Association of Utrophin and Multiple Dystrophin Short Forms with the Mammalian Mr 58,000 Dystrophin-associated Protein (Syntrophin)*

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Electric tissue syntrophin, originally described as an Mr 58,000 postnysptic protein having homologs in mammalian muscle, was previously shown to associate with dystrophin in Triton extracts of Torpedo postysptic membranes. It also associates with the Torpedo Mr 87,000 postnysptic protein (87K), the core of which is a superdomain homologous to the cysteine-rich (CR) and COOH-terminal (CT) domains of human dystrophin. Using immunoaffinity purifications from various rat tissues and immunoblotting, we find that syntrophin associates with dystrophin, utrophin (the chromosome 6-encoded dystrophin homolog formerly known as dystrophin-related protein), multiple proteins which are cross-reactive with 87K, and two subfamilies of 71K-like proteins (CRCT-containing proteins encoded by the dystrophin gene under the control of an alternative promoter in intron 62). One 71K subfamily contains the dystrophin COOH-terminal sequence; the other has an alternative COOH-terminal sequence caused by deletion of the penultimate exon by alternative splicing. The relative mass of the members of the subfamilies suggest they arise by alternative splicing at other previously described sites within CT. These results establish that syntrophin is a general ligand for the CRCT domain in mammalian dystrophin and its homologs. They also reveal a greater diversity in 71K proteins than has previously been apparent.

Dystrophin, the protein product of the Duchenne muscular dystrophy gene, is a member of the spectrin-a-actinin family of actin-binding proteins. Although this affiliation alone would be likely to assure a wide distribution, the degree to which dystrophin and related proteins are widely distributed over tissues and often interestingly distributed on cell surfaces has proven surprising. The products of the dystrophin gene are also remarkably varied and include short forms of a sort unknown in other branches of the spectrin-a-actinin family. This family has recently been characterized as being more subject to alternative splicing than any other cytoskeletal family (Ahn and Kunkel, 1993). The full-length forms of the dystrophin family are dystrophin (Hoffman et al., 1987) and utrophin (previously known as dystrophin-related, or DRP; Love et al., 1989; Khurana et al., 1990). Dystrophin is expressed principally in the three muscle types and in brain (Hoffman et al., 1987; Chelly et al., 1988). Utrophin is encoded by a separate gene and is expressed in most tissues, although at low levels in normal skeletal muscle (Khurana et al., 1990; Love et al., 1991). These large peripheral membrane proteins have an NH2-terminal actin binding domain, a central rod region containing 24 (22 in utrophin) spectrin-like triple helical repeats; a cysteine-rich EF hand-containing third domain (CR) with homology to the COOH-terminal domain of Dictostelium o-actinin, and a COOH-terminal domain (CT) without homology to proteins outside the dystrophin subfamily (Koenig et al., 1988; Davison and Critchley, 1988; Tinsley et al., 1992). Dystrophin is shown schematically in Fig. 1. In skeletal muscle, dystrophin and utrophin are associated with a complex of transmembrane glycoproteins, including an extracellular laminin-binding protein (Ervasti et al., 1990; Yoshiha and Ozawa, 1990; Ibraghimov-Beskrovnaya et al., 1992; Matsumura et al., 1992). The CRCT domain, which is highly conserved across species in both dystrophin and utrophin (Lemaire et al., 1988; Love et al., 1991), is believed to contain the glycoprotein binding site (Suzuki et al., 1992). Thus, dystrophin and utrophin appear to be long flexible bridges between the extracellular matrix and the actin-based cytoskeleton. Although the exact roles of these proteins have not been determined, they are believed to be involved in mechanical stabilization and molecular organization of the plasma membrane and cell cortex and possibly in regulation of calcium channel activity (reviewed in Lansman and Franco, 1991 and in Ahn and Kunkel, 1993).

The known normally occurring short forms in this family include several which are encoded by the dystrophin gene plus an Mr 87,000 phosphoprotein (87K) first identified in electric tissue of electric rays (Carr et al., 1989). All are based on the cysteine-rich (CR) and COOH-terminal (CT) domains (see Fig. 1). Of those encoded by the dystrophin gene, the only one to have been fully sequenced is a 71K protein expressed under the direction of an internal promoter lying between exons 62 and 63 (Lederfein et al., 1992; Rapaport et al., 1992). It has a core domain containing 583 out of the 610 amino acids of the CRCT superdomain flanked by unique NH2- and COOH-terminal sequences. The latter arises by excision of the penultimate exon of dystrophin, resulting in a shift of the reading frame (Bar et al., 1990). In this paper, we call this the “71R founder sequence” because of its primal role in defining the 71K family (Bar et al., 1990).

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The abbreviations used are: CR and CT, the cysteine-rich and COOH-terminal domains of dystrophin and homologous sequences; Ab, rabbit polyclonal antibody; mAb, mouse monoclonal antibody; 87K, the 87-kDa postnysptic protein described by Carr et al. (1989); 71K, the sequence described by Lederfein et al. (1992) and related sequences.
Association of Syntrophin and Dystrophin Homologs

RESULTS

Synaptotagmin Associates with Dystrophin and Utrrophin—To identify proteins with which mammalian syntrophin may be associated, we immunopurified syntrophin from Triton X-100 extracts of crude particulate fractions of various rat tissues using Sepharose-bound anti-syntrophin mAb 1351SYN. Upon

MATERIALS AND METHODS

Monoclonal Antibodies

Anti-syntrophin mAb—Monoclonal (mAb) 1808DYS was raised against Torpedo dystrophin in the Froehner laboratory and shown to recognize mammalian dystrophin specifically; it was previously design-
Fig. 2. Co-Purification of dystrophin and utrophin with syntrophin. A, Coomassie Blue-stained gel of syntrophin preparations from Torpedo electric tissue (T) and rat skeletal muscle (Sk), cardiac muscle (C), stomach/smooth muscle (Sm), brain (Br), lung (Lu), liver (Li), kidney (K), and testis (Te). The positions of dystrophin and utrophin (D/U), Torpedo 87K and Torpedo syntrophin (Syn) are indicated. Molecular mass markers are indicated on the left. Proteins that were major species in both the initial extracts and control preparations are designated M (putative myosin, in the three muscle preparations), A (actin, in the muscle and kidney preparations), and L (in liver). Molecular weight markers are indicated on the left, Torpedo syntrophin, originally identified as a 58K protein (Froehner et al., 1987), in fact migrated as an M, 53,000 protein; similar low values have been found by others (Carr et al., 1989; Chen et al., 1990). B-D, immunoblots of syntrophin preparations probed for syntrophin with mAb 1351SYN (B), for dystrophin with mAb 1808DYS (C), and for utrophin with Ab UTR3165 (D).

denaturing gel electrophoresis and Coomassie staining, the resulting preparations gave one or more major bands at the position expected for syntrophin ("Syn" in Fig. 2A) and at the position (D/U) of dystrophin and utrophin (which are not distinguishable in our gel system), plus other bands. Only three major proteins (M, A, and L in Fig. 2A) could be readily identified as nonspecific components, since they were also present in control preparations (see "Materials and Methods") and were major proteins in the starting extracts. M is presumably myosin, and A is presumably actin; immunoblotting with a pan-actin monoclonal antibody confirmed the presence of actin at the appropriate M, in all preparations (not shown). Liver protein L was not identified.

Immunoblotting with mAb 1351SYN confirmed the presence of syntrophin in all the preparations, as expected (Fig. 2B). Typically, it occurred in two, and sometimes more, closely spaced bands (cf. also Froehner et al., 1987). This multiplicity is consistent with the results of CDNA sequencing by Adams et al. (1993), who have identified two forms of mammalian syntrophin (syntrophin-1 and -2) encoded by separate genes. Since we had no way of linking bands to specific sequences, all mAb 1351SYN-reactive species have been taken together as syntrophin in this paper.

Blotting with anti-dystrophin mAb 1808DYS (Butler et al., 1992) showed strong reactivity in preparations from skeletal, cardiac, and smooth muscle and weak reactivity in lung (Fig. 2C). Preparations from brain, liver, kidney, and testis were essentially negative with this antibody. Anti-dystrophin mAbs DYS2 and MANDRA1 (see "Materials and Methods") gave similar results. The antibody DY51, against the central rod region of dystrophin, detected weak reactivity in preparations from brain (not shown). The low amounts in preparations from brain may be due to localization of brain dystrophin in postsynaptic densities (Lidov et al., 1990, 1993; Kim et al., 1992), which are a detergent-resistant fraction. None of the antibodies detected dystrophin in control preparations (data not shown).

In the reverse experiment, mAb 1808DYS-purified dystrophin from rat skeletal, cardiac, and smooth muscle contained syntrophin (not shown), as previously found with Torpedo dystrophin from electric tissue postsynaptic membranes (Butler et al., 1992). Syntrophin is thus associated with dystrophin in extracts of all three types of rat muscle.

Blots were probed for utrophin using polyclonal antibody (Ab) UTR3165, raised against a peptide specific to the utrophin CT domain. Utrophin was readily detected in all rat syntrophin preparations, although the amounts were low in preparations from skeletal and cardiac muscle and from brain (Fig. 2D). Utrophin could not be detected in control preparations Ab UTR3165 was specific for utrophin, since its reactivity was blocked by preincubation with 2.5 μg peptide antigen, and it failed to react with mAb 1808DYS-purified dystrophin (from all three muscle types (data not shown)). These results suggest that syntrophin associates with utrophin in extracts of normal skeletal, cardiac, and smooth muscle and of several non-muscle tissues. The failure of ab UTR3165 to label dystrophin preparations also suggests that dystrophin and utrophin do not form heterocomplexes, even when one is present in excess over the other (skeletal and smooth muscle; cf. Figs. 2, C and D; Chelly et al., 1988; Nguyen thi Man et al., 1992).

Syntrophin Associates with Multiple 71K-like Proteins—The syntrophin preparations contained proteins which appeared to correspond to many of the short CRCT-containing alternative products of the dystrophin gene. The anti-dystrophin mAb MANDRA1 detected prominent bands in the region of M, 70,000–80,000 in preparations from all rat tissues, although in lower amounts in the skeletal muscle preparation (Fig. 3A). These characteristics mimic those described for the 71K founder sequence in whole tissue samples and that protein is known to be recognized by MANDRA1 (Lederfein et al., 1992). Except in the preparations from skeletal muscle, 71K-like proteins were also detected by Ab 71Knt, raised against a peptide having the 71K NH₂-terminal sequence (Fig. 3B). Reactivity was essentially eliminated by prior incubation of the antibody with the amino-terminal peptide (25 μm), but not by incubation with a control peptide (the COOH-terminal peptide of the 71K founder sequence; data not shown). Neither Ab 71Knt nor MANDRA1 reacted detectably with blots of control preparations. Kidney syntrophin preparations contained a low molecular weight Ab 71Knt-reactive form not recognized by MANDRA1 (cf. Fig. 3, A and B, lane K); whether this is a new form or a proteolytic fragment is not known.

Blots were also probed with Ab 71KFSc, raised against a peptide corresponding to the COOH-terminal 18 amino acids of the 71K founder sequence. Although this antibody detected 71K-like proteins in preparations from all the tissues, these proteins unexpectedly constituted only a high molecular mass subpopulation of the MANDRA1- and Ab 71Knt-positive proteins in the 71K region (not shown). To facilitate comparison, Ab 71KFSc-reactive proteins were purified from a liver extract using a Sepharose-bound IgG fraction, then electrophoresed next to a mAb 1351SYN-purified liver syntrophin preparation and blotted. The two preparations appeared to contain the same subset of proteins when probed by ab 71KFSc, as expected if all the Ab 71KFSc-reactive forms in liver also asso-
proteins. The observed range of molecular masses is compatible with the several alternative splices in CT which have already been described (Feener et al., 1989; Bies et al., 1992). When discrete bands were resolved on immunoblots, four or five bands could typically be discerned (not explicitly shown). Liver therefore appears to contain a large population of 71K-like, syntrophin-associated proteins encoded by the dystrophin gene under the control of the promoter located between exons 62 and 63, but retaining the dystrophin COOH-terminal sequence. While this manuscript was in preparation, Cox et al. (1993) also reported that DYS2 recognizes 71K-like proteins on immunoblots.

Multiple DYS2-reactive bands were found in the 71K region in the syntrophin preparations from all the tissues studied, including skeletal muscle. Only in kidney were very weak signals obtained (Fig. 3D). In view of these results, we attach little significance to the failure of Ab 71Knt to detect 71K-like proteins in skeletal muscle we were able to apply to the gels was limited. On the other hand, the ease with which we could detect 71K-like proteins in skeletal and cardiac muscle tissues using DYS2 and MANDRA1 appeared to be at variance with the impression given by several studies of mRNA levels that 71K proteins are expressed at extremely low levels in adult skeletal muscle tissue, although they are abundant in fetal muscle. However, whether the skeletal muscle 71K-like proteins we identified were products of muscle cells or of non-muscle components of the tissues is not known.

Whether electric tissue contains 71K-like proteins is unknown. However, the failure of Torpedo preparations to react with DYS2 (Fig. 3D, lanes T) is without significance, since the COOH-terminal sequence of Torpedo dystrophin is substantially different from that of mammalian dystrophin (Yeadon et al., 1991).

To determine whether the co-purification of 71K founder sequence-like proteins and syntrophin was specific, 71K was purified from liver extracts using Ab 71KFSc as before, but in the presence and absence of the founder sequence COOH-terminal peptide. The product obtained in the absence of the peptide was reactive with MANDRA1 and Abs 71Knt and 71KFSc and contained syntrophin (Fig. 3E, lanes indicated by “−”), the preparations done in the presence of the peptide were negative for all these antibodies (lanes indicated by “+”). The co-purification was therefore specific.

87K-like Proteins Co-purify with Syntrophin—All syntrophin preparations, but no control preparations, also contained reactivity to mAb 13H1, raised against Torpedo 87K (Carr et al., 1989). The reactive species (Fig. 4) occurred in a single band (liver, M_r, app 62,000) or in multiple bands (M_r, app 83,000–46,000). Multiple mAb 13H1-reactive bands were also observed

![Diagram](image-url)
in preparations from electric tissue. This could possibly have been due to proteolysis during preparation, since Carr et al. (1987) noted that 87K was particularly sensitive to proteolysis. However, our anti-protease mixture included the precautions suggested by Carr et al. (1987) plus additional ones (see “Materials and Methods”) and appeared to be adequate. It protected dystrophin (known to be highly protease-sensitive; Koenig and Kunkel, 1990), since mAb 1808DYS detected only signals were obtained from all tissues. Torpedo 87K protein is indicated directly with all these proteins, suggesting that syntrophin is a general ligand of the CRCT superdomain in dystrophin and homologous proteins. Unfortunately, the information on cellular distributions which is required as a first test for the in situ existence of these associations is available only for muscle. Dystrophin and syntrophin are both general sarcolemmal proteins but are particularly concentrated at the neuromuscular and myotendinous junctions (Froehner et al., 1987; Chen et al., 1990; Shimizu et al., 1989; Byers et al., 1991). Where it has been examined, their distributions are co-extensive at a very high level of detail (Kramarcy and Sealock, 1995; Bloch et al., 1991). Syntrophin is also a sarcolemmal component in cardiac (Butler et al., 1992) and smooth muscle tissue: as is dystrophin. These results support in situ association of syntrophin and dystrophin.

Rabbit and mouse muscle dystrophins and mouse muscle utrophin are associated with a cytoplasmic 59K peripheral membrane protein in addition to the glycoprotein complex (Ervasti et al., 1990; Yoshida and Ozawa, 1990; Matsumura et al., 1992). The 59K protein runs as multiple bands on gels (Ervasti et al., 1991), and its concentration on sarcolemma is reduced in dystrophin-minus muscle (Ohlendieck and Campbell, 1991; Ohlendieck et al., 1993), although it remains concentrated at the neuromuscular junction (Matsumura et al., 1992). Syntrophin shares these properties (Froehner et al., 1987; Butler et al., 1992). These similarities suggest that the 59K protein and syntrophin could be identical, but this has not been tested in any direct experiment. If they are identical, the speculative prediction of Ervasti and Campbell (1991) that the 59K protein associates with CRCT will prove to have been correct.

The association of syntrophin with CRCT has implications for the possible activities of the protein. A syntrophin molecule bound to CRCT would lie near the glycoprotein complex (Suzuki et al., 1992), and could possibly modulate the interaction of dystrophin with the complex. This could be an important function, since the complex is one, although not the only, membrane

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3 J. Holder and R. Sealock, unpublished observations.

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binding site for dystrophin (Hoffman et al., 1991; Helliwell et al., 1992; Récan et al., 1992). This activity could be general. The available evidence suggests that utrophin and dystrophin associate with the same glycoprotein complex in muscle (Matsumura et al., 1992). The 87K protein is membrane-bound, although its membrane receptor is unknown (Carr et al., 1989), and the proteins of the 71K family appear to be membrane-bound (they occur in particulate fractions and are solubilized by Triton; Lederfein et al., 1992; see “Materials and Methods”). The short forms are presumably bound to the membrane via the same glycoprotein complex as dystrophin or a similar one. Although there is presently no demonstrated reason for which association of these proteins with the α-glycoprotein complex would require modulation (but see below), the facts that syntrophin and the 87K protein in electric tissue can be phosphorylated at serine/threonine and the 87K protein in electric tissue can be phosphorylated at serine/threonine and the 87K protein in electric tissue could have included forms with the dystrophin COOH-terminus (see Fig. 3C and accompanying discussion in “Results”). This result has the interesting implication that retention of the penultimate exon allows fuller expression of alternative splicing events in CT.

No direct experiment has yet revealed the functional role or importance of syntrophin at the cellular or organismal level. However, in the mdx3Cy mouse, a single point mutation in intron 65 of the dystrophin gene causes truncation and failure of expression of normal dystrophin and of normal 71K-like proteins of both subfamilies (Cox et al., 1993). In addition to suffering from the skeletal and cardiac muscular dystrophy characteristic of the mdx mouse, the mdx3Cy mouse produces a reduced number of progeny and has a strikingly reduced neonatal survival rate. Cox et al. (1993) concluded that the absence of the 71K proteins is the probable cause of this phenotype. This would also implicate syntrophin, since binding of syntrophin and membrane association are the only known activities of the 71K proteins.

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