Dystrophin-associated Proteins Are Greatly Reduced in Skeletal Muscle from mdx Mice

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Abstract. Dystrophin, the protein product of the human Duchenne muscular dystrophy gene, exists in skeletal muscle as a large oligomeric complex that contains four glycoproteins of 156, 50, 43, and 35 kD and a protein of 59 kD. Here, we investigated the relative abundance of each of the components of the dystrophin-glycoprotein complex in skeletal muscle from normal and mdx mice, which are missing dystrophin. Immunoblot analysis using total muscle membranes from control and mdx mice of ages 1 d to 30 wk found that all of the dystrophin-associated proteins were greatly reduced (80–90%) in mdx mouse skeletal muscle. The specificity of the loss of the dystrophin-associated glycoproteins was demonstrated by the finding that the major glycoprotein composition of skeletal muscle membranes from normal and mdx mice was identical. Furthermore, skeletal muscle membranes from the dystrophic dy/dy mouse exhibited a normal density of dystrophin and dystrophin-associated proteins. Immunofluorescence microscopy confirmed the results from the immunoblot analysis and showed a drastically reduced density of dystrophin-associated proteins in mdx muscle cryosections compared with normal and dy/dy mouse muscle. Therefore, our results demonstrate that all of the dystrophin-associated proteins are significantly reduced in mdx skeletal muscle and suggest that the loss of dystrophin-associated proteins is due to the absence of dystrophin and not due to secondary effects of muscle fiber degradation.

THE X-linked recessive neuromuscular disorder Duchenne muscular dystrophy (DMD)[1] is the most common and devastating of the human muscular dystrophies (4, 15). Dystrophin, the high molecular weight protein product of the human DMD gene (19, 24, 25), localizes to the cell periphery of normal skeletal muscle, while biopsy specimens of DMD patients are characterized by a complete absence of dystrophin (3, 5, 20, 42, 45). The predicted primary structure of dystrophin and its subcellular localization suggest that dystrophin is a membrane-associated cytoskeletal protein of the skeletal muscle sarcolemma (25). Early histopathological events in DMD are characterized by persistent skeletal muscle necrosis. In comparison, the animal model mdx mouse (8) is also missing dystrophin (5, 19) due to a point mutation in the dystrophin gene (38) and exhibits necrosis of skeletal muscle fibers (39). The absence of dystrophin accompanied by skeletal muscle necrosis makes the mdx mouse an excellent model system to study how muscle fiber necrosis is caused by the lack of dystrophin. In addition, the mdx mouse is currently being used in the development of potential therapies for the replacement of dystrophin (35).

Dystrophin exists in a large oligomeric complex tightly associated with four glycoproteins of 35, 43, 50, and 156 kD and a protein component at 59 kD (9, 17). Components of the dystrophin–glycoprotein complex are highly enriched in the sarcolemma from rabbit skeletal muscle (34) and dystrophin comprises 5% of total cytoskeletal protein of sarcolemma (32). Recent biochemical analysis of the membrane organization of the dystrophin–glycoprotein complex revealed that the dystrophin-associated glycoproteins (DAGs) of 35, 43, and 50 kD are integral membrane proteins (16). Dystrophin and its 59-kD associated protein were found to be cytoskeletal elements that are tightly linked to a heteromeric integral membrane complex, which spans the sarcolemma membrane and binds tightly to an extracellular glycoprotein of 156 kD (16). These results suggest that the membrane-spanning dystrophin–glycoprotein complex could have an important structural role in the muscle cell periphery.

It is known from the study of other genetic diseases involving the cytoskeleton that a deficiency in a major cytoskeletal component can be accompanied by the loss of other cytoskeletal components. For example, spectrin deficiency in hereditary elliptocytosis is also associated with a reduced abundance in protein-4.1 and minor sialoglycoproteins (1). In view of these findings, it is important to investigate the status of dystrophin-associated proteins (DAPs) in mdx skeletal muscle fibers that are missing dystrophin. Previ-

1. *Abbreviations used in this paper*: DAG, dystrophin-associated glycoprotein; DAP, dystrophin-associated protein; DMD, Duchenne muscular dystrophy; DRP, dystrophin-related protein; WGA, wheat germ agglutinin.

ously, we have shown that the dystrophin-associated glycoprotein of apparent 156 kD (156-DAG) was significantly reduced in membranes from mdx mice (17). Here, we investigate the abundance of all the DAPs using affinity-purified antibodies. DAPs were found to be drastically reduced in membranes from dystrophin-deficient mdx mouse muscle compared with control and dy/dy mouse muscle, which has dystrophic fibers but contains dystrophin (2). Immunofluorescence microscopy confirmed that the density of DAPs is greatly reduced in skeletal muscle cryosections from mdx mice. These data support the hypothesis that the absence of dystrophin leads to a deficiency in all of the components of the dystrophin–glycoprotein complex and suggest that a lack in DAPs may render skeletal muscle fibers more susceptible to muscle cell necrosis.

Materials and Methods

Isolation of Skeletal Muscle Membranes

Total skeletal muscle membranes were prepared from age-matched normal control mice and mdx mice. Hind leg and back muscles were dissected and homogenized in 7.5 vol of homogenization buffer (20 mM sodium pyrophosphate, 20 mM sodium phosphate monohydrate, 1 mM $MgCl_2$, 0.303 M sucrose, and 0.5 mM EDTA, pH 7.0) by a Polytron PTS-10-S (Kinematic GmbH, Luzern, Switzerland) in the presence of a protease inhibitor cocktail (34) to minimize protein degradation. Homogenates were centrifuged for 15 min at 1,100 *g* and the supernatant was filtered through four layers of cheesecloth. The pellets from this initial centrifugation step were rehomogenized and centrifuged as described above. The supernatants of four repeated homogenization cycles were combined and total muscle membranes were obtained by centrifugation of the combined supernatants for 35 min at 140,000 *g*. The final membrane preparation was KCl-washed as previously described (34) to remove actomyosin contamination. Skeletal muscle membranes from control and dystrophic dy/dy mice (C57BL/6J-dy; Jackson Laboratory, Bar Harbor, ME) were prepared as described for control and mdx mouse muscle.

Purified rabbit skeletal muscle sarcolemma (34) was isolated using a previously described wheat germ agglutination (WGA) procedure and dystrophin–glycoprotein complex was prepared from rabbit skeletal muscle as described (18). Protein was determined as described (36) using BSA as a standard.

Antibodies to the Dystrophin-associated Proteins

Specific antibodies against the different components of the dystrophin–glycoprotein complex were produced in a sheep using the purified dystrophin–glycoprotein complex (9, 18) as previously performed with guinea pigs (16). Subcutaneous injections of 1 ml of the native dystrophin–glycoprotein complex (0.7 mg protein) (9, 18) mixed with Freund's complete adjuvant (Sigma Chemical Co., St. Louis, MO) were administered into a sheep at numerous sites. 8 wk after the initial injection the animal was boosted intravenously with 0.7 mg of dystrophin–glycoprotein complex and 7 d later a blood sample was taken. After testing the crude sheep antisera for the presence of antibodies against the dystrophin–glycoprotein complex, specific antibodies to 35-DAG, 43-DAG, 50-DAG, and 59-DAP were affinity purified from individual Immobilon-P transfer membrane strips of the various components of the dystrophin–glycoprotein complex as described (16, 37). Specificity of affinity-purified antibodies was subsequently determined by immunoblot analysis with rabbit sarcolemma and rabbit dystrophin–glycoprotein complex.

mAbs XIXC2 to dystrophin (17), $VIA4_1$ to 156-DAG (17), $IVD3_1$ to 50-DAG (17, 21), and McB2 to Na/K-ATPase (41) were previously characterized by extensive immunofluorescence and immunoblot analysis (34). Rabbit polyclonal antibodies against the COOH-terminal sequences of human dystrophin and human dystrophin-related protein (DRP) were affinity purified and characterized as described (18, 33).

Gel Electrophoresis and Immunoblot Analysis

Proteins were fractionated on 3–12% gradient SDS polyacrylamide gels according to Laemmli (26). Protein bands were visualized by Coomassie blue staining and also analyzed by Stains-all staining (10). Transfer of proteins to nitrocellulose was performed according to Towbin et al. (40) and immunoblot staining with antibodies as described (17). After blocking in Blotto (5% nonfat dry milk in PBS, pH 7.4) for 2 h, immunoblots were incubated overnight at room temperature with 1:1,000 diluted crude antisera, 1:50 diluted affinity-purified antibodies, or 1:5 diluted hybridoma supernatant. After washing nitrocellulose membranes three times for 10 min in Blotto, immunoblots were incubated with 1:1,000 diluted, affinity-purified, peroxidase-conjugated goat anti-mouse IgG or goat anti-rabbit IgG or rabbit anti-sheep IgG (Boehringer Mannheim Corp., Indianapolis, IN). Finally immunoblots were again washed three times in Blotto, briefly rinsed in distilled water, and then developed in 20 mM Tris-Cl, pH 7.5, 200 mM NaCl using 4-chloro-1-naphthol as substrate (21). Densitometric scanning of radioactively labeled immunoblots was carried out on a computing densitometer (model 300S; Molecular Dynamics, Sunnyvale, CA) (34). After primary labeling with sheep antibody, immunoblots of mouse muscle membranes were incubated with 1:1,000 diluted rabbit anti-sheep secondary antibody followed by incubation with ^{125}I-labeled protein A (~200,000 cpm/ml; Amersham Corp., Arlington Heights, IL). This procedure gave a strong reproducible signal in autoradiography and enabled densitometric scanning of antibody binding to control and mdx mouse muscle membranes. Membrane preparations used for comparative purposes in autoradiography were obtained from individual mice and not combined during the isolation procedure. Antibody staining intensity from autoradiographs of immunoblots after incubation with ^{125}I-labeled protein A revealed a linear relationship between the amount of membrane protein (0.05–300 μg protein) and the relative labeling intensity of the specific antibody. Prestained molecular weight standards were obtained from Bethesda Research Laboratories (Gaithersburg, MD) and the apparent molecular masses were as follows: myosin, 224 kD; phosphorylase b, 109 kD; albumin, 72 kD; ovalbumin, 46 kD; and carbonic anhydrase, 29 kD.

Lectin staining of immunoblots was carried out under optimized conditions as described (9, 34). Blots were incubated for 1 h with 1:1,000 diluted peroxidase-labeled WGA, Con A (Sigma Chemical Co.), and jacalin (Vector Laboratories, Inc., Burlingame, CA) and developed in 20 mM Tris-Cl, pH 7.5, 200 mM NaCl using 4-chloro-1-naphthol as substrate.

Immunofluorescence Microscopy

Immunofluorescence microscopy of 7-μm transverse cryosections from control, mdx, and dy/dy mouse skeletal muscle (gastrocnemius) was performed as described previously (33, 34). After preincubation for 20 min with 5% normal goat serum in PBS (50 mM sodium phosphate, pH 7.4, 0.9% NaCl), cryosections were incubated for 1 h at 37°C with primary antibodies (affinity-purified antibodies or 1:1,000 dilution of crude antisera). After extensive washing in PBS the sections were labeled with 1:200 diluted, affinity-purified, fluorescein-labeled goat anti-rabbit IgG or rabbit anti-sheep IgG (Boehringer Mannheim Corp.) and subsequently examined in a Zeiss Axioplan fluorescence microscope.

For labeling of skeletal muscle specimens with WGA, cryosections were incubated with 1:1,000 diluted fluorescein-conjugated WGA (Sigma Chemical Co.) for 30 min in the presence and absence of 0.3 M *N*-acetylglucosamine. Sections were extensively washed in PBS and then examined for specific labeling in a fluorescence microscope. Histochemical examination of control, mdx, and dy/dy mouse skeletal muscle cryosections was performed by haematoxylin and eosin staining as described by Dubowitz (14).

Results

Affinity Purification of Antibodies to DAPs

Sheep antiserum raised against the native dystrophin–glycoprotein complex was used to affinity purify antibodies to the individual components of the tightly associated dystrophin–glycoprotein complex (9, 16, 17). The immunoblot analysis of Fig. 1 demonstrates that crude sheep antiserum reacted with all of the components of the dystrophin–glycoprotein complex (dystrophin, 156-DAG, 59-DAP, 50-DAG, 43-DAG, 35-DAG) in sarcolemma and purified dystrophin–glycoprotein complex. After affinity purification, sheep antibodies to 35-DAG, 43-DAG, 50-DAG, and 59-DAP exhibited

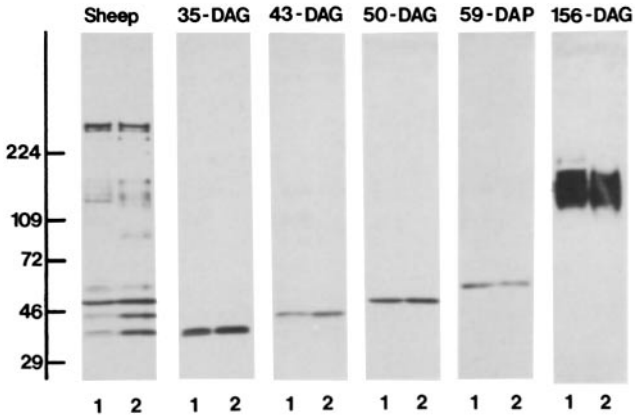

Figure 1. Characterization of affinity-purified sheep antibodies to DAPs. Shown are identical immunoblots labeled with crude sheep antiserum to dystrophin–glycoprotein complex (*sheep*), as well as affinity-purified sheep antibodies to DAPs 35-DAG, 43-DAG, 50-DAG, and 59-DAP and mAb VIA4$_1$ to 156-DAG. Lanes *1* and *2* consist of purified rabbit skeletal muscle sarcolemma (50 μg protein/lane) and dystrophin–glycoprotein complex, respectively. For comparative purposes, an experimentally evaluated amount of purified dystrophin–glycoprotein complex was chosen for lane *2* in order to exhibit the same peroxidase staining intensity as the sarcolemma proteins in lane *1*. All five antibodies are highly specific for their respective antigen, which is an important prerequisite for the comparative immunoblot analysis of skeletal muscle membranes from normal, dy/dy, and mdx mice (see Figs. 2, 3, 4, and 6). Molecular weight standards ($M_r \times 10^{-3}$) are indicated on the left.

strong labeling of their respective antigen in sarcolemma and isolated dystrophin–glycoprotein complex from rabbit skeletal muscle. The results from Fig. 1 indicate the specificity of the affinity-purified antibodies for the different components of the dystrophin–glycoprotein complex in skeletal muscle and this is a crucial prerequisite for the characterization of DAPs in control, mdx, and dy/dy mouse muscle (see Figs. 2–4 and 6). Affinity purification of sheep antibodies to 156-DAG was complicated due to contaminating fragments from degraded dystrophin molecules. We therefore used the previously characterized mAb VIA4$_1$ (17, 34) for the analysis of 156-DAG, which is a highly specific probe for 156-DAG and exhibits strong labeling in immunoblotting (34 and Fig. 1).

Protein and Glycoprotein Composition of Skeletal Muscle Membranes from mdx Mice

To examine the status of DAPs in mdx mouse muscle, total membrane isolates from skeletal muscle homogenates were prepared by a successive homogenization procedure. Total membrane preparations exhibited a highly reproducible protein composition as judged by Coomassie blue and Stains-all staining as well as immunoblot analysis using highly specific antibodies for marker proteins of the various skeletal muscle membrane systems (34) (results not shown). Coomassie blue staining of control and mdx membranes revealed that both membrane preparations are very similar in overall protein composition (Fig. 2). Furthermore, the plasma membrane marker Na/K-ATPase was found to be equally distributed in both membrane preparations (Fig. 2), demonstrating that both membrane preparations contained equal amounts of sarcolemma. Total skeletal muscle membranes from mdx mice contain DRP (22, 28) in normal size and abundance as already previously described for purified sarcolemma (33) but lacked dystrophin (Fig. 2). Before the examination of the DAGs in mdx mouse muscle we evaluated the general status of glycoproteins in the membrane preparation of normal and mdx mouse muscle using lectin staining. WGA, Con A, and jacalin staining showed that the glycoprotein composition with respect to these three lectins is nearly identical in control and mdx mouse muscle membranes (Fig. 2). These results indicate that the major glycoprotein components are not severely affected in mdx mouse muscle. Lectin staining is an important control experiment to account for possible secondary effects on sarcolemma proteins caused by the ongoing degeneration and regeneration cycles in mdx skeletal muscle fibers.

Dystrophin-associated Proteins in Skeletal Muscle Membranes from mdx Mice

Identical immunoblots were examined for the relative abundance of DAPs in skeletal muscle membranes from control and mdx mice. Immunoblot analysis of normal mouse skeletal muscle with antibodies to DAPs demonstrated the presence of DAPs of apparent 35, 43, 50, 59, and 156 kD (Fig. 2) as previously described for rabbit skeletal muscle (9, 16, 17). However, the relative abundance of all of the DAPs was significantly reduced in mdx muscle membranes (Fig. 2). Although peroxidase staining showed very low levels of DAPs in mdx skeletal muscle membranes, the exact amount of remaining DAPs was quantitated using ^{125}I-protein A–labeled immunoblots. Densitometric scanning of autoradiographs, carried out as described in Materials and Methods, revealed an 84 $\pm$ 3% reduction for 35-DAG, an 80 $\pm$ 5% reduction for 43-DAG, an 83 $\pm$ 5% reduction for 50-DAG, an 86 $\pm$ 6% reduction for 59-DAP, and an 85 $\pm$ 6% reduction for 156-DAG in mdx muscle membranes when compared with control membranes (see Table I). The comparative densitometric scanning was performed with individually isolated membranes from five 10-wk-old control mice and five 10-wk-old mdx mice. The same results were obtained with crude skeletal muscle membranes, which had not been washed with 0.6 M KCl, and also with microsomal membranes prepared as described previously (34) (results not shown). DAPs were reduced to a similar extent in membranes isolated from 1-, 3-, and 5-d-old mice (Fig. 3 *a*), as well as 2-, 5-, and 30-wk-old mdx mice (Fig. 3 *b*) as compared with age-matched control mice. The immunoblot analysis illustrated in Fig. 3 covers the time period before, during, and after segmental necrosis in mdx skeletal muscle fibers (39). Dystrophin is completely lacking from mdx skeletal muscle of all ages, while DRP was found to be present in mdx mice of all ages (Fig. 3). Plasma membrane marker Na/K-ATPase is equally distributed in the membrane preparations of differently aged normal and mdx mice (Fig. 3), showing that approximately equal amounts of sarcolemma proteins are present in the individual membrane preparations. It should be noted that 156-DAG exhibited, with an increase in age of mice, a slower electrophoretic mobility (Fig. 3), possibly caused by the different glycosylation of 156-DAG at different points in the development of mouse skeletal muscle. These findings demonstrate the specific loss

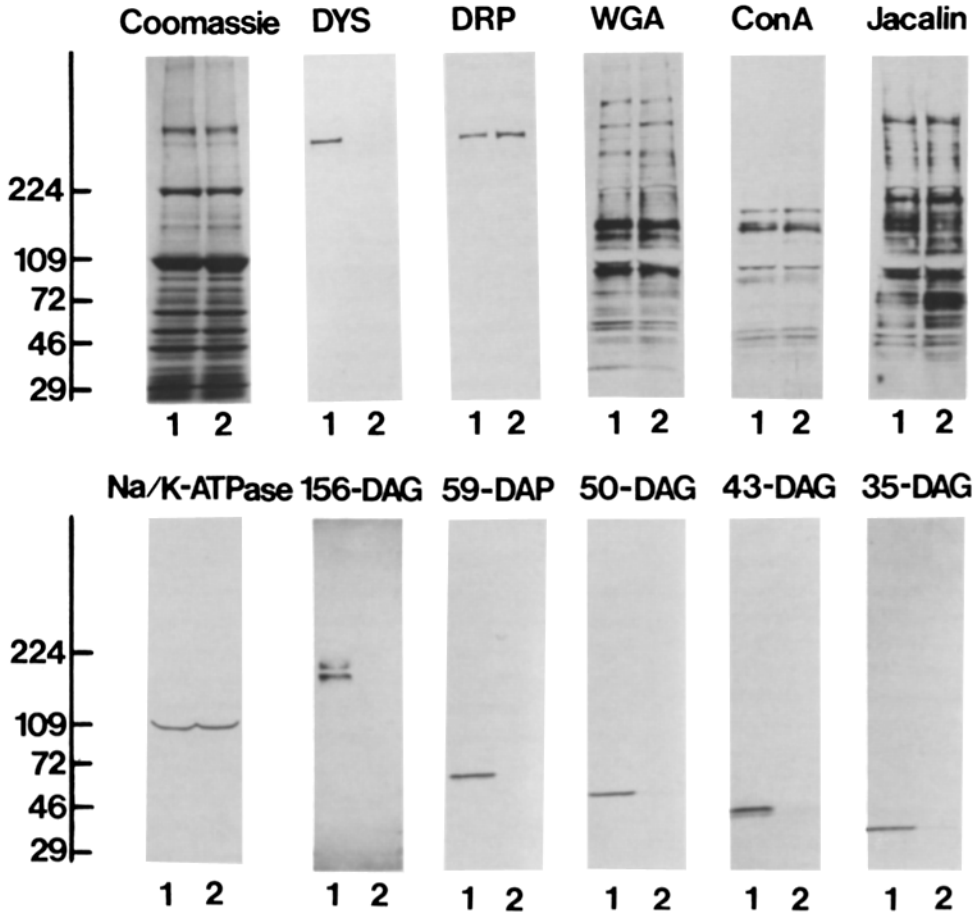

Figure 2. Immunoblot analysis of DAPs in skeletal muscle membranes from mdx mice. Shown are a Coomassie blue-stained gel and identical immunoblots labeled with rabbit antibodies to dystrophin (*DYS*) and DRP, mAb McB2 to Na/K-ATPase, mAb VIA4$_1$ to 156-DAG, and affinity-purified sheep antibodies to DAPs 35-DAG, 43-DAG, 50-DAG, and 59-DAP, as well as blots stained with WGA, Con A, and jacalin. Lanes *1* and *2* consist of membranes prepared from 10-wk-old normal and mdx mouse skeletal muscle (250 μg protein/lane), respectively. Both preparations are very comparable with respect to protein and glycoprotein composition. However, in mdx skeletal muscle membranes dystrophin is absent and all of the DAPs are greatly reduced when compared with control skeletal muscle. A reduction of 84% for 35-DAG, 80% for 43-DAG, 83% for 50-DAG, 86% for 59-DAP, and 85% for 156-DAG was revealed by densitometric scanning of ^{125}I-protein A–labeled immunoblots. Molecular weight standards ($M_r \times 10^{-3}$) are indicated on the left.

of the DAPs in mdx skeletal muscle, while the overall glycoprotein composition is unaffected.

DAPs in Skeletal Muscle Membranes from dy/dy Mouse

The murine mutant *Dystrophia muscularis* dy/dy, which has an autosomal-recessive mode of inheritance, is another animal model for muscular dystrophy which exhibits progressive and severe degeneration of skeletal muscle fibers (6). The genetic defect is not known but the genetic locus for the neuromuscular disorder dystrophia muscularis is on mouse chromosome 10 (7). Histochemical examination of skeletal muscle fibers from dy/dy mice reveal necrotic fibers (6, and see Fig. 5 *c* for haematoxylin and eosin staining of skeletal muscle cryosections from dy/dy mouse). Muscle membranes from dy/dy mice contain dystrophin of normal size and abundance (Fig. 4), making this animal model a very good control for the status of DAPs in necrotic muscle which

Table I. Dystrophin-associated Proteins in Skeletal Muscle Membranes from Normal, dy/dy, and mdx Mice

Component	M_r	Immunoblotting (total skeletal muscle membranes)			Ab (reference)
		Normal	dy/dy	mdx	
Dystrophin	427 kD	+	+	ND	Rb-DYS (18)
DRP	>427 kD	+	+	+	Rb-DRP (33)
156-DAG	156 kD	+	+	−	VIA4$_1$ (17)
59-DAP	59 kD	+	+	−	Sh-DAP/59 (*)
50-DAG	50 kD	+	+	−	Sh-DAP/50 (*)
43-DAG	43 kD	+	+	−	Sh-DAP/43 (*)
35-DAG	35 kD	+	+	−	Sh-DAP/35 (*)
25-DAP	25 kD	NE	NE	NE	**

Rabbit antibodies Rb-DYS and Rb-DRP are directed against the unique COOH-terminal sequences of dystrophin and dystrophin-related protein, respectively. Antibodies against DAPs were affinity purified from sheep antiserum, which had been raised against the native dystrophin–glycoprotein complex.
+, Present; − reduced (80–90%); ND, not detected; NE, not examined.
* This study.
** No antibody to this protein has been produced.

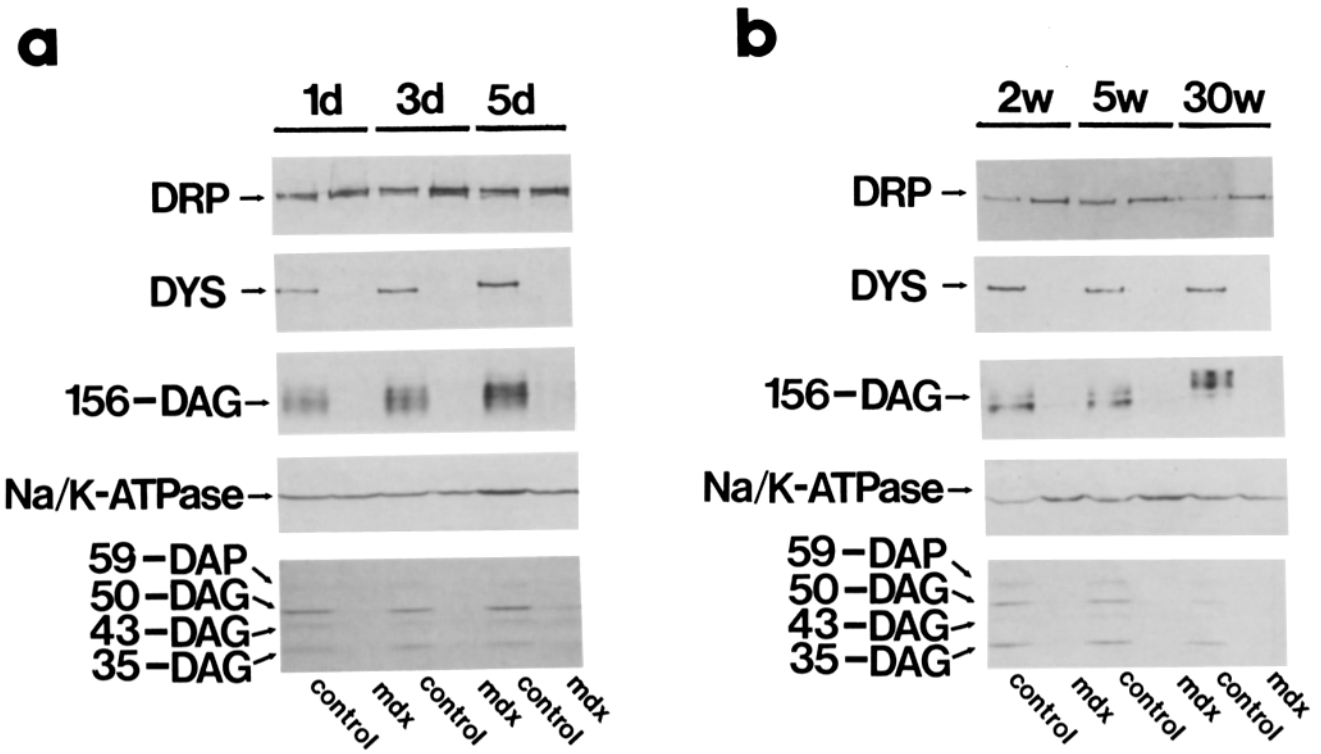

Figure 3. Immunoblot analysis of DAPs in skeletal muscle membranes from mdx mice of differing ages. Shown is a summary of the immunoblot analysis of control and mdx skeletal muscle membranes from mice of different ages. Immunoblots were stained with rabbit antibodies to dystrophin (*DYS*) and DRP, mAb McB2 to Na/K-ATPase, mAb VIA4$_1$ to 156-DAG, and affinity-purified sheep antibodies to DAPs 35-DAG, 43-DAG, 50-DAG, and 59-DAP. The individual lanes consist of control and mdx skeletal muscle membranes of 1-, 3-, and 5-d-old mice (*a*) and of 2-, 5-, and 30-wk-old mice (*b*). Membranes of dystrophin-deficient mdx skeletal muscle from mice of different ages showed comparable amounts of Na/K-ATPase, while all of the DAPs were found to be drastically reduced in the time period before, during, and after segmental skeletal muscle fiber necrosis. DRP was present in control and mdx mice of all ages. Molecular weight standards ($M_r \times 10^{-3}$) are indicated on the left.

contains dystrophin. Coomassie blue staining revealed no apparent differences between membranes isolated from control and dy/dy mouse skeletal muscle, and the density of dystrophin and dystrophin-related protein is also comparable between both membrane preparations (Fig. 4). Most importantly, antibodies to the different DAPs showed approximately equal amounts of these proteins in skeletal muscle membranes from control and dy/dy mice (Fig. 4). These findings demonstrate that DAPs are not affected by secondary events in necrotic muscle and suggest that the reduced density of DAPs in skeletal muscle membranes from mdx mice is probably due to the absence of dystrophin from the membrane cytoskeleton of mdx muscle.

Distribution of DAGs in Skeletal Muscle from mdx Mice

After investigating the relative abundance of DAPs in total membranes from mdx skeletal muscle, it was important to confirm these results in studies of whole muscle tissue. We therefore used indirect immunofluorescence microscopy to evaluate the status of DAPs in skeletal muscle cryosections from control, mdx, and dy/dy mice. Affinity-purified sheep antibodies to DAPs exhibited strong immunofluorescence labeling of mouse skeletal muscle. For a reproducible and reliable comparison of all muscle samples, cryosections were treated in an identical manner during all incubation and washing steps. Photographs were taken under identical conditions with the same exposure time so that a direct comparison of immunofluorescent staining intensity could be undertaken.

The upper panel of Fig. 5 shows histochemical staining of skeletal muscle cryosections from normal, mdx, and dy/dy mice with haematoxylin and eosin to outline the general histological differences between these tissues. While normal mouse skeletal muscle cells exhibit peripheral nucleation (Fig. 5 *a*), mdx mouse skeletal muscle cells have many centrally localized nuclei (Fig. 5 *b*) which are a consequence of the ongoing degeneration and regeneration occurring in mdx muscle. Segmental necrosis of mdx skeletal muscle fibers starts at ~15 d of age and is followed by fiber regeneration whereby regenerated fibers are characterized by central nucleation (39). In contrast, cryosections from dy/dy mouse skeletal muscle exhibit muscle fibers with rounded contours, marked variability of fiber size diameter, an increase of the interstitial connective tissue, and many fibers with central nucleation (Fig. 5 *c*) (6). Staining of mouse cryosections with fluorescein-labeled WGA demonstrated that mdx and dy/dy mouse muscle exhibits comparable levels of WGA binding components in the cell periphery when compared with normal mouse muscle (Fig. 5, *d–f*). These results agree with the findings from the comparative immunoblot analysis of Fig. 2, which illustrates an equal amount of WGA-positive glycoproteins in muscle membranes from control and mdx mice. Dystrophin antibodies labeled the entire cell periphery

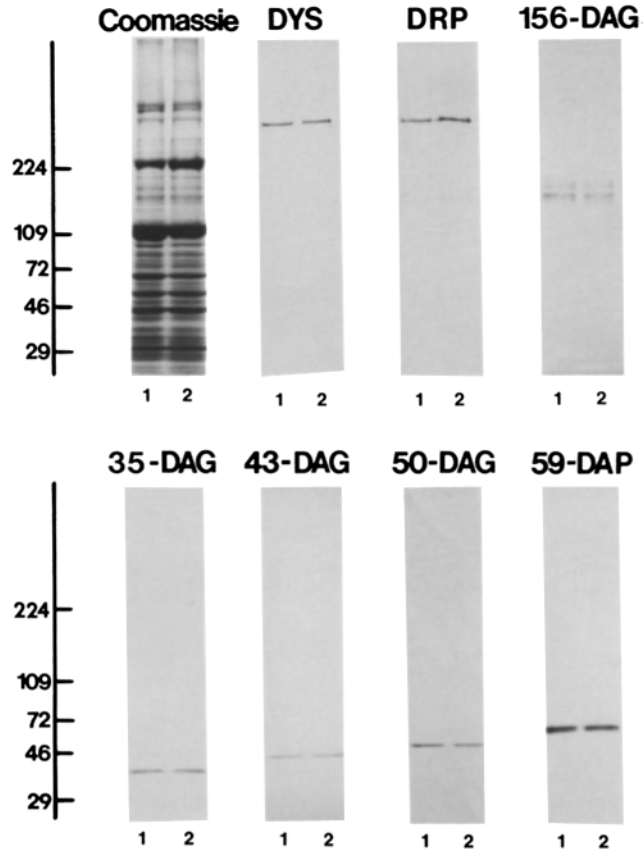

Figure 4. Immunoblot analysis of DAPs in skeletal muscle membranes from dy/dy mouse. Shown is a Coomassie blue–stained gel and identical immunoblots labeled with rabbit antibodies to dystrophin (*DYS*) and DRP, mAb VIA4$_1$ to 156-DAG, as well as affinity-purified sheep antibodies to 35-DAG, 43-DAG, 50-DAG, and 59-DAP. Lanes *1* and *2* consist of membranes prepared from 10-wk-old normal and dy/dy mouse skeletal muscle (250 μg protein/lane), respectively. Components of the dystrophin–glycoprotein complex are found with approximately equal abundance in normal and dy/dy muscle membranes. Molecular weight standards ($M_r \times 10^{-3}$) are indicated on the left.

of control and dy/dy skeletal muscle cryosections (Fig. 5, *g* and *i*), illustrating normal distribution and abundance of this sarcolemmal cytoskeleton protein in dystrophic muscle from dy/dy mouse. These results are in agreement with the immunoblot analysis of Fig. 4, which shows that dystrophin is present in normal size and abundance in skeletal muscle membranes from dy/dy mouse. In contrast, mdx mouse skeletal muscle fibers are completely missing dystrophin (Fig. 5 *h*), which is a consequence of a point mutation in the dystrophin gene (38).

After this characterization, mouse skeletal muscle cryosections were labeled with affinity-purified sheep antibodies to DAPs (Fig. 6). Immunofluorescence labeling of DAPs was restricted to the muscle cell periphery and the staining intensity was comparable between normal and dy/dy skeletal muscle (Fig. 6). On the other hand, the immunofluorescence staining intensity for 35-DAG, 43-DAG, 50-DAG, and 59-DAP was greatly reduced in skeletal muscle fibers from mdx mouse (Fig. 6). Immunofluorescence labeling of the muscle cell surface was not only markedly reduced but also occasionally of a patchy nature, demonstrating that abundance and distribution of DAGs is severely affected in mdx skeletal muscle fibers. The fact that low levels of DAPs are detectable in the mdx muscle cell periphery by immunofluorescence microscopy and mdx skeletal muscle membranes by immunoblotting suggests that the remaining DAPs are not present in the cytoplasm but remain with the mdx sarcolemma membrane. DAGs were deficient in mdx skeletal muscle cryosections before, during, and after necrosis as revealed by immunofluorescence microscopy of similarly aged control and mdx mice as used in the immunoblot analysis of Fig. 3. The reduced density of 50-DAG in mdx skeletal muscle was confirmed by immunofluorescence microscopy with mAb IVD3$_1$ to 50-DAG (34) (results not shown). Sheep antibodies to 156-DAG did not exhibit strong enough immunofluorescence labeling for a comparative analysis of skeletal muscle cryosections from control, mdx, and dy/dy mice. In agreement with the immunoblotting results of Figs. 2 and 3 the findings from immunofluorescence microscopy indicate that the suppressed density of these proteins is a consequence of the absence of dystrophin in the mdx muscle. If the reduced amount of DAPs would be independent of the status of dystrophin and due to secondary effects in skeletal muscle degradation, one would also expect reduced levels of DAPs in dystrophic dy/dy skeletal muscle. This is not the case, as illustrated by strong labeling of the dy/dy skeletal muscle surface by antibodies to DAPs (Fig. 6).

Discussion

The membrane cytoskeletal component dystrophin is tightly associated to a large oligomeric complex containing several sarcolemmal glycoproteins in skeletal muscle. A central question of current muscular dystrophy research is how the absence of dystrophin causes muscle cell necrosis. To learn more about the early events in the molecular pathogenesis of muscular dystrophy, here we investigated the relative abundance of all of the components of the dystrophin–glycoprotein complex in skeletal muscle membranes from mdx mice, a dystrophin-deficient animal model for DMD which exhibits necrotic skeletal muscle fibers. Immunoblot analysis using total muscle membrane preparations and affinity-purified antibodies was combined with data obtained by immunofluorescence microscopy. We have presented evidence that the DAPs of apparent 35, 43, 50, 59, and 156 kD are specifically and significantly reduced in skeletal muscle from mdx mice. The major glycoprotein composition of skeletal muscle fibers was not affected in mdx mice, which indicates that the loss of glycoproteins in mdx mice is specific for those glycoproteins that are normally linked to dystrophin. Control experiments with dystrophin-containing membranes from necrotic dy/dy mouse muscle exhibited a normal abundance of DAPs, which demonstrates that the loss of these proteins in mdx muscle is not caused by secondary effects of muscle degradation.

It is known from studies of other genetic diseases involving membrane cytoskeletal proteins, as well as enzyme or ion channel complexes, that the deficiency in one subunit or component of a complex can result in the loss of other subunits or components of the protein complex. Hereditary elliptocytosis, characterized by a defect in spectrin, is also associated with deficiencies in protein-4.1 and minor sialoglycoproteins (1). Skeletal muscle phosphorylase kinase de-

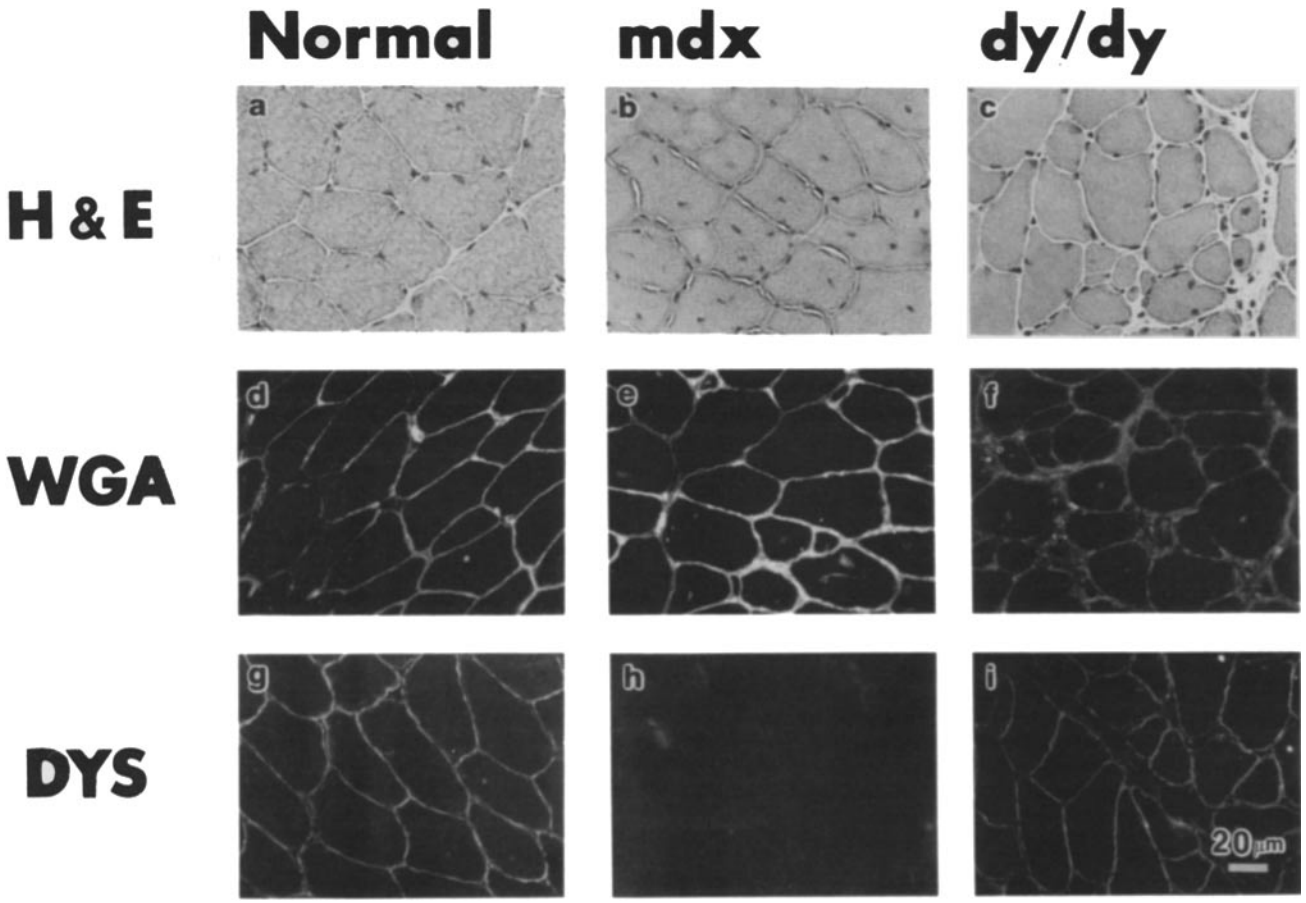

Figure 5. Characterization of skeletal muscle cryosections from control, mdx, and dy/dy mice. Shown are transverse cryosections of normal (*a*, *d*, *g*), mdx (*b*, *e*, *h*), and dy/dy (*c*, *f*, *i*) mouse skeletal muscle stained with haematoxylin and eosin (*a*, *b*, *c*) and labeled with WGA (*d*, *e*, *f*) and rabbit antisera to the COOH-terminal decapeptide of dystrophin (*g*, *h*, *i*). Skeletal muscle from 10-wk-old mdx and dy/dy mice exhibit comparable amounts of WGA-binding components in the cell periphery when compared with skeletal muscle cryosections from normal mice. Bar, 20 μm.

ficiency, which is caused by a single gene defect on the X chromosome, is characterized by the combined loss of all four subunits of this enzyme (12). However, a generalized loss of components in a protein complex is not observed in the genetic disease muscular dysgenesis. This disorder results in a complete absence of skeletal muscle contraction due to the failure of depolarization of the transverse tubular membrane to trigger calcium release from sarcoplasmic reticulum. Interestingly, only the α_1 subunit of the dihydropyridine receptor is absent in dysgenic mice, while the α_2 subunit of the receptor is present (23). Based on the variety of findings for the molecular basis of genetic disorders, it was not possible to conclude from our initial finding of greatly reduced 156-DAG in mdx muscle (17) that all the other DAPs are also affected in skeletal muscle fibers from mdx mice. Consequently, highly specific antibodies to the DAPs had to be developed to evaluate the status of all components of the dystrophin–glycoprotein complex in mdx muscle tissue.

The loss of DAPs from the muscle cell surface could principally occur in two different ways: (*a*) due to a decrease in synthesis and/or assembly, or (*b*) due to an increase in degradation. The absence of dystrophin may result in the decrease in synthesis of DAPs similarly to that seen for phosphorylase kinase subunits (12). DAPs could be synthesized in normal amounts but may not be properly assembled into an oligomeric complex due to the lack of dystrophin. Both of these possibilities could cause greatly reduced amounts of DAPs in skeletal muscle sarcolemma. On the other hand, DAPs may be synthesized and assembled correctly, but due to the deficiency in dystrophin, the membrane complex will lack the proper interaction with the actin cytoskeleton. This could result in greater mobility of the membrane complex, which may render the protein components of the complex more vulnerable to degradation.

The molecular events that lead to muscle cell necrosis in dystrophin-deficient muscle fibers and finally cause muscle cell death have not yet been established. Membrane biochemical studies demonstrated that dystrophin is a major component of the membrane cytoskeleton of skeletal muscle sarcolemma (32, 34). By analogy to observations in other cytoskeletal diseases (29, 44), dystrophin appears to be an important structural component of the subsarcolemmal cytoskeleton. In addition, components of the dystrophin–glycoprotein complex could be important adhesion factors in linking the subsarcolemmal actin cytoskeleton to an extracellular glycoprotein of skeletal muscle fibers, as recently suggested by an extensive biochemical and structural analysis of the dystrophin–glycoprotein complex (16). Dystrophin and its 59-kD associated protein were identified as cytoskeletal elements which are tightly linked to a heteromeric inte-

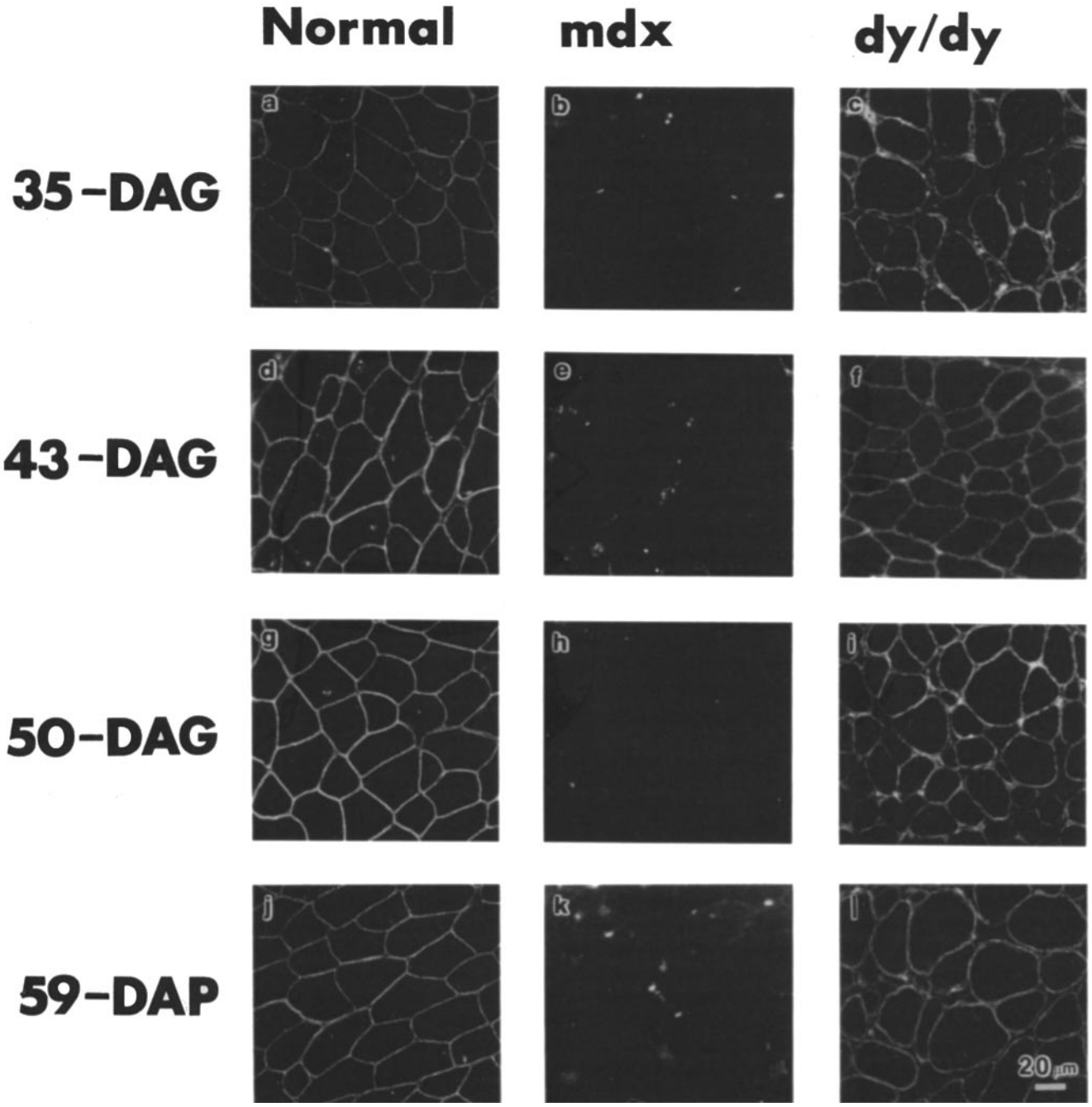

Figure 6. Distribution of DAGs in normal, mdx, and dy/dy mouse skeletal muscle. Shown are transverse cryosections of skeletal muscle from 10-wk-old normal (*a*, *d*, *g*, *j*), mdx (*b*, *e*, *h*, *k*), and dy/dy (*c*, *f*, *i*, *l*) mice labeled with affinity-purified sheep antibodies to DAPs 35-DAG, 43-DAG, 50-DAG, and 59-DAP. Immunofluorescence staining intensity for DAPs is greatly reduced in mdx skeletal muscle when compared with skeletal muscle cryosections from normal and dy/dy mice. Bar, 20 μm.

gral membrane complex (16). The DAGs of apparent 35, 43, and 50 kD were found to be the integral protein components of the complex which spans the sarcolemma membrane, while 156-DAG appears to be an extracellular glycoprotein (16). Disruption of the various components involved in the structural link between subsarcolemmal cytoskeleton and an extracellular glycoprotein may severely weaken the sarcolemma membrane. This hypothesis is supported by the histopathological finding that DMD muscle fibers exhibit an early separation between muscle cell surface and basal lamina (Bonilla, E., and E. Moggio. 1986. *Neurology.* 36 [suppl 1]: 171). Comprehensive analysis of DMD skeletal muscle shows that muscle cell necrosis is preceded by a breakdown of the plasma membrane (11, 15, 31). Changes in muscle cell surface morphology were observed in cultured skeletal muscle cells from DMD patients and a decrease in basal lamina components described (13). These findings suggest that deficiency in dystrophin and loss of DAPs may have secondary effects on components of the basal lamina. In addition, skeletal muscle fibers from mdx mice exhibited an enhanced

vulnerability in contraction experiments, which may render the sarcolemma more susceptible to suffering focal breaks (43). Recent findings demonstrated that skeletal muscle fibers from mdx mouse are more fragile and have a decreased osmotic stability (30).

In addition to being a good animal model for studying segmental necrosis in dystrophin-deficient skeletal muscle fibers, mdx mice are currently being used to validate potential therapies for the replacement of dystrophin such as myoblast transfer therapy (35) or gene therapy (27). It will be very important to determine the status of DAPs in skeletal muscle fibers in which dystrophin has been replaced by these experimental therapies. If the loss of DAPs is a significant factor in muscle cell necrosis, then experimental therapies will only succeed when, in addition to dystrophin, all DAPs are restored.

In conclusion, we find that the density of DAPs is drastically suppressed in skeletal muscle membranes and cryosections from mdx mice. Our results strongly support the hypothesis that the absence of dystrophin leads to a deficiency in all of the DAPs. These findings, combined with results of biochemical and structural analysis of the dystrophin–glycoprotein complex (16), suggest that muscle fibers may be rendered more susceptible to necrosis due to the loss of DAPs.

The authors would like to thank Suzanne W. Sernett, Steven D. Kahl, and Kristin Stang for their excellent technical assistance, and Dr. James M. Ervasti and Dr. Kiichiro Matsumura for their helpful comments on this paper. We are indebted to Dr. Kathleen Sweadner for her generous gift of monoclonal antibodies to Na/K-ATPase.

Kevin P. Campbell is an Investigator of the Howard Hughes Medical Institute. This study was also supported by the Muscular Dystrophy Association.

Received for publication 29 July 1991 and in revised form 5 September 1991.

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