Direct binding of *Torpedo* syntrophin to dystrophin and the 87 kDa dystrophin homologue

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Abstract Syntrophin, a 58-kDa membrane-associated protein, is one component of a protein complex associated with dystrophin and other members of the dystrophin family, including the 87-kDa homologue (87K protein). To characterize interactions between syntrophin and 87K protein, we used an in vitro overlay binding assay. We demonstrate that purified *Torpedo* syntrophin binds directly to dystrophin and 87K. By expressing overlapping regions of the 87K protein as bacterial fusion proteins for binding targets, we show that a 52-amino acid region of 87K (residues 375–426) is sufficient for binding syntrophin.

Key words: Syntrophin; Dystrophin; Binding site; Torpedo

1. Introduction

Dystrophin is the gene product of the Duchenne Muscular Dystrophy locus [1]. The 14Kb dystrophin mRNA encodes a 427-kDa protein that is expressed in both muscle and neuronal tissues [2–4]. In skeletal muscle, dystrophin is localized to the cytoplasmic side of the sarcolemmal membrane [5] where it interacts with several transmembrane and peripheral proteins [6–10]. Although the exact role of dystrophin and the complex in skeletal muscle is not known, they are believed to link the internal actin cytoskeleton to the extracellular matrix, thereby stabilizing the membrane during contractions (for reviews, see [11,12]).

Molecular cloning studies have revealed that dystrophin is but one member of a growing family of related proteins. Several shorter forms of dystrophin are derived from the dystrophin gene [11,12]. These include Dp 240, Dp 116 and Dp 71, all of which contain the cysteine-rich, C-terminal region (CRCT) of the protein. Two other dystrophin-related proteins, utrophin and the 87K protein, are products of different genes. The 87K protein, a substrate for tyrosine phosphorylation, was first discovered as a postsynaptic protein of *Torpedo* electric organ [13]. The 87K protein shows modest but significant homology to the CRCT region of dystrophin [14]. One feature in common among dystrophin, utrophin, Dp 71 and the 87K protein (and presumably the other short dystrophin proteins) is their association with syntrophin, a 58-kDa peripheral protein [9,10].

Syntrophin, like 87K, was first identified as a component of the postsynaptic membrane of *Torpedo* electric organ where it could play a role in synapse formation [15]. Recently, three independent genes encoding three distinct syntrophins have been identified [16–18]. The syntrophins can be loosely divided into two groups based on isoelectric point: acidic (α, pI = 6.7) or basic (β1 and β2, pI = 9+) [19]. In skeletal muscle, α-syntrophin is found on the sarcolemma, in a distribution like that of dystrophin and the 87K protein, while β2-syntrophin is localized to the neuromuscular junction [20].

Here, we characterize the binding of *Torpedo* syntrophin to dystrophin and 87K. Using an in vitro blot overlay assay, we show that *Torpedo* syntrophin (an α-syntrophin) binds directly to dystrophin and 87K. Using fusion proteins encoding portions of 87K, we map a binding site of syntrophin to residues 375–426.

2. Materials and methods

2.1. Materials

Liquid nitrogen frozen electric organ from *Torpedo nobiliana* was purchased from Biofish (Georgetown, MA). Production and characterization of the antisyntrophin mAb 1351 and antidystrophin mAb 1958 have been described previously [15,21]. Lithium diiodosalycilate (LIS) was purchased from Eastman Kodak (Rochester, NY). Oligonucleotides for PCR amplification were purchased from Macromolecular Resources (Fort Collins, CO). The pET14b expression vector and BL21(DE3)pLysS bacteria were purchased from Novagen (Madison, WI). The pGEX-3X GST fusion vector and anti-GST antibody were purchased from Pharmacia (Piscataway, NJ). The 87K cDNA and anti-87K Ab 638 were gifts of Dr. R. Huganir (Johns Hopkins University, Baltimore, MD). Anti-87K mAbs 13H1 and 24H3 were gifts of Dr. J. Cohen (Harvard University, Cambridge, MA).

2.2. Purification of syntrophin and dystrophin complex from T. nobiliana

Membranes were prepared from the electric organ of *T. nobiliana* by the method of Porter et al. [22]. Syntrophin, and other peripheral membrane proteins, were solubilized from the membranes with 10 mM lithium diiodosalycilate (LIS) [22], a treatment that also dissociates syntrophin from dystrophin. The LIS extracts were diluted with 5 vols. of column buffer (10 mM $NaPO_4$, pH 7.6, 150 mM NaCl, 1% Triton-X 100, 5 mM EDTA, 5 mM EGTA, 1 μg/ml aprotinin, 5 μg/ml leupeptin, 2.5 μg/ml phenylmethylsulfonyl fluoride, 5 μg/ml pepstatin A) and incubated for 2 h at 4°C with a 3-ml Affigel 10 column derivatized with mAb 1351. The column was then washed successively with 10 ml column buffer, 10 ml column buffer containing 1 M NaCl and 10 ml column buffer without Triton-X 100. Syntrophin was then eluted with 0.1 M triethylamine (pH 11.5) and immediately neutralized with Tris-HCl (pH 6.0). Tween-20 was added to a final concentration of 0.1% and the purified syntrophin was dialyzed against overlay buffer (see below) for $\geq$2 h.

Dystrophin complex was isolated by the method of Butler et al. [9].

2.3. Construction of pET14-87Ka, pET14-87Kb, pET14-87Kc and pGEX-87K52

Eight oligonucleotide primers were used in the polymerase chain reaction (PCR) to create four overlapping cDNAs of 87K from the full-length cDNA [14]:
primer 87Ka, 5′-
CAGAAAATCATATGGCCGAGAGAAGACAGTTG 3′;
primer 87Kb, 5′-

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GCTGGTACATATGACACTGCTGGCAACGATATCG 3′;
primer 87Kc, 5′-
GTTTCTACGACATATGCTGAAGTTGCCAACATCTG 3′;
primer 87Kd, 5′-
GTGAGGATCCTGCTTCAGCAGCTAGTCGTGC 3′;
primer 87Ke, 5′-
CAGCCTTCATATGGCTGATAGACTGGGTGATG 3′;
primer 87Kf, 5′-
GTCAATGGATCCTGAGGTGTATGAGTTGGAG 3′;
primer 87K52a, 5′-
ACAGGATCCATATTGCTGA TAGACTGGGTG 3′;
primer 87K52b, 5′-
GTGAATCCCGCTGCTTCAGCAGCTAGTCGTGC 3′;

Each primer had two or four mismatch nucleotides (underlined above) that created a restriction enzyme recognition site necessary for subcloning. PCR was performed using the full-length cDNA encoding *Torpedo* 87K as a template with the following primer pairs: 87Ka and 87Kb, 87Kc and 87Kd, 87Ke and 87Kf, and 87K52a and 87K52b. Each primer pair produced a single DNA band of the appropriate length that was digested and subcloned into the bacterial expression vector pET14b (first three pairs) or pGEX-3X (last pair) to create pET14-87Ka, pET14-87Kb, pET14-87Kc and pGEX-87K52. Each construct was sequenced to verify correct subcloning and sequence integrity (UNC DNA Sequencing Facility, Chapel Hill, NC). When expressed in BL21(DE3)pLysS bacteria using the manufacturers protocol (Novagen, Madison, WI), the three pET14 constructs, respectively, produced the fusion proteins 87Ka, 87Kb and 87Kc (Fig. 3) fused to a 21-amino acid leader sequence encoded by the vector. Expression of pGEX-3X and pGEX-87K52 in JM109 bacteria as per the manufacturers protocol (Pharmacia, Piscataway, NJ), respectively, produce glutathione S-transferase (GST) and GST87K52 containing amino acids 375–426 of the 87K protein fused to GST (Fig. 4). Bacteria expressing the fusion proteins were pelleted and resuspended in 1/20 vol. PBS for SDS gel electrophoresis.

2.4. Overlay analysis

Blot overlay analysis was done using a modified method of Suzuki et al. [23]. Purified dystrophin complex or bacteria containing expressed fusion proteins were separated on an 8% SDS-PAGE gel and blotted to nitrocellulose as described previously [10]. Nitrocellulose blots were blocked overnight in PBS (pH 7.4), 0.1% Tween 20, and 2% Carnation instant milk and then incubated with purified syntrophin at a concentration of 2–20 nM (estimated from band staining intensity on SDS gels) for 3–4 h at room temperature in overlay buffer (20 mM HEPES pH 7.5, 150 mM NaCl, 2 mM $MgCl_2$, 1 mM DTT, 2% carnation instant milk, 1 μg/ml aprotinin, 5 μg/ml leupeptin, 2.5 μg/ml phenylmethylsulfonyl fluoride, 5 μg/ml pepstatin A). Blots were then washed and transferred to TBST, 0.1% fish gelatin (0.5 M NaCl, 10 mM Tris-HCl (pH 8.0), 0.5% Tween 20, 0.002% NaN_3, 0.1% fish gelatin). Detection of bound syntrophin was accomplished by Western blotting with antisyntrophin mAb 1351, alkaline-phosphatase conjugated antimouse secondary antibody and Western Blue substrate (Promega, Madison, WI).

3. Results

Previous studies have shown that syntrophin is associated with dystrophin and 87K protein in detergent extracts of *Torpedo* electric organ [9] and co-immunoprecipitated with 87K [14]. To determine if this association is mediated by direct interaction, we developed a blot overlay assay with *Torpedo* syntrophin. Syntrophin was purified by immunoaffinity chromatography from lithium diiodosalycilate extracts of *Torpedo* membranes. As previously shown [16], LIS dissociates syntrophin from *Torpedo* membranes and other proteins of the dystrophin complex. Extensive washing of the column removes contaminating proteins, such that by SDS-PAGE/Coomassie blue staining analysis, syntrophin preparations isolated in this manner contain a single band of approximately 58,000 molecular weight (data not shown). This purified syntrophin (an α-syntrophin based on its isoelectric point) was used to probe nitrocellulose strips containing dystrophin complex.

Control overlay blots, done in the absence of antisyntrophin mAb1351 (Fig. 1, lane b) or of syntrophin (Fig. 1, lane c), produced the expected results. No non-specific binding of the secondary antibody was seen in the absence of mAb 1351 and only a single band, representing the syntrophin endogenous to the dystrophin complex, was visualized in the absence of syntrophin (Fig. 1, lane c, lower arrow). In contrast, when blots were first incubated with purified syntrophin and then probed with antisyntrophin mAb 1351, two additional reactive bands

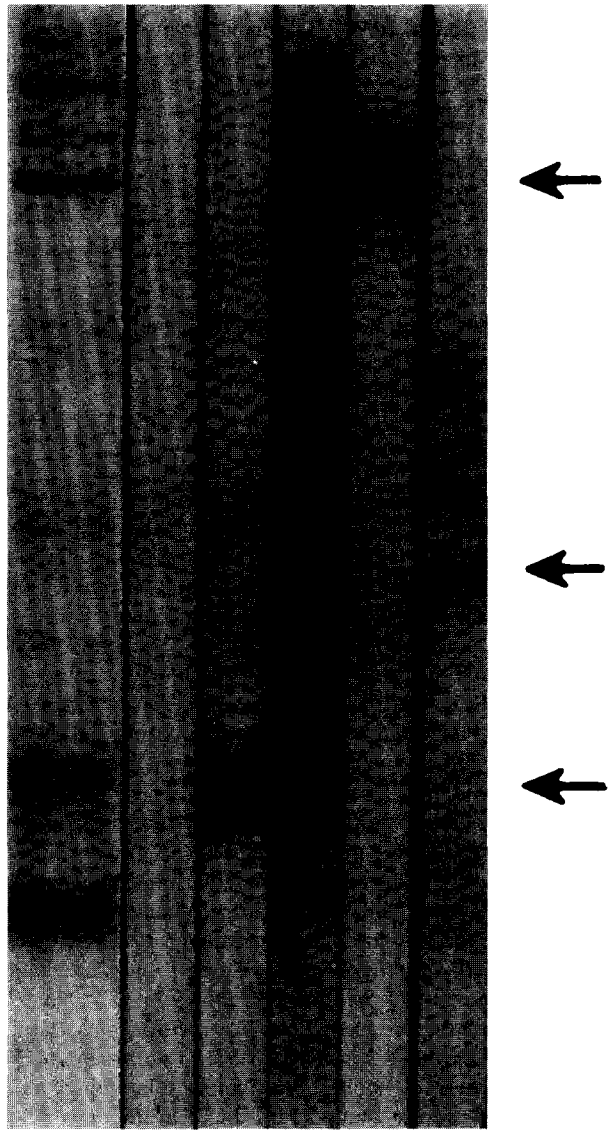

Fig. 1. Overlay analysis on dystrophin complex. *Torpedo* dystrophin complex was subjected to SDS-PAGE and either stained with coommassie (lane a) or transferred to nitrocellulose strips (lane b–f). Overlay analysis was done in the absence of mAb1351 (control lane b), absence of syntrophin (control lane c) or presence of both (experimental lane d). Western analysis was done with antidystrophin mAb 1958 (lane e) or anti-87K mAb 13H1 (lane f). From bottom to top, arrows denote the positions of syntrophin, 87K and dystrophin.

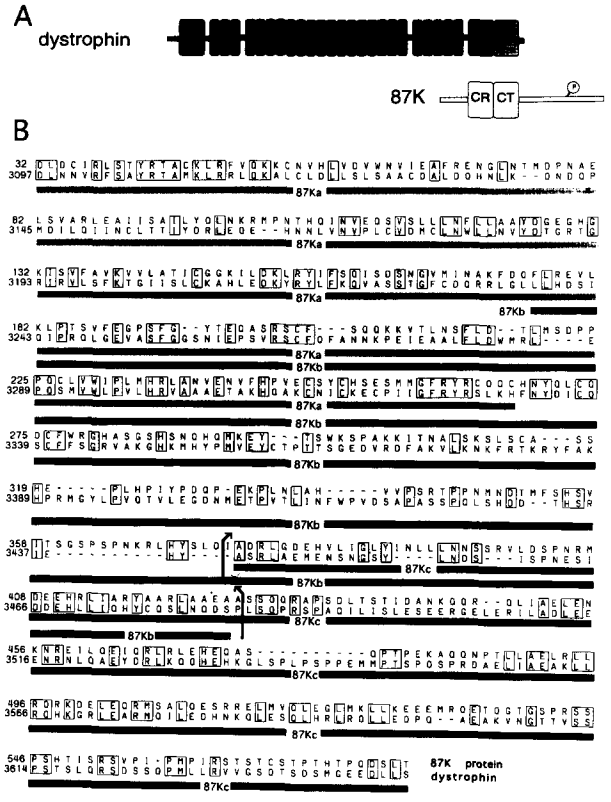

Fig. 2. Amino acid conservation between 87K and dystrophin and location of sequence encoded by the fusion proteins 87Ka, 87Kb, 87Kc and 87K52. (A) Schematic diagram showing region of conservation between dystrophin and 87K. (AB) actin-binding domain of dystrophin. (CRCT) Cysteine-rich and C-terminal domain conserved in both dystrophin and 87K. (B) Comparison of the CRCT domain sequence from 87K (top) and dystrophin (bottom). Boxed residues are identical in both sequences. Amino acids underlined by the light bar (residues 32–267) were expressed in the fusion protein 87Ka. Amino acids underlined by the black bar (residues 176–426) were expressed in the fusion protein 87Kb. Amino acids underlined by the dark grey bar (residues 376–578) were expressed in the fusion protein 87Kc. Residues between the arrows (375–426) form the syntrophin-binding site found in 87K52.

were observed at the positions expected for 87K protein and dystrophin, respectively (Fig. 1, lane d, middle and upper arrows). Western analysis using antidystrophin (Fig. 1, lane e) and anti-87K (lane f) confirmed that these bands are dystrophin and the 87K postsynaptic protein. Syntrophin binding appears to be specific to 87K and dystrophin since other proteins in the preparation, including the major 43,000-Da protein (Fig. 1, lane a) showed no binding. Thus, *Torpedo* α-syntrophin binds directly to dystrophin and 87K in a specific manner.

To map the syntrophin-binding site on 87K protein, we produced overlapping recombinant fragments as fusion proteins. As previously shown [14], the CRCT region of dystrophin is homologous with the 87K protein (approximately 500 residues). cDNA constructs encoding three overlapping fusion proteins, 87Ka, 87Kb and 87Kc (Fig. 2) were produced by PCR and subcloned into pET-14b bacterial expression vector. SDS-PAGE analysis of bacterial cell lysates showed that each expression vector produced a fusion protein of the appropriate molecular weight (Fig. 3A). These proteins were not produced by the pET14b vector alone.

Bacterial cell lysates containing each of the three fusion proteins, or the pET14b expression vector alone as a control, were then used in the overlay assay. Syntrophin showed no binding to proteins of bacterial lysates expressing the vector alone or fusion peptide 87Ka (Fig. 3B, lanes a and b, respectively). However, lysates containing fusion peptides 87Kb and 87Kc each contained a single band that bound syntrophin (Fig. 3B, lane c and d). The syntrophin reactive protein in the 87Kb lysates also bound the anti-87K Ab 638 (Fig. 3B, lane e). Similarly, the syntrophin reactive protein in the 87Kc sample was recognized by the anti-87K mAbs 24H3 (Fig. 3B, lane f) and 13H1 (data not shown). We conclude that a specific interaction, not affected by the addition of 21 amino acids encoded by the vector, occurs between syntrophin and both 87Kb and 87Kc. Thus, the sequence in common between these fusion proteins (the 50 residues from 376–425) contains the probable binding site for syntrophin.

To determine directly whether these 50 amino acids form a syntrophin-binding site, the 87K52 fusion protein was tested for syntrophin binding. This fusion protein contains residues 375–426 of the 87K protein fused to GST. SDS-PAGE analysis of bacterial cell lysates shows that, as expected, 87K52 is slightly larger than GST alone and is found only in bacteria induced to produce fusion protein (Fig. 4A).

Overlay analysis on bacterial cell lysates expressing either GST or 87K52 shows that a single syntrophin reactive band is found in the 87K52 lysates but not in the GST lysates (Fig. 4B). The same band is also recognized in Western analysis with anti-GST antibodies (data not shown). Thus, syntrophin interacts specifically with 87K52 and not GST, demonstrating that this interaction is mediated by the 52-amino acid addition to GST.

4. Discussion

Recent work from several laboratories has shown that mammalian syntrophins are capable of binding directly to different members of the dystrophin family [24–27]. The binding to dystrophin was characterized in particular and, in each case,

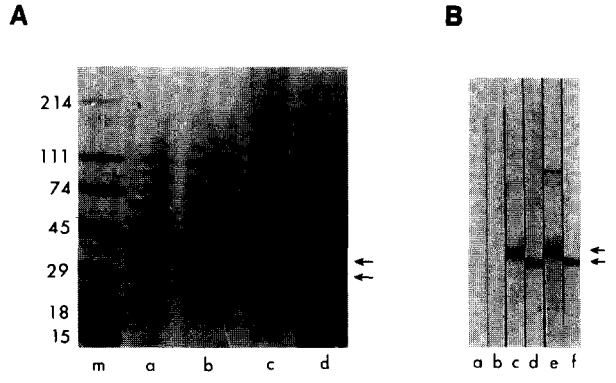

Fig. 3. Expression and overlay analysis of 87Ka, 87Kb and 87Kc. (A) Coommassie staining of an 8% SDS-PAGE gel containing bacterial lysates expressing the pET14b expression vector alone (lane a), pET14–87Ka (lane b), pET14–87Kb (lane c) or pET14–87Kc (lane d). Lane M denotes molecular weight markers. (B) Nitrocellulose blots containing bacterial lysates expressing the pET14b expression vector alone (lane a), pET14–87Ka (lane b), pET14–87Kb (lanes c and e) or pET14–87Kc (lanes d and f) were subjected to overlay analysis with syntrophin (lanes a–d) or Western analysis with anti-87K Ab 638 (lane e) or anti-87K mAb 24H3 (lane f).

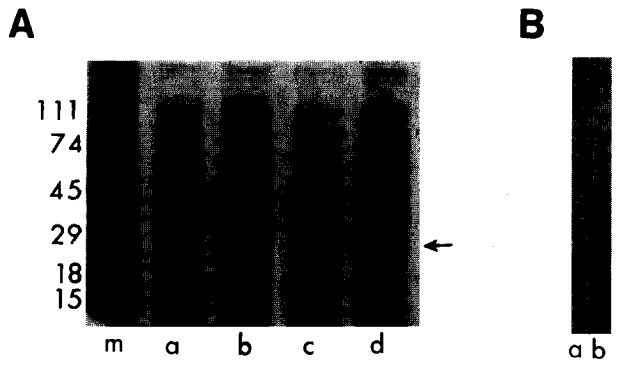

Fig. 4. Expression and overlay analysis of GST and 87K52. (A) Coommassie staining of an 8% SDS-PAGE gel containing bacterial lysates of uninduced pGEX-3X (GST) alone (lane a), induced pGEX-3X (lane b), uninduced pGEX-87K52 (lane c) or induced pGEX-87K52 (lane d). Lane M denotes molecular weight markers. The arrow denotes the position of the 87K52 fusion peptide. (B) Nitrocellulose blot containing bacterial lysates expressing pGEX-3X (lane a) or pGEX-87K52 (lane b) were subjected to overlay analysis with syntrophin.

the syntrophin-binding site in dystrophin was mapped. Using immunoprecipitation of in vitro translated proteins, Ahn et al. have mapped the human β1-syntrophin-binding site to the alternatively spliced exon 74 of the dystrophin gene and to the analogous region of utrophin and 87K protein [24]. Suzuki et al. [25] obtained a similar but slightly different result. Using portions of dystrophin as a probe in overlay analysis, they present evidence that α-syntrophins and β-syntrophins have slightly different binding sites. They conclude that α-syntrophin binds amino acids 3444–3494 of dystrophin, an area partially encoded by exon 74, while β1-syntrophin binding is blocked by a peptide of amino acids 3494–3544, encoded partially by exon 74 and exon 75. Finally, Yang and colleagues [26] found that in vitro translated α-syntrophin binds amino acids 3435–3482 of dystrophin.

We have studied the binding of *Torpedo* syntrophin to two CRCT containing proteins, dystrophin and the 87K postsynaptic protein. In our blot overlay assay, *Torpedo* syntrophin was shown to bind specifically to *Torpedo* dystrophin, thus demonstrating that, at least for dystrophin binding, *Torpedo* syntrophin behaves like the mammalian syntrophins.

Torpedo α-syntrophin also bound to an additional member of the dystrophin family, the 87K postsynaptic protein. The 87K protein has 27% amino acid identity to dystrophin scattered throughout the CRCT domain ([14], see also Fig. 2). By expressing portions of 87K CRCT domain as fusion proteins in bacteria, we have mapped the syntrophin-binding site to amino acids 375–426 of the 87K protein. These residues are homologous to residues 3441–3483 of dystrophin. Thus, the α-syntrophin-binding site in 87K is essentially the same as that found by others for dystrophin.

The α-syntrophin-binding site for 87K described here has 19 amino acids in common with the β1-syntrophin-binding site found in 87K by Ahn and colleagues [24] (amino acids 408–426). The highest degree of conservation between 87K and dystrophin is also found within this region (amino acids 408–417), suggesting that these residues may form the core syntrophin-binding site. However, a peptide containing amino acids 405–419 was unable to block binding of syntrophin to 87Kb (T.M. Dwyer and S.C. Froehner, unpubl. data). Similarly, Ahn et al. observed no binding of syntrophin to the first half of the dystrophin exon 74 in their assay [24]. While these somewhat disparate results may be due to differences in the methods used, it is also possible that α- and β-syntrophins have slightly different binding sites on 87K, as seen by Suzuki et al. for dystrophin [25]. In the 52-amino acid binding site, both 87K and dystrophin are predicted to have similar structures: two alpha helices interrupted by either beta and turn structures (87K) or turn structures alone (dystrophin). Thus, the binding of syntrophin may depend on secondary structure of the proteins in this region and not strictly on the linear sequence of amino acids.

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