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Dystrophin-Related Protein Is Localized to Neuromuscular Junctions of Adult Skeletal Muscle

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Summary

Dystrophin-related protein (DRP) is an autosomal gene product with high homology to dystrophin. We have used highly specific antibodies to the unique C-terminal peptide sequences of DRP and dystrophin to examine the subcellular localization and biochemical properties of DRP in adult skeletal muscle. DRP is enriched in isolated sarcolemma from control and mdx mouse muscle, but is much less abundant than dystrophin. Immunofluorescence microscopy localized DRP almost exclusively to the neuromuscular junction region in rabbit and mouse skeletal muscle, as well as mdx mouse muscle and denervated mouse muscle. DRP is also present in normal size and abundance and localizes to the neuromuscular junction region in muscle from the dystrophic mouse model *dy/dy*. Thus, DRP is a junction-specific membrane cytoskeletal protein that may play an important role in the organization of the postsynaptic membrane of the neuromuscular junction.

Introduction

The inherited neuromuscular disorder Duchenne muscular dystrophy (DMD) is characterized by a single gene defect on the X-chromosome. Dystrophin, the large protein product of the human DMD gene, is localized to the cytoplasmic face of the sarcolemma membrane in normal skeletal muscle (Arahata et al., 1988; Bonilla et al., 1988; Watkins et al., 1988; Zubrzycka-Gaarn et al., 1988), but is absent from skeletal muscle of DMD humans (Hoffman et al., 1987, 1988) and mdx mice (Bonilla et al., 1988; Hoffman et al., 1987). Sequence similarities between dystrophin and spectrin/α-actinin suggest that dystrophin is a membrane cytoskeletal protein (Koenig et al., 1988). Dystrophin, isolated from skeletal muscle membranes using wheat germ agglutinin (WGA) chromatography (Campbell and Kahl, 1989), was found to exist in a large, tightly associated oligomeric complex that contains four glycoproteins of 156, 50, 43, and 35 kd and a protein triplet at 59 kd (Ervasti et al., 1990). We have recently shown that components of the dystrophin–glycoprotein complex are highly enriched in purified sarcolemma from rabbit skeletal muscle and that dystrophin accounts for approximately 2%–5% of total sarcolemma protein (Ohlendieck and Campbell, 1991; Ohlendieck et al., 1991). These results suggest that dystrophin is a major constituent of the subsarcolemmal cytoskeleton in skeletal muscle.

Recently, Love et al. (1989) cloned from skeletal muscle an autosomal transcript similar in size and with high homology to dystrophin. Contrary to the localization of the DMD gene to chromosome Xp21, the dystrophin-related protein (DRP) gene maps to chromosome 6 (Love et al., 1989). Immunoblot analysis of whole skeletal muscle homogenates showed that the protein product of the DRP gene is similar in size to dystrophin (Khurana et al., 1990). Based on sequence homology, DRP was hypothesized to be a newly identified member of the spectrin/α-actinin/ dystrophin superfamily (Khurana et al., 1990; Love et al., 1989). Here we compare the subcellular localization and cytoskeletal properties of DRP and dystrophin using highly sequence-specific antibodies to the C-terminal sequences of both proteins. We report that DRP has a slightly higher relative molecular mass than dystrophin using comparative SDS-PAGE and immunoblot analysis of purified sarcolemma from normal and mdx mouse skeletal muscle. The relative density of DRP is approximately equal in both membrane preparations, but DRP is much less abundant than dystrophin in adult mouse muscle. Interestingly, immunofluorescence microscopy localized DRP almost exclusively to the neuromuscular junction region in normal skeletal muscle and dystrophin-deficient mdx mouse skeletal muscle. The dystrophic mouse model *dy/dy* was also found to contain DRP of normal size and abundance, suggesting that a mutation in the DRP gene is not the genetic basis for the autosomal neuromuscular disease dystrophia muscularis. Our results suggest that DRP represents a specialized form of dystrophin which is restricted to the membrane cytoskeleton underlying the neuromuscular junction.

Results

Specificity of Rabbit Antibodies to the C-Termini of DRP and Dystrophin

To study the subcellular localization and cytoskeletal properties of DRP and compare these findings with the properties of dystrophin, we developed highly specific antibodies to the C-terminal sequences of human dystrophin and human DRP. The amino acid sequences of the C-terminal peptides used for the production of specific polyclonal antibodies are shown in Figure 1a. The specificity of rabbit antisera against the C-terminus of dystrophin was previously established (Ervasti et al., 1990; Ohlendieck et al., 1991) and is illustrated by the fact that the antiserum strongly labels the dystrophin protein band of 400 kd on immunoblots but does not cross-react with spectrin or α-actinin. In addition, this antiserum does not stain mdx or DMD skeletal muscle, but does stain the entire cell

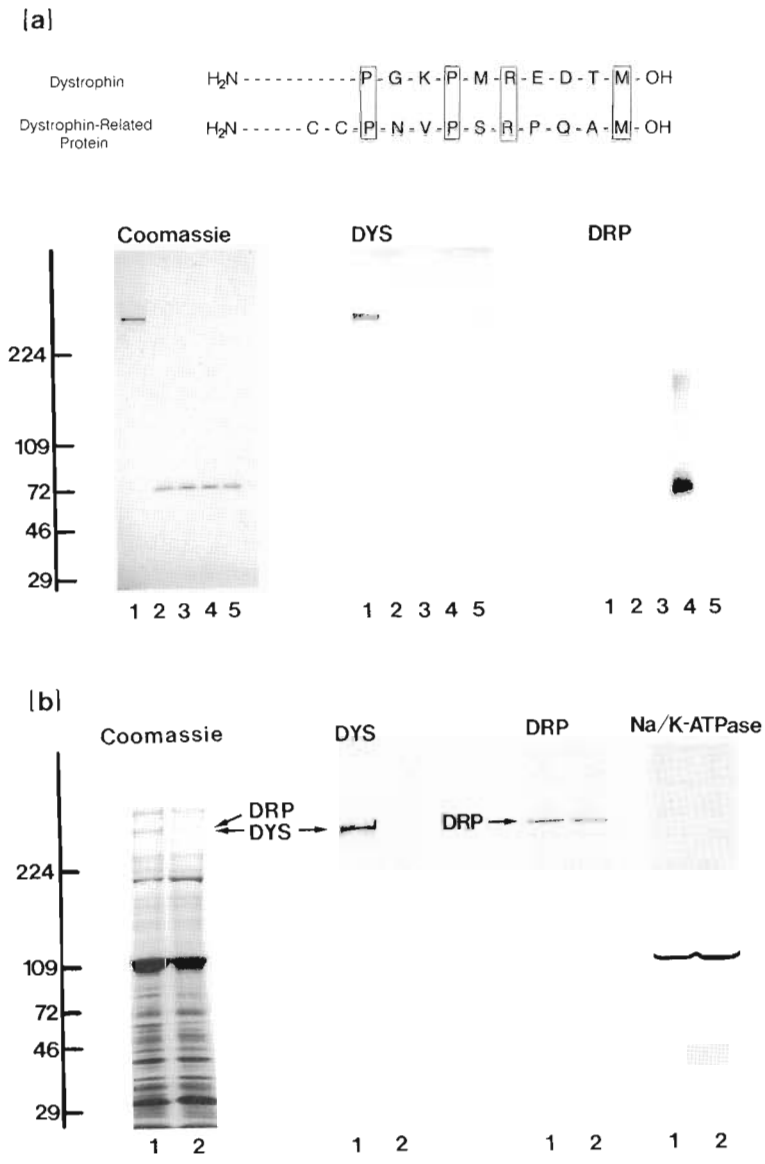


Figure 1. Characterization of Rabbit Antibody to the C-Terminus of DRP

(a) Comparison of the amino acid sequence of the C-termini of dystrophin and DRP. Shown is a Coomassie blue-stained gel and identical immunoblots labeled with rabbit antisera to the C-terminus of dystrophin (DYS) and the C-terminus of dystrophin-related protein (DRP). Lanes 1–5 contain 1 μg of pure dystrophin protein, BSA-conjugated C-terminus and N-terminus of dystrophin, BSA-conjugated C-terminus of DRP, and unconjugated BSA, respectively.

(b) Coomassie blue-stained gel and identical immunoblots labeled with rabbit antisera to the C-terminus of dystrophin (DYS), affinity-purified rabbit antibody against the C-terminus of dystrophin-related protein (DRP), and MAb McB2 against Na/K-ATPase. Lanes 1 and 2 consist of purified sarcolemma from normal control and mdx mouse skeletal muscle. Dystrophin is represented by a Coomassie blue-stained protein band of 400 kd in normal control mouse muscle only, whereas DRP is found in sarcolemma of normal control and mdx mouse muscle. Because of the absence of the dystrophin band in mdx mouse muscle sarcolemma, a faint Coomassie blue-stained protein band of slightly higher apparent molecular mass than dystrophin can be related to DRP when compared with the comigrating DRP band on immunoblots. Dystrophin and DRP bands are indicated by arrows. Molecular weight standards ($M_r \times 10^{-3}$) are indicated on the left.

periphery of normal skeletal muscle cells (Ervasti et al., 1990; Ohlendieck et al., 1991).

To establish the sequence specificity of rabbit antibodies against the C-terminus of DRP, immunoblot analysis was carried out with purified rabbit skeletal muscle dystrophin (Ervasti et al., 1991), BSA-conjugated C-terminus and N-terminus of dystrophin (Ervasti et al., 1991), and BSA-conjugated C-terminus of DRP, as well as unconjugated BSA (Figure 1a, Coomassie). Antibodies to the C-terminal decapeptide of dystrophin exclusively labeled purified dystrophin and the BSA-conjugated C-terminus of dystrophin and showed no immunological cross-reactivity with the N-terminus of dystrophin, the C-terminus of DRP, or BSA (Figure 1a, DYS). Antibodies to DRP recognized the DRP peptide; pure dystrophin, the C- and N-terminal peptide sequences of dystrophin, and BSA were not recognized (Figure 1a, DRP). Specificity of antisera to dystrophin and DRP was also shown using immunoblot analysis of crude extracts from muscle (data not shown). This high degree of specificity makes these antibodies very useful tools to study and compare the localization and properties of DRP with the known characteristics of dystrophin.

DRP in mdx Mouse Skeletal Muscle Sarcolemma

To identify and characterize DRP, sarcolemmal vesicles derived from rabbit skeletal muscle, as well as from normal control and mdx mouse skeletal muscle, were prepared by a WGA procedure as described (Ohlendieck et al., 1991) and probed with antibodies to DRP and dystrophin. Immunoblot analysis of rabbit skeletal muscle membrane fractions established that DRP is enriched in purified sarcolemma and not present in a WGA void fraction that contains vesicles derived from transverse tubules and sarcoplasmic retic-

ulum (Ohlendieck et al., 1991; data not shown). Comparative SDS-PAGE and immunoblot analysis of normal and mdx mouse skeletal muscle sarcolemma demonstrated that both membrane preparations have a similar protein composition (Figure 1b) and exhibit equal staining intensity for the surface membrane marker Na/K-ATPase (Figure 1b). Immunoblotting revealed that the 400 kd protein band of dystrophin present in normal control sarcolemma is absent from mdx sarcolemma (Figure 1b, DYS). DRP of slightly higher apparent molecular mass than dystrophin is found in approximately equal density in both membrane preparations, but is much less abundant than dystrophin (Figure 1b, DRP). The identification of DRP in isolated sarcolemma, which has been extensively washed with high salt to remove peripheral proteins (Ohlendieck et al., 1991), indicates that DRP is tightly associated with the sarcolemmal membrane, as are dystrophin and other membrane cytoskeletal proteins. The results of the immunoblot labeling correlate well with the Coomassie blue-stained SDS-PAGE profile of both sarcolemmal preparations. The dystrophin protein band of 400 kd is present exclusively in normal sarcolemma, whereas a faint protein band of slightly slower electrophoretic mobility than dystrophin in mdx sarcolemma comigrates with the DRP band on immunoblots (Figure 1b, DRP). DRP was also found to exhibit properties characteristic of a membrane cytoskeletal protein, i.e., DRP was found in the detergent-insoluble cytoskeletal fraction of skeletal muscle sarcolemma, whereas alkaline treatment removed DRP from the bilayer in the absence of detergent (data not shown).

DRP Is Localized to the Neuromuscular Junction

Immunofluorescence microscopy of skeletal muscle was carried out to investigate further the subcellular distribution of DRP. Muscle sections were double-labeled with fluorescein-conjugated antibodies and rhodamine-conjugated α-bungarotoxin. This allowed simultaneous visualization of the subcellular distribution of the proteins under investigation and the distribution of acetylcholine receptor, which is confined to the postsynaptic membrane of the neuromuscular junction (Anderson and Cohen, 1974). Immunofluorescence staining with each antibody was done under identical conditions, and photographs were obtained using the same exposure time. Rabbit antibodies to the C-terminus of DRP intensely labeled the neuromuscular junction region of mouse skeletal muscle (Figures 2a and 2e), as visualized by double labeling with α-bungarotoxin (Figures 2c and 2e). In contrast, antibodies to the C-terminus of dystrophin exhibit strong immunofluorescence staining of the entire skeletal muscle cell periphery and an increased labeling of the neuromuscular junction region (Figures 2b and 2f).

The specificity of each antibody was confirmed by peptide competition experiments. DRP labeling of the neuromuscular junction region was not affected by incubation with dystrophin peptide, but was completely suppressed by the presence of DRP peptide (Figures 3a and 3b). On the other hand, dystrophin peptide inhibits the staining of rabbit antibodies to dystrophin, but DRP peptide had no effect on the labeling of the cell periphery or neuromuscular junction by dystrophin antibodies (Figures 3c and 3d).

Comparison of dystrophin staining (Figure 4c) to the immunofluorescence labeling of a series of other sarcolemmal markers (Figures 4e, 4g, and 4i), which were previously characterized by extensive immunofluorescence and immunoblot analysis (Ohlendieck et al., 1991), shows that the increased staining of the neuromuscular junctions is a property of all the sarcolemmal markers investigated. This comparative immunofluorescence study was performed with rabbit tissue because the monoclonal antibodies (MAbs) against the sarcolemmal markers did not immunologically cross-react with mouse skeletal muscle. The relative position of neuromuscular junctions in cryosections was visualized by α-bungarotoxin staining (Figures 4b, 4d, 4f, 4h, and 4j). In comparison, DRP localizes exclusively to the neuromuscular junction region (Figure 4a), suggesting a true restriction of DRP in the neuromuscular junction of skeletal muscle cells.

Immunofluorescence localization of DRP and dystrophin in normal and mdx mouse muscle was performed to investigate the distribution of DRP in muscle with no dystrophin. For direct comparison, cryosections of quadriceps femoris from both normal and mdx mouse muscle were placed on the same microscope slide and treated in the same way during all incubation and washing steps. Double labeling with α-bungarotoxin and rabbit antibodies to DRP revealed that DRP is highly enriched in the neuromuscular junction of normal control and mdx mouse skeletal muscle (Figure 5a). In comparison, dystrophin antibodies did not stain sarcolemma or neuromuscular junction in mdx mouse muscle (Figure 5c), although neuromuscular junctions appear normal in mdx mouse muscle (Figure 5d). Occasionally the double labeling technique revealed weak immunofluorescence staining for DRP in the cell periphery located near the bright labeling of neuromuscular junction region of cryosections from older (20-week-old) mdx mice (data not shown).

DRP Is Localized to the Postsynaptic Membrane of the Neuromuscular Junction

To determine whether DRP is associated with the pre- or postsynaptic membrane of the neuromuscular junction region in skeletal muscle, DRP was examined in control and denervated muscle. Mouse skeletal muscle was surgically denervated and immunocytochemically labeled for DRP after 3, 7, and 21 days. Figure 6a illustrates the results of immunofluorescence labeling for DRP after 21 days. Neuromuscular

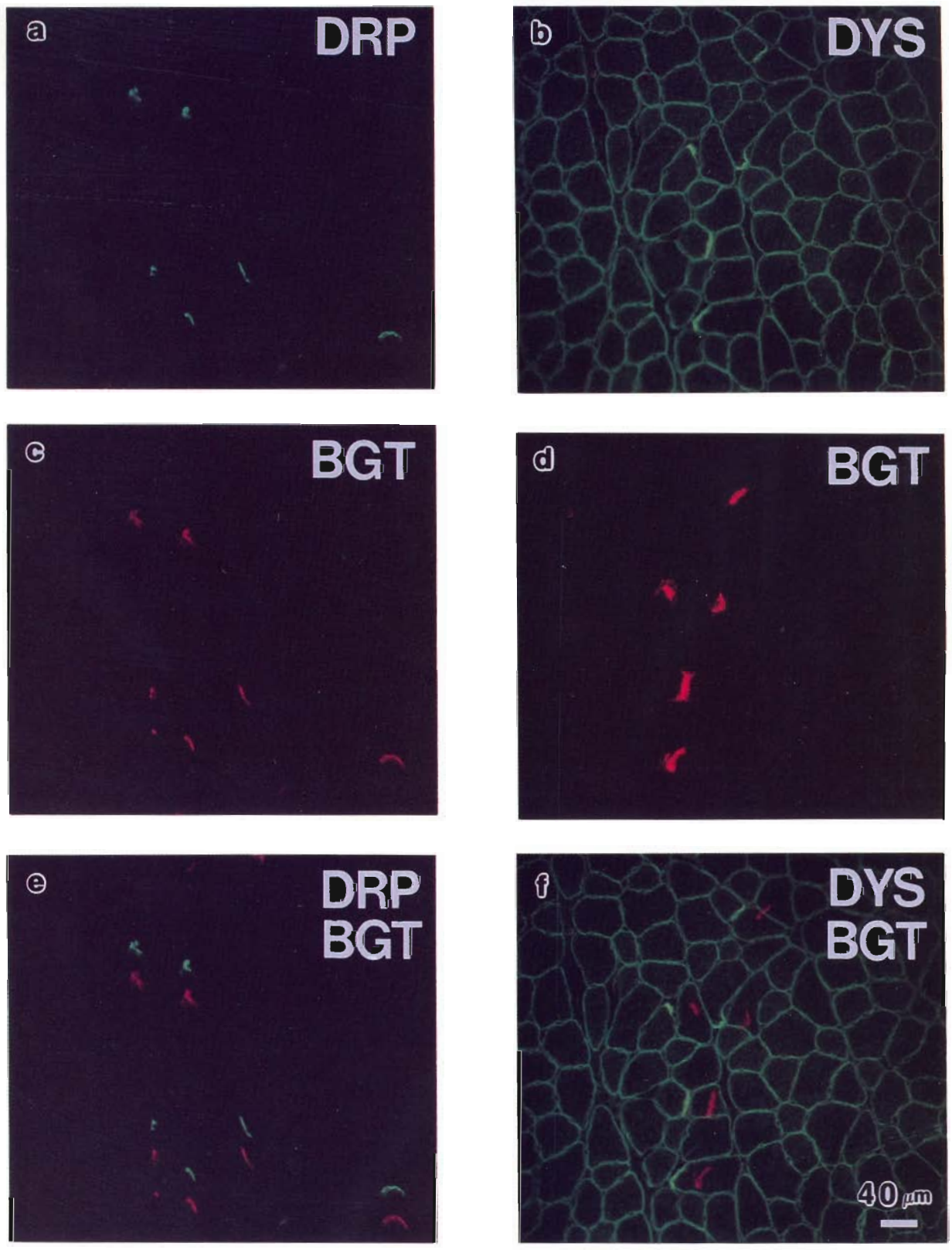


Figure 2. Distribution of DRP and Dystrophin in Mouse Skeletal Muscle

Immunofluorescence labeling of transverse cryosections of the muscle midsection from quadriceps femoris, stained with secondarily fluorescein-labeled rabbit antibodies to the C-terminus of dystrophin-related protein (DRP) (a) and the C-terminus of dystrophin (DYS) (b). To outline the neuromuscular junction regions, sections were simultaneously stained with rhodamine-labeled α-bungarotoxin (BGT)

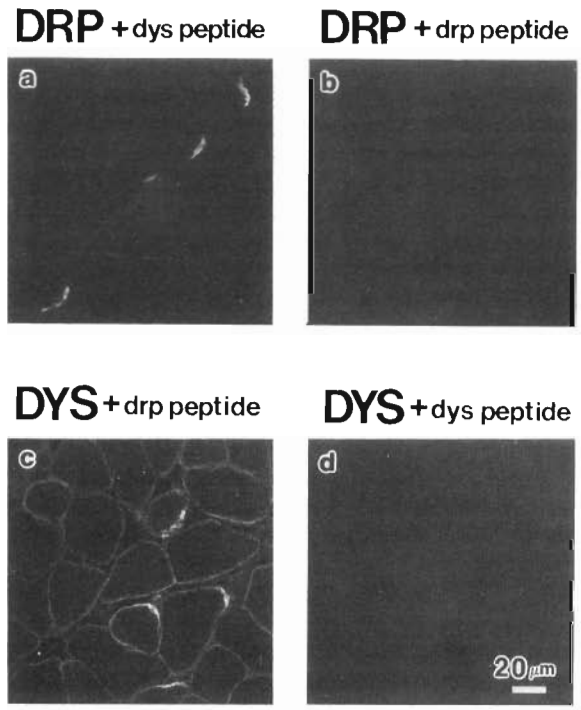


Figure 3. Specificity of Rabbit Antibody to the C-Termini of Dystrophin and DRP

Cryosections from normal mouse quadriceps femoris were labeled with preincubated antibody solutions. Antibodies against the C-termini of dystrophin-related protein (DRP) (a and b) and dystrophin (DYS) (c and d) were incubated with BSA-conjugated DRP peptide (drp) (b and c) or dystrophin peptide (dys) (a and d) to estimate the specificity of the antibodies and to account for any cross-reactivity between them. Labeling of both antibodies was suppressed only by its competitive peptide and not by the control peptide, demonstrating the extremely high degree of specificity and the lack of immunological cross-reactivity between the two different rabbit antibodies.

junction regions were visualized by double labeling with α-bungarotoxin (Figure 6c). In all three cases, after denervation DRP staining remained associated with the neuromuscular junction region, which strongly suggests that this cytoskeletal protein is localized to the postsynaptic membrane and not the presynaptic nerve ending.

DRP in Dystrophic *dy/dy* Mice

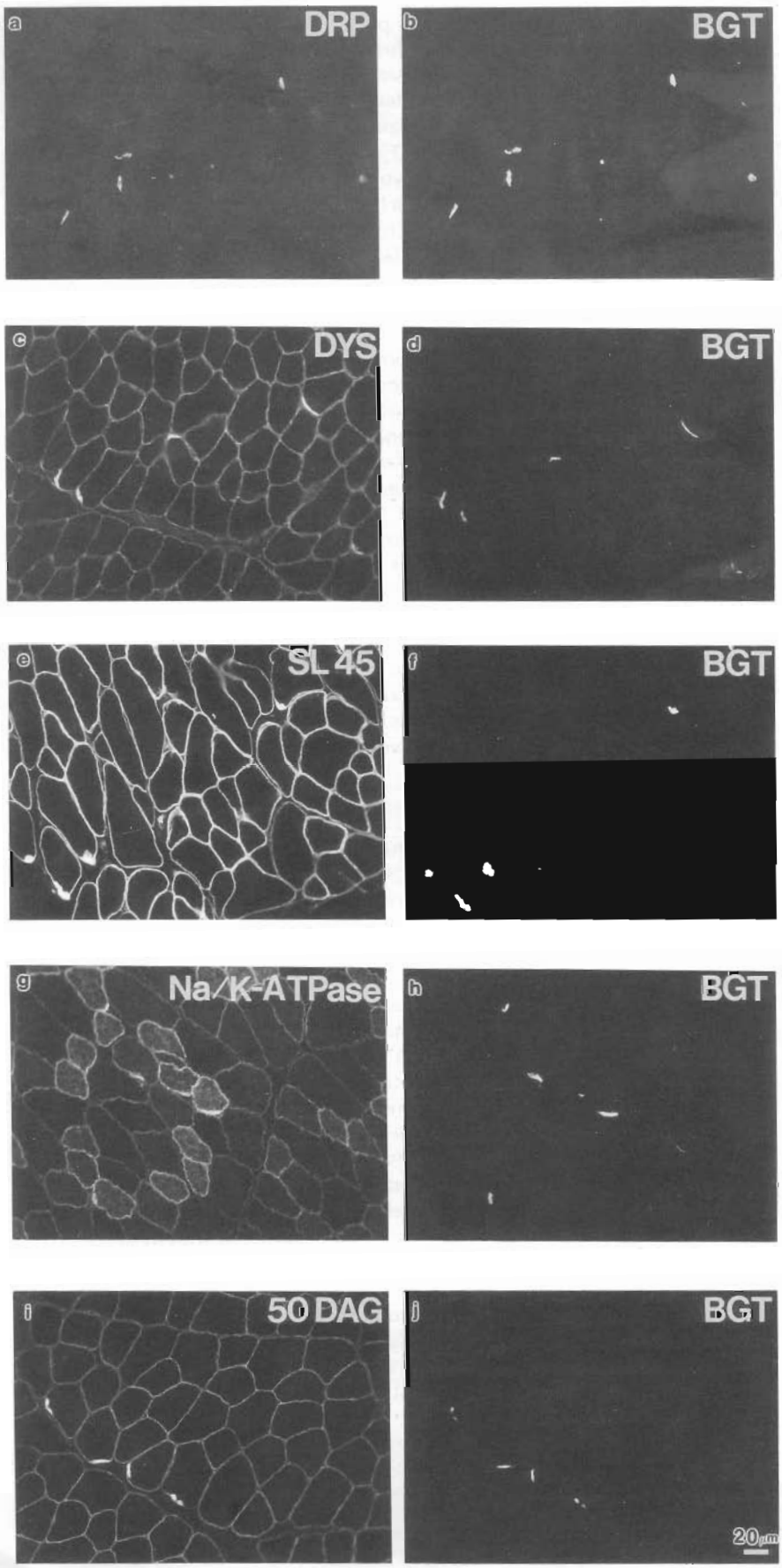
The sequence homology between DRP and dystrophin suggests that a genetic defect in the expression of DRP could be the basis of an autosomal recessive neuromuscular disorder. Buckle et al. (1990) recently localized the autosomal gene for DRP to chromosome 6 in humans and chromosome 10 in mice. The genetic locus for the neuromuscular disorder dystrophia muscularis was previously assigned to the same region of mouse chromosome 10. Therefore we investigated the expression of DRP in dystrophic muscle of the animal model *dy/dy* mouse, which presents a progressive necrotizing myopathy similar to human muscular dystrophy (Figure 6; Figure 7). Histological examination of cryosections from *dy/dy* mouse muscle, stained with hematoxylin and eosin, revealed muscle fibers with rounded contours, marked variability of fiber size diameter, increased amount of fibers with central nucleation, and increased interstitial connective tissue (data not shown) typical for dystrophic *dy/dy* mouse muscle (Bray and Banker, 1970). Immunofluorescence microscopy showed that antibodies to DRP strongly labeled the neuromuscular junction region in *dy/dy* muscle cryosections (Figure 6b). Immunoblot analysis showed that DRP is found in approximately equal amounts and is of comparable relative molecular mass in control and *dy/dy* muscle membranes. Furthermore, components of the dystrophin-glycoprotein complex (dystrophin, 156-DAG) and proteins involved in excitation-contraction coupling (ryanodine and dihydropyridine receptors), as well as albumin and the plasma membrane marker Na/K-ATPase, are equally abundant in control and *dy/dy* mouse muscle (Figure 7). Thus, DRP is present in normal size and abundance in *dy/dy* muscle membranes and is localized to the neuromuscular junction region in cryosections of *dy/dy* mouse muscle.

Discussion

An affinity-purified polyclonal antibody to the C-terminus of DRP was found to be highly specific for DRP and showed no cross-reactivity with dystrophin. This specificity enabled us to learn more about the cytoskeletal properties and subcellular localization of DRP in skeletal muscle and compare it with the much better characterized dystrophin. The DRP protein band, detectable as a faint but discrete Coomassie blue-stained band in purified sarcolemma of dystrophin-deficient mdx mouse skeletal muscle, has a slightly higher apparent molecular mass than dystrophin, which agrees with previous findings (Khurana et al., 1990). SDS-PAGE and immunoblot analysis of cytoskeletal fractions from sarcolemma of adult skeletal muscle revealed that DRP has similar cytoskeletal properties when compared with dystrophin, but is present in much lower abundance.

Since the first identification of dystrophin (Hoffman et al., 1987), several reports described dystrophin-like proteins (Chang et al., 1989; Fardeau et al., 1990; Hoffman et al 1989; Ishiura et al., 1990; Jasmin et al., 1990; Khurana et al., 1990; Love et al., 1989; Tanaka et al.,

(c and d). Micrographs (e) and (f) are double exposed and show simultaneously red rhodamine fluorescence for α-bungarotoxin and slightly shifted green immunofluorescence for DRP or dystrophin. DRP localized exclusively to the neuromuscular junction region; whereas dystrophin labeled the entire cell periphery and the neuromuscular junction region.


a
DRP
b
BGT
c
DYS
d
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e
SL 45
f
BGT
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Na/K-ATPase
h
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50 DAG
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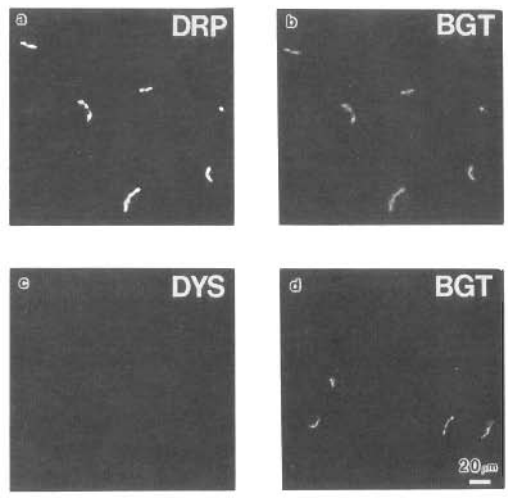

Figure 5. Immunofluorescence Labeling of Skeletal Muscle Cryosections from mdx Mice with Antibody to the C-Terminus of DRP

Cryosections of the midsection of control mdx mouse quadriceps femoris were fluorescently double-labeled with α-bungarotoxin (BGT) (b and d) and rabbit antibodies to the C-termini of dystrophin-related protein (DRP) (a) or dystrophin (DYS) (c). DRP strongly labeled the neuromuscular junction region of mdx mouse muscle sections. In comparison, dystrophin exhibited no staining of mdx mouse cryosections.

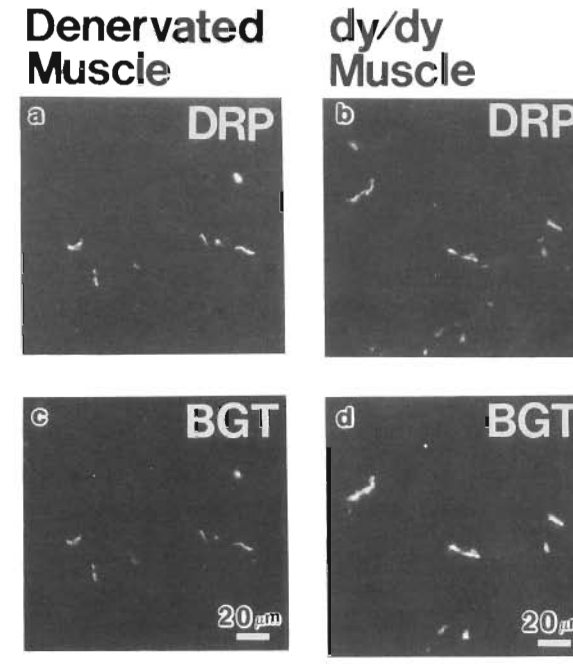

Figure 6. Immunofluorescence Labeling of Cryosections from Denervated Mouse Skeletal Muscle and *dy/dy* Mouse Skeletal Muscle with Antibody to the C-Terminus of DRP

Cryosections of lower leg muscles from denervated (a and c) and *dy/dy* (b and d) mice were fluorescently double-labeled with α-bungarotoxin (BGT) (c and d) and rabbit antibodies to the C-terminus of dystrophin-related protein (DRP) (a and b). DRP labeled the neuromuscular junction regions of both denervated and *dy/dy* mouse skeletal muscle.

1989). A 400 kd protein with homology to dystrophin was found in the electric organ of Torpedo (Chang et al., 1989) closely associated with acetylcholine receptor-rich membranes (Jasmin et al., 1990). The highly sequence-specific antibodies raised in this study enabled a reproducible and objective comparison of DRP and dystrophin. Our results suggest that DRP is a specialized form of dystrophin in mammalian muscle that could play an important role in the structure and function of the neuromuscular junction. DRP appears to be tightly associated with the neuromuscular junction and is found in the postsynaptic membrane. It could therefore possibly link membrane proteins of the neuromuscular junction to the underlying membrane cytoskeleton.

The genetic locus for the DRP gene was recently assigned to the same region as the neuromuscular disorder dystrophia muscularis on mouse chromosome 10 (Buckle et al., 1990), suggesting that a genetic defect in the DRP gene could possibly be the basis of this disorder. Our immunoblot analysis clearly demonstrates that DRP is present in normal abundance and size in membranes prepared from *dy/dy* mice. Furthermore, immunofluorescence microscopy demonstrated that DRP localizes to the neuromuscular junction region of *dy/dy* muscle cryosections. Therefore, we conclude that the suppressed expression of DRP based on a mutation in the DRP gene, comparable to the loss of dystrophin in DMD, is not the cause for the autosomal neuromuscular disorder dystrophia muscularis.

The more intense labeling of the neuromuscular junction with antibodies to dystrophin and other established sarcolemmal markers, compared with the equally distributed staining of the entire cell periphery, could possibly be due to increased folding of the skeletal muscle surface membrane and not to a true

Figure 4. Distribution of DRP and Dystrophin in Rabbit Skeletal Muscle

Immunofluorescence labeling of transverse cryosections of the muscle midsection from quadriceps femoris, stained with rabbit antibodies to the C-terminus of dystrophin-related protein (DRP) (a) and the C-terminus of dystrophin (DYS) (c), MAb XVB9 against sarcolemmal protein of 45 kd(SL45) (e), MAb McB2 against the α2 subunit of Na/K-ATPase (g), and MAb IVD3$_1$ against DAG of 50 kd (50DAG) (i). For the purpose of fluorescence double labeling, sections were simultaneously stained with rhodamine-labeled α-bungarotoxin (BGT) (b, d, f, h, and j). DRP localized exclusively and very intensively to the motor endplate region. Dystrophin and the other three sarcolemmal markers exhibited, besides strong and evenly intensive staining of the cell periphery, a more intense labeling of the neuromuscular junction region.

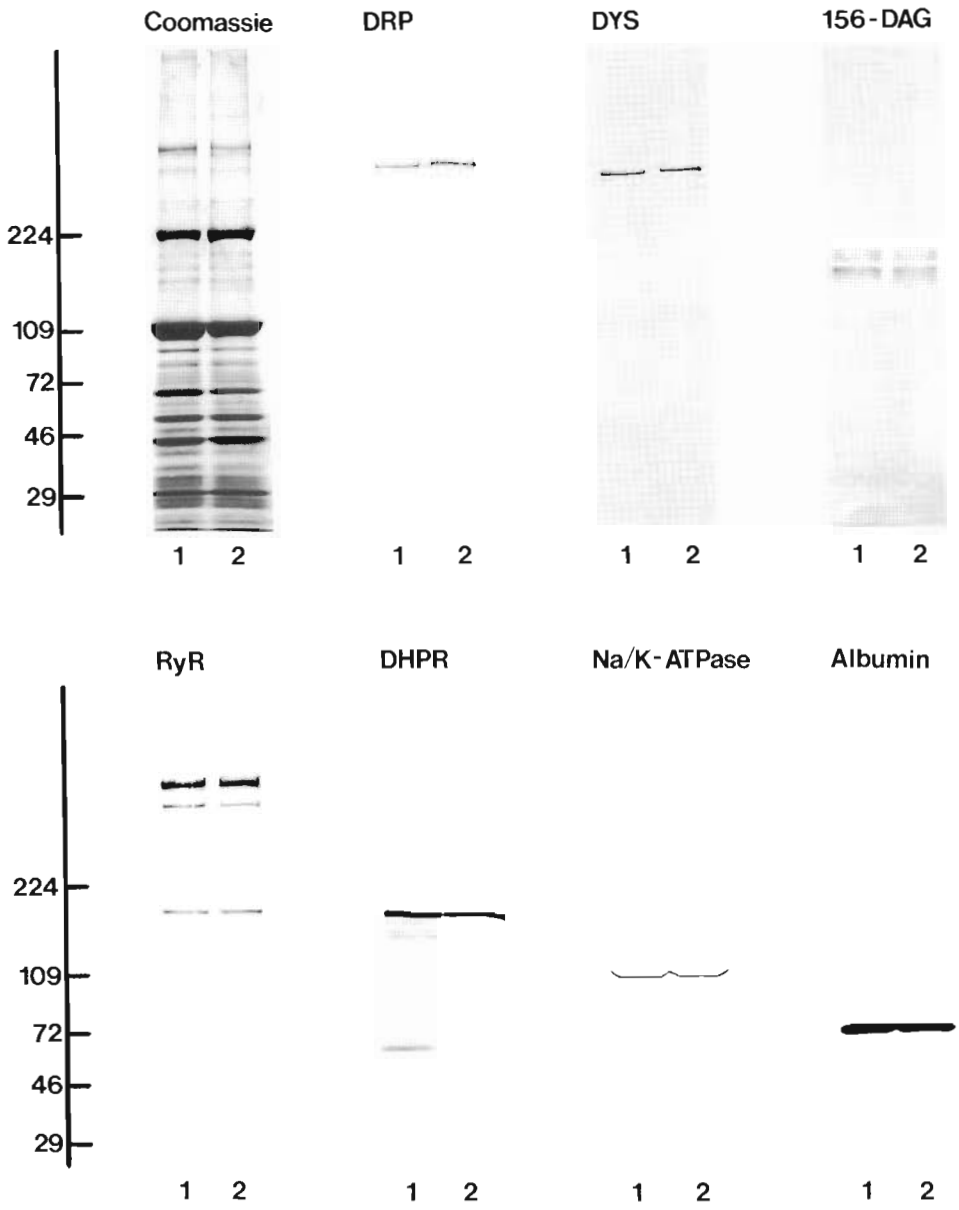

Figure 7. Immunoblot Analysis of Membranes from Control and *dy/dy* Mice

Coomassie blue-stained gel and identical immunoblots, labeled with rabbit antibodies to the C-terminus of dystrophin-related protein (DRP) and dystrophin (DYS), MAb VIA4$_1$ to dystrophin-associated glycoprotein of 156 kd (156-DAG), polyclonal sheep antibodies to the ryanodine receptor (RyR), dihydropyridine receptor (DHPR), albumin, and MAb McB2 to the Na/K-ATPase. Lanes 1 and 2 contain membranes from normal and *dy/dy* mouse muscle, respectively. Molecular weight markers ($M_r \times 10^{-3}$) are indicated on the left.

enrichment of these sarcolemmal proteins in the neuromuscular junction. The ultrastructure of the neuromuscular junction clearly reveals the fine structure of junctional folds in the postsynaptic muscle surface membrane (Birks et al., 1960). Recently, Sanes et al. (1990) reported that antibodies to the basal lamina component laminin-B2 stained synaptic sites more intensely than extrasynaptic areas, which probably reflects an increased amount of basal lamina in synaptic junctional folds (Sanes and Chiu, 1983). Thus, the question of whether dystrophin is equally distributed between synaptic and extrasynaptic regions cannot be fully answered using immunofluorescence microscopy techniques. However, our results demonstrate that DRP is truly restricted to the neuromuscular junction of skeletal muscle cells because of its intense and almost exclusive labeling of the neuromuscular junction in immunofluorescence microscopy. Therefore, DRP may be a specialized form of dystrophin that could play an important role in the integrity of the neuromuscular junction during muscle contraction, as hypothesized for dystrophin in the muscle surface membrane (Koenig and Kunkel, 1990).

Differences in the subcellular distribution of re-

lated muscle proteins are also found for basal lamina components in skeletal muscle. Sanes et al. (1990) described molecular heterogeneity of basal lamina isoforms of laminin and collagen IV at the neuromuscular junction. Isoform levels of the different basal lamina components studied showed widespread differences between synaptic and extrasynaptic basal lamina. The specific localization of S-laminin at the neuromuscular junction (Sanes et al., 1990) is comparable to the restricted localization of DRP at the neuromuscular junction region. This is an interesting analogy because DRP is a cytoskeletal component and S-laminin is associated with the extracellular matrix.

In conclusion, we find that the chromosome 6–encoded dystrophin-related protein is localized to the postsynaptic membrane of the neuromuscular junction. DRP has a slightly higher apparent molecular mass than dystrophin and exhibits cytoskeletal properties comparable to those of dystrophin, but it is less abundant in isolated sarcolemma from adult muscle. Most importantly, DRP is localized to neuromuscular junctions of control and mdx mouse skeletal muscle. Our results suggest that the mdx mouse muscle could be very useful for future studies to isolate DRP and to characterize interaction of DRP with other proteins in the neuromuscular junction. The localization and abundance of DRP suggest that it may play an important cytoskeletal function in the neuromuscular junction. Although DRP is most likely not involved in the neuromuscular disorder dystrophia muscularis, a defect in the DRP gene could possibly be the genetic basis of another autosomal neuromuscular disease involving the neuromuscular junction.

Experimental Procedures

Isolation of Skeletal Muscle Sarcolemma

Highly purified rabbit sarcolemma was isolated by a newly developed WGA procedure as described (Ohlendieck et al., 1991). MAbs specific for protein components of the surface membrane of rabbit skeletal muscle were used as markers in the isolation and characterization of skeletal muscle sarcolemma (Ohlendieck et al., 1991). Mouse skeletal muscle sarcolemma from age-matched, normal control mice and dystrophin-missing mdx mice was isolated by the same WGA procedure as described for rabbit skeletal muscle (Ohlendieck et al., 1991). Purification was monitored by the enrichment of the plasma membrane marker Na/K-ATPase.

Membrane Preparation from Skeletal Muscle

Crude microsomes were prepared from control and dystrophic *dy/dy* mice (C57BL/6J-dy) obtained from Jackson Laboratory, Bar Harbor, ME. Hindleg and back muscles were dissected as quickly as possible and homogenized by a Polytron (Kinematic GmbH, Luzern, Switzerland) in the presence of a protease inhibitor cocktail (Ohlendieck et al., 1991) to minimize protein degradation. KCl-washed membranes were subsequently prepared as described (Ohlendieck et al., 1991).

Antibodies

Polyclonal antisera against chemically synthesized peptides representing either the C-terminal 12 amino acids (CCPNVPSRPQAM) of the chromosome 6–encoded dystrophin-related protein (Love et al., 1989) or the last 10 carboxy-terminal amino acids (PGKPMREDTM) of the predicted human skeletal muscle dystrophin sequence (Koenig et al., 1988) were raised in New Zealand White rabbits (Ervasti et al., 1990; Ohlendieck et al., 1991) and affinity purified against the synthetic peptides as described by Sharp and Campbell (1989). Characterization of MAbs against sarcolemmal markers SL-45, 50-DAG (SL-50), and 156-DAG (SL-156) by immunofluorescence and immunoblot analysis was described previously (see Table I of Ohlendieck et al., 1991). Sheep polyclonal antibodies to skeletal muscle ryanodine and dihydropyridine receptors were previously characterized (Ervasti et al., 1990). MAb McB2 against Na/K-ATPase (Urayama et al., 1989) was a generous gift from Dr. Kathleen Sweadner, Harvard Medical School, Boston, MA. Sheep antiserum to albumin was obtained from Sigma Chemical Co.

Gel Electrophoresis and Immunoblot Analysis

Protein samples were fractionated on 3%–12% gradient SDS polyacrylamide gels (Laemmli, 1970) and stained with Coomassie blue, or transferred to nitrocellulose paper (Towbin et al., 1979) and stained with antibodies as described (Ervasti et al., 1990). Prestained molecular weight standards were from Bethesda Research Laboratories. Apparent molecular masses are as follows: myosin, 224 kd; phosphorylase b, 109 kd; albumin, 72 kd; ovalbumin, 46 kd; carbonic anhydrase, 29 kd. Protein was determined according to Peterson (1977) using BSA as standard. Coomassie blue–stained gels contained 50 μg of protein in each lane; immunoblots contained 150 μg of protein in each lane. Purification of dystrophin and preparation of BSA-conjugated peptides of the C-terminus and N-terminus of dystrophin and the C-terminus of DRP were carried out as recently described (Ervasti et al., 1991).

Immunofluorescence Microscopy

Immunofluorescence microscopy of 8 μm transverse cryosections from rabbit and mouse skeletal muscle was performed as described previously (Ohlendieck et al., 1991). The midsection of quadriceps femoris was carefully dissected to obtain cryosections enriched in motor endplate regions. Transverse cryosections of skeletal muscle were preincubated for 20 min with 5% normal goat serum in PBS (50 mM sodium phosphate [pH 7.4], 0.9% NaCl), followed by a 1 hr incubation at 37°C with the primary antibody (1:10 dilution of hybridoma supernatant, 1:50 dilution of affinity-purified antibodies, or 1:1000 dilution of crude antisera). After washing in PBS, the sections were double-labeled with a 1:1000 dilution of rhodamine-labeled α-bungarotoxin (Molecular Probes) and a 1:100 dilution of affinity-purified fluorescein-labeled goat anti-mouse, goat anti-rabbit, or rabbit anti-sheep IgG (Boehringer-Mannheim). Sections were subsequently examined in a Zeiss Axioplan fluorescence microscope. In the case of nonspecific background staining due to fluorescently labeled secondary antibodies, a biotin–streptavidin system was employed for immunodetection. Affinity-purified primary antibodies were biotinylated according to the instructions in the commercially available biotinylation kit from Amersham. Cryosections were incubated with biotinylated primary antibody, as already described for unlabeled primary antibody, and then extensively washed in PBS. Finally, sections were fluorescently labeled by incubation with a 1:100 dilution of affinity-purified fluorescein-conjugated avidin (Sigma Chemical Co.). Fluorescently labeled α-bungarotoxin was used as an established marker for visualizing the distribution of acetylcholine receptor, which is confined to the neuromuscular junction and associated with the postsynaptic membrane (Anderson and Cohen, 1974). For recording fluorescence double labeling on the same photograph, colored film was exposed with fluorescein immunofluorescence without advancing the film roll. Subsequently, the fluorescence image was slightly shifted to the left and reexposed with rhodamine fluorescence to outline α-bungarotoxin staining in the same micrograph.

To test the specificity and the possible cross-reactivity of the two different rabbit antibodies raised against the C-termini of DRP and dystrophin, each 1 ml of sufficiently diluted antibody solution was incubated for 30 min at room temperature with 0.1 mg of pure BSA-conjugated peptide. Antibodies to DRP were incubated with competitive DRP peptide and control dystrophin

peptide; antibodies to dystrophin were incubated with competitive dystrophin peptide and control DRP peptide. Cryosections from normal mouse skeletal muscle were subsequently incubated with the antibody-peptide solutions as already described.

Denervated mice were a generous gift from Dr. John P. Merlie, Washington University School of Medicine, St. Louis, MO. The left hindleg of mice was denervated by cutting the sciatic nerve at the upper portion of the thigh, and the untreated right leg was used as a control. Following denervation (3, 7, and 21 days), control and denervated muscles of the lower leg were carefully dissected and prepared for immunocytochemical analysis of DRP.

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