Integrins ($\alpha7\beta1$) in Muscle Function and Survival

Disrupted Expression in Merosin-deficient Congenital Muscular Dystrophy

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Abstract

Mutations in genes coding for dystrophin, for α, β, γ, and δ-sarcoglycans, or for the α2 chain of the basement membrane component merosin (laminin-2/4) cause various forms of muscular dystrophy. Analyses of integrins showed an abnormal expression and localization of α7β1 isoforms in myofibers of merosin-deficient human patients and mice, but not in dystrophin-deficient or sarcoglycan-deficient humans and animals. It was shown previously that skeletal muscle fibers require merosin for survival and function (Vachon, P.H., F. Loechel, H. Xu, U.M. Wewer, and E. Engvall. 1996. *J. Cell Biol.* 134:1483–1497). Correction of merosin deficiency in vitro through cell transfection with the merosin α2 chain restored the normal localization of α7β1D integrins as well as myotube survival. Overexpression of the apoptosis-suppressing molecule Bcl-2 also promoted the survival of merosin-deficient myotubes, but did not restore a normal expression of α7β1D integrins. Blocking of β1 integrins in normal myotubes induced apoptosis and severely reduced their survival. These findings (*a)* identify α7β1D integrins as the de facto receptors for merosin in skeletal muscle; (*b)* indicate a merosin dependence for the accurate expression and membrane localization of α7β1D integrins in myofibers; (*c)* provide a molecular basis for the critical role of merosin in myofiber survival; and *(d)* add new insights to the pathogenesis of neuromuscular disorders. (*J. Clin. Invest.* 1997. 100:1870–1881.) Key words: apoptosis • basement membrane • dystrophin–glycoprotein complex • extracellular matrix • laminin receptor

Introduction

Striated muscle function is dependent on a strong and stable attachment of myofibers to their basement membrane (1–4). Several of the muscular dystrophies characterized to date are caused by mutations in the genes coding for proteins that are thought to link the cytoskeleton to the extracellular matrix (ECM)[1] (2–5). The presence of these proteins is presumed to be required for the maintenance of the cytoarchitecture of myofibers as well as for their anchorage and viability (2–5). The proposed critical proteins include those of a complex of membrane glycoproteins referred to as the dystrophin-associated glycoprotein (DAG) complex, to which the cytoskeletal component dystrophin is tightly associated (2, 3, 5). The DAG complex is composed of two major subcomplexes, the dystroglycan (α/β) and the sarcoglycan (α/β/γ/δ) transmembrane complexes (2, 3, 5). Examples of muscular dystrophies in which these proteins are affected include the X-linked Duchenne/Becker muscular dystrophy (DMD/BMD) and the mutant *mdx* mouse, which involve a deficiency in dystrophin, and the autosomal recessive limb-girdle muscular dystrophies and the mutant BIO14.6 hamster, which involve defects in components of the sarcoglycan complex (2, 3, 5, 6). A characteristic of these diseases is that the absence or defect in dystrophin or any one component of the DAG complex often causes the abnormal expression of the other components, thereby pointing to a direct association between them (3, 5). For instance, the absence of dystrophin causes a reduction or loss of sarcoglycans and dystroglycans, whereas a defect in any one of the four sarcoglycans affects the expression of the other three (2, 3, 5, 6).

Laminins are a large family of basement membrane components consisting of a heavy chain (α) and two light chains (β and γ). The principal laminin variants in basement membranes of mature muscle fibers, laminin-2 (α2-β1-γ1) and laminin-4 (α2-β2-γ1), are collectively referred to as merosin (4, 7–9). Mutations in the gene for the α2 chain (*LAMA2*) of merosin, which lead to either a loss of expression or to the expression of a defective molecule, have been shown to cause a severe form of autosomal recessive congenital muscular dystrophy (CMD) termed merosin-deficient CMD (MCMD; 2, 4, 8, 10). A reduction in merosin α2 and/or β1/β2 chains has been observed in other muscular dystrophies, such as the Fukuyama congenital muscular dystrophy (FCMD), the Finnish muscle–eye–brain disease (MEB), and the Walker-Warburg syndrome (4, 11–14). However, the genetic defects responsible for these other diseases have yet to be identified. Two dystrophic mutant mice serve as animal models of MCMD: the *dy*, which exhibits the merosin-negative form, and the allelic variant dy^{2J}, which exhibits a merosin-positive, truncated α2 chain form (15–18). De-

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1. *Abbreviations used in this paper:* BMD, Becker muscular dystrophy; CMD, congenital muscular dystrophy; DAG, dystrophin-associated glycoprotein; DMD, Duchenne muscular dystrophy; ECM, extracellular matrix; FCMD, Fukuyama CMD; MCMD, merosin-deficient CMD; MEB, muscle–eye–brain disease; RT, reverse transcription.

fects in merosin in vivo have been shown to result in severely defective basement membranes (16–21). Furthermore, we have demonstrated recently that merosin performs a critical function in differentiated muscle cells by promoting myofiber survival (22).

The DAG complex is widely regarded as being of major importance for the attachment of myofibers to the basement membrane (2, 3, 5). The dystroglycan complex has been shown to bind to purified merosin in in vitro assays (15, 23, 24). Consequently, it has been proposed that dystroglycan is the specific membrane protein that links dystrophin to merosin (2, 3, 5, 23, 24). However, there has been no firm evidence in vivo for this linkage; for example, merosin expression is not affected in DMD or in limb-girdle muscular dystrophy, and none of the proteins mutated in these diseases are affected in MCMD (2, 4, 11, 13, 15).

Little attention has been given to the role of integrins in neuromuscular disorders despite the wide recognition of integrins as primary mediators of ECM–cell interactions (25, 26). Integrins are transmembrane, heterodimeric (α/β) receptors which play a central role in establishing ECM–cytoskeleton linkages as well as transducing ECM–cell signals (25–27). Hence, integrins are pivotal in the regulation of fundamental cellular processes such as adhesion, proliferation, cytoskeletal organization, migration, differentiation, and survival (25–29). The importance of integrins in skeletal muscle development and myofiber attachment has been well-demonstrated in *Drosophila, Caenorhabditis elegans*, and avians (30–35). In mammalian striated muscle, the α7β1 complex is regarded as the principal integrin-type receptor for laminins (36–38). Indeed, binding studies and adhesion assays using muscle cells have demonstrated that this integrin acts as a receptor not only for laminin-1, but for merosin as well (37, 38). Furthermore, alternative splicing forms of the α7 and β1 subunits are expressed differentially during myogenic differentiation and muscle development (39–42). Hence, the β1D subunit is the sole β1 isoform expressed in mature fibers, and it forms complexes with α7A and α7B at the sarcolemma (41, 42).

To address the roles of the integrin α7β1D and the DAG complex in muscle function, we analyzed the expression and localization of these proteins in skeletal muscle of human patients with MCMD as well as in the mutant *dy* and dy^{2J} mouse models of MCMD. For comparison, we analyzed skeletal muscle from patients and animals with other forms of muscular dystrophy but with normal merosin expression. The analyses included patients with DMD/BMD, and the mutant mouse *mdx* and BIO14.6 hamster models of dystrophin- and sarcoglycan-deficient muscular dystrophies. We show that merosin deficiency causes a severe disruption in the sarcolemmal expression and localization of the α7β1D integrins but not of the dystroglycan and sarcoglycan complexes. In contrast, defects in dystrophin or sarcoglycans do not have such effects on α7β1D integrins. We demonstrate further that restoration of merosin expression results in the accurate expression and localization of α7β1D integrins in myofibers, and provides evidence for their role in the mediation of merosin-directed myofiber survival.

Methods

Animals and skeletal muscle tissues. Heterozygous mouse breeding pairs (+/*mdx*, +/*dy*, and +/dy^{2J}) on strain C57BL/6J background were purchased from The Jackson Laboratory (Bar Harbor, ME). BIO14.6 founder hamsters were a kind gift of Dr. G. Jasmin (Université de Montréal, Québec, Canada). Homozygous (*dy*/*dy* and dy^{2J}/dy^{2J}) and control (+/*?*; either wild-type or heterozygous) mice as well as BIO14.6 hamsters and male +/*mdx* heterozygous mice were bred in the animal facility at The Burnham Institute. Hind leg striated muscle specimens from 4–8-wk-old mice or hamsters were collected and embedded in OCT (Optimum Cutting Temperature compound; Tissue Tek, Miles, Inc., Elkhart, IN) as previously described (16, 17). At least three different specimens from wild-type individuals (or heterozygous +/*dy*, +/dy^{2J}), heterozygous males (*mdx*), or homozygous individuals (*dy*, dy^{2J}, and BIO14.6) were analyzed.

Human skeletal muscle samples embedded in OCT were obtained by biopsy or at autopsy. Pathologies and disease identities were established and confirmed by clinical, biochemical, immunocytochemical, and/or genetic criteria as described elsewhere (11, 13, 15, 21, 43). Specimens analyzed herein were the following (see also Table II): merosin-negative MCMD (n = 3), merosin-positive (truncated molecule) MCMD (n = 1), FCMD (n = 2), MEB (n = 1), dystrophin-negative DMD (n = 2), and dystrophin-reduced BMD (n = 1). Normal specimens (n = 2) were also analyzed. This study was approved by The Burnham Institute's ethical committee on the use of laboratory animals for research purposes, as well as by the ethical committees on the use of human biological materials for research purposes of all the institutions involved.

Cell lines, clonal variants, and cell culture. The human RD rhabdomyosarcoma and mouse C2C12 myogenic cell lines were obtained from the American Type Culture Collection (Rockville, MD). Their myogenic differentiation properties under our culture conditions have been characterized previously (22). These cells express differentially laminin and merosin as a function of their myogenic differentiation program, where laminin is downregulated once fusion has occurred, and merosin is upregulated in association with formed myotubes (22, 44). The differentiation-defective clonal variants of RD and C2C12 cells used herein were generated by limiting dilution cloning and were characterized previously (22). The RD.B8 clonal variant cells express laminin-1 but are deficient in merosin α2 chain expression; they fuse into myotubes which undergo apoptosis and subsequently degenerate (22). The fusion-deficient C2C12.B4 clonal variant cells express the β1 and γ1 chains of laminin, but are deficient in the expression of both the α1 and α2 chains (22). Cells and clonal variants were grown and subcultured in DME (GIBCO BRL, Gaithersburg, MD) containing 10% FBS (Hyclone Laboratories, Inc., Logan, UT). Myogenic differentiation of cells was initiated at confluence by replacing the growth medium with DME containing only 1% FBS (differentiation medium) and maintaining the cells in this medium for 6 d (22, 44, 45). Growth and differentiation of cell cultures were routinely monitored by phase contrast microscopy.

Antibodies. Specific rabbit polyclonal, affinity-purified antipeptide antibodies directed to the individual cytoplasmic domains of β1A (46), β1B (47), and β1C (48) integrins were characterized elsewhere and kindly provided by Drs. E. Ruoslahti (The Burnham Institute, La Jolla, CA), G. Tarone (University of Torino, Torino, Italy), and L.R. Languino (Yale University, New Haven, CT), respectively. To generate antibodies specific for α7β1 integrin subunits, the following cytoplasmic domain sequences were used for peptide synthesis: NH_2-CNNFKNPNYGRKAGL for β1D (41), NH_2-CDSSSGRSTPRP-PCPSTTQ for α7A (39, 40), and NH_2-CDWHPELGPDGHPVSVTA for α7B (39). Antipeptide antibodies directed to the 14-most COOH-terminal amino acids of human and mouse α-sarcoglycans (NH_2-CRVDSAQVPLILDQH; reference 49), and against the cytoplasmic domain of β-dystroglycan (NH_2-CKNMTPYRSPPPYVPP; reference 50), were also raised. In all cases, peptides were synthesized, purified, coupled to keyhole limpet hemocyanin (Sigma Chemical Co., St. Louis, MO) with maleimide, and/or mixed with methylated BSA (Sigma Chemical Co.), for immunization of New Zealand White rabbits as described (42, 51, 52). Immunoglobulins from antisera were precipitated with 18% sodium sulfate, dialyzed against PBS, and then

affinity-purified as previously described (9, 52) using peptides coupled to Sepharose 4B (Pharmacia Biotech AB, Uppsala, Sweden). The specificity of antipeptide antibodies was verified by immunoblotting, as well as by their failure to stain normal mouse skeletal muscle cryosections after preincubation with their respective peptides (not shown).

Other antibodies were also used. Antibodies to various merosin subunits, to the β4 integrin subunit, and to dystrophin, were characterized elsewhere and used as described (11, 13–17, 22, 50, 51). The rat mAb GoH3 directed to the α6 integrin subunit was a kind gift of Dr. A. Sonnenberg (The Netherlands Cancer Institute, Amsterdam, The Netherlands). Finally, mouse mAbs against talin and sarcomeric α-actinin were purchased from Sigma Chemical Co.

Indirect immunofluorescence. Cryosections (4–6 μm thick) of skeletal muscle samples or cells grown onto 13-mm glass coverslips (22) were fixed and stained as described previously (16, 17, 22, 42). Rabbit antisera were used at 1:100–1:1,000 dilution, affinity-purified antibodies were used at 10–50 μg/ml, hybridoma supernatants were used at 1:1–1:5 dilution, and ascites were used at 1:100–1:800 dilution. All dilutions were made in PBS (pH 7.4) containing 2% BSA (Sigma Chemical Co.). FITC-conjugated goat anti–rabbit or anti–rat IgG (Boehringer Mannheim, Indianapolis, IN) was used as a secondary antibody. Sections or cells were counterstained with 0.01% Evans blue in PBS, mounted in Vecta-Shield (Vector Labs., Inc., Burlingame, CA), and viewed with an Axiovert 405M microscope (Carl Zeiss, Inc., Thornwood, NY) equipped for epifluorescence. In all cases, no immunofluorescent staining was observed when primary antibodies were omitted or replaced by nonimmune immunoglobulins (not shown).

Protein expression levels. Cell cultures were washed with PBS and scraped in 1× sample buffer [2.3% SDS, 10% glycerol, 5% β-mercaptoethanol, and 0.001% bromophenol blue, in 0.06 M Tris-HCl (pH 6.8)], boiled, and cleared by centrifugation (13,000 rpm, 5 min, room temperature). SDS-PAGE was performed on 12% gels (Novex, San Diego, CA) as described (17, 22). High molecular mass markers (14.3–200-kD range; GIBCO BRL) were used as standards. Protein contents of all samples were estimated using the Bio-Rad Laboratories (Hercules, CA) protein assay. Total proteins (30 μg/well) were separated by electrophoresis and then electrotransferred to nitrocellulose membranes (Nitrocellulose-1; GIBCO BRL) for subsequent immunoblotting (17, 22). Affinity-purified antibodies were used at 5–25 μg/ml, and antisera were used at 1:200–1:1,000 dilution. Immunoreactive bands were visualized by the enhanced chemiluminescence method (ECL system; Amersham Corp., Arlington Heights, IL).

RNA purification and reverse transcription PCR (RT-PCR). Cell cultures were washed with cold PBS before total RNA extraction by the acid guanidinium thiocyanate-phenol-chloroform method, as previously described (16, 17, 22). RT-PCR was carried out as described (16, 17, 22); PCR products were resolved on agarose gels (1.5%) and stained with ethidium bromide. A 100-bp ladder was used as standard (GIBCO BRL). Primers used for integrin β1D were: 5′.2β1D+ (5′-TAGAATTCAAACTTTTAATGATAATTCATGACAGA-3′), and 3′.1β1D− (5′-TAGGATCCAGTTGTCACGGCGCTCTTGTAAATA-3′) (41). These primers are predicted to amplify a 200-bp fragment from the β1D mRNA and a 130-bp fragment from the β1A transcript (41). Differentiated myoblast cultures contain both β1D-expressing myotubes as well as β1A-expressing unfused myoblasts (41). For clarity, only the β1D band was shown in this study. Primers for β-actin (Stratagene Inc., La Jolla, CA) were also used to amplify either mouse or human β-actin mRNA.

Expression constructs and transfections. The construction of a full-length human merosin α2 chain cDNA was described elsewhere (22). For the construction of a full-length human laminin α1 chain cDNA, the 5.0-kb XhoI fragment of pLA7 containing the 3′ half of the human laminin α1 chain cDNA (53) was ligated to the 7.3-kb XhoI fragment of pLA123, which contains the 5′ half of the α1 chain (53). This yielded a near full-length cDNA with a 179-bp deletion between nucleotides 1045 and 1225 (deriving from pLA123). The 1.7-kb SpeI/SphI fragment containing this deletion was then replaced with the 1.9-kb SpeI/SphI fragment from pLA129 (53), therefore yielding the full-length human laminin α1 chain cDNA (beginning at nucleotide 89) flanked by NotI sites. A full-length human Bcl-2 cDNA was described elsewhere (54).

Cells in culture were transfected by the lipofection method (Lipofectamine; GIBCO BRL) as described (22). The expression vectors used were pmer, plam, and pc, which are pcDNA3-based vectors (Invitrogen Corp., San Diego, CA) containing either the full-length human merosin α2 chain cDNA (pmer), the full-length human laminin α1 chain cDNA (plam), or no insert (pc) under regulation of the human cytomegalovirus promoter. Bcl-2 constructs (pBcl-2) were inserted in the puro-pBABE retroviral cDNA vector (54). Stable transfectants were selected with 400 μg/ml G418 sulfate (22) or 2.5 μg/ml puromycin (54); clones were isolated, expanded, and thereafter cultured and analyzed as already described (22). At least 15 clones were analyzed for each type of transfection. RD.B8/pmer clones are rescued from the phenotype of myotube apoptosis and degeneration, whereas RD.B8/pc clones are not (22). C2C12.B4/pmer are rescued from the phenotype of fusion deficiency in contrast to C2C12.B4/pc clones, and form nondegenerating and nonapoptotic myotubes (22).

Antibody inhibition assays. 5-d postconfluent, differentiated RD cell cultures were incubated overnight at 37°C (5% CO_2-95% air) in differentiation medium containing 0–200 μg/ml of either mouse IgG (Sigma Chemical Co.) or the mouse mAb P4C10 (kindly provided by E. Ruoslahti), which blocks the ligand-binding activity of human β1 integrin (55, 56). Apoptosis was detected by the presence of apoptotic bodies and degenerating myotubes, as previously described (22). Since both unfused myoblasts and myotubes were affected by anti–β1 integrin treatment (not shown), myotube survival was evaluated by counting remaining viable myotubes (myotubes/mm^2 of culture surface; reference 22) and expressed as percentage of control cultures (±SD).

Table I. Expression of Sarcolemmal ECM–Cytoskeleton Linkage Components in Skeletal Muscle of Normal and Dystrophic Mouse and Hamster

	*wt**	*dy/dy*	*dy^{2J}/dy^{2J}*	*mdx*	BIO14.6
Integrins					
α7A‡	+§	−	−	+	+
α7B‡	++	⇕P	⇕P	+++	+++
β1D	+++	↓P	↓P	+++	+++
Other integrins					
α6‖	±	++	++	++	++
β1A‖	±	++	++	++	++
β1B	−	−	−	−	−
β1C	−	−	−	−	−
β4	−	−	−	−	−
α-Sarcoglycan	+++	+++	+++	±	−
β-Dystroglycan	+++	+++	+++	↓P	+++

Cryostat sections of normal and mutant mouse or hamster striated muscle were stained with specific antibodies to subunits of laminin-binding integrins, to α-sarcoglycan, or to β-dystroglycan. **wt*, wild-type. Other entries indicate homozygous animals or heterozygous males (*mdx*). ‡Staining predominant at neuromuscular junctions.§Relative staining intensity at the sarcolemmal membrane: −, no detectable staining; ±, weak; + to +++, positive staining; ↓*P*, greatly reduced and irregular (patchy); ⇕*P*, highly irregular and variable in staining. ‖Staining restricted to blood vessels, satellite cells, and/or proliferating myoblasts.

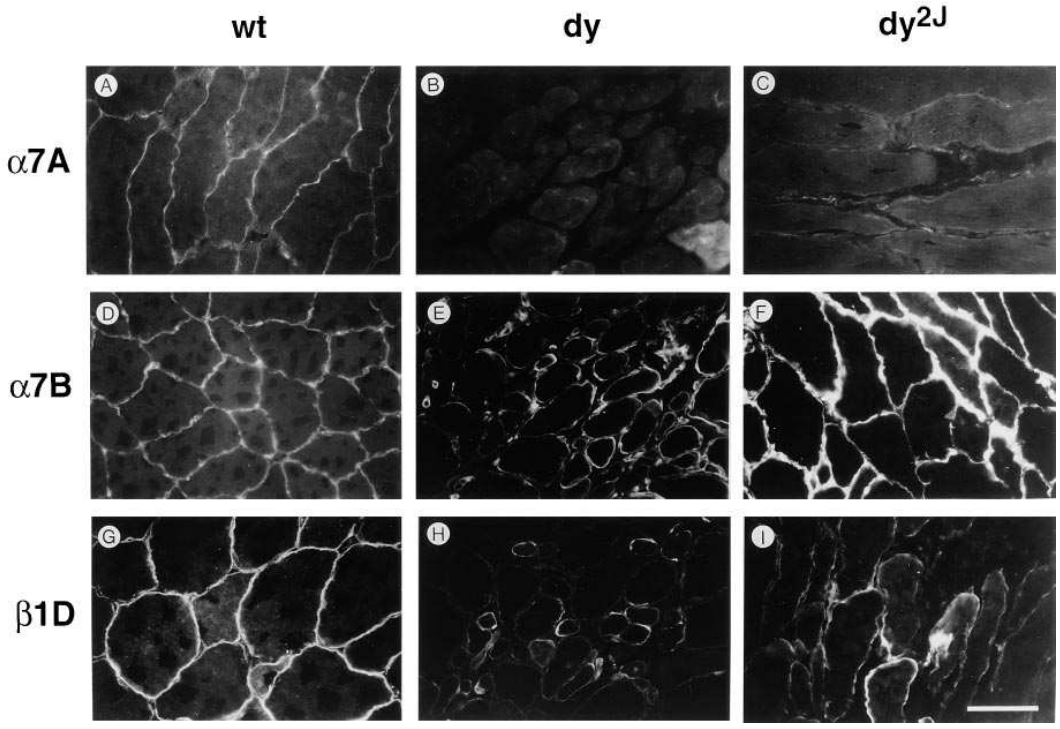

Figure 1. Disruption of α7β1 integrins in mouse striated muscle correlates with merosin deficiency. Immunolocalization of α7A (*A–C*), α7B (*D–F*) and β1D (*G–I*) integrin subunits in skeletal muscle from wild-type mouse (*wt*; *A*, *D*, and *G*), or from *dy*/*dy* (*dy*; *B*, *E*, and *H*) and dy^{2J}/dy^{2J} (dy^{2J}; *C*, *F*, and *I*) mouse models of MCMD. The sarcolemmal expression and localization of α7β1D integrins is severely disrupted in merosin-deficient myofibers, as evidenced by a loss of α7A (*B* and *C*), an irregular (patchy) expression of α7B (*E* and *F*), and a reduced as well as irregular expression of β1D (*H* and *I*). *Bar*, 75 μm.

Results

The distribution of α7β1D integrin isoforms is abnormal in MCMD. To analyze α7β1 integrin in relation to merosin expression in striated muscle, the in vivo distribution and localization of its isoforms were assessed by immunofluorescence in animal specimens (Table I). Staining for the α7A, α7B, and β1D isoforms in normal mouse muscle showed the expected distribution patterns, with β1D (Fig. 1 *G*) localized throughout the sarcolemma, and α7A and α7B predominant at neuromuscular junctions but also found throughout the membrane (Fig. 1, *A* and *D*). Furthermore, the three integrin subunits were detected at myotendinous junctions (not shown; references 41 and 42). In contrast, the expression and localization of all isoforms of the α7β1D integrin were severely disrupted in striated muscle of the *dy*/*dy* and dy^{2J}/dy^{2J} mouse models of MCMD (Fig. 1 and Table I). The sarcolemmal and neuromuscular junctional localization of α7A was consistently lost (Fig. 1, *B* and *C*). The overall staining intensity for α7B appeared to be increased in some areas, but its sarcolemmal localization was highly irregular (patchy) among myofibers, with staining ranging in intensity from negative and weakly positive to strongly positive (Fig. 1, *E* and *F*). The expression of β1D (Fig. 1, *H* and *I*) was drastically reduced as well as irregular. Interestingly, the localization of α7A, α7B, and β1D at the myotendinous junctions appeared intact in merosin-deficient samples analyzed (Fig. 2).

Analyses of α7β1D isoforms in human specimens with merosin deficiencies also revealed abnormalities in expression and localization (Fig. 3 and Table II). In each MCMD case studied, the expression of α7A was lost (Fig. 3 *A*), whereas that of α7B (Fig. 3 *C*) and β1D (Fig. 3 *E*) was drastically irregular and/or reduced. A disruption of α7β1D integrins was also observed in other muscular dystrophies with secondary deficiencies in merosin, namely, in FCMD and MEB (Table II). Therefore, these data show a correlation between merosin deficiency and abnormal distribution of α7β1D integrins in striated muscle.

The distribution of α7β1D integrins is normal in dystrophin and sarcoglycan deficiencies. The in vivo distribution of the α7 and β1D integrin subunits was also analyzed in muscular dystrophies involving defects in dystrophin and/or in other components of the DAG complex but with normal merosin expres-

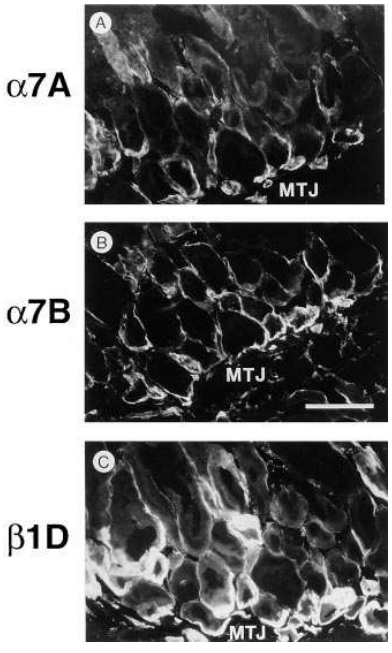

Figure 2. α7β1 integrins are maintained at the myotendinous junction of merosin-deficient striated muscle. Immunolocalization of α7A (*A*), α7B (*B*), and β1D (*C*) integrin subunits at myotendinous junctions of skeletal muscle from the *dy*/*dy* mouse model of MCMD. Although the expression of α7A, α7B, and β1D is disrupted in merosin-deficient myofibers (see Fig. 1), these integrin subunits are still detected at myotendinous junctions (*MTJ*). *Bar*, 75 μm.

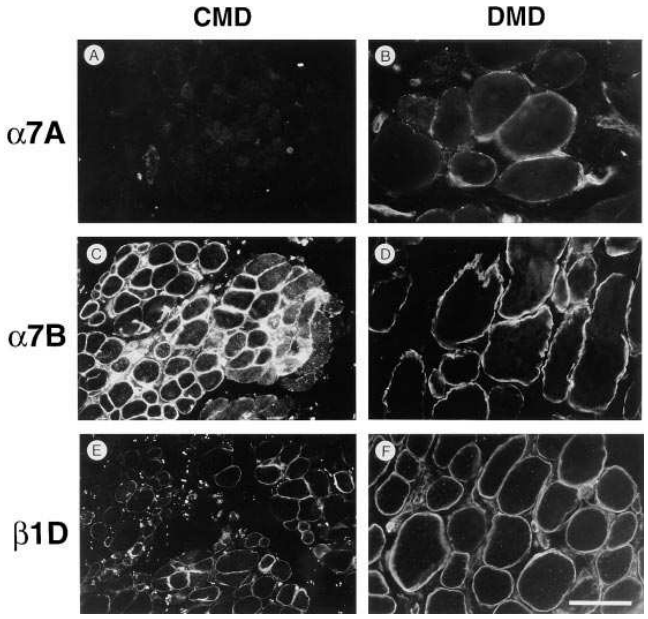

Figure 3. Abnormal distribution of α7β1 integrins in human MCMD but not DMD. Immunolocalization of α7A (*A* and *B*), α7B (*C* and *D*), and β1D (*E* and *F*) integrin subunits in skeletal muscle from a child patient with MCMD (*A*, *C*, and *E*), or from an adult patient with DMD (*B*, *D*, and *F*). The expression and localization of α7β1D integrins is severely disrupted in merosin-deficient myofibers, as evidenced by a loss of α7A (*A*), an irregular (patchy) expression of α7B (*C*), and a reduced as well as irregular expression of β1D (*E*). There is no apparent disruption of α7β1D integrin localization in dystrophin-deficient myofibers (*B*, *D*, and *F*), although the staining for α7B appears to be increased (*D*). *Bar*, 50 μm.

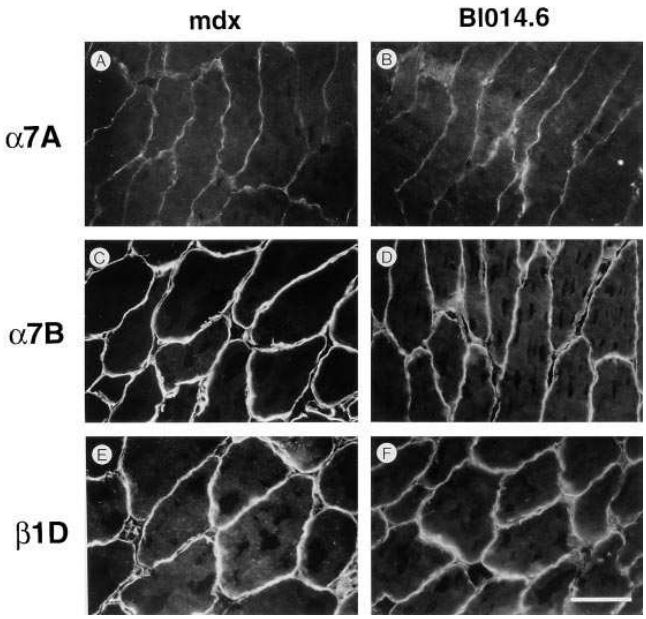

Figure 4. Dystrophin- and sarcoglycan-deficient muscle display no disruption in the localization of α7β1 integrins. Immunolocalization of α7A (*A* and *B*), α7B (*C* and *D*), and β1D (*E* and *F*) integrin subunits in skeletal muscle from the dystrophin-deficient *mdx* (*A*, *C*, and *E*) mouse model of DMD, or from sarcoglycan-deficient BIO14.6 hamster (*B*, *D*, and *F*). The localization of α7β1D integrins is not disrupted in either type of deficiency (compare with wild-type skeletal muscle, Fig. 1), although staining for α7B appears to be increased (*C* and *D*). *Bar*, 75 μm.

sion (Tables I and II). The sarcolemmal expression and localization of α7A, α7B, and β1D were normal in muscle of the dystrophin-deficient *mdx* mice (Fig. 4, *A*, *C*, and *E*) and of the sarcoglycan-deficient BIO14.6 hamsters (Fig. 4, *B*, *D*, and *F*). Likewise, α7β1D integrin isoforms were normal in human samples of DMD (Fig. 3, *B*, *D*, and *F*) and BMD (Table II). The myofiber staining for α7B appeared to be somewhat increased (Fig. 4, *C* and *D*) in dystrophin- or sarcoglycan-deficient animals and humans, as it was in the case of merosin deficiency (Fig. 1, *E* and *F*, and Fig. 3 *C*). However, no irregular patterns of sarcolemmal distribution were noted (Fig. 3 *D*, and Fig. 4, *C* and *D*). Since both proliferative myoblasts and differentiated myotubes express the α7B integrin subunit (37–41), the apparent increased sarcolemmal expression of this subunit may be the result of enhanced myoblast fusion with actively regenerating dystrophic myofibers (11–17). Thus, these data indicate that the loss and/or disruption of α7β1D integrin isoforms in striated muscle is correlated specifically to merosin deficiency, and is not an overall consequence of muscular dystrophy.

Table II. Expression of Components for Sarcolemmal ECM–Cytoskeleton Linkage in Normal and Dystrophic Human Skeletal Muscle

	Merosin α2 chain	Integrin α7A	Integrin α7B	Integrin β1D	Dystrophin	α-Sarcoglycan	β-Dystroglycan
Normal (n = 2)	+++*	+	++	+++	+++	+++	+++
MCMD− (n = 3)	−	−	↕P	↓P	+++	+++	+++
MCMD+ (n = 1)	±	−	↕P	↓P	+++	+++	+++
FCMD (n = 2)	↓P	n.d.	n.d.	↓P	↕P	↕P	↕P
MEB (n = 1)	↓P	−	↕P	↓P	n.d.	n.d.	n.d.
DMD (n = 2)	+++	+	++	+++	−	↓P	↓P
BMD (n = 1)	+++	n.d.	n.d.	+++	↓P	↓P	↓P

Cryostat sections of normal and pathological human muscle were stained with specific antibodies to the α2 chain of merosin, to α and β subunit isoforms of the merosin-binding integrin α7β1, or to components of the DAG complex. *Normal*, nonpathological specimen, *MCMD*−, merosin-negative MCMD, *MCMD*+, merosin-positive (truncated form) MCMD. *Relative staining intensity: −, no detectable staining; ±, weak; + to +++, positive staining; *n.d.*, not determined; ↓*P*, greatly reduced and irregular (patchy); ↕*P*, highly irregular and variable in staining.

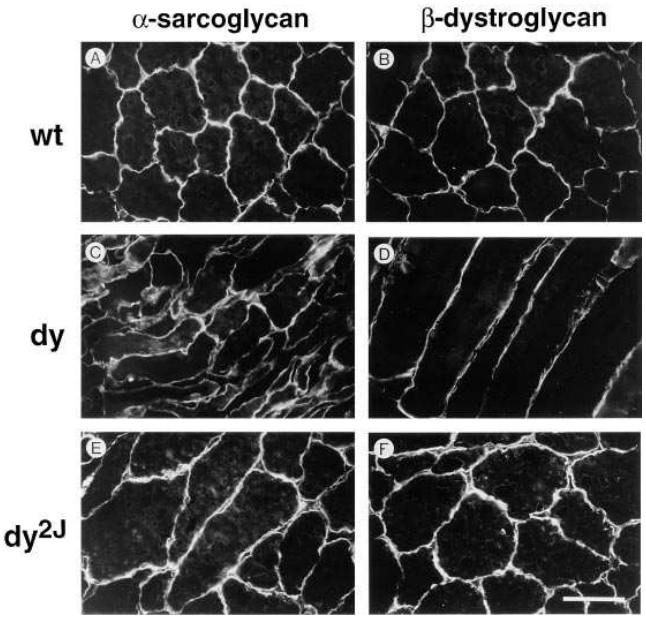

Figure 5. The DAG complex is not affected in striated muscle of merosin-deficient mice. Immunolocalization of α-sarcoglycan (*A*, *C*, and *E*), and β-dystroglycan (*B*, *D*, and *F*) of the DAG complex in wild-type (*wt*; *A* and *B*), *dy*/*dy* (*dy*; *C* and *D*), and dy^{2J}/dy^{2J} (dy^{2J}; *E* and *F*) mice. Both components of the DAG complex are expressed at the sarcolemma of merosin-deficient fibers (*C–F*), despite the disease state. *Bar*, 75 μm.

The disruption of α7β1D integrins in merosin deficiency is not compensated by laminin-binding α6 integrins or by integrins with other β1 isoforms. The presence of other integrins which bind exclusively to laminins was also analyzed, namely, α6β1 and α6β4 (25–29). In all normal human and animal specimens tested, the α6 and β1A integrin subunits were undetectable at the sarcolemmal membrane of myofibers, but detected instead in association with blood vessels and satellite cells (Table I) as documented by others (41, 42). An increased staining for both α6 and β1A subunits was observed in dystrophic muscle tissues regardless of the species and type of muscular dystrophy (Table I). However, such staining was found to be associated with mononucleated cells, presumably myoblasts involved in the active regeneration process which is known to occur under these pathological conditions (Table I; references 2–4, 11–20). The β1B, β1C, and β4 integrin subunits were not detected in normal myofibers either (Table I; references 41 and 47), nor were they detected under the different pathological conditions analyzed in both mice (Table I) and humans (not shown). Hence, other laminin-binding integrins or integrins with other known β1 subunit isoforms do not appear to compensate for the disruption of α7β1D integrins in merosin-deficient myofibers.

The dystroglycan and sarcoglycan complexes are unaffected in MCMD. The DAG complex was analyzed in relation to merosin expression in striated muscle (Tables I and II). In agreement with previous reports (2, 4, 11, 13, 15), the dystroglycan and sarcoglycan subcomplexes appeared to be expressed normally in homozygous *dy*/*dy* (Fig. 5, *C* and *D*) and dy^{2J}/dy^{2J} (Fig. 5, *E* and *F*) mutant mice. Indeed, the staining for α-sarcoglycan and β-dystroglycan was continuous and similar to that observed in wild-type and/or heterozygous +/*dy* and $+/dy^{2J}$ specimens (Fig. 5, *A* and *B*). Dystrophin was also expressed normally in these dystrophic mice, as expected (not shown; references 2, 4, 11, and 15). In contrast, both β-dystroglycan and α-sarcoglycan were drastically reduced and irregular (patchy) in muscle of *mdx* mice, and α-sarcoglycan was greatly reduced in the BIO14.6 dystrophic hamster, as reported (Table I; references 6, 11, 13, and 15).

The dystroglycan and sarcoglycan complexes appeared normal in striated muscle samples from patients diagnosed

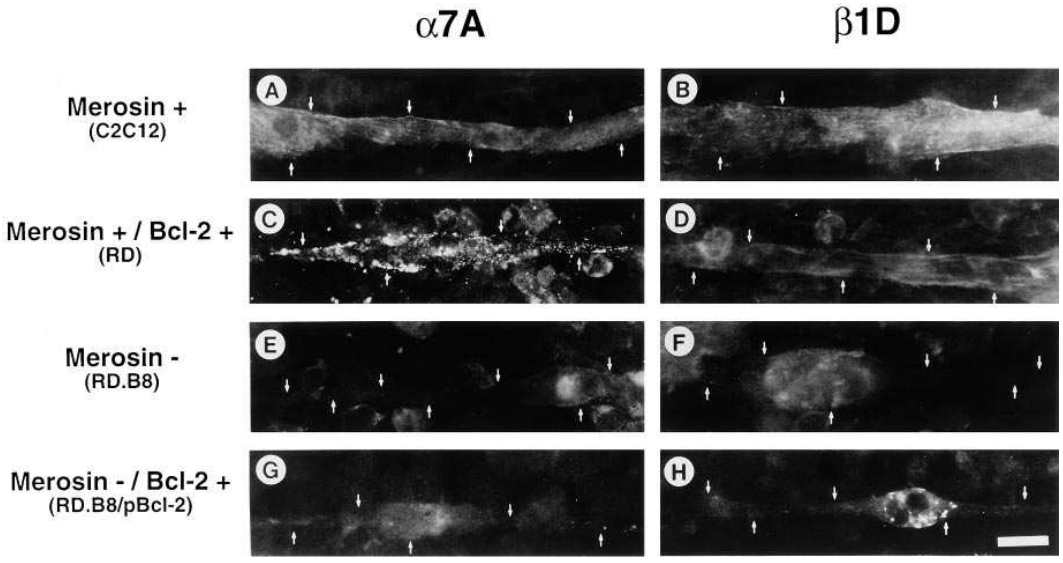

Figure 6. Expression of α7β1D integrins is disrupted in merosin-deficient myotubes in vitro. For clarity, single myotubes (outlined by arrows) are shown. (*A–F*) Immunostaining analysis of α7A (*A*, *C*, and *E*) and β1D (*B*, *D*, and *F*) integrin subunits in cultured myotubes from C2C12 (*Merosin +*; *A* and *B*), RD (*C* and *D*), or merosin-deficient RD.B8 cells (*Merosin −*; *E* and *F*). Weak cytoplasmic staining for α7A (*E*) and β1D (*F*) is seen in RD.B8 myotubes. (*G* and *H*) The expression and localization of α7β1D integrins are not restored in merosin-deficient myotubes rescued from apoptosis with Bcl-2. α7A and β1D integrin subunits in cultured myotubes from RD (*Merosin +*/*Bcl-2 +*; *C* and *D*) or merosin-deficient RD.B8/pBcl-2 cells (*Merosin −*/*Bcl-2 +*; *E* and *F*) stably transfected with a full-length human Bcl-2 cDNA. In contrast to Bcl-2–transfected RD myotubes (*C* and *D*), the weak cytoplasmic expression for α7A and the prominent Golgi staining for β1D persist in RD.B8/pBcl-2 myotubes (*G* and *H*), as in apoptotic merosin-deficient myotubes (*E* and *F*). *Bar*, 25 μm.

Table III. Expression of Membrane-associated Components in Myotubes of Normal and Merosin-deficient Cells

	RD	RD.B8/pc*	RD.B8/pmr	RD.B8/pBcl-2	C2C12	C2C12.B4/plam	C2C12.B4/pmr
Merosin	++‡	−	+++	−	+++	−	+++
Integrin							
α7A	+	±	+	±	++	±	++
α7B	+++	⇕P	+++	⇕P	+++	⇕P	+++
β1D	++	±	++	±	+++	±	+++
Sarcomeric							
α-actinin§	+++	+++	+++	+++	+++	+++	+++
Talin§	++	++	++	++	++	++	++

Human (*RD*, *RD.B8*) or mouse (*C2C12*, *C2C12.B4)* myoblasts were cultured until 6 d postconfluence under differentiation conditions, fixed, permeabilized, and then stained with specific antibodies to the α2 chain of merosin, to integrin α7β1 subunits, or to the integrin-associated cytoskeletal components talin and sarcomeric α-actinin. *Cells stably transfected either with pc (control vector), plam (vector containing the full-length human laminin α1 chain cDNA), pmer (vector containing the full-length human merosin α2 chain cDNA), or pBcl-2 (vector containing the full-length human Bcl-2 cDNA).‡Relative staining intensity associated with myotubes only: −, no detectable staining; ±, weak; + to +++, positive staining; ⇕*P*, highly irregular in staining. §Staining associated with sarcomeres and sarcomeric actin filaments (*α-actinin*), or to focal adhesion plaques which concentrate at myotube termini (*talin*).

with MCMD, as they did in merosin-deficient mice (Table II), in agreement with previous data (2, 4, 5, 11, 15). This suggests that the dystroglycan and sarcoglycan subcomplexes do not serve as the primary receptors for merosin in myofibers. In contrast, a severe disruption or absence of expression of dystrophin, β-dystroglycan, and/or α-sarcoglycan was noted in patients diagnosed with DMD and/or BMD (Table II), as expected (2, 3, 5). An abnormal expression of these three components was also observed in patients with merosin-deficient FCMD (Table II; references 2, 4, 5, and 11–13), a disorder for which a complex pathogenesis is suspected (2, 4, 5).

Expression and localization of α7β1D in differentiated muscle cells in vitro; verification of merosin dependency. The localization of α7β1D integrins was investigated further in cultured myoblast cell lines and merosin-deficient clonal variants (Fig. 6 and Table III). When induced to differentiate, α7Bβ1A-expressing myoblasts fuse to form myotubes which express α7A and β1D (37–40). During this process, laminin-1 expression is downregulated, whereas that of merosin is upregulated (22, 44, 45). Consequently, differentiated cultures contain unfused α7Bβ1A-expressing myoblasts as well as α7(A,B)β1D- and merosin-expressing myotubes (22, 38–41). As shown here for normal C2C12 (Fig. 6, *A* and *B*) and RD cells [Fig. 6, *C* and *D*; normal RD cells and RD cells transfected with Bcl-2 (see below) were identical], staining for α7A and β1D was associated predominantly with the surface of myotubes, whereas staining for α7B was associated with the membranes of both myotubes and unfused myoblasts (not shown). Furthermore, strong staining for α7A, α7B, and β1D was found at myotube termini (not shown), as reported (32, 41). Variable cytoplasmic and/or Golgi staining could also be observed for α7A and β1D in unfused myoblasts (Fig. 6, *A–C*), as reported previously (38, 41).

In contrast to the normal C2C12 and RD cell lines (Table III and Fig. 6), myotubes formed by the merosin-deficient RD.B8 cells showed a disrupted expression and localization of α7β1D integrins (Table III and Fig. 6, *E* and *F*). Indeed, staining for α7A was absent from the surface of myotubes (Fig. 6 *E*), whereas that for α7B was irregular and patchy (Table III). In addition, staining for β1D was greatly reduced and predominant in the Golgi apparatus of merosin-deficient myotubes (Fig. 6 *F*). Some staining for all three integrin isoforms was still observed at myotube termini (not shown), where talin-positive focal adhesion plaques are found (Table III; references 31, 35, and 41). Thus, the distribution of these integrins in merosin-deficient myotubes in vitro resembled that of MCMD in vivo.

Immunoblotting analyses of α7β1D integrin subunit levels in differentiated cultures of merosin-deficient RD.B8 cells showed a drastic reduction in α7A (not shown), a slight reduction in α7B (Fig. 7 *A*, lane *2*), and a great reduction in β1D (Fig. 7 *B*, lane *2*), as compared with similar cultures of normal RD (Fig. 7, *A* and *B*, lane *1*) and C2C12 (Fig. 7, *A* and *B*, lane *4*) cells. In keeping with the predominant Golgi staining observed in myotubes formed by RD.B8 cells (Fig. 6 *D*), only the precursor form of the β1D polypeptide was detected in RD.B8 cultures. This precursor migrates at an apparent lower molecular weight than the mature form (Fig. 7 *B*, lane *2*). The effects at the protein levels of α7β1D subunits in merosin-deficient myotubes were not apparent at the mRNA level, as evidenced by RT-PCR for β1D (Fig. 8).

The reduced α7β1D integrin protein but not mRNA levels suggest that merosin deficiency may result in an increased turnover and/or degradation of these integrins in the absence of their ligand. This was substantiated by the reinstatement of merosin expression in RD.B8 cells through stable transfection with a full-length human merosin α2 chain cDNA (22). Indeed, protein levels of α7β1D integrins were restored to normal in RD.B8/pmer transfectants (Fig. 7, *A* and *B*, lane *3*), but not in the control-transfected RD.B8/pc cells (Table III; see also Fig. 7, *A* and *B*, lane *2*, for comparison). The mature β1D polypeptide was again detected in the merosin-transfected cells (Fig. 7 *B*, lane *3*). Accordingly, immunofluorescence staining confirmed that the membrane localization of these integrin isoforms is restored in RD.B8/pmer myotubes (Table III).

The apparent dependence on merosin for appropriate expression and localization of α7β1D integrins appears to be specific for this member of the laminin family. The merosin-deficient RD.B8 cells express laminin-1 (22), and yet the myotubes they formed did not maintain a normal expression of the α7β1 integrin subunits (see above). In addition, the fusion-deficient C2C12.B4 clonal variant cells can fuse after transfection with

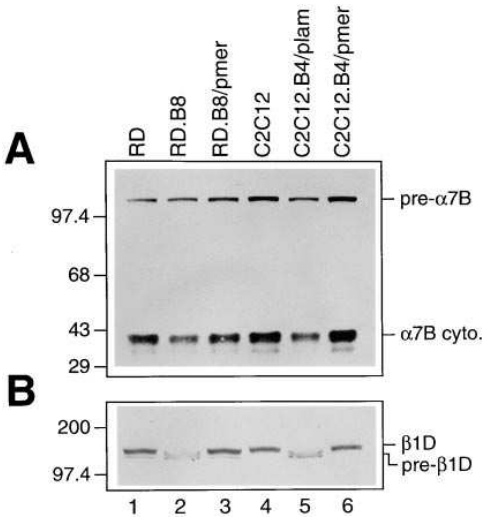

Figure 7. The abnormal expression of α7β1 integrin isoforms in cultured merosin-deficient myotubes is corrected when merosin expression is reinstated. Immunoblot analysis of α7B (*A*) and β1D (*B*) integrin subunit expression levels in differentiated cultures of normal RD (lane *1*) and C2C12 (lane *4*) cells, of merosin-deficient RD.B8 (lane *2*) and C2C12.B4/plam (lane *5*) cells, and of merosin-transfected RD.B8/pmer(lane *3*) and C2C12.B4/pmer (lane *6*) cells. Total proteins (50 μg/well) were separated by SDS-PAGE under reducing conditions, transferred to nitrocellulose membranes, and blotted with specific antibodies directed to the cytoplasmic domains of α7B or β1D integrins. These conditions allow for the detection of (*A*) the unprocessed 121-kD α7B precursor (*pre-α7B*) and the 38-kD cytoplasmic domain of the processed molecule (*α7B cyto.*), or of (*B*) the mature 140-kD β1D polypeptide or its ~ 125-kD precursor (pre-β1D). *pmer*, cells stably transfected with an expression vector containing the human merosin α2 chain cDNA; *plam*, cells stably transfected with an expression vector containing the human laminin α1 chain cDNA. Molecular masses are in kilodaltons.

either the merosin α2 chain or the laminin α1 chain (22), but the expression of α7β1D integrins was restored only in C2C12.B4/pmer cells (Fig. 7, *A* and *B*, lane *5* vs. lane *6*; Table III).

β1 integrins mediate myotube survival. Since merosin is crucial for myofiber survival (4, 22), the possible role of α7β1D integrins in mediating this function was addressed. Treatment of differentiated RD cell cultures with blocking antibodies to integrin β1 decreased myotube survival in a dose-dependent manner (Fig. 9 *A*). Cell death was also induced in remaining unfused myoblasts (not shown). It is of note that myoblasts but not myotubes express fibronectin and laminin-1 as well as the integrins α7Bβ1A and α5β1A (22, 35, 40, 44, 45, 57). The α5β1 integrin was shown recently to promote fibronectin-mediated cell survival (56). Hence, the results indicate that β1 integrins mediate the survival of both myotubes and myoblasts. We did not evaluate the contribution of α7 integrins to myoblast or myotube survival, for lack of blocking antibodies of the α7 extracellular binding domain and because isoform-specific antibodies cannot be used.

Bcl-2 rescues merosin-deficient myotubes from apoptosis but does not restore α7β1D integrin expression. To understand further the role of α7β1D integrins in merosin-directed myotube survival, transfections of merosin-deficient myoblasts with the apoptosis-suppressing molecule Bcl-2 were performed. We have observed that normal merosin-positive myotubes formed by RD and C2C12 cells express high levels of Bcl-2, whereas Bcl-2 expression is undetectable in myotubes formed by the merosin-deficient RD.B8 and by the laminin α1 chain–transfected C2C12.B4/plam cells (Vachon, P.H., and E. Engvall, manuscript in preparation). Overexpression of Bcl-2 restored the viability of myotubes from RD.B8 cells (Fig. 9 *B*, RD.B8/pBcl-2) to a similar degree as merosin α2 chain transfection (reference 22; see also Fig. 9 *B*, RD.B8/pmer and C2C12.B4/pmer cells). Similarly, C2C12.B4/plam cells formed myotubes that were also rescued from apoptosis by forced expression of Bcl-2 (Fig. 9 *B*, C2C12.B4/plam/pBcl-2). In contrast, myotubes formed by control-transfected RD.B8 cells (RD.B8/pc) or by C2C12.B4 cells transfected only with the laminin α1 chain (C2C12.B4/plam/pc) showed extensive apoptosis and degeneration (Fig. 9 *B*). Therefore, myofiber apoptosis caused by merosin deficiency can be corrected by overexpression of Bcl-2.

Although Bcl-2 promoted the survival of merosin-deficient myotubes, proper expression and localization of α7β1D integrins was not restored (Table III; Fig. 6). In contrast to myotubes formed by the parental RD or Bcl-2–transfected cells (Fig. 6 and Table III), staining for α7A remained weak and absent from the surface of myotubes (Fig. 6 *G*), that for α7B was irregular and patchy (Table III), and that for β1D was still reduced as well as predominant in the Golgi apparatus (Fig. 6 *H*). This indicates that Bcl-2 acts downstream of α7β1D integrins in the merosin-directed pathway of myofiber survival, and that the disruption of integrins in merosin-deficient muscle is a direct consequence of the merosin deficiency rather than a consequence of cell death and/or degeneration.

Discussion

α7β1D integrins are major mediators of myofiber attachment and survival. We have identified the α7β1D integrins as primary receptor complexes for merosin in mammalian striated muscle fibers. Undifferentiated myoblasts express a relatively wide repertoire of integrins, of which α7β1 is predominant (35, 38, 41). In contrast, mature myofibers display fewer known integrins at their surface. The collagen-binding α1β1 integrin is restricted to neuromuscular junctions; α3β1, α9β1, and αvβ1

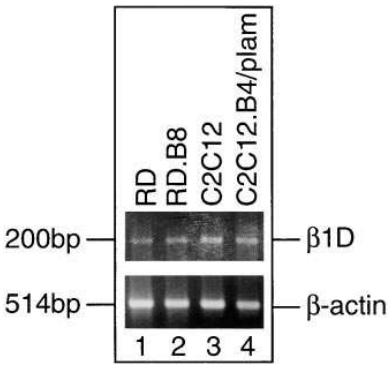

Figure 8. RT-PCR analysis of the mRNA expression for the β1D integrin subunit and for β-actin in differentiated cultures of RD (lane *1*), RD.B8 (lane *2*), C2C12 (lane *3*), and C2C12.B4/plam (lane *4*) cells. Amplified products were resolved on 1.5% agarose gels and stained with ethidium bromide. There are no apparent differences in β1D mRNA levels between normal (*RD* and *C2C12*) and merosin-deficient (*RD.B8* and *C2C12.B4/plam*) myotubes. The β-actin mRNA was amplified to ensure equal quantities of starting RNA before performing RT-PCR. The sizes of the amplified fragments are indicated on the left.

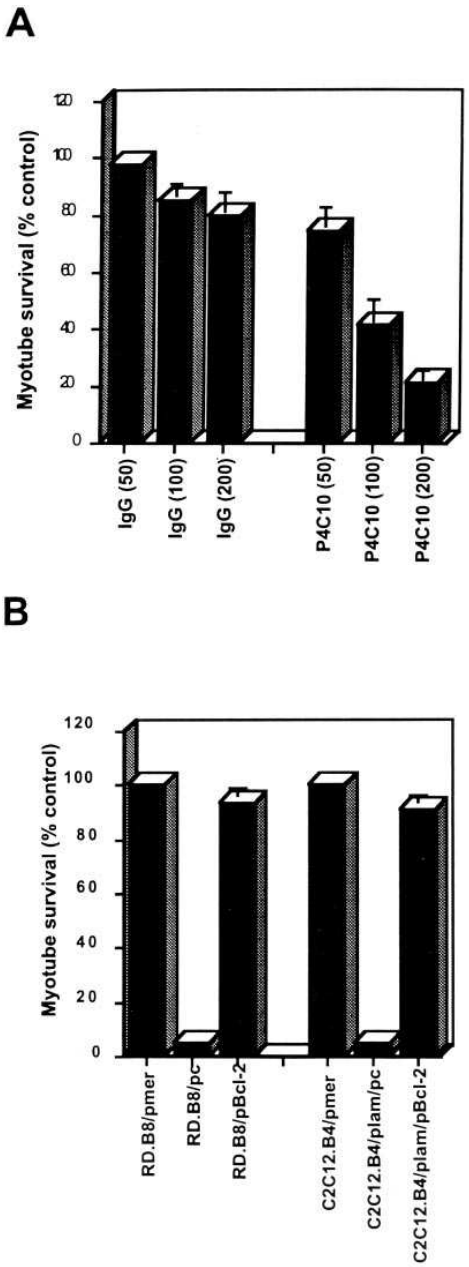

Figure 9. (*A*) Blocking of β1 integrin induces myotube apoptosis in a dose-dependent manner. 5-d postconfluent RD cells were incubated overnight with 0 (control), 50, 100, or 200 μg/ml of mouse IgG or the mouse anti–human β1 integrin–blocking mAb P4C10. Remaining myotube densities were scored, and the myotube survival index (percentage of control) was established (±SD). (*B*) Forced expression of Bcl-2 rescues merosin-deficient myotubes from apoptosis. RD.B8 and C2C12.B4 cells were transfected with the expression vectors pc (control vectors without inserts), pmer (human α2 chain cDNA), plam (human α1 chain cDNA), pBcl-2 (human Bcl-2 cDNA), or with both plam and pBcl-2. Cells were induced to differentiate, and myotube densities were scored to establish the myotube survival index (percentage of control; ±SD), using RD.B8/pmer and C2C12.B4/pmer as controls (i.e., 100% myotube survival).

are weakly expressed in association with sarcomeres; and α7β1 is again the predominant integrin expressed throughout the sarcolemma (35, 38, 41, 42). Considering that myofibers interact with a merosin-rich basement membrane, it is not surprising that α7β1 has been demonstrated to be a fully functional receptor for merosin (38). On the other hand, the dystroglycan subcomplex has been shown also to bind to merosin through its extracellular α subunit (15, 23, 24). Because of this ability and its association with dystrophin, dystroglycan was viewed as a link to the extracellular matrix and thus thought of as the major receptor for merosin in myofibers (2, 3, 5, 15, 23, 24). Several lines of evidence now argue against this premise. First, we found that deficiencies in the α2 chain of merosin cause severe disruptions in α7β1D integrin isoforms but not in dystroglycans or sarcoglycans. Second, defects in components of the DAG complex as observed in DMD and sarcoglycan deficiency do not affect α7β1 integrins. Third, the sarcolemmal expression of the dystroglycan complex as well as that of the sarcoglycan subcomplex are less affected than α7β1 integrins by genetic defects that indirectly cause merosin deficiency, as seen in FCMD and MEB (this study and references, 2, 4, 11, 13, and 14). Fourth, our α2 chain–restoration experiments confirm that the appropriate expression and localization of α7β1D integrin isoforms are merosin dependent. Finally, we also showed that β1 integrins play a major role in mediating myotube survival, which is in keeping with the crucial function performed by merosin in this process (4, 22). Therefore, we conclude that the α7β1D integrins are the de facto receptors for merosin (laminin-2/-4) in mammalian striated muscle fibers.

β1 integrins have been shown previously to mediate cell adhesion–dependent survival in epithelial cells, endothelial cells, and fibroblasts (28, 29, 56, 58–60). In some cases, this mediation involves signaling events which ultimately affect the expression and/or function of Bcl-2 and its homologues (29, 56, 58, 61, 62). So far, α2β1, α4β1, α5β1, and α6β4 have been identified as mediators of cell adhesion–dependent survival (29, 56, 63–65). Our study adds another likely candidate to this list, α7β1. Although direct evidence for α7 subunit involvement in muscle survival has yet to be obtained, our data provide nonetheless a molecular basis by which a distinct basement membrane component (merosin) functions as a survival factor for a specific differentiated cell type (striated muscle fibers). In this respect, our observation that Bcl-2 suppresses apoptosis in merosin-deficient myotubes suggests that the promotion of myofiber survival by merosin may be Bcl-2–dependent, as in the case of fibronectin-α5β1–mediated fibroblast survival (56).

A major role for integrins in merosin-dependent myofiber adhesion and survival does not exclude any merosin- or basement membrane–binding functions on the part of dystroglycans and/or sarcoglycans. Myofibers are assumed to require enhanced attachment and stability by virtue of the severe mechanical stress brought about by contraction and work, and may therefore require multiple adhesion receptors (3–5). To this effect, the DAG complex has been proposed to contribute to muscle stability by providing binding to merosin and other basement membrane components (3, 5, 23, 24). The importance of such anchorage-strengthening function is emphasized clearly by the observations that dystrophic muscle of the *mdx* mouse and BIO14.6 hamster undergo apoptosis, degeneration, and subsequent tissue necrosis as disease progresses (references 66 and 67 and our unpublished data). Consequently, it appears that myofibers may necessitate at least two separate but parallel attachment systems for their anchorage-dependent stability and survival: (*a*) the α7β1D integrin–merosin anchorage system, which would confer both mechanophysical stability and ECM-cell–survival signaling; and (*b*) the basement

membrane–DAG anchorage system, which would also provide mechanophysical stability.

Merosin directs the sarcolemmal expression and localization of α7β1D integrins. Depending on developmental situation, cell type, and integrin complex studied, the composition of the extracellular matrix has been shown to influence integrin mRNA levels, posttranslational modification, membrane localization, and/or turnover (42, 51, 68–71). In developing muscle, the expression of integrin subunits is regulated at the transcriptional level as well as through differential mRNA splicing events (39–42). Previous studies suggested that the localization of integrins is also regulated through cytoskeletal and intracellular (inside-out) signals during myofiber formation (33, 35, 41, 72). From our studies, it appears that specific extracellular (outside-in) cues generated by merosin are primarily responsible for the sarcolemmal localization and turnover of α7β1D integrins. Several lines of evidence support this hypothesis. First, the membrane localization of α7β1 integrin isoforms is established in parallel to the appearance of merosin laminin-2 and -4 during muscle development and maturation (9, 42–44, 73, 74). Second, we found that the expression and localization of α7β1D integrins are lost and/or disrupted in conditions of merosin α2 chain deficiencies. Supposedly, the lack of ligand binding causes degradation and increased turnover of the receptors. Similarly, the α7B isoform has been shown to be lost selectively from neuromuscular junctions of mice carrying a null-mutation for the β2 chain of laminin-4 (42), which is concentrated at these specialized sarcolemmal domains (41, 72–75). Third, we demonstrated that only the reinstatement of α2 chain expression, but not overexpression of laminin α1 chain or Bcl-2, can restore a normal expression of α7β1D integrins in merosin-deficient myotubes in vitro. Furthermore, we also found that the muscle-specific expression of an α2 chain transgene in merosin-deficient *dy*^{2J} mice restored the accurate expression and localization of these integrins in vivo (Xu, H., P.H. Vachon, L. Liu, F. Loechel, U.M. Wewer, and E. Engvall, manuscript submitted for publication). Finally, we have also observed that the expression of integrin–cytoskeletal linkage proteins, such as talin and sarcomeric α-actinin, appears normal in merosin-deficient myofibers, indicating that these intracellular components are not driving the sarcolemmal membrane localization of α7β1D integrins in muscle.

The localization of α7β1D integrins may not always be dependent on merosin. Indeed, the presence of α7Aβ1D and α7Bβ1D at the myotendinous junctions and myofiber termini of muscle deficient in the merosin α2 (this study) or β2 chain (42) may be indicative of integrin interactions at these specialized sites with laminins other than merosin or with other extracellular ligands. mRNAs coding for the recently discovered α4 and α5 laminin chains have been demonstrated in striated muscle tissues. However, the cell-type specificity and protein distribution of α4- and α5-containing laminin molecules remain to be fully characterized (4, 8, 76).

The persistence of α7β1D integrins at merosin-deficient myotendinous junctions and myofiber termini is also reminiscent of the situation observed in *Drosophila engrailed* and *invected* double mutants, which lack segmental tendons but nonetheless localize the $\alpha_{PS2}\beta_{PS}$ integrin at muscle fiber termini (72). Myotendinous junctions and myofiber termini are thought of as the striated muscle equivalents of focal adhesion sites (32, 35, 41). Indeed, focal adhesion plaques and their associated proteins, such as talin and p125^{FAK}, are concentrated at these specialized domains both in vivo and in vitro (this study, and references 31, 32, 35, and 41). Studies indicate that the localization of integrins at myotendinous junctions may be a process that is primarily dependent upon inside-out mechanisms, and occurs through interactions with the cytoplasmic tail of the β subunit (26, 27, 35, 41, 71, 72). The β1D cytoplasmic domain confers focal adhesion recruitment properties similar to the homologous β1A, in contrast to the nonhomologous β1B and β1C counterparts (41, 47, 48). Similarly, α7A and α7B share homologies in their cytoplasmic tails (38–40, 42). Therefore, the recruitment and persistent localization of α7Aβ1D and α7Bβ1D at myofiber termini regardless of the presence or absence of merosin may also involve inside-out signaling via a common domain within the cytoplasmic tails of the two α7 isoforms. Further analyses will be required to address these questions.

Pathogenesis of MCMD: perspectives and conclusions. Analyses of mutant phenotypes, whether natural or generated in the laboratory, can provide abundant evidence for the function of molecules in cellular and tissue processes (31, 63, 72, 77). In this respect, this study brings new insights to the biological functions of merosin in striated muscle attachment and survival, as well as to some important molecular interactions involved in these functions. Our identification of α7β1D integrins as primary receptors for merosin in muscle adds to our understanding of the pathogenesis of MCMD by providing a molecular basis for the myofiber survival functions of merosin. Indeed, the disruption of α7β1D integrin–mediated signaling is likely to be a significant factor in the increased susceptibility of merosin-deficient myofibers to undergo apoptosis in vitro as well as in vivo. This in turn brings additional insights to other less-characterized muscular dystrophies with secondary deficiencies in merosin, namely, FCMD and MEB, which now appear to involve an abnormal expression of α7β1D integrins as well.

Finally, these findings provide the first evidence of a role for integrins in neuromuscular diseases. Defects in the genes coding for the integrin subunits α3, α6, and β4 have been identified as novel causes for severe blistering skin diseases, such as junctional epidermolysis bullosa (63–65, 77, 78). Considering these data and the fact that β1 integrins mediate myotube survival, one may predict that defects in the β1 or α7 integrin genes leading to the loss of the β1D isoform or any one of the α7 isoforms are likely to cause some form of muscular dystrophy. In this respect, knockout mice lacking the α7 gene were reported recently to develop dystrophic symptoms (79). Therefore, closer scrutiny of α7β1D integrin expression in less-characterized subgroups of CMD or in other unclassified muscular disorders may help the identification of such putative genetic defects or any other which may involve muscle-specific molecules that associate with these integrins.

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