Human Amnion Contains a Novel Laminin Variant, Laminin 7, Which Like Laminin 6, Covalently Associates with Laminin 5 to Promote Stable Epithelial–Stromal Attachment

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Abstract. Stable attachment of external epithelia to the basement membrane and underlying stroma is mediated by transmembrane proteins such as the integrin α6β4 and bullous pemphigoid antigen 2 within the hemidesmosomes along the basolateral surface of the epithelial cell and their ligands that include a specialized subfamily of laminins. The laminin 5 molecule (previously termed kalinin/nicein/epiligrin) is a member of this epithelial-specific subfamily. Laminin 5 chains are not only considerably truncated within domains III–VI, but are also extensively proteolytically processed in vitro and in vivo. As a result, the domains expected to be required for the association of laminins with other basement membrane components are lacking in the mature laminin 5 molecule. Therefore, the tight binding of laminin 5 to the basement membrane may occur by a unique mechanism. To examine laminin 5 in tissue, we chose human amnion as the source, because of its availability and the similarity of the amniotic epithelial basement membrane with that of skin. We isolated the laminin 5 contained within the basement membrane of human amnion. In addition to monomeric laminin 5, we find that much of the laminin 5 isolated is covalently adducted with laminin 6 (α3β1γ1) and a novel laminin isotype we have termed laminin 7 (α3 β2 γ1). We propose that the association between laminin 5 and laminins 6 and 7 is a mechanism used in amnion to allow stable association of laminin 5 with the basement membrane. The β2 chain is seen at the human amniotic epithelial–stromal interface and at the dermal–epidermal junction of fetal and adult bovine skin by immunofluorescence, but is not present, or only weakly present, in neonatal human skin.

THE attachment of external epithelia to the underlying stroma is mediated by a unique set of ultrastructural entities within the basement membrane zone called the attachment complex (Gipson et al., 1987). This complex includes hemidesmosomes on the basolateral surface of the epithelium (Kelly, 1966), anchoring filaments that bridge the hemidesmosomes with the lamina densa (Komura, 1973), and anchoring fibrils that form an extended network surrounding stromal fibrous elements and insert into the basement membrane (Bruns, 1969). Characterization of the components of the complex show that the hemidesmosomes contain an intracellular protein, bullous pemphigoid antigen (BPAG)[1]-1, with homology to desmoplakin that is assumed to mediate the interaction of the hemidesmosomes with the cytokeratin network (Labib et al., 1986; Tanaka et al., 1991), HD-1 (Hieda et al., 1992), and BPAG-2, a transmembrane protein whose exodomain contains amino acid sequences that predict potential triple-helical structure (Li et al., 1991). Therefore, BPAG-2 has also been called type XVII collagen. The hemidesmosome also contains the integrin α6β4 (Carter et al., 1990; Stepp et al., 1990). The anchoring fibrils are condensations of type VII collagen dimers (Sakai et al., 1986; Morris et al., 1986; Keene et al., 1987). The anchoring filaments contain laminin 5 (previously called kalinin [Rousselle et al., 1991]/nicein [Verrando, et al., 1987], and likely to be contained in the preparation termed epiligrin [Carter et al., 1991; see Burgeson et al., 1994 for nomenclature]), the exodomain of type XVII collagen and perhaps other proteins (Fine et al., 1989; Chan et al., 1993).

Laminin 5 is a unique laminin variant composed of three nonidentical subunits (Rousselle et al., 1991), α3 (Ryan et al., 1994), β3 (Gerecke et al., 1994; Utani et al., 1995), and

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1. *Abbreviation used in this paper*: BPAG, bullous pemphigoid antigen.

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γ2 (Vailly et al., 1994*a*; Gerecke et al., 1994; Sugiyama et al., 1995), each of which is substantially truncated within the short arm domains relative to laminin 1. In addition, laminin 5 is proteolytically processed after secretion (Marinkovich et al., 1992*b*). In addition to domains I and II, the proteolytic product retains only the G domain at the COOH terminus of the α3 chain, a globular domain at the end of the β3 chain that is homologous to the VI domain of β1, and portions of the EGF-like domains. The molecule lacks the ability to bind nidogen/entactin (Mayer et al., 1995).

Laminin 5 is essential for epidermal–dermal adhesion. Gene defects in the β3 (Pulkkinen et al., 1994*a*) and γ2 chains (Pulkkinen et al., 1994*b*; Aberdam et al., 1994; Vailly et al., 1994) underlie the Herlitz's variety of junctional epidermolysis bullosa, a lethal disease causing extensive dermal–epidermal separation in the plane of the lamina lucida. Therefore, laminin 5 must interact with the epithelial cell surface and with components of the basement membrane. There are now abundant data from both in vitro and in vivo studies that indicate that cells bind to laminin 5 via α6β4 (Sonnenberg et al., 1993; Niessen et al., 1994) and α3β1 (Carter et al., 1990, 1991). However, how an association with the basement membrane occurs in the absence of the short arm domains thought to be involved in the assembly of laminins 1, 2, 3, and 4 with the lamina densa is unclear. Here we report studies of the molecules associated with laminin 5 in human amniotic membrane and observe a novel laminin variant containing α3, β2 (S-chain) (Hunter et al., 1989*a*), and γ1. The description of this molecule and its association with laminin 5 is the focus of this report. We suggest the name laminin 7 for the trimeric assembly of α3, β2, γ1. Laminin 5 is also found covalently associated with laminin 6 in amnion.

β2 has been described by Hunter et al. (1989*a*) as having overall structural similarity to β1. β2 is contained in laminin 3 (S-laminin) together with α1 and γ1, and in laminin 4 (S-merosin) combined with α2 and γ1 (Green et al., 1992). Unlike laminin 1 that is present in most basement membranes, the distribution of the β2 is considerably restricted to the motor neuron synapse, blood vessels, and the kidney glomerulus (Hunter et al., 1989*a*; Engvall et al., 1990; Engvall, 1993). To date, no functional differences have been reported between laminins 1, 2, 3, and 4 relative to the epithelial basement membrane.

The basement membrane zone underlying the amniotic epithelium is ultrastructurally and biochemically similar to the structures underlying other external epithelia. As shown in Fig. 1, typical anchoring complex structures are visualized that include hemidesmosomes, anchoring filaments, and anchoring fibrils. The components of these complexes and the association of these components have previously been considered typical of external epithelial basement membranes as well, as exemplified by proposed requirement of dimerization of type VII collagen before anchoring fibril formation (Keene et al., 1987; Lunstrum et al., 1987), which was based upon interactions of type VII collagen molecules extracted from amnion. This model, now eight years old, has continued to be consistent with data accumulated from biochemical studies and with models of pathogenesis resulting from COL7A1 mutations. The processing of laminin 5 observed in keratinocyte culture and skin organ culture also occurs in amnion (Marinkovich et al., 1992*a*). Therefore, we have used amnion as a source on epithelial laminin in this study.

Materials and Methods

Antibodies

Preparations of the mAb BM 165, mAb K140 (Rousselle et al., 1991), and mAb 545 specific of the β1 chain of human laminin (Marinkovich et al., 1992*a*) have been described. mAbs C1 and C4 were the generous gift of Dr. Joshua Sanes (Washington University School of Medicine, St Louis, MO). mAb 1924 specific of the α1 chain of laminin was purchased from Chemicon Intl., Inc. (Temecula, CA). Anti-α3 peptide antibody was prepared in New Zealand White rabbits by standard procedures to a peptide with the amino acid sequence KANDITDEVLDGLNPIQTD present in the domain I of α3. Anti-γ2 polyclonal antibodies were prepared by affinity purification of polyclonal anti-laminin 5 on nitrocellulose containing the bound product of a bacterial lawn expressing a λgt11 cDNA clone encoding sequences from the γ2 I/II domain. The bound and eluted antibodies showed no cross-reactivity with β1, β2, β3, α1, or α3 by Western analysis, although minor cross-reactivity with γ1 was detected at high antibody concentration.

Protein Purification

The purification of the laminin 5–laminin 7 complex was carried out as follows: human amnions were frozen in liquid nitrogen, ground in a blender (Waring Products Div., New Hartford, CT), and washed in 1 M NaCl. The final tissue pellet (200 g, wet wt) was suspended in 1 liter of extraction buffer (50 mM Tris-HCl, pH 7.8, 5 mM $CaCl_2$, 625 mg/l of *N*-ethylmaleimide, 150 mg/l of PMSF, and 4,000 U of bacterial collagenase (CLSPA; Worthington Biochemical Corp., Freehold, NJ). The suspension was incubated at room temperature with stirring, and after 24 h, an additional 4,000 U of enzyme was added. The extraction was continued for another 24 h. Unless otherwise noted, all subsequent steps were performed at 4°C. The soluble fraction was collected after centrifugation (30,000 *g*, 60 min) and precipitated by 300 g/liter of ammonium sulfate). The precipitated proteins were collected by centrifugation (30,000 *g*, 60 min) and redissolved into chromatography buffer (2 M urea, 25 mM NaCl, 5 mM EDTA, and 50 mM Tris-HCl, pH 7.8). The sample was then treated with diisopropyl fluorophosphate (5 mg/liter) and dialyzed against the same buffer. After dialysis, 0.5 vol of buffer-equilibrated DEAE-cellulose (DE-52; Whatman Inc., Clifton, NJ) was added, and the mixture was shaken overnight. Material not bound to DEAE-cellulose was collected by filtration on a Buchner funnel (filter 4, Whatman Inc.) and precipitated by addition of 300 g/liter of ammonium sulfate. The proteins were collected by centrifugation (30,000 *g*, 60 min), redissolved in the ConA buffer (0.5 M NaCl, 5 mM $CaCl_2$, 5 mM $MgCl_2$, and Tris-HCl 50 mM, pH 7.8), and dialyzed against the same buffer overnight. The fraction was applied to a 2.5 × 5 cm ConA–Sepharose column (Pharmacia Fine Chemicals, Piscataway, NJ), and unbound material was removed by extensive washing. Bound proteins were first eluted with 10 mM α-D-Mannopyrannoside (Sigma Chemical Co., St. Louis, MO) and secondly with 1 M α-D-Glucopyrannoside (Sigma Chemical Co.). A third elution with 1M α-D-Manno-pyrannoside allowed the recovery of the proteins of interest. This fraction was concentrated to 10 ml on a concentrator (30 kD membrane; Amicon Corp., Danvers, MA) and applied to a 2.5 × 100 cm Sephacryl S-500 column in a 0.5 M NaCl, 50 mM Tris-HCl, pH 7.8, buffer. The fractions of interest were pooled, dialyzed against Mono-Q buffer (0.1 M NaCl, 25 mM Tris-HCl, pH 7.8), and applied to the 1 × 5 cm Mono-Q column (Pharmacia Fine Chemicals). Elution was achieved with a 60-ml, 0.1–0.5-M NaCl gradient. The laminin 5–6 complex was purified from the materials not bound to DEAE (as described above) by clearing the preparation of fibronectin using gelatin-Sepharose and immunoaffinity chromatography using an mAb 545 Sepharose column (anti-β1).

Protein Sequencing

Protein sequencing was done according to Aebersold et al. (1987). The laminin 5–laminin 7 complex was run on a polyacrylamide gel in the presence of 2-mercaptoethanol and blotted onto a nitrocellulose membrane (Bio-Rad Laboratories, Richmond, CA). The 190-kD band (β2) and the 165-kD band (α3) were separately excised and digested by the protease

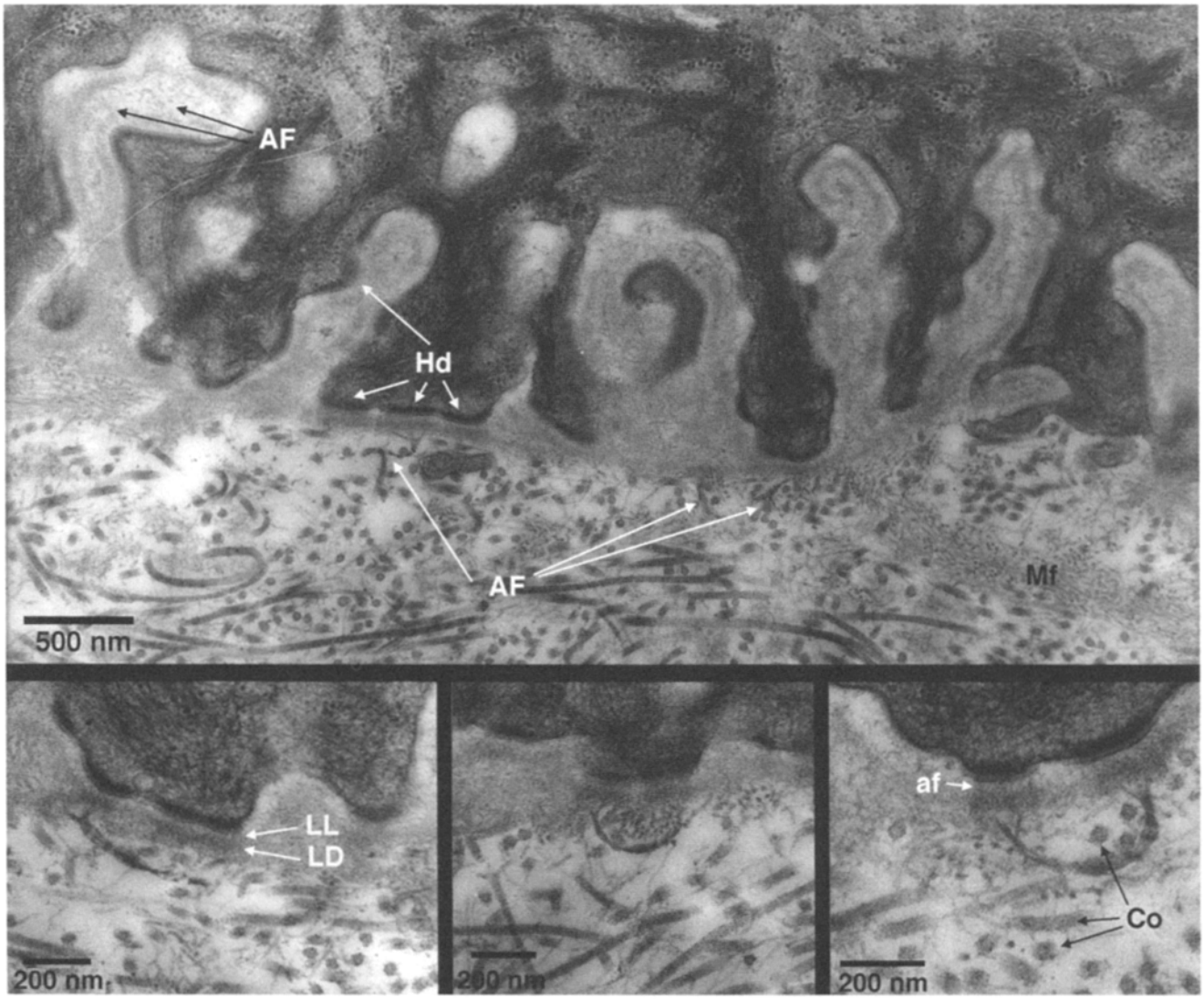

Figure 1. Transmission electron micrographs of the amniotic epithelial–stromal junction show extreme fenestration of the epithelial basolateral surface. Typical anchoring complex structures are readily seen including hemidesmosomes (*Hd*), anchoring filaments (*af*), and anchoring fibrils (*AF*). Anchoring fibrils are prominent and show insertion of the ends of the fibrils into the lamina densa (*LD*) at hemidesmosomes. In addition, anchoring fibrils are seen within the clefts of the fenestrae, where they appear to stabilize the convolutions of the epithelial surface.

Lys-C. The digest product was separated by HPLC, and one fragment was sequenced on a sequenator (Applied Biosystems, Foster City, CA).

Other Methods

The following procedures were performed as previously described: SDS-PAGE (Laemmli, 1970), electrophoretic transfer of proteins to nitrocellulose with immunoblot analysis (Lunstrum et al., 1986), indirect immunofluorescent microscopy of frozen sections of human tissue and bovine skin (Sakai et al., 1986), and visualization of rotary-shadowed images by EM (Morris et al., 1986).

Results

Laminin 5–containing complexes from human amnion were partially fractionated by FPLC Mono-Q ion exchange chromatography. The materials eluted as two overlapping peaks at ~0.075 and 0.125 M NaCl (Fig. 2). Gel electrophoretic analysis of alternate fractions across the peaks (Fig. 2) indicated two distinct patterns. The materials contained in Mono-Q peak 1 were evaluated by SDS-PAGE before disulfide bond reduction (Fig. 3). Three major bands were observed. The slowest band (*B*) migrated only a short distance into the running gel, and while its mass could not be accurately estimated, it is greater than observed for monomeric laminin. The second band (*A*) migrated to a position expected for a laminin 5 monomer (~600–700 kD). The third band was very near the dye front and was not further characterized. The chain compositions of bands A and B were determined after electrophoretic resolution and Coomassie blue staining of the reduction product of the excised gel band (Fig. 3), followed by immunoblotting (Fig. 4, *A* and *B*). Before reduction band B was immunoreactive with mAb C4 (anti-β2), polyclonal anti–laminin 1, BM-165 (anti-α3), and BM-140 (anti-β3); band A was also immunoreactive with anti-β2,

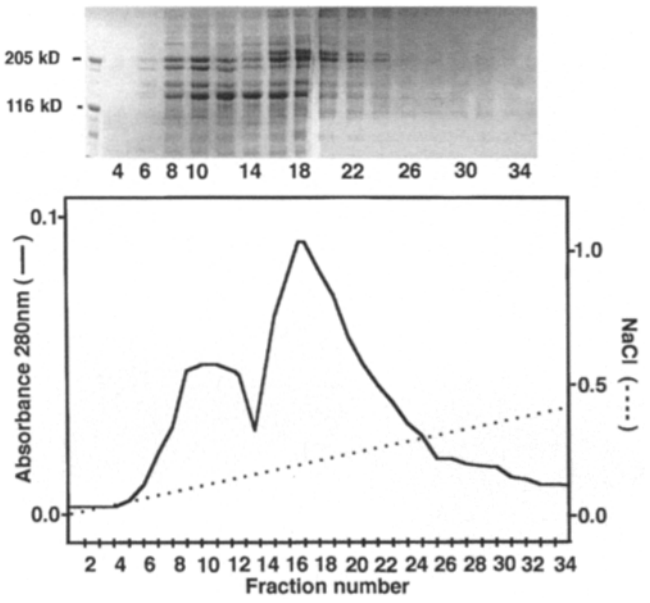

Figure 2. Separation of laminin 5–containing complexes by ion exchange FPLC on Mono-Q. (*Lower panel*) the A_{280} profile for Mono-Q elution showing the separation into two peaks. (*Upper panel*) alternate fractions were analysed by SDS-PAGE on a 5% gel under reducing conditions. Proteins were visualized by Coomassie blue staining. M_r markers are shown at left.

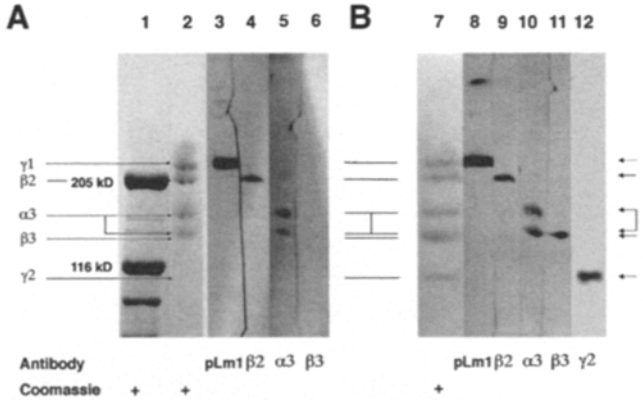

Figure 4. Immunoblot characterization of the reduced form of the bands A and B (Fig. 3). After transfer to nitrocellulose, band A or B proteins were visualized using: lanes *2* and *7*, Coomassie blue; lanes *3* and *8*, polyclonal anti-laminin 1 (*pLm1*) (Sigma Chemical Co.) that identifies the γ1 chain; lanes *4* and *9*, mAb C4 that identifies the β2 (a gift of Dr Sanes); lanes *5* and *10*, mAb BM-165 that is specific for α3; lanes *6* and *11*, mAb BM-140 that is specific for β3; and lane *12*, polyclonal anti-γ2 serum. Prestained molecular weight markers are in lane *1*. The results show that band A contains a disulfide-bonded complex of α3, β2, and γ1, while band B contains α3, β2, β3, γ1, and γ2, suggesting a complex of two molecules of [α3, β2, γ1]s-s[α3, β3, γ2].

anti–laminin 1, and anti-α3, but not with anti-β3 (data not shown). After reduction of band A, major bands at 210, 190, 165, and 140 kD were observed (Fig. 4, lane *2*). The 210-kD band in the position of γ1 was reactive with anti–laminin 1 (Fig. 4, lane *3*), and the 190-kD band was recognized by anti-β2 (Fig. 4, lane *4*). The identity of the 190-kD chain as human β2 was confirmed by amino acid sequencing of a fragment derived from the gel band. The peptide sequence was identical to human β2 (Wewer et al., 1994) over 24 amino acids (Table I). In no case was a band in the position of α1 or β1 recognized by anti–laminin 1 antibodies in either peak. The remaining unidentified bands at 165 and 145 kD in peak A were identified as the α3 chain by immunoblotting (Fig. 4, lane *5*), but neither the β3 chain (Fig. 4, lane *6*) nor the γ2 chain was detected by immunoblot analysis with polyclonal anti–laminin 5 or γ2-specific antibody (data not shown), or by Coomassie blue staining (Fig. 4, lane *2*). The identity of the 165-kD band as α3 was confirmed by amino acid sequence determination. The sequence obtained matched a sequence contained in α3EpA in domain IIIa very near the domain I/II border (Table I). Therefore, band A contains a disulfide-bonded aggregate of α3, β2, and γ1 chains. The amounts of 165 kD plus 145 kD (Fig. 4, lane *2*) are approximately equal to that of the 210- or 190-kD species, suggesting that each chain is present in equimolar amounts, consistent with their assembly into a laminin molecule. Rotary-shadowed images of the materials in Mono-Q peak 1 (Fig. 5 *A*) showed Y-shaped molecules consistent with the molecular shape expected for a molecule containing these chains. We suggest that this molecule (α3β2γ1) be called laminin 7, and it will be referred to as such below.

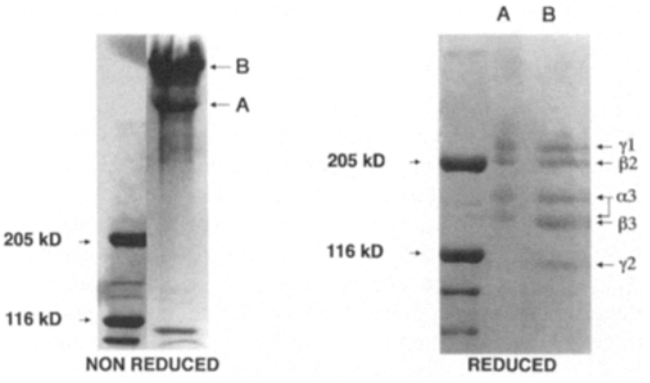

Figure 3. Evaluation of the disulfide-bonded complexes contained within the first Mono-Q peak (Fig. 2). Fraction 12 of the first peak was analyzed by SDS-PAGE on a 3–5% gel under non–disulfide bond reducing conditions. The upper band (*B*) and the lower band (*A*) were cut from the gel, reequilibrated in sample buffer in presence of 10% 2-mercaptoethanol, and run in a 5% SDS-PAGE gel. Proteins were visualized by Coomassie blue. M_r markers are shown at left of each analysis.

After disulfide bond reduction, electrophoretic band B from Fig. 3 contained bands in the positions of γ1, β2, α3 (165/145 kD), β3 (140 kD), and γ2 (105 kD). The identification of these chains was confirmed by Western blotting (Fig. 4 *B*). The 210-kD band was recognized by polyclonal anti–laminin 1 and is in the position of the γ1 chain. The 190-kD chain was recognized by mAb C4 as β2. The 165- and 145-kD bands are recognized by mAb BM-165 as the α3 chain. The 140-kD band reacted with mAb BM-140 specific for β3. The 105-kD chain was recognized by polyclonal anti-γ2. The simplest interpretation of these data is that band A represents a disulfide-bonded dimer of one molecule of laminin 5 with one molecule of laminin 7.

Table I. Confirmation of the Identification of the 190-kD band of Laminin 5–7 Complex as Human LAMB2 and the 145-kD α3 Chain as LAMA3

Rat LAMB2* res#1641	KRAGNSLAASTAEETAGSAQSRAREAE
Human LAMB2‡	KRAGNSLAASTAEETAGSAQGRAQEAE
Human laminin β2 peptide	−AGNSLAASTAEETAGSAQGRAQEA−
Mouse LAMA1§	CDCSGNVDPSEA.GHCDSVTGECLKCLGNTDGAHCERCAD
Mouse LAMA3‖	CSCNSNGQ LGPCDPLTGDCVNQEPK DGSPAEECDD
Human LAMA3¶	CNCNSNGQ....LGSCHPLTGDCINQEPK DSSPAEECDD
Human laminin α3 peptide	DSSPAEECD

A peptide sequence obtained from the 190-kD band of the laminin 5–7 complex is located within the cDNA-predicted amino acid sequence of β2 from rat (Hunter et al., 1989*a*) with which it has 92%, and with human (Wewer et al., 1994) with which it has 100% amino acid identity. A peptide sequence isolated from the 165-kD α3 chain of the laminin 5–7 complex shows 100% identity to the predicted human sequence (Ryan et al., 1994) and 98% identity to mouse α3 (Galliano et al., 1995), but shows only 33% identity to the homologous region (domain IIIa, EGF repeat 9) of α1 (Sasaki et al., 1988).

*Hunter et al., 1989.
‡Wewer et al., 1994.
§Sasaki et al., 1988.
‖Galliano et al., 1995.
¶Ryan et al., 1994.

Both the electrophoretic mobility of nonreduced B and the rotary-shadowed image analysis of Mono-Q peak 1 are consistent with this interpretation. As shown in Fig. 5 *B*, in addition to Y-shaped molecules, the other prominent image appears as a molecule with two long arms and two short arms. Globular domains similar to the α-chain G domains are present at the ends of the long arms. The images suggest that laminin 5 associates with the laminin 7 molecule through its truncated short arms at the intersection of the long and short arms of laminin 7.

Preliminary analyses of Mono-Q peak 2 (Fig. 2) materials by immunoblotting indicated the presence of both laminin 1 and laminin 5 chains but a reduced relative amount of β2 (data not shown). Gel electrophoresis before disulfide reduction indicated that like peak 1 materials, peak 2 laminins were present as aggregates (data not shown). To further characterize these materials, β1 chains containing laminins were isolated from amnion extracts by immunoaffinity chromatography using monoclonal anti-β1 antibody 545. Three major electrophoretic species were detected by Coomassie blue staining (Fig. 6). Two of these (*A* and *B*) have electrophoretic mobilities expected to be multimers, and band C is predicted to be monomeric. The identities of bands A and C were analyzed as detailed below. The characterization of band B is not yet complete. A minor band can also be seen in the same electrophoretic analysis with a slower mobility than band A. This band is in the position expected for laminin 1 and has not been further analyzed.

The disulfide bond reduction product of band A contains bands in the electrophoretic positions of β1, γ1, α3 (165 kD), α3 (145 kD), and γ2 (Fig 6). The band in the position of γ1 is recognized by anti–laminin 1 antiserum (Fig. 7, lane *2*); the β1-position band is recognized by mAb 545 (anti-β1) (Fig. 7, lane *3*); the presumptive α3-position bands are recognized by polyclonal anti-α3 serum (Fig. 7, lane *4*); the β3-position band is recognized by BM-140 (anti-β3) (Fig. 7, lane *5*); the γ2-position band is recognized by polyclonal anti-γ2 (Fig. 7, lane *6*). These characteristics are consistent with an identification of nonreduced band A as a complex of laminin 5 with laminin 6 (α3, β1, γ1). Therefore, laminin 5 appears to be able to form covalent complexes with other α3-containing laminins, although we have no evidence for a laminin 5–laminin 5 covalent complex at this time.

Nonreduced band C was similarly excised and reduced. The reduction product contains four electrophoretic species (Fig. 7, lane *7*). The band in the position of β1 is recognized by mAb 545 (anti-β1) (Fig. 7, lane *9*); the band in the position of γ1 is recognized by anti–laminin 1 (Fig. 7, lane *9*). The α3-position bands are immunoblotted by anti-α3 (Fig. 7, lane *10*) (the 140-kD–position band is underrepresented due to a defect in the electrophoretic gel, but the portion of the band transferred is immunoblot positive). No Coomassie blue staining is seen in the positions of β3 and γ2 (Fig. 7, lane *7*), nor is there reactivity with BM-140 (anti-β3) (Fig. 7, lane *11*) or polyclonal anti-γ2 (data not shown). These data are consistent with the identification of unreduced band C as monomeric laminin 6. The nonreduced sample does not contain monomeric laminin 7 because it was isolated by anti-β1 immunoaffinity.

The observation of β2 in the amniotic extracts was unexpected since no β2 was seen in similar preparation from human skin or in the culture medium of human keratinocytes; therefore, the distribution of β2 was examined in skin and in amnion. Full-term human amnion was immunostained using antibodies specific for laminin 5 (BM-165; Fig. 8 *A*), laminin β2 (C1, C4; Fig. 8, *B* and *E*, respectively), type VII collagen (NP-185; Fig. 8 *D*), laminin α1 chain (mAb 1924; Fig. 8 *C*), and laminin β1 chain (mAb 545; Fig. 8 *F*). The β2 chain is visualized only at the epithelial–stromal interface, equivalent to the distribution of type VII and laminin 5, and is not present in the capillary beds stained by antibodies recognizing laminin α1 and β1.

Staining of human foreskin with the same antibodies showed the expected distributions for laminin 5 (Fig. 9 *A*), type VII collagen (Fig. 9 *D*), and laminin 1 (α1 chain; Fig. 9 *C*; β1 chain, Fig. 9 *F*). Laminin 5 and collagen type VII

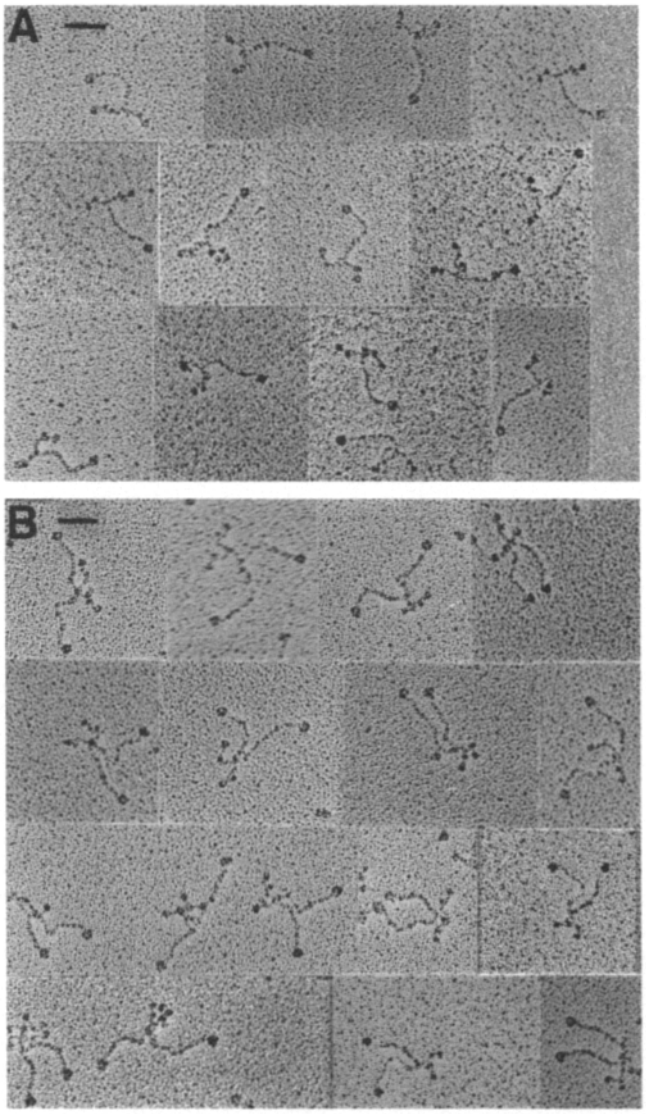

Figure 5. Peaks A and B were visualized by EM after rotary shadowing. The images indicate that peak A contains a monomeric Y-shaped molecule with the features predicted for laminin 7. Peak B contains more complex images. The images are consistent with what would be predicted for a covalent aggregate of laminins 5 and 7. Bars, 100 nm.

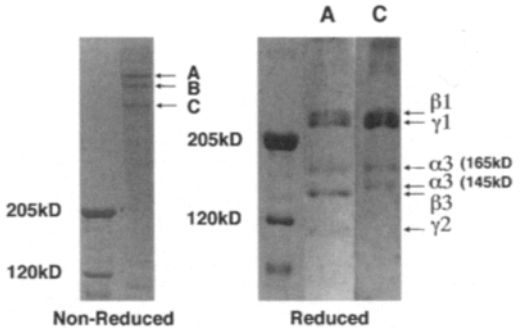

Figure 6. Laminins were solubilized from human amnion and partially purified by immunoaffinity chromatography using a Sepharose column complexed with monoclonal anti-laminin β1 antibody 545. The purification products were resolved by 3–5% PAGE under non-disulfide bond reducing conditions, and three high molecular weight bands, A, B, and C, were visualized by Coomassie blue staining. Bands A and C were individually excised from the gel and the disulfide bond reduction products were resolved by 5% PAGE. The reduction products of band A are in the positions expected for β1, γ1, α3, β3, and γ2; those of B are in the positions of β1, γ1, and α3.

collagen show crisp, brilliant staining of the dermal–epidermal junction. Anti-α1 antibody staining is very strong for the dermal vasculature, but relatively less intense at the dermal–epidermal junction. Anti-β1 antibodies stain the dermal–epidermal junction more intensely and also recognize the dermal vasculature, consistent with the possibility of β1 being present in laminin 1 and laminin 6. β2 staining (C1 and C4; Fig. 9, *B* and *E*, respectively) was observed around nerves, but was absent or only weakly present at the dermal–epidermal junction. This is in contrast to what is seen in bovine skin (Fig. 10). In this case, β2 (C1 and C4; Fig. 10, *B* and *E*, respectively) is distributed around nerves and at the dermal–epidermal junction of both adult (10 *B*) and fetal (10 *E*) bovine skin. As in human skin, β1 staining (10 *F*) is stronger at the dermal–epidermal junction than is α1 (10 *C*), while α1 staining of blood vessels is stronger than β1 staining.

Discussion

The previous description of laminin 6 indicated that the α3 chain, or a closely related chain, is capable of forming coiled-coil interactions with the β1 and γ1 chains. In this study we confirm the existence of a laminin containing authentic α3 together with β1 and γ1 and suggest that this molecule is laminin 6. Since β2 can combine with α1 to form laminin 3, it is perhaps predictable that since β1 can associate with α3, β2 could also associate with α3 to form laminin 7. However, as β2 is primarily associated with the basement membranes of blood vessels and associated with nerves, it was thought that the functional specialty of β2

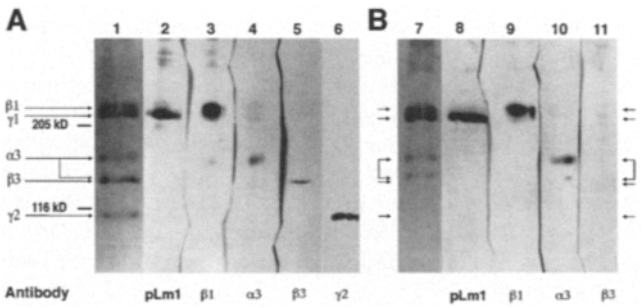

Figure 7. Nonreduced bands A and C (Fig. 6) were further characterized after excision from the gel, disulfide bond reduction, resolution of the reduction products on a 5% SDS-PAGE gel, transfer to nitrocellulose, and Western analysis using: lanes *1* and *7*, Coomassie blue; lanes *2* and *8*, polyclonal anti-laminin 1 antiserum (Sigma Chemical Co.); lanes *3* and *9*, mAb 545 (anti-β1); lanes *4* and *10*, polyclonal anti-α3 serum; lanes *5* and *11*, mAb BM-140 (anti-β3); and lane *6*, polyclonal anti-γ2. The results indicate that band B contains monomeric laminin 6 (α3β1γ1), and band A contains a mixture of laminins 5 and 6.

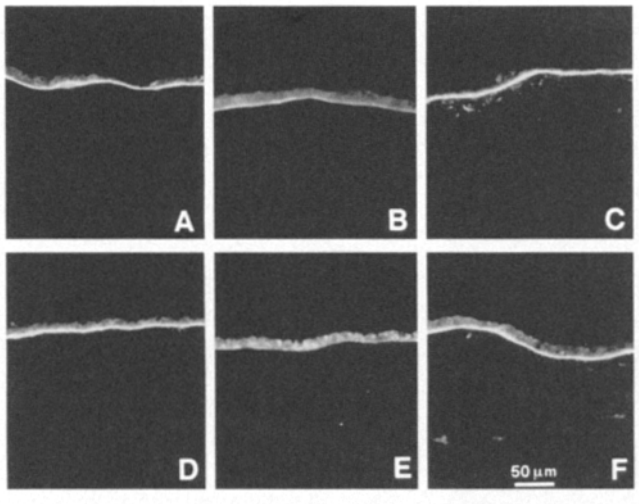

Figure 8. Cryosections of full-term human amnion were processed for indirect immunofluorescence using: (*A*) mAb BM-165 specific for α3; (*B*) mAb C1 specific for β2; (*C*) mAb 1924 specific for α1; (*D*) mAb NP-185 specific for type VII collagen; (*E*) mAb C4 specific for β2; and (*F*) mAb 545 specific for β1.

was related to those tissues. The aberrant neuromuscular junctions in β2-deficient mice (Noakes et al., 1995*a*) are consistent with that expectation. Therefore, the role of β2 in amnion is unclear.

The β1 chain has been shown to mediate laminin polymerization in vitro through interactions of the VI domain (Yurchenco et al., 1985; Paulsson, 1988; Schittney and Yurchenco, 1990). The rat β2-chain VI domain shares 70% sequence identity with the human β1 VI domain, strongly suggesting that β2 participates in laminin 3 polymerization. β1 has also been implicated in cell binding through the sequences PDSGR and YIGSR in the ninth EGF repeat of domain III (Graf et al., 1987; Kleinman et al., 1989). These sequences are not found in β2, yet the cell attachment activity of β1 and β2–containing laminins 2 and 4 are indistinguishable (Brown et al., 1994). Two activities have been reported to be specific to the β2 chain: adhesion of ciliary ganglion neurons to the sequence LRE in β2 (Hunter et al., 1989*b*), and the in vitro binding of β2 (probably laminin 4) containing placental laminin to BM-90 (Brown et al., 1994). The physiological significance of these observations is not known. In view of this current state of understanding of the laminin 3 and 4 function, it is not possible to predict any functional differences between laminin 6 and laminin 7.

The complex of laminins 6 or 7 with laminin 5 is most likely to derive from an interaction of the β3-chain VI domain with the α3-chain short arm domain III in laminin 7. This prediction reflects our interpretation of the rotary-shadowed image of the complex as the short arm of laminin 5 interacting with a laminin 6/7 domain near the intersection of the laminin 6/7 short arms. Support for a role of the β3-chain VI domain comes from the presence of an unpaired cysteinyl residue in that domain (Gerecke et al., 1994) and the absence of any other globular domain at the NH_2 terminus of the fully processed laminin 5 molecule (Marinkovich et al., 1992*b*). This unpaired cysteine is conserved between human (Gerecke et al., 1994) and mouse (Sasaki et al., 1988). The contributor of the other unpaired cysteine from laminin 7 is not known, but since complex

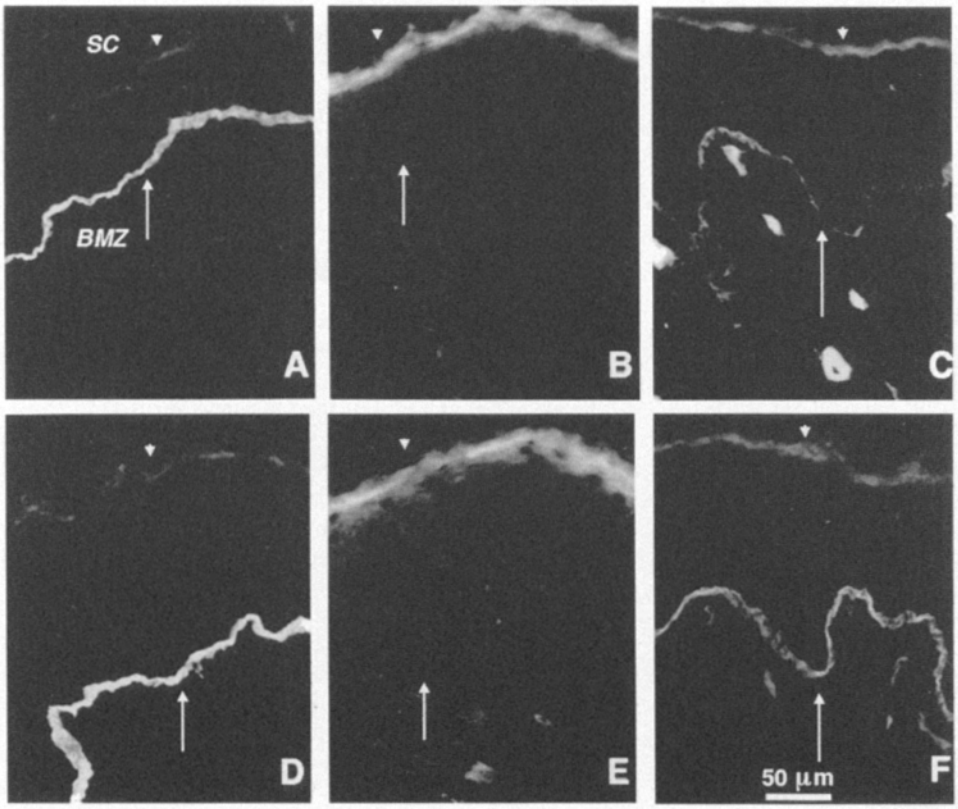

Figure 9. Cryosection of human foreskin processed for immunofluorescence using: (*A*) mAb BM-165 specific for α3; (*B*) mAb C1 specific for β2; (*C*) mAb 1924 specific for α1; (*D*) mAb NP-185 specific for type VII collagen; (*E*) mAb C4 specific for β2; and (*F*) mAb 545 specific for β1. *SC*, stratum corneum; *BMZ*, basement membrane zone.

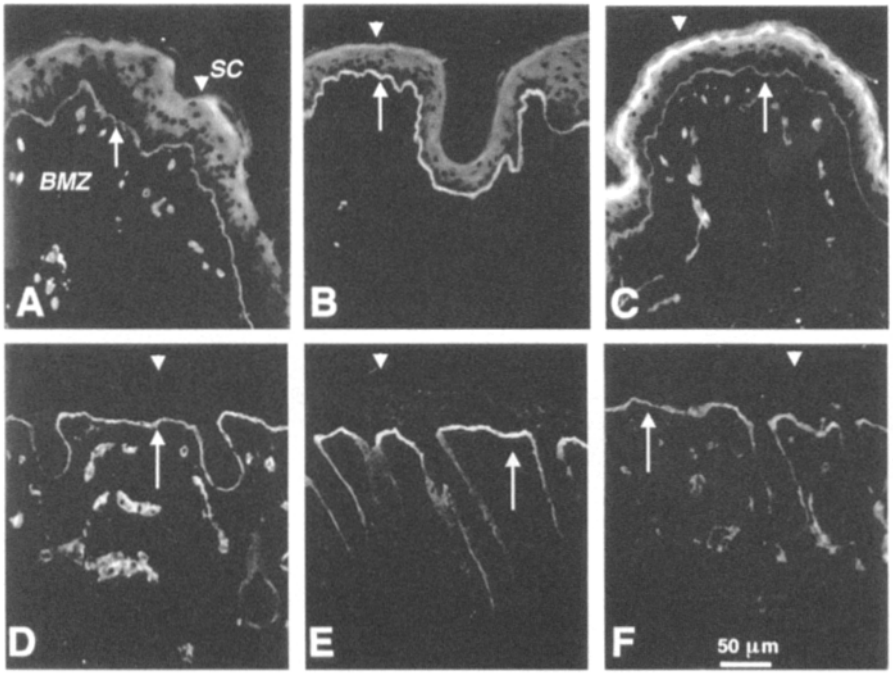

Figure 10. Cryosection of adult bovine skin (*A–C*) and fetal bovine skin (*D–F*) processed for immunofluorescence using: (*A, D*) polyclonal antibody against human laminin 1; (*B, E*) polyclonal antibody against laminin 5; and (*C, F*) mAb C4 specific for β2.

formation between laminin 5 and other laminins has only been seen between truncated α-chain–containing laminins (i.e., laminin 6 and laminin 7), despite the fact that other laminins are synthesized by keratinocytes and present within the basement membranes of skin and amnion, the most likely bonding partner within laminin 7 for the β3 VI domain is the α3 chain. The exact location of the required α3 cysteinyl residue is not known; however, the final EGF repeat in domain IIIa of both α3 (Ryan et al., 1994) and α4 (Iivanainen et al., 1995) is only a half repeat. As it is unclear if the half EGF repeat is capable of correctly folding to form any of the typical disulfide bridges, any of the contained cysteine residues could remain unpaired. One of the presumptive unpaired cysteine residues is contained within the determined peptide sequence from the 145-kD α3, so this cysteinyl residue is retained even within the most fully processed form of α3. We have thus far been unable to confirm the identification of the 165-kD α3 as $\alpha3_{EpA}$; as we have not identified protein sequences specific to either splice variant, there is a formal possibility that the 165-kD α3 derives from $\alpha3_{EpB}$. However, several lines of evidence argue against this possibility. The 200-kD precursor of the 165-kD chain is the only α3 chain detected within cultured epithelial cells (Marinkovich et al., 1992*b*). The 200- and 165-kD (and very minor amounts of 145 kD) forms are the only α3 species found in the culture medium of normal human keratinocytes and of SCC-25 cells (a line derived from a squamous cell carcinoma). The 165- and 145-kD α3 forms are only found extracellularly and appear sequentially in pulse-chase experiments (Marinkovich et al., 1992*b*) in both keratinocyte and WISH cell cultures. $\alpha3_{EpB}$ is only a minor transcription product of keratinocytes (Kallunki et al., 1992; Galliano et al., 1995), and there is no evidence that $\alpha3_{EpB}$ is translated by keratinocytes. The 200-kD intracellular precursor corresponds well with the calculated mass of 184 kD plus an additional mass of ~20 kD due to glycosylation (Champliaud, M.-F., unpublished observations) for $\alpha3_{EpA}$. No protein product of the size predicted to correspond to $\alpha3_{EpB}$ has been detected in these studies despite the fact that the α3 antibodies used here for immunoisolation recognize epitopes predicted to exist in all α3 splice variants.

It is not entirely clear where 200-kD α3 is cleaved to produce the 165- and 145-kD forms. However, since at least the domain III–II junction is present within α3 145 kD, the calculated mass of α3 is insufficient to include the entire G domain sequence. Instead, assuming that domains I and II are not cleaved, repeats 4 and 5 of domain G cannot be be present in the 145-kD form. Furthermore, the mass difference between the 200 kD and 165 kD is too large to be only domain IIIa. Therefore, the most likely consequence of the α3 processing is an initial loss of G repeats 4 and 5 during the 200–265-kD conversion and loss of NH_2-terminal domain IIIa during the conversion from 165 to 154 kD. The observed mass changes correspond quite well to masses calculated from the predicted amino acid sequence, assuming an average addition of 10% due to glycosylation. A similar proteolytic processing between G repeats 3 and 4 has been observed for α2 (Wewer et al., 1994).

The severe truncation of the laminin 5 short arms, especially after processing, deprives the molecule of the domains believed to be necessary for self-polymerization. In addition, the γ2 chain does not bind nidogen, and therefore cannot interact with type IV collagen, perlecan, or the fibulins (Brown et al., 1995; Mayer et al., 1995). This is consistent with the model (Fig. 11) that laminin 5 alone cannot generate epithelial–stromal stability. The model assumes that α6β4 in the hemidesmosome binds the α3 G domain. This derives from published studies showing that α6β4 binds laminin 5 (Sonnenberg et al., 1993; Niessen et al., 1994) and that the antibody BM-165 that can deepithelize fragments of human skin in vitro, as well as inhibit cell attachment of epithelial cells in vitro, specifically immuno-

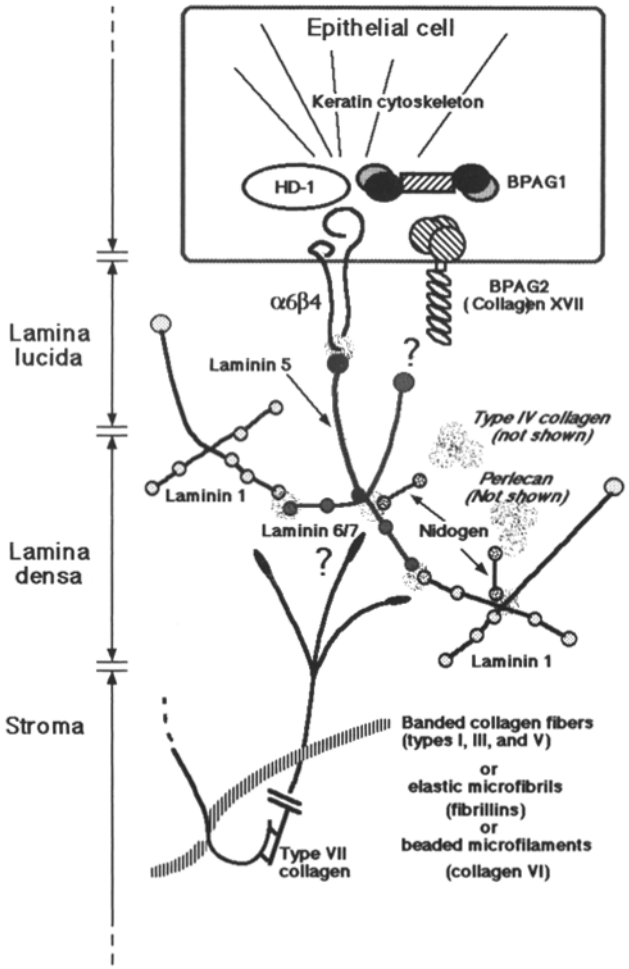

Figure 11. Model of the proposed function of the laminin 5–7 complex in epidermal–stromal adhesion. The laminin 5–7 complex is shown mediating the interactions of the epithelial transmembrane hemidesmosomal protein α6β4 through the G domain of α3 to polymerized laminin, through the VI domains of laminin 6 or 7, and with the type IV collagen network and perlecan, through nidogen binding to the γ1 chain of laminin 6 or 7.

blots the α3 chain. The epitope recognized by this antibody resides in the first repeat of the G domain (Champliaud, M.-F., unpublished results). Assuming the G domain of α3 is the primary site of cell binding, the NH_2 terminus of the laminin 5 molecule must strongly interact with another species to bind components of the basement membrane. The observed covalent associations with laminin 6 and laminin 7 fill this requirement. The γ1 chains of laminins 6 and 7 provide the binding site for nidogen. The VI domains of β1, β2, and γ1 provide potential sites for polymerization. Thus, in combination with laminins 6 and 7, laminin 5 is able to mediate binding of the epithelial cell to the basement membrane. However, complex formation with laminins 6 and 7 need not provide the only mechanism. The anchoring fibrils secure the basement membrane to the stroma through interactions of the NC-1 domain of type VII collagen. It is likely that type VII collagen interacts directly with monomeric laminin 5 or with the laminin 5–6 or laminin 5–7 complexes, but this has not yet been reported.

Since the basement membranes of both the dermal–epidermal junction and the amniotic epithelium contain the structures and proteins characteristic of the contained anchoring complexes, it is surprising that the amnion, but not skin, is rich in β2-containing laminins. Even more perplexing is the presence of β2-containing laminins in both fetal and adult calf skin. We have observed only one ultrastructural correlate with these differences, that being that the basolateral plasma membranes and adjacent basement membrane zone of the fetal calf and the human amnion are highly fenestrated relative to human skin, with many of the anchoring fibrils appearing to stabilize the infoldings by spanning the matrix between the folds. Consistent with the possibility that β2-containing laminins determine or stabilize basement membrane infolding is the observation that $\beta2^{-}/_{-}$ mice show significantly reduced junctional folds within the neuromuscular junctions (Noakes et al., 1995*a*). Recently, a defect in the visceral epithelial cells of the β2-deficient mouse glomerulus has been described (Noakes et al., 1995*b*). The foot processes of these cells are frequently fused. This is again consistent with the possibility that β2-containing laminins generate or stabilize fenestrated cellular structures. However, it is not at all clear how a laminin repertoire could influence this architecture.

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