Developmental Regulation of the Laminin $\alpha_5$ Chain Suggests a Role in Epithelial and Endothelial Cell Maturation

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We have previously shown that mouse and bovine endothelial cells express a novel 400-kDa laminin $\alpha$ chain complexed to $\beta_1$ and $\gamma_1$ laminin chains. We describe here purification of this laminin isoform from the conditioned medium of a mouse peripheral lymph node endothelial cell line, SVEC. The laminin $\alpha$ chain was isolated from the laminin complex, subjected to Edman digestion, and the amino acid sequences of the resulting peptides were determined. Amino acid sequence revealed 100% identity to the predicted amino acid sequence of the recently reported laminin $\alpha_5$ gene. A monoclonal antibody to the laminin $\alpha_5$ chain was raised (4G6), allowing investigation of its distribution in embryonic, newborn, and mature mouse tissues. The laminin $\alpha_5$ chain was expressed mainly by epithelial, endothelial, and myogenic cells: In both embryonic and mature tissues the laminin $\alpha_5$ chain was strongly expressed by epithelial cells, the bronchi of the lungs and the developing kidney tubules being the sites of strongest expression. However, laminin $\alpha_5$ was not associated with early stages of epithelial cell development, but rather with epithelial cell maturation. Widespread expression of laminin $\alpha_5$ in endothelial cells was apparent only in tissues of mature mice, its appearance correlating approximately with sexual maturity. During embryogenesis and in newborn tissues, laminin $\alpha_5$ occurred in basement membranes of larger blood vessels only, excluding a role in angiogenic processes. Smooth muscle and skeletal muscle cells were the only other cell types which showed considerable laminin $\alpha_5$ expression, with skeletal muscle exhibiting a developmentally regulated pattern of expression: The laminin $\alpha_5$ chain occurred in skeletal muscle fiber basement membranes early in embryogenesis (E13–E15) but decreased with development, remaining strongly expressed only at the neuromuscular junction. The data show that laminin $\alpha_5$ expression is associated with epithelial and endothelial cell maturation, implicating a role for this laminin chain in the maintenance of differentiated epithelial and endothelial cell phenotype.

INTRODUCTION

The laminins are a family of basement membrane proteins, each member of which is a heterotrimer composed of an $\alpha$, $\beta$, and $\gamma$ chain, linked by disulphide bonds. To date 10 genetically distinct laminin chains have been identified which may combine to form at least 10 different laminin isoforms (Delwel and Sonnenberg, 1996; Miner et al., 1995; Iivanainen et al., 1995). Laminin-1 was the first of the laminin isoforms to be identified (Timpl et al., 1979). This together with the relative ease of isolation of large amounts of this protein from a mouse tumor (Engelbreth-Holm-Swarm tumor) has led to the accumulation of considerable information on laminin-1 protein and gene structure and on its functional significance (reviewed in Engel, 1993; Delwel and Sonnenberg, 1996). Laminin $\alpha_1$, $\beta_1$, and $\gamma_1$ chains share homologous structures which include globular domains (domains IV and VI), rod-like domains containing EGF-like repeats (domains III and V), and domains forming the $\alpha$-helical coiled-coil of the long arm of the molecule (domains I and II). In addition, the laminin $\alpha$ chain contains a large C-terminal globular domain with five internal repeat motifs (G domain). This domain structure is conserved in all of the variant laminin chains identified to date. Gene sequence data is available for many of the laminin chains, allowing comparisons of domain organization with laminin-1 and consequently deduction of structure–function relationships. In this way,
the G-domain of laminin α chains has been associated with cell binding, while EGF-like repeats in domain III are crucial for laminin assembly into basement membranes via nidogen binding, and domain VI is necessary for self assembly of the molecule (Yurchenco and Cheng, 1993; reviewed in Engel, 1993). Despite some overall structural similarity between different laminin isoforms, their tissue-specific distribution suggests that they have distinct functions which largely remain to be elucidated.

Recent data on inherited diseases involving laminin gene defects and on laminin β2-knockout mice have provided clues to the function of some laminin isoforms and have clearly shown that laminins play crucial roles in vivo. In epidermolysis bullosa junctionalis, a blistering skin disease, mutations in the human genes coding for laminin α3, γ2, and β3 chains which comprise the laminin-5 isoform have been described, demonstrating that this protein is essential for attachment of keratinocytes to their basement membrane (Aberdam et al., 1994; Pulkkinen et al., 1994; Kivirikko et al., 1995; Vidal et al., 1995; Valley et al., 1995; Gagnoux-Palacios et al., 1996). In the dy/dy mouse strain, a mutation has been described in the gene coding for laminin α2 resulting in significant reduction in the expression of the protein and a marked muscular dystrophy (Sunada et al., 1994; Xu et al., 1994). These results indicate that laminin α2 is necessary for terminal differentiation and/or function of muscle fibers. Eliminating the expression of the gene coding for laminin β2 has resulted in mice with impaired synapse formation and glomerular disfigurement (Nokes et al., 1995).

We have shown that endothelial cells in vitro express at least two different laminin α chains of 400 and 240 kDa (Sorokin et al., 1994). Mouse endothelial cell lines derived from mesenteric and peripheral lymph nodes, skin, embryonic hemangiomas, and brain capillaries all express the 400-kDa laminin α chain coupled to β1 and γ1 chains, while only a subpopulation of these cells express the truncated α chain which was recently identified as the laminin α4 chain (Frieser et al., 1997). In particular, mouse endothelial cell lines derived from skin hemangiomas and mesenteric lymph nodes, but not from brain or from embryonic hemangiomas, express the laminin α4 chain. Bovine aortic cells in culture also secrete predominantly the laminin α4 chain (Frieser et al., 1997; Sorokin et al., 1994; Tokida et al., 1990). These results suggest a functional distinction between the two laminin isoforms which may be related to the different functions carried out by endothelial in different organs. Here we report on the purification of the 400-kDa laminin α chain from the conditioned medium of a peripheral lymph node-derived cell line (SVEC). Amino acid sequence data reveals that this 400-kDa laminin chain is the product of the recently reported laminin α5 gene (Minner et al., 1995). Production of a monoclonal antibody against the laminin α5 chain (4G6) is described and its immunofluorescent staining pattern, in comparison to expression of laminin α5 mRNA, during mouse embryogenesis is reported. The temporal and spatial expression of laminin α5 in vivo provide important clues to the function of this molecule.

**MATERIALS AND METHODS**

**Cell Lines Employed and Culture Conditions**

Endothelial cell lines employed were derived from different mouse organs including skin (sEND1), mesenteric lymph nodes (mEND), and peripheral lymph nodes (SVEC) as described previously (Sorokin et al., 1994). A control cell line, PYS, derived from a mouse parietal yolk sac carcinoma which produces predominantly the laminin-1 isoform (Wewer et al., 1987) was also employed. All cells were grown in Dulbecco's minimal essential medium (DMEM) supplemented with 10% fetal calf serum, 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 tumor as previously described (Paulsson et al., 1987; M. Paulsson (University of Cologne, Germany) kindly supplied a mixture of laminin-2 and -4 isolated from mature mouse hearts (Lindblom et al., 1994). **Isolation of SVEC Laminin**

Most endothelial cells secrete insufficient laminin into culture to allow purification from conditioned medium as has been possible with other laminin isoforms. One exception was the SVEC cell line. Conditioned medium was therefore collected from this cell line and laminin isolated as follows: Cell culture supernatants (batches of 500 ml) from confluent SVEC cells were dialyzed against 50 mM NaHCO₃ containing 0.02% sodium azide and passed over a Heparin-Sepharose column (7 × 2.6 cm, Pharmacia). The flow through fraction was discarded, and bound proteins were eluted with 50 mM NaHCO₃ containing 0.5 M NaCl, 0.02% azide. This laminin-enriched fraction (ca. 35 ml) was further purified by affinity chromatography. For this purpose, affinity-purified polyclonal laminin-1 antibodies were coupled to BrCN-activated Agarose 150m (Bio-Rad) (with an exclusion limit of 150 million Daltons). It proved essential to use a packing with very large pores to allow laminin molecules, which tend to aggregate, to penetrate into the gel matrix. The heparin binding fraction was passed over the anti-laminin-1 column (10-ml bed volume), washed extensively with PBS buffer containing 0.5 mM NaCl, and eluted with 0.1 M triethylamine, 0.5 M NaCl, and immediately neutralized with 1 M Tris buffer, pH 7.5. Typically, about 40 μg of laminin was isolated from 1 liter of culture supernatant. Alternatively, preparative immunoprecipitation was performed to obtain smaller amounts of laminin for preparation of monoclonal antibodies. Affinity purified anti-laminin-1 antibodies (300 μg/ml) were incubated with formaldehyde-fixed Staphylococcus aureus cells for 3 h at 25°C, and subsequently crosslinked with 20 mM dimethylidipropionamidate in 0.2 M triethanolamine/HCl, pH 8.5. After purification by Heparin-Sepharose or ion exchange chromatography on Q-Sepharose, 100 ml fractions enriched in SVEC laminin were incubated overnight at 4°C with 4 ml of a 10% suspension of the adsorbent, centrifuged, washed with TBS buffer containing 1 mM

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Preparation of Peptides for Edman Degradation

Immunopurified laminin was precipitated by addition of an equal volume of acetone. After overnight incubation at 4°C, the precipitate was collected by centrifugation and the wet pellet dissolved in 0.2 M NH₄HCO₃ followed by 0.2 M NH₄HCO₃/acetonitril (1:1). The procedure was repeated twice. The gel pieces were lyophilized for 2 h and then rehydrated by adding three portions of 0.2 M NH₄HCO₃, 0.02% Tween 20, at 5-min intervals. The first aliquot added contained 1 μg trypsin per 100-μl gel pieces. Only as much liquid was used as was necessary to restore the original gel volume. Protease digestion was performed overnight at 37°C, the peptides were extracted twice with 5% trifluoracetic acid (TFA) and once with 2.5% TFA in 50% aqueous acetonitril. Alternatively, the gel pieces were repeatedly washed with 50 mM Tris buffer, pH 9.0, and 50 mM Tris buffer/acetonitril (1:1) and cleavage was carried out with a lysine-specific protease from Achromobacter (Wako) at pH 9.0 and 30°C. The peptides were then separated by HPLC on a reversed phase Nucleosil-120 C18 column using a linear gradient of 0–70% aqueous acetonitril in 20 mM ammonium acetate buffer. Because of the complexity of the peptide mixtures obtained the collected peptides were further purified by a second HPLC separation on a Vydac TP54 C18 column using the standard TFA/acetonitril gradient system.Sequence analysis was done on a Procise Protein Sequencing system (Applied Biosystems) according to the manufacturer's instructions.

Antibodies

An affinity purified rabbit antibody against mouse laminin-1 (308) which recognizes α1, β1, and γ1 chains (Klein et al., 1988) was employed in immunofluorescence and immunoprecipitation experiments. Rat monoclonal antibodies (MAb) specific for mouse laminin α1 (198) (Sorokin et al., 1992) and mouse laminin α2 (4H8-2) (Schuler and Sorokin, 1995) were used in ELISA and immunofluorescence experiments. Trimethyl-rhodamine isothiocyanate (TRITC)-conjugated α-bungarotoxin (Molecular Probes) and FITC-labeled goat anti-rat IgG. Sections were examined under a Zeiss Axiophot microscope equipped with epifluorescent optics.

Reversibility of Peptide Degradation

Cells were cultured for 1 h in methionine-free MEM and then labeled for 4 h with 50–100 μCi/ml of [35S]methionine (60 μCi/mmol). Culture medium was collected and the cells were washed and solubilized by gentle stirring overnight in lysis buffer (50 mM Tris–HCl, pH 7.4, 150 mM NaCl, 10 mM EDTA, 0.1% NP-40, 0.1% SDS plus protease inhibitors) (Paulsson et al., 1987). Labeled proteins were immunoprecipitated from cell lysates or conditioned medium using the affinity-purified anti-laminin-1 antiserum (308) or monoclonal antibodies and protein G-Sepharose (Pharmacia). All antibody incubations were performed overnight at 4°C. To test for the specificity of the 4G6 monoclonal antibody, immunodepletion experiments were performed as follows: Labeled proteins in cell lysates or conditioned medium were immunoprecipitated with 4G6 and protein G-Sepharose, samples were centrifuged at 4°C, the supernatant was removed and subsequently immunoprecipitated with affinity-purified anti-laminin-1 antiserum (308) and protein G-Sepharose. Immunoprecipitated proteins were analyzed by 3–10% SDS-polyacrylamide gradient gels. Gels were fixed with methanol/acetic acid, enhanced with Amplify TM (Amersham-Buchler), dried, and exposed to Kodak XAR films at –70°C.

Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR) Analysis

RT-PCR analysis was employed to investigate the expression of the laminin α5, α2, and α3 mRNA in mouse endothelial cells, and to obtain a laminin α5-specific cDNA probe for use in Northern blot and in situ hybridization experiments. Five micrograms total RNA isolated from sEND1, mlEND, SVEC, and PYS was transcribed to cDNA using Moloney murine leukemia virus RNase H-reverse transcriptase (Gibco) and random primers. The cDNA was amplified by Taq DNA polymerase (Perkin-Elmer) at 1.5 mM MgCl₂. The primers used for amplification of α2 and α3 sequences are those described in Schuler and Sorokin (1995) and Galliano et al. (1995), respectively. Primers for amplification of laminin α5 cDNA were constructed from the mouse laminin α5 sequence (Miner et al., 1995), covering nucleotides 8477–8931 (DNA sense strand 5' TGAATGAGGAGGTGGTCAGC; antisense strand 5'CATGTATATCCTGGTGTCACG). Nucleotides 3962–4623 (DNA sense strand 5'ACTTCATGCCCAGTGTG; antisense strand 5'AGCATCCAAAGGAGGAT). The primers were used under the following conditions: 35 cycles at 94°C for 1 min, 55°C for 1 min, 72°C for 1 min. PCR products were analyzed by electrophoresis in 1% agarose gels. All PCR products were cloned into pGEM 3Z and sequenced (T7-sequencing kit, Pharmacia) to
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**FIG. 1.** Coomassie blue staining of SVEC and EHS tumor laminins separated on SDS–PAGE under reducing conditions. Note the presence of laminin α chains at 400 kDa and slightly lower molecular weight in the SVEC laminin preparation. Position of the approximately 200-kDa laminin β1 and γ1 chains is marked.

allow comparisons to be made with published laminin α chain cDNA sequences.

**Northern Blot Analysis**

Total RNA was isolated from the cell lines by guanidinium thiocyanate (Chromczynski and Sacchi, 1987). 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. Five micrograms of mRNA was denatured with formaldehyde and electrophoresed on a 1% agarose gel (Maniatis et al., 1982). After transfer to Hybond-N filters (Amersham-Buchler), the filters were fixed by exposure to UV light and preincubated for 1 h at 42°C in 50% formamide, 2.5× Denhardt’s solution, 2× SSC, 0.1% SDS, and 50 μg/ml Herring sperm DNA. Hybridizations were performed in the same solution for 16 h at 42°C with the above described 454-bp mouse laminin α5-specific cDNA obtained by RT-PCR or with a GAPDH-specific probe. Probes were labeled to a specific activity of 0.5–1.0 × 10⁶ cpm/μg cDNA by the random priming method. Following hybridization, filters were washed several times with a final stringency of 2× SSC, 0.1% SDS at 50°C, and exposed to Kodak XAR films at −70°C. The sizes of the mRNAs detected were determined using DNA molecular weight markers (Boehringer, Mannheim, FRG).

**FIG. 2.** ELISA for specificity of monoclonal antibodies 4G6 (△), 198 (●), and 4H8-2 (□) and the polyclonal anti-laminin-1 antibody, 308 (○), for laminin-1, laminin-2, or SVEC laminin. The MAb 4G6 raised against SVEC laminin showed specific reaction only with this laminin isoform, while the laminin α1 chain-specific MAb, 198, reacted only with laminin-1, and the laminin α2 chain-specific MAb reacted only with laminin 2/4. 308, a polyclonal antibody which reacts with laminin α1, β1, and γ1 chains, showed specific reaction with all laminin isoforms.

**TABLE 1**

<table>
<thead>
<tr>
<th>List of Laminin α5 Chain Peptides Sequenced</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>LNVTSPDL . . .</td>
<td>(842)</td>
</tr>
<tr>
<td>GQLQLVEG . . .</td>
<td>(1678)</td>
</tr>
<tr>
<td>TWEMVVRQR . . .</td>
<td>(2468)</td>
</tr>
<tr>
<td>NVERWOSQ . . .</td>
<td>(2575)</td>
</tr>
<tr>
<td>TLOFGHMS . . .</td>
<td>(2757)</td>
</tr>
<tr>
<td>QLFPSGGS . . .</td>
<td>(3000)</td>
</tr>
</tbody>
</table>

Note. In parenthesis are the positions of the first amino acids of the peptides according to Miner et al. (1995).
Days 13, 15, and 18 of gestation (E13, E15, and E18) or of tissues SVEC laminin, but not mouse laminin-1 or... with 200 kDa and nido-6-gene (ND) from endothelial cell-conditioned media. In the PYS cell line, polyclonal anti-laminin-1 precipitated the 400-kDa α1 chain, and β1 and γ1 chains of 200 kDa, while 4G6 showed only nonspecific precipitation of fibronectin (FN), as was observed without primary antibody. The same pattern of results as observed for SVEC was found with mlEND and sEND1 (not shown). (B) Immunodepletion experiments involved immunoprecipitation of [35S]-methionine-labeled SVEC conditioned medium with 4G6 (4) and subsequently with a polyclonal antibody against laminin-1 (5) or protein G–Sepharose alone (6). 4G6 immunoprecipitated the major laminin complex from the SVEC-conditioned medium.

Production of Monoclonal Antibodies

Initial screening of the monoclonal antibodies raised against the SVEC laminin involved specific reaction with SVEC laminin, but not mouse laminin-1 or laminin-2/4, in ELISA (Fig. 2). The SVEC laminin employed both for immunization of rats and in ELISA contained predominantly laminin α5, β1, and γ1 chains as revealed by amino acid sequence analysis described above. In this way, clones

FIG. 4. Northern blot hybridization of 32P-labeled cDNA specific for laminin α5 to 5–10 μg mRNA isolated from sEND1, mlEND, SVEC, and PYS cells. Results shown were obtained with a cDNA probe obtained from the SVEC cell line using RT-PCR with primers constructed from laminin α5 gene sequences covering nucleotides 8477–8931. The approximately 12-kb laminin α5 mRNA (LamA5) was strongly expressed in all three endothelial cell lines, but not in PYS. The same pattern of results was found with a cDNA probe covering nucleotides 3962–4623 of the mouse laminin α5 gene sequence (not shown). Control hybridizations with a GAPDH-specific probe is also shown.

RESULTS

SVEC Laminin Characterization

We have previously shown by rotary shadowing that laminin isolated from culture supernatants of confluent SVEC cells exhibits a cruciform structure similar to laminin-1, indicating the presence of an isoform with a long α chain (Sorokin et al., 1994). By immunopurification on anti-laminin-1 columns we have now isolated this isoform in quantities sufficient for structural characterization. SDS gel-electrophoresis of the reduced protein showed two doublets centered at 400 and 200 kDa with a mobility slightly faster than the α1 and β/γ chains of laminin-1 (Fig.1). The 200-kDa bands comprised the β/γ chains since they were detected in immunoblots by polyclonal anti-laminin-1 antibodies (Sorokin et al., 1994). To further assess the identity of the 200- and 400-kDa polypeptides we performed in-gel digestions of the bands in these two molecular weight ranges to generate peptides for sequence analysis. Peptides obtained from the 200-kDa bands revealed only laminin β1 and γ1 amino acid sequences (not shown). Both bands of the 400-kDa doublet showed identical peptide patterns by reversed phase HPLC, and sequence analysis of peptides revealed 100% identity to the predicted protein sequence of the recently described laminin α5 gene (Miner et al., 1995). A list of representative peptide sequences is given in Table 1. The lower 400-kDa peptide, therefore, probably arises by degradation of the upper band or may represent a differently glycosylated form of the upper band. Amino acid sequences of other laminin α chains were not detected.
which reacted with laminin-1 or laminin-2/4 in ELISA, and hence with laminin α1, α2, β1, β2, or γ1 chains, were excluded from further characterization studies. Only clones reacting exclusively with SVEC laminin and, therefore, probably the laminin α5 chain were chosen for further characterization. One clone, 4G6, reacted exclusively with SVEC laminin in ELISA and in immunofluorescence recognized endothelial cell basement membranes in sections of mature mouse skeletal (Fig. 9D) and cardiac muscle. 4G6 did not function in immunoblots of reduced SVEC laminin, but did react in dot blots with the nonreduced protein (data not shown) which was used for amino acid sequencing. Immunoprecipitation was therefore used to further characterize this monoclonal antibody. In immunoprecipitations of [35S]methionine-labeled conditioned medium collected from SVEC, mlEND, sEND1, and PYS, 4G6 precipitated the 400-kDa novel laminin α chain together with laminin β1 and γ1 chains only from the endothelial cell lysates. Results for SVEC are shown in Fig. 3A. The same pattern of immunoprecipitated polypeptides was obtained with polyclonal anti-laminin-1 antibody (Fig. 3A), but not with laminin α1 chain-specific MAb 198 (Sorokin et al., 1994). 4G6, however, failed to immunoprecipitate laminin complexes from the PYS cell line which expresses predominantly the laminin-1 isoform (Fig. 3A).

To ensure that the laminin complex immunoprecipitated by 4G6 was the major laminin secreted by SVEC and not a minor component, too rare to be caught by peptide sequencing but common enough to be detected by dot blotting, immunodepletion experiments were performed: Radiolabeled SVEC conditioned medium was first immunoprecipitated with 4G6 and protein G-Sepharose, the supernatant being used as the first immunoprecipitation. The supernatant was subsequently immunoprecipitated with anti-laminin-1 serum (308) and protein G-Sepharose, or with protein G-Sepharose alone. Since the anti-laminin-1 serum recognizes laminin α1, β1, and γ1 chains, radiolabeled laminin complexes containing any one of these chains and not immunoprecipitated by 4G6 would have been immunoprecipitated by the antiserum. Results of the immunodepletion experiments are illustrated in Fig. 3B and clearly show that the major laminin complex secreted by SVEC was indeed recognized by the 4G6 antibody. Subsequent immunoprecipitation with the anti-laminin-1 serum resulted in only non-specific bands, one of which is fibronectin as shown by immunoblotting experiments (Sorokin et al., 1994). In immunoprecipitation, the 400-kDa α chain often appeared as a 400-kDa doublet, as was the case with the isolated protein, suggesting that some proteolytic breakdown occurs or that differently glycosylated forms exist.

**Laminin α5 mRNA Expression**

Using RT-PCR and subsequent sequencing, 454- and 661-bp laminin α5 cDNA sequences between nucleotides 8477–8931 and 3962–4623, respectively, were amplified from SVEC, mlEND, and sEND1 RNA, but not from PYS RNA (not shown). No laminin α2 or α3 cDNA sequences could be amplified from SVEC, mlEND, or sEND1 RNA (not shown). Using both laminin α5 cDNA fragments in Northern blot analysis, the 12-kb laminin α5 mRNA was found to be strongly expressed in SVEC, sEND, and mlEND, but not in PYS cells (Fig. 4).

The amino acid sequence data from the SVEC laminin, together with the strong expression of the laminin α5 mRNA in the mouse endothelial cells in Northern blot analysis suggests that the novel 400-kDa laminin α chain expressed in mouse endothelial cell lines is the laminin α5 chain. This is strong evidence that the 4G6 monoclonal antibody raised against SVEC laminin reacts with the mouse laminin α5 polypeptide. The final proof was provided by comparison of the in vivo distribution of 4G6 with the in situ hybridization pattern of laminin α5-specific probes in E13–E18 mouse embryos and in newborn mouse tissues as shown below.

**In Vivo Expression of Laminin α5**

(a) Epithelial cells. In E13–E18 mouse embryos the in vivo staining pattern obtained with 4G6 and the in situ hybridization pattern of the laminin α5-specific probes were tightly correlated and showed an unexpected association predominantly with epithelial cells. At the earliest stages of development examined (E13–E15), laminin α5 was most strongly expressed in epithelial cells of the lung and of the kidney (Figs. 5 and 6). Figure 5 illustrates 4G6 staining of basement membranes underlying bronchial epithelium in the lung of an E15 embryo (compare A and B), while laminin α5-specific probes hybridize specifically to the bronchial epithelial cells on an adjacent section (C, D). With further embryonic development (E15–E18) expression of laminin α5 protein and mRNA became apparent in most epithelial cells, including the stratified epithelium of the skin, gland-
FIG. 6. Comparison of laminin α1 and α5 chain expression in kidneys of E14 (A–C, G, I) and E17 (D–F, H, J) mouse embryos. Sections were stained with polyclonal anti-laminin-1 antibody (A,D), or MAbs specific for laminin α1 (B,E) or α5 (4G6) (C,F). Polyclonal anti-laminin-1 stains all epithelial and endothelial cell basement membranes in both E14 (A) and E17 (D) mouse kidneys. Laminin α1-specific MAb-stained ureter (u) and developing tubuli basement membranes in E14 embryos (B), while staining for laminin α5 was restricted to ureter basement membranes at this stage of development (C). Structures stained in the cortex of the E14 kidney in (C) are cross-sections through the branching tip of the ureter. In E17 kidneys, laminin α1 was no longer detectable in distal tubule and collecting duct basement membranes, in glomerular basement membranes, or in blood vessel basement membranes (E), where laminin α5 was strongly expressed (F). Note the absence of laminin α1 and α5 in capillary basement membranes in both E14 and E17 kidneys. Positions of glomeruli in A–F are marked by arrows. Open arrows in E and F mark large blood vessels which express laminin α5, but not laminin α1. In situ hybridizations for laminin α5 mRNA expression in E14 (G is dark field, I is light field) and E17 (H is dark field, J is light field) kidney sections revealed strong laminin α5 mRNA expression in epithelial cells of the ureter, collecting ducts and distal tubules. "c" is calx; "u" is ureter. Original mag. x200 (A–C); x100 (D–J).
lar, gut, and sensory epithelium. An example of this expression pattern is shown for the intestine of an E17 embryo in Figs. 5E-5H.

Closer investigation of the laminin α5 expression in epithelium revealed that it was not associated with newly forming epithelial cells. This was best illustrated in the kidney: In E14 kidneys laminin α1 but not laminin α5, was expressed by condensing mesenchyme, comma-, and S-shaped tubules which characterize the earliest steps in epithelial cell development (Figs. 6B and 6C) (Ekblom et al., 1990). Laminin α5, in contrast, was restricted to the ureter basement membrane (Fig. 6C). Figure 6G illustrates in situ hybridization of a laminin α5-specific probe to an adjacent section of E14 kidney, revealing strong expression of the laminin α5 mRNA in epithelial cells of the ureter only. Not only was laminin α5 absent from sites of epithelial cell formation, but its expression was inversely correlated with that of laminin α1. For example, laminin α1 was no longer detectable in basement membranes of the collecting ducts or distal tubules in the kidney of E17 embryos, but was predominantly associated with newly forming epithelial cells in the kidney cortex and with proximal tubules (Fig. 6E) (Ekblom et al., 1990). In contrast, laminin α5 polypeptide (Fig. 6F) and mRNA expression (Fig. 6H) had broadened in the kidney at this stage of development and occurred predominantly in association with the collecting ducts and distal tubules. In addition, laminin α5 expression within the glomerular basement membrane and in large blood vessel basement membranes increased markedly during this period of development, while laminin α1 was not detectable at these sites (compare Figs. 6E and 6F). A similar inverse association of laminin α5 and α1 expression was noted during epithelial cell development in other organs such as the lung, intestine, and salivary gland (manuscript in preparation), which may reflect a role for this laminin chain in maturation of epithelium.

(b) Endothelial cells. In all E13–E18 embryonic and newborn tissues examined, the laminin α5 chain occurred in basement membranes of larger blood vessels, but not of capillaries. An example is shown in Fig. 7, which illustrates 4G6 staining of basement membranes of surrounding large blood vessels in the heart of an E18 embryo (Fig. 7B). Hybridization of a similar section with a laminin α5-specific probe revealed an even distribution of the signal over the entire blood vessel (Fig. 7D), suggesting expression of laminin α5 mRNA by smooth muscle cells surrounding arteries and probably also by the endothelial cells.

Laminin α5 chain expression in capillary basement membranes became evident only in tissues isolated from mice older than 3–4 weeks of age. This was most evident in the brain where 4G6 stained only the choroid plexus basement membranes during embryogenesis and in newborn tissues, and not capillary basement membranes (Fig. 7F). The choroid plexus are composed of two cell layers, an endothelial and neuroepithelial cell layer, between which lies the basement membrane composed of proteins secreted by both cell layers. The neuroepithelial cell layer extends beyond the choroid plexus to encape the brain. In situ hybridization revealed the epithelial cells of the choroid plexus to be the only source of laminin α5 mRNA in the brains of embryonic and new born mice (Fig. 7H); however, by 4 weeks after birth the laminin α5 mRNA expression was also associated with capillaries within and surrounding the brain (Fig. 7I).

In mature mice, extensive laminin α5 chain expression was also apparent in lymph nodes where it was localized in basement membranes of capillaries and high endothelial postcapillary venules (Fig. 8B). Interestingly, in lymph nodes and thymus (not shown) laminin α5 also occurred in a fine reticular pattern probably associated with the reticular fibers which stain for other basement membrane components (van der Berg et al., 1993). The laminin α5 chain occurred in basement membranes of the central vessels of the liver (Fig. 8D), and of arteries, veins, and capillaries in the brain (Fig. 8F), intestine (Fig. 8H), lung, adrenal cortex (not shown) of mature mice. In mature skeletal and cardiac muscle, endothelial cell basement membranes of capillaries, arteries, and veins were recognized by the 4G6 antibody (Fig. 9D); however, in embryonic and newborn animals 4G6 also stained muscle fiber basement membranes.

(c) Myogenic cells. The only other tissue types which showed considerable laminin α5 expression were smooth and skeletal muscle. 4G6 staining of smooth and skeletal muscle basement membranes was evident in E13–E15 embryos, while weak laminin α5 mRNA expression was detectable in the corresponding myogenic cells. In smooth muscle, the laminin α5 chain was detected in both embryonic (Fig. 5F) and mature tissues (Fig. 8H), while expression in skeletal muscle was developmentally regulated: The laminin α5 chain was located in skeletal muscle fiber basement membranes of embryonic mice (Fig. 9B), but was down regulated during development. Skeletal muscle fiber basement membranes remained only very weakly positive for laminin α5 in the mature mouse, but accumulation was evident at the neuromuscular junction. Figures 9E and 9F illustrate double staining of mature skeletal muscle for α-bungarotoxin and laminin α5, showing colocalization of acetylcholine receptors and laminin α5 at the neuromuscular junction. Very weak laminin α5 expression was also observed in the basement membranes of cardiac muscle in embryonic and new born, but not mature mice (not shown). At no stage of development was laminin α5 expression detected in nervous or fat tissue.

DISCUSSION

In a previous paper we showed that various mouse endothelial cells express a laminin isoform composed of three main polypeptides including laminin β1 and γ1 chains, and a 400-kDa novel α chain which often occurs as a doublet (Sorokin et al., 1994). Isolation of the laminin isoform from conditioned medium of a peripheral lymph node cell line, SVEC, and amino acid sequences of the 400-kDa α chain doublet revealed its identity with the recently reported lam-
Distinct developmentally regulated pattern of laminin α5 chain (Miner et al., 1995). This is supported by amplification of laminin α5 gene sequences by PCR from mouse endothelial cell lines, SVEC, mlEND, and sEND1, and strong expression of the 12-kb laminin α5 mRNA in Northern blot analysis of these cells. Gene sequence data reported by Miner et al. (1995) suggests that the laminin α5 gene codes for an approximately 440-kDa polypeptide; however, no protein data are available from this study. The protein expressed by the mouse endothelial cell lines studied here always appeared as a 400-kDa or slightly smaller band in SDS-PAGE. It may be therefore that this chain is proteolytically processed, in the same way as the laminin α2 chain (Leivo and Engvall, 1988; Ehrig et al., 1990; Paulsson et al., 1991), and that this may represent the physiologically important form of the polypeptide.

Monoclonal antibodies were raised to the laminin α5 chain using heterotrimeric SVEC laminin as antigen: Based on specific reaction with SVEC laminin in ELISA and not with laminin-1 or laminin-2/4, clone 4G6 was chosen for further characterization. Immunoprecipitation of laminin complexes from mouse endothelial cells (SVEC, mlEND, sSEND1), but not from laminin-1 expressing PYS cells, and the immunodepletion of laminin complexes by 4G6 from SVEC-conditioned medium, suggest that 4G6 recognizes the 400-kDa laminin α5 chain of endothelial cell laminin. The absence of expression of other full-length laminin α chains (400 kDa), such as α1, α2, or α3, by the mouse endothelial cell lines employed in this study, and the close correlation between the laminin α5 mRNA in vivo expression and the 4G6 staining pattern further supports the specificity of 4G6 for the laminin α5 chain. The 4G6 antibody recognizes the non-denatured form of laminin α5 since it did not react with reduced SVEC laminin in immunoblots, but did react in dot blots with the nonreduced protein which was used for amino acid sequencing. Despite the large amount of evidence which suggests that 4G6 reacts with the laminin α5 chain there, nevertheless, remains a possibility that 4G6 recognizes a complex epitope that requires expression of the laminin α5 chain together with α1 and/or γ laminin chain.

Immunofluorescence and in situ hybridization revealed a distinct developmentally regulated pattern of laminin α5 expression in epithelial, endothelial, and myogenic cells. Surprisingly, even though the laminin α5 chain was isolated from an endothelial cell line, in embryonic and newborn tissues it occurred in basement membranes of large blood vessels only. Laminin α5 expression in capillary basement membranes became evident only in tissues collected from animals older than 3–4 weeks of age. Capillary endothelial cell basement membranes are also not recognized by antibodies to laminin α1 (Sorokin et al., 1994), α2 (Sanes et al., 1990; Schuler and Sorokin et al., 1995), or α3 (Marinkovich et al., 1992). Although no laminin α4 antibodies exist for use in immunohistochemical investigations, we have recent evidence that laminin α4 mRNA is expressed by only a subset of endothelial cells in vivo (Frieser et al., 1997). The data therefore suggest the existence of another/other laminin α chains in capillary endothelium during embryogenesis and excludes the possibility that laminin α5 plays a role in angiogenesis. The appearance of the laminin α5 chain in capillary basement membranes in the present study correlated approximately with sexual maturity of the animals (approximately 4 weeks of age). It has been previously reported that the expression of endothelial cell laminins is influenced by progesterone derivatives which also increase in the circulation of both sexes at sexual maturity. Tokida et al. (1990) showed that the expression of a 400-kDa laminin α chain by bovine aortic endothelial cells is stimulated by medroxyprogesterone, while expression of a truncated α chain is suppressed. At the time the identity of this 400-kDa chain was not clear, however, we have unpublished evidence that it is the laminin α5 chain. Hence, at sexual maturity increased levels of progesterone in the circulation may induce expression of laminin α5 in capillary endothelium via a progesterone-sensitive element in the laminin α5 gene. This possibility is currently under investigation.

Most epithelial cells strongly expressed laminin α5 throughout development and in mature mouse tissues, the bronchi of the lungs and the kidney tubules being the sites of earliest and strongest expression. Analysis of the laminin α5 expression pattern during development, however, clearly showed that it is unlikely to play a role in epithelial cell development. This was demonstrated by the expression of laminin α5 in the villi tips of mature mouse intestine, but not in the crypts where epithelial cell development occurs and where laminin α2 is localized (Simon-Assman et al., 1995).

**FIG. 7.** Expression of the laminin α5 chain in association with mouse endothelial cells. Sections of E18 embryonic heart and brain were stained with polyclonal anti-laminin-1 (A, E) or laminin α5-specific MAb, 4G6 (B, F). In situ hybridization for laminin α5 mRNA expression was performed on sections of E18 heart (C is light field, D is dark field) and brain (G is light field, H is dark field), and of 4-week-old mouse brain (I is dark field). In E18 heart, polyclonal anti-laminin-1 stained basement membranes of blood vessels and cardiac muscle (A), while 4G6 stained mainly the basement membranes of large blood vessels (B). In a similar section, hybridization of a laminin α5-specific probe to large blood vessels suggest expression of laminin α5 mRNA in smooth muscle cells surrounding arteries and possibly also in endothelial cells (D); "α" is artery; arrowheads in C and D mark the position of an additional large blood vessel. Polyclonal anti-laminin-1 stained basement membranes of capillaries within the brain of an E18 embryo, of the choroid plexus, and of the neuroepithelium (arrows in E and F) encasing the brain (E), while 4G6 stained exclusively the choroid plexus basement membrane (F). In situ hybridization of an adjacent section demonstrated the expression of laminin α5 mRNA only in the epithelial cells of the choroid plexus (marked by arrows in G and H). By 4 weeks after birth, laminin α5 mRNA expression was also associated with small blood vessels within the brain (I). Arrow in I marks a blood vessel which has invaded the brain. Original mag. x100 (E, F); x200 (A–D, G, H); x300 (I).
In the embryonic kidney the epithelium of the ureter grows into the metanephric mesenchyme inducing conversion of mesenchyme to epithelium, the first signs of which include mesenchymal condensates, comma-, and S-shaped tubules. Laminin α1 is expressed at these sites of early epithelial cell development (Ekblom et al., 1990), while laminin α5 was restricted to the ureter basement membrane. During kidney development laminin α5 expression broadened to become the predominant laminin α chain in basement membranes of the ureter and distal tubules, glomeruli, and blood vessels in the mature organ. This expression pattern is approximately inversely correlated to that of laminin α1, a feature that was noted in other developing epithelial cells, in particular those of the lung, salivary gland, and intestine. The inverse association of laminin α5 and laminin α1 chain expression which occurs with maturation of epithelium, and the fact that they have an overlapping distribution at certain stages of development, may reflect similar functions for isoforms containing these laminin α chains. Laminin α1 has been shown to be important for the development of the polarized state of various epithelial cell membranes. The smooth muscle underlying the intestinal epithelium, and the fact that they have an overlapping distribution at certain stages of development, may reflect similar functions for isoforms containing these laminin α chains. Laminin α5 has been shown to be important for the development of the polarized state of various epithelial cells (Klein et al., 1988; Kadoya et al., 1995), its gradual replacement by laminin α5 during maturation of epithelium may reflect a role for laminin α5 in maintenance of the fully differentiated or polarized phenotype. This possibility is supported by the observation that laminin α5 is expressed in basement membranes of mature endothelial cells only, and not of newly forming endothelial cells. The earlier expression of laminin α5 in large vessel endothelium, such as arterial endothelium, also supports this possibility: It has been shown that large vessel endothelium matures earlier in development than capillary endothelium, which attains the fully differentiated state approximately 25 days after birth (Robertson et al., 1985; Risau, 1995). Alternatively, the expression of laminin α5 may be related to the barrier function of epithelium and endothelium. Both of these cell types form tight sheets which are not penetrable except in cases of inflammation or metastasis. Hence, the presence of laminin α5, either directly or indirectly, may control the permeability or penetrability of basement membranes.

An unexpected finding was the expression of the laminin α5 chain in smooth and skeletal muscle basement membranes. The smooth muscle underlying the intestinal epithelium and surrounding arteries expressed the laminin α5 chain at all stages of development examined, while in skeletal muscle a developmentally regulated pattern of expression was observed: Laminin α5 was located in basement membranes of skeletal muscle fibers up to birth, but was barely detectable in mature animals with the exception of the neuromuscular junction. At all stages of embryonic development weak in situ hybridization signal of the laminin α5-specific probes to skeletal muscle was apparent (not shown), suggesting either a low level of expression at these sites or expression only in a subset of the nuclei in the muscle fibers. Various molecules have been shown to be expressed preferentially by subsynaptic nuclei in muscle fibers, including the neural cell adhesion molecule, rapsyn, and the laminin β2 chain (Martin et al., 1995; Moscoso et al., 1995). Since the subsynaptic nuclei constitute a minor fraction of all nuclei in muscle fibers, the expression of laminin α5 mRNA might not be detectable in the cross-sections used in this study.

It was previously thought that laminin isoforms containing the α2 chain were the only laminins of muscle fiber basement membranes (Sanes et al., 1990; Schuler and Sorokin, 1995). However, the expression of laminin α5 in myogenic tissues described here shows that this is not the case. This is the first report of expression of an additional laminin α chain in myogenic tissues and may explain why laminin α2-deficient dy/dy mice (Sunada et al., 1994; Xu et al., 1994) exhibit normal skeletal and smooth muscle development. It may be, therefore, that the expression of laminin α5 in myogenic tissues principally during embryogenesis reflects a role in myogenesis, but clearly not in the maintenance of the myogenic phenotype as it is no longer present in muscle fiber basement membranes in the mature tissue. The concentration of laminin α5 at the neuromuscular junction in mature skeletal muscle is particularly noteworthy. It has been suggested that the neuromuscular junction contains a signal which arrests outgrowth of axons at this site and thereby controls formation of this specialized structure. The laminin β2 chain has been implicated in this “stop” function (Martin et al., 1995); however, mice lacking this chain still develop neuromuscular junctions, albeit fewer in number and with impaired function as compared to normal animals (Noakes et al., 1995). These results suggest either the involvement of an as yet unidentified laminin isoform containing the laminin β2 chain, or some other factor together with the laminin β2 chain in neuromuscular formation. The concentration of laminin α5 at the neuromuscular junction in mature skeletal muscle may, therefore, reflect the missing axon arresting signal.

The immunofluorescence and in situ hybridization data presented here correlate well with the previously reported

**FIG. 8.** Staining patterns obtained in mature mouse tissues with polyclonal anti-laminin-1 (left) and the laminin α5-specific MAb, 4G6 (right). Tissues shown are mesenteric lymph node (A, B), liver (C, D), brain (E, F), and intestine (G, H). In the lymph node 4G6 stained basement membranes of capillaries and high endothelial postcapillary venules (arrows in A and B), and of reticular fibers. Note extensive 4G6 staining of capillary basement membranes, but not the meningeal basement membrane in the brain (arrows in E and F). 4G6 stained epithelial cell basement membranes at the tips of the intestinal villi but not in the crypts, and the basement membranes of blood vessels within the villi and at their base. 4G6 also stained basement membranes of the smooth muscle underlying the intestine. Original mag. ×200.
FIG. 9. Immunofluorescent localization of laminin $\alpha_5$ in skeletal muscle of E15 mouse embryos (A, B) and of mature mice (C–F). Sections were stained with polyclonal anti-laminin-1 (A, C), the laminin $\alpha_5$-specific MAb, 4G6 (B, D, F), or with TRITC-conjugated $\alpha$ bungarotoxin (E). E and F are the same section double stained with 4G6 and anti-$\alpha$ bungarotoxin. Early in development laminin $\alpha_5$ was strongly expressed in basement membranes of muscle fibers and of larger blood vessels (B). In the mature tissue, very weak laminin $\alpha_5$ expression in the muscle fiber basement membranes was detectable, but strong expression occurred in blood vessel basement membranes (D) and at the neuromuscular junction as revealed by colocalization of 4G6 and anti-$\alpha$ bungarotoxin (compare E and F). Mag. $\times$100 (A–D); $\times$300 (E,F).
laminin α5 N orthern blot data which showed strong signals in mature mouse heart, lung, and kidney (in order of intensity), and weaker signals in skeletal muscle and brain (Miner et al., 1995). However, the previous study did not provide any information on the cellular localization of the laminin α5 chain. The data presented here show that the high laminin α5 mRNA signal observed by Miner et al. (1995) in lung and kidney was probably due to its expression in epithelium and endothelium, while in mature heart, skeletal muscle, and brain the laminin α5 mRNA signal probably stems principally from endothelial cells. In addition, a 375-kDa laminin α chain has been reported to be the predominant laminin α chain isolated from mature bovine kidneys (Lindblom et al., 1994). In view of molecular weight similarities and the widespread 4G6 staining in the kidney, it is possible that this bovine kidney laminin chain is the homologue of the mouse laminin α5 chain. Whether 4G6 cross-reacts with bovine tissues in immunofluorescence or with purified bovine kidney laminin in ELISA or immunoprecipitation is currently under investigation.

In conclusion, the data presented here reveal a developmentally regulated expression of the laminin α5 chain in epithelium, endothelium, and skeletal muscle, suggesting a role for this laminin chain in maturation of epithelial and endothelial cells and possibly also in neuromuscular junction formation.

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