Merosin and Laminin in Myogenesis; Specific Requirement for Merosin in Myotube Stability and Survival

Pierre H. Vachon,* Frosty Loechel,‡ Hong Xu,* Ulla M. Wewer,‡ and Eva Engvall*§

*The Burnham Institute (La Jolla Cancer Research Center), La Jolla, California 92037; ‡Laboratory of Molecular Pathology, University Institute of Pathological Anatomy, University of Copenhagen, 2100 Copenhagen, Denmark; §Department of Developmental Biology, The Wenner-Gren Institute, University of Stockholm, 10691 Stockholm, Sweden

Abstract. Laminin (laminin-1; α1-β1-γ1) is known to promote myoblast proliferation, fusion, and myotube formation. Merosin (laminin-2 and -4; α2-β1/β2-γ1) is the predominant laminin variant in skeletal muscle basement membranes; genetic defects affecting its structure or expression are the causes of some types of congenital muscular dystrophy. However, the precise nature of the functions of merosin in muscle remain unknown. We have developed an in vitro system that exploits human RD and mouse C2C12 myoblastic cell lines and their clonal variants to study the roles of merosin and laminin in myogenesis. In the parental cells, which fuse efficiently to multinucleated myotubes, merosin expression is upregulated as a function of differentiation while laminin expression is downregulated. Cells from fusion-deficient clones do not express either protein, but laminin or merosin added to the culture medium induced their fusion. Clonal variants which fuse, but form unstable myotubes, express laminin but not merosin. Exogenous merosin converted these myotubes to a stable phenotype, while laminin had no effect. Myotube instability was corrected most efficiently by transfection of the merosin-deficient cells with the merosin α2 chain cDNA. Finally, merosin appears to promote myotube stability by preventing apoptosis. Hence, these studies identify novel biological functions for merosin in myoblast fusion and muscle cell survival; furthermore, these explain some of the pathogenic events observed in congenital muscular dystrophy caused by merosin deficiency and provide in vitro models to further investigate the molecular mechanisms of this disease.

A large part of our current understanding of the cellular and molecular mechanisms involved during myogenesis has been gained through the use of in vitro models (Weintraub et al., 1991; Edmondson and Olson, 1993; Weintraub, 1993). It is well established that the sequence of myoblast proliferation, myoblast fusion, and myotube formation, which characterizes the myogenic differentiation process, is regulated primarily by muscle-specific transcription factors, such as MyoD, Myf-5, and myogenin (Weintraub et al., 1991; Olson, 1992; Edmondson and Olson, 1993; Weintraub, 1993; Lassar et al., 1994). It is also recognized that other types of molecules likewise play a pivotal role in the regulation of muscle differentiation; these include growth factors (Olson, 1992; Lassar et al., 1994) as well as extracellular matrix (ECM)[1] components (Mayne and Sanderson, 1985; Engvall, 1993; Lassar et al., 1994) and their cell surface receptors (Volk et al., 1990; Engvall, 1994; McDonald et al., 1995; Belkin et al., 1996; Sastry et al., 1996).

Laminins are a family of basement membrane components that are composed of a heavy chain (α) and two similar but not identical light chains (β and γ) (Burgeson et al., 1994; Wewer and Engvall, 1994). The "classical" laminin, laminin-1, is composed of the α1 chain (400 kD) and the two β1/γ1 subunits (of ~205–220 kD). The predominant laminin variants in the muscle basement membrane contain the α2 heavy chain and are collectively referred to as merosin (Leivo and Engvall, 1988; Engvall, 1993; Wewer and Engvall, 1994). The α2 chain is resolved as two fragments of 300 and 80 kD by electrophoresis under reducing conditions. In muscle basement membranes, this subunit associates predominantly with the β1/γ1 chains as laminin-2, whereas it associates with the β2/γ1 chains as laminin-4 at neuromuscular junctions (Sanes et al., 1990; Wewer and Engvall, 1994; Martin et al., 1995).

Laminin-1 as well as fibronectin have been studied extensively in myogenesis. Myogenic cell lines and myoblast primary cell cultures express both of these proteins (Kühl et al., 1982; Mayne and Sanderson, 1985; Sanes et al., 1986; Kroll et al., 1994; Schuler and Sorokin, 1995). Laminin-1 is thought to play a prominent role in promoting myoblast

Address all correspondence to Eva Engvall, The Burnham Institute, La Jolla Cancer Research Center, Bldg. 400, 10901 North Torrey Pines Rd., La Jolla, CA 92037. Tel.: (619) 646-3100. FAX (619) 587-6089. E-mail: eengvall@ljcrf.edu

1. *Abbreviations used in this paper*: CMD, congenital muscular dystrophy; DAPI, 4′-6-diamine-2′-phenylindole dihydrochloride; ECM, extracellular matrix; TdT, terminal deoxynucleotidyl transferase; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling.

adhesion, migration, proliferation and myotube formation (Kühl et al., 1982; Foster et al., 1987; Öcalan et al, 1988; Goodman et al., 1989*b*; von der Mark and Öcalan, 1989; Kroll et al., 1994) whereas fibronectin, while stimulating myoblast adhesion and proliferation, retards or inhibits myogenic differentiation (Podelski et al., 1979; Foster et al., 1987; von der Mark and Öcalan, 1989). Less is known of the function of merosin in myogenesis. Its expression appears quite early during human and mouse muscle development in vivo (Sewry et al., 1995; Schuler and Sorokin, 1995) and the mRNA and protein for the α2 and β2 chains have recently been found in cultured myoblasts (Martin et al., 1995; Schuler and Sorokin, 1995). The laminin-2 heterotrimer is the predominant species synthesized by such cells and was found to promote faster myoblast cell spreading than laminin-1 (Schuler and Sorokin, 1995).

Merosin is also implicated in muscle pathologies such as autosomal recessive congenital muscular dystrophies (CMD) in humans and in *dy* and dy^{2J} mice. Dystrophic symptoms in the dy^{2J} mouse as well as in specific cases of CMD are caused by mutations in the merosin α2 chain gene, which result in the synthesis of a truncated protein or of a complete absence of a protein (Hillaire et al., 1994; Sunada et al., 1994, 1995; Tomé et al., 1994; Xu et al., 1994*a*,*b*; Hebling-Leclerc et al., 1995). This in turn can lead to severely defective muscle basement membranes (Xu et al., 1994*a*). In some other cases of CMD, there is a reduction of merosin α or β chains as a result of mutations in genes which have not yet been identified (Hayashi et al., 1993; Higuchi et al., 1994; Wewer et al., 1995). Because of the association of merosin with severe degenerative muscle diseases, the question of the precise roles of merosin in myogenic differentiation and skeletal muscle fiber maintenance is therefore an important one.

In the present study, we have investigated the role of merosin in myogenic differentiation in vitro in comparison to that of laminin-1. Our data show that both proteins promote myoblast fusion and myotube formation, but only merosin is essential for the stability and survival of myotubes. These results identify novel functions for merosin in muscle and provide key insight to the role of merosin-deficiency in the pathogenesis of CMD.

Materials and Methods

Myogenic Cell Lines and Cell Culture

The human RD rhabdomyosarcoma and mouse C2C12 myogenic cell lines were obtained from the American Type Culture Collection (Rockville, MD). Cells between passages 32 and 52 were maintained in DMEM (GIBCO BRL, Gaithersburg, MD) containing 10% FBS (Hyclone, Logan, Utah), 20 mM Hepes, 2% Eagle's non-essential amino acids solution (with glutamine; GIBCO BRL), 1 mM sodium pyruvate (GIBCO BRL) and antibiotics (growth medium), at 37°C in a humidified atmosphere of 5% CO_2 in air. Cultures were refed every 48 h and subcultured when ~70–80% confluent (−1 d postconfluence). Differentiation of cells was initiated at confluence (0 d postconfluence) by replacing the growth medium with complete DMEM but containing only 1% FBS (differentiation medium) and maintaining the cells in this medium for 6 d (Collo et al., 1993; Katagiri et al., 1994; Kroll et al., 1994; Schuler and Sorokin, 1995). Inhibition of myogenic differentiation was achieved by adding to the differentiation medium human recombinant transforming growth factor beta-1 (TGF-β1; a generous gift from Dr. Daniel R. Twardzik), at the effective concentration of 10 ng/ml (Brunetti and Goldfine, 1990; Katagiri et al., 1994), or human recombinant basic fibroblast growth factor (bFGF) (GIBCO BRL), at the effective concentration of 100 ng/ml (Brunetti and Goldfine, 1990). Growth and differentiation of cell cultures were routinely monitored by phase contrast microscopy. The degree of myogenic morphological differentiation was evaluated at each stage by scoring the number of elongated bi- and multinucleated myotubes per mm^2 of culture surface (expressed as mean ± SD), as described (Podelski et al., 1979; Foster et al., 1987; Katagiri et al., 1994; Kroll et al., 1994).

Myogenic Clonal Variant Cell Lines

RD and C2C12 clonal variants were obtained by limiting dilution cloning from parental cells at passage 35. Subconfluent cultures were trypsinized according to standard procedures and single-cell suspensions were plated at ~0.5 cells/8-mm well of 96-well culture clusters (Costar Corp., Cambridge, MA), then grown for 10–15 d. For RD cells, clones from 18 wells were subcultured and maintained, and 15 of them were expanded (Table I). For C2C12 cells, clones from 13 wells were subcultured and maintained, and 11 of them were expanded (Table I). Culture conditions of the clonal variants were as for the parental cell lines. In the present study, clonal variants were used between passages 5 and 15 after cloning; no changes in their behavior or in their expression of the proteins analyzed herein were observed.

Extracellular Matrix Components (ECM) and ECM Protein-Overlay Cultures

Laminin-1, merosin, and fibronectin were purified as previously described (Ehrig et al., 1990; Paulsson et al., 1991). Commercial preparations of laminin-1 and merosin (both from GIBCO BRL) were also used. Composition and purity of preparations were evaluated by SDS-PAGE followed by Coomassie blue staining (see Fig. 2 *B*, lanes *3* and *4*). The effects of these ECM components on the myogenic differentiation properties of some clonal variants were analyzed according to the ECM protein-overlay method (Roskelley et al., 1994). Briefly, cells were plated in 24-well culture clusters (Corning, New York, NY) and, beginning at −1 d postconfluence, were cultured in presence of 0–100 μg/ml of either laminin-1, merosin, fibronectin, or with a mixture of laminin-1 and merosin at equal concentrations, in growth medium (from −1 to 0 d postconfluence) or differentiation medium (from 0 to 6 d postconfluence). Medium with or without exogenous ECM components was renewed every 48 h and cultures were scored for myotube densities by phase contrast microscopy (see above).

Antibodies

The following monoclonal antibodies directed to laminin subunits were used: 4C7, directed to the G domain of the human α1 chain (Engvall et al., 1990); 4E10, directed to the human β1 chain (Engvall et al., 1990); 2E8, directed to the human γ1 chain (Engvall et al., 1990); 5H2, directed to the G domain of the human α2 chain (Leivo and Engvall, 1988; Engvall et al., 1990); and mAb 200, a rat monoclonal directed to the G domain of mouse α1 chain (Sorokin et al., 1992; a gift from Dr. Peter Ekblom). Rabbit polyclonal antibodies used in the present study were: Ab 363, an antiserum that recognizes several laminin subunits of both human and mouse (Ehrig et al., 1990); Ab 804, an affinity-purified antibody that recognizes the 80-kD fragment of the α2 chain of human and mouse merosin (Xu et al., 1994*a*,*b*); Ab 1301, an antiserum to a synthetic peptide derived from a sequence of the second repeat in the G domain of the α2 chain (Ehrig et al., 1990; Paulsson et al., 1991), and which detects the 300-kD fragment of this chain (Xu et al., 1994*a*,*b*); Ab 1471, an affinity-purified antiserum directed to the E3 fragment of laminin-1 (Paulsson et al., 1991); and anti-A2G, an affinity-purified antiserum directed to a recombinant G domain of the α2 chain (a gift from Dr. Peter D. Yurchenc). Antisera directed to skeletal muscle myosin heavy chain and to desmin (Sigma Chem. Co., St. Louis, MO) were also used.

Indirect Immunofluorescence

Immunofluorescence was performed on cells grown onto 13-mm glass coverslips as described (Vachon and Beaulieu, 1995). Rabbit antisera were used at 1:100 to 1:500 dilution, affinity purified antibodies were used at 10–30 μg/ml and hybridoma-conditioned media were used at 1:1 to 1:5 dilution. All dilutions were made in PBS (pH 7.4) containing 2% BSA (Sigma). FITC-conjugated goat anti-rabbit IgG, anti-mouse IgG, or anti-rat IgG (Boehringer Mannheim, Indianapolis, IN) were used as sec-

ondary antibodies. Cells were counter-stained with 0.01% Evans blue in PBS, mounted in Vecta-Shield (Vector, Burlingame, CA) and viewed with an Axiovert 405M microscope (Carl Zeiss, Thornwood, NY) equipped for epifluorescence. Micrographs and histological counterparts were obtained as previously described (Beaulieu and Vachon, 1994). In all cases, no immunofluorescent staining was observed when primary antibodies were omitted or replaced by rabbit or mouse immunoglobins (not shown).

RNA Purification and Reverse Transcription PCR (RT-PCR)

Total RNA was isolated by the acid guanidinium thiocyanate-phenol-chloroform extraction method, as previously described (Xu et al., 1994*a*,*b*). Cell cultures were washed three times with cold PBS before RNA extraction. Reverse transcription and subsequent PCR was carried out with 1.0 μg of total RNA, as described elsewhere (Xu et al., 1994*a*,*b*). PCR products (20 μl out of a 60-μl final reaction mixture) were resolved on 1.5% agarose gels stained with ethidium bromide, using a 100-bp ladder (GIBCO BRL) as standard. For the α1 chain of laminin-1, the following primers were used: the mouse-specific MA8243+ (5′-ACTTGGTGCTCCCTCTGAATC-3′) and MA9041− (5′-ATGCGGTGCTTGCTTTTGTGG-3′), which amplify a 803-bp fragment (Sasaki et al., 1988); and the human-specific HA1489+ (5′-GTGTTTGTAAGGAAAACGTT-3′) and HA2088− (5′-TCAAAAGATGTGTCACATTG-3′), which generate a 600-bp product (Haaparanta et al., 1991). Primers for the α2 chain of merosin were: the mouse-specific MM191+ (5′-AGTTGGTGGAACATGTCC-3′) and MM802− (5′-GTGTGACAATGGGATCGA-3′), which amplify a 610-bp fragment (Xu et al., 1994*b*); and the human-specific HM6480+ (5′-TCAAAGTATCTGTGTCTTCAGGA-3′) and HM6951− (5′-CCAGTGAATGTAATCACACGTACAGC-3′), which generate a 470-bp product (Vuolteenaho et al., 1994). Primers for myogenin (Collo et al., 1993) and β-actin (Stratagene, La Jolla, CA) were also used.

^{35}S-Radiolabeling and Immunoprecipitation

Cells were washed with DMEM lacking methionine and cysteine (GIBCO BRL) and were incubated for 24 h (37°C) in the same medium supplemented with 1% FBS and 100 μCi/ml of Expre^{35}S^{35}S protein labeling mix (NEN-Dupont, Wilmington, DE). Radiolabeled or unlabeled conditioned media (24 h) from duplicate cell cultures at each stage studied were collected (5 ml/100-mm dish) and centrifuged (3,000 g, 10 min, room temperature) to remove cell debris. One-fourth of fivefold concentrated immunoprecipitation buffer (final concentration: 1% Triton X-100, 0.1% SDS, 20 mM EDTA, 5 mM *N*-ethylmaleimide, 2 mM PMSF, 1 μg/ml pepstatin, 1 μg/ml leupeptin, and 0.05% sodium azide in PBS, pH 7.4) was added to the media, followed by incubation (2 h, room temperature) with 1/20 volume of gelatin–sepharose beads (Sigma) prewashed with immunoprecipitation buffer, to remove excess fibronectin. Samples were centrifuged again and the supernatants were aliquoted (1 ml) for storage (−70°C) until used. Immunoprecipitation of laminin molecules was performed as described previously (Leivo and Engvall, 1988; Engvall et al., 1990; Paulsson et al., 1991), using protein A-coupled agarose beads (Sigma). Controls included the preimmune serum corresponding to each rabbit antiserum used or 2% BSA-PBS (not shown). Immunoprecipitates were boiled (5 min, 105°C) in 1× solubilization buffer (2.3% SDS, 10% glycerol, and 0.001% bromophenol blue in 62.5 mM Tris-HCl (pH 6.8) containing 5% β-mercaptoethanol), and samples were cleared by centrifugation (13,000 rpm, 5 min, room temperature) before storage at −70°C until used.

Gel Electrophoresis and Immunoblotting

SDS-PAGE was performed on 4–12% gels (NOVEX, San Diego, CA) as described (Beaulieu and Vachon, 1994). High molecular mass markers (14.3–200 kD range; GIBCO BRL) were used as standards. Protein contents of all samples were estimated using the BioRad (Hercules, CA) protein assay. Total protein (25–50 μg/well) from radiolabeled immunoprecipitates were separated, then gels were dried and exposed overnight at room temperature to X-Omat Kodak films. Total protein (25–50 μg/well) from non-radiolabeled immunoprecipitates or from cell cultures solubilized in 1× solubilization buffer (Vachon and Beaulieu, 1995) were separated by electrophoresis and then electrotransfered to nitrocellulose membranes (Nitrocellulose-1; GIBCO BRL) for subsequent immunoblotting (Beaulieu and Vachon, 1994; Vachon and Beaulieu, 1995). Affinity-purified antibodies were used at 5–10 μg/ml and antisera were used at 1:500–1:1,000 dilution. Immunoreactive bands were visualized using the enhanced chemiluminescence method (ECL system; Amersham, Arlington Heights, IL).

In Situ Detection of Nuclear Condensation and Apoptosis-associated DNA Strand Breaks

Apoptotic nuclear condensation was visualized by staining cells cultured onto 13-mm cover slips with 4′-6-diamine-2′-phenylindole dihydrochloride (DAPI; Sigma) and counterstaining with Evans blue. Preparations were then mounted and viewed for epifluorescence. For in situ detection of apoptosis-associated DNA strand breaks, cover slip cell cultures were washed with PBS, fixed with 2% formaldehyde (Sigma) in PBS (45 min, 4°C) and quenched in 100 mM glycine-PBS (pH 7.4, 45 min, 4°C). Terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end-labeling (TUNEL) (Gavrieli et al., 1992) was then carried out using the ApopTag apoptosis detection kit (Oncor, Gaithersburg, MD) according to the manufacturer's instructions, followed by counterstaining with Evans blue. Preparations were then mounted and viewed with a Zeiss LSM 410 confocal microscope.

DNA Laddering Assays

DNA was isolated from 100-mm cell cultures as described (Frisch and Francis, 1994). DNA contents of all samples were estimated by OD_{260}. For the visualization of apoptosis-associated DNA inter-nucleosomal fragmentation (DNA laddering), each sample was then resolved by electrophoresis (20 μg DNA/lane) on 2% agarose gels stained with ethidium bromide, using a 100-bp DNA ladder (GIBCO BRL) as standard.

Construction of a Full-length Human Merosin α2 Chain cDNA

A DNA fragment containing nucleotides 13–268 of the α2 chain was amplified by PCR using *Pfu* DNA polymerase (Stratagene), the cDNA clone M6-16 (Vuolteenaho et al., 1994) as template and the following primers: 5′-GTGTCTAGATTTAAATTGGCTCCCGAGAAGGTGG-3′ and 5′-GACATGTTCTACCAATTTGC-3′. An overlapping DNA fragment containing nucleotides 238–1081 was synthesized by PCR using the cDNA clone M5-1 (Vuolteenaho et al., 1994) as template and the following primers: 5′-TGAAATGTACTGCAAATTGG-3′ and 5′-GGAGTGCATGCTTCACATTCAGTTTTAGTTAGAAAAGTTCCAGCTCTCCAGGG-3′. The two fragments were then joined by strand-overlap PCR to generate a fragment containing nucleotides 13–1081 with an XbaI and a SwaI restriction site immediately upstream of the translation initiation codon and extending to the SphI site at nucleotide 1080. This fragment was sequenced to confirm that no mutations had been introduced by PCR. It was then inserted between the XbaI and SphI sites of the cDNA clone M10-22 (Vuolteenaho et al., 1994), a 3.2-kb fragment cloned into Bluescript II (Stratagene), therefore generating the plasmid p933. The MscI/SacII fragment from clone M1-1 (Vuolteenaho et al., 1994), consisting of nucleotides 3940–6927, was in turn inserted between the MscI/SacII sites of p933. This gave the plasmid p936, containing nucleotides 13–6927. The BstBI/PacI fragment from the cDNA clone MER3′ (Ehrig et al., 1990), consisting of nucleotides 6510–9500, was subcloned in the BstBI/PacI sites of pSL1190 (Pharmacia, Uppsala, Sweden) after first converting the NruI site of pSL1190 to a PacI site by filling with the Klenow fragment of *Escherichia coli* DNA polymerase I followed by ligation to a PacI linker. The cDNA fragment was then cut back out of this plasmid with BstBI and SacII, then inserted between the BstBI/SacII sites of p936, thus generating the plasmid p937. This plasmid contains a unique SwaI site, followed by nucleotides 13–9500 of the full-length human α2 chain cDNA with a unique PacI site at nucleotide 9500.

A vector for the expression of the full-length human α2 chain cDNA under the control of the human cytomegalovirus promoter was constructed as follows. First, the XbaI site of the expression vector pcDNA3 (Invitrogen, San Diego, CA) was converted to a PacI site by filling with Klenow fragment of *E. coli* DNA polymerase I followed by ligation to a PacI linker, thus giving the plasmid p938. Then, the full length SwaI/PacI cDNA fragment from p937 was inserted between the EcoRV/PacI sites of p938, finally giving the plasmid p941. To verify the capacity for expression, COS-7 cells were transfected with p938 or p941 (see below) and stained by immunofluorescence with monoclonal antibodies directed to the human merosin α2 chain. Intense cytoplasmic staining was observed in the COS-7/p941 cells, whereas no staining was evidenced in COS-7/p938

cells (not shown). In the present studies, the p938 and p941 constructs were renamed pc (control) and pmer (merosin) respectively, for simplicity.

Stable Transfection of Myogenic Cells

Cell cultures at 50–60% confluence were transfected by the lipofection method (Lipofectamine; GIBCO BRL) according to the manufacturer's instructions with 10 μg of plasmid DNA. After the transfection, cells were kept in growth medium until 80–90% confluence, then split 1:5 and grown further for 2 d. Thereafter, the growth medium was replaced with the same medium supplemented with 400 μg/ml of G418 sulfate (Geneticin; GIBCO BRL) for selection of stable transfectants. After 10–14 d, the surviving colonies were isolated by the cloning cylinder technique and expanded. Transfected cell lines were thereafter routinely cultured in growth or differentiation medium as for the non-transfected cell lines but in the continuous presence of the selection drug. Clones were used beginning at passage 4 after cloning.

Results

Myogenic Differentiation of RD and C2C12 Cells

We first characterized the myogenic differentiation properties of the human RD rhabdomyosarcoma and mouse C2C12 myoblast cell lines. Cultures of growing RD cells typically consisted of small spindle-shaped and polygonal cells, while proliferating C2C12 cells exhibited a myoblast-like polygonal morphology (Fig. 1). Despite their cancerous origins, RD cells underwent extensive myoblast fusion and myotube formation when cultured under conditions that promote differentiation (Collo et al., 1993; Katagiri et al., 1994; Kroll et al., 1994), leading to an increase in the number of myotubes within the first 6 d of postconfluent culture (Fig. 1; Table I). The cultures showed numerous elongated, irregularly arranged multinucleated myotubes of various sizes and length (Fig. 1). C2C12 myotube densities also increased rapidly between 0 and 6 d postconfluence under differentiation conditions, the cultures exhibiting a large number of elongated, regularly arranged multinucleated myotubes of various sizes (Fig. 1; Table I).

Merosin Expression Correlates with Myogenic Differentiation

To ascertain any potential function for merosin in myogenesis, we analyzed its expression in relation to the myogenic differentiation of both cell lines. RT-PCR was carried out on total RNA extracted from RD and C2C12 cell cultures at various stages to examine the time-course appearance of merosin α2 chain mRNA (Fig. 2 *A*). For comparison, the presence of the laminin α1 chain and myogenin mRNAs were analyzed in the same experiments. Expression of the β-actin transcript was also assessed to ensure cDNA integrity and amounts of cell-derived RNA as starting material (Fig. 2 *A*). In both RD and C2C12 cells, the levels of merosin α2 chain mRNA increased gradually with time of postconfluent culture (Fig. 2 *A*). The laminin α1 transcript was barely detectable at −1 d and increased after 1 d postconfluence; thereafter, the levels decreased drastically as the cells completed their myogenic differentiation (Fig. 2 *A*). The apparent gradual increase of the merosin α2 chain mRNA and the concomitant decrease of the laminin α1 chain transcript were found concurrent with the emergence of the myogenin mRNA (Fig. 2 *A*) and myotube formation (Table I). As expected from previous studies (Collo et al., 1993; Katagiri et al., 1994), myogenin mRNA was not detected in prolif-

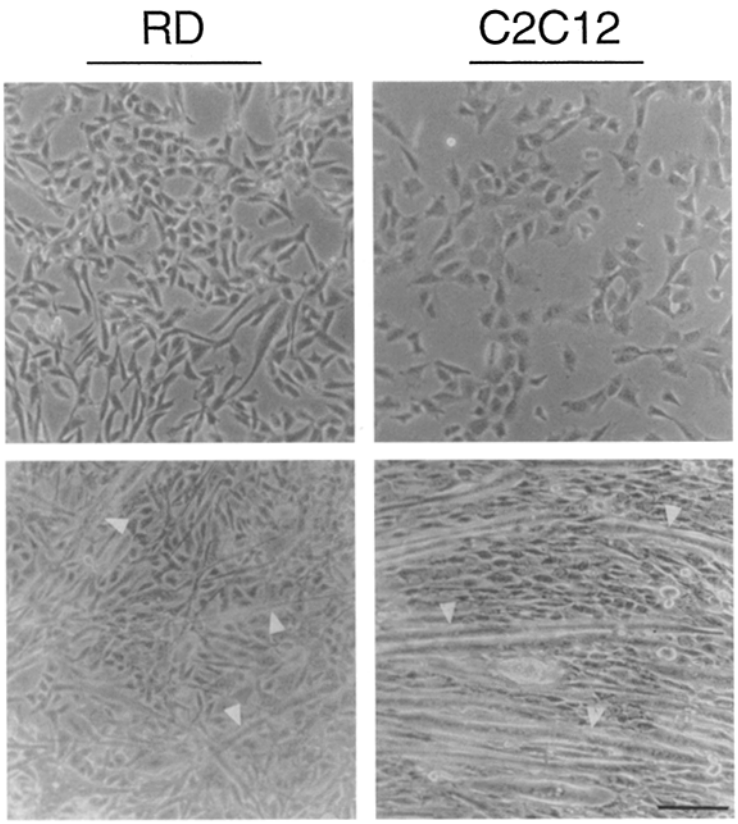

Figure 1. Morphological characterization of the myogenic differentiation of human and mouse myoblastic cells. Representative phase contrast micrographs of the human RD and mouse C2C12 cells on culture after −1 d (undifferentiated) or 6 d postconfluence (differentiated). Arrowheads point to myotubes. Bar, 50 μm.

Table I. Myogenic Differentiation of RD and C2C12 Cells, and Clonal Variants: Number of myotubes/mm² as a Function of Days of Postconfluent (PC) Culture

Type	Clone	Number of myotubes/mm² at −1 PC	1 PC	6 PC	Clone	Number of myotubes/mm² at −1 PC	1 PC	6 PC
Parental	RD	0.2 ± 0.1	1.5 ± 0.2	9.0 ± 1.2	C2C12	0.0 ± 0.0	1.9 ± 0.7	13.2 ± 2.3
1	RD.A4	0.2 ± 0.1	n.d.	8.7 ± 1.1	C2C12.A2	0.0 ± 0.0	n.d.	11.3 ± 3.2
	RD.B3	0.1 ± 0.1	1.0 ± 0.1	7.4 ± 2.2	C2C12.B6	0.0 ± 0.0	n.d.	12.5 ± 1.2
	RD.C2	0.1 ± 0.1	1.0 ± 0.3	7.6 ± 1.3	C2C12.C9	0.0 ± 0.0	n.d.	12.7 ± 2.2
	RD.C6	0.2 ± 0.1	n.d.	9.0 ± 2.4	C2C12.D2	0.0 ± 0.0	2.1 ± 0.4	15.4 ± 3.1
	RD.D1	0.1 ± 0.1	n.d.	8.2 ± 2.1	C2C12.E2	0.0 ± 0.0	2.0 ± 0.7	14.3 ± 2.2
	RD.E1	0.1 ± 0.1	1.0 ± 0.2	7.3 ± 1.5	C2C12F.9	0.0 ± 0.0	n.d.	16.4 ± 2.8
	RD.F5	0.1 ± 0.1	n.d.	7.5 ± 2.0	C2C12.H7	0.0 ± 0.0	1.0 ± 0.3	13.0 ± 1.3
	RD.G4	0.1 ± 0.1	n.d.	8.5 ± 1.2				
2	RD.B8	0.1 ± 0.1	0.8 ± 0.1	0.5 ± 0.1*	C2C12.C1	0.0 ± 0.0	n.d.	1.9 ± 0.7
	RD.C3	0.1 ± 0.1	n.d.	0.3 ± 0.1*	C2C12.E6	0.0 ± 0.0	0.5 ± 0.1	2.1 ± 0.5
	RD.C4	2.2 ± 0.4	0.8 ± 0.2*	0.6 ± 0.1*				
	RD.D6	0.1 ± 0.1	0.7 ± 0.1	0.3 ± 0.1*				
	RD.H1	0.1 ± 0.1	n.d.	0.4 ± 0.2*				
3	RD.A9	0.0 ± 0.0	n.d.	0.0 ± 0.0	C2C12.B4	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
	RD.D2	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	C2C12.E5	0.0 ± 0.0	n.d.	0.0 ± 0.0

Cells were cultured in growth medium (until 0-day postconfluence) or differentiation medium (from 0 to 6 d postconfluence). At each culture stage, the number of myotubes/mm² of culture surface was scored; values presented are mean ± SD from three separate cultures ($n \geq 30$). n.d., not determined.
*Indicates evidence of extensive myotube degeneration and cell death.

erating (undifferentiated) C2C12 cells; rather, its expression coincided with the onset of fusion and myotube formation in these cells (Fig. 2 *A*; Table I).

The α2 chain of merosin was also detected at the protein level. Analysis of ^{35}S-radiolabeled proteins, immunoprecipitated with α2 chain-specific antibodies, from the media of RD and C2C12 cell cultures indicated the presence of major polypeptides of 300, 205–220 and 80 kD after separation by SDS-PAGE under reducing conditions (Fig. 2 *B*, lanes *1* and *2*). In agreement with previous reports (Kroll et al., 1994; Schuler and Sorokin, 1995), these were found to correspond to the 300- and 80-kd segments of the α2 chain of purified merosin (Fig. 2 *B*, lane *3*) as well as to the common 205–220-kD β1 and γ1 subunits of both laminin-1 and merosin (Fig. 2 *B*, lanes *3* and *4*). By using immunoblotting in time-course studies, we found that the amounts of α2 chain increased gradually during myogenic differentiation of RD and C2C12 cells (Fig. 2 *C*), in a manner that paralleled that of the α2 chain mRNA (Fig. 2 *A*). This increase likewise followed that of myosin heavy chain and desmin (Fig. 2 *C*), the expression of which are known to be upregulated as a function of myogenic differentiation (Foster et al., 1987; Bandman, 1992; Katagiri et al., 1994).

Laminin, as revealed in immunofluorescence by antiserum reactive with multiple subunits, was found around myotubes as well as in an irregular network among unfused myoblasts (Fig. 3 *A*). Intracellular staining was also observed. Detection for the individual β1 (Fig. 3 *C*), γ1 (not shown), or α1 (Fig. 3 *E*) chains revealed similar localization and staining patterns. However, the staining for the α1 chain at this terminal stage of differentiation was generally weaker as compared to other subunits (Fig. 3 *E*), in agreement with the decrease in α1 chain mRNA observed in differentiated cultures (Fig. 2 *A*). The merosin α2 chain was found concentrated around myotubes (Fig. 3 *G*) and staining was generally much stronger than that for the α1 chain (compare Fig. 3, *E* and *G*). Hence, these results altogether show that the expression of merosin is upregulated as myoblasts undergo myogenic differentiation, whereas that of laminin-1 is downregulated. Furthermore, the merosin heterotrimers synthesized by differentiated RD and C2C12 cells associate primarily with myotubes.

Merosin Expression is Downregulated when Myogenic Differentiation is Inhibited

To further investigate the correlation between the upregulation of merosin and myogenic differentiation, as well as its inverse relationship with the downregulation of laminin-1, we analyzed the effects of TGF-β1 and bFGF on the expression of the α1 and α2 chains. The dose-dependent inhibitory action of these two growth factors on myogenic differentiation has been well documented (Brunetti and Goldfine, 1990; Olson, 1992; Katagiri et al., 1994; Lassar et al., 1994). The presence of 10 ng/ml of TGF-β1 or 100 ng/ml of bFGF completely inhibited myoblast fusion and myotube formation (not shown). RT-PCR analysis of cells treated with either growth factor also revealed significant changes in laminin α1, merosin α2, and myogenin mRNA levels (Fig. 4). In both TGF-β1 and bFGF-treated cells, the α1 transcript was greatly increased. In contrast, treatment prevented the induction of merosin α2 chain mRNA accumulation observed in untreated cultures. The myogenin transcript was absent in growth factor-treated cells whereas it was readily detectable in controls, as expected from previous reports (Brunetti and Goldfine, 1990; Olson, 1992; Katagiri et al., 1994). These results indicate that the differential expression of the laminin α1 and the merosin α2 chains is a function of the myogenic differentiation program.

Defective Myogenic Differentiation Is Correlated with Abnormal Laminin Expression

We sought to examine whether merosin is actually required for proper differentiation. For this purpose, we generated clonal variants of RD and C2C12 cells by limit-

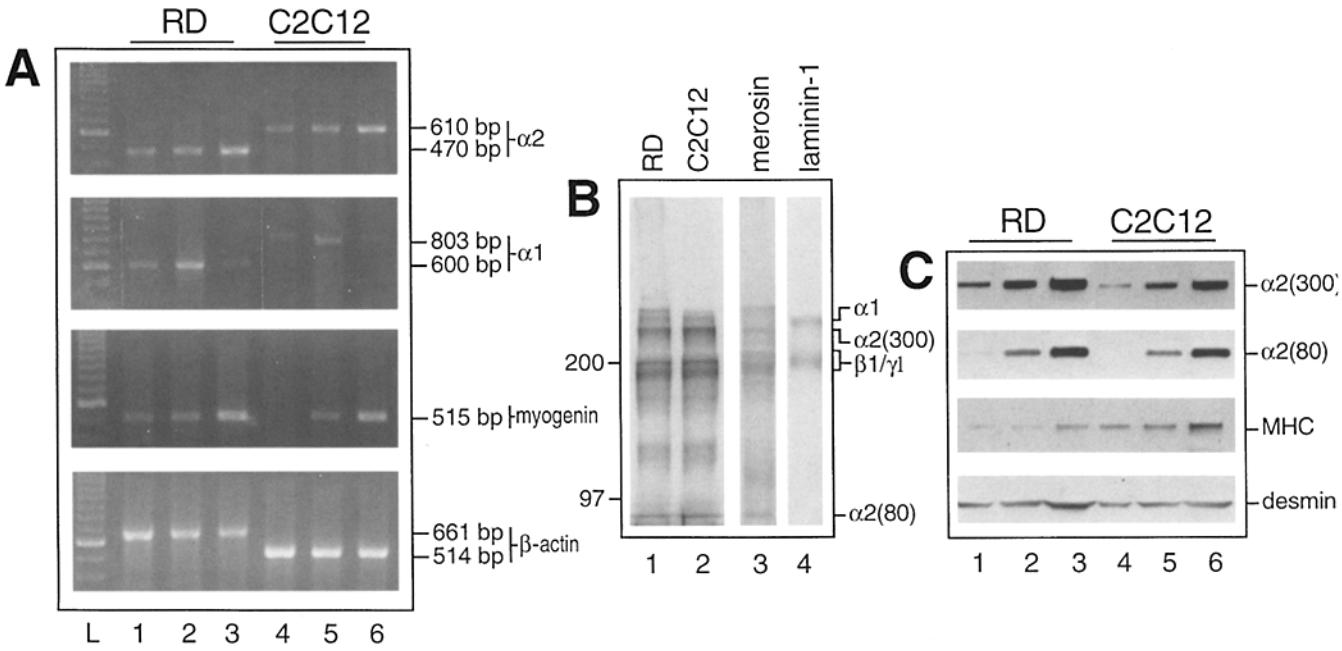

Figure 2. Molecular characterization of the myogenic differentiation of human and mouse myoblastic cells. (*A*) Expression of merosin α2, laminin α1, myogenin, and β-actin mRNA's during myogenic differentiation. RT-PCR analyses of RD (lanes *1–3*) and C2C12 (lanes *4–6*) cells after −1 (lanes *1* and *4*), 1 (lanes *2* and *5*) and 6 d (lanes *3* and *6*) of postconfluence. The experiment shown is representative of three experiments. L, 100-bp DNA size markers (DNA-ladder), the 600-bp marker being the most intense. (*B*) Representative immunoprecipitation and autoradiography of ^{35}S-radiolabeled merosin synthesized by RD (lane *1*) and C2C12 (lane *2*) cells after 1 day of postconfluent culture. For comparison, purified human merosin (lane *3*) and mouse laminin-1 (lane *4*) were separated on the same gels and stained with Coomassie blue. The α1 chain (400 kD), the 300- and 80-kD fragments of the α2 chain ($\alpha2_{300}$; $\alpha2_{80}$), and the β1/γ1 chains (205–220 kD) are identified. Molecular masses are in thousands. (*C*) Expression of merosin during myogenic differentiation. Immunoblotting analysis of immunoprecipitated merosin α2 chain synthesized by RD (lanes *1–3*) and C2C12 (lanes *4–6*) cells after −1 (lanes *1* and *4*), 1 (lanes *2* and *5*) and 6 d (lanes *3* and *6*) of postconfluence, using specific antibodies for the 300-kD ($\alpha2_{300}$) and the 80-kD ($\alpha2_{80}$) fragments. Total protein from cell lysates were also probed with antibodies specific for the myosin heavy chain (MHC; ~200 kD) and desmin (~55 kD). Immunoblot data shown are representative of three separate experiments.

ing dilution cloning and assayed these for their myogenic differentiation properties and for the expression of the laminin and merosin α chains. As summarized in Table I, the clonal variants generated could be classified into three general types. Type-1 RD and C2C12 clonal variants displayed myogenic differentiation properties similar to their parental cell lines. These comprised the highest proportion of clonal variants obtained (≥53%), indicating a relatively good chromosomal stability and genetic homogeneity of the parental cell lines. Type-2 RD cell variants (5/15 of total RD clones) also underwent fusion and myotube formation, but the myotubes degenerated over the time-period studied (6 d postconfluence). This degeneration was evidenced by the appearance of cellular blebs protruding from myotubes and by the presence of dead cells at (and around) remaining phantom myotube-like structures (see Fig. 5 *A*, 7 *B*, and 8 *B* for examples), resulting in significantly lower myotube densities (Table I). Type-2 C2C12 cell variants (2/11 of total C2C12 clones) proved to be fusion-ineffective relative to the parental cells. Finally, Type-3 RD (2/15 clones) and C2C12 (2/11 clones) variants were completely fusion-defective.

We analyzed in some detail the properties of the RD.B8 (Type-2), RD.D2 (Type-3) and C2C12.B4 (Type-3) clonal variants. The RD.B8 cells are able to fuse but show extensive myotube degeneration (or instability) after 6 d postconfluence (Fig. 5 *A*). Differentiated cell cultures contained high levels of the α1 chain mRNA but lacked detectable α2 chain mRNA and protein (Fig. 5 *B*, and *C*), unlike the parental RD cells. The RD.D2 cells, which do not undergo myoblast fusion (Fig. 5 *A*), did not contain detectable α1 or α2 chain mRNA (Fig. 5 *B*). Lack of expression of both chains was also noted in the fusion-defective C2C12.B4 cells (Fig. 5 *A–C*). To ensure that the behavior of these variants was not caused by a deficiency in myogenic regulatory factors, each was assayed for the expression of myogenin which lies downstream of the hierarchy of regulatory proteins that drive the myogenic differentiation program (Weintraub et al., 1991; Olson, 1992; Edmondson and Olson, 1993; Lassar et al., 1994) and which is required for myoblast fusion to occur (Weintraub, 1993; Lassar et al., 1994). As shown in Fig. 5 *B*, each of these three clonal cell lines were positive for the myogenin transcript. These data show that myotube instability can be associated with merosin deficiency, while fusion-deficiency can be correlated to a lack of expression of both laminin-1 and merosin.

Roles of Laminin-1 and Merosin in Myotube Formation and Stability

We then analyzed directly the function of laminin-1 and

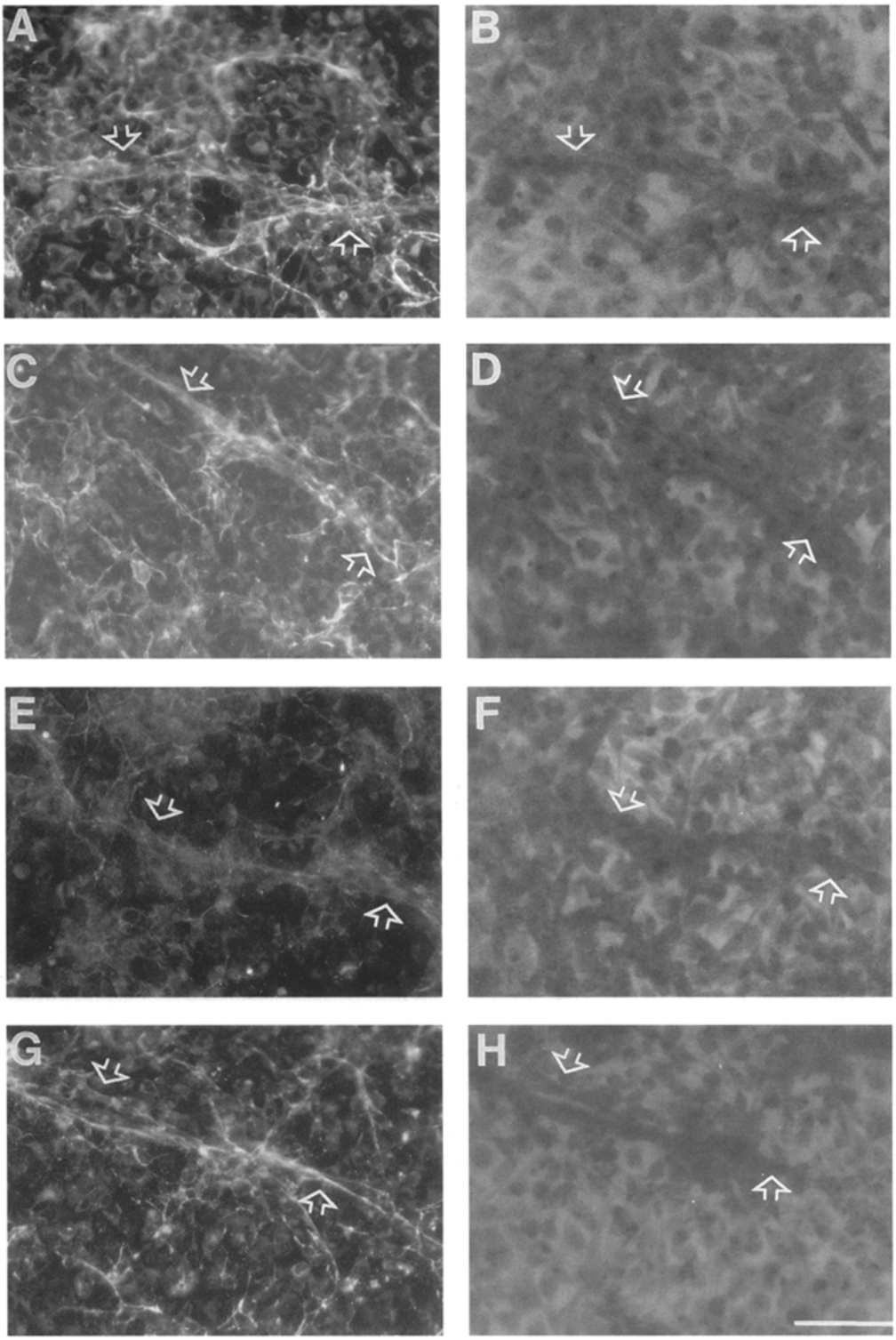

Figure 3. Localization of laminin and merosin subunits in differentiated myogenic cell cultures by immunofluorescence. Representative micrographs of RD cells at 6 d postconfluence after fixation, permeabilization and staining for multiple laminin subunits (*A*), the β1 chain (*C*), the α1 chain (*E*), or the α2 chain (*G*), with human specific polyclonal (*A*) or monoclonal (*C*, *E*, *G*) antibodies. (*B*, *D*, *F*, *H*) Corresponding histological counterparts to (*A*, *C*, *E*, *G*). Arrows point to a single myotube. Bar, 25 μm.

merosin in myogenic differentiation, using the differentiation-defective RD.B8, RD.D2, and C2C12.B4 cells. Culturing the myotube-unstable RD.B8 cells (Fig. 6 *A*) with either laminin-1 or fibronectin as ECM protein-overlays had no effect on the stability of the myotubes formed, regardless of the concentration used. In contrast, a clear dose-dependent effect of merosin on RD.B8 myotube stability was observed, which was reflected by increased myotube densities. Addition of both laminin-1 and merosin at equal concentrations resulted in similar dose-dependent effects on myotube stability, but not significantly different from that exhibited by merosin alone, therefore indicating a merosin-specific influence on myotube stability. It is of note that merosin, alone or in combination with laminin-1, almost completely rescued the unstable myotubes in RD.B8 cells: myotube densities formed under these conditions were ~0.5 times those found in cultures of the parental RD cells (for example, 4.6 myotubes/mm^2, in the pres-

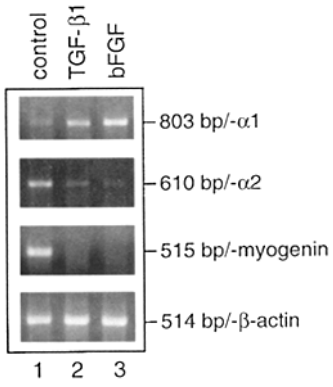

Figure 4. Inhibition of myogenic differentiation by TGF-β1 and bFGF affect laminin α chains and myogenin expression in C2C12 cells. RT-PCR analyses of laminin α1, merosin α2, myogenin, and β-actin mRNA levels after 6 days of postconfluent culture without added growth factors (lane *1*) or with 10 ng/ml of TGF-β1 (lane *2*) or 100 ng/ml of bFGF (lane *3*). The experiment shown is representative of three separate experiments.

ence of 100 μg/ml merosin, versus 9.0 for the parental cells at the same culture stage; Fig. 6 *A* and Table I).

Both laminin-1 and merosin induced myotube formation in a dose-dependent manner in the fusion-defective RD.D2 (Fig. 6 *B*) and C2C12.B4 (Fig. 6 *C*) cells. In contrast, fibronectin had no effect as expected (Podelski et al., 1979; Foster et al., 1987; von der Mark and Öcalan, 1989). Using laminin-1 and merosin together induced myotube formation at a lower protein concentration than either one alone and yielded more myotubes (about twofold). Moreover, there was no evidence of myotube degeneration and cell death, whereas these were evidenced when laminin-1 was used alone. Hence, these results suggest a synergistic effect by laminin-1 and merosin on myogenesis, and further support a specific role for merosin in myotube stability.

Rescue of Differentiation-defective Myoblasts by Transfection with a Full-length Human Merosin α2 Chain cDNA

To confirm the roles of merosin in myotube formation and stability, we transfected some of the differentiation-defective, merosin-negative cell lines with an expression vector (pmer) containing a full-length cDNA encoding the human α2 chain, under the regulation of the human cytomegalovirus promoter. The expression vector without insert (pc) was used as control in parallel experiments. RT-PCR analysis of selected clones showed the presence of the human α2 chain mRNA in the pmer-transfected but not in control-transfected cells (not shown). Immunofluorescence using monoclonal anti-human α2 chain antibodies revealed the expression of the merosin protein and its association with myotubes, indicating proper assembly of the human α2 chain with the endogenous β and γ chains (Fig. 7 *A*).

The parental C2C12 cells transfected with the human merosin α2 chain exhibited a 1.5-fold increase in their capacity to form myotubes (Table II). In contrast, control-transfected C2C12 cells showed no notable difference in their differentiation properties, as compared to non-transfected cells (Tables I and II). The C2C12.B4 variant cells were then stably transfected with the pmer vector, in an attempt to rescue their fusion-defective phenotype. As shown above, these cells express neither the α1 nor the α2 chains, but do express the β1/γ1 chains (not shown) and can be induced to form myotubes by adding exogenous laminin-1, merosin, or both (see Fig. 6 *C*). The C2C12.B4/pmer transfectants underwent fusion and myotube formation (Fig. 7 *B*; Table II) nearly as efficiently as the parental C2C12 cells. Furthermore, the transfection of the merosin α2 chain cDNA was more efficient in rescuing C2C12.B4 cells than the addition of high concentrations of purified merosin, or of an equal mixture of laminin-1 and merosin as protein-overlays (see Fig. 6 *C*). In contrast, the C2C12.B4/pc controls remained fusion-defective (Fig. 7 *B*; Table II). These findings confirm a capacity of merosin to support and promote myoblast fusion.

The α2 chain transfection also corrected the myotube instability in the RD.B8 cells (Fig. 7 *B*; Table II), fully restoring the phenotype of the parental RD cells. Transfection with the control vector had no effect. Again, the transfection with merosin α2 chain was significantly more efficient in restoring myotube stability than the addition of merosin to the culture medium (see Fig. 6 *A*). These observations confirm a role for merosin in promoting and maintaining the stability of myotubes.

Unstable, Merosin-deficient Myotubes Undergo Apoptosis

To gain additional insights into the mechanisms by which merosin promotes myotube stability, we tested the hypothesis that the degeneration of myotubes in the α2 chain-deficient RD.B8 cells was linked to apoptosis. Numerous dead cells at (and around) degenerating myotubes were observed in differentiated RD.B8 cell cultures; these cells were shrunken and rounded, and became detached from the plates or were only loosely adherent (Fig. 5 *A*), typical of apoptotic cells. Staining with the DNA-binding fluorochrome DAPI revealed chromatin condensation and shrunken, compact nuclei in degenerating myotubes as early as 3 d postconfluence (not shown). This was not observed in unfused myoblasts. Probing for the presence of DNA strand breaks using the TUNEL method showed strong incorporation of labeled nucleotides in nuclei of myotubes that had not yet begun to degenerate (Fig. 8 *B*), whereas weak to strong labeling was also noted in myotubes already advanced in their degeneration process (Fig. 8 *B*), indicating that apoptosis precedes the myotube degeneration in these cells. Unfused myoblasts did not incorporate labeled nucleotides (Fig. 8 *B*). Apoptosis-associated inter-nucleosomal fragmentation (DNA laddering) was also evident as early as 3 d postconfluence in RD.B8 cells (Fig. 9, lane *2*), and this DNA fragmentation was increased with time after confluence (Fig. 9, lane *3*), consistent with an increased number of apoptotic cells. In contrast, no evidence of myotube degeneration (Fig. 1), incorporation of labeled nucleotides (Fig. 8 *A*), or DNA laddering (Fig. 9, lane *1*) were observed in parental RD cells at the same stages.

Finally, the restoration of α2 chain expression in the transfected RD.B8/pmer cells corrected their phenotype of myotube instability and consequently abolished the accumulation of apoptotic cells in cultures (Fig. 7 *B*) and inhibited apoptosis-associated DNA fragmentation (Fig. 9, lane 5). In contrast, dead cells (Fig. 7 *B*) and DNA laddering (Fig. 9, lane *4*) were observed consistently in control-

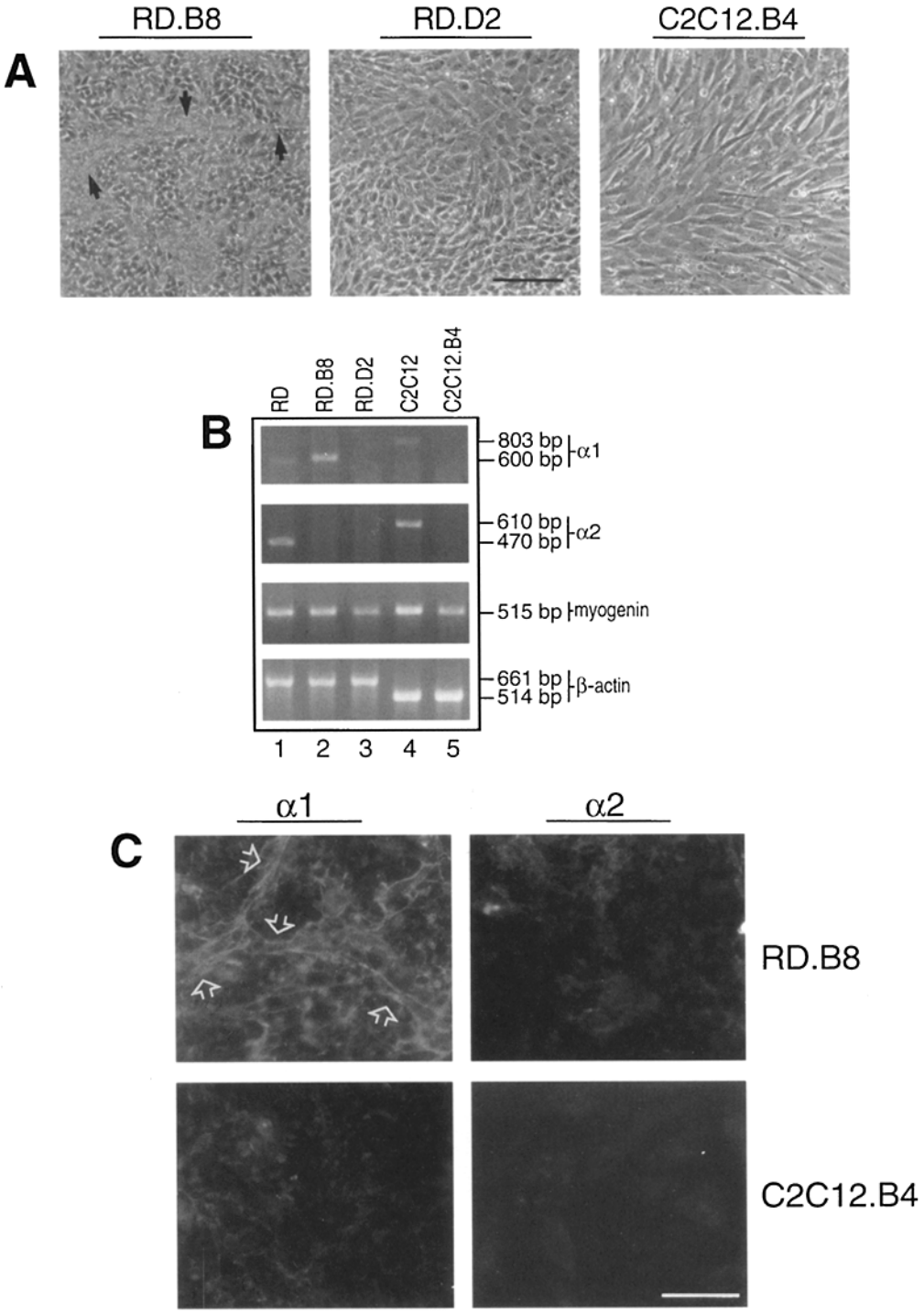

Figure 5. Myogenic differentiation-defective cell lines. Clonal variants of RD and C2C12 cells were generated by dilution limiting cloning and evaluated for their myogenic differentiation properties. Shown here in *A* are representative phase contrast micrographs of 6 d postconfluent RD.B8 cells, which exhibit extensive myotube instability and degeneration (arrows point to degenerating myotubes, which are surrounded by dead cells); and RD.D2 and C2C12.B4 cells, which are fusion-defective. (*B*) Laminin α1, merosin α2, myogenin, and β-actin gene expression as detected by RT-PCR in 6 d postconfluent RD (lane *1*), RD.B8 (lane *2*), RD.D2 (lane *3*), C2C12 (lane *4*) and C2C12.B4 (lane *5*) cells. The experiment shown is representative of three separate experiments. (*C*) Immunofluorescence micrographs of 6 d postconfluent RD.B8 and C2C12.B4 cells after fixation, permeabilization and staining for the detection of the laminin α1 chain or the merosin α2 chain, with specific monoclonal antibodies. Arrows point to a single myotube. Bars: (*A*) 50 μm; (*C*) 25 μm.

transfected RD.B8/pc cells. Therefore, these data altogether indicate that merosin promotes myotube stability by preventing apoptosis.

Discussion

The myogenic differentiation process is essentially comprised of three distinct stages: myoblast proliferation, myoblast fusion into myotubes, and myotube stabilization and survival. In the present study, we have examined the roles of merosin and laminin-1 in this process, particularly in the two latter stages. We found that merosin expression is upregulated as a function of myogenic differentiation, while laminin-1 expression undergoes simultaneous downregulation. Our results also provide clear evidence that both proteins can promote myoblast fusion and myotube formation, but only merosin promotes the structural stability as well as the survival of myotubes. Accordingly, a lack of merosin and laminin expression precludes fusion in myoblasts even in the presence of the muscle regulatory factor myogenin, whereas a lack of merosin expression causes myotube apoptosis, instability and degeneration. Hence,

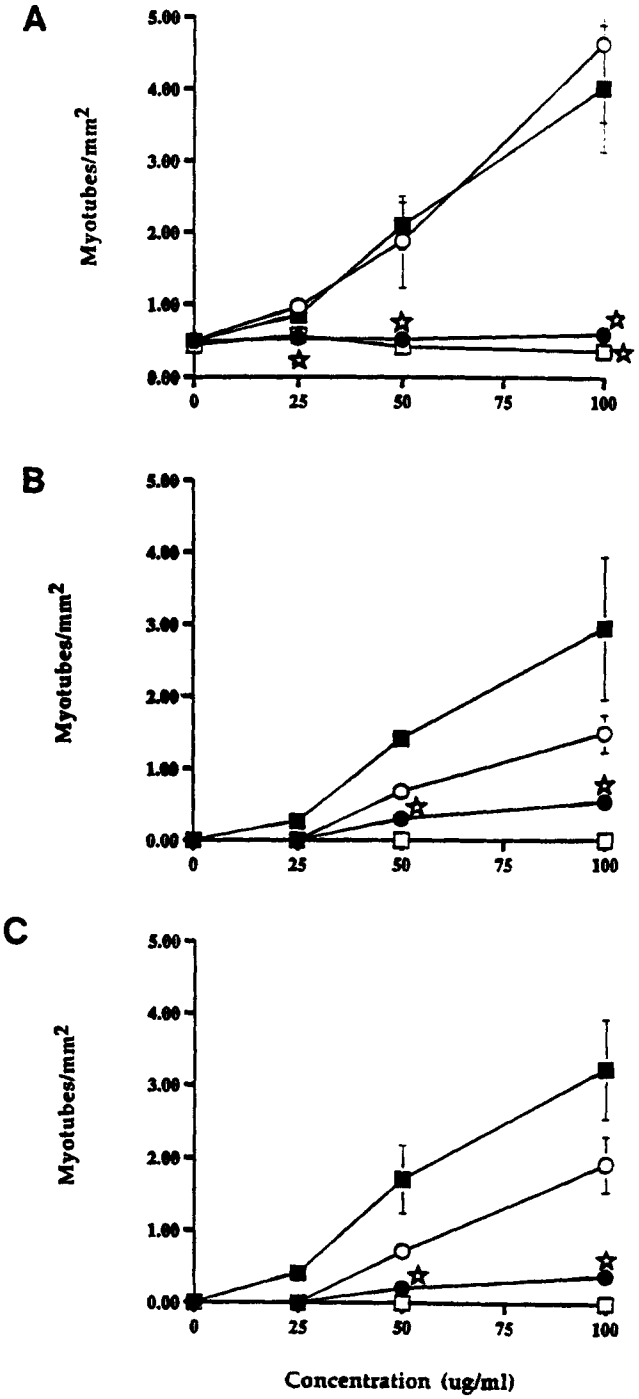

Figure 6. Effects of merosin and laminin on myotube formation and stability. The RD.B8 (*A*), RD.D2 (*B*) and C2C12.B4 (*C*) cells were cultured in the presence of extracellular matrix protein-overlays consisting of 0–100 μg/ml of purified merosin (*open circles*), laminin-1 (*filled circles*), an equal mixture of merosin and laminin-1 (*filled squares*), or fibronectin (*open squares*), beginning at −1 d postconfluence (under growth conditions) and up to 6 d postconfluence (under differentiation conditions), at which time the numbers of myotubes/mm^2 of culture surface were scored. Values presented are mean ± SD from three separate experiments ($n \geq 15$). A star indicates evidence of extensive myotube degeneration and cell death.

these findings indicate that both molecules are crucial players in the myogenic differentiation process.

Merosin and Laminin-1 Are Expressed as Part of the Myogenic Differentiation Program

Myogenesis is primarily driven by a fine-tuned program of gene regulation which causes the withdrawal of myoblasts from the cell cycle and leads to the emergence of the terminally differentiated, multinucleated, post-mitotic myotubes (Weintraub et al., 1991; Olson, 1992; Edmondson and Olson, 1993; Weintraub, 1993; Lassar et al., 1994). Several lines of evidence now suggest that the differential expression of laminin-1 and merosin in differentiating myoblasts constitutes an integral part of this program. First, the two molecules are expressed according to a developmental sequence where laminin-1 preceeds the onset of myogenesis (Kühl et al., 1982; Mayne and Sanderson, 1985; Sanes et al., 1986; Foster et al., 1987; Klein et al., 1990; Kroll et al., 1994), while merosin is associated with differentiated myofibers (this study; Leivo and Engvall, 1988; Ehrig et al., 1990; Schuler and Sorokin, 1995; Sewry et al., 1995). Second, we found that the expression of the muscle regulatory factor myogenin is directly correlated with that of merosin and inversely correlated with that of laminin-1. Third, TGF-β1 and bFGF upregulated the expression of laminin-1 and downregulated that of merosin, while also inhibiting myogenin expression, myoblast fusion, and myotube formation. Finally, fibroblasts transfected with MyoD, another muscle regulatory transcription factor, undergo myogenic differentiation and concomitantly acquire the ability to express laminin-1, as well as a 350-kD polypeptide that could be the α2 chain of merosin (Kroll et al., 1994). These observations also suggest a direct action of muscle regulatory factors on the differential regulation of the expression of laminin-1 and merosin during muscle development.

Merosin and Laminin-1 Perform Shared as Well as Unique Roles in Myogenesis

Our use of differentiation-defective myogenic clonal variant cell lines have provided new evidence on the functions of laminin-1 and merosin in myogenic differentiation. The precise causes of the defective properties exhibited by these cells are unknown and likely to be complex. However, that the correction of their phenotypes could be achieved by the addition of either laminin-1 or merosin as ECM protein-overlays, or by transfection with the merosin α2 chain cDNA, indicates that the lack of expression of laminin α1 and/or merosin α2 chains observed in these variants were in fact the critical defects.

The present study, along with others which have focused on the role of laminin-1 in myogenesis, indicate that merosin and laminin-1 play both similar and distinct roles at specific stages of the myogenic differentiation process: laminin-1 promotes myoblast proliferation, both proteins promote fusion, but only merosin promotes myotube stability. A specific role for laminin-1 in myoblast proliferation is supported by the demonstration that it acts as a potent promoter of this process in vitro (Foster et al., 1987; Öcalan et al., 1988; Goodman et al., 1989*b*; von der Mark and Öcalan, 1989) and by the observations that it is the

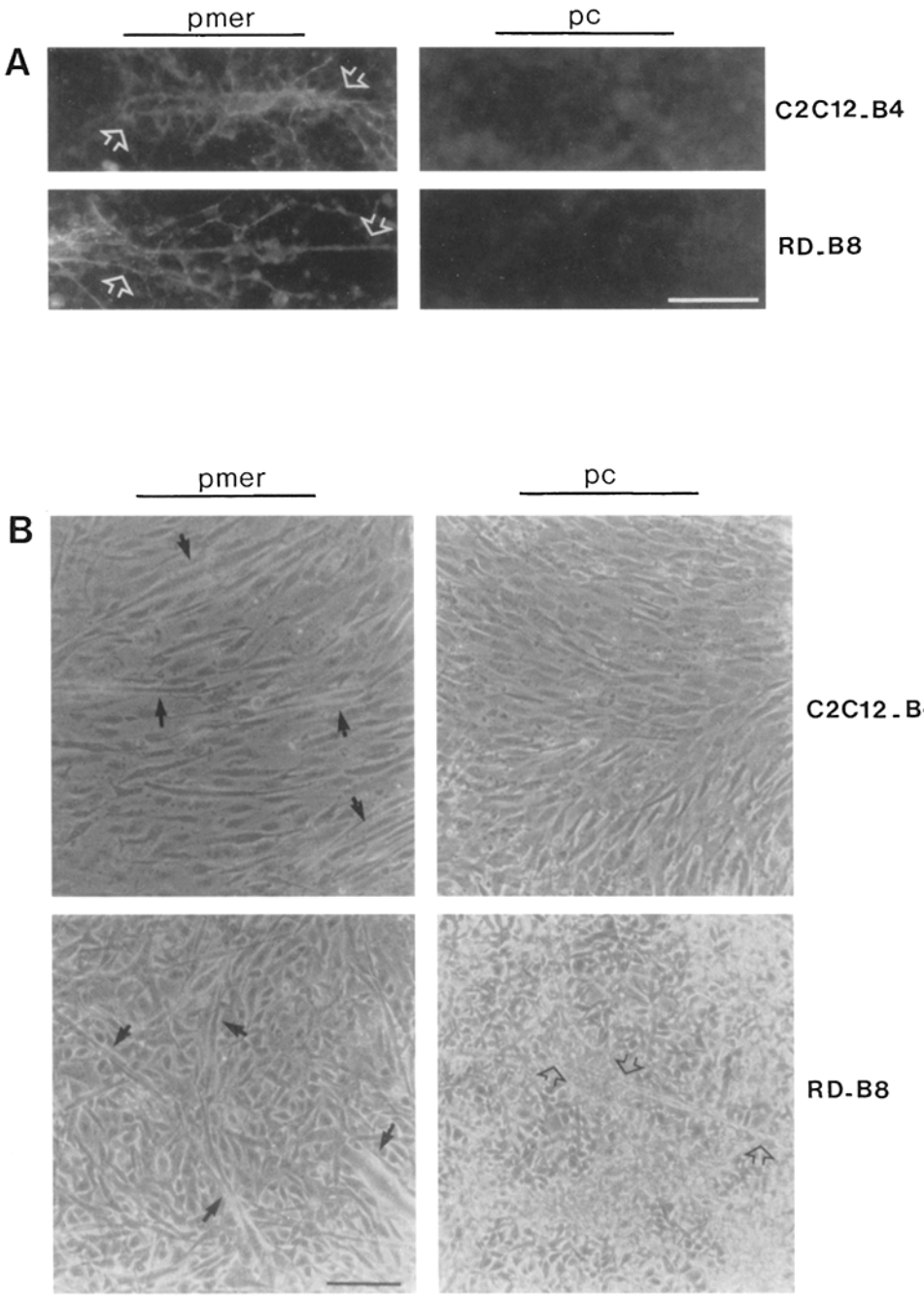

Figure 7. Effect of transfection of myogenic differentiation-defective cells with a full length human α2 chain cDNA. Selected clones stably transfected with either pc, the expression vector without any insert (control), or pmer, the expression vector containing the full-length human α2 chain cDNA under regulation of the human cytomegalovirus promoter. (*A*) Clones of transfected cells were cultured under differentiation conditions until 6 d postconfluence, fixed, permeabilized and then stained for the α2 chain of human merosin with a human-specific monoclonal antibody. Representative clones of the C2C12.B4 and RD.B8 cells are shown. Arrows point to a single myotube. (*B*) Morphology of C2C12.B4 and RD.B8 transfected cells. Arrows point to multinucleated, stable myotubes or to degenerating, unstable myotubes which are surrounded by dead cells. Bars: (*A*) 25 μm; (*B*) 50 μm.

predominant laminin molecule expressed until myoblast fusion is initiated (this study; Kühl et al., 1982; Mayne and Sanderson, 1985; Sanes et al., 1986; Foster et al., 1987; Klein et al., 1990; Kroll et al., 1994). Furthermore, laminin-1 expression is a common feature of proliferating myoblasts (Kühl et al., 1982; Sanes et al., 1986; Foster et al., 1987; Kroll et al., 1994) and regenerating muscle fibers (Hayashi et al., 1993; Tomé et al., 1994; Xu et al., 1994*a*). Although it remains possible that merosin shares with laminin-1 a role in the proliferation of myoblasts, the fact that it is expressed later than laminin-1 and coincident with fusion and myotube formation (this study; Schuler and Sorokin, 1995; Sewry et al., 1995) implies that its primary role is different.

Our data do indicate that both proteins can perform similar functions in myogenesis, specifically in the promotion of myoblast fusion. A role for laminin-1 in this process has been suggested previously (Kühl et al., 1982; Foster et al., 1987; von der Mark and Öcalan, 1988; Volk et al., 1990), but such a function for merosin is demonstrated for the first time here. That these two molecules are capable of exerting similar activities is not surprising, considering the similarities in their structural and functional domains (Leivo and Engvall, 1988; Ehrig et al., 1990; Paulsson et al., 1991; Vuolteenaho et al., 1994; Wewer and Engvall, 1994). Accordingly, myoblasts adhere equally well to them (Schuler and Sorokin, 1995) and both proteins have been shown to perform similar functions in other biological pro-

Table II. Effects of Stable Transfection with a Full-length Human Merosin α2 Chain cDNA on Myotube Formation and Stability

Transfectant*	No. of clones selected	Myotubes/ mm² at 6 PC‡	Myotube degeneration§
C2C12/pc	15	13.6 ± 2.6	no (15/15)
C2C12//pmer	30	21.5 ± 2.4	no (30/30)
C2C12.B4/pc	15	0.0 ± 0.0	n.a.‖
C2C12.B4/pmer	20	8.0 ± 1.3	no (20/20)
RD.B8/pc	10	0.5 ± 0.2	yes (10/10)
RD.B8/pmer	20	9.7 ± 1.2	no (20/20)

*Cells were stably transfected with either pc, the expression vector without insert (control), or pmer, consisting of the expression vector containing the full-length human merosin α2 chain cDNA under the regulation of the human cytomegalovirus promoter. Selected clones were cultured in growth medium (until 0-d postconfluence) or differentiation medium (from 0 to 6 d postconfluence), in the presence of 400 μg/ml G418 sulfate.

‡The number of myotubes/mm² of culture surface was scored at 6 d PC for each clone analyzed. Values presented are mean ± SD from three separate cultures ($n \geq 50$). PC, postconfluence.

§Evidence (*yes*) or absence (*no*) of extensive myotube degeneration and cell death after 6 d postconfluence. Parentheses indicate the number of clones scored positive or negative over the total number of clones analyzed.

‖n.a., not applicable.

cesses, such as in the promotion of neurite outgrowth (Engvall et al., 1992). However, the biological relevance of the functional overlap between merosin and laminin-1 in myoblast fusion remains unclear. Our observations that the laminin- and merosin-deficient RD.D2 and C2C12.B4 cells fail to undergo fusion, whereas the laminin-expressing but merosin-deficient RD.B8 cells do fuse, suggest that merosin may not be necessary for fusion while laminin-1 would be required. This is further supported by the fact that muscle fiber development apparently occurs normally in merosin-deficient cases of CMD (Hayashi et al., 1993; Tomé et al., 1994), as well as in the dystrophic *dy* and dy^{2J} mice (Sunada et al., 1994, 1995; Xu et al., 1994*a*,*b*). On the other hand, myoblasts lose their adhesion and responsiveness to laminin-1 just prior to fusion (Goodman et al., 1989*a*), a change in cell behavior which may occur in response to the emergence of merosin (this study) and to concomitant changes in the expression of cell surface receptors (Volk et al., 1990; Collo et al., 1993; Song et al., 1993; McDonald et al., 1995; Zhidkova et al., 1995; Belkin et al., 1996). Our protein-overlay assays further indicated a potential synergy of merosin and laminin-1 in myoblast fusion. Such synergy could possibly optimize fusion events and thus potentiate myotube formation during myogenesis in vivo. That myofibers are consistently smaller at birth in merosin-deficient cases of CMD (Hayashi et al., 1993; Tomé et al., 1994; Campbell, 1995) seems to support this. Therefore, further analysis of the respective contributions of laminin-1 and merosin in the fusion process will be required in order to resolve the inconsistencies between these observations.

A specificity of function for merosin in myogenic cellular processes has been inferred by the fact that it is the predominant laminin variant in basement membranes of developing and mature muscle fibers (Leivo and Engvall, 1988; Engvall, 1993; Schuler and Sorokin, 1995; Sewry et al., 1995). This assumption is further supported by the recent observations that myoblasts display faster cell spreading on laminin-2 than on laminin-1 (Schuler and Sorokin, 1995) and that merosin can exert functions in other tissues that are distinct from that of laminin-1, such as in the case of the differential modulation of brush border membrane hydrolase expression in intestinal epithelial absorptive cells (Beaulieu and Vachon, 1994; Vachon and Beaulieu, 1995). Herein, our results clearly established that merosin is essential for the stabilization of myotubes, whereas laminin-1 does not exert any effect on this process. Hence, this indicates that merosin performs a unique function in the promotion and maintenance of the structural integrity of myofibers. This novel, but critical, role of merosin partly explains the progressive deterioration of muscle fibers seen in CMD with merosin deficiency (Hayashi et al., 1993; Sunada et al., 1995; Tomé et al., 1994; Xu et al., 1994*a*,*b*; Hebling-Leclerc et al., 1995), which leads ultimately to severe histological abnormalities and degeneration.

That merosin and laminin-1 perform shared and distinct functions at specific stages of myogenesis raise the question of the cell surface receptors involved. Integrins are recognized as the principal mediators of ECM-cell interactions and signaling (Hynes, 1992; Ruoslahti and Reed, 1994; Roskelley et al., 1995), and their importance in myogenesis has been demonstrated in *Drosophilia*, *C. elegans* and avians (Volk et al., 1990; Engvall, 1994; Lassar et al., 1994; McDonald et al., 1995; Sastry et al., 1996). In mammalian muscle, the α7β1 integrin is regarded as the primary laminin-binding receptor (Collo et al., 1993; Song et al., 1993; Echtermeyer et al., 1996) and splicing variant forms of its two subunits are expressed differentially as a function of myogenic differentiation (Collo et al., 1993; Song et al., 1993; Zhidkova et al., 1995; Belkin et al., 1996). Hence, the α7Bβ1A complex is found in proliferating/undifferentiated myoblasts but is gradually replaced by the α7Aβ1D variant integrin as cells undergo fusion and myotube formation (Collo et al., 1993; Song et al., 1993; Zhidkova et al., 1995; Belkin et al., 1996). Such a switch appears strongly coincidental with the differential expression and stage-specific roles of laminin-1 and merosin described here. It is therefore likely that these variant integrins are mainly responsible for the mediation of the functions exerted by merosin and laminin-1 during the myogenic differentiation process.

Role of Merosin Deficiency in the Pathogenesis of CMD

Several of the muscular dystrophies characterized so far may be regarded as diseases of muscle cell adhesion and are caused by mutations in a number of proteins that link the cytoskeleton of the cell to the ECM (Engvall, 1994; Tinsley et al., 1994; Campbell, 1995), thus disrupting the cytoarchitecture of myofibers as well as compromising their binding to the basement membrane. There is accumulating evidence that the deterioration of myofibers seen in these diseases results from an increase in programmed cell death (Lockshin et al., 1995; Matsuda et al., 1995; Smith et al., 1995; Tidball et al., 1995). This may be expected, considering the importance of the ECM for the survival of cells in vitro and the demonstration that the disruption of cell attachment induces apoptosis (Meredith et al., 1993; Frisch and Francis, 1994; Ruoslahti and Reed, 1994; Boudreau et al., 1995). In this context, the correla-

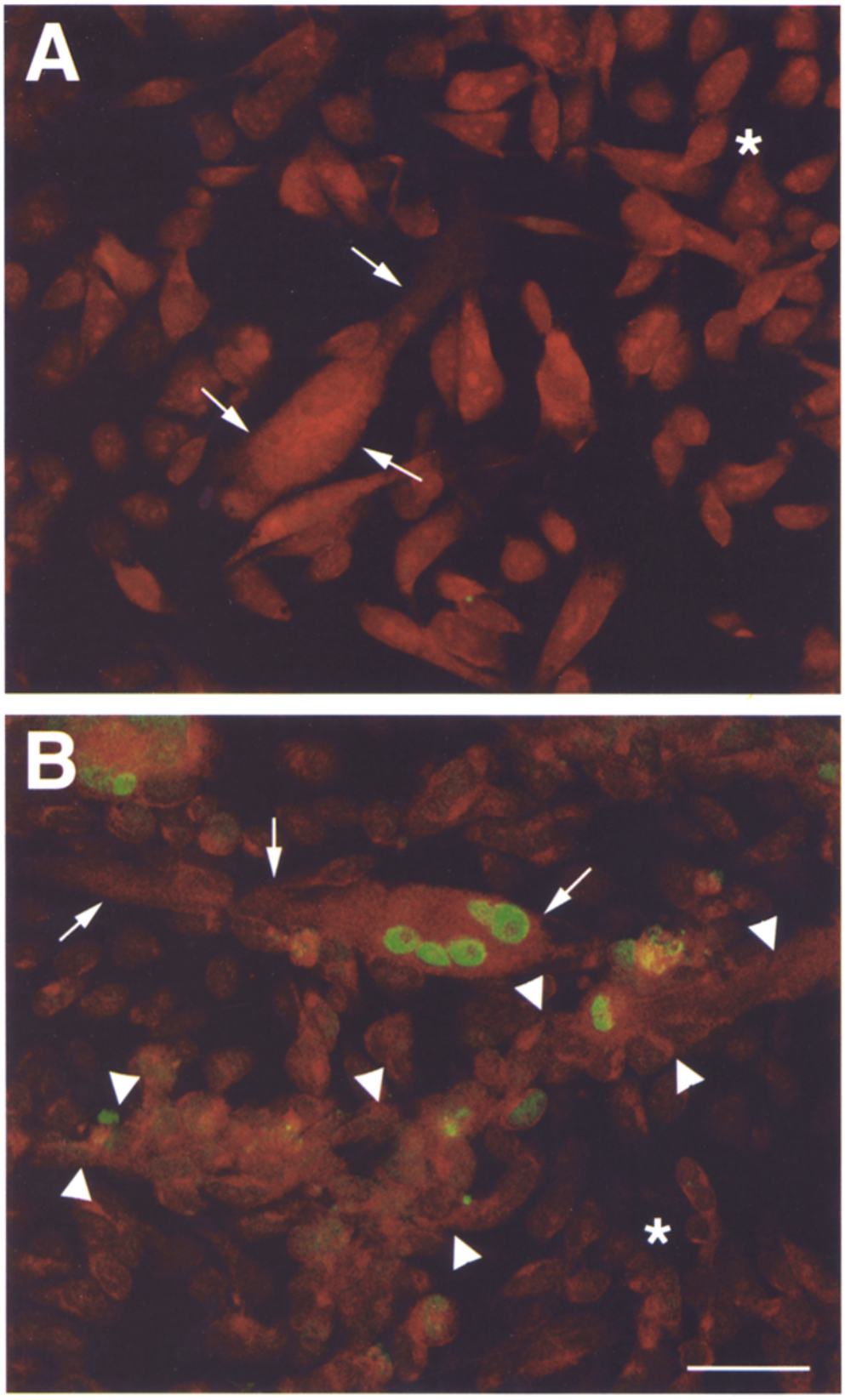

Figure 8. Myotubes formed by merosin-deficient myoblasts undergo apoptosis. Representative double staining immunofluorescence micrographs of 3 d postconfluent cultures of the parental, myotube-stable RD cells (*A*) and the myotube unstable, merosin-deficient RD.B8 cells (*B*) after in situ dioxigenin-conjugated dUTP labeling of apoptosis-associated DNA strand breaks by the TUNEL method, followed by incubation with an FITC-conjugated anti-dioxigenin antibody (*green*) and counterstaining with Evans blue (*red*). Arrows point to a single myotube (*A* and *B*); arrowheads point to a degenerated myotube (*B*); and asterisks indicate unfused myoblasts (*A* and *B*). Bar, 10 μm.

tion between increased apoptosis and lack of merosin expression as revealed in the present study, in addition to the observation that extensive apoptosis does occur in *dy* and dy^{2J} muscle in vivo and in primary cultures (Vachon, P.H., and E. Engvall, unpublished observation), strongly support the concept that merosin deficiency in CMD results in undue apoptosis in muscle fibers, thus leading to their degeneration and subsequent necrosis.

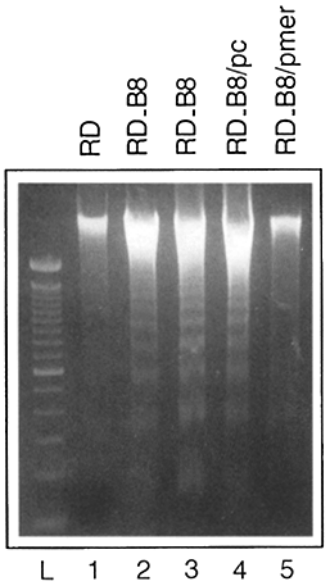

Figure 9. Lack of merosin is correlated with apoptosis in myogenic cells. DNA (20 μg/lane) was isolated from 3 (lane *2*) or 6 d (lanes *1*, *3–5*) postconfluent RD (lane *1*), RD.B8 (lanes *2–3*), RD.B8/pc (lane *4*) and RD.B8/pmer (lane *5*) cell cultures, and assayed for evidence of apoptosis-associated internucleosomal DNA fragmentation (DNA laddering). *L*, 100-bp DNA size markers.

The mechanisms involved in merosin-mediated survival of muscle cells remain unknown at this time. For instance, myotubes underwent rapid and massive degeneration in our merosin-deficient in vitro models, whereas such accelerated pace of the degenerative process of diseased muscle fibers clearly does not occur in vivo. In fact, CMD with merosin deficiency is a relatively slowly progressing disease. This points to the existence of multiple compensatory factors which may include continuous regeneration, other ECM components, and growth factors derived from myoblasts and/or other cell types in muscle tissues (Lockshin et al., 1995). As an example, the insulin-like growth factor II (IGF-II) was recently shown to inhibit muscle cell apoptosis in the dystrophin-deficient *mdx* mouse (Smith et al., 1995). Therefore, the identification of trophic factors that can functionally synergize with merosin in the prevention of apoptosis may provide a better understanding of myofiber survival in vivo.

In conclusion, the present findings provide key insight into the distinct roles of merosin and laminin-1 in myogenesis and CMD caused by merosin deficiency, and identify novel functions of merosin in myoblast fusion, myofiber stability, and muscle cell survival. The in vitro model systems featured here will be useful for delineating the signaling pathways underlying these and for further dissecting the molecular bases of muscle development and disease, as well as investigating potential treatments. In this respect, the rescue of the differentiation-defective RD.B8 and C2C12.B4 cells by transfection with a full-length merosin α2 chain cDNA shows promise in the development of therapeutic strategies involving myoblast transplantation coupled to gene therapy for the treatment of merosin-deficient CMD.

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