NC1 Domain of Type VII Collagen Binds to the β3 Chain of Laminin 5 Via a Unique Subdomain Within the Fibronectin-Like Repeats

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Type VII collagen, the major component of anchoring fibrils, consists of a central collagenous triple-helical domain flanked by two noncollagenous, globular domains, NC1 and NC2. Approximately 50% of the molecular mass of the molecule is consumed by the NC1 domain. We previously demonstrated that NC1 binds to various extracellular matrix components including a complex of laminin 5 and laminin 6 (Chen *et al*, 1997a). In this study, we examined the interaction of NC1 with laminin 5 (a component of anchoring filaments). Both authentic and purified recombinant NC1 bound to human and rat laminin 5 as measured by enzyme-linked immunosorbant assay and by binding of ^{125}I-radiolabeled NC1 to laminin 5-coated wells, but not to laminin 1 or albumin. NC1 bound predominantly to the β3 chain of laminin 5, but also to the γ2 chain when examined by a protein overlay assay. The binding of ^{125}I-NC1 to laminin 5 was inhibited by a 50-fold excess of unlabeled NC1 or de-glycosylated NC1, as well as a polyclonal antibody to laminin 5 or a monoclonal antibody to the β3 chain. In contrast, the NC1–laminin 5 interaction was not affected by a monoclonal antibody to the α3 chain. Using NC1 deletion mutant recombinant proteins, a 285 AA (residues 760–1045) subdomain of NC1 was identified as the binding site for laminin 5. IgG from an epidermolysis bullosa acquisita serum containing autoantibodies to epitopes within NC1 that colocalized with the laminin 5 binding site inhibited the binding of NC1 to laminin 5. Thus, perturbation of the NC1–laminin 5 interaction may contribute to the pathogenesis of epidermolysis bullosa acquisita. *Key words: autoantibodies/epidermolysis bullosa/extracellular matrix components. J Invest Dermatol 112:177–183, 1999*

The epidermis and dermis of human skin are separated by a complex zone called the dermal–epidermal junction (DEJ) that contains a basement membrane (Briggaman and Wheeler, 1975). Many DEJ components are synthesized by keratinocytes within the epidermis, including type VII collagen (Stanley *et al*, 1985; Woodley *et al*, 1985). When viewed by electron microscopy, discrete structures within the DEJ are identified. These include hemidesmosomes associated with the plasma membrane of the basal keratinocytes apposed to the DEJ; a 35–50 nm lucent space called the lamina lucida that contains delicate filaments called anchoring filaments; a 35–50 nm electron dense zone called the lamina densa and wheat-stack shaped structures called anchoring fibrils that emanate perpendicularly beneath the lamina densa (Briggaman and Wheeler, 1975). The main component of anchoring fibrils is type VII collagen (Sakai *et al*, 1986; Keene *et al*, 1987), whereas the main component of anchoring filaments is laminin 5 (formerly known as kalinin, nicein, and epiligrin) (Rousselle *et al*, 1991).

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Abbreviations: FNIII, fibronectin type III-like repeats; VWF-A, A domain of von-Willebrand factor.

Recently, using unfixed frozen skin, this traditional organization of the DEJ has been challenged (Keene and McDonald, 1993; Kivirikko, 1993). This neatly organized zone viewed by electron microscopy may be an artifact of the fixation process, and the true DEJ organization may allow much closer apposition of various DEJ components. In the traditional view of the DEJ, anchoring filaments are separated from anchoring fibrils by the lamina densa, a structure that is rich in type IV collagen and proteoglycans (Stanley *et al*, 1982). It may be, however, that in reality there is apposition between anchoring filaments and anchoring fibrils, both structures that are thought to be critical for maintenance of epidermal–dermal adherence. Gene defects in components of either structure, namely laminin 5 or type VII collagen, are associated with severe incurable blistering disorders of the skin (Uitto *et al*, 1994).

Laminin 5 consists of three nonidentical chains: α3 (200 kDa), β3 (140 kDa), and γ2 (155 kDa), each encoded by a distinct gene (Kallunki *et al*, 1992; Gerecke *et al*, 1994; Ryan *et al*, 1994). Laminin 5 supports epithelial cell adhesion by interacting with the α6β4 and α3β1 integrin receptors on basal keratinocytes (Carter *et al*, 1990, 1991; Niessen *et al*, 1994). It also forms complexes with other laminin isoforms such as laminin 6 and laminin 7 (Champliaud *et al*, 1996). Therefore, laminin 5 has both matrix and cell binding capabilities. Autoantibodies to the α3 chain are associated with a severe autoimmune blistering disease of the mucosa called cicatricial pemphigoid (Domloge-Hultsch *et al*, 1992; Kirtschig *et al*, 1995; Chan *et al*, 1997). Defects in the genes

encoding for laminin 5 result in a severe inherited blistering disease of the skin called junctional epidermolysis bullosa (Aberdam *et al*, 1994; Pulkkinen *et al*, 1994a, b). The cleavage plane of junctional epidermolysis bullosa blisters is through the lamina lucida where anchoring filaments are localized. Because junctional epidermolysis bullosa patients also have perturbations in hemidesmosomes associated with a paucity of laminin 5 and anchoring filaments, there is direct evidence that anchoring filaments and laminin 5 may have a role in the initiation or maintenance of hemidesmosome structures, possibly by interactions with the $\alpha 6\beta 4$ and $\alpha 3\beta 1$ integrins (Baker *et al*, 1996b).

Type VII collagen is composed of three identical alpha chains encoded by chromosome 3. Each chain consists of a central helical domain flanked by a large amino-terminal noncollagenous domain (NC1) and a smaller carboxyl-terminal noncollagenous domain (NC2) (Lunstrum *et al*, 1986, 1987; Burgeson *et al*, 1990; Parente *et al*, 1991; Christiano *et al*, 1992, 1994). The larger NC1 domain (Mr = 145,000) contains motifs that are similar to fibronectin type III-like repeats (FNIII), the A domain of von-Willebrand factor (VWF-A), and cartilage matrix protein, all elements with known adhesive properties (Christiano *et al*, 1992, 1994). Therefore, it is conceivable that NC1 serves to mediate interactions between type VII collagen and other matrix components. Mutations in the gene encoding type VII collagen result in a family of severe blistering skin diseases called dystrophic epidermolysis bullosa, characterized by a paucity of normal anchoring fibrils (Uitto and Christiano, 1993, 1994). Autoantibodies to type VII collagen are associated with an acquired autoimmune form of EB, epidermolysis bullosa acquisita (EBA) (Woodley *et al*, 1984, 1988).

Previously, using full-length recombinant NC1, we demonstrated that its structural and functional properties were identical to authentic NC1 (Chen *et al*, 1997a). We (Chen *et al*, 1997a) and others (Burgeson *et al*, 1990) have shown that NC1 binds to fibronectin, type I collagen, and type IV collagen. We also demonstrated that NC1 binds to a complex of laminin 5 and laminin 6 (Chen *et al*, 1997a). In the study described herein, we focused on the interaction between NC1 and purified laminin 5. Using three independent binding assays, combined with the use of deletional mutants within NC1, we demonstrate that NC1 binds to laminin 5 and that this binding is mediated predominantly by the $\beta 3$ chain of laminin 5 and selected FN motifs within NC1. These data, taken together with the clinical observations that patients with gene defects in either laminin 5 or type VII collagen exhibit incurable blistering disorders of the skin, suggest that the interaction between laminin 5 and type VII collagen may be very important for the maintenance of epidermal–dermal adherence in human skin. In addition, we demonstrated that autoantibodies against the NC1 domain of type VII collagen in the serum of a patient with EBA inhibit the binding of NC1 to laminin 5, whereas IgG from normal human serum does not inhibit this interaction. Therefore, if the NC1–laminin 5 interaction is important for epidermal–dermal adherence, the presence of EBA autoantibodies may be detrimental to this adherence.

MATERIALS AND METHODS

Materials Mouse laminin 1 was prepared from the EHS tumor as described (Woodley *et al*, 1983) and human laminin 1 was obtained from Gibco-BRL (Gaithersburg, MD). Human laminin 5 was purified from keratinocyte medium by antibody affinity chromatography using MoAb K140-sepharose (Marinkovich *et al*, 1992) or prepared from cultures of human keratinocytes by sequential extraction as described previously (Carter *et al*, 1991). Rat laminin 5 was isolated from the medium of 804G cells by column chromatography and was provided by Desmos (San Diego, CA) (Baker *et al*, 1996a).

The recombinant NC1 domain of human type VII collagen was purified from serum-free culture medium of human 293 cells stably transfected with NC1 cDNA as described (Chen *et al*, 1997a). Purification of the authentic NC1 from collagenase digests of human amnion was accomplished as described previously using affinity chromatography with coupled monoclonal antibodies NP185 and NP32 (Bachinger *et al*, 1990).

Antibodies The experimental antibodies used in this study include: (i) a murine monoclonal antibody, LH 7.2, which specifically recognizes the NC1 domain of type VII collagen (Lapiere *et al*, 1994); (ii) a murine monoclonal antibody, K140, which specifically recognizes the $\beta 3$ chain of human laminin 5 (Marinkovich *et al*, 1992); (iii) a murine monoclonal antibody, BM165, which specifically recognizes the $\alpha 3$ chain of human laminin 5 (6); (iv) a rabbit polyclonal antibody, J-18, which recognizes both rat and human laminin 5 (Langhofer *et al*, 1993); (v) a polyclonal antibody to the $\beta 3$ chain and a polyclonal antibody to the $\gamma 2$ chain of human laminin 5 (generously supplied by Dr. Warren Hoeffler, Stanford University); and (vi) sera obtained from a patient with EBA and from a healthy volunteer, who served as a control. This EBA serum demonstrated strong reactivity with immobilized NC1 in an enzyme-linked immunosorbant assay and by immunoblot analysis (Chen *et al*, 1997b). The IgG fractions from these two sources were partially purified by affinity chromatography using Protein G Sepharose 4 (Pharmacia, Piscataway, NJ) according to the manufacturer's instructions. The IgG fractions, after dialysis against phosphate-buffered saline, were concentrated by ultrafiltration to 100 mg per ml (Amicon, Beverly, MA) and stored at –20°C.

Proteins binding assays

Enzyme-linked immunosorbant assay Binding of soluble NC1 to immobilized ligands followed by a colorimetric enzyme-linked antibody reaction was performed as previously described (Woodley *et al*, 1983; Chen *et al*, 1997a). Multiwell plates (96 wells, Dynatech, Chantilly, VA) were coated overnight with purified laminin isoforms (1 μg) in 100 mM carbonate buffer, pH 9.3. The wells were then blocked with 1% bovine serum albumin (BSA) in phosphate-buffered saline, 0.05% Tween-20 (PBST). Coated wells were subsequently incubated with purified recombinant NC1 at a concentration of 20 μg per ml overnight at 4°C. The binding of NC1 to each laminin isoform was detected with a monoclonal anti-type VII collagen antibody, LH 7.2 (Sigma, St. Louis, MO), at a dilution of 1:300, followed by incubation with alkaline phosphatase-conjugated goat anti-mouse IgG (1:400) (Organon Teknika-Cappel, Durham, NC). The development of the colorimetric reaction using p-nitro-phenylphosphate as a substrate (Bio-Rad, Melville, NY) was measured by reading the absorbance of the product at 405 nm (Labsystems Multiskan Multisoft, Finland).

Radiolabeled protein binding assay Purified recombinant NC1 was labeled with ^{125}I by iodination with IODO-BEADS according to the manufacturer's instructions (PIERCE, Rockford, IL). ^{125}I Labeled NC1 was then separated from free iodine by chromatography over a D-Salt Excellulose column (PIERCE). The radiolabeled NC1 preparations were characterized for purity by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) on a 6% slab gel. Dried gels were subjected to autoradiography. The iodinated NC1 was stored in aliquots at –70°C.

Ninety six well plates were coated with 1 μg of albumin or various purified laminin isoforms in 100 mM carbonate buffer, pH 9.3 overnight at 4°C. The wells were then blocked with 1% albumin in PBST for 2 h. ^{125}I-labeled NC1 was added to each well in 100 μl PBST at concentrations between 10 and 150 ng and incubated for 4 h at 4°C. Unbound protein was removed and the wells washed three times with PBST. Radiolabeled NC1 bound to the substratum of each well was detected by solubilizing the proteins by the addition of 100 μl of 2% SDS, 25 mM Tris-HCl, 150 mM NaCl, 5 mM ethylenediamine tetraacetic acid to each well, incubating the wells for 2 h at room temperature and counting the bound radioactivity in scintillation fluid with a gamma-counter (Beckman).

Western blots labeled with ^{125}I-NC1 Protein-to-protein affinity by western blot analysis was performed as previously described (Woodley *et al*, 1987). Briefly, 2 μg of human or rat laminin 5, human or mouse laminin 1, BSA, fibronectin, and type IV collagen were reduced with 2 mM dithiothreitol followed by SDS-PAGE and electrophoretic transfer to nitrocellulose. The blots were blocked with PBST/3% BSA overnight at 4°C, washed three times in PBST, and incubated with ≈500,000 cpm per ml of ^{125}I-labeled NC1 in PBST containing 1% BSA for 4 h at room temperature. After the incubation, the blots were washed extensively in PBST, air-dried, and subjected to autoradiography.

For the EBA serum inhibition assay, ^{125}I-labeled NC1 was preincubated with IgG from EBA serum or control IgG from normal human serum (100 μg per ml) for 2 h at room temperature before being added to the nitrocellulose strips containing laminin 5.

Construction and expression of deletion recombinant NC1 proteins The eukaryotic expression vector containing the full-length human NC1 domain was described previously (Chen *et al*, 1997a). In this study, we used the expression vector containing the full-length NC1

cDNA in the TA vector (Invitrogen, San Diego, CA) as a template to prepare two additional deletional constructs by digesting the template with various restriction enzymes, including Xho I and Bst XI. All constructs were then inserted into the Hind III/Xba I-digested eukaryotic expression vector pRC/CMV (Invitrogen). cDNA constructs were transfected into the human embryonic kidney cell line 293 (ATCC, Rockville, MD). G418 resistant cell clones were then selected and screened for expression of the deletion proteins by immunoblot analysis with a mouse monoclonal antibody, LH 7.2 (1:200) (Sigma), against the NC1 domain of type VII collagen. Secreted recombinant proteins were collected from conditioned medium from confluent cell cultures grown for 2 d in serum-free culture medium and purified as described previously (Chen *et al*, 1997a). These recombinant proteins include full-length NC1 (residues 1–1253), and two diminutive NC1 proteins created by deletions in the NC1 cDNA: NC1-S1 (residues 1–1045) and NC1-S2 (residues 1–760).

Bacterial fusion proteins corresponding to discrete segments within the NC1 domain of type VII collagen were developed and purified by a glutathione-Sepharose 4B column (Pharmacia, Uppsala, Sweden) as described (Lapiere *et al*, 1993). These fusion proteins included FP1 (residues 201–602), FP3 (residues 595–826), FP7 (residues 814–1028), and FP8 (residues 1022–1253).

RESULTS

Binding of NC1 to laminin 5 immobilized on plastic We previously demonstrated that recombinant and authentic NC1 interact with a laminin 5/6 complex (Chen *et al*, 1997a). In this study, we sought to determine if NC1 could bind directly to laminin 5. We examined the binding of both recombinant NC1 and authentic NC1 to immobilized laminin 5 by enzyme-linked immunosorbant assay. As shown in **Fig 1**(***A***), both recombinant NC1 and authentic NC1 bound to human laminin 5 or rat laminin 5. In contrast, little or no binding occurred to human laminin 1 or mouse laminin 1. There was no significant difference in the binding affinity for laminin 5 when authentic and recombinant NC1 were compared. Both rat laminin 5 and human laminin 5 bound to NC1 with comparable potency.

The binding of ^{125}I-NC1 to immobilized laminin 5 was also examined with a second type of solid phase assay. As shown in **Fig 1**(***B***), ^{125}I-labeled NC1 bound to both human laminin 5 and rat laminin 5 in a concentration-dependent manner. The binding of NC1 to control wells coated with BSA was negligible. The level of binding to laminin 1 and type V collagen was equivalent to BSA (data not shown).

We next examined the ability of unlabeled NC1 and anti-laminin 5 antibodies to inhibit specifically the interaction between NC1 and laminin 5. J-18 is an anti-laminin 5 polyclonal antibody that strongly recognizes the β3 and γ2 chains of human laminin 5 and weakly recognizes the α3 and α3* (processed α3 chain) of human laminin 5 (Langhofer *et al*, 1993) (**Fig 2***A*, *lane 3*). The identities of the β3 and γ2 chain were confirmed by immunoblotting with chain specific polyclonal antibodies (**Fig 2***A*, *lanes 4* and *5*). As shown in **Fig 2**(***B***), the binding of ^{125}I-labeled NC1 was completely inhibited by the J-18 antibody (compare bar NONE with bar J-18). Like the presence of the J-18 antibody, the presence of a 50-fold excess of unlabeled NC1 also completely blocked the interaction between labeled NC1 and laminin 5 (compare bar NONE with bar NC1). NC1 binding to laminin 5 was also inhibited by ≈60% with K140, a monoclonal antibody to the β3 chain (compare bar NONE with bar K140). In contrast, MoAb BM165, which specifically recognizes only the α3 chain and control normal rabbit serum, did not inhibit the interaction between laminin 5 and NC1 (compare bar NONE with bars BM165 and NIgG). These data suggest that the β3 chain of laminin 5 is involved in the NC1–laminin 5 interaction.

We have previously shown that NC1 is N-linked glycosylated (Chen *et al*, 1997a). To examine whether N-linked glycosylation plays a role in NC1 binding to laminin 5, the purified recombinant NC1 was deglycosylated with peptide N-glycosidase F and then tested for its ability to bind laminin 5. As shown in **Fig 2**(***B***), the presence of a 50-fold excess of unlabeled deglycosylated NC1 completely abolished the interaction between labeled NC1 and laminin 5 (compare bar NONE with bar NC1*). This indicates that N-linked glycosylation of NC1 is not required for its affinity for laminin 5.

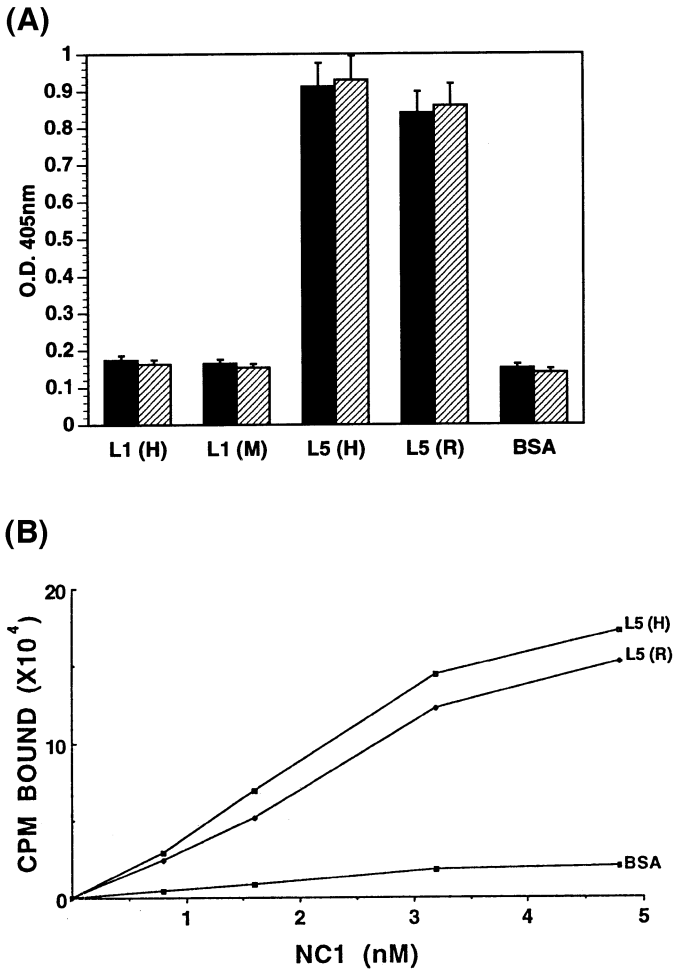

Figure 1. Solid phase binding of NC1 to laminin 5. (*A*) BSA, human laminin 1 [L1 (H)], mouse laminin 1 [L1 (M)], human laminin 5 [L5 (H)], rat laminin 5 [L5 (R)] were plated onto an enzyme-linked immunosorbant assay plate at 10 μg per ml. Two micrograms of purified authentic NC1 (*solid bar*) or recombinant NC1 (*shadow bar*) were incubated with each protein overnight in PBST at 4°C. Binding was detected using a monoclonal anti-NC1 antibody LH 7.2 (1:300), followed by an alkaline phosphatase-conjugated secondary antibody (1:400). These data represent the mean ± SD of triplicate determinations in one representative experiment. Similar results were obtained in two other independent experiments. (*B*) Enzyme-linked immunosorbent assay plates were coated with either human laminin 5 [L5 (H)], rat laminin 5 [L5 (R)], or BSA (10 μg per ml) and incubated with the indicated concentrations of ^{125}I-NC1. Each data point is an average of three independent observations. The variance between these observations was less than 8%.

Binding of NC1 to β and γ chains of laminin 5 in the protein overlay assay Laminin-5 is a heterotrimer composed of three nonidentical α, β, and γ polypeptide chains. To further investigate which chain(s) of laminin 5 interacts with NC1, the binding of ^{125}I-NC1 to laminin 5 was studied by a protein overlay assay using laminin 5 that had been reduced and electrophoretically separated on SDS polyacrylamide gels and transferred to nitrocellulose. The specificity of this assay was tested by evaluating the relative binding of albumin, fibronectin, type IV collagen, human laminin 1, and mouse laminin 1 to ^{125}I-NC1 on the same blot. As shown in **Fig 3**(***A***), ^{125}I-NC1 bound strongly to the 140 kDa β3 chain of laminin 5. It also bound weakly to the 155 kDa γ2 chain. No detectable binding, however, was observed to the 200 kDa α3 chain or the 165 kDa processed α3* chain (**Fig 3***A*, *lanes 6* and *7*). Albumin, type IV collagen, human laminin 1, and mouse laminin 1 also failed to bind NC1 (**Fig 3***A*, *lanes 1*, *3–5*).

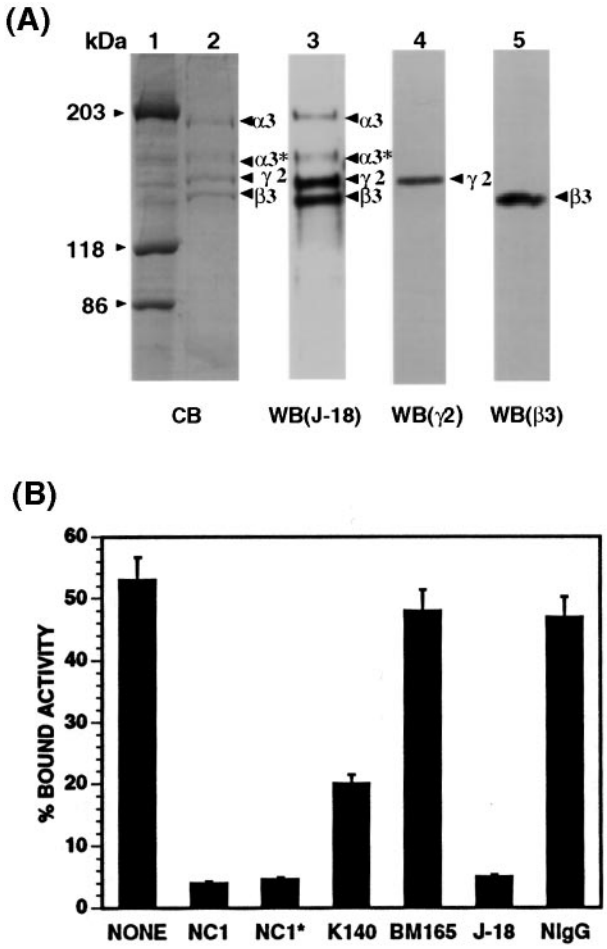

Figure 2. Inhibition of ^{125}I-NC1 binding to immobilized laminin 5. (*A*) Two micrograms of human laminin 5 purified from keratinocyte ECM was subjected to 6% SDS-PAGE and Coomassie Blue staining (*lane 2*), or immunoblotting with a polyclonal antibody J-18 (*lane 3*), a polyclonal antibody to γ2 chain (*lane 4*), and a polyclonal antibody to β3 chain (*lane 5*). *Lane 1* is CB staining of molecular mass markers. The positions of the molecular mass markers and the α3, α3*, β3, and γ2 chains of laminin 5 are indicated. (*B*) Microtiter wells coated with laminin 5 (10 μg per ml) were preincubated with a β3 chain-specific monoclonal antibody, K140 (0.15 mg per ml); an α3 chain-specific monoclonal antibody, BM165 (0.15 mg per ml); a polyclonal antibody, J-18 (1:100); normal rabbit serum NIgG (1:100); or a 50-fold excess of unlabeled NC1 or deglycosylated NC1* for 2 h at room temperature, followed by incubation with 50 ng of radiolabeled ^{125}I-NC1. Results are shown as the percentage of radioactivity bound to the wells after substracting the BSA coated wells after 4 h incubation at 4°C. These data represent the mean ± SD of triplicate determinations in a representative experiment. Similar results were obtained in two other independent experiments.

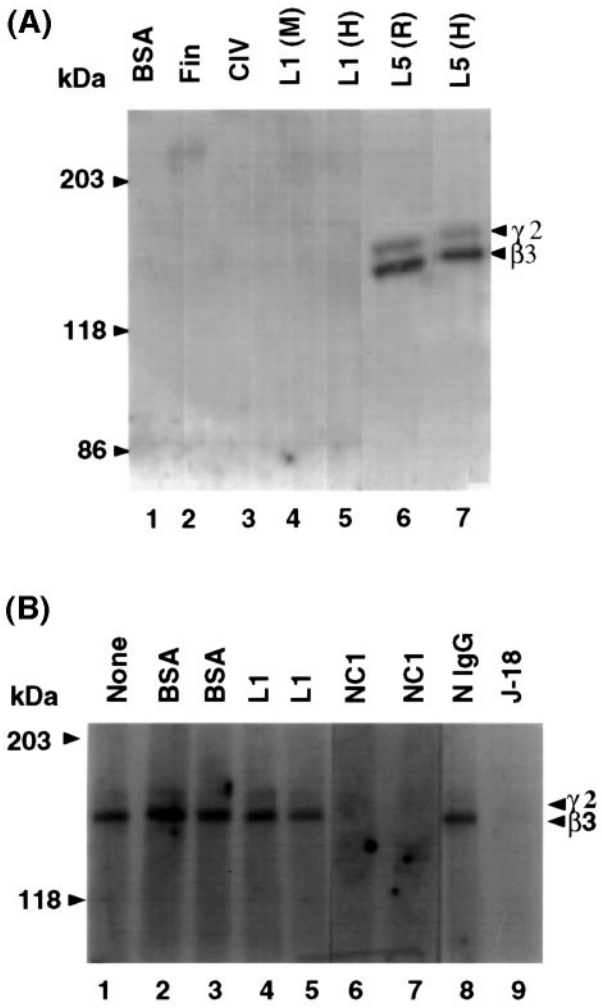

Figure 3. Binding of ^{125}I-NC1 to laminin 5 in protein overlay assay. (*A*) Shown are nitrocellulose transfers containing SDS-polyacrylamide gel electrophoretically separated proteins overlaid with ^{125}I-NC1. These proteins include BSA (*lane 1*), fibronectin (Fin) (*lane 2*), type IV collagen (CIV) (*lane 3*), mouse laminin 1 [L1 (M)] (*lane 4*), human laminin 1 [H (L1)] (*lane 5*), rat laminin 5 [L5 (R)] (*lane 6*), and human laminin 5 [L5 (H)] (*lane 7*). The positions of the molecular mass markers and the β3 and γ2 chains of laminin 5 are indicated. (*B*) Shown are nitrocellulose transfers containing 2 μg of human laminin 5 in each lane, which were overlaid with ^{125}I-NC1 in the absence (None) (*lane 1*) or presence of 1000-fold or 2000-fold excesses (wt/wt) of BSA (*lanes 2* and *3*) and laminin 1 (L1) (*lanes 4* and *5*), or in the presence of 50- or 100-fold excesses of unlabeled NC1 (*lanes 6* and *7*), or in the presence of normal rabbit serum NIgG (1:200) (*lane 8*) and a polyclonal antibody J-18 to the laminin 5 (1:200) (*lane 9*).

To further evaluate the specificity of NC1 binding to the β3 and γ2 chains of laminin-5, ^{125}I-NC1 incubations were carried out in the presence of a 0-, 50-, or 100-fold excess of unlabeled NC1 or a 1000-fold and 2000-fold excess of unrelated proteins such as BSA and laminin 1. As demonstrated in **Fig 3(*B*)**, the presence of unlabeled NC1 inhibits the ^{125}I-NC1 binding to the β3 or γ2 chains of human laminin 5 (**Fig 3*B***, *lanes 6* and *7*), whereas the presence of albumin and laminin 1 does not (**Fig 3*B***, *lanes 2–5*). Furthermore, the binding of ^{125}I-NC1 to the laminin 5 chains was inhibited by the presence of J-18, the anti-laminin 5 antibody, but not by control normal rabbit serum (**Fig 3*B***, *lanes 8* and *9*).

Deletion analysis of the laminin 5 binding site within NC1 Sequence analysis of the NC1 revealed the presence of multiple submodules with homology to adhesive proteins (Christiano *et al*, 1992, 1994). This suggested that these submodules of the NC1 domain might mediate interactions with other matrix components within the lamina densa and papillary dermis. In order to identify which of these submodules are necessary for NC1 to bind to laminin 5, we made two deletion cDNA constructs, NC1-S1 and NC1-S2, from the carboxyl-terminal of the NC1 domain (**Fig 4*A***), transfected them into 293 cells, and isolated stably transfected 293 clones. Recombinant proteins were then purified from serum free culture medium, as described for full-length NC1 (Chen *et al*, 1997a). As shown in **Fig 4(*B*)**, purified recombinant proteins had apparent molecular masses of 145 kDa for NC1, 120 kDa for NC1-S1, and 86 kDa for NC1-S2. These were the masses expected from the respective cDNA inserts. The binding activities of these recombinant deletion proteins were then analyzed by the protein overlay assay. As shown in **Fig 5(*A*)**, the binding of ^{125}I-NC1 to laminin 5 was inhibited by the inclusion of a 50-fold excess of unlabeled full-length NC1 or NC-S1 containing a

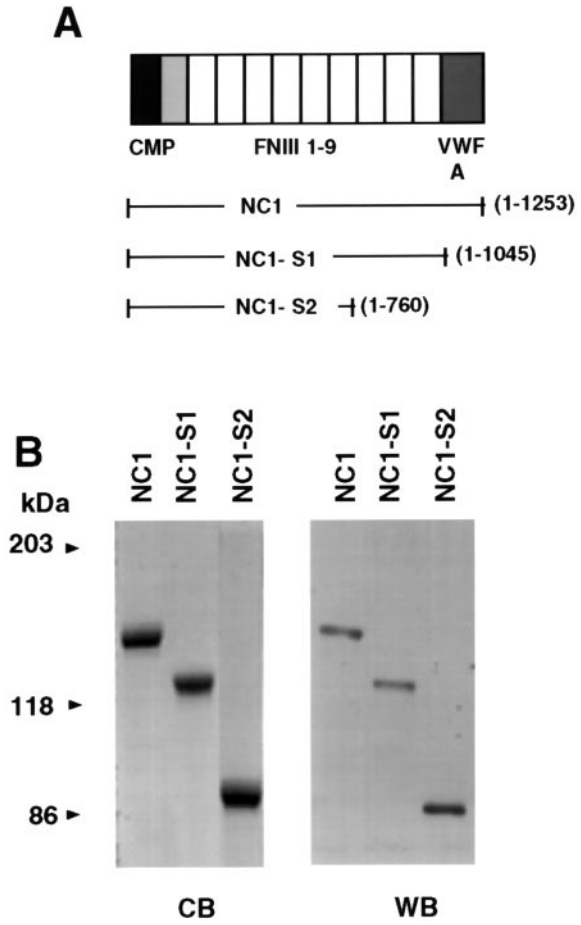

Figure 4. Domain organization and construction of NC1 mutants recombinant proteins. (*A*) Schematic diagram of the recombinant proteins created by selected deletions from the NC1 cDNA. The 1253-amino acid sequence of NC1 has motifs with homology to known adhesive proteins including cartilage matrix protein, nine FNIII 1–9, and the VWF-A. Two additional deletion mutant recombinant proteins are shown in the schematic with the amino acid residue numbers indicated. (*B*) NC1 mutant proteins were purified from 293 cell clones and subjected to 6% SDS-PAGE followed by Coomassie Blue staining (CB) and western blot analysis (WB) with monoclonal antibody LH7.2. The positions of the molecular mass marker are indicated.

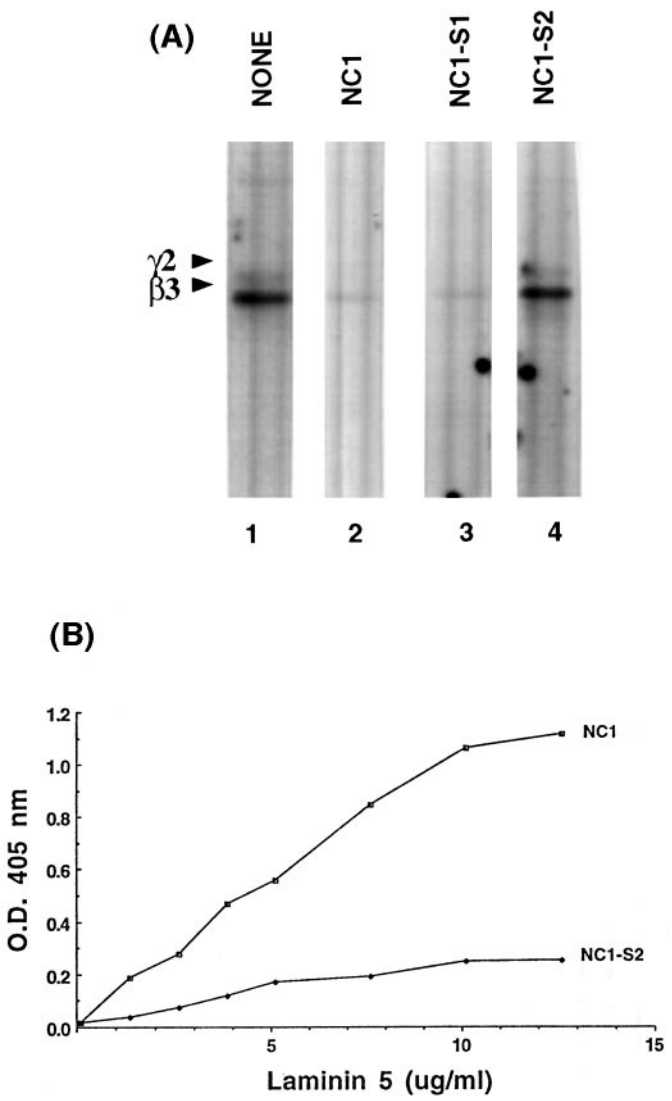

Figure 5. Laminin-binding properties of deletion NC1 mutant proteins. (*A*) Shown are nitrocellulose transfers containing 2 μg of human laminin 5 in each lane that were overlaid with ^{125}I-NC1 in the absence (NONE) (*lane 1*) or presence of 40-fold excesses (wt/wt) of nonradioactive NC1 (*lane 2*), NC1-S1 (*lane 3*), or NC1-S2 (*lane 4*). (*B*) Enzyme-linked immunosorbant assay plates were coated with 2 μg of purified recombinant NC1 and NC1-S2 and incubated with the indicated concentrations of purified laminin 5. A polyclonal antibody to laminin 5 and an alkaline phosphatase-conjugated secondary antibody, were used to detect the amount of bound substrates. Each data point is an average of three independent observations.

deletion of the VWF-A domain (**Fig 5*A***, *lanes 2* and *3*). In contrast, the NC1-S2 containing a further deletion (residues 760–1045) did not compete with the ^{125}I-NC1 binding to laminin 5 (**Fig 5*A***, *lane 4*). The ability of NC1-S2 to bind to laminin 5 was further examined using the solid-phase ligand binding assay. As shown in **Fig 5(*B*)**, consistent with the overlay protein binding assay, NC1 again demonstrated affinity for laminin 5. In contrast, NC1-S2 was incapable of binding to laminin 5. Taken together, these results indicate that the active region for laminin 5 binding within the NC1 domain is located between the sixth and ninth FNIII repeats (residues 760–1045).

Inhibition of NC1/laminin 5 interaction with EBA serum EBA is a severe blistering skin disease in which the epidermis separates from the dermis at the BMZ. Because EBA often occurs with minimal clinical or histologic inflammation, it has been hypothesized that the defective epidermal–dermal adherence in EBA involves autoantibody targeting of functional epitopes causing interference with the normal interactions between NC1 and its ECM ligands (Woodley *et al*, 1987; Chen *et al*, 1997a). In the context of this investigation, we sought to determine if IgG from EBA serum would perturb the interaction between NC1 and laminin 5. To examine this possibility, ^{125}I-NC1 was preincubated with IgG from EBA serum or control normal human serum and then reacted with nitrocellulose strips containing 2 μg of immobilized laminin 5. As demonstrated in **Fig 6(*A*)**, the presence of 100 μg EBA IgG per ml, but not normal serum IgG, inhibited the binding of ^{125}I-NC1 to laminin 5. This suggests that the presence of EBA IgG perturbs the interaction between NC1 and laminin 5.

We have previously produced a series of GST fusion proteins encompassing the complete NC1 domain of type VII collagen (Lapiere *et al*, 1993). These recombinant type VII collagen fusion proteins (**Fig 6*B***) were then used to identify regions within NC1 that were recognized by EBA serum. As shown in **Fig 6(*C*)**, the EBA IgG used in this study recognized two major antigenic epitopes within NC1, because it bound to FP1 and FP7 by western analysis (**Fig 6*C***, *lanes 1* and *3*). This binding was specific, because the EBA IgG did not recognize FP4 and FP8 (**Fig 6*C***, *lanes 2* and *4*). Furthermore, control normal human serum did not react with any of the fusion proteins tested (data not shown). Interestingly,

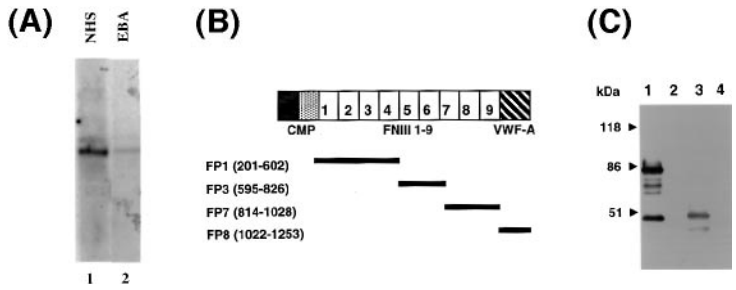

Figure 6. Inhibition of ^{125}I-NC1 binding to laminin 5 by EBA serum. (*A*) Shown are nitrocellulose transfers containing 2 μg of human laminin 5 that were overlaid with ^{125}I-NC1. ^{125}I-NC1 was preincubated for 2 h at room temperature with 100 μg EBA serum (EBA) per ml or normal human serum (NHS) before testing the ^{125}I-NC1 binding to laminin 5. (*B*) The positions of four fusion proteins that correspond to the NC1 are shown in the schematic with the amino acid residue numbers indicated. (*C*) Western immunoblot of the fusion proteins with EBA serum. Purified recombinant fusion proteins FP1 (*lane 1*), FP3 (*lane 2*), FP7 (*lane 3*), and FP8 (*lane 4*) were subjected to 10% SDS-PAGE followed by western blot analysis with EBA serum (1:100). The positions of the molecular mass markers are indicated.

the FP7 epitope region lies within residues 814 and 1028 that colocalize with the NC1 region that binds to laminin 5 (residues 760–1045).

DISCUSSION

In this study, we demonstrated that the NC1 domain of the type VII collagen alpha chain binds to laminin 5. This extends a recent study by Rousselle *et al* (1997) and our earlier study (Chen *et al*, 1997a), which demonstrated specific binding between NC1 and a complex of laminin 5 and laminin 6. The specificity of this interaction was demonstrated by the lack of NC1 interaction with laminin 1 and BSA and by the lack of binding of labeled NC1 to laminin 5 in the presence of excess unlabeled NC1, but not other proteins. The interaction also could be inhibited with a monoclonal antibody to the β3 chain of laminin 5 (K140) and an anti-laminin 5 polyclonal antibody, but not by a monoclonal antibody (BM165) to the α3 chain. Additionally, in an independent gel-overlay protein binding assay, we directly demonstrated that NC1 binds to laminin 5 predominately via the β3 chain. Using a panel of purified recombinant NC1 deletion proteins, we also mapped the laminin 5-binding site within the NC1 domain to a region between FNIII 7 and FNIII 9.

It has been suggested that anchoring fibrils associate with hemidesmosomes via putative interactions between the NC1 domain of type VII collagen and the components of anchoring filaments (Burgeson, 1987) such as laminin 5. From the available evidence, it has been hypothesized that laminin 5 is oriented in the basement membrane with its carboxyl-terminal α3 G-domain located adjacent to the basal cell surface and apposed to the hemidesmosome integrin receptor, α6β4 (Baker *et al*, 1996b; Green and Jones, 1996). In this model, the amino-terminal truncated short arms of laminin 5 face the lamina densa and the β3 and γ2 chains interact with NC1 (Rousselle *et al*, 1997). Our data directly demonstrate that the β3 and γ2 chains of laminin 5 are involved in this interaction. Antibody blocking experiments showed that a β3 chain-specific monoclonal antibody, K140, was able to inhibit the binding of NC1 to laminin 5. In contrast, an α3 chain-specific monoclonal antibody, BM 165, had no inhibitory effect. This strongly suggests that one of the NC1 binding sites on laminin 5 is within the β3 chain.

The K140 monoclonal antibody to the β3 chain did not completely abolish the NC1–laminin 5 interaction. There may be multiple NC1-binding sites on the β3 chain, and the K140 monoclonal antibody may only block one of them. In addition, our data suggest that although the β3 chain may predominantly mediate the NC1 binding, the γ2 chain also may contribute to the NC1 affinity. In the protein overlay binding assay, the labeled NC1 bound strongly to the β3 chain, and also bound to the γ2 chain, but much less so. In addition, in the antibody blocking experiments, a polyclonal antibody to laminin 5 (which reacts with both the β3 and γ2 chains) completely abolished the NC1–laminin 5 interaction. Taken together, these data support the notion that although the β3 chain may contain the predominant NC1-binding domain, the γ2 chain also plays a role in mediating the NC1-laminin 5 affinity. The fact that NC1 interacts with both human and rat laminin 5 suggests that the NC1-binding domains of laminin 5 are conserved between these two species.

In our previous study (Chen *et al*, 1997a), we demonstrated that both authentic and recombinant NC1 specifically bound to type I collagen, type IV collagen, and fibronectin by a solid-phase binding assay. In the present overlay-protein binding assay, however, NC1 did not bind to type IV collagen and fibronectin (**Fig 3*A***). Because type IV collagen and fibronectin were reduced and denatured in the protein overlay assay, our data suggest that a native conformation of type IV collagen and fibronectin is required for the NC1 interaction.

Laminin 5 binding sites within NC1 were mapped to the 285 amino acid residues between the seventh and nineth FNIII repeats within NC1, an area distinct from the collagen binding site (unpublished observation). In keeping with these data, a recent study (Rousselle *et al*, 1997) showed that type XII and XIV collagens do not interact with laminin 5, although they share both FNIII and VWF-A motifs with the NC1 domain of type VII collagen. Therefore, the subdomain within the seventh and nineth FNIII may contain the specific sequences that are responsible for binding to laminin 5. In this regard, it is interesting to note that previous epitope mapping studies (Gammon *et al*, 1993; Lapiere *et al*, 1993) identified four major antigenic epitopes recognized by autoantibodies in the sera from EBA patients. One antigenic epitope resides within the seventh and nineth FNIII repeats.

Furthermore, in this study, we demonstrated that the NC1/laminin 5 interaction could be abrogated by the presence of EBA IgG but not IgG from normal human serum. Epitope mapping of this EBA serum showed that the autoantibodies recognized two distinct NC1 sites, and one of these colocalized precisely with the binding site for laminin 5. These data support the hypothesis that EBA autoantibodies may contribute to defective epidermal–dermal adhesion by the relatively novel pathomechanism of targeting functional epitopes within important matrix adherence proteins. Thus, autoantibodies to the NC1 domain of type VII collagen may interfere with the normal interactions between type VII collagen and its ECM ligand(s) in the basement membrane zone or the papillary dermis; in this case to laminin 5, a component of anchoring filaments.

The biologic implication of the *in vivo* NC1–laminin 5 interaction remains an open question. Laminin 5 and NC1 can be isolated as a complex from collagenase-digested amnion (Rousselle *et al*, 1997). These data suggest that the strong affinity of NC1 to laminin 5 seen *in vitro* is physiologically significant. Molecular defects in genes encoding for type VII collagen or laminin 5 in humans result in severe blistering disorders of the skin. Likewise, autoantibodies against either type VII collagen or laminin 5 result in acquired incurable blistering diseases of the skin. These naturally occurring diseases demonstrate the importance of type VII collagen and

laminin 5 in maintenance of the DEJ and epidermal–dermal adherence.

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