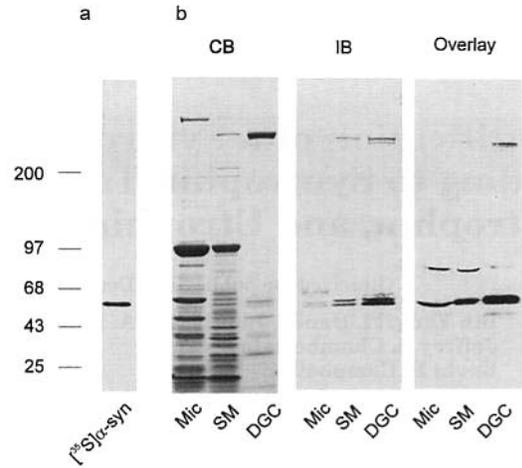
FIG. 1. **Identification of syntrophin-binding proteins in skeletal muscle cell membrane and purified dystrophin-glycoprotein complex.** *a*, autoradiogram of SDS-polyacrylamide gel of *in vitro* translated ^{35}S-labeled α-syntrophin ([^{35}S]α-syn). *b*, Coomassie Blue (*CB*)-stained SDS-polyacrylamide gel of rabbit skeletal muscle crude membranes (*Mic*), surface membrane (*SM*), and purified dystrophin-glycoprotein complex (*DGC*); *IB*, corresponding immunoblot stained with a mix of affinity-purified anti-dystrophin and anti-syntrophin triplet antibodies; *Overlay*, autoradiogram of an identical nitrocellulose transfer overlaid by *in vitro* translated [^{35}S]α-syntrophin probe.

To further define syntrophin-dystrophin interactions, the murine non-muscle 71-kDa dystrophin isoform corresponding to the sequence of DP-71 was *in vitro* translated with [^{35}S]methionine as described above (20, 21). This probe was used to overlay skeletal muscle surface membranes and purified DGC separated by SDS-PAGE. This dystrophin isoform bound the syntrophin triplet suggesting that all the syntrophins including α- as well as β-type may directly bind to dystrophin (Fig. 2). Previous work by Ozawa's group indicated that one of 43-DAGs was overlaid by dystrophin fusion protein containing cysteine-rich and C-terminal domains (16). This interaction was not detected in our experiments, probably because of the lower concentration of *in vitro* translated dystrophin in the overlay assay. Interaction of dystrophin with β-dystroglycan (one of the 43-DAGs) can be seen using another protein binding assay.[2]

To determine regions important for syntrophin binding in dystrophin, seven overlapping human dystrophin GST fusion proteins were synthesized. Protein overlay assay on these fusion proteins was performed with *in vitro* translated ^{35}S-labeled α-syntrophin (Fig. 3a). The relative positions of these fusion proteins in human dystrophin molecule are indicated in Fig. 3b. The association between these fusion proteins and syntrophin is also described in Fig. 3b. The largest dystrophin fusion protein, which contains both the cysteine-rich and the C-terminal domains, bound α-syntrophin. In this region, only the C-terminal domain (amino acids 3435-3685), covering the last 251 amino acids, is responsible for this binding. Comparison of the association of syntrophin and the dystrophin fusion proteins, which contain amino acids 3435-3482 or 3481-3685, confines the syntrophin binding motif to amino acids 3447-3481. Interestingly, two other fusion proteins (3054-3463 and 3464-3685), which divide this motif in half, were able to independently bind syntrophin, which suggests that there are more than one binding site in this motif. If all syntrophins directly bind dystrophin as a complex *in vivo*, the presence of more than one syntrophin interaction sites may be required. Fusion proteins containing the dystrophin N terminus or rod domain were also synthesized, but showed no binding activity to syntrophin

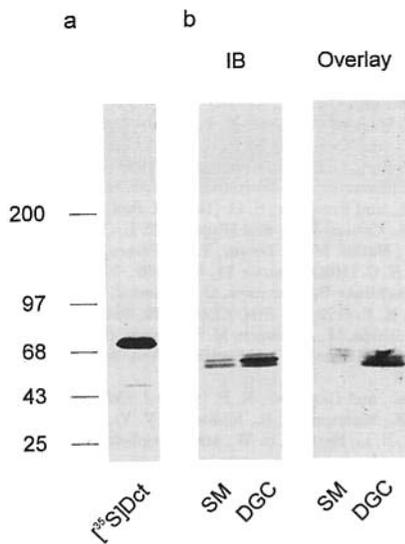[2] D. Jung, B. Yang, and K. P. Campbell, unpublished data.

FIG. 2. **Identification of dystrophin-binding proteins in skeletal muscle cell membrane and purified dystrophin-glycoprotein complex.** *a*, autoradiogram of SDS-polyacrylamide gel of *in vitro* translated [^{35}S]-labeled dystrophin C terminus ([^{35}S]*Dct*). *b*, *IB*, corresponding immunoblot stained with affinity-purified anti-syntrophin triplet antibodies; *Overlay*, Autoradiogram of the same nitrocellulose transfer overlaid by *in vitro* translated ^{35}S-labeled dystrophin C terminus probe.

(data not shown). This is consistent with the observation that all syntrophin isoforms bind to the dystrophin C terminus (Fig. 2).

Previously, we reported that utrophin associates with the same complex of dystrophin-associated proteins (23). In order to test the direct interaction of syntrophin and utrophin, mouse utrophin C terminus was expressed as a MBP fusion protein. Fig. 4*a* shows that this fusion protein was also bound by *in vitro* translated [^{35}S]α-syntrophin in an overlay assay. As the negative control, mouse dystrophin N-terminal MBP fusion protein containing amino acids 1-246 shows no binding on syntrophin (Fig. 4*a*). This suggests that syntrophin may interact with utrophin at sites homologous to the corresponding sequence in dystrophin. Comparing the deduced protein sequences of dystrophin and utrophin, many amino acid residues are conserved in this syntrophin binding motif (22, 24). These amino acid residues are probably critical for syntrophin interactions in both dystrophin and utrophin.

α-Syntrophin is present throughout the sarcolemma in control mouse skeletal muscle (Fig. 4*b*). However, the staining of α-syntrophin is greatly reduced in the sarcolemma of *mdx* mice which lack dystrophin. Notably, α-syntrophin remains enriched at the neuromuscular junction (Fig. 4*b*) where utrophin is located (25). This further suggests that α-syntrophin associates with both dystrophin and utrophin *in vivo*.

Thus far, our results indicate that syntrophin isoforms bind to each other, perhaps forming a syntrophin complex. This complex may directly associate with dystrophin or utrophin, which may be involved in anchoring dystrophin and utrophin to muscle sarcolemma. Another member of the syntrophin triplet, β-A1, contains an extended N-terminal hydrophobic sequence (12). It is possible that the syntrophin complex associates with the cell membrane by the β-A1 N-terminal hydrophobic domain, thereby anchoring dystrophin or utrophin to the cytoskeleton. Syntrophin also contains two pleckstrin homology domains (26). Pleckstrin homology domains are found in many intracellular signaling or cytoskeletal proteins and have been shown to bind the βγ subunits of G proteins and phosphatidylinositol 4,5-bisphosphate (27-29). Thus, the pleckstrin homology domain of syntrophin may also be involved in membrane
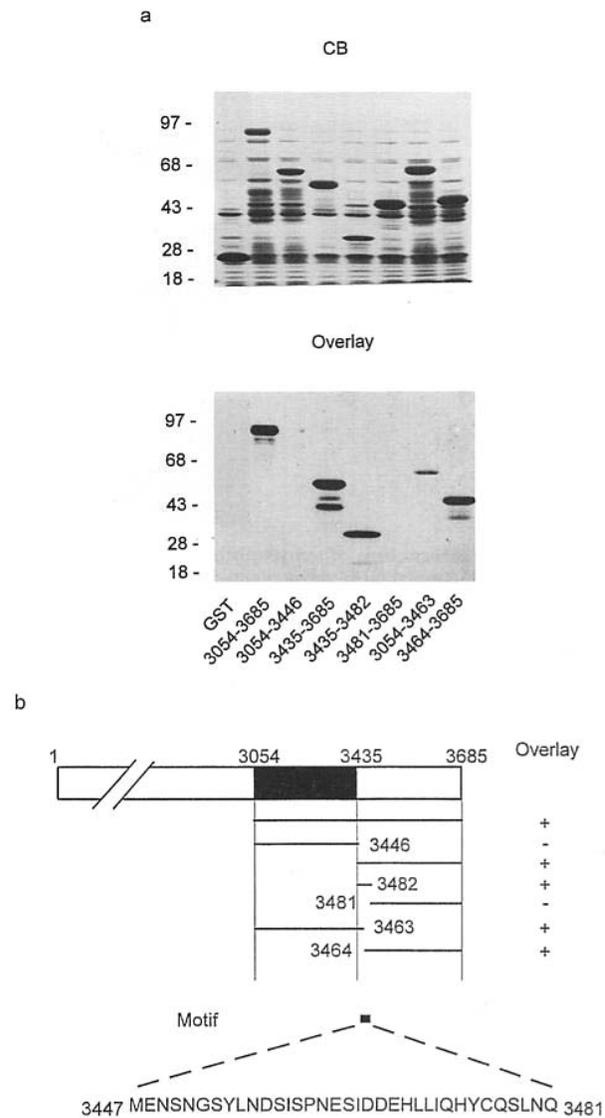

FIG. 3. **Characterization of the syntrophin binding motif in dystrophin.** *a*, Coomassie Blue-stained SDS-polyacrylamide gel of GST control and seven different human dystrophin C-terminal domains expressed as GST fusion proteins in total bacterial lysates (*top*), and autoradiogram of corresponding overlay with [^{35}S]α-syntrophin on these immobilized fusion proteins (*bottom*). The relative locations of these fusion proteins in human dystrophin are indicated below, *b*, alignment of these fusion proteins against the dystrophin C-terminal region identifying a dystrophin-syntrophin interaction motif containing more than one independent syntrophin binding site. The first and last amino acids of each fusion protein and the interaction motif were numbered based on their locations in the primary structure. The *black box* represents the cysteine-rich domain. The interactions between these fusion proteins and α-syntrophin are indicated on the *right*.

association of syntrophin.

The correct localization of the dystrophin-glycoprotein complex in the sarcolemma is crucial for its function, based on Duchenne muscular dystrophy studies (30-32). Identification of mutations in the DMD gene demonstrated the importance of the unique C-terminal domain in dystrophin (33-35). Most mutations in the C-terminal domain cause severe Duchenne-type dystrophy, probably due to deficient interactions between dystrophin and its associated proteins. However, the exact function of this domain remains elusive. Previously, two patients with small C-terminal domain truncations were analyzed (36). One patient with truncated C-terminal domain from
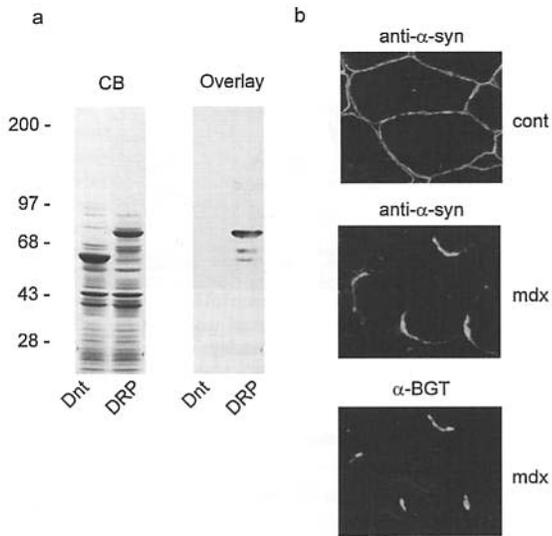
FIG. 4. **Direct interaction of syntrophin and utrophin.** *a,* Coomassie Blue (*CB*)-stained SDS-polyacrylamide gel of a control MBP fusion protein containing dystrophin N-terminal domain (*Dnt*) and utrophin C-terminal domain expressed as MBP fusion protein (DRP); *Overlay,* Corresponding overlay with ^{35}S-labeled α-syntrophin. *b,* immunofluorescence on control (*cont; top*) and *mdx* (*middle*) mouse quadriceps muscle stained with anti-α-syntrophin antibody (*anti-α-syn*). Neuromuscular junctions were identified by double staining with rhodamine-labeled α-bungarotoxin (*α-BGT*) in *mdx* mouse muscle (*bottom*).

ammo acids 3444 to 3685 had a severe DMD phenotype, whereas another patient with a truncation from amino acids 3485 to 3685 had a milder phenotype. By comparing these two truncations, it was suggested that the domain that contains amino acids 3444-3485 in dystrophin molecule is of functional importance and may be linked to certain severe DMD phenotypes (36). Interestingly, this sequence exactly matches the syntrophin-binding domain reported here, which covers amino acids 3447-3481. This may indicate that the inability of dystrophin to associate with syntrophin may be partially responsible for the severe DMD phenotypes. However, transgenic mice expressing a murine dystrophin mini-gene lacking exons 71-74 display normal muscle function and express all the dystrophin-associated proteins, including α-syntrophin, in the muscle sarcolemma (37). This suggests that there may be more syntrophin binding sites beyond exons 71-74 in dystrophin that were not detected by overlay experiments. Alternatively, syntrophin may have been recruited by other unknown syntrophin-binding proteins other than dystrophin.

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