

Characterization and Expression of the Laminin γ 3 Chain: A Novel, Non-Basement Membrane-associated, Laminin Chain

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Abstract. Laminins are heterotrimeric molecules composed of an α , a β , and a γ chain; they have broad functional roles in development and in stabilizing epithelial structures. Here, we identified a novel laminin, composed of known α and β chains but containing a novel γ chain, γ 3. We have cloned gene encoding this chain, LAMC3, which maps to chromosome 9 at q31-34. Protein and cDNA analyses demonstrate that γ 3 contains all the expected domains of a γ chain, including two consensus glycosylation sites and a putative nidogen-binding site. This suggests that γ 3-containing laminins are likely to exist in a stable matrix.

Studies of the tissue distribution of γ 3 chain show that it is broadly expressed in: skin, heart, lung, and the reproductive tracts. In skin, γ 3 protein is seen within

the basement membrane of the dermal-epidermal junction at points of nerve penetration. The γ 3 chain is also a prominent element of the apical surface of ciliated epithelial cells of: lung, oviduct, epididymis, ductus deferens, and seminiferous tubules. The distribution of γ 3-containing laminins on the apical surfaces of a variety of epithelial tissues is novel and suggests that they are not found within ultrastructurally defined basement membranes. It seems likely that these apical laminins are important in the morphogenesis and structural stability of the ciliated processes of these cells.

Key words: laminin • testis • oviduct • lung • chromosome 9q31-34

LAMININS are large glycoproteins found in all basement membranes (Ekblom, 1996). In overall appearance, laminins are cross-shaped with a single long arm arising from the coiled-coil interaction of three genetically distinct polypeptide chains and three NH₂-terminal short arms, each originating from the individual polypeptide chains (Maurer, 1996). The three subunit chains are termed α , β , and γ according to the current nomenclature (Burgeson et al., 1994). The complete primary structure for each of the nine human laminin subunit chains has been elucidated: α 1 (Haaparanta et al., 1991), α 2, (Vuolteenaho et al., 1994), α 3 (Ryan et al., 1994), α 4 (Iivanainen et al., 1995a), β 1 (Pikkarainen et al., 1987), β 2 (Wewer et al., 1994; Iivanainen et al., 1995b), β 3 (Gerecke et al., 1994), γ 1 (Pikkarainen et al., 1988), and γ 2 (Kallunki et al., 1992). The complete cDNA sequence for a fifth laminin α

chain has been determined in mouse (Miner et al., 1995); partial cDNA sequences of human α 5 (Durkin et al., 1997), and a novel chicken β chain (Ybot-Gonzalez et al., 1995) have been reported. All three chains have globular domains separated by multiple epidermal growth factor-like domains within the NH₂-terminal short arms. Their long arm portions are composed of heptad repeats that are typical for α -helical coiled-coil proteins. In addition, the COOH terminus of each α chain is composed of five globular (G)¹ domains (Engel, 1992).

The many functions ascribed to laminins are thought to derive from their structural and signal transduction roles, by which they contribute to the formation and stability of basement membranes, to the stability of cellular attachments to basement membranes, and to cytoskeletal rearrangements mediated by their occupancy of cell surface receptors (Ryan et al., 1994). These activities at least partially result from: (a) the binding of the COOH-terminal laminin G domains to integrins in most cells (Deutzmann

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1. *Abbreviations used in this paper:* FCMD, Fukuyama congenital muscular dystrophy; FISH, fluorescent in situ hybridization; G, globular.

et al., 1990; Drago et al., 1991; Goodman, 1992; Matter and Laurie, 1994; Rousselle et al., 1995; Chen et al., 1997), and/or to dystroglycan in muscle cells (Henry and Campbell, 1996; Pall et al., 1996; Wewer and Engvall, 1996; Cohen et al., 1997); (b) from the self-assembly of the laminins into a pericellular extracellular matrix through interactions of domains VI, which are present at the ends of the short arms of the subunit chains (Yurchenco et al., 1992; Yurchenco and Cheng, 1993, 1994); and (c) from assembly of the pericellular laminin network with a conceptually separate network of type IV collagen molecules specifically mediated by the molecule nidogen, one end of which binds the laminin $\gamma 1$ chain, and the other end of which binds type IV collagen and other basement membrane matrix components (Fox et al., 1991; Battaglia et al., 1992; Aumailley et al., 1993; Reinhardt et al., 1993).

The role of laminin 5 ($\alpha 3\beta 3\gamma 2$) in stabilization of epithelial-stromal interactions is the exception to this generalized scheme. While the 5 laminin G domains of $\alpha 3$ bind integrins $\alpha 6\beta 4$ and $\alpha 3\beta 1$ on the epithelial basolateral surface (Niessen et al., 1994), the absence of domains VI on the truncated short arms of $\alpha 3$ and $\gamma 2$ (Kallunki et al., 1992; Ryan et al., 1994), and the absence of a nidogen binding site on $\gamma 2$ (Mayer et al., 1995) prevent their participation in the above-described model. Instead, the NH_2 terminus of the epithelial cell-associated laminin 5 binds type VII collagen present within the subjacent stromal matrix (Rousselle et al., 1997). Thus, laminin 5 appears to play a unique role in epithelial frictional resistance, rather than a direct role in overall basement membrane structure.

The primary functions of the laminin $\gamma 1$ chain in the above generalized scheme is the contribution of a domain VI which shares high amino acid sequence identity to domains VI of the laminin α and β chains and a unique sequence required for the binding of nidogen. This sequence, NIDPNAV, is present in the fourth EGF-like repeat of domain III (Mayer et al., 1979; Poschl et al., 1994). In the $\gamma 2$ chain, the analogous sequence NVDPSAS is present, but does not support high affinity nidogen binding (Mayer et al., 1995).

In this report, we describe a novel human laminin γ chain. Its predicted structure indicates the presence of all the domains homologous to $\gamma 1$, including a domain VI homologue and a nidogen-binding site containing a single conservative amino acid substitution. We call this chain the laminin $\gamma 3$ chain. The predicted structure of this chain suggests that it should be capable of associations with other laminin chains for basement membrane assembly; however, immunolocalization studies in several tissues indicate the presence of laminin $\gamma 3$ chains in regions lacking ultrastructurally identifiable basement membranes.

Materials and Methods

Isolation of a Novel Laminin 12 ($\alpha 2\beta 1\gamma 3$)

The purification of the novel laminin 12 ($\alpha 2\beta 1\gamma 3$) was carried out as follows. Human placental chorionic villi were frozen in liquid nitrogen, ground in a Waring blender, and then washed in 1 M NaCl. Unless otherwise noted, all subsequent steps were performed at 4°C. The final tissue pellet (200 g, wet weight) was suspended by stirring for 48 h in 1 liter of extraction buffer (0.5 M NaCl, 10 mM EDTA, and 625 mg/liter *N*-ethylmaleimide, 150 mg/liter phenylmethylsulfonyl fluoride, and 50 mM Tris-

HCl, pH 7.8). The soluble fraction was collected after centrifugation (30,000 *g*, 60 min) and precipitated with 300 g/liter ammonium sulfate. The precipitated proteins were collected by centrifugation (30,000 *g*, 60 min) and dissolved in chromatography buffer (2 M urea, 25 mM NaCl, 5 mM EDTA, and 50 mM Tris-HCl, pH 7.8). The sample was then dialyzed against the same buffer. After dialysis, 0.5 vol of buffer-equilibrated DEAE-cellulose (DE-52; Whatman) was added and the mixture was shaken overnight. Material not bound to DEAE-cellulose was collected by filtration on a Buchner funnel (Whatman; filter 4) and precipitated by addition of 300 g/liter ammonium sulfate. The proteins were collected by centrifugation (30,000 *g*, 60 min), redissolved in Concanavalin A buffer (0.5 M NaCl, 5 mM CaCl_2 , 5 mM MgCl_2 , and 50 mM Tris-HCl, pH 7.8), and dialyzed against the same buffer overnight. The fraction was applied to a 2.5×5 cm Concanavalin A-Sepharose column (Pharmacia). Unbound material was removed by extensive washing while bound proteins were eluted by successive washing with 10 mM α -D-mannopyranoside, 1 M α -D-glucopyranoside, and finally 1 M α -D-mannopyranoside (Sigma Chemical Co.). Laminins are typically recovered in the latter two fractions; each fraction was independently concentrated to 10 ml with an Amicon concentrator (30-kD membrane) and applied to a 2.5×100 cm Sephacryl S-500 column in 0.5 M NaCl, 50 mM Tris-HCl, pH 7.8. The fractions of interest were pooled, dialyzed against Mono-Q buffer (0.1 M NaCl and 25 mM Tris-HCl, pH 7.8), and applied to a 1×5 cm Mono-Q column (Pharmacia). Elution was achieved with a 60-ml 0.1–0.5-M NaCl gradient. The fraction eluted at 250 mM NaCl was taken for further study.

Protein Sequencing

Protein sequencing was performed with minor modifications of published methods (Aebbersold et al., 1987). In brief, laminin 12 was resolved on a polyacrylamide gel in the presence of 2-mercaptoethanol. The bands at 205, 185, and 170 kD were excised separately, digested with trypsin, and then separated by HPLC and sequenced on an Applied Biosystem sequenator. Analysis of a trypsin digest of laminin $\alpha 2$ isolated from laminin 12 was performed with matrix-assisted laser desorption time-of-flight mass spectrometry performed on a Finnigan Lasermat 2000 (Chait and Kent, 1992).

cDNA Cloning

By comparison of the laminin $\gamma 1$ amino acid sequence (SWISS-PROT accession number, P11047) with the dbEST database (Boguski et al., 1993; NCBI) using the program BLAST (Altschul et al., 1990) one clone (these data are available from GenBank/EMBL/DBJ under accession number AA297192) was chosen as a possible candidate for a new laminin γ chain. To extend the cDNA, specific primers for 5' or 3' extension were deduced from a previously published expressed sequence tag (clone, AA297192). Nested PCR on placental Marathon-Ready cDNA (Clontech) were performed following the manufacturer's instructions using the supplied nonspecific primers with the following gene-specific primers: for the 5' extension; in the first round, (5'-dCGCATGTGCCGTTCTCGTG-GCACTGG); in the second round, (5'-dGCGGCAGGTGCACTGTC-CAGTCTTGG); for the 3' extension, in the first round, (5'-dTGCACGGGACTGCAGCCGCTGCTACCC); in the second round (5'-dGCTGCTACCCTGGCTTCTTCGACCTCC). For PCR, the Long Expand PCR Kit (Boehringer Mannheim) was used with the following conditions: denaturation, 94°C for 3 min; 10 cycles of 94°C for 30 s, 63°C (–0.5°C per cycle) for 30 s, 68°C for 4 min; 25 cycles of 94°C for 30 s, 58°C for 30 s, 68°C for 4 min (+10 s per cycle); a final extension period at 68°C of 8 min.

The PCR samples from the first round were purified (PCR Purification Kit; Qiagen) and 2% of the sample volume was used in the second round of PCR using the same PCR protocol. These PCR products were purified from an agarose gel (Gel Purification Kit; Qiagen) and either subcloned (into PCR II or PCR 2.1 vectors; Invitrogen) or directly used for sequencing. To reconfirm the nucleotide sequence and control for PCR-induced nucleotide substitutions, gene-specific primers were used to reamplify the entire $\gamma 3$ cDNA. A first strand cDNA synthesis kit (Clontech) was used to synthesize cDNA from total placental RNA using oligo dT, random, or specific human laminin $\gamma 3$ antisense primers following the manufacturer's protocol; PCR was used to generate overlapping clones complementary to the entire human laminin $\gamma 3$ chain. Sequencing of all the obtained PCR products revealed the nucleotide sequence of laminin $\gamma 3$ from what we eventually inferred as nt 297 to nt 5020. However, all further 5' extensions failed to extend the sequence further toward the 5' end.

Genomic DNA

The sequence of the 5' end of the cDNA was determined from the human genomic P1 clone DMPC-HFF#1-1461F2, which was obtained from a PCR-based library screen performed at Genome Systems, Inc. The oligonucleotide primers that were provided to Genome Systems specifically amplify exon 2 of the human $\gamma 3$ gene (sense primer, 5'-dCCCCG-CAGGGGAAGCGGGTCTCTG; antisense primer, 5'-dGGCTTAT-GAGATCACGTATGTGAG). To obtain the sequence of the missing 5' end, the genomic clone was sequenced (in 4% DMSO) with gene-specific antisense primers. The sequence of the laminin $\gamma 3$ 5' untranslated region was confirmed by RT-PCR from placental RNA in 4% DMSO, (sense primer, 5'-dCGCGCGGGCTCGGTGCCCTTGACC; antisense primer 5'-dGCTTGTAGATGGCAAAGCTCTCAGG).

Nucleotide Sequencing

Nucleotide sequences were determined with a Thermo Sequenase cycle sequencing kit and ^{33}P -ddNTP (Amersham Pharmacia) using either the M13 forward or reverse primers or gene-specific primers synthesized in our laboratory. A 1:1 ratio of inosine to guanosine was included in the sequencing mix. Sequence data were assembled and manipulated using Genetyx-Max 8.0 and Genestream-1 at <http://genome.eerie.fr/home.html> (Software Development Co., Ltd.). The signal peptide cleavage site was predicted using <http://genome.cbs.dtu.dk/services/SignalP/> (Nielsen et al., 1997).

Northern Blot Analysis

A 956-bp PCR product (nt 1316–2271) was generated (Long Expand PCR Kit; Boehringer Mannheim) from placental cDNA, purified (PCR purification kit; Qiagen), and labeled with [^{33}P]dCTP (NEN) using the rediprime DNA labeling system (Amersham). Without further purification, the probe was denatured in the same buffer containing 1/10 (vol/vol) human Cot-1 DNA (Boehringer Mannheim), and 1/10 (vol/vol) sheared salmon testes DNA (GIBCO BRL) at 94°C for 5 min then chilled before use. Northern blots (Clontech) were prehybridized in 50% formamide, 5× SSPE, 1× Denhardt's, 1% SDS, 10% Dextran-sulfate, 0.1 mg/ml salmon sperm DNA (GIBCO BRL) at 42°C for 2 h the probe was added and hybridized for 20 h. The blot was washed three times in 2× SSC, 1% SDS at 42°C and two times in 0.1× SSC, 1% SDS at 42°C. Blots were placed on a BioMax MR film (Kodak) with a BioMax TranScreen-LE intensifying screen (Kodak) for 20 h at -70°C.

Recombinant Expression of Domain I of the $\gamma 3$ Chain

A cDNA encoding the COOH terminus of human $\gamma 3$ was cloned into the HisTrx and pPEP-T vectors (kindly provided by Richard Kammerer, Biozentrum, Basel, Switzerland; based on the pET system; Novagen). The HisTrx vector has a histidine-tagged bacterial thioredoxin cDNA as a carrier in front of the cloning site; pPEP-T has a piece of the coiled-coil domain of mouse $\beta 1$ in front of the cloning site. The $\gamma 3$ cDNA fragment used was amplified by PCR from human placenta cDNA (see cDNA cloning) using primers that include the EcoRI adapters: forward, 5'-GCGGATC-CGAGGAAGCTGAGCGGGTGGGTGCTG-3'; reverse, 5'-GCGAA-TTCTTACTGCCAGCTGGCACAGTTCTCGGG-3'. The resultant plasmids were transformed into BL21(DE3) pLysS bacteria (Novagen) and fusion proteins were isolated according to the pET System manual (Novagen). A recombinant fragment containing only histidine-tagged thioredoxin was similarly expressed and purified.

Antibody Production

The 170-kD band (i.e., $\gamma 3$ chain) was excised from the reducing SDS-PAGE gel described above and injected into a rabbit for antibody production following standard procedures (Harlow and Lane, 1988). The resulting serum (R16) was evaluated by Western analysis and shown to react with the 170-kD $\gamma 3$ chain, and showed minor cross-reactivity with other laminin chains at high antibody concentrations. All antibody-related studies presented in this communication were conducted at concentrations well below those where cross-reactivity was observed. The histidine-tagged, thioredoxin- $\gamma 3$ fusion protein was used for the production of a second rabbit antiserum (R21) which reacted with a single band in Western blots of placental extracts.

Affinity Purification

The R16 antiserum was affinity-purified by binding to gel-purified $\gamma 3$ that had been transferred to nitrocellulose and then eluted with 1 M acetic acid followed by immediate neutralization. The R21 serum was purified by binding to the histidine-tagged, mouse $\beta 1$ -human $\gamma 3$ fusion protein coupled to activated CNBr-Sepharose; the bound antibodies were eluted with 2 M urea in PBS, or with 1 M acetic acid which was immediately neutralized. The immunofluorescent patterns produced by these two affinity-purified antibody pools were indistinguishable, and were similar to whole R21 serum with reduced background staining. Only affinity-purified preparations of R21 serum were used for these studies. Antibodies made against histidine-tagged thioredoxin were similarly isolated by affinity chromatography from R21 serum; immunofluorescent patterns with these controls were blank.

Immunofluorescent Analyses

Most tissues were obtained from various colleagues using specimens for other purposes: these include tissues from male and female rats; from normal human tissues discarded after surgery; and from rhesus monkey, *Macaca mulatta*. Bovine tissues were purchased from a local slaughterhouse. Dissected and blocked tissues were placed directly in embedding compound (O.C.T.; Sakura Finetek) and frozen by immersion in liquid nitrogen-cooled isopentane. 10- μm sections were made on a Leica CM 3000 or 3050 and collected on Superfrost slides (Fisher Scientific). Sections were air dried and stored at -20°C until use. Just before use, sections were immersed in acetone at -20°C and then rinsed three times in PBS at room temperature. Sections were incubated with primary antibodies diluted in PBS containing: 2% normal goat serum, 0.25% sodium azide, and 0.1% Triton X-100. Sections were incubated overnight at 4°C; they were washed in three changes of PBS (5 min per wash) and then incubated for 45–60 min with secondary antibody coupled to either Cy3, FITC, or Texas red. After incubation, sections were washed and coverslipped in Prolong (Molecular Probes). The sections were imaged on a Leica confocal laser scanning microscope (Leica TCS-NT). The gain was adjusted in each channel of the confocal to assure that there was no bleeding across the channels; this adjustment is performed at the outset of each confocal session. Images were transferred to Adobe Photoshop and cropped for reproduction. The brightness and contrast were adjusted to make printed images similar to that obtained on the microscope monitor.

Other primary laminin reactive primaries used were: polyclonal anti-EHS-laminin-1 (Sigma Chemical Co.); monoclonal anti-laminin $\alpha 2$ chain (mAb 1922, Chemicon); polyclonal anti-laminin $\alpha 4$ (Miner et al., 1997); polyclonal anti-laminin $\alpha 5$ chain (Miner et al., 1995); two monoclonal anti-laminin $\beta 1$ chain (545, Marinkovich et al., 1992; clone C21, Green et al., 1992); monoclonal anti-laminin $\gamma 2$ chain (Verrando et al., 1987). Monoclonal anti-PGP 9.5 (Ultrasclone, Ltd.) was used to identify nerves in skin. Secondary antibodies used were: goat anti-rabbit FITC (ICN Pharmaceuticals); goat anti-rabbit-Cy3 (Jackson ImmunoResearch Laboratories).

In Situ Hybridizations

Paraffin sections were processed for in situ hybridizations as previously described in detail (Libby et al., 1997). In brief, cRNA probes for the laminin $\gamma 3$ chain were generated from human $\gamma 3$ clones; cRNAs were labeled during transcription by the incorporation of digoxigenin-UTP (Boehringer Mannheim); $\sim 1 \mu\text{g/ml}$ of cRNA was used for hybridization; hybridizations were performed at high stringency (50% formamide and 5× SSC, 60°C; see Libby et al., 1997 for complete details). After overnight hybridization, sections were washed (50% formamide, 1× SSC, for 30 min at 60°C) and the unhybridized probe was destroyed by RNase A. The hybrids were detected with an anti-digoxigenin antibody coupled to alkaline phosphatase (Boehringer Mannheim). Sections were incubated overnight with anti-digoxigenin diluted 1:1,000 in blocking solution (Boehringer Mannheim). After washing to remove unbound antibody, endogenous alkaline phosphatase activity was blocked by washing in levamisole for 10 min; the alkaline phosphatase reaction was carried out overnight at room temperature.

Other Methods

SDS-PAGE (Laemmli, 1970) and electrophoretic transfer of proteins to nitrocellulose with immunoblot analysis were performed essentially as previously described (Lunstrum et al., 1986). For the FISH analysis, a

1217 bp cDNA fragment was generated by RT-PCR from placental RNA, using the sense primer 5'-dAGTGCCACTATAACGGCACATGCG and antisense primer 5'-dCTCGTGTCTGCAAGGAGTCTGTCA. The gel band was purified and subcloned (PCR II vector; Invitrogen). After the sequence of the fragment was verified, the resultant plasmid was used for the fluorescent in situ localization of the LAMC3 gene (SeeDNA Biotech, Inc.).

Results

Characterization of Laminin 12 ($\alpha 2\beta 1\gamma 3$)

Laminin 12 was extracted from human chorionic villi using EDTA and partially purified by a combination of DEAE-cellulose, Concanavalin A, Sephacryl S-500, and Mono-Q chromatography (see Materials and Methods). The final fraction of interest resulting from the above protocol contains multiple laminins. Laminin 12 was resolved from this mixture by SDS-PAGE (3–5% polyacrylamide) under nonreducing conditions. Six bands were resolved (Fig. 1, Unreduced). Only the bands at ~ 560 kD and at the top of the gel were reactive with a polyclonal anti-laminin antiserum (Sigma Chemical Co.; not shown). Therefore, the resolved band at 560 kD was excised, reduced in 10% 2-mercaptoethanol SDS-PAGE sample buffer, and resolved by 5% SDS-PAGE. Three bands were observed with masses of ~ 205 , 185, and 170 kD (Fig. 1, Reduced). The band at 185 kD reacted with a monoclonal antibody (clone 545; Marinkovich et al., 1992) specific to the laminin $\beta 1$ chain (Fig. 1, Western blot). Each of the three bands was digested with trypsin, the peptides were resolved by HPLC, and selected resolved peptides were sequenced. The sequences obtained are shown in Table I. The 205-kD chain contained three peptides sequence identical to human laminin $\alpha 2$ (published residues 536, 70, 1367; Vuolteenaho

Table I. Comparison of Peptide Sequences Obtained from Placental Laminin 12 205- and 185-kD Bands and Published Sequences of $\alpha 2$ and $\beta 1$ Residues

Human $\alpha 2^*$ res. no. 536	KIQMSGWYL
Peptide from 205-kD band	IQMSG-YL
Human $\alpha 2^*$ res. no. 70	KLVEHVPGPQVPR
Peptide from 205-kD band	LVEHVPGPQVPR
Human $\alpha 2^*$ res. no. 1367	RGTTMTPPADLIEK
Peptide from 205-kD band	GTTMTPPADLIEK
Human $\beta 1^\dagger$ no. 171	RYSDIEPSTEGEVIFR
Peptide from 185-kD band	YSDIEPSTEGEVIFR
Human $\beta 1^\dagger$ res. no. 558	RIPSWTGAGFVR
Peptide from 185-kD band	IPSWTGAGFVR

*Vuolteenaho et al., 1994.

†Pikkarainen et al., 1987.

et al., 1994). On that basis, the band was identified as human laminin $\alpha 2$, despite our observation that the 205-kD band did not react with anti- $\alpha 2$ mAb (mAb 1922; Chemicon). The band at 185 kD produced two peptides identical to human $\beta 1$, and was thereby confirmed as human $\beta 1$.

In contrast to the easy identification of the other two bands, the band at 170 kD contained three sequences not contained within any known laminin chain. The NH_2 -terminal sequence of the 170-kD chain was determined, and it also was novel; i.e., nonidentical to known laminin sequences. As these four sequences from the 170-kD band were derived from an unknown laminin and we had identified the laminin α and β chains, we assumed these sequences were derived from a novel laminin γ chain that we call $\gamma 3$.

The apparent molecular masses for the 205- and 185-kD bands are not consistent with the literature values published for the $\alpha 2$ and $\beta 1$ chains, respectively. Thus, these bands are indicated in Fig. 1 as $\alpha 2t$, $\beta 1t$, and $\gamma 3t$ to indicate that they have been processed (truncated). Laminin 2 and laminin 4 were also present in these preparations; when characterized by similar procedures (not described here in detail) they showed molecular masses consistent with literature predictions, suggesting that our preparations were not extensively and nonspecifically degraded. Together these observations suggest that the truncations observed for the $\gamma 3$ -containing molecules may be physiologically relevant.

Characterization of the $\gamma 3$ cDNA

The cDNA sequences of human $\gamma 1$ and $\gamma 2$ were used to probe the National Center for Biomedical Information (NCBI) expressed-sequence-tag database (dbEST), and a clone was identified that was homologous, but not identical, to $\gamma 1$ and $\gamma 2$. The sequence of this clone was used to design PCR primers for extensions at 3' and 5' ends (see Materials and Methods) using human placental cDNA, and additional sequence information was obtained by a combination of genomic DNA and placental cDNA sequencing. The resulting sequence is shown in Fig. 2. The

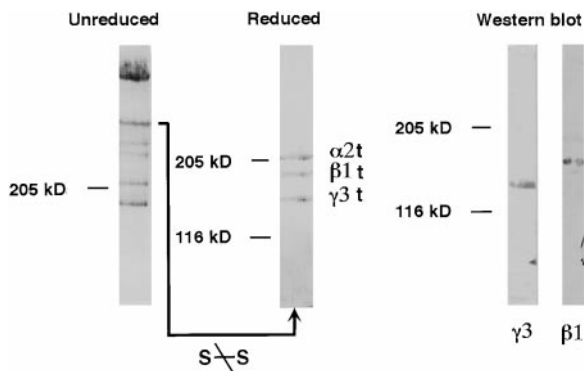


Figure 1. Identification of laminin 12 isolated from human placenta. A partially purified preparation of laminins isolated from an EDTA extract of placenta was subjected to 3–5% SDS-PAGE (Unreduced). The gel band in the indicated position (bar) was excised from the gel and resolved by 5% SDS-PAGE after disulfide-bond reduction. Three bands were seen from the single unreduced band (Reduced). The 185-kD band was recognized by an anti- $\beta 1$ antibody, while the 170-kD band was specifically recognized by a polyclonal antibody (R16; which used the same band as an antigen; Western blot). Each of the three bands (Reduced gel) was subsequently identified as laminin $\alpha 2$, $\beta 1$, and $\gamma 3$ (see text for details). As they have faster electrophoretic mobilities than expected for these chains they are marked $\alpha 2t$, $\beta 1t$, and $\gamma 3t$ to indicate that they are truncated.

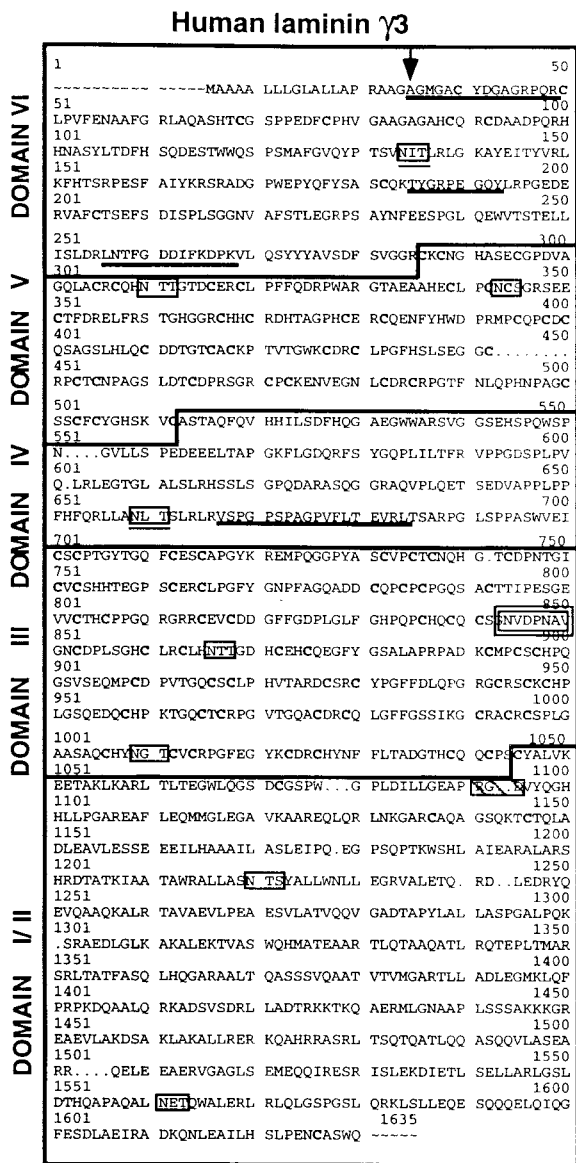


Figure 2. The complete amino acid sequence of human $\gamma 3$ as predicted from the corresponding cDNA sequence. The amino acid positions are numbered in accordance to the homologous locations within laminin $\gamma 1$ (Pikkarainen et al., 1988). A 19-amino acid signal sequence precedes the $\gamma 3$ NH₂ terminus (arrow marks the signal peptide cleavage site). Peptide sequences obtained from Edman analyses of the fragmented $\gamma 3$ t gel band (Fig. 1, Reduced) are underlined. Potential glycosylation sites are boxed, and those conserved between $\gamma 1$ and $\gamma 3$ are also underlined. The nidogen binding consensus sequence is boxed in bold type. The sequence RGD is boxed and hatched.

deduced amino acid sequence contains regions with 100% identity to all three of the peptide sequences obtained from the 170-kD band (underlined in Fig. 2). The nucleotide sequence reported in this paper has been submitted to GenBank/EMBL Data Bank with the accession number AF041835.

The DNA sequence contains an open reading frame predicting 1620 amino acids, including a 19-amino acid-

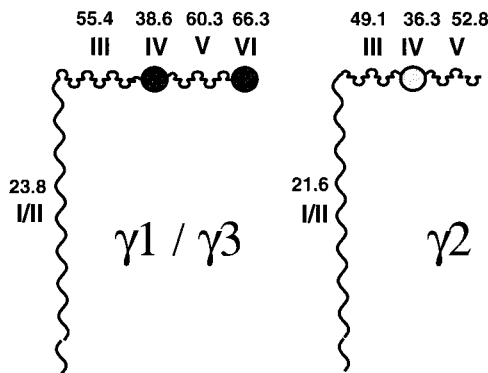


Figure 3. Comparison of the predicted domain structures and the percent amino acid identity of $\gamma 3$ with $\gamma 1$ and with $\gamma 2$. The $\gamma 3$ cDNA predicts a full-length laminin chain where all the domains found in $\gamma 1$ are represented. On the left side a diagram of the domain structure of both $\gamma 1$ and $\gamma 3$ is given; on the right, the domain structure of $\gamma 2$. Above each diagram is noted by domain the percent amino acid identity between $\gamma 1$ or $\gamma 2$ and $\gamma 3$. The degree of amino acid identity of $\gamma 3$ to $\gamma 1$ is higher than that of $\gamma 3$ to $\gamma 2$, suggesting that $\gamma 3$ has a closer evolutionary relationship with $\gamma 1$ than $\gamma 2$.

long putative signal peptide that closely meets the criteria described by Nielsen et al. (1997). The predicted cleavage site was confirmed by protein sequencing of the $\gamma 3$ NH₂ terminus; this sequence exactly matched the predicted amino acid sequence following the signal peptide. The overall sequence of $\gamma 3$ is most similar to that of $\gamma 1$, sharing 52% amino acid similarity with human $\gamma 1$ (Pikkarainen et al., 1988). In addition, the amino acid sequence predicted by the $\gamma 3$ cDNA contains a domain distribution most like that of the $\gamma 1$ chain. All six domains are represented.

Overall, the $\gamma 3$ chain has 43.6% amino acid identity with the $\gamma 1$ chain and 34% identity with the $\gamma 2$ chain. The highest conservation is seen between domains $\gamma 1$ VI and $\gamma 3$ VI (Fig. 3). Domains $\gamma 3$ V and III also show considerable similarity to domains $\gamma 1$ V and III and $\gamma 2$ V and III.

The predicted $\gamma 3$ sequence contains nine potential glycosylation sites (Fig. 2, boxed), only two of which (Fig. 2, boxed and underlined) are conserved in both human and mouse $\gamma 1$. As these conserved sites are contained within the globular domains IV and VI, it is likely that these sites are used physiologically. There is a single RGD sequence (boxed, hatched) within domain II, but this site is not conserved in either human or mouse $\gamma 1$ and $\gamma 2$ proteins. The sequence NVDPNNAV (Fig. 2, double boxed) occurs within the fourth EGF-like repeat of domain III and is a homologue of the nidogen binding site (NIDPNNAV) within the same domain of $\gamma 1$. These sequences differ by only a single conservative amino acid substitution.

LAMC3 Maps to Chromosome 9q31-q34

The $\gamma 3$ chromosomal location was determined by searching the NCBI Human Genomic Sequencing Index data base with the $\gamma 3$ cDNA sequence. The sequence is identical to a database, Sequence Tagged Sites (clone WI-14302), that has been localized to chromosome 9q33-q34. A 1.2-kb $\gamma 3$ cDNA probe within domains I and II of the predicted

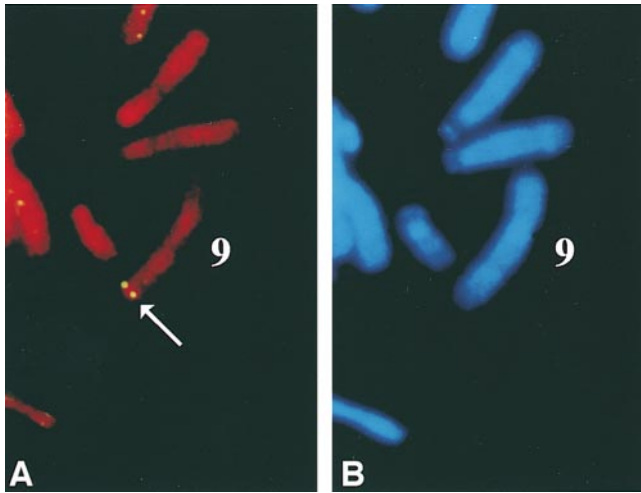


Figure 4. Localization of LAMC3 to chromosome 9, band q31-q34 by FISH. The position of LAMC3 was probed using a 1.3-kb cDNA probe within predicted protein domains I and II of γ 3. The FISH signal (A) was superimposed over the DAPI-banded chromosomes (B) to identify the location of the γ 3 gene. Both alleles of the γ 3 gene are labeled (A, arrow) at the end of the long arm of chromosome 9 (B, 9).

protein, the regions of least homology among the γ chains, was used to localize LAMC3 by fluorescent in situ hybridization (FISH) analysis (SeeDNA Biotech, Inc.). The results confirm the localization to chromosome 9q31-q34 (Fig. 4).

Laminin γ 3 Associates with α 2 β 1 to Form Laminin 12 which, in Placenta, Is Lacking Part of the I/II Domains of All Three Chains

To determine the domains present within α 2t, the 205-kD gel band, purified from placenta, was fragmented with trypsin and the resulting peptides were fractionated by HPLC; the masses of the eluted peptides were determined by mass spectroscopy. The ion chromatograms were then evaluated relative to the masses predicted from the published amino acid sequence for α 2 in order to determine the NH₂- and COOH-terminal peptides present within the digest. The results identified a number of tryptic peptides; among these, the peptide LVEHVPGQP(VR), beginning at residue 70 within domain VI, was the most NH₂-terminal; the peptide GTTMTPPADLIEK, beginning at residue 1367 within domain III, was the most COOH-terminal. These results indicate that α 2t is a fragment containing the short arm of the laminin α 2 chain. This conclusion is consistent with the observation that the initial peptide sequence identified from α 2t was within the short arm domains (above).

β 1t and γ 3t are also short arm fragments, as all the peptide sequences determined for both species are present within the short arm domains. However, the masses of α 2t, β 1t, and γ 3t are greater than predicted for the short arms alone. In addition, α 2t, β 1t, and γ 3t are not separable by gel electrophoresis without the reduction of disulfide bonds. Therefore, this truncated laminin 12 molecule is very likely to contain portions of domain II of all three

chains, as the interchain disulfide bonds should lie between these domains. It is of interest to note that domain II of γ 3 contains three cysteinyl residues whose bonding partners are not readily identified and are not present in domain II of other laminin chains. These three cysteinyl residues are conserved in mouse γ 3 (Albus, A., and R.E. Burgeson, unpublished observation). Whether these cysteinyl residues could form intrachain, interchain, or intermolecular disulfide bonds that in some way contribute to the cleavage of γ 3 chain-containing laminins is unknown.

Tissue Distribution of γ 3 Expression by Northern Analysis

Tissue RNA blots and Master Blot dot blots (Clontech) were probed with a γ 3 nucleotide probe (nt 1316 to 2277). A single major transcript of \sim 5 kb, consistent in size with other laminin γ chains, is present in several of the tissues examined (Fig. 5 A). A small amount of a second larger transcript can also be detected. This larger transcript is most likely due to differences in polyadenylation or due to inefficient splicing. The γ 3 chain RNA is abundant in spleen, testis, placenta, lung, and liver; lesser amounts are seen in kidney and ovary (Fig. 5 A). The predominance of a single transcript allowed use of the RNA Master Blot (Clontech) to determine expression in a large number of other tissues. On this dot blot, tissue RNA concentrations have been normalized to housekeeping genes. The Master Blot (Fig. 5 B) confirms the abundant presence of γ 3 transcripts in placenta, adrenal gland, testis, lung, and fetal kidney, but also shows detectable levels of γ 3 transcripts in numerous additional tissues, including brain and skeletal muscle.

Characterization of the Immunospecificity of Anti-Laminin γ 3 (R16; R21)

A polyclonal antiserum, R16, was made in a rabbit to the γ 3 chain excised from a reduced SDS-PAGE gel similar to that shown in Fig. 1. Another, R21, was made to recombinant γ 3 protein (see Methods). The R16 antiserum recognizes the γ 3 chain on immunoblots of placental extracts, but at very high antibody concentrations, it shows some reactivity with the β 1 and γ 1 chains as well. Thus, as a control, human neonatal foreskin was immunostained with anti-laminin γ 1 (polyclonal anti-laminin 1; Sigma Chemical Co.), anti-laminin γ 2 (GB3, Verrando et al., 1987), and with anti-laminin γ 3 (R16). Crisp, brilliant fluorescence was observed along the dermal-epidermal junction, and around capillaries with the anti- γ 1 antibodies (data not shown), and in the basement membrane at the dermal-epidermal junction with anti- γ 2 (data not shown); in contrast, no signal above background was detected using the anti- γ 3 reagent (R16) when it was applied at dilutions of 1:250 or more (data not shown). The antigen could not be unmasked by treatment of the cryosections with 2, 4, or 6 M urea, or with 2 M guanidinium-HCl (data not shown). As all known laminin chains have been detected in skin within either the epithelial basement membranes or the vascular basement membranes, these results indicate that the cross-reactivity detected by Western blot analyses using the polyclonal anti- γ 3 (R16) antibody was either not apparent by immunohistochemistry, or was below detection at the

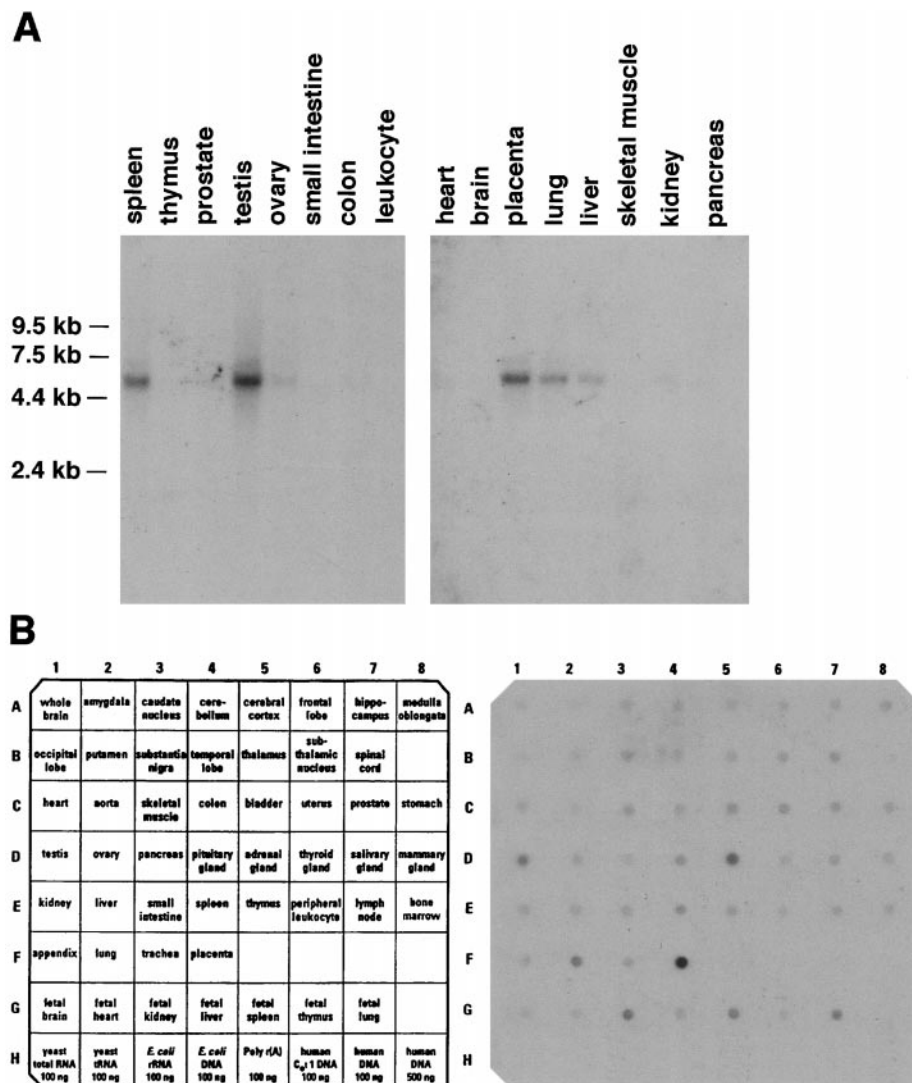


Figure 5. Laminin $\gamma 3$ chain is expressed widely in human tissues. The same cDNA probe described in Fig. 4 was used to probe tissue blots (A) and a dot blot (B). Only a single major RNA species is detected on the tissue blots; strong signals are obtained from testis, spleen, placenta, and lung. Less intense signals are obtained from ovary, kidney, liver, and skeletal muscle. Dot blots indicate a more widespread tissue distribution, including thyroid, gut, and some fetal tissues.

antibody concentrations used. For the subsequent anatomical experiments (below), R16 was diluted 1:250 or greater to assure no cross-reactivity was occurring. R21, the affinity-purified antiserum to recombinant $\gamma 3$, was also tested on sections of neonatal foreskin. As with R16, no immunoreactivity was seen (data not shown); thus we conclude that this antiserum has no cross-reactivity with other known γ chains. Neither R21 nor R16 antisera label the blood vessel basement membranes (see below) consistent with a lack of cross-reactivity to other γ chains.

$\gamma 3$ -containing Laminins Are Localized to Peripheral Nerves and to Ciliated Epithelial Apical Surfaces

Unlike the lack of anti-laminin $\gamma 3$ chain immunoreactivity seen in neonatal foreskin, laminin $\gamma 3$ chain immunoreactivity was detected in human leg skin. As shown in Fig. 6 A, and consistent with published results, laminin $\gamma 1$ chain reactivity is seen at the dermal-epidermal junction and within the basement membranes of the vasculature, while laminin $\gamma 2$ chain immunoreactivity is restricted to the dermal-epidermal junction (Fig. 6 B). The laminin $\gamma 3$ chain

immunoreactivity is further restricted to distinct patches widely spaced along the dermal-epidermal junction (Fig. 6 C). In experiments not shown, the immunoreactivity did not correlate positively or negatively with sites of cell proliferation, nor did it correlate with fixed positions relative to the rete ridges. However, there is a direct correlation of the laminin $\gamma 3$ chain immunoreactivity (Fig. 6 D) with sites where nerves cross the dermal-epidermal junction as detected by an antibody to the neuronal marker PGP9.5 (Fig. 6 E), which reacts with ubiquitin COOH-terminal hydrolase (Day et al., 1990). The results in skin suggest that $\gamma 3$ -containing laminins may be deposited into the dermal-epidermal junction by nerve or nerve associated cells, or that its expression by epithelial cells is induced by the adjacent nerve.

Laminin $\gamma 3$ is also expressed in the neural retina at the apical surface of the retina and in the outer synaptic layer (Libby, R.T., Y. Xu, E.P. Gibbons, M.-F. Champlaud, M. Koch, R.E. Burgeson, D.D. Hunter, and W.J. Brunken, manuscript submitted for publication); in the retina, the $\gamma 3$ chain is coexpressed with the $\alpha 4$, $\alpha 3$, and $\beta 2$ chains. Native $\gamma 3$ -containing laminins have not been isolated as yet from

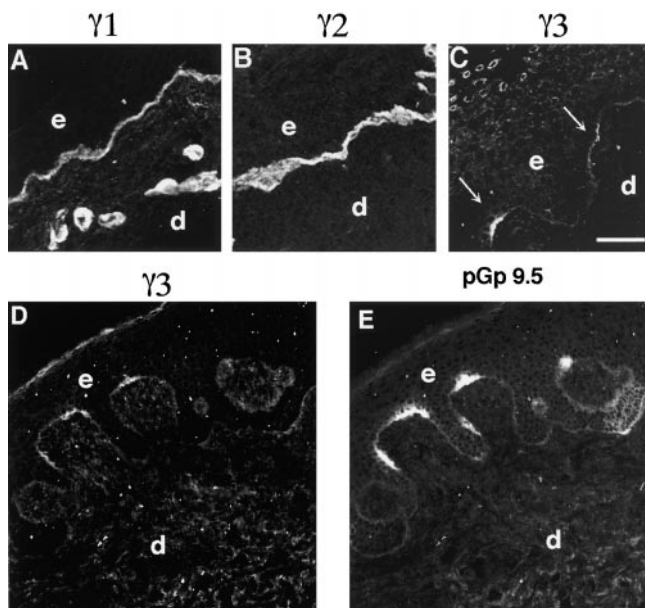


Figure 6. Laminin $\gamma 3$ chain is expressed in restricted sites, correlated with nerve terminations, in human skin. Laminin $\gamma 1$, $\gamma 2$, and $\gamma 3$ chains and nerve-specific ubiquitin COOH-terminal hydrolase (PGP 9.5) in human leg skin were localized by immunohistochemistry and confocal laser microscopy. Laminin $\gamma 1$ (A) immunoreactivity is present throughout the dermal-epidermal junctional basement membrane, as well as in the basement membranes of the glands and hair follicles. Laminin $\gamma 2$ immunoreactivity (B), on the other hand, is restricted to the basement membrane of the dermal-epidermal junction and that underlying the epithelial cells of the outer root sheath of the hair follicles. Finally, laminin $\gamma 3$ immunoreactivity (C) is most restricted and is found in distinct patches within the dermal-epidermal junction basement membrane. Laminin $\gamma 3$ immunofluorescence (D) and a nerve-specific marker (E, anti-PGP 9.5) are colocalized when both primaries are applied to the same section.

the retina; however, they have from another region of the central nervous system, the cerebellum, from which we have obtained two novel laminins, $\alpha 3\beta 2\gamma 3$ and $\alpha 4\beta 2\gamma 3$ (Champlaud, M.-F., unpublished observations). In addition, anatomical methods (immunohistochemistry and *in situ* hybridization), demonstrate the expression of $\gamma 3$ in cerebellum and forebrain structures (Brunken, W.J., unpublished observations). It seems likely that $\gamma 3$ -containing laminins will be a general feature of the matrix in the CNS.

The Northern analysis indicated that the laminin $\gamma 3$ chain was most strongly expressed in placenta, testis, lung, liver, spleen, and ovary. Therefore, we examined the localization of $\gamma 3$ chains within testis, lung, and ovary. The reactivity within the epididymis and the fallopian tube were particularly striking. Thus, the distribution of $\gamma 3$ in these tissues was extensively studied. In the female reproductive system, the oviduct was strongly reactive. Cryosections of the bovine (Fig. 7, A–F) or rat (Fig. 7, G–I) ampulla reacted for $\gamma 3$ using R16 (Fig. 7, A, D, and G–I), or R21 (Fig. 7, B and E) showed brilliant immunoreactivity at the apical surfaces of the tubal mucosa. Double immunofluorescent studies performed with laminin $\gamma 3$ and either lami-

nin $\alpha 2$ (Fig. 7, A and D) or laminin $\alpha 5$ antibodies (Fig. 7 E) demonstrated that both of these α chains are restricted to the basement membranes of the tubal epithelial and the subjacent endothelium whereas $\gamma 3$ is expressed at the apical surface. The pre-immune serum from rabbit 21 (Fig. 7 C) was negative, as was the reactivity of anti-thioredoxin antibodies purified from the R21 serum by immunoaffinity (Fig. 7 F).

The pattern of immunoreactivity for R16 in the rat oviduct was identical to that seen in bovine tissue (Fig. 7 G). Higher magnification micrographs of the epithelial apical surface of the rat ampulla (Fig. 7, H and I) show the $\gamma 3$ chain to be localized to the apical surface of the epithelial cells at the base of the cilia.

It should be noted that the labeling pattern of R21 differed somewhat from that of R16. In general, the pattern with R21 was somewhat punctate, showing large deposits of immunoreactivity at the apical surface, and increased cytoplasmic labeling of the tubal epithelium, whereas the R16 immunoreactivity was more restricted to the apical extracellular surface. These observations suggest that the R21 antiserum, made to recombinant domain I, may recognize the unfolded $\gamma 3$ chain better than the R16 antiserum, which should recognize primarily short arm domains.

The male monkey reproductive tract was examined also. Like the fallopian tube, the epithelium in the epididymis is a single columnar epithelium (Fig. 8, A, H, and E). *In situ* hybridization performed on adjacent sections of the monkey epididymis (Fig. 8 B; $\gamma 3$) localized transcripts for the $\gamma 3$ chain to the apical region of the epithelial cells (compare Fig. 8, A with B). R16 (data not shown) and R21 (Fig. 8, C and D, R21) sera gave similar patterns, reacting with both the basal and apical surfaces of the epithelial cells. The R21 antiserum reacted with apparently intracellular stores of $\gamma 3$, as was seen in the bovine fallopian tube. The preimmune control serum from R21 showed only punctate autofluorescence (Fig. 8 E, Pre).

Potential chain partners were explored by examination of the same tissue with antibodies specific for a variety of other laminin chains: $\alpha 2$ (Fig. 8 F), $\alpha 4$ (G), $\beta 1$ (H), and $\beta 2$ (I). We used two monoclonal antibodies to test for the presence of $\beta 1$ at the apical surface (clones 545; and C21) both gave the same pattern of immunolabeling; only the results with clone 545 are shown. As can be seen readily, $\alpha 2$ and $\beta 2$ were restricted to the basal surface of the epithelial cells, while staining for $\alpha 4$ and $\beta 1$ were also seen at the apical surface. Thus, in contrast to the results from placental extracts, $\alpha 4$ (and not $\alpha 2$) appears to be a candidate chain partner for $\gamma 3$ in the epididymis. These observations suggest that a wide variety of $\gamma 3$ -containing laminins will be expressed in a tissue-specific pattern.

Expression of laminin $\gamma 3$ chain was examined in the rat as well and the tissue distribution of $\gamma 3$ in the rat epididymis was similar to that described for the monkey (data not shown); namely, $\gamma 3$ immunoreactivity was localized to the apical surface of the epithelium. We also studied other regions of the rat reproductive system. Unlike laminin-1 immunoreactivity, which is localized to the basement membrane of the seminiferous tubules (Fig. 9 A), $\gamma 3$ immunoreactivity is not present within the basement membrane of the seminiferous tubules nor is it found around the interstitial cells (Fig. 9 A, arrows; Fig. 9 B, asterisk).

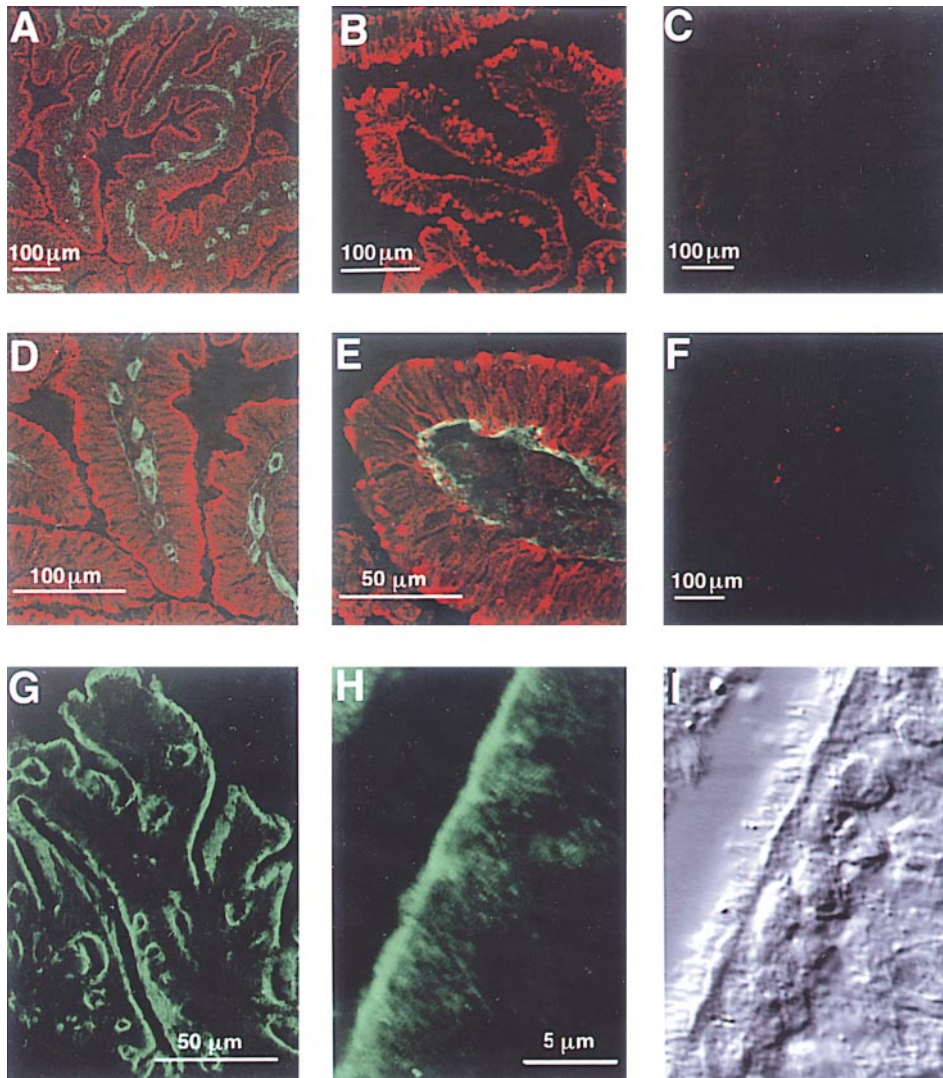


Figure 7. Laminin $\gamma 3$ is expressed on the apical surface of the ciliated epithelium of the bovine and rat fallopian tube. Confocal laser microscope images of freshly frozen tissue sections of fallopian tube; in each image, five optical sections were superimposed. The laminin $\gamma 3$ -reactive antiserum R16 (A and D, red) and anti-laminin $\alpha 2$ chain (A and D, green) were applied to bovine tissue together; $\gamma 3$ immunoreactivity is confined to the apical surface of the epithelium, whereas $\alpha 2$ is present in the basement membrane; D is a higher magnification of one of the folia in A. Application of the $\gamma 3$ -reactive R21 serum alone (B) also labels the apical surface of the bovine epithelium, but in plaque-like structures. R21 pre-immune serum is nonreactive (C). Simultaneous visualization of R21 immunoreactivity (E, red) and $\alpha 5$ immunoreactivity (E, green) shows that the $\gamma 3$ is found on the apical side of the epithelium and in cytoplasmic stores in the long processes of these columnar tubal cells whereas $\alpha 5$ -immunoreactivity is confined to the basement membrane. Antibodies purified from the R21 serum with the carrier protein, His-thioredoxin are negative (F). $\gamma 3$ shows a similar distribution in rat fallopian tube (G and H): R16 reactivity is present at the apical surface over the whole of the ampulla (G) and in a higher power view of the epithelium (H); comparison of the fluorescent image in H with the differential interference contrast image in I demonstrates that $\gamma 3$ immunoreactivity is associated with the ciliated surface of the epithelium.

epithelium (H); comparison of the fluorescent image in H with the differential interference contrast image in I demonstrates that $\gamma 3$ immunoreactivity is associated with the ciliated surface of the epithelium.

Within the seminiferous tubules, only the occasional tubule reacted strongly with the laminin $\gamma 3$ reactive serum (R16, Fig. 9 B); it was our impression that those tubules identified by the antibody contained nearly mature spermatids. Further along the male reproductive system, in the ductus deferens, laminin-1 immunoreactivity (Fig. 9 C; arrows mark the apical surface of the epithelium) was seen along the epithelial basement membrane, in the lamina propria and ensheathing the smooth muscle cells of the muscular layer. In contrast, $\gamma 3$ immunoreactivity (R16) was found at the apical and basal surfaces of the epithelial cells, as well as intracellularly (Fig. 9 D).

The apical distribution of the $\gamma 3$ chain is not confined to the reproductive system; in rat lung, the ciliated epithelial cells lining the bronchi were also strongly reactive with the anti-laminin $\gamma 3$ antiserum, R16 (Fig. 9 E). Again, the fluorescence was apparent along the apical surface, as determined by differential interference contrast microscopy (Fig. 9 F). No $\gamma 3$ -immunoreactivity was seen in respiratory

epithelium nor in the pulmonary capillary bed (not shown).

Discussion

The laminin $\gamma 3$ chain described here is the eleventh laminin subunit to be identified. The predicted primary and secondary structure of this chain suggests that $\gamma 3$ is more closely related to human $\gamma 1$ than $\gamma 2$. Unlike $\gamma 2$, the $\gamma 3$ cDNA sequence predicts a laminin subunit without the short-arm truncations predicted for $\gamma 2$. Perhaps more significantly, $\gamma 3$ contains a $\gamma 1$ -like nidogen binding motif with only a single conservative amino acid substitution, suggesting that $\gamma 3$ -containing laminins should be capable of associating with other basement membrane molecules through nidogen interactions (Mayer et al., 1995; Poschl et al., 1996). In addition, domain VI of $\gamma 3$ shares the highest sequence identity with domain VI of the $\gamma 1$ chain. As this latter domain has been shown to support laminin self-

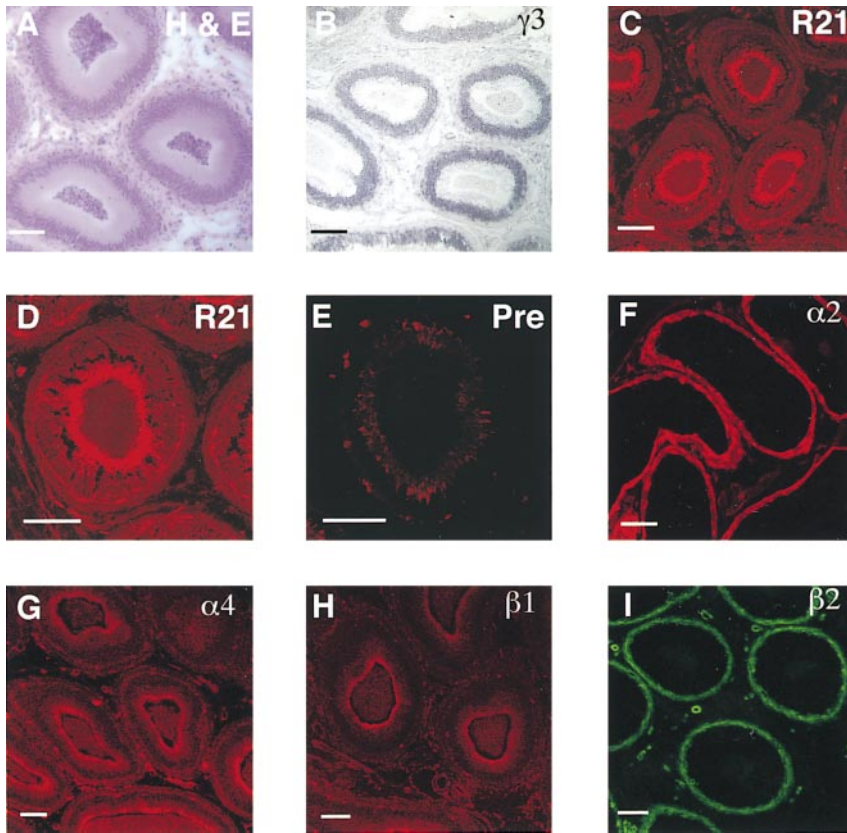


Figure 8. Laminin $\gamma 3$ is expressed on the surface of male reproductive epithelium. Light (A and B) and confocal (C–I) images of the epididymis from monkey (rhesus). In A, a section was fixed and stained with H&E for orientation; the epididymis is composed of a columnar epithelium, with considerable interstitial matrix between adjacent folds. (B) By in situ hybridization, a $\gamma 3$ -specific probe is localized to the epithelium; the reaction product (dark blue) is concentrated above the nuclear region of the cell; this section is not counter-stained. (C and D) $\gamma 3$ immunoreactivity (R21) is present at the surface of the epithelium; (E) pre-immune (Pre) serum is nonreactive in this tissue. (F–I) Immunolocalization of other laminin chains; both $\alpha 2$ and $\beta 2$ are confined to the basement membranes of the epithelium and blood vessels, whereas both $\alpha 4$ and $\beta 1$ are seen at the apical surface of the epithelium; $\alpha 4$ is also expressed in the interstitial matrix and the basement membrane. Bars, 100 μm .

assembly (Yurchenco and Cheng, 1994), it seems reasonable to suggest that domain VI of the $\gamma 3$ chain may also support self-assembly.

Two of the predicted glycosylation sites in the $\gamma 3$ chain are also found in human and in mouse $\gamma 1$; these are within short-arm globular domains, i.e., VI and IV. Interestingly, the glycosylation site in domain IV is also found in human (Kallunki et al., 1992) and in mouse (Sugiyama et al., 1995) $\gamma 2$. This remarkable conservation of glycosylation sites among these three chains and between these species suggests that these sites are indeed important and glycosylated; they are likely to be critical in the folding of this region or may play another important function.

The RGD sequence within domain II of the $\gamma 3$ chain is found in neither $\gamma 1$ nor in $\gamma 2$. Moreover, it seems likely that this sequence is not functional within native $\gamma 3$ -containing laminins as it is located within the coiled-coil region of $\gamma 3$. However, it very well may promote integrin-mediated recognition of non-native molecules or of protein fragments.

In placenta, the $\gamma 3$ chain can combine with the laminin $\alpha 2$ and $\beta 1$ chains. This observation suggests that, unlike $\gamma 2$ which pairs preferentially with $\beta 3$, $\gamma 3$ may pair with any β chain, with the possible exception of $\beta 3$, and with any of the known α chains. This prediction suggests the existence of an additional 10 laminins with the following chain compositions: $\alpha 1\beta 1\gamma 3$, $\alpha 1\beta 2\gamma 3$, $\alpha 2\beta 1\gamma 3$, $\alpha 2\beta 2\gamma 3$, $\alpha 3\beta 1\gamma 3$, $\alpha 3\beta 2\gamma 3$, $\alpha 4\beta 1\gamma 3$, $\alpha 4\beta 2\gamma 3$, $\alpha 5\beta 1\gamma 3$, and $\alpha 5\beta 2\gamma 3$. In both the epididymis and the fallopian tube, $\gamma 3$ is not combined with $\alpha 2$. In the epididymis, the $\alpha 4$ and $\beta 1$ chains appear to

be potential partners. Given that the total number of human laminins is not known, at least one additional β chain has been identified in chicken (Ybot-Gonzalez et al., 1995; Liu et al., 1998) and in mammals (Olson, P.F., unpublished observations), assigning a final laminin numerical identifier to these laminins is premature. However, as we have shown $\alpha 2\beta 1\gamma 3$ to be the twelfth laminin to be identified, we provisionally call this heterotrimer, laminin 12.

The masses of the chains of laminin 12 as approximated by electrophoretic mobility are considerably less than predicted by the amino acid sequences and from prior experience with the $\alpha 2$ and $\beta 1$ chains. They are also less than the $\alpha 2$, $\beta 1$, and $\beta 2$ chains present in laminins 2 and 4 obtained from the same preparations. The reason for these more rapid electrophoretic migration rates appears to be proteolysis within the domains II of the chains comprising this molecule. In placenta, this proteolysis may be physiological, since laminins 2 and 4 isolated from the same preparations are apparently intact. The significance of this observation awaits considerable additional experimentation before it is understood. However, we have observed three cysteinyl residues within domain II of the $\gamma 3$ chain that are not present in other human α , β , or γ chains. It is possible that a disulfide bond between two of these residues distorts the coiled-coil conformation making molecules containing this chain more susceptible to proteolysis. At this time, we do not know if truncation of laminins containing the $\gamma 3$ chain can be generalized to tissues other than placenta; however, this seems unlikely in that our isolation of $\gamma 3$ -containing laminins from the CNS do not show

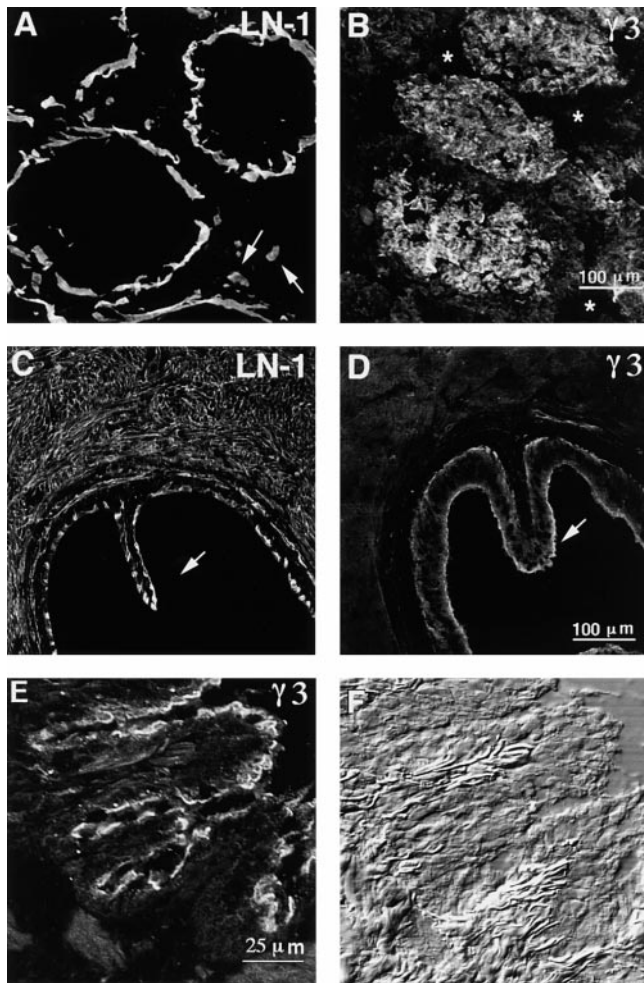


Figure 9. Laminin $\gamma 3$ is expressed on the surface of rat ciliated epithelia in testis and lung. Various tissues from the rat male reproductive system (A–D) were incubated with either laminin-1 ($\alpha 1/\beta 1/\gamma 1$) antiserum (left column) or $\gamma 3$ antiserum (right column). (A and B) testis; (C and D) ductus deferens. The laminin-1 immunoreactivity is present in the basement membrane in these structures (left column); arrows denote interstitial cells in A, and the apical borders of the epithelium in C; the basal lamina surrounding the smooth muscle is also easily distinguished (C). In contrast, laminin $\gamma 3$ expression is distinctly not in the basement membrane of the testis (B) nor of the ductus deferens (D). $\gamma 3$ expression is limited to the seminiferous tubules themselves and does not surround the interstitial cells (B, asterisk). Within the seminiferous tubules, $\gamma 3$ expression appears to change with the state of maturation of sperm (see text for details). In the ductus deferens, $\gamma 3$ expression (D) is limited to the surface of epithelial cells (basolateral and apical). No $\gamma 3$ expression is seen in the smooth muscle of the ductus deferens. In lung (E and F), laminin $\gamma 3$ is expressed on the apical surface of nonrespiratory airways. $\gamma 3$ immunoreactivity is limited to the apical surface of the epithelium (E; compare to the differential interference contrast image of the same section in F). Scale bars are given for each pair of figures (A and B, C and D, E and F).

the same truncation (Champlaud, unpublished observations). The COOH-terminal truncation of $\alpha 2t$ explains its lack of reactivity with an anti- $\alpha 2$ antibody, mAb 1922, which is specific for the $\alpha 2$ G domain (Engvall et al., 1990).

The antiserum to the recombinant $\gamma 3$ domain I fusion

protein (R21) was originally made to evaluate this potential processing, since epitopes contained within this domain should be absent from the processed molecule. In this regard, the immunohistochemical data is not definitive. Like R16, R21 immunoreactivity is seen at the apical surface; however, the apical reactivity is distinctly different than that for R16. Specifically, R21 immunoreactivity appears as a plaque-like structure at the cell surface, with some reactivity within the cells. Thus, it seems possible that R21 epitopes are entirely intracellular but it is also possible that some of the R21 epitopes are present at the apical surface of these epithelial cells. Further experimentation beyond the scope of this report is required to address this question. However, laminins containing $\gamma 3$ chains have been immunisolated from the medium of A204 cells derived from a human rhabdomyosarcoma (Champlaud, M.-F., unpublished data), indicating that $\gamma 3$ -containing laminins are capable of being secreted in vitro. These $\gamma 3$ -containing laminins from A204 are a mixture of processed (truncated) and unprocessed (untruncated) molecules.

Reports of laminins in tissue locations not identified as basement membranes are increasingly frequent. In the brain, laminins have been observed not only within the basement membrane of capillaries, but also at other sites not conceptualized as basement membranes (Higuchi et al., 1991; Jucker et al., 1992, 1996a,b; Mori et al., 1992; Tian et al., 1996, 1997; Hagg et al., 1997; Yamamoto et al., 1997). In the eye, the laminin $\beta 2$ chain has been identified in both basement membrane and non-basement membrane locations (Hunter et al., 1992; Libby et al., 1996; Libby et al., 1997; Toti et al., 1997). Laminins have also been observed in cartilage (Durr et al., 1996).

Intriguingly, the laminin $\gamma 3$ chain appears most commonly to be associated with non-basement membrane structures. In the cerebellum, $\gamma 3$ chains are detected in the pericellular nets surrounding both neurons and glia (Brunken, W.J., unpublished observations). Reported elsewhere (Libby et al., manuscript submitted for publication, see above), $\gamma 3$ is present within the neural retina at two extracellular sites: between the outer segments of the photoreceptors, and at the synapses of the photoreceptors with the bipolar and horizontal cells. In the retina, these $\gamma 3$ -containing molecules are the products of the Müller glial cells which, like the tubal epithelium, contain a considerable store of intracellular $\gamma 3$ chain. The laminin $\alpha 3$ and $\beta 2$ chains are also present at these sites, whereas the $\gamma 1$ and $\gamma 2$ chains are absent. The functions fulfilled by these laminins are unclear, but possibilities include: stabilization of neural architecture; induction and stabilization of differentiated neural phenotypes (Hunter et al., 1992b; Libby et al., 1996; Hunter and Brunken, 1997); and stabilization of synaptic junctions.

However, the most abundant expression of $\gamma 3$ as detected by Northern analyses is not within neural tissues, but rather is in the testis, the placenta, the spleen, the lung, and the ovary. $\gamma 3$ immunoreactivity is present at the bases of the epithelial cilia of the epididymis, the trachea, the bronchi, and the oviduct. There are no structures resembling basement membrane at these sites. However, $\gamma 3$ chains may be present within the basement membranes along the basolateral surfaces of some of these epithelia.

The chain partners for the $\gamma 3$ chain in these apical laminins are not yet known with certainty. However, in epididymis, the laminin $\alpha 2$ chain does not colocalize with $\gamma 3$ at the apical epithelial surface; rather, the $\alpha 4$ and $\beta 1$ chains are present at that location and, thereby, are potential chain partners. Thus, it seems likely that $\gamma 3$ will be as promiscuous as $\gamma 1$ with respect to partner choice during laminin assembly.

The presence of laminins along ciliated epithelial surfaces was unexpected and their functions there are unknown. Perhaps a modified basement membrane containing at least laminin helps organize or stabilize the specialized cytoskeleton of the cilia. Laminins at these apical surfaces may also participate in the anchorage of mucins to the surface. Alternatively, laminins might stabilize the outfoldings of the plasma membranes of the cilia. Similar functions for laminins have been postulated to stabilize the junctional folds beneath synapses at neuromuscular junctions (Noakes et al., 1995), and contribute to the organization of epithelial hemidesmosomes (Langhofer et al., 1993; Baker et al., 1996). Laminins expressed at the apical surface of the retina are thought to play a role in photoreceptor morphogenesis, specifically outer segment formation and synapse development (Libby et al., 1997, 1998).

Consistent with the above speculations regarding an essential function for $\gamma 3$ containing laminins in neural tissues, the chromosomal locus of LAMC3 at 9q31-q34 is shared with four diseases having various degrees of neural dysfunction in common: Fukuyama congenital muscular dystrophy (FCMD); muscle-eye-brain syndrome; Walker-Warburg syndrome; and retinitis pigmentosa-21 with deafness (RP-21). The genetic cause of the latter three of these conditions is unclear. While $\gamma 3$ expression may be affected in FCMD, the LAMC3 cannot be the genetic cause of the problem as a retrotransposal insertion in a different gene has recently been identified in 87% of the FCMD alleles (Kobayashi et al., 1998). However, LAMC3 is an excellent candidate for mutations underlying one or more of remaining syndromes in this cluster of human diseases, particularly RP-21.

We have identified the laminin $\gamma 3$ chain together with the $\alpha 2$ and $\beta 1$ chains within laminin 12 from human placenta, but multiple other combinations are possible in other tissues. It would be of particular interest if $\gamma 3$ were to associate with the $\beta 2$ chain and well as with the $\alpha 2$ chain, as both these chains have been reported to show neural and muscle-associated expression and function. The $\gamma 3$ -containing laminins are likely to be the subject of considerable interest as they constitute a novel class of laminin molecules distributed outside of the traditional basement membrane. The identification of the function of this diverse family of laminins remains to be elucidated by future experiments.

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