

A Novel Laminin E8 Cell Adhesion Site Required for Lung Alveolar Formation In Vitro

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Abstract. Basement membrane-adherent type II alveolar cells isolated from lung assemble into lumen-containing cellular spheres which retain the correct polarity and thereby approximate the earliest fetal stage of alveolar morphogenesis. The molecular basis of this process, determined in initial experiments to be attributable mainly to the large heterotrimeric glycoprotein laminin, was probed with laminin proteolytic fragments, antibodies, and synthetic peptides. The carboxy-terminal fragment E8, but not equimolar amounts of fragment P1, blocked alveolar formation. To pursue this observation, we used several anti-E8 antibodies and identified one, prepared against A chain

residues 2179–2198 (“SN-peptide”) from the first loop of the G domain, as inhibitory. These results were confirmed by use of SN-peptide alone and further defined by trypsin digestion of SN-peptide to the sequence SINNNR. This conserved site promoted divalent cation dependent adhesion of both type II alveolar and HT1080 cells, was inhibitable with equimolar amounts of fragment E8 but not P1, and derives from a form of laminin present in fetal alveolar basement membranes. These studies point to an important novel cell adhesion site in the laminin E8 region with a key role in lung alveolar morphogenesis.

MORPHOGENESIS of lung alveoli, the functional unit of bi-directional gas exchange, occurs mainly postnatally with an increase of 280 million alveoli during the first eight years of human life (Thurlbeck, 1975). Alveoli arise initially from outgrowths of terminal air ducts, a process coincident with the appearance of basement membrane-adherent type II alveolar cells (Burri, 1991). Type II cells subsequently proliferate, coassemble, and serve both as progenitors for attenuated type I alveolar cells (Mason and Williams, 1991) and later as the only source of pulmonary surfactant (Hawgood, 1991). A mature alveolus consists of a central air space lined by type II and type I alveolar cells which are adherent basally to a thin basement membrane (McGowan, 1992; Sannes, 1991; Lwebuga-Mukasa, 1991).

Mechanistic investigations of this complex and important phenomenon have historically been restricted to in vivo studies in fetal or neonatal sheep and rodents (Ballard, 1986) for which molecular information is limited. An alternative approach is to develop an in vitro model system (Diglio and Kikkawa, 1977) which, despite limitations inherent in simplification, could effectively identify active molecules whose presence or absence in vivo may later be determined. This approach is made feasible by both the relative ease of type II alveolar cell isolation (Rannels and Rannels, 1988) and the retention of a remarkable capacity for basement membrane

dependent alveolar-like morphogenesis in vitro (Adamson et al., 1989; Blau et al., 1988; Edelson et al., 1989). Here we report on the use of such an in vitro model system to identify a key alveolar activating sequence within the carboxy terminal region of the basement membrane glycoprotein laminin.

Materials and Methods

Preparation of Substrates

Basement membrane substrate (BMS)¹ was prepared at 4°C in the presence of NEM (0.5 mM) and PMSF (0.5 mM) by extraction of Engelbreth-Holm-Swarm (EHS) mouse tumor with 10 mM EDTA in 50 mM Tris, 150 mM NaCl, pH 7.4 according to the method of Paulsson et al. (1987). Briefly, EHS tumor, collected from C57Bl or ICR (Hilltop Lab Animals, Inc., Scottsdale, PA) mice, was homogenized, washed in 150 mM NaCl, 50 mM Tris, pH 7.4 (TBS), and extracted overnight in TBS containing 10 mM EDTA (1 ml/gm tumor starting material). BMS, comprising the solubilized material, was then sterilized by dialysis against TBS containing chloroform (5 ml/l). Subsequent dialysis steps were against TBS and finally against three changes of DME. BMS protein concentration (8–10 mg/ml) was determined by lyophilization versus an equal volume of DME. BMS was stored as 1-ml aliquots at –80°C.

For gel filtration (4°C) BMS was passed over a Biogel A 1.5-m column (2.5 × 100 cm; Bio Rad Laboratories, Melville, NY) equilibrated in TBS containing 10 mM EDTA and proteolytic inhibitors. Fractions making up each of the two peaks (Paulsson et al., 1987) were pooled, concentrated (if necessary on an Amicon YM1 membrane [Amicon Corp., Beverly, MA]), sterilized with chloroform, and dialyzed against DME.

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1. *Abbreviations used in this paper:* BMS, basement membrane substrate; EHS, Engelbreth-Holm-Swarm; JMEM, Joklik's modified minimal medium.

Mouse laminin and collagen IV were kindly supplied by Dr. R. Ogle (University of Virginia, Charlottesville, VA). Mouse entactin was purchased from Upstate Biotechnology, Inc. (Lake Placid, NY). Rat tail collagen I was purchased from Collaborative Research Inc. (Bedford, MA). Protein concentration of laminin and collagen IV was determined using their respective extinction coefficients [laminin: $8.3 (A^{1\%} 1 \text{ cm}_{280})$, (McCarthy et al., 1983); collagen IV: $5.48 (A^{1\%} 1 \text{ cm}_{230})$]. Protein concentration of entactin and collagen I was used as supplied by the manufacturer.

Preparation of Antibodies

Rabbit anti-mouse laminin (ab-Ln) and rabbit anti-mouse collagen IV antisera were produced on contract with Hazelton Labs (Denver, PA). Rabbit anti-mouse entactin antibodies were obtained from Upstate Biotechnology, Inc. All antisera were purified on protein A-Sepharose (Pharmacia Fine Chemicals, Piscataway, NJ) before use; concentration of eluted antibody was calculated using the extinction coefficient for rabbit IgG ($13.5 [A^{1\%} 1 \text{ cm}_{280})$). Ab-Ln and anti-collagen IV antibodies inhibit cell adhesion to laminin and collagen IV, respectively. Chain specific rabbit anti-mouse laminin polyclonal antibodies were kindly provided by Dr. Y. Yamada (NIDR, Bethesda, MD), purified on protein A-Sepharose (Pharmacia Fine Chemicals), and checked for specificity by Western blotting. These antibodies are: (a) ab-B1 (antigen is a fusion protein spanning amino acids 925–933 which includes the YIGSR sequence; previously designated HK-58); (b) ab-B2 (antigen is a synthetic peptide consisting of amino acids 1420–1439; previously designated YY-13); (c) ab-A[IK] (antigen is a synthetic peptide consisting of amino acids 2097–2108; previously designated PA22-2 in Sephel et al. [1989]); (d) ab-A[SN] (antigen is a synthetic peptide consisting of residues 2179–2198; previously designated PA10 in Sephel et al. [1989]). The rat anti-mouse monoclonal anti-laminin antibodies 5D3, 5A2, and 5C1 were kindly provided by Dr. D. Abrahamson (University of Alabama, Birmingham, AL) (Abrahamson et al., 1989).

For Western blotting, DTT-reduced laminin was separated on 5% SDS-PAGE gels, transferred to nitrocellulose, blocked, incubated with anti-laminin antibody, washed, and detected with peroxidase-labeled goat anti-rabbit IgG (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) using the chemiluminescent ECL method (Amersham Corp., Arlington Heights, IL). Preabsorption of secondary antibody on a BMS-Sepharose column was necessary to eliminate background.

Laminin Fragments and Synthetic Peptides

Fragments E8 and P1, isolated from mouse laminin, were both kindly provided by Drs. Rupert Timpl (Max Planck Institut für Biochemie, Martinsried, Germany) and Peter Yurchenco (Robert Wood Johnson Medical School, Piscataway, NJ). SN-peptide (SINNNRWHSIYTRFGNMG; amino acids 2179–2198 from mouse laminin A chain) was synthesized by the Biomolecular Research Facility (University of Virginia), purified by reverse phase HPLC, and verified through NH_2 -terminal sequencing. To attempt to determine the minimal active length, SN-peptide (10 mg) was incubated with TPCK treated trypsin (Washington Biochemical Corp., Freehold, NJ) for 18 h at 37°C giving rise to three smaller peptides. Digestion was terminated by lowering the pH to 2.0; fragments were purified by reverse phase HPLC and sequenced. Examination of SN-peptide and SINNNR conservation among species made use of FastA and BestFit searches (Dayhoff et al., 1978).

The HPLC purified peptide AASIKVAVSADR (amino acids 2097–2108 from mouse laminin A chain) and its scrambled control AASVVIKASADR were kindly supplied by Dr. Y. Yamada (Tashiro et al., 1989). Peptide KQNCLSSRASFRGCVRLRLSR (amino acids 3011–3032 from mouse laminin A chain, previously designated GD-6 (Gehlsen et al., 1992), corresponding to the proposed $\alpha_3\beta_1$ integrin binding site, was kindly provided by Dr. K. Gehlsen (California Institute for Biological Research, La Jolla, CA). RGDG (functionally identical to RGDN; amino acids 1123–1126 from mouse laminin A chain) was provided by Dr. R. Ogle (University of Virginia).

Isolation of Type II Alveolar Cells

Type II alveolar cells were isolated from 250-g Sprague Dawley rats (Hilltop Laboratory Animals, Inc.) according to the method of Rannels (Rannels and Rannels, 1988). Briefly, an initial cardiac perfusion with 0.9% saline was followed by instillation of the airways with 0.1% elastase (Calbiochem Corp., La Jolla, CA) in Joklik's modified minimal medium (JMEM) containing 0.05% BaSO_4 . Elastase was inactivated by instillation of JMEM

containing soybean trypsin inhibitor (0.08%; Sigma Chemical Co., St. Louis, MO), DNase (0.08%; Sigma Chemical Co.), and 50% newborn calf serum (GIBCO BRL, Gaithersburg, MD). Lung tissue was minced, vortexed, and filtered through 160- μm nylon mesh (Tetko, Elmsford, NY). Released cells were centrifuged for 10 min at 500 g, resuspended in JMEM containing 0.08% DNase, and layered on a Percoll (Pharmacia Fine Chemicals) discontinuous density gradient. After centrifugation for 20 min (4°C), cells were collected at the 1.04/1.08 interface, washed in JMEM, brought up in DME containing 10% fetal bovine serum (FBS) (GIBCO BRL), and incubated for 30 min (37°C) in 75- cm^2 tissue culture flasks to eliminate contaminating macrophage cells which rapidly adhere to the plastic surface. The resultant type II alveolar cell preparations were 95% viable as determined by trypan blue exclusion; purity was 93% as assessed by the presence of lamellar bodies visible with Hoffman optics at a magnification of 40 and by tannic acid staining. Analysis of total cellular DNA/well on each day of an experiment was performed in triplicate on trypsin/dispase released cells using a DNA fluorometry assay (Labarca and Paigen, 1980). No contaminating DNA could be detected in wells containing BMS alone, which after dissolution with dispase and trypsin did not contribute to the cell pellet.

Alveolar Formation Studies

Freshly isolated type II alveolar cells were plated in 96-well plates at 20×10^3 cells/well on 500 $\mu\text{g}/\text{well}$ (1.8 mg/cm^2) of gelled BMS or collagen I. Cells were cultured over 5 d with one media change performed on day three. Alveolar formation was analyzed at 24-h intervals over 5 d from photographic negatives ($4 \times$ original magnification of central portion of each well) of triplicate wells. Images from negatives were transferred via video camera to an Image I imaging system (Universal Imaging Corp., West Chester, PA) and viewed on a color video monitor. The area of cellular structures was then determined and expressed as the mean \pm standard deviation. In some cases two size categories were distinguished: (a) single cells ($200\text{--}300 \mu\text{m}^2$) and (b) model alveoli ($20 \times 10^3 \mu\text{m}^2$ or greater) with data expressed as percent of area occupied by each category.

To examine sectioned cultures, type II alveolar cells were plated on gelled BMS (500 $\mu\text{g}/\text{insert}$) supported by Millicell 0.4- μm filter inserts (Millipore Corp., Bedford, MA). After 5 d, cultures were fixed for 1 h with 2% formaldehyde/2% glutaraldehyde in 0.05 M sodium phosphate buffer, pH 6.8, and washed. Filters were cut out, treated for 1 h with 1% osmium tetroxide, acetone dehydrated, and embedded in Spurr's resin (Electron Microscopy Sciences, Ft. Washington, PA). Semi-thin or thin sections were then cut, stained, and examined in the light or electron microscope, respectively.

In alveolar inhibition studies, antibodies (50 $\mu\text{g}/\text{well}$) were incubated with gelled BMS (500 $\mu\text{g}/\text{well}$) in wells of 96-well plates for 60 min at 37°C . Unbound antibody was removed by three DME washes (200 $\mu\text{l}/\text{well}$) and then freshly isolated cells were added. Dose-response assays were performed on all antibodies. Laminin fragments and synthetic peptides (micromolar amount indicated on figures) were preincubated with freshly isolated type II alveolar cells in suspension for 30 min at 37°C with gentle agitation every 5 min; cells together with fragment or peptide were then plated on BMS. Cell viability in the presence of each antibody, fragment, or synthetic peptide was assessed using the MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide) assay (Chemicon International, Inc., Temecula, CA).

Cell Adhesion Studies

Plates coated overnight (4°C) with equimolar amounts of laminin, laminin fragment, or synthetic peptide were blocked with 1% BSA (Sigma Chemical Co.) for 4 h (4°C) and cells were subsequently added ($2 \times 10^4/\text{well}$) in serum-free media and incubated for 60 min (37°C) according to the method of Aumailley and Timpl (1986). Inhibition studies were carried out by preincubating cells for 30 min (37°C); gentle agitation every 5 min) with equimolar amounts of soluble fragment or peptide. Cells, together with soluble inhibitor, were then added to the coated plates. After a 60-min (37°C) incubation, plates were washed twice with PBS, fixed with 1% glutaraldehyde (Electron Microscopy Service Laboratories, Westmont, NJ), PBS washed, stained with 0.1% crystal violet (Serva Biochemicals, Hauppauge, NY), washed twice with distilled water, solubilized in 0.5% Triton X-100 (Sigma Chemical Co.), and read on an ELISA plate reader (Molecular Devices Corp., Menlo Park, CA) equipped with a 595-nm filter.

Immunohistochemistry

Immunostaining was performed on unfixed frozen sections of late gestation

rat lung. Sections were blocked with 3% BSA (Sigma Chemical Co.) and then incubated with ab-A[IK] (rabbit anti-laminin A chain peptide [2097-2108]; 1 $\mu\text{g}/\text{ml}$) overnight at 4°C. Detection was through Cy3-labeled goat anti-rabbit IgG (1/50; Jackson Immunoresearch) that had been preadsorbed to gelled BMS for 30 min at 37°C (Streuli et al., 1991). Slide preparations were washed and examined with a fluorescence microscope using a Rhodamine far red filter. ab-A[SN] (rabbit anti-laminin A chain peptide [2179-2198]), gave the same pattern but was much lighter.

Results

Role of Basement Membrane in Model Alveolar Formation

Alveoli-like structures become (Figs. 1 and 2) apparent 3–5 d after plating dispersed type II alveolar cells on gelled BMS, a 10-mM EDTA extract of mouse EHS tumor basement membrane. These structures have a central lumen (Fig. 1 C, *inset*; and Fig. 2 A) lined by a cuboidal epithelium of lamellar body containing (Fig. 2, A and B) type II alveolar cells. Type II cells are of appropriate polarity and therefore bear a striking resemblance to their *in vivo* fetal counterparts prior to the appearance of type I cells (Adamson and Bowden, 1975). Morphogenesis was not dependent on cell proliferation (Fig. 1 C) and was basement membrane specific since replacement with an equal milligram amount of gelled collagen I was completely ineffective (compare (Fig. 1, A and B).

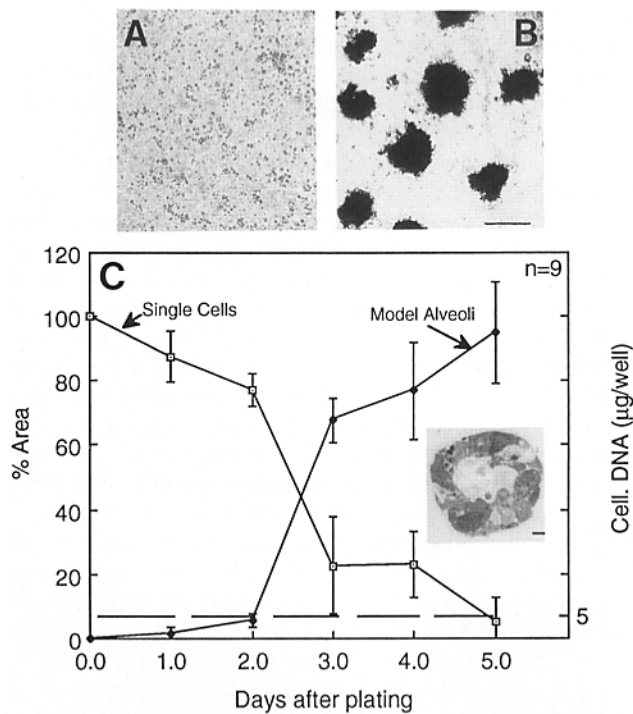


Figure 1. Alveolar morphogenesis *in vitro*. (A) Negative control illustrating type II alveolar cells dispersed five days after plating on rat tail collagen I. (B) Alveoli formed five days after plating type II alveolar cells on BMS. Gelling concentration of all substrates here and throughout article is 1.8 mg/cm². (C) Percentage of single cells declined as alveolar formation progressed (*continuous lines*). Proliferation was absent as revealed by daily analysis of cellular DNA content per well (*dashed line*). (*Inset*) Light micrograph of a representative sectioned day five alveolus illustrating central lumen. Values represent the mean \pm SD; $n = 9$. Bars: (A and B) 100 μm ; (C) 10 μm .

Basement membranes are a partially characterized source of cell attachment, structural, and growth factor-like molecules (Paulsson, 1992). To determine which molecule or combination of molecules contributed to this process, we separated BMS into high and low molecular weight peaks by gel filtration and plated type II alveolar cells on each peak. Only the high molecular weight peak was active (not shown). Since constituents of the high molecular weight peak are laminin and entactin, interspersed with collagen IV (Paulsson et al., 1987), we plated type II alveolar cells on equal

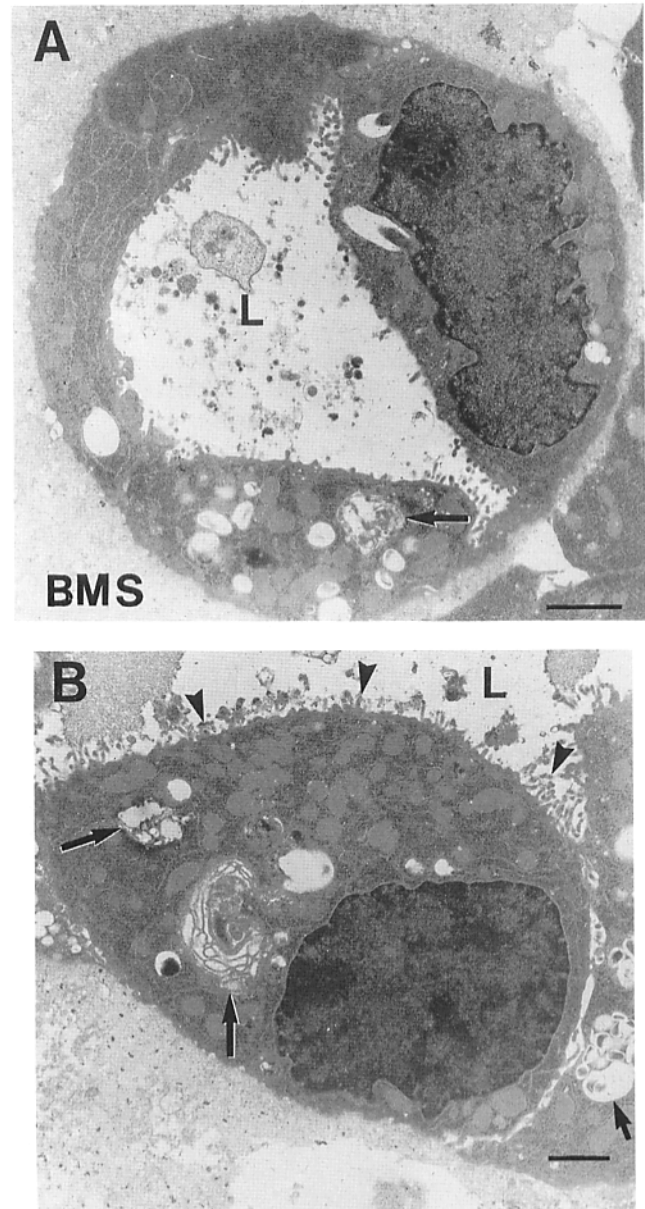


Figure 2. (A) Electron micrograph of a sectioned day five alveolus illustrating cuboidal type II alveolar cell lining similar to fetal alveoli. Alveolus is surrounded by BMS. Arrow points to characteristic lamellar body. L, central lumen. (B) Electron micrograph of type II alveolar cell within day five alveolus. Presence of circular lamellar bodies (*arrows*) and apical microvilli (*arrowheads*) are characteristic of type II alveolar cells *in vivo*. Bars: (A) 30 μm ; (B) 50 μm .

Table I. Effect of Laminin and Anti-Laminin Antibodies on Alveolar Formation In Vitro

Protein or antibody	Area ($\mu\text{m}^2 \times 10^3$)
BMS	108 \pm 23
Laminin	35 \pm 3
Entactin	1 \pm 0.5
Collagen IV	0.4 \pm 0.2
Anti-Laminin (ab-Ln)	12 \pm 3.9
Anti-Entactin	105 \pm 15
Anti-Collagen IV	110 \pm 25

Type II alveolar cells were plated on 1.8 mg/cm² gelled BMS, laminin, entactin, or collagen IV; or on the same concentration of BMS which had been preincubated with 50 $\mu\text{g}/\text{well}$ ab-Ln, anti-entactin, or anti-collagen IV antibodies. 5 d later, area of cellular structures was determined. Data is expressed as the mean of three experiments performed in triplicate \pm SD. Alveoli are defined as structures $\geq 20 \times 10^3 \mu\text{m}^2$.

milligram amounts of each of these substrates and found that alveoli formed only on laminin (Table I), although smaller in size (since alveoli which formed on peak 1 or on laminin were smaller than alveoli on intact BMS, molecules of the low molecular weight peak may have an accessory role in modulating alveolar size) than alveoli that formed on undivided BMS. These experiments were supported by parallel use of BMS preincubated with IgGs purified from either anti-laminin, anti-entactin, or anti-collagen IV sera. Only ab-Ln was inhibitory (Table I), a result consistent with an earlier observation that polyclonal anti-laminin antiserum blocks Matrigel (Matrigel [Collaborative Research Inc.] is a 2 M urea extract of EHS tumor [Kleinman et al., 1986]) inhibition of type II alveolar cell spreading (Rannels et al., 1987).

Inhibition of Alveolar Formation by Fragment E8

Laminin is a large cross-shaped cell adhesive heterotrimer (Fig. 3; Beck et al., 1990; Engel, 1992). Several large laminin domains have been partially characterized through the preparation of functional proteolytic fragments, particularly the P1 pepsin fragment and the E8 elastase fragment, whose respective origins on the intact molecule are known (Fig. 3).

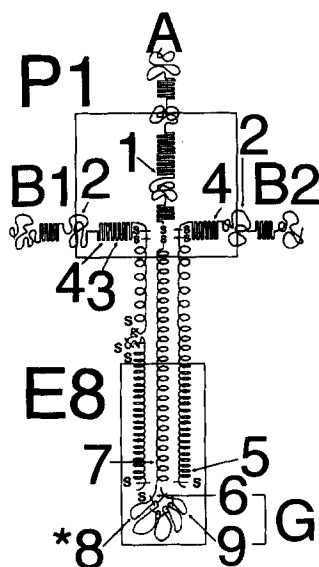


Figure 3. Schematic diagram of laminin illustrating constituent B1, B2, and A chains; and origin of P1 and E8 fragments. G domain is the large A chain carboxy-terminal globule. Arrows indicate antibody binding sites or origin of peptides tested: 1, RGDS; 2, 5A2; 3, ab-B1; 4, 5C1; 5, ab-B2; 6, AASIKVAVSADR or ab-A[IK]; 7, 5D3; 8, ab-A[SN] or SN-peptide; 9, KQNCLS-SRASFRGCVRNLRSLR. See Materials and Methods for details on antibodies and peptides. Laminin diagram modified from Sasaki et al. (1988), with permission.

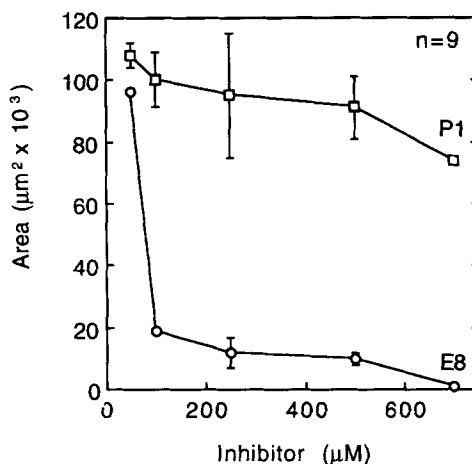


Figure 4. Alveolar formation is inhibited by E8 but not P1 fragment. Dose-dependent inhibition of alveolar formation by soluble E8 but not P1 fragment. Freshly isolated type II alveolar cells were preincubated with E8 or P1 fragment prior to plating on BMS. Analysis was performed on day five. Values represent the mean \pm SD; $n = 9$.

To determine whether in vitro alveolar promoting activity resides in laminin P1 or E8 region(s), we preincubated type II alveolar cells with increasing micromolar amounts (Fig. 4) of soluble P1 or E8 fragment prior to plating on BMS. Only fragment E8 was inhibitory (Fig. 4), an effect which was not due to lower cell viability (viability $85 \pm 3\%$ at $700 \mu\text{M}$) nor to a decrease in the number of adherent cells (not shown); adhesion is presumably mediated through alternative sites in laminin or compensated by collagen IV or attachment factors present in the lower molecular weight peak (Laurie, G. W., J. O. Glass, R. A. Ogle, C. M. Stone, J. R. Sluss, and L. Chen, manuscript submitted for publication).

Fragment E8 represents the 250-kD carboxy-terminal one third of laminin with its constituent B1, B2, and A chains. To locate the active site within E8, we obtained a number of chain specific (Fig. 5 A, inset) antibodies prepared against synthetic peptides or fusion proteins, and several monoclonal antibodies whose binding sites (Fig. 3) had been mapped through rotary shadowing. We preincubated BMS with equal microgram amounts of purified antibody, washed away unbound antibody, and plated cells. As a positive control, we used equal microgram amounts of ab-Ln against intact laminin which, as mentioned above (Table I), was inhibitory (Fig. 5). All antibodies prepared against sites within the P1 region were inactive, as were all but one of the anti-E8 fragment antibodies (Fig. 5; Table II). Complete inhibition occurred with ab-A[SN] raised against a 20-amino acid synthetic peptide (2179-2198) corresponding to a site (Fig. 3, *8) within the first loop of the large globule (designated "G domain") at the terminus of the laminin A chain (Sephel et al., 1989).

Alveolar Formation Inhibited by SINNNR

To test this observation directly, we synthesized the 20-amino acid peptide (SINNNRWHSIYITRFGNMGS; designated "SN-peptide") and preincubated it at increasing micromolar amounts with freshly isolated dispersed type II alveolar cells prior to plating on BMS. SN-peptide inhibited alveolar formation in a dose dependent fashion (Fig. 6; $\text{IC}_{50} = 50$

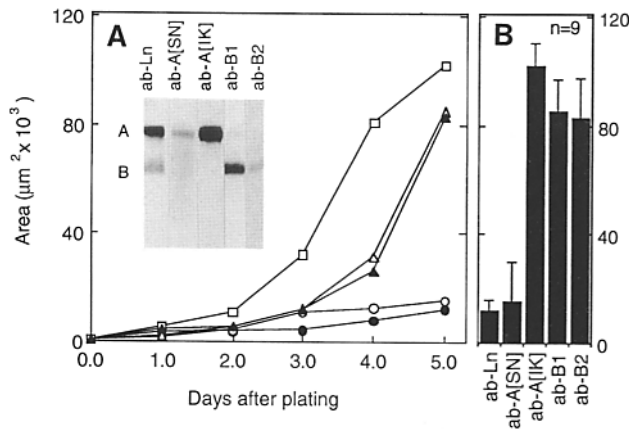


Figure 5. Alveolar formation is inhibited by an antibody directed against SN-peptide in the first loop of the E8 region G domain. (A) Time course inhibition of alveolar formation by ab-Ln (●) and ab-A[SN] (○). Ab-A[IK] (□), ab-B1 (△), and ab-B2 (▲) had little or no effect; *t* test for ab-B1 and ab-B2 on day four vs BMS alone revealed *p* values of 0.3, whereas *p* value for ab-A[SN] on day four was 0.015. Antibodies were protein A-Sepharose purified prior to incubation with BMS; type II alveolar cells were plated after washing away unbound antibody. (Inset) Western blot analysis of antibody specificity. (B) Inhibition of alveolar formation by ab-Ln and ab-A[SN], expressed as the mean ± SD of analysis performed on day five; *n* = 9.

μM) without affecting cell viability, even at the highest micromolar concentration tested (viability 97 ± 1%). Studies were also performed with equimolar amounts of other laminin A chain synthetic peptides including: AASIKVAVSADR (antigen of ab-A[IK]), AASVVIKASADR (scrambled IKVAV), KQNCLSSRASFRGCVRNLRSLR (proposed binding site for α3β1 integrin), and RGD (functionally equivalent to P1 fragment cell adhesion site RGDN) however, all had no effect on alveolar formation (Table II). To define further the alveolarization site, we trypsin digested SN-peptide generating the smaller peptides SINNNR, WHSIYTR, and FGNMGS. Each was HPLC purified, sequenced, and preincubated with type II alveolar cells at increasing micromolar concentrations. Only SINNNR was inhibitory (Fig. 6; IC50 = 68 μM).

Table II. Lack of Effect of Several Monoclonal Anti-Laminin Antibodies and Laminin A Chain Synthetic Peptides on Alveolar Formation In Vitro

Antibody or peptide	Area (μm ² × 10 ³)
None	108 ± 23
5D3	63 ± 18
5A2	82 ± 25
5C1	103 ± 20
AASIKVAVSADR	98 ± 22
AASVVIKASADR	100 ± 18
KQNCLSSRASFRGCVRNLRSLR	110 ± 19
RGDS	105 ± 24

Antibodies (50 μg/well) were preincubated with gelled BMS (1.8 mg/cm²). Peptides (100 μM) were preincubated with cells prior to plating on BMS. Area was determined 5 d after plating. *t* test of 5D3 and 5A2 values vs no antibody control indicated no significant difference. Data represents mean ± SD from three experiments performed in triplicate.

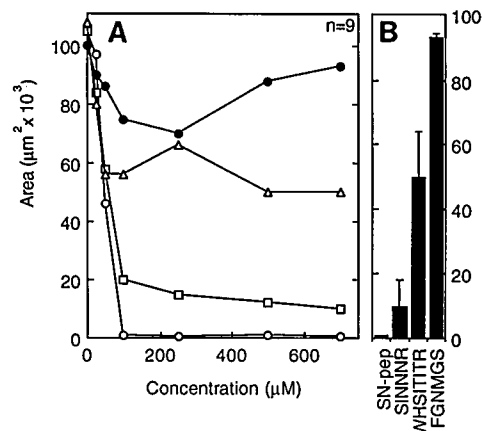


Figure 6. Alveolar formation is inhibited by SN-peptide, further defined by trypsin digestion to SINNNR. (A) Dose-dependent inhibition of alveolar formation by SN-peptide (○) and its amino-terminal 6-mer SINNNR (□). The carboxy-terminal 6-mer FGNMGS (●) and middle 8-mer WHSIYTR (△) have minimal or partial effect, respectively. Cells were preincubated with peptides in the same manner as laminin fragments and analyzed on day five. (B) Mean ± SD at 700 μM on day five; *n* = 9.

SN-Peptide and SINNNR have Cell Adhesion Activity

How might the SN-peptide site drive alveolar morphogenesis? One possibility is via cell adhesion, a fundamental requirement of kidney epithelial morphogenesis for which E8 fragment is thought to play a key role (Klein et al., 1988). To examine this possibility, we carried out cell adhesion assays using SN-peptide and SINNNR in the presence or absence of soluble inhibitors, or after preincubation with antibody. Both type II alveolar (Fig. 7 A) and HT1080 (Fig. 7 B) human fibrosarcoma cells adhered to SN-peptide and SINNNR (Table III) at levels similar to E8 fragment or intact laminin, an interaction which was inhibited by preincubation with equimolar amounts of laminin E8 or SN-peptide but not P1 fragment (Fig. 7, A and B). Similarly, ab-A[SN] but not ab-A[IK] inhibited adhesion to SN-peptide, SINNNR, and E8 without affecting adhesion to P1 (not shown). In reciprocal experiments, adhesion to E8 was completely inhibited by an equimolar amount of SN-peptide (Fig. 7 B). In addition, preincubation with 2 mM EDTA was inhibitory (Fig. 7 B) suggesting that SN-peptide adhesion was perhaps mediated via an integrin receptor which requires divalent cations for function (Hynes, 1992).

To determine whether SN-peptide and SINNNR were conserved among different species (Table IV), we used the FastA and BestFit programs revealing that SN-peptide has 65% identity and 85% similarity over the same 20-amino acid residues in human (Haaparanta et al., 1991; Nissinen et al., 1991) laminin A chain. SINNNR displayed 50% identity and 83% similarity. Compared with merosin and Drosophila laminin A chain (Garrison et al., 1991; Hortsch and Goodman, 1991), SN-peptide was 30% and 21% identical and 47% and 40% similar, respectively (Table IV).

Since the laminin A chain is replaced by the A chain homologue, merosin, in some organs (Ehrig et al., 1990; Engvall et al., 1990; Sanes et al., 1990), we investigated whether laminin A chain was indeed present in rat lung alve-

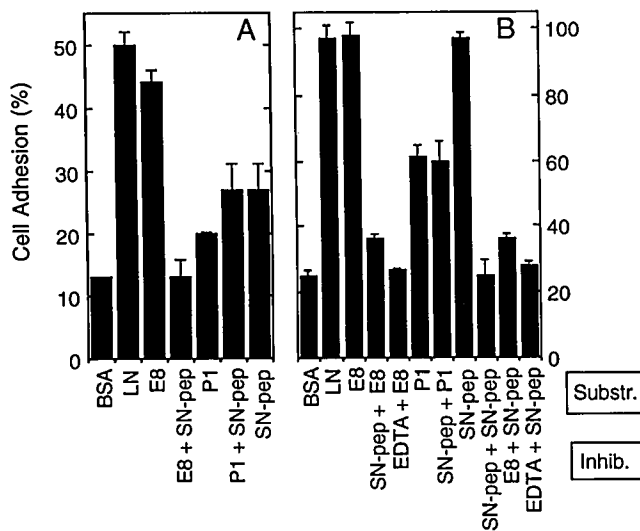


Figure 7. Cells adhere to SN-peptide in an E8 fragment and 2 mM EDTA inhibitable manner. (A) Adhesion of type II alveolar cells to laminin, E8 fragment, and SN-peptide but not BSA. Preincubation of type II cells with E8 fragment competitively inhibited SN-pep adhesion whereas P1 fragment did not. (B) Adhesion of HT1080 cells to SN-peptide and laminin, with BSA as the negative control. E8 fragment inhibited HT1080 cell adhesion to SN-peptide. Similarly, SN-peptide inhibited adhesion to E8 fragment and SN-peptide but not to P1 fragment. Preincubation of 2 mM EDTA with HT1080 cells inhibited adhesion to SN-peptide. Coating and inhibiting concentrations were both 100 μ M corresponding to the inhibiting amounts used in alveolar formation five day time course experiments. Values in A and B represent the mean \pm SD; $n = 9$.

olar basement membranes. We probed fetal lung with antibody ab-A[IK] (Fig. 8) and ab-A[SN], through Western blotting (Fig. 8 A) and immunofluorescence (Fig. 8 C), revealing that laminin A chain was readily detectable in late gestation rat alveolar basement membranes, as had been observed earlier for mouse lung (Klein et al., 1990; Schuger et al., 1991).

Discussion

The results of this study point to a conserved cell adhesion site within the laminin E8 region G domain which plays a key role in alveolar formation in vitro and is present in basement membranes of developing alveoli in vivo. SN-peptide derives from the first of five G domain loops, in keeping with evidence that a major cell adhesion site exists at an un-

Table III. SN-peptide and SINNNR Adhesion Activity

Substrate	Percent cell attachment
Laminin	80 \pm 0.02
E8 fragment	78 \pm 0.05
SN-peptide	76 \pm 0.02
SINNNR	65 \pm 0.09
BSA	21 \pm 0.03

Adhesion of HT1080 cells to SN-peptide, SINNNR, laminin, and E8 fragment with BSA as the negative control; coated at 35 μ M. Data represents mean \pm SD from three experiments performed in triplicate.

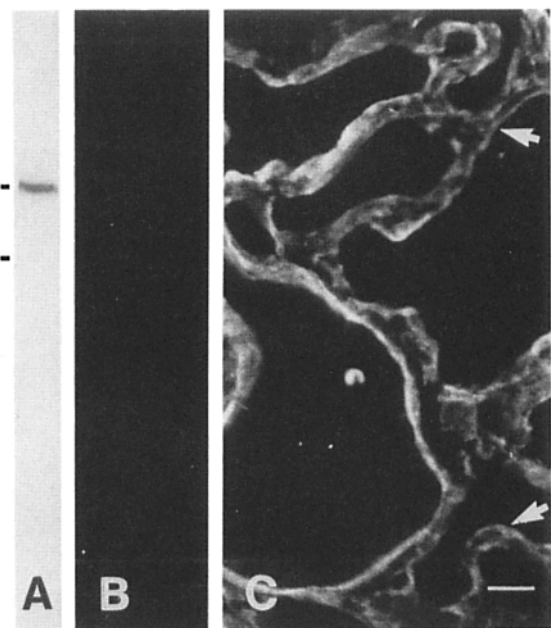


Figure 8. Presence of laminin A chain in fetal rat alveolar basement membranes. (A) Laminin A chain detected in fetal lung homogenate blot using ab-A[IK]. (B) Light micrograph of fetal rat lung incubated with Cy3-labeled secondary antibody alone, as compared with (C) incubation with ab-A[IK] followed by Cy3-labeled secondary antibody. Arrows indicate immunoreactive alveolar basement membranes. Bar, 50 μ m.

identified location within the first three loops (Yurchenco et al., 1993).

We initially determined that isolated type II alveolar cells assembled into alveolar-like structures on gelled BMS, as previously described using Matrigel (Shannon et al., 1987; Adamson et al., 1989). BMS was fractionated by size exclusion chromatography and the high molecular weight peak supported alveolar formation, an activity subsequently identified as laminin using inhibitory antibodies and laminin gels; in agreement with an earlier observation of Rannels who described how an anti-laminin antiserum neutralized the capacity of Matrigel to inhibit alveolar cell spreading (Rannels et al., 1987). Use of proteolytic fragments, inhibitory antibodies, and synthetic peptides progressively localized the alveolarization site first within the laminin E8 region, then to a novel cell adhesive 20 residue sequence (amino acids 2179–2198) within the first loop of the carboxy

Table IV. Conservation of Laminin A Chain G Domain Sequence Between Species and Homologue

Laminin A chain	Sequence
MOUSE	SINNNRWHSIYITRFGNMGS
HUMAN	PIDDNRWHSIHVARFGNIGS
DROS	YVADGRWYQAVVDRMGPNAK
MEROSIN	TIDDSYWYRIVASRTGRNGT

Shaded regions indicate amino acids identical to the mouse laminin sequence. Conservatively substituted amino acids with a comparison value equal to or greater than 0.50 are shown by two dots (:). A single dot (.) indicates a comparison value equal to or greater than 0.10 as defined by the BestFit program.

terminal G domain, and finally to the sub-sequence SINNNR (amino acids 2179–2184). In this manner, and eliminating the possibility of inhibition via cell toxicity, we followed three of Yamada's four criteria for proof of synthetic peptide specificity (Yamada, 1991).

Curiously, SN-peptide was first tested by Sephel et al. (1989; designated "PA10") in a 16-peptide screen for a laminin neurite outgrowth site, wherein SN-peptide was found not to support PC12 cell adhesion (also our own unpublished observations) and neurite outgrowth. In contrast, type II alveolar and HT1080 fibrosarcoma cells adhered to SN-peptide and SINNNR which at a coating concentration of 35 μ M was equivalent to fragment E8 adhesion, and when presented to cells in soluble form at this level completely inhibited adhesion to E8. Moreover soluble fragment E8 but not P1 inhibited SN-peptide dependent adhesion, and divalent cation dependency raised the interesting possibility that an integrin surface receptor may mediate the interaction, the identity of which is under investigation. Whether this site is active for other cells and whether it is non-neuronal specific remains to be determined.

Laminin-driven morphogenesis has been documented in other in vitro lung systems which have examined branching of embryonic airways (attributed by partially neutralizing antibodies to the center of the laminin cross and ends of the short arms; Schuger et al., 1991), and reaggregation of mixed fetal epithelial and mesenchymal lung cells (active region of laminin unknown; Schuger et al., 1992). These studies are made relevant by the early and sustained in vivo presence of embryonic airway and alveolar basement membrane laminin (Gil and Martinez-Hernandez, 1984; Chen et al., 1986) containing A, B1, and B2 chains (Klein et al., 1990; Schuger et al., 1991).

The laminin E8 region has proven to be the most adhesive of all parts of laminin (Timpl, 1989; Aumailley et al., 1990; Drago et al., 1991), an activity used by numerous different cell types in which the $\alpha_6\beta_1$ integrin serves as the most common surface receptor (Sonnenberg et al., 1990; Aumailley and Timpl, 1990; Sorokin et al., 1990; Akiyama, 1990). Since fragment E8 is large, attempts have been made to precisely define adhesive site(s). Initial studies with synthetic peptides identified: (a) IKVAV (Tashiro et al., 1989), a highly conserved A chain adhesive sequence located on the amino side of the G domain whose surface receptor is now known to be the same as that for amyloid precursor protein (Kibbey et al., 1993), and (b) the proposed laminin binding site of the $\alpha_3\beta_1$ integrin receptor, KQNCLSSRASFRGCV-RNLRLSR (amino acids 3011–3032; Gehlsen et al., 1992), located at the carboxy terminus of the G domain; neither of which had any effect on alveolar formation. Another approach has been to systematically test proteolytic subfragments of E8 (Deutzmann et al., 1990) and a recombinant G domain (Yurchenco et al., 1993). These studies have given rise to the interesting conclusion that a key cell adhesion site exists somewhere within the first three loops of the G domain. The manner by which this site may be presented to the cell surface is the subject of discussion. One suggestion, based on experiments with E8 subfragments but apparently incompatible with our data, depicts that a site is formed by folding all or part of the first three loops with the rod domain formed by B1 and B2 chain carboxy termini and associated A chain. An alternative interpretation is that both the site in

the first three loops and another in the rod domain are required for complete E8 adhesion activity (Deutzmann et al., 1990). Differing from these models is the recent observation that full myoblast cell adhesion and spreading activity resides in a recombinant G domain fragment consisting of the first three loops (Yurchenco et al., 1993); whether this property applies to other cell types remains to be determined. The two site possibility would be in keeping with dose response experiments (not shown) in which SN-peptide is less active than E8 at low coating concentrations (such as 10 μ M), much as has been observed in the case of fibronectin wherein RGD plus a second synergistic site are required for full adhesive activity of the central cell binding domain (Obara et al., 1988; Nagai et al., 1991). Identification of SINNNR should greatly facilitate understanding the mechanism by which the E8 region signals cell surface integrin receptors, for which combined use of SINNNR with E8 subfragments and recombinant G domain could be very revealing.

In summary, the combined morphogenic/cell adhesive role of the laminin A chain sequence SINNNR in vitro, taken together with the early appearance of laminin A chain in alveolar basement membranes in vivo, raises the possibility that through receptor interaction the SINNNR site may serve as an important extracellular trigger in early lung alveolar development.

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