Soleus muscle in glycosylation-deficient muscular dystrophy is protected from contraction-induced injury

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Submitted 20 May 2010; accepted in final form 13 September 2010

Gumerson JD, Kabaeva ZT, Davis CS, Faulkner JA, Michele DE. Soleus muscle in glycosylation-deficient muscular dystrophy is protected from contraction-induced injury. Am J Physiol Cell Physiol 299: C1430–C1440, 2010. First published September 15, 2010; doi:10.1152/ajpcell.00192.2010.—The glycosylation of dystroglycan is required for its function as a high-affinity laminin receptor, and loss of dystroglycan glycosylation results in congenital muscular dystrophy. The purpose of this study was to investigate the functional defects in slow- and fast-twitch muscles of glycosylation-deficient Largemyd/myd mice. While a partial alteration in glycosylation of dystroglycan in heterozygous Large/mouse was not sufficient to alter muscle function, homozygous Large/myd mice demonstrated a marked reduction in specific force in both soleus and extensor digitorum longus (EDL) muscles. Although EDL muscles from Large/myd mice were highly susceptible to lengthening contraction-induced injury, Large/myd soleus muscles surprisingly showed no greater force deficit compared with wild-type soleus muscles even after five lengthening contractions. Despite no increased susceptibility to injury, Large/myd soleus muscles showed loss of dystroglycan glycosylation and laminin binding activity and dystrophic pathology. Interestingly, we show that soleus muscles have a markedly higher sarcolemma expression of β1-containing integrins compared with EDL and gastrocnemius muscles. Therefore, we conclude that β1-containing integrins play an important role as matrix receptors in protecting muscles containing slow-twitch fibers from contraction-induced injury in the absence of dystroglycan function, and that contraction-induced injury appears to be a separable phenotype from the dystrophic pathology of muscular dystrophy.

LARGE; extracellular matrix; dystroglycan; integrin

THE MUSCULAR DYSTROPHIES comprise a heterogeneous group of genetic diseases characterized by progressive degeneration of myofibers, severe weakness, and impaired skeletal muscle function (33). Although these diseases can arise from a single mutation in any of several known causative genes, a subset of congenital muscular dystrophies known as the dystroglycanopathies are caused by distinct mutations that result in loss of function of the membrane protein dystroglycan as an extracellular matrix (ECM) receptor (39). Mutations have been identified in several genes that appear to encode glycosyltransferases (37). While specific glycan structures have been proposed to be important for the function of dystroglycan as an ECM receptor (31), the precise pathway by which each of these glycosyltransferases functions is unclear. In each of the dystroglycanopathies, the glycosylation of dystroglycan is reduced or lost completely (34). The reduction or loss of the glycosylation of dystroglycan impairs the function of dystroglycan, which is thought to lead to the observed phenotypes in the many tissues where dystroglycan is expressed.

Dystroglycan is transcribed from the DAG1 gene, and the protein is cleaved posttranslationally into α- and β-subunits (25), which then remain associated with one another at the cell membrane (35). The β-dystroglycan (β-DG) subunit contains a single transmembrane domain and associates with dystrophin in its intracellular COOH terminus. In contrast, the extracellular α-DG subunit localizes to the exterior of the sarcolemma because of its noncovalent association with β-DG. The extensive glycosylation enables α-DG to function as a receptor for several ligands in the ECM that include laminin, agrin, and perlecan (9, 14, 42). The interaction of α-DG with laminin appears to be critical for the normal functioning of skeletal muscles in that both reduction of α-DG glycosylation, leading to loss of laminin binding affinity, and null mutations in laminin α2, leading to loss of the predominant laminin expressed in skeletal muscle, result in severe congenital muscular dystrophy (21, 34). Dystroglycan is an essential component of the dystrophin-glycoprotein complex (DGC) in muscle, a multisubunit complex that links the intracellular actin cytoskeleton to the ECM through the interactions of β-DG with dystrophin and α-DG with the ECM (10, 11). Null mutations in several components of the DGC, including dystrophin, that disrupt DGC expression also cause several forms of muscular dystrophy in humans.

While the complete function of the DGC in muscle is unknown, dystroglycan is hypothesized to provide a mechanical link that helps maintain integrity of the sarcolemma during cycles of contraction and relaxation (9). Evidence that the DGC plays a role in stabilizing the sarcolemma during contraction comes from observations that dystrophic muscle, with genetic disruption of the DGC, is highly susceptible to contraction-induced injury (30). The mdx mouse contains a null mutation in dystrophin, and mdx muscle demonstrates an increased propensity for muscle injury as measured by a significantly elevated force deficit when maximal force production is measured before and after a series of lengthening contractions (8). Additionally, increased force deficit in mdx muscle following injury is associated with an increase in membrane permeability when lengthening contractions are performed in the presence of a membrane-impermeant dye (2). Such defects at the membrane are thought to either directly or indirectly contribute to the observed degeneration of myofibers that ultimately results in impaired muscle function and weakness. The increased fragility of the sarcolemma in mdx mice is

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also supported by the observation that treatment with membrane sealants in vitro leads to a decrease in the magnitude of injury produced by repetitive isometric contractions (41). Therefore, susceptibility to contraction-induced injury appears to be a hallmark feature in many forms of DGC-related muscular dystrophy. Although the presence of dystroglycan is hypothesized to be essential for normal function of the DGC, the role of dystroglycan glycosylation that specifically regulates its function as a matrix receptor in the preservation of sarcolemma integrity during lengthening contractions is not as well defined. Therefore, our goal was to study the functional deficits in a mouse model of glycosylation-deficient muscular dystrophy to provide further insight into the role of the interaction of dystroglycan with the ECM in skeletal muscle function.

The Large<sup>mvd</sup> mouse is a spontaneously arising mouse model of muscular dystrophy that contains an autosomal recessive mutation in the gene that encodes the glycosyltransferase LARGE and consequently demonstrates reduced glycosylation of α-DG (16, 23). Although enzymatic activity for LARGE has not been confirmed, it contains two predicted catalytic domains (16) and has been shown to directly bind to the NH<sub>2</sub> terminus of dystroglycan (26). Additionally, overexpression of LARGE in myoblasts from patients with defects in other glycosyltransferases restores dystroglycan function (1). Although several studies have identified specific glycan structures that may be necessary for dystroglycan function (6, 45, 47, 52), the precise glycan on dystroglycan that is transferred by LARGE and its role in normal skeletal muscle function are presently unknown.

While mice provide important genetic models of human disease, the composition of fiber types in muscles of humans and mice differ significantly. The limb muscles most commonly studied in dystrophic mice, the extensor digitorum longus (EDL) and tibialis anterior, are composed almost exclusively of fast-twitch fibers with an approximately equal proportion of glycolytic and oxidative fibers. In contrast, the soleus muscle is one of the few skeletal muscles in the mouse to contain a large percentage of slow-twitch fibers (>50% of the total) (50). In contrast, many human skeletal muscles involved in locomotion are composed of an approximately 50–50 ratio of both fast- and slow-twitch fiber types, which is more comparable to the ratio observed in soleus muscle of the mouse (44). Muscle fiber types are determined by activation of distinct genetic programs that regulate both myosin isoform composition and oxidative capacity. Whether the complex glycosylation pathway necessary for dystroglycan glycosylation and function or the impact of the loss of its function is similar in both fast-twitch and slow-twitch muscle fiber types is not known. Therefore, we compared the contractile defects associated with loss of LARGE-dependent glycosylation of dystroglycan in fast- and slow-twitch muscles. In doing so, we identified differences in expression of key matrix receptor proteins between fast-twitch and slow-twitch fibers that might underlie fiber type-specific differences in the susceptibility to contraction-induced injury of the skeletal muscles of the dystrophic Large<sup>mvd</sup> mouse. Furthermore, while susceptibility to contraction-induced injury is often considered a hallmark of DGC-related muscular dystrophies, our data show that dystrophic pathology and muscle weakness is a clearly separable phenotype from contraction-induced injury in muscles containing a mixture of fast-twitch and slow-twitch muscle fibers.

**METHODS**

**Animals.** All animals were housed in a specific pathogen-free (SPF) barrier facility in the Unit for Laboratory Animal Medicine at the University of Michigan, and all procedures were approved by the University of Michigan Committee for the Use and Care of Animals. Large<sup>mvd</sup> and wild-type (WT) littermates used for all experiments were aged 12–36 wk and taken from a maintained colony. Sprague-Dawley rats were obtained from Charles River Laboratories.

**Measurement of contractile properties.** Contractile measurements were performed as previously described (4). For in vitro measurements, the EDL or soleus muscle was isolated from anesthetized mice. A 5–0 silk suture was tied to the proximal and distal tendons. The muscle was placed in Krebs mammalian Ringer solution maintained at 25°C and bubbled continuously with 95% O<sub>2</sub> and 5% CO<sub>2</sub> to stabilize pH at 7.4. One tendon was tied to a servomotor (Aurora Scientific, model 300) and the other to a force transducer (Kulite Semiconductor, model BG-50). The muscle was stimulated by square wave pulses delivered between two platinum electrodes connected to a high-power biphasic current stimulator (Aurora Scientific, model 701B). An IBM-compatible personal computer and custom-designed software (LabVIEW 7.1, National Instruments, Austin, TX) controlled electrical pulse properties and servomotor activity and recorded data from the force transducer. The voltage of pulses was increased, and optimal muscle length (L<sub>o</sub>) was subsequently adjusted to the length that resulted in maximum twitch force (4). L<sub>o</sub> was measured with digital calipers. Muscles were held at L<sub>o</sub> and subjected to trains of pulses to generate an isotonic contraction. Pulse trains were 300 ms for EDL muscles and 900 ms for soleus muscles. Stimulus frequency was increased until maximum isometric force (P<sub>i</sub>) was achieved (4). The muscle was weighed, and the mean cross-sectional area (CSA) was estimated by dividing the muscle wet mass by the product of fiber length (L<sub>f</sub>) and the density of mammalian muscle (1.06 g/cm<sup>3</sup>). Specific force (sP<sub>i</sub>) was determined by dividing P<sub>i</sub> by CSA.

**Muscle injury protocol.** After measurement of maximum twitch force and P<sub>i</sub>, muscles were stimulated and held at L<sub>f</sub>, for 100 ms for EDL muscles and 300 ms for soleus muscles to allow muscles to develop P<sub>c</sub>. After the isometric contraction, muscles were stretched through a 30% strain relative to L<sub>o</sub>. The velocity of the stretch was 1 L<sub>f</sub>/s. The total time of stimulation was 400 ms for EDL muscles and 600 ms for soleus muscles. After stimulation, muscles were returned to L<sub>o</sub> and then were subjected to four additional 30% stretches, each with 12 s in between, for a total of five stretches per muscle. The muscle was allowed to rest 1 min, and P<sub>c</sub> was measured. The force deficit was calculated as the decrease in P<sub>c</sub> observed after the stretch protocol as a percentage of P<sub>c</sub> before the protocol.

**Membrane preparation, SDS-PAGE, and Western blotting.** Mouse and rat EDL and soleus muscles were dissected from anesthetized animals, the myotendinous regions were removed, and samples were immediately frozen on dry ice until further processing. Individual (rat) or pooled (mouse) samples were homogenized in ice-cold homogenization buffer containing (in mM) 20 sodium pyrophosphate, 20 sodium phosphate (monobasic), 1 magnesium chloride, and 5 EDTA, pH 7.1, with 0.303 M sucrose. Samples were subjected to a low-speed (10,000 g) and a high-speed (45,000 g) spin in order to isolate the membrane fraction, and final pellets were resuspended in buffer I (0.303 M sucrose, 20 mM Tris-maleate, pH 7.0) and quantified with the Bio-Rad Bradford assay. All buffers contained protease inhibitors (0.5 μg/ml pepstatin A, 2 kallikrein inhibitor units/ml aprotinin, 1 μg/ml leupeptin, 0.4 mM PMSF, 0.6 mM benzamidine). Samples were separated with 3–15% gradient polyacrylamide gels and were transferred via Western blot to polyvinylidene fluoride membrane (Millipore). After membranes were blocked in Tris-buffered saline (TBS) + 0.05% Tween 20 (TBS-T) + 5% nonfat dry milk for 1 h,
membranes were incubated with primary antibody for at least 2 h up
to overnight. Primary antibodies included rabbit polyclonal antibodies
to β-DG and α5-integrin (H40) (Santa Cruz), α5-integrin and β1-
integrin (Chemicon/Millipore), and laminin (L-9393, Sigma), mouse
monoclonal antibody to dysferlin (Hamlet, Novacava), slow myosin
(A4.840, Developmental Studies Hybridoma Bank, Iowa City, IA),
and glycosylated dystroglycan (IIH6, gift from Dr. Kevin Campbell,
University of Iowa, Iowa City, IA), and rat monoclonal antibodies to
α6-integrin (eBiosciences) and β1-integrin (BD Pharmingen). After
three 10-min washes in TBS-T, membranes were incubated with
secondary antibody conjugated to horseradish peroxidase (HRP) for
1.5 h. Membranes were washed for 10 min with TBS-T and
incubated in chemiluminescent substrate (Thermo Scientific) 1–2
min before exposure. Membranes used for reprobing were washed in
TBS and incubated in a stripping buffer (TBS + 2% SDS + 7 µM
β-mercaptoethanol) for 30 min. Membranes were rinsed several times
in TBS and were reblocked for 1–2 h in TBS + 5% nonfat dry milk
before a second round of antibody staining.

Solid-phase laminin binding assay. Wheat germ agglutinin
(WGA)-purified skeletal muscle samples were diluted in TBS and
coated onto 96-well microplates at a final concentration of 0.1 µg/well
overnight. After washing in binding buffer (TBS + Ca2+), plates were
coated with a blocking buffer of 3% bovine serum albumin (BSA) in
binding buffer for 1 h. Wells were aspirated, rinsed in binding buffer,
replaced with dilutions of 0.02–20 nM laminin (Invitrogen), and
diluted in blocking buffer with and without the presence of 20 mM
EDTA for 2 h. After four washes in binding buffer, wells were
replaced with anti-laminin antibody (L-9393) diluted 1:5,000 in
blocking buffer and incubated at room temperature for 1.5 h. Wells
were aspirated, washed four times in binding buffer, and replaced
with goat anti-rabbit IgG-HRP diluted 1:5,000 in blocking buffer for 1 h
at room temperature. Again, plates were washed four times in binding
buffer. For developing, 100 µl of o-phenylenediamine dihydrochloride
(OPD)-citrate phosphate buffer (CPB) was added to each well
and incubated 5–25 min. The reaction was stopped with 50 µl of 2 M
H2SO4, and plates were read at 495 nm.

Immunofluorescence and histology. Soleus, EDL, and gastrocne-
mius muscles were dissected together from anesthetized mice,
mounted in OCT, and immediately frozen in liquid nitrogen-cooled
isopentane. Frozen samples were cut into 8-µm cross sections with a
cryostat and stored at −80°C until the slices were used for immuno-
fluorescent microscopy or stained with either hematoxylin and eosin
or 0.1% sirius red-0.1% fast green in picric acid. For immunohisto-
chemical experiments, slides were rehydrated with PBS and blocked
1–2 h in 5% BSA in PBS. Slides were then incubated with primary
and secondary antibody for 1–2 h each with four 5-min washes of PBS
between incubations. Final slides were mounted in Permafluor (In-
vitrogen) and imaged with an Olympus BX-51 fluorescence micro-
scope.

RESULTS

Partial loss of dystroglycan glycosylation in heterozygous
Largemyd/myd mice. Homozygous Largemyd/myd mice have previ-
ously been described as having a complete loss of α-DG
glycosylation as demonstrated by the loss of reactivity with the
glycosylation-specific antibody IIH6 (34). Western blot analy-
isis from skeletal muscle whole lysates (WT, Largemyd/myd, and
Largemyd/myd) revealed altered glycosylation in mice heterozy-
uous for the LARGE mutation as indicated by a slight reduc-
tion in the molecular mass of α-DG (Fig. 1A). Partial loss of
α-DG glycosylation has also been detected in human patients
with mild limb-girdle muscular dystrophy (39). Although het-
eryozygous Largemyd/myd mice did not show pathology consistent
with muscular dystrophy (Fig. 1B; Supplemental Fig. S1), the
potential functional effects of the partial glycosylation of
dystroglycan remain unknown. Therefore, the contractile
function of heterozygous mice was studied to determine
whether the altered glycosylation due to the presence of a
single functional LARGE gene was sufficient to cause changes
in skeletal muscle function. Although absolute and specific
forces were both reduced in the soleus and EDL muscles of
homozygous LargeLargemyd/myd mice, heterozygous mice exhibited no
significant differences compared with WT littermates (Fig. 2, A
and B). In response to muscle injury induced by two lengthen-
ing contractions of 30% strain, force deficits measured in
heterozygous mice displayed no differences from in WT mice
for either the EDL muscle (WT 17%, heterozygous 18%) or the
soleus muscle (WT 22%, heterozygous 21%) (Fig. 2C). There-
fore, while the loss of a single functional LARGE gene was
sufficient to reduce the glycosylation of dystroglycan in skel-
etal muscle slightly, this alteration was not sufficient to cause
a change in muscle function or susceptibility to contraction-
induced injury following lengthening contractions.

Effects of complete loss of functional dystroglycan glycosyla-
tion on muscle function are fiber type specific. The complete
loss of dystroglycan glycosylation in Largemyd/myd mice leads to
dystrophic pathology (Fig. 1B; Supplemental Fig. S1) and
results in a profound reduction in both absolute and specific
force production (Fig. 2, A and B). The maximum specific
force measured for muscles from homozygous LargeLargemyd/myd
mice was only 67% of that measured in EDL muscle and 66% of
that measured in soleus muscle of WT mice, indicating that
maximal force production in skeletal muscle requires the pres-
ence of LARGE-mediated glycosylation of dystroglycan. Addi-
tionally, a significantly elevated force deficit of 36% was
measured in the EDL muscle of homozygous LargeLargemyd/myd
mice after two lengthening contractions, which was nearly
twofold greater than the force deficit observed in EDL muscles
from WT and heterozygous mice (Fig. 2C). Interestingly, the
degree of injury due to lengthening contractions in muscles
containing functionally impaired dystroglycan seemed to be
fiber type specific. A force deficit of 22% measured in the
soleus muscle of homozygous LargeLargemyd/myd mice after two
lengthening contractions was not significantly different from
that of WT mice. To further stress the muscle we performed
three additional lengthening contractions, and after a total of
five lengthening contractions force deficits measured in EDL
muscles from WT and heterozygous mice were not signifi-
cantly different (WT 35%, heterozygous 36%), nor were those
in soleus muscles (WT 26%, heterozygous 30%) (Fig. 2D).
While EDL muscles from homozygous LargeLargemyd/myd
mice showed a further increase in force deficit to 88% (compared
to 32% in WT), the 30% force deficit measured in the soleus of
LargeLargemyd/myd mice after five lengthening contractions was still
not significantly different from WT measurements. These
results suggest that while the complete loss of functional glyco-
sylation of dystroglycan in LargeLargemyd/myd skeletal muscle results
in reduced maximal force production in both soleus and EDL
muscle, the soleus muscle of LargeLargemyd/myd mice is protected
completely from the high degree of susceptibility to lengthen-
ing contraction-induced injury observed in the EDL muscle.

1 Supplemental Material for this article is available online at the Journal
website.
Large<sup>myd/myd</sup> soleus is dystrophic and demonstrates reduced glycosylation of dystroglycan concomitant with reduction in laminin binding activity. Because susceptibility to contraction-induced injury in skeletal muscle is considered to be a hallmark of DGC-related muscular dystrophies, we sought to determine whether the soleus of Large<sup>myd/myd</sup> mice was also protected from dystrophy. Hematoxylin and eosin staining of gastrocnemius, EDL, and soleus sections of Large<sup>myd/myd</sup> mice revealed that, similar to gastrocnemius and EDL muscles, the soleus contained several features of muscular dystrophy including a high percentage of fibers with internalized nuclei, heterogeneity in fiber size, and infiltration of mononuclear cells (Fig. 1B).

Additionally, all three muscles isolated from Large<sup>myd/myd</sup> mice contained increased sirius red staining indicative of interstitial fibrosis (Supplemental Fig. S1).

An alternative hypothesis for the lack of susceptibility to contraction-induced injury in the soleus muscle of Large<sup>myd/myd</sup> animals was that perhaps a partial glycosylation of dystroglycan is preserved in soleus muscle through the actions of either the LARGE homolog LARGE2 or alternative glycosyltrans-
Fig. 2. Large^myd/my^d soleus muscle is weak but not sensitive to contraction-induced injury. A and B: absolute (A) and specific (B) force were measured in vitro in soleus and EDL muscles isolated from 4- to 5-mo-old wild-type, heterozygous, or Large^myd/my^d mice. While absolute and specific force were significantly reduced in both Large^myd/my^d soleus and EDL muscles, heterozygous mice showed no difference in either muscle compared with wild-type animals. C and D: contraction-induced injury was performed by subjecting each muscle to 2 (C) or 5 (D) lengthening contractions (LC) of 30% strain. In both soleus and EDL muscles from heterozygous mice, measured force deficits were not different from wild type. Although force deficits measured in EDL muscles of Large^myd/my^d mice were significantly higher than wild type, no such difference was observed in the soleus muscle.
tinct α-integrin isoforms. During development, β1-integrin dimerizes with α3-integrin to form a receptor for fibronectin. However, in adult muscle, α5-integrin expression is downregulated and replaced with α7-integrin to dimerize with β1-integrin to form a laminin receptor that is predominantly localized to the myotendinous junction (40). With both a WGA-purified fraction (Fig. 5A) and a microsome fraction (Fig. 5B) isolated from EDL and soleus muscles of rats, a heavy chain-specific α7-integrin antibody detected bands at both 100 kDa and 120 kDa. The 120-kDa isoform is reported to be a glycosylated form of α7-integrin (46), and in support of this conclusion, only this isoform was detected in the WGA-purified fraction. While no differences were observed in expression levels of the 100-kDa α7-integrin isoform in microsome fractions from either EDL or soleus, α7-integrin detected in the WGA-purified fraction appeared to be more highly expressed in soleus muscle. Additionally, immunofluorescent staining detected α7-integrin expression predominantly at the sarcolemma in soleus in a pattern consistent with previously shown β1-integrin expression (Figs. 4C and 5C). To determine whether it may be possible that β1-integrin combines with other α-integrins in the soleus to form receptors for additional extracellular ligands, Western blots and/or soleus/gastrocnemius muscle cross sections were also stained for α5- and α6-integrin. The α6-integrin was not expressed at detectable levels in fibers of either the soleus or the gastrocnemius of WT muscle and instead appeared to be concentrated only in blood vessels (not shown). The α5-integrin appeared to be expressed in both the EDL and soleus muscles, but levels were lower than those in neonatal muscles and did not differ between the two muscle types (Fig. 5A).

**DISCUSSION**

Our results demonstrate for the first time that, although the skeletal muscles of Large<sup>myd/myd</sup> mice are weaker than those of WT control mice, the soleus is protected from contraction-induced injury despite the loss of the glycosylation-dependent function of dystroglycan as an ECM receptor. We also show that soleus muscle has markedly higher expression of β1-integrin compared with other limb muscles composed of fast-twitch muscle fibers. This observation suggests that αβ1-integrin may be playing an important role as an alternative matrix receptor capable of protecting the sarcolemma from contraction-induced injury in slow-twitch muscle. Finally, although susceptibility to contraction-induced injury is often considered a hallmark of DGC-related muscular dystrophies, our results indicate that a susceptibility to contraction-induced injury is not required, and that muscle weakness and dystrophy can occur in the soleus of Large<sup>myd/myd</sup> homozygous mice in its absence.

The exact mechanism by which the DGC protects muscle from injury following lengthening contractions and the relationship of muscle injury to the development of dystrophy are still unclear. In whole muscle, lengthening contractions may produce injury by straining sarcomeres with preexisting heterogeneity in length within muscle fibers, or strain lateral connections from fiber to fiber, resulting in fiber damage and

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**Fig. 3.** Loss of glycosylated dystroglycan occurs in both soleus and gastrocnemius muscle of Large<sup>myd/myd</sup> mice and results in decreased laminin binding activity. Gastrocnemius and soleus muscles were dissected from 36-wk-old wild-type and Large<sup>myd/myd</sup> mice. Frozen sections were stained separately for β dystroglycan (bDG), glycosylated α dystroglycan (IIH6), or laminin and secondary antibodies conjugated to either Alexa 488 or Cy3. A: sections were imaged at regions where the gastrocnemius (bottom right) and soleus (top left) muscles lie adjacent to one another; the boundary of each is indicated by the dashed line. Loss of dystroglycan glycosylation in both muscles is indicated by a loss of reactivity with IIH6. Despite the loss of dystroglycan glycosylation, the presence of laminin, an important dystroglycan ligand in skeletal muscle, is retained. Scale bar, 200 μm. B: loss of dystroglycan glycosylation results in loss of laminin binding activity in both soleus and EDL muscles. Wheat germ agglutinin (WGA)-purified fractions of pooled muscle tissue from wild-type and Large<sup>myd/myd</sup> (myd) mice were isolated, quantified, and coated onto 96-well plates. Laminin binding activity was determined by overlaying wells with a range of concentrations of laminin between 0.2 and 20 nM.
damage to sarcomeres (13). Alternatively, lengthening contractions may impart large mechanical strain on connections of muscle fibers to the matrix, disrupting those connections that stabilize the sarcolemma. Recently, Han et al. (17) demonstrated that dystroglycan is important for enabling skeletal muscle fibers to bind the sarcolemma tightly to the basal lamina, the loss of which results in muscle that is prone to contraction-induced sarcolemma injury. However, our results show that this is not universally true for all muscles. The loss of glycosylation and function of dystroglycan is not sufficient

Fig. 4. β1-Integrin is highly expressed in sarcolemma of muscle containing a large percentage of slow-twitch fibers. Soleus (S) and EDL (E) muscles were isolated from Sprague-Dawley rats or wild-type mice. Myotendinous regions were removed, and microsome fractions were isolated from either single rat muscles or pooled mouse muscle (3 groups, each containing 6 muscles). Immunoblots were stained with IIH6 and anti-β-dystroglycan. A and B: in both rat (A) and mouse (B), α-dystroglycan from soleus muscle appears to run at a higher molecular mass than α-dystroglycan from EDL muscle. Immunoblots were also stained for β1-integrin (β1 ltgn) and dysferlin (Dysf). In both rat and mouse, β1-integrin appears to be more highly expressed in soleus muscle. No differences were observed in the expression of dysferlin, a protein known to be involved in membrane repair. Numbers on right are molecular masses in kDa. C: immunofluorescent staining of soleus (S)/gastrocnemius(G) muscle sections (top) shows that the increased expression of β1-integrin observed in the soleus muscle is due to increased expression at the sarcolemma. Costaining with an antibody specific to slow myosin (bottom) demonstrates that increased expression of β1-integrin in the soleus muscle corresponds to muscle that contains a high proportion of slow-twitch fibers. D: the decreased expression of β1-integrin in predominantly fast-twitch fibers is also demonstrated by reduced staining in the EDL muscle (bottom) compared with the soleus (top) when both sections were imaged at the same exposure. Scale bar, 200 μm.
Dystrophic soleus muscle is protected from injury

Fig. 5. Skeletal muscle contains multiple α-integrin isoforms capable of forming dimers with β1-integrin. Microsome or WGA-purified fractions were isolated from either soleus and EDL muscle from Sprague-Dawley rats or whole muscle from neonatal mice. A and B: staining with an antibody to α7-integrin resulted in the detection of multiple bands, 2 of which have molecular masses corresponding to a nonglycosylated (100 kDa) and a glycosylated (120 kDa) form of α7-integrin. While no difference in expression of α7-integrin was detected in the 100-kDa band, the 120-kDa band appeared to be more highly expressed in soleus (S) muscle than in EDL (E) muscle. Additionally, α7-integrin was expressed at equally low levels in both muscles compared with neonatal muscle (N) (A, B). C: in a pattern similar to β1-integrin, immunofluorescent staining of mouse soleus (S)/gastrocnemius (G) muscle sections showed that α7-integrin is more highly expressed at the sarcolemma of soleus muscle. Scale bar, 200 μm.

to result in increased susceptibility to contraction-induced injury in Large^myd/myd^ soleus muscle. Interestingly, the soleus muscles of Large^myd/myd^ mice still have pathological features of muscular dystrophy including increased variability of fiber size and centrally nucleated fibers consistent with ongoing muscle degeneration and regeneration. The soleus still showed marked deficits in specific force and contained dystroglycan that lacked functional glycosylation and was unable to bind extracellular laminin. This implies that contraction-induced injury is not a primary cause for dystrophy and weakness observed in this muscle. While dystroglycan may have an important role in mechanically stabilizing fast-twitch muscle against contraction-induced injury, our results suggest that dystroglycan may also have additional essential functions in muscle that when impaired contribute strongly to the dystrophic phenotype and weakness in muscles containing slow-twitch fibers.

Several groups have hypothesized that defects in cell signaling might contribute to the dystrophic pathology, but the physiological significance has not been demonstrated. Muscles isolated from laminin 211-deficient dy/dy mice are dystrophic and weak but have been shown to be no more susceptible to contraction-induced injury compared with WT animals (20). dy/dy animals have increased activation of apoptotic death pathways, and it was suggested that altered signaling may contribute to the dystrophic pathology in laminin 211 deficiency (15, 38). Consistent with this hypothesis, several studies have demonstrated that defects in the specific interaction of laminin with dystroglycan result in altered cellular signaling important for normal cell growth. Disruption of the laminin/dystroglycan interaction in vitro with blocking antibodies against α-DG resulted in altered AKT and GSK-3β activation and an increase in apoptotic cell death (27). More recently, the DGC has also been shown to interact with subunits of heterotrimeric G proteins in a laminin-dependent manner (51), which has been suggested to underlie the altered Ca^2+^ homeostasis observed in several forms of muscular dystrophy. While the precise mechanism by which defects in dystroglycan result in impaired signaling is unclear, our results suggest that such signaling may be physiologically relevant and may have significant implications for other dystrophies associated with genetic disruption of the DGC that lead to a concomitant reduction in dystroglycan expression at the sarcolemma.

Previous studies have shown that lengthening contractions of soleus muscles of mdx mice fail to cause higher force deficits or sarcolemma damage compared with WT mice (36). While this has led to the predominant use of muscles composed primarily of fast-twitch fibers such as EDL in studying contraction-induced injury in the mdx mouse, an underlying molecular mechanism that accounts for the lack of increased contraction injury in soleus muscle has not yet been elucidated. Muscle impairment due to the loss of dystrophin expression in mdx mice can be compensated for by the upregulation of utrophin, and differences in utrophin expression might be a reasonable hypothesis to explain the lack of susceptibility of the mdx soleus muscles to contraction-induced injury. However, in the present study, the Large^myd/myd^ sarcolemma contains normal expression of all DGC components including dystrophin, and only the posttranslational processing of α-DG and its laminin binding activity are compromised (17). Therefore, we hypothesized that differences in expression of other ECM receptors between the EDL and soleus muscles may be responsible for the protection against contraction-induced injury. In addition to dystroglycan, α7β1-integrin has also been shown to be an important laminin receptor essential for normal skeletal muscle function, and mutations have been associated with muscular dystrophy in patients (19) and in animal models. Although knockout of β1-integrin in the mouse is embryonically lethal because of its essential role in embryogenesis (12, 48), the tissue-specific loss of α7-integrin in skeletal muscle results in myopathy and initial
experiments demonstrated that α7-integrin-deficient animals exhibited a myopathy predominantly affecting the myotendinous junction (32). While most of the α7-integrin-null skeletal muscle evaluated exhibited very few histological characteristics of muscular dystrophy, the authors noted that only the soleus showed histological signs of myopathy. These results support our observation that the α7β1 receptor, being more highly expressed in soleus, functions as an important laminar receptor along the lateral membrane of skeletal muscle fibers that can function in the prevention of contraction-induced injury specifically in slow-twitch fibers.

Han et al. (17) recently showed that loss of either the DGC or α7β1-integrin can result in deficits in force production but that only loss of the DGC resulted in susceptibility to contraction-induced injury. This was based on the observation that although a greater force deficit was observed in EDL muscles taken from Large<sup>mdx/mdy</sup> animals compared with WT control animals, no such difference existed in EDL muscles from α7-integrin-null mice. Although the soleus was not studied in these experiments, on the basis of our results we would hypothesize that α7-integrin-null mice would indeed have a measured force deficit after contraction-induced injury because of the increased expression and requirement of α7β1-integrin in this muscle.

Since our data indicate that soleus muscles of Large<sup>mdx/mdy</sup> mice are protected from contraction-induced injury and that this is correlated with increased α7β1-integrin expression in soleus muscle, we would predict that increased expression of α7β1-integrin in fast-twitch muscles might also provide protection against contraction-induced injury. While this remains to be formally tested in Large<sup>mdx/mdy</sup> mice, previous studies have shown that transgenic overexpression of this receptor in dystrophin-deficient mouse models can partially alleviate the dystrophic pathology. Expression of rat α7-integrin in mdx:<i>utr</i>−/− mice resulted in an increase in muscle integrity and a decrease in mononuclear cell infiltrate and correlated with an increase in life span (5). Additionally, when α7-integrin was overexpressed in muscles of normal animals, exercise-induced muscle injury was minimized as demonstrated by a reduction in Evans blue dye uptake after exercise (3). This suggests that an increase in α7-integrin expression can provide protection against sarcrolemmal damage in fast-twitch muscles. Finally, the combined loss of dystrophin and α7-integrin causes an even more severe muscular dystrophy than the loss of either alone (43), which suggests that the DGC and α7β1-integrin have compensatory functions in skeletal muscle.

The striking differences in susceptibility to contraction-induced damage in Large<sup>mdx/mdy</sup> soleus and EDL muscles and the markedly higher expression of α7β1-integrin in soleus muscle compared with EDL and gastrocnemius muscles indicate that there are important fiber type-specific differences in the interactions between the basal lamina and the cytoskeleton in muscle fibers. Specifically, the functions of the DGC and α7β1-integrin complexes may be fiber type specific. Peroxisome proliferator-activated receptor-γ coactivator-1α (PGC-1α) is a transcriptional activator that has been shown to play a critical role in driving the slow-twitch muscle fiber gene program. This protein is highly expressed in slow-twitch fibers and when transgenically overexpressed throughout skeletal muscle results in a conversion to a slow-twitch fiber phenotype (28). Interestingly, overexpression of PGC-1α in mdx mice partially improved the muscular dystrophy phenotype, although the mechanism of action was not clearly identified (18). However, mdx/PGC-1α mice were shown to have decreased levels of serum creatine kinase and decreased levels of Evans blue dye uptake after downhill running, which supports the hypothesis that part of the effect may be mediated by an increase in protection of the sarcrolemma. This suggests that although most of the downstream transcriptional events related to PGC-1α expression are focused on metabolic genes, the induction of a slow-twitch muscle gene program may also produce downstream changes that affect expression of ECM receptors, particularly that of α7β1-integrin, resulting in its increased sarcrolemmal expression and leading to protection from contraction-induced injury. While the overexpression of PGC-1α likely results in a number of changes in gene transcription, the specific overexpression of α7β1-integrin in skeletal muscle can increase muscle fiber viability without significantly affecting the transcription of other proteins and may be beneficial as a potential therapeutic for muscular dystrophy patients (29). This also supports the conclusion that the alleviation of muscle damage and dystrophic phenotype observed when α7-integrin is transgenically expressed in animal models is due to the specific function of α7β1-integrin at the sarcrolemma and not to altered expression of other proteins that may also contribute to enhanced viability of muscle fibers.

While a substantial portion of soleus muscle from Large<sup>mdx/mdy</sup> mice contains fast-twitch muscle fibers, we did not observe a partial deficit in contraction-induced injury. This suggests that interactions of slow-twitch muscle fibers with the ECM through β1-containing integrins may also help stabilize neighboring fast-twitch muscle fibers from contraction-induced injury. Previous studies have shown that striated muscle from animals with an impaired DGC typically has a higher degree of uptake of Evans blue dye in large patches of neighboring fibers rather than randomly distributed in single fibers throughout the muscle (49). This supports the hypothesis that strong lateral interactions of muscle fibers with the ECM between neighboring fibers, through either dystroglycan or integrin, are critical for the protection of both individual fibers and the integrity of the entire muscle during normal muscle contractile activity. Finally, it is important to remember that human skeletal muscle is composed of a mixture of fiber types, and in some respects the mouse soleus better models this typical composition of human skeletal muscle. Therefore, understanding the complete functions of dystroglycan in both fast-twitch and slow-twitch skeletal muscle, and the relative functional contributions of dystroglycan and integrins in these types of muscle, will have significant implications for future therapeutics aimed at treating muscular dystrophy.

**GRANTS**

This work was supported by funds provided to support the Contractility Core of the Nathan Shock Center from National Institutes of Health Grant P30-AG-13283 to J. A. Faulkner and the Functionality Core from Grant PO1-AG-15434 to J. A. Faulkner and by funds from Grant HL-080388 to D. E. Michele.

**DISCLOSURES**

No conflicts of interest, financial or otherwise, are declared by the author(s).
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