

Laminin 5 Binds the NC-1 Domain of Type VII Collagen

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Abstract. Mutational analyses of genes that encode components of the anchoring complex underlying the basolateral surface of external epithelia indicate that this structure is the major element providing for resistance to external friction. Ultrastructurally, laminin 5 ($\alpha 3\beta 3\gamma 2$; a component of the anchoring filament) appears as a thin filament bridging the hemidesmosome with the anchoring fibrils. Laminin 5 binds the cell surface through hemidesmosomal integrin $\alpha 6\beta 4$. However, the interaction of laminin 5 with the anchoring fibril (type VII collagen) has not been elucidated. In this study we demonstrate that monomeric laminin 5 binds the NH_2 -terminal NC-1 domain of type VII collagen. The binding is dependent upon the native conformation of both laminin 5 and type VII collagen NC-1. Laminin 6 ($\alpha 3\beta 1\gamma 1$) has no detectable affinity for type VII collagen NC-1, indicating that the binding is medi-

ated by the $\beta 3$ and/or $\gamma 2$ chains of laminin 5. Approximately half of the laminin 5 solubilized from human amnion or skin is covalently complexed with laminins 6 or 7 ($\alpha 3\beta 2\gamma 1$). The adduction occurs between the NH_2 terminus of laminin 5 and the branch point of the short arms of laminins 6 or 7. The results are consistent with the presumed orientation of laminin 5, having the COOH-terminal G domain apposed to the hemidesmosomal integrin, and the NH_2 -terminal domains within the lamina densa. The results also support a model predicting that monomeric laminin 5 constitutes the anchoring filaments and bridges integrin $\alpha 6\beta 4$ with type VII collagen, and the laminin 5–6/7 complexes are present within the interhemidesmosomal spaces bound at least by integrin $\alpha 3\beta 1$ where they may mediate basement membrane assembly or stability, but contribute less significantly to epithelial friction resistance.

ELECTRON microscopic evaluation of the epithelial-stromal junction of many external tissues after conventional fixation and dehydration protocols shows a lamina densa and lamina lucida much like that seen for other basement membranes. In addition, spaced along the length of the basal lamina are structures termed the adhesion complex (for reviews see Burgeson, 1993a,b; Gerecke et al., 1994a). These complexes consist of electron dense thickenings of the basolateral plasma membrane called hemidesmosomes, due to their resemblance to half of the desmosomal plaques present on the plasma membranes at sites of cell–cell contact. Cytoskeletal keratin filaments insert into both the hemidesmosomes and the desmosomes bridging all aspects of the cellular plasma membranes within a given cell, and provide a continuous intercellular network throughout the entire epidermis.

Thin filaments, termed “anchoring filaments,” span the lamina lucida and insert into the lamina densa. At high resolution, the anchoring filaments appear to be continuous with centrosymmetrically banded structures, termed “anchoring fibrils” (Ellison and Garrod, 1984). The anchoring fibrils originate within the lamina densa and project into the upper regions of the papillary dermis. About 800 nm in length, the anchoring fibrils either loop back to reinsert into the lamina densa, or insert into structures termed “anchoring plaques” (Keene et al., 1987) that appear as junctions between two or more anchoring fibrils. Additional anchoring fibrils originate in the plaques and extend further into the papillary dermis. The resulting network engulfs banded collagen fibers and other fibrous elements of the papillary dermis, thus assuring the strength of the union of the basement membrane and the upper dermis.

The molecules contained in these structures have been partly identified and characterized. Laminin 5 localizes to the anchoring filaments (Rousselle et al., 1991); it contains three unique subunits, $\alpha 3$ (Ryan et al., 1994), $\beta 3$ (Gerecke et al., 1994b), and $\gamma 2$ (Kallunki et al., 1992). In skin and

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amion, laminin 5 is proteolytically processed after secretion (Marinkovich et al., 1992a). The 200-kD $\alpha 3$ chain is cleaved first to 165 kD, and finally to 145-kD. The $\gamma 2$ chain is processed from 155 kD to 105 kD, while the $\beta 3$ chain remains intact. Thus, three forms of laminin 5 are seen: an intracellular form ($\alpha 3$, 200 kD; $\beta 3$, 140 kD; $\gamma 2$, 155 kD), and two tissue forms ($\alpha 3$, 165 kD; $\beta 3$, 140 kD; $\gamma 2$, 105 kD) and ($\alpha 3$, 145 kD; $\beta 3$, 140 kD; $\gamma 2$, 105 kD). The $\gamma 2$ chain cannot bind nidogen under physiological conditions (Mayer et al., 1995), and therefore laminin 5 alone cannot associate with perlecan or the collagen IV network (Yurchenco et al., 1985). Laminin 5 also lacks the short arm domains believed to be required to promote assembly into laminin networks (Schittny and Yurchenco, 1990; Yurchenco et al., 1992). Laminin can be incorporated into the basement membrane through a unique mechanism involving cross-linking of laminin 5 with laminin 6 ($\alpha 3\beta 1\gamma 1$) or laminin 7 ($\alpha 3\beta 2\gamma 1$) (Champliaud et al., 1996).

Anchoring fibrils are disulfide bond-stabilized dimers of type VII collagen (Morris et al., 1986; Sakai et al., 1986). The biosynthetic product is a monomer containing a large NH_2 -terminal globular domain (Parente et al., 1991), NC-1 (Lunstrum et al., 1986), an unusually long and interrupted triple-helical domain (Bentz et al., 1983), and a relatively small COOH-terminal globular domain, NC-2, that is proteolytically removed during maturation of the anchoring fibrils, and is probably involved in the dimerization process (Morris et al., 1986; Lunstrum et al., 1987; Bruckner-Tuderman et al., 1994). Immunoelectron microscopic studies of the location of well-characterized epitopes within type VII collagen indicate that the NC-1 domain is contained within the lamina densa (Keene et al., 1987). Therefore, a model of the interactions that stabilize the epithelial-stromal attachment has evolved that postulates epithelial cell binding to the laminin 5-6/7 complex $\alpha 3$ G domains, binding of the laminin 5 complex to the collagen network via nidogen, and binding of the type VII collagen NC-1 domain to the basement membrane through interactions of NC-1 with type IV collagen (Burgeson et al., 1990).

Recently, the gene defects underlying Herlitz's junctional epidermolysis bullosa have been identified as mutations in genes encoding laminin 5 chains that result in premature termination codons in both alleles. The resulting laminin 5 null phenotype is characterized by extensive blistering of the skin and the mucosal surfaces of the respiratory and digestive tracts, leading to death during the early months of life. The Herlitz phenotype can be due to mutations in the genes encoding $\alpha 3$ (Kivirikko et al., 1995), $\beta 3$ (Pulkkinen et al., 1994b; Pulkkinen et al., 1995; Vailly et al., 1995), or $\gamma 2$ (Pulkkinen et al., 1994a). This was surprising since the model predicted that laminin 6 or 7 could partially substitute for laminin 5 since it can bind both epithelial cells (through the $\alpha 3$ chains) and basement membranes (through $\gamma 1$ -mediated interactions with nidogen). Therefore, only $\alpha 3$ mutations were predicted to cause Herlitz's junctional epidermolysis bullosa. These findings indicate that additional and uncharacterized interactions must be required for epithelial-stromal stability. The ultrastructure of the anchoring complex (Ellison and Garrod, 1984) suggests a direct interaction of the anchoring filaments with the anchoring fibrils. Hence, we evaluated the binding of type VII collagen to laminin 5.

Materials and Methods

Antibodies

Preparation and characterization of laminin 5 and 6 antibodies mAb BM165 (anti- $\alpha 3$ chain), mAb SF12 (anti- $\gamma 2$), mAb 545 (anti- $\beta 1$), and polyclonal antibody (pAb)¹ anti-laminin 5 have been described elsewhere (Rousselle et al., 1991; Marinkovich et al., 1992b). mAb NP32 to the NC-1 domain of type VII procollagen, and pAb made to the whole type VII procollagen molecule have been previously described (Sakai et al., 1986; Lunstrum et al., 1986). pAb anti-laminin 1 was purchased from Sigma Chimie (St. Quentin Fallavier, France).

Cell Culture and Immunopurification of Type VII Procollagen, Laminin 5, and Laminin 6

WISH cells derived from human amnion (certified cell line [CCL] 25; American Type Culture Collection, Rockville, MD) were grown in DME (GIBCO BRL, Gaithersburg, MD) supplemented with 10% FCS, 2 mM glutamine, 1 mM sodium pyruvate, 50 U/ml penicillin, 50 ng/ml streptomycin. Squamous carcinoma cells (SCC) 25 (CCL 25; American Type Culture Collection) were grown in 50% Ham's F-12 and 50% DME-F12 (GIBCO BRL), supplemented with 10% FCS 2 mM glutamine, hydrocortisone (0.4 $\mu\text{g}/\text{ml}$), and the same cocktail of antibiotics. Normal human keratinocytes (NHK) were isolated from neonatal foreskin (Rousselle et al., 1991) and grown in monolayer culture in synthetic serum-free medium (keratinocyte-SFM; GIBCO BRL).

For type VII procollagen purification, proteins secreted from confluent WISH monolayers were collected during a 24-h incubation in serum-free medium containing 100 ng/ml ascorbic acid as previously described (Lunstrum et al., 1986). For laminin 5 and laminin 6 purification, culture media were regularly harvested from confluent SCC25 culture. In all cases, after collection, unattached cells were removed by centrifugation (2,000 g/min), and EDTA, *N*-methylmaleimide, and phenylmethanesulfonyl fluoride were added to final concentration of 5 mM, 50 mM, and 50 mM, respectively. Spent culture media (500 ml) from WISH cells was passed sequentially over 25 ml of gelatin-Sepharose (Pharmacia Fine Chemicals, Piscataway, NJ) and 2 ml of NP32-Sepharose (Pharmacia Fine Chemicals). Spent culture media from SCC25 cells was passed sequentially over 25 ml of gelatin-Sepharose, and then over 5 ml of 545-Sepharose (anti-laminin $\beta 1$) for laminin 6 purification, and then over 10 ml of BM165-Sepharose (anti-laminin $\alpha 3$) for laminin 5 purification. Affinity chromatography on gelatin Sepharose was necessary and sufficient to remove fibronectin from the conditioned media as indicated by immunoblotting with a pAb against fibronectin (not shown). The antigens were eluted with 1M acetic acid, immediately neutralized, and fractions were monitored for absorbance at 280 nm. Pooled fractions were dialyzed against PBS and kept frozen at -20°C . Protein concentration was determined by microprotein assay using bicinchoninic acid (Pierce Interchim, Montlucon Cedex, France).

Extraction of NC1 (Type VII Collagen) and Laminin 5-Laminin 6 Complex from Amniotic Membranes

Extraction and purification of NC-1 (type VII collagen) and laminin 5-laminin 6 complex from collagenase extract of amniotic membrane have been described previously (Bachinger et al., 1990; Marinkovich et al., 1992b). Antibodies used during the affinity chromatography step include mAbs NP32, 545, and 6F12. Bound material was eluted with 1 M acetic acid and immediately neutralized. Eluted fractions were analyzed by electrophoresis, pooled, and dialyzed against PBS and kept at -20°C .

Extraction of the Laminin 5-NC1 Complex from Amniotic Membranes

The type VII collagen-NC1 preparation immunopurified from collagenase extracts of amniotic membranes was dialyzed against PBS and passed over an mAb 6F12 (anti-laminin $\beta 3$) affinity chromatography column. Bound material was eluted with 1 M acetic acid, immediately neutralized, and eluted fractions were ethanol precipitated and analyzed by Western blotting with polyclonal anti-laminin 5 and with mAb NP-32 (anti-VII NC1).

1. Abbreviations used in this paper: NHK, normal human keratinocytes; pAb, polyclonal antibody; SCC, squamous carcinoma cells.

Sources of Other Proteins

Collagens XII and XIV were extracted from bovine tendon (Aubert-Foucher et al., 1992) and kindly donated by Dr. E. Aubert-Foucher (Institut de Biologie et Chimie des Proteines [IBCP], Lyon, France). Laminin 1–nidogen complex and collagen IV extracted from the Engelbreth-Holm-Swarm tumor of the mouse were kindly provided by Dr. M. Aumailley (IBCP), and purchased from Collaborative Biomedical Products (TEBU, Perray en Yvelines, France), respectively. Bovine collagen I was a generous gift of Dr. F. Ruggiero (IBCP).

Collagenase and Pepsin Treatment of Type VII Collagen

Incubations with bacterial collagenase (10 $\mu\text{g/ml}$; CLSPA; Worthington Biochemical Co., Freehold, NJ) were for 8 h at 37°C in 50 mM Tris-HCl, pH 7.5, 500 mM NaCl, and 5 mM CaCl_2 . Pepsin digestion (100 $\mu\text{g/ml}$) was for 45 min at 4°C in 0.5 M acetic acid, 0.1 M NaCl. After enzymatic treatments, samples were dialyzed against PBS before interaction studies.

Protein Binding and Cell Adhesion Assays

The assay used was based on proteins immobilized on a plastic surface that were then exposed to varying concentrations of dissolved ligands followed by an enzyme immunoassay detection system. Multiwell plates (96 wells; Greiner, Dutscher, France) were incubated with solutions of the first ligand (10 $\mu\text{g/ml}$, 100 μl per well) dissolved in 20 mM Na_2CO_3 , pH 9.2, for 18 h at 4°C. The wells were then blocked with 2% milk powder dissolved in 0.05 M Tris-HCl, pH 7.2, 0.2 M NaCl (TBS). All further steps were carried out in 2% milk powder in TBS, 0.02% Tween 20, and washes between steps were with TBS, 0.02% Tween 20. Wells were incubated with soluble ligand (0.6–40 $\mu\text{g/ml}$) for 2 h at 22°C and exposed (1 h, 22°C) to a constant amount of diluted pAb against the soluble ligand followed by a typical enzyme-immunoassay reaction with peroxidase conjugate of goat anti-rabbit immunoglobulin (DAKOPATTS, Copenhagen, Denmark) as second antibody and 2,2'-azino-bis(3-ethylthiazoline-6-sulfonic acid) as the chromogenic substrate. Color yields were determined at 405 nm in an ELISA reader (MR5000; Dynatech Labs., Ltd., Guernsey, Channel Islands, UK). Negative control values, from wells in which the soluble ligands were omitted, were subtracted from the binding data. For inhibition assays, soluble ligands were incubated with inhibitors (0.2–60 $\mu\text{g/ml}$) for 6 h at 4°C before being added to the immobilized ligand. For experiments testing effects of cations, 1% BSA dissolved in TBS was used instead of 2% milk powder.

Measurements of cell adhesion to test protein-coated 96-well plates were performed using colorimetric detection assays as have been previously described (Aumailley et al., 1989). Adhesion of NHK to laminin 5 (0.5 $\mu\text{g/well}$) was prevented by preincubation of the substrate with various concentrations of mAb BM165 (0–10 $\mu\text{g/ml}$) as previously described (Rousselle and Aumailley, 1994).

Formation of the Laminin 5-NC1 (Type VII Collagen) Complex In Vitro and Immunoprecipitation

Preparations of purified laminin 5 (20 $\mu\text{g/ml}$), purified NC-1 type VII collagen (10 $\mu\text{g/ml}$), a mixture of laminin 5 (20 $\mu\text{g/ml}$) and NC-1 (10 $\mu\text{g/ml}$), and a mixture of laminin 5 (20 $\mu\text{g/ml}$) and thermal denatured NC-1 (10 $\mu\text{g/ml}$) were obtained after dilution in PBS to a final volume of 300 μl . To perform thermal denaturation of NC-1, the protein was heat denatured for 10 min at 80°C, immediately cooled and mixed with the laminin 5. All the samples were incubated 16 h at 4°C under gentle agitation. Each sample was then incubated 1 h at room temperature with a specific immunobead complex prepared as follows: 200 μl GammaBind G Sepharose (Pharmacia Biotech S.A., St. Quentin Yvelines, France) were incubated overnight with 2 μg of mAb BM165 specific to $\alpha 3$ chain of laminin 5, washed four times with PBS, and then incubated with each of the samples described above. The beads were collected by centrifugation, washed five times with PBS containing 0.05% Tween, and two times with PBS. The beads were resuspended in SDS-lysis buffer and boiled for 3 min. The released materials were analyzed by SDS-PAGE and immunoblotting.

Rotary Shadowing

Solutions of single proteins and of protein complexes were diluted to 10

$\mu\text{g/ml}$ with 0.2 M ammonium bicarbonate, and after addition of an equal volume of glycerol, the solutions were sprayed onto freshly cleaved mica sheets. The samples were immediately placed on the holder of a MED 010 evaporator (Balzers S.p.A., Milan, Italy) and rotary shadowing was carried out as previously described (Ruggiero et al., 1993). Observations of replicas were performed with a Philips CM120 microscope (Phillips Technologies, Cheshire, CT) at the Centre de Microscopie Electronique Appliquée à la Biologie et à la Géologie, Université Claude Bernard, Lyon, France).

Enbloc (Diffusion) Immunolabeling

Fresh neonatal foreskin was immunolabeled as previously described (Sakai and Keene, 1994). Briefly, fresh tissue was rinsed in PBS and then submerged overnight in mAb BM-165 (anti-laminin $\alpha 3$) diluted 1:5 in PBS or a mixture of BM-165 and rabbit pAb 3959 (anti-type VII collagen) at a ratio of 1:1:4 parts PBS. After an extensive rinse in PBS, the tissue was submerged overnight in secondary antibodies conjugated to different size gold particles (Amersham International, Little Chalfont, UK) as described in the legend for Fig. 9. Silver enhancement was performed on samples labeled with 1 nm gold. The samples were rinsed extensively in PBS and 0.1 M cacodylate buffer and then fixed, dehydrated, and embedded in Spurr's epoxy resin. 90-nm thick sections, which included the epithelium, papillary, and reticular dermis, were stained in uranyl acetate and lead citrate and examined using a Philips EM 410 transmission electron microscope.

Results

Laminin 5 was purified from the culture medium of SCC25 cells. The preparation was homogeneous as judged by SDS-PAGE (Fig. 1, lane 1), and contained the proteolytically processed $\alpha 3$ chain at 165 kD, the unprocessed $\beta 3$ chain (140 kD), and unprocessed $\gamma 2$ chain (155 kD), which

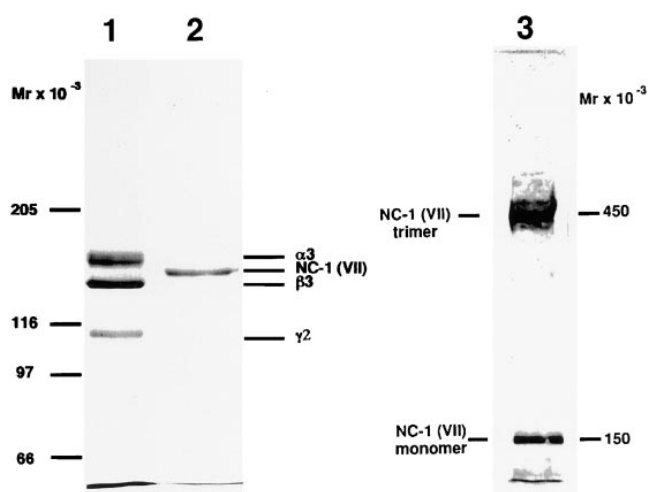


Figure 1. Electrophoretic analysis of immunoaffinity-purified laminin 5 from cell culture media and type VII collagen–NC-1 from collagenase extracts of amniotic membranes. 2 μg of materials affinity purified on mAb 6/F12 from SCC25 cell culture medium (lane 1), and 2 μg of materials affinity purified on mAb NP-32 from collagenase extracts of amniotic membranes (lane 2) were separated by SDS-PAGE after reduction on a 3–7.5% gradient polyacrylamide gel and visualized by Coomassie blue staining. Consistent with previous results, the reduced bands representing subunits of laminin 5 ($\alpha 3$, 165 kD; $\beta 3$, 140 kD; $\gamma 2$, 155 and 105 kD) and type VII collagen–NC-1 (150 kD). The electrophoretic migration position of purified NC-1 before disulfide-bond reduction is shown in lane 3. Migration positions of the molecular weight markers are indicated to the left of each gel.

fail to resolve on this gel, and the fully processed $\gamma 2$ chain (105 kD). Solid phase protein binding assays were first optimized for signal strength and specificity using the documented affinity of collagen IV for the laminin 1–nidogen complex (Aumailley et al., 1989; and data not shown). The assay demonstrated saturable binding of collagen IV to laminin–nidogen, four- to fivefold stronger than to collagen I. Under these conditions, laminin 5, collagens XII and XIV, and the NC-1 domain of collagen VII showed minimal binding.

To test the potential binding of laminin 5 to type VII collagen, type VII procollagen was partially purified from WISH cell (spontaneously transformed amniotic epithelial cell line) culture medium by immunoaffinity chromatography using NP-32–Sepharose. Solutions of 0–20 $\mu\text{g/ml}$ of laminin 5 were incubated with multiwell plates precoated with partially purified type VII procollagen, a clostridial collagenase digest of the type VII procollagen preparation, a pepsin digest of the type VII procollagen preparation, and purified type VII collagen NC-1. Saturable binding of type VII procollagen to laminin 5 was observed (Fig. 2), indicating that the two molecules did interact either directly or indirectly. To localize the binding activity within type VII procollagen to the triple-helical or nontriple-helical domains, the procollagen solution was digested separately with pepsin or collagenase before coating multi-chamber wells. Incubation with pepsin completely eliminated binding of laminin 5, while removal of the triple helix augmented the interaction. These observations suggest that the laminin 5–type VII collagen interaction occurs through either NC-1 or NC-2 of type VII procollagen. Since NC-2 is proteolytically removed during anchoring fibril formation (Lunstrum et al., 1987; Bruckner-Tuderman et al., 1994), if the interaction has physiological relevance, it is most likely an interaction of NC-1 with laminin 5. Therefore, NC-1 was purified from human amnion using established methods. The product was >95% pure as judged by SDS-PAGE (Fig. 1, lane 2) and by rotary shadowing (Bachinger et al., 1990) (not shown). The majority of the purified NC-1 remained trimeric as shown by electrophoretic separation without disulfide bond reduction (Fig. 1, lane 3). NC-1 showed comparable binding to laminin 5 as was seen with collagenase-digested type VII procollagen. The results show a direct interaction of laminin 5 with type VII collagen mediated by the large NC-1 domain. We believe that the difference in binding seen be-

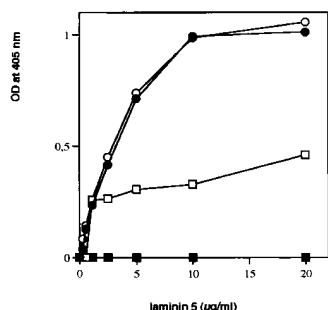


Figure 2. Binding of soluble laminin 5 to immobilized type VII procollagen. Microtiter wells were coated with 1 μg of type VII–NC-1 purified from amnion (●) or intact type VII procollagen immunopurified from WISH cell culture medium (□), or type VII procollagen digested with either bacterial collagenase (○) or pepsin (■). pAb to laminin 5 were used for detecting the amount of bound substrate.

tween pure NC-1 or collagenase-treated type VII procollagen and intact type VII procollagen is not significant as it most likely results from removal of collagenous contaminants (primarily type VI collagen aggregates) from the procollagen preparation by collagenase digestion thereby increasing the effective NC-1 concentration.

The specificity of this interaction was tested by evaluating the relative binding of type VII–NC-1, laminin 1–nidogen, and collagens I-, IV-, XII-, and XIV-coated wells to soluble laminin 5. As illustrated in Fig. 3 A, only type VII–NC-1 strongly bound laminin 5. The laminin 1–nidogen complex showed background affinity for laminin 5. When type VII–NC-1 was added in solution to laminin 5-coated wells (Fig. 3 B), again NC-1 bound most strongly to laminin 5. Under these conditions, NC-1 showed no affinity for laminin 1–nidogen, but type VII–NC-1 did bind collagen IV as previously shown (Burgeson et al., 1990), but only to about half the extent of the binding to laminin 5. Of particular interest, laminin 6 showed little binding to type VII–NC-1 relative to laminin 5 even though both laminins share the same $\alpha 3$ subunit (Champlaud et al., 1996). Consistent with this finding, NC-1 showed diminished binding to laminin 5 when complexed with laminin 6. However, the molar amounts of laminin 5 present in the complex solutions are $\sim 50\%$ the molar concentration of laminin 5 in solutions of laminin 5 alone. Since laminin 6 does not bind NC-1, the laminin 5–6 complex binds NC-1 with the same efficiency or only slightly diminished efficiency relative to laminin 5 alone. The binding of 5 μg of NC-1 to laminin 5 was inhibited by preincubation of NC-1 with increasing concentrations of laminin 5, but not by preincubation with laminin 1–nidogen (Fig. 4).

The dependence of binding upon the native conformation of either laminin 5 or NC-1 was tested by separately heating each of the ligands to 85°C for 10 min, and then rapidly cooling them before the solid phase binding assay.

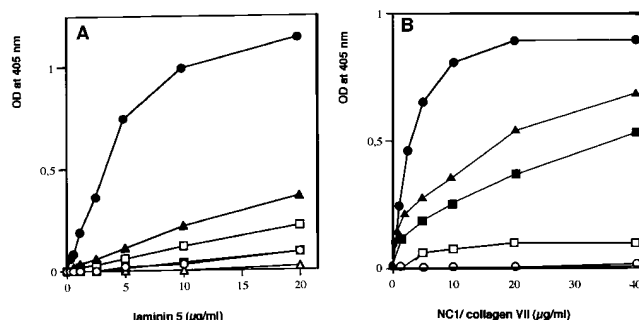


Figure 3. (A) Interaction of laminin 5 with various immobilized extracellular matrix components. Binding of soluble laminin 5 to immobilized collagen I (□), collagen IV (■), collagen XII (▲), collagen XIV (△), NC-1–VII (●), and laminin 1–nidogen complex (○) at a concentration of 1 μg per well was determined by a pAb in an enzyme-coupled second antibody reaction as described in Materials and Methods. (B) Interaction of type VII–NC-1 with type IV collagen and with various laminin isoforms. Binding of soluble type VII–NC-1 to immobilized collagen IV (■), laminin 1–nidogen complex (○), laminin 5 (●), laminin 5–laminin 6 complex (▲) at the concentration of 1 μg per well was determined by a pAb in an enzyme-coupled second antibody reaction as described in Materials and Methods.

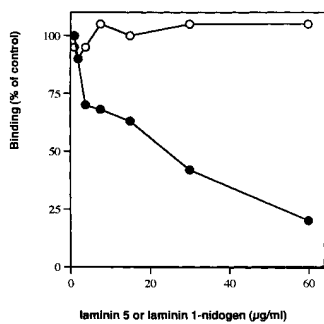


Figure 4. Inhibition of type VII-NC-1 binding to immobilized laminin 5 by preincubation with various competitors. The test system consisted of laminin 5 as the immobilized phase at the concentration of 1 µg per well, and a fixed amount of soluble type VII collagen-NC-1 (5 µg/ml). Type VII-NC-1 was incubated at 4°C for 8 h with varying concentration of laminin 5 (●) or laminin 1-nidogen complex (○) before testing on the insoluble ligand.

The denaturation products were not degraded during the heat treatment (data not shown). Denaturation of either ligand completely abolished binding (data not shown). The temperature at which the laminin 5 conformation necessary for binding to NC-1 is lost was then examined. The midpoint of the temperature where loss of active conformation occurs was ~65°C (Fig. 5 A). mAb BM165 inhibits the binding of epithelial cells to laminin 5 (Rousselle et al., 1991). Therefore, the ability of BM165 to disrupt the binding of laminin 5 to NC-1 was evaluated. As shown in Fig. 5 B, BM165 very effectively inhibits cell binding, but has no effect upon the laminin 5-NC-1 interaction. Divalent cations are also required for the binding of type VII collagen and laminin 5, as the addition of 10 mM EDTA completely inhibits the interaction (data not shown).

To be certain that the binding of laminin 5 to type VII

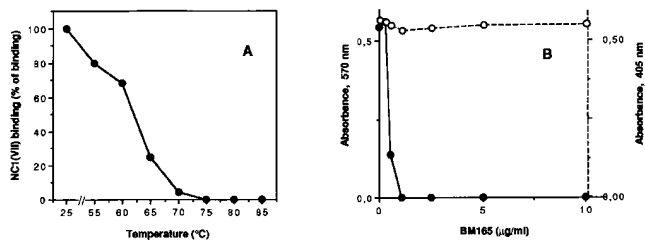


Figure 5. (A) Effect of heat denaturation on laminin 5 interaction with type VII-NC-1. Multiwell plates were coated with laminin 5 (●) at concentration of 1 µg before or after heating at the indicated temperatures. Soluble type VII-NC-1 was then added at concentration of 10 µg/ml and bound material was determined by a pAb in an enzyme-coupled second antibody reaction as described in Materials and Methods. (B) Effects of mAb BM165 on adhesion of normal human keratinocytes and on binding of type VII-NC-1 to laminin 5. Multiwell plates were coated with laminin 5 at 10 µg/ml. After saturation, the wells were incubated with indicated concentrations of mAb BM165 for 60 min before the cell adhesion experiment (●), or together with soluble type VII-NC-1 (10 µg/ml) for the binding assay (○). Extent of cell adhesion was measured with a colorimetric reaction at 570 nm. Each point represents the average of triplicate wells. In the ELISA assay, bound type VII-NC-1 was determined by a pAb in an enzyme-coupled second antibody reaction as described in Materials and Methods. Because the OD values in both systems were in the same range, the data were presented on a single graph.

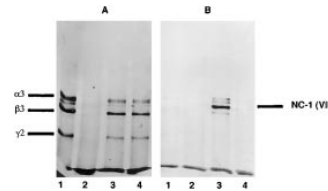


Figure 6. Western blot analysis of the complex of laminin 5 and NC-1 produced in solution. Laminin 5 alone (lanes A1 and B1), NC-1 alone (lanes A2 and B2), a mixture of laminin 5 and NC-1 (lanes A3 and B3), or a mixture of laminin 5 and heat denatured NC-1 (lanes A4 and B4) were immunoprecipitated with anti-α3 mAb (BM-165). The contents of the precipitates were separated by SDS-PAGE after disulfide bond reduction and visualized by Western analysis using (A) polyclonal anti-laminin 5 or (B) monoclonal anti-NC-1 (mAb NP-32).

collagen NC-1 was not artifactually influenced by the solid-phase conditions, purified laminin 5 was coincubated in solution with and without NC-1, and with heat-denatured NC-1, and then complexed and uncomplexed laminin 5 was isolated by immunoprecipitation using anti-laminin α3 (BM-165). The immunoprecipitation products were separated by SDS-PAGE after disulfide bond reduction. Laminin 5 and NC-1 were identified by Western blot analysis using polyclonal anti-laminin 5 and monoclonal anti-NC-1 (NP32). Immunoprecipitated laminin 5 in the absence of NC-1 gave the expected pattern by Western analysis (Fig. 6, lane A1) and did not react with anti-NC-1 (Fig. 6, lane B1). Immunoprecipitation of NC-1 in the absence of laminin 5 produced no product (Fig. 6, lanes A2 and B2). Both laminin 5 (Fig. 6, lane A3) and NC-1 (Fig. 6, lane B3) were immunoprecipitated from the mixture of the two, while denatured NC-1 did not precipitate with laminin 5 (Fig. 6, lanes A4 and B4). The results obtained using these conditions confirm the solid-phase binding data and again emphasize the importance of the native conformation of NC-1.

Images of NC-1-laminin 5 adduct produced in solution as described above were examined by transmission electron microscopy after rotary shadowing. Three types of images were seen (Fig. 7). One was identical to the images previously reported for laminin 5 (Rousselle et al., 1991). The second image appeared as a somewhat irregular spheroid, with its shortest diameter being ~41 nm (41 ± 9 nm; $n = 53$). Occasionally spheroids with contiguous single rod-like structures resembling collagen triple helices were seen. We interpret these structures to be trimeric NC-1 with some partial digestion products, consistent with the electrophoretic band pattern (Fig. 1). The third image seen appears as complexes of the first two forms. The images are consistent with visualization of the NC-1 and laminin 5 monomers, and of the complex of one laminin 5 molecule with each NC-1 fragment.

If laminin 5 and type VII collagen NC-1 interact in vivo, it may be possible to solubilize the complex if the tissue is solubilized under nondenaturing conditions. NC-1 was prepared as a collagenase digest of amnion and partially purified by NP32 (anti-NC-1) immunoaffinity chromatography. If a complex exists in this mixture, it should be retained by 6F12 (anti-laminin β3) immunoaffinity columns. Thus, partially purified NC-1 eluted from the anti-NC-1 column was applied to a column containing anti-β3. The flow-through (Fig. 8, lanes 2 and 6, *nonbound*) is Western

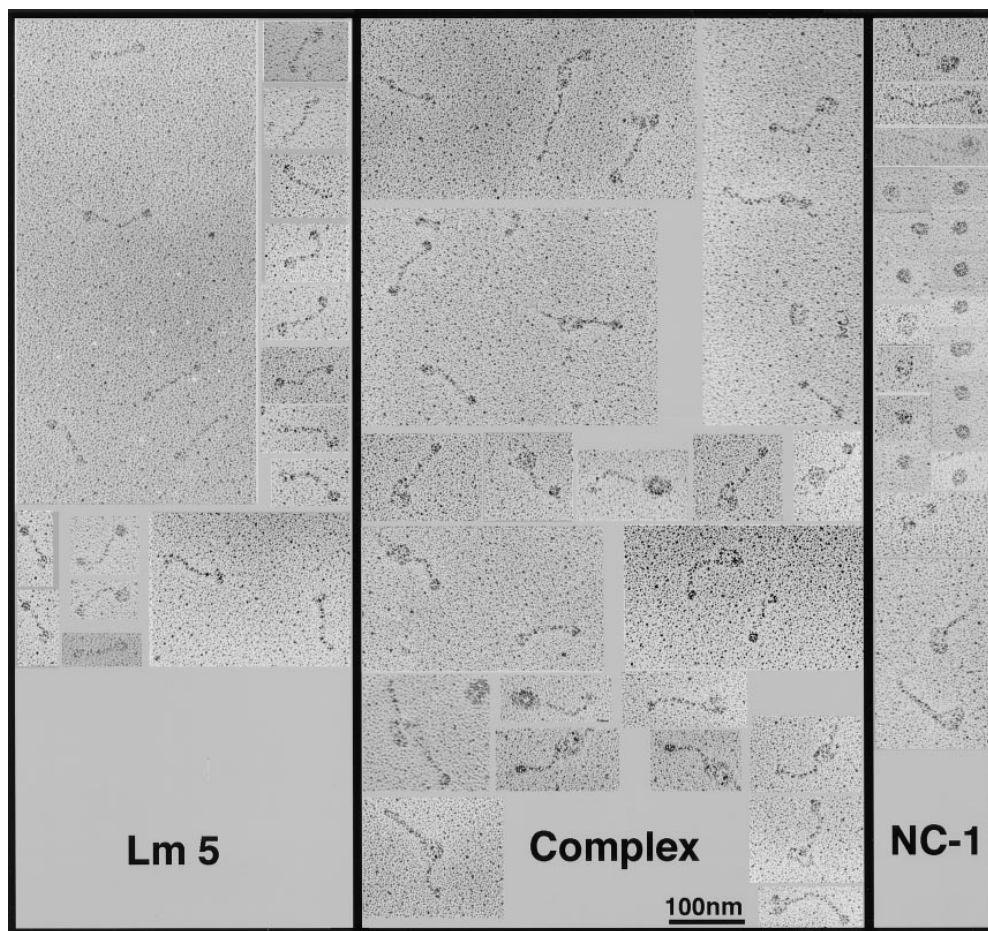


Figure 7. Visualization of the laminin 5–NC-1 complex formed in solution by transmission electron microscopy after rotary shadowing. Collagen VII–NC-1, laminin 5, and a mixture of both molecules were dialyzed against 0.2 M ammonium bicarbonate, diluted 1:1 with glycerol, and rotary shadowed. (*Lm5*) The images visualized are characteristic of laminin 5 molecules. The 107-nm molecules appear as extended rods with globular knobs at each terminus. (*NC1*) Under the conditions used to maintain the stability of the complex, NC-1 appears as an asymmetrical spheroid, ~41 nm in narrowest diameter. Due to incomplete digestion, helical domains of type VII collagen can be seen on some NC-1 molecules. (*Complex*) Images of the laminin 5–type VII collagen–NC-1 complex preparation contains uncomplexed laminin 5 and NC-1, and images that appear as a complex of one laminin 5 molecule with one NC-1 molecule, in which the long arm may correspond to laminin 5 and the big globular domain to NC-1.

blotted by anti-NC-1 (Fig. 8, lane 6) but not by anti-laminin 5 (Fig. 8, lane 2) indicating that the nonbound fraction contains only NC-1 and is equivalent to the NC-1 used in the binding studies described above. If complexed laminin 5 and NC-1 are present in the extract, they should be bound by the anti- $\beta 3$ column. As seen in Fig. 8, lanes 3 and

4, the materials eluted from the anti- $\beta 3$ column are Western blotted by both anti-laminin 5 (Fig. 8, lane 3) and anti-NC-1 (Fig. 8, lane 4) indicating that the bound fraction contains both NC-1 and laminin 5.

In previous studies, Keene et al. (1987) showed that the anchoring fibrils in the papillary dermis of skin terminated in junctions, the anchoring plaques. These plaques showed the inclusion of type IV collagen by ultrastructural immunolocalization. Studies conducted at the same time, but not included in the publication, showed the absence of anchoring plaque labeling with mAbs to laminin $\alpha 1$, but minor labeling with polyclonal anti-laminin antibodies. At that time we were unsure how to interpret the result. We have repeated the immunolocalization of laminin 5 in skin with particular attention to the anchoring plaques. As shown in Fig. 9, *A–D*, the plaques are clearly recognized by anti- $\alpha 3$ antibodies. Further, in double-labeling experiments, the reactivity of monoclonal anti- $\alpha 3$ is colocalized to the plaques with the reactivity of polyclonal anti-NC-1 (Fig. 9, *E* and *F*).

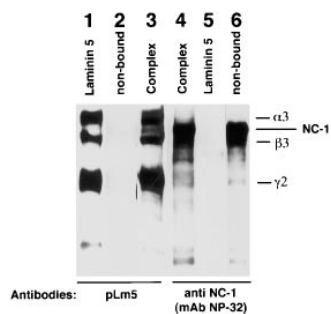


Figure 8. Western blot analysis of the laminin 5–type VII collagen–NC-1 complex immunoaffinity purified from collagenase extracts of amniotic membranes. Type VII collagen–NC-1 was first affinity purified on mAb NP-32 from collagenase extracts of amniotic membranes. Eluted fractions of NC-1 were pooled, dialyzed against PBS, and passed over a mAb

6F12 affinity chromatography column. Bound materials were immunoblotted with polyclonal anti-laminin 5 (*pLm5*; lane 3), and anti-NC-1 (*mAb NP-32*, lane 4). The bound material shows the pattern obtained for purified laminin 5 (lane 1), which does not contain NC-1 (lane 2) and for purified NC-1 (not shown, but identical to lane 6). The nonbound fraction contains NC-1 only (lane 6) but no laminin 5 (lane 2).

Discussion

The data presented here suggest that at least two mechanisms promote epithelial–stromal attachment. The first is the previously suggested (Champlaud et al., 1996) binding

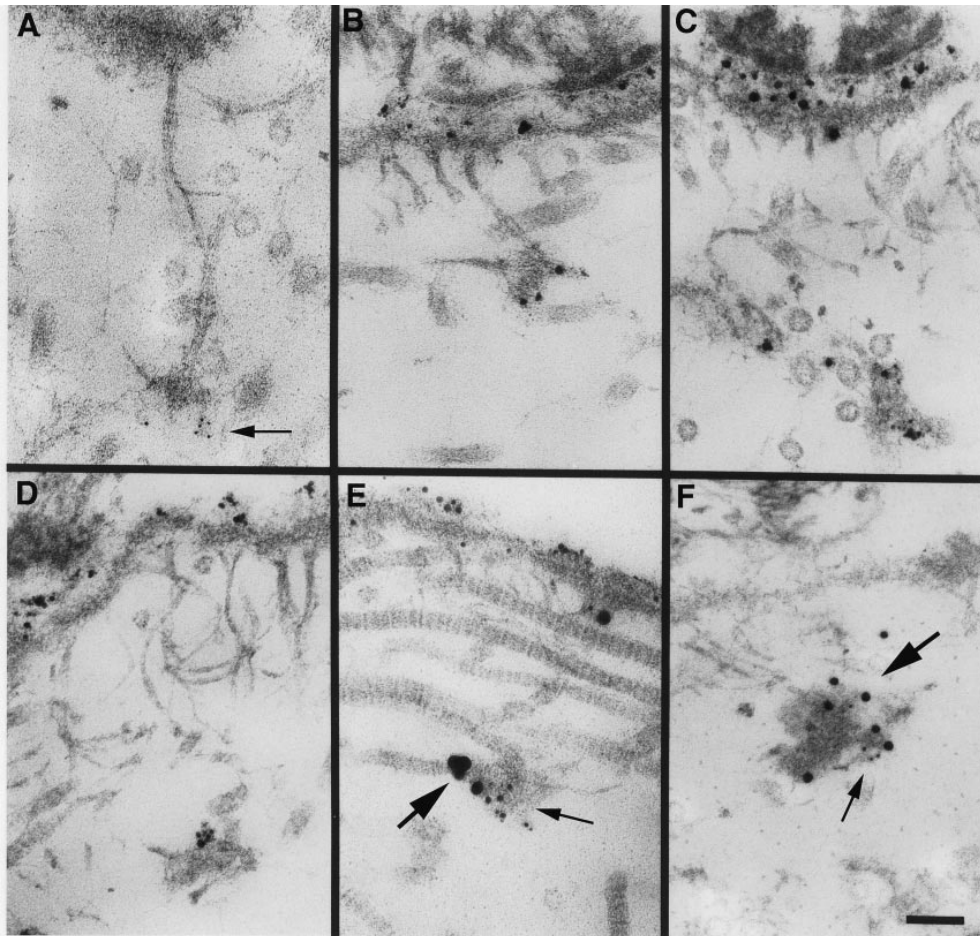


Figure 9. Localization of laminin 5 to anchoring plaques. Laminin 5 is localized to anchoring plaques in human skin using mAb BM165 and 5-nm (A, arrow) or 1 nm silver-enhanced colloidal gold (B–D). Labeling of the lamina lucida and anchoring plaques is intense using silver-enhanced 1 nm colloidal gold in comparison to 5 nm colloidal gold. Using a mixture of mAb BM165 (anti-laminin $\alpha 3$) and pAb 3959 (anti-type VII collagen NC-1), laminin 5 is seen to colocalize with type VII collagen in anchoring plaques (E, 1-nm silver-enhanced particles specific for mAb BM165 [small arrow] and 10-nm silver-enhanced particles are specific for pAb 3959 [large arrow]; F, 5-nm particles specific for mAb BM165 [small arrow] and 15-nm particles specific for pAb 3959 [large arrow]. Bar, 100 nm.

of the laminin 5–6/7 complex to lamina densa components of the epithelial basement membrane. That model suggests that the presence of $\gamma 1$ chains in laminin 6 or 7 provides a nidogen binding site, and the VI domains of both $\beta 1/\beta 2$ and $\gamma 1$ allow for assembly of the complex with the laminin 1 network. The model also suggests that null phenotypes resulting from mutations in laminin $\alpha 3$ would result in significant destabilization of epithelial–stromal adhesion, as these mutations would be null for laminin 5, 6, and 7. However, the model also predicts that mutations in $\beta 3$ or $\gamma 2$ would only affect the presence of laminin 5, and not either laminin 6 or 7. Therefore, since both laminins 6 and 7 contain an $\alpha 3$ chain and promote adhesion of keratinocytes, laminin 6 or 7 should at least partially substitute for the absence of laminin 5. This prediction is incorrect, as mutations in genes encoding $\alpha 3$, $\beta 3$, or $\gamma 2$ all result in Herlitz’s junctional epidermolysis bullosa. Therefore, a second and equally (or more) important mechanism by which laminin 5 interacts with a basement membrane or stromal component must exist.

The present study indicates a second mechanism involving the binding of monomeric laminin 5 to type VII collagen NC-1 domain. These findings suggest a model (Fig. 10) that predicts that monomeric laminin 5 is the primary bridge between hemidesmosomal $\alpha 6\beta 4$ and type VII collagen, and the laminin 5–6/7 complex is present within the interhemidesmosomal spaces. This model is consistent with the following observations: (a) Monomeric laminin 5

binds integrin $\alpha 6\beta 4$ (Niessen et al., 1994) and type VII collagen strongly, but has minimal affinity for other basement membrane components; (b) approximately half of the extractable laminin 5 is complexed with laminins 6 and 7 (Champlaud et al., 1996); (c) the laminin 5–6/7 complex binds collagen IV strongly through nidogen (Mayer et al., 1995); (d) laminin 5 is uniformly distributed along the epithelial basement membrane (Rousselle et al., 1991), while anchoring fibrils (Ellison and Garrod, 1984) and type VII collagen (Sakai et al., 1986) are concentrated under hemidesmosomes. This model is also consistent with (e) the ultrastructure of the anchoring complex and the spatial orientation of the involved molecules (Ellison and Garrod, 1984); and (f) with the phenotypes of null mutations in BPAG2 (McGrath et al., 1995; Jonkman et al., 1995), integrin $\beta 4$ (Vidal et al., 1995; Dowling et al., 1996; van der Neut et al., 1996) or $\alpha 6$ (Georges-Labouesse et al., 1996), laminin 5 chains, and type VII collagen (Uitto et al., 1994; Bruckner-Tuderman, 1994), as the absence of laminin 5 results in the lethal Herlitz’s form of epidermolysis bullosa. The absence of type VII collagen results in severe generalized dystrophic epidermolysis bullosa, and the lack of $\alpha 6\beta 4$ integrin causes lethal blistering in the mouse (Dowling et al., 1996; van der Neut et al., 1996; Georges-Labouesse et al., 1996) although the absence of integrin $\alpha 6\beta 4$ in humans appears to be less severe (Vidal et al., 1995). Absent mouse BPAG1 (Guo et al., 1995), BPAG2 (McGrath et al., 1995, 1996), or plectin (Gache et al., 1996;

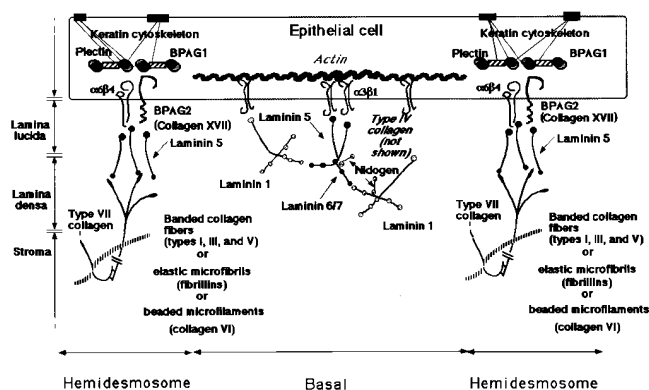


Figure 10. Model for the presence of monomeric laminin 5 and complexed laminin 5 within an epithelial-stromal junction. The cartoon depicts monomeric laminin 5 as the bridge between hemidesmosomal integrin $\alpha 6 \beta 4$ and the type VII collagen NC-1 domain. The tight binding of laminin 5 to $\alpha 6 \beta 4$ and to type VII collagen provides the primary resistance to frictional forces. The laminin 5-6/7 complex is shown within the basement membrane between hemidesmosomes, bound by integrin $\alpha 3 \beta 1$ where it potentially functions to maintain basement membrane stability and contact with the epithelial basolateral surface.

McLean et al., 1996; Smith et al., 1996) results in relatively mild blistering, suggesting that their roles in epidermal adhesion can be partially compensated by other hemidesmosomal components.

The precise role of laminin 5-6/7 complex-nidogen binding is now less clear. As the presence of laminins 6 or 7 cannot substitute for laminin 5 in stabilizing epithelial attachment to the basement membrane, the characteristics of monomeric laminin 5 best fit its direct role in the anchoring complex. Since monomeric laminin 5 has no known mechanism for binding to components of the lamina densa other than to type VII collagen, it is difficult to imagine how it might function outside the hemidesmosome, given our current level of understanding. It is likely that the laminin 5 detected within the basement membrane between hemidesmosomes is the laminin 5-6/7 complex. Within this interhemidesmosomal space it is capable of associations with the basolateral plasma membrane through integrin $\alpha 3 \beta 1$ (Delwel et al., 1994; DiPersio et al., 1995). Its presence there may stabilize the association of the basement membrane with the basolateral keratinocyte surface. The laminin 5-6/7 complex may play additional important roles in basement membrane assembly and stability.

We have previously shown that NC-1 can bind collagen IV and, to a considerably lesser extent, laminin 1 (Burgesson et al., 1990). As shown in Fig. 3 B, in this assay the relative binding of NC-1 to the laminin 1-nidogen complex is minimal, the binding to type IV collagen is moderate, and NC-1 binds laminin 5 most strongly. The failure of NC-1 to bind laminin 6 was informative. As laminin 5 and laminin 6 share the $\alpha 3$ chain, this observation suggests that either $\beta 3$ or $\gamma 2$ mediate the interaction with type VII collagen. Binding to $\beta 3$ or $\gamma 2$ could occur within the α -helical long arm domains I/II, or within the short arms. In this regard, the measurements of conformational stability of the NC-1 binding domain are informative. NC-1 binding is lost if

laminin 5 is subjected to temperatures above 65°C . As this is well below the midpoint transition temperature for denaturation of the coiled-coil long arm (72°C) (Rousselle et al., 1995), the data suggest that binding of laminin 5 to NC-1 occurs within the $\beta 3$ or $\gamma 2$ short arms. Of interest, recently a case of junctional epidermolysis bullosa has been described that is due to a homozygous inframe exon skip resulting in the deletion of part of domains III and VI of the $\gamma 2$ chain (Pulkkinen et al., 1994a). The processing cleavage site is retained within domain III, but lies only six residues after the deletion, and therefore the authors speculate that the phenotype may result from failure of $\gamma 2$ chain processing. If only processed $\gamma 2$ interacts with NC-1, this would be consistent with the observed phenotype. The laminin 5 used in this present study consists of a mixture of molecules containing both full-length, unprocessed $\gamma 2$ (155 kD), and processed 105 kD $\gamma 2$. Therefore, we are unable to determine if $\gamma 2$ processing influences NC-1 binding.

The rotary-shadowed images of the NC-1-laminin 5 complex are consistent with the proposed interaction of NC-1 with an end of laminin 5, but not with the coiled-coil domain. We were unable to unambiguously identify the globule at the extended end of laminin 5 within the complex as the $\alpha 3\text{G}$ domain since the monomeric laminin 5 in the preparation is very heterogeneous at one end, consistent with the electrophoretic pattern indicating the presence of a mixture of processing intermediates, and consistent with the observed heterogeneity being at the NH_2 -terminus. However, our impression is that the globule seen at the free end of laminin 5 in the complex is less heterogeneous than seen for the NH_2 terminus of monomeric laminin 5, and is therefore likely to be the G domain. It is interesting that under the lower glycerol concentrations used in the spreading buffer NC-1 appears as a spheroid rather than in an extended trident shape as we have previously reported (Lunstrum et al., 1986). It is possible that the observed globular structure is due to noncovalent intrachain interactions of the von Willebrand factor domains (Fretto et al., 1986). The lower glycerol concentrations were needed to visualize the complex, suggesting either that glycerol at high concentration destabilizes the complex, or that spreading of the short arms disrupts the complex. It is also surprising that all the complexes visualized appeared to be between a single molecule of trimeric NC-1, and a single laminin 5 molecule. Since NC-1 is a homotrimer, we expected that up to three laminin 5 molecules might be bound by each NC-1 domain.

These studies do not directly address the subdomain(s) within NC-1 to which laminin 5 binds. However, type XII and XIV collagens were selected as candidates for the solid-phase binding studies since they share both fibronectin type III repeats and von Willebrand factor A repeats with NC-1. Therefore, since laminin 5 does not bind either of these collagens, the subdomain responsible for binding must contain sequences that are unique to type VII collagen.

If laminin 5 binds type VII collagen NC-1 domains with high affinity, then laminin 5 could mediate the interaction of anchoring fibrils and would be expected within anchoring plaques. Previous attempts to localize laminin to the anchoring plaques with monoclonal anti- $\alpha 1$ or polyclonal anti-laminin 1 were ambiguous. The present study shows

the localization of anti- $\alpha 3$ reactivity to the anchoring plaques, colocalized with anti-type VII collagen NC-1. This strongly suggests that the tight binding of NC-1 and laminin 5 seen in vitro is physiologically significant.

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