Genetic compensation for sarcoglycan loss by integrin α7β1 in muscle

Michael J. Allikian1, Andrew A. Hack1, Stephanie Mewborn2, Ulrike Mayer3 and Elizabeth M. McNally1,2,*

1Department of Medicine and 2Department of Human Genetics, The University of Chicago, 5841 South Maryland Avenue, Chicago, IL 60637, USA
3Wellcome Trust Centre for Cell-Matrix Research, School of Biological Sciences, University of Manchester, 2.205 Stopford Building, Oxford Road, Manchester, M13 9PT, UK
*Author for correspondence (e-mail: emcnally@medicine.bsd.uchicago.edu)

Accepted 23 March 2004
Journal of Cell Science 117, 3821-3830 Published by The Company of Biologists 2004
doi:10.1242/jcs.01234

Summary

Disruption of the sarcoglycan complex leads to muscle membrane instability and muscular dystrophy in humans and mice. Through the dystrophin glycoprotein complex, sarcoglycan participates in connecting the internal cytoskeleton to the membrane and the extracellular matrix. Integrin α7β1 is also a transmembrane protein of skeletal and cardiac muscle that similarly links the cytoskeleton to the extracellular matrix. Mice lacking integrin α7 develop mild muscle degeneration, while sarcoglycan mutant mice display overt muscle degeneration and muscular dystrophy. In sarcoglycan-deficient muscle, integrin α7 protein was upregulated at the plasma membrane. To ascertain whether integrin α7 upregulation compensates for the loss of the transmembrane sarcoglycan linkage in sarcoglycan-deficient muscle, we generated mice lacking both integrin α7 and γ-sarcoglycan (gxi). These double-mutant gxi mice exhibit profound, rapid muscle degeneration leading to death before one month of age consistent with a weakened cellular attachment to the extracellular matrix. The regenerative capacity of gxi muscle was intact with increased embryonic myosin heavy chain expression, myofiber central nucleation and normal in vivo myoblast differentiation. Therefore, upregulation of integrin α7β1 compensates as a transmembrane muscle cell attachment for sarcoglycan consistent with overlapping roles for sarcoglycan and integrins in mediating cytoskeletal-membrane-extracellular matrix interaction.

Movie available online

Key words: Sarcoglycan, Dystrophin, Integrin, Membrane, Muscle

Introduction

Integrins are heterodimeric αβ transmembrane proteins that bind extracellular matrix (ECM) proteins such as laminin and fibronectin as well as cytoskeletal proteins such as talin and filamin (Hynes, 1992). Integrins serve in a mechanosignaling capacity allowing integrin to conduct both inside-out as well as outside-in signaling. In skeletal muscle, integrin α7β1 serves as the major laminin-binding integrin. Mice deficient for integrin β1 die early in development, but integrin β1-deficient cells can effectively contribute to developing muscle (Fassler and Meyer, 1995; Hirsch et al., 1998). Integrin α7 is expressed highly in cardiac and skeletal muscle where it binds the α2 chain of laminin-2 in the extracellular matrix (von der Mark et al., 1991). In humans, mutations in the integrin α7 gene lead to congenital muscular dystrophy (Hayashi et al., 1998). Gene targeting of the murine integrin α7 gene produces a mild, progressive muscular dystrophy characterized by comparatively little muscle degeneration and regeneration (Mayer et al., 1997). The interaction of integrin α7β1 with laminin-2 (merosin) appears to be a specialized mechanosignaling transducer essential for the maintenance of mature skeletal muscle (Mayer et al., 1997; Vachon et al., 1997).

In striated muscle, the sarcoglycan complex is intimately associated with dystrophin and dystroglycan to form the dystrophin glycoprotein complex (DGC) (Ervasti, 1993). Dystrophin mutations lead to Duchenne Muscular Dystrophy (DMD) and secondarily destabilize the sarcoglycan complex from the skeletal muscle plasma membrane (Rafael and Brown, 2000). In humans, mutations in sarcoglycan genes lead to a Duchenne-like muscular dystrophy (Bonnemann, 1996; Hack et al., 2000a). Gene targeting of murine sarcoglycan genes recapitulates the human muscular dystrophy phenotype where muscle degeneration is accompanied by muscle regeneration (Allamand and Campbell, 2000; Heydemann et al., 2001; Ozawa et al., 2001). Both δ- and γ-sarcoglycan bind the cytoplasmic actin binding protein, filamin C (Thompson et al., 2000). Moreover, mutations that disrupt sarcoglycan cause redistribution of filamin C. Sarcoglycan also stabilizes the interaction of dystroglycan subunits (Durbeej et al., 2000; Straub et al., 1998). Dystroglycan is a broadly expressed transmembrane protein with its α subunit binding directly to the G domains of laminin-α2 in the ECM and its β subunit binding dystrophin in the cytoplasm (Henry and Campbell, 1999). Biochemical preparations of the DGC from sarcoglycan mutant muscle reveal a less tightly adherent α-dystroglycan subunit suggesting abnormal interaction between α- and β-dystroglycan in the absence of sarcoglycan (Durbeej et al., 2000; Straub et al., 1998). Additionally, the absence of sarcoglycan alters membrane integrity in that the muscle membrane becomes abnormally permeable to small molecular mass tracers such as Evans blue dye (EBD) (Hack et al., 1998;
Matsuda et al., 1995; Straub et al., 1997). Thus, the sarcoglycan-dystroglycan complex mediates at least two links to the cytoskeleton, to dystrophin and filamin C, and coordinates interaction with laminin α2 in the ECM. Like the integrin complex, the sarcoglycan-dystroglycan complex mediates interactions between the ECM and membrane cytoskeleton.

Upregulation of integrin α7β1 has been observed in DMD muscle biopsies (Hodges et al., 1997). A transgenic mouse model of integrin α7BX2 overexpression was bred with mice lacking dystrophin and utrophin where it ameliorated the severe phenotype seen in these mice (Burkin et al., 2001). The presence of the integrin α7BX2 transgene on the dystrophin/utrophin mutant background reduced phagocytic cell infiltration and embryonic myosin heavy chain expression and improved lifespan (Burkin et al., 2001). Upregulation of integrin α7 produced from the transgene was relatively modest and the mechanism by which this increase reduced muscular dystrophy was not known.

Because both integrin α7β1 and the sarcoglycans are transmembrane proteins that mediate laminin interactions in muscle, upregulation of integrin may compensate for the loss of sarcoglycan. A conditional allele that targets the integrin β1 gene in skeletal muscle results in late embryonic lethality (Schwander et al., 2003). Integrin β1 muscle displays delayed myoblast fusion and disorganized sarcomere structure. Dystroglycan, dystrophin and laminin α2 appear to be normally localized in integrin β1 null muscle highlighting the importance of integrin complexes in muscle development. We found that integrin α7 was upregulated in mice lacking sarcoglycans. To determine whether integrin α7 upregulation is compensatory, we bred mice mutant for γ-sarcoglycan (gsg−/−) with mice mutant for integrin α7 (Itgα7−/−) to create mice lacking both proteins (gxi). Double-mutant gxi mice died within one month of birth and examination of these mice showed pervasive muscle degeneration. gxi muscle showed more EBD uptake than gsg−/− muscle indicating greater muscle degeneration. As loss of integrins may impair muscle regeneration, we determined that muscle regeneration was intact in gxi mice as gxi myoblasts showed normal in vitro and in vivo differentiation. These results argue that integrin and sarcoglycan have overlapping roles in maintaining muscle membrane stability and that integrin upregulation compensates for sarcoglycan loss.

Materials and Methods
Mice lacking γ-sarcoglycan (gsg−/−) and integrin α7 (Itgα7−/−) γ-sarcoglycan mutant mice were generated (Hack et al., 1998) by replacing exon 2 of the murine γ-sarcoglycan gene with a neomycin resistance gene. γ-sarcoglycan mutant mice (gsg−/−) produce no γ-sarcoglycan protein and are a null allele. Mice lacking integrin α7 (Itgα7−/−) were generated by targeting the 5′ region of the murine integrin α7 locus (Mayer et al., 1997) and are null for integrin α7 expression. Both the gsg−/− and Itgα7−/− alleles were bred through multiple generations onto a C57B1/6J background. Genotypes were confirmed using primers as follows: For gsg the primers used were 1. mgsx neoF GCCTGCTCTTCTTACTGAAAGCTCTTT; 2. mgsxE2-1 GGAAGAAGGCGGCCTAATCTTT; 3. mgsx 9R CAAATGCTTTGGCTCAGTATTCC. For Itgα7 the primers used were 1. lα7F TAGCTGCTTTGGCTCAGCAGC; 2. lα7neo CTCTCTCTTACTGAAGGCTC; 3. lα7R GCCCGTGTAAGAAACAGTCCAGCG. All animals used in this study were housed and treated in accordance with standards set by the University of Chicago Animal Care and Use Committee.

Microsome preparation and immunoblotting
Heavy microsomes were purified as described (Ohlendieck and Campbell, 1991) with modifications (Duclos et al., 1998; Hack et al., 2000b). Microsomes were prepared from a minimum of three animals, using six distinct muscle groups from each animal (quadriceps, gastrocnemius, soleus, biceps, triceps and pectoralis). Microsomal protein content was determined for each sample using the BioRad (Hercules, CA) protein assay. Protein was subjected to denaturing and reducing conditions, resolved by SDS-PAGE using either 4-12% or 4-20% linear gradient gels (Novex, San Diego, CA) and transferred to Immobilon P membranes (Millipore, Bedford, MA). Equal loading was confirmed by Coomassie blue staining. Immunoblotting was performed as described previously (Hack et al., 1998) with antibodies (listed below). Detection was performed with ECL-Plus (Amersham-Pharmacia, Piscataway, NJ) and visualized on film or using a Storm 860 (Molecular Dynamics, Sunnyvale, CA) and quantified using ImageQuant software (Molecular Dynamics, Sunnyvale, CA).

For laminin α2 immunoblotting, laminin was extracted from muscle tissue essentially as described (Xu et al., 1994). Briefly, 0.1 g (wt weight) of frozen quadriceps muscle was ground in liquid nitrogen and transferred into 1 ml extraction buffer without EDTA [150 mM NaCl, 50 mM Tris-HCl, pH 7.4 and protease inhibitor cocktail (Roche Diagnostics, Mannheim, Germany)]. This mixture was homogenized briefly and then centrifuged at 16,000 g for 15 minutes at 4°C. The supernatant was kept and frozen while the pellet was resuspended in 0.3 ml of extraction buffer with 10 mM EDTA and incubated on ice for 1 hour with periodic mixing. The mixture was centrifuged as before, and the supernatant was kept while the pellet was discarded. The protein content of the EDTA extract was quantified using the BioRad protein assay. 20 μg of protein was separated on 4-10% SDS/PAGE gradient gels under non-reducing conditions at 160V for approximately 20 hours and then transferred to Immobilon P membrane. The membrane was blocked in 10% dry milk in phosphate buffered saline with 0.1% Tween-20 and then incubated with polyclonal anti-laminin α2 at 1:500 (Ab 1301) (Kuang et al., 1998) in fresh blocking buffer. Immunoreactive protein bands were visualized as described above.

Antibodies
Embryonic myosin heavy chain monoclonal antibody (F1.652) was obtained from the Developmental Studies Hybridoma Bank (Iowa City, IA). Integrin α7A (used at 1:2500) and α7B (used at 1:5000) affinity-purified polyclonal antibodies were previously described (Mayer et al., 1997). The rabbit polyclonal antibody to dystrophin (AB6-10) was described previously (Lidov et al., 1990) and used at a concentration of 1:1000. β-dystroglycan was detected with NCL-bDG (Novocastra, Newcastle upon Tyne, UK) and used at 1:50. The anti-skeletal muscle actin antibody was used at 1:1000 (Sigma-Aldrich, St Louis, MO), and the anti-integrin α5 antibody was used at 1:5000 for immunoblotting and 1:500 for immunostaining of acetone-fixed muscle sections (Chemicon, Temecula, CA). Secondary antibodies (Jackson ImmunoResearch, West Grove, PA) used were goat anti-rabbit conjugated to FITC (1:2500) for dystrophin, goat anti-mouse conjugated to Cy3 (1:2500) for embryonic myosin heavy chain, and goat anti-rabbit conjugated to horseradish peroxidase (1:2500).

Immunocytochemistry
Mice from representative genotypes were sacrificed, and skeletal muscle was dissected from gsg−/−, wild-type (WT), Itgα7−/− and gxi.
animals and frozen in liquid nitrogen-cooled isopentane. 7 μm sections were prepared using a cryostat at −20°C, fixed in ice-cold methanol for 2 minutes and blocked in a solution of phosphate buffered saline (PBS) with 5% fetal bovine serum (FBS) for one hour at room temperature (RT). Primary antibodies (see above) were diluted in blocking solution and incubated overnight at 4°C. Cy-3 and FITC-conjugated secondary antibodies (Jackson ImmunoResearch) were diluted in blocking solution at 1:2500 at RT for 2 hours. Sections were mounted with Vectashield containing DAPI (Vector Laboratories, Burlingame, CA) and photographed using a Zeiss Axiopt microscope equipped with an Axioscan (Carl Zeiss, Germany). Where double staining with a polyclonal and monoclonal antibody was necessary, serial sections were taken. In order to minimize the background caused by the anti-mouse secondary antibody, the mouse on mouse (MOM) immunodetection kit was used in conjunction with the avidin/biotin blocking kit (Vector Laboratories, Burlingame, CA). The experiment was carried out according to manufacturer’s protocol.

Central nuclei quantification

Hematoxylin- and eosin-stained quadriceps sections of each genotype were analyzed for number of centralized nuclei. Sections from 6-12 animals were used for each genotype and two sections from each slide were counted. Eight random microscopic fields were analyzed per section, per slide. This resulted in 5000-7000 myofibers being analyzed per genotype.

Primary myoblast cultures

Primary myoblasts were isolated as described (Rando and Blau, 1997). Briefly, muscle was dissected from 1-3 day old mice and placed in PBS where it was minced using a razor blade. Approximately 2 ml collagenase/dispase/CaCl2 per gram of tissue was added and the mixture was incubated at 37°C for 30-45 minutes. This slurry was passed through an 80 μm nylon mesh filter and centrifuged for 5 minutes at 350 g. The pellet was resuspended in 8 ml F10-based primary myoblast growth medium and plated in a collagen-coated dish. The cells underwent a variable number of rounds of preplating to reduce fibroblast contamination and for enrichment of myoblasts. Differentiation was induced with Dulbecco’s modified Eagle medium (DMEM) supplemented with 5% horse serum. Fusion indices were calculated as described (van der Putten et al., 2002). Briefly, myoblast cultures were allowed to differentiate for 6 days prior to fusion index calculation. A minimum of six low-power microscopic fields was counted for each genotype. Fusion index was calculated as the number of nuclei in myotubes divided by the total number of nuclei. A myotube was defined as having three or more nuclei. Fusion indices were analyzed per genotype.

Quantitative RT-PCR

Total RNA was prepared using 100 mg quadriceps tissue from each genotype. Frozen, powdered muscle was added directly to 1.5 ml Trizol reagent (Invitrogen, Carlsbad, CA). The mixture was homogenized through successively smaller needles (16, 18, 20 and 21 gauge). The remainder of the protocol followed the manufacturer’s recommendations. 4 μg total RNA was added as a template for the Superscript II Reverse Transcription kit (Invitrogen, Carlsbad, CA). Following reverse transcription, 2 μl of a 1:4 dilution of each cDNA was used as a template for quantitative PCR using primers to integran α7 (Igta7F GCTGATAACCGTCGTCTGTTTC and Igta7R TCATGGTTGTTGCTCAGCCAC) and glyceraldehyde-3-phosphate dehydrogenase (G3PDH) G3PDHF ACCACAGTCCATGCCATCAC and G3PDHR GCCACCGAGGTTGCTCAG. PCR was carried out using the DyNAmo SYBR Green qPCR kit using the manufacturer’s recommended parameters (Finnzymes, Espoo, Finland) and an MJ Research Opticon Monitor. Cycle thresholds were determined for integrin α7 and GAPDH mRNA levels in wild-type and gsg−/− muscle. The experiment was performed in duplicate.

Results

Integrin is upregulated in sarcoglycan-deficient mice

We assessed the effect of sarcoglycan loss on the major sarcolemmal integrin complex, α7β1, using antibodies to the integrin α7 subunits. Integrin α7A and α7B derive from alternative splicing that alters the cytoplasmic domain of integrin α7; integrin α7B is the major form of integrin α7 expressed in skeletal muscle (Crawley et al., 1997). We studied microsomal membrane fractions from mice with null alleles in δ-sarcoglycan (dsg−/−) or γ-sarcoglycan (gsg−/−) (Hack et al., 2000b; Hack et al., 1998). Microsomal fractions enriched for membrane-associated proteins and removed contamination by sarcomeric proteins. We also studied mdx mice that lack full length dystrophin (Sicinski et al., 1989) and as a secondary consequence, show a decrease of the sarcoglycan subunits at the plasma membrane (Ohlendieck and Campbell, 1991). In common to each of these three mutants, dsg−/−, gsg−/− and mdx is sarcoglycan complex loss from the plasma membrane. Each of these three mutants showed an increase in integrin α7 protein levels (Fig. 1). We evaluated whether integrin α7 mRNA was increased to account for the upregulation seen on immunoblotting. Using quantitative RTPCR in two separate experiments, we found that integrin α7 mRNA was increased around threefold (average 3.1) in gsg−/− muscle compared to wild-type muscle. Interestingly, the degree of integrin protein upregulation was more marked in δ-sarcoglycan mutant mice. Although the phenotype is identical between gsg−/− and dsg−/− mice, dsg−/− mice have a more complete loss of the sarcoglycan complex (Hack et al., 2000b) potentially accounting for the greater increase of integrin α7 in dsg−/− compared to gsg−/− and mdx.

Double-mutant (gxi) mice display early lethality

To determine if integrin α7 upregulation compensates for the
In absence of sarcoglycan, \textit{gsg}^{+/–} \textit{Itg}α7^{+/–} double-mutant mice (\textit{gxi}) were generated by crossing \textit{gsg}^{+/–} \textit{Itg}α7^{+/–} mice. We chose \textit{γ}-sarcoglycan mice for this analysis as the defect in these mice derives wholly from a striated muscle-intrinsic defect, as opposed to a vascular smooth muscle defect as has been hypothesized for \textit{δ}-sarcoglycan null mice (Coral-Vazquez et al., 1999). It was difficult to discern a phenotype in very young animals, as activity levels are very limited in all genotypes at this young age. By 10-14 days of age most \textit{gxi} animals were 21 days, \textit{gxi} mice display reduced movement and a hopping gait with limb stiffness. (B) Radiographic images of wild-type and \textit{gxi} mice showing kyphosis affecting the cervicothoracic spine in the double mutant (arrows). (C) Survival curve showing relative life span of wild-type, \textit{gsg}^{+/–} and \textit{gxi} mice. \textit{gxi} mice have markedly reduced survival rates (\textit{n}=10 for \textit{gsg}^{+/–} and \textit{gxi} mice, \textit{n}=3 for control and \textit{Itg}α7^{+/–} mice). Integrin α7 mutants do not have reduced lethality less than 6 months of age (Mayer et al., 1997).
Severe muscle degeneration results from loss of both integrin α7 and γ-sarcoglycan

Histological examination of quadriceps skeletal muscle demonstrated a severe degenerative process in all muscle groups of gxi mice (Fig. 3). Muscular dystrophy is characterized by ongoing necrosis accompanied by regeneration giving rise to variation in myofiber size. An increase in the number of myofibers with centrally placed nuclei is consistent with regeneration. The degenerative process exceeds the regenerative potential so that replacement by connective and adipose tissue ensues. In dystrophin and sarcoglycan mutant muscle, the degenerative process is focal in nature. That is, normal-appearing areas of muscle are found neighboring foci of degeneration (Fig. 3, pale blue area, lower left panel). In contrast, double-mutant mouse muscle (gxi) has widespread degeneration affecting all parts of the muscle. At this low magnification view, fibrosis is seen interspersed throughout gxi muscle (note blue areas in lower right panel). Bar, 200 μm.

Enhanced muscle degeneration in gxi mice

The severe muscular dystrophy in gxi muscle may arise from increased degeneration from lack of muscle membrane integrity or decreased regeneration, or a combination thereof. To distinguish these possibilities, we studied gxi mice using the vital tracer EBD. Normal muscle is impermeable to EBD whereas muscle that lacks sarcoglycan or dystrophin becomes abnormally permeable to this tracer (Matsuda et al., 1995). Since gxi mice do not survive beyond 21-25 days, we studied 21-day-old gxi mice and gsg−/− mice and found significant uptake of EBD in gxi muscles and comparatively little EBD uptake in gsg−/− muscle (Fig. 4). Normal mice and 3-week-old Itgα7−/− mice show no EBD uptake grossly (data not shown) or microscopically (Fig. 4A, upper panels and Fig. 4B). The loss of both sarcoglycan and integrin α7 results in enhanced membrane permeability and degeneration.

To determine whether the major extracellular matrix attachment for integrin was present, we evaluated expression of laminin α2 (Fig. 5). Laminin α2 upregulation is seen in
Regenerative properties of gxi muscle are intact

To evaluate whether doubly deficient gxi mice have impaired muscle regeneration, we quantified the number of central nuclei in the myofibers of each genotype since centrally placed nuclei indicate myofibers that have undergone regeneration. We found that gsg<sup>-/-</sup> and gxi muscle had a statistically significant increase in centrally nucleated myofibers compared to either wild-type or Itgα7<sup>-/-</sup> muscle (Fig. 6). We also studied the expression of embryonic myosin heavy chain (eMyHC) as expression of this myosin isoform reflects myofiber regeneration. In gsg<sup>-/-</sup> muscle, expression of eMyHC is noted surrounding regions of degeneration (Fig. 7A, lower left panel). In contrast, eMyHC expression was present diffusely throughout gxi muscle reflecting regeneration in concert with diffuse degeneration (Fig. 7A, lower right panel). This pattern parallels the widespread degeneration seen with histology and reflects the close coupling of degeneration and regeneration. Normal muscle displays no eMyHC expression and Itgα7<sup>-/-</sup> muscle taken from young mice also does not express eMyHC (Fig. 7A, upper panels).

We evaluated the contribution of programmed cell death to the gxi phenotype with TUNEL labeling. An increase in TUNEL-positive nuclei characterizes dystrophin and sarcoglycan mutant muscle compared to normal muscle (Hack et al., 1998; Matsuda et al., 1995). The number of TUNEL-positive nuclei appears insufficient to account for the degree of degeneration seen in sarcoglycan or dystrophin mutants, but the increase is nonetheless a consistent feature that occurs near regions of muscle degeneration. In gxi muscle, TUNEL-positive nuclei were seen dispersed throughout the entire muscle consistent with an enhanced and widespread degenerative process (Fig. 7B).

In vitro fusion is normal in gxi myoblasts

To evaluate myofiber regeneration in gxi muscle further, we...
Integrin and sarcoglycan cultured primary myoblasts from normal, gsg\(^{-/-}\), Itg\(a_7\)\(^{-/-}\) and gxi muscle. In each case myoblasts were cultured from neonatal mice and upon serum starvation showed normal properties of differentiation and fusion to myotubes. These differentiated cultures were immunostained using antibodies directed against both eMyHC (red) and dystrophin (green) as expression of these proteins reflects normal differentiation of myotubes in culture (Fig. 8A). Normal and mutant cultures from each of the genotypes were able to express eMyHC and dystrophin in developing myotubes, indicating that gxi mice retained the ability to regenerate damaged muscle. We determined the fusion index for each genotype to measure the timeframe of myotube development. We found no statistically significant difference between any of the genotypes examined consistent with intact regenerative potential of gxi myotubes (Fig. 8B).

Upregulation of integrin \(\alpha 5\)
Integrin \(\alpha 5\) is highly expressed in developing myotubes and serves as the major fibronectin linkage for myofibers (Muschler and Horwitz, 1991). We found that integrin \(\alpha 5\) was increased in both dystrophin and sarcoglycan mutant mice (Fig. 9A). We examined the expression of integrin \(\alpha 5\) in gxi, \(\gamma\)-sarcoglycan and integrin \(\alpha 7\) mutant muscle and found that integrin \(\alpha 5\) was upregulated more in gxi compared to gsg\(^{-/-}\) muscle. Immunostaining of gsg\(^{-/-}\) and gxi muscle revealed that integrin \(\alpha 5\) upregulation was seen in fibers positive for embryonic myosin heavy chain expression (Fig. 9D). In gxi muscle, the increase in integrin \(\alpha 5\) expression was also seen in areas with increased connective tissue deposition as can be noted by the cluster of DAPI positive nuclei in the right hand side of the image taken from gxi muscle (Fig. 9D, lower right panel). As integrin \(\alpha 5\) upregulation is generally not associated with mature myotubes, it is less likely to contribute to myofiber stability or instability in this model.

Discussion
Muscle is a dynamic tissue that undergoes marked cell shape changes associated with significant stress and strain during muscle contraction. Mutations in cytoskeletal proteins such as dystrophin implicate the cortical cytoskeleton as important for maintenance of cell stiffness as disruption of this network leads to changes in muscle membrane properties that render muscle more susceptible to damage during muscle contraction (Harper et al., 2002; Petrof et al., 1993). The role of the remainder of the DGC, particularly the sarcoglycan subcomplex, is less well understood. Dystroglycan is a major cell surface receptor for laminin-2, and it is thought that the sarcoglycan complex stabilizes the \(\alpha\) and \(\beta\) subunits of dystroglycan (Allamand and Campbell, 2000). Biochemical dissociation studies of sarcoglycan null muscle suggest that \(\alpha\)-dystroglycan is less tightly adherent to the remainder of the complex resulting in a weakened connection from the dystrophin cytoskeleton to the ECM (Durbeej et al., 2000; Straub et al., 1998). Therefore, a complete sarcoglycan-dystroglycan complex is required for normal membrane stability.

The severe phenotype seen in gxi double-mutant mice provides genetic evidence that integrin and sarcoglycan are
parallel pathways for the maintenance of muscle membrane integrity. This point is underscored by recent work showing the phenotype of integrin β1-null muscle. Integrin β1-null muscle has aberrant sarcomere patterning and is defective in myofiber development despite normal expression and localization of dystroglycan, dystrophin and laminin-2 (Schwander et al., 2003). Therefore, it may be that sarcoglycan and integrin α7β1 are compensatory proteins with regard to mature muscle membrane integrity but that integrin has a unique additional role in muscle development. Additional support for this assertion is the observed upregulation of the DGC protein γ-sarcoglycan seen in integrin α7-deficient muscle as assayed by immunoblot (data not shown). Taken together, the available data seem to point to a functional redundancy between sarcoglycan and integrin for maintenance of muscle membrane integrity but not for actual fusion of myoblasts and development into myotubes. In the absence of both transmembrane linkages, widespread degeneration occurs. In

dystrophin or sarcoglycan-deficient muscle, there is focal necrosis with normal-appearing muscle immediately adjacent to areas of muscle damage. In contrast, muscle damage is seen spread throughout gxi muscle with little evidence of spared areas of muscle. gxi double-mutant muscle showed marked

Fig. 8. In vitro myoblast differentiation and fusion to myotubes is normal in gxi mutant mouse. (A) To assess the regenerative capacity of myoblasts, we cultured myoblasts from wild-type (WT), integrin α7 mutant (Itgα7+/−), γ-sarcoglycan mutant (gsg−/−) and double mutant (gxi) neonatal mice. Each culture was differentiated to myotubes, and stained for dystrophin (green) and embryonic myosin heavy chain expression (red). Bar, 50 μm. (B) The fusion index was determined as described in Materials and Methods for each genotype and no significant differences were noted in the timing or degree of myoblast fusion to myotubes (P>0.05 for all comparisons).

Fig. 9. Upregulation of integrin α5 is found in regenerating fibers. (A) Immunoblot and graphical representation of integrin α5 expression in wild-type (normal), δ-sarcoglycan mutant (dsg−/−), γ-sarcoglycan mutant (gsg−/−) and dystrophin mutant (mdx) muscle protein. Integrin α5 is a fibronectin receptor in muscle and is upregulated in DGC mutant muscle. β-actin labelling is shown in the lower panels as a loading control. (B) Immunoblot showing that integrin α5 is upregulated in double-mutant gxi and gsg−/− mutant muscle compared to muscle from integrin α7 mutant (Itgα7−/−). β-actin labelling is again shown in the lower panel as a loading control. (C) Immunostaining with an antibody to integrin α5 shows little to no increase in Itgα5−/− muscle compared to wild-type (WT) muscle. (D) Immunostaining shows that integrin α5 is found in regions of regeneration as the same fibers that are positive for integrin α5 also stain with embryonic myosin heavy chain (green). The increased fibrosis in gxi muscle also reacts with the integrin α5 antibodies consistent with its expression outside of muscle. Bar, 50 μm (C,D).
EBD uptake consistent with enhanced muscle damage. The early lethality in gxi mice appears to be the result of muscle weakness itself and probably involves a decline in the function of respiratory muscles. Examination of the hearts from gxi mice did not reveal evidence of focal necrosis like that seen in older gsg−/− mice (Heydemann et al., 2001) (data not shown). In the case of gsg−/− mice, cardiomyopathy typically develops by 6 months of age and often is associated with sudden unpredictable death probably arising from cardiac arrhythmias. In contrast, gxi mice appear well with reduced ambulation and an increased respiratory rate. The absence of overt cardiomyopathy may be explained because the mice do not survive long enough for focal degenerative cardiomyopathy to develop.

Within a myofiber, there may be regional roles for sarcoglycan and integrin in the maintenance of muscle membranes. Shear-type injury of muscle resulted in increased integrin expression at the ends of myofibers where integrins participate in forming myotendinous junctions. shear stress that disrupted the long axis of the myofiber membrane produced upregulation of integrin and sarcoglycan, suggesting that these transmembrane linkages may both be important to lateral interactions of myofibers with the extracellular matrix and neighboring myofibers (Kaariainen et al., 2000; Kaariainen et al., 2001). The severe phenotype in gxi mice may arise from a fully defective ECM attachment along the lateral aspects of myofibers, although based on the known functions of integrins and the hypothesized roles of sarcoglycan, downstream signaling is likely to be impaired. Normal signals, transmitted perhaps through the nucleus and gene expression, are unable to respond and repair in the face of the fully defective ECM connection.

In muscle development, the fusion of myoblasts to myotubes is associated with an increase in integrin expression, notably integrin α5β1 (McDonald et al., 1995). In mature muscle, integrin α7β1 predominates (Crawley et al., 1997), although integrin α5 may also be important for a fibronectin interaction that stabilizes muscle membrane integrity (Taverna et al., 1998). We noted an increase in the fibronectin receptor, integrin α5, in sarcoglycan-deficient muscle. This receptor may also be capable of compensating for the loss of a functional sarcoglycan-dystroglycan unit, or alternatively, the increase in integrin α5 could be pathologic to muscle. Immunolocalization data demonstrated that integrin α5 expression is predominantly coincident with embryonic myosin heavy chain expression and therefore reflects regeneration. While it could be expected that gxi muscle derives its phenotype in part from ineffective regeneration, gxi muscle development does not appear significantly impaired since in vivo features of regeneration are normally present. Furthermore, in vitro myoblast fusion of gxi myoblast cultures was indistinguishable from normal muscle making ineffective regeneration unlikely to account for the severe muscle findings in gxi mice.

The compensatory role of integrin was suggested by recent work showing that overexpression of rat integrin α7β1 can ameliorate the muscular dystrophy phenotype as well as increase the life span of mdx/utr−/− DKO mice (Burkin et al., 2001). Interestingly, only a modest upregulation of integrin subunits was necessary to produce this improvement in phenotype (2.3-fold upregulation of integrin α7B and 1.5-fold upregulation of β1D integrin). Integrin α7β1 RNA levels are upregulated in both mdx mice and human Duchenne and Becker muscular dystrophy patients (Hodges et al., 1997). More recently, integrin α7B was shown to be upregulated in muscle fibers deficient in dystroglycan, despite dystrophin being present in some of the fibers (Cote et al., 2002). The sarcoglycan complex is secondarily destabilized in dystrophin deficiency. Thus, it is likely that upregulation of integrin is compensating for the secondary loss of sarcoglycan in each of these cases.

The role of integrins as mechanosignaling molecules for cell adhesion has been established. Signals from extracellular matrix proteins such as laminin and fibronectin are transmitted through integrin complexes to intracellular proteins such as focal adhesion kinase (FAK) and phosphatidylinositol (PtdIns) 3-kinase (Howe et al., 2002; Schwartz, 2001). Like the integrins, the sarcoglycan complex may participate in both mechanical and signaling roles at the plasma membrane. Supporting this, the cytoplasmic tails of γ- and δ-sarcoglycan have conserved tyrosine residues that can be phosphorylated. Furthermore, the cytoplasmic domains of γ- and δ-sarcoglycan bind filamin C (Thompson et al., 2000). In other tissues, filamins have been shown to interact with integrin subunits, specifically to the β1 subunit of integrin. Thus, filamin may be one of the signaling mechanisms onto which both integrins and sarcoglycans converge. The synthetic lethal phenotype produced by the combined sarcoglycan and integrin loss from the surface of myofibers highlights not only the downstream signaling mechanisms that may function in parallel between these two cellular attachments, but also emphasizes the extracellular component, laminin, with which each of these complexes interacts. These findings help elucidate the role of the sarcoglycan complex, drawing parallels to the integrin complex, in its role in cytoskeletal-membrane-matrix interactions.

The authors thank the McNally and Svensson laboratories for helpful discussions. This work was supported by the Muscular Dystrophy Association, the Burroughs Wellcome Fund, the American Heart Association and HL61322 (E.M.M.) and HL8472 (M.J.A.) and by the Wellcome Trust (#060549 to U.M.).

References
Allamand, V. and Campbell, K. P. (2000). Animal models for muscular dystrophy: valuable tools for the development of therapies. Hum. Mol. Genet. 9, 2459-2467.
Bonnewmann, C. G., McNally, E. M. and Kunkel, L. M. (1996). Beyond dystrophin: current progress in the muscular dystrophies. Curr. Opin. Pediatr. 8, 569-582.
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 viability in dystrophic mice. J. Cell Biol. 152, 1207-1218.
Coral-Vazquez, R., Cohn, R. D., Moore, S. A., Hill, J. A., Weiss, R. M., Davison, R. L., Straub, V., Barresi, R., Bansal, D., Hrstka, R. F. et al. (1999). Disruption of the sarcoglycan-sarcospan complex in vascular smooth muscle: a novel mechanism for cardiomyopathy and muscular dystrophy. Cell 98, 465-474.
Cote, P. D., Moukhles, H. and Carbonetto, S. (2002). Dystroglycan is not required for localization of dystrophin, syntrophin, and neuronal nitric-oxide synthase at the sarcolemma but regulates integrin alpha 7B expression and caveolin-3 distribution. J. Biol. Chem. 277, 4672-4679.
Crawley, S., Farrell, E. M., Wang, W., Gu, M., Huang, H. Y., Huynh, V., Hodges, B. L., Cooper, D. N. and Kaufman, S. J. (1997). The alpha7beta1
integrin mediates adhesion and migration of skeletal myoblasts on laminin. Exp. Cell Res. 235, 274-286.

Duclos, F., Straub, V. C., Moore, S. A., Venzke, D. P., Hrstka, R. F., Crosbie, R. H., Durbeje, M., Lebakken, C. S., Ettinger, A. J., van der Meulen, J. et al. (1998). Progressive muscular dystrophy in alpha-sarcoglycan-deficient mice. J. Cell Biol. 142, 1461-1471.

Durbeje, M., Cohn, R. D., Hrstka, R. F., Moore, S. A., Allamand, V., Davidson, B. L., Williamson, R. A. and Campbell, K. P. (2000). Disruption of the beta-sarcoglycan gene reveals pathogenetic complexity of limb-girdle muscular dystrophy type 2E. Mol. Cell 5, 141-151.

Ervasti, J. M. and Campbell, K. P. (1993). A role for the dystrophin-glycoprotein complex as a transmembrane linker between laminin and actin. J. Cell Biol. 122, 809-823.

Fassler, R. and Meyer, M. (1995). Consequences of lack of beta 1 integrin gene expression in mice. Genes Dev. 9, 1896-1908.

Hack, A. A., Ly, C. T., Jiang, F., Clendenin, C. J., Sigrist, K. S., Wollmann, Fassler, R. and Meyer, M. (2000). Domain requirements for association of the dystrophin-glycoprotein complex with the actin cytoskeleton. J. Cell Sci. 113, 2535-2544.

Harper, S. Q., Hauser, M. A., DelboRusso, C., Duan, D., Crawford, R. W., Phelps, S. E., Harper, H. A., Robinson, A. S., Engelhardt, J. F., Brooks, S. V. et al. (2002). Modularity of dystrophin: implications for gene therapy of Duchenne muscular dystrophy. Nat. Med. 8, 253-261.

Hayashi, Y. K., Chou, F. L., Engvall, E., Ogawa, M., Matsuda, C., Hirabayashi, S., Yokochi, K., Zlober, B. L., Kramer, R. H., Kaufman, S. J. et al. (1998). Mutations in the integrin alpha 7 gene cause congenital myopathy. Nat. Genet. 19, 94-97.

Henry, M. D. and Campbell, K. P. (1999). Dystroglycan inside and out. Curr. Opin. Cell Biol. 11, 602-607.

Heydemann, A., Wheeler, M. T. and McNally, E. M. (2001). Cardiomyopathy in animal models of muscular dystrophy. Curr. Opin. Cardiol. 16, 211-217.

Hirsch, E., Lokhikangas, L., Gullberg, D., Johansson, S. and Fassler, R. (1998). Mouse myoblasts can fuse and form a normal sarcomere in the absence of beta 1 integrin expression. J. Cell Sci. 111, 2397-2409.

Hodges, B. L., Hayashi, Y. K., Nonaka, I., Wang, W., Arahata, K. and Kaufman, S. J. (1997). Altered expression of the alpha beta 1 integrin in human and murine muscular dystrophies. J. Cell Sci. 110, 2873-2881.

Howe, A. K., Aplin, A. E. and Juliano, R. L. (2002). Anchorage-dependent ERK signaling – mechanisms and consequences. Curr. Opin. Genet. Dev. 12, 30-35.

Hynes, R. O. (1992). Integrins: versatility, modulation, and signaling in cell adhesion. Cell 69, 11-25.

Kaariainen, M., Kaariainen, J., Jarvinen, T. L., Nissinen, L., Heino, J., Jarvinen, M. and Kalimo, H. (2000). Integrin and dystrophin associated protein complexes during regeneration of shearing-type muscle injury. Neuromuscul. Disord. 10, 121-132.

Kaariainen, M., Liljamo, T., Pelto-Huikko, M., Heino, J., Jarvinen, M. and Kalimo, H. (2001). Regulation of alpha 7 integrin by mechanical stress during skeletal muscle regeneration. Neuromuscul. Disord. 11, 360-369.

Kuang, W., Xu, H., Vachon, P. H., Liu, L., Loechel, F., Wewer, U. M. and Engvall, E. (1998). Merosin-deficient congenital muscular dystrophy: partial genetic correction in two mouse models. J. Clin. Invest. 102, 844-852.

Lidov, H. G., Byers, T. J., Watkins, S. C. and Kunkel, L. M. (1990). Localization of dystrophin to postsynaptic regions of central nervous system cortical neurons. Nature 348, 725-728.

Matsuda, R., Nishikawa, A. and Tanaka, H. (1995). Visualization of dystrophic muscle fibers in mdx mouse by vital staining with Evans blue: evidence of apoptosis in dystrophin-deficient muscle. J. Biochem. (Tokyo) 118, 959-964.

Mayer, U., Saher, G., Fassler, R., Bornemann, A., Echtnermeyer, F., von der Mark, H., Miosge, N., Poschl, E. and von der Mark, K. (1997). Absence of integrin alpha 7 causes a novel form of muscular dystrophy. Nat. Genet. 17, 318-323.

McDonald, K. A., Lokonishok, M. and Horwitz, A. F. (1995). Alpha v and alpha 3 integrin subunits are associated with myofibrils during myotubulogenesis. J. Cell Sci. 108, 2573-2581.

Muschler, J. L. and Horwitz, A. F. (1991). Down-regulation of the chicken alpha 5 beta 1 integrin fibronectin receptor during development. Development 113, 327-337.

Ohlendieck, K. and Campbell, K. P. (1991). Dystrophin-associated proteins are greatly reduced in skeletal muscle from mdx mice. J. Cell Biol. 115, 1685-1694.

Ozawa, E., Nishino, I. and Nonaka, I. (2001). Sarcolemmemopathy: muscular dystrophies with cell membrane defects. Brain Pathol. 11, 218-230.

Petrof, B. J., Shrager, J. B., Stedman, H. H., Kelly, A. M. and Sweeney, H. L. (1993). Dystrophin protects the sarcolemma from stresses developed during muscle contraction. Proc. Natl. Acad. Sci. USA 90, 3710-3714.

Rafael, J. A. and Brown, S. C. (2000). Dystrophin and utrophin: genetic analyses of their role in skeletal muscle. Microsc. Res. Tech. 48, 155-166.

Rando, T. A. and Blau, H. M. (1997). Methods for myoblast transplantation. Methods Cell Biol. 52, 261-272.

Schwander, M., Leu, M., Stumm, M., Dorchies, O. M., Ruegg, U. T., Schittny, J. and Muller, U. (2003). Betal integrins regulate myoblast fusion and sarcosome assembly. Dev. Cell 4, 673-685.

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

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.

Straub, V., Rafael, J. A., Chamberlain, J. S. and Campbell, K. P. (1997). Animal models for muscular dystrophy show different patterns of sarcolemmal disruption. J. Cell Biol. 139, 375-385.

Straub, V., Duclos, F., Venzke, D. P., Lee, J. C., Cutshall, S., Leveille, C. J. and Campbell, K. P. (1998). Molecular pathogenesis of muscle degeneration in the delta-sarcoglycan-deficient hamster. Am. J. Pathol. 153, 1623-1630.

Taverna, D., Disatnik, M. H., Rayburn, H., Bronson, R. T., Yang, J., Rando, T. A. and Hynes, R. O. (1998). Dystrophic muscle in mice chimeric for expression of alpha 5 integrin. J. Cell Biol. 143, 849-859.

Thompson, T. G., Chan, Y. M., Hack, A. A., Brosius, M., Rajala, M., Lidov, H. G., McNally, E. M., Watkins, S. and Kunkel, L. M. (2000). Filamin 2 (FLN2): a muscle-specific sarcoglycan interacting protein. J. Cell Biol. 148, 115-126.

Vachon, P. H., Xu, H., Liu, L., Loechel, F., Hayashi, Y., Arahata, K., Reed, J. C., Wewer, U. M. and Engvall, E. (1997). Integrins (alpha beta 1) in muscle function and survival. Disrupted expression in merosin-deficient congenital muscular dystrophy. J. Clin. Invest. 100, 1870-1881.

van der Putten, H. H., Joosten, B. J., Klaren, P. H. and Everts, M. E. (2000). Sarcolemmal F8 fragments of laminin. Journal of Cell Science 117 (17).