

# $\alpha 3\beta 1$ Integrin Is Required for Normal Development of the Epidermal Basement Membrane

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**Abstract.** Integrins  $\alpha 3\beta 1$  and  $\alpha 6\beta 4$  are abundant receptors on keratinocytes for laminin-5, a major component of the basement membrane between the epidermis and the dermis in skin. These integrins are recruited to distinct adhesion structures within keratinocytes;  $\alpha 6\beta 4$  is present in hemidesmosomes, while  $\alpha 3\beta 1$  is recruited into focal contacts in cultured cells. To determine whether differences in localization reflect distinct functions of these integrins in the epidermis, we studied skin development in  $\alpha 3\beta 1$ -deficient mice. Examination of extracellular matrix by immunofluorescence microscopy and electron microscopy revealed regions of disorganized basement membrane in  $\alpha 3\beta 1$ -deficient skin. Disorganized matrix was first detected by day 15.5 of embryonic development and became progressively more extensive as development proceeded. In neonatal skin, matrix disorganization was frequently accompa-

nied by blistering at the dermal-epidermal junction. Laminin-5 and other matrix proteins remained associated with both the dermal and epidermal sides of blisters, suggesting rupture of the basement membrane itself, rather than detachment of the epidermis from the basement membrane as occurs in some blistering disorders such as epidermolysis bullosa. Consistent with this notion, primary keratinocytes from  $\alpha 3\beta 1$ -deficient skin adhered to laminin-5 through  $\alpha 6$  integrins. However,  $\alpha 3\beta 1$ -deficient keratinocytes spread poorly compared with wild-type cells on laminin-5, demonstrating a postattachment requirement for  $\alpha 3\beta 1$  and indicating distinct roles for  $\alpha 3\beta 1$  and  $\alpha 6\beta 4$ . Our findings support a novel role for  $\alpha 3\beta 1$  in establishment and/or maintenance of basement membrane integrity, while  $\alpha 6\beta 4$  is required for stable adhesion of the epidermis to the basement membrane through hemidesmosomes.

**I**NTEGRINS are heterodimeric, transmembrane proteins consisting of an  $\alpha$  and a  $\beta$  subunit that are receptors for cell adhesion to the extracellular matrix (ECM)<sup>1</sup> or to other cells (Hynes, 1992). In the epidermis, basal keratinocytes adhere to the basement membrane through integrins. Human keratinocytes express a number of integrins, including  $\alpha 2\beta 1$ ,  $\alpha 3\beta 1$ ,  $\alpha 5\beta 1$ ,  $\alpha 6\beta 4$ , and  $\alpha v\beta 5$  (reviewed in Watt and Hertle, 1994). Integrin expression is normally restricted to the basal, proliferative cell layer, both in the epidermis and in stratified cultures of keratinocytes (Peltonen et al., 1989; Carter et al., 1990a,b; Hertle et al., 1991; Adams and Watt, 1991). When keratinocytes of the basal

layer withdraw from the cell cycle and become committed to terminal differentiation, they detach from the basement membrane and migrate into the suprabasal layers. This process appears to involve both changes in the ligand-binding activities of certain integrins and changes in expression of integrins on the cell surface (Adams and Watt, 1990; Hotchin and Watt, 1992), and may involve complex interactions between integrins and other classes of adhesion receptors, such as cadherins (Hodivala and Watt, 1994). Changes in integrin expression and function may also be important for keratinocytes during wound healing (Clark, 1990; Cavani et al., 1993), psoriasis (Pelligrini et al., 1992; Carroll et al., 1995), and tumorigenesis (Tennenbaum et al., 1992).

The integrins  $\alpha 3\beta 1$  and  $\alpha 6\beta 4$  are abundant in keratinocytes and function as cell adhesion receptors for laminin-5 (Niessen et al., 1994; Delwel et al., 1994), a member of the laminin family of ECM proteins that was originally described as kalinin (Rousselle et al., 1991), nicein (Verardo et al., 1988), and a component of epiligrin (Carter et al., 1991). Laminin-5 consists of three distinct, multido-

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1. *Abbreviations used in this paper:* ECM, extracellular matrix; HEK, human epidermal keratinocytes; JEB, junctional epidermolysis bullosa.

main chains designated  $\alpha 3$ ,  $\beta 3$ , and  $\gamma 2$  (Burgeson et al., 1994), and is a major component of the epidermal basement membrane. Keratinocyte adhesion to laminin-5 is critical for maintenance of the dermal-epidermal junction in the skin, since mutations in laminin-5 present in inherited junctional epidermolysis bullosa (JEB), and anti-laminin-5 antibodies present in acquired autoimmune diseases such as cicatricial pemphigoid cause blistering of the epidermis from the basement membrane (Domloge-Hultsch, 1992; Aberdam et al., 1994; Pulkkinen et al., 1994a,b; Uitto et al., 1994; Kivirikko et al., 1995; Kirtschig et al., 1995).

Despite their common functions as laminin-5 receptors,  $\alpha 3\beta 1$  and  $\alpha 6\beta 4$  are recruited to distinct cell adhesion structures.  $\alpha 6\beta 4$  is a component of hemidesmosomes (Stepp et al., 1990; Jones et al., 1991), the adherens junctions that anchor basal keratinocytes of the epidermis to the basement membrane;  $\alpha 6\beta 4$  thereby links laminin-5 anchoring filaments outside the cell with the keratin filament network inside the cell. The distribution of  $\alpha 6\beta 4$  to hemidesmosomes is reflected in keratinocytes cultured on laminin-5, where  $\alpha 6\beta 4$  localizes to hemidesmosome-like structures called stable anchoring contacts (Carter et al., 1990b, 1991). In contrast,  $\alpha 3\beta 1$  is recruited to focal contacts in keratinocytes and other cells in culture, and thereby links the ECM to components of the actin cytoskeleton (Carter et al., 1990b; Grenz et al., 1993; DiPersio et al., 1995). These differences in localization between  $\alpha 3\beta 1$  and  $\alpha 6\beta 4$  appear to reflect differences in adhesion-related functions. For example,  $\alpha 6\beta 4$  mediates stable anchorage of keratinocytes to the substrate, while  $\alpha 3\beta 1$  appears to function in cell spreading and migration (Carter et al., 1990b; Xia et al., 1996). Furthermore, cell adhesion through  $\alpha 6\beta 4$  or  $\alpha 3\beta 1$  results in tyrosine phosphorylation of distinct sets of cellular proteins (Kornberg et al., 1991; Mainiero et al., 1995; Jewell et al., 1995; Xia et al., 1996), indicating the activation of distinct signal transduction pathways via these integrins.

In addition to its function as a receptor for laminin-5,  $\alpha 3\beta 1$  has been implicated as a weak receptor for a variety of other ECM proteins, including fibronectin, collagens, laminin-1, entactin/nidogen, and thrombospondin (Wayner and Carter, 1987; Gehlsen et al., 1988; Tomaselli et al., 1990; Elices et al., 1991; Dedhar et al., 1992; Wu et al., 1995; DeFreitas et al., 1995; DiPersio et al., 1995). Although the physiological significance of these weaker interactions is not known, some of these ECM proteins are present in the epidermal basement membrane (Martin, 1987; Timpl, 1989) or in the provisional matrix during wound healing (Raugi et al., 1987; Clark, 1990; Cavani et al., 1993) and, therefore, may be ligands for  $\alpha 3\beta 1$  in skin.

Mutation or altered expression of components of hemidesmosomes and the keratin filament network have been shown to be important for cell adhesion and/or mechanical integrity at the dermal-epidermal junction. These include either subunit of integrin  $\alpha 6\beta 4$  (Georges-Labouesse et al., 1996; van der Neut et al., 1996; Dowling et al., 1996), bullous pemphigoid antigen 1 (BPAG1; Guo et al., 1995), BPAG2 (McGrath et al., 1995), and keratin 14 (Coulombe et al., 1991). In contrast, the role of integrin  $\alpha 3\beta 1$  in skin has been unclear. We have studied the skin of  $\alpha 3\beta 1$ -deficient mice generated by null mutation of the gene encoding the  $\alpha 3$  integrin subunit (Kreidberg et al., 1996). Mice homozygous for the  $\alpha 3$ -null mutation die shortly after birth, possi-

bly due to defects in kidney and/or lung organogenesis (Kreidberg et al., 1996). In this report, we used immunofluorescence and ultrastructural analysis to show that the epidermal basement membrane becomes disorganized during skin development in  $\alpha 3$ -null mice. By birth, matrix disorganization was correlated with blistering of the epidermis from the dermis. Surprisingly, all basement membrane proteins tested localized to both the epidermal and dermal sides of these blisters, identifying a novel form of epidermal blistering caused by rupture of the basement membrane itself rather than by detachment of the epidermis from basement membrane ligands. Consistent with this blistering mechanism, primary keratinocytes from  $\alpha 3$ -null, neonatal mice could still attach to laminin-5 through  $\alpha 6$  integrins; however,  $\alpha 3\beta 1$  was required for postattachment spreading of keratinocytes on laminin-5. Our results support distinct but overlapping roles, rather than redundant functions, for  $\alpha 3\beta 1$  and  $\alpha 6\beta 4$  in keratinocytes and reflect distinct roles for these integrins in the epidermis.

## Materials and Methods

### Antibodies

Rabbit antisera against the cytoplasmic domains of the human  $\beta 1$  subunit and the chicken  $\alpha 3A$  subunit were prepared as described (Marcantonio and Hynes, 1988; Hynes et al., 1989; DiPersio et al., 1995). Rabbit antiserum against the cytoplasmic domain of the  $\alpha 6A$  subunit, and the monoclonal antibody 346-11A against the  $\beta 4$  subunit, were kindly provided by V. Quaranta (Scripps Research Institute, La Jolla, CA) and Stephen Kennel (Oak Ridge National Laboratory, Oak Ridge, TN), respectively. The monoclonal antibody GoH3 against the  $\alpha 6$  integrin subunit was purchased from Immunotech (Westbrook, ME). Rabbit antisera against laminin-5 and type VII collagen were gifts from R. Burgeson (Massachusetts General Hospital, Charlestown, MA). Rabbit antiserum against entactin was a gift from A. Chung (University of Pittsburgh, Pittsburgh, PA). Rabbit antiserum against rat plasma fibronectin was prepared as described (Mautner and Hynes, 1977). Rabbit antiserum specific for the E11B segment of fibronectin was raised against an E11B-GST fusion protein and immunopurified, as described (Peters et al., 1995).

### Genotyping of Mice by PCR

Embryonic or neonatal offspring were collected from matings of mice heterozygous for a null mutation of the  $\alpha 3$  integrin gene (Kreidberg et al., 1996), and their tails were removed and digested overnight at 56°C in 0.2% SDS, 100 mM Tris, pH 6.8, 200 mM NaCl, 5 mM EDTA, and 0.1 mg/ml proteinase K. DNA was precipitated with isopropanol, dissolved in 50  $\mu$ l–100  $\mu$ l of 10 mM Tris (pH 7.5) and 0.1 mM EDTA. PCR analysis was carried out using 1  $\mu$ l of template DNA and the following oligonucleotide primers: wild-type primer (from  $\alpha 3$  gene): 5'-CCGCTATGTCTTCATGAACC-3';  $\alpha 3$ -knockout primer (neomycin-resistance gene): 5'-GGG-GAACTCCTGACTAG-3'; and common primer ( $\alpha 3$  gene): 5'-GGA-ATCCATCCTGGTTGATGTC. PCR reaction conditions were as follows: denaturation at 94°C for 30 s; extension at 55°C for 40 s; and annealing at 72°C for 30 s. Thirty amplification cycles were performed. The wild-type and common primers amplified a 130-bp fragment from the wild-type  $\alpha 3$  gene; and the  $\alpha 3$ -knockout and common primers amplified a 285-bp fragment from the targeted allele. Amplified PCR products were separated by electrophoresis on a 1.5% agarose gel and visualized by staining with ethidium bromide.

### Preparation of Frozen Sections and Immunofluorescence

Limbs were removed from neonatal mice after sacrifice by CO<sub>2</sub> narcosis, and then embedded and frozen in O.C.T. compound and stored at -80°C. Embryos were collected by Cesarean section at days 11.5, 15.5, or 17.5 postcoitum, and limbs or torso skin (day 17.5) or whole embryos (days

11.5 and 15.5) were embedded as described above. 8- $\mu$ m sections were cut on a Reichert-Jung cryostat (model 2800 Frigocut-E), and were either stained with hematoxylin and eosin or prepared for immunofluorescence. For immunofluorescence, frozen sections were hydrated in PBS plus 0.9 mM CaCl<sub>2</sub> and 0.5 mM MgCl<sub>2</sub> (PBS/Ca<sup>2+</sup>Mg<sup>2+</sup>) for 5 min. Since recognition of fibronectin by the anti-E11B serum is inhibited by N-linked oligosaccharides, sections to be stained with anti-E11B were incubated at 37°C for 2 h with N-glycanase (PNGase F, New England Biolabs, Beverly, MA) at 50,000 U/ml in reaction buffer provided by the manufacturer, as described previously (Peters and Hynes, 1996). Sections were fixed in 3.7% formaldehyde for 10 min and then incubated in blocking buffer (0.1% BSA, 0.2% Triton X-100, 0.1% glycine in PBS/ Ca<sup>2+</sup>Mg<sup>2+</sup>) for at least 30 min. Sections were stained with rabbit antisera (diluted in blocking buffer at 70  $\mu$ g/ml for immunopurified anti-E11B, or at 1:100 for other antisera) for at least 1 h, followed by biotin-conjugated goat anti-rabbit Ig (1:200 dilution; TAGO Biosource, Camarillo, CA) for 30 min and then avidin-FITC (1:100 dilution; Sigma Chemical Co., St. Louis, MO) for 30 min. For double-label immunofluorescence, a monoclonal antibody against either the  $\alpha$ 6 integrin subunit (GoH3) or the  $\beta$ 4 integrin subunit (346-11A) was included in the incubation with primary antibodies, followed by Texas red-conjugated goat anti-rat IgG (Oncogene Science, Manhasset, NY) at a concentration of 1  $\mu$ g/ml. Representative fields were photographed on a Zeiss Axiophot microscope (Thornwood, NY). Control experiments showed that neither bleed-through of fluorescein to Texas red nor bleed-through in the opposite direction occurs using our conditions.

### Ultrastructural Analysis of the Epidermal Basement Membrane

Limbs from neonatal mice were prepared as described above, and feet were removed and prepared for electron microscopy as follows: samples were fixed in 2.5% glutaraldehyde/2.5% paraformaldehyde in 0.1 M cacodylate buffer, pH 7.2, for 1 h at 4°C, washed in 0.1 M cacodylate buffer, postfixed in 2% osmium tetroxide in cacodylate buffer for 2 h at 4°C, and then washed in cacodylate buffer. Samples were then dehydrated in ethanol and propylene oxide and embedded in Spurr resin for 48 h at 60°C. Ultrathin sections (70 nm) were cut on a Leica microtome, mounted onto 200 mesh copper grids, stained with uranyl acetate and lead citrate, and viewed on a JEOL 1200EX electron microscope (Peabody, MA).

### Isolation and Culture of Primary Mouse Keratinocytes

Epidermal keratinocytes were prepared from neonatal mice essentially as described previously (Dlugosz et al., 1995). Briefly, newborn mice were sacrificed by CO<sub>2</sub> narcosis, washed in 10% iodine solution (Mallinckrodt, Paris, KY) in PBS for 10 min, rinsed with PBS, washed in 70% ethanol for 10 min, and then rinsed in PBS. Tails were removed and used for genotyping by PCR; limbs were removed and used to prepare frozen skin sections (see above). Skins were removed from the torso and head, and then floated on 0.25% trypsin solution (GIBCO BRL, Gaithersburg, MD) overnight at 4°C, with the epidermis facing upward. Skins were then transferred to a dry, sterile surface with the epidermis facing down, and the dermis was separated from the epidermis and discarded. The epidermis was minced, suspended in growth medium (see below), and agitated to release keratinocytes. Suspensions were passed through a sterile, 70- $\mu$ m nylon filter (Becton Dickinson, Mountain View, CA) to remove cornified sheets. Keratinocytes were seeded onto tissue culture plates coated with 30  $\mu$ g/ml denatured rat tail collagen (Collagen Corp., Palo Alto, CA) at a density of  $\sim$ 2–4  $\times$  10<sup>5</sup> cells/cm<sup>2</sup>. To prevent differentiation of keratinocytes, cultures were grown in low calcium medium consisting of Eagle's Minimum Essential Medium (EMEM; BioWhittaker, Walkersville, MD) supplemented with 4% FBS (Intergen, Purchase, NY) from which Ca<sup>2+</sup> had been removed by chelation (Brennen et al., 1982), 0.05 mM CaCl<sub>2</sub>, "HICE" mix (0.5  $\mu$ g/ml hydrocortisone [Calbiochem-Novabiochem Corp., La Jolla, CA], 5  $\mu$ g/ml insulin [Sigma], 10<sup>-10</sup> M cholera toxin [ICN Biomedicals, Inc., Costa Mesa, CA], 10 ng/ml epidermal growth factor [Upstate Biotechnology, Lake Placid, NY], 2  $\times$  10<sup>-9</sup> M T3 [Sigma]), 100 U/ml penicillin and 100  $\mu$ g/ml streptomycin (GIBCO BRL). Mouse keratinocytes were cultured at 34°C, 7.5% CO<sub>2</sub> for 5–7 d before use in experiments.

### Iodination and Immunoprecipitation of Integrins

Mouse keratinocyte cultures were grown for 7 d, and monolayers were

surface-labeled with 0.5 mCi/10-cm plate of Na<sup>125</sup>I (New England Nuclear, Boston, MA) using the lactoperoxidase-glucose oxidase method (Hynes, 1973). Cells were washed four times with 50 mM NaI in PBS/ Ca<sup>2+</sup>Mg<sup>2+</sup> and lysed for 15 min on ice in 1 ml of a detergent buffer containing 200 mM octyl- $\beta$ -D-glucopyranoside (Calbiochem), 50 mM Tris, pH 7.5, 150 mM NaCl, 1 mM CaCl<sub>2</sub>, 1 mM MgCl<sub>2</sub>, 2 mM PMSF (Sigma), 0.02 mg/ml aprotinin (Sigma), and 0.0125 mg/ml leupeptin (Calbiochem). Lysates were sedimented for 10 min at 10,000 g. Supernatants were preincubated with 100  $\mu$ l of protein A-Sepharose (1:1 slurry; Pharmacia LKB, Piscataway, NJ) for 1 h and the beads sedimented for 2 min at 10,000 g. Protein concentrations of supernatants were determined using a Bio-Rad kit, and equal amounts of protein were immunoprecipitated with anti-integrin antibodies as described (Marcantonio and Hynes, 1988). Briefly, BSA was added to lysates (180  $\mu$ g total protein) to a final concentration of  $\sim$ 3 mg/ml, followed by 5–10  $\mu$ l of antiserum. After incubation at 4°C for 1 h, 50  $\mu$ l of protein A-Sepharose (1:1 slurry preabsorbed with 10 mg/ml BSA in lysis buffer) was added to reactions. Reactions were incubated overnight at 4°C. Samples were washed four times with cold lysis buffer plus protease inhibitors, and samples were suspended in sample buffer (2% SDS, 80 mM Tris-HCl, pH 6.8, 2 mM EDTA, 10% glycerol, and bromophenol blue) and boiled for 5 min. Nonreducing SDS-PAGE was performed by the method of Laemmli (1970) using 5% acrylamide and a 3% stacking gel.

### Preparation of Laminin-5-rich Extracellular Matrix from Keratinocytes

To prepare laminin-5-rich ECM, human epidermal keratinocytes (HEKs) were either prepared from neonatal foreskin as described (Rheinwald and Green, 1975) or purchased from Clonetics (San Diego, CA) and grown, respectively, in either FAD medium (1:3 mix of Ham's F12 and DMEM), 1.8  $\times$  10<sup>-4</sup> M adenine, 10% FBS, HICE mix, 100 U/ml penicillin, and 100  $\mu$ g/ml streptomycin) or serum-free Keratinocyte Growth Medium (GIBCO BRL) supplemented with EGF and bovine pituitary extract, as directed by the manufacturer. Cells were grown on 35-mm tissue culture plates (Becton Dickinson) for several days, removed with 0.05% trypsin, 1 mM EDTA in PBS, and discarded. Surfaces coated with HEK-secreted ECM were treated with 0.5 mg/ml soybean trypsin inhibitor (Sigma) in PBS and then blocked with 1 mg/ml BSA in PBS.

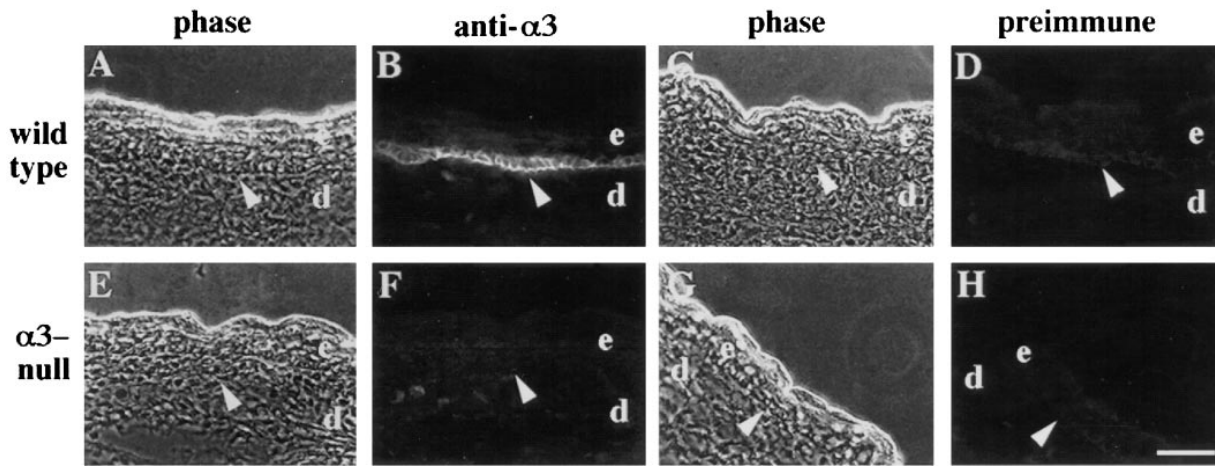
### Cell Spreading Assays

Purified human laminin-5 was kindly provided by R. Burgeson (Massachusetts General Hospital, Charlestown, MA). 96-well tissue culture plates (Costar Corp., Cambridge, MA) were coated overnight at 4°C with laminin-5 dissolved in PBS at a concentration of 20  $\mu$ g/ml; in some cases rat fibronectin (GIBCO BRL) was included at a concentration of 20  $\mu$ g/ml. Coated plates were blocked with 2 mg/ml heat-denatured BSA in PBS for at least 1 h. Cultures of primary mouse keratinocytes were split with 0.05% trypsin and subcultured onto coated plates. For subculture onto laminin-5-rich, HEK-secreted matrix, mouse keratinocytes were seeded at a density of 10<sup>5</sup> cells/cm<sup>2</sup>, allowed to adhere for 1.5 h at 34°C, and then photographed. For spreading assays on purified laminin-5, mouse keratinocytes were seeded at a density of 3  $\times$  10<sup>4</sup> cells/well, allowed to adhere for 1 h at 34°C, and nonattached cells were washed gently away with PBS. In some cases, the monoclonal antibody GoH3, which specifically blocks adhesion through  $\alpha$ 6 integrins, was added to cells before plating at a concentration of 2  $\mu$ g/ml. Adhered cells were then fixed with 4% paraformaldehyde, stained with 0.02% Giemsa (Sigma), and photographed.

## Results

### Formation of Skin Blisters in Mice Deficient for $\alpha$ 3 $\beta$ 1 Integrin

Frozen sections were prepared from the limbs of  $\alpha$ 3-null or wild-type mice and stained by immunofluorescence with an antiserum against the cytoplasmic domain of the  $\alpha$ 3 subunit (DiPersio et al., 1995). In wild-type mice,  $\alpha$ 3 expression was restricted to the basal layer of keratinocytes in the epidermis (Fig. 1, A and B, arrows), as expected for this integrin (Peltonen et al., 1989; Carter et al., 1990a,b;

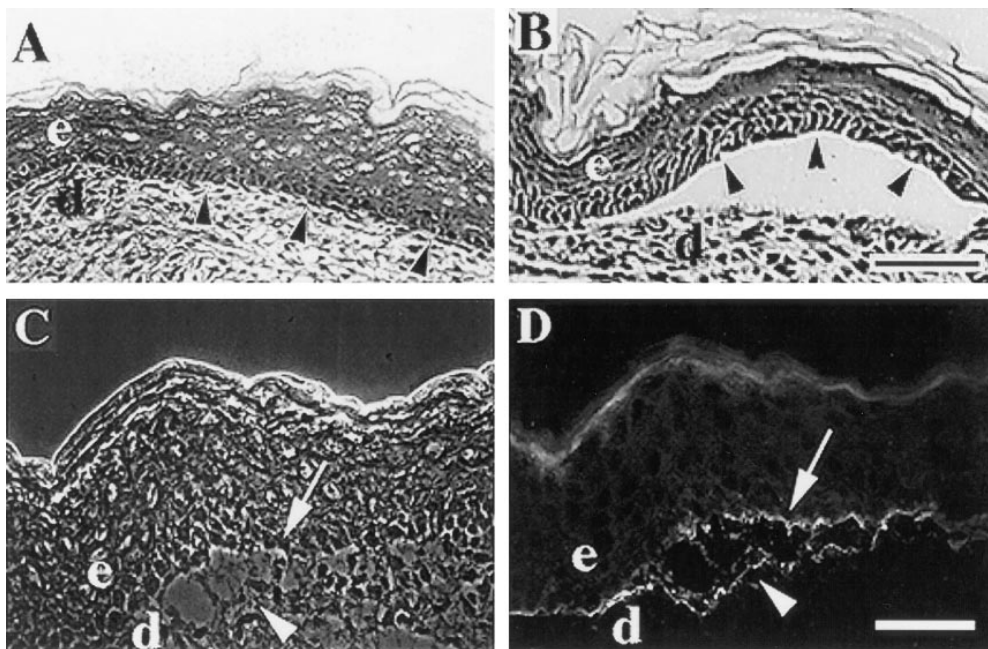


**Figure 1.**  $\alpha 3\beta 1$  integrin is absent from the skin of  $\alpha 3$ -null mice. Frozen skin sections were prepared from the limbs of wild-type (A–D) or  $\alpha 3$ -null (E–H) mice and stained by immunofluorescence with an antiserum against the cytoplasmic domain of  $\alpha 3$  (B and F), or with the preimmune serum (D and H). The corresponding phase contrast (A, C, E, and G) is shown to the left of each immunofluorescence panel. e, epidermis; d, dermis. Arrowheads point to basal keratinocytes of the epidermis. Bar, 50  $\mu\text{m}$ .

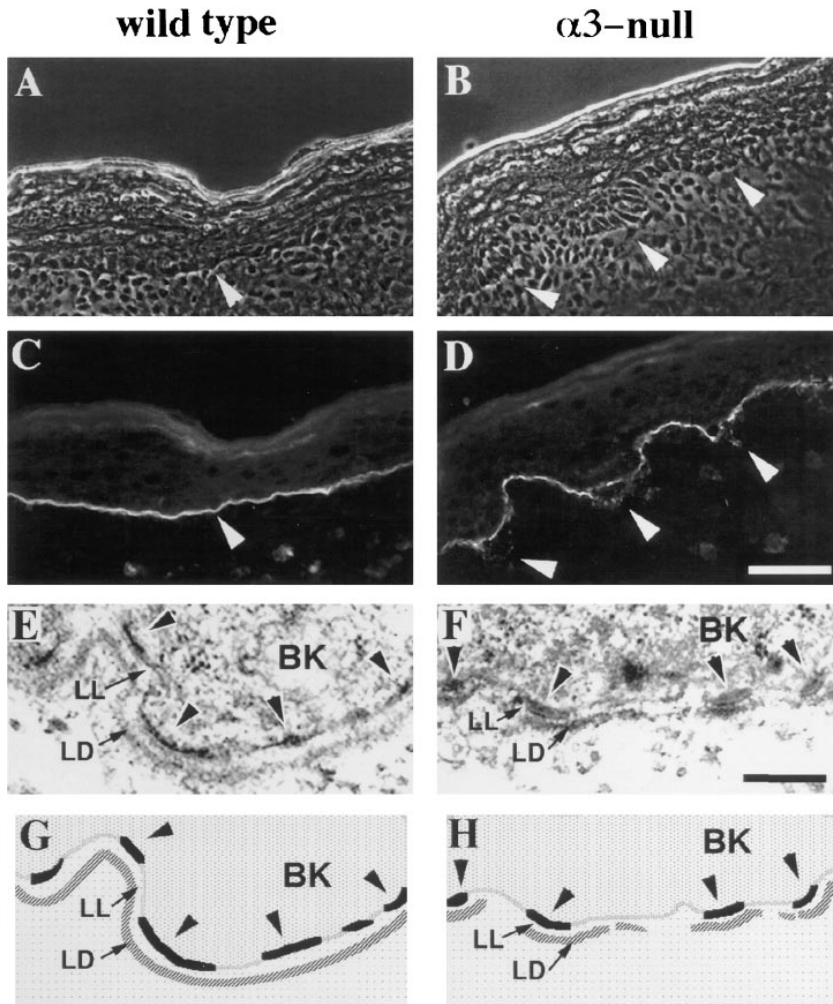
Hertle et al., 1991). Preimmune serum did not stain the epidermis (Fig. 1, C and D). In  $\alpha 3$ -null mice, stratification of the epidermis appeared normal (Figs. 1 E and 2 B). However, staining of the basal keratinocytes of  $\alpha 3$ -null epidermis with anti- $\alpha 3$  (Fig. 1 F, arrow) was comparable to background staining with preimmune serum (Figs. 1, G and H). The absence of  $\alpha 3\beta 1$  from  $\alpha 3$ -null skin was confirmed by Western blotting (data not shown).  $\alpha 3$  staining intensity of basal keratinocytes in mice that were heterozygous for the  $\alpha 3$  mutation was intermediate (data not shown).

Examination of frozen skin sections from  $\alpha 3$ -null mice revealed occasional blisters where the epidermis was separated from the dermis (Fig. 2 B). Basal keratinocytes of the epidermis were intact over blistered regions (Fig. 2 B, arrowheads). Blisters appeared to be concentrated on the

legs and footpads, possibly due to physical trauma to these regions within the first several hours after birth, and were never found in skin sections from wild-type mice (Fig. 2 A) or heterozygous mice (not shown). Epidermal blistering in the  $\alpha 3$ -null mice is reminiscent of more severe blistering phenotypes associated with loss of  $\alpha 6\beta 4$  integrin through null mutations of either the  $\alpha 6$  (Georges-Labouesse et al., 1996) or the  $\beta 4$  subunit (van der Neut et al., 1996; Dowling et al., 1996) and with human blistering diseases caused by defects in laminin-5 (Domloge-Hultsch, 1992; Baudoin et al., 1994; Kirtschig et al., 1995), the ligand for both  $\alpha 6\beta 4$  and  $\alpha 3\beta 1$ . In these cases, extreme blistering appears to result from detachment of basal keratinocytes from the basement membrane due either to the absence of  $\alpha 6\beta 4$  integrin or to deficiencies in its ligand laminin-5. Indeed, in



**Figure 2.**  $\alpha 3$ -null mice form skin blisters. (A and B) Frozen skin sections from wild-type (A) or  $\alpha 3$ -null (B) mice were stained with hematoxylin and eosin and the epidermal-dermal junctions were compared. Arrowheads point to basal keratinocytes of the epidermis. (C and D) A frozen skin section from an  $\alpha 3$ -null mouse showing a blister viewed by phase contrast (C) or stained by immunofluorescence with an antiserum against laminin-5 (D). Arrowheads and arrows point to areas of laminin-5 staining at the dermal and epidermal sides of the blister, respectively. e, epidermis; d, dermis. Bars, 50  $\mu\text{m}$ .



**Figure 3.** The basement membrane in  $\alpha 3$ -null skin is disorganized. (A–D) Frozen skin sections from wild-type (A and C) or  $\alpha 3$ -null (B and D) mice were viewed by phase contrast (A and B) or stained by immunofluorescence with an antiserum against laminin-5 (C and D). Arrowheads point to areas of laminin-5 staining at the basement membrane in the wild-type skin (A and C) or at regions of disorganized basement membrane in  $\alpha 3$ -null skin (B and D). (E and F) Electron micrographs comparing ultrastructure of the basement membrane zone in wild-type (E) and  $\alpha 3$ -null (F) skin. (G and H) Schematic illustration of relevant structures seen in E and F. BK, basal keratinocyte; LL, lamina lucida; LD, lamina densa; arrowheads point to hemidesmosomes along the basal aspect of the plasma membranes in the basal keratinocytes. Bars: (D) 50  $\mu\text{m}$ ; (F) 200 nm.

$\alpha 6$ -null or  $\beta 4$ -null skin, laminin-5 remains associated with the dermal sides of blisters (Georges-Labouesse et al., 1996; van der Neut et al., 1996; Dowling et al., 1996). Therefore, the blistering phenotype in the  $\alpha 3$ -null epidermis appeared consistent with a role for  $\alpha 3\beta 1$  as a receptor for laminin-5 or other basement membrane ligands.

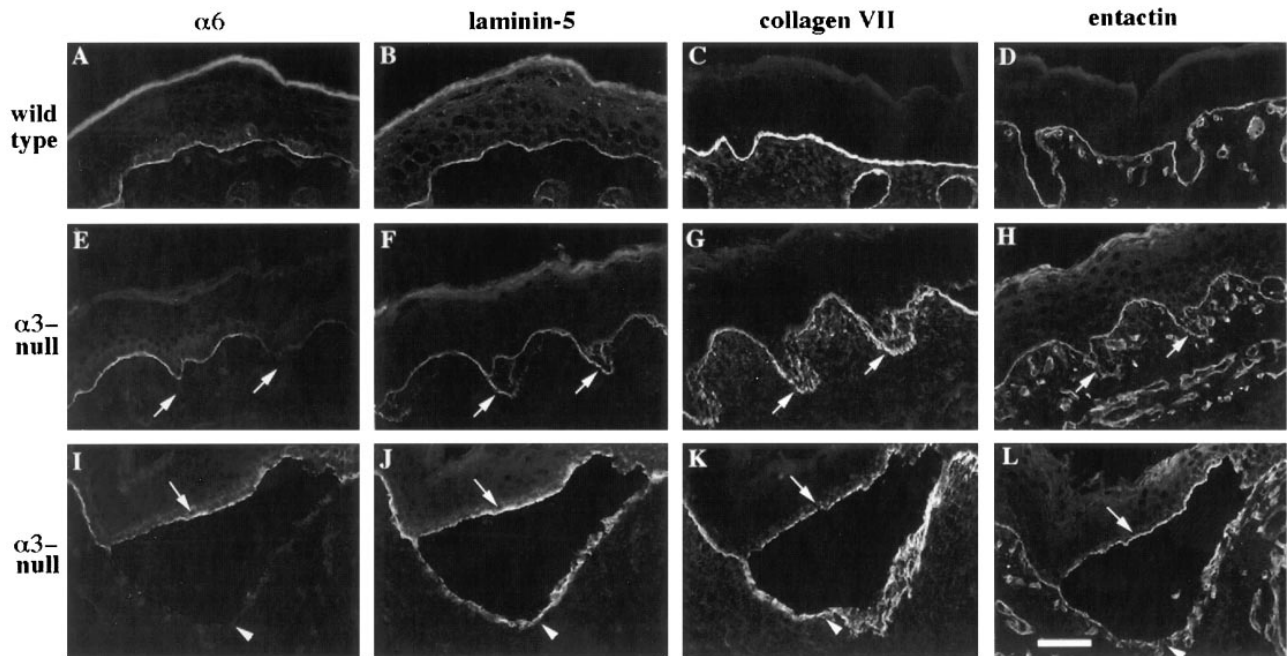
To determine the distribution of laminin-5 within blisters of  $\alpha 3$ -null skin, we stained frozen skin sections from neonatal mice with a polyclonal antiserum against laminin-5 (Marinkovich et al., 1992). Surprisingly, laminin-5 was detected at both the epidermal and dermal sides of blisters, and occasionally in what appeared to be remnants of matrix extending through the blisters (Fig. 2, C and D), suggesting that the split occurred within the basement membrane rather than between the basal keratinocytes and the basement membrane. Therefore, skin blisters in mice lacking  $\alpha 3\beta 1$  are distinct from those seen in mice lacking  $\alpha 6\beta 4$ .

#### Defects in Basement Membrane Organization in $\alpha 3$ -Null Mice

Given the rupture of the basement membrane in blistered regions of  $\alpha 3$ -null skin, we also examined basement membrane organization in nonblistered regions. As expected, laminin-5 in wild-type skin was restricted to the basement

membrane directly below the basal keratinocytes of the epidermis (Fig. 3, A and C, arrowheads); the basement membrane appeared intact throughout each section examined. In marked contrast, staining of skin from  $\alpha 3$ -null mice revealed extensive regions of matrix disorganization, where laminin-5 was also detected below the plane of the basement membrane (Fig. 3 D, arrowheads). Phase contrast of the same field showed that the epidermis and dermis were intact through these regions (Fig. 3 B). In some cases, dense concentrations of laminin-5, resembling continuous remnants of basement membrane, extended down into the dermal regions (for example, see Fig. 4 F). Laminin-5 staining of the basement membrane appeared normal in mice heterozygous for the  $\alpha 3$ -null mutation, where  $\alpha 3\beta 1$  levels were reduced but still easily detectable (not shown). Based on the disorganized matrix/blistering phenotype, we were routinely able to identify skin sections from  $\alpha 3$ -null mice before confirmation of genotype by PCR or Southern blot analysis.

We also examined the ultrastructure of the dermal-epidermal junction. The results of these analyses, shown in Fig. 3, E and F, are illustrated schematically in Fig. 3, G and H, respectively. In neonatal skin from wild-type mice (Fig. 3, E and G), the electron-dense hemidesmosomes (arrowheads) were detected along the basal surfaces of keratinocytes adjacent to the lamina lucida (LL) of the base-



**Figure 4.** Entactin and type VII collagen codistribute with laminin-5 over regions of disorganized basement membrane and to both dermal and epidermal sides of blisters in  $\alpha 3$ -null skin. Frozen sections from neonatal skin were stained by double-label immunofluorescence with GoH3 monoclonal antibody against the  $\alpha 6$  integrin subunit and antiserum against laminin-5 (A, E, I, and B, F, J, respectively), or by immunofluorescence with antisera against type VII collagen (C, G, and K) or entactin (D, H, and L). (A–D) Representative fields from wild-type skin. (E–H) Adjacent sections through a region of disorganized basement membrane in  $\alpha 3$ -null skin; arrows point to areas in the dermis, outside the basement membrane zone as defined by  $\alpha 6$ -staining (E) where basement membrane proteins are detected (F–H). (I–L) Adjacent sections through a blister in  $\alpha 3$ -null skin; arrowheads and arrows point to the dermal and epidermal sides of the blister, respectively. Bar, 50  $\mu\text{m}$ .

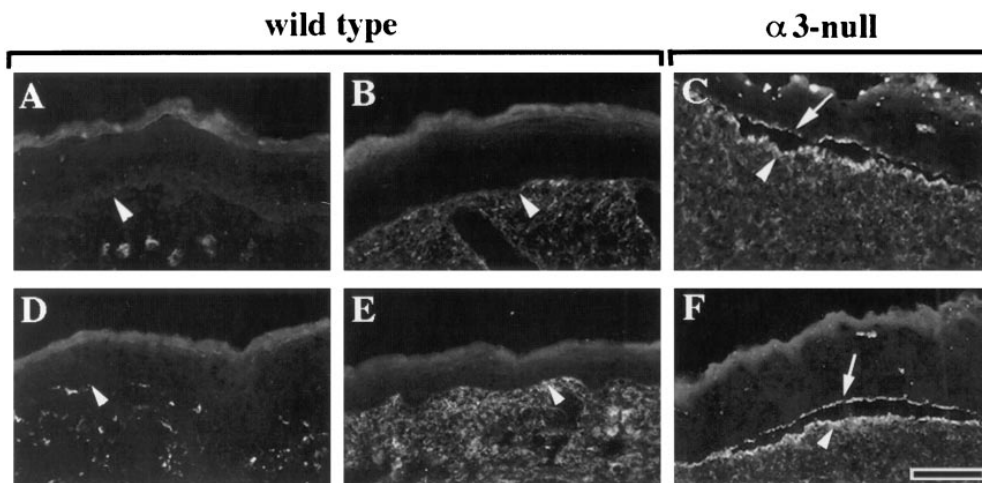
ment membrane zone, and the lamina densa (LD) of the basement membrane was visible as a continuous, electron-dense zone below the lamina lucida. In neonatal skin from  $\alpha 3$ -null mice (Fig. 3, F and H), hemidesmosomes were present on the basal keratinocytes and were of comparable size and frequency to those seen in wild-type skin. However, in contrast with wild-type skin, the lamina densa of  $\alpha 3$ -null skin appeared discontinuous in sections from several animals; electron-dense material was associated with regions directly beneath and adjacent to hemidesmosomes but was reduced in or absent from regions between hemidesmosomes. Therefore, disorganization of the basement membrane is also evident at the ultrastructural level in  $\alpha 3$ -null skin.

Since hemidesmosomes appeared normal at the ultrastructural level in  $\alpha 3$ -null skin, we also examined the expression and distribution of  $\alpha 6\beta 4$ , a component of hemidesmosomes (Stepp et al., 1990; Jones et al., 1991). The  $\alpha 6$  subunit also associates with the  $\beta 1$  subunit in some cells (Hynes, 1992). However, most or all  $\alpha 6$  in the epidermis appears to be associated with  $\beta 4$  (Watt and Hertle, 1994), and little or no  $\alpha 6$  was detected in skin from  $\beta 4$ -null mice (van der Neut et al., 1996; Dowling et al., 1996). In addition, we detected no  $\alpha 6\beta 1$  in primary mouse keratinocytes (discussed below), and the distributions of  $\alpha 6$  and  $\beta 4$  were identical in wild-type and  $\alpha 3$ -null skin. Double-label immunofluorescence of wild-type skin, using anti-laminin-5 serum and the anti- $\alpha 6$  monoclonal antibody GoH3 (Sonnenberg et al., 1987), showed that  $\alpha 6$  subunit was concen-

trated at the basal surfaces of keratinocytes (Fig. 4 A) adjacent to laminin-5 in the basement membrane (Fig. 4 B), as expected for  $\alpha 6\beta 4$  (Carter et al., 1990b). Although  $\alpha 6$  showed the same distribution in  $\alpha 3$ -null epidermis, it did not codistribute with laminin-5 when the latter was detected below the plane of the basement membrane in regions of disorganized matrix (compare Fig. 4, E and F). Therefore, double-label immunofluorescence, with antibodies against either the  $\alpha 6$  or the  $\beta 4$  integrin subunit and laminin-5, was used in all subsequent analyses to confirm the presence of disorganized basement membrane.

In contrast with laminin-5,  $\alpha 6$  remained confined to the basal surfaces of basal keratinocytes on the epidermal sides of blisters (compare Fig. 4, I and J) and occasionally showed a discontinuous distribution over blisters (not shown). Distribution of laminin-5 to the epidermal sides of blisters was likely due to its binding to  $\alpha 6\beta 4$  on the basal keratinocytes, consistent with our adhesion studies using cultured keratinocytes, described below.

Type VII collagen and entactin, both components of the epidermal basement membrane (Martin, 1987; Timpl, 1989; Burgeson, 1993), were each detected in the basement membrane of wild-type epidermis (Fig. 4, C and D, respectively); entactin was also detected in the basement membranes of capillaries throughout the dermis (Fig. 4 D). Staining of adjacent sections from  $\alpha 3$ -null skin showed that type VII collagen and entactin each codistributed with laminin-5 to regions of disorganized basement membrane (compare Fig. 4, F–H, arrows). In addition, staining of ad-



**Figure 5.** Fibronectin distributes to both epidermal and dermal sides of blisters in  $\alpha 3$ -null skin. Frozen sections from wild-type (A, B, D, and E) or  $\alpha 3$ -null (C and F) skin were stained with an antiserum against fibronectin (B and C) or the preimmune serum (A), or with antiserum specific for the EIIIB segment of fibronectin (D–F). Recognition of fibronectin by anti-EIIIB requires treatment with N-glycanase (E and F), as described previously (Peters and Hynes, 1996); as a control, the section in D was not treated

with N-glycanase. (A, B, D, and E) Arrowheads point to the dermal-epidermal junction. (C and F) Arrowheads and arrows point to the dermal and epidermal sides of blisters, respectively. Bar, 50  $\mu$ m.

Adjacent sections through a blister showed that collagen VII and entactin, like laminin-5, each distributed to both the epidermal and dermal sides of the split (compare Fig. 4, J–L). Similar staining (not shown) was seen with an antiserum expected to recognize laminin isoforms containing the  $\alpha 1$ ,  $\beta 1$ , or  $\gamma 1$  chain (see Burgeson et al., 1994), including the basement membrane protein laminin-6 ( $\alpha 3$ ,  $\beta 1$ , and  $\gamma 1$ ), but not laminin-5 ( $\alpha 3$ ,  $\beta 3$ , and  $\gamma 2$ ).

An antiserum against fibronectin produced a fibrillar pattern of staining throughout the dermis (Fig. 5, A and B) and occasionally showed concentrated staining in the basement membrane zone (not shown), as reported previously during embryonic development (Peters and Hynes, 1996). An antiserum specific for the EIIIB segment of fibronectin produced similar staining patterns (Fig. 5, D and E). In  $\alpha 3$ -null skin, total fibronectin (Fig. 5 C) and EIIIB+ fibronectin (Fig. 5 F) were each detected at both the epidermal and dermal sides of blisters. Since levels of EIIIB+ fibronectin are normally very low in serum (Peters et al., 1995), these staining patterns suggest that fibronectin present in the blisters is derived from extracellular matrix rather than from infiltrating serum. However, we cannot rule out some contribution of serum fibronectin to the fibronectin detected in blisters. Although the role of fibronectin as a component of the epidermal basement membrane has been controversial (Hynes, 1990), our findings imply an association of fibronectin with the basal keratinocytes of the epidermis. Considered together, the distributions of the various matrix proteins indicate that blister formation in the  $\alpha 3$ -null epidermis is associated with disorganization and rupture of the basement membrane itself rather than with a simple defect in adhesion of the epidermis to the basement membrane.

#### **Distributions of Basement Membrane Proteins in Developing Skin**

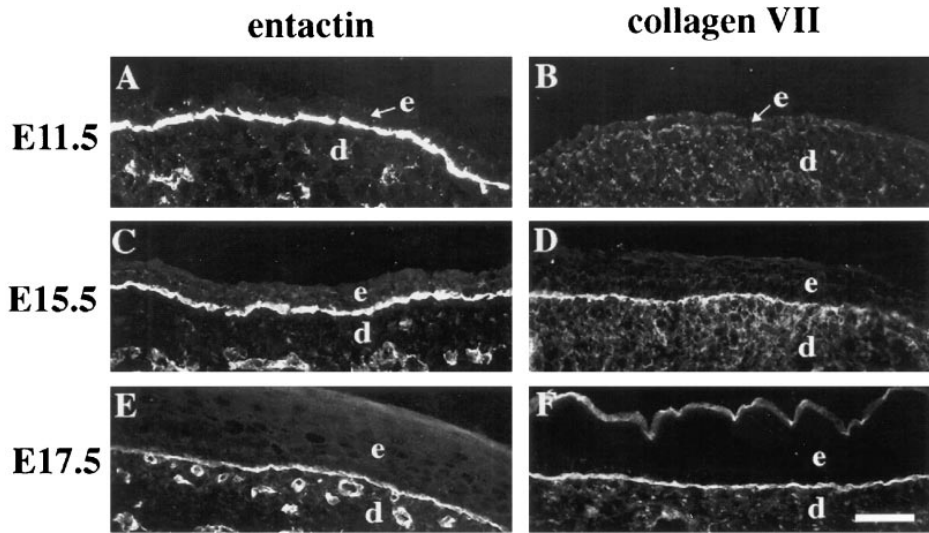
To compare skin development and basement membrane formation in normal and  $\alpha 3$ -null mice, frozen sections were prepared from embryos at days 11.5 (E11.5), 15.5 (E15.5), and 17.5 (E17.5) postcoitum. Phase contrast mi-

croscopy (not shown) revealed that skins from  $\alpha 3$ -null, wild-type, or heterozygous embryos were morphologically indistinguishable at each of these developmental stages, and appeared to stratify normally (for a review of skin development, see Watt and Hertle, 1994). The embryonic epidermis at E11.5 is composed of a basal cell layer and an outer cell layer (the periderm) and was distinguishable as a thin sheet of cells surrounding the embryo. At E15.5, the epidermis showed varying degrees of stratification. By E17.5, 2–3 d before birth, the epidermis was clearly stratified and resembled that of a newborn mouse.

The developmental expression and general distributions of several ECM proteins were identical in skin from wild-type, heterozygous, and  $\alpha 3$ -null embryos at each developmental stage examined; therefore, panels in Fig. 6 show representative data for entactin and type VII collagen. Entactin clearly defined a basement membrane zone at the dermal-epidermal junction at E11.5 (Fig. 6 A), E15.5 (Fig. 6 C), and E17.5 (Fig. 6 E). Type VII collagen was detected throughout the dermis at each of these stages, as it was at birth, but it became more concentrated in the basement membrane zone as development proceeded (compare Fig. 6 B, D, and F). The distribution of fibronectin at each stage was similar to that seen at birth; fibronectin was detected throughout the dermis up to the dermal-epidermal junction, and in some sections it was considerably more concentrated at this junction (data not shown), as reported previously (Peters and Hynes, 1996).

#### **Distributions of Laminin-5 and Its Integrin Receptors during Development of Normal and $\alpha 3$ -Null Skin**

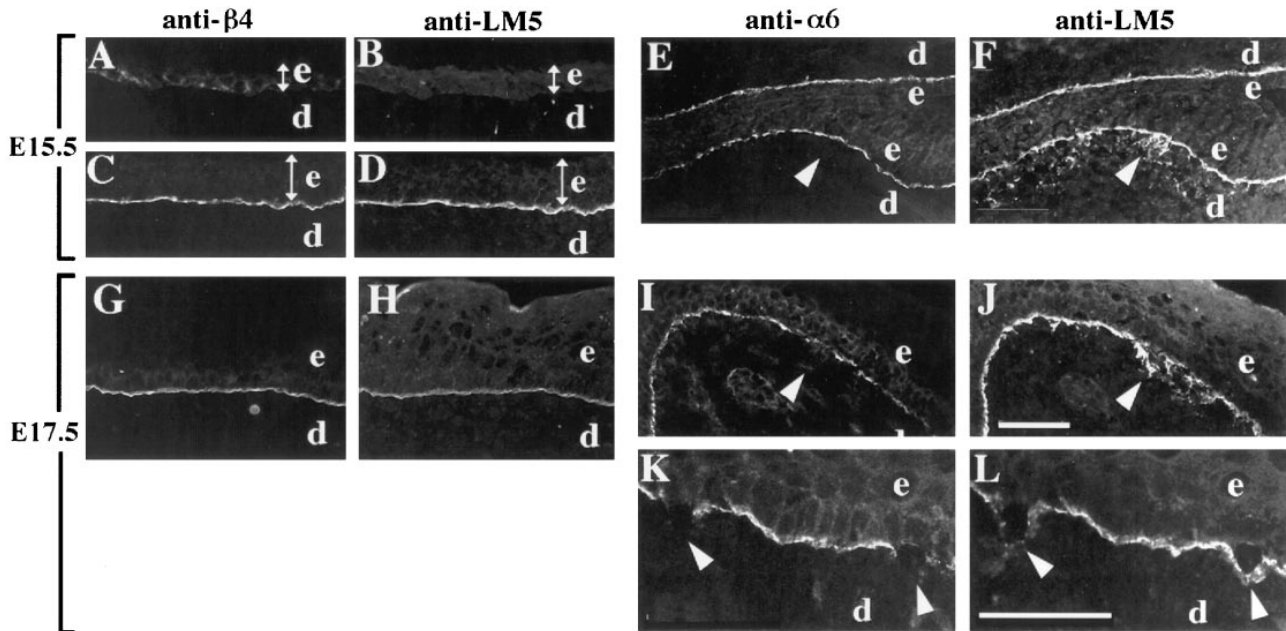
To compare developmental expression of laminin-5 and its receptors  $\alpha 3\beta 1$  and  $\alpha 6\beta 4$ , we first examined expression of these proteins in wild-type or heterozygous mice.  $\alpha 3\beta 1$  was expressed in the embryonic epidermis as early as E9.5 and remained restricted to basal keratinocytes, in a basolateral distribution, throughout development (Fig. 1 B and data not shown). The relative distributions of  $\alpha 6\beta 4$  and laminin-5 were determined by double-label immunofluorescence using a monoclonal antibody against the  $\beta 4$  sub-



**Figure 6.** Distributions of entactin and type VII collagen in the developing skin. Frozen sections from mouse embryonic skin at days E11.5 (A and B), E15.5 (C and D), or E17.5 (E and F) of development were stained with antisera against entactin (A, C, and E) or type VII collagen (B, D, and F). Staining patterns for entactin or type VII collagen were identical in wild-type (A, C, and D) and heterozygous (E and F) embryos; type VII collagen staining in B is from an  $\alpha 3$ -null, E11.5 embryo, but was identical to that of a wild-type embryo at this stage. e, epidermis; d, dermis. Bar, 50  $\mu$ m.

unit (346-11A; Kennel et al., 1989) and anti-laminin-5 serum (Fig. 7, A–D, G, and H). Laminin-5 was not detected in the unstratified skin of E11.5 embryos (data not shown). At E15.5, the degree of stratification varied throughout the embryo, and the appearance of laminin-5 in the basement membrane zone coincided roughly with increased epidermal stratification; anti-laminin-5 did not stain the

basement membrane in less stratified regions (Fig. 7 B), but clearly stained the basement membrane in more stratified regions (Fig. 7 D). The distribution of  $\beta 4$  at E15.5 was similar; in less stratified regions it was present in a basolateral distribution within the basal keratinocyte layer (Fig. 7 A), similar to the distribution of  $\alpha 3\beta 1$  (not shown), and in more stratified regions it was concentrated at the basal



**Figure 7.** Distributions of  $\alpha 6\beta 4$  and laminin-5 in the developing skin of normal and  $\alpha 3$ -null embryos. Frozen sections from mouse embryonic skin at days E15.5 (A–F) or E17.5 (G–L) of development were stained by double-label immunofluorescence with either monoclonal antibody 346-11A against the  $\beta 4$  integrin subunit (A, C, and G) or GoH3 monoclonal antibody against the  $\alpha 6$  integrin subunit (E, I, and K), and anti-laminin-5 serum (B, D, H, and F, J, L, respectively). Control sections were from wild-type embryos (A–D) or heterozygous embryos (G and H). In wild-type E15.5 embryos,  $\alpha 6\beta 4$  and laminin-5 codistributed to the basement membrane zone in more stratified regions (C and D), but not in less stratified regions (A and B); the width of the epidermis in each panel is indicated by a double-headed arrow. In  $\alpha 3$ -null embryos at E15.5 (E and F) and E17.5 (I and J), arrowheads point to areas of laminin-5 staining in areas of disorganized basement membrane, below the  $\alpha 6$ -positive basal keratinocytes; the skin in E and F is folded back on itself. (K and L) Higher magnification of  $\alpha 3$ -null skin at E17.5 showing  $\alpha 6$ -negative, basal keratinocytes that have separated from the laminin-5 positive basement membrane, marked by arrowheads. e, epidermis; d, dermis. Bars: (shown in J for A–J) 50  $\mu$ m; and (in L for K and L) 50  $\mu$ m.



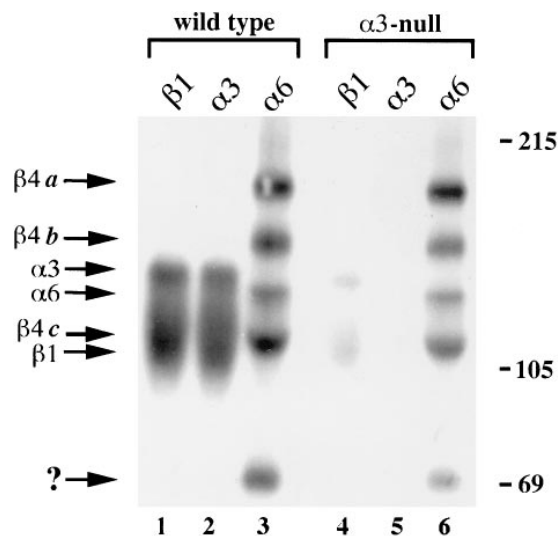
surfaces of the basal keratinocytes, adjacent to laminin-5 in the basement membrane (compare Fig. 7, *C* and *D*). Therefore, recruitment of  $\alpha\beta4$  to the basal aspects of basal keratinocytes appeared to coincide with incorporation of laminin-5 into the basement membrane, and may reflect the establishment of cell adhesion to laminin-5 mediated by  $\alpha\beta4$ . In E17.5 epidermis,  $\alpha\beta4$  was always concentrated in the basal surfaces of basal keratinocytes adjacent to the laminin-5-rich basement membrane (Fig. 7, *G* and *H*), as in newborn skin (Fig. 4, *A* and *B*).

The temporal expression and distribution of  $\alpha\beta4$  (i.e.,  $\alpha6$  or  $\beta4$  subunit) during development of  $\alpha3$ -null embryos were identical to those in wild-type or heterozygous embryos. However, staining of E15.5 embryos with anti-laminin-5 revealed occasional regions of basement membrane disorganization in  $\alpha3$ -null skin (Fig. 7, *E* and *F*). By E17.5 these regions were more common (Fig. 7, *I* and *J*), and became progressively more disorganized and more extensive by birth. Disorganized basement membrane was found on skin from both legs and torso in  $\alpha3$ -null embryos, but was not detected in skin from wild-type or heterozygous embryos.

Although we cannot rule out prenatal blister formation in  $\alpha3$ -null mice, we did not detect skin blisters greater than a few cells in diameter in  $\alpha3$ -null embryos. In E17.5 embryos, we occasionally observed individual basal keratinocytes in which  $\alpha6$  staining was absent (Fig. 7 *K*). As expected for basal keratinocytes lacking  $\alpha\beta4$  (van der Neut et al., 1996; Georges-Labouesse et al., 1996; Dowling et al., 1996), these cells were detached from the basement membrane, since laminin-5 distributed only to the dermal side of the separation (compare arrowheads in Fig. 7, *K* and *L*). However, we did not detect blisters at E17.5 that resembled the larger splits seen in neonatal skin. Embryos were collected by Cesarean section, thereby reducing the mechanical stress associated with birth. Otherwise, skin sections from E17.5 embryos and neonatal mice were prepared in the same manner, suggesting that blisters in  $\alpha3$ -null neonates were caused by physical trauma during or after birth, rather than by mechanical stress during sample preparation.

### Isolation of Primary Keratinocytes from $\alpha3$ -Null, Neonatal Mice

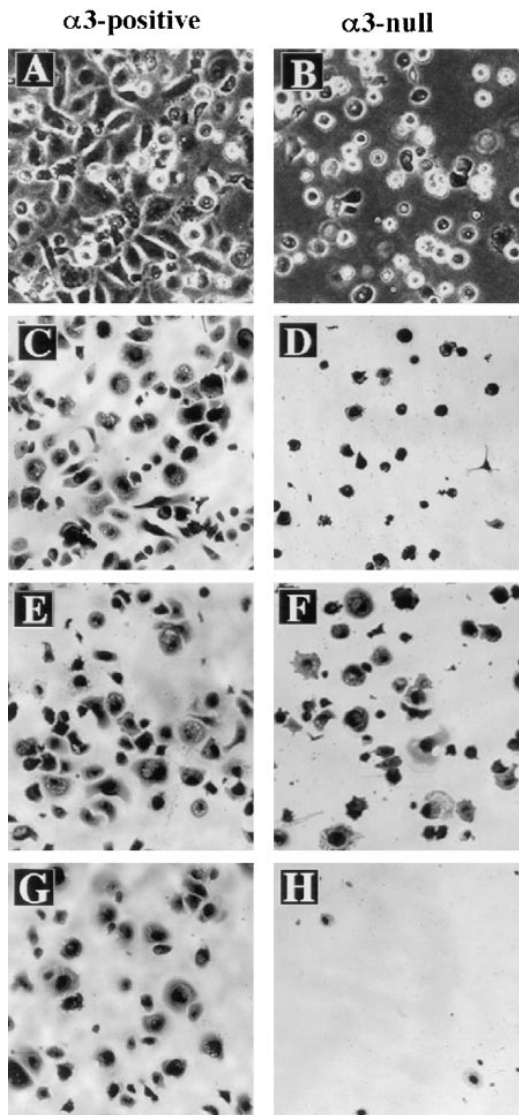
To address directly the importance of  $\alpha3\beta1$  as a laminin-5 receptor in the epidermis, we isolated primary keratinocytes from wild-type and  $\alpha3$ -null, neonatal mice (Dlugosz et al., 1995; for details see Materials and Methods). To compare surface expression of integrins that bind to laminin-5 in wild-type and  $\alpha3\beta1$ -deficient keratinocytes, 7-d, primary cultures were surface-iodinated with  $^{125}\text{I}$ , and detergent lysates were immunoprecipitated with antibodies against integrin subunits and analyzed by SDS-PAGE under nonreducing conditions (Fig. 8). As expected, antiserum against  $\alpha3$  immunoprecipitated an abundance of the  $\alpha3$  subunit ( $\sim 150$  kD) associated with the  $\beta1$  subunit ( $\sim 110$  kD) from lysates of wild-type cells (lane 2), but did not detect  $\alpha3\beta1$  in lysates from  $\alpha3$ -null cells (lane 5). An anti- $\beta1$  serum (lane 1) coimmunoprecipitated  $\beta1$  and associated proteins of  $\sim 150$  kD, corresponding to  $\alpha3$  and other comigrating  $\alpha$  subunits present in keratinocytes (Watt and Hertle, 1994).



**Figure 8.** Surface expression of integrins in primary keratinocytes isolated from wild-type (lanes 1–3) or  $\alpha3$ -null (lanes 4–6), neonatal mice. Detergent lysates from  $^{125}\text{I}$  surface-labeled cells were immunoprecipitated with antisera against the cytoplasmic domains of the  $\beta1$ ,  $\alpha3$ , or  $\alpha6$  integrin subunits, as indicated at the top of each lane. Molecular weight markers are shown to the right of the autoradiograph. Migratory positions of certain integrin subunits are indicated to the left, including proteolytic fragments of  $\beta4$ , *a*, *b*, and *c*, described previously by Hemler et al. (1989). The  $\beta1$ -associated band in  $\alpha3$ -null cells that comigrates with  $\alpha3$  (lane 4) represents other integrin  $\alpha$  subunits that dimerize with  $\beta1$  in keratinocytes, since  $\alpha3$  was not detected in  $\alpha3$ -null cells (lane 5). *?*, an unidentified band that may represent a proteolytic fragment of  $\beta4$  (see text).

Surface levels of  $\beta1$  integrins dropped significantly in  $\alpha3$ -null cells compared to wild-type cells (compare lanes 1 and 4), demonstrating that  $\alpha3\beta1$  constitutes the major proportion of total  $\beta1$  integrins in mouse keratinocytes.

$\alpha\beta4$  was detected using a polyclonal antiserum against the  $\alpha6$  integrin subunit (lane 3), which coimmunoprecipitated a series of bands expected for the  $\alpha6$  subunit ( $\sim 140$  kD) and various derivatives of the  $\beta4$  subunit (Hemler et al., 1989; Kajiji et al., 1989; Adams and Watt, 1991). In nonreduced immunoprecipitates from human cells, the full-length  $\beta4$  subunit migrates at  $\sim 210$  kD (*a* fragment), and smaller fragments of 165 kD (*b* fragment), 125 kD (*c* fragment), and 85 kD are thought to result from proteolysis of  $\beta4$  (Hemler et al., 1989). Indeed, this pattern of proteolysis has been useful as a diagnostic tool in studies of  $\beta4$  integrins (Hemler et al., 1989). Bands corresponding to the *a*, *b*, and *c* fragments of  $\beta4$  in mouse keratinocytes are indicated in Fig. 8; an unidentified band of  $\sim 70$  kD may correspond to the 85-kD fragment seen in human cells. Each of these fragments showed faster migration in the mouse keratinocytes than has been reported in human cells (Hemler et al., 1989), possibly due to species-specific differences in glycosylation of  $\beta4$ . Importantly,  $\alpha6$ -associated bands were identical in lysates from wild-type cells and  $\alpha3$ -null cells (compare lanes 3 and 6). A band corresponding to the  $\alpha6$  subunit was not detected in  $\beta1$  immunoprecipitations (lanes 1 and 4), suggesting that primary mouse keratinocytes have little, if any,  $\alpha6\beta1$ . Consistent with this notion,  $\alpha6\beta1$  was not detected in mouse keratinocytes either



**Figure 9.**  $\alpha 3\beta 1$  is required for postadhesion spreading of mouse keratinocytes on laminin-5. (A and B) Primary keratinocytes from neonatal mice heterozygous (A) or homozygous (B) for the  $\alpha 3$ -null mutation were subcultured on HEK-secreted, laminin-5-rich ECM (see Materials and Methods) and then photographed after 1.5 h. (C–H) Primary keratinocytes from wild-type (C, E, and G) or  $\alpha 3$ -null (D, F, and H) neonatal mice were subcultured on purified laminin-5 for 1 h and then fixed in 4% paraformaldehyde and stained with Giemsa. (E and F) Fibronectin was included with laminin-5 in the substrate. (G and H) Cells were treated with the monoclonal antibody GoH3, which blocks adhesion by  $\alpha 6$  integrins.

by immunoprecipitation of  $\alpha 6$  integrins with GoH3 followed by Western blotting with anti- $\beta 1$  serum, or by immunoprecipitation of  $\beta 1$  integrins followed by Western blotting with anti- $\alpha 6$  serum (data not shown).

#### ***$\alpha 3\beta 1$ Is Required for Spreading of Keratinocytes on Laminin-5***

We subcultured primary mouse keratinocytes on laminin-5-rich ECM prepared from human keratinocyte cultures, as described in the Materials and Methods section; this

ECM supports  $\alpha 3\beta 1$ -mediated cell attachment (Carter et al., 1991; Weitzman et al., 1993). Both  $\alpha 3$ -positive cells (in this case from a mouse heterozygous for the  $\alpha 3$ -null mutation) and  $\alpha 3$ -null cells attached to laminin-5-rich ECM within 15 min. After attachment,  $\alpha 3$ -positive keratinocytes spread rapidly on the laminin-5-rich ECM (Fig. 9 A). In contrast,  $\alpha 3$ -null keratinocytes remained unspread for up to 2 h following attachment to this matrix (Fig. 9 B). The same results were obtained in spreading assays using purified laminin-5 (Fig. 9, C and D).  $\alpha 3$ -null keratinocytes were able to spread when fibronectin was present in the substrate with laminin-5 (Fig. 9, E and F), demonstrating that  $\alpha 3\beta 1$ -deficient cells were not generally deficient in the ability to spread. The antibody GoH3, which blocks cell adhesion mediated by  $\alpha 6$  integrins (Sonnenberg et al., 1987), only slightly inhibited attachment, and did not affect spreading, of wild-type keratinocytes on laminin-5 (Fig. 9 G). In contrast, the same concentration of GoH3 completely inhibited attachment of  $\alpha 3\beta 1$ -deficient cells to laminin-5 (Fig. 9 H). Therefore, while  $\alpha 6\beta 4$  and  $\alpha 3\beta 1$  are each sufficient for attachment of mouse keratinocytes to laminin-5,  $\alpha 3\beta 1$  is essential for postattachment cell spreading on this ligand, consistent with previous, integrin-blocking studies using human foreskin keratinocytes (Xia et al., 1996).

#### ***Discussion***

The importance of integrin  $\alpha 3\beta 1$  as an ECM receptor in the epidermis has been unclear. The generation of mutant mice lacking the  $\alpha 3$  integrin subunit provides a useful animal model for the study of  $\alpha 3\beta 1$  functions in the epidermis. In addition, since  $\alpha 3$ -null mice develop fully until perinatal death, they provide a rich source of  $\alpha 3\beta 1$ -deficient, epidermal keratinocytes for study in culture. We have examined both  $\alpha 3$ -null mice and  $\alpha 3$ -deficient keratinocytes isolated from these mice for defects in adhesion-related functions.

#### ***$\alpha 3\beta 1$ Is Required for Basement Membrane Integrity in the Skin***

Although stratification of the skin from  $\alpha 3$ -deficient mice was comparable to that of wild-type mice, both immunofluorescence microscopy and electron microscopy of skin sections from  $\alpha 3$ -null mice revealed a basement membrane with extensive regions of disorganization. Furthermore, matrix disorganization was often associated with blistering at the dermal-epidermal junction in neonatal mice. A deficiency in  $\alpha 6\beta 4$  through null mutation of either the  $\alpha 6$  subunit (Georges-Labouesse et al., 1996) or the  $\beta 4$  subunit (van der Neut et al., 1996; Dowling et al., 1996) also causes epidermal blistering, although the blistering phenotype in these mice is much more severe than in the  $\alpha 3$ -null mice. In addition, the  $\alpha 3$ -null phenotype differs from those of the  $\alpha 6$ -null and  $\beta 4$ -null mice in another important way. The latter two mutations appear to cause detachment of basal epidermal keratinocytes from the basement membrane, since laminin-5 localized only to the dermal sides of blisters in both cases (Georges-Labouesse et al., 1996; van der Neut et al., 1996; Dowling et al., 1996). In contrast, blistering in  $\alpha 3$ -null epidermis appeared to result from de-

fects within the basement membrane itself, since laminin-5 distributed to both the epidermal and the dermal sides of the split. Indeed, the  $\alpha 3$ -null epidermis appeared to retain its ability to bind to the basement membrane, probably via  $\alpha 6\beta 4$ , since laminin-5 colocalized with  $\alpha 6\beta 4$  to the epidermal sides of blisters, and  $\alpha 3$ -null keratinocytes attached to laminin-5 in an  $\alpha 6$ -dependent manner. Thus, the epidermal phenotypes associated with  $\alpha 3\beta 1$ -deficient and  $\alpha 6\beta 4$ -deficient mice appear distinct from one another, and suggest an involvement of  $\alpha 3\beta 1$  in establishing and/or maintaining integrity of the basement membrane.

The chemical composition and physical organization of the epidermal basement membrane are complex (for reviews see Borradori and Sonnenberg, 1996; Uitto et al., 1996). At the ultrastructural level, the dermal-epidermal junction is resolved into the lamina lucida, which abuts the plasma membranes of basal keratinocytes in the epidermis, and the underlying lamina densa. Entactin/nidogen localizes to the lamina densa along with type IV collagen, laminin-5, and other laminin isoforms, including laminin-6 and -7. Some basement membrane structures are specifically associated with hemidesmosomes. For example, anchoring fibrils, which consist of type VII collagen, extend from the dermis into the lamina densa of the basement membrane adjacent to hemidesmosomes. The anchoring filaments, which contain laminin-5, extend from the anchoring fibrils across the lamina lucida to the hemidesmosomes at the basal cell surface. Interestingly, type VII collagen, entactin, and laminin-5 each distributed to both sides of blisters in  $\alpha 3$ -null skin. These distributions suggest that the basement membrane does not rupture between distinct domains of the basement membrane but, instead, ruptures unevenly through different layers and structures. Alternatively, distributions of these proteins within blisters may reflect distinct fractions that are associated with different domains of the basement membrane. For example, type VII collagen present at the epidermal side of a blister may represent a fraction that has not been properly assembled into anchoring fibrils. In either case, the distributions of matrix proteins within blisters appear to reflect a general disorganization and weakening of the basement membrane in  $\alpha 3$ -null skin. Although the presence of fibronectin in the basement membrane has been controversial (discussed in Hynes, 1990), this matrix protein also distributed to both sides of blisters, suggesting either a direct or an indirect interaction between fibronectin and basal keratinocytes.

### ***Disorganized Basement Membrane Appears during Development of $\alpha 3$ -Null Skin***

Our observations suggest that the temporal expression patterns and general distributions of  $\alpha 6\beta 4$ , laminin-5, and several other ECM proteins in the developing skin of  $\alpha 3$ -null mice are comparable to those in the skin of wild-type and heterozygous mice. However, examination of skin sections from  $\alpha 3$ -null embryos stained with anti-laminin-5 serum revealed defects in basement membrane organization by day E15.5 of development, which became progressively more evident as development proceeded. E15.5 was also the stage at which laminin-5 was first detected in the basement membrane beneath more stratified regions of the

epidermis, where most of the  $\alpha 6\beta 4$  had relocated to the basal surface of the basal keratinocytes. Therefore, this stage of development appears to represent a time when laminin-5 is recruited into the basement membrane and, presumably, when maintenance of the dermal-epidermal junction begins to switch over to a laminin-5-dependent adhesion mechanism.  $\alpha 3\beta 1$  may be required at this time to help assemble and/or maintain this new laminin-5-dependent basement membrane (see below).

The causal relationships of the disorganized matrix and epidermal blistering seen in  $\alpha 3$ -null skin are unknown. However, as discussed above, it seems likely that matrix disorganization reflects a weak basement membrane that eventually ruptures and leads to blister formation. In an alternative but not exclusive model, it is possible that small blisters occur during skin development (i.e., see Fig. 7, *K* and *L*) and that, during fetal repair of the dermal-epidermal junction, remnants of old basement membrane are displaced below the plane of the newly deposited matrix, producing the disorganized appearance of the basement membrane. Indeed, dense concentrations of laminin-5 and other matrix proteins that resembled remnants of continuous basement membrane were often seen extending from the dermal-epidermal junction down into the dermal regions.

### ***The Laminin-5 Receptors $\alpha 3\beta 1$ and $\alpha 6\beta 4$ Have Distinct Functions in Keratinocytes***

Keratinocytes isolated from  $\alpha 3$ -null epidermis retained the ability to attach to laminin-5 in culture but, in contrast to wild-type or heterozygous keratinocytes, did not spread well under our experimental conditions. Attachment of  $\alpha 3$ -null keratinocytes to laminin-5 was dependent on  $\alpha 6\beta 4$ , since the monoclonal antibody GoH3 completely blocked attachment and  $\alpha 6\beta 1$  was not detected in these cells. On the other hand, the same concentrations of GoH3 had little effect on attachment of wild-type keratinocytes to laminin-5, suggesting that  $\alpha 3\beta 1$  was sufficient for attachment. These results show that  $\alpha 3\beta 1$  and  $\alpha 6\beta 4$  have distinct but overlapping functions in mouse keratinocytes; both can support initial cell attachment to laminin-5, but  $\alpha 3\beta 1$  is required for cell spreading. These roles for  $\alpha 3\beta 1$  and  $\alpha 6\beta 4$  have been demonstrated previously in function-blocking studies of human foreskin keratinocytes (Xia et al., 1996), and have been suggested for a murine mammary tumor cell line (Sonnenberg et al., 1993). Differences in function between these two integrins reflect their distinct roles *in vivo*;  $\alpha 6\beta 4$  is required for stable adhesion of the epidermis to the basement membrane, and  $\alpha 3\beta 1$  appears to have a post-adhesion role in basement membrane integrity. Indeed, although  $\alpha 3\beta 1$  may partially compensate for the absence of  $\alpha 6\beta 4$  in  $\beta 4$ -null mice by relocalizing to the basal surfaces of basal keratinocytes (van der Neut et al., 1996),  $\alpha 3\beta 1$  was clearly not sufficient to maintain adhesion of the epidermis to the basement membrane in these mice.

Ultrastructure of the basement membrane in  $\alpha 3$ -null skin reflects these distinct functions for  $\alpha 6\beta 4$  and  $\alpha 3\beta 1$ . In  $\alpha 3$ -null skin, the lamina densa was present directly beneath and adjacent to hemidesmosomes, but was not formed properly in regions between hemidesmosomes, suggesting that formation of the lamina densa in hemidesmosome-

free regions is  $\alpha 3\beta 1$ -dependent. Alternatively, hemidesmosomes may be associated with an electron-dense component of the basement membrane that is distinct from the rest of the lamina densa. Thus,  $\alpha 3\beta 1$  is necessary for formation of a continuous lamina densa, but an intact basement membrane is not required for hemidesmosome assembly. In contrast,  $\alpha 6\beta 4$  is necessary for assembly of hemidesmosomes but not for formation of a continuous lamina densa (Georges-Labouesse et al., 1996; van der Neut et al., 1996; Dowling et al., 1996).

### *$\alpha 3\beta 1$ May Function as a Regulator of Basement Membrane Assembly/Remodeling*

Analysis of kidneys from  $\alpha 3$ -null mice also revealed disorganization of the glomerular basement membrane (Kreidberg et al., 1996), suggesting that  $\alpha 3\beta 1$  may be generally important for organization of extracellular matrices. Consistent with this notion,  $\alpha 3\beta 1$  can interact weakly with a broad range of matrix proteins, including fibronectin (Wayner and Carter, 1987; Elices et al., 1991), laminin-1 (Wayner and Carter, 1987; Gehlsen et al., 1988; Elices et al., 1991), collagens (Wayner and Carter, 1987; Elices et al., 1991), and entactin/nidogen (Dedhar et al., 1992; Wu et al., 1995), and shows post-adhesion localization to focal contacts in cells cultured on most of these proteins (DiPersio et al., 1995).

There are several ways in which  $\alpha 3\beta 1$  might serve as a secondary receptor that functions in matrix organization. One possibility is that  $\alpha 3\beta 1$  is involved in initial assembly of the basement membrane by binding to laminin-5 or other matrix proteins during their processing or recruitment into sites of cell adhesion. Indeed, Wu et al. (1995) recently showed that  $\alpha 3\beta 1$  in transfected cells can mediate assembly of both fibronectin and entactin into the ECM. In the epidermal basement membrane, entactin binds to the type IV collagen network (Aumailley et al., 1989). Therefore, certain bridging interactions between matrix proteins that require  $\alpha 3\beta 1$  may be absent from  $\alpha 3$ -null skin, thereby compromising basement membrane integrity and weakening the dermal-epidermal junction. Thus,  $\alpha 6\beta 4$  may be a primary receptor for keratinocyte attachment to the basement membrane, while  $\alpha 3\beta 1$  functions as a relatively weak, postadhesion receptor (DiPersio et al., 1995) required for basement membrane assembly. A recent study demonstrated such distinct functions for fibronectin receptors in an embryonic stem cell line, where  $\alpha v\beta 3$  was dominant in focal contact formation, while  $\alpha 5\beta 1$  was primarily responsible for fibronectin matrix assembly (Wennerberg et al., 1996).

A second possibility is that  $\alpha 3\beta 1$  binds to laminin-5 or other matrix proteins in the pre-assembled basement membrane in order to stabilize primary interactions of cells with the ECM (i.e., via  $\alpha 6\beta 4$  in hemidesmosomes) and/or between ECM proteins themselves. In this way,  $\alpha 3\beta 1$  may be required to maintain strength and integrity of the basement membrane independently of its initial assembly.

Finally, interactions of  $\alpha 3\beta 1$  with basement membrane ligands may induce signal transduction events in keratinocytes that regulate matrix organization.  $\alpha 3\beta 1$ -mediated adhesion can induce the tyrosine phosphorylation of focal adhesion kinase, or FAK (Kornberg et al., 1991; Jewell et al., 1995), as well as of other unidentified proteins in mouse

keratinocytes (DiPersio, C.M., and R.O. Hynes, unpublished data). It is possible that  $\alpha 3\beta 1$ -dependent signaling pathways regulate the production of matrix proteins or the activities of metalloproteinases or other enzymes that regulate matrix assembly and/or remodeling. Indeed, a balance between ECM structure/composition and the activities of matrix degrading enzymes has been demonstrated in other systems (Sympson et al., 1994; Tremble et al., 1994) and can be regulated through integrins (Huhtala et al., 1995; Brooks et al., 1996). Ligand binding by  $\alpha 3\beta 1$  may be required to signal the presence of basement membrane and provide positive or negative feedback to the cell that regulates basement membrane production.

A number of blistering skin diseases in humans are caused by defective basement membrane proteins or hemidesmosomal components that interfere with normal adhesion of the epidermis to the dermis. For example, some forms of JEB have been attributed to defects in the  $\alpha 3$ ,  $\beta 3$ , or  $\gamma 2$  chain of laminin-5 (Aberdam et al., 1994; Pulkkinen et al., 1994a,b; Uitto et al., 1994; Kivirikko et al., 1995), and dystrophic forms of JEB are caused by mutations in the gene for type VII collagen (Uitto et al., 1994). Patients with cicatricial pemphigoid that develop anti-laminin-5 antibodies also form skin blisters (Kirtschig et al., 1995). Similarly, patients with bullous pemphigoid, an acquired blistering disease, develop antibodies against either BPAG-1e (bullous pemphigoid antigen-1e) or BPAG-2, both components of hemidesmosomes (Diaz et al., 1977; Mueller et al., 1989; Sawamura et al., 1991). At least two cases of JEB with pyloric atresia are associated with loss of  $\alpha 6\beta 4$  integrin through mutations in the  $\beta 4$  integrin subunit (Vidal et al., 1995; Niessen et al., 1996). The blistering phenotype associated with mice lacking integrin  $\alpha 3\beta 1$  suggests that this basement membrane receptor may also be a potential target of human blistering diseases.  $\alpha 3$ -null mice provide a useful system in which to study the roles of integrin receptors both in normal epidermal development and function, and in epidermal blistering diseases.

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