

β 4 Integrin Is Required for Hemidesmosome Formation, Cell Adhesion and Cell Survival

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Abstract. The integrin heterodimer α 6 β 4 is expressed in many epithelia and in Schwann cells. In stratified epithelia, α 6 β 4 couple with BPAG1-e and BPAG2 to form hemidesmosomes, attaching externally to laminin and internally to the keratin cytoskeleton. To explore the function of this atypical integrin, and its relation to conventional actin-associated integrins, we targeted the removal of the β 4 gene in mice. Tissues that express α 6 β 4 are grossly affected. Stratified tissues are devoid of hemidesmosomes, display only a very fragile attachment to the basal lamina, and exhibit signs of degenera-

tion and tissue disorganization. Simple epithelia which express α 6 β 4 are also defective in adherence, even though they do not form hemidesmosomes. In the absence of β 4, α 6 is dramatically downregulated, and other integrins do not appear to compensate for the loss of this heterodimer. These data have important implications for understanding integrin function in cell-substratum adhesion, cell survival and differentiation, and for understanding the role of α 6 β 4 in junctional epidermolysis bullosa, an often lethal human disorder with pathology similar to our mice.

INTEGRINS are transmembrane receptors implicated in mediating cell-substratum attachment or cell-cell adhesion, and in transducing signals that regulate such diverse processes as growth, differentiation, and migration (for review see Hynes, 1992; Watt et al., 1993). Most integrins use specific components of extracellular matrix (ECM) to recognize and receive cues from their environment. Intracellularly, integrins associate dynamically with the cytoskeleton to transmit and translate these cues. Hemidesmosomes are specialized, integrin-mediated adherens junctions characteristic of stratified epithelia (for review see Garrod, 1993). These junctions are composed of α 6 β 4 integrin heterodimers (Stepp et al., 1990; Sonnenberg et al., 1991), which use laminin 5 anchoring filaments to attach an epithelium to the underlying basal lamina (Carter et al., 1991; Rousselle et al., 1991). Interactions between integrins and anchoring filaments appear to be essential for cell-substratum adhesion, as judged by the fact that a number of patients with the severe (Herlitz) form of junctional epidermolysis bullosa (JEB)¹ have mutations in both alleles of any one of the three genes encoding laminin 5 (Aberdam et al., 1994; Pulkkinen et al., 1994a,b; Kivir-

ikko et al., 1995; McGrath et al., 1996). Patients with this disorder display trauma-induced denuding of skin, and whole-scale mesenchymal-epithelial separations in tissues that attach to a basal lamina through laminin-anchoring filaments (Fine et al., 1991).

Hemidesmosomes differ from conventional integrin adherens junctions in several ways. Specifically, they connect with the keratin filament rather than actin filament network, and they associate with two unique proteins, BPAG1-e (230 kD) and BPAG2 (180 kD), first identified as antigenic determinants of circulating antibodies of patients with the acquired autoimmune disease bullous pemphigoid (BPAG, bullous pemphigoid antigen; for review see Stanley, 1993). Based on immunoelectron microscopy, BPAG1-e localizes to a region referred to as the inner plate, on the cytoplasmic surface of the hemidesmosome. Ablation of BPAG1-e in mice removes this plate and severs the connection between hemidesmosomes and keratin filaments, creating a cytoplasmic zone of mechanical fragility just above the hemidesmosomes (Guo et al., 1995). Surprisingly, neither hemidesmosome stability and structure nor cell substratum adhesion appear to be weakened in these mice.

In contrast to BPAG1-e, BPAG2 is a novel member of the collagen superfamily. It has a transmembrane domain, lacks a significant cytoplasmic domain, and has a prominent extracellular domain with classical collagen repeat sequences (Giudice et al., 1991; Li et al., 1993). Gene targeting of BPAG2 in mice has not yet been conducted, although mutations in both BPAG2 alleles have recently been described in two different patients with generalized

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1. *Abbreviations used in this paper:* BPAG, bullous pemphigoid antigen; GABEB, generalized atrophic benign epidermolysis bullosa; JEB, junctional epidermolysis bullosa.

atrophic benign epidermolysis bullosa (GABEB), a rare, milder variant of JEB (McGrath et al., 1995). If these mutations are causative, BPAG2 is involved in maintaining adhesion between the epidermis and the dermis. This said, hemidesmosomes and anchoring filaments are still present in the basal epidermal layer of GABEB patients, although the lamina lucida is wider than normal (McGrath et al., 1995).

Even though hemidesmosomes occur only in cells that express both $\alpha 6\beta 4$ integrin and BPAG proteins (Owaribe et al., 1990; Sonnenberg et al., 1990; Stepp et al., 1990), $\alpha 6\beta 4$ integrins exist in simple epithelia and Schwann cells, neither of which have hemidesmosomes (Sonnenberg et al., 1990; Natali et al., 1992; Einheber et al., 1993). All cells that express $\beta 4$ also express $\alpha 6$, although $\alpha 6$ is found in some tissues where $\beta 4$ is not expressed; in these cases, $\alpha 6$ probably binds to $\beta 1$, which it can do both in vitro and also in transfected cells (Hall et al., 1990; Cooper et al., 1991; Natali et al., 1992; Kennel et al., 1992; deCurtis and Reichardt, 1993).

$\beta 4$'s functions in the various tissues that express it have not yet been demonstrated, although $\beta 4$ has been implicated in many different processes. Recently, a patient with a severe case of junctional epidermolysis bullosa and pyloric atresia (gastrointestinal blockage) was found to contain a frameshift mutation in one $\beta 4$ allele and a potential internal deletion mutation in the other (Vidal et al., 1995). The basal epidermal cells of this patient displayed fewer and seemingly rudimentary hemidesmosomes, which still attached to keratin filaments. However, in the absence of functional evidence and chromosomal linkage analysis, it remains unclear whether $\beta 4$ mutations are causative for severe junctional EB. Moreover, given the nature of the $\beta 4$ mutations, even if causative, it cannot be assessed whether the phenotype and pathology reflect gain of function or partial loss of function mutations, or whether they are representative of a true $\beta 4$ null mutation.

In addition to unanswered questions regarding the role of $\beta 4$ in hemidesmosome formation, keratin filament attachment, cell adhesion, and JEB, issues still remain that relate to $\beta 4$ function in hemidesmosome and nonhemidesmosome containing tissues. What is the fate of $\alpha 6$ in the absence of $\beta 4$? Can other integrin heterodimers compensate for the loss of $\beta 4$? What is the role of $\beta 4$ in non-hemidesmosomal tissues? Is $\alpha 6\beta 4$ necessary for cell survival? In this report, we describe the isolation of the mouse $\beta 4$ integrin gene and its ablation using homologous recombination and embryonic stem cell technology. Our findings have led to the answers to many of the questions outlined above.

Materials and Methods

Screening the 129/sv Genomic Library

A radiolabeled 558-bp PCR fragment encompassing the 5' end of the mouse $\beta 4$ coding sequence (Kennel et al., 1993) was used to screen a 129/sv mouse genomic library (Stratagene, Palo Alto, CA). Processed filters were prehybridized at 42°C in a solution containing 50% deionized formamide, 0.02 M Hepes, 5× SSC, 1× Denhardt's mixture, 0.1 $\mu\text{g}/\text{ml}$ tRNA, and 0.1 $\mu\text{g}/\text{ml}$ denatured salmon sperm DNA. A radiolabeled probe was made using Stratagene PrimeItII kit, and 2×10^6 cpm/ml were added to fresh hybridization solution. After 12 h, hybridized filters were then washed and exposed to X-ray film as described by Guo et al. (1995). Five

hybridizing clones were identified and subsequently purified. One clone, m $\beta 4$ intAC, was subcloned as a 16-kb NotI restriction fragment into blue-script KS+. This clone, referred to as pKSC10, was subjected to extensive restriction map analyses, and was subsequently used for the preparation of the targeting vector.

Electroporation and Analysis of ES Cells and Knockout Mice

Embryonic stem cells (R1 strain from Andreas Nagy and Janet Rossant, University of Toronto) were transfected by electroporation, followed by culture and selection in the presence of G418 (250 $\mu\text{g}/\text{ml}$) and gancyclovir (1 μM) (Hogan et al., 1994). A fraction of each surviving colony was assayed for the presence of the desired homologous recombination event. To isolate DNAs, cells were incubated for 12 h at 55°C in the presence of TE buffer (1 mM EDTA, 50 mM Tris-HCl, pH 8) containing 20 mM NaCl, 1% SDS and 1 mg/ml Proteinase K. Cell solutions were then extracted with an equal volume of buffer-equilibrated phenol, followed by precipitation with 20 mM sodium acetate in 100% ethanol. After 75% ethanol washes, DNAs were resuspended in TE buffer. DNAs were assayed by Southern Blot analysis, and ES cells harboring the desired targeting event were injected into mouse SV129 blastocysts, which were then transferred to C57BL/6 mothers. After breeding, heterozygous and homozygous mice were identified by PCR and Southern Blot analysis of tail DNAs, isolated as outlined above.

For Southern analysis, DNAs were digested with EcoRI restriction endonuclease and the fragments were separated by electrophoresis through 0.8% agarose gels. DNAs were transferred to nitrocellulose, and then hybridized with ^{32}P -labeled cDNA probes corresponding to m $\beta 4$ sequences just 3' from those taken for the targeting vector. After hybridization, blots were washed and exposed to X-ray film for 1 d.

Immunofluorescence Microscopy on Frozen Tissue Sections

Frozen tissue sections (10 μm) were cut onto Superfrost plus slides. Sections were briefly fixed with methanol (-20°C) for 10 min, and then washed 2× with PBS. Sections were preblocked with a solution containing 1% BSA, 0.1% Triton X-100 and 1% gelatin in PBS. Primary antibodies were then added to fresh solution and incubated with sections at r.t. for 1 h. Antibody concentrations used were anti- $\beta 4$ antibody against a segment of the $\beta 4$ extracellular domain not included in the region chosen for targeting, 1:100 (Kennel et al., 1989, 1990); mAb-5E (anti-BPAG1), 1:50; anti- $\alpha 6$ (Kennel et al., 1989), 1:50; anti-BPAG2 (J17; Jones et al., 1991), 1:50; anti-laminin 5 (J18; Jones et al., 1991), 1:50; anti-K5, 1:50; and anti-K6, 1:400 (gift from D.R. Roop, Baylor University, Houston, TX); anti-K10, 1:500 (gift from S. Yuspa). After washing the slides 3× with PBS for 10 min each, sections were incubated with fresh solution containing secondary Texas red or FITC-conjugated antibodies (1:100 dilution) for 30 min before washing as before and mounting. Sections were examined using a Zeiss Axiophot immunofluorescence microscope.

Routine Histology and Ultrastructural Analyses

Animals were taken shortly after birth or by Caesarean of pregnant mothers. Tissues were gently removed from the animal, being careful not to apply any mechanical stress or pressure to the regions of interest. Tissues were processed for either (a) electron microscopy, by fixing in 2.5% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.4, followed by partial oxidation with H_2O_2 , followed by the same fixation without H_2O_2 , both at r.t. (stratified tissues), or (b) paraffin embedding, sectioning and hematoxylin-eosin staining, by fixing in Bouin's fixative. Semithin sections were stained with toluidine blue, and these and hematoxylin-eosin-stained sections were visualized by light microscopy. Ultrathin sections on copper grids were treated with uranyl acetate and lead citrate, and these grids were examined in a JEOL-CX electron microscope.

Results

Isolation of the Murine $\beta 4$ Gene and Generation of Mutant Mice

The mouse $\beta 4$ integrin cDNA has been cloned and characterized in its entirety (Kennel et al., 1993). Based on the

published sequence, specific oligonucleotide primers were designed to encompass a 558-bp sequence beginning at the ATG translation start codon of the mouse $\beta 4$ integrin mRNA and extending 3' to this codon. These primers were used in combination with reverse transcribed mouse skin mRNA to generate this fragment by polymerase chain reaction (PCR). The fragment was then subcloned, sequenced to verify its identity, radiolabeled, and used as a probe to isolate genomic clones from a mouse 129/sv genomic DNA library. One clone, m $\beta 4$ int λ C, was found which included the 5' end of the m $\beta 4$ integrin gene within 16 kb of genomic sequence. A restriction map of this sequence is shown in Fig. 1 A. This map extends and modifies the partial 5' map of a murine genomic clone reported previously (Kennel et al., 1993). Sequence analyses revealed that the two EcoRI sites indicated in Fig. 1 A encompass exons I-IV of the $\beta 4$ gene.

The m $\beta 4$ targeting vector was constructed from m $\beta 4$ int λ C genomic DNA (Fig. 1 A). A 4-kb Eco47III-Afl II fragment was targeted for deletion. This fragment begins in exon I (at nucleotide 173), 115 nucleotides 5' from the ATG translation start codon. The fragment ends in intron III, 3' from nucleotide 758 of the $\beta 4$ mRNA. This fragment was replaced with a 1.8-kb fragment containing a pgk1-neomycin resistance gene for positive selection. The pgk1 neo gene was flanked 5' with a 2.8-kb m $\beta 4$ fragment and 3' with a 1-kb m $\beta 4$ fragment. A pgk1-Herpes thymidine kinase gene was used for negative selection as outlined in Fig. 1 A (Adra et al., 1987).

The targeting vector was transfected into embryonic stem (ES) cells, and the transfectants were subjected to positive (G418) and negative (Gancyclovir) selection. DNAs from surviving colonies were isolated and screened for the desired homologous recombinants. Fig. 1 B shows representative Southern blot data from targeted ES clones illustrating the diagnostic bands for a homologously recombined $\beta 4$ allele. Of a total of 20 clones analyzed, three scored positive for homologous recombination and displayed identical hybridization patterns. A PCR strategy

was then devised to facilitate screening. The 1.8-kb fragment diagnostic for the homologous recombination event was present in the clones that had been identified initially on the basis of Southern blot analysis (Fig. 1 C).

The mutant clones were injected separately into C57BL/6 blastocysts to produce chimeric animals, which were mated with C57BL/6 females. Heterozygous offspring were then mated to produce homozygous mutants, and offspring were tested for the m $\beta 4$ null mutation (Fig. 1 D). These data verified that the targeting of both $\beta 4$ alleles had been successful.

The (-/-) $\beta 4$ Mice Display Extremely Severe Skin Blistering over Their Entire Body Surface

The phenotype of the (-/-) $\beta 4$ mice was unmistakable. These animals were born with large sections of the skin peeled from their body surface (Fig. 2 A). Animals died within a few hours after birth, apparently from multiple complications, including respiratory failure, gastrointestinal problems, and gross skin denuding. The blistering was extremely severe, and following our initial set of observations, we subsequently conducted Cesarean deliveries of offspring to minimize the mechanical stress to their skin.

Skin sections of these animals revealed a marked separation at the dermo-epidermal junction (Fig. 2 B, control; Fig. 2 C, -/-). Other $\beta 4$ (-/-) tissues that possess hemidesmosomes, such as tongue and esophagus, were similarly affected, except that for internal tissues, the separation at the epithelial-mesenchymal junction was not as pronounced, presumably because internal tissues were not as exposed to as much mechanical stress as external tissues. Abnormalities were found in complex epithelial tissues such as trachea and bronchus. In all of these tissues, the basal layer, i.e., the cells known to express $\beta 4$ and contain hemidesmosomes, displayed some signs of detachment from the underlying mesenchyme.

Taken together, the severity of the skin blistering in mice, the involvement of internal epithelial tissues, and

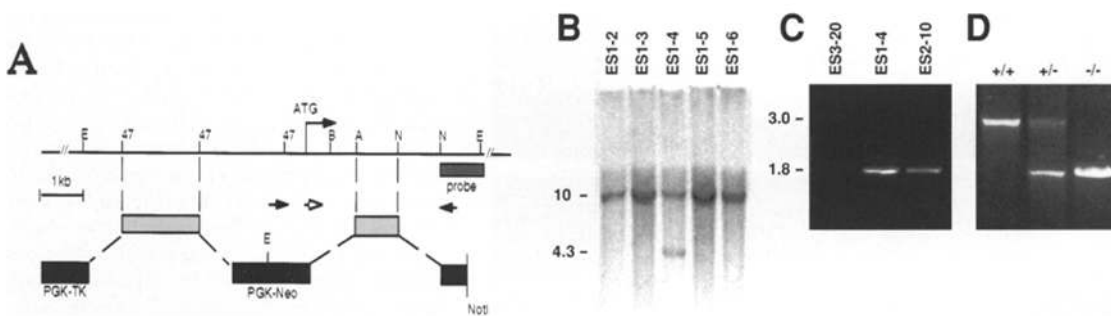


Figure 1. Targeting vector, Southern, and PCR analyses of ES cells and mice lacking the $\beta 4$ integrin gene. (A) Restriction map of clone m $\beta 4$ int λ C (top) and the targeting vector used for gene ablation (bottom). The 2.8-kb and 1-kb $\beta 4$ arms are shown (light gray boxes), along with the ~ 4 -kb $\beta 4$ genomic sequence replaced with PGK-NEO. Black arrow set denote primers used for PCR of targeted alleles. NotI denotes site of linearization of the targeting vector; white arrow is the 5' primer for the endogenous allele screen. E, EcoRI; 47, Eco47 III; A, AflIII; N, NheI. (B) Southern blot analyses of EcoRI-digested genomic DNAs from five representative control and targeted ES clones from the first plate of colonies (ES1, clones 2-6). The blot was probed with the 3' fragment indicated in the gray box in the upper right in A. The ~ 4.3 -kb hybridizing band is diagnostic for the homologous recombination event, and the ~ 10 -kb band is characteristic of the wild-type $\beta 4$ allele (see A). (C-D) PCR data from 3' probe analysis of representative DNAs of ES clones (C) and of an embryo litter from a heterozygous mating of mice germline for transmission of clone ES2-10. (D) Genomic DNAs were subjected to PCR using primers specific for the mouse $\beta 4$ gene and targeted event. The 3-kb band is diagnostic of the wild-type DNA (primer set only used in D); the 1.8-kb band is diagnostic of the targeted event.

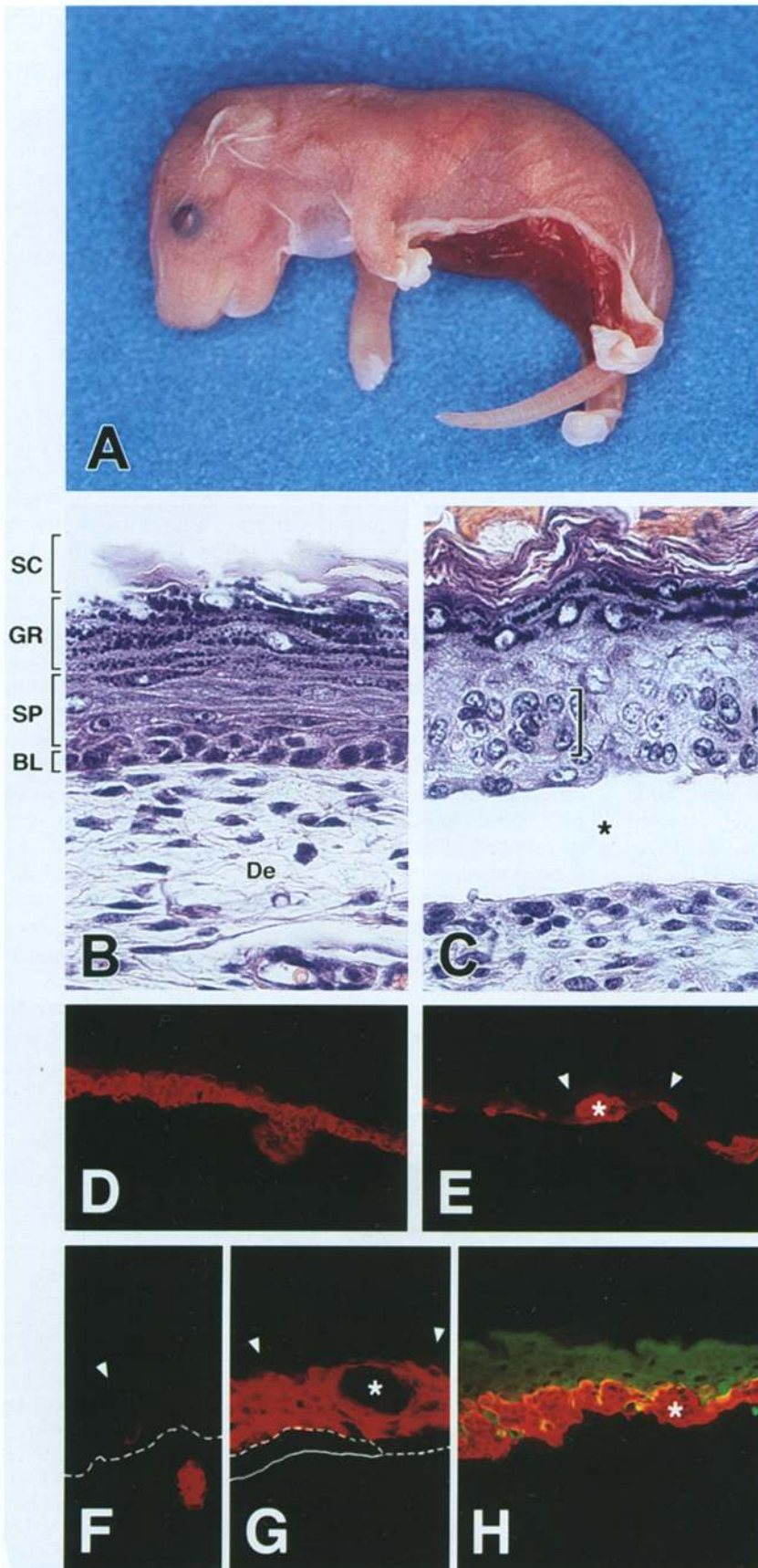


Figure 2. Separation at the mesenchymal-epithelial junctions in skin of $\beta 4$ integrin ($-/-$) mice. (A) Newborn $\beta 4$ null mouse. Note gross denuding of the body trunk skin. This occurred at birth of all animals homozygous for the $\beta 4$ null allele, and it resembled denuding seen in junctional epidermolysis bullosa in humans. (B–C) Hematoxylin-eosin stained sections of skin from control (B) and null (C) mice. In C, note (1) gross separation at the dermo-epidermal junction (asterisk); (2) pearl-like organization of some groups of cells in spinous layers (bracket); (3) general disorganization of cells in basal and spinous layers; and (4) the difference in thickness of the stratum corneum in B and C is not real; the upper layers frequently detach during sectioning. (D–H) Newborn control (D and F) and null (E, G, and H) skin sections stained with antibodies against K5 (D and E), K6 (F and G), or K10 (green) and K5 (red) (H). Note discontinuous layer of anti-K5 positive cells in E, presumably due to degeneration of basal cells (discussed later in text). B–C were taken with a $63\times$ objective and (D–G) were taken with a $40\times$ objective. Abbreviations: BL, basal layer; SP, spinous layer; GR, granular layer; SC, stratum corneum; De, dermis; white dotted line, dermo-epidermal junction; white line, a small blister separating the epidermis from the underlying dermis. White arrowheads point to stratum corneum; white asterisk in E, G, and H highlight a pearl of suprabasal keratinocytes displaying basal-like properties.

the tissue separations at mesenchymal-epithelial junctions bore a striking resemblance to the severe form of junctional epidermolysis bullosa in humans (Fine et al., 1991). Additional parallels are the autosomal recessive pattern of inheritance in JEB and its high rate of infant mortality. The mutant mouse skin differed from the skin of most JEB cases in that cells within the basal and spinous layers of the epidermis were disorganized (Fig. 2 C). Most striking was the presence of pearls of cells with closely spaced, different-sized nuclei, and very little cytoplasm (an example is bracketed in Fig. 2 C). These pearls were found in a number of stratified squamous epithelia, and included regions that were at least partially attached to their substratum. The cells within these pearls appeared basal-like in character and stained with antibodies against keratin 5, typically found only in basal cells (Fig. 2, D and E; pearl marked by asterisk). These cells did not stain with antibodies against keratin 6, typically induced in suprabasal cells during wound-healing (Fig. 2, F and G; Mansbridge and Knapp, 1987), nor did they stain with antibodies against the suprabasal marker, keratin 10 (Fig. 2 H; double label anti-K5, red; anti-K10, green). In addition to these biochemical differences, spinous layer cells did not flatten out as they typically do during the course of terminal differentiation. This said, granular and stratum corneum layers seemed normal, and gave a normal pattern of staining with anti-loricrin, a granular layer marker (data not shown). These differences were most prominent, but not exclusive to, areas where the epidermis was detached from the underlying dermis.

The Skin of the (-/-) Mice Do Not Have Detectable $\beta 4$ Integrin mRNA or $\beta 4$ Integrin Protein

To assess whether the targeted homologous recombination resulted in ablation of the normal $\beta 4$ integrin mRNA, we conducted RT-PCR on total RNAs extracted from skins of these mice. To verify the amount and quality of each RNA sample, we used primers selective for mouse β actin. To test for expression of the normal $\beta 4$ RNA, we used a primer set corresponding to a segment of $\beta 4$ sequence encompassing the segment expected to be missing in the $\beta 4$ null genome. As shown in Fig. 3, no $\beta 4$ band was amplified in the skin isolated from the $\beta 4$ knockout mouse. These data indicated that normal $\beta 4$ integrin mRNA expression is fully compromised in the skin of the (-/-) mice.

While normal $\beta 4$ mRNAs were not produced by our knockout mice, we did detect aberrant $\beta 4$ integrin RNAs when PCR primers were used to 3' downstream regions of the $\beta 4$ transcript (not shown). However, as judged by immunoblot analysis, no $\beta 4$ protein was produced in the knockout mouse (Fig. 4 A). In this case, we used an anti- $\beta 4$ antibody against an epitope encoded by a cytoplasmic $\beta 4$ segment that is still present within the (-/-) genome (Tamura et al., 1990). This anti- $\beta 4$ antibody recognized an ~200-kD band in skin protein extracts from a (+/-) animal, but not in the extracts from a (-/-) animal. Taken together, the RNA and protein analyses demonstrated convincingly that the targeting event to ablate the $\beta 4$ gene was successful.

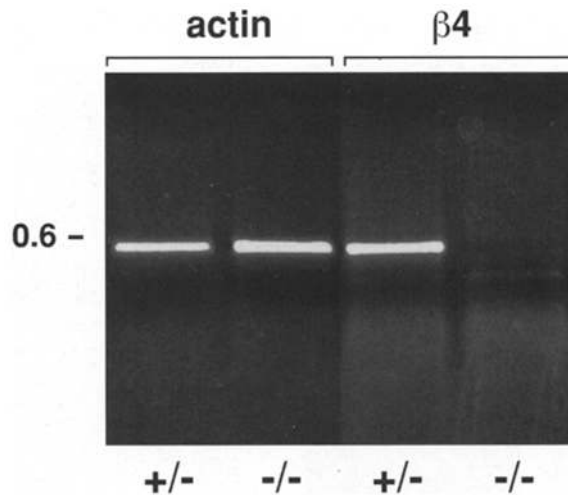


Figure 3. PCR analysis of RNAs reveals the absence of normal $\beta 4$ integrin mRNA in the knockout mice. For analyzing $\beta 4$ mRNA expression in putative knockout mice, total RNAs were isolated from the skins of E15.5 control (+/-) and knockout (-/-) mice using the Trizol reagent and procedure described by the manufacturer (GIBCO BRL, Gaithersburg, MD). RNAs were reverse transcribed, and then subjected to PCR using primers specific for lanes 1-2, actin mRNA; and lanes 3-4, $\beta 4$ integrin mRNA. Each primer set was designed to generate an ~600-bp PCR fragment.

Anti- $\beta 4$ Antibody Staining Is Absent Near the Dermo-Epidermal Junction of (-/-) Mice, and Some Other Immunodeterminants of Hemidesmosomes Are Perturbed

To further examine the loss of $\beta 4$ integrin in our knockout mice, we conducted immunofluorescence microscopy. In control skin, the $\beta 4$ antibody localized to the dermo-epidermal junction as expected (Fig. 5 A). In contrast, anti- $\beta 4$ showed no reactivity to (-/-) mouse skin (Fig. 5 B). These data are consistent with the RNA and immunoblot data, and verify that the homologous recombination resulted in the loss of $\beta 4$ integrin expression.

To assess whether the loss of $\beta 4$ protein affected the localization of any of the other hemidesmosome components, we subjected the mouse skin sections to further immunofluorescence analysis. Interestingly, antibodies against $\alpha 6$ integrin displayed strong staining in control skin, but staining was barely detectable in the (-/-) skin (Fig. 5, C and D, respectively). A reduction of $\alpha 6$ staining was also observed in the skin of the JEB-PA patient with $\beta 4$ mutations (Vidal et al., 1995). To test whether the reduction in $\alpha 6$ antibody staining reflected a downregulation in $\alpha 6$ protein, we conducted immunoblot analysis. In the absence of $\beta 4$, the levels of $\alpha 6$ protein were markedly reduced relative to the wild-type control (Fig. 4 B, $\alpha 6$; Fig. 4 C, K14 control for equal loading of protein). Taken together, our studies suggest that $\alpha 6$ integrin may be unstable in the absence of $\beta 4$.

The marked decline in $\alpha 6$ in the complete absence of $\beta 4$ integrin was surprising given that (a) $\alpha 6$ integrin can partner with $\beta 1$ integrin in cells that do not contain $\beta 4$ (Natali et al., 1992 and references therein), and (b) $\alpha 6\beta 4$ and $\alpha 6\beta 1$ seem to co-exist under certain circumstances in vivo (Na-

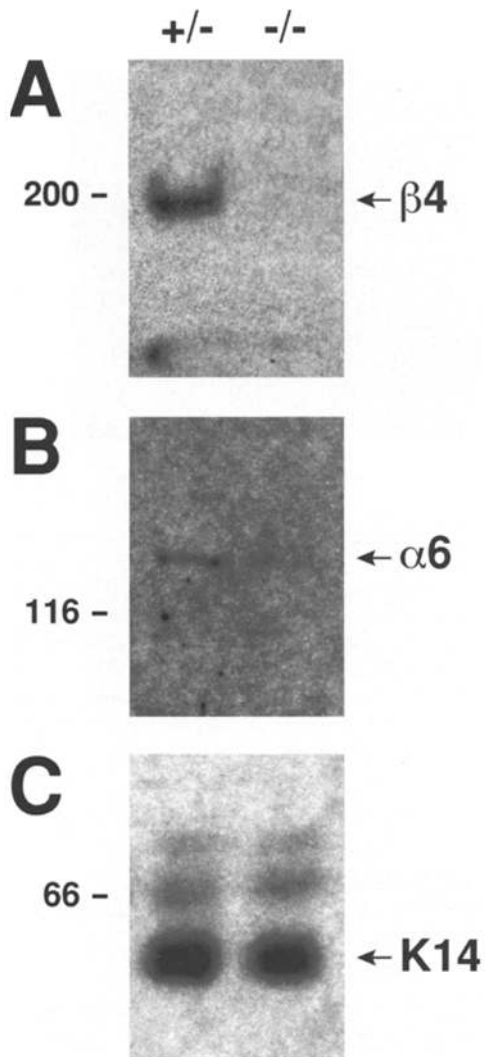


Figure 4. Immunoblot analysis reveals the absence of $\beta 4$ and downregulation of $\alpha 6$ integrin proteins in the knockout mice. Skins of E15.5 control (+/-) and knockout (-/-) mouse embryos were minced, frozen in liquid nitrogen, and then pulverized. Total proteins were extracted and resolved by electrophoresis through SDS polyacrylamide gels (6%). Proteins were transferred to nitrocellulose paper by electroblotting. Transfer efficiency of blots was verified using Ponceau S staining (Sigma Biochemicals, St. Louis, MO; data not shown). Blots were developed using an antibody (1:3,000 dilution) against an epitope of $\beta 4$ within the cytoplasmic tail portion of $\beta 4$ (Tamura et al., 1990). Blots were also developed with an anti- $\alpha 6$ antibody (1:1,000; Curtis and Reichardt, 1993; see Results section) and an anti-keratin 14 antibody (control; 1:2,000). In all cases, horseradish peroxidase-conjugated secondary antibodies were used to detect bound antibody, and the blots were processed with a chemiluminescent substrate (ECL method; Amersham Corp., Arlington Heights, IL). Relevant molecular mass standards are indicated in kilodaltons at left. Known sizes: $\beta 4$ (200 kD); $\alpha 6$ (130 kD); K14 (52 kD).

tali et al., 1992; Niessen et al., 1994). Since $\beta 1$ is normally expressed in stratified epithelia (Hynes, 1992), we wondered whether $\beta 1$ does not stabilize $\alpha 6$ because it is downregulated. As judged by immunohistochemistry with an anti- $\beta 1$ antibody, no apparent difference was detected between the control and the (-/-) skin (not shown). Thus, even in the presence of $\beta 1$, $\alpha 6$ levels dropped in the absence of $\beta 4$. It remains to be established whether this is because $\beta 1$ has a stronger affinity for the other α integrins present, or alternatively, because $\alpha 6$ adopts a conformation that is unable to interact with $\beta 1$ in the $\beta 4$ null cells.

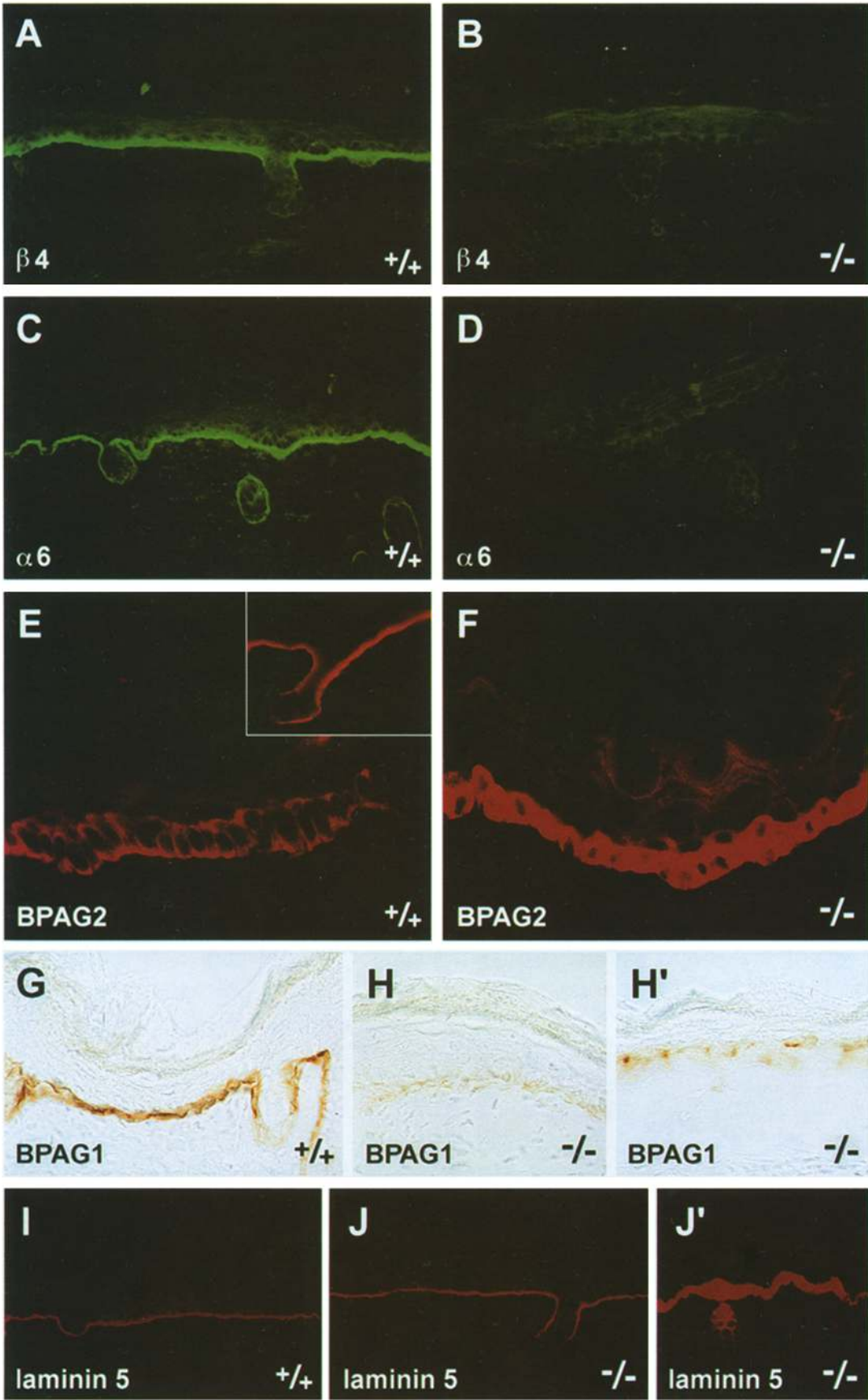
Surprisingly, anti-BPAG2 stained strongly the basal layer of the epidermis in both control and (-/-) skin (Fig. 5, E-F). In fact, the staining was somewhat stronger in the knockout skin than in control skin. Interestingly, at this early stage in neonatal development, BPAG2 staining was not restricted to the base of the basal keratinocyte, as it is in older animals (Fig. 5, E; compare with inset). While the (-/-) animals did not survive long enough to analyze their skin at an age where BPAG2 became more restricted in its localization, these data suggest that at least at this early age, BPAG2's presence is not dependent upon $\beta 4$, nor does it appear to be dependent upon $\alpha 6$. This is intriguing in light of recent studies suggesting that $\alpha 6$ interacts with the noncollagenous extracellular domain of BPAG2 (Hopkinson et al., 1995).

In contrast to anti-BPAG2 staining patterns, antibodies against BPAG1 displayed a markedly reduced staining pattern (Fig. 5, G-H). The protein was barely detectable, and in blistered areas, it appeared to form aggregates within the cytoplasm (Fig. 5, H'). These data suggest that in the absence of $\beta 4$, BPAG1-e expression is either reduced or unable to maintain its location within the cell. If protein levels are affected, this could either be due to decreased transcription or translation, or most likely to a decrease in stability. This result was expected given that BPAG1-e resides in the inner plate of the hemidesmosomes, and attaches the hemidesmosomes to keratin filaments (Guo et al., 1995 and references therein). Finally, antibodies against laminin 5 displayed a pattern that was indistinguishable between control and (-/-) skin sections (Fig. 5, I-J). Only upon skin blistering was the anti-laminin staining pattern more diffuse than normal (Fig. 5, J'). These data suggest that laminin 5 remains at or near the basement membrane in the absence of $\beta 4$.

Marked Ultrastructural Differences between the Epithelium of $\beta 4$ Integrin Null Mice and That of Patients with Severe JEB: Loss of $\beta 4$ Results in Loss of Hemidesmosomes

To examine in more detail the consequences of ablating $\beta 4$ integrin, we performed ultrastructural analyses on skin and other stratified squamous epithelial tissues of wild-

Figure 5. Immunohistochemistry of mutant mouse skin. Newborn mice were frozen in OCT compound, sectioned, and subjected to immunohistochemistry (Albers and Fuchs, 1987). First section of each pair is from control skin and second is from (-/-) skin. A and B, anti- $\beta 4$ integrin; C and D, anti- $\alpha 6$ integrin; E and F, anti-BPAG2; inset to E, anti-BPAG2 of adult skin; G and H, anti-BPAG1 (mAb-5E); H', anti-BPAG1 of region where skin has separated at the dermo-epidermal junction (note punctate staining); I, J and J', anti-laminin 5; J', region where epidermis has separated from the laminin and basal lamina. All staining is near or at the dermo-epidermal junction. All frames were photographed at the same magnification (40 \times objective), except BPAG1 (100 \times objective).



type and (-/-) mice taken at birth (Fig. 6). Wild-type cells at the mesenchymal-epithelial junction of these tissues display numerous electron dense hemidesmosomal plaques at their basal surface (Fig. 6 A, schematic of hemidesmosome; Fig. 6 B, control mouse skin).

Attached to the dense plaque is the inner plate, composed of BPAG1-e, which attaches the keratin filament framework to the membrane (Guo et al., 1995). In striking contrast, hemidesmosomes were completely absent in $\beta 4$ null keratinocytes (Fig. 6, C and D). This was true both for regions

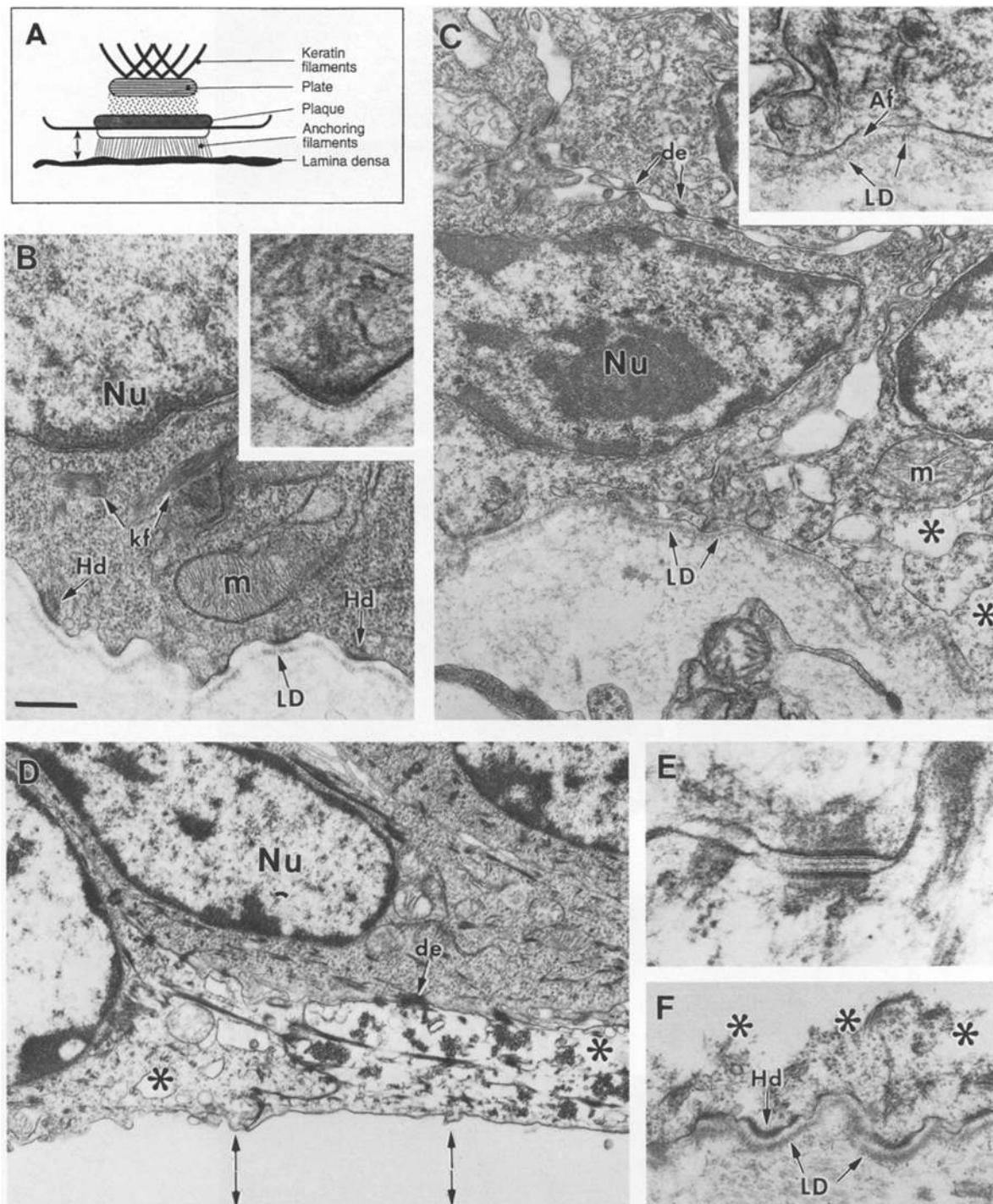


Figure 6. The absence of hemidesmosomes in $\beta 4$ mutant mice. (A) Schematic of hemidesmosome (for review see Schwarz et al., 1990; also see Guo et al., 1995). Double arrow denotes lamina lucida. (B) Electron microscopy of basal layer of control newborn mouse skin, fixed and embedded in Epon. Inset shows hemidesmosome at higher magnification to illustrate the features characteristic of the diagram in A (the plaque is the most electron dense structure). (C) Region of newborn esophagus of $\beta 4$ null mouse, illustrating the complete absence of hemidesmosomes at the cell membrane, even though the cell is still attached to the underlying lamina densa. Inset, higher magnification of mesenchymal-epithelial junction to show that fine filaments, resembling anchoring filaments, are still present.

where the epithelium was still attached to its underlying lamina densa (Fig. 6 C), and for regions where it had completely separated from the underlying mesenchyme (Fig. 6 D). Despite the lack of hemidesmosomes, some connecting fibers still spanned the lamina lucida in areas where the epithelium had not detached from the lamina densa (Fig. 6, inset to C). These thin filaments resembled anchoring filaments, i.e., the specialized laminin fibers responsible for attaching the hemidesmosome to the lamina densa (see Fig. 6 A). When taken together with our immunofluorescence data demonstrating that laminin 5 is still present in the (-/-) skin, we surmise that the lack of $\beta 4$ does not prevent laminin 5 from assembling into anchoring filaments. This said, the fragility of the attachment between the lamina densa and the epithelium underscored the fact that these filaments were not able to anchor efficiently in the absence of the hemidesmosomes.

A remarkable and obvious feature of the stratified squamous epithelia of $\beta 4$ null animals was the degeneration within the innermost layer (Fig. 6 D; see also Fig. 2). That the degeneration was a consequence of the lack of hemidesmosomes, rather than the converse, was clear from comparing $\beta 4$ null epidermis with trauma-induced intracellular rupturing in BPAG1-e null epidermis: in BPAG1-e null cells, normal numbers of hemidesmosomes are retained at the base of each ruptured cell despite gross signs of degeneration (Fig. 6 F). These data demonstrate unequivocally that loss of $\beta 4$, rather than cytolysis, leads to a loss of hemidesmosomes.

In contrast to the basal layer, most regions of the suprabasal layers of stratified $\beta 4$ null epithelia displayed fewer signs of degeneration (Fig. 6, cell with nucleus marked in D). Moreover, desmosomes appeared unaffected by the ablation of $\beta 4$ integrin or by the resulting degeneration (Fig. 6 E). This is consistent with the knowledge that despite their similar ultrastructure, hemidesmosomes and desmosomes are composed of entirely different proteins (for review see Garrod, 1993).

Some of the degeneration seen in the basal layer appeared to be mediated by apoptosis (Fig. 7). While biochemical studies have not yet been conducted, classical signs of apoptosis, including striking condensation of both nucleus and cytoplasm, were prevalent in basal cells (Fig. 7, A and B). The most condensed cells were easily recognized by their electron dense appearance. Three of the seven cells shown in Fig. 7 A displayed highly condensed nuclei and cytoplasm (also see Fig. 7 B). Other basal cells showed milder signs of nuclear and/or cytoplasmic degeneration (remaining four cells in Fig. 7 A). In addition, suprabasal cells sometimes displayed apoptotic bodies and late-stage signs of apoptosis (not shown). Typical of apoptosis, no neutrophils or inflammatory cells were seen in the vicinity of the dying basal cells. These data imply that sur-

vival of mitotically active cells of stratified squamous epithelia is dependent upon $\beta 4$ integrin.

The absence of hemidesmosomes and the presence of epidermal cell degeneration was different from skin of PA-JEB patients, where rudimentary hemidesmosomes and keratin filament attachments are still seen, and where cell degeneration in nonlesional skin is minimal (Nazzaro et al., 1990; Lacour et al., 1992; Phillips et al., 1994; Vidal et al., 1995). To assess whether such differences may be attributed to variations in mechanical stress, exerted either on newborn mice during delivery or upon movement of the embryos in the womb, we repeated our studies on E15.5 mouse embryos, carefully isolated by Cesarean section of pregnant females. While the skin was largely attached in these embryos, it was still very fragile (not shown). Moreover, stratified squamous epithelia still lacked hemidesmosomes and displayed signs of degeneration (not shown). Therefore, unless species specific variations account for the differences in severity between the epithelium of PA-JEB patients and $\beta 4$ null mice, we would anticipate that bona fide null mutations in $\beta 4$ genes in humans will produce very severe JEB, with a complete lack of hemidesmosomes.

A final intriguing aberration in the epidermis of newborn $\beta 4$ null mouse skin was the presence of mitotic cells in the suprabasal layers. An example is shown in Fig. 7 C (*large arrow*). This mitotic cell was located within one of the pearls of basal-like cells (see also Fig. 2), and was many cells removed from the basal layer. Such suprabasal mitoses were never seen in normal control skin. Moreover, the pearls of cells contained very peculiar cell organization. Often, these cells were small and very closely spaced, with very little cytoplasm (Fig. 7 D: typical spinous cell is at left; cytoplasm of three pearl cells are at right). While pearl cells displayed desmosomes and keratin filament bundles (*inset*), such structures were fewer in number than typical suprabasal cells. While further experiments will be necessary to examine the sequence of events that lead to these remarkable changes within the epidermis, the initial event that led to these differences was ablation of $\beta 4$.

Degeneration in Cells That Express $\beta 4$ Integrin but Do Not Possess Hemidesmosomes

Most single layered epithelia, including pyloric epithelium and the epithelium of the small intestine, do not possess hemidesmosomes and yet are known to express $\alpha 6\beta 4$ integrins (Owaribe et al., 1990; Sonnenberg et al., 1990; Natali et al., 1992). Interestingly, cell adhesion was also grossly perturbed in these nonhemidesmosomal, $\alpha 6\beta 4$ positive, tissues (Fig. 8; shown is transverse section of small intestine; A, control; B, -/-). As judged by immunohistochemistry, $\beta 4$ integrin was not detectable in these knockout tissues

Note: Hemidesmosomes were absent in all (-/-) stratified squamous epithelia examined. (D) Newborn paw epidermis of $\beta 4$ null mouse, showing a region where the basal layer has separated from the underlying lamina densa. Note degeneration in basal cell. (E) Desmosome from $\beta 4$ null mouse skin. No differences in desmosome number or structure were detected. (F) Basal epidermal layer from tail skin of a BPAG1 null mouse (Guo et al., 1995). Note zone of rupturing just above hemidesmosomes. Note also that despite cytolysis, hemidesmosomes are intact. Abbreviations: Hd, hemidesmosome; LD, lamina densa; Nu, nucleus; de, desmosome; Af, anchoring filaments; kf, keratin filaments; m, mitochondria; *double arrows*, mesenchymal-epidermal separation; *asterisks*, cell degeneration. Bar in B represents 0.3 μm in B, 0.6 μm in C, 1 μm in D; 0.1 μm in inset to B and in E; 0.24 μm in inset to C; 0.55 μm in F.

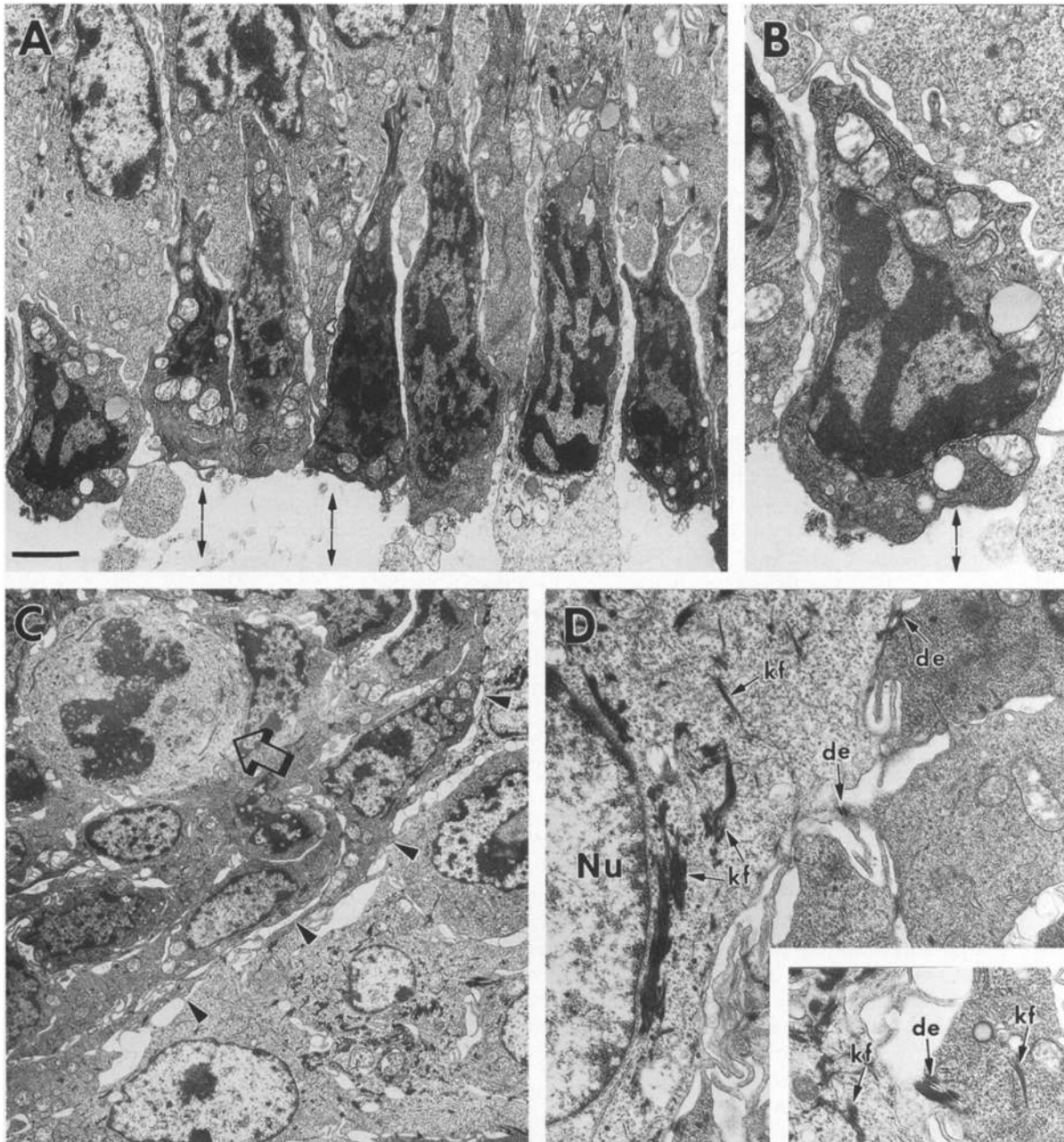


Figure 7. Ultrastructure showing apoptosis in basal epidermal layer and mitotically active basal-like cells in suprabasal pearls. Electron microscopy of basal layer of $\beta 4$ null newborn mouse skin, fixed, and embedded in Epon. (A–B) Basal layer, separated from underlying basal lamina (double arrowheads denote separation), showing numerous examples of cells displaying highly condensed chromatin and condensed cytoplasm, giving the cells an electron dense appearance. All seven cells show some signs of apoptosis, and the first, fourth, and seventh cells in the row are in the most advanced stages. Cell in B is a higher magnification of first cell in A. Note the highly condensed chromatin and signs of degeneration of mitochondria. (C) Boundary of a pearl of cells within the suprabasal layer. Arrowheads denote border: cells above are within the pearl; those below are normal looking spinous cells. Note cell in prophase (*big arrow*). (D). Higher magnification of a boundary region, showing normal looking spinous cell at left and cytoplasm of three different pearl cells to the right. Note abundance of keratin filaments (*kfs*) and desmosomes (*de*) in the spinous cell, but their paucity in the immature looking cells within the pearl. Inset shows keratin filaments and desmosome within a pearl cell, demonstrating that these cells are keratinocytes. Bar in A represents 1.9 μm in A; 0.8 μm in B; 3.3 μm in C, and 1 μm in D; 1.6 μm inset to D.

and $\alpha 6$ integrin was markedly reduced (Fig. 8, C–F). The lesions in gastrointestinal epithelia were relevant in light of the fact that these tissues are affected in the severe forms of JEB, and in patients displaying JEB with pyloric atresia. Such defects were also seen in the one patient thus far found to contain mutations in the $\beta 4$ integrin alleles (Vidal et al., 1995).

Interestingly, our mice displayed a partial restriction of epithelial passages in esophagus and tongue, where the outer layers from a detached area were often fused through extracellular material to an opposing attached area, a feature reflecting the natural ability of outer layer cells to interconnect.

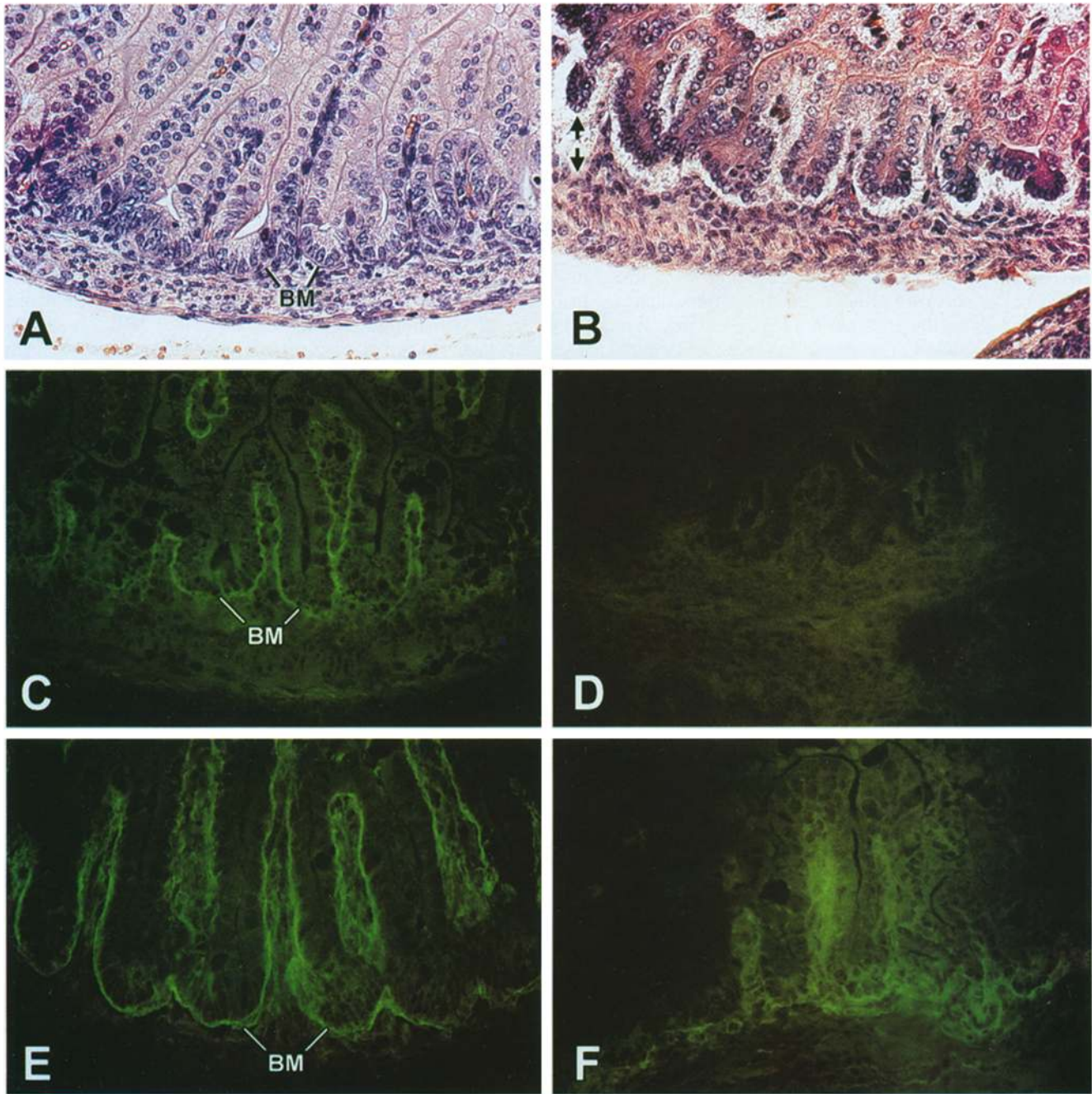


Figure 8. Degeneration in epithelia lacking hemidesmosomes. (A–B) Hematoxylin- and eosin-stained sections of intestinal epithelium from newborn control and $\beta 4$ null mouse, respectively. Note signs of degeneration at the epithelial-mesenchymal junction in B (double arrowhead denotes separation/degeneration). (C–F) Immunofluorescence of intestinal epithelium of newborn control (C and E) and $\beta 4$ null (D and F) mouse. C and D, anti- $\beta 4$; E and F, anti- $\alpha 6$. BM, basement membrane. Frames were photographed at 40 \times objective.

We could not readily evaluate the effects of $\beta 4$ ablation on Schwann cell myelination and axonal integrity. Although it appeared from our preliminary studies that Schwann cell development may be affected in these mice, tissue culture studies on Schwann cells from newborn $\beta 4$ null mice, and/or studies on Schwann cells in older $\beta 4$ null mice (if possible) will be necessary to assess the extent to which the nervous system may be affected by $\beta 4$ ablation.

Discussion

What Does the $\beta 4$ Integrin Knockout Tell Us about the Structure of the Hemidesmosome?

Our results demonstrate that hemidesmosomal formation is dependent upon $\beta 4$ integrin expression in stratified epithelia. Given the lack of discernable hemidesmosomes in our $\beta 4$ null mice, it is perhaps not surprising that the localization of some other hemidesmosomal components is also

perturbed by ablation of $\beta 4$. Remarkable in this regard is the dramatic reduction of $\alpha 6$ integrin, which is barely detectable by immunofluorescence staining or by immunoblot analysis.

Previous researchers have shown that $\alpha 6$ can pair with $\beta 1$ in cells that do not possess $\beta 4$ naturally (Hall et al., 1990; Cooper et al., 1991; Sonnenberg et al., 1990; Niessen et al., 1994). It has always been assumed that if $\beta 4$ were absent in epithelial cells, then $\alpha 6$ would pair with $\beta 1$. Moreover, since both $\alpha 6\beta 1$ and $\alpha 6\beta 4$ are receptors for laminin 5 (Niessen et al., 1994), it seemed possible that the complete loss of $\beta 4$ might affect hemidesmosome structure, but would not necessarily affect cell-substratum adhesion. In our studies, we show that when $\beta 4$ is absent, a marked reduction in $\alpha 6$ occurs in epithelial cells whose normal counterparts express $\beta 4$. In addition, reduction of $\alpha 6$ occurs under conditions where $\beta 1$ seems to be present in normal amounts. Based on these observations, it would appear that $\alpha 6$ does not pair appreciably with $\beta 1$ in mutant epithelial cells lacking $\beta 4$, and that the bulk of $\alpha 6$ seems to be unstable in epithelia under conditions where $\beta 4$ is absent.

The substantial loss of cell adhesion in the absence of hemidesmosomes provides a graphic illustration that hemidesmosomes are essential structures for cell-substratum adhesion in stratified squamous epithelia. Yet basal epidermal keratinocytes express a number of integrins, including $\alpha 2\beta 1$ and $\alpha 3\beta 1$, in addition to $\alpha 6\beta 4$ (Marchisio et al., 1991; Hertle et al., 1991; Watt et al., 1993). In vitro, these other integrins participate in cell-substratum adhesion (Carter et al., 1990, 1991). We do not yet know why these other integrins are not able to provide sufficient adhesion in vivo to counteract the loss of hemidesmosomes in our knockout mice.

The $\beta 4$ Null Mice Have a Severe Form of Junctional Epidermolysis Bullosa

JEB is a rare autosomal recessive disorder affecting approximately 1:100,000 in the human population. With the exception of generalized atrophic benign EB, a high mortality rate is seen in JEB, previously referred to as EB letalis. In addition to severe blistering at the dermo-epidermal junction, recurrent erosions occur in the gastrointestinal and genitourinary tracts, as well as in the cornea and in the respiratory tract. Approximately 15% of patients with severe junctional EB have pyloric atresia, i.e., blockage of the gastroduodenal tract (Hayashi et al., 1991; Fine et al., 1991). This blockage appears to arise from fusion of the detached epithelial lining of the atretic pyloric segment. The common link between the affected tissues in severe cases of JEB appears to be an expression of $\alpha 6\beta 4$ and laminin 5 and not merely hemidesmosomes (Sonnenberg et al., 1990; Natali et al., 1992).

In the majority of JEB cases, antibodies against laminin 5 label the dermoepidermal junction weakly, if at all (Schofield et al., 1990). In many of these cases, homozygous or compound heterozygous mutations have been detected in both alleles encoding any one of the laminin 5 chains (Pulkkinen et al., 1994a,b; Christiano et al., 1996; McGrath et al., 1996, and references therein). In contrast, skins from several JEB patients with pyloric atresia stain with antibodies against laminin 5, but not against $\beta 4$ inte-

grin (Nazzaro et al., 1990; Lacour et al., 1992). Weak anti- $\beta 4$ labeling has also been reported in the one JEB-PA case analyzed genetically (Vidal et al., 1995), as well as in a number of conventional Herlitz JEB cases (Phillips et al., 1994).

Our results now provide functional demonstration of causality for a gene whose mutations have been implicated in JEB. This functional evidence is extremely important in light of the fact that chromosomal linkage studies have not been possible, due to the often small size of JEB families. Our findings reveal compelling similarities between the skin of $\beta 4$ integrin null mice and severe junctional epidermolysis bullosa. In addition, internal epithelia typically affected in severe JEB, including the pyloric epithelium, are grossly affected in $\beta 4$ null mice. Whether JEB-PA should be classified as a distinct genetic subtype is a separate issue that awaits further analysis of human patients.

Differences between Severe JEB in Humans and in Our Knockout Mice and New Functions for Hemidesmosomes

Unexpectedly, our mice exhibited a significantly more severe phenotype than most reported human JEB cases. Denuding of the skin occurred with the mildest of mechanical stress, and the animals were unable to survive for more than a few hours after birth. Ultrastructurally, hemidesmosomes were completely lacking, even in esophageal tissue and in embryos, where mechanical trauma should be minimal relative to that exerted on neonatal skin. In contrast, the JEB patients known to have reduced anti- $\beta 4$ integrin staining survived at least 6–8 mo, and hemidesmosomes, albeit rudimentary and sparser, were still present (Nazzaro et al., 1990; Lacour et al., 1992; Vidal et al., 1995).

While we cannot exclude the possibility that species-specific differences account for these differences in disease severity, we think that the difference may reside in the fact that we have generated a true null mutation, while the genetic lesions thus far identified in either laminin 5 chains or $\beta 4$ integrins of JEB patients are either splicing defects, frameshifts, internal deletion mutations, or premature termination codons that are well within the coding segment of the hemidesmosomal gene. One of the two alleles of the only patient thus far reported to have $\beta 4$ mutations was a T nucleotide insertion just 3' to the *gt* 5' donor splice site at nucleotide 3801 of the coding sequence (Vidal et al., 1995). It was suggested that this insertion might lead to an in-frame deletion of 51 nucleotides from the mRNA transcript (Vidal et al., 1995). Despite reports that anti- $\beta 4$ antibody staining is weak if present at all in this and in some other patients, even small amounts of a $\beta 4$ protein predicted to have a 17-amino acid residue internal deletion in a 200-kD protein could account for the presence of the aberrant hemidesmosomes seen in these patients. Taken together, we predict that in humans as in mice, hemidesmosomes cannot form in epithelial cells in the absence of $\beta 4$ integrin.

New Insights into Functions of $\alpha 6\beta 4$ Integrins

Our results provide the first insights into the loss of cellular functions in the complete absence of hemidesmosomes. Our findings imply that in the absence of hemidesmosomes, cells are not only weakly adhesive, as described

above, but in addition, they are susceptible to degeneration and at least in some cases, apoptotic cell death. Cell degeneration and loss of cell-substratum adhesion was even seen in simple epithelia which express $\alpha 6 \beta 4$ but do not have hemidesmosomes.

While degeneration was clearly associated with loss of cell adhesion, it was interesting that even cells that remained attached to their underlying basal lamina still showed signs of degeneration. In nonadhering cells, signs of apoptosis, including chromatin condensation, cytoplasmic condensation, and eventually the appearance of apoptotic bodies, were observed. These signs were similar to those described when cultured keratinocytes were placed in suspension so that they could not adhere to a substratum (Frisch and Francis, 1994). We cannot attribute the degeneration to defects in structural integrity caused by the inability of keratin filaments to anchor to hemidesmosomes: in our BPAG1 knockout mice, we severed the connection between keratin filaments and $\alpha 6 \beta 4$ integrin, and stratified squamous epithelia were still healthy, unless subjected to very severe mechanical stress (Guo et al., 1995). Thus, the inability of other integrins to compensate cannot readily be explained by the differences between the cytoskeletons to which different integrins attach. Rather, it seems more likely that $\alpha 6 \beta 4$ interacts with laminin 5 to mediate an additional, as yet unidentified signal, which is essential for cell survival in the animal. Such a function has typically been ascribed to the integrins that attach to actin-based cytoskeletons and form focal contacts, and the importance of $\alpha 6 \beta 4$ in cell survival has not been hitherto appreciated. The underlying reason for this may reside in the fact that many of the studies on the relative importance of different integrins in cell adhesion and survival have been conducted on tissue culture cells, where adhesion and tissue architecture are very different from that which exists in vivo.

Finally, it was intriguing that the stratified layers of $\beta 4$ null tissues often contained pearls of basal-like keratinocytes, sometimes mitotic, and with very immature cytoplasm. Further studies will be necessary before we can assess whether all these basal keratin-expressing cells are mitotically active, or whether the prophase and metaphase cells that we observe are only aberrations within the population of immature pearl cells. This said, the ability of some suprabasal $\beta 4$ null cells to maintain expression of basal cell markers and to undergo mitosis was remarkable. In this regard, it is interesting that spatial reorganization of basal cells and apparent loss of cell polarity occurs 8–24 h following detachment of epidermis from its substratum in culture (Poumay et al., 1994 and references therein). This spatial reorganization correlated with an internalization of hemidesmosomes; our studies now provide evidence that loss of spatial organization within the epidermis is directly linked to a loss of hemidesmosomes. Moreover, the fact that such reorganization occurs even in attached $\beta 4$ (–/–) cells argues against this process arising merely from a mechanical induced catapulting of basal cells into the suprabasal layers. Finally, it is interesting that squamous cell carcinomas, which contain similar cellular organizations, appear to lose $\beta 4$ expression, but retain $\alpha 3 \beta 1$ and $\alpha 6 \beta 1$ (for review, see Cress et al., 1995). As further studies are conducted, we should be able to address the possibility

that a pearl of keratinocytes in $\beta 4$ null epidermis might represent a clonal expansion of a basal keratinocyte which somehow escaped a cell death mechanism.

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