Dystrophin Is a Component of the Subsynaptic Membrane

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Abstract. A subsynaptic protein of M_r ~300 kD is a major component of *Torpedo* electric organ postsynaptic membranes and copurifies with the AChR and the 43-kD subsynaptic protein. mAbs against this protein react with neuromuscular synapses in higher vertebrates, but not at synapses in dystrophic muscle. The *Torpedo* 300-kD protein comigrates in SDS-PAGE with murine dystrophin and reacts with antibodies against murine dystrophin. The sequence of a partial cDNA isolated by screening an expression library with mAbs against the *Torpedo* 300-kD protein shows striking homology to mammalian dystrophin, and in particular to the b isoform of dystrophin. These results indicate that dystrophin is a component of the postsynaptic membrane at neuromuscular synapses and raise the possibility that loss of dystrophin from synapses in dystrophic muscle may have consequences that contribute to muscular dystrophy.

MUTATIONS in the gene encoding dystrophin can result in myopathies termed Duchenne or Becker muscular dystrophy (Koenig et al., 1989). Dystrophin expression is restricted to muscle and nervous tissue, and in skeletal muscle, the protein is found at the intracellular surface of the nonsynaptic plasma membrane (Watkins et al., 1988; Zubrzycka-Gaarn et al., 1988; Bonilla et al., 1988; Sugita et al., 1988). It is not clear how loss of dystrophin results in myopathies.

Recent studies have demonstrated that antibodies against dystrophin cross-react with a protein enriched at neuromuscular synapses (Chang et al., 1989; Jasmin et al., 1990; Fardeau et al., 1990). However, because antibodies against dystrophin can cross-react with other proteins that contain homologous domains (Hoffman et al., 1989), it is difficult to ascertain whether the cross-reacting molecule at the synapse is dystrophin rather than another molecule which shares epitopes with dystrophin. Indeed, a recent study has shown that dystrophin-like immunoreactivity persists at synaptic sites in dystrophic muscle (Fardeau et al., 1990). In this study we provide strong evidence that dystrophin itself is present at synaptic sites.

We have shown previously that a peripheral membrane protein of M_r ~300 kD copurifies with postsynaptic membranes isolated from *Torpedo* electric organ and appears concentrated at neuromuscular synapses (Burden et al., 1983; Woodruff et al., 1987). We have now isolated a cDNA by screening an expression library with mAbs against the *Torpedo* 300-kD protein, and show that the amino acid sequence encoded by this partial cDNA is homologous to dystrophin. The cDNA encodes a protein with repeat units that have 60–70% amino acid sequence identity with the repeat units in human dystrophin and a carboxy-terminal region with 90% homology to the dystrophin *b* isoform. Moreover, antibodies against murine dystrophin react with a *Torpedo* 300-kD protein, which comigrates in SDS-PAGE with murine dystrophin, and mAbs against the *Torpedo* 300-kD protein react with neuromuscular synapses in normal, but not dystrophic, mouse muscle. These results provide strong evidence that dystrophin is present at neuromuscular synapses and raise the possibility that the absence of dystrophin from synapses in dystrophic muscle may perturb the structure and/or function of the synapse and contribute to muscular dystrophy.

Materials and Methods

Isolation of AChR-rich Membranes and Western Blotting

AChR-rich and AChR-poor membranes were isolated from *Torpedo* electric organ as described previously (Burden et al., 1983). The M_r of 300-kD protein was determined in 6% polyacrylamide SDS gels with human erythrocyte spectrin and rabbit macrophage actin-binding protein as molecular weight standards (Woodruff et al., 1987). Peripheral proteins from AChR-rich membranes were fractionated by two-dimensional gels as described previously (Burden, 1985). Western blots were probed with mAb hybridoma supernatant, affinity-purified antibodies against a trpE+60 kD mouse dystrophin fusion protein (encoding the first four repeats in murine dystrophin; Hoffman et al., 1987) diluted 1:2,000, and affinity-purified antibodies against a trpE fusion protein containing the carboxy-terminal region of human dystrophin (antibody 11; Koenig and Kunkel, 1990) diluted 1:500, followed by alkaline phosphatase–coupled secondary antibodies.

Immunohistochemistry

Staining of unfixed frozen sections from *Torpedo* electric organ was performed as described previously (Woodruff et al., 1987). Affinity-purified antibodies against a trpE+60-kD murine dystrophin fusion protein dystrophin (Hoffman et al., 1987) were used at 1:2,000, and hybridoma supernatant containing mAb 602 was used undiluted. Frozen sections (8 μm) from unfixed intercostal muscles were stained with biotinylated mAb 601 (identical results were obtained with a different mAb against the *Torpedo* 300-kD protein, mAb 607) or with antibodies against the trpE+60-kD dystrophin

fusion protein (Hoffman, et al., 1987) diluted 1:2,000 for 1 h at room temperature. After incubation with either fluorescein-labeled avidin or fluorescein-labeled goat-anti-sheep IgG and TMR-BGT, sections were visualized with optics selective for either rhodamine or fluorescein (Woodruff et al., 1987).

Rat myotubes were fixed (1% paraformaldehyde in PBS) for 15 min, washed (in PBS), permeabilized with 0.1% NP-40 (in PBS), incubated with hybridoma supernatant containing mAb 601 (2 h at room temperature) and subsequently with fluorescein-labeled goat-anti-mouse IgG and TMR-BGT. AChR clusters in the murine C2 muscle cell line are labeled with mAb 601 as well (J. A. Theriot and S. J. Burden, unpublished results).

Molecular Biological Methods

300,000 recombinant phage from a λgt11 *Torpedo* electric organ cDNA library (Baldwin et al., 1988*a*) were screened with four different mAbs (601, 602, 603, 604) that react exclusively with the *Torpedo* electric organ 300-kD protein (Woodruff et al., 1987). One positive phage was detected with mAb 602, which identified a phage harboring a 4.1-kb cDNA insert. Our other mAbs against the *Torpedo* 300-kD protein do not react with the fusion protein, and these antibodies presumably react with regions of *Torpedo* dystrophin that are not encoded by the partial cDNA. Affinity-purified antibodies against the carboxy-terminal region of human dystrophin (antibody 10; Koenig and Kunkel, 1990) also react with the fusion protein and with the *Torpedo* 300-kD protein (data not presented). cDNA from the purified phage was mapped with restriction endonucleases and sequenced (Sanger et al., 1977; Baldwin et al., 1988*b*). The cDNA clone encodes an additional ~1.5 kb beyond the termination codon and ends with a 44-bp poly A tract, which presumably represents the 3′ end of the mRNA. The deduced amino acid sequences of *Torpedo* 300-kD protein and human dystrophin were aligned (Wilbur and Lipmann, 1983).

An additional seven cDNAs have been detected by screening 80,000 recombinant phage with a 920-bp probe derived from the 4.1-kb cDNA (hybridization in 5× SSC, 50°C, washed in 0.2× SSC, 56°C) (Ravin et al., 1991). Thus, the relative abundance of cDNAs in this library encoding dystrophin (0.9:10,000), 43-kD protein (4.5:10,000) (Baldwin et al., 1988*a*) and AChR alpha subunit (11/10,000) (Baldwin et al., 1988*b*) is similar to the relative abundance of the corresponding proteins (1:10:20; Burden et al., 1983).

^{32}P-labeled, random-primed probe derived from the 4.1-kb cDNA encoding the *Torpedo* 300-kD protein was hybridized to a Northern blot of total RNA (30 μg per lane) isolated from *Torpedo* electric organ. The blot was hybridized overnight (in 5× SSPE) at 55°C and washed (in 0.2× SSC, 0.1% SDS) at 57°C (Baldwin et al., 1988*b*). An RNA ladder of 9.5, 7.5, 4.4, 2.4, 1.4, 0.2 kb was used to estimate the size of the *Torpedo* transcript.

Results

The Torpedo 300-kD Protein and Dystrophin Share Epitopes

Antibodies against the first four repeats in murine dystrophin react with a protein concentrated in AChR-rich membranes

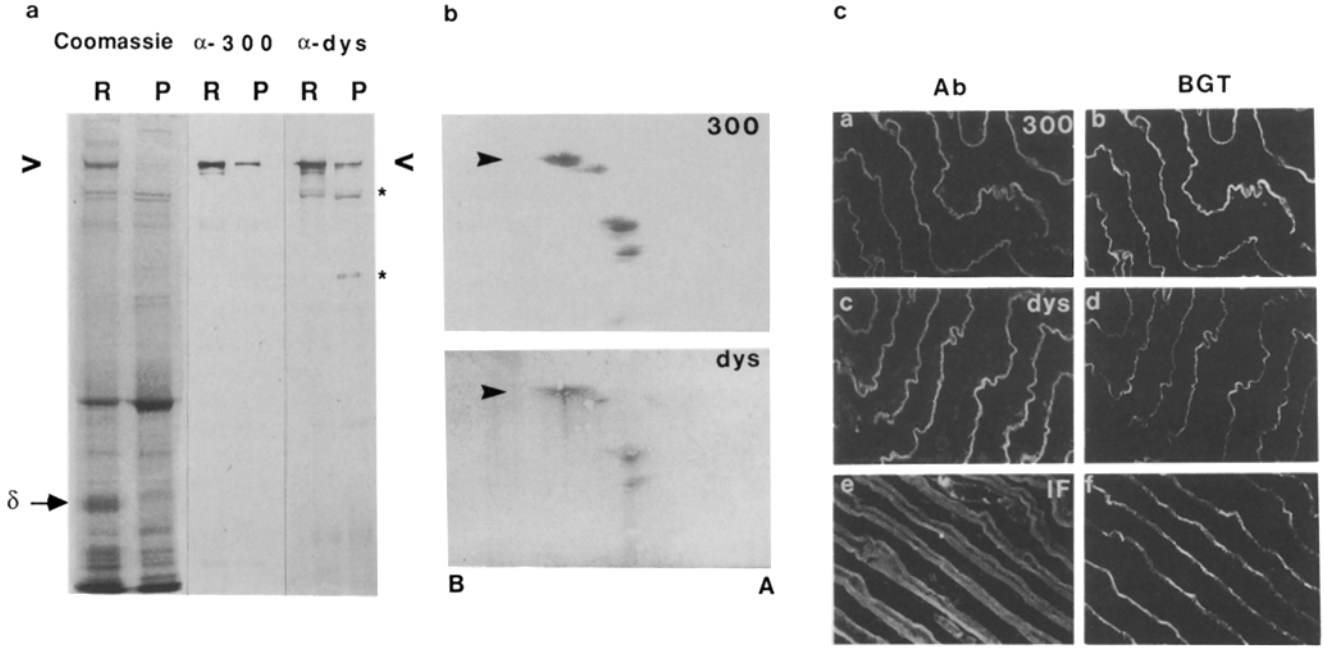


Figure 1. Antibodies against dystrophin cross-react with the *Torpedo* 300 kD subsynaptic protein, which copurifies with AChR-rich membranes. (*a*) Postsynaptic membranes from *Torpedo* electric organ were fractionated in an equilibrium density sucrose gradient and the protein composition was analyzed by SDS-PAGE. Proteins in AChR-rich (*R*) and AChR-poor (*P*) membranes were fractionated by SDS-PAGE (6% polyacrylamide) and either stained with Coomassie brilliant blue or transferred to nitrocellulose and probed with mAb (602) to the *Torpedo* 300-kD protein or antibodies to murine dystrophin (trpE+60 kD). Both antibodies react with the 300-kD protein (*arrowhead*), which is more abundant in AChR-rich than in AChR-poor membranes. Labeling of the protein band just beneath the 300-kD protein with mAbs against the 300-kD protein is variable, and is likely due to partial proteolysis of the 300-kD protein. Antibodies against dystrophin, but not mAbs against the *Torpedo* 300-kD protein, react with several proteins (*) that are enriched in AChR-poor membrane fractions and are thus not likely to be proteolytic fragments of dystrophin; these proteins are not labeled with secondary antibody alone, and therefore cross-react with the antibodies against murine dystrophin. (*b*) Peripheral membrane proteins from AChR-rich were resolved by two-dimensional electrophoresis, transferred to nitrocellulose, and probed either with mAb (602) to the *Torpedo* 300-kD protein or with affinity-purified antibodies to the carboxy-terminal region of human dystrophin (antibody 11). The different antibodies react with the same protein, since the 300-kD protein (*arrowhead*) as well as the same set of proteolytic fragments are labeled with both antibodies. The basic (*B*) and acidic (*A*) directions are indicated. (*c*) The innervated surface of the *Torpedo* electrocyte is labeled with mAbs against the *Torpedo* 300-kD protein and antibodies against dystrophin. Single frozen sections of *Torpedo* electric organ were labeled with tetramethylrhodamine-labeled α-bungarotoxin (*BGT*) (*b*, *d*, and *f*) and with either mAb 602 (*a*), antibodies against dystrophin (*c*), or mAb against a *Torpedo* intermediate filament protein (*e*) (Burden, 1982). The magnification is 400×.

isolated from *Torpedo* electric organ (Fig. 1 *a*). The cross-reacting protein comigrates in SDS-PAGE with the 300-kD subsynaptic protein that we identified previously as a component of the postsynaptic membrane in *Torpedo* electric organ and in skeletal myofibers (Fig. 1 *a*) (Burden et al., 1983; Woodruff et al., 1987).

Because several proteins could comigrate at 300-kD, proteins from AChR-rich membranes were resolved further by IEF, and blots from two dimensional gels were probed either with antibodies against human dystrophin or with a mAb against the *Torpedo* 300-kD protein. Fig. 1 *b* shows that the different antibodies react with the same protein, since the 300-kD protein as well as the same set of proteolytic fragments are labeled with both antibodies. Thus, antibodies against dystrophin react with the 300-kD protein that we identified previously.

Furthermore, all of our mAbs against the 300-kD protein (Woodruff et al., 1987), and antibodies against murine dystrophin (Hoffman et al., 1987), react in situ with the postsynaptic membrane of the electrocyte (Fig. 1 *c*; see also Chang et al., 1989).

mAbs Against the Torpedo 300-kD Protein React with Synaptic Sites in Normal, but Not Dystrophic, Muscle

Among the mAbs that we produced against the *Torpedo* 300-kD protein, mAb 601 cross-reacts with neuromuscular synapses in both amphibian and mammalian muscle (Woodruff et al., 1987). We used mAb 601 to determine whether the molecule recognized by this antibody is absent from synaptic sites in dystrophic (*mdx*) mouse muscle (Bulfield et al., 1984). Fig. 2 demonstrates that mAb 601 reacts with synaptic sites in normal, but not in dystrophic, mouse muscle. Extrasynaptic staining is also detectable with mAb 601 in normal, but not dystrophic, mouse muscle (Fig. 2). In addition, antibodies against murine dystrophin react with synaptic sites in normal (Fig. 2; see also Chang et al., 1989), but not dystrophic, muscle (Fig. 2). Thus, a protein in normal mouse muscle that reacts with mAb 601 is absent from dystrophic muscle.

The protein product of an autosomal gene has homology to the product of the X-linked dystrophin gene (Love et al., 1989), and this protein has been termed dystrophin-related protein (DRP)[1] (Khurana et al., 1990). However, since DRP is retained in muscle from *mdx* mice (Khurana et al., 1990), neither mAb 601 nor the antibodies against murine dystrophin used in this study react with DRP.

Clusters of AChRs occur at synaptic sites in innervated skeletal muscle and can also form in the absence of innervation in cultured embryonic myotubes (Vogel et al., 1972; Fischbach and Cohen, 1973). We examined whether the molecule recognized by mAb 601 is concentrated at AChR clusters in myotube cultures as well as at synaptic sites. Fig. 3 demonstrates that mAb 601 staining is present throughout the myotube, but is concentrated at AChR clusters. Moreover, there is an intricate arrangement of AChRs within a cluster, and a similar, but not identical, arrangement is seen with mAb 601 (see also Sealock et al., 1991). Since mAb 601 reactivity is concentrated at AChR clusters in noninnervated

1. *Abbreviations used in this paper*: AchR, acetylcholine receptor; DRP, dystrophin-related protein.

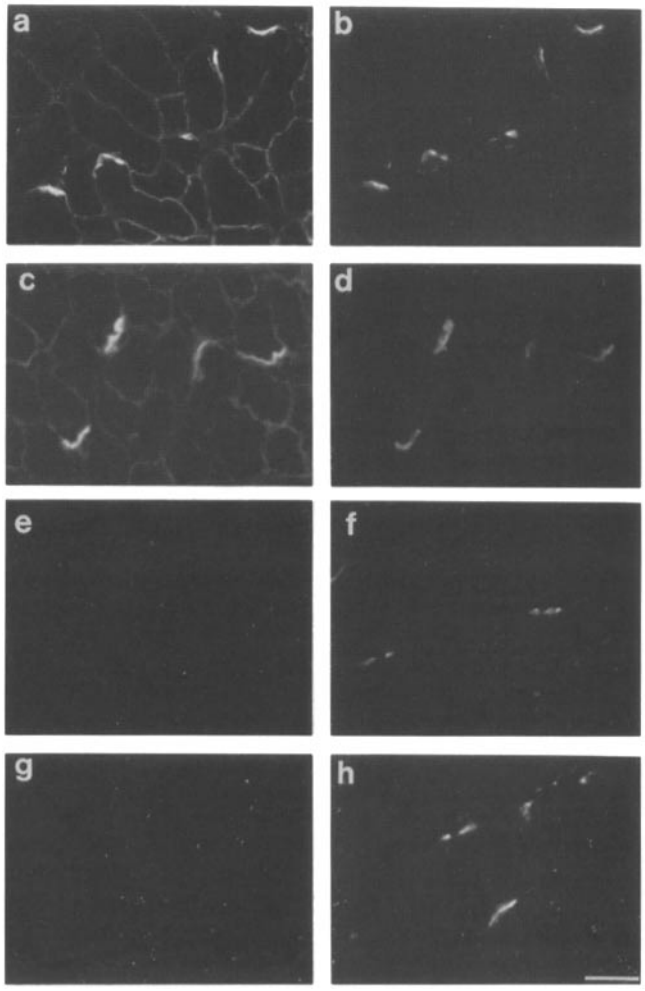


Figure 2. Both antibodies against murine dystrophin and mAb against the *Torpedo* 300-kD protein label synaptic sites in normal, but not in dystrophic, mouse muscle. Frozen sections of normal (*a–d*) and *mdx* (*e–h*) mouse muscle were labeled with biotinylated mAb 601 (*a* and *e*), or to dystrophin antibodies (*c* and *g*), followed by appropriate fluorescein-labeled secondary reagents and TMR-BGT (*b*, *d*, *f*, and *h*). We have not investigated whether the number or size of endplates is altered in *mdx* muscle. Bar, 20 μm.

embryonic myotubes and at synapses in newborn rats (data not presented), the association of the 300-kD subsynaptic protein with the postsynaptic membrane is likely to be an early event during synaptogenesis. Furthermore, since folding of the plasma membrane at AChR clusters in cultured myotubes is rare (Vogel and Daniels, 1976), the accumulation of the 300-kD protein at these sites cannot be attributed entirely to an increase in membrane folding.

Dystrophin and the Torpedo 300-kD protein

In our previous studies we used SDS-PAGE to estimate an M_r of 300 kD for the *Torpedo* electric organ subsynaptic protein (Woodruff et al., 1987). The molecular mass of human dystrophin, calculated from the amino sequence deduced from cDNA, is 427 kD (Koenig et al., 1988). Fig. 4 demonstrates that the *Torpedo* electric organ 300-kD protein and murine dystrophin comigrate in SDS-PAGE, since antibodies against murine dystrophin react with a protein of

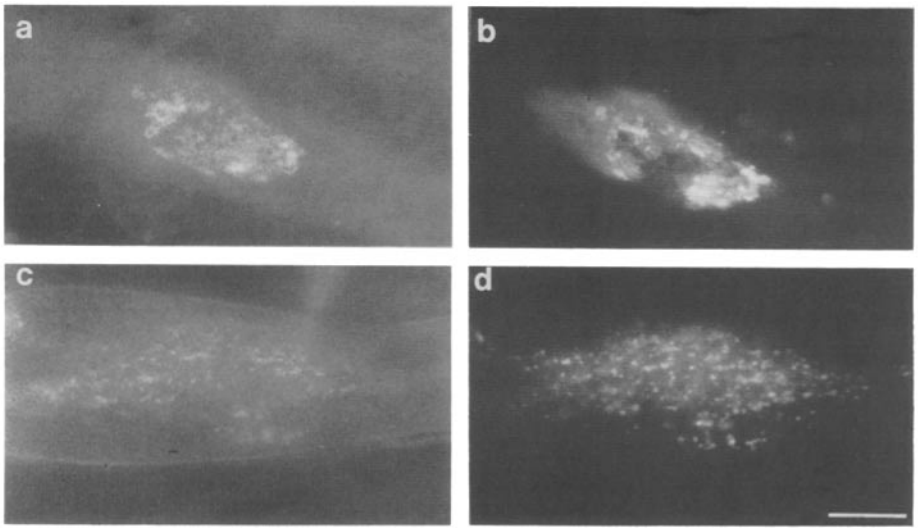


Figure 3. AChR clusters in primary rat myotubes are labeled with mAb against the *Torpedo* 300-kD protein. Rat myotubes were labeled with mAb 601 (*a* and *c*), followed by fluorescein-labeled goat-anti-mouse IgG and TMR-BGT (*b* and *d*). Antibody labeling is present throughout the myofiber, but is concentrated at AChR clusters. Within AChR clusters, a similar pattern of labeling is seen with TMR-BGT and mAb 601. Bar, 10 μm.

identical size in *Torpedo* AChR-rich membranes and in normal, but not *mdx*, mouse muscle.

Dystrophin is composed of 24 repeats (Koenig and Kunkel, 1990), which have structural similarity to repeats in spectrin and α-actinin (Davison and Critchley, 1988; Hammond, 1987; Koenig et al., 1988). Each repeat is thought to be organized into three alpha helixes which form coil-coil interactions, and this common structural feature can be detected with antibodies raised against dystrophin, which can cross-react with α-actinin (Hoffman et al., 1989). Thus, we were concerned that antibodies against dystrophin could cross-react with other proteins that share these repeats (see also Fig. 1 *b*). Antibodies against α-actinin (Bloch and Hall, 1983) and β-spectrin (Bloch and Morrow, 1989), as well as antibodies against dystrophin (Fig. 2; see also Chang et al., 1989), react with synaptic sites in skeletal muscle; however, identification of the molecule(s) recognized by these antibodies is complicated by the possibility of cross-reactivity due to the conserved structural feature described above.

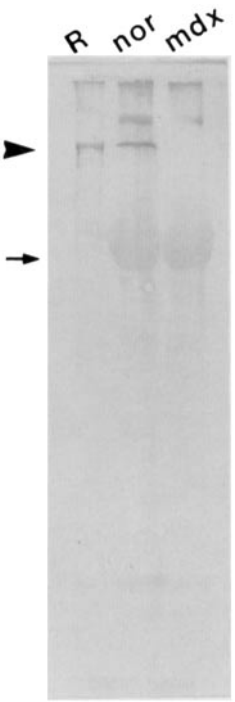


Figure 4. The *Torpedo* 300-kD protein and murine dystrophin comigrate in SDS-PAGE. Proteins from *Torpedo* electric organ AChR-rich membranes and total protein (~70 μg) from normal and *mdx* mouse muscle were fractionated by SDS-PAGE (6% polyacrylamide), transferred to nitrocellulose, and probed with antibodies to murine dystrophin (trpE+60kD; Hoffman et al., 1987). The *Torpedo* electric organ 300-kD protein and murine dystrophin comigrate in SDS-PAGE (*arrowhead*), since antibodies against murine dystrophin react with a protein of identical size in *Torpedo* AChR-rich membranes (*R*) and in normal (*nor*), but not *mdx* (*mdx*) mouse muscle. Labeling of skeletal muscle protein(s) that migrate more slowly than dystrophin is nonspecific, since labeling is detected with secondary antibody alone; however, the antibodies to dystrophin cross-react weakly with myosin (*arrow*), which is present at similar levels in normal and *mdx* muscle.

Although the lack of reactivity of mAb 601 in dystrophic muscle strongly suggests that the antibody recognizes dystrophin, it remains possible, however, that the mAb cross-reacts with another molecule whose loss is an indirect consequence of the absence of dystrophin. Indeed, it is clear that other proteins, in addition to dystrophin, are absent from skeletal muscle in the *mdx* mouse (Ervasti et al., 1990) and in individuals with Duchenne muscular dystrophy (Hoffman et al., 1987). Thus, we sought an additional and different method to establish a relationship between dystrophin and the 300-kD subsynaptic protein.

The Sequence of a Torpedo Electric Organ cDNA Is Homologous to Dystrophin

We used mAbs against the *Torpedo* 300-kD subsynaptic protein to screen an expression library from *Torpedo* electric organ. We isolated a cDNA that encodes a protein that reacts with one of these mAbs (602), and the protein-coding portion of this cDNA was sequenced. Figs. 5 and 6 demonstrate that the amino acid sequence deduced from the *Torpedo* cDNA, is strikingly homologous to human dystrophin. Furthermore, affinity-purified antibodies against the carboxy-terminal region of human dystrophin (antibody 10; Koenig and Kunkel, 1990) also react with the fusion protein and with the *Torpedo* 300-kD protein (data not presented). Since the isolated cDNA is 4.1 kb and hybridizes to RNA which is ~14 kb in length (Fig. 7), the sequence encoded by the cDNA is incomplete (seven additional cDNAs from sequences further 5′ have been isolated, see Materials and Methods).

The amino acid sequence deduced from the 5′ end of the cDNA has 59 and 72% homology, respectively, with the 23rd and 24th repeat in human dystrophin (Koenig and Kunkel, 1990; Davison and Critchley, 1988). This same repeat region has more limited sequence homology with spectrins (~20%), α-actinin (28%), and myosins (~20%). Moreover, alignment of the sequences encoded by the *Torpedo* cDNA and dystrophin requires no gaps, whereas alignment

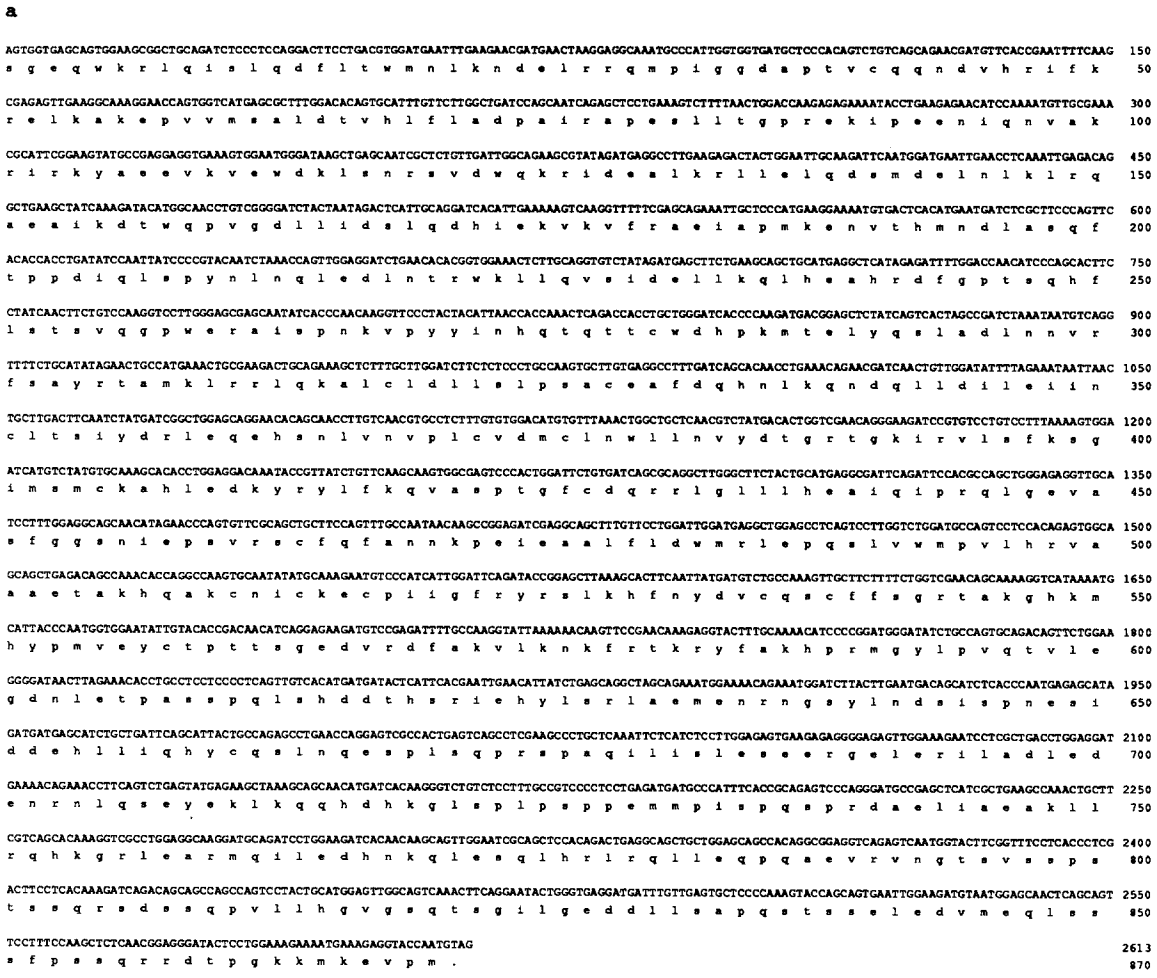
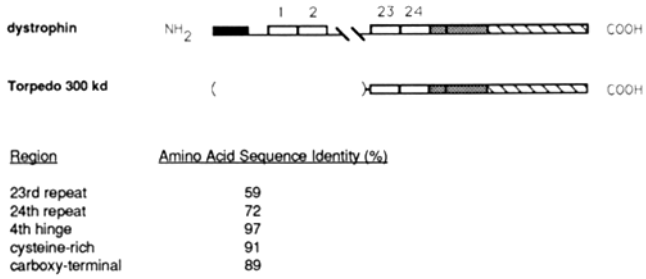


Figure 5. The amino acid sequences of the *Torpedo* 300-kD subsynaptic protein and human dystrophin are similar. The nucleic acid and amino acid sequence of the protein coding portion of the 4.1 kb cDNA are illustrated in *a*. (*b*) The protein coding portion of the *Torpedo* 4.1-kb cDNA (*TORDYS*) is aligned with the human dystrophin (*HUMDYS*) sequence that extends from amino acid 2803 to the carboxy terminus of the *a* isoform. Identical residues in the HUMDYS sequence are indicated with a dash (−) and amino acid substitutions are shown by the one-letter amino acid notation. A gap in the TORDYS sequence, which is indicated with asterisks (*), aligns with amino acids 3409–3421 in HUMDYS and signifies the *b* isoform of dystrophin; homology of the human dystrophin *b* isoform with the *Torpedo* 300-kD protein, which is encoded by the partial cDNA, is 84%. The DNA sequence of the partial cDNA encoding the *Torpedo* 300-kD protein is available from EMBL/GenBank/DDBJ under accession number M37645.



Region	Amino Acid Sequence Identity (%)
23rd repeat	59
24th repeat	72
4th hinge	97
cysteine-rich	91
carboxy-terminal	89

Figure 6. The domain structure of dystrophin and the domain organization of the *Torpedo* 300-kD protein are identical. The cartoon illustrates the location of the amino terminal region (*solid box*), the repeat units (*open boxes*), the fourth hinge and cysteine-rich region (*stippled*) and the carboxy-terminal region (*hatched*) in dystrophin (Koenig and Kunkel, 1990). The cartoon depicts the first two and last two repeats in dystrophin. Parentheses illustrate the region of the 300-kD protein that is not encoded by the cDNA. Homology between human dystrophin and the *Torpedo* 300-kD protein is indicated separately for each domain: 23rd repeat, amino acids 2803–2931; 24th repeat, 2932–3040; 4th hinge, 3041–3112; cysteine-rich, 3113–3360; and carboxy-terminal, 3361–3685 (Koenig and Kunkel, 1990; Koenig et al., 1988).

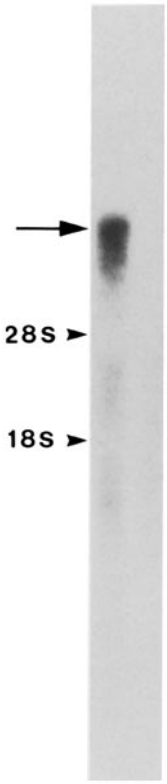


Figure 7. cDNA encoding the *Torpedo* 300-kD protein hybridizes to a ~14-kb RNA (*arrow*) in *Torpedo* electric organ. The positions of 18S and 28S rRNA are indicated with arrowheads. The filter was exposed to x-ray film with an intensifying screen for 3 d at −70°C.

of either the *Torpedo* cDNA or dystrophin with spectrin requires two single amino acid gaps within each repeat (Koenig and Kunkel, 1990; Davison and Critchley, 1988). Thus, the region of sequence homology between the *Torpedo* cDNA and human dystrophin includes a region of dystrophin that is thought to be fundamental for establishing its tertiary structure (Koenig and Kunkel, 1990; Davison and Critchley, 1988).

Since the dystrophin sequence that follows the repeat units is unrelated to spectrin and vertebrate α-actinin, comparison within this region is a more stringent criterion for establishing structural similarity to dystrophin (Koenig et al., 1988; Feener et al., 1989). Fig. 6 shows that there is 97% homology between the fourth hinge region of dystrophin and the proline-rich sequence following the predicted 24th repeat encoded by the *Torpedo* cDNA. This degree of conservation suggests that the fourth hinge region of dystrophin has a critical functional role, and that this domain has an importance in addition to disruption of the alpha helical organization. Moreover, beyond the hinge region of dystrophin is a cysteine-rich region followed by the carboxy-terminal region. Both of these regions are conserved in the *Torpedo* cDNA (91 and 89% identity, respectively). Thus, the sequence encoded by the cDNA is highly homologous to dystrophin, and is only distantly related to spectrin, α-actinin, and myosin.

Since humans possess an autosomal gene that encodes a protein, DRP, with homology to the product of the X-linked dystrophin gene (the partial cDNA has 80% amino acid sequence identity with the dystrophin *a* isoform) (Love et al., 1989), it seemed possible that the protein encoded by *Torpedo* cDNA could be the homologue of this autosomal gene, rather than of dystrophin. However, alignment of the corresponding sequence encoded by the *Torpedo* cDNA with the protein product of this autosomal gene reveals 72% identity in the region of overlap and requires several gaps, whereas alignment with dystrophin shows 88% identity and requires no gaps. Moreover, antibodies against mammalian dystrophin (antibody 11; Koenig and Kunkel, 1990) react with the *Torpedo* 300-kD protein, but not with mammalian DRP (Hoffman et al., 1989). Thus, we think it likely that the protein encoded by the *Torpedo* cDNA is *Torpedo* dystrophin.

Multiple dystrophin transcripts, which are generated by alternative splicing, yield several isoforms (Feener et al., 1989). The *b* isoform of dystrophin lacks 13 amino acids present in the carboxy-terminal region of the *a* isoform and is generated by splicing and removal of 39 nucleotides (Feener et al., 1989). The *Torpedo* cDNA sequence illustrated in Fig. 5 corresponds to the *b* isoform, and this alignment supports further the conclusion that the protein encoded by the *Torpedo* cDNA is dystrophin. The *b* isoform, however, is not the only isoform present in the electric organ, since we have isolated an electric organ cDNA that encodes the *a* isoform of dystrophin (Ravin et al., 1991).

Discussion

This study demonstrates that a protein in *Torpedo* electric organ is highly homologous to mammalian dystrophin. The extent of sequence homology with mammalian dystrophin indicates that this *Torpedo* protein is *Torpedo* dystrophin rather than a dystrophin-related protein. Consistent with this interpretation, antibodies against murine dystrophin, which do not cross-react with murine DRP, react with the same 300-kD electric organ protein that copurifies with AChR-rich postsynaptic membranes, and a mAb (601) directed against the electric organ 300-kD subsynaptic protein reacts with synaptic and nonsynaptic membrane in normal, but not dystrophic mouse muscle.

We think it is likely that the protein encoded by the 4.1-kb cDNA, which we designate *Torpedo* dystrophin, is the 300-

kD subsynaptic protein that we identified previously on the basis of copurification with AChR-rich postsynaptic membranes from *Torpedo* electric organ. First, the cDNA encodes a protein that reacts with a mAb (602) directed against the electric organ 300-kD subsynaptic protein. Second, antibodies against dystrophin (antibody 10; Koenig and Kunkel, 1990) react both with the fusion protein and with the *Torpedo* 300-kD protein (data not presented). These results support the conclusion that the 300-kD subsynaptic protein which copurifies with electric organ postsynaptic membranes is *Torpedo* dystrophin.

Furthermore, we have screened our electric organ cDNA library at moderate stringency with a probe derived from the 4.1-kb cDNA and isolated seven additional cDNAs that encode *Torpedo* dystrophin (see Materials and Methods) and no cDNAs that encode a dystrophin-like protein. Thus, the abundance of dystrophin cDNAs in the electric organ library is similar to the abundance of 300-kD protein in the electric organ, and dystrophin-like sequences are likely to be present at much lower abundance than dystrophin, if at all, in the electric organ.

Recent studies have demonstrated that antibodies against dystrophin cross-react with a *Torpedo* electric organ protein of ~400 kD that is enriched at the postsynaptic membrane (Chang et al., 1989; Jasmin et al., 1990). Moreover, a recent study showed that antibodies against murine dystrophin cross-react with a protein at neuromuscular synapses in normal rat muscle, and that antibodies against the *Torpedo* 400-kD protein react with nonsynaptic membrane in normal, but not dystrophic, human muscle (Chang et al., 1989). Because antibodies against dystrophin can cross-react with other proteins that contain homologous domains, as described above, and because these studies did not determine whether antibody staining was absent from synaptic sites in dystrophic muscle, it is difficult to ascertain whether the cross-reacting molecule at the synapse is dystrophin, rather than another molecule that shares epitopes with dystrophin. Indeed, a recent study demonstrated that some antibodies against dystrophin do react with synaptic sites in dystrophic muscle, and these authors suggest that a dystrophin-related protein is present at synaptic sites in normal muscle and persists in dystrophic muscle (Fardeau et al., 1990; Pons et al., 1991). Our study demonstrates that there is extensive homology between the amino acid sequences for mammalian dystrophin and the *Torpedo* 300-kD subsynaptic protein, and that antibodies against the *Torpedo* subsynaptic protein react with synaptic sites in normal, but not dystrophic muscle. Thus, we conclude that dystrophin itself is present in the electric organ and at synaptic sites in skeletal muscle.

In skeletal muscle dystrophin is found in the synaptic and nonsynaptic myofiber membrane, but is more concentrated at synaptic sites. It is unclear to what extent the increase in membrane surface area at the synaptic site, due to postjunctional folds, contributes to the higher dystrophin concentration observed by light microscopy (Woodruff et al., 1987; Sealock et al., 1991), and immunoelectron microscopy will be necessary to resolve this issue.

How does dystrophin associate with the postsynaptic membrane and with the nonsynaptic membrane of skeletal muscle fibers? We showed previously that postsynaptic membranes isolated from *Torpedo* electric organ contain stoichiometric quantities of AChR and the 43-kD subsynaptic protein, and ~10-fold lower levels of the 300-kD protein (Burden et al., 1983). We postulated that the 300-kD protein could serve as a scaffold upon which multiple copies of the 43-kD protein could associate, and that this assembly could serve to stabilize the structure of the postsynaptic membrane in the electric organ (Woodruff et al., 1987). One prediction of this model was that the 300-kD protein would have a repeat unit structure upon which multiple copies of the 43-kD protein could be arranged, and indeed the 300-kD protein does have repeated structural units. Nevertheless, the repeat units in the 300-kD protein may not be involved in targeting the protein to the postsynaptic membrane, and the actin-binding domain or the carboxy-terminal region may serve this function. Further, there is evidence that a 58-kD subsynaptic protein associates with dystrophin, and this protein could be important for membrane targeting (Sealock et al., 1991). Nevertheless, because the AChR-rich postsynaptic membrane from *Torpedo* electric organ contains largely the four AChR subunits and two peripheral membrane proteins of 43 and 300 kD (Froehner, 1986; Burden, 1987), this strikingly simple membrane should be a particularly favorable system to study how dystrophin becomes membrane-associated.

The distribution of dystrophin and AChR/43-kD protein is less well correlated in skeletal muscle than in the electric organ. First, dystrophin is present in the nonsynaptic membrane of myofibers, where neither AChR nor 43-kD protein are detectable. Second, the precise distribution of dystrophin and AChR within AChR-rich domains found in cultured embryonic muscle cells and at synaptic sites can be distinct (Sealock et al., 1991). Thus, proteins other than AChR/43-kD must link dystrophin to nonsynaptic membrane (Ervasti et al., 1990; Campbell and Kahl, 1989), and could link dystrophin to the synaptic membrane as well.

We do not know what function dystrophin has at the synapse, nor how loss of dystrophin from skeletal muscle results in myopathies. Since AChRs are concentrated at synaptic sites in dystrophic muscle (Fig. 2), it seems clear that dystrophin is not required for the formation of AChR clusters. Because dystrophin is a component of neuromuscular synapses and is suitably positioned to have a role in stabilizing the structure of the postsynaptic membrane, loss of dystrophin may nevertheless have consequences for synaptic transmission. Indeed, electron microscopic studies of dystrophic muscle have illustrated simplification of the synapse, notably a reduction in the number of postjunctional folds (Jerusalem et al., 1974; Torres and Duchen, 1987; Nagel et al., 1990). However, these changes may reflect properties of degenerating or regenerating muscle and may only be an indirect consequence of loss of dystrophin.

The presence of dystrophin in the electrocyte, a cell which is specialized for synaptic transmission and does not contract, suggests that dystrophin can have a role other than stabilizing the plasma membrane from structural distortion during contraction. Furthermore, the location of dystrophin exclusively at the postsynaptic membrane of the electrocyte suggests that dystrophin's function in this cell is associated with synaptic structure and/or function.

Current ideas for therapeutic treatment of patients with muscular dystrophy include injections of normal myoblasts into dystrophic muscle to promote formation of chimeric myotubes containing normal nuclei. Since the synaptic region of a skeletal myofiber is <1% of the myofiber volume,

and dystrophin may not diffuse freely in a myofiber, reconstitution of synaptic dystrophin may require that normal nuclei be situated in the synaptic region. If dystrophin has an important role at the synapse, successful therapeutic treatment may require a substantial increase in the efficiency of normal myoblast incorporation into chimeric myotubes.

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