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Isolated dystrophin molecules as seen by electron microscopy

(smooth muscle/chicken gizzard/spectrin/actinin)

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ABSTRACT **Dystrophin, the protein product of the Duchenne muscular dystrophy locus [Hoffman, E. P., Brown, R. H., Jr., & Kunkel, L. M. (1987) *Cell* 51, 919–928], is expressed in striated and smooth muscles as well as in nonmuscle tissues. Examination of its primary structure has revealed that the molecule is composed of four domains, three of which share many features with the membrane cytoskeletal proteins spectrin and actinin. Dystrophin has thus been predicted to adopt a rod shape [Koenig, M., Monaco, A. P. & Kunkel, L. M. (1988) *Cell* 53, 219–228]. In the present study, we describe its isolation from the chicken gizzard smooth muscle and present electron microscopic images of the molecule. Polyclonal antibodies were first prepared from a dystrophin fragment derived from the chicken skeletal muscle gene (residues 1173–1728). A dystrophin-enriched membrane preparation from chicken gizzard muscle was then purified by passing it through an affinity chromatography column made with the anti-dystrophin antibodies. Electron microscopy of isolated and rotatory-shadowed dystrophin molecules revealed that the lengths measured for the dystrophin monomers (175 ± 15 nm) are compatible with a structural arrangement of the repeat sequence segments in triple-barrel α-helices connected by short-turn regions, as was earlier postulated for the repeat domains of spectrin and actinin. Electron microscopic images indicate that in addition the dystrophin molecules could present the same capacity of self-association in oligomeric structures as these cytoskeletal proteins and may thus be a part of a complex molecular meshwork essential to muscle cell function.**

The Duchenne/Becker muscular dystrophy (DMD) gene (1, 2) encodes a large protein (about 400 kDa) of low abundance (0.01% of total skeletal muscle protein), called dystrophin (for review, see refs. 3 and 4). It is expressed in striated and smooth muscles as well as in other nonmuscle tissues (5–7). Optical and electron microscopic (EM) studies demonstrate that dystrophin is localized to the inner surface of the muscle sarcolemma in normal human skeletal muscles but is absent in skeletal muscles of DMD patients (8–11). A four-domain structural arrangement can be predicted from the human and chicken skeletal dystrophin amino acid sequences (2, 12). The N-terminal domain is structurally homologous with the actin binding domain of actinin (13, 14). It is followed by a large domain that is postulated to be rod shaped and formed by a succession of 25 triple-helical 109-residue segments, similar to the repeat domains of spectrin (15, 16). A third domain contains a cysteine-rich segment that is partially similar to the entire C-terminal domain of *Dictyostelium* α-actinin (17). The 420-amino acid C-terminal domain of dystrophin, which is highly conserved in man and chicken, to our knowledge, shows no similarity to any reported protein. Another amino acid sequence homology between dystrophin (position 1834–1936) and caldesmon has been reported (18). Dystrophin thus shares many structural (functional) features with the cytoskeletal proteins spectrin and actinin and could adopt a rod shape (2, 19, 20). No experimental evidence has yet confirmed this hypothetical structure of native dystrophin. Until now, dystrophin has been isolated in tight association with either an integral membrane glycoprotein (21) or with proteins of the muscle triad transverse tubular system (22) or in an SDS-denatured form (23).

In this paper, we describe the isolation of dystrophin from the chicken gizzard smooth muscle by using polyclonal antibodies raised against a piece of the central domain of dystrophin from chicken skeletal muscle (12). We then present EM images of isolated and rotatory-shadowed dystrophin molecules.

MATERIALS AND METHODS

Anti-Dystrophin Antibodies. The 1.65-kilobase cDNA sequence used to produce antibodies specific to dystrophin was excised by *Nsi* I from the dystrophin cDNA subclone 2, originally isolated from a λgt10 library of chicken skeletal muscle cDNA (12). After blunt-ending by using the Klenow fragment of DNA polymerase I and dATP, the resulting sequence was inserted in the dephosphorylated *Sma* I site of vector pEX2 (24). The plasmid construction resulted in the fusion of a 555-residue dystrophin segment (amino acids 1173–1728) to the C-terminal end of a 110-kDa β-galactosidase fragment. The fusion protein, hereafter designated fusion protein C, was induced in *Escherichia coli* pop 2136 and purified from cell homogenates after treatment with lysozyme and DNase (25). The fusion protein was solubilized by boiling in 2.5% (wt/vol) SDS. Two New Zealand female rabbits were immunized at monthly intervals with three or four subcutaneous injections of 1 mg of fusion protein C dialyzed against 0.1% SDS and emulsified with Freund's complete adjuvant for the first injection and then with Freund's incomplete adjuvant for the others. The injected fusion protein C caused rapid production of antisera, consistently and persistently containing high amounts of dystrophin-specific antibodies. Sera specificity and affinity were tested by ELISAs (26).

Preparation of Chicken Gizzard Muscle Dystrophin. One chicken gizzard muscle (about 10 g, from a 12-month-old chicken) was dissected immediately after the chicken was killed, cut up, and homogenized in a Waring Blendor twice for 30 sec at high speed in 20 vol of 0.1 M Tris·HCl (pH 9.0) containing soybean trypsin inhibitor at 1 mg/ml, leupeptin at 1 mg/ml, 10 mM iodoacetamide, 5 mM phenylmethylsulfonyl

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Abbreviations: DMD, Duchenne muscular dystrophy; EM, electron microscopic.

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fluoride, and 1% Triton X-100. The insoluble fragments were immediately removed by a 20-min centrifugation at 8000 × *g*. The supernatant was then precipitated by solid ammonium sulfate to a final concentration of 20% (wt/vol). After a 20-min centrifugation at 8000 × *g*, the pellet was dissolved by homogenizing with a Potter–Elvehjem grinding mortar in 1 vol of the homogenization buffer except without Triton X-100. After a 20-min centrifugation at 20,000 × *g*, the corresponding supernatant was concentrated 7–10 times on an Amicon cell using PM30 membranes and immediately applied to a 0.5 × 3.0 cm column of Sepharose 4B coupled to the polyclonal antibodies raised against the dystrophin fusion protein C that had been equilibrated with 0.1 M sodium phosphate (pH 7.5). The column was washed with 10 vol of 0.5 M NaCl/10 mM Tris·HCl, pH 7.5 and then with 3 vol of 0.1 M ammonium formate (pH 7.5) to eliminate nonvolatile salts. The bound proteins were eluted with 10 ml of 0.1 M formic acid (pH 3.0) and collected in 1-ml fractions. The fractions were immediately neutralized in 30 μl of 2 M Tris (pH 11) and diluted 1:1 either with SDS buffer for polyacrylamide gel and immunoblot analyses (27, 28) or with 70% (vol/vol) glycerol for rotatory-shadowing and EM experiments (29–30).

Immunofluorescence Detection. Transverse cryostat sections (8 μm thick) from human and chicken muscle biopsies were processed for indirect immunofluorescence analysis (26). Each sample was labeled with polyclonal anti-dystrophin antibodies directed against fusion protein C used at a 1:100 dilution and fluorescein-conjugated anti-rabbit IgG.

SDS/PAGE and Immunoblot Analyses. Total muscle homogenates and dystrophin preparations were separated in a 2.5–7.5% gradient resolving polyacrylamide gel (27) containing 25% glycerol and no stacking gel. The following proteins were used as molecular mass markers: myosin (200 kDa), phosphorylase *b* (94 kDa), bovine serum albumin (67 kDa), and ovalbumin (43 kDa). Fractionated proteins were electrotransferred onto nitrocellulose sheets in the transfer buffer containing 0.1% SDS (28) and labeled with polyclonal antibodies directed against fusion protein C used at a 1:100 dilution and with anti-mouse IgG coupled to alkaline phosphatase. The monoclonal antibody specific for actin was provided by Biogenex Laboratories (San Ramon, CA).

EM Analysis. Isolated dystrophin, derived from the anti-dystrophin IgG-Sepharose 4B column and diluted in a 70% glycerol/0.5 M ammonium formate, pH 7.5, was sprayed at 20 μg/ml on a freshly cleaved mica and low-angle rotatory-shadowed with platinium/carbon, as described (29, 30).

RESULTS

Anti-Dystrophin Antibodies. Antibodies were raised against a 555-residue dystrophin segment derived from the N-terminal end of the dystrophin central domain composed of repeat sequence segments (2, 12). Immunofluorescence and immunoblot analyses were made to establish the specificity of the polyclonal antibodies for the protein product of the human DMD locus and to determine how these antibodies detected dystrophin in chicken skeletal and gizzard muscles.

Transverse cryostat sections of human skeletal muscles from a normal individual and a 6-year-old child with DMD and of chicken soleus and gizzard muscles were incubated with anti-dystrophin antibodies and then with fluorescein-coupled anti-rabbit antibodies. The clear positive staining observed in fibers of the normal human muscle compared to the almost nonexistent staining of muscle fibers from the patient with a clinical diagnosis of DMD (Fig. 1 *A* and *B*) demonstrated the specificity of the anti-dystrophin sera. The same antibodies intensely stained the periphery of every gizzard muscle small fiber in the same way as it did for chicken and human skeletal muscle large fibers (Fig. 1 *D*, *C*, and *A*, respectively) (8, 11, 31, 32). Total SDS homogenates of human skeletal muscles from a normal individual and a 6-year-old child with DMD (Fig. 1*E*, lanes 1 and 2) and chicken skeletal and gizzard muscle (Fig. 1*E*, lanes 3 and 4) were compared on Western immunoblots using the same anti-dystrophin antibodies (Fig. 1*F*, lanes 1–4). Doublet protein bands of about 400 kDa were immunologically detected in total homogenates of all normal muscles (22). In contrast, no immune reactivity was observed in the muscle homogenates of the DMD patient. Immunoblot and immunofluorescence analyses indicated thus that chicken smooth muscles expressed a large amount of a 400-kDa dystrophin immunologically similar to human and chicken skeletal muscle dystrophins, at least with respect to the repeat sequence segment domain of dystrophin used for raising antibodies (2, 12).

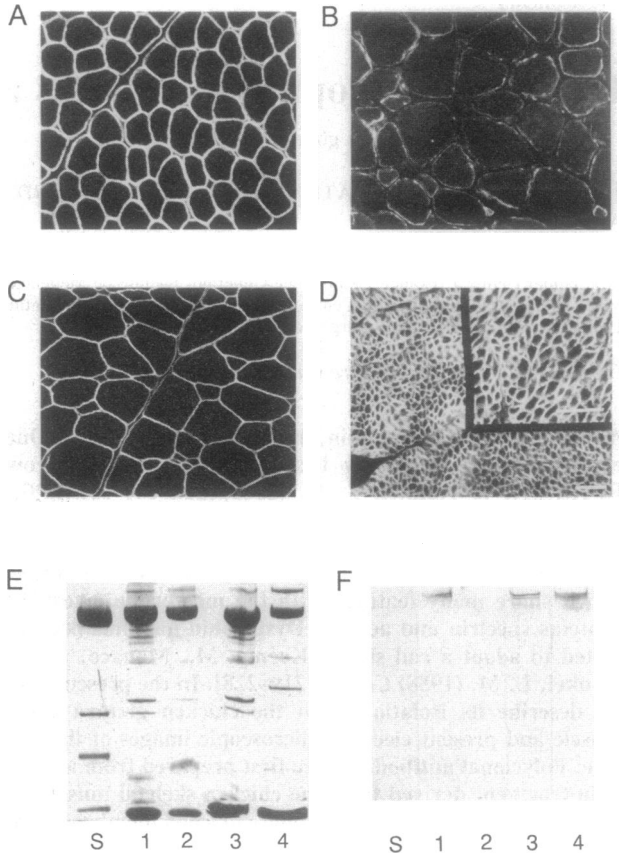

FIG. 1. Immunodetection of dystrophin in human skeletal muscles and in chicken skeletal and smooth muscles, using polyclonal antibodies against a piece of the central domain of the chicken skeletal muscle dystrophin. Transverse cryostat sections of normal human deltoid muscle (*A*), a deltoid muscle from a 6 year old with DMD (*B*), chicken soleus muscle (*C*), and chicken gizzard muscle (*D*) labeled with anti-dystrophin antibodies and a second antibody coupled with fluorescein are shown. (Bars in *D* and its *Inset* = 10 μm.) Coomassie blue-stained gels (*E*) and immunoblot analysis (*F*) of identical gels labeled with anti-dystrophin antibodies and a second antibody coupled with alkaline phosphatase are shown. Lanes: S, molecular mass standards; 1–4, SDS-solubilized homogenates of normal human deltoid muscle, deltoid muscle from a 6-year-old child with DMD, chicken soleus muscle, and chicken gizzard muscle, respectively. The Coomassie-stained 400-kDa band contained other proteins in addition to the dystrophin molecule (22).

Isolation of Chicken Gizzard Muscle Dystrophin. We rapidly purified the chicken gizzard muscle dystrophin by passing a dystrophin-enriched membrane preparation through an affinity chromatography column made with the anti-dystrophin fragment C antibodies. The general procedure for isolating

dystrophin from chicken gizzard muscle is presented as a schematic diagram in Fig. 2. The subcellular fractionation was followed by Coomassie blue staining after SDS/PAGE and by immunoblot analysis (Fig. 2). Dystrophin was present in soluble and insoluble membrane fractions, which originated from one freshly dissected chicken gizzard homogenized in a low ionic strength solution containing a detergent (Triton X-100) and a strong anti-protease mixture (Fig. 2 *A* and *B*). Most of the dystrophin was found in the soluble fraction although a small amount remained in the insoluble membrane fraction with most of the myosin. Large amounts of filamin and actin were present in the pellet obtained from the previous soluble membrane fraction precipitated at 20% ammonium sulfate and solubilized at low ionic strength without Triton X-100 (Fig. 2*C*). This dystrophin-enriched fraction was then applied to the Sepharose 4B column coupled to the specific anti-dystrophin antibodies. After extensive washing with 0.5 NaCl, the protein adsorbed to the anti-dystrophin antibodies was eluted. Filamin was absent, as it had been removed by the high ionic strength column wash.

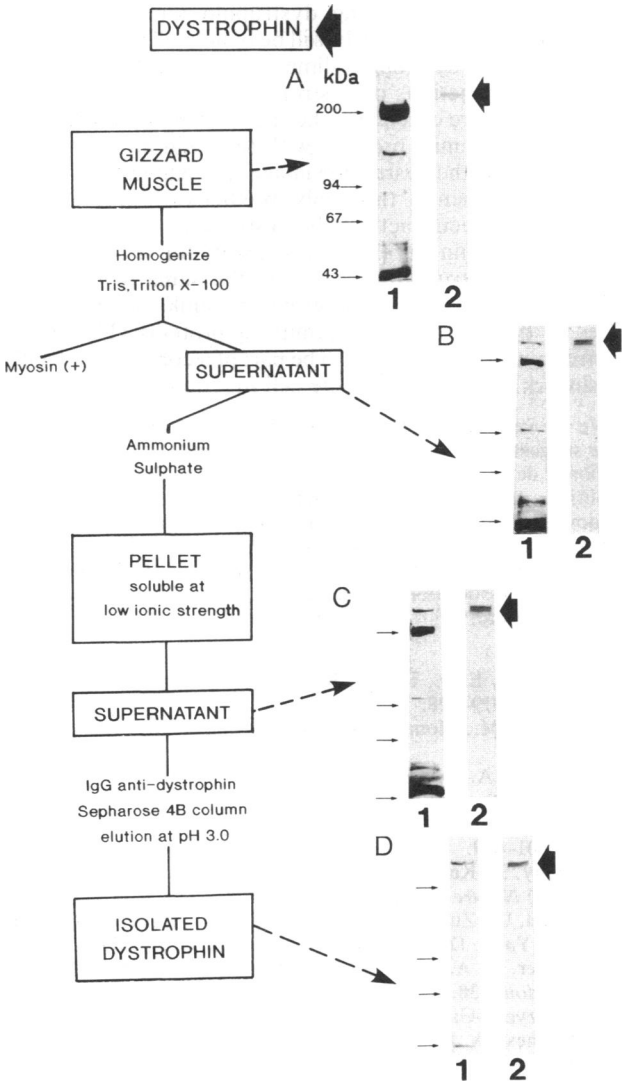

FIG. 2. Schematic diagram of subcellular fractionations of chicken gizzard muscle for isolating dystrophin. Details of data in *A–D* are described in the text. Identification of dystrophin in the subcellular protein fractions. Lanes: 1, Coomassie blue-staining lanes; 2, immunoblots of the same lanes with anti-dystrophin antibodies and a second antibody coupled with alkaline phosphatase. Arrows indicate the position of dystrophin.

In the final eluate, dystrophin and variable amounts of a 43-kDa polypeptide were detected by Coomassie blue staining (Fig. 2*D*). The 43-kDa band, which coeluted with dystrophin, comigrated with actin as detected by immunoblot analysis with an anti-actin monoclonal antibody (data not shown). The anti-dystrophin antibodies stained only the 400-kDa band of dystrophin.

EM Images of Dystrophin. Various preparations of chicken gizzard muscle dystrophin molecules, eluted from the anti-dystrophin affinity column, were next spread onto freshly cleaved mica and rotatory-shadowed at low angle with metal. The resulting EM images are presented in Fig. 3. The dystrophin molecules appeared as long flexible rod-shaped molecules exhibiting a wide variety of configurations relative to their lengths and thicknesses (Fig. 3 *A* and *B*). The majority of the rotatory-shadowed molecules observed, which we presume represent dystrophin monomers, appeared as relatively flexible rods (mean length, 175 ± 15 nm; $n = 80$) (Fig. 3*C*). The rod diameter was roughly constant over the entire length and yielded a value slightly higher than that of the myosin molecule rod [2 nm (29); Fig. 3*H*]. Some of these dystrophin monomers showed an enlargement at one end of their rod, a diameter reduction at the other end, or both characteristics. The enlargements did not always have the same shape. They sometimes appeared as single globular or helicoidal heads of variable sizes. In other images, two adjacent monomer chains were found to be beside one another over most of their lengths (mean length, 180 ± 15 nm; $n = 25$) (Fig. 3*D*). They will be referred to hereafter as side-by-side dystrophin dimers. Other images showed dys-

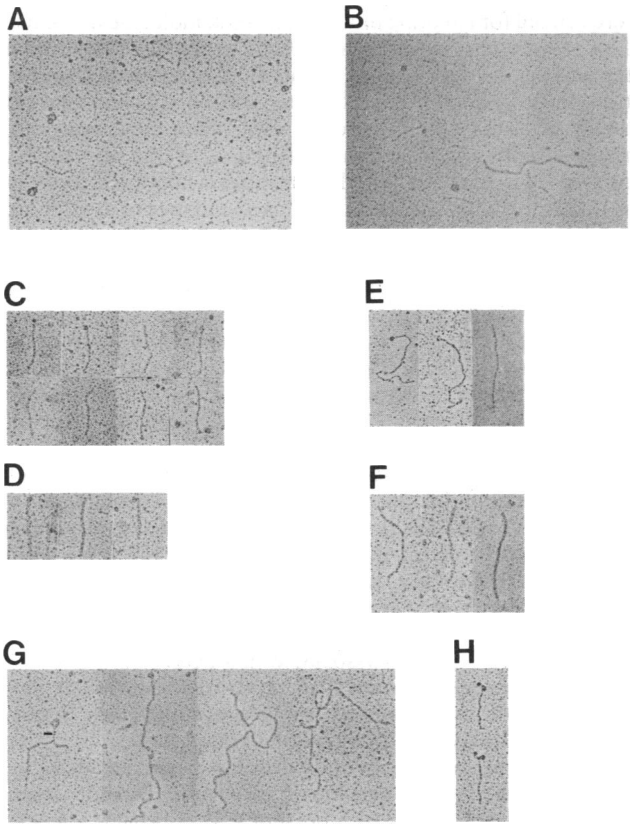

FIG. 3. EM images of isolated chicken gizzard muscle dystrophin after rotatory shadowing. (*A*) Individual dystrophin molecules. (*B*) Vicinity of a complex dystrophin arrangement. (*C*) Dystrophin monomers. (*D*) Side-to-side dystrophin dimers. (*E*) End-to-end dystrophin dimers. (*F*) Dystrophin tetramers. (*G*) Least frequent/complex dystrophin arrangements. (*H*) Myosin molecules, added as standard (29, 30). (×55,300.)

trophins that were twice the length of the previous monomers (345 ± 20 nm; $n = 35$) and either the same or double the thickness. They will be referred to hereafter as end-to-end dystrophin dimers or tetramers, respectively (Fig. 3 *E* and *F*). Enlargements, similar to those of monomers, were observed on the side-to-side and end-to-end dimers and on the tetramers. More complex configurations, representing higher levels of dystrophin aggregation, were also observed. Some, such as the one on the left of Fig. 3*G*, were highly informative. This dystrophin aggregate, given the lengths and thicknesses of simple dystrophin monomers, dimers, and tetramers, could be composed of at least three dystrophin molecules: one end-to-end dimer bound with one monomer. Both individual strands are twisted together to form an abnormally large head. Monomers and more complex oligomers were also detected in some dystrophin-enriched fractions as well as in the most purified dystrophin preparations containing very little (if any) of the contaminating 43-kDa component. However, no attempt was made to control the relative proportions of the various dystrophin oligomeric structures.

DISCUSSION

This paper presents EM images of isolated and rotatory-shadowed dystrophin molecules. The simple and rapid (3–5 hr) procedure presented here for preparing chicken gizzard muscle dystrophin is an adaptation of procedures for mammalian skeletal muscle dystrophins (21, 22). The chicken gizzard muscle dystrophin is at least partially solubilized in detergent solutions. Like its skeletal counterparts, smooth muscle dystrophin is highly prone to proteolytic breakdown. The use of various protease inhibitors and a reduced preparation time were crucial for isolating intact dystrophin molecules. The use of an affinity chromatography column in the last preparation step was critical for avoiding further time-consuming fractionation steps. This last preparation step involving a short acid pH treatment of the antibody-bound dystrophin may, however, induce changes in the native structure of the dystrophin molecule. The yield of eluted dystrophin is about 100 μg from the initial 10 g of gizzard muscle. This abundance of dystrophin in chicken gizzard muscle is, therefore, about 10-fold higher than that reported in human skeletal muscle (1, 7). The expression of dystrophin in smooth muscles was initially reported at the RNA level and it is presumed that the gene coding for it is the same as that coding for the DMD protein in skeletal muscle (1, 5, 7). Dystrophin has also been detected by immunofluorescence in rat stomach and aortic smooth muscle (32). We observed that, like the skeletal muscle dystrophin, chicken gizzard muscle dystrophin appears as a doublet of about 400 kDa even in total muscle homogenates (22). This dystrophin doublet could result from the rapid proteolysis of a single native 400-kDa dystrophin. It could also correspond to two isozymic forms of dystrophin coded by the same gene through an alternative splicing mechanism (7). Additional experimental evidence is still required to determine if dystrophin, like spectrin and actinin molecules, exists under highly similar isoforms that are associated in a heterodimeric way.

The EM images obtained from dystrophin molecules isolated from an avian smooth muscle are probably representative of the overall topology of all other muscle or tissue dystrophins, since all known dystrophin molecules actually have the same highly conserved structure. Despite some limitations of resolution, the present EM approach appears uniquely suitable for visualizing the large central rod-shaped domain of the molecule and its key role in the process of dystrophin oligomerization. The lengths measured for the dystrophin monomers are compatible with a structural arrangement of the repeat dystrophin segments in triple-barrel α-helices connected by short-turn regions, as earlier postulated for the repeat domains of spectrin or actinin (13, 14, 22). Such highly folded tertiary conformations for the central dystrophin region, corresponding to a ratio of about 2.0–2.4 kDa/nm, are confirmed here by the diameter values for the dystrophin monomers. Their diameters are slightly higher than those observed for the myosin rod, which is a rod-shaped double-helix structure of two 110-kDa regions that are 165 nm long (ratio, 1.4 kDa/nm) (29, 30). The topology of dystrophin aggregates observed here is close to that observed (15, 19, 20) for spectrin and actinin, at least under the conditions used for rotatory-shadowing experiments that can modify conformations of dystrophin or of any other structurally related molecules existing in solution. The least frequent complexes shown in Fig. 3 *B* and *G*, which correspond to a linear elongation process of the dystrophin side-to-side dimers beyond the tetramer structure, suggest that the end-to-end dystrophin association takes place by a head-to-tail rather than a head-to-head process. In contrast, it is unclear how each dystrophin monomeric chain is arranged in the side-to-side dimers. Additional information will be required to elucidate whether the antiparallel arrangements observed in the corresponding spectrin, actinin, or caldesmon dimers exist in the corresponding dystrophin dimers. Anti-dystrophin antibodies obviously will be of help. The nature of the arrangement of dystrophin dimers is important for defining the possible functions of dystrophin since such antiparallel arrangements are crucial for the regular interactions of other spectrin superfamily proteins with the actin molecule.

In summary, the dystrophin images presented demonstrate at the experimental (not only predictive) level that the dystrophin molecule actually has a structure similar to that of spectrin or actinin and the same capacity for self-association in oligomeric structures. By using the same (even further extended) mechanisms of internal crosslinks as other molecules of the spectrin superfamily of proteins (15, 19, 20), dystrophin molecules seem to be part of a complex molecular meshwork essential to muscle cell function.

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