Severe muscular dystrophy in mice that lack dystrophin and α7 integrin

Jachinta E. Rooney1,*, Jennifer V. Welser1,*, Melissa A. Dechert1, Nichole L. Flintoff-Dye1, Stephen J. Kaufman2 and Dean J. Burkin1,3,‡

1Department of Pharmacology, University of Nevada, Reno, NV 89557, USA
2Department of Cell and Developmental Biology, University of Illinois, Urbana, IL 61801, USA
3Nevada Transgenic Center, University of Nevada, Reno, NV 89557, USA

*These authors contributed equally to this work
‡Author for correspondence (e-mail: dburkin@med.unr.edu)

Summary

The dystrophin glycoprotein complex links laminin in the extracellular matrix to the cell cytoskeleton. Loss of dystrophin causes Duchenne muscular dystrophy, the most common human X-chromosome-linked genetic disease. The αβ1 integrin is a second transmembrane laminin receptor expressed in skeletal muscle. Mutations in the α7 integrin gene cause congenital myopathy in humans and mice. The αβ1 integrin is increased in the skeletal muscle of Duchenne muscular dystrophy patients and mdx mice. This observation has led to the suggestion that dystrophin and αβ1 integrin have complementary functional and structural roles. To test this hypothesis, we generated mice lacking both dystrophin and α7 integrin (mdx/α7−/−). The mdx/α7−/− mice developed early-onset muscular dystrophy and died at 2-4 weeks of age. Muscle fibers from mdx/α7−/− mice exhibited extensive loss of membrane integrity, increased centrally located nuclei and inflammatory cell infiltrate, greater necrosis and increased muscle degeneration compared to mdx or α7-integrin null animals. In addition, loss of dystrophin and/or α7 integrin resulted in altered expression of laminin-α2 chain. These results point to complementary roles for dystrophin and αβ1 integrin in maintaining the functional integrity of skeletal muscle.

Key words: α7 integrin, Dystrophin, Transgenic mice, Double knockout, Skeletal muscle, Muscular dystrophy

Introduction

Duchenne muscular dystrophy (DMD) is the most common X-chromosome-linked human disease that affects approximately 1 in 3,500 males. DMD patients suffer from severe, progressive muscle wasting with clinical symptoms first detected between 2 and 5 years of age. As the disease progresses, patients are confined to a wheelchair in their early teens and die in their early twenties from cardiopulmonary failure (Emery, 1993; Moser, 1984). DMD patients and mdx mice have dystrophin gene mutations that result in an absence of the dystrophin protein (Bulfield et al., 1984; Campbell, 1995; Matsumura et al., 1992; Monaco et al., 1986; Sicinski et al., 1989). Dystrophin is localized to the cytoplasmic face of the plasma membrane and mediates the interaction between the cell cytoskeleton and the extracellular matrix (ECM) through a complex of associated glycoproteins called the dystrophin-glycoprotein complex (DGC). In skeletal muscle, the DGC is localized along muscle fibers and at myotendinous and neuromuscular junctions (MTJs and NMJs, respectively). This complex includes dystroglycans, sarcoglycans and syntrophins (Suzuki et al., 1994). Dystrophin interacts with F-actin through its N-terminus (Way et al., 1992). The C-terminus interacts with syntrophins and with the cytoplasmic tail of β-dystroglycan (Henry and Campbell, 1996). α-dystroglycan binds to laminin in the extracellular matrix. Overall, the DGC links the muscle cell cytoskeleton to the extracellular matrix and maintains sarcolemmal integrity.

In DMD patients, loss of dystrophin results in a disruption of the DGC-extracellular matrix linkage. This leads to the loss of sarcolemmal integrity, unregulated influx of Ca2+ into muscle cells, activation of Ca2+-dependent proteases and muscle degeneration (Gillis, 1996). Muscle degeneration is accompanied by regeneration through the activation of muscle satellite cells; however, regenerative capacity in DMD patients is exceeded by progressive muscle degeneration. This results in the replacement of muscle fibers with adipose and connective tissue (O'Brien and Kunkel, 2001).

The mdx mouse is a valuable model for DMD and has been used extensively to analyze the progression of muscle disease. Although DMD patients (Monaco et al., 1986) and mdx mice (Bulfield et al., 1984; Sicinski et al., 1989) both lack dystrophin, the mdx mouse develops less severe muscle pathology compared with DMD patients, and has a normal life span. Several factors may explain the reduced pathology in mdx mice including differences in muscle use between humans and captive mice. In addition, mdx mice might compensate for the loss of dystrophin, possibly by overexpressing one or more proteins with functional similarity to dystrophin. One candidate protein is utrophin, an autosomal homolog of dystrophin that is expressed at the post-synaptic membrane of the NMJ in adult skeletal muscle. Utophin is upregulated in the muscle of both DMD patients and mdx mice and is localized at extrajunctional sites (Ohlendiek et al., 1991; Pons et al., 1991).
Overexpression of utrophin can rescue the dystrophic phenotype in *mdx* mice (Deconinck et al., 1997b; Rafael et al., 1998; Squire et al., 2002; Tinsley et al., 1998; Tinsley et al., 1996). Mice that lack utrophin show mild neuromuscular defects (Deconinck et al., 1997a; Grady et al., 1997a). By contrast, mice lacking both utrophin and dystrophin (*mdxutr* mice) develop severe muscular dystrophy and cardiomyopathy similar to that of DMD patients, and die between 4-14 weeks of age (Deconinck et al., 1997b; Grady et al., 1997b).

The α7β1 integrin is a second laminin receptor in skeletal muscle (Burkin and Kaufman, 1999). Like the DGC, α7β1 integrin serves as a structural link between the cell cytoskeleton and laminin in the basal lamina and, therefore, contributes to the overall integrity of the sarcolemma. Mutations in the α7-integrin gene are responsible for human congenital myopathy, in which patients exhibit delayed motor milestones (Hayashi et al., 1998). Mice that lack α7 integrin develop myopathy and demonstrate altered force transmission, compliance and viscoelasticity in diaphragm muscle (Lopez et al., 2005; Mayer et al., 1997). In addition, α7β1 integrin plays an important role in the development of NMJs (Burkin et al., 1998; Burkin et al., 2000) and MTJs (Mayer et al., 1997; Nawrotzki et al., 2003). These junctional sites are often damaged in diseased muscle (Lyons and Slater, 1991; Nagel et al., 1990; Nawrotzki et al., 2003; Rafael et al., 2000).

Increased amounts of α7 integrin are found in DMD patients and *mdx* mice (Hodges et al., 1997). These observations suggest that enhanced α7 integrin expression is a mechanism by which muscle can compensate for the loss of dystrophin. This hypothesis was supported by transgenic overexpression of the α7-integrin chain which alleviated the severe muscular dystrophy and extended the life-span of mice that lacked dystrophin and utrophin (Burkin et al., 2001; Burkin et al., 2005). These observations suggest potential structural and functional overlap between the α7β1 integrin and DGC.

To explore the structural and functional overlap between dystrophin and α7β1 integrin, we generated mice that lack both laminin-binding complexes (*mdxα7−/−* mice). These mice exhibited more severe muscular dystrophy than either *mdx* or α7-integrin-deficient animals and died at 2-4 weeks of age. In addition, the muscle of 3-week-old *mdxα7−/−* mice showed inflammation and centrally located nuclei, indicative of severe muscle damage and increased requirement for muscle repair. Finally, loss of dystrophin and/or α7 integrin resulted in reduced laminin-α2-chain expression in skeletal muscle, demonstrating regulation of laminin expression by α7β1 integrin. Collectively, these studies support functional and structural overlap between DGC and α7β1 integrin in maintaining the integrity of skeletal muscle.

**Results**

*mdxα7−/−* mice exhibit muscle wasting and reduced viability

To determine whether functional overlap exists between dystrophin and α7β1 integrin, *mdx* mice were bred with mice that lack the α7-integrin gene (Flintoff-Dye et al., 2005) to produce mice that lack both dystrophin and α7 integrin (*mdxα7−/−*). Mutations in the dystrophin and α7 integrin genes were confirmed by genotyping (Fig. 1A). Multiplex PCR
detected a 482 bp product representing the targeted α7-integrin allele in both the mdx/α7−/− and mdx/α7−/− mice, whereas the wild-type allele was 727 bp. Amplification-resistant mutation system (ARMS) PCR yielded a 275 bp product and confirmed the presence of the mdx mutation in mdx and mdx/α7−/− mice (Fig. 1A). Loss of α7 protein was confirmed by immunoblotting. Western analysis demonstrated that both α7A-integrin and α7B-integrin isoforms were missing in mdx/α7−/− mice (Fig. 1B). The absence of both dystrophin and α7 integrin in the skeletal muscle of mdx/α7−/− mice was confirmed by immunofluorescence (Fig. 1C).

At 10 days of age, the average weight of male wild-type, mdx, α7−/− or mdx/α7−/− pups was 7.9±0.2 g, 6.3±0.9 g, 7.8±0.17 g or 5.6±0.45 g, respectively (Fig. 2B). The weight of mdx and mdx/α7−/− littermates was statistically different from wild-type and α7−/− mice (P<0.05), but was not different from each other. By day 18, mdx/α7−/− mice exhibited a failure to thrive and appeared smaller compared with mdx littermates (Fig. 2A). By day 21, male mdx/α7−/− mice weighed only 5.05±0.51 g (n=8), less than half the weight of wild-type (11.89±0.83 g, n=5), mdx (9.86±0.15 g, n=7) or α7−/− (13.04±0.54 g, n=9) animals, indicating that double-knockout mice failed to gain weight (Fig. 2C). mdx/α7−/− animals displayed kyphosis, joint contractures of the limbs, an abnormal waddling gate, tremors and reduced mobility (a movie that contrasts mobility, joint contractures, tremor and gait in mdx and mdx/α7−/− mice can be viewed as supplementary material, Movie 1). This debilitating phenotype progressed rapidly, resulting in the death of these mice at day 16-26 (Fig. 2D).

Muscle membrane integrity is compromised in mdx/α7−/− mice

To evaluate skeletal muscle integrity, mice were injected with Evan’s Blue dye (EBD). EBD binds albumin, fluoresces red and can enter cells with compromised plasma membranes. At 3 weeks, wild-type and α7−/− mice showed no evidence of EBD uptake, signifying that the sarcolemma was undamaged in these animals (Fig. 3A). By contrast, myofibers from both mdx and dystrophin/α7 double-knockout mice showed considerable EBD uptake (Fig. 3A). Muscle from mdx and mdx/α7−/− mice had 14.6% and 18.9% EBD-positive fibers, respectively; however, these were not significantly different from each other (Fig. 3B). These data indicate that the loss of dystrophin but not α7 integrin, in the skeletal muscle of 3-week-old mice decreases sarcoglycan integrity.

mdx/α7−/− mice exhibit severe muscle pathology

To investigate whether the skeletal muscle from mdx/α7−/− mice exhibited greater pathology compared with controls, muscle sections were stained with hematoxylin and eosin. Skeletal muscle from 3-week-old wild-type and α7−/− mice showed no pathology (Fig. 4A). By comparison, skeletal muscle from 3-week-old mdx mice exhibited small necrotic lesions (Fig. 4A). By contrast, skeletal muscle from mdx/α7−/− mice exhibited severe pathology including variation in myofiber size, large necrotic regions and areas of mononuclear cell infiltration (Fig. 4A). These data demonstrate that the skeletal muscle of 3-week-old mdx/α7−/− mice has undergone increased muscle degeneration compared with wild-type, mdx or α7-integrin null animals.

Earlier studies have implicated inflammation in the pathogenesis of muscular dystrophy (McDouall et al., 1990; Tews and Goebel, 1996). To assess the inflammatory response in mice lacking dystrophin and α7 integrin, tissue cryosections from the tibiceps muscle of 3-week-old wild-type, mdx, α7−/− and mdx/α7−/− mice were incubated with anti-CD4 antibody. CD4 is a type-I membrane glycoprotein expressed on thymocytes, and to some extent, monocytes and macrophage-related cells. There was on average less than 1 CD4-positive cell per field of view from each of the control genotypes (Fig. 4B). By contrast, an average of 44 CD4-positive cells per field was observed in the muscle of mdx/α7−/− mice. These results

Fig. 2. Muscle-wasting and reduced viability in mdx/α7−/− mice. (A) Photograph of mdx and mdx/α7−/− littermates aged 18 days. Double-knockout mice appear smaller than mdx littermates and show outward signs of kyphosis. These mice also exhibit joint contractures, reduced mobility and tremors (see supplementary material, Movie 1). (B) The average weight of 10-day-old male mdx/α7−/− mice was indistinguishable from that of mdx littermates. The weight of both mdx and mdx/α7−/− mice was significantly different from wild-type and α7−/− animals (*P<0.05). (C) The average weight of 21-day-old male mdx/α7−/− mice was significantly different from wild-type, mdx, and α7−/− animals (*P<0.05), indicating that double-knockout mice failed to gain weight. (D) The viability of mdx/α7−/− mice (n=21) is severely reduced compared with wild-type (n=12), α7−/− (n=148) and mdx (n=19) mice. All mdx/α7−/− mice died at 16-26 days.
demonstrate that mdx/α7−/− mice experienced an increased inflammatory response in skeletal muscle compared with wild-type, mdx or α7-integrin null animals at 3 weeks of age.

**Increased muscle regeneration in mdx/α7−/− mice**

To examine the extent of skeletal muscle regeneration in mdx/α7−/− mice, fibers containing centrally located nuclei in triceps and tibialis anterior muscles were counted and compared with control genotypes. As expected, skeletal muscle from wild-type mice had few centrally located nuclei (Fig. 5A-B). By comparison, the tibialis anterior muscle from 3-week-old mdx and mdx/α7−/− animals showed 4.1% and 3.2% fibers with centrally located nuclei, respectively (Fig. 5B). Their triceps muscle showed 2.8% and 0.3% of muscle fibers with centrally located nuclei, respectively, which was not statistically significant from the wild-type controls (Fig. 5B). By contrast, 17.8% of muscle fibers in the tibialis anterior muscle and 40.1% of muscle fibers from the triceps muscle from mdx/α7−/− contained centrally located nuclei (Fig. 5B). These results suggest an earlier onset of regeneration in response to muscle fiber damage in mdx/α7−/− mice.

Embryonic myosin heavy chain (eMyHC) expression was examined as a second measure of muscle regeneration. Consistent with data obtained from the analysis of centrally located nuclei in muscle fibers, a significant increase in the number of eMyHC-positive fibers was observed in 3-week-old

---

**Fig. 3.** Reduced sarcolemmal integrity in muscle from mdx/α7−/− mice. (A) Evan's Blue dye (EBD) uptake was used to assess membrane integrity of the tibialis anterior skeletal muscle. Myofibers were outlined with Oregon Green-488-conjugated WGA (green). EBD uptake (red) was not detected in wild-type and α7−/− myofibers. By contrast, significant EBD uptake was observed in muscle from mdx and mdx/α7−/− animals. Bar, 10 μm. (B) The number of EBD-positive muscle fibers was assayed. Wild-type and α7−/− muscle had less than 1% EBD-positive fibers. By contrast, 18.9% of fibers were positive for EBD in mdx/α7−/− mice, but this was not significantly different from the number of EBD-positive fibers seen in mdx animals.

**Fig. 4.** Severe muscle pathology in mdx/α7−/− mice. (A) Hematoxylin and eosin staining of tibialis anterior muscle from 3-week-old mice shows the characteristically uniform fiber diameters in wild-type mice. Small necrotic regions and variations in myofiber size were observed in muscle of 3-week-old mdx mice. At this age, muscle from α7−/− mice appeared to be similar to wild-type. By contrast, muscle from mdx/α7−/− mice exhibited extreme variations in myofiber size and large areas of mononuclear cell infiltration (arrow). Bar, 10 μm. (B) Inflammation in the muscle of mdx/α7−/− mice was quantified by counting the number of CD4-positive immune cells per field of view in triceps muscle of 3-week-old mice. Muscle of wild-type, mdx and α7−/− animals showed less than 1% CD4-positive cells. By contrast, an average of 43.88 CD4-positive immune cells per field was observed in the triceps muscle of mdx/α7−/− mice, which was significantly higher when compared with control genotypes (*P<0.05).
Dystrophin and α7-integrin null mice

mdx/α7−/− muscle. As expected, wild-type muscle showed only 0.3 eMyHC-positive fibers per field. Muscle from 3-week-old mdx and α7−/− mice showed 0.2 and 0.1 eMyHC-positive fibers per field, respectively. By contrast, 7.5 eMyHC-positive fibers per field of view were counted in muscle from mdx/α7−/− mice (Fig. 6B). Collectively, these data indicate that loss of dystrophin and α7 integrin results in more muscle regeneration in mdx/α7−/− mice at the age of 3 weeks compared with wild-type, mdx or α7 integrin deficient animals.

Altered expression of laminin-α2-chain expression in α7−/− and mdx/α7−/− mice

In mature skeletal muscle, the α7-integrin chain preferentially associates with the β1D subunit and binds to laminin-211 and laminin-221 in the extracellular matrix. The α7β1 integrin has also been shown to regulate laminin deposition (Li et al., 2003). Immunoblotting and immunofluorescence were undertaken to determine whether the absence of α7, coupled with the loss of dystrophin, affected the expression and localization of β1D integrin, utrophin and/or laminin. Wild-type, mdx, α7−/− and mdx/α7−/− mice exhibited similar amounts of β1D integrin in skeletal muscle (Fig. 7A), which was confirmed by western analysis (data not shown).

Immunofluorescence revealed an increase in laminin-α2-chain expression in mdx mice and downregulation in the muscle of α7−/− and mdx/α7−/− mice (Fig. 7A). This was confirmed by quantitative western analysis with laminin-α2 bands normalized for protein loading to the constitutively and ubiquitously expressed protein cyclooxygenase-1 (Cox-1). Expression of Cox-1 did not appear to change in wild-type or

Journal of Cell Science
diseased states. In mdx mice an increase in laminin-α2 chain was detected compared with wild-type muscle. By contrast, a 7.4-fold decrease in laminin-α2 protein was observed in the muscle of α7−/− and mdx/α7−/− mice compared with both wild-type and mdx mice. Bar, 10 μm. (B) Laminin-α2 expression in the gastrocnemius muscle was quantitated by western analysis. mdx mice showed a significant increase in laminin-α2 expression compared with wild-type. α7−/− and mdx/α7−/− mice showed a decrease in laminin-α2-chain expression compared with wild-type and mdx animals (*P<0.05).

Fig. 7. Altered expression of laminin-α2 chain in the skeletal muscle of mdx/α7−/− mice. (A) Immunofluorescence was used to detect the localization of β1D and laminin-α2 chain in triceps muscle of 3-week-old mice. A similar pattern of β1D localization at the myofiber sarcolemma was observed in wild-type, α7−/−, mdx and mdx/α7−/− mice. Immunofluorescence revealed a decrease in laminin-α2 chain in the muscle of α7−/− and mdx/α7−/− mice compared with wild-type and mdx mice. Bar, 10 μm. (B) Laminin-α2 expression in the gastrocnemius muscle was quantitated by western analysis. mdx mice showed a significant increase in laminin-α2 expression compared with wild-type. α7−/− and mdx/α7−/− mice showed a decrease in laminin-α2-chain expression compared with wild-type and mdx animals (*P<0.05).

Discussion

The dystrophin glycoprotein complex and α7β1 integrin are transmembrane receptors in skeletal muscle that provide molecular continuity between laminin in the extracellular matrix and the cell cytoskeleton. Disruption of either receptor leads to neuromuscular disease in both humans and mice reflecting the importance of both complexes in maintaining muscle integrity (Emery, 1993; Hayashi et al., 1998; Mayer et al., 1997; Monaco et al., 1986; Moser, 1984; Sicinski et al.,...
Dystrophin and α7-integrin null mice

2191

Fig. 8. Increased utrophin expression in mdx but not mdx/α7−/− mice. (A) Utrophin localization was examined by immunofluorescence in the gastrocnemius muscle of 3-week-old wild-type, mdx, α7−/− and mdx/α7−/− mice. As expected, utrophin was localized at the NMJs of muscle from wild-type mice and at neuromuscular and extrajunctional sites of muscle from mdx animals, confirming previous studies. Utrophin localization appeared at both junctional and extrajunctional sites in α7+/− or mdx/α7−/− mice, Bar, 10 μm. (B) Utrophin expression in the gastrocnemius muscle was quantitated by western analysis. mdx mice showed a statistically significant increase in utrophin expression compared with wild-type (*P<0.05). α7−/− and mdx/α7−/− mice showed no increase in utrophin compared with wild-type animals.

Levels of the α7-integrin chain are increased twofold in the muscle of DMD patients and mdx mice (Hodges et al., 1997). Transgenic overexpression of α7 integrin in the skeletal muscle of severely dystrophic mdx/utr−/− mice can partially rescue the disease phenotype and extend the lifespan of these animals without restoring the expression of DGC components (Burkin et al., 2001; Burkin et al., 2005). Together, these results suggest the DGC and α7β1 integrin have overlapping and compensatory functions. The severe muscle phenotype and premature death of the mdx/α7−/− mice reported in this study further extends these studies, and supports the hypothesis that dystrophin and the α7β1 integrin have overlapping roles in maintaining the structural and functional integrity of skeletal muscle. We observed only mild muscle pathology in 3-week-old α7+/− and mdx mice, which is consistent with previously reported studies (Grady et al., 1997b; Mayer et al., 1997). By contrast, the absence of both proteins in mdx/α7−/− mice results in severe muscle degeneration, variation in myofiber size and inflammatory cell infiltration.

The muscle pathology of mice that lack dystrophin and utrophin is similar to that seen in DMD patients (Deconinck et al., 1997b; Grady et al., 1997b). Utrophin is an autosomal homolog of dystrophin localized to junctional sites in adult skeletal muscle (Blake et al., 1996). In mdx mice, utrophin is increased and is localized at extrajunctional sites were it replaces dystrophin and associates with an otherwise normal dystrophin-associated protein complex (Matsumura et al., 1992). Mice that lack dystrophin and utrophin have a mean lifespan of 14 weeks (Burkin et al., 2001; Deconinck et al., 1997b; Grady et al., 1997b), whereas mice that lack dystrophin and α7 integrin all die before the age of 4 weeks. These results indicate

Fig. 9. Ultrastructure of MTJs in muscle from 3-week-old wild-type, mdx, α7+/− and mdx/α7+/− mice. The sarcolemma of the MTJs of wild-type and mdx mice is highly folded, increasing the surface area between muscle and tendon (arrows); the extracellular matrix at this site is highly organized and electron-dense. By contrast, MTJs of α7+/− and mdx/α7+/− mice show little or no sarcolemmal folding (arrows) and the extracellular matrix appeared less dense and organized compared with those of wild-type and mdx mice (arrowheads). Bar, 1 μm.
that compensation by α7 integrin allows mdx/utr–/– mice to live longer. This hypothesis is supported by the partial rescue of the dystrophic phenotype in mdx/utr–/– mice by transgenic expression of α7 integrin, which extends the life span of these animals approximately threefold (Burkin et al., 2001; Burkin et al., 2005). By contrast, endogenous expression of utrophin in mdx/α7–/– mice appears to be unable to compensate to the same extent for the loss of α7 integrin and dystrophin. Interestingly, the loss of dystrophin and α7 integrin does not result in increased utrophin expression as seen in the skeletal muscle of mdx mice.

The mdx/α7–/– mice analyzed in this study exhibit a phenotype similar to mice deficient in α7-integrin and γ-sarcoglycan (Allikian et al., 2004). The phenotypic similarity between these mice suggests that functional overlap exists between γ-sarcoglycan in the dystrophin complex and α7β1 integrin. Alternatively, loss of γ-sarcoglycan might affect the stability of the dystrophin-glycoprotein complex, resulting in the observed phenotypic similarity (Hack et al., 2000b; Hack et al., 2000a; Zhu et al., 2001). The production and analysis of double-knockout mice lacking α7 integrin and other sarcoglycan subunits may elucidate further the functional overlap between members of the sarcoglycan complex and α7 integrin.

EBD uptake in the muscle of mdx/α7–/– mice was similar to that observed in mdx animals, implying that loss of dystrophin is responsible for the observed membrane damage in both groups of animals. Prior investigations have shown that the height of muscle degeneration and its ensuing regeneration in mdx mice occurs at 4-6 weeks of age (Grady et al., 1997b; McGeachie et al., 1993). By conducting the present studies in 3-week-old mice, only the onset of changes in membrane permeability in mdx muscle fibers was observed. This concept of age-dependency was further supported by the extensive muscle damage and centrally located nuclei observed in mdx/α7–/– mice compared with the single-knockout phenotypes. The absence of both dystrophin and α7 integrin resulted in significantly more centrally located nuclei and eMyHc expression in skeletal muscle fibers compared with control genotypes. Therefore, our data indicate that mdx/α7–/– mice have a requirement for increased muscle regeneration compared with mdx or α7-integrin null animals. Compromised contractile function or too little muscle regeneration might be mechanisms underlying the severe muscle damage observed in the mdx/α7–/– mice.

The severe muscle pathology observed in mdx/α7–/– mice is similar to that reported in laminin-α2-deficient mice (Miyagoe-Suzuki et al., 2000). The skeletal muscle of laminin-α2 null mice show reduced expression of α7 integrin (Hodges et al., 1997). Our data showed increased endogenous expression of both α7-integrin and laminin-α2 proteins in the muscle of mdx mice. Although α7 deficient mice exhibit decreased levels of laminin-α2 in skeletal muscle, these mice live beyond 20 weeks of age. These observations suggest that levels of laminin, dystrophin and utrophin in α7-integrin-deficient mice are sufficient to maintain muscle integrity. A further reduction in laminin-α2-chain expression was not observed in mdx/α7–/– mice compared with α7–/– animals, indicating that the loss of the laminin-α2 chain is primarily due to the loss of α7 integrin. Together, these data support the idea that, in skeletal muscle, a regulatory mechanism exists between the expression of α7 integrin and the laminin-α2 chain. This mechanism might also be involved in the rescue of severely dystrophic mice by the transgenic overexpression of α7 integrin (Burkin et al., 2001). Interestingly, utrophin and laminin expression are upregulated in mdx mice, but not mdx/α7–/– mice, suggesting the possibility that utrophin expression is regulated by laminin.

The inflammatory response to chronic muscle-fiber damage is believed to be a major factor contributing to the progression of pathology observed in DMD patients (Gorospe et al., 1994; Gosselin and McCormick, 2004). Muscle of mdx mice shows changes in the expression of genes involved in the inflammatory response (Porter et al., 2002). A significant inflammatory response was observed in the skeletal muscle and MTJs of mdx/α7–/– mice compared with mdx or α7-integrin-deficient animals at 3 weeks of age. These observations suggest that loss of dystrophin and integrin protein complexes lead to decreased extracellular matrix-to-muscle-cell contacts within muscle and at critical junctional sites, resulting in increased muscle-fiber damage and inflammation.

Although our data support the model of overlapping structural roles for dystrophin and α7 integrin in maintaining the integrity of the sarcolemma, it is also possible that these proteins have functionally intersecting cell-signaling pathways that regulate muscle survival because both mdx and laminin-deficient mice show increased apoptosis (Ruegg et al., 2002; Tidball et al., 1995; Tombes et al., 1995). Integrins exhibit mechanism transduction ability, and the binding of extracellular ligands or integrin-clustering can initiate cell signaling through proteins such as focal adhesion kinase or phosphoinositol-3-kinase (Schlaepfer et al., 1999; Schwartz, 2001). Interestingly, signaling through FAK and the adaptor molecule growth-factor-receptor-binding protein 2 (Grb2) can lead to activation of the mitogen-activated protein kinase (MAPK) pathway (Schlaepfer et al., 1994), which promotes cell survival (Bonni et al., 1999).

Evidence that dystrophin and other members of the DGC have cell-signaling capacities that regulate cell survival and growth is beginning to emerge. Dystrophin itself can become phosphorylated, which can alter its affinity for actin (Senter et al., 1999). Calmodulin binding sites are present on both dystrophin and syntrophin (Madhavan et al., 1992), which link the DGC to Ca2+/calmodulin-dependent protein kinase II (CaMKII)-mediated signaling (Rando, 2001). Neuronal nitric oxide synthase (nNOS), which regulates many signaling pathways, is also associated with DGC (Abdelmoity et al., 2000). Finally, Grb2 can bind to β-dystroglycan (Yang et al., 1995) and may therefore be a point of convergence between dystrophin and integrins.

Recently, several genes have been identified that appear to compensate for the loss of dystrophin. Using a complementary gene therapy approach, these ‘booster’ genes have been overexpressed in skeletal muscle of dystrophic mouse models and were shown to rescue the diseased phenotype (Engvall and Wewer, 2003). These complementary proteins include utrophin, α7 integrin, GalNac, nNOS and Adam12 (Burkin et al., 2001; Moghadaszadeh et al., 2003; Nguyen et al., 2002; Tidball and Wehling-Henricks, 2004; Tinsley et al., 1998). By taking advantage of single- and double-knockout mice, this study has identified areas of structural and functional convergence and independence between the α7β1 integrin and dystrophin protein complexes. Understanding the functional
overlap between each of the complementary proteins and dystrophin might identify new pathways that can be targeted to muscle dystrophy.

Materials and Methods

Generation of mdx/α7−/− mice

α7−/− mice (C57BL6-α/β gal strain) produced in the Nevada Transgenic Center (Finlayson et al., 2009) were crossed with mdx (C57BL10ScSn-Dmd strain) female animals. The resulting F1 males, which were heterozygous at the α7 locus, were backcrossed with mdx (C57BL10ScSn-Dmd strain) female animals. Males and females produced in the F2 generation that were heterozygous at the α7 locus were bred to generate mdx/α double-knockout mice. The wild-type control strain used in this study was C57BL10ScSn. Male littermates or age-matched mice were used for analysis.

To genotype mice, genomic DNA was isolated from tail tips using a Wizard SV DNA purification system (Promega, Madison, WI) following the manufacturer’s instructions. Mice were genotyped by multiplex PCR to detect the wild-type and targeted α7-integrin alleles with the following primers: α7Pf10 (5’-TGAAG-GAATGAGTCCAAGTGC-3’), α7exon1R1 (5’-AGATGCTGGTGGGAGCATAC-3’) and Bgαι2R (5’-GACCTGACGCGATGACGAC-3’). PCR conditions were as follows: 95°C for 4 minutes then 34 cycles at 95°C for 1 minute, 62°C for 1 minute and 72°C for 1 minute. A wild-type band was 727 bp, whereas the α7-integrin-targeted allele produced a 482 bp band.

The mutation in the dystrophin gene was detected by a modified ARMS assay (Amalfitano and Chamberlain, 1998; Burkein et al., 2001). The following primers were used: p259E (5’-GTCATCTAGATGTTAGACATTTAA-3’), p260E (5’-GTCATCTAGATGTTAGACATTTAA-3’), p306E (5’-CATGATTTATCCTGATGTTAGACATTTAA-3’). PCR conditions were as follows: 95°C for 4 minutes then 34 cycles at 95°C for 1 minute, 55°C for 1 minute and 72°C for 1 minute. Primer set p259 and p306 produced a 275 bp wild-type dystrophin allele. Primer set p260 and p306 detects the mdx point mutation and produced a 275 bp product. To genotype the dystrophin gene, separate PCR reactions were performed because the product sizes are identical in wild-type and mdx mice.

Isolation of skeletal muscle

Three-week-old wild-type, mdx, α7−/− and mdx/α7−/− male mice were euthanized by CO2 inhalation in accordance with a protocol approved by the University of Nevada, Reno Institutional Animal Care and Use Committee (IACUC). Tissue was dissected, gastrocnemius, tibialis anterior muscles from these mice were dissected, flash-frozen in liquid nitrogen and stored at −80°C.

Immunoblotting

The gastrocnemius muscle from 3-week-old male mice was ground in liquid nitrogen and stored at −80°C. Protein was extracted in 200 mM octyl-β-D-glucopyranoside (Sigma Aldrich, St Louis, MO), 50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1 mM CaCl2, 1 mM Na3VO4, 25 mM Hepes pH 7.4, 150 mM NaCl, 1 mM Na3VO4, 10 mM NaF, 0.5% Triton X-100, 0.5% NP40, 10% glycerol, 2 mM PMSF and a 1:200 dilution of Protease Inhibitor Cocktail Set III and quantified by a Bio-Rad protein assay. To prepare samples for Western blot analysis, protein was extracted from the gastrocnemius muscle with RIPA buffer (50 mM Hepes pH 7.4, 150 mM NaCl, 1 mM Na3VO4, 10 mM NaF, 0.5% Triton X-100, 0.5% NP40, 10% glycerol, 2 mM PMSF and a 1:200 dilution of Protease Inhibitor Cocktail Set III) and quantified by Bradford assay. Protein was separated on 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gels and transferred to nitrocellulose membranes. Membranes were blocked in Odyssey blocking buffer for 1 hour followed by a 1:1000 dilution of mouse monoclonal anti-Cox-1 antibody (Santa Cruz Biotechnology, Santa Cruz, CA) at 1:2000 followed by a 1:500 dilution of rabbit polyclonal antibody followed by a 1:500 dilution of FITC-conjugated anti-rabbit secondary antibody. The β1D integrin was detected with a 1:500 dilution of rabbit polyclonal antibody followed by a 1:500 dilution of FITC-conjugated anti-rabbit antibody. Lammin-α2 chain was detected with a 1:500 dilution of rabbit anti-α2G polyclonal antibody (a kind gift from Peter Yurchenco, Robert Wood Johnson Medical School, Department of Pathology, Piscataway, NJ). Dystrophin was detected with the mouse monoclonal anti-Dys2 antibody (Novacstra Laboratories, Ltd, Newcastle upon Tyne, UK) and utrophin was detected with MANCHOT7 F3 monoclonal antibody against utrophin (a kind gift from Glenn Morris, Center for Inherited Neuromuscular Disease, Shropshire, UK) at a dilution of 1:200. Anti-C4D monoclonal antibody (a kind gift from Dorothy Hudig, University of Nevada, Reno) was used to assay muscle inflammation at a dilution of 1:200. The mouse monoclonal antibodies were used in conjunction with a mouse-on-mouse (MOM) immunodetection kit (Vector Laboratories, Burlingame, CA) to block mouse immunoglobulin and a 1:500 dilution of FITC-conjugated anti-mouse secondary antibody. Acetylated low-density lipoprotein (LDL) and its metabolites were detected with Rhodamine-labeled α-bungarotoxin (1:1000 (Molecular Probes). Fluorescence was observed with a Zeiss Axioskop 2 Plus fluorescent microscope and images were captured with a Zeiss Axiocam MRc digital camera and Axiosvision 4.1 software.

Embryonic myosin heavy chain

Embryonic myosin heavy chain (eMyHC) was used in immunofluorescence experiments to measure muscle regeneration. Immunofluorescence was performed on 10-μm sections from tibialis anterior muscle from 3-week-old wild-type, mdx, α7−/− and mdx/α7−/− mice with a 1:500 dilution of anti-eMyHC antibody (F1,652, Developmental Studies Hybridoma Bank, University of Iowa, LA). The primary antibody was detected with a 1:500 dilution of FITC-conjugated anti-mouse secondary antibody. A 1 mg/ml concentration of tetramethylrhodamine-conjugated wheat-germ agglutinin (WGA) (Molecular Probes, Eugene, OR) was used to define muscle fibers. Multiple adjacent sections were analyzed with ten random, non-overlapping microscopic fields that were counted per animal at 630X magnification. Data was reported as the number of eMyHC-positive fibers per field.

Hematomyelin and eosin staining

Tibialis anterior and triceps muscles were cryosectioned and 10-μm sections were placed on nitrocellulose microscope slides. Tissue sections were fixed in ice-cold 4% ethanol for 2 minutes followed by 70% ethanol for 2 minutes and then re-hydrated in running water for 5 minutes. The tissue sections were then stained with Gill’s hematomyelin (Fisher Scientific, Fair Lawn, NJ) and rinsed in water for 5 minutes. Tissue sections were placed in Scott’s solution (0.024 M NaHCO3, 0.17 M MgSO4) for 3 minutes and rinsed in water for 5 minutes. Tissue sections were stained in eosin solution (Sigma-Aldrich, St Louis, MO) for 2 minutes. Tissue sections were then dehydrated in ice-cold 70% and 95% ethanol for 30 seconds each, followed by 100% ethanol for 2 minutes. Tissue sections were then cleared in xylene for 5 minutes prior to mounting with DePeX mounting medium (Electron Microscopy Sciences, Washington, PA). Centrally located nuclei were counted at 630X magnification by bright-field microscopy. The number of central nuclei per muscle fiber was determined by counting 1000 muscle fibers for triceps muscle and 700 muscle fibers for tibialis anterior muscle per animal. At least five animals from each genotype (wild-type, mdx, α7−/− and mdx/α7−/−) were analyzed.

Evans’ Blue dye uptake

Mice were injected intraperitoneally with 50 μl per 10 g of body weight sterile Evans Blue dye solution (10 mg/ml). After 3 hours, the gastrocnemius muscle was harvested and flash-frozen in liquid nitrogen. 10-μm cryosections were placed on microscope slides and fixed in 4% paraformaldehyde. To outline muscle fibers, tissue sections were incubated with 2 μg/ml Oregon Green-488-conjugated WGA (Molecular Probes, Eugene, OR). Muscle fibers positive for Evans Blue dye were counted in a minimum of 1000 fibers per animal, and at least three animals from each phenotype were analyzed. Counting was conducted and images captured at 630X magnification.

Electron microscopy

The gastrocnemius muscle and tendon were dissected from 3-week-old animals and fixed in 2% glutaraldehyde and 2.5% paraformaldehyde in 0.2% Sorenson’s phosphate buffer. Tissue was embedded in LX-112 epoxy (Ladd Research
Industries), sectioned at 0.1 μm with a Reichert Ultracut E Ultramicrotome, stained with uranyl acetate and lead citrate and viewed with a Hitachi H-600 transmission electron microscope at 5000× magnification.

Statistical analysis
All averaged data are reported as the mean ± s.d. Comparisons between multiple groups were performed by one-way-analysis of variance (ANOVA) for parametric data or by Kruskal-Wallis one-way-analysis of variance on ranks for non-parametric data using SigmaStat 1.0 software (Jandel Corporation, San Rafael, CA). P<0.05 was considered statistically significant.

The authors thank Glenn Morris (Center for Inherited Neuromuscular Disease, Shropshire, UK) for the MAN Cho3 and MAN Cho7 anti-urotrophin antibodies, Peter Yurchenco (Robert Wood Johnson Medical School, Piscataway, NJ) for the anti-laminin-α2G antibody, Woo Keun Song (Gwanju Institute for Science and Technology, South Korea) for the anti-β1D antibody and Dorothy Hudig (University of Nevada, Reno) for the anti-CD4 antibody. The authors also thank Eric Chaney and Paul Scowen for technical assistance and Dr Heather Burkin for critically reading the manuscript. This work was supported by grants from the NIH/NCCR P20 RR018751-01 and P20 RR15581-04 to D.J.B.

References
Abdolmey, A., Padre, R. C., Stull, J. T. and Lau, K. S. (1998). Neuronal nitric oxide synthase localizes through multiple structural motifs to the cytoskeleton-extracellular matrix linkage. J. Cell Sci. 111, 2873-2881.

Burkin, D. J. and Kaufman, S. J. (1999). The alpha7beta1 integrin in muscular dystrophy and disease. Cell Tissue Res. 296, 183-190.

Burkin, D. J., Gu, M., Hodges, B. L., Campellone, J. T. and Kaufman, S. J. (1998a). A functional role for specific spliced variants of the alpha7beta1 integrin in acetylcholine receptor clustering. J. Cell Biol. 143, 1067-1075.

Burkin, D. J., Kim, J. E., Gu, M. and Kaufman, S. J. (2000). Laminin and alpha7beta1 integrin mediate agrin-induced clustering of acetylcholine receptors. J. Cell Sci. 113, 2877-2886.

Burkin, D. J., Wallace, G. Q., Nicol, K. J., Kaufman, D. J. and Kaufman, S. J. (2001). Enhanced expression of the alpha 7 beta 1 integrin reduces muscular dystrophy and restores
restoration of the dystrophic mice. J. Cell Biol. 152, 1207-1218.

Burkin, D. J., Wallace, G. Q., Milner, D. J., Chaney, E. J., Mulligan, J. A. and Kaufman, S. J. (2005). Transgenic expression of [alpha]7[beta]1 integrin maintains laminin matrix deposition. Dev. Biol. 281, 209-219.

Lopez, M. A., Mayer, U., Hwang, W., Taylor, T., Hashmi, M. A., Jannapureddy, S. R. and Boriek, A. M. (2005). Force transmission, compliance, and viscoelasticity are altered in the alpha7-integrin-null mouse diaphragm. Am. J. Physiol. Cell Physiol. 288, C278-C289.

Lyons, P. R. and Slater, C. R. (1991). Structure and function of the neuromuscular junction in young adult mdx mice. J. Neurol. Sci. 107, 1477-1483.

Li, J., Rao, H., Burkin, D., Kaufman, S. J. and Wu, C. (2003). The muscle integrin binding protein (MBP) interacts with alpha7beta1 integrin and regulates cell adhesion and laminin matrix deposition. Dev. Biol. 261, 209-219.

Miyagoe-Suzuki, Y., Nakagawa, M. and Takeda, S. (2000). Merosin and congenital muscular dystrophy. Microsc. Res. Tech. 48, 181-191.

Moghadamzadeh, B., Albrechtsen, R., Guo, T. L., Zaik, M., Kawaguchi, N., Borup, R. C., Knapskog, L., P. Scherfig and B. Madsen (2003). Compensatory adaptation for dystrophin-deficiency: ADAM12 overexpression in skeletal muscle results in increased alpha 7 integrin, utrophin and associated glycoproteins. Hum. Mol. Genet. 12, 2467-2479.

Monacle, A. P., Neve, R. L., Colletti-Freener, C., Bertelson, C. J., Kurnit, D. M. and Kunel, L. M. (1986). Isolation of candidate CDNSAs for portions of the Duchenne muscular dystrophy gene. Nature 323, 646-650.

Moser, H. (1984). Duchenne muscular dystrophy: pathogenetic aspects and genetic prediction. Hum. Genet. 66, 17-40.

Nagel, A., Lehmann-Horn, F. and Engel, A. G. (1990). Neuromuscular transmission in the mdx mouse. Muscle Nerve 13, 742-749.

Nawrotzki, R., Willem, M., Miosge, N., Brinkmeier, H. and Mayer, U. (2003). Defective integrin switch and matrix composition at alpha 7-deficient muscular dystrophic junctions precede the onset of muscular dystrophy in mice. Hum. Mol. Genet. 12, 483-495.

Nguyen, H. H., Jayasinha, V., Xia, B., Hoyte, K. and Martin, P. T. (2002). Overexpression of the cytosotaxin T cell GalNAc transerase in skeletal muscle inhibits muscular dystrophy in mdx mice. Proc. Natl. Acad. Sci. USA 99, 5616-5621.

O’Brien, K. F. and Kunel, L. M. (2001). Dystrophin and muscular dystrophy: past, present, and future. Mol. Genet. Metab. 74, 75-88.

Oleksiewicz, K., Ervasti, J. M., Matsumura, K., Kahl, S. D., Leveille, C. J. and Engle, A. G. (1997). Skeletal and cardiac myopathies in mice lacking utrophin and dystrophin: a model for Duchenne muscular dystrophy. J. Cell Biol. 139, 729-738.
Skeletal muscle-specific expression of a utrophin transgene rescues utrophin-dystrophin deficient mice. *Nat. Genet.* 19, 79-82.

Rafael, J. A., Townsend, E. R., Squire, S. E., Potter, A. C., Chamberlain, J. S. and Davies, K. E. (2000). Dystrophin and utrophin influence fiber type composition and post-synaptic membrane structure. *Hum. Mol. Genet.* 9, 1357-1367.

Rando, T. A. (2001). The dystrophin-glycoprotein complex, cellular signaling, and the regulation of cell survival in the muscular dystrophies. *Muscle Nerve* 24, 1575-1594.

Ruegg, U. T., Nicolas-Metral, V., Challet, C., Bernard-Helary, K., Dorchies, O. M., Wagner, S. and Baetler, T. M. (2002). Pharmacological control of cellular calcium handling in dystrophic skeletal muscle. *Neuromuscul. Disord.* 12, S155-S161.

Schlaepfer, D. D., Hanks, S. K., Hunter, T. and van der Geer, P. (1994). Integrin-mediated signal transduction linked to Ras pathway by GRB2 binding to focal adhesion kinase. *Nature* 372, 786-791.

Schlaepfer, D. D., Hauck, C. R. and Sieg, D. J. (1999). Signaling through focal adhesion kinase. *Prog. Biophys. Mol. Biol.* 71, 435-478.

Schwartz, M. A. (2001). Integrin signaling revisited. *Trends Cell Biol.* 11, 466-470.

Senter, L., Luise, M., Presotto, C., Betto, R., Teresi, A., Ceoldo, S. and Salviati, G. (1993). Interaction of dystrophin with cytoskeletal proteins: binding to talin and actin. *Biochem. Biophys. Res. Commun.* 192, 899-904.

Sicinski, P., Geng, Y., Ryder-Cook, A. S., Barnard, E. A., Darlison, M. G. and Barnard, P. J. (1989). The molecular basis of muscular dystrophy in the mdx mouse: a point mutation. *Science* 244, 1578-1580.

Squire, S., Raymackers, J. M., Vandebrouck, C., Potter, A., Tinsley, J., Fisher, R., Gillis, J. M. and Davies, K. E. (2002). Prevention of pathology in mdx mice by expression of utrophin: analysis using an inducible transgenic expression system. *Hum. Mol. Genet.* 11, 3333-3344.

Suzuki, A., Yoshida, M., Hayashi, K., Mizumo, Y., Hagiwara, Y. and Ozawa, E. (1994). Molecular organization at the glycoprotein-complex-binding site of dystrophin.

Three dystrophin-associated proteins bind directly to the carboxy-terminal portion of dystrophin. *Eur. J. Biochem.* 220, 283-292.

Tows, D. S. and Goebel, H. H. (1996). DNA fragmentation and BCL-2 expression in infantile spinal muscular atrophy. *Neuromuscul. Disord.* 6, 265-273.

Tidball, J. G., Albrecht, D. E., Lokensgard, B. E. and Spencer, M. J. (1995). Apoptosis precedes necrosis of dystrophin-deficient muscle. *J. Cell Sci.* 108, 2197-2204.

Tidball, J. G. and Wehling-Henricks, M. (2004). Expression of a NOS transgene in dystrophin-deficient muscle reduces muscle membrane damage without increasing the expression of membrane-associated cytoskeletal proteins. *Mol. Genet. Metab.* 82, 312-320.

Tinsley, J. M., Potter, A. C., Phelps, S. R., Fisher, R., Trickett, J. I. and Davies, K. E. (1996). Amelioration of the dystrophic phenotype of mdx mice using a truncated utrophin transgene. *Nature* 384, 349-353.

Tinsley, J., Deconinck, N., Fisher, R., Kahn, D., Phelps, S., Gillis, J. M. and Davies, K. (1998). Expression of full-length utrophin prevents muscular dystrophy in mdx mice. *Nat. Med.* 4, 1441-1444.

Tombes, R. M., Grant, S., Westin, E. H. and Krystal, G. (1995). G1 cell cycle arrest and apoptosis are induced in NIH 3T3 cells by KN-93, an inhibitor of CaMK-II (the multifunctional Ca2+/CaM kinase). *Cell Growth Diff.* 6, 1063-1070.

Way, M., Pope, B., Cross, R. A., Kendrick-Jones, J. and Weeds, A. G. (1992). Expression of the N-terminal domain of dystrophin in E. coli and demonstration of binding to F-actin. *FEBS Lett.* 301, 243-245.

Yang, S. S., Van Aelst, L. and Bar-Sagi, D. (1995). Differential interactions of human Sos1 and Sos2 with Grb2. *J. Biol. Chem.* 270, 18212-18215.

Zhu, X., Hadhazy, M., Groh, M. E., Wheeler, M. T., Wollmann, R. and McNally, E. M. (2001). Overexpression of gamma-sarcoglycan induces severe muscular dystrophy. Implications for the regulation of Sarcoglycan assembly. *J. Biol. Chem.* 276, 21785-21790.