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*

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Dystroglycan is part of the dystrophin-associated protein complex, which joins laminin in the extracellular matrix to dystrophin within the subsarcolemmal cytoskeleton. We have investigated how mutations in the components of the laminin-dystroglycan-dystrophin axis affect the organization and expression of dystrophin-associated proteins by comparing mice mutant for merosin (α7-laminin, dy), dystrophin (mdx), and dystroglycan (Dag1) using immunohistochemistry and immunoblots. We report that syntrophin and neuronal nitric-oxide synthase are depleted in muscle fibers lacking both dystrophin and dystroglycan. Some fibers deficient in dystroglycan, however, localize dystrophin at the cell surface at levels similar to that in wild-type muscle. Nevertheless, these fibers have signs of degeneration/regeneration including increased cell surface permeability and central nuclei. In these fibers, syntrophin and nitric-oxide synthase are also localized to the plasma membrane, whereas the sarcoglycan complex is disrupted. These results suggest a mechanism of membrane attachment for dystrophin independent of dystroglycan and that the interaction of sarcoglycans with dystrophin requires dystroglycan. The distribution of caveolin-3, a muscle-specific component of caveolae recently found to bind dystroglycan, was affected in dystroglycan- and dystrophin-deficient mice. We also examined alternative mechanisms of cell-extracellular matrix attachment to elucidate how the muscle basement membrane may subsist in the absence of dystroglycan, and we found the α7B splice variant of the α7 integrin receptor subunit to be up-regulated. These results suggest the possibility that α7 integrin compensates in mediating cell-extracellular matrix attachment but cannot rescue the dystrophic phenotype.

Striated muscle fibers interact with a specialized extracellular matrix, the basement membrane, upon which they depend for survival and function. This interaction is mediated primarily by a large oligomeric dystrophin-associated protein (DAP)1

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‡ The abbreviations used are: DAP, dystrophin associated protein; DAPI, 4',6-diamidino-2-phenylindole; CK, creatine kinase; ES, embryonic stem; GPI, glucose-phosphate isomerase; NOS, neuronal nitric-oxide synthase; DG, dystroglycan; PBS, phosphate-buffered saline.
lin-3 was recently found to bind directly to the same domain as dystrophin on the cytoplasmic tail of β-dystroglycan (24). Mutations in caveolin-3 lead to limb girdle muscular dystrophy 1C (21), and it was also found to be overexpressed in muscle from Duchenne muscular dystrophy patients and from mdx mice (25, 26). The pathogenic mechanism of caveolin-3 remains elusive, but precise regulation of expression levels appears to be important for normal muscle function because a null mutation (27, 28) and induced overexpression (29) both lead to muscle pathology in mice.

In a previous report (30), we demonstrated that chimeric mice with little or no dystroglycan had a severe histopathology closely resembling that of patients with Duchenne muscular dystrophy and that was more severe than that of the mdx mouse. In the present study, we have explored the composition of the DAP complex in dystroglycan-deficient muscle to determine whether interactions inferred from biochemical studies or from mutations of dystroglycan-interacting proteins are confirmed by deletion of dystroglycan in situ. We find that the deletion of dystroglycan-interacting proteins results not only from displacement from the sarcolemma toward the cytosol but also from a decrease in overall protein levels. Furthermore dystrophin was found not to be absolutely dependent on β-dystroglycan for membrane targeting. We have also examined the distribution of caveolin-3 in dystroglycan-deficient muscle and found its distribution identical to that of the mdx mouse. Finally, the α7β1 splice variant of the integrin α7β subunit was found to be selectively up-regulated. Because dystroglycan-deficient muscles have basement membranes that are indistinguishable from controls (30), this receptor may compensate for the loss of dystroglycan in basement membrane assembly.

EXPERIMENTAL PROCEDURES

Animals—Embryonic stem (ES) cell lines rendered null for dystroglycan were described previously (30). Briefly, we generated two targeting vectors, one harboring a hygromycin resistance gene at the 5’ end of the second exon of the dystroglycan gene (Dag1) and one with the neomycin resistance gene also at the 5’ end of the second exon. RI ES cells (31) were electroporated with the hygromycin resistance vector and submitted to drug selection, and homologous recombinants were identified by Southern blotting. The same procedure was repeated on single copy Dag1 mutants with the neomycin resistance targeting vector to target the other copy. Two dystroglycan null clones, 3C12 and 3H1, were chosen for blastocyst injection. Mice from the resulting litters were selected initially on the basis of their coat color for further analysis. Mice with mutations in the dystrophin (B6Ras.Cg-Dmdmdx), α1-syntrophin (B6.Ras.Cg-Dmdmdx.Lam1) and α2-laminin (B6.Ras.Cg-Dmdmdx.Lam2) genes were obtained from The Jackson Laboratory (Bar Harbor, ME). The mdx-3Cv mice are deficient in muscle and non-muscle dystrophin isoforms as a result of a mutation in a consensus splice acceptor site (32). The animals were maintained and humanely sacrificed in accordance to the guidelines of the Canadian Council on Animal Care.

Creatine Kinase Assay—Approximately 0.5 ml of blood was collected from mice that were anesthetized by using 13 mg/kg xylazine and 100 mg/kg ketamine. Total creatine kinase (CK) levels in serum were measured with an Hitachi 917 automated clinical chemistry analyzer (HITACHI, Tokyo, Japan). Ultrathin sections were collected (60 nm) on 100 or 200 mesh grids, contrasted with lead citrate, and examined with a CM10 Philips transmission electron microscope.

[Text continues with detailed experimental procedures and results]
immunoblotting and GPI assays, revealed that in muscle that was dystroglycan-deficient by 4674 and 5C36. We have also observed...

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Fig. 1. Elevated levels of serum creatine kinase and pathological ultrastructural features in muscle deficient in dystroglycans. A, serum CK levels in dystroglycan-deficient chimeric mice (ch) are 20 times more elevated than in control (wt) animals. Dystroglycan-deficient chimeric mice have an abnormal ultrastructure (B and C). Degenerating fibers are electron dense and have dilated sarcoplasmic reticulum vesicles. Cytomembrane swirls (C, arrowheads) are often observed at close proximity to the sarcolemma. Bar indicates 1.3 μm in C and 5 μm in B.

36). We have also observed “cytomembrane swirls” (Fig. 1C) that appear as concentric layers of intracellular membranes with a morphology distinct from “fingerprint bodies” that are seen in several myopathies (37, 38).

Dystroglycan Is Not Required for the Localization of Dystrophin and the Cytoplasmic DAP Complex to the Sarcolemma—In a previous study (30) we had observed several fibers that were positively labeled for dystrophin even within chimeric muscle that appeared to be null for dystroglycan when muscle extracts were assayed by immunoblotting or by GPI electrophoresis. At the time we were unable to monitor dystroglycan expression within single myofibers because the anti-mouse IgG secondary antiserum used to recognize the dystroglycan monoclonal antibody also reacted with endogenous immunoglobulins that had infiltrated the dystroglycan-depleted muscle (30). Thus, the localization of dystrophin to the sarcolemma of a subset of these fibers could be due to dystroglycan expression in chimeric myofibers with a small number of wild-type nuclei. Therefore, we generated a rabbit anti-β-dystroglycan antiserum, which revealed that in muscle that was dystroglycan-deficient by immunoblotting and GPI assays, ∼1–3% of the fibers were dystroglycan-positive (Fig. 2G and not shown).

By staining serial sections of muscle and identifying the same region on all the sections, it was possible to determine the make-up of the whole DAP complex in a single fiber. In the vast majority of myofibers in dystroglycan-deficient chimeras dystroglycan was undetectable (Fig. 2G) as were dystrophin (Fig. 2J), the sarcoglycans (Fig. 2, H and I), and the cytoplasmic complex (α-syntrophin and nNOS) (Fig. 2, K and L). These fibers had an immunohistochemical profile similar to dystrophin null muscle (Fig. 2, S–X) but distinct from merosin null muscle (M–R) where DAPs are largely unaffected (12). In a few instances where fibers in dystroglycan-deficient muscle had detectable levels of dystroglycan, dystrophin, the sarcoglycans (α and γ), and the cytoplasmic DAP complex (syntrophin and nNOS) were localized at the plasmalemma (Fig. 2, G–L, arrowheads) at levels similar to that in wild-type mice (Fig. 2, A–F).

However, even when dystroglycan was poorly expressed, it was often concentrated at neuromuscular junctions, identified by double labeling with α-bungarotoxin (39; not shown), and co-localized with molecules of the DAP complex. Presumably, recruitment of residual dystroglycan to junctions during development (40) can stabilize the complex at those sites (39) and may help render these regions resistant to disruption.

In some fibers where there was no trace of dystroglycan or α-sarcoglycan (Fig. 3, A and B), dystrophin was clearly detectable at the plasmalemma, and these fibers were typically found in small groups in the muscle (Fig. 3C). In these instances syntrophin and nNOS co-distributed with dystrophin at the cell surface (Fig. 3, D and E). Thus, dystrophin and a cytoplasmic complex of at least syntrophin and nNOS can be localized at the plasma membrane independently of dystroglycan or the sarcoglycans. The membrane anchor responsible for this interaction is unknown as are member(s) of the cytoplasmic complex that can interact with it (discussed below). It is important to note, however, that the complex lacking dystroglycan was insufficient to prevent muscle degeneration because these myofibers were atrophic and/or had central nuclei typical of myofiber regeneration (Fig. 3F) and were occasionally found to contain high levels of endogenous immunoglobulins (not shown) indicative of a breakdown in the sarcolemma.

Absence of Dystroglycan Leads to a Reduction in Expression of the DAP Complex—To assess whether the reduced immunohistochemical labeling at the sarcolemma of mutant muscle reflects a reduction in expression of the DAPs or simply a displacement from the sarcolemma to the cytosol, we performed immunoblots for dystrophin and members of the DAP complex. The expression level of the DAP complex in total protein extracts (1% Triton X-100) of dystroglycan-deficient chimeric mice was compared with that of mdx and dy mice. Replicate blots were made and probed with antibodies to α-dystroglycan, β-dystroglycan, α-sarcoglycan, β-sarcoglycan, γ-sarcoglycan, dystrophin, α1-syntrophin, β1-syntrophin, and nNOS (Fig. 4B). As shown previously, the absence of dystrophin in the mdx mouse leads to a reduction in the expression of DAP components including dystroglycan, sarcoglycans, syntrophin, dystrobrevin, and nNOS (12, 41–43). Dystrophin levels were considerably reduced in dystroglycan-deficient chimeric muscle, and the effect of dystroglycan depletion mirrored that of dystrophin depletion in the mdx mouse resulting in a significant reduction of the sarcoglycan complex and α1-dystrobrevin. nNOS, which binds to syntrophin, was also substantially decreased in dystroglycan-deficient mice but only moderately affected in mdx mice. The reduction in these DAPs did not appear merely to result from the degeneration of myofibers in these animals because dy mice, which have a severe muscular dystrophy, expressed these DAPs in amounts equal to those in wild-type skeletal muscle (Fig. 4B and Fig. 2, M–R).

In contrast, α1-syntrophin showed little change in expression levels in dystroglycan-deficient muscle. Thus targeting to the membrane of α1-syntrophin rather than expression, per se,
Fig. 2. Syntrophins and nNOS are absent from the sarcolemma in the absence of dystrophin and dystroglycan. Immunohistochemistry was performed on fresh-frozen sections of gastrocnemius muscle of control (wt; A–F), dystroglycan-depleted chimeric (ch; G–L), dy laminin-α 2-deficient (dy; M–R), and mdx dystrophin-deficient (mdx; S–X) muscle for β-dystroglycan (β-DG; A, G, M, and S), α-sarcoglycan (α-SG; B, H, N, and T), γ-sarcoglycan (γ-SG; C, I, O, and U), dystrophin (dyst; D, J, P, and V), α1-syntrophin (α1-SY; E, K, Q, and W), and neuronal nitric-oxide synthase (nNOS; F, L, R, and X). In mdx dystrophin-deficient muscles all the components of the DAP complex are absent. In contrast, the components of the DAP complex in the dy laminin-α mutant mice are all localized at the sarcolemma and near control levels. Serial sections were used for the analysis of dystroglycan-depleted muscle. The dystroglycan-negative fibers are also negative for all the DAP complex molecules as well as for dystrophin. Fibers positive for dystroglycan (arrowheads) provide an internal positive control. Bar in X indicates 75 μm.

seems disrupted in the absence of dystroglycan. Consistent with this an internal pool of α-syntrophin is visible immunohistochemically in some myofibers (Fig. 2K). This pattern is different from that in dystrophin null muscle where α1-syntrophin is essentially undetectable (Fig. 2W). In mdx, dy, and dystroglycan-deficient muscle the anti-β1-syntrophin antibody revealed a protein of 56 kDa in addition to the 59-kDa protein. This protein is most likely a product of proteolysis that occurs in all types of dystrophy.

Finally, the predominantly synaptic isoform of α-dystrobrevin, α1-dystrobrevin, was not affected in dy, mdx, or chimeric mice, which again appears to reflect the stability of the postsynaptic density at neuromuscular junctions. These differences in DAPS in dystroglycan-deficient, mdx, and dy muscles reveal distinct interactions for dystroglycan, dystrophin, and merosin in the DAP complex as well as in regulation of expression of other members of the DAP complex.

Redistribution of Caveolin-3 in Dystroglycan-deficient Chimeric Muscle—Caveolin-3 is a muscle-specific component of small membrane invaginations (caveolae) found in many cell types and has recently been implicated in human myopathies (21, 22). Because caveolin-3 binds to dystroglycan (24), we investigated its distribution in dystroglycan-deficient mice. Immunostaining with antisera specific to caveolin-3 stained the sarcolemma of chimeric muscle in a fashion closely resembling that of mdx mice (compare Fig. 5, H, I, K with F and G). Not only was the staining in chimeras and mdx mice more intense than in wild-type and dy mice (Fig. 5, A, B, D, and E), but the antigen was in a wider band at the cell surface. In immunoblots, caveolin-3 levels were increased by ~36%, suggesting that dystrophin and dystroglycan are in a genetic pathway that regulates the distribution of caveolin-3 at the cell surface (Fig. 5L).

The α7B Integrin Is a Candidate for Compensation in Dystroglycan-deficient Chimeric Muscle—Dystroglycan is thought to be a laminin receptor essential for basement membrane assembly (44, 45). Yet the function of basement membranes in maintaining the integrity of the cell surface and in the etiology of muscular dystrophies is complex. For example, in its composition and ultrastructure, the muscle basement membrane from dystroglycan-depleted chimeric mice appears to be normal (30). Because the α7β1 integrin is a major integrin receptor for laminins in muscle and the α7B integrin is up-regulated in cases of Duchenne and Becker muscular dystrophy (18, 19), we examined the expression and the distribution of α7 integrin subunit splice variants in dystroglycan-deficient muscle. Immunoblot analysis of total protein extracts pooled with anti-α7A integrin and anti-α7B integrin antisera revealed that levels of α7A integrin are decreased in dystroglycan-deficient chimeric muscle, whereas levels of α7B integrin are increased (Fig. 6, A and B). Immunofluorescence with anti-α7B antibody revealed areas of increased immunoreactivity, which in longitudinal sections appeared to follow the whole length of the fiber (Fig. 6, C and D). These areas of increased α7B staining often contained atrophic/centrally nucleated fibers and were interspersed with degenerating fibers. Thus, in the absence of dystroglycan a splice variant of the α7 integrin subunit is selectively up-regulated and may help assemble and maintain the basement membrane, but this is insufficient to sustain muscle fiber integrity.

DISCUSSION

α- and β-dystroglycan are thought to form the functional core of the DAP complex, but detailed study of these proteins has been hampered by the absence of natural mutations in the DAG1 gene as well as by the early embryonic lethality of targeted mutations of DAG1 in mice (46). In previous work we
have generated chimeric mice with skeletal muscle deficient in dystroglycan, which have a muscular dystrophy and defects in nerve-muscle synapses (30, 39). Here we present evidence on interactions in situ of members of the DAP complex in the absence of /H9251- and /H9252-dystroglycan. Dystrophin interacts with /H9252-dystroglycan primarily via amino acids 3054–3271, although amino acids 3271–3446 are necessary for optimal binding (47, 48). These regions are near the C terminus and encompass a WW domain, two putative calcium-binding EF hand motifs, and a putative zinc finger domain (49, 50). Mutations within the C-terminal of dystrophin are rare and usually result in the absence of detectable dystrophin protein. However, at least five patients have been reported who completely lacked the C terminus, including the cysteine-rich region, but have dystrophin localized correctly to the sarcolemma (51–55). In three of these patients proteins of the DAP complex were greatly reduced (55). Here we describe for the first time a situation where dystrophin is localized to the sarcolemma in myofibers that have undetectable dystroglycan. These data complement the data discussed above and reveal that the sarcolemma contains a non-dystroglycan anchor for dystrophin. The nature of this anchor is obscure. nNOS is known to bind caveolin-3 (56) and syntrophin (57, 58) and may provide a means for dystrophin.
independent of dystroglycan, to associate with the sarcolemma. Alternatively, the syntrophins have pleckstrin homology domains that are known to direct membrane targeting of their host proteins by binding to polyphosphoinositides (59, 60). Indeed, we have found that nNOS and \( \alpha \)-syntrophin were localized at the sarcolemma in dystroglycan-negative/dystrophin-positive fibers (Fig. 3), consistent with the possibility that either or both of these proteins anchor dystrophin to the sarcolemma in the absence of dystroglycan. Other possibilities include direct binding of dystrophin to the lipid bilayer through its spectrin-like domains (61) or maintenance of dystrophin at the periphery via its interaction with actin or via an unidentified membrane protein.

It is important to note that this non-dystroglycan anchor is insufficient to maintain muscle integrity. Dystroglycan-negative/dystrophin-positive myofibers have increased cell-surface permeability reflected by the infiltration of immunoglobulins into the sarcoplasm\(^2\) despite localization of dystrophin, syntrophin, and nNOS at the sarcolemma. Consistent with this, forced expression of dystrophin constructs lacking the entire cysteine-rich domain in \( \textit{mdx} \) mice did not alter dystrophin targeting to the sarcolemma (62). Also consistent with our observations in chimeric mice, the muscular dystrophy progressed unabated in \( \textit{mdx} \) mice expressing these dystrophin constructs (62).

The interactions of the sarcoglycans are manifold. For exam-
ple, loss of any one of the sarcoglycans leads to abnormal expression of all others at the sarcolemma (63–66). Also reduction of dystrophin levels in cases of γ-sarcoglycan deficiency (67) together with chemical cross-linking studies (2) indicate that dystrophin and γ-sarcoglycan interact directly. α-Sarcoglycan seems to be only loosely associated with the other sarcoglycans, whereas δ-sarcoglycan appears to be most closely associated with β-dystroglycan (68). The disruption of γ-sarcoglycan only results in a mild reduction of α- and ε-sarcoglycans with no effect on the expression of α- and β-dystroglycan (65).

In chimeric muscle with reduced dystroglycan, α- and γ-sarcoglycan are found exclusively in myofibers that are dystroglycan-positive (Fig. 2). Thus, unlike syntrophin and nNOS, the presence of dystrophin at the sarcolemma in the absence of dystroglycan does not restore the sarcoglycan complex (Fig. 3). Matsumura et al. (69) have predicted a direct interaction of sarcoglycans with the extracellular matrix and proposed a model in which the sarcoglycans bind both the dystroglycans and the extracellular matrix to stabilize the DAP complex. If this is so, our results suggest that the absence of dystroglycan may destabilize the complex or, perhaps, that it is required for assembly or insertion of the sarcoglycan complex at the cell surface.

Caveolin oligomers are major structural components of caveolae, small (50–100 nm) vesicle-like structures at the plasma membrane that have been implicated in endocytosis, signal transduction, and organization of lipids in the membrane (28, 70). Mutations in caveolin-3 give rise to limb girdle muscular dystrophy 1C (21, 22), and mice with a targeted mutation in the caveolin-3 gene or mice overexpressing caveolin-3 have dystrophic-like muscles. Biochemical studies indicate that in skeletal muscle caveolin-3 (71) can interact with nNOS (56) as well as with the C terminus of β-dystroglycan (24). By comparing different muscular dystrophies, one finds that in mice null for caveolin and chimeric mice deficient in dystroglycan there is a substantial increase in serum levels of creatine kinase indicative of muscle degeneration (Ref. 28, Fig. 1). Although in dystroglycan-deficient mice (30) muscle degeneration is considerably more severe (cf. Ref. 28). Moreover, mice (28) or human (72) mutants in caveolin-3 show no changes in the amount of β-dystroglycan, dystrophin, or α-sarcoglycan. α-Dystroglycan, however, may be absent at the sarcolemma (72). This suggests a common pathway for muscle pathology in these mutants and emphasizes the value of genetic studies in analyzing the interactions of DAPs in situ because biochemical studies have suggested that α- and β-dystroglycan are obligate partners (4). Mice lacking caveolin also do not appear to target β-dystroglycan, dystrophin, or α-sarcoglycan to cholesterol-sphingolipid rafts (28) which are normally rich in caveolin. In mice lacking dystroglycan, caveolin expression is increased and appears microscopically as a broad band at the sarcolemma with occasional whispy extensions into the proximal sarcoplasm (Fig. 5). Immunohistochemically and in Western blots the increase in caveolin expression appears similar to that seen in Duchenne muscular dystrophy (26). This mislocalization of caveolin may reflect its interaction with the PXXY motif in β-dystroglycan (24). Also in dystroglycan-deficient muscle, nNOS is essentially absent from the sarcolemma, whereas caveolin is increased, again suggesting that the interaction of these molecules may be necessary for localization to caveolae rather than for targeting to the sarcolemma per se. We are currently exploring whether dystroglycan regulates the formation of caveolae and whether dystroglycan-deficient mice may have other features of mice null for caveolin (28).

The notion that DAPs are involved in the assembly of basement membranes is supported by observations in skeletal muscle (45, 73) and other tissues (44, 46). Consistent with this, merosin, perlecan, and acetylcholinesterase are depleted from the basement membrane at neuromuscular junctions of dystroglycan-deficient muscle (39). In contrast, basement membranes are not similarly affected in the extrajunctional regions of skeletal muscle deficient in dystroglycan (30). This may result from compensation in extrajunctional regions by other extracellular matrix receptors, most obviously the αβ integrin that forms the dominant laminin-binding integrin in mature skeletal muscle. The α2 integrin subunit has also been reported to be up-regulated in cases of Duchenne muscular dystrophy and in the mdx mouse (18, 19), both of which have apparently normal basement membranes. On the other hand, dy mice that have disrupted basement membranes (74, 75) have decreased levels of α7 integrins (18, 19, 76) with no effect on dystroglycan expression. In our studies with dystroglycan-deficient muscle, the α7A splice variant was found to be up-regulated both in extracts of chimeric muscle, and the α7A variant was down-regulated. The α2B subunit was also increased immunohistochemically at the surface of the gastrocnemius muscle (Fig. 6, C and D). This muscle was chosen for analysis because it is composed primarily of type IIB and type IId fibers (77) which normally express low or moderate levels, respectively, of α2B integrin at the sarcolemma. These results support others (18, 19) demonstrating a regulatory pathway between the DAP complex and integrin receptors and are consistent with the idea that dystroglycans are involved in selective regulation of integrin translocation (18).

Mice and humans with mutations in the α7 integrin gene are myopathic (15, 16). In a recent study (78), the α7 integrin was overexpressed on a dystrophin (mdx)/utrophin double knockout genetic background, which resulted in a considerable amelioration of the dystrophy in the double knockout. One can speculate that in dystroglycan-deficient muscle where levels of α7 integrin are maintained, there may be compensatory effects in basement membrane assembly and muscle degeneration, which raises questions about whether these alterations affect the etiology of other muscular dystrophies where dystroglycan expression is compromised.

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