Syntrophin Binds to an Alternatively Spliced Exon of Dystrophin

Andrew H. Ahn* and Louis M. Kunkel†

*Program in Neuroscience and Medical Scientist Training Program, Harvard Medical School; and †Howard Hughes Medical Institute at Children's Hospital, Boston, Massachusetts 02115

Abstract. Dystrophin, the protein product of the Duchenne muscular dystrophy locus, is a protein of the membrane cytoskeleton that associates with a complex of integral and membrane-associated proteins. Of these, the 58-kD intracellular membrane-associated protein, syntrophin, was recently shown to consist of a family of three related but distinct genes. We expressed the cDNA of human Bα-syntrophin and the COOH terminus of human dystrophin in reticulocyte lysates using an in vitro transcription/translation system. Using antibodies to dystrophin we immunoprecipitated these two interacting proteins in a variety of salt and detergent conditions. We demonstrate that the 53 amino acids encoded on exon 74 of dystrophin, an alternatively spliced exon, are necessary and sufficient for interaction with translated Bα-syntrophin in our assay. On the basis of its alternative splicing, dystrophin may thus be present in two functionally distinct populations. In this recombinant expression system, the dystrophin relatives, human dystrophin related protein (DRP or utrophin) and the 87K postsynaptic protein from Torpedo electric organ, also bind to translated Bα-syntrophin. We have found a COOH-terminal 37-kD fragment of Bα-syntrophin sufficient to interact with translated dystrophin and its homologues, suggesting that the dystrophin binding site on Bα-syntrophin occurs on a region that is conserved among the three syntrophin homologues.
Of the two basic isoforms, β-syntrophin is expressed in liver, pancreas, kidney, skeletal muscle, lung, placenta, and in lower levels in brain and heart (Ahn et al., 1994). The β-syntrophin (previously identified as mouse syntrophin-2) is present in low levels in many tissues (Adams et al., 1993). Subcellular localization with isoform-specific antibodies shows that α-syntrophin is expressed throughout the entire muscle membrane. On the other hand, β-β-syntrophin is found only at the postsynaptic densities of the muscle membrane (Peters et al., 1994). The cellular localization of β-syntrophin is yet to be shown.

Syntrophin purified from the electric organ of Torpedo (Frohner et al., 1987) interacts directly with dystrophin in immuno-affinity and blot-overlay experiments (Butler et al., 1992; Cartaud et al., 1993). Quantitative immunabsorption of dystrophin followed by immuno-absorption of syntrophin from Torpedo electroplaque extract showed that there is a distinct non-dystrophin-associated pool of syntrophin that contains the 87K protein (Butler et al., 1992). A similar immuno-affinity purification of syntrophin from several rat tissues shows that dystrophin, Dp71 isoforms, DRP, and 87K-like proteins all copurify with syntrophin (Kramarcy et al., 1994). The syntrophin binding site on dystrophin has been localized to the COOH-terminus of dystrophin (Suzuki et al., 1992, 1994).

The widespread distribution of the syntrophins and their coincident expression with Dp71 and DRP in non-muscle tissues suggests that these proteins may play an analogous role to dystrophin in the membrane cytoskeleton of these other cell types (Kramarcy et al., 1994). It is possible that the specific subcellular localization of the syntrophin isoforms accounts for the specificity of localization and function of dystrophin, Dp71, and DRP.

The observation that anti-87K protein or anti-syntrophin antibodies copurify 87K, dystrophin, and syntrophin led to the proposal that an amphipathic repeated heptad motif on both dystrophin and 87K could be the basis for their association (Wagner et al., 1993). This proposal does not exclude the possibility of a distinct syntrophin binding site on both dystrophin and the 87K protein, which is supported by the copurification of 87K with syntrophin in a dystrophin-depleted extract (Butler et al., 1992).

To more fully understand these relationships, we have used recombinant techniques to study the syntrophin–dystrophin interaction by using an in vitro expression system. We have expressed portions of the human dystrophin gene and have mapped its site of interaction with a defined isoform of human syntrophin, β-syntrophin, by coimmunoprecipitation. In our assay, the 53 amino acids encoded on exon 74 of dystrophin are sufficient to bind to in vitrotranslated β-syntrophin. In the accompanying paper, Suzuki et al. have independently obtained a similar localization of this binding site by using a blot-overlay approach (Suzuki et al., 1995).

We have also expressed naturally occurring spliced isoforms of dystrophin, in which 110 amino acids (including the 53 amino acids of exon 74) are internally deleted from the COOH terminus of dystrophin, and this isoform does not bind β-syntrophin in our assay. This characterization of the syntrophin binding site on dystrophin suggests that, on the basis of its alternative splicing, dystrophin is present in two functionally distinct populations. In addition, we have found in our in vitro assay that the dystrophin relatives DRP and the 87K postsynaptic protein also interact with syntrophin, demonstrating that this binding site on dystrophin is functionally conserved. The finding that syntrophin binds the three dystrophin family members suggests that they may play functionally analogous roles.

Materials and Methods

Construction of a Vector for Transcription and Translation

To facilitate efficient and processive translation of cDNAs, an expression cassette was designed and constructed from synthetic oligonucleotides by phosphoramidite synthetic chemistry (Applied Biosystems Inc., Foster City, CA). A series of partial, overlapping, and complementary oligonucleotides with BssHII complementary ends were ligated to the plasmid Bluescript II KS+ (Stratagene Inc., CA), in which the RNA polymerase and multiple cloning site was removed by digestion with BssHII. The resulting expression vector, pMG7, contains the following features (Fig. 1 a): a T7 RNA polymerase late promoter (Panagiotatos and Wells, 1979); the 5′ untranslated region (U) of the tobacco mosaic virus (TMV) (Gallie et al., 1987, 1988); an initiator ATG codon in the context of a Kozak consensus (M) (Kozak, 1986); an extensive polylinker (MCS); stop codons in all three reading frames; a short poly(A)+ region; a polyadenylation signal; and other unique restriction sites for linearization of the plasmid (L).

The expression vector pMG7 was further modified to accommodate an EcoRI fragment in another reading frame (Table I). This modified vector, TR-3, was used to produce the expression construct TDR (see below). To express the octapeptide FLAG antigen (bi) at the NH2 terminus of the expressed polypeptide, complementary oligonucleotides (Table I) were ligated to the NcoI/BamHI site on pMG7. The resulting vector, pHFR, permitted the introduction of inserts as BamHI/EcoRI fragments, and the translation of a fusion polypeptide with the sequence beginning MDYKDDDD KGS . . . (Table I).

Subcloning of cDNA’s into Expression Vector

PCR primers were synthesized to amplify regions of specific cDNAs. The oligonucleotides used in the subcloning of dystrophin and all other constructs used in this report are listed in Table I (Sambrook et al., 1989). For dystrophin, 100 ng of plasmid p9-14, encoding the entire COOH terminus of dystrophin (Koenig et al., 1987), was used as template with 100 pmol of each primer. To maintain the fidelity of the amplified DNA, we used the thermo-stable DNA polymerase Vent polymerase (New England Biolabs, Sharon, MA) in all amplifications. Amplified DNA was cloned into pMG7 using restriction enzymes indicated (Table I). To confirm the accuracy of the amplification and cloning steps, partial sequence was obtained by the dyeoxy-terminator method using Taq polymerase and analyzed on an Applied Biosystems automated sequencer.

Because the COOH terminus of dystrophin has numerous NcoI sites, some constructs were produced by partial digestion with NcoI and XhoI, isolated by electrophoresis, and then ligated into the expression vector. In other cases, the inserts were cloned into the KpnI to SpeI site of pMG7, resulting in a fusion protein that contains the additional amino acids MGT . . . at the NH2 terminus of the native sequence. To introduce the seven unique amino acids at the NH2 terminus of Dp71, a two-step amplification was used. The oligonucleotide F1 was used in the first round to amplify cDNA from exon 63 and then F2 in the second round to add the novel sequence (Table I).

To construct Dp71Δ110, a naturally occurring splice variant of Dp71 missing exons 71 through 74 was isolated from a human fetal brain cDNA library. We isolated the SacI restriction fragment which spans the alternative spliced exons 71-74 and replaced the SacI fragment in the expression plasmid encoding Dp71.

The cDNA clone to the COOH terminus of human DRP containing the region homologous to the entire COOH terminus of dystrophin, DRP2, was kindly provided by T. S. Khurana (in our laboratory). This EcoRI-flanked clone begins at nucleotide 8257 of the DRP cDNA encoding the amino acids (2753–3432) starting with NDLSQSGSP . . . (Tinsley et al., 1992). We subcloned this EcoRI fragment of DRP2 into a modification of pMG7, in which the EcoRI reading frame was modified to accommodate the reading frame (TR-3, Table I). The insert within expression plasmid FDR, coding for the region of DRP that is homologous to exon 74 of dystrophin, was PCR-amplified from this same DRP cDNA.

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Table I. Construction of Expression Vectors

| Clone name | Forward and reverse oligonucleotides* | Expressed protein sequence† |
|------------|--------------------------------------|----------------------------|
| C2979      | ACT TCC ACC ATG GCA CTG CGA GGA GAA ATT GTG GAG TCG ACT TCC TAC ATT GTG TCC TGT CTC ATT GGC T | M (dystrophin 2980-3685) |
| C3145      | NcoI partial digestion product of C2979 | dystrophin 3145-3685 |
| Dp71       | Fl: AGG GAA CAG CTC AAA GGC CAC GAG ACT CAA ACA ACT F2: ATG GGT ACC ATG AGG GAA CAG CTC AAA GGC R: TCA CTA GAT GTA AAA CAT TTA TTC TGC TCC | MGTMREQLKG (dys 3076-3685) |
| Dp71Δ148   | Fl and F2 GAC ACT AGT CAG GAC AGG CCT TTA TGT TCG | MGTMREQLKG (dys 3076-3537) |
| Dp71Δ276   | Fl and F2 GAT ACT AGT TCA GGG AGT TTC CAT GTT GTC | MGTMREQLKG (dys 3076-3537) |
| C3356Δ148  | Dp71 Δ148 digested with NcoI and recircularized | dystrophin 3356-3537 |
| Dp71Δ110   | replaced the internal SacI fragment from Dp 71 with that of 19A5 pFHR C ATG GAC TAC AAG GAC GAC GAC GAA G GAA TCC CTT GTC GTC GTC CTT GTC GTC | M DYKD1DDKS GS |
| F72-74     | GAT GGA TCC GCC CCT GCC TCG GCC CCT GG GGA ATT CTA TTT ATG TTC GTG CTC GTG | MDYKD1DDKS (dys 3421-3534) |
| F73-74     | TAT GGA TCC AGG CTA GCA GAA ATG GAA GG GGA ATT CTA TTT ATG TTC GTG CTC GTG | MDYKD1DDKS (dys 3443-3534) |
| F74        | AAT GGA TCC ATA GAT GAT GAA CAT TTG GG GGA ATT CTA TTT ATG TTC GTG CTC GTG | MDYKD1DDKS (dys 3465-3534) |
| F74-01     | AAT GGA TCC ATA GAT GAT GAA CAT TTG GTG AAA TCC AAT TGG TTT CTT CTT CCA | MDYKD1DDKS (dys 3465-3519) |
| F74A       | F74 digested with BglII and re-circularized | MDYKD1DDKS (dys 3465-3496) N |
| F74B (F74-32) | AAG GGA TCC TTA GAG AGT GAG GAA AGA GG GG GGA ATT CTA TTT ATG TTC GTG CTC GTG | MDYKD1DDKS (dys 3498-3534) |
| F8/S       | βA1 digested with BamHI and EcoRI and ligated into FHR | MDYKD1DDDKGS (β1 syntro 205-537) |
| FDR        | ACA GGA TCC GTG GAA GAG GAC CAC GC CAT GAA TTC ACT GTA CAT TTC TCT CTC | MDYKD1DDDKGSVEDEH . . . |
| F87        | AGT GGA TCC AGA ATG GAT GAA GAA C TCT GAA TTC ACT GCA GGA TT CTC TAT | MDYKD1DDKKSRMDEE . . . |
| T87        | C ATG GTG AAA ACT GCA NcoI to PstI linker GT TTT CAC | native 87K sequence |
| TR3        | C ATG GGA ATG NcoI to EcoRI linker A ATT CAT TCC | MGML . . . |
| TDR        | Eco RI fragment of DRP-2 cloned into TR3 | MGML (DRP 2753-3432) |

* Oligonucleotides are written in the conventional 5’ to 3’ direction. In cases where PCR products were not used to generate the construct, the cloning strategy is briefly described.
† Single letter amino acid symbols are used. Amino acid numbers for human dystrophin, human β1-syntrophin, human DRP, and Torpedo 87K protein are respectively from Koenig et al., 1988; Ahn et al., 1994; Tinsley et al., 1993; and Wagner et al., 1993.

In the expression of β1-syntrophin, the cDNA clone βA1 was used directly from within the Bluescript vector (Ahn et al., 1994). This plasmid expressed protein at much lower levels than the expression vectors described above. To construct F8/S, the BamHI/EcoRI fragment of the plasmid βA1 was subcloned into pFHR, so that the last 335 of the 537 amino acids of β1-syntrophin were expressed as a fusion protein with the FLAG antigen.

The entire Torpedo 87K protein cDNA was kindly provided by J. B. Cohen (Wagner et al., 1993). The cDNA was subcloned into the NcoI to EcoRI site within pGEM7 in three parts: (a) an oligonucleotide linker coding for the first five amino acids of the 87K protein (see Table I); (b) the PstI to BamHI fragment (nucleotides 128-345); and (c) the BamHI to EcoRI fragment (nucleotides 346-2594) from the 87K cDNA. The insert within expression plasmid F87, coding for the region of 87K that is homologous to exon 74 of dystrophin, was PCR-amplified from this 87K cDNA (Table I).

**Transcription and Translation of cDNA Sequences**

The polypeptides of interest were produced from plasmid DNA in the TNT T7 coupled reticulocyte lysate system (Promega Corp., Madison, WI) in a reaction volume of 50 μl as per the manufacturer's protocol. When indicated, the reaction was carried out in a reaction buffer in which 0.2 μCi (4 μl) of L-[3-14C]leucine (>300 mCi/mmol; Amersham Corp., Arlington Heights, IL) was added to a 50-μl reaction mix that lacked leucine. In general, the protein which is recognized by the antibody (cognate protein) was expressed without the incorporation of radioactivity, and the protein which did not interact with the antibody (probe protein) was separately expressed with [14C]leucine (as above). In some cases the cognate protein (which binds the antibody) was synthesized with 0.05 μCi of [14C]leucine added to the synthesis reaction so that the presence of the polypeptide could be followed through the immunoprecipitation and SDS-PAGE.

Polypeptide secondary structure predictions were made with the University of Wisconsin GCG Sequence Analysis Software Package (Madison, WI).

**Immunoprecipitation.** Protein interactions were assayed by their coprecipitation in the following assay. Directly from the translation reaction above, 10 μl of both cognate and probe proteins were incubated together with 20 μl of 10 mM Tris-buffered saline (pH 8), 0.1% Tween-20 (TBST). After 2-h incubation at room temperature, 10 μl of unpurified immune serum raised against the COOH terminus of dystrophin (dll) (Koenig and Kunkel, 1990) or 1 μl of anti-FLAG monoclonal antibody (M2; IBI/Kodak, Ayn and Kunkel Syntrophin Binds a Spliced Exon of Dystrophin 365
Interaction by Immunoprecipitation

electroplaque (Butler et al., 1992) or from several rat tissues

tated with the M2 anti-FLAG monoclonal antibody (IBI/

giving rise to the vector pFHR. Both FLAG-dystrophin (see

Materials and Methods), the pMGT vector permits the ex-

pression of analytical quantities of proteins of up to 150 kD

translated region and a strong initiator of translation (see

A. H. Ahn, unpublished observations).

While determining the syntrophin-binding site on dystro-

phin in this present study, our collection of region-specific

antibodies to dystrophin could no longer immunoprecipitate

some of the smaller expressed domains of dystrophin. Hence

it became necessary to produce translated proteins that were

tagged with a uniformly defined, unique antigen. A synthetic

antibodies to dystrophin could no longer immunoprecipitate

Results

Expression of Dystrophin and Syntrophin In Vitro

The expression vector pMGT (Fig. 1 a) directs the high level

synthesis of a variety of stable, soluble polypeptides in an in

vitro transcription/translation system. These expressed pro-

teins are synthesized in the presence of [14C]leucine and

analyzed by denaturing polyacrylamide gel electrophoresis

and autoradiography (Fig. 1 b). The cDNA translated from

pMGT incorporates 10–1,000 times more label than from

similar eDNA in the Bluescript polylinker (Fig. 1 b, lane 8

is from Bluescript). Presumably due to a favorable 5' un-

translated region and a strong initiator of translation (see

Materials and Methods), the pMGT vector permits the ex-

pression of analytical quantities of proteins of up to 150 kD

(A. H. Ahn, unpublished observations).

Figure 1. Expression of dystrophin and syntrophin polypeptides. (a) schematic diagram of the expression cassette used for in vitro tran-
scription/translation and the nucleotide sequence (below; see

methods); the T7 RNA polymerase late promoter (T7), the β' re-

tion of TMV (TMV 5'UTR); an ATG start codon in a Kozak context (M); a multiple cloning site (MCS), including Ncol at the start

ATG, Kpnl, Spel, Xhol, BamHI, Smal, Clal, HindIII, PstI, EcoRI, Xbal, and BglII; a poly(A) region, including a polyadenylation sig-

al; and further restriction sites for linearization of the plasmid (L),

including Sall, MluI, and Ncol were cloned into Bluescript digested

with BssHII. The arrows mark the beginning of transcription by the

RNA polymerase and the Ncol site with the initiator ATG is under-

linesequence (below; see

Materials and Methods) of a representative set of in vitro translated proteins

within the vector pMGT (see Table 1; βA1 is translated from the

T7 promoter within Bluescript II SK+). Molecular weight standards

(C2979 (2), Dp71 (4), Dp71Δ148 (4), Dp71Δ276 (5),

C355Δ148 (6), TDR (7), βA1 (8), and F5S (9).

Characterization of Dystrophin–Syntrophin Interaction by Immunoprecipitation

The COOH terminus of dystrophin interacts with purified

syntrophin immobilized onto a solid support (Cartaud et al.,

1993; Suzuki et al., 1994), or when immuno-affinity

purified from the receptor-rich membranes of the Torpedo
electroplaque (Butler et al., 1992) or from several rat tissues

(Kramarcy et al., 1994). To confirm and better understand this

interaction with a single isoform of syntrophin in the soluble

phase, we translated various portions of the dystro-
Coprecipitation of COOH-terminal dystrophin polypeptide (C2979) with translated radiolabeled syntrophin, using the anti-dystrophin antibody d11. After incubation with protein G–Sepharose, the samples were washed three times in Tris buffer and 0.1% Tween-20 with 0 mM NaCl (I), 150 mM NaCl (2), 1,000 mM NaCl (3), TBST with 1% Triton X-100 (4), or RIPA buffer (5). No translated syntrophin was precipitated in the absence of dystrophin polypeptide (6), or antiserum (7), or when a non-specific antibody to the NH2 terminus of dystrophin, 1-2a (8) (Koenig and Kunkel, 1990) was used.

Translated Syntrophin Interacts with the Differentially Spliced Area of Dystrophin

The structural requirements of the dystrophin–syntrophin interaction were more precisely characterized by creating a set of nested dystrophin products of the COOH terminus of dystrophin and Dp71 (Fig. 3 a). Like sequences derived from full sized dystrophin, Dp71, which contains only seven unique amino acids at its NH2 terminus, also binds translated syntrophin in a similar assay. COOH-terminal deletions of Dp71, up to 148 amino acids (Dp71Δ148), continued to bind translated syntrophin, while a protein with a deletion of 276 amino acids (Dp71Δ276) failed to bind (Fig. 2, lanes 6–8).

Figure 2. Coprecipitation of COOH-terminal dystrophin polypeptide (C2979) with translated radiolabeled syntrophin, using the anti-dystrophin antibody d11. After incubation with protein G–Sepharose, the samples were washed three times in Tris buffer and 0.1% Tween-20 with 0 mM NaCl (I), 150 mM NaCl (2), 1,000 mM NaCl (3), TBST with 1% Triton X-100 (4), or RIPA buffer (5). No translated syntrophin was precipitated in the absence of dystrophin polypeptide (6), or antiserum (7), or when a non-specific antibody to the NH2 terminus of dystrophin, 1-2a (8) (Koenig and Kunkel, 1990) was used.

Figure 3. Localization of the syntrophin binding site to exon 74 of dystrophin. Anti-dystrophin antibody d11 (a) or anti-FLAG monoclonal antibody (b) coprecipitated dystrophin polypeptides with translated syntrophin. After incubation with protein G–Sepharose, the pellets were washed three times, boiled in loading buffer, electrophoresed in SDS-PAGE, and translated syntrophin detected by autoradiography (see Materials and Methods). The upper portion of each figure is a schematic representation of the polypeptides tested and their interpretation ("+" for coprecipitation of translated syntrophin and "−" for no detected signal). The lower portion shows the autoradiographic data from the assay (see Materials and Methods). (a) A series of COOH-terminal dystrophin polypeptides (1 and 2) and a translated Dp71 polypeptide (3) coprecipitate translated syntrophin. A 148-amino acid COOH-terminal deletion of Dp71 still binds translated syntrophin (4), but a larger deletion of 276 amino acids fails to bind (5). A polypeptide spanning the alternatively spliced region binds translated syntrophin (6), whereas an expressed polypeptide that represents a naturally occurring splice variant of Dp71 which is missing this region fails to bind (7). Control assays in which the dystrophin polypeptide (-dys) or the antibody (-Ab) were withheld are negative. (b) FLAG-dystrophin fusion polypeptides encoding exons 72–74 coprecipitate translated syntrophin (1), as do FLAG-fusion polypeptides representing exons 73–74 (2) or exon 74 alone (3 and 4). FLAG-fusion polypeptides of only portions of exon 74 (5 and 6) failed to coprecipitate translated syntrophin. Control assays in which the dystrophin polypeptide (-dys) or the antibody (-Ab) were withheld are negative.
Dystrophin amino acids 3356–3537 include those encoded on exons 71 through 74, which are known to be alternatively spliced in skeletal muscle, brain, and heart (Feener et al., 1989; Bies et al., 1992). The alternative splicing generates transcripts that are missing various in-frame combinations of these exons, including forms that lack this entire region, amino acids 3409–3518 of dystrophin. We expressed a Dp71 protein in which the 110 amino acids encoded by exons 71–74 were internally deleted (Dp 71Δ110) and showed that it failed to bind syntrophin in our assay (Fig. 3 a, lane 7).

A nested series of expression constructs were used to express progressively smaller portions of this alternatively spliced region of dystrophin (Fig. 3 b). Since the available antibodies to dystrophin do not immunoprecipitate this region alone, we cloned amplified cDNA into pFHR, introducing amino-terminal fusions of the FLAG octapeptide with the proteins of interest. The FLAG-dystrophin fusion proteins also bound translated syntrophin when immunoprecipitated with the anti-FLAG monoclonal antibody M2 (Fig. 3 b). In each case, precipitation of translated syntrophin depended upon the inclusion of dystrophin fusion protein to the assay, and the dystrophin–syntrophin complex did not precipitate in the absence of M2 antibody (Fig. 3 b, lanes “dys” and “Ab”). A FLAG-dystrophin fusion protein including the amino acids encoded on exon 74 (F74-01) was sufficient to bind translated syntrophin (Fig. 3 b, lane 4). FLAG-dystrophin fusion proteins of the first or second half of exon 74 (F74A and F74B) failed to coprecipitate translated syntrophin in our assay (Fig. 3 b, lanes 5 and 6).

As a further control of specificity, we performed the converse immunoprecipitation, in which a radiolabeled dystrophin COOH terminus and an unlabeled syntrophin polypeptide were combined and precipitated with an antibody to the translated syntrophin polypeptide. In this instance, we subcloned the cDNA encoding the last 335 amino acids of β1-syntrophin into the FLAG fusion vector (pFHR). This FLAG-syntrophin fusion protein, F/3S (Fig. 1 b, lane 9), together with the anti-FLAG monoclonal antibody M2, specifically immuno precipitated the COOH terminus of dystrophin (Fig. 4 a, lane 7).

**DRP and 87K Polypeptides Also Bind Translated Syntrophin**

To examine whether the exon 74-encoded region on dystrophin is functionally preserved in the two other dystrophin relatives, human DRP and the *Torpedo* 87K postsynaptic protein, we tested translated DRP and 87K probes in a similar assay. A large COOH terminal portion of DRP and all of the 87K protein were produced as radiolabeled polypeptides. The FLAG-syntrophin fusion protein coprecipitated both the translated DRP and 87K proteins, and fails to precipitate when the FLAG-syntrophin fusion protein or the anti-FLAG antibody are omitted from the assay (Fig. 4 a).

The converse experiment was also performed, in which the FLAG-antigen was switched to the other polypeptide. Based upon the localization of the syntrophin binding site on dystrophin, the exon 74 homologous regions of DRP and 87K were expressed as FLAG-fusion proteins. All three FLAG-fusion proteins of dystrophin, DRP, and 87K immunoprecipitated the translated full-length β1-syntrophin probe, whereas their complexes do not precipitate in the absence of M2 antibody (Fig. 4 b).

**Figure 4.** The syntrophin binding site is functionally conserved among the three dystrophin family members. (a) The FLAG-syntrophin fusion protein F/3S was incubated with radiolabeled polypeptides of the COOH terminus of dystrophin (1), the COOH terminus of DRP (2), and the 87K protein (3) and precipitated with anti-FLAG monoclonal antibody. The control reactions in which all three dystrophin family members were used in the absence of F/3S (4) or in the absence of anti-FLAG monoclonal antibody (5) are negative. (b) The interaction is preserved when the FLAG antigen has been switched to the other protein. Radiolabeled syntrophin (dark arrow) was incubated with FLAG fusions of the exon 74-encoded region of dystrophin (1) the FLAG-fusion protein (the lower band incorporated the radiolabel poorly, and is not easily seen in this exposure), the FLAG fusion of the homologous region of DRP (2) and the FLAG fusion of the homologous region of 87K protein (3) and precipitated with anti-FLAG monoclonal antibody. The presence of the FLAG-fusion proteins (light arrows) were followed by the addition of a small amount of [14C]leucine to their translation reactions (see Materials and Methods). Precipitation of syntrophin depended on the addition of the anti-FLAG monoclonal antibody (4). An assay-equivalent of translated syntrophin is shown for comparison (5). (c) alignment of the amino acid sequences of exon 74 of dystrophin and their homologous region from DRP and the 87K protein. Gaps in the sequence were made to maintain the alignment. The consensus line (con) lists the identical residues of the alignment (single letter abbreviation) and conservative differences (*).
To look for sequence trends that may define a $\beta$-syntrophin binding motif, an alignment of the 53 amino acids homologous to exon 74 of dystrophin in DRP and the 87K protein was prepared (Fig. 4 c). Exon 74-encoded amino acids are represented in capital single-letter amino acid abbreviations. (An additional 2–4 amino acids from either side of exon 74 were expressed from the constructs F74-01, FDR, and F87 and are described in Table I.) Only 24% of the residues in the area shown are identical in all three proteins, and they are 45% similar when conservative differences are considered. The homology is dispersed throughout the exon-encoded region, and the highly acidic residues in dystrophin and DRP are not always conserved in the 87K protein.

Discussion

The 58-kD peripheral intracellular dystrophin-associated phosphoprotein called syntrophin is a biochemically heterogeneous (Yamamoto et al., 1993) family of three isoforms encoded on three distinct genes (Adams et al., 1993; Ahn et al., 1994; Yang et al., 1994). These proteins are expressed in a wide variety of tissues, each in an overlapping and distinct pattern of expression. Furthermore, the immunohistochemical localization of $\alpha$-syntrophin and $\beta$-syntrophin has shown that they have a segregated subcellular distribution. Localization of $\alpha$-syntrophin to the entire sarcolemma and $\beta$-syntrophin to the neuromuscular junction has led to the hypothesis that the syntrophins may in part account for the distinct localization and perhaps functioning of dystrophin and DRP (Peters et al., 1994). Immunooaffinity isolation of syntrophin from a large range of rat tissues shows that dystrophin, Dp71, and DRP each copurify with the syntrophins, and the respective converse immuno-affinity purifications show that dystrophin and DRP probably interact with syntrophin independently of each other (Kramarcy et al., 1994). The biochemical heterogeneity of both the dystrophins and the syntrophins isolated from tissues, however, makes this interaction difficult to study in further detail.

To reconstruct and test these proposed interactions individually, we have expressed these components from cDNA in vitro. A defined isoform of human syntrophin, $\beta$-syntrophin (Ahn et al., 1994), interacts with the COOH terminus of human dystrophin in an immunoprecipitation assay. The specificity of the dystrophin–syntrophin interaction was verified in a number of conditions (Fig. 2). First, the interaction is stable in a wide variety of salt and detergent conditions. Second, syntrophin remains in the supernatant when dystrophin COOH terminus is omitted from the precipitation. Third, the dystrophin–syntrophin complex remains in the supernatant in the absence of antibody to the COOH terminus of dystrophin, and does not precipitate when a non-specific antibody is added. Finally, in the converse precipitation, a FLAG-syntrophin fusion protein of the COOH terminal 37 kD of $\beta$-syntrophin can co precipitate a translated, radio-labeled dystrophin fragment (Fig. 4 a).

Since Dp71 differs from dystrophin by only seven amino acids at its NH$_2$ terminus, we were not surprised to find that Dp71 also coprecipitates translated syntrophin (Fig. 3 a). This result is in agreement with an independent set of experiments, in which Dp71 immuno-affinity purified from rat liver copurified with syntrophin (Kramarcy et al., 1994).

We have mapped a syntrophin binding site on dystrophin to exon 74, a region which is alternatively spliced in skeletal muscle, brain, and heart. In our assay, this region of dystrophin is necessary to coprecipitate translated syntrophin, since the translated product of a splice variant which lacks this region does not coprecipitate translated syntrophin probe (Fig. 3 a, lane 7). Since the translated polypeptide Dp71A110 represents a naturally occurring splice variant of dystrophin and Dp71, this polypeptide is more likely to fold correctly than an arbitrary deletion mutant. It is still possible that another region of dystrophin also interacts with syntrophin, but in our assay, its interaction alone is not strong enough to coprecipitate translated syntrophin in the absence of exons 71–74. Another possibility is that our polyclonal anti-dystrophin antibody (d1 I) interferes with the binding activity of another COOH-terminal region of dystrophin. This other part of dystrophin may play a regulatory or modulatory role in the interaction between dystrophin and one or more of the syntrophins.

In the accompanying paper, an independent series of experiments by Suzuki and colleagues suggest that $\alpha$-syntrophin and $\beta$-syntrophin have slightly different binding sites, but both reside substantially within exon 74. They also suggest that a further weak interaction exists between syntrophin and the far COOH terminus of dystrophin (Suzuki et al., 1995).

The Exon 74-encoded Polypeptide Is Functionally Conserved

The syntrophin-binding site on dystrophin is functionally conserved in the dystrophin relatives DRP and 87K protein (Fig. 4, a and b). We have not ruled out the possibility that other regions of DRP and 87K also interact with syntrophin. The functional conservation of this domain is particularly revealing in that the 87K protein is so distantly related to dystrophin (Fig. 4 c). Whereas the syntrophin-binding domains of dystrophin and DRP contain many acidic residues, the 87K protein conserves only a few of these. The stability of the dystrophin–syntrophin interaction in high salt conditions also argues against the interaction being purely electrostatic in nature (Fig. 2). Rather, the conserved sequence is dispersed throughout the exon-encoded region and includes both charged and hydrophobic residues (Fig. 4 c). Pairwise comparison of the 87K protein to the homologous region of dystrophin shows that this particular region is no more conserved between the two proteins than the rest of the protein (Wagner et al., 1993).

The 53 amino acids encoded on exon 74 are predicted to be alpha-helical, bisected by at least one proline that is conserved in all three proteins. Expression constructs that divide exon 74 into these two smaller helical regions alone, however, do not bind syntrophin (Fig. 3 b). This exon-encoded region may thus form a helix-turn-helix domain which is necessary for the formation of a binding site to syntrophin. It is also possible that this relatively small region does not form any significant tertiary structure, and instead acts as a signal peptide for its association with syntrophin. This kind of interaction is observed between the src SH2 domain and six residues of a high-affinity 11-residue phosphopeptide (PQ(pY)EEI), whose crystal structure was recently determined (Waksman et al., 1993). The exon 74-encoded region is distinct from the amphipathic helical region identified by Wagner as a possible site of dystrophin interaction with the
87K protein (Wagner et al., 1993). Although several leucines and other hydrophobic residues are conserved in the consensus sequence, the repeated heptad motif characteristic of the leucine zipper is not strongly evident in the helical regions of this exon.

The Dystrophin–Syntrophin Interaction in the Membrane Cytoskeleton

The exon 74-encoded region on dystrophin may help localize dystrophin and its relatives to the membrane cytoskeleton. On the other hand, it may be dystrophin and its relatives that direct the segregated distribution of the syntrophins. We have found that a recombinant COOH-terminal 37-kD fragment of syntrophin is sufficient to interact with translated dystrophin and its homologues in our assay (Fig. 4 a). Since the COOH termini of all three syntrophins are relatively well-conserved compared to their NH2 termini (Ahn et al., 1994), their affinities for each of the dystrophins and dystrophin relatives should be preserved.

The specialized subcellular localization of the syntrophins and dystrophins may thus be determined by either quantitative differences in pairwise affinity or in other modifying factors. One modifying factor that may address qualitative or quantitative differences in the dystrophin–syntrophin interaction may reside in the phosphorylation of syntrophin. Syntrophin is phosphorylated at both serine and tyrosine residues (Yamamoto et al., 1993; Wagner and Huganir, 1994). In addition, dystrophin itself may be phosphorylated at a serine and/or threonine residue at a site distal to the exon 74-encoded region (Milner et al., 1993; Madhavan and Jarrett, 1994).

The role of the dystrophin–syntrophin interaction may be elucidated by the relationship of this complex to other components of the DGC and membrane cytoskeleton. Results from other groups suggest that the molecular organization of dystrophin with other components of the DGC is complicated. An mdx mouse bearing a transgene of dystrophin with a truncated COOH-terminal 100-kD fragment of dystrophin does not associate directly with syntrophin. Their relative scarcity, however, does not rule out the possibility that they play a significant role in some specialization of the cytoskeleton.

The authors would like to thank E. M. McNally, H. S. Selig, M. Bowe, E. Gussoni, T. J. Byers, A. Viel, S. Grossman, J. B. Cohen, and S. C. Harrison for their advice and comments, and A. Suzuki, M. Yoshida, E. Ozawa, K. P. Campbell, J. A. Rafael, J. S. Chamberlain, D. C. Gorecki, S. C. Froehner, and R. Sealock for sharing their results before publication. The Torpedo 87K cDNA was a kind gift of J. B. Cohen, and that of DRP2 cDNA by T. S. Khurana. The authors are deeply indebted to C. A. Feener, R. R. Bennett, and S. Bruno for their technical assistance.

This work has been supported by National Institutes of Health HD18658 to L. M. Kunkel. L. M. Kunkel is an Investigator of the Howard Hughes Medical Institute.

Received for publication 23 August 1994 and in revised form 18 October 1994.

References

Adams, M. E., M. H. Butler, T. M. Dwyer, M. F. Peters, A. A. Murnane, and S. C. Froehner. 1993. Two forms of mouse syntrophin, a 58 kD dystrophin-associated protein, differ in primary structure and tissue distribution. Neuron 11:531-540.
Ahn, A. H., M. Yoshida, M. D. S. Anderson, C. A. Feener, S. Selig, Y. Hagiwara, E. Ozawa, and L. M. Kunkel. 1994. Cloning of a novel dystrophin family member from human fetal brain cDNA libraries. Neuron 14:161-172.
Arahata, K., S. Ishiura, T. Ishiguro, T. Tsukahara, Y. Subara, C. Eguchi, T. Ishibashi, I. Nomura, E. Ozawa, and H. Sugita. 1988. Immunostaining of skeletal and cardiac muscle surface membrane with antibody against Duchenne muscular dystrophy dystrophin. J. Cell Biol. 108:421-428.
Bies, D. S., D. S. Phelps, M. D. Corez, R. Roberts, C. T. Caskey, and J. S. Chamberlain. 1992. Human and murine dystrophin mRNA transcripts are differentially expressed during skeletal muscle, heart, and brain development. Nucleic Acids Res. 20:1725-1731.
Butler, M. H., K. Douville, A. A. Murnane, N. R. Kramarcy, J. B. Cohen, R. Sealock, and S. C. Froehner. 1992. Association of the M, 87,000 postsynaptic protein of electric tissue with dystrophin and the M, 87,000 postsynaptic protein. J. Biol. Chem. 267:6213-6218.
Campbell, K. P., and S. D. Kahl. 1989. Association of dystrophin and an integral membrane glycoprotein. J. Biol. Chem. 264:13515-13522.
Car,
Gallie, D. R., D. E. Sleat, J. W. Watts, P. C. Turner, and T. M. A. Wilson. 1987. The 5′-leader sequence of tobacco mosaic virus RNA enhances the expression of foreign gene transcripts in vitro and in vivo. Nucleic Acids Res. 15:3257–3273.

Gallie, D. R., D. E. Sleat, J. W. Watts, P. C. Turner, and T. M. A. Wilson. 1988. Mutational analysis of the tobacco mosaic virus 5′-leader for altered ability to enhance translation. Nucleic Acids Res. 16:883–893.

Khurana, M., S. C. Watkins, P. Chafey, J. Chelly, F. M. Tome, M. Fardeau, J. C. Kaplan, and L. M. Kunkel. 1991. Immunolocalization and developmental expression of dystrophin related protein in skeletal muscle. Neuro- mus. Dis. 1:185–196.

Koenig, M., and L. M. Kunkel. 1990. Detailed analysis of the repeat domain of dystrophin reveals four potential hinge segments that may confer flexibility. J. Biol. Chem. 265:4560–4566.

Koenig, M., E. P. Hoffman, C. J. Bertelson, A. P. Monaco, C. Feener, and L. M. Kunkel. 1987. Complete cloning of the Duchenne muscular dystrophy (DMD) CDNA and preliminary genomic organization of the DMD gene in normal and affected individuals. Cell. 50:509–517.

Kozak, M. 1986. Point mutations define a sequence flanking the AUG initiator codon that modulates translation by eukaryotic ribosomes. Cell. 44:283–292.

Kramarcy, N. R., A. Vidal, S. C. Froehner, and S. Sealock. 1994. Association of utrophin and multiple dystrophin short forms with the mammalian M, 58,000 dystrophin-associated protein (syntrophin). J. Biol. Chem. 269:2870–2876.

Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (Lond.). 227:680–685.

Lederfein, D., Z. Levy, N. Augier, D. Mornet, G. Morris, O. Fuchs, D. Yaffe, and U. Nudel. 1992. A 71-kilodalton protein is the major product of the Duchenne muscular dystrophy gene in brain and other nonmuscle tissues. Proc. Natl. Acad. Sci. USA. 89:5346–5350.

Lidov, H. G., T. J. Byers, S. C. Watkins, and L. M. Kunkel. 1990. Localization of dystrophin to postsynaptic regions of central nervous system cortical neurons. Nature (Lond.). 348:725–728.

Lidov, H. G. W., T. J. Byers, and L. M. Kunkel. 1993. The distribution of dystrophin in the murine central nervous system: an immunocytochemical study. Neurosciences. 54:167–187.

Love, D. R., D. F. Hill, G. Dickson, N. K. Spurr, B. C. Byth, R. F. Marsden, F. S. Walsh, Y. H. Edwards, and K. E. Davies. 1989. An autosomal transcript in skeletal muscle with homology to dystrophin. Nature (Lond.). 339:55–58.

Love, D. R., G. E. Morris, J. M. Ellis, U. Fairbrother, R. F. Marsden, J. F. Bloomfield, Y. H. Edwards, C. P. Slater, D. J. Parry, and K. E. Davies. 1991. Tissue distribution of the dystrophin-related gene product and expression in the mdx and dmy mouse. Proc. Natl. Acad. Sci. USA. 88:3243–3247.

Madhavan, R., and H. W. Jarrett. 1994. Calmodulin-activated phosphorylation of dystrophin. Biochemistry. 33:5797–5804.

Matsumura, K., J. M. Ervasti, K. Ohlendieck, S. D. Kahl, and K. P. Campbell. 1992. Association of dystrophin-related protein with dystrophin-associated proteins in mdx mouse muscle. Nature (Lond.). 360:588–591.

Milner, R. E., J. L. Besaan, C. F. B. Holmes, J. H. Wang, and M. Michalak. 1993. Phosphorylation of dystrophin: the carboxyl-terminal region of dystrophin is a substrate for in vitro phosphorylation by p34cdc2 protein kinase. J. Biol. Chem. 268:21901–21905.

Ohlendieck, K., J. M. Ervasti, K. Matsumura, S. D. Kahl, C. J. Leveille, and K. P. Campbell. 1991. Dystrophin-related protein is localized to neuromuscular junctions of adult skeletal muscle. Neuron. 7:499–508.

Panayotatos, N., and R. D. Wells. 1979. Recognition and initiation site for four late promoters of phage T7 is a 22-base pair DNA sequence. Nature (Lond.). 280:35–39.

Peters, M. F., N. R. Kramarcy, R. Sealock, and S. C. Froehner. 1994. β2-syntrophin: localization at the neuromuscular junction in skeletal muscle. Neuron. 5:1577–1580.

Rafael, J. A., Y. Sunada, N. M. Cole, K. P. Campbell, J. A. Faulkner, and J. S. Chamberlain. 1994. Prevention of dystrophic pathology in mdx mice by a truncated dystrophin isoform. Hum. Mol. Genet. 1725–1735.

Sambrook, J., E. F. Fritsch, and T. Maniatis. 1989. Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.

Suzuki, A., M. Yoshida, H. Yamamoto, and O. Ozawa. 1992. Glycoprotein-binding site of dystrophin is confined to the cysteine-rich domain and the first half of the carboxy-terminal domain. FEBS (Fed. Eur. Biochem. Soc.) Lett. 308:154–160.

Suzuki, A., M. Yoshida, K. Hayashi, Y. Mizuno, Y. Hagiwara, and O. Ozawa. 1994. Molecular organization at the glycoprotein complex-binding site of dystrophin: Three dystrophin-associated proteins bind directly to the carboxy-terminal portion of dystrophin. Eur. J. Biochem. 220:283–292.

Suzuki, A., M. Yoshida, and O. Ozawa. 1995. Mammalian α- and β1-syntrophin bind to the alternative splice-prone region of the dystrophin COOH-Terminus. J. Cell Biol. 128:373–381.

Tinsley, J. M., D. J. Blake, A. Roche, U. Fairbrother, J. Rist, B. C. Byth, A. E. Knight, J. Kendrick-Jones, G. K. Sutters, D. R. Love et al., 1992. Primary structure of dystrophin-related protein. Nature (Lond.). 360:591–593.

Wagner, K. R., and R. L. Huganir. 1994. Tyrosine and serine phosphorylation of dystrophin and the 58-kD protein in the postsynaptic membrane of Torpedo electric organ. J. Neurochem. 62:1947–1952.

Wagner, K. R., J. B. Cohen, and R. L. Huganir. 1993. The 87K postsynaptic membrane protein from Torpedo is a protein-tyrosine kinase substrate homologous to dystrophin. Neuron. 10:511–522.

Wakasugi, G., S. E. Shoelson, N. Pant, D. Cowburn, and J. Kuriyan. 1993. Binding of a high affinity phosphotyrosyl peptide to the src SH2 domain: crystal structures of the complexed and peptide-free forms. Cell. 72:779–790.

Yamamoto, H., Y. Hagiwara, Y. Mizuno, M. Yoshida, and O. Ozawa. 1993. Heterogeneity of dystrophin-associated proteins. J. Biochem. (Tokyo). 114:132–139.

Yang, B., O. Braghinov-Bezurovnya, C. R. Moonaw, C. A. Slaughter, and R. F. Campbell. 1994. Heterogeneity of the 59-kD dystrophin-associated protein revealed by CDNA cloning and expression. J. Biol. Chem. 269:6040–6044.

Yoshida, M., and O. Ozawa. 1990. Glycoprotein complex anchoring dystrophin to sarcolemma. J. Biochem. (Tokyo). 108:748–752.