THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 269, No. 4, Issue of January 28, pp. 2870-2876, 1994
Printed in U.S.A.

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

(Received for publication, July 22, 1993, and in revised form, September 9, 1993)

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Electric tissue syntrophin, originally described as an M_r 58,000 postsynaptic protein having homologs in mammalian muscle, was previously shown to associate with dystrophin in Triton extracts of *Torpedo* postsynaptic membranes. It also associates with the *Torpedo* M_r 87,000 postsynaptic 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 retains 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 masses 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/α-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/α-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 functions of most of these products are totally unknown. However, their wide distribution suggests appropriate studies in almost any tissue could potentially aid in identifying those functions and in explaining why quantitative or qualitative deficiencies in dystrophin are catastrophic for muscle cells.

The full-length forms of the dystrophin family are dystrophin (Hoffman *et al.*, 1987) and utrophin (previously known as dystrophin-related protein, 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 proteins have an NH_2-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)[1] with homology to the COOH-terminal domain of Dictyostelium α-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; Yoshida 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 M_r 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 NH_2- 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 "71K founder sequence" because of its primal role in defining the 71K family (Bar *et al.*,

* This work was supported by National Institutes of Health Grants NS15293 (to R. S.) and NS14871 (to S. C. F.), by grants from the Muscular Dystrophy Foundation (to R. S. and S. C. F.), and by Fogarty International Center Fellowship 1-F05-TW04733 (to A. V.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] 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 postsynaptic protein described by Carr *et al.* (1989); 71K, the sequence described by Lederfein *et al.* (1992) and related sequences.

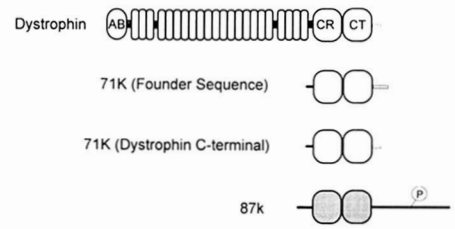

FIG. 1. **Schematic representation of dystrophin and related proteins (from Koenig *et al.* (1988) and Koenig and Kunkel (1990)).** *AB*, actin binding domain; *rounded rectangles*, spectrin-like triple helical repeats. *Black rectangles*, hinge regions. *CR*, cysteine-rich domain. *CT*, COOH-terminal domain. Utrophin would be represented like dystrophin but with a slightly shorter repeat region. The 71K founder sequence is the sequence of Lederfein *et al.* (1992); it has a unique COOH-terminal. The other form of 71K shares NH_2 and COOH termini with the founder sequence and dystrophin, respectively. The NH_2- and COOH-terminal sequences of the 87K protein are unique; the latter can be phosphorylated. For other details, see text.

1990; Rapaport *et al.*, 1992; Lederfein *et al.*, 1992). It is believed to be encoded by a 6.5-kilobase transcript. In addition, recent immunoblotting (Cox *et al.*, 1993) and partial sequencing of a 4.8-kilobase transcript (Blake *et al.*, 1992) have suggested the existence of forms which are similar but retain the dystrophin COOH-terminal sequence (Fig. 1). All of these shorter forms may also occur in multiple isoforms, since the CT domain is subject to extensive alternative splicing (Feener *et al.*, 1989; Bies *et al.*, 1992).

The 87K protein is a component of acetylcholine receptor-rich postsynaptic membranes from electric tissue (Carr *et al.*, 1989). It has a core domain of 547 amino acids which has modest, but clear, homology to the dystrophin CRCT superdomain. Interestingly, its 150-amino acid COOH-terminal sequence, which is not homologous to any known sequence, is subject to tyrosine and serine phosphorylation by kinases endogenous to the postsynaptic membranes (Wagner *et al.*, 1993; Fig. 1). Anti-87K antibodies label rat skeletal muscle at the neuromuscular junction and on the extrajunctional sarcolemma (Carr *et al.*, 1989), suggesting the existence of a mammalian 87K homolog distributed in muscle very much like dystrophin.

To understand the functions of the long and short forms of the dystrophin family, it will be essential to identify all the proteins with which they associate. Electric tissue of electric rays contains an M_r 58,000 protein (58K) which was originally identified as a cytoplasmic component of acetylcholine receptor-rich domains in electric tissue and cultured skeletal muscle (Froehner *et al.*, 1987). With the development of our knowledge of dystrophin, it became apparent that many features of the distribution of 58K in muscle cells are shared with dystrophin (see "Discussion"). The protein was then shown by biochemical means to associate with both dystrophin and the 87K protein in Triton extracts of electric tissue membranes (Butler *et al.*, 1992). These results suggested that 58K, whose mammalian homologs are widely expressed in muscle and non-muscle tissues (Froehner *et al.*, 1987; Adams *et al.*, 1993) could be a general ligand for dystrophin and related proteins. In this paper, we show that 58K does in fact associate with mammalian dystrophin, utrophin, and multiple short CRCT-containing homologs. Because it accompanies all known proteins of the dystrophin family which have been tested, Adams *et al.* (1993) have given to 58K the name "syntrophin" (from *συντροφος*, companion, associate).

MATERIALS AND METHODS

Monoclonal Antibodies

Anti-dystrophin mAbs—Monoclonal (mAb) 1808DYS was raised against *Torpedo* dystrophin in the Froehner laboratory and shown to recognize mammalian dystrophin specifically; it was previously designated mAb 1808 (Sealock *et al.*, 1991). NCL-DYS2 (also designated Dy8/6C5; purchased from Vector Laboratories) was raised against a peptide corresponding to the last 17 amino acids of human dystrophin (Nicholson *et al.*, 1992). NCL-DYS1 (also designated Dy4/6D3; Vector) recognizes the central rod region of dystrophin (Nicholson *et al.*, 1990). MANDRA1 (a generous gift of G. E. Morris) reacts within the COOH-terminal domain of dystrophin (Nguyen thi Man *et al.*, 1992) and has been shown to recognize the 71K founder sequence (Lederfein *et al.*, 1992). It does not recognize utrophin.

Anti-syntrophin—mAb 1351SYN was raised against *Torpedo* syntrophin (Froehner *et al.*, 1987; originally identified as mAb 1351) but shows broad cross-species reactivity (Froehner *et al.*, 1987; Chen *et al.*, 1990; Adams *et al.*, 1993).

Anti-87K—mAb 13H1 (a generous gift of J. B. Cohen) was raised against *Torpedo* 87K and shown by immunofluorescence to recognize a protein of the sarcolemma and the neuromuscular junction in rat and chick skeletal muscle (Carr *et al.*, 1989).

Anti-actin—The pan-actin antibody was obtained from Boehringer Mannheim.

Peptides and Polyclonal Antibodies

Polyclonal antibodies (Abs) against high performance liquid chromatography-purified synthetic peptides (Multiple Peptide Systems) were prepared in rabbits according to standard methods. Ab UTR3165 was raised against the peptide SMWPEHYDPSC (amino acids 3165–3174 of human utrophin plus a COOH-terminal Cys; Love *et al.*, 1989; Tinsley *et al.*, 1992). Ab 71Knt was raised against REHLKGHEC (the N-terminal sequence encoded under the control of the alternative promoter in intron 62). Ab 71KFSct was raised against CGRAMESLVSVMTDEEGAE (the 71K founder sequence carboxy-terminal plus an NH_2-terminal cysteine). Antibodies in antiserum UTR3165 were affinity-purified by standard methods using Affi-Gel 15-coupled SMWPEHYDPSC. The IgG fraction of serum 71KFSct was prepared by ammonium sulfate fractionation and DEAE chromatography (Fujiwara and Pollard, 1976) and coupled to Sepharose as described (Butler *et al.*, 1992). Each of these antibodies was shown to be a specific probe on blots by competition experiments in which incubation with the appropriate peptide, but not an inappropriate peptide, eliminated immunoreactivity.

Immunoaffinity Purifications

Frozen tissues (5 g) were homogenized 30 s in a Polytron homogenizer in 50 ml of ice-cold 10 mM sodium phosphate, 5 mM EDTA, 0.4 M NaCl, pH 7.8, containing protease inhibitors (Sigma; aprotinin, leupeptin, and aprotinin at 0.5 μg/ml each, pepstatin A, 0.05 μg/ml, and 2 mM phenylmethylsulfonyl fluoride). After centrifugation (10 min at 12,000 × *g*), the pellet was rehomogenized, centrifuged, and resuspended in 15 ml of buffer. Proteins were solubilized by addition of Triton X-100 (Pierce Chemical Co.) to 1%, incubation on ice 15 min, and centrifugation. Extracts were subjected to immunoaffinity purifications on Sepharose-bound antibodies and the resulting fractions prepared for electrophoresis as described previously (Butler *et al.*, 1992). For control preparations, resins were coupled to a monoclonal antibody of unrelated specificity.

Torpedo dystrophin was immunoaffinity purified from electric organ postsynaptic membranes as described (Butler *et al.*, 1992).

Gel Electrophoresis and Immunoblotting

For Coomassie Blue staining and blotting, standard Laemmli SDS gels contained 8% acrylamide and 0.13% bisacrylamide (0.04% bisacrylamide when blotting dystrophin or utrophin). Prestained molecular weight standards (Sigma) were calibrated with normal standards (Sigma). Proteins and prestained standards were transferred to Immobilon P (Millipore) in 25 mM Tris, 192 mM glycine in a semidry apparatus (Bio-Rad). Blots were blocked with 5% milk protein, then probed by standard methods. The alkaline phosphatase-conjugated second antibodies (Jackson ImmunoResearch) were revealed with Western Blue (Promega).

RESULTS

Syntrophin Associates with Dystrophin and Utrophin—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

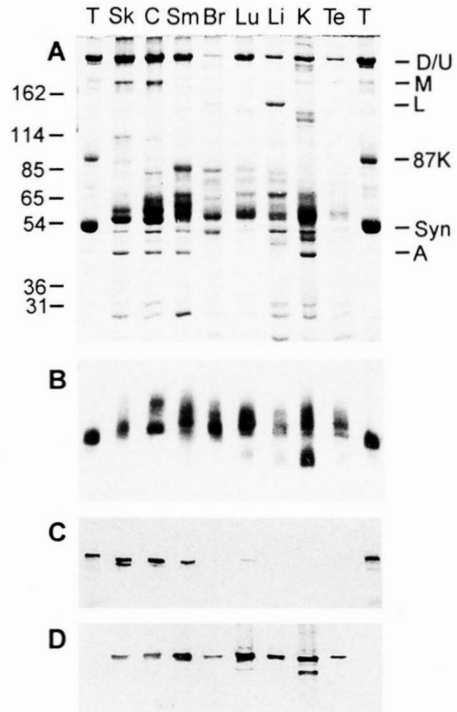

FIG. 2. **Co-Purification of dystrophin and utrophin with syntrophin.** *A*, Coomassie Blue-stained gel of syntrophin preparations from *Torpedo* electric tissue (*T*) and rat skeletal muscle (*Sk*), cardiac muscle (*C*), stomach/smooth muscle (*Sm*), brain (*Br*), lung (*Lu*), liver (*Li*), kidney (*K*), 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_r 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. 2*A*) 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. 2*A*) 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_r 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. 2*B*). 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. 2*C*). 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 DYS1, 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. 2*D*). Utrophin could not be detected in control preparations. Ab UTR3165 was specific for utrophin, since its reactivity was blocked by preincubation with 2.5 μM 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_r 70,000–80,000 in preparations from all rat tissues, although in lower amounts in the skeletal muscle preparation (Fig. 3*A*). 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 NH_2-terminal sequence (Fig. 3*B*). 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 71KFSct, 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 71KFSct-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 71KFSct, as expected if all the Ab 71KFSct-reactive forms in liver also asso-

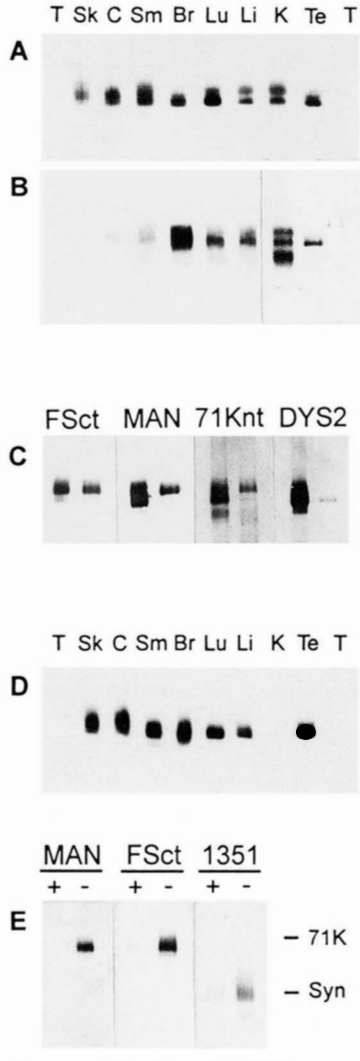

FIG. 3. ***A* and *B*, co-purification of 71K-like proteins with syntrophin.** Syntrophin preparations were probed for 71K-like proteins with anti-dystrophin/anti-71K mAb MANDRA1 (*A*) and with Ab 71Knt, raised against the NH_2-terminal sequence common to all known 71K proteins (***B***). Only the 71K regions of the blots are shown. *C*, comparison of 71K-like proteins in liver syntrophin preparations with founder sequence-like 71K proteins directly purified from liver. In each pair, the *left lane* shows the liver syntrophin preparation; the *right lane* shows 71K proteins purified with Ab 71KFSct, raised against the founder sequence COOH-terminal. The blots were probed with Ab 71KFSct (abbreviated *FSct*), the anti-dystrophin CT domain MANDRA1 (*MAN*), Ab 71Knt, and mAb DYS2, which recognizes the last 17 amino acids of dystrophin. 71K-like proteins having the founder sequence-specific and dystrophin-specific COOH termini form distinct subpopulations. *D*, syntrophin preparations from all tissues were probed for 71K proteins having the dystrophin COOH-terminal with mAb DYS2. Readily detectable or strong signals were obtained from all the mammalian tissues except kidney. *E*, co-purification of syntrophin with 71K founder sequence-like proteins. 71K proteins in liver extracts repurified on affinity resins made with Ab 71KFSct in the presence (lanes indicated "+") or absence (lanes indicated "–") of 25 μM founder sequence COOH-terminal peptide. Blots were probed for 71K-like proteins with MANDRA1 (*MAN*) and Ab 71KFSct (abbreviated *FSct*), and for syntrophin with mAb 1351SYN (abbreviated *1351*). Syntrophin co-purified with 71K proteins. The co-purifiecation was specific, since purifications in the presence of the peptide yielded undetectable amounts (*MAN*) or barely detectable amounts (*71KFSct, 1351SYN*) of antibody reactivity. Tissue identifications in *A* and *D* are the same as in Fig. 2.

ciate with syntrophin (Fig. 3*C*, *lanes* indicated *71KFSct*). However, probing with MANDRA1 or the anti-71K NH_2 terminus Ab 71Knt showed clearly that the Ab 71KFSct-purified sample contained only a subset of the 71K-like proteins found in the syntrophin sample (Fig. 3*C*, *lanes MAN* and *71Knt*, respectively). This subset could usually be resolved into two bands running at apparent M_r 80,000 and 77,000 in our gels. To explore the possibility that the Ab 71KFSct-negative forms could be 71K-like proteins that retain the dystrophin COOH-terminal sequence, blots were probed with mab DYS2, a commercially available antibody raised against the last 17 amino acids of dystrophin (see "Materials and Methods"). This antibody should not react with the 71K founder sequence, and the Ab 71KFSct-purified proteins were in fact not labeled, although a minor band not coincident with ab 71KFSct-labeled protein was detected (Fig. 3*C*, *right lane* under *DYS2*). In the syntrophin preparation, however, DYS2 recognized a number of proteins of approximate $M_{r,\,app}$ ranging from 80,000 to 71,000 (Fig. 3*C*, *left lane* under *DYS2*). The envelope of proteins labeled with DYS2 corresponded closely with those labeled by MANDRA1 and Ab 71Knt, eliminating the possibility that the multiplicity arose by proteolytic nicking. 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. 3*D*). In view of these results, we attach little significance to the failure of ab 71Knt to detect 71K-like proteins in skeletal muscle (Fig. 3*B*). That antibody typically gives a weak immunoblot signal, and the amounts of material from 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. 3*D*, *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 71KFSct 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 71KFSct and contained syntrophin (Fig. 3*E*, *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

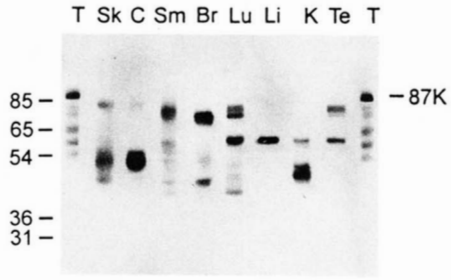

FIG. 4. **Co-purification of 87K-like proteins with syntrophin.** Syntrophin preparations from tissues (see Fig. 2) were probed with mAb 1^3H1, raised against the *Torpedo* 87K protein. One or more strong signals were obtained from all tissues. *Torpedo* 87K protein is indicated.

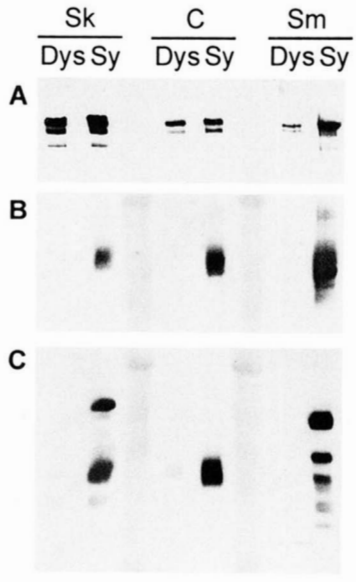

FIG. 5. **Failure of CRCT homologs to co-purify with dystrophin.** Dystrophin (lanes marked *Dys*) was purified from extracts of rat skeletal (*Sk*), cardiac (*C*), and stomach/smooth muscle (*Sm*) using anti-dystrophin mAb 1808DYS and compared with the corresponding syntrophin preparations (*Syn*). Blots were probed with mab MANDRA1 for both dystrophin (*A*) and 71K (*B*) and with mAb 1^3H1 for 87K-like protein (*C*). Unlabeled lanes, prestained standards. The 71K- and 87K-like proteins were significantly present only in the syntrophin preparations.

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 full-length forms on blots (Fig. 2*C*). (mAb 1808DYS recognizes an extremely large population of dystrophin fragments in samples of *Torpedo* dystrophin which are known to have been proteolyzed).[2] In addition, the lower molecular weight forms of *Torpedo* 87K in our preparations were minor components essentially undetectable on Coomassie-stained gels (Fig. 2*A*, *lanes T*). Our results, therefore, suggest that the mAb 13H1-reactive proteins in the rat constitute a family of similar proteins and that most rat tissues contain one or more members of the family.

71K and 87K Proteins Form Independent Complexes with Syntrophin—To determine whether the 71K- and 87K-like proteins could have occurred in syntrophin preparations indirectly via association with dystrophin, the syntrophin preparations were compared to mAb 1808DYS-purified dystrophin. At similar high dystrophin loadings (Fig. 5*A*), 71K-like proteins (founder sequence type) and 87K-like proteins gave strong signals in skeletal, cardiac, and smooth muscle syntrophin preparations, but could not (71K; Fig. 5*B*) or could barely (87K; Fig. 5*C*) be detected in the corresponding dystrophin preparations. A similar result was obtained with electric tissue postsynaptic membranes (Butler *et al.*, 1992), where syntrophin-87K co-purification via independent association with a third, non-dystrophin, protein was rendered unlikely by the simple protein composition of the final product. Syntrophin thus appears to interact directly with both the 71K- and 87K-like proteins in extracts of rat muscle.

DISCUSSION

We have shown that dystrophin and 71K founder sequence-like proteins co-purify with syntrophin from Triton extracts of several rat tissues and that syntrophin co-purifies with dystrophin and with 71K. Utrophin, 71K-like proteins which appear to retain the dystrophin COOH-terminal sequence, and proteins immunologically related to the 87K phosphoprotein of electric tissue also co-purified with syntrophin, although in these cases we did not have the means to do the reverse purifications. None of these proteins was purified in significant quantity in control purifications. For utrophin, 71K founder sequence-like proteins, and 87K-like proteins, we documented that the co-purification was independent of dystrophin. These results constitute strong evidence that syntrophin associates 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 subcellular 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, 1990; Bloch *et al.*, 1991). Syntrophin is also a sarcolemmal component in cardiac (Butler *et al.*, 1992) and smooth muscle tissue,[3] 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

[2] J. Holder and R. Sealock, unpublished observations.

[3] N. R. Kramarcy and R. Sealock, unpublished results.

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/a glycoprotein complex would require modulation (but see below), the facts that syntrophin and the 87K protein in electric tissue can be phosphorylated by endogenous kinases (Wagner and Huganir, 1993; Wagner *et al.*, 1993) and that recombinant CR binds Ca^{2+} (presumably on the EF hands; Milner *et al.*, 1992) suggest that syntrophin-CRCT complexes are loci of regulatory activity.

If dystrophin forms an antiparallel homodimer, as ultrastructural images (Sato *et al.*, 1992) and analogy to spectrin suggest, CRCT-bound syntrophin on one dystrophin molecule would lie near to and could potentially modulate activity of the actin binding domain of the sister dystrophin molecule. This seems unlikely to be an obligatory activity, however, since at present there is no known way to fit the short CRCT homologs into such a scheme.

The present results provide a basis for interpretation of studies of the possible involvement of syntrophin in acetylcholine receptor clustering (Froehner *et al.*, 1987; Bloch *et al.*, 1991; Froehner, 1993). When acetylcholine receptor clusters are induced on cultured *Xenopus* muscle cells by application of latex beads, syntrophin appears at clusters on a far more rapid time course (a few hours *versus* a few days) and with a more receptor-specific distribution than does dystrophin (Chen *et al.*, 1990; Peng and Chen, 1992). Similarly, syntrophin localizes both to focal adhesions and to spontaneously occurring acetylcholine receptor clusters in the *Xenopus* cells, but dystrophin localizes only to the focal adhesions (Kramarcy and Sealock, 1990). In the mdx mouse, syntrophin incorporation on the general sarcolemma is decreased relative to normal muscle, but the protein remains concentrated at the neuromuscular junction (Butler *et al.*, 1992), which is known to contain dystrophin-related protein or proteins, including utrophin (Fardeau *et al.*, 1990; Ohlendieck *et al.*, 1991; Bewick *et al.*, 1993). Thus, there must be one or more non-dystrophin-binding proteins for syntrophin in muscle and, in particular, at acetylcholine receptor clusters. Any of the CRCT-containing proteins discussed here are possibilities, although since utrophin has been shown to be a component of the receptor-rich domains at the junction (Bewick *et al.*, 1993), it would appear to be a leading candidate.

The existence and tissue distribution of the 71K founder sequence protein were originally demonstrated using the antibody MANDRA1 (Lederfein *et al.*, 1992; Hugnot *et al.*, 1992). This antibody does not distinguish forms which differ in COOH-terminal sequence (see Fig. 1), so that these initial results could have included forms with the dystrophin COOH-terminal sequence. Using an antibody against the COOH-terminal peptide of the founder sequence, we have shown that syntrophin-associated founder sequence-like 71K proteins do exist in substantial quantities in most tissues. Using antibody DYS2, against the last 17 amino acids of dystrophin, we found even larger amounts of syntrophin-associated 71K-like proteins which retain the dystrophin COOH-terminal sequence. This subfamily appears to have more members than the subfamily having the alternative COOH-terminal sequence (see Fig. 3*C* 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 mdx^{3Cv} 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 mdx^{3Cv} 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.

Acknowledgments—We thank Glenn E. Morris and Jonathan B. Cohen for generous gifts of monoclonal antibodies MANDRA1 and 13H1, respectively.

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