The Dermal–Epidermal Junction of Human Skin Contains a Novel Laminin Variant

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Abstract. We report the identification of a novel laminin variant that appears to be unique to a subset of epithelial basement membranes. The variant contains two chains electrophoretically and immunologically identical to the B1 and B2 chains. Epitopes contained in the laminin A chain are absent from the molecule, and a 190-kD chain substitutes for the A chain. V8 protease analysis and Western blotting studies indicate that the variant 190-kD chain shows structural and immunological similarity to the 200-kD chain of kalinin. Rotary shadowing analysis indicates that the 190-kD chain contributes a large globular structure to the variant long arm, but lacks the short arm contributed to laminin by the A chain. The variant is produced by cultured skin explants, human keratinocytes and a squamous cell carcinoma line, and is present in human amniotic fluid. Polyclonal antibodies raised to kalinin, a recently characterized novel component of anchoring filaments, and mAb BM165 which recognizes a subunit of kalinin (Rousselle et al., 1991) cross react with the variant under nonreducing conditions. Immunohistological surveys of human tissues using the cross-reacting antikalinin antiserum indicate that the distribution of this laminin variant is at least restricted to anchoring filament containing basement membranes. We propose the name K-laminin for this variant.

LAMININ purified from the murine Engelbreth-Holm-Swarm (EHS)[1] tumor, is a disulfide-bonded trimer consisting of a 400-kD A chain, a 220-kD B1 chain, and a 210-kD B2 chain (Cooper et al., 1981). By rotary shadowing EM, EHS laminin has the image of an asymmetric cross with one long arm and three short arms (Engel et al., 1981). Laminin is a component of all basement membranes (Foidart et al., 1980)

Fragmentation studies of the large EHS laminin molecule have facilitated the localization of several of its properties to individual molecular domains. The large size and multidomain structure of this molecule give it the potential to span the basement membrane, mediate the interactions of multiple basement membrane components, and interact with receptors at basal cell surfaces adjacent to basement membrane (Beck et al., 1990). Several extracellular matrix proteins are capable of interacting with EHS laminin, including type IV collagen, nidogen, and heparin sulfate proteoglycan (Martin and Timpl, 1987). Of these, the nidogen–laminin complex is the most stable (Paulsson et al., 1987). EHS laminin also has the ability to self aggregate (Yurchenco et al., 1985).

Many types of cells including keratinocytes (Stanley et al., 1982) and dermal fibroblasts (Woodley et al., 1988) have both been shown to synthesize laminin in culture. Some cell lines, including choriocarcinoma cells (Peters et al., 1985) and HT 1080 fibrosarcoma cells (Alitalo et al., 1980) synthesize an excess of B chains relative to A chain. Pertinent to these observations, recent in situ hybridization experiments of human skin samples has revealed abundant expression of B1 and B2 chain genes, but undetectable expression of A chain gene (Olsen et al., 1989). It is possible that in both skin and cultured cells, B1 and B2 chains are synthesized in relative excess and that synthesis of A chain serves as the rate-limiting step for laminin assembly.

Additionally, it is becoming increasingly clear that laminin chains are assembled into a variety of structures. Merosin is a laminin variant which contains a B1 chain, a B2 chain, and a third chain distinct from the A chain, although it shares 40% homology by sequence analysis (Ehrig et al., 1990). Mouse heart laminin (Paulsson and Saladin, 1989) is a laminin variant with a substituted A chain of a size similar to the one in merosin. S-laminin, another laminin variant, contains a normal A chain, B2 chain, and a variant chain that shows some sequence homology to the B1 chain (Hunter et al., 1989). Recently, the merosin variant chain and the S-laminin variant chain have been found complexed together with B2 in certain tissues, including the myotendonous junction (Engvall et al., 1990). Two other laminin variants which apparently lack an A chain have been reported, but unlike merosin and S-laminin, it is not known whether they are present in tissue. These include rat RN22 schwannoma laminin (Davis et al., 1985; Edgar et al., 1988) and 3T3 adipocyte laminin

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1. *Abbreviations used in this paper:* EHS, Engelbreth-Holm-Swarm; KGM, keratinocyte growth medium; SCC, squamous cell carcinoma.

(Aratani and Kitigawa, 1988). These forms contain B1 and B2 subunits, but lack electrophoretically normal A subunits. Thus laminin exists as a family of proteins, and its individual members have restricted tissue distributions, for example merosin and S-laminin localize to muscular and neural basement membranes, respectively, but not to epithelial basement membranes (Engvall et al., 1990).

Laminin has been shown to influence the growth and differentiation of many types of cells, and is present at the earliest stages of human development (Timpl and Dziadek, 1986). Laminin is also a component of the extracellular matrix deposited by keratinocytes onto culture substratum (Carter et al., 1990; Marchisio et al., 1991). Exogenously supplied EHS laminin facilitates the attachment of a variety of epithelial cell types (Terranova et al., 1980; Goodman et al., 1987), including human keratinocytes (Wilke and Skubitz, 1991) but markedly decreases motility of cultured keratinocytes (Woodley et al., 1988).

Anchoring filaments are thin threadlike structures which localize to the lamina lucida of a subset of basement membranes (Ellison and Garrod, 1984). An anchoring filament component, kalinin, has recently been described (Rousselle et al., 1991). Kalinin is similar to laminin in that it is a large heterotrimeric protein which localizes to the lamina lucida, plays a role in cell attachment and is a component of the matrix deposited by keratinocytes onto culture substratum (Rousselle et al., 1991). It is composed of a disulfide-bonded complex of 200-, 155-, and 140-kD subunits. In tissue and cell culture, the 200-Kd subunit is processed to 165 kD, and the 155-kD subunit is processed to 105 kD (Marinkovich et al., 1992). The mAb BM165 recognizes the kalinin 200/165-kD subunit (Rousselle et al., 1991) and the recently characterized mAb K140 recognizes the kalinin 140-kD subunit (Marinkovich et al., 1992).

Previously, basement membranes at areas of epithelial-mesenchymal interface, such as the one found at the dermal-epidermal junction, were believed to contain only laminin of the EHS prototype. We show here a subset of epithelial basement membranes contain a second novel variant form of laminin, in addition to laminin of the EHS prototype. Skin in organ culture and epidermal cells in primary culture produce the variant, which has a "Y" shaped rotary-shadowed image. We demonstrate that this variant is composed of a B1 chain, a B2 chain, and a third 190-kD chain which is immunologically distinct from the laminin A chain but immunologically and structurally related to the 200/165-kD subunit of kalinin. We propose the name K-laminin for this variant.

Materials and Methods

Keratinocyte growth medium (KGM) was purchased from Clonetics Corporation (San Diego, CA). Hydrocortisone, cholera toxin, and tissue culture grade epidermal growth factor were purchased from Sigma Chemical Company (St. Louis, MO). Mouse EHS laminin, DME, heat inactivated FBS, Ham's F-12 nutrient mixture, and penicillin/streptomycin 100× solution were purchased from Gibco Laboratories (Grand Island, NY). Rabbit anti-mouse IgG immunobeads, goat anti-rabbit IgG immunobeads, and pre-stained high molecular weight markers for electrophoresis were purchased from Bio-Rad Laboratories (Richmond, CA). CNBr-activated sepharose Cl-4B beads were purchased from Pharmacia/LKB (Pleasant Hill, CA). V8 protease was purchased from ICN Biomedicals, Inc., Irvine, CA. [35S]-

cysteine and [35S]methionine were purchased from Amersham Corp. (Danvers, MA).

Antibodies

The mAb BM165 reacts with the 165-kD chain of kalinin, as previously described (Rousselle et al., 1991). Preparation and specificity of mAb K140 which reacts with the 140-kD subunit of kalinin and rabbit polyclonal antiserum against kalinin is described elsewhere (Marinkovich et al., 1992). The laminin A chain specific mAbs 1F5, 11D5 (Engvall et al., 1990), 4C7 (Engvall et al., 1986), laminin B2 chain specific mAb 2E8 (Engvall et al., 1986), and anti merosin mAb 5H2 (Leivo et al., 1989) were kindly provided by Dr. Eva Engvall of the La Jolla Cancer Research Foundation. Affinity-purified polyclonal antibody against mouse laminin was obtained from Sigma Chemical Co. mAbs were purified from hybridoma medium as previously described (Keene et al., 1991).

During the course of these studies a new mAb, 545, was developed. Antigen for mAb 545 was obtained from the reduced product of the PF3 fraction of human amniotic membrane prepared as previously described (Maddox et al., 1989). Briefly, disulfide bonds of the PF3 fraction were reduced and alkylated with vinyl pyridine. Peptides containing cysteine residues were reduced in 100-fold molar excess of 2-mercaptoethanol overnight at room temperature in 0.5 M Tris-HCl buffer, pH 7.5, containing 0.2 M NaCl and 5 mM EDTA. Equimolar amounts of vinyl pyridine to mercaptoethanol were added and after a further 90-min incubation at room temperature, the peptides were separated from excess reagent by gel filtration, and used for immunization of a BALB/c mouse as previously described (Sakai et al., 1986). mAb 545 has been shown to specifically immunoprecipitate laminin from a complex mixture of proteins in radiolabeled keratinocyte-conditioned medium (not shown). Additionally, this antibody has been shown to have a staining pattern identical to polyclonal antilaminin antibody on human skin sections by indirect immunofluorescent microscopy (not shown).

Cell Culture

Keratinocytes were cultured from newborn foreskins by a modification of an established method (Rheinwald and Green, 1975; O'Keefe et al., 1988). Before the first passage cultures were incubated in sterile PBS containing 0.02% EDTA for 5 min, gently pipetted to remove dermal fibroblasts and 3T3 cells, then washed and treated with trypsin 0.05% and EDTA 0.02% wt/vol to suspend keratinocytes. Subsequently, cells were grown in KGM containing 0.15 mM CaCl2 and subcultured according to the manufacturer's instructions. Squamous cell carcinoma line SCC-25 (ATCC No. CRL 1628) was cultured in 50% Ham's F-12 medium, 50% DME with 0.5 µg/ml hydrocortisone, and 10% FBS, and was routinely subcultured with 0.05% trypsin, 0.02% EDTA in PBS.

Cell Labeling

For 24-h labeling experiments, dissociated third passage keratinocytes were allowed to attach to tissue culture plastic dishes, 5×10^5 cells/cm^2, in complete KGM for 2 h. Adherent cells were briefly washed with methionine and cysteine-deficient KGM. Labeling was performed in deficient KGM containing 50 µCi/ml each of [35S]methionine and [35S]cysteine for 24 h under standard culture conditions.

In organ culture experiments, fetal bovine skin was removed from a 10-in. (crown to rump length) calf <4 h out of the uterus. Skin was cut into 1×1-mm sections and cultured in suspension for 24 h in methionine- and cysteine-deficient DME containing hydrocortisone (0.5 µg/ml) cholera toxin (10 ng/ml), EGF (10 ng/ml), penicillin (100 U/ml), streptomycin (100 µg/ml) FBS 2%, [35S]methionine (50 µCi/ml), and [35S]cysteine (50 µCi/ml). Subsequently, tissue was washed and incubated in complete non-radioactive DME containing the above factors and 10% FBS for 72 h. Aliquots of tissue were removed after the 24-h labeling period and again after the 72-h nonradioactive chase period and processed for immunoprecipitation as described below for cell samples.

Antibody Precomplexing and Radioimmunoprecipitation

For each sample to be analyzed 10 µg protein-G purified mAb was added to 100 µl rabbit anti-mouse IgG immunobeads or 10 µl polyclonal rabbit antisera was added to 400 µl "second antibody" goat anti-rabbit IgG immunobeads. For polyclonal control conditions 10 µl normal rabbit serum was used. For monoclonal control conditions, no primary antibody was

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used. The mixtures were incubated at 37°C for 2 h with mild agitation. The antibody–immunobead complexes were pelleted by centrifugation at 2,500 rpm, washed once with radioimmunoprecipitation assay (RIPA) buffer (10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 2 mM EDTA, 250 μM PMSF, 1 mM n-ethylmaleimide, 2 mM l-methionine, 2 mM l-cysteine, 0.3% NP-40, 0.05% triton X-100, 0.3% sodium deoxycholate, 0.1% BSA, and repelleted before use with labeled sample.

Cell layers were washed once with nonradioactive culture medium then harvested with a cell scraper and ice cold RIPA buffer containing 0.1% SDS. All subsequent steps were performed at 4°C. Labeled cell material was solubilized in a Dounce homogenizer and spun at 14,000 rpm for 10 min. Labeled medium was removed from culture and centrifuged at 2,000 rpm. Preclearing of each sample was accomplished by adding aliquots of labeled cell or medium supernatants to the centrifuged pellets of either 400 μl goat anti-rabbit IgG immunobeads precomplexed with 10 μl nonimmune rabbit serum, or 100 μl rabbit anti–mouse IgG immunobeads. Each sample was vortexed briefly, then left on a rocking platform for 1 h. The mixture was then centrifuged at 14,000 rpm for 10 min, and the supernatant combined with the centrifuged pellet of immunobeads precomplexed with specific antibody. Each sample was incubated with specific antibody for 18 h on a rocking platform, then pelleted by centrifugation at 2,500 rpm for 10 min. After centrifugation, the supernatant was removed and the pellet was washed with RIPA buffer (medium samples) or RIPA buffer containing 0.1% SDS (cells), briefly vortexed, then recentrifuged. After five washes, the pellets were mixed with sample buffer, heated to 95°C for 3 min, recentrifuged, and the supernatant was analyzed by SDS-PAGE. In one experiment, kalinin was removed from precleared labeled keratinocyte medium by passage over a mAb K140-sepharose column before immunoprecipitation with mAb BM165.

Immunoaffinity Purification of the Laminin Variant

Human amniotic fluid obtained from first and second trimester amniocentesis or squamous cell carcinoma–conditioned medium were each centrifuged at 1,000 rpm to remove cell debris. Supernatants were brought to 250 μm PMSF, 1 mM n-ethylmaleimide, 2 mM EDTA, and 0.02% sodium azide, then centrifuged at 18,000 rpm for 90 min. The supernatants were then passed over either mAb BM165-sepharose (Rousselle et al., 1991), or mAb 2E8-sepharose columns (1 mg antibody per 1 ml matrix, coupled to CL4B sepharose per manufacturer's instructions) (Pharmacia Fine Chemicals, Pleasant Hill, CA), washed with 50 column volumes PBS, then eluted with 1 M acetic acid. Peak fractions were determined by UV 280 absorbance and Western blotting, treated with diisopropyl fluorophosphate (5 μg/ml), and dialyzed against water. Samples for rotary shadowing analysis were dialyzed against 0.2 M ammonium bicarbonate and concentrated fivefold on a centricon-30 microconcentrator (Amicon, Beverly, MA).

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), visualization of rotary-shadowed images by EM (Morris et al., 1986), indirect immunofluorescent microscopy of frozen sections of human tissue (Sakai et al., 1986), fluorography of acrylamide gels containing radioactive proteins (Bonner and Laskey, 1974), and V8 protease digestion of excised gel bands (Cleveland et al., 1977).

Results

Biosynthetically radiolabeled keratinocyte-conditioned culture medium was immunoprecipitated using several antibodies. Polyclonal anti-EHS laminin specifically precipitates two electrophoretic species before disulfide bond reduction (Fig. 1, lane *1*). No bands are precipitated from the same medium in the absence of primary antibody (Fig. 1, lane *8*). The same two electrophoretic species are precipitated by monoclonal antilaminin B2 chain antibody 2E8 (Fig. 1, lane *2*). In contrast, monoclonal antilaminin A chain antibodies 1F5, 4C7, and 11D5, precipitate only the slower electrophoretic species (Fig. 1, lanes *3*, *4*, and *5*, respectively).
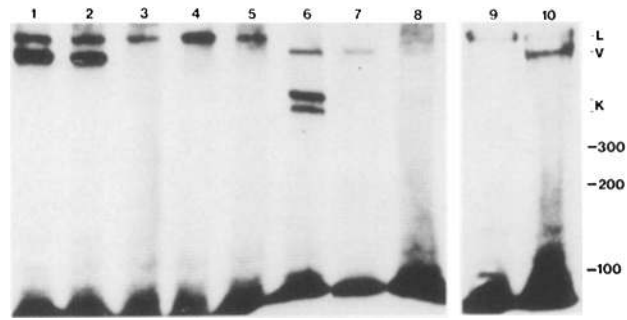

Figure 1. Nonreduced immunoprecipitation of keratinocyte medium using various antibodies. Human keratinocyte-conditioned medium derived from a 24-h radiolabeling period was immunoprecipitated with the following antibodies: lane *1*, polyclonal antilaminin; lane *2*, 2E8 (monoclonal antilaminin B2 chain); lane *3*, 1F5 (monoclonal antilaminin A chain); lane *4*, 4C7 (monoclonal antilaminin A chain); lane *5*, 11D5 (monoclonal antilaminin A chain); lane *6*, BM165 (monoclonal antikalinin 200/165-kD chain); lane *7*, BM165 immunoprecipitation of medium which has been cleared of kalinin by mAb K140 (monoclonal antikalinin 140-kD chain); lane *8*, control (no primary antibody); and lanes *9* and *10*, medium sequentially immunoprecipitated, first with 4C7 monoclonal antilaminin A chain (lane *9*) then with polyclonal antilaminin (lane *10*). Samples were analyzed by nonreduced SDS-PAGE on 3–5% acrylamide gradient gels and visualized by autoradiography. *L*, laminin; *V*, variant; and *K*, kalinin. Right margin indicates M_r × 10^{-3}.

mAb BM165 coprecipitates kalinin and the faster migrating species (Fig. 1, lane *6*). The mAb K140 precipitates kalinin but not the laminin variant from labeled keratinocyte medium (not shown). When kalinin is precleared from labeled medium with an excess of K140, BM165 only precipitates the faster migrating laminin variant (Fig. 1, lane *7*). Given the characterized specificity of the immunological reagents, the results suggest that human keratinocytes secrete a lower molecular weight variant of laminin containing at least a B2 chain, but not a normal A chain. These results also show that mAb BM165 cross reacts with the faster-migrating laminin species, suggesting that immunologically the variant chain contained in this laminin species is more closely related to the kalinin 200/165-kD chain than to the laminin A chain. Antimerosin mAb 5H2 failed to precipitate either species from keratinocyte medium (not shown) consistent with the absence of merosin from the dermal–epidermal basement membrane of human skin (Leivo and Engvall, 1988).

To further verify the identity of the faster electrophoretic species as a laminin variant, radiolabeled keratinocyte medium was precleared of normal laminin using antilaminin A chain antibody and then reprecipitated using polyclonal antilaminin serum. The anti–A chain antibody removes only laminin, leaving the variant which is specifically removed by the polyclonal serum plus a small amount of conventional laminin (Fig. 1, lanes *9* and *10*).

When the antilaminin immunoprecipitation product from keratinocytes medium fractions (Fig. 2, lane *1*) is compared with that of keratinocyte cell fractions (Fig. 2, lane *2*) by nonreduced SDS-PAGE, it is apparent that after a 24-h labeling period, there is a greater fraction of laminin (Fig. 2, *L*) present in the cell fraction, than in the medium fraction. The
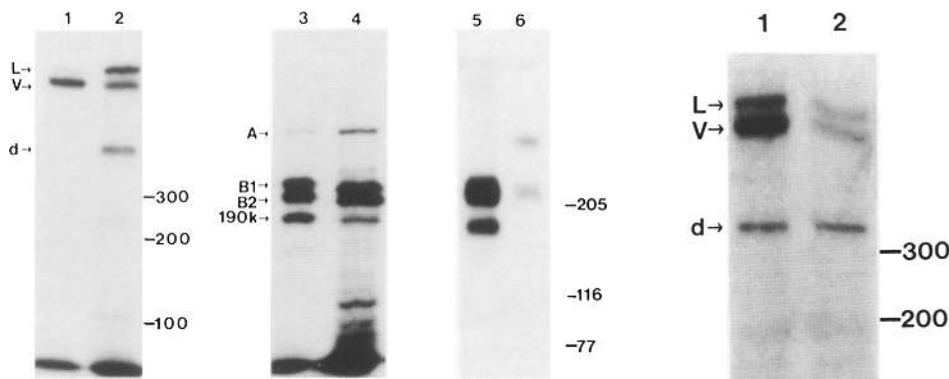
Figure 2. Comparison of laminin in keratinocyte cell and medium fractions. Following a 24-h metabolic labeling period, keratinocyte-conditioned medium (lanes *1* and *3*), and keratinocyte cell fraction (lanes *2* and *4*), were each immunoprecipitated with polyclonal antilaminin. Precipitated materials were then separated by nonreduced SDS-PAGE on a 3–5% gradient gel (lanes *1* and *2*) and separated by reduced SDS-PAGE on a 5% acrylamide gel (lanes *3* and *4*). Nonreduced gel bands containing the variant (*V*, lane *1*) and laminin (*L*, lane *1*) were excised from the dried gel, rehydrated with sample buffer containing 2% 2-mercaptoethanol, and separated by second dimension reduced SDS-PAGE on a 5% acrylamide gel; variant band (lane *5*), laminin band (lane *6*). All samples were visualized by fluorography. Right margins indicate $M_r \times 10^{-3}$.
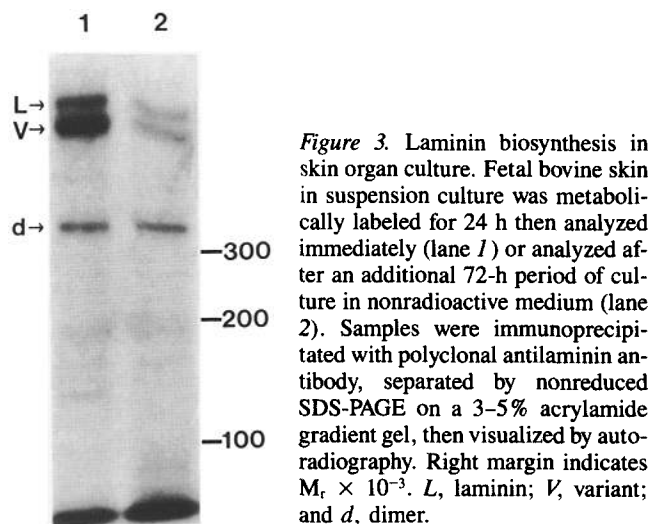
Figure 3. Laminin biosynthesis in skin organ culture. Fetal bovine skin in suspension culture was metabolically labeled for 24 h then analyzed immediately (lane *1*) or analyzed after an additional 72-h period of culture in nonradioactive medium (lane *2*). Samples were immunoprecipitated with polyclonal antilaminin antibody, separated by nonreduced SDS-PAGE on a 3–5% acrylamide gradient gel, then visualized by autoradiography. Right margin indicates $M_r \times 10^{-3}$. *L*, laminin; *V*, variant; and *d*, dimer.

cell fraction also contains a strong 400-kD band (Fig. 2, *d*) which reduces to a pair of bands at 220–210 kD (not shown). This 400-kD band is therefore interpreted to be a B1–B2 chain dimer, whose existence has been previously proposed (Cooper et al., 1981; Morita et al., 1985; Peters et al., 1985). No laminin A chain was detected in the second dimension analysis of the 400-kD band (not shown).

Reduction of the medium derived immunoprecipitates (Fig. 2, lane *1*) clearly shows the predominance of bands in the positions of the laminin B1 and B2 chains, and in the 190-kD position (Fig. 2, lane *3*). Consistent with the presence of a minor amount of laminin, only small amounts of laminin A chain are seen. In contrast, reduction of the cell-derived materials (Fig. 2, lane *4*) shows amounts of A, B1, B2, and 190-kD chains expected from a nearly equal mixture of laminin and the variant indicated by the unreduced gel pattern (Fig. 2, lane *2*). The pattern of bands of apparent molecular weight <190 kD in lanes *3* and *4* are identical to those of control samples where no primary antibody was used (not shown).

When the nonreduced variant gel band (Fig. 1, lane *1*, band *V*) is excised and analyzed by SDS-PAGE after disulfide bond reduction (Fig. 2, lane *5*), a broad band containing the B chains and a distinct 190-kD band are present. Alternatively, when the nonreduced laminin gel band is similarly treated, a 400-kD A chain and no 190-kD band are observed (Fig. 2, lane *6*). Together with the results presented in Fig. 1, these data indicate that the variant does contain chains electrophoretically identical to both the B1 and B2 chains, and contains a third chain of 190 kD that is not immunoreactive with three mAbs each directed toward different epitopes within the A chain, but which is reactive to a mAb directed against the 200/165-kD subunit of kalinin.

Organ culture studies with fetal bovine skin reveal that after a 24-h labeling period, the nonreduced pattern of

materials immunoprecipitated with polyclonal laminin antisera from cultured skin (Fig. 3, lane *1*) is similar to that obtained from 24-h labeled keratinocyte cell fractions (Fig. 2, lane *2*). This finding indicates that production of the variant species is not an artifact of primary cell culture and production also occurs in skin organ culture. When the labeled skin is cultured in nonradioactive medium for an additional 72 h there is a diminution of the intensity of the laminin and variant species but there is no significant loss of intensity of the presumed B1-B2 dimer (Fig. 3, lane *2*). Although there is a diminution of the intensity of the bands representing the variant and laminin, indicating turnover or increased insolubilization of these materials to assay procedures, there is no evidence of one form being processed into the other. The preserved intensity of the B1–B2 dimer band after a period of nonradioactive chase suggests that the dimer may not simply represent incompletely disulfide bonded laminin, but that it may represent a stable entity that is sequestered for future use either in the assembly of laminin or for some other purpose.

Conditioned medium from human squamous cell carcinoma cultures, and human amniotic fluid both were found to be useful for immunoaffinity purification of biochemical quantities of laminin and variant. When conditioned squamous carcinoma cell medium is purified using 2E8-sepharose and the peak elution fraction is separated by nonreduced SDS-PAGE, two high molecular weight laminin species, as well as a 150-kD band are stained by Coomassie blue (Fig. 4 *A*, lane *1*). Because the 150-kD band failed to yield lower molecular weight bands on second dimension reduced SDS-PAGE (not shown), we interpret this band to represent nidogen. When the band representing the faster migrating variant species is excised, separated by second dimension reduced SDS-PAGE, and stained by Coomassie blue a broad 220–210-kD band as well as a distinct 190-kD band are visualized (Fig. 4 *B*, lane *1*) analogous to the results obtained with radiolabeled materials in Fig. 2. Thus Coomassie blue staining reveals that stoichiometrically, the B1, B2, and 190-kD chains of the faster migrating variant species are present in equal amounts. Several minor faintly staining bands of lower molecular weight are also noted in Fig. 4 *B*, lane *1*. Similarly treated samples were immunoblotted and found to contain
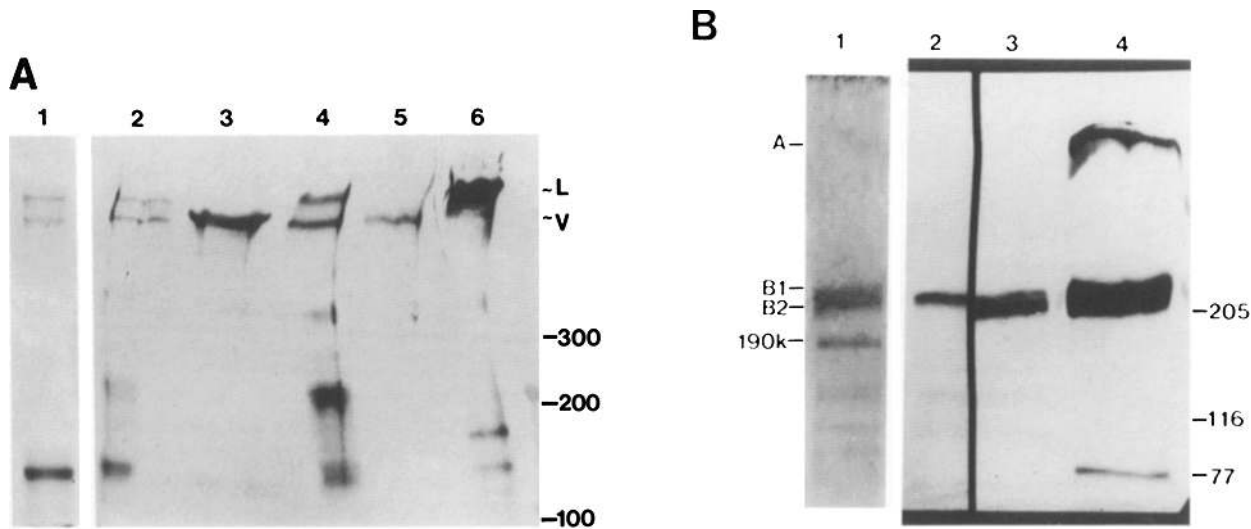
Figure 4. Immunochemical comparison of laminin and the variant. (*A*) Nonreduced analysis of laminin and the variant. Human amniotic fluid and squamous cell carcinoma (SCC)-conditioned medium were used for affinity chromatography with either 2E8-sepharose (monoclonal antilaminin B2 chain), or BM165-sepharose (monoclonal antikalinin). Lanes *1* and *4*, 2E8 purified SCC-conditioned medium; lane *2*, 2E8 purified human amniotic fluid; lane *3*, BM165 purified human amniotic fluid; lane *5*, BM165 purified SCC-conditioned medium; lane *6*, laminin from EHS tumor. Samples were separated by nonreduced SDS-PAGE on a 3–5% acrylamide gradient gel and visualized either by Coomassie blue staining (lane *1*) or Western blotting with polyclonal antilaminin antibody (lanes *2*, *3*, *4*, *5*, and *6*). Right margin indicates $M_r \times 10^{-3}$. *L*, laminin; and *V*, variant. (*B*) Reduced analysis of laminin and the variant. The nonreduced variant band derived from SCC-conditioned medium shown in *A*, (lane *1*, *V*) was excised from the gel, separated by second dimension SDS-PAGE under reducing conditions and visualized by Coomassie blue staining (lane *1*). Peak fractions from BM165-sepharose purification of SCC-conditioned medium (lanes *2* and *3*) and EHS laminin (lane *4*) were separated by SDS-PAGE on a 5% acrylamide gel under reducing conditions and transferred to nitrocellulose. The lane containing the SCC-derived material (lanes *2* and *3*) was cut in half and one half was incubated with mAb 545 (lane *2*). The other half (lanes *3* and *4*) were incubated with polyclonal antilaminin antibody. Right margin indicates $M_r \times 10^{-3}$.

several faint lower molecular weight bands recognized by laminin but not kalinin antisera (not shown). We interpret these bands to represent degradation products of K-laminin produced during fixation and destaining.

Nonreduced Western blot analysis (Fig. 4 *A*) of the peak fractions from conditioned squamous carcinoma cell medium and amniotic fluid purified with 2E8-sepharose (Fig. 4 *A*, lanes *1*, *3*, and *5*) and BM165-sepharose (Fig. 4 *A*, lanes *2* and *4*) was done with polyclonal laminin antiserum used as primary antibody. Amniotic fluid (Fig. 4 *A*, lanes *2* and *3*) and squamous carcinoma cell medium (Fig. 4 *A*, lanes *1*, *4*, and *5*) produce essentially identical results in that purification over an antilaminin B2 chain column produced two nonreduced species and purification over an antikalinin column produced only a single nonreduced species. The higher molecular weight nonreduced species (Fig. 4 *A*, lanes *1*, *2*, and *4*) comigrates with laminin purified from EHS tumor (Fig. 4 *A*, lane *6*). Analogous to the results with radioactive keratinocyte medium in Fig. 1, the lower molecular weight nonreduced laminin variant species is the only form affinity purified by BM165-sepharose.

The variant plus kalinin were purified from squamous carcinoma cell culture medium by BM165 affinity chromatography. The disulfide-bond reduced kalinin and variant chains were separated by SDS-PAGE using a wide comb, and transferred to nitrocellulose. The nitrocellulose was then cut through the center of the electrophoretic lane, and one half (Fig. 4 *B*, lane *2*) was Western blotted using mAb 545, specific for the laminin B1 chain. The other half of the lane (Fig. 4 *B*, lane *3*) and a second lane containing EHS laminin

chains (Fig. 4 *B*, lane *4*) were blotted with polyclonal antilaminin. The anti-B1 antibody blots only the upper half of the wide band containing the B chains, while both the B1 and B2 chains are recognized by the polyclonal serum. This result, together with the observation that the variant is immunoprecipitated by anti-B2 mAb (Fig. 1, lane *2*) indicates that the variant contains authentic B1 and B2 chains. No reaction was noted in the position of the 190-kD chain, further verifying the immunological distinction of this chain from other EHS laminin subunits.

Anti-B2 chain affinity-purified variant and laminin from squamous cell carcinoma conditioned medium as shown in Fig. 4 *A*, lane *1*, were visualized by rotary shadowing. Molecules closely resembling laminin were readily identified (Fig. 5 *A*). In addition, molecules shown in Fig. 5 *B* were frequently seen. These appear as Y-shaped molecules similar in size and conformation to the usual cross-shaped laminin molecule in that each has one long arm with a large globular domain at its distal end, and two short arms each with two globular domains at their distal ends. The Y-shaped molecule which we interpret as representing the variant differs from the cross shape in that there is no third short arm, although some images show that there is a small globular domain present at the intersection of the two small arms and the long arm. The difference in molecular mass between the 400-kD A chain and the 190-kD variant chain would therefore need to be contained in the third short arm of the cross. The 190-kD substitution for the A chain contributes the large globule at the distal end of the long arm similar to the structure contributed to laminin by the A chain. The fact that this
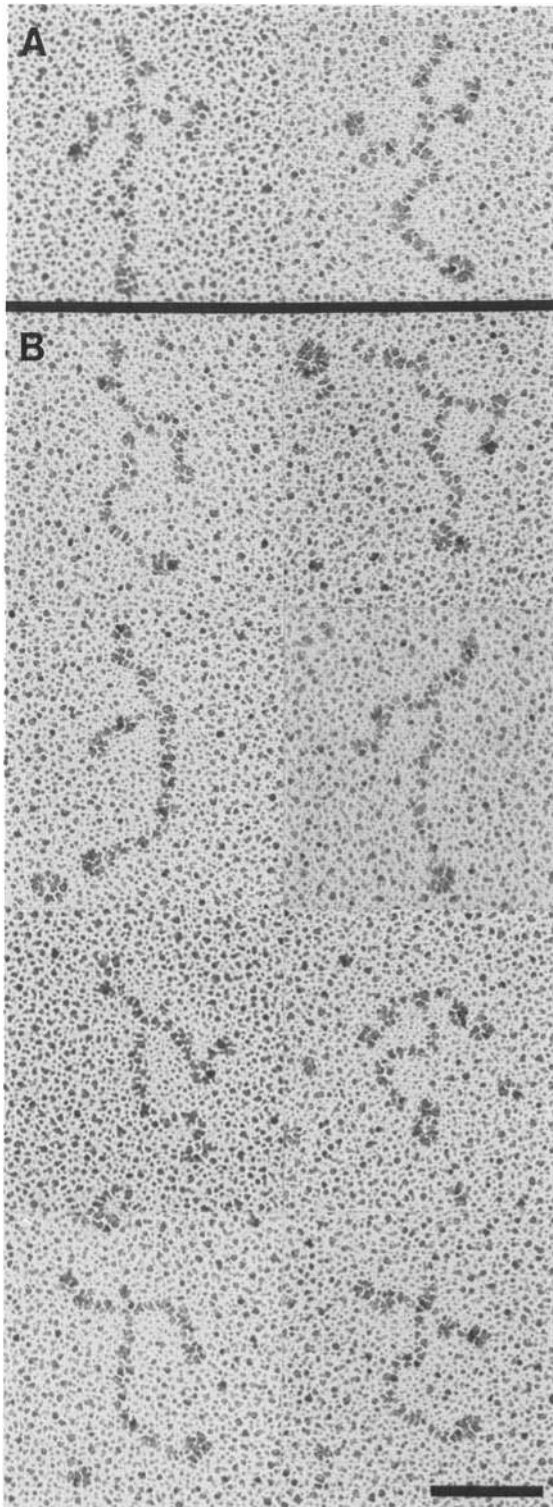
Figure 5. Rotary shadowing analysis of variant and laminin from affinity-purified SCC medium. SCC-conditioned medium was purified by 2E8-sepharose (antilaminin B2 chain). Pooled peak elution fractions analyzed by Coomassie staining and Western blot in Fig. 4 *A* (lanes *1* and *4*) were imaged by rotary shadowing EM. (*A*) images representing human laminin. (*B*) images representing the laminin variant. Bar, 50 nm.

globule is apparently intact in the variant and antibody 4C7 specifically recognizes this globule on laminin (Engvall et al., 1986) is further evidence that this chain is a true substitution for the A chain, and not a degradation product.

A polyclonal antibody was raised in rabbits to the 400-kD nonreduced kalinin gel band (Rousselle et al., 1991). This antibody identified all kalinin chains by Western blotting after disulfide-bond reduction (Marinkovich et al., 1992). Laminin and variant were purified from squamous carcinoma cell medium using 2E8-sepharose (anti-B2 chain) affinity chromatography, and the product was evaluated by SDS-PAGE and Western blotting before and after disulfide bond reduction. The nitrocellulose was cut down the center of the two lanes, and one half of each lane was incubated with polyclonal anti-laminin, and the other was incubated with the polyclonal antikalinin (Fig. 6 *A*). The antilaminin serum identified both laminin and the variant before reduction, while the antikalinin serum recognizes only the variant. The lack of reactivity of the antikalinin serum with laminin indicates that the cross-reactivity with the variant must be with the 190-kD chain. This cross-reactivity is lost upon disulfide bond reduction, and the polyclonal antikalinin serum fails to react with the 190-kD chain. The immunological cross-reactivity suggests that the chains share conformational epitopes but not sequence specific epitopes. The reactivity of the variant with the polyclonal antikalinin serum indicates that the 190-kD variant chain contains epitopes present in the 200-kD kalinin chain, but absent from the laminin A chain, supporting the conclusion that the variant chain is not a degradation product of the A chain. Comparison of the peptides generated from V8 protease digestion (Fig. 6 *B*) of the 190-kD variant chain (*V*), and the 200-kD kalinin chain (*K*), each excised from polyacrylamide gels, indicates nearly identical electrophoretic mobilities. In total, these data indicate that these chains are very similar but not identical.

Since the polyclonal antikalinin antiserum cross reacts with the variant, we examined the distribution of this reactivity in skin and in brachial plexus nerve. Similar to results seen for kalinin with mAb BM165 (Rousselle et al., 1991), the reactivity was restricted to the basement membrane zone of the dermal–epidermal junction of skin (Fig. 7 *D*), no reactivity above preimmune serum control was seen in brachial plexus nerve (Fig. 7, *C* and *F*). The reactivity of the polyclonal antilaminin serum reacts with all basement membranes in nerve and skin (Fig. 7, *A* and *B*). The nerve was also intensely stained with B2 chain specific antibodies (not shown) and with antimerosin (Fig. 7 *E*). Assuming the epitopes recognized by the polyclonal antikalinin serum are accessible in tissue, the result indicates that the variant is not distributed beyond the dermal–epidermal junction in skin and is not present in peripheral nerve.

We examined the distribution of kalinin, laminin, and variant in several other human tissues (Table I). Laminin which is specifically recognized by polyclonal laminin antiserum is clearly reactive in the basement membranes of skeletal muscle, blood vessel endothelium, and as seen in Fig. 7, peripheral nerve. Neither kalinin nor variant are present in these distributions evidenced by lack of immunoreactivity with mAb K140 or with polyclonal kalinin antiserum which recognizes both kalinin and variant. In trachea, large and small intestine, amnion, lung, and skin, polyclonal laminin antiserum reacted with all basement membrane containing
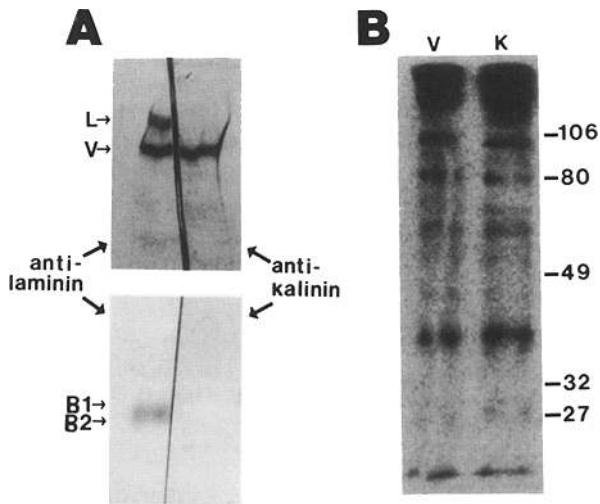
Figure 6. Immunological and structural analysis of the laminin variant. (A) Comparative immunoblotting of laminin and the variant. Laminin and variant derived from 2E8-Sepharose (antilaminin B2 chain) purification of SCC-conditioned medium, were separated on 3–5% acrylamide gels under nonreducing (upper panel) or under reducing conditions (lower panel) and electrophoretically transferred to nitrocellulose. The nitrocellulose containing the lanes of both the nonreduced and reduced samples were cut in half. Western blot analysis was then completed with both polyclonal laminin antisera (left halves) and polyclonal kalinin antisera (right halves). (B) Peptide mapping studies using V8 protease. Bands containing the 190-kD subunit of the variant (lane V) derived from polyclonal antilaminin immunoprecipitation of radiolabeled keratinocyte medium, and the 200-kD subunit of kalinin, (lane K) derived from BM165 immunoprecipitation of radiolabeled keratinocyte cell fraction were excised from gels, partially digested with V8 protease (2 μg/ml for 30 min, at room temperature) and the digestion products separated by SDS-PAGE side by side on a 10% acrylamide gel. Right margin indicates $M_r \times 10^{-3}$.

structures, while mAb K140 and polyclonal kalinin antiserum reacted only at areas of epithelial–mesenchymal interface. These results indicate that, in the tissues examined, the variant represents a distinct subset of the total laminin distribution, and that the distribution of the variant does not appear to extend beyond that of kalinin.

Discussion

We have identified a variant of laminin that is restricted in its distribution to the basement membrane zone of the dermal–epidermal junction in skin, and is absent in nonepithelial basement membranes including those of peripheral nerve, skeletal muscle, and blood vessels. The molecule is synthesized by human and bovine keratinocytes, and by a squamous cell carcinoma line. The molecule is also present in amniotic fluid, as is the epithelial-specific molecule kalinin (not shown). This variant has a 190-kD chain substituted for the A chain, which is shorter than the A chain by ~240 kD, and is immunologically unrelated to the laminin A chain. Instead, the variant 190-kD chain shows a structural and immunological similarity to the 200/165-kD chain of kalinin. Visualization of the variant by rotary shadowing indicates that the short arm normally contributed by the A chain to
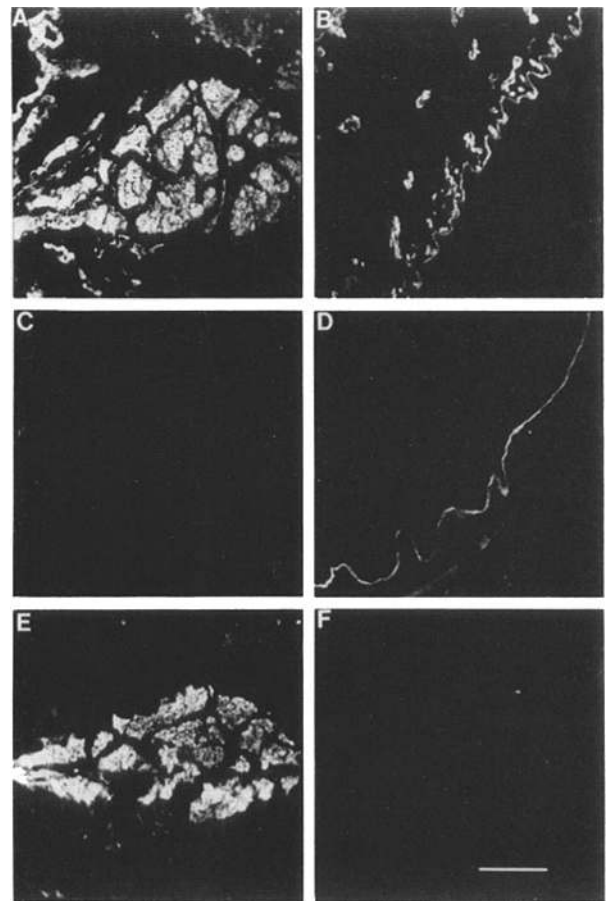

Figure 7. Comparison of laminin distribution in peripheral nerve and neonatal foreskin. Frozen 8-μm-thick sections of either brachial plexus nerve from a 20-wk human fetus (A, C, E, and F), or human neonatal foreskin, (B and D) were analyzed by indirect immunofluorescent microscopy. (A and B) Polyclonal antilaminin; (C and D) polyclonal antikalinin; (E) mAb 5H2 antimerosin; (F) polyclonal antikalinin, preimmune serum. Bar, 160 μm.

laminin is missing in this molecule. However, the substituted 190-kD chain contributes a large globule at the end of the long arm that appears very much like the domain contributed to normal laminin by the A chain. The presence of this large

Table I. Tissue Survey[‡]

	Antibodies		
Tissue	mAb K140	Polyclonal kalinin	Polyclonal laminin
Skin	+*	+*	+
Trachea	+*	+*	+
Large intestine	+*	+*	+
Small intestine	+*	+*	+
Lung	+*	+*	+
Amnion	+*	+*	+
Peripheral nerve	−	−	+
Blood vessels	−	−	+
Skeletal muscle	−	−	+

* Indicates that staining was present only at epithelial–mesenchymal interfaces.
‡ For this survey indirect immunofluorescent microscopy was performed on frozen sections of tissue obtained from a 20-wk (estimated gestational age) normal human fetus, except the human amnion which was obtained after a term delivery.

globule rules out the possibility that the rotary shadowed images represent B1–B2 chain dimers, consistent with the observation that none of these dimers were detected in the cell media preparations.

A Y-shaped laminin variant has been previously reported as a product of rat schwannoma cells. This molecule lacks the laminin A chain and instead contains additional peptides of 130 and 35 kD (Davis et al., 1985). Polyclonal antikalinin cross reacts with skin cell–derived variant, but is unreactive with peripheral neural tissue suggesting that the skin variant is not related to the schwannoma produced variant. Rat astrocytes also synthesize a laminin variant lacking the A chain but no substituted chain was observed (Liesi and Risteli, 1989). 3T3-L1 adipocytes produce a molecule with a 200-kD chain substituted for the A chain (Aratani and Kitigawa, 1988). The relationship of any of these molecules to the variant described here in skin is not yet known. Other laminin variants with substituted A chains include merosin (Ehrig et al., 1990) and mouse heart laminin (Paulsson and Saladin, 1989), but both of these molecules retain the three short arms reminiscent of EHS laminin.

Previous evaluation of laminin synthesized by both malignant and nonmalignant keratinocytes showed association of laminin with additional glycoproteins (Frenette et al., 1988). The data support the coprecipitation of laminin, the keratinocyte variant, and kalinin by the authors' antilaminin serum. It is of particular interest that the authors conclude that laminin becomes covalently associated with the glycoprotein we believe to be kalinin under the culture conditions that they use. We have been unable to demonstrate binding of the variant to kalinin in materials derived from cell culture as the variant is not coprecipitated or copurified with mAb K140 and kalinin is not coprecipitated or copurified with any of the laminin antibodies used in this study (not shown). However, when kalinin is isolated directly from human amnion we consistently find kalinin chains and laminin variant chains which are complexed into a disulfide-bonded aggregate that enters only dilute polyacrylamide gels (Lunstrum et al., manuscript in preparation).

Woodley et al. (1988) have reported that laminin inhibits keratinocyte migration. Recently, the inhibitory activity has been reported to reside in a fragment of the A chain (D. Woodley, personal communication). We have recently reported that kalinin is synthesized by growing and migrating keratinocytes, but not by confluent keratinocytes (Rousselle et al., 1991). Since the variant and kalinin appear to be associated, it is tempting to speculate that the absence of the A chain in the variant may facilitate cell migration, but the variant may substitute for other functions provided by laminin.

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