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Neural Regulation of α-Dystroglycan Biosynthesis and Glycosylation in Skeletal Muscle

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Abstract: α-Dystroglycan (α-DG) is part of a complex of cell surface proteins linked to dystrophin or utrophin, which is distributed over the myofiber surface and is concentrated at neuromuscular junctions. In laminin overlays of muscle extracts from developing chick hindlimb muscle, α-DG first appears at embryonic day (E) 10 with an apparent molecular mass of 120 kDa. By E15 it is replaced by smaller (~100 kDa) and larger (~150 kDa) isoforms. The larger form increases in amount and in molecular mass (>200 kDa) as the muscle is innervated and the postsynaptic membrane differentiates (E10–E20), and then decreases dramatically in amount after hatching. In myoblasts differentiating in culture the molecular mass of α-DG is not significantly increased by their replication, fusion, or differentiation into myotubes. Monoclonal antibody IIH6, which recognizes a carbohydrate epitope on α-DG, preferentially binds to the larger forms, suggesting that the core protein is differentially glycosylated beginning at E16. Consistent with prior observations implicating the IIH6 epitope in laminin binding, the smaller forms of α-DG bind more weakly to laminin affinity columns than the larger ones. In blots of adult rat skeletal muscle probed with radiolabeled laminin or monoclonal antibody IIH6, α-DG appears as a >200-kDa band that decreases in molecular mass but increases in intensity following denervation. Northern blots reveal a single mRNA transcript, indicating that the reduction in molecular mass of α-DG after denervation is not obviously a result of alternative splicing but is likely due to posttranslational modification of newly synthesized molecules. The regulation of α-DG by the nerve and its increased affinity for laminin suggest that glycosylation of this protein may be important in myofiber–basement membrane interactions during development and after denervation. **Key Words:** α-Dystroglycan—Dystrophin-associated proteins—Denervation—Glycosylation—Laminin—Muscle development.
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Denervation substantially alters the metabolism of skeletal muscles, affecting the turnover of synaptic and nonsynaptic proteins alike. For example, biosyntheses of acetylcholine receptors (AChRs) (Brockes and Hall, 1975; Devreotes and Fambrough, 1976; Linden and Fambrough, 1979; Merlie et al., 1984), acetylcholinesterase (Weinberg and Hall, 1979; Lømo and Slater, 1980), and several dystrophin-associated proteins (DAPs) are increased soon after denervation (Biral et al., 1996; Mitsui et al., 1996), indicative of control by the nerve. In the longer term, denervation leads to a host of changes resulting in muscle atrophy that is similar in some respects to degeneration in dystrophic muscle. At one point this led some to hypothesize that muscular dystrophies might result from a primary defect in motoneurons (Bradley, 1974). More recently, extensive progress has been made in our understanding of the degeneration of skeletal muscle in muscular dystrophies (reviewed by Campbell, 1995). Although these studies have revealed that Duchenne and related muscular dystrophies are myogenic, the details of myofiber degeneration in these myopathies may well bear on muscle degeneration following denervation.

Duchenne and Becker muscular dystrophies result from mutations in the gene for dystrophin (Hoffman et al., 1987). Dystrophin and its homologue utrophin are cytoskeletal proteins found beneath the plasmalemma of muscle fibers where they interact with a complex of membrane proteins that extend to the basement mem-

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Abbreviations used: AChR, acetylcholine receptor; DAP, dystrophin-associated protein; α-DG, α-dystroglycan; β-DG, β-dystroglycan; E, embryonic day; ECM, extracellular matrix; Mab, monoclonal antibody; NMJ, neuromuscular junction; P, posthatching day; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulfate; SSC, saline sodium citrate.

brane (Campbell and Kahl, 1989; Yoshida and Ozawa, 1990; Ervasti and Campbell, 1991). This complex of DAPs consists of at least 13 polypeptides, seven of which [α, β, γ, δ, and ε sarcoglycans, sarcospan, and β-dystroglycan (β-DG)] are transmembrane proteins, whereas the others are peripheral membrane proteins found at the outer [α-dystroglycan (α-DG)] or inner (α-, β1-, and β2-syntrophins, dystrobrevin, nitric oxide synthase, and Grb 2) surface of the plasma membrane (reviewed by Campbell, 1995; Crosbie et al., 1997; McNally et al., 1998). Duchenne and Becker muscular dystrophies are associated with a concomitant decrease in levels of DAPs at the cell surface (Ervasti et al., 1990). Moreover, mutations in the laminin α_2 chain, a major component of the muscle basement membrane (Helbling-Leclerc et al., 1995), or in α, β, γ, or δ sarcoglycan (Roberds et al., 1993, 1994; Mizuno et al., 1994; Bönnemann et al., 1995; Lim et al., 1995; Noguchi et al., 1995; Jung et al., 1996; McNally et al., 1996; Nigro et al., 1996; Sakamoto et al., 1997; Duclos et al., 1998; Hack et al., 1998) lead to dystrophic phenotypes of varying severity (Campbell, 1995; Worton, 1995; Lim and Campbell, 1998), strongly indicating that interactions between the dystrophin complex and basement membrane are required for maintenance of muscle integrity. The degeneration of muscle in some of these dystrophies involves apoptotic cell death (Matsuda et al., 1995; Tidball et al., 1995; Tews and Goebel, 1997; Hack et al., 1998). Recent studies in cell culture have implicated laminin in muscle cell apoptosis (Vachon et al., 1996) acting through cell surface receptors of the integrin superfamily (Vachon et al., 1997) and α-DG (Montanaro et al., 1999).

Prompted by these studies, we sought to investigate whether changes in α-DG regulated by innervation might contribute to denervation atrophy by altering muscle extracellular matrix (ECM) interactions. We have used monoclonal antibody (Mab) IIH6 to monitor changes in α-DG during muscle development and following innervation and correlated these changes with altered laminin binding in ligand blots and by laminin affinity chromatography. Mab IIH6 recognizes a carbohydrate epitope on α-DG and blocks laminin binding (Ervasti and Campbell, 1993). During development of chick muscle, α-DG increases in amount and in size from 120 to >200 kDa owing to increased glycosylation, and this is reflected by a pronounced increase in binding of Mab IIH6. The higher-molecular-weight forms of α-DG bind more tightly to laminin affinity columns. Conversely, denervation of hindlimb muscles in adult rats results in increased expression of smaller forms of α-DG. Thus, not only do incoming axons appear to regulate the size of α-DG in developing muscle, but denervation of adult muscle leads to a partial reversal of that increase and the appearance of smaller forms of α-DG that overlap in size with those expressed transiently during development. Again, this change is posttranslational and is likely due to the regulation of glycosylation. α-DG has been implicated in the formation of basement membranes in muscle (Cohen et al., 1997; Montanaro et al., 1998, 1999; Colognato et al., 1999), and the appearance of these more heavily glycosylated forms of α-DG may contribute to this process. In denervated muscle the decrease in size of α-DG may result in the expression of forms that bind less tightly to laminin, possibly contributing to disruptions in basement membranes known to accompany denervation (Lu et al., 1997).

MATERIALS AND METHODS

Reagents

Laminin was purified from the Engelbreth–Holm–Swarm sarcoma as published previously (Douville et al., 1988) and was assayed for purity by sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis (PAGE). The E3 fragment of laminin was generated by elastase treatment of native laminin and has been characterized extensively by Dr. Peter Yurchenco (Robert Wood Johnson Medical School, Piscataway, NJ, U.S.A.), who generously supplied this reagent. Mab IIH6, a mouse Mab directed against a unique carbohydrate (Ervasti and Campbell, 1993) on several glycoisoforms of α-DG, was used in the form of hybridoma culture supernatant or ascites fluid. Antisera to dystroglycan fusion proteins (anti-FP-B and anti-FP-D) were produced and characterized previously (Ibraghimov-Beskrovnaya et al., 1992). Horseradish peroxidase-labeled anti-mouse antiserum was obtained commercially (Sigma).

Surgical procedures and immunohistochemistry

The experimental protocols were approved by the Committee for Animal Care of the Montreal General Hospital Research Institute and conform to the guidelines of the MRC. Adult Sprague–Dawley rats were anesthetized with 3% chloral hydrate, and the sciatic nerve of the right hindlimb was transected midway along the thigh. The proximal end of the cut nerve was sutured to block regeneration. Fourteen days after surgery animals were killed with a lethal dose of CO_2, and the gastrocnemius, soleus, extensor digitorum longus, and tibialis anterior muscles were removed from denervated and contralateral control hindlimbs. For preparation of protein extracts, the muscles were frozen immediately at −80°C and stored for subsequent homogenization. For northern blots, RNA was extracted as described below. Immunohistochemistry of frozen muscle sections followed conventional procedures that have been published previously (Montanaro et al., 1995).

Cell culture

Primary cultures of chick myoblasts were prepared from the hindlimbs of 12-day chick embryos after the methods of Devreotes and Fambrough (1976). Skin fibroblast cultures were also prepared from chick embryos using similar procedures. After several days when myoblasts began to fuse, the cultures were switched to fusion medium (Dulbecco's modified Eagle's medium plus high glucose, 2.5% horse serum, and 2 m*M* L-glutamine; all from GIBCO). In mature myotube cultures, some unfused myoblasts remained, and we refer to these cultures as mixed myotube/myoblast cultures.

Protein extraction and analysis

In vivo studies of α-DG used skeletal muscles from chick embryos from embryonic day (E) 10 until hatching and from adult chickens. These offer an abundant supply of tissue with well-defined developmental stages. Fresh or fresh-frozen tissues were homogenized in a Waring blender in buffer contain-

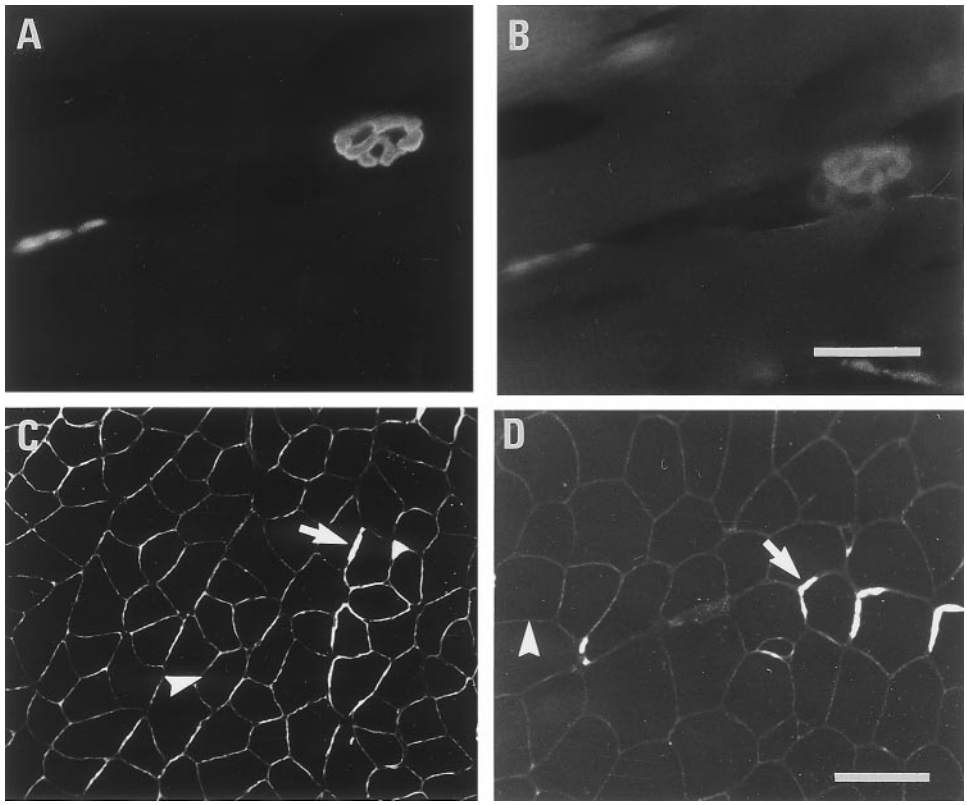


FIG. 1. α-DG is concentrated at NMJs. Frozen sections of rat skeletal muscles (**A** and **B**) or wild-type (**C**) and homozygous *mdx* mouse skeletal muscles (**D**) were double-labeled with rhodamine-conjugated α-bungarotoxin to identify AChRs and polyclonal antisera to either α-DG alone (anti-FP-D; C and D) or both α- and β-DG (anti-FP-B; B) (Ibraghimov-Beskrovnaya et al., 1992). In rat skeletal muscles α- and β-DGs are diffusely distributed over the extrajunctional region of the myofiber but are concentrated at junctional regions (B) identified by their high density of AChRs (A). Bar in B = 20 μm. In cross-sections of wild-type mouse muscles (C), α-DG is also found at the cell surface of both extrajunctional (arrowhead) and synaptic (arrow) regions of muscle fibers. In skeletal muscle from homozygous *mdx* mice the level of α-DG is substantially diminished (arrowhead), and its concentration at endplate regions (arrow) is more prominent (D). Bar in D = 50 μm.

ing 10% sucrose, 20 m*M* $Na_4P_2O_7$, 40 m*M* NaOH, 20 m*M* NaH_2PO_4, 1 m*M* $MgCl_2$ with *N*-ethylmaleimide, 1 m*M* benzamidine, 1 m*M* phenylmethylsulfonyl fluoride, and 1 m*M* iodoacetamide. Homogenates were centrifuged for 30 min at 100,000 *g* at 4°C. Fivefold reducing SDS-sample buffer [250 m*M* Tris-HCl (pH 6.8), 500 m*M* dithiothreitol, 10% (wt/vol) SDS, 0.5% (wt/vol) bromophenol blue, and 50% (vol/vol) glycerol] was added to the samples, and they were heated for 5 min at 95°C. After SDS-PAGE and electroblotting onto nitrocellulose membranes, tissue extracts were probed with either ^{125}I-laminin (ligand blot) or with antibodies. Ligand blots were carried out as described previously (Douville et al., 1988; Gee et al., 1993). For western blots, electroblotted proteins on nitrocellulose filters were blocked with 5% skim milk in 0.1% Tween in Tris-buffered saline [10 m*M* Tris (pH 7.5) and 150 m*M* NaCl], incubated with primary antibodies for 30 min at room temperature, washed (three times for 10 min each), then incubated with horseradish peroxidase-conjugated secondary antiserum for 1 h at room temperature, washed (four times for 10 min each), and exposed to x-ray film for chemiluminescence detection (New England Nuclear). In some experiments α-DG was further purified from soluble extracts by lectin affinity chromatography on succinylated wheat germ agglutinin-Sepharose and/or laminin affinity chromatography, performed essentially as described by Gee et al. (1993). Fractions from affinity columns were analyzed by SDS-PAGE and western blotting as described above.

RNA preparation, probe labeling, and hybridization

Total RNA from tissue and cell culture samples was prepared using the one-step guanidinium thiocyanate technique (Chomczynski and Sacchi, 1989). RNA (10–20 μg) was denatured and electrophoresed on 0.8% agarose gels containing 2.2 *M* formaldehyde, 40 m*M* MOPS (pH 7.0), 10 m*M* sodium acetate, and 1 m*M* EDTA at 70 V for 5 h. The gel was then soaked for 15 min in 50 m*M* NaOH and then for 45 min in 20× saline sodium citrate (SSC), subsequently capillary-blotted onto nylon membranes (Hybond N; Amersham), and fixed by UV cross-linking (Stratalinker; Stratagene).

A 400-bp *Bam*HI/*Sac*I mouse dystroglycan cDNA probe (Gee et al., 1994) was labeled to high specific activity with [γ-^{32}P]CTP (3,000 Ci/nmol; Amersham) using a Megaprime labeling kit (Amersham-USB). Labeled probes were then further purified by gel filtration on push columns (Stratagene). Northern blots were prehybridized for 2 h at 42°C in a solution consisting of 50% formamide, 5× SSC, 5× Denhardt's solution, 50 m*M* sodium phosphate (pH 6.8), 250 μg/ml denatured salmon sperm DNA, 0.2% SDS, and 10% dextran sulfate and then hybridized with the denatured labeled probe in the same solution for 18 h at 42°C. After hybridization, the blots were

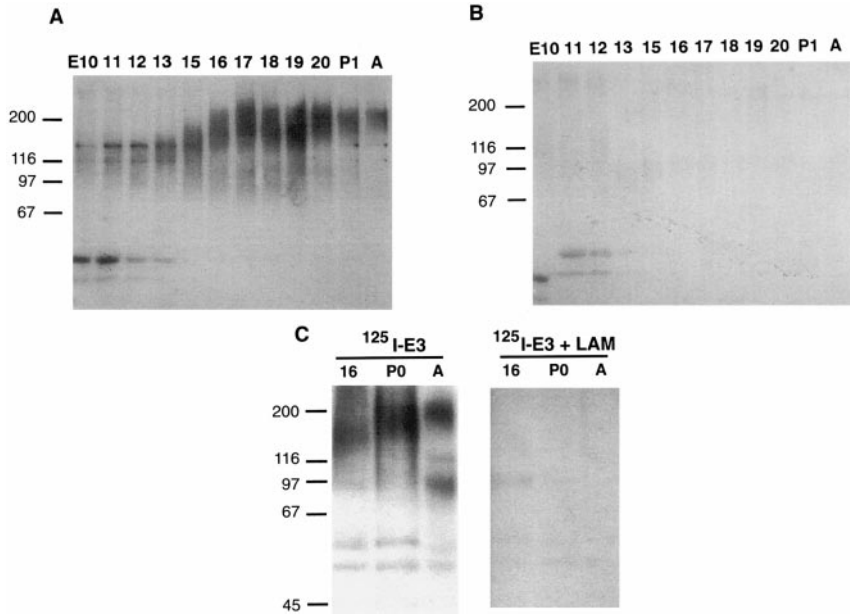


FIG. 2. Expression of α-DG is regulated in developing chick skeletal muscle. Extracts were prepared from chick hindlimb muscles at times ranging from E10 to adult (A). These were ligand-blotted with the ^{125}I-E3 fragment of laminin in the absence (**A**) or presence (**B**) of excess unlabeled intact laminin (LAM). A prominent band appears at E13, and by E16 two bands, one at 100 kDa and a second at ~150 kDa, are seen. The larger band increases in size and in laminin binding until hatching, when it decreases dramatically in intensity. Similar results are seen with microsomal fractions (**C**) containing membrane-bound α-DG, although the 100-kDa band is more difficult to see in this somewhat underexposed autoradiograph. Molecular masses are indicated on the left margin in kDa.

washed twice in 2× SSC containing 0.1% SDS for 30 min at 65°C and twice in 0.2× SSC containing 0.1% SDS for 30 min at 65°C. They were then wrapped in Saran Wrap and exposed overnight at −70°C to x-ray film (XAR 5; Eastman Kodak) with intensifying screens (Cronex Lighting Plus; DuPont-NEN).

RESULTS

Localization of α-DG in skeletal muscle

Immunohistochemistry revealed that α-DG was visible over the entire sarcolemma of rat skeletal muscle but was more evident at neuromuscular junctions (NMJs) (Fig. 1B), as demonstrated by colocalization with AChRs labeled with α-bungarotoxin (Fig. 1A). In the *mdx* mouse, where the dystrophin gene is mutated and the protein is not expressed, α-DG was substantially deficient in the extrajunctional sarcolemma (Ohlendieck and Campbell, 1991), when compared with wild-type mice (Fig. 1C), but was retained at NMJs (Fig. 1D, arrow). This is consistent with previous findings that α-DG coimmunoprecipitates with utrophin (Matsumura et al., 1992), which is localized to motor endplates (Ohlendieck et al., 1991; Bewick et al., 1992), and colocalizes with clusters of AChRs on skeletal muscle myotubes in culture (Campanelli et al., 1994; Gee et al., 1994; Sugiyama et al., 1994; Montanaro et al., 1998) as well as at nascent synapses (Cohen et al., 1995). Thus, although α-DG is concentrated at NMJs, most of it covers the much larger extrajunctional surface, and therefore in biochemical studies most α-DG is derived from extrajunctional regions.

Developmental regulation of α-DG in skeletal muscle

Ligand blotting of membrane proteins with laminin was used initially to identify α-DG as a major laminin receptor in various tissues, including skeletal muscle and brain (Smalheiser and Schwartz, 1987; Douville et al., 1988; Ibraghimov-Beskrovnaya et al., 1992; Ervasti and Campbell, 1993; Gee et al., 1993). These same blotting procedures were used to determine whether α-DG is regulated in developing skeletal muscle during the period in which it is being innervated. Skeletal muscle extracts from chick embryos were probed with intact laminin or an elastase fragment (E3) consisting of the last two globular domains in the α chain, which encompass the binding site for α-DG (Gee et al., 1993). The E3 fragment rather than full laminin was used because no integrin binds to this region of laminin and α-DG is the only laminin receptor known to bind to the E3 fragment. At E10–12, both laminin and the E3 fragment bound specifically to a faint, broad band (~110–150 kDa) encompassing two sharper bands at ~120 and ~150 kDa (Fig. 2A and B). By E13 the broad band was somewhat more intense and larger in size (~97–155 kDa), and by E15, there was a further increase in intensity and breadth (~85–180 kDa) that could be resolved 1 day later (E16) into an intense upper band of ~130 to >200 kDa and a fainter band at ~85–100 kDa. At E20 the upper band had reached the size of the adult form (>200 kDa) and had become less broad, whereas the lower band was barely detectable. In posthatching day (P) 1 and adult animals the intensity of both bands in skeletal muscle was diminished (Fig. 2A and C), and the upper band had become much less broad, ranging from ~180 to 210 kDa, compared with 130 to >200 kDa at E19. Although α-DG associates tightly with skeletal muscle membranes (Ervasti and Campbell, 1991), there is also a more soluble pool, which is released by vigorous homogenization in isotonic buffers (Gee et al., 1993). Immunohistochemical studies showed α-DG localized at the cell surface, where

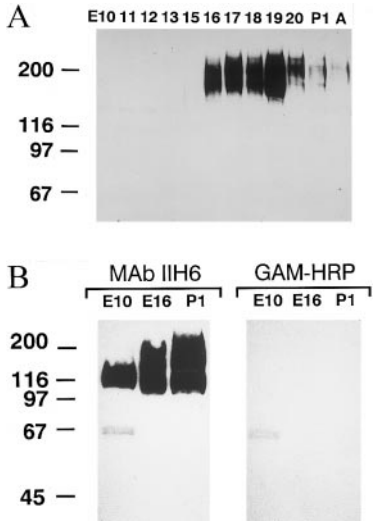


FIG. 3. Western blot analysis of α-DG expression in developing chick skeletal muscle. **A:** Extracts of chick hindlimb muscle prepared from stages E10 to adult (A) were fractionated by SDS-PAGE, electrophoretically transferred to a nitrocellulose membrane, and probed with Mab IIH6. Mab IIH6 preferentially recognizes the larger isoforms of α-DG, which appear by E16 and peak by E19, decreasing substantially thereafter. **B:** E10, E16, and P1 muscle extracts were enriched for α-DG by laminin affinity chromatography and then analyzed as above. Note that Mab IIH6 is now able to recognize the smaller-molecular-mass forms of α-DG, suggesting that they are in relatively low abundance in the muscle extracts before enrichment. No α-DG staining was apparent when blots were incubated with secondary antibody alone [goat anti-mouse IgG conjugated to horseradish peroxidase (GAM-HRP)]. The faint band at ~67 kDa is a nonspecific band also seen with the secondary antibody alone. Molecular masses are indicated on the left margin in kDa.

it codistributes with laminin in the ECM (Klietsch et al., 1992). Nevertheless, forms of α-DG extracted by vigorous homogenization are identical to the membrane-associated forms in electrophoretic mobility, laminin binding (Fig. 2A and C), and recognition by Mab IIH6 (data not shown). Laminin itself is extracted similarly by vigorous homogenization (David et al., 1995), suggesting that this pool represents cell surface-associated ECM. Similar developmental changes in the size of α-DG were evident in extracts from microsome fractions (Fig. 2C), which are enriched in the pool of α-DG tightly bound to membranes, or from fractions containing the pool of α-DG more weakly bound to the cell surface (data not shown).

As shown previously (Campanelli et al., 1994; Gee et al., 1994; Sugiyama et al., 1994), in extracts of cultured mouse skeletal muscle cells (C2 cells) Mab IIH6 to α-DG binds to a single, broad band in western blots and blocks laminin binding to α-DG (Gee et al., 1994). We therefore used this antibody to determine the developmental expression of α-DG in chick muscle. Figure 3A shows that in western blots of chick skeletal muscle probed with Mab IIH6, only bands of 150–200 kDa are detected. These bands are broad and span the same range of molecular masses as the upper, intense bands detected in E3 fragment ligand blots (Fig. 2), and their intensity also decreases after hatching. Between E19 and E20, there is a clear decrease in breadth of the upper band in both western blots (Fig. 3A) and the laminin binding band (Fig. 2). In both cases the range of sizes goes from ~130–220 kDa at E19 to ~180–210 kDa after hatching. It is interesting that at E15 and earlier there is little detectable immunoreactivity for α-DG (Fig. 3A) with Mab IIH6. Mab IIH6 has been shown to recognize a glycosylated epitope on α-DG (Ervasti and Campbell, 1993), and it appears that the smaller forms of α-DG detected by ligand blotting (100–120 kDa; Fig. 2) are isoforms of α-DG that bind Mab IIH6 weakly or lack its epitope altogether.

Given the preference of Mab IIH6 for higher-molecular-mass forms, α-DG was concentrated from crude muscle extracts (E10, E16, and P1) by laminin affinity column chromatography and eluted with low salt (0.2 *M* NaCl; Fig. 3B). Under these conditions, Mab IIH6 specifically recognized a broad band of 110–150 kDa in E10 skeletal muscle, indicating that a low-molecular-mass form of α-DG is expressed at early embryonic stages in chick skeletal muscle. The molecular mass of this isoform of α-DG corresponds to the molecular mass of the laminin-binding band detected at E10 by ligand blot assays (Fig. 2). In western blots of protein extracts from E16 and P1 muscles eluted from laminin affinity columns, a broad band ranging from 110 to ~200 kDa was detected (Fig. 3B). When muscle extracts were not purified over a laminin affinity column, Mab IIH6 detected bands ranging from ~150–200 kDa at E16 and ~180–200 kDa at P1. This enrichment allowed detection of lower-molecular-mass forms of α-DG ranging from 110 to 150 kDa in size, and these, unlike larger forms of α-DG, were expressed throughout the developmental period studied. The protein core of α-DG has a calculated molecular mass of 74 kDa (Deyst et al., 1995; Smalheiser and Kim, 1995), but the native protein is heavily glycosylated and varies in size from 100 to 220 kDa on SDS-PAGE (Ervasti and Campbell, 1993; Chiba et al., 1997; Ervasti et al., 1997), and the large increase in size of α-DG during development likely results from increased glycosylation of α-DG. Reflecting this directly, Mab IIH6, which recognizes a carbohydrate epitope (Ervasti and Campbell, 1993), had a clear preference for higher-molecular-mass isoforms of α-DG and recognized smaller forms of α-DG only after enrichment over a laminin affinity column. Furthermore, comparison with laminin blots (Fig. 2) suggested that these lower-molecular-mass forms of α-DG also bind laminin with lower affinity than higher-molecular-mass forms of α-DG. To test this, glycoproteins were concentrated from crude protein extracts from E18 chick muscle over a lectin column (wheat germ agglutinin) and fractionated over a laminin affinity column. Fractions were eluted first at low salt (0.2 *M* NaCl) and then at high salt (0.5 *M* NaCl) and probed for the presence of α-DG by western blotting with Mab IIH6. Smaller forms of α-DG eluted at 0.2 *M* NaCl (Fig. 4, lane 1, arrow), whereas the larger forms stayed tightly bound until the salt concentration

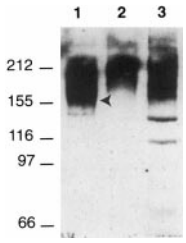


FIG. 4. Laminin affinity chromatography of E18 chick muscle α-DG. α-DG was concentrated from E18 chick muscle extracts by succinylated wheat germ agglutinin (sWGA) chromatography and fractionated on a laminin-Sepharose column (see Materials and Methods). Bound proteins were eluted with a two-step salt gradient, and peak fractions were analyzed by western blotting with mab IIH6. Low-molecular-mass forms of α-DG (arrowhead) are eluted at low salt (lane 1), whereas high salt concentrations are required to elute completely the higher-molecular-mass forms (lane 2). Lane 1, peak of 0.2 *M* salt fraction; lane 2, peak of 0.5 *M* salt fraction; lane 3, sWGA eluate (column load). Molecular masses are indicated on the left margin in kDa.

was increased to 0.5 *M* (Fig. 4, lane 2), indicating that smaller forms of α-DG bind laminin with lower affinity.

We conclude that early in development α-DG exists as an immature form with an apparent molecular mass of 120 kDa and that during the most active period of innervation of hindlimb muscles [E13–16 (Burden, 1977)] these forms are replaced by ones >200 and ~100 kDa. The larger forms are preferentially recognized by Mab IIH6, indicative of developmentally regulated glycosylation. The epitope recognized by Mab IIH6 functions in binding to laminin, and its presence correlates with observations that the larger forms of α-DG bind more tightly to laminin.

Changes in α-DG isoforms in muscle cells in culture

Over the period E8–E20 in ovo, chick skeletal myoblasts proliferate, fuse, and differentiate into multinucleated myofibers, which are then innervated (Burden, 1977). To help ascertain which of these developmental events may contribute to the observed changes in expression of α-DG isoforms, we used cultures of chick myoblasts and myotubes. Aneural cultures of embryonic chick myoblasts express α-DG that is equivalent in size (120 kDa) to the earliest form expressed in skeletal muscle in vivo (Fig. 5, arrowheads). About 7–10 days

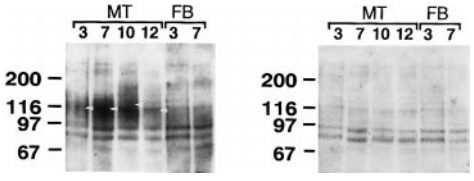


FIG. 5. Ligand blot analysis of chick primary muscle and fibroblast (FB) cultures. Extracts from primary cultures of chick myotubes (MTs) maintained for up to 12 days after fusion or skin FBs maintained for up to 1 week in culture were probed with the ^{125}I-E3 fragment of laminin in the absence (**left**) or presence (**right**) of excess unlabeled laminin. Binding to α-DG in MTs is evident in a band at 120 kDa (arrowheads) that is competed by excess unlabeled laminin. This band increases somewhat in intensity and very slightly in size by 10 days after the beginning of fusion and then declines in intensity at 12 days. By contrast, extracts of skin FBs show little evidence of α-DG expression. Molecular masses are indicated on the left margin in kDa.

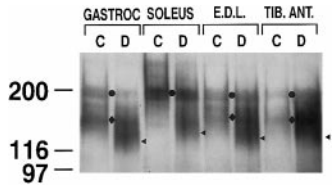


FIG. 6. Laminin binding to α-DG is altered following denervation of skeletal muscle. Extracts from control (C) or denervated (D) gastrocnemius (GASTROC), soleus (SOLEUS), extensor digitorum longus (E.D.L.), and anterior tibialis (TIB. ANT.) muscles were separated by SDS-PAGE, transferred onto a nitrocellulose membrane, and ligand-blotted with radioiodinated laminin. In all control muscles there is an upper band of laminin binding centered at ~200 kDa (circles), and with the exception of the soleus muscle, there is a second broader band of laminin binding at ~150 kDa (diamond). Following denervation there is an increase in intensity of the 150-kDa band (diamond) and the appearance of laminin-binding proteins with a molecular mass of <150 kDa (arrowheads) in all muscles. Molecular masses are indicated on the left margin in kDa.

after the start of myoblast fusion, there was an increase in the amount of α-DG and a small increase in its size. An increase in dystroglycan mRNA has also been shown to occur following myoblast fusion (Gee et al., 1994; Kostrominova and Tanzer, 1995) and correlates well with the increased intensity of α-DG in laminin ligand blots (Fig. 5). After 12 days in culture, when myoblast fusion is over, the level of α-DG expression decreases as it does in vivo. In culture, however, there is no detectable expression of the most mature form of α-DG seen in vivo (>200 kDa; Fig. 2) even when myotubes were maintained in culture for several weeks (data not shown). Primary myoblast cultures are typically contaminated with fibroblasts; however, α-DG was barely detectable in cultures of connective tissue fibroblasts (Fig. 5), suggesting that fibroblasts do not contribute significantly to the changes in α-DG expression seen in myotube cultures.

α-DG is increased in amount and reduced in size following denervation

To test whether α-DG expression is regulated by innervation, the sciatic nerves of adult rats were severed, and 14 days later extracts prepared from denervated and contralateral control muscles (soleus, gastrocnemius, extensor digitorum longus, and anterior tibialis) were ligand-blotted with radioiodinated laminin. All control muscles had an upper band of laminin binding >200 kDa (Fig. 6, lanes C, circles) and, with the exception of the soleus, a broader, less intense band at 120–150 kDa (Fig. 6, diamonds). After denervation there was an increase in the intensity of the lower band (120–150 kDa; Fig. 6, lanes D, diamonds) and the appearance of laminin-binding bands <150 kDa (~110–150 kDa; Fig. 6, lanes D, arrowheads). To confirm that α-DG was changing with denervation, similar extracts were probed with either ^{125}I-laminin or by western blotting with Mab IIH6. In control soleus muscles (Fig. 7A), Mab IIH6 binds to a broad band above 200 kDa that also binds laminin, consistent with the identity of these bands as α-DG

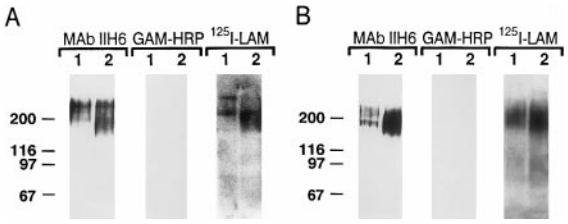


FIG. 7. Comparison of western blots and ligand blots of control (lanes 1) and denervated (lanes 2) rat skeletal muscle. Extracts from (**A**) soleus and (**B**) gastrocnemius muscle were probed for the presence of α-DG with Mab IIH6, whereas parallel blots were ligand-blotted with ^{125}I-laminin (^{125}I-LAM). In both sets of muscle extracts, Mab IIH6 and ^{125}I-LAM recognize bands with similar molecular masses whose size is decreased following denervation. No staining is visible with secondary antibody alone [goat anti-mouse IgG conjugated to horseradish peroxidase (GAM-HRP)]. Molecular masses are indicated on the left margin in kDa.

(discussed below). Following denervation, a population of smaller forms of α-DG is evident. This population is centered around 150 kDa in blots of soleus extracts probed with Mab IIH6 or with laminin (Fig. 7A) and is larger than the smallest forms seen during development, which are weakly labeled by radioactive laminin or Mab IIH6 (Figs. 2A and 3A). Similar results were seen for extensor digitorum longus and anterior tibialis muscles (data not shown). In the gastrocnemius (Fig. 7B) there is a smaller shift downward in the size of α-DG following denervation but a more pronounced increase in both the binding of laminin and reactivity with Mab IIH6.

Because Mab IIH6 recognizes a carbohydrate side chain(s) on α-DG (Ervasti and Campbell, 1993), the increase in labeling seen in blots of denervated muscle probed with either Mab IIH6 or laminin (Figs. 2 and 3) could result from increased expression of α-DG, altered glycosylation, or both. Northern blots of mRNA from denervated muscles normalized by levels of ribosomal RNA showed no obvious change in the size or amount of α-DG mRNA when compared with control muscles (Fig. 8). On the other hand, 1B7, a Mab raised to the α-DG protein core that recognizes a subpopulation of smaller α-DG isoforms (<160 kDa) than Mab IIH6 (H. Moukhles, R. Roque, and S. Carbonetto, unpublished

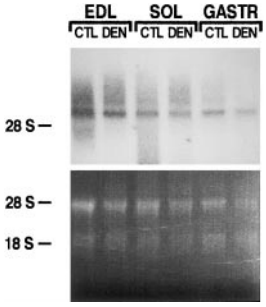
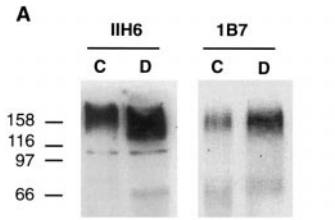


FIG. 8. Northern blots of α-DG RNA in normal [control (CTL)] and denervated (DEN) rat skeletal muscle. Total RNA extracted from DEN or CTL rat extensor digitorum longus (EDL), soleus (SOL), and gastrocnemius (GASTR) muscles was probed (**top panel**) by northern blot with a labeled mouse cDNA probe for α-DG. The probe hybridizes to an mRNA of 5.5 kb, corresponding to the reported size of dystroglycan mRNA (Ibraghimov-Beskrovnaya et al., 1992) and that is present in equivalent amounts in CTL and DEN muscles. RNA loading is comparable for each sample as revealed by ethidium bromide staining of the gel (**bottom panel**).



B

	% increase
Rat 1	27
Rat 2	111
Rat 3	454
Rat 4	248
Mean ± SEM	210.2 ± 93.23 *

FIG. 9. Expression of α-DG in denervated (D) rat muscles. **A:** Western blots of rat muscle extracts from D or control (C) muscles were probed for α-DG with either Mab IIH6, to a carbohydrate epitope, or 1B7, to the core protein. Both Mabs show an increase in the intensity of immunoreactivity following denervation. Forms of α-DG recognized by Mab IIH6 decrease in size with denervation, whereas the subpopulation recognized by Mab 1B7 shifts little if at all. Molecular masses are indicated on the left margin in kDa. **B:** Densitometric quantification of autoradiographs from several experiments reveals a variable but statistically significant increase in immunoreactivity of Mab 1B7 with denervation (210 ± 93.2% of control; $^*p < 0.05$ by unpaired *t* test).

data; Fig. 9A), shows a clear increase in intensity in extracts of denervated hindlimb muscles averaging 210% above control hindlimb muscles (Fig. 9B). There is little decrease in size of these intermediate-sized forms of α-DG recognized by Mab 1B7 (Fig. 9A), suggesting that the downward shift observed with Mab IIH6 or in laminin ligand blots is not due to proteolysis of larger forms in extracts of denervated muscle. Thus, denervation results in a prominent increase in the amount of smaller isoforms of α-DG expressed in skeletal muscle, and this regulation occurs posttranscriptionally.

DISCUSSION

Previous immunohistochemical studies have shown that several dystrophin/utrophin-associated proteins, including β-DG, are found on the surface of skeletal muscle, with a particularly high concentration at NMJs (Ohlendieck et al., 1991; Matsumura et al., 1992). β-DG is known to bind tightly to α-DG (Bowe et al., 1994), but the two are not always coordinately expressed in situ (Vater et al., 1995; Tian et al., 1997). We show here that α-DG is concentrated in the postsynaptic membrane. This preferential localization of α-DG is more evident in *mdx* mice, which do not express dystrophin and where the levels of α-DG and other associated proteins at the cell surface are diminished and undetectable immunohistochemically at extrajunctional regions (Ervasti et al., 1990; Ohlendieck and Campbell, 1991). In *mdx* mice, the residual α-DG may be associated with utrophin, which is

highly homologous to dystrophin and can form a complex with the same cell surface proteins (Ohlendieck et al., 1991). However, the NMJ accounts for <0.1% of the surface area of a typical myofiber, so that in the biochemical studies reported here, it is likely that α-DG is derived largely from extrajunctional regions of the muscle.

α-DG is regulated by innervation

In embryonic chick skeletal muscle (E10), α-DG first appears in ligand blots as a 120-kDa protein. By E13, this band begins to increase in apparent size, and by E15 there is a broad band centered at 150 kDa (Fig. 5) as well as a smaller band at 100 kDa. The upper band appears to increase in size to attain the mature size (200 kDa) in chick skeletal muscle, reaching peak intensity between days E17 and E19 and decreasing substantially in intensity after hatching. Comparing Figs. 2A and 3A, it is clear that Mab IIH6 preferentially recognizes mature forms of α-DG that appear at E16. This recognition by Mab IIH6 appears qualitatively different between E15 and E16 (Fig. 3A), but other data indicate that Mab IIH6 binds to these smaller forms when they are present in larger amounts following purification (Fig. 3B). In principle, the increase in size and intensity could result entirely from the turnover of α-DG followed by increased glycosylation of newly synthesized molecules because both Mab IIH6 and laminin are thought to bind to carbohydrate side chains (Ervasti and Campbell, 1993; Chiba et al., 1997; Ervasti et al., 1997). However, Mab 1B7, which recognizes the rat α-DG core protein, shows a similar increase in intensity in developing muscle (H. Moukhles and S. Carbonetto, unpublished data), suggesting that there is a concomitant increase in expression of α-DG. In culture, there is an increase in α-DG mRNA level following fusion and differentiation of myoblasts (Gee et al., 1994), which may vary in amount with culture conditions (Kostrominova and Tanzer, 1995), as well as a similar increase in laminin binding (Fig. 5). The much larger increase in laminin binding (Fig. 2) in vivo coincides closely with the expression of AChRs, which starts increasing at E10, peaks at E16, and then declines by E20 (Burden, 1977). Expression of AChRs is known to be neurally regulated, which suggested to us that α-DG may be similarly controlled. During differentiation of myoblasts into myotubes there is no change in size of the α-DG mRNA (Gee et al., 1994) and little increase in the protein size (Fig. 5). Moreover, the increase in size of α-DG seen in laminin blots (Fig. 2) is not due to the small amount of α-DG derived from the ingrowing nerve because neural α-DG has a molecular mass of 120 kDa (Douville et al., 1988; Gee et al., 1993), whereas muscle α-DG increases in size to >200 kDa. At this time (E20) there is no band of 120 kDa visible (Fig. 5). In primary cultures of differentiating myoblasts where the nerve is absent, α-DG never reaches its mature size (>200 kDa), confirming that the expression of highly glycosylated forms of α-DG depends on innervation. However, innervation does not appear to be required for the general decrease in the expression of α-DG observed both in vivo and in vitro after the period of myoblast fusion. Therefore, the increase in size of α-DG during muscle development appears to be regulated by the nerve, correlating closely with the most active period of synaptogenesis, basement membrane assembly, and muscle agrin expression in chick muscle (Burden, 1977; Fallon and Gelfman, 1989).

Denervation leads to increased expression of several muscle proteins (reviewed by Hall and Sanes, 1993). Of particular interest here is that Biral et al. (1996) and Mitsui et al. (1996) have reported increases in levels of β-DG, α sarcoglycan, and dystrophin but not utrophin (Biral et al., 1996) in denervated skeletal muscle. We have found that denervation results in an increase in the total amount of α-DG detected in ligand blots as well as with Mabs IIH6 and 1B7. In addition, there is a significant shift downward in the size of α-DG expressed in denervated muscle from >200 to ~150 kDa in western blots probed with Mab IIH6 and in ^{125}I-laminin overlays. In northern blots of denervated muscle there is no decrease in the size of α-DG mRNA consistent with post-translational control of the protein size. It is surprising that there is no obvious increase in amount of the α-DG mRNA (Fig. 8), and we are currently investigating whether the increase in levels of α-DG that is detectable immunochemically and in laminin blots results from increased rates of translation.

Evidence for neural control of glycosylation of α-DG

Dystroglycan is encoded by a single gene and has a single-sized message (5.5 kb; Fig. 8) in northern blots of all tissues studied to date. The core protein of α-DG has an estimated molecular mass of 74 kDa and is extensively glycosylated to attain its final size of >200 kDa in skeletal muscle (Ervasti and Campbell, 1993), 120 kDa in brain and nerve (Gee et al., 1993; Smalheiser and Kim, 1995; Yamada et al., 1996), and 190 kDa in *Torpedo* electric organ (Bowe et al., 1994). Studies on the carbohydrate side chains indicate that α-DG is a mucin-like protein (Smalheiser and Kim, 1995) with N-linked carbohydrate side chains (Smalheiser and Kim, 1995) and a novel O-linked carbohydrate side chain rich in sialic acid (Chiba et al., 1997), which may be involved in binding to laminin (Chiba et al., 1997; cf. Ervasti et al., 1997). In addition to the tissue-specific regulation of α-DG glycosylation, we have found that glycosylation in skeletal muscle varies with development and is neurally controlled. This is reflected in a large increase in the molecular mass of α-DG from E12 to E17–20 as well as in the increase in level of a carbohydrate epitope recognized by Mab IIH6. There are multiple potential sites for glycosylation in α-DG, but the size, number, and composition of the carbohydrate chains are unknown, as are the sites recognized by Mab IIH6. As a result it is difficult to establish a precise relationship between apparent molecular mass of α-DG and the binding of Mab IIH6 and laminin. In blots the smallest forms of α-DG (120 kDa)

bind Mab IIH6 and laminin weakly, whereas the largest forms of >200 kDa bind both strongly. Intermediate forms of ~150 kDa that are prominent following denervation also bind laminin and Mab IIH6 in blots, but forms of this size from normal muscle bind relatively weakly to laminin affinity columns. Quantitative studies of laminin binding to α-DG are necessary to resolve this issue but would be premature without identification and characterization of the carbohydrate side chains. Nevertheless, our data point to a trend in binding of laminin and Mab IIH6 that appears to be directly proportional to the size of α-DG, at least during muscle development.

Following denervation there is a decrease in size of α-DG in many, but not all, hindlimb muscles. That this is generally the case is shown in Fig. 9A, where the shift in size is detectable with Mab IIH6 in extracts prepared form entire hindlimbs, albeit less pronounced than the decrease in the soleus muscle alone. The decrease in size is the result of posttranscriptional events because the mRNA size is not affected by denervation. The extensive glycosylation of α-DG and the dramatic reduction of ≥30 kDa in some muscles make it likely that there is altered glycosylation. Alternatively, the shift in size may result from increased proteolysis of α-DG in denervated muscles. However, Mab 1B7, which recognizes the core protein of a subpopulation of α-DG (Fig. 9; H. Moukhles and S. Carbonetto, unpublished data), revealed no decrease in the size of α-DG, nor is β-DG decreased in size (H. Moukhles and S. Carbonetto, unpublished data), which together argue against proteolysis.

Functional consequences of neural regulation of α-DG

Genetic approaches have strongly implicated dystrophin and associated proteins in several muscular dystrophies (reviewed by Campbell, 1995; Worton, 1995). One hypothesis is that α-DG serves to link the basement membrane to the muscle cell surface and ultimately the intracellular cytoskeleton, forming a superstructure that maintains the integrity of the cell surface in this highly contractile tissue. Recent studies have confirmed the requirement for α-DG in the assembly of laminin-rich basement membranes during embryogenesis (Williamson et al., 1997; Henry and Campbell, 1998) as well as in differentiated skeletal muscle (Colognato et al., 1999; Montanaro et al., 1999). In skeletal muscle, the biochemical composition of the basal lamina changes considerably during muscle development and following innervation. For example, laminins 4, 9, and 11 are expressed only at the NMJ (Sanes et al., 1990; Patton et al., 1997). Extrasynaptically, laminin 1 is replaced by laminin 2, both of which are ligands for α-DG (Patton et al., 1997). It is interesting that, in skeletal muscle, glycosylation of α-DG regulates the binding of laminin 1 but not laminin 2 in a heparin-sensitive manner (Pall et al., 1996). Conceivably, a switch in basal lamina composition from laminin 1 to laminin 2 and a concomitant increase in α-DG glycosylation during skeletal muscle development might ensure a tight binding of the muscle plasmalemma to laminin in the basement membrane, in the face of heparan sulfates expressed therein. Alternatively, changes in glycosylation might differentially affect binding of α-DG to isoforms of laminin and agrin or to proteoglycans such as perlecan (Peng et al., 1998), allowing discrimination among its ligands and creating microdomains on the surface of the muscle fiber. Indeed, Ervasti et al. (1997) hypothesized the existence of a highly abundant form of α-DG with sialic acid residues masking its binding site for the plant lectin VVA-B_4 and a less abundant form of α-DG with an exposed *N*-acetylgalactosamine residue that is involved in synapse formation. Glycosylation of α-DG at the synapse might modulate its interaction with isoforms of laminin and agrin, possibly influencing the choice of intracellular binding partners of β-DG. The reduced glycosylation of α-DG that accompanies denervation may weaken the interaction of the sarcolemma with laminin, perlecan, and agrin in the basement membrane, contributing to disruptions in basement membranes following denervation (Lu et al., 1997) and possibly the atrophy of skeletal muscle.

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