Characterization and Expression of the Laminin γ3 Chain: A Novel, Non-Basement Membrane-associated, Laminin Chain

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Abstract. Laminins are heterotrimeric molecules composed of an α, β, and 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, γ3. We have cloned gene encoding this chain, LAMC3, which maps to chromosome 9 at q31-34. Protein and cDNA analyses demonstrate that γ3 contains all the expected domains of a γ chain, including two consensus glycosylation sites and a putative nidogen-binding site. This suggests that γ3-containing laminins are likely to exist in a stable matrix.

Studies of the tissue distribution of γ3 chain show that it is broadly expressed in: skin, heart, lung, and the reproductive tracts. In skin, γ3 protein is seen within the basement membrane of the dermal-epidermal junction at points of nerve penetration. The γ3 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 γ3-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: α1 (Hapaparanta et al., 1991), α2, (Volteenaho et al., 1994), α3 (Ryan et al., 1994), α4 (Iivanainen et al., 1995a), β1 (Pikkarainen et al., 1987), β2 (Wewer et al., 1994; Iivanainen et al., 1995b), β3 (Gerecke et al., 1994), γ1 (Pikkarainen et al., 1988), and γ2 (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 α5 (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) 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...
et al., 1990; Dragó et al., 1991; Goodman, 1992; M. attter and Laurie, 1994; Roussel et al., 1995; Chen et al., 1997), and/or to dystroglycan in muscle cells (Heunry 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 (Urchenco 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 γ1 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; R. einhardt et al., 1993).

The role of laminin 5 (α3β3γ2) in stabilization of epithelial-stromal interactions is the exception to this generalized scheme. While the 5 laminin G domains of α3 bind integrins α6β4 and α3β1 on the epithelial basolateral surface (Nissen et al., 1994), the absence of domains VI on the truncated short arms of α3 and γ2 (Kallunki et al., 1992; R. Yan et al., 1994), and the absence of a nidogen binding site on γ2 (M. ayer et al., 1995) prevent their participation in the above-described model. Instead, the NH₂ terminus of the epithelial cell–associated laminin 5 binds type VII collagen present within the subjacent stromal matrix (Roussel 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 γ1 chain in the above generalized scheme is the contribution of a domain V1 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 (M. ayer et al., 1979; Poschl et al., 1994). In the γ2 chain, the analogous sequence NVDPSAS is present, but does not support high affinity nidogen binding (M. Ayer 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 γ1, including a domain V1 homologue and a nidogen-binding site containing a single conservative amino acid substitution. We call this chain the laminin γ3 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 γ3 chains in regions lacking ultrastructurally identifiable basement membranes.

**Materials and Methods**

**Isolation of a Novel Laminin 12 (α2β1γ3)**

The purification of the novel laminin 12 (α2β1γ3) 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 glit heterosumum 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 the laminins were eluted by addition of 300 glit heterosumum sulfate. The proteins were collected by centrifugation (30,000 g, 60 min), redissolved in Concanavalin A buffer (0.5 M NaCl, 5 mM CaCl₂, 5 mM MgCl₂, 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-glucopyranosides, 1 M α-glucopyranosides, and finally 1 M α-mannopyranosides (Sigma Chemical Co.). Laminins were 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 fraction of interest was 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 (Abersold 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 kDa were excised separately, digested with trypsin, and then separated by HPLC and sequenced using an Applied Biosystem sequenator. Analysis of the tryptic digest of laminin γ2 isolated from laminin 12 was performed with matrix-assisted laser desorption time-of-flight mass spectrometry performed on a Finngan Lasermat 2000 (Chait and Kent, 1992).

**cDNA Cloning**

By comparison of the laminin γ1 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 AA 297192) 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 (clone, AA 297192). Nested PCR on placental Marathon-Ready cDNA (Clontech) were performed following the manufacturer’s instructions using the supplied non-specific primers with the following gene-specific primers for the 5′ extension; in the first round, (5′-dCGCATTGTGGCTCGTGTCCG-GCACTGG) in the second round, (5′-dGCGACAATGCATCTGGCCATGTTGCCG-CACTTGG); for the 3′ extension, in the first round, (5′-ATG-CAACGGACTCGACCGCTGCTACACCC); in the second round (5′-dCTGGCTACATCCCCGTCTCGACACTCC). 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 agarose gel (Gel Purification Kit; Qiagen) and either subcloned (into PCR II or PCR 2.1 vectors; Invitrogen) or directly used for sequencing. To confirm the nucleotide sequence and control for PCR-induced nucleotide substitutions, gene-specific primers were used to reamplify the entire γ3 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 γ3 antisense primers following the manufacturer’s protocol; PCR was used to generate overlapping clones complementary to the entire human laminin γ3 chain. Sequencing of all the obtained PCR products revealed the nucleotide sequence of laminin γ3 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 PI clone DMPC-HFF #1-1463F2, 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 γ3 gene (sense primer, 5'-dCCCGG-CAGGGAAAGGCGGTCTTG; antisense primer, 5'-dGGTTAT-TAGAATCAGGATGAGCCAAGGCTCG). 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-γ3 5' untranslated region was confirmed by RT-PCR from placental RNA in 4% DMSO, (sense primer, 5'-dGGCTTGGAGATGCGCAAAGGGCTCAGG).

Nucleotide Sequencing
Nucleotide sequences were determined with a Thermo Sequenase cycle sequencing kit and dideoxyTP (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 GeneEdit-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 [32P]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× Denatron-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 TransScan-LE intensifying screen (Kodak) for 20 h at ~70°C.

Recombinant Expression of Domain I of the γ3 Chain
A cDNA encoding the COOH terminus of human γ3 was cloned into the Histx and pPEP-T vectors (kindly provided by Richard Kammerer, Biozentrum, Basel, Switzerland; based on the pET system; Novagen). The Histx 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 p1 in front of the cloning site. The γ3 cDNA fragment used was amplified by PCR from human placenta cDNA (see cDNA cloning) using primers that include the EcoRI adapters: forward, 5'-GCGGAATCCGAGGAAAGGCGGTCTTG; reverse, 5'-GGTATGCThrCACCAGCTCGGAGGCGGTCTTG; 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., γ3 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 γ3 chain, and showed minor cross-reactivity with other laminin chains at high antibody concentrations. A II antibody-related studies presented in this communication were conducted at concentrations well below those where cross-reactivity was observed. The histidine-tagged, thioredoxin-γ3 fusion protein was used for the production of a second rabbit antiserum (R21) which reacted with a single band in Western blots of placentally extracts.

Affinity Purification
The R16 antiserum was affinity-purified by binding to gel-purified γ3 that had been transferred to nitrocellulose and then eluted with 1 M acetic acid following by immediate neutralization. The R21 serum was purified by binding to the histidine-tagged, mouse μ1-human γ3 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, M acaca 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 α2 chain (mAb b2, 1992, Chemicon); polyclonal anti-laminin α4 (M Imner et al., 1997); polyclonal anti-laminin α5 chain (M Imner et al., 1995); two monoclonal anti-laminin β1 chain (345, M arinkovich et al., 1992, clone C21, Green et al., 1992); monoclonal anti-laminin β2 chain (Vartrand et al., 1987); monoclonal anti-PGP 9.5 (Ultrarclone, Ltd.) was used to identify nerves in skin. Secondary antibodies used were: goat anti-rabbit FITC (ICN Pharmaceuticals); goat anti-rabbit-Cy3 (J akson 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-γ3 chain were generated from human γ3 clones; cRNA were labeled during transcription by the incorporation of digoxigenin-UTP (Boehringer Mannheim); ~1 μg/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). A filter 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 hybridization was detected with an anti-digoxigenin antibody coupled to alkaline phosphatase (Boehringer Mannheim). Sections were incubated overnight with anti-digoxigenin diluted 1:10,000 in blocking solution (Boehringer Mannheim). A filter 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 (Lundstrom et al., 1986). For the FISH analysis, a
Results

Characterization of Laminin 12 (α2β1γ3)

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 M ono-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, U n reduced). 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, R educed). The band at 185 kD reacted with a monoclonal antibody (clone 545; Marinkovich et al., 1992) specific to the laminin β1 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 band contained three peptides sequence identical to human laminin α2 (published residues 536, 70, 1367; Vuolteenaho et al., 1994). On that basis, the band was identified as human laminin α2, despite our observation that the 205-kD band did not react with anti-α2 mAb (α2b 1922; Chemicon). The band at 185 kD produced two peptides identical to human β1, and was thereby confirmed as human β1.

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 NH2-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 γ3.

The apparent molecular masses for the 205- and 185-kD bands are not consistent with the literature values published for the α2 and β1 chains, respectively. Thus, these bands are indicated in Fig. 1 as α2t, β1t, and γ3t 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 γ3-containing molecules may be physiologically relevant.

Characterization of the γ3 cDNA

The cDNA sequences of human γ1 and γ2 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 γ1 and γ2. 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
The 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-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 g3 NH2 terminus; this sequence exactly matched the predicted amino acid sequence following the signal peptide. The overall sequence of g3 is most similar to that of g1, sharing 52% amino acid similarity with human g1 (Pikkarainen et al., 1988). In addition, the amino acid sequence predicted by the g3 cDNA contains a domain distribution most like that of the g1 chain. All six domains are represented. Overall, the g3 chain has 43.6% amino acid identity with the g1 chain and 34% identity with the g2 chain. The highest conservation is seen between domains g1VI and g3VI (Fig. 3). Domains g3V and III also show considerable similarity to domains g1V and III and g2V and III.

The predicted g3 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 g1. 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 g1 and g2 proteins. The sequence NVDPNAV (Fig. 2, double boxed) occurs within the fourth EGF-like repeat of domain III and is a homologue of the nidogen binding site (NIDPNAV) within the same domain of g1. These sequences differ by only a single conservative amino acid substitution.

**LAMC3 Maps to Chromosome 9q31-q34**

The g3 chromosomal location was determined by searching the NCBI Human Genomic Sequencing Index data base with the g3 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 g3 cDNA probe within domains I and II of the predicted...
published amino acid sequence for evaluated relative to the masses predicted from the pub-
by mass spectroscopy. The ion chromatograms were then HPLC; the masses of the eluted peptides were determined
trypsin and the resulting peptides were fractionated by
gel band, purified from placenta, was fragmented with
NH2- and COOH-terminal peptides present within the
at residue 70 within domain VI, was the most NH2-ter-
aminal; the peptide GTTMTPPADLIEK, beginning at
of All Three Chains
which, in Placenta, Is Lacking Part of the I/II Domains
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 γ3. The FISH signal (A) was superimposed over the DAPI-banded chromosomes (B) to identify the location of the γ3 gene. Both alleles of the γ3 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 hybrid-
ization (FISH) analysis (SeedaDNA Biotech, Inc.). The re-
sults confirm the localization to chromosome 9q31-q34 (Fig. 4).

Laminin γ3 Associates with α2β1 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 α2t, 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 pub-
ished amino acid sequence for α2 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 LVEHVPGQPR (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 α2t is a fragment con-
taining the short arm of the laminin α2 chain. This conclu-
sion is consistent with the observation that the initial
peptide sequence identified from α2t was within the short
arm domains (above).

β1t and γ3t are also short arm fragments, as all the pep-
tide sequences determined for both species are present
within the short arm domains. However, the masses of α2t,
β1t, and γ3t are greater than predicted for the short arms
alone. In addition, α2t, β1t, and γ3t 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 I of all three
chains, as the interchain disulfide bonds should lie be-
tween these domains. It is of interest to note that domain
II of γ3 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 γ3 (A Ibbs, A., and R.E. 
Burgeson, unpublished observation). Whether these cyste-
inyl residues could form intrachain, interchain, or inter-
molecular disulfide bonds that in some way contribute to
the cleavage of γ3 chain-containing laminins is unknown.

Tissue Distribution of γ3 Expression by
Northern Analysis
Tissue RNA blots and Master Blot dot blots (Clontech)
were probed with a γ3 nucleotide probe (nt 1316 to 2277).
A single major transcript of ~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 γ3 chain RNA is abundant in
spleen, tests, 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 γ3 tran-
scripts in placenta, adrenal gland, tests, lung, and fetal
kidney, but also shows detectable levels of γ3 transcripts in
numerous additional tissues, including brain and skeletal
muscle.

Characterization of the Immunospecificity of
Anti-Laminin γ3 (R16; R21)
A polyclonal antiserum, R 16, was made in a rabbit to the
γ3 chain excised from a reduced SDS-PAGE gel similar to
that shown in Fig. 1. A nother, R 21, was made to recombi-
nant γ3 protein (see Methods). The R 16 antiserum recog-
nizes the γ3 chain on immunoblots of placental extracts,
but at very high antibody concentrations, it shows some re-
activity with the β1 and γ1 chains as well. Thus, as a con-
hom, human neonatal foreskin was immunostained with
anti-laminin γ1 (polyclonal anti-laminin 1; Sigma Chemi-
cal Co.), anti-laminin γ2 (GB 3, Verrando et al., 1987), and
with anti-laminin γ3 (R 16). Crisp, brilliant fluorescence
was observed along the dermal-epidermal junction, and
around capillaries with the anti-γ1 antibodies (data not
shown), and in the basement membrane at the dermal-epi-
dermal junction with anti-γ3 (data not shown); in contrast,
no signal above background was detected using the anti-γ3
reagent (R 16) when it was applied at dilutions of 1:250 or
more (data not shown). The antigen could not be un-
masked by treatment of the cryosections with 2, 4, or 6 M
urea, or with 2 M guanidinium-HCl (data not shown). A s
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-γ3 (R 16) antibody was either not apparent
by immunohistochemistry, or was below detection at the
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 g3, 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 g chains. Neither R21 nor R16 antisera label the blood vessel basement membranes (see below) consistent with a lack of cross-reactivity to other g chains.

**g3-containing Laminins Are Localized to Peripheral Nerves and to Ciliated Epithelial Apical Surfaces**

Unlike the lack of anti-laminin g3 chain immunoreactivity seen in neonatal foreskin, laminin g3 chain immunoreactivity was detected in human leg skin. As shown in Fig. 6 A, and consistent with published results, laminin g1 chain reactivity is seen at the dermal-epidermal junction and within the basement membranes of the vasculature, while laminin g2 chain immunoreactivity is restricted to the dermal-epidermal junction (Fig. 6 B). The laminin g3 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 g3 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 g3-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 g3 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. Champliaud, M. Koch, R.E. Burgeson, D.D. Hunter, and W.J. Brunken, manuscript submitted for publication); in the retina, the g3 chain is coexpressed with the a4, a3, and b2 chains. Native g3-containing laminins have not been isolated as yet from
immunoreactivity is present throughout the dermal-epidermal junctional basement membrane. Laminin-g3 immunoreactivity (A) is present throughout the dermal-epidermal junctional basement membrane, as well as in the basement membranes of the glands and hair follicles. Laminin-g2 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-g3 immunoreactivity (C) is most restricted and is found in distinct patches within the dermal-epidermal junction basement membrane. Laminin-g3 immunofluorescence (D) and a nerve-specific marker (E, anti-PGP 9.5) are colocalized when both primaries are applied to the same section.

The Northern analysis indicated that the laminin-g3 chain was most strongly expressed in placenta, testis, lung, liver, spleen, and ovary. Therefore, we examined the localization of g3 chains in testis, lung, and ovary. The reactivity within the epididymis and the fallopian tube were particularly striking. Thus, the distribution of g3 in these tissues was extensively studied. In the female reproductive system, the 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) showed the g3 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 antisera, made to recombinant domain I, may recognize the unfolded g3 chain better than the R16 antisera, 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; g3) localized transcripts for the g3 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 antisera reacted with apparently intracellular stores of g3, as was seen in the bovine fallopian tube. The preimmune control serum from R21 showed only punctate autofluorescence (Fig. 8 E, Pre).

Potential chains partners were explored by examination of the same tissue with antibodies specific for a variety of other laminin chains: a2 (Fig. 8 F), a4 (G), b1 (H), and b2 (I). We used two monoclonal antibodies to test for the presence of b1 at the apical surface (clones 545; and C21) both gave the same pattern of immunolabeling; only the results with clone 545 are shown. a2 and b2 were restricted to the basal surface of the epithelial cells, while staining for a4 and b1 were also seen at the apical surface. Thus, in contrast to the results from placental extracts, a4 (and not a2) appears to be a candidate chain partner for g3 in the epididymis. These observations suggest that a wide variety of g3-containing laminins will be expressed in a tissue-specific pattern.

Expression of laminin-g3 chain was examined in the rat as well and the tissue distribution of g3 in the rat epididymis was similar to that described for the monkey (data not shown); namely, g3 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), g3 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).
Within the seminiferous tubules, only the occasional tu-
bule reacted strongly with the laminin γ3 reactive serum
(R16, Fig. 9 B); it was our impression that those tubules
identified by the antibody contained nearly mature sper-
matids. Further along the male reproductive system, in the
ductus deferens, laminin-1 immunoreactivity (Fig. 9 C;
arrow 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, γ3 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 γ3 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 γ3 antiserum, R16 (Fig. 9 E). A gain, the fluo-
rescence was apparent along the apical surface, as deter-
mined by differential interference contrast microscopy
(Fig. 9 F). No γ3-immunoreactivity was seen in respiratory
epithelium nor in the pulmonary capillary bed (not shown).

Discussion
The laminin γ3 chain described here is the eleventh lami-
nin subunit to be identified. The predicted primary and
secondary structure of this chain suggests that γ3 is more
closely related to human γ1 than γ2. Unlike γ2, the γ3
cDNA sequence predicts a laminin subunit without the
short-arm truncations predicted for γ2. Perhaps more sig-
nificantly, γ3 contains a γ1-like nidogen binding motif with
only a single conservative amino acid substitution, suggest-
ing that γ3-containing laminins should be capable of asso-
ciating with other basement membrane molecules through
nidogen interactions (Mayer et al., 1995; Poschl et al.,
1996). In addition, domain VI of γ3 shares the highest se-
quence identity with domain VI of the γ1 chain. As this
latter domain has been shown to support laminin self-

assembly (Yurchenco and Cheng, 1994), it seems reasonable to suggest that domain VI of the γ3 chain may also support self-assembly.

Two of the predicted glycosylation sites in the γ3 chain are also found in human and in mouse γ1; 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). 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 γ3 chain is found in neither γ1 nor in γ2. Moreover, it seems likely that this sequence is not functional within native γ3-containing laminins as it is located within the coiled-coil region of γ3. However, it very well may promote integrin-mediated recognition of non-native molecules or of protein fragments.

In placenta, the γ3 chain can combine with the laminin α2 and β1 chains. This observation suggests that, unlike γ2 which pairs preferentially with β3, γ3 may pair with any β chain, with the possible exception of β3, and with any of the known α chains. This prediction suggests the existence of an additional 10 laminins with the following chain compositions: α1β1γ3, α1β2γ3, α2β1γ3, α2β2γ3, α3β1γ3, α3β2γ3, α4β1γ3, α4β2γ3, α5β1γ3, and α5β2γ3. In both the epididymis and the fallopian tube, γ3 is not combined with α2. In the epididymis, the α4 and β1 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 (Y bot-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 α2β1γ3 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 α2 and β1 chains. They are also less than the α2, β1, and β2 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 γ3 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 γ3 chain can be generalized to tissues other than placenta; however, this seems unlikely in that our isolation of γ3-containing laminins from the CNS do not show...
The COOH-terminal truncation (Champliaud, unpublished observations) of laminin γ3 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 (α1/β1/γ1) antiserum (left column) or γ3 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 γ3 expression is distinctly not in the basement membrane of the testis (B) nor of the ductus deferens (D). γ3 expression is limited to the seminiferous tubules themselves and does not surround the interstitial cells (B, asterisk). Within the seminiferous tubules, γ3 expression appears to change with the state of maturation of sperm (see text for details). In the ductus deferens, γ3 expression (D) is limited to the surface of epithelial cells (basolateral and apical). No γ3 expression is seen in the smooth muscle of the ductus deferens. In lung (E and F), laminin γ3 is expressed on the apical surface of nonrespiratory airways. γ3 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 (Champliaud, unpublished observations). The COOH-terminal truncation of α2t explains its lack of reactivity with an anti-α2 antibody, mAb 2A2 1922, which is specific for the α2 G domain (Engvall et al., 1990).

The antiserum to the recombinant γ3 domain I fusion protein (R 21) 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 R 16, R 21 immunoreactivity is seen at the apical surface; however, the apical reactivity is distinctly different than that for R 16. Specifically, R 21 immunoreactivity appears as a plaque-like structure at the cell surface, with some reactivity within the cells. Thus, it seems possible that R 21 epitopes are entirely intracellular but it is also possible that some of the R 21 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 γ3 chains have been immunoisolated from the medium of A 204 cells derived from a human rhabdiosarcoma (Champliaud, M.-F., unpublished data), indicating that γ3-containing laminins are capable of being secreted in vitro. These γ3-containing laminins from A 204 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 β2 chain has been identified in both basement membrane and non-basement membrane locations (Unter et al., 1992; Libby et al., 1996; Libby et al., 1997; Töti et al., 1997). Laminins have also been observed in cartilage (Durr et al., 1996).

Intriguingly, the laminin γ3 chain appears most commonly to be associated with non-basement membrane structures. In the cerebellum, γ3 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), γ3 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 γ3-containing molecules are the products of the Müller glial cells which, like the tubal epithelium, contain a considerable store of intracellular γ3 chain. The laminin α3 and β2 chains are also present at these sites, whereas the γ1 and γ2 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 (Unter et al., 1992b; Libby et al., 1996; Unter and Brunken, 1997); and stabilization of synaptic junctions.

However, the most abundant expression of γ3 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. γ3 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, γ3 chains may be present within the basement membranes along the basolateral surfaces of some of these epithelia.
The chain partners for the γ3 chain in these apical laminins are not yet known with certainty. However, in epidermis, the laminin α2 chain does not colocalize with γ3 at the apical epithelial surface; rather, the α4 and β1 chains are present at that location and, thereby, are potential chain partners. Thus, it seems likely that γ3 will be as promiscuous as γ1 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 in neuromuscular junctions (Oakes 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 γ3-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 γ3 is present in these neural tissues, the chromosomal locus of LAMC3 at 9q31-q34 is essential for the HisTrx and a pPEP-T vector. The authors gratefully acknowledge the excellent technical support provided by Ms. Carol Milbury, and the expert assistance of Dr. Yimin Ge confoocal microscopy. The authors also thank Dr. R. Richard Kammerer for the HiStRx and a pPEP-T vector.

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