Expression of laminin isoforms in mouse myogenic cells in vitro and in vivo

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SUMMARY

The expression of laminin-1 (previously EHS laminin) and laminin-2 (previously merosin) isoforms by myogenic cells was examined in vitro and in vivo. No laminin α2 chain-specific antibodies react with mouse tissues, so rat monoclonal antibodies were raised against the mouse laminin α2 chain: their characterization is described here. Myoblasts and myotubes from myogenic cell lines and primary myogenic cultures express laminin β1 and γ1 chains and form a complex with a 380 kDa α chain identified as laminin α2 by immunofluorescence, immunoprecipitation and PCR.

PCR from C2C12 myoblasts and myotubes for the laminin α2 chain gene (LamA2) provided cDNA sequences which were used to investigate the in vivo expression of mouse LamA2 mRNA in embryonic tissues by in situ hybridization. Comparisons were made with specific probes for the laminin α1 chain gene (LamA1). LamA2 but not LamA1 mRNA was expressed in myogenic tissues of 14- and 17-day-old mouse embryos, while the laminin α2 polypeptide was localized in adjacent basement membranes in the muscle fibres. In situ hybridization also revealed strong expression of the LamA2 mRNA in the dermis, indicating that laminin α2 is expressed other than by myogenic cells in vivo. Immunofluorescence studies localized laminin α2 in basement membranes of basal keratinocytes and the epithelial cells of hair follicles, providing new insight into basement membrane assembly during embryogenesis.

In vitro cell attachment assays revealed that C2C12 and primary myoblasts adhere to laminin-1 and -2 isoforms in a similar manner except that myoblast spreading was significantly faster on laminin-2. Taken together, the data suggest that laminins 1 and 2 play distinct roles in myogenesis.

Key words: laminin, mouse muscle cell, basement membrane

INTRODUCTION

Myoblasts migrate in two successive waves from the myotomes of the somites to the limb anlagen and trunk during myogenesis in vivo. There they fuse to form the primary and the secondary myotubes which will form the bulk of the mature muscle (Rugh, 1968; Christ et al., 1986). Different factors including cytokines (Florini et al., 1991) and the cell adhesion molecules NCAM, M-cadherin, VCAM-1 and VLA4 (Rosen et al., 1992; Donalies et al., 1991; Hamshere et al., 1991) play crucial roles in such processes. In addition, the results of in vitro experiments suggest that extracellular matrix proteins influence the migration of myoblasts and their development to terminally differentiated myotubes. Primary mouse myoblasts preferentially migrate on mixtures of laminin/collagen type IV coated onto culture dishes, as opposed to fibronectin or collagen type I substrates (Kühl et al., 1986; Öcalan et al., 1988; Goodman et al., 1989). Furthermore, on binding to laminin, but not to fibronectin or collagen, murine myoblasts rapidly differentiate to myotubes (Kühl et al., 1986; von der Mark and Öcalan, 1989).

However, an anomaly exists: the laminin isoform used in these studies was extracted from the mouse Engelbreth-Holm-Swarm (EHS) tumour and is composed of α1, β1 and γ1 chains (nomenclature according to Burgeson et al., 1994; Timpl, 1989). The migratory and myogenic activity of this laminin-1 isoform is contained in the α1 chain in a proteolytic fragment known as E8 (Deutzmann et al., 1988; Goodman et al., 1987, 1989). However, in vivo the basement membranes of mature muscle fibres do not contain the α1, but rather the laminin α2 polypeptide (Leivo and Engvall, 1988; Sanes et al., 1990). In combination with either β1 and γ1 or β2 and γ1 chains, α2 characterizes laminins 2 (merosin) and 4 (S-merosin) (Leivo and Engvall, 1988; Paulsson and Saladin, 1989; Burgeson et al., 1994). The sequence identity of the laminin α2 and α1 chains in human and mouse does not exceed 47% (Vuolteenaho et al., 1994; Bernier et al., 1995). The question therefore arises: what is the significance of in vitro assays using laminin-1 and myogenic cells, and what might be the function of α2 containing laminins in myogenic tissues.

Myoblasts may migrate on and differentiate in response to the α1 chain of laminin-1 synthesized by other cells in vivo. However, there is evidence that both during normal development (Hughes and Blau, 1990) and in muscle regeneration (e.g. muscular dystrophy or muscle damage; Brand-Saberi et al., 1989; Bischoff, 1990), myoblasts migrate along and traverse muscle fibre basement membranes lacking laminin α1. It is also not clear whether α2-laminins are expressed during myogenic differentiation in vivo, in the myotome and limb anlage when myoblasts fuse to form myotubes. To investigate
its role in myogenesis, the expression of laminin α2 was studied in mouse myogenic cell lines and primary cultures during differentiation. Laminin-2 was expressed by both myoblasts and myotubes in vitro. A 0.5 kb section of the 3’ coding region of the gene coding for mouse laminin α2 chain (LamA2) was characterized and used to study the in vivo expression of LamA2 mRNA in murine embryonic tissues. mRNA expression was compared to the distribution of the laminin α2 polypeptide using monoclonal antibodies raised against mouse laminin α2 chain.

MATERIALS AND METHODS

Cell culture
Mouse myoblast cell lines (C2C12, G7 and G8) were from the American Tissue Culture Collection (access numbers CRL1772, CRL1447; CRL1456). They had the spindle-shaped morphology typical of myoblasts when maintained at subconfluency in Dulbecco’s minimal essential medium (DMEM) supplemented with 4.5 g/l glucose, 2 mM glutamine and 10% fetal calf serum (FCS). At confluency, the myoblasts spontaneously fused to form multinucleate myotubes, a process promoted by changing the culture medium to DMEM supplemented with 5% horse serum (HS). The % fusion ratio (% nuclei contained in myotubes/total nuclei) was consistently highest in C2C12 (70%). This cell line was, therefore, used to obtain myotube cultures. In addition, primary cultures of myoblasts were prepared from the thigh muscle of three day post-natal Balb/c mice as previously described (Kühl et al., 1982, 1986; Goodman et al., 1987). Cells were grown in DMEM supplemented with 20% HS, 1% chick embryo extract; fusion was induced by reducing the content of HS to 5% and chick embryo extract to 0.5%. Chick embryo extract is a homogenate of 9- to 10-day-old chick embryos diluted 1:1 with serum free DMEM and subsequently centrifuged to pellet debris. A mouse cell line derived from a parietal yolk sac sarcoma (PYS), known to express the laminin-1 isoform, was also employed (Weaver et al., 1987). The Rugli rat glioblastoma cell line (Goodman et al., 1987; Öcalan et al., 1988) was used in cell attachment assays. Both PYS and Rugli were maintained in DMEM supplemented with 4.5 g/l glucose, 2 mM glutamine and 10% FCS. All cell lines and primary myogenic cultures were maintained at 37°C and 7.5% CO2 in a water saturated environment.

Proteins and peptides
Laminin-1 was isolated from the Engelbreth-Holm-Swarm (EHS) mouse tumour as previously described (Paulsson et al., 1982, 1986; Goodman et al., 1987). Cells from the thigh muscle of three day post-natal Balb/c mice as previously described (Kühl et al., 1982, 1986; Goodman et al., 1987). Cells were grown in DMEM supplemented with 20% HS, 1% chick embryo extract; fusion was induced by reducing the content of HS to 5% and chick embryo extract to 0.5%. Chick embryo extract is a homogenate of 9- to 10-day-old chick embryos diluted 1:1 with serum free DMEM and subsequently centrifuged to pellet debris. A mouse cell line derived from a parietal yolk sac sarcoma (PYS), known to express the laminin-1 isoform, was also employed (Weaver et al., 1987). The Rugli rat glioblastoma cell line (Goodman et al., 1987; Öcalan et al., 1988) was used in cell attachment assays. Both PYS and Rugli were maintained in DMEM supplemented with 4.5 g/l glucose, 2 mM glutamine and 10% FCS. All cell lines and primary myogenic cultures were maintained at 37°C and 7.5% CO2 in a water saturated environment.

Polyclonal sera and monoclonal antibodies
Anti-desmin polyclonal antibodies used for the identification of myogenic cells were from Eurodiagnostics (Amsterdam). An affinity purified rabbit antibody against mouse laminin-1 which recognizes α1, β1 and γ1 chains (Klein et al., 1990) was employed in immunofluorescence, immunoprecipitation and immunoblotting experiments. Rat monoclonal antibodies raised against the E3 fragment of laminin-1 (nos 198, 200, 201) (Sorokin et al., 1992) were used as markers for the laminin α1 chain.

To investigate the distribution of the laminin α2 polypeptide in embryonic mouse tissues monoclonal antibodies specific for the mouse laminin α2 chain were raised. Lewis rats were immunized with laminin-2 purified from mouse hearts (Lindblom et al., 1994). Hybridomas were produced as previously described (Sorokin et al., 1992). Initial selection of hybridomas was based on specific reaction with mouse laminin-2 and not laminin-1 in ELISA. Further characterization of monoclonal antibodies included immunoblotting of the laminin α2 polypeptide and specific immunoprecipitation of 125I-labelled laminin-2.

Immunohistochemistry
Cell lines grown on glass coverslips and 5 μm sections of embryonic or adult mouse tissues were fixed in methanol at ~20°C, nonspecific protein binding was saturated by PBS containing 1% bovine serum albumin (BSA), and incubations with antibodies were subsequently carried out at room temperature. Bound antibodies were visualized using goat anti-rat IgG conjugated with FITC and goat anti-rabbit IgG conjugated with rhodamine second antibodies (Dianova GmbH, Hamburg, FRG). Slides were examined under a Zeiss Axiophot microscope equipped with epiluminescence.

To investigate the possibility that the epitopes recognized by the monoclonal antibodies specific for laminin α1 or α2 chains were masked in vivo, the cells/sections were subjected to various unmasking treatments following fixation. These included 0.1% acetic acid (Merck); 0.1% trypsin (Sigma) at pH 5; 2 ng/ml pepsin (Boehringer Mannheim) at pH 5; 1 mg/ml hyaluronidase (Boehringer Mannheim) at pH 5; 0.118 mg/ml neuraminidase (Boehringer Mannheim) at pH 5; or 1 U/ml heparitinase (Sigma) at pH 7.5. Immunofluorescence studies were subsequently carried out as described above.

ELISA
The specificity of the newly produced monoclonal antibodies for laminin-2 was tested in ELISA, as previously described (Dziadek et al., 1983).

Immunoprecipitation
Cell cultures were incubated for 1 hour in methionine-free MEM and then labelled for 4 hours (for immunoprecipitations from cell lysates) or overnight (for immunoprecipitations from culture media) with 50 μCi/ml [35S]methionine (Amersham, specific activity 1190 Ci/mmol). Culture medium was collected and employed directly for immunoprecipitations experiments. To ensure efficient extraction of radio-labelled laminin complexes, cells were solubilized overnight in lysis buffer (150 mM NaCl, 10 mM EDTA, 50 mM Tris-HCl, pH 7.4, 0.1% NP-40, 0.1% SDS, plus 1 mM N-ethylmaleimide and phenylmethyl-sulphonyl fluoride) (Paulsson et al., 1987). Characterization of the monoclonal antibodies raised against mouse laminin-2 also involved immunoprecipitations performed with 125I-labelled laminin-1 or laminin-2. Proteins were labelled using the iodogen-coated tube method (Fraker and Speck, 1978). 35S- or 125I-labelled proteins were immunoprecipitated using the affinity purified anti-laminin-1 antisera, laminin α1 or α2 chain specific monoclonal antibodies and Protein G-Sepharose (Pharmacia), and were subsequently analysed by SDS-polyacrylamide gradient gels under reducing conditions. All gels were fixed with methanol/acetic acid and, in the case of immunoprecipitations of 35S-labelled proteins, gels were enhanced with Amplify TM (Amersham-Buchler), dried and exposed to Kodak XAR films at ~70°C.

Immunoblotting
Cell lines were directly dissolved in an electrophoresis sample buffer containing SDS and DTT (Laemmlli, 1970). Approximately 4-5 μg of total protein extracted from PYs, myoblast or myotube cultures was separated on SDS-polyacrylamide gradient gels under reducing conditions and transferred to a nitrocellulose membrane (Towbin et al., 1979). Nonspecific binding to filters was blocked with 3% BSA containing 0.1% Tween-20. Filters were incubated for 2 hours at room temperature with anti-laminin-1 antisera, α1 or α2 chain specific monoclonal antibodies. Bound antibodies were visualized by biotinylated anti-rabbit IgG or anti-rat IgG, followed by peroxidase-
Northern blot analysis

Total RNA was isolated from the myogenic cells as described by Chirgwin et al. (1979). Poly(A)+ mRNA was isolated by two passages through an oligo (dT)-cellulose column (Pharmacia mRNA purification kit, Uppsala, Sweden) and the final poly(A)+ fraction was 1-3% of the total RNA loaded; 15 μg of total RNA or 5 μg mRNA was denatured with formamide and electrophoresed on a 1% agarose gel (Maniatis et al., 1982). After transfer (Thomas, 1980) to Hybond-N filter (Amersham-Buchler) the filter was fixed by exposure to UV light. Filters were preincubated for 2 hours at 42°C in 50% formamide, 2.5x Denhardt’s solution, 2x SSC, 0.1% SDS and 50 μg/ml herring sperm DNA. Hybridizations were performed in the same solution for 16-20 hours at 42°C with the following mouse cDNA probes: a 1.5 kb cDNA covering nucleotides 7,786-9,286 in the 3’ end of the coding region of the mouse LamA1 mRNA (LAC) (Deutzmann et al., 1988); a 1.1 kb cDNA corresponding to nucleotides 267-1,367 near the 5’ end of the LamA1 mRNA (clone LA1) (Hartl et al., 1988) and a 4.5 kb cDNA covering nucleotides 6,382-7,642 (Sasaki et al., 1988) and for LamB1 mRNA, a 2.7 kb cDNA covering nucleotides 2,580-5,300 (Oberbäumer, 1986). Control hybridizations with the same filters were performed using the 1.2 kb PstI fragment of murine β-actin cDNA clone pAL41 (Minty et al., 1983). Probes were labelled to a specific activity of 0.5-1.0×10⁶ cpm/μg cDNA by the primer extension method (Sambrook et al., 1989). Following hybridization, filters were washed several times with a final stringency of 2x SSC, 0.1% SDS at 52-62°C, and exposed to Kodak XAR films at −70°C. mRNA sizes were estimated using DNA molecular mass markers (Boehringer).

Reverse transcriptase-polymerase chain reaction (PCR) analysis

PCR analysis was employed to investigate the LamA2 mRNA and possible low expression of LamA1: 5 μg total RNA from G7, G8, C2C12 and PYS was transcribed to cDNA using Moloney murine leukemia virus RNAse H− reverse transcriptase (Gibco) and hexameric random primers. The cDNA was amplified by Taq DNA polymerase (Appligene) at 1.5 mM MgCl₂ for LamA1 primers and at 7.5 mM MgCl₂ for the LamA2 primers. The primers used for the amplification were constructed from: (1) sequences of the mouse LamA1 mRNA covering nucleotides 6,430 to 6,907: DNA sense strand 5’CACAGGCCAATCCGATTTGCC3’; antisense strand 5’CACAGGCCTTCCGATTTGCC3’; (2) and (2) sequences of the rat LamA2 mRNA (Engvall et al., 1992): DNA sense strand 5’AAAG-TATCTGTGCTTCAAGGA3’; antisense strand 5’AGTGAAATT- TTACACACGTTACG3’. The LamA1 primers were used under the following conditions: 5 cycles at 94°C for 0.7 minute, 50°C for 1 minute, 72°C for 1.5 minutes, followed by 30 cycles at 94°C for 0.7 minute, 65°C for 1 minute and 72°C for 1.5 minutes. LamA2 primers were used as follows: 30 cycles at 94°C for 0.7 minute, 55°C for 1 minute and 72°C for 1.5 minutes. Control PCR reactions were performed using primers constructed from the rat β-actin sequence. PCR products were analysed by electrophoresis in 1-2% agarose gels.

Sense primers contained an EcoRI site and the antisense primers contained a BamHI site to allow cloning of PCR products into pUC18 vectors for subsequent sequence analysis, or into pGEM3-blue vector (Promega Biotech) for in situ hybridization.

In situ hybridization

Probes used for in situ hybridization studies included the LamA2 PCR product described above, and a 1.5 kb cDNA covering nucleotides 7,786-9,286 in the 3’ end of the coding region of the mouse LamA1 mRNA (LAC). Mid-sagittal frozen sections of mouse embryos were employed for in situ hybridization which was carried out according to the method of Aigner et al. (1993). No signals above background level were observed in the routinely performed control hybridizations employing sense transcripts of comparable specific activity. Slides were exposed to Ilford K2 emulsion for 10-15 days and after development were stained with 0.02% toluidine blue. Slides were examined on a Zeiss Axiophot microscope using bright-field and dark-ground illumination.

Functional assays

To investigate the roles of lamins 1 and 2 in myoblast attachment, in vitro cell attachment assays were performed as previously described (Goodman et al., 1987). The Rugli rat glioblastoma cell line, attachment of which on laminin-1 is well characterized, was also employed in these experiments. Microtiter plates were coated with varying concentrations (0.3-30 μg/ml) of mouse laminin-1, mouse laminin-2, human laminin-2, or BSA as described by Ocalan et al. (1988). After 90 minutes, the number of adhering cells was determined by colorimetric analysis of lysosomal hexosaminidase (Landegren, 1984). The time course of adhesion to laminins was studied on plates coated with 10 μg/ml protein.

RESULTS

Characterization of laminin α2 chain-specific monoclonal antibodies

To investigate the expression of laminin α2 polypeptide in the mouse, monoclonal antibodies were raised against mouse laminin-2. It was necessary to produce specific antibodies since those available do not react in mouse and function only in immunoblots of the purified laminin-2 isoform. Based on specific reaction with laminin-2 in ELISA, the 4H8-2, 4H8-4, 4H8-9 and 8G11D10 hybridomas were chosen. ELISA results obtained with 4H8-2 are illustrated in Fig. 1. All monoclonal antibodies immunoprecipitated exclusively 125I-labelled-laminin-2 but not laminin-1, while the laminin α1 chain specific monoclonal antibody, 198, immunoprecipitated only laminin-1 (Fig. 2A). Two antibodies, 4H8-2 and 8G1-D10, were functional in immunoblots of purified laminin-2 and gave distinct patterns of results: 4H8-2 stained predominantly a polypeptide of 300 kDa, while 8G11-D10 recognized a 150 kDa polypeptide (Fig. 2B). It has been shown that a 150 kDa fragment is the major proteolytic fragment of the 300 kDa laminin α2 chain (Paulsson et al., 1991). The results, therefore, suggest that 8G11-D10 recognizes this 150 kDa fragment, while 4H8-2 reacts with the intact 300 kDa molecule. Both antibodies reacted weakly or not at all on cell lysates of myoblasts or myotubes suggesting a low affinity for denatured laminin α2 chain (results not shown).

In vitro expression of laminin chains by myogenic cells

Immunofluorescence revealed that myoblasts derived from G7, G8, C2C12, and primary myogenic cultures express laminin extracellularly and intracellularly around the nucleus. A similar staining pattern was seen with the laminin α2 chain specific monoclonal antibodies, 4H8-2 and 8G11-D10, and is illustrated in Fig. 3A. Myotubes generated from C2C12 and from primary myoblast cultures also express laminin but, in contrast
to myoblasts, staining was principally extracellular and occurred in patches on the cell surface (Fig. 3C,D). Laminin α1 chain specific monoclonal antibody (198, 200, 201) did not stain myoblast or myotube cultures, regardless of various pretreatments designed to unmask inaccessible epitopes (see Materials and Methods) (Fig. 3B).

To investigate the chain composition of the laminin isoforms expressed by myoblasts and myotubes in vitro, immunoblotting was performed on cell lysates with an anti-laminin-1 polyclonal antibody which reacts with α1, β1 and γ1 chains. Using equal amounts of protein, only polypeptides of approximately 200 kDa were detected in myoblast and myotube cell lysates, and no 400 kDa laminin α1 chain was visible (Fig. 4). Control immunoblots of adult mouse skeletal and cardiac muscle extracts using the same polyclonal antibody also revealed the 200 kDa bands which probably represent the laminin β1 and γ1 chains (results not shown). Polyclonal anti-laminin-1 immunoprecipitated a complex of [35S]methionine-labelled proteins from myoblast and myotube cell lysates and conditioned media with molecular masses of approximately 380 kDa, 200 kDa and 150 kDa (Fig. 5A). The same pattern was obtained with laminin α2 chain specific antibodies (shown in Fig. 5B for C2C12 myoblasts). Immunoprecipitations performed with laminin α1 chain specific monoclonal antibody (198) revealed the 400 kDa α1 chain and coprecipitated β1 and γ1 chains at approximately 200 kDa from PYS cell lysates and from conditioned medium (Fig. 5C). In myoblast or myotube cell lysates or conditioned media only a band non-specifically precipitated at 250 kDa was evident which was shown to be fibronectin by immunoblotting (results not shown). Fibronectin was also non-specifically precipitated with polyclonal anti-laminin-1 serum and anti-laminin α2 monoclonal antibodies (Fig. 5A,B).
Northern blot analysis confirmed the expression of the genes coding for laminin β1 (LamB1) and γ1 (LamC1) chains in C2C12 myoblast and myotube cultures, and in the control cell line PYS (Fig. 6). Expression of the laminin α1 chain gene (LamA1) was investigated with three different cDNA probes which together code for the entire LamA1 mRNA; these failed to reveal a signal on myoblast or myotube mRNA despite a strong reaction with the 10 kb LamA1 mRNA in PYS (Fig. 6).

Human LamA2 specific primers have been used in RT-PCR of rat cell lines (Engvall et al., 1992) and mouse thymus samples (Chang et al., 1993). RT-PCR with these primers revealed the predicted 490 bp fragment with RNA from C2C12 myoblast and myotube cultures, and new born mouse skeletal muscle (Fig. 7). In contrast, the LamA2 primers gave no signal from the control PYS cell line (results not shown). LamA1 primers amplified the predicted 480 bp fragment from PYS cells, but not from G7, G8 (results not shown) or C2C12 myoblasts or myotubes (Fig. 7). Subsequent sequence analysis of the cDNA fragments obtained with LamA2 primers revealed higher homology to the C terminus of the mouse (95%) and human (89%) LamA2 mRNAs (Vuolteenaho et al., 1994; Bernier et al., 1995) than to the mouse LamA1 gene (59%) (data not shown).

**In vivo expression of laminin chains in myogenic tissues**

The biochemical and molecular data described above show that the G7, G8, C2C12 cell lines, primary myoblast cultures, and myotubes derived from C2C12 express the laminin-2 isoform consisting of β1, γ1 and α2 chains. To investigate whether a similar expression occurs in myogenic tissues in vivo, in situ hybridization and immunofluorescence were employed. Myogenesis occurs in two waves in the mouse, one at approximately 10-14 days of embryonic development and another between day 16 of embryonic development and day 3 of postnatal life (Christ et al., 1986). Mid-sagittal sections of mouse embryos of 14 and 17 days of gestation were therefore examined. The 490 bp PCR fragment corresponding to the 3′ coding region of LamA2 mRNA was used for in situ hybridization. For comparison, in situ hybridization was also performed with the LAC cDNA which codes for the equivalent region of LamA1 mRNA (Sasaki et al., 1988).

In both day 14 and 17 embryos, LamA2 and not LamA1 mRNA was expressed in the newly forming muscles of the limbs and torso and in cardiac muscle. Fig. 8 illustrates the similar distribution pattern of desmin-positive myogenic cells and LamA2 positive cells in the rib region of a day 17 embryo (compare Fig. 8B,E). The distribution of the laminin α2 polypeptide closely paralleled this pattern as revealed by immunofluorescent staining of muscle fibre basement membranes by 4H8-2 (Fig. 8D), but not 198, 200 or 201 (Fig. 8A).
In both embryonic and mature muscle, the distribution of the laminin α2 polypeptide was similar and was restricted to basement membranes of muscle fibres and peripheral nerves (Fig. 9A,B). Axons of peripheral nerves are ensheathed by myelinating or nonmyelinating Schwann cells and surrounded by the endoneurial basement membrane. Bundles of axon-Schwann cell units are encircled by a multilamellar cellular sheath called the perineurium which is also bounded by a basement membrane. Laminin α2 chain specific monoclonal antibodies stained the endoneurial but not the perineurial basement membranes of the peripheral nerves in immunofluorescence (Fig. 9B). No laminin α2 polypeptide was detected in blood vessel basement membranes (Fig. 9C), as previously reported (Sorokin et al., 1994).

In situ hybridization also revealed expression of LamA2 mRNA in various mesenchymal tissues (our unpublished data). In particular, LamA2 was strongly expressed in the mesenchymal cells of the dermis of the skin (Fig. 10A). At both stages of development examined, this expression was widespread throughout the dermis with some concentration in mesenchymal cells.

**Fig. 5.** Immunoprecipitation of [35S]methionine labelled laminin complexes from conditioned medium of PYS, and C2C12 myoblast (Mb) and myotube (Mt) cultures using polyclonal anti-laminin-1 (A), monoclonal antibodies specific for laminin α2, 4H8-2 (B), and α1, 198, (C), and without primary antibody (−). (A) Polyclonal anti-laminin-1 immunoprecipitated β1/γ1 laminin chains of approximately 200 kDa, and a 380 kDa polypeptide from myogenic cells. The same serum precipitated α1, β1 and γ1 laminin chains from the control PYS cell line. (B) Immunoprecipitation of myoblasts with 4H8-2 revealed β1/γ1 laminin chains and the 380 kDa polypeptide, confirming the identity of the 380 kDa band with laminin α2. The same results were found with myotubes. For comparison, immunoprecipitation of laminin-1 from PYS using polyclonal anti-laminin-1 is shown. (C) 198 immunoprecipitated laminin α1, β1 and γ1 chains only from PYS and showed no specific immunoprecipitation of laminin complexes from myoblasts; the same pattern of results was observed with myotubes. Non-specific precipitation of fibronectin (FN) from myoblast and myotube conditioned medium was evident when immunoprecipitations were performed with either polyclonal anti-laminin-1 or monoclonal antibodies and is probably due to non-specific reaction with Protein G-Sepharose.

**Fig. 6.** Northern blot analysis of genes coding for laminin α1 (LamA1), β1 (LamB1), γ1 (LamC1) and β-actin in C2C12 myoblasts (Mb), C2C12 myotubes (Mt) and PYS cells. The 10 kb LamA1 mRNA was detected with the LAC probe in PYS cells only. LamB1 and LamC1 specific probes hybridized to the expected 6 kb and 8 kb mRNAs, respectively, in myogenic and PYS cells.

**Fig. 7.** PCR analysis of LamA1 and LamA2 gene expression in myogenic cells. mRNA was isolated from C2C12 myoblasts (Mb) and myotubes (Mt), new born mouse skeletal muscle (M) and the control PYS cell line. RT-PCR using primers specific for the genes coding for laminin α1 and α2 revealed only LamA2 gene sequences in myogenic cells and LamA1 gene sequences only in the control PYS cell line. Φ, DNA size markers.
mal cells at the base of hair follicles. Immunofluorescence localized the laminin α2 polypeptide in basement membranes underlying the basal keratinocytes of the epidermis and surrounding the epithelial follicle cells (Fig. 10C). In contrast, LamA1 mRNA expression was weak in the skin of 14- and 17-day-old embryos and was restricted to the epithelial cells of the epidermis (Fig. 10B). It was often difficult to interpret whether expression of LamA1 occurred in the epidermal cells because most probes concentrated at the edge of the section. However, the laminin α1 polypeptide was found in basement membranes of the basal keratinocytes of the epidermis (Fig. 10D), suggesting that hybridization of the LAC probe to the epidermis was indeed higher than background. In addition, laminin α1 polypeptide was detected in basement membranes of the epithelial cells constituting hair follicles and glands, but not in endothelial cell basement membranes (results not shown).

**Functional assays**

Since laminin-1 plays a significant role in myoblast attachment and differentiation, the ability of C2C12 and primary myoblasts to attach to laminin-2 as compared to laminin-1 was investigated using in vitro assays. The Rugli glioblastoma cell line was also employed since the attachment of this and similar glioblastoma cell lines (Engvall et al., 1992; Brown and Timpl, 1994) to mouse laminin-1 and human laminin-2 substrates has been characterized. Hence, Rugli in attachment assays served as an internal control for the integrity of the laminin substrates. C2C12 myoblasts and primary myoblasts bound with similar affinities to both laminin isoforms regardless of the species of origin (Fig. 11A). In contrast, Rugli cells bound to mouse laminin-1 and human laminin-2 with higher affinity than to mouse laminin-2 (Fig. 11A). These differences may be due to species differences in laminin-2 substrate. The time course of myoblast attachment was comparable between all three laminin sources, but the time required for cell spreading varied considerably according to substrate: Myoblasts spread within 5-10 minutes of incubation on mouse or human laminin-2 (10 μg/ml), while on the same concentration of mouse laminin-1 they maintained a rounded morphology for up to 20-30 minutes of incubation (Fig. 11B). The time required for spreading of Rugli cells did not vary with laminin substrate, and ranged from 20-40 minutes of incubation.
Fig. 9. Immunofluorescence staining of mature mouse skeletal muscle sections by polyclonal anti-laminin-1 (A), or monoclonal antibodies specific for laminin α2, 4H8-2 (B), laminin α1, 198 (C). Comparison of polyclonal anti-laminin-1 and 4H8-2 staining patterns localizes laminin α2 polypeptide in basement membranes of muscle fibres, and in endoneurial but not perineurial (arrows in A) basement membranes of peripheral nerves. No laminin α2 was detectable in basement membranes of arteries (a) or veins (v). Bar, 50 μm.

Fig. 10. In situ hybridization of LamA2 (A) and LamA1 (B) probes to a section through the limb of a 17-day-old mouse embryo. LamA2 expression was detected in skeletal muscle layers (m), but also in mesenchymal cells of the dermis (d) of the skin. Note that epithelial cells of hair follicles and the epidermis do not express LamA2 (arrowheads). Immunofluorescence localized the laminin α2 polypeptide (C) in basement membranes of the basal keratinocytes and epithelial cells of the hair follicles (arrowheads). The LamA1 specific probe hybridized only weakly to epidermal cells (B), while laminin α1 polypeptide occurred in basement membranes of the basal keratinocytes (D). The same pattern of results was observed for 14-day-old embryonic mice. Bar, 20 μm.
**DISCUSSION**

Immunofluorescence, immunoprecipitation and immunoblot experiments revealed that the major laminin isoform expressed by both myoblasts and myotubes in vitro is laminin-2 (previously merosin), whilst laminin-1 is not expressed. This was confirmed by northern blot analysis which demonstrated the expression of the genes coding for laminin β1 (LamB1) and γ1 (LamC1) chains, but not for the α1 chain (LamA1). Immunoprecipitation of radiolabelled laminins from myogenic cell lysates and from conditioned medium revealed that laminin β1 and γ1 chains were complexed with a 380 kDa α chain. Similarities in molecular mass suggest that this polypeptide is the laminin α2 chain. The recent sequencing of the mouse laminin α2 gene (LamA2) revealed a mRNA coding for a protein with a predicted molecular mass of 390 kDa (Bernier et al., 1995) that may be cleaved into a 300 kDa N-terminal segment and a 80 kDa C-terminal segment (Ehrig et al., 1990; Paulsson et al., 1991). The presence of laminin α2 in myogenic cells was substantiated by the monoclonal antibodies 4H8-2 and 8G19-D10, raised against mouse laminin-2 and shown here to be specific for the laminin α2 chain. Both monoclonal antibodies stained myoblasts and myotubes in vitro and immunoprecipitated radiolabelled laminin complexes from these cells. In addition, RT-PCR of myoblast and myotube mRNA using primers specific for the LamA2 gene resulted in a 490 bp fragment with higher homology to the human (89%) and mouse (95%) LamA2 genes than to the mouse LamA1 gene (59%).

In situ hybridization demonstrated the expression of LamA2 but not LamA1 mRNA in developing myogenic tissues of day 14 and 17 embryonic mice, while immunofluorescence localized the laminin α2 polypeptide in muscle fibre basement membranes at both stages of development. In contrast, the laminin α1 polypeptide was not expressed in developing muscle of the embryos, indicating that the α2 and not the α1 laminin chain is present when myogenic differentiation and migration occurs in vivo. In skeletal or cardiac muscle of adult mice, however, expression of LamA2 mRNA was not detectable. The corresponding expression pattern of laminin α2 polypeptide in these tissues revealed its localization in the basement membranes of the mature muscle fibres and in endoneurial but not perineurial basement membranes of peripheral nerves.

For want of appropriate tools, few studies have attempted to analyse the chain composition of laminins expressed by myogenic cells in vitro. Only Kroll et al. (1994) have made the attempt using MyoD-transfected C3H10T1/2 fibroblasts. Transfection with MyoD was associated with induction of cells along the myogenic lineage and, in contrast to the data presented here, expression of a polypeptide designated laminin Ac3H (350 kDa) which the authors state is distinct from laminin α2. In addition, the laminin α1 chain was shown to be expressed by various clones of cells arising from MyoD-transfected cultures. A likely explanation for such discrepancies is differences in the types of cells used. It cannot yet be excluded, however, that the 350 kDa laminin chain expressed by the MyoD transfected cells is not the laminin α2 chain, since immunoblots or PCR experiments were not performed to investigate the possible expression of the laminin α2 polypeptide or mRNA.

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**Fig. 11.** (A) In vitro attachment of C2C12 myoblasts and the Rugli glioblastoma cell line to varying concentrations of mouse laminins 1 (○) and 2 (▲), and human laminin-2 (■). Cell attachment was measured after 90 minutes of incubation with the substrates. (B) The degree of myoblast spreading after 5 minutes incubation on 10 μg/ml mouse laminin-1, mouse laminin-2 or human laminin-2.
The expression of laminin-2 by myoblasts and myotubes in vitro is consistent with the predominant distribution of this laminin isoform in mature myogenic tissues of man, rat, rabbit and guinea pig (Ehrig et al., 1990; Sanes et al., 1990). The in vivo expression of laminin α2 in basement membranes of mature muscle fibres and peripheral nerves observed here is also comparable to those previously described in rabbit (Ehrig et al., 1990; Sanes et al., 1990). However, the present study is first to correlate expression of the LamA2 mRNA and its corresponding protein in embryonic muscle. Using an antibody directed to the 80 kDa fragment of human α2 laminin chain (4F11), Leivo and Engvall (1988) investigated expression of the laminin α2 polypeptide in skeletal muscle of day 15 embryos and new born rats. In contrast to the data presented here, laminin α2 polypeptide was detected only in new born rat muscle. Such discrepancies may relate to species differences or, more likely, to antibodies employed and the developmental stages investigated. It is unlikely that the epitope recognized by 4F11 is masked in tissues, since various unmasking techniques were used. Rather, the affinity of this anti-human antibody for the rat epitope may be low so that significant staining was observed only when sufficient laminin α2 chain was deposited into the basement membranes at relatively late stages of development. We found here that the intensity of immunofluorescent staining for α2 laminin is indeed considerably weaker in embryonic than in mature muscle. Furthermore, in vitro laminin is organized in patches on the myotube cell surface and only with fibre maturation does the basement membrane form a continuous envelope (Kühl et al., 1982). In vivo, the muscle fibres continue development after birth (Christ et al., 1986) and laminin is being deposited into the forming basement membranes throughout fibre development.

The expression of α2-containing laminins in embryonic muscle is not incongruous with a role for the laminin α1 chain in myogenesis, as suggested by in vitro studies (Öcalan et al., 1988; Kühl et al., 1986; Goodman et al., 1989). It is possible that myoblasts migrate to the sites in the body where they will eventually form muscle by adhering to α1 chain containing laminins expressed by other cells. This is supported by the observation made in the present study that both laminins 1 and 2 support myoblast attachment to similar extents in in-vitro assays, and that the affinity of myoblast binding to both isoforms is comparable. However, the observation that myoblasts spread more quickly on mouse or human laminin-2, as compared to laminin-1, may hint that the two isoforms are functionally distinct. Myoblasts bind to laminin-1 via the α7β1 integrin (von der Mark et al., 1991). At present, no conclusions can be made on whether the same receptor mediates binding to laminin-2. However, the spreading results show that the intracellular signals which arise from myoblast adhesion to laminins 1 and 2 differ. It has been shown that muscle fibres can attach to the basement membrane via the laminin α2 chain and α-dystroglycan on the surface of muscle fibres, and the absence of the laminin α2 chain from muscle fibre basement membranes in congenital muscular dystrophies results in muscle degeneration (Sunada et al., 1994; Xu et al., 1994, 1995). These results suggest that laminin α2 interactions with α-dystroglycan are essential for muscle fibre stability and the consequent maintenance of the myogenic phenotype. Therefore, laminin-2 may play a more significant role in myoblast differentiation.

Apart from its expression in myogenic cells, LamA2 mRNA is strongly expressed in mesenchymal cells, in particular those of the dermis of the skin. Clearly, non-myoogenic cells also have the capacity to secrete α2 containing laminins. This supports the mRNA data reported by Vuolteenaho et al. (1994) for human fetal tissues. Similarly, Simon-Assman et al. (1994) have reported that laminin-2 is located in the basement membrane of intestinal epithelial cells in the developing rat, surrounding the base of the crypts where cell proliferation and differentiation occur. Here we correlate the distribution of the laminin α2 mRNA and polypeptide, a comparison which showed that although LamA2 mRNA is expressed by mesenchymal cells, the protein is deposited into adjacent basement membranes. This is true not only of the skin, but also of other organs including lung, kidney and testis (our unpublished data). Such observations provide new information on the assembly of basement membranes during embryogenesis, since similar data is available only for the laminin α1 polypeptide and mRNA both of which are expressed principally by epithelial cells. The restricted deposition of laminin α2 polypeptide in comparison to the widespread expression of its mRNA suggests successful expression only in the vicinity of the basal keratinocytes and hair follicles where laminin α2 is deposited into basement membranes. Alternatively, a balance between protein synthesis and degradation may be involved in establishing the observed laminin α2 distribution pattern.

In conclusion, the data presented here show that laminin α2 containing isoforms are expressed by myoblasts and myotubes in vitro and in vivo, and that laminins 1 and 2 play distinct roles in myogenesis in the mouse. In addition, the in vivo expression of LamA2 mRNA and its polypeptide provides new information on mechanisms of basement membrane assembly during embryonic development.

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