Journal of Cellular Biochemistry 58:527–534 (1995)

Temporal and Spatial Appearance of α-Dystroglycan in Differentiated Mouse Myoblasts in Culture

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Abstract The dystrophin–glycoprotein complex plays an important role in muscle function. One of the components of the complex, a 156-kDa cell surface glycoprotein (α-dystroglycan) binds to laminin, thereby connecting the basal lamina and muscle cells. We have examined the progressive appearance of α-dystroglycan and laminin in muscle cells that differentiate in culture. We find that nondifferentiated cultures of C2C12 myoblasts express low amounts of dystroglycan mRNA and, in contrast, this gene is prominently expressed in differentiated myotubes. Immunofluorescence analysis with a monoclonal antibody against α-dystroglycan shows its progressive appearance during myoblast differentiation into myotubes. Immunostaining with a monoclonal antibody against laminin shows that it is not present on the surface of undifferentiated myoblasts. Subsequently, laminin becomes apparent on the surface of differentiated myotubes where it codistributes with immunostained α-dystroglycan. Immunoblotting of isolated surface membrane proteins of differentiated myotubes with antibodies against α-dystroglycan identifies a broad band of about 140–160 kDa, resembling α-dystroglycan from rabbit muscle. The composite results indicate that α-dystroglycan and laminin appear and become co-distributed on the surface of cultured C2C12 during the progression of differentiation.

Key words: α-dystroglycan, laminin, myoblasts, differentiation

Each skeletal myofiber in vertebrates is ensheated within an extracellular matrix (ECM) that is organized into two layers: outer reticular lamina and inner basal lamina. Interactions between cytoskeleton, sarcolemma, and surrounding ECM are critical for muscle structure and function. A major component of basal lamina is laminin, a large glycoprotein of about 800 kDa with a characteristic cruciform shape. Several isoforms of laminin occur in different tissues and at different stages of development [Tryggvason, 1993]. Nine laminin subunit chains are now characterized and they form six molecular heterotrimer isoforms. Merosin is the most abundant laminin isoform of striated muscle and its presence is important for myogenesis. Laminin substrata stimulate myoblast locomotion and proliferation [Ocalan et al., 1988; Goodman et al., 1989a,b], as well as myoblast differentiation and fusion into myotubes [Foster et al., 1987; von der Mark and Ocalan, 1989].

Several non-integrin and integrin receptors for laminin have been described in muscle cells: α7β1 integrin and 67 kDa non-integrin laminin receptors were identified in myoblast cell cultures [Lesot et al., 1983; von der Mark et al., 1991; Song et al., 1992]. Recently, Ibraghimov-Beskrovnaya et al. [1992] described α-dystroglycan, one of the components of the dystrophin-glycoprotein complex as a new laminin receptor in striated muscle; merosin was shown to be a native ligand for α-dystroglycan [Sunada et al., 1994].

Skeletal muscle dystrophin (427 kDa) forms a complex with six sarcolemmal proteins. Dystrophin and its 59-kDa associated protein are cytoskeletal elements linked to α-dystroglycan through a complex of 50-, 43-, 35-, and 25-kDa transmembrane proteins [Matsumura and Campbell, 1994]. Dystroglycan is synthesized as a large precursor polypeptide (97 kDa) and is post-translationally processed into both 43-kDa and 156-kDa dystrophin-associated glycopro-

Received January 3, 1995; accepted January 25, 1995.

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teins (β- and α-dystroglycans, respectively) [Ibraghimov-Beskrovnaya et al., 1992]. One of the functions of the dystrophin–glycoprotein complex is to provide a linkage between the subsarcolemmal cytoskeleton and the ECM in skeletal muscle [Ervasti and Campbell, 1993]. The disruption of this linkage, caused by a deficiency of either the components of the dystrophin–glycoprotein complex (Duchenne muscular dystrophy and *mdx* mice) or of merosin (*dy/dy* mice) leads to muscular dystrophy [Ervasti et al., 1990; Ibraghimov-Beskrovnaya et al., 1992; Ohlendeick and Campbell, 1991; Xu et al., 1993; Ohlendeick et al., 1993; Sunada et al., 1994].

In this study, we investigated the appearance of α-dystroglycan and laminin (merosin) during myoblast differentiation. Using C2C12 mouse myoblasts, capable of differentiating in culture, we show that the appearance of both α-dystroglycan and laminin on the surface of myotubes correlates with the progression of differentiation.

MATERIALS AND METHODS

Cell Culture

C2C12 mouse muscle myoblasts were obtained from ATCC. They were grown in DMEM (GIBCO BRL) supplemented with 15% fetal bovine serum (FBS) (Gemini Bio-Products, Calabuses, CA) 0.5% chick embryo extract (Gibco BRL) and 1% antibiotic/antimycotic mixture (Gibco BRL). After forming a monolayer, myoblast cultures were induced to differentiate in DMEM with 2% horse serum (Hy Clone) and 1% antibiotic/antimycotic mixture for 5–7 days.

Immunofluorescence Analysis

C2C12 myoblasts were plated at 200,000 cells per well in 400 ml of DMEM/15% FBS/1% antibiotic/antimycotic mixture into 4 wells of 8-well slide culture chambers (Nunc). Cells were allowed to grow for 2–3 days in a CO_2 incubator until they formed a monolayer; myoblasts were stimulated to differentiate by changing the medium to DMEM with 2% horse serum. After 5–7 days of differentiation, when cultures formed myotubes, nondifferentiated C2C12 myoblasts were plated into the remaining empty 4 wells and cultured under nondifferentiating conditions, described above. After 1–2 days, cultures were washed with phosphate-buffered saline (PBS) and fixed with 2% paraformaldehyde in PBS for 30 min on ice. Immunofluorescence assays were done according to standard methods. Spent culture medium from the mouse IIH6 hybridoma (generous gift of Dr. K.P. Campbell, Howard Hughes Institute, University of Iowa) and rat 5D3 hybridoma (generously provided by Dr. D. Abrahamson, University of Alabama) were used as a source of primary antibody against α-dystroglycan and laminin (merosin), respectively. Fluorescein-conjugated donkey antirat IgG or Texas Red-conjugated donkey antimouse IgG (Jackson ImmunoResearch Laboratories, West Grove, PA) were used for immunodetection.

Northern Blot Analysis

Total cellular RNA was obtained from differentiated and nondifferentiated C2C12 myoblasts using TRIzol TM Reagent (Gibco BRL) according to the manufacturer's protocol. After visualizing the purified total RNA using a 1.5% agarose–formamide gel, the Poly(A^+)RNA component was isolated using an mRNA purification kit (Pharmacia). Aliquots of 5 μg of mRNA were then electrophoresed on a 1.5% agarose–formamide gel. The gel was stained with ethidium bromide and photographed; mRNA was then transferred to nylon membranes (Boehringer Mannheim) as described by Sambrook et al. [1987]. The filters were prehybridized for 4 h in 50% formamide, 6 × SSPE, 5× Denhardt's, 0.5% sodium dodecyl sulfate (SDS), 100 μg/ml salmon sperm DNA at 42°C and hybridized overnight in the same buffer containing a probe at 1×10^6 cpm/ml. Membranes were washed in 2 × SSC, 1.0% SDS at 55°C and exposed to X-ray film at −70°C. We used a 1.5-kb cDNA probe specific for α-dystroglycan, which detects a 5.8-kb transcript in rabbit and mouse muscle (R43-B, a generous gift of Dr. K.P. Campbell, Howard Hughes Institute, University of Iowa). A 2.2-kb mouse β-actin probe (received from Dr. Carroll, NYU Medical Center) was used as a control.

Membrane Isolation

Cell surface membranes were purified from C2C12 myoblasts according to the method of Ohlendeick et al. [1991]. Briefly, cells were homogenized in phosphate buffer, 0.303 M sucrose, 0.5 mM EDTA, at pH 7.0, in the presence of protease inhibitors and centrifuged for 15 min at 14,000*g*. The supernatant was centrifuged for 30 min at 30,000*g*. Solid KCl was added to the supernatant to a final concentration 0.6 M and centrifuged for 35 min at 142,000*g*. The final pellet of light microsomes

was extracted overnight at 4°C in buffer A (10 mM triethanolamine, 140 mM NaCl, 1 mM CaCl, 1 mM $MgCl_2$, 50 mM *n*-octyl β-D-glycopyranoside (Sigma), pH 7.6) and used for laminin-affinity chromatography and Western blotting.

Immunoblotting

Samples of light microsomes and acetone precipitated fractions from a laminin-affinity column were separated on 3–10% gradient SDS polyacrylamide gels under reducing conditions and transferred to a nitrocellulose membrane (Schleicher and Shuell). After overnight blocking with 5% bovine serum albumin (BSA) in Tris-buffered saline pH 8.0 (TBST), the membrane was reacted with a primary antibody IIH6 against α-dystroglycan. Then it was reacted with horseradish peroxidase (HRP) conjugates of goat antimouse IgG (BioRad) and visualized using an ECL detection kit (Amersham).

Chromatography on Laminin-Agarose

Laminin was isolated from EHS sarcoma by the method of Timpl et al. [1979]. Reacti-Gel (Pierce) was used according to the manufacturer's protocol to produce laminin-agarose. Light microsomes were lysed in buffer A, then mixed with laminin-agarose at 4°C for 48 h, poured into a column, washed with the same buffer, and then eluted with Ca^{2+}, Mg^{2+}-free buffer A containing 10 mM EDTA. Eluted fractions were concentrated by acetone precipitation and used for immunoblot analysis.

RESULTS

Expression of the α-Dystroglycan Gene in Myoblasts and Myotubes

To investigate whether the expression of dystroglycan mRNA in cultures of C2C12 mouse myoblasts depends upon the state of differentiation we used Northern blotting analysis with a cDNA probe coding for rabbit dystroglycan. The data show that while myotubes express abundant amounts of dystroglycan mRNA, myoblasts express very low amounts (Fig. 1). A cDNA probe of mouse β-actin, as well as ethidium bromide staining of RNA in the gel, reflects the loading of equal amounts of mRNA from differentiated and differentiated cultures into the wells. The dystroglycan mRNA from C2C12 myoblasts has a similar size, 5.8 kb, as that from rabbit muscle [Ibraghimov-Beskrovnaya et al., 1992].

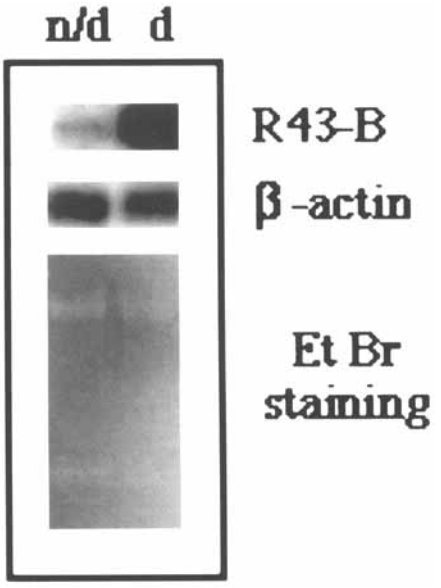

Fig. 1. Expression of α-dystroglycan in myoblasts and myotubes. Northern blot analysis of mRNA from myoblasts and myotubes was performed with R43-B cDNA encoding rabbit α- and β-dystroglycans according to the protocol described in Experimental Procedures. Hybridization with a mouse β-actin probe as well as ethidium bromide staining confirmed equivalent mRNA abundance.

Characterization of α-Dystroglycan and Laminin Immunostaining on the Surface of Myoblasts and Myotubes

Indirect immunofluorescence analysis of myoblasts and myotubes with monoclonal antibodies against α-dystroglycan (II H6) and laminin (merosin)(5D3) shows intense immunostaining of differentiated cultures with both types of antibodies (Fig. 2E,F). A characteristic patchy staining pattern on the surface of myotubes is observed. The unique appearance of either laminin or α-dystroglycan immunostaining, as well as control staining in which either primary antibody was omitted (Fig. 2G,H), verifies the specificity of the dual-labeling analyses. The same pattern of immunostaining of myotubes is seen using single-labeling analyses for laminin (merosin) or α-dystroglycan (not shown). By contrast, immunostaining of myoblasts with the same antibodies is near the background staining seen with secondary antibodies alone (Fig. 2C,D). At higher magnification, immunostaining patterns for α-dystroglycan and laminin (merosin) on the surfaces of myotubes are very similar but not entirely identical (Fig. 3A,B). Most immunostained areas coincide for both antigens; occasionally there is lack of coincidence.

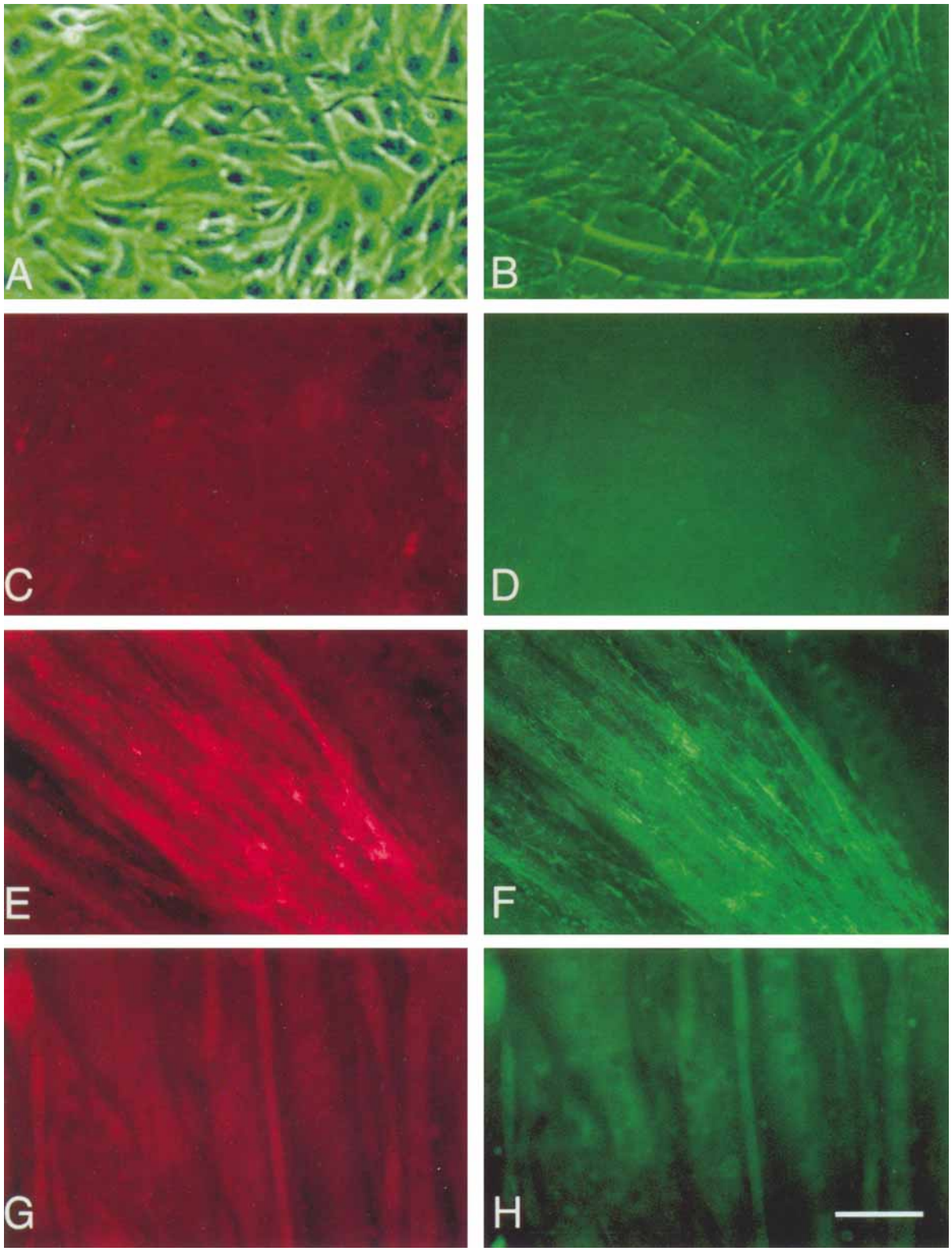

Fig. 2. Appearance of α-dystroglycan and laminin on the surface of myotubes. Mononucleated C2C12 myoblasts (**A**) are able to fuse and form myotubes in culture (**B**). Immunofluorescence analysis with monoclonal antibodies against α-dystroglycan (**C,E**) and laminin (**D,F**) shows no staining on the surface of nondifferentiated myoblasts (**C,D**), but substantial immunostaining for both antigens is seen on the surface of myotubes (**E,F**). Controls in which either primary antibody was omitted (**G,H**) showed background staining for myotubes. Control immunostaining of nondifferentiated myoblasts (not shown) was the same as staining with either primary antibody. The conditions of the experiment are described in Experimental Procedures. *Bar* = 50 μm.

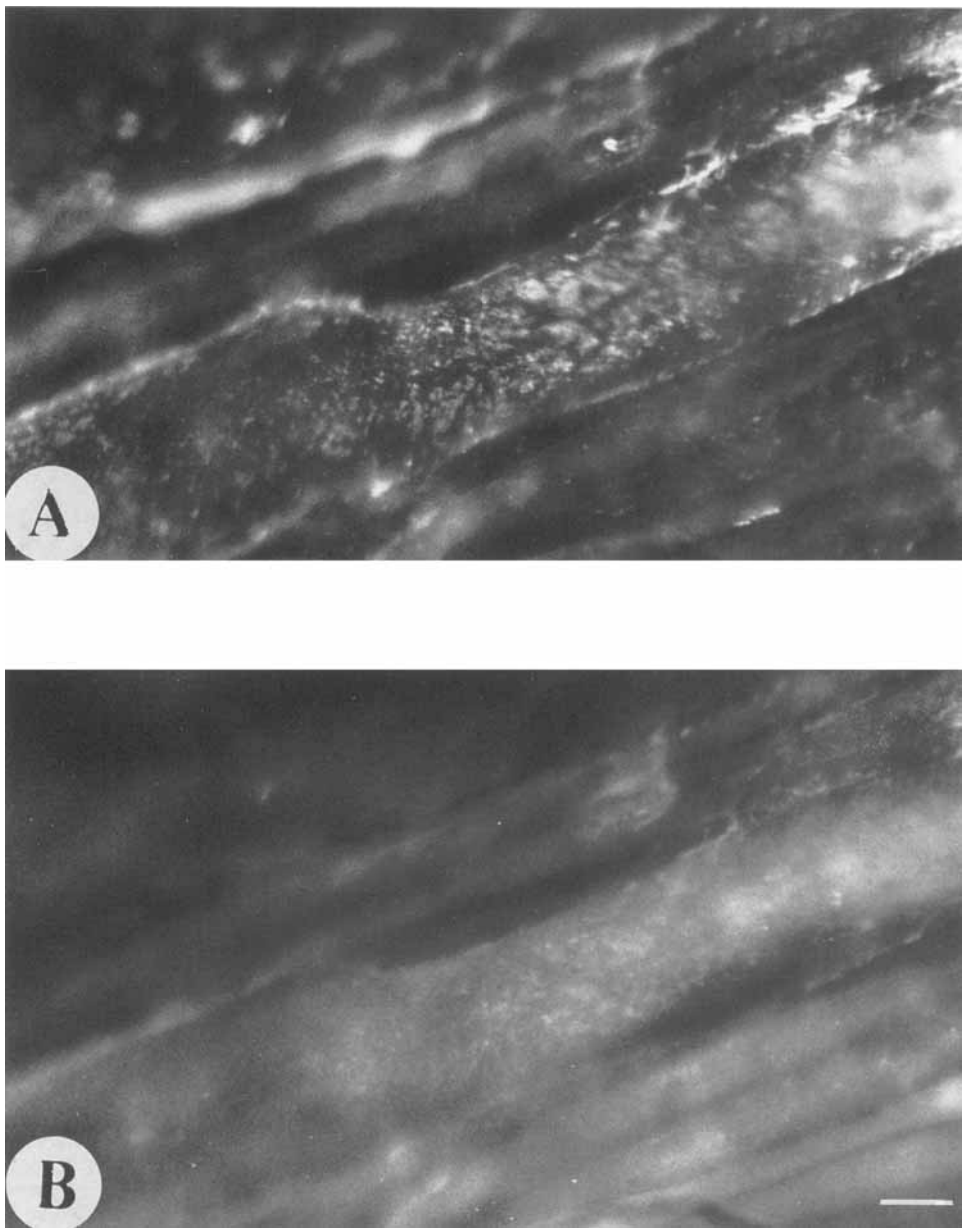

Fig. 3. Co-localization of α-dystroglycan and laminin on the surface of myotubes. Immunofluorescence analysis with monoclonal antibody against α-dystroglycan (**A**) and laminin (**B**) was performed as described in Experimental Procedures. **A,B:** Identical fields of view in the culture of C2C12 myotubes on the 5th day after the onset of differentiation. Bar = 10 μm.

Occurrence of α-Dystroglycan in Membrane Preparations of Myotubes and Its Ability to Bind Laminin

In order to detect α-dystroglycan in membrane fractions of differentiated myotubes immunoblotting analysis with an antibody specific for α-dystroglycan was done (Fig. 4). A wide band of 140–160 kDa in membrane fractions of myotubes reacts with the antibody. It resembles the previously described α-dystroglycan from rabbit muscle, both in size and in appearance [Ibraghimov-Bescrovnaya et al., 1992]. A bound fraction, that elutes from a laminin-affinity column, shows greater immunostaining due to enrichment of the protein by this chromatographic procedure (Fig. 4).

DISCUSSION

It is well known that the ECM plays an important role in the function of muscle cells. Of the

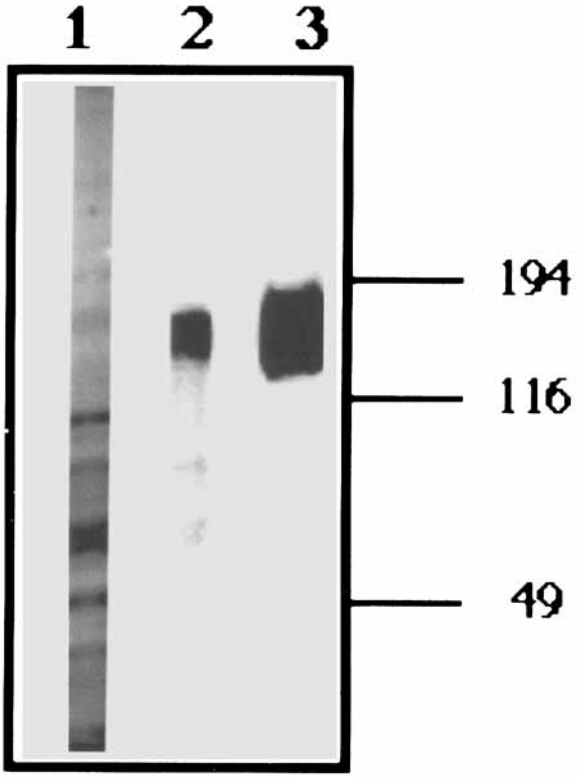

Fig. 4. Identification of α-dystroglycan in membrane fractions of myotubes. *Lane 1,* Coomassie blue-stained SDS polyacrylamide gel of separated surface membrane proteins. *Lane 2,* immunoblot corresponding to *lane 1* stained with antibody against α-dystroglycan. *Lane 3,* immunoblot of membrane proteins eluted from a laminin-affinity column and stained with antibody against α-dystroglycan. The detailed conditions of the experiment are described in Experimental Procedures. The positions of the molecular weight standards, in kDa, are indicated on the right.

known components of the ECM, laminin was shown to be the most effective in stimulating growth and differentiation of muscle cells in culture [Goodman et al., 1989a; von der Mark and Ocalan, 1989]. Agents that perturb cell–matrix interactions have been shown to disrupt myogenesis. For example, a mAb recognizing β1 integrin subunits prevented terminal differentiation of chick embryonic myoblasts in culture [Menco and Boettinger, 1987].

A recently described dystrophin–glycoprotein complex provides a linkage between the subsarcolemmal cytoskeleton and the ECM in skeletal muscle [Matsumura and Campbell, 1994]. On the inner surface of the sarcolemma dystrophin interacts with F-actin while on the outer surface α-dystroglycan interacts with laminin (merosin). Disruption of this continuous actin cytoskeleton/dystrophin–glycoprotein complex/merosin interaction is known to cause severe muscular dystrophy [Ervasti et al., 1990; Ibraghimov-Beskrovnaya et al., 1992; Ohlendeick and Campbell, 1991; Xu et al., 1993; Ohlendeick et al., 1993; Sunada et al., 1994].

Myoblast cell lines provide a useful model for studying the process of muscle differentiation. Our experiments show that nondifferentiated C2C12 mouse myoblasts express low amounts of α-dystroglycan mRNA. By contrast, differentiated C2C12 myotubes prominently express α-dystroglycan mRNA. It has the same size, 5.8 kb, previously demonstrated for muscle and nonmuscle tissues [Ibraghimov-Beskrovnaya et al., 1992, 1993]. Immunofluorescence analyses using an mAb against α-dystroglycan shows that only myotubes are stained. Dickson et al. [1992] showed that immunostaining of cultures of normal mouse muscles for dystrophin revealed the most intense staining in mature, well-differentiated cultures and generally was not observed in myoblasts. The pattern of immunostaining for dystrophin in myotubes showed streaks and patches; we see a similar pattern in differentiated myotubes when α-dystroglycan is stained. Our data show that the expression of α-dystroglycan in myoblasts is minimal whereas during differentiation it progressively accumulates on the surface of myotubes.

Two recent articles show the role of α-dystroglycan as an agrin receptor in cultures of C2 myoblasts [Campanelli et al., 1994; Gee et al., 1994]. Both groups showed α-dystroglycan immunostaining on the surface of agrin-stimulated myotubes. Campanelli et al. [1994] also showed the presence of α-dystroglycan on the surface of C2 myoblasts by flow cytometry. A small amount of α-dystroglycan is seen on the surface of proliferating C2C12 myoblasts but immunofluorescence microscopy, used in our experiments, is not sensitive enough to detect it. Gee et al. [1994] showed a 50% increase in dystroglycan mRNA after fusion of C2 myoblasts into myotubes. The difference in dystroglycan mRNA between myoblasts and myotubes in our experiments is greater than 50%. Differences in experimental conditions may explain this point. Gee et al. [1994] used confluent cultures of C2 myoblasts to isolate total RNA while we used subconfluent cultures. We also used a rabbit cDNA probe, not the mouse cDNA probe used by Gee et al. [1994], and we used Poly(A^+) RNA while Gee et al. [1994] used total RNA.

α-Dystroglycan codistributes with laminin on the surface of papillary myofibers [Klietsch et al., 1993]. Immunostaining of L6 rat myoblasts

in cultures with antibody against laminin shows accumulation of laminin on the surface of myotubes about 5–7 days after the onset of fusion [Kuhl et al., 1982; Olwin and Hall, 1985]. Recent studies of myoblast differentiation using cultures of mouse C2C12, mouse BC3H1, rat L6, and primary mouse myoblasts as well as induced cultures of multipotent C3H10T1/2 mouse embryo fibroblasts demonstrated that expression of Ae and Ac3h laminin chains is associated with expression of MyoD [Kroll et al., 1994]; in our experiments, a similar progression was found. Nondifferentiated C2C12 myoblasts did not show any surface immunostaining with antibody against laminin. Later, the surface of C2C12 myotubes showed intense immunostaining. Moreover, laminin and α-dystroglycan immunostaining patterns generally coincide, indicating that they are co-distributed.

We isolated α-dystroglycan from mature myotube surface membranes by laminin-affinity chromatography. Analogous membrane preparations from younger myotubes did not show this band, although cultures from this stage were stained with this antibody. Probably, there is sufficient loss of α-dystroglycan during the membrane preparative procedures to account for the discrepancy. The IIH6 antibody used in our experiments is thought to recognize the carbohydrates of α-dystroglycan [Ervasti and Campbell, 1993]. Consequently, during early stages of differentiation α-dystroglycan is properly glycosylated, since it reacts with the antibody.

In summary, we show that the cellular appearance of α-dystroglycan correlates with developmental progression of cultures of mouse C2C12 myoblasts. It binds to laminin and is codistributed with extracellular laminin on the surface of C2C12 myotubes.

ACKNOWLEDGMENTS

We thank Drs. K.P. Campbell and Oxana Ibraghimov-Beskrovnaya (Howard Hughes Medical Institute, University of Iowa College of Medicine) for providing IIH6 monoclonal antibody and cDNA (R43-B) for α-dystroglycan. This work was supported by NIH grant AR17220.

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