

# Forced Expression of Dystrophin Deletion Constructs Reveals Structure–Function Correlations

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**Abstract.** Dystrophin plays an important role in skeletal muscle by linking the cytoskeleton and the extracellular matrix. The amino terminus of dystrophin binds to actin and possibly other components of the subsarcolemmal cytoskeleton, while the carboxy terminus associates with a group of integral and peripheral membrane proteins and glycoproteins that are collectively known as the dystrophin-associated protein (DAP) complex. We have generated transgenic/*mdx* mice expressing “full-length” dystrophin constructs, but with consecutive deletions within the COOH-terminal domains. These mice have enabled analysis of the interaction between dystrophin and members of the DAP complex and the effects that perturbing these associations have on the dystrophic process. Deletions within the cysteine-rich region disrupt the interaction between dystrophin and the DAP complex, leading to a severe

dystrophic pathology. These deletions remove the  $\beta$ -dystroglycan-binding site, which leads to a parallel loss of both  $\beta$ -dystroglycan and the sarcoglycan complex from the sarcolemma. In contrast, deletion of the alternatively spliced domain and the extreme COOH terminus has no apparent effect on the function of dystrophin when expressed at normal levels. The proteins resulting from these latter two deletions supported formation of a completely normal DAP complex, and their expression was associated with normal muscle morphology in *mdx* mice. These data indicate that the cysteine-rich domain is critical for functional activity, presumably by mediating a direct interaction with  $\beta$ -dystroglycan. However, the remainder of the COOH terminus is not required for assembly of the DAP complex.

**D**UCHENNE muscular dystrophy (DMD)<sup>1</sup> and the milder Becker muscular dystrophy (BMD) are caused by mutations in the dystrophin gene (24). Although dystrophin is expressed from a variety of promoters in a wide array of tissues, disruption of dystrophin function in striated muscle leads to the most devastating effects of these diseases (for review see reference 2). The complete function of the dystrophin protein in skeletal muscle has not yet been fully elucidated, although it is thought to provide a crucial link between the intracellular actin cytoskeleton and the extracellular matrix (15). The *mdx* mouse contains a nonsense mutation in the dystrophin gene that leads to a complete absence of dystrophin in muscle, which causes a similar muscle degeneration as is

seen in DMD patients. As a result, the *mdx* mouse is a useful system for studying the function of the dystrophin protein.

The full-length 427-kD dystrophin protein that is localized at the sarcolemmal membrane in skeletal muscle is often described as consisting of four structural domains (24). These include an NH<sub>2</sub>-terminal actin binding domain, a central rod domain consisting of 4 hinge regions and 24 units with homology to those in  $\beta$ -spectrin, a cysteine-rich domain, and a COOH-terminal domain that contains regions of alternative splicing and two leucine zipper motifs. We have recently observed that expression of a full-length dystrophin construct deleted for the actin-binding domain located in exons 3–7 of the NH<sub>2</sub> terminus improves the pathology of the *mdx* mouse to a mild “BMD-like” phenotype (Corrado, K., and J.S. Chamberlain, manuscript submitted for publication). We and others have shown that deletion of two-thirds of the central-rod domain results in an almost fully functional dystrophin protein in skeletal muscle of *mdx* mice (37, 43). Both of these observations correlate well with patient data (13, 44). Deletions within the COOH-terminal domains of dystrophin are relatively rare, and most of these mutations result in the absence of detectable dystrophin protein caused by instability at the

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1. *Abbreviations used in this paper:* BMD, Becker muscular dystrophy; DAP, dystrophin-associated protein; DMD, Duchenne muscular dystrophy; GMA, glycol methacrylate; LGMD, limb girdle muscular dystrophy.

RNA and/or protein level. As a result, there exists little patient data relating to structure–function correlations of the COOH terminus of dystrophin. There are few reported cases where a truncated dystrophin protein lacking all of the COOH-terminal domains is able to localize correctly to the sarcolemmal membrane (5, 18, 19, 29). However, these situations have resulted in severe DMD in patients who are old enough for a diagnosis. Furthermore, point mutations and frameshifting deletions that prevent translation of the dystrophin COOH terminus almost invariably lead to DMD, but without detectable accumulation of a mutant dystrophin. These studies indicate that the dystrophin COOH terminus is critical for its function. However, patient studies have not enabled a clear description of whether subregions within the COOH-terminal domains are important for stabilization of the dystrophin protein and allowing its accumulation in muscle fibers as opposed to playing a more direct role such as mediating protein–protein interactions.

Dystrophin is known to associate with a large complex of integral and peripheral membrane proteins and glycoproteins collectively known as the dystrophin-associated protein (DAP) complex (34). All of the DAPs are greatly reduced in the skeletal muscle of DMD patients and the *mdx* mouse (16, 33). The DAPs have recently been shown to be composed of three subcomplexes. Laminin-2, a major component of the extracellular matrix in muscle tissue, binds to  $\alpha$ -dystroglycan, which binds directly to  $\beta$ -dystroglycan (14). This link to the extracellular matrix has been named the dystroglycan complex. The sarcoglycan complex consisting of the integral membrane proteins  $\alpha$ -,  $\beta$ -, and  $\gamma$ -, sarcoglycan is thought to associate with  $\beta$ -dystroglycan (22, 41), although the exact contacts between the complexes have not been identified. The third subcomplex consists of one or more of the 59-kD peripheral membrane proteins encoded by individual yet homologous genes, known as the syntrophins ( $\alpha$ 1,  $\beta$ 1, and  $\beta$ 2).  $\alpha$ 1-syntrophin is present uniformly around the sarcolemmal membrane and is thought to be present in most dystrophin complexes (36).  $\beta$ 1-syntrophin is present at low levels in skeletal muscle, but its precise localization pattern has not been reported (1).  $\beta$ 2-syntrophin colocalizes at the neuromuscular junction with utrophin, and is hypothesized to associate in complexes with this dystrophin homologue (36).

The identification of the primary defects for forms of muscular dystrophy other than DMD has provided some of the evidence for these subcomplexes. Limb girdle muscular dystrophies (LGMD) 2D, 2E, and 2C are caused by mutations in members of the sarcoglycan complex (8, 22, 27, 28, 32). All of the members of the sarcoglycan complex are missing from the sarcolemmal membrane in these patients; however, dystrophin and the dystroglycan complex remain correctly localized. No patient mutations have yet been found in the dystroglycans or the syntrophins. Recent *in vitro* data suggests that  $\beta$ -dystroglycan binds directly to the dystrophin cysteine-rich domain (23, 41), and that no other members of the dystroglycan or sarcoglycan complexes bind directly to dystrophin.  $\alpha$ 1-syntrophin has been shown to bind to the portion of dystrophin encoded by exons 73 and 74 *in vitro* (3, 42, 45), although we have previously shown that deletion of this region of dystrophin has no effect on localization of syntrophin or the assembly

of the DAP complex and produces a fully functional dystrophin protein in young transgenic/*mdx* mice (39). These data, together with the fact that mutations in the known DAP members cannot account for several forms of limb girdle muscular dystrophy, suggest that other members of the DAP complex remain to be identified. In addition, *in vitro* data may not provide a complete picture with which to predict endogenous protein function, thus underscoring the importance of an *in vivo* approach.

We have previously shown that expression of the cysteine-rich and COOH-terminal domains of dystrophin in the skeletal muscle of transgenic/*mdx* mice is able to restore proper localization of all members of the DAP complex, but is unable to prevent the dystrophic phenotype in these mice (12, 17). In the present study, we generated transgenic/*mdx* mice that express full-length dystrophin constructs deleted for consecutive regions of the COOH terminus to enable an *in vivo* analysis of the function of the dystrophin COOH terminus and to determine how it interacts with the members of the DAP complex.

## Materials and Methods

### Dystrophin Deletion Transgenes

All deletions were constructed by recombinant PCR using the murine full-length dystrophin cDNA as a template (26). Although exon numbers were used to name these constructs, the extent of each deletion does not precisely follow exon boundaries.  $\Delta$ 64-67 deletes amino acids 3145-3300,  $\Delta$ 68-70 deletes amino acids 3301-3443, and  $\Delta$ 75-78 deletes amino acids 3570-3717, leaving the last three amino acids of the mouse dystrophin protein intact (9).  $\Delta$ 65-66 is a precise deletion of exons 65 and 66, based on the *mdx*<sup>3cv</sup> mutation (11).  $\Delta$ 71-74 precisely deletes these exons and has been described previously (39). Dystrophin constructs were cloned into expression vectors containing the -3300 mouse muscle creatine kinase promoter and enhancer elements, either a VPI intron from the pSVL plasmid (Pharmacia Fine Chemicals, Piscataway, NJ) ( $\Delta$ 64-67 and  $\Delta$ 65-66) or the synthetic minx intron derived from adenovirus (31), as well as the SV-40 polyadenylation site.

### Generation of Transgenic Mice

Wild-type hybrid embryos from C57Bl/6J SJL/J were injected with dystrophin expression vectors as previously described (20) ( $\Delta$ 64-67,  $\Delta$ 65-66, and  $\Delta$ 68-70). F0 mice were screened by PCR with primers to the mouse dystrophin cDNA. Positive transgenic mice were bred onto an *mdx* null dystrophin background for analysis. The  $\Delta$ 75-79 transgene was injected directly into C57Bl/10J *mdx*  $\times$  (SJL/J  $\times$  C57Bl/10J *mdx*) embryos to reduce the number of generations of breeding needed to obtain animals on the *mdx* background for analysis. The females used for superovulation to produce these embryos were 4 wk old and weighed between 10 and 12 g.

### Western Analysis

Total protein was extracted from the quadriceps of control and transgenic mice, and protein concentrations were determined using the Coomassie Plus Protein Assay Reagent (Pierce Chemical Co., Rockford, IL). 100  $\mu$ g of each sample was electrophoresed on a 6% polyacrylamide/SDS gel (29.7:0.3 acryl/bis), transferred for 2 h at 75 V onto Biotrace Nitrocellulose (Gelman Science, Ann Arbor MI) at a 1/1,000 dilution for 2 h at room temperature, washed, and then probed with HRP-conjugated anti-mouse antibodies at a 1/2,000 dilution (Cappel Laboratories, Durham, NC). Blots were developed using the ECL chemiluminescence system (Amersham, Arlington Heights, IL). All incubations contained 1% normal goat serum and 0.1% Tween 20.

## Production of the NH<sub>2</sub>-terminal Dystrophin Antisera

New Zealand White rabbits were injected subcutaneously with gel-purified, bacterially expressed recombinant protein containing the first 423 amino acids of murine dystrophin fused to the FLAG peptide (IBI). Initial injections were with 500 µg of protein mixed 1:1 (vol/vol) with Freund's complete adjuvant. Rabbits were boosted eight times at ~4 wk intervals with 100–300 µg of the fusion protein mixed 1:1 (vol/vol) with Freund's incomplete adjuvant. Serum was obtained 11 d after the eighth booster injection.

To affinity purify the rabbit antidystrophin antibodies, a bacterially expressed fusion protein containing the first 246 amino acids of murine dystrophin fused to the maltose-binding protein was purified according to the manufacturer's instructions (New England Biolabs, Beverly, MA) and coupled to a glutaraldehyde-activated Acti-Disk 25 according to the manufacturer's instructions (Whatman Chemical Co., Clifton, NJ). Immune serum was chromatographed over the disk, washed with PBS, followed by PBS containing 1 M NaCl. Antibodies were eluted with 0.1 M glycine-HCl, pH 2.8, into a tube containing 0.25 vol of 1 M Tris. The antibodies were concentrated and resuspended in PBS with a Centricon-30 concentrator (Amicon, Inc., Beverly, MA).

## Immunohistochemistry

Skeletal muscle was removed from control and transgenic animals, cut into strips, embedded in Tissue-tek OCT mounting media (Miles, Inc.), and frozen quickly in liquid nitrogen-cooled isopentane. 7-µm sections were blocked with 1% gelatin in KPBS for 15 min, washed in KPBS + 0.2% gelatin (KPBSG), and incubated for 2 h in KPBSG + 1% normal goat serum with each affinity-purified primary antibody at the following dilutions: dystrophin NH<sub>2</sub> terminus 1/1,300, β-dystroglycan 1/200 (21), syntrophin 1/15 (this polyclonal antibody recognizes all three members of the syntrophin triplet on Western analysis, but cross-reactivity with individual isoforms has not been further characterized [33, 45]), α-sarcoglycan 1/20 (40), and γ-sarcoglycan 1/5 (22). After washing, the slides were incubated for 1 h with either biotin-labeled goat anti-rabbit polyclonal antibodies (for dystrophin, β-dystroglycan, and α-sarcoglycan; Pierce) or biotin-labeled donkey anti-sheep polyclonal antibodies (for syntrophin and γ-sarcoglycan; Sigma Immunochemicals, St. Louis, MO) washed again, and incubated with FITC-conjugated streptavidin. After a final wash, Vectashield (Vector Laboratories, Burlingame, CA) with 4,6-diamidino-2-phenylindole (DAPI) was applied and sections were photographed through a dual bandpass filter (Chromatek) under 50× magnification using an Optiphot-2 microscope (Nikon Inc., Melville, NY).

## Histology

Skeletal muscle was removed from control and transgenic animals, cut into strips, and fixed overnight at 4°C in 3.5% formaldehyde/0.5% glutaraldehyde. The sections were dehydrated in 70% ethanol, incubated overnight in glycol methacrylate (GMA) reagents, and embedded in liquid GMA that was subsequently polymerized. 4-µm sections of the tissue-containing GMA blocks were affixed to slides and stained with hematoxylin and eosin.

## Results

We generated transgenic/*mdx* mice that express full-length dystrophin constructs deleted for consecutive regions of the COOH terminus to analyze the function of the dystrophin COOH terminus and how it interacts with the members of the DAP complex (Fig. 1). Transgene constructs were either injected directly into *mdx* embryos or onto a wild-type background that required breeding of positive transgenic animals onto an *mdx* background for analysis. Total protein extracts of quadriceps muscles from each transgenic/*mdx* line were analyzed by Western analysis with an antibody raised against the rod domain of dystrophin (Fig. 2). Each transgenic/*mdx* line produced a single isoform of dystrophin in muscle that corresponded to the deletion construct used to generate the line. No degradation products were observed for any of the dystrophin deletion proteins, suggesting that all proteins were being stably produced at levels sufficient for correction in the case of a functional dystrophin protein (except for the Δ65–66 line, see below). This latter conclusion is derived from previous observations indicating that 20–30% of normal dystrophin levels are sufficient to prevent dystrophic pathology in *mdx* mice (37, 43). In addition, the Δ64–67 mice and the Δ75–78 mice accumulate similar dystrophin levels (Figs. 2 and 3), and the latter line of mice displays no pathology (see below). To determine the effect of each of the

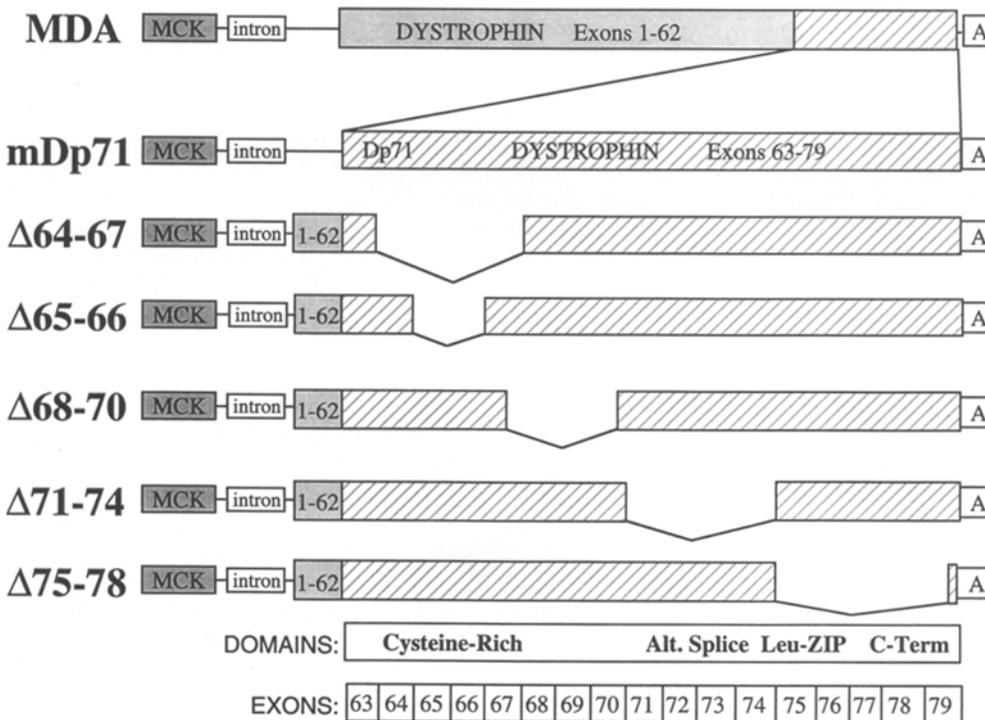
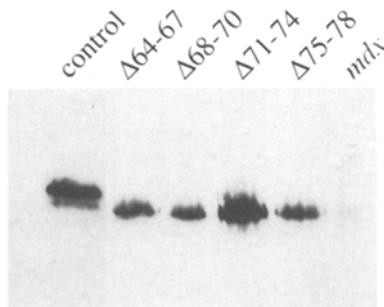


Figure 1. Diagram of the transgenes used to generate dystrophin COOH-terminal deletion proteins in *mdx* mice. The MDA full-length dystrophin construct (10) and the Dp71 COOH-terminal dystrophin construct (12) are shown as a reference for the position of the deletions in the full-length COOH-terminal deletion transgenes. The -3300 muscle creatine kinase (*MCK*) promoter and either the VPI or minx intron was used to express each of the COOH-terminal deletion dystrophin proteins. An SV-40 poly A addition site was inserted downstream from each dystrophin cDNA. COOH-terminal domains and exon numbers are labeled.



**Figure 2.** Immunoblot analysis of transgenic/*mdx* mice. An affinity-purified mAb raised against the rod domain of dystrophin detects the 427-kD protein in control (C57Bl/10) quadriceps muscle and the truncated dystrophin proteins produced in each of the transgenic lines. No dystrophin protein is detected in extracts from *mdx* quadriceps muscle.

deletions on dystrophin localization, as well as the uniformity of expression of each of the deleted proteins, transgenic/*mdx* mice were analyzed by immunohistochemistry. Each of the deleted dystrophin proteins properly localized to the sarcolemmal membrane (Fig. 3).

Proper localization and accumulation of the members of the DAP complex has been shown to be disrupted in *mdx* mice (33). We have previously shown that expression of the COOH terminus of dystrophin (Dp71), which consists of exons 63-79 (Fig. 1), is able to restore proper localization of the members of the DAP complex (12). To determine the effect of the consecutive COOH-terminal deletions of dystrophin on the localization of the DAPs, immunohistochemical analysis was carried out on quadriceps sections from 3-mo-old transgenic/*mdx* animals using antibodies to each of the identified members of the dystroglycan, sarcoglycan, and syntrophin subcomplexes.

Skeletal muscle from 3-mo-old *mdx* mice is also characterized by a high percentage of central nuclei, which is indicative of rounds of degeneration and regeneration of muscle fibers, as well as infiltration of small necrotic regions with macrophages and a progressive accumulation of connective tissue, particularly in the diaphragm. To determine the effect that each of the COOH-terminal deletion dystrophin proteins had on the dystrophic phenotype of the *mdx* mouse, fixed quadriceps sections from 3-mo-old transgenic/*mdx* mice from each uniform expressing line were stained with hematoxylin and eosin to assess the function of the dystrophin-associated complex that was formed with each deletion protein. In addition, the quadriceps sections analyzed by immunohistochemistry were counterstained with the DNA dye DAPI to observe the presence of central nuclei within the myofibers.

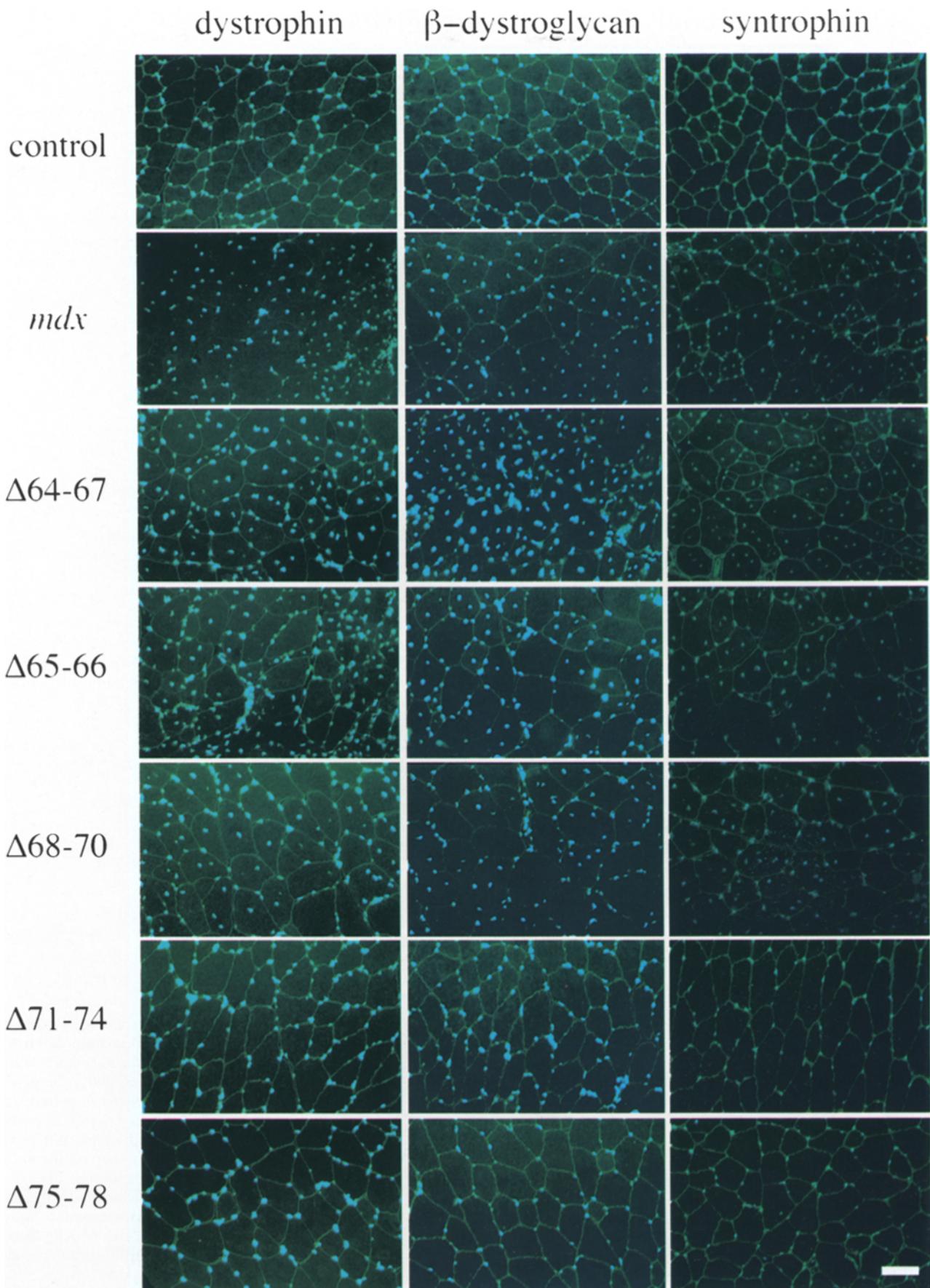
### Deletion of Exons 64-67

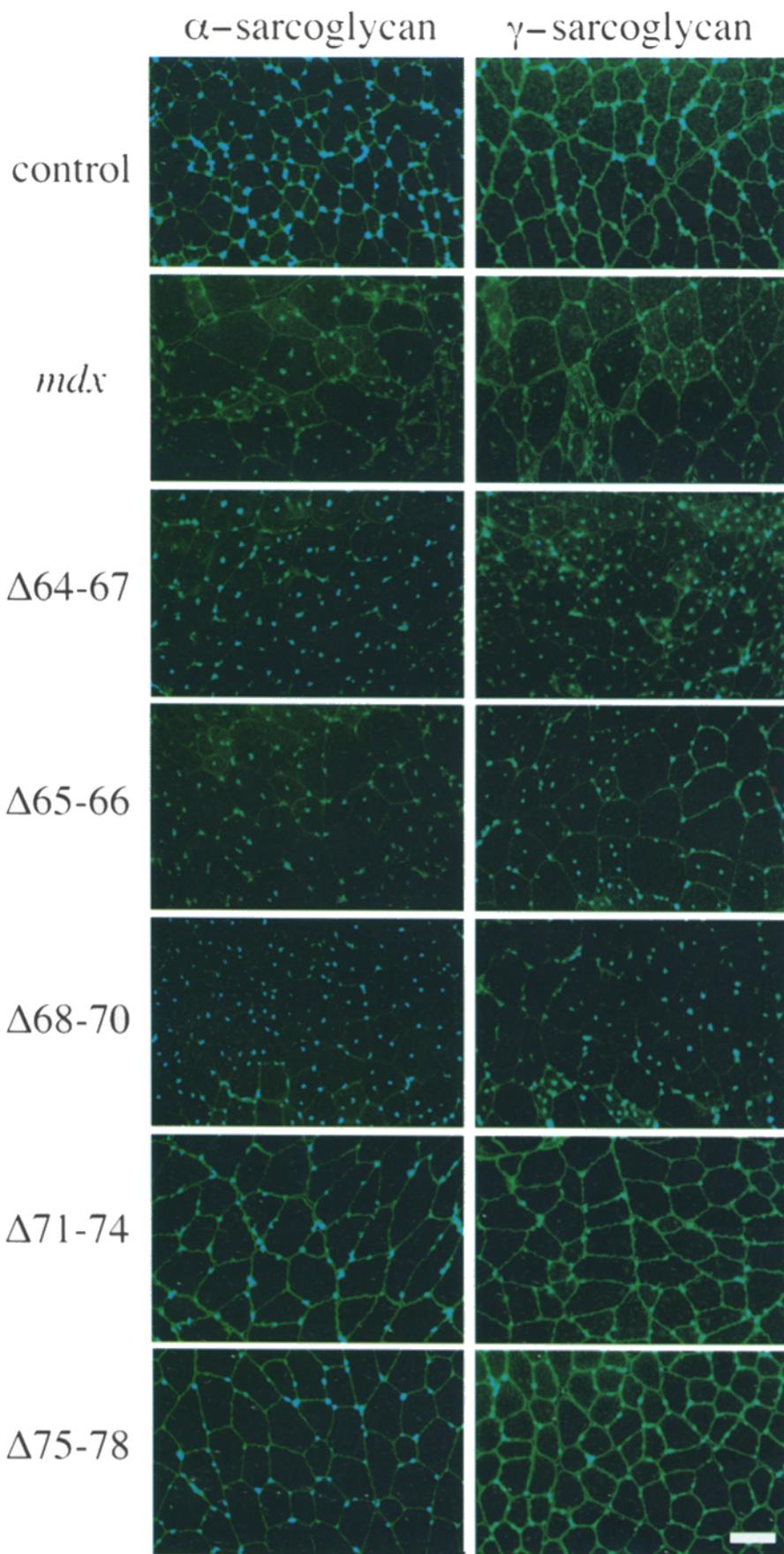
The  $\Delta 64-67$  construct is deleted for the region of homology with the cysteine-rich region of  $\beta$ -spectrin (Fig. 1). Only one line of transgenic mice with this construct displayed expression of the transgene, and this expression was observed only in limb muscles at levels  $\sim 50\%$  of wild-type expression (Fig. 2). Localization of  $\beta$ -dystroglycan and all members of the sarcoglycan complex at the sarcolemmal membranes of mice expressing the  $\Delta 64-67$  dystrophin protein is reduced to levels equal to or less than those present in *mdx* mice (Fig. 3).  $\alpha 1$ -syntrophin, however, is present at levels similar to that of the dystrophin deletion protein.  $\Delta 64-67$  was unable to function to prevent the development of a dystrophic pathology. Skeletal muscle from these mice was characterized by patches of necrosis and fibrosis larger than those seen in age-matched *mdx* mice (Fig. 5).

### Deletion of Exons 65-66

The  $\Delta 65-66$  construct corresponds to the major spliced form of dystrophin observed in the *mdx*<sup>3cv</sup> mouse (12). It was not known whether the dystrophic pathology seen in these mice was caused by the extremely low levels of protein produced, or whether this protein was nonfunctional. The only transgenic lines expressing this construct displayed a mosaic pattern of expression, making a direct analysis of the function of this deleted protein impossible (Fig. 3). These mosaic-expressing mice, however, were able to be analyzed for proper localization of the members of the DAP complex. To establish that formation of the dystroglycan complex is independent of uniform dystrophin expression, we analyzed the pattern of  $\beta$ -dystroglycan localization in a line of mice with mosaic expression of the  $\Delta 71-74$  dystrophin protein, which we have already shown to interact normally with all of the DAPs. These mice show a mosaic pattern of localization of  $\beta$ -dystroglycan, similar to that observed for dystrophin (data not shown). This result shows that the localization of the DAPs in a fiber is dependent only on expression of dystrophin in that same fiber, and that no higher order structure between fibers is needed for the formation of the dystrophin-glycoprotein complex.  $\beta$ -dystroglycan (Fig. 3) and the members of the sarcoglycan complex (Fig. 4) were all observed to be greatly reduced at the sarcolemmal membrane of transgenic/*mdx* mice expressing the  $\Delta 65-66$  dystrophin protein, compared with control animals. In contrast, syntrophin was present in a similar mosaic pattern as dystrophin expression (Fig. 3). We thus conclude that the  $\Delta 65-66$  protein cannot associate with the dystroglycan complex, and is therefore unlikely to be functional.

**Figure 3.** Immunostaining of quadriceps muscle sections from control, *mdx*, and transgenic/*mdx* mice with antibodies against dystrophin,  $\beta$ -dystroglycan, and syntrophin. All truncated dystrophin proteins localize normally to the sarcolemma.  $\beta$ -dystroglycan and syntrophin colocalize with dystrophin in control animals and are greatly reduced in *mdx* fibers. Syntrophin is present at the sarcolemmal membrane of fibers from all transgenic/*mdx* lines.  $\beta$ -dystroglycan localization at the sarcolemma is reduced to levels equal to or less than those present in *mdx* fibers in all transgenic/*mdx* lines containing a deletion in the cysteine-rich domain, but is normal in  $\Delta 71-74$ /*mdx*, and  $\Delta 75-78$ /*mdx* lines. All photographs were taken at the same exposure. Bar, 100  $\mu\text{m}$ .





*Figure 4.* Immunostaining of quadriceps muscle sections from control, *mdx*, and transgenic/*mdx* mice with antibodies against  $\alpha$ - and  $\gamma$ -sarcoglycan.  $\alpha$ - and  $\gamma$ -sarcoglycan colocalize with dystrophin in control animals and with truncated dystrophin proteins that are able to correct the *mdx* phenotype. Both proteins are reduced at the sarcolemmal membrane in *mdx* mice and transgenic/*mdx* lines containing cysteine-rich domain deletions. All photographs were taken at the same exposure. Bar, 100  $\mu$ m.

### Deletion of Exons 68-70

The  $\Delta 68-70$  construct is deleted for the region of dystrophin containing the second half of the cysteine-rich domain (Fig. 1). Expression of the transgene in these mice results in essentially wild-type levels of dystrophin in quadriceps muscles (Fig. 2). The  $\Delta 68-70$  mice show a reduction in levels of  $\beta$ -dystroglycan,  $\alpha$ -, and  $\gamma$ -sarcoglycan at the sarcolemmal membrane equivalent to that seen in *mdx* skeletal muscle (Figs. 3 and 4). In contrast, syntrophin localization is unaffected in these mice (Fig. 3). The  $\Delta 68-70$  protein is also unable to prevent the phenotype of the *mdx* mouse, suggesting again the importance of the interaction of dystrophin with the dystroglycan and sarcoglycan complexes (Fig. 5).

### Deletion of Exons 71-74

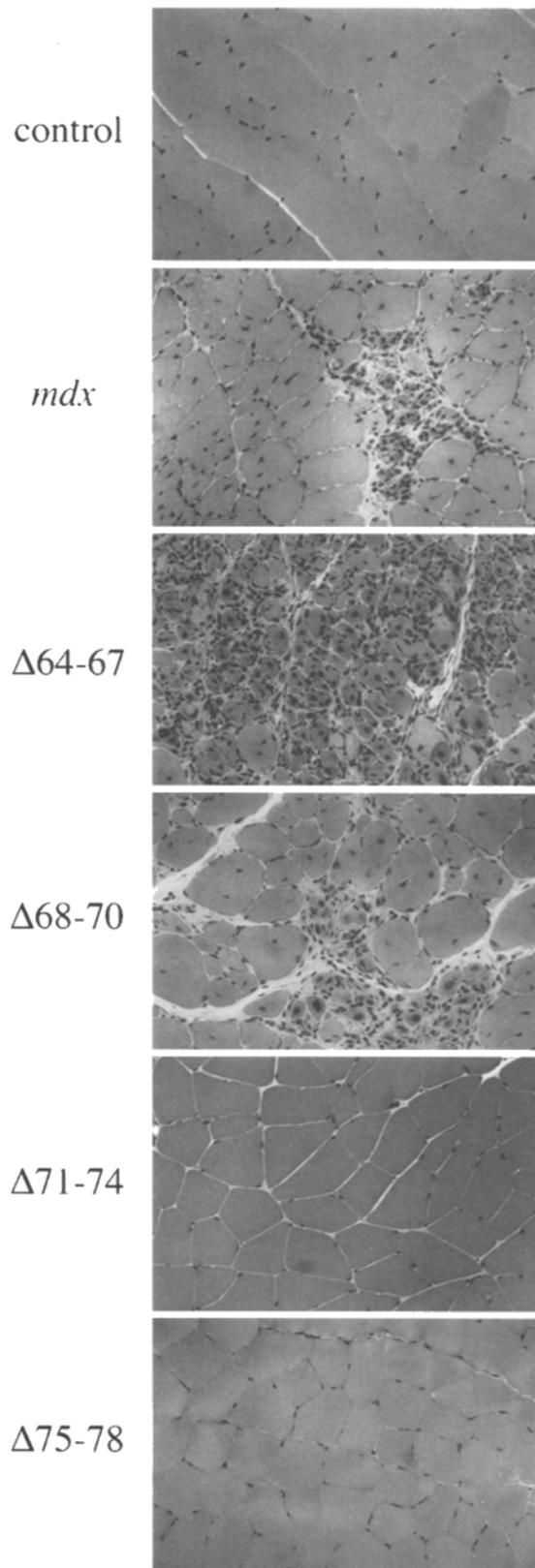
As we have previously reported, deletion of the alternatively spliced domain and disruption of the first leucine zipper motif,  $\Delta 71-74$  (previously referred to as  $\Delta 330$ ), has no effect on the function of dystrophin (39). We have now observed that this dystrophin deletion protein functions completely normally up to at least 2 yr of age (Rafael, J.A., and J.S. Chamberlain, unpublished observations). We have also previously shown that the  $\Delta 71-74$  dystrophin protein is able to associate with some members of the DAP complex. To expand this analysis to the newly identified members of the sarcoglycan complex, we used an antibody to  $\gamma$ -sarcoglycan for immunohistochemical analysis of quadriceps sections from these same mice. All of the known DAP complex members were observed to associate normally with the  $\Delta 71-74$  dystrophin protein (Figs. 3–5).

### Deletion of Exons 75-78

The  $\Delta 75-78$  construct is deleted for the second leucine zipper motif and all except the final three amino acids of the COOH terminus of dystrophin. Multiple lines of mice showed expression of this transgene in either diaphragm or quadriceps muscles or in both. The  $\Delta 75-78$  dystrophin protein is also able to localize correctly all of the members of the DAP complex and was able to prevent the appearance of any dystrophic symptoms in 3-mo-old transgenic/*mdx* mice (Figs. 3–5).

### Discussion

Although efforts are being made to develop treatments for patients with DMD, the primary pathogenesis leading to this disease is not fully understood. By studying structure–function correlations of the critical COOH-terminal domain of dystrophin, we may achieve a greater understanding of the degenerative process seen in DMD. Previously, we showed that expression only of the dystrophin COOH-terminal region (Dp71) in skeletal muscles of *mdx* mice supported normal assembly of the DAP complex, but the dystrophic pathology was not prevented, indicating that amino-terminal dystrophin sequences are also required for dystrophin function (12). To explore the COOH-terminal domains in the context of an otherwise fully functional dystrophin, we have now generated transgenic mice ex-



**Figure 5.** Morphology of quadriceps sections from control, *mdx*, and transgenic/*mdx* mice. Haematoxylin and eosin staining of 4- $\mu$ m fixed sections from 3-mo-old mice shows a high percentage of central nuclei and patches of necrosis and fibrosis in *mdx* and transgenic/*mdx* animals where the truncated dystrophin protein is not able to associate with the dystroglycan and sarcoglycan complexes.  $\Delta 71-74$ /*mdx* and  $\Delta 75-78$ /*mdx* quadriceps sections do not show signs of any dystrophic pathology.

pressing full-length dystrophin constructs with deletions of consecutive regions of the COOH terminus of dystrophin. Our data show that the cysteine-rich region of dystrophin is critical for interacting with the DAP complex in vivo, and that disruption of this interaction alone is able to render a membrane-localized, full-length dystrophin protein completely nonfunctional. In contrast, deletions of the alternatively spliced region and the extreme COOH terminus have no apparent effect on the function of dystrophin. Both of these latter dystrophin proteins are able to associate properly with all known DAP complex members.

These transgenic studies provide information about a region of dystrophin that was not well understood from studies of patient data. Most DMD patients with COOH-terminal deletions have undetectable levels of dystrophin protein, making structure-function studies impossible. Few patients have been described that contribute to an understanding of the dystrophin COOH terminus. One patient (patient CM) has a deletion of exons 72-79 that is predicted to make a truncated protein (30). This patient has a very mild BMD phenotype, although it is not known at what level the truncated protein is expressed. We have deleted this region in two parts, and have shown that both deleted proteins support normal muscle development and function when expressed near normal levels. A second reported patient has a deletion of exons 64-78 that encompasses the  $\beta$ -dystroglycan-binding domain in addition to the remainder of the COOH terminus (5, 19). This patient displays a mosaic expression pattern, and multiple degradation products of the truncated dystrophin protein were observed by Western analysis. The patient with the exon 64-78 deletion is very severely affected, as are the Dp71 (12) and the  $\Delta$ 64-67 transgenic mice (Fig. 5), suggesting that the presence on the sarcolemmal membrane of a nonfunctional dystrophin protein is more deleterious than a lack of dystrophin expression. It is possible that either utrophin or the 87-kD dystrophin-like phosphoprotein (dystrobrevin), which have both been shown to bind some of the DAPs in vitro (3, 6, 45), are able to compensate partially for dystrophin in its absence. A nonfunctional dystrophin localized to the sarcolemmal membrane, however, may out-compete such interactions.

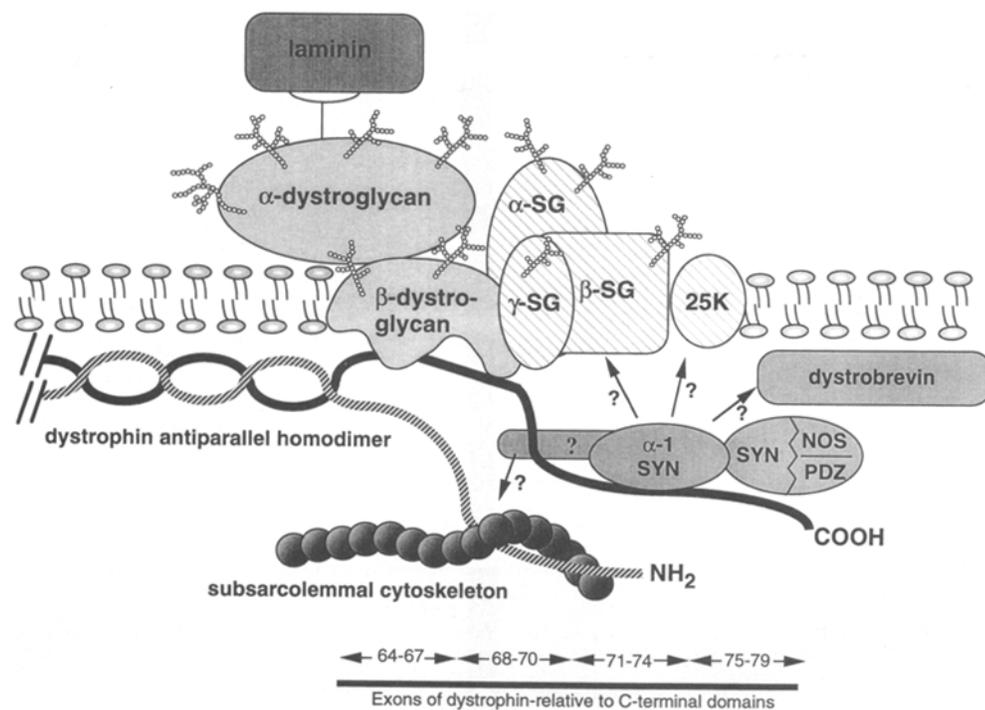
Our in vivo data, in combination with recent in vitro binding assays between dystrophin and some members of the DAP complex, supports a model for the structure of this complex. The in vitro binding studies suggest that  $\beta$ -dystroglycan may be the only member of the dystroglycan and sarcoglycan complexes that binds directly to dystrophin.  $\beta$ -dystroglycan is able to bind to fusion proteins containing only exons 63-67 of dystrophin, but this binding appears to be enhanced by protein sequences contained within exons 68-70 of dystrophin (23, 41). This in vitro data is supported by our in vivo evidence. The  $\Delta$ 68-70 line appears less affected than the  $\Delta$ 64-67 line. This observation suggests that the low level of  $\beta$ -dystroglycan in  $\Delta$ 68-70 mice may be able to form partially functional complexes via binding to the exon 64-67 region of dystrophin. Interestingly, the critical region between exons 63-67 represents the COOH-terminal extent of the conserved region among dystrophin,  $\alpha$ -spectrin, and  $\alpha$ -actinin (25). While the exon 68-70 region is not conserved with these latter proteins, it does contain a zinc finger-like motif that could

participate in strengthening the interaction with  $\beta$ -dystroglycan (38).

Results from the  $\Delta$ 64-67 mice, together with patient data, implies that the sarcoglycan complex associates with dystrophin via  $\beta$ -dystroglycan. Studies of LGMD patients has revealed that LGMD types 2D, 2E, and 2C are caused by defects in  $\alpha$ -,  $\beta$ -, and  $\gamma$ -sarcoglycan, respectively (8, 22, 27, 28, 32). In each of these sarcoglycanopathies, deficiency of one sarcoglycan protein results in the absence of each of the remaining two sarcoglycan proteins, indicating that these proteins are mutually dependent on one another for stability. The absence of the sarcoglycans do not, however, affect the stability or localization of either dystrophin or dystroglycan. Nonetheless, a complete absence of either dystrophin, or the sarcoglycans leads to a severe, DMD-like phenotype. Our data indicate that the absence of a direct link between dystrophin and  $\beta$ -dystroglycan similarly leads to the absence of the sarcoglycan proteins and a severe phenotype. Thus, even though no human diseases have been observed to result from the absence of dystroglycan, our data indicate that defective expression of dystrophin, dystroglycan, or the sarcoglycans is incompatible with normal muscle function.

$\alpha$ 1-syntrophin is highly expressed in skeletal muscle and displays an identical localization pattern to dystrophin (36). Both  $\alpha$ 1-syntrophin and  $\beta$ 1-syntrophin, the latter expressed predominantly in liver but at moderate levels in skeletal muscle, have been shown to bind the exon 73-74 regions of dystrophin in vitro (3, 42, 45). The deletion of these exons in vivo does not affect the proper localization of syntrophin (reference 39 and Fig. 4). The  $\Delta$ 71-74 protein is also completely functional in *mdx* mice, suggesting that these exons are not critical for dystrophin function in skeletal muscle. It is possible that these exons, which are alternatively spliced in nonskeletal muscle tissues such as brain and cardiac muscle, are involved in modulating the interaction between dystrophin isoforms and the syntrophins in these tissues.

The normal expression pattern of syntrophin in the  $\Delta$ 71-74 mice indicates that there must be additional factors involved in the localization of the syntrophins. One possibility is that  $\alpha$ 1 and/or  $\beta$ 1-syntrophin binds to a member of the dystroglycan or sarcoglycan complexes and that this association leads to the proper localization of syntrophin in the  $\Delta$ 71-74 mice. Another alternative is that syntrophin binds as a homo- or hetero-dimer to dystrophin, both to the exon 71-74 region and perhaps weakly to a region elsewhere in dystrophin, possibly exons 75-78. Yang et al. (45) have shown that  $\alpha$ 1-syntrophin is able to bind all three syntrophin isoforms in vitro, suggesting that dimer or trimer formation is possible in vivo. Although syntrophin was not found to bind to a downstream region in vitro, such an interaction could occur in vivo. Finally, syntrophin that is bound to dystrophin may also interact with additional partners that could help anchor it into the DAP complex (Fig. 6). Support for this latter hypothesis comes from the observation that protein A0 copurifies with the syntrophin subcomplex (41), and A0 has recently been identified as an isoform of dystrobrevin (6, 46). Neuronal nitric oxide synthase has recently been shown to bind  $\alpha$ 1-syntrophin in type II muscle fibers via conserved PDZ domains (7), although nNOS may only bind a subset of the



**Figure 6.** A model for the interaction of dystrophin-associated proteins with the COOH terminus of dystrophin, based on in vitro and in vivo data. The dystroglycan and sarcoglycan (SG) proteins require the COOH-terminal portions of dystrophin encoded on exons 64-67 for assembly and stability. Removal of dystrophin exons 68-70 also destabilizes the dystroglycan and sarcoglycan complexes but to a lesser degree, as evidenced by the milder phenotype of *mdx* animals expressing the  $\Delta 68-70$  construct, as compared with *mdx* mice expressing the  $\Delta 64-67$  construct (Figs. 3-5). These observations are consistent with a model in which the sarcoglycan proteins do not directly bind dystrophin, but are dependent on the  $\beta$ -dystroglycan-dystrophin interaction for stability and as-

sociation with the complex. Syntrophin (*SYN*) isoforms in vitro are able to bind themselves and to the portion of dystrophin encoded by exons 73-74, but association of  $\alpha 1$ -syntrophin with the dystrophin complex is stable in the absence of these exons in vivo (Fig. 3). These observations suggest that a direct interaction between syntrophin and another protein can stabilize the localization of syntrophin and allow normal muscle function in the absence of a direct link with dystrophin. This additional interaction might be with another member of the DAP complex, with other syntrophin isoforms, with dystrophin homologues such as dystrobrevin, or with PDZ domain containing proteins, including nitric oxide synthase (*NOS*), some of which might link syntrophin to the membrane or to the subsarcolemmal cytoskeleton. Question marks indicate hypothetical interactions.

DAP complexes in these fibers. Syntrophin might also interact with additional PDZ domain proteins to form attachments either to membrane-bound DAPs or to other components of the subsarcolemmal cytoskeleton (Fig. 6).

Through any of these mechanisms, syntrophin isoforms would still be able to form a functional interaction with the DAP complex, even in the absence of a direct link with dystrophin. It is possible that syntrophin plays a fundamentally distinct role in the DAP complex than do the dystroglycan and sarcoglycan proteins. This role may be distinct from the primary structural role of the DAP complex in linking the subsarcolemmal cytoskeleton to the extracellular matrix. One possibility is that syntrophin may play more of a regulatory role, modulating the interaction between the remaining DAP members, or between the DAP complex and the cytoskeleton, in response to varying physiological states of the muscle fibers.

Other protein interactions could also be occurring in the COOH-terminal regions of dystrophin. The  $\Delta 75-78$  region contains a leucine zipper motif that has been observed to bind troponin T in the yeast two-hybrid system (35). Disruptions of this leucine zipper motif and the one encoded in exons 74-75, however, appear to have no effect in our transgenic mice. Aciculin has also been shown to associate with dystrophin in a muscle cell line (4). However, this protein has not been shown to be the primary defect in any muscular dystrophy, nor is it clear whether it binds to dystrophin or to a member of the DAP complex.

The analysis of transgenic/*mdx* mice expressing dystrophin proteins with consecutive deletions of the COOH-terminal region has provided insight into the formation of the dystrophin-associated protein complex and the pathogenesis of DMD. This study provides additional in vivo evidence that a critical function of dystrophin in skeletal muscle is to provide a link to the DAP complex via a direct association with  $\beta$ -dystroglycan. Severing this link via small deletions in the cysteine-rich domain, without disrupting the remainder of the dystrophin molecule, is enough to lead to the severe muscle pathology seen in DMD. In contrast, a direct link between dystrophin and syntrophin does not appear critical for prevention of the dystrophic pathology observed in DMD and in *mdx* mice. A complete understanding of the formation of the dystrophin-associated protein complex will help to provide a basis for a greater understanding of muscular dystrophies.

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