Cell cycle and adhesion defects in mice carrying a targeted deletion of the integrin $\beta 4$ cytoplasmic domain

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The cytoplasmic domain of the integrin $\beta 4$ subunit mediates both association with the hemidesmosomal cytoskeleton and recruitment of the signaling adaptor protein Shc. To examine the significance of these interactions during development, we have generated mice carrying a targeted deletion of the $\beta 4$ cytoplasmic domain. Analysis of homozygous mutant mice indicates that the tail-less $\alpha 6\beta 4$ binds efficiently to laminin 5, but is unable to integrate with the cytoskeleton. Accordingly, these mice display extensive epidermal detachment at birth and die immediately thereafter from a syndrome resembling the human disease junctional epidermolysis bullosa with pyloric atresia (PA-JEB). In addition, we find a significant proliferative defect. Specifically, the number of precursor cells in the intestinal epithelium, which remains adherent to the basement membrane, and in intact areas of the skin is reduced, and post-mitotic enterocytes display increased levels of the cyclin-dependent kinase inhibitor p27^{Kip}. These findings indicate that the interactions mediated by the $\beta 4$ tail are crucial for stable adhesion of stratified epithelia to the basement membrane and for proper cell-cycle control in the proliferative compartments of both stratified and simple epithelia.

Keywords: blistering skin disease/cell cycle/gene targeting/integrin/signaling

Introduction

Basement membranes are thin, continuous sheets of specialized extracellular matrix (ECM), which support epithelial and other cells and separate them from the underlying interstitial connective tissue (Yurchenco and O'Rear, 1994; Timpl and Brown, 1996). In addition to promoting cell adhesion and cytoskeletal organization, basement membranes influence the proliferation and differentiation of cells. The effects of basement membranes on cellular behavior are likely to be mediated by integrins (Hynes, 1987; Ruoslahti and Pierschbacher, 1987; Hemler, 1990). Upon binding to ECM ligand, integrins cluster on the

plasma membrane and interact with the cytoskeleton, thereby promoting the assembly of adhesive junctions, such as focal adhesions and hemidesmosomes (Borradori and Sonnenberg, 1996; Burridge and Chrzanowska-Wonidka, 1996). In addition, integrins are coupled to intracellular signaling pathways potentially able to regulate the cell cycle (Giancotti, 1997). In particular, a class of integrins, which include the $\alpha 6\beta 4$ laminin receptor, the $\alpha 1\beta 1$ collagen IV/laminin receptor, the $\alpha 5\beta 1$ fibronectin receptor and the broad specificity receptor $\alpha v\beta 3$, are linked to the Ras-ERK signaling pathway and control the cell cycle by the signaling adaptor protein Shc (Mainiero *et al.*, 1995, 1997; Wary *et al.*, 1996).

The integrin $\alpha 6\beta 4$ is a receptor for various isoforms of the basement membrane component laminin and binds with the highest apparent affinity to laminin 5 (Giancotti, 1996). *In vivo*, $\alpha 6\beta 4$ is expressed in a number of tissues and cell types that are in contact with a basement membrane, including both simple and stratified epithelia (Kajiji *et al.*, 1989), Schwann cells (Sonnenberg *et al.*, 1990; Einheber *et al.*, 1993), and a subset of endothelial cells (Kennel *et al.*, 1992; Klein *et al.*, 1993) and thymocytes (Wadsworth *et al.*, 1992). In contrast to all the other known α and β subunit cytoplasmic domains, which are relatively short, the intracellular portion of the $\beta 4$ subunit measures over 1000 amino acids in length and contains, in its C-terminal half, two pairs of type III fibronectin-like repeats separated by a 142 amino acid connecting segment (Hogervorst *et al.*, 1990; Suzuki and Naitoh, 1990). While $\beta 1$ and αv subunit-containing integrins interact with the actin cytoskeleton and localize to focal adhesions, $\alpha 6\beta 4$ is found concentrated at hemidesmosomes both in cultured cells and *in vivo* (Carter *et al.*, 1990; Stepp *et al.*, 1990), suggesting that the $\beta 4$ tail specifies association with the hemidesmosomal cytoskeleton.

Hemidesmosomes are complex adhesive junctions that link the basement membrane to the intracellular keratin cytoskeleton and are found exclusively in the basal cell layer of stratified and transitional epithelia (Borradori and Sonnenberg, 1996). Gene transfer studies in cultured cells have provided evidence that the unique cytoplasmic domain of $\beta 4$, and specifically a region comprising the first pair of type-III fibronectin-like modules and the connecting segment, is required for association of $\alpha 6\beta 4$ with the hemidesmosomal cytoskeleton (Spinardi *et al.*, 1993). Subsequent studies have indicated that ligation of $\alpha 6\beta 4$ activates an integrin-associated kinase and causes phosphorylation of the $\beta 4$ cytoplasmic domain at multiple tyrosine residues (Mainiero *et al.*, 1995). Phosphorylation of a tyrosine activation motif (TAM) located in the connecting segment is likely to be required for association with the hemidesmosomal cytoskeleton, because mutations at either one of the two tyrosine residues in the TAM abolish the incorporation of $\alpha 6\beta 4$ in hemidesmosomes

Fig. 1. Targeted deletion of the $\beta 4$ cytoplasmic domain. (A) Strategy used to introduce a stop codon in the $\beta 4$ gene. The structures of replacement vector, wild-type (WT) locus, mutant locus and tail-less $\beta 4$ protein are shown. The star indicates the Stop codon, which is followed by an SV40 polyadenylation signal and a neomycin resistance expression cassette. Restriction sites are indicated: E, *EcoRI*; N, *NcoI*. The sequences encoding the transmembrane segment of $\beta 4$ are indicated (Tm). P5' and P3' are DNA probes used for Southern hybridization. (B) Southern analysis on genomic DNA from wild-type (+/+) and heterozygous mutant (+/-) ES clones. (C) PCR analysis on the intercrosses between heterozygous mutant mice: wild-type (+/+), heterozygous mutant (+/-) and homozygous mutant (-/-) mice. The 0.8 kb band originates from the mutant allele, the 0.5 kb band from the wild-type allele. (D) A wild-type ($\beta 4^{WT/WT}$), a heterozygous mutant ($\beta 4^{WT/TL}$) and two homozygous mutant mice ($\beta 4^{TL/TL}$) with different degrees of epidermal blistering (arrows) are shown.

(Mainiero *et al.*, 1995). The ability of a truncated $\beta 4$ subunit to exert a dominant-negative effect on hemidesmosome assembly without inhibiting initial adhesion to laminin 5 (Spinardi *et al.*, 1995) and the absence of hemidesmosomes in the skin of $\alpha 6$ and $\beta 4$ knock-out mice (Dowling *et al.*, 1996; Georges-Labouesse *et al.*, 1996; van der Neut *et al.*, 1996) have indicated that $\alpha 6\beta 4$ plays a crucial role in the assembly of hemidesmosomes and their linkage to the keratin filament system. Although it is widely assumed that integrin interaction with the cytoskeleton at adhesive junctions such as hemidesmosomes is necessary to consolidate cell adhesion (Alberts *et al.*, 1994), this hypothesis has not yet been tested directly *in vitro* or *in vivo*. For example, although mice carrying null mutations at the $\beta 4$ or $\alpha 6$ locus die a few hours after birth from a severe form of epidermal blistering (Dowling *et al.*, 1996; Georges-Labouesse *et al.*, 1996; van der Neut *et al.*, 1996), it is not clear whether their cell adhesion defect is caused by the absence of $\alpha 6\beta 4$ -mediated binding to laminin 5, the lack of integrin association with the cytoskeleton, or both.

Several observations suggest that ligation of $\alpha 6\beta 4$ may also promote cell proliferation. In the skin and other

stratified squamous epithelia, the expression of $\alpha 6\beta 4$ is restricted to the actively proliferating basal keratinocytes (Kajiji *et al.*, 1989). When these cells detach from the laminin-rich basement membrane to migrate to the upper epidermal layers, they exit from the cell cycle and begin to differentiate (Hall and Watt, 1989). Similarly, depriving cultured keratinocytes of anchorage to their ECM, which is rich in laminin 5 (Carter *et al.*, 1991; Rousselle *et al.*, 1991), results in withdrawal from the cell cycle and differentiation (Green, 1977). Finally, squamous carcinoma cells endowed with high proliferative potential often express elevated levels of $\alpha 6\beta 4$ (Kimmel and Carey, 1986; Wolf *et al.*, 1990), and overexpression of the integrin augments the invasive potential of breast carcinoma cells (Shaw *et al.*, 1997). These results suggest that $\alpha 6\beta 4$ can facilitate both invasion and growth in tumor cells.

In accordance with these observations, recent biochemical studies have indicated that $\alpha 6\beta 4$ activates signaling pathways able to influence cell proliferation. In particular, ligation of $\alpha 6\beta 4$ results in recruitment of the adaptor protein Shc. Upon association with $\alpha 6\beta 4$, Shc is phosphorylated on tyrosine and thereby combines with the Grb2/mSOS complex (Mainiero *et al.*, 1995). This process

Fig. 2. Expression analysis. (A) An aliquot (10 μ g) of total RNA extracted from the skin of wild-type (+/+), heterozygous (+/-) and homozygous mutant (-/-) E18.5 embryos was probed with a 0.5 kb mouse cDNA fragment complementary to the extracellular portion of β 4 mRNA. (B) Equal amounts of total epidermal proteins extracted from the skin of wild-type and homozygous mutant E18.5 embryos were immunoprecipitated with anti- α 6 mAb GoH3 (α 6) or control anti-MHC mAb W6.32 (C) and probed by immunoblotting with a rabbit antiserum to the ectodomain of β 4. Arrows point to wild-type and tail-less β 4. The two lower molecular weight forms of wild-type β 4 correspond to the proteolytic processing products A and B described previously (Giancotti *et al.*, 1992). (C) CD25⁺ (solid lines) and CD25⁻ (dashed lines) thymocytes from wild-type and homozygous mutant E16.5 embryos were subjected to FACS analysis with mAb 346-11A, which binds to the extracellular domain of mouse β 4. Mean intensities of fluorescence (MIF) for CD25⁺ cells are indicated.

results in the activation of both the Ras-ERK and the Rac-JNK mitogen-activated protein (MAP) kinase pathways (Mainiero *et al.*, 1997). The α 6 β 4 integrin has also been shown to activate phosphatidylinositol 3-OH kinase (PI-3K) (Shaw *et al.*, 1997). Since inhibition of PI-3K blocks the activation of JNK by α 6 β 4, it is likely that this lipid kinase couples α 6 β 4-mediated activation of Ras to the Rac-JNK pathway (Mainiero *et al.*, 1997). In cultured keratinocytes, adhesion to laminin 5 mediated by α 6 β 4 induces transcription from the Fos serum response element (SRE) and promotes progression through the G₁ phase of the cell cycle in the presence of epidermal growth factor (EGF). In contrast, adhesion mediated by α 2 β 1, which is not linked to Shc signaling, results in exit from the cell cycle even in the presence of otherwise mitogenic

concentrations of EGF (Mainiero *et al.*, 1997). These findings suggest that the signals from α 6 β 4 and the EGF receptor converge on the Ras-ERK pathway, ultimately resulting in transcription of immediate-early genes and progression through G₁. Although it is clear that α 6 β 4 can promote cell proliferation under defined conditions *in vitro*, it remains to be established whether α 6 β 4 signaling influences cell proliferation *in vivo*.

In order to examine the biological significance of the intracellular functions of α 6 β 4 *in vivo*, we have generated mice carrying a targeted deletion of the β 4 cytoplasmic domain. Analysis of these mice indicates that the β 4 cytoplasmic domain is necessary for stable adhesion of stratified epithelia to the basement membrane and for proper cell cycle control in both simple and stratified epithelia.

Results and discussion

Targeted deletion of the β 4 cytoplasmic domain

Homologous recombination in embryonic stem (ES) cells was used to introduce a stop codon in the mouse β 4 gene immediately after the sequences encoding the transmembrane domain. As illustrated in Figure 1A, the replacement vector consisted of a fragment of $\sim$ 7 kb of the mouse β 4 gene interrupted immediately after the exon encoding the transmembrane segment by an insertion cassette containing a stop codon followed by an SV40 polyadenylation site and a neomycin resistance gene. Homologous recombination at the wild-type locus was expected to generate a mutant locus encoding a β 4 subunit truncated after Lys734, the halt-transfer stop signal. Gene transfer experiments in cultured cells have indicated that this tail-less β 4 subunit combines efficiently with α 6 and is exported regularly to the cell surface, but is unable to associate with Shc (Mainiero *et al.*, 1997) or the hemidesmosomal cytoskeleton (data not shown). Southern blot analysis indicated successful incorporation of the desired mutation in $\sim$ 10% of the doubly selected ES cell clones (Figure 1B and data not shown). PCR analysis on the intercrossovers between heterozygous mice carrying the targeted deletion revealed that the mutation was transmitted with the expected Mendelian frequency (Figure 1C). While heterozygous mice appeared completely normal, all homozygous mutant animals presented with extensive blistering of the skin, particularly in the abdomen, the ventral portion of the torso, the paws and the tip of the tail (Figure 1D). They were unable to feed, and died within a few hours after birth, presumably from dehydration. To minimize damage to the skin, all subsequent analyses were conducted on mice delivered by Cesarean section by embryonic day (E) 18.5.

Expression analysis

To examine if the tail-less β 4 subunit encoded by the mutant allele was expressed correctly, we subjected the skin of wild-type and mutant mice to Northern blotting and immunoprecipitation analysis. As shown in Figure 2A, the recombinant truncated mRNA had the expected size, but was significantly more abundant than the wild-type mRNA, presumably as a result of increased stability and/or transcription. If the biosynthesis of β 4 exceeds that of α 6, the fraction of unpaired β 4 is degraded rapidly in

Fig. 3. The stratified epithelium of mutant mice detaches from the basement membrane leaving a fraction of tail-less $\alpha 6\beta 4$ behind. (A) Hematoxylin–eosin staining of skin and pyloric junction and periodic acid Schiff (PAS)–hematoxylin staining of proximal duodenum from wild-type ($\beta 4^{WT}$) and homozygous mutant ($\beta 4^{TL}$) E18.5 embryos. PAS-positive intestinal goblet cells are stained in red. (B) Sections from the skin of $\beta 4^{WT}$ embryos and attached and unattached areas of the skin of $\beta 4^{TL}$ embryos at E18.5 were subjected to immunofluorescent staining with mAb 346-11A which binds to the extracellular domain of mouse $\beta 4$ (anti- $\beta 4$), mAb GoH3 (anti- $\alpha 6$), affinity-purified rabbit antibodies to mouse keratin 5, which at this stage is expressed in both basal and suprabasal layers of epidermis (anti-Ker 5), and a rabbit antiserum to human laminin 5 (anti-Lam 5). The stars mark the areas of separation of the epidermis from the basement membrane.

Fig. 4. Tail-less $\alpha 6 \beta 4$ does not mediate stable adhesion to laminin 5 *in vitro*. (A) Static adhesion assay. Primary keratinocytes from $\beta 4^{\text{WT}}$ and $\beta 4^{\text{TL}}$ E18.5 embryos were incubated in the presence of the inhibitory anti-mouse $\beta 1$ mAb HM β 1-1 on microtiter wells coated with the indicated concentrations of purified laminin 5 for 30 min. (B) Laminar flow detachment assay. Cells were drawn into glass capillaries coated with 10 $\mu\text{g}/\text{ml}$ purified laminin 5 and allowed to adhere under static conditions for 30 min in the presence of the inhibitory anti- $\beta 1$ mAb HM β 1-1. The cells were then subjected to increasing flow rates. The number of cells remaining adherent after each step is expressed as a percentage of cells adhering at time 0 (T_0).

the endoplasmic reticulum (ER) (Giancotti *et al.*, 1992). In accordance with this finding, immunoprecipitation experiments indicated that the amount of tail-less $\beta 4$ associated with $\alpha 6$ in homozygous mutant mice was comparable with that of wild-type $\beta 4$ paired with $\alpha 6$ in wild-type mice (Figure 2B). To verify that the levels of tail-less $\alpha 6 \beta 4$ at the cell surface were comparable with those of wild-type integrin, we performed fluorescence-activated cell sorting (FACS) analysis on CD25⁺ thymocytes derived from homozygous mutant and wild-type mice. These cells express homogeneous levels of $\alpha 6 \beta 4$ at their surface (Wadsworth *et al.*, 1992) and can be analyzed easily without subculturing. The results demonstrated that the level of expression of tail-less $\alpha 6 \beta 4$ at the cell surface was similar to that of wild-type integrin (Figure 2C). Similar results were obtained with primary keratinocytes (not shown). We concluded that the tail-less integrin encoded by the mutant allele was expressed correctly at the cell surface.

Characterization of the cell adhesion defect

Histological analysis of the skin of mutant mice showed wide areas of separation of the epidermis from the dermis (Figure 3A). In several instances, the epidermis remained connected to the dermis by short filaments emanating from the basal keratinocytes. Cross-sections at the pyloric junction revealed that the stratified epithelium of the esophagus had detached from the underlying connective tissue and degenerated (Figure 3A). Similar signs of degeneration were also observed in cross-sections of the largest (and presumably oldest) epidermal blisters. No signs of apoptosis in either affected or unaffected areas of the skin were revealed by TdT-mediated dUTP-biotin nick end labeling (TUNEL) analysis (data not shown), and electron microscopy indicated that cell death in the oldest blisters was due to necrosis, presumably from the interruption of nutrient exchange with the dermal interstitium (Figure 6, bottom left panel). These results are consistent with the observation that primary keratinocytes denied anchorage to the ECM exit from the cell cycle and begin to differentiate rather than undergoing apoptosis (Gandarillas *et al.*, 1997). The histological abnormalities of tail-less $\beta 4$ mice resembled those observed in the human disease junctional epidermolysis bullosa with

pyloric atresia (PA-JEB), which is caused by mutations in the $\alpha 6$ or $\beta 4$ gene (Vidal *et al.*, 1995; Ruzzi *et al.*, 1997), as well as in $\alpha 6$ and $\beta 4$ knock-out mice (Dowling *et al.*, 1996; Georges-Labouesse *et al.*, 1996; van der Neut *et al.*, 1996). In contrast to the epidermis, the intestinal epithelium of homozygous mutant mice did not detach from the underlying mesenchyme (Figure 3A), suggesting that the cytoplasmic domain of $\beta 4$ is essential for stable adhesion of stratified, but not simple, epithelia. No signs of apoptosis were detected in either wild-type or mutant intestinal epithelium at E18.5 (data not shown). Thus, signaling by the cytoplasmic domain of $\beta 4$ may contribute to, but does not appear to be required for, epithelial survival.

We next examined the mechanism of epidermal detachment in mutant mice. Immunofluorescent staining of unaffected areas of the skin indicated that the tail-less $\alpha 6 \beta 4$ was concentrated normally at the basement membrane junction. This suggests that the tail-less integrin binds efficiently to laminin 5, and this binding is sufficient for recruitment to the basal surface in the absence of cytoskeletal interactions (Figure 3B). The immunostaining for laminin 5 (as well as collagen IV and laminin 1, data not shown) in mutant mice was linear along the basement membrane and had the same intensity as that observed in normal mice. The pattern of expression of other epidermal integrins, including $\alpha 1 \beta 1$, $\alpha 2 \beta 1$ and the basement membrane assembly receptor $\alpha 3 \beta 1$ (DiPersio *et al.*, 1997), was also unchanged in the skin of mutant mice (data not shown). Finally, the ultrastructure of basement membrane in the skin of mutant mice appeared normal at the electron microscopic level (Figure 6, top right). These observations suggest that the mutation in $\beta 4$ does not affect the expression of other integrins or the assembly of the basement membrane.

Immunofluorescent analysis of detached areas of the skin of mutant mice indicated that the anti-laminin 5 staining demarcated the floor of the lesions, indicating that the skin had split above the basement membrane. However, a significant fraction of tail-less $\alpha 6 \beta 4$ remained attached to the basement membrane, while keratin 5 was confined entirely to the epidermis above the roof of the blister (Figure 3B). In accordance with this finding, electron microscopic analysis indicated that remnants of basal cell projections remained attached to the basement

Fig. 5. Abnormal recruitment of HD1/plectin to the basement membrane zone in the epidermis of tail-less $\beta 4$ mice. Skin sections from $\beta 4^{WT}$ and $\beta 4^{TL}$ E18.5 embryos were subjected to immunofluorescent staining with human mAb 5E (anti-BPAG1), rabbit antiserum to BPAG2 (anti-BPAG2) and mAb 121 (anti-HD1).

Fig. 6. Absence of hemidesmosomes in the epidermis of tail-less $\beta 4$ mice. The epidermal-dermal junction of $\beta 4^{WT}$ and $\beta 4^{TL}$ E18.5 embryos was examined by transmission electron microscopy. Note the presence of hemidesmosomes in the skin of $\beta 4^{WT}$ embryos (top left) and their absence in the skin of $\beta 4^{TL}$ embryos (top right). Desmosomes are intact in $\beta 4^{WT}$ embryos (top right, insert). In the skin of $\beta 4^{TL}$ embryos, basal keratinocytes remaining in contact with the basement membrane do not show signs of apoptosis, while those which have detached from the basement membrane display cytoplasmic and nuclear features of necrosis. Arrows point to the nuclei of two necrotic keratinocytes above a small blister (B) (bottom left). Isolated detaching keratinocytes often left fragments of their basal portion attached to the basement membrane (bottom right, arrow). The separation between epidermal cells in the lower panels is an artifact of fixation. Magnifications are indicated in the lower left corner of each panel.

Fig. 7. Proliferation defects in the epidermis and intestinal epithelium of tail-less $\beta 4$ mice. (A) Sections from the skin and duodenum of $\beta 4^{WT}$ and $\beta 4^{TL}$ E18.5 embryos were subjected to immunoperoxidase staining with the anti-Ki-67 mAb MM1 and counterstained with hematoxylin. (B) Duodenal sections from $\beta 4^{WT}$ and $\beta 4^{TL}$ E18.5 embryos were subjected to immunoperoxidase staining with affinity-purified anti-p27 antibodies and counterstained with hematoxylin. Arrows point to p27-positive cells in the intervillar region.

membrane (Figure 6, bottom right panel). The observation that a significant fraction of tail-less $\alpha 6\beta 4$ appears to be pulled out of the membrane of basal keratinocytes when these cells detached from the basement membrane is consistent with the finding that the tail-less integrin binds efficiently to laminin 5, but cannot integrate with the cytoskeleton.

To investigate further the adhesive function of tail-less $\alpha 6\beta 4$, we performed adhesion assays on primary keratinocytes derived from wild-type and homozygous mutant mice. The cells were plated on microtiter wells coated with different concentrations of laminin 5 in the presence of a monoclonal antibody that blocks the adhesive function of all $\beta 1$ integrins, including $\alpha 3\beta 1$, the only other known laminin 5 receptor in keratinocytes. As shown in Figure 4A, the keratinocytes expressing tail-less $\alpha 6\beta 4$ adhered to laminin 5 as well as cells expressing the wild-type integrin in an adhesion assay. However, the results of a laminar flow detachment assay revealed that the adhesive strength mediated by tail-less $\alpha 6\beta 4$ was significantly lower than that of the wild-type integrin (Figure 4B). These results indicate that while the tail-less integrin binds efficiently to laminin 5, it is unable to mediate stable adhesion.

To examine the role of the $\beta 4$ tail in hemidesmosome

assembly, non-lesional areas of the skin of mutant mice were subjected to immunofluorescence and electron microscopic analysis. Immunofluorescent staining indicated that the cytoskeletal component HD1/plectin, which interacts with the $\beta 4$ cytoplasmic domain (Niessen *et al.*, 1997; Rezniecek *et al.*, 1998), was distributed diffusely in the cytoplasm of basal keratinocytes instead of being concentrated at the basement membrane junction (Figure 5). The putative adhesion receptor bullous pemphigoid antigen 2 (BPAG2), which is thought to be recruited to hemidesmosomes in response to a signal generated by the $\beta 4$ cytoplasmic domain (Borradori *et al.*, 1997), was partially polarized at the basal surface of keratinocytes in both wild-type and mutant mice, suggesting that the ability of BPAG2 to bind to an extracellular ligand may be sufficient for targeting at the basement membrane junction (Figure 5). The hemidesmosomal plaque component BPAG1, which is involved in interaction with the keratin cytoskeleton (Guo *et al.*, 1995), was concentrated at the basement membrane junction despite the absence of the $\beta 4$ tail (Figure 5), perhaps because it can interact at least indirectly with BPAG 2. These observations indicate that the cytoplasmic domain of $\beta 4$ is necessary for the recruitment of HD1/plectin, but not BPAG2 and BPAG1, to the basement membrane zone.

Fig. 8. Normal expression of differentiation markers in the epidermis and intestinal epithelium of tail-less $\beta 4$ mice. Skin sections from $\beta 4^{\text{WT}}$ and $\beta 4^{\text{TL}}$ E18.5 embryos were subjected to either double immunostaining with antibodies to loricrin (red) and laminin $\beta 2$ subunit (green) or single immunostaining with antibodies to filaggrin. Duodenal sections from $\beta 4^{\text{WT}}$ and $\beta 4^{\text{TL}}$ E18.5 embryos were assayed for lactase activity (Stoward and Everson Pearce, 1991) (blue).

Finally, electron microscopic analysis revealed that the epidermis of mutant mice completely lacked hemidesmosomes (Figure 6, top right). The desmosomes were instead intact (Figure 6, top right, insert). Taken together, these results demonstrate that the $\beta 4$ cytoplasmic domain is necessary for nucleation of hemidesmosomes and indicate that the adhesive defect of mutant mice is caused by the inability of tail-less $\alpha 6\beta 4$ to mediate this function.

Analysis of the cell signaling defect

In the next series of experiments, we analyzed if $\alpha 6\beta 4$ signaling regulated cell proliferation in stratified and simple epithelia. In the adult epidermis, the expression of $\alpha 6\beta 4$ is restricted to the basal layer, which contains cells with proliferative capacity (Giancotti, 1996). To examine the role of $\alpha 6\beta 4$ signaling in epidermal proliferation, skin sections from wild-type and mutant mice at E18.5 were stained with monoclonal antibody (mAb) Ki-67, which is directed toward a nuclear protein expressed exclusively during the S, G₂ and M phase of the cell cycle (Schlüter *et al.*, 1993). To rule out effects caused by disrupted adhesion, we examined only non-lesional areas of mutant mice. As shown in Figure 7A, the epidermis of mutant mice contained significantly fewer Ki-67-positive cells per linear millimeter of basement membrane (33.7 ± 4.0) than that of wild-type mice (61.7 ± 8.1). In addition, in normal mice at E18.5, both the basal and immediately suprabasal layers contained a large fraction of Ki-67-positive cells, but in mutant mice cell proliferation was largely restricted to the basal layer (Figure 7A). This result indicates that the cytoplasmic domain of $\beta 4$ is required for optimal proliferation of epidermal precursor cells.

The integrin $\alpha 6\beta 4$ is also expressed in several simple epithelia including the gastrointestinal tract (Simon-Assmann *et al.*, 1994). The epithelium of the intestine is renewed continually from a small number of proliferating cells concentrated in invaginated structures named crypts.

Proliferation ceases in the upper segment of the crypt, and post-mitotic enterocytes migrate toward the tip of the villus and begin to differentiate (Gordon and Hermiston, 1994). Different laminin variants are expressed along the crypt-villus axis: laminin 2 is enriched in the crypts, laminin 1 is more abundant in villi and laminin 5 is expressed in both (Simon-Assmann *et al.*, 1994; Orian-Rousseau *et al.*, 1996). Laminin 1 has been shown recently to contribute to the establishment and maintenance of differentiation in enterocyte-like CaCo2 cells (De Arcangelis *et al.*, 1996). The role of other laminin isoforms and their integrin receptors in intestinal function remains to be determined.

To determine if $\alpha 6\beta 4$ signaling also regulated proliferation in simple epithelia, we focused on the intestine. In the proximal duodenum at E18.5, $\alpha 6\beta 4$ and laminin 5 were concentrated at the basement membrane junction in both intervillar spaces and villi (data not shown), and cell proliferation was confined to the intervillar spaces, which invaginate later to form the crypts (Figure 7A). We observed that the total number of Ki-67-positive cells per intervillar space was lower in mutant mice (8.2 ± 1.4) than in wild-type mice (12.9 ± 3.0). In addition, while in normal mice at this stage of development the basal portion of most villi contained a large fraction of Ki-67-positive enterocytes, cell proliferation was largely restricted to the intervillar spaces in mutant mice. Since adhesion in these sections was intact, these findings indicate that signals transduced by the $\beta 4$ tail are necessary to maintain a normal proliferative compartment in both the epidermis and intestine.

Previous studies have indicated that cells denied adhesion to the ECM, and thereby arrested in mid-G₁, contain elevated levels of cyclin-dependent kinase (cdk) inhibitors such as p27^{Kip} (Fang *et al.*, 1996; Zhu *et al.*, 1996). We therefore stained sections of duodenum from wild-type and mutant mice with antibodies to p27^{Kip}. While in normal mice the enterocytes of intervillar spaces and the

basal portion of villi did not contain detectable levels of p27^{Kip}, in mutant mice the immunostaining for p27^{Kip} extended to the base of villi with frequent p27^{Kip}-positive cells in the intervillar spaces (Figure 7B, arrows). Accordingly, the intervillar spaces of mutant mice contained fewer p27-negative cells (9.7 ± 2.6) than those of wild-type mice (15.6 ± 4.2). In addition, the nuclei of post-mitotic enterocytes in mutant mice were stained more intensely by anti-p27 antibodies than those in wild-type mice. Although the suprabasal layers of skin contain another cdk inhibitor, p21^{Cip}, we did not detect increased accumulation of this inhibitor in the suprabasal layers of mutant mice (data not shown). These results suggest that $\alpha 6\beta 4$ signaling contributes to the regulation of p27^{Kip}, but not p21^{Cip}. Taken together, the reduced Ki-67 labeling and increased p27^{Kip} staining in the intestine of mutant mice are consistent with the conclusion that $\alpha 6\beta 4$ signaling is necessary for optimal proliferation *in vivo*.

Finally, we examined whether the reduction of progenitor cells associated with the tail-less B4 defect affected differentiation in the epidermis or intestine. Immunofluorescent analysis indicated that the pattern of expression of epidermal differentiation markers involucrin (data not shown), filaggrin and loricrin was similar in wild-type and mutant mice at E18.5 (Figure 8). In addition, assays of enzymatic activity *in situ* indicated that the level of expression and localization of lactase in the intestine were similar in wild-type and mutant mice at E18.5 (Figure 8). These results, together with those of histological analysis, suggest that $\alpha 6\beta 4$ signaling is not required for normal differentiation of skin and intestine during embryogenesis.

Biological implications

It is well established that normal cells must adhere to the ECM in order to proliferate *in vitro* (Giancotti and Mainiero, 1994). The phenotype of mice lacking the $\beta 4$ tail provides direct evidence that integrin signaling regulates the cell cycle *in vivo*. In particular, the observation that the number of proliferating cells in the basal layer of epidermis and intervillar space of intestine is significantly reduced in mutant mice, despite the presence of other Shc-linked integrins in these cells, suggests that the combined input from multiple Shc-linked integrins is required for optimal proliferation. Previous studies have indicated that the basal layer of human epidermis contains two types of precursor cells that can be identified on the basis of their $\beta 1$ integrin expression levels: the slow-cycling stem cells, which express high levels of $\alpha 3\beta 1$ and $\alpha 2\beta 1$; and the more rapidly proliferating transit amplifying cells, which display lower levels of $\beta 1$ integrins (Jones and Watt, 1993; Jones *et al.*, 1995). Notably, $\alpha 6\beta 4$ is expressed at similar levels on both stem cells and transit-amplifying cells (Jones and Watt, 1993; Jones *et al.*, 1995), and may thus affect the proliferation of both types of precursor cells.

In accordance with the observation that anchorage to the ECM is required for proper down-regulation of the cdk inhibitor p27^{Kip} in early-to-mid G₁ (Zhu *et al.*, 1996), we have found that in tail-less $\beta 4$ mice the enterocytes located at the base of villi display increased nuclear levels of p27^{Kip}. In contrast, the post-mitotic keratinocytes did not contain increased levels of the other cdk inhibitor, p21^{Cip}, expressed in the epidermis. These observations

suggest that increased levels of p27^{Kip} in the intestinal precursor cells of mutant mice may contribute to the premature withdrawal from the cell cycle. However, the presence of a significant proliferation defect in the basal layer of the epidermis suggests that the down-regulation of cdk inhibitors is not the only mechanism by which integrin signaling promotes cell cycle progression. Other mechanisms, such as the ability of integrins and growth factor receptors to coordinately regulate the expression of immediate-early genes, including D-type cyclins, are likely to play a crucial role.

The proliferation defect observed in mice lacking the $\beta 4$ tail does not result in a reduced production of differentiated cells in the skin and intestine of E18.5 embryos. This is not surprising because the precursor cell compartment in normal skin and intestine at this stage of development is much larger than in the adult, and thus presumably exceeds the physiological requirements of the tissues. Based on the approach illustrated here, it should now be possible to introduce in mice a $\beta 4$ mutation that abolishes signaling without affecting linkage to the cytoskeleton, and thus to examine if $\alpha 6\beta 4$ signaling is necessary for homeostasis of adult mouse skin and intestine.

It has been speculated widely that the association of integrins with the cytoskeleton is necessary to stabilize adhesion to the ECM (Alberts *et al.*, 1994). The phenotype of mice lacking the $\beta 4$ tail provides a particularly vivid illustration of the importance of cytoskeletal anchorage for stable adhesion. Because of the association of $\beta 4$ tail with the keratin cytoskeleton, stresses applied to the $\alpha 6\beta 4$ -laminin 5 bond may be distributed to the entire cytoskeleton. This view is consistent with the results of static and laminar flow adhesion assays and the observation that a significant fraction of tail-less $\alpha 6\beta 4$ appears to be pulled out of the membrane when the epidermis of mutant mice detaches from the basement membrane.

The phenotype of tail-less $\beta 4$ mice closely resembles that of humans affected by PA-JEB, including the blistering of the skin and the detachment and degeneration of the pyloric epithelium. Therefore, the cell adhesion defect in PA-JEB patients is most likely derived from the disruption of the transmembrane link between the basement membrane and keratin cytoskeleton mediated by $\alpha 6\beta 4$. Our observation that $\alpha 6\beta 4$ also regulates epidermal proliferation may explain another important feature of PA-JEB. In contrast to individuals with classical JEB, which have a severe form of skin blistering caused by mutations in the $\alpha 6\beta 4$ ligand laminin 5, but never display areas of skin hypoplasia or aplasia (Aberdam *et al.*, 1994; Pulkkinen *et al.*, 1994), newborns affected by PA-JEB display extensive areas of skin aplasia, especially in the lower limbs (Vidal *et al.*, 1995; Ruzzi *et al.*, 1997). Since the production of differentiated keratinocytes appears to be normal in our mutant mice, it is possible that the precursor cell compartment in certain areas of human fetal skin is smaller than in mice so that the $\alpha 6\beta 4$ mutation results in neonatal aplasia only in humans.

In conclusion, the results of this study indicate that the intracellular interactions mediated by the $\beta 4$ cytoplasmic domain are necessary for stable adhesion of stratified epithelia to the basement membrane and for proper cell cycle control in both simple and stratified epithelia. These findings reveal that normal cells are also exquisitely

anchorage-dependent *in vivo* and help to understand the molecular basis of the human blistering skin disease PA-JEB.

Materials and methods

Targeted deletion of the $\beta 4$ cytoplasmic domain

A 7 kb fragment of the mouse gene $\beta 4$ was isolated by screening a 129Sv library with a human cDNA probe complementary to the sequence encoding the transmembrane domain. Site-directed mutagenesis was used to introduce a stop codon followed by a novel *EcoRI* site immediately after the sequence encoding the transmembrane domain. The *EcoRI* site was then used to subclone an SV40 polyadenylation signal followed by a neomycin resistance expression cassette downstream of the stop codon. Finally, the modified fragment was introduced into the targeting vector pPNT (Tybulewicz *et al.*, 1991) in two steps (left arm 5 kb and right arm 1.8 kb). Prior to electroporation in ES cells (Swiatek and Gridley, 1993), the targeting vector was linearized by digestion with *NorI*. Positively transfected cells which had undergone homologous recombination were selected in 0.5 mg/ml G418 and 0.2 mM gancyclovir and identified by Southern blotting. Six distinct ES cell lines were found to carry the expected mutation, and four were injected into blastocyst-stage C57BL/6 mouse embryos which were then transplanted into the uteri of pseudopregnant C57BL/6 mice. Two lines produced extensively chimeric male mice which were crossed to C57BL/6 females. Heterozygous offspring were then used to generate embryos homozygous for the targeted deletion. Embryos were genotyped by PCR using genomic DNA isolated from the forelimb. The primers used for amplification were: right arm primer, 5'-GATCTTCCAGCGG-ACTGTGTC-3'; neomycin cassette primer, 5'-GCTCCCGATTGCGA-GCGCCATCG-3'; and wild-type specific primer, 5'-AGAGGATGGTGTCTCCTCA-3'. The wild-type allele yielded a 0.5 kb fragment and the mutant allele gave rise to a 0.8 kb fragment.

Northern blotting

Total RNA was extracted from mouse skin and hybridized to a 0.5 kb cDNA probe complementary to sequences in the $\beta 4$ ectodomain. The probe was generated by amplifying reverse-transcribed RNA from mouse PAM keratinocytes with the following primers: 5'-CTGTGAGC-AAGGAAGTT-3' and 5'-CGTGTAGAGCGACTGCTGTT-3'.

Antibodies

The rabbit antiserum SE144 recognizes the $\gamma 2$ subunit of laminin 5 (Vailly *et al.*, 1994) and mAb 4E10 the $\beta 2$ subunit of laminin 1, 2, 6 and 8 (Engvall *et al.*, 1986). The rat anti-mouse $\alpha 6$ mAb GoH3 and hamster anti-mouse $\beta 1$ mAb HM β 1-1 were purchased from Pharmingen (Los Angeles, CA). The rabbit antiserum to a GST fusion protein comprising amino acids 31–217 of human $\beta 4$ has been described (Maniero *et al.*, 1997). mAb 346-11A reacts with the ectodomain of mouse $\beta 4$ (Kennel *et al.*, 1989). The antibodies to BPAG2 were raised by immunizing rabbits with a GST fusion protein comprising amino acids 1–250 of mouse BPAG2 and affinity purified on the antigen. MAb5E reacts with BPAG1 (Shimizu *et al.*, 1991) and mAb 121 with HD1/plectin (Hieda *et al.*, 1992). Affinity-purified rabbit antibodies to synthetic peptides from mouse filaggrin, loricrin, keratin 1, keratin 5 and involucrin were characterized previously (Calautti *et al.*, 1995). The anti-Ki-67 mAb MM1 was produced at the Monoclonal Antibody Facility of Sloan Kettering Institute. Affinity-purified antibodies to a GST fusion protein comprising full-length p27^{Kip} were described previously (Casaccia-Bonnel *et al.*, 1997). Secondary antibodies were purchased from Jackson ImmunoResearch Laboratories (West Grove, PA).

Immunoprecipitation

Skins from wild-type and homozygous mutant E18.5 embryos were extracted in RIPA buffer containing protease inhibitors. Total epidermal proteins were normalized by immunoblotting with anti-keratin 1 antibodies. Equivalent amounts of total epidermal proteins were immunoprecipitated with the anti- $\alpha 6$ mAb GoH3 and subjected to immunoblotting with the antiserum to the $\beta 4$ ectodomain (Mainiero *et al.*, 1997).

Adhesion assays

Primary mouse keratinocytes were isolated as described (DiPersio *et al.*, 1997). For static adhesion assay, primary keratinocytes from wild-type and mutant E18.5 embryos were pre-incubated with 10 μ g/ml of anti- $\beta 1$ mAb HM β 1-1, to block the adhesive function of $\alpha 3\beta 1$, and plated

for 30 min in the presence of the same antibody on microtiter wells coated with the indicated concentrations of purified laminin 5. For laminar flow adhesion assay (Brieher *et al.*, 1996), primary keratinocytes from wild-type and mutant E18.5 embryos were pre-incubated with 10 μ g/ml anti- $\beta 1$ mAb HM β 1-1 and drawn into a glass capillary coated with 10 μ g/ml purified laminin 5. The cells were allowed to bind under static conditions for 30 min in the presence of the same antibody and then subjected to increasing flow rates. The number of cells remaining adherent after each step was evaluated microscopically.

Immunohistochemistry and lactase assay

Skin and intestine samples were harvested from embryos and embedded in either OCT or paraffin. Tissue sections (10 μ M) were stained with hematoxylin and eosin or subjected to either immunofluorescence or immunoperoxidase staining. Lactase activity was assayed on tissue sections as previously described (Stoward and Everson Pearse, 1991).

Acknowledgements

We thank V. Soares and W. Mark of the Transgenic Mouse Facility, K. Manova of the Molecular Cytology Facility, and N. Lampen of the Electron Microscopy Facility for their invaluable help; E. Calautti, G. P. Dotto and C. M. DiPersio for their advice on the isolation of primary keratinocytes; W. M. Brieher for assistance in the laminar flow assays; P. Rousselle and R. E. Burgeson for providing purified laminin 5; E. Calautti, G. P. Dotto, E. Engvall, T. Hashimoto, S. J. Kennel, A. Koff, G. Meneguzzi, K. Owaribe, M. Park and F. Watt for antibodies; and C. Blobel and B. Gumbiner for comments on the manuscript and discussions. This work was supported by DAMD grant 17-94-J4306 and NIH grants R01-CA58976 and P30-CA08748. C.M. was on leave of absence from the Istituto Nazionale della Nutrizione in Rome. F.G.G. is an Established Investigator of the American Heart Association.

References

- Alberts, B., Bray, D., Lewis, J., Raff, M., Roberts, K. and Watson, J. D. (1994) Extracellular matrix receptors on animal cells: the integrins. In *Molecular Biology of the Cell*. 3rd edn. Garland Publishing, Inc., New York and London, pp. 995–999.
- Aberdam, D. *et al.* (1994) Herlitz's junctional epidermolysis bullosa is genetically linked to mutations in the *nicotin/kalinin (laminin-5) LAMC2* gene. *Nature Genet.*, **6**, 299–304.
- Borradori, L. and Sonnenberg, A. (1996) Hemidesmosomes: roles in adhesion, signaling and human diseases. *Curr. Opin. Cell Biol.*, **8**, 647–656.
- Borradori, L., Koch, P. J., Niessen, C. M., Erkeland, S. and van Leusden, M. R. (1997) The localization of bullous pemphigoid antigen 180 (BP180) in hemidesmosomes is mediated by its cytoplasmic domain and seems to be regulated by the $\beta 4$ integrin subunit. *J. Cell Biol.*, **136**, 1333–1347.
- Brieher, W. M., Yap, A. S. and Gumbiner, B. M. (1996) Lateral dimerization is required for the homophilic binding activity of C-cadherin. *J. Cell Biol.*, **135**, 487–496.
- Burridge, K. and Chrzanowska-Wonidka, M. (1996) Focal adhesions, contractility, and signaling. *Annu. Rev. Dev. Biol.*, **12**, 463–519.
- Calautti, E., Missero, C., Stein, P. L., Ezzel, R. M. and Dotto, G. P. (1995) Fyn tyrosine kinase is involved in keratinocyte differentiation control. *Genes Dev.*, **9**, 2279–2291.
- Carter, W. G., Kaur, P., Gil, S. G., Gahr, P. J. and Wayner, E. A. (1990) Distinct functions for integrins $\alpha 3\beta 1$ in focal adhesions and $\alpha 6\beta 4$ /bullous pemphigoid antigen in a new stable anchoring contact (SAC) of keratinocytes: relation to hemidesmosomes. *J. Cell Biol.*, **111**, 3141–3154.
- Carter, W. G., Ryan, M. C. and Gahr, P. J. (1991) Epiligrin, a new cell adhesion ligand for integrin $\alpha 3\beta 1$ in epithelial basement membranes. *Cell*, **65**, 599–610.
- Casaccia-Bonnel, P., Tikoo, R., Kiyokawa, H., Friedrich, V., Jr, Chao, M. V. and Koff, A. (1997) Oligodendrocyte precursor differentiation is perturbed in the absence of the cyclin-dependent kinase inhibitor p27^{Kip1}. *Genes Dev.*, **11**, 2335–2346.
- De Arcangelis, A., Neuville, P., Boukamel, R., Lefebvre, O., Keding, M. and Simon-Assmann, P. (1996) Inhibition of laminin alpha 1-chain expression leads to alteration of basement membrane assembly and cell differentiation. *J. Cell Biol.*, **133**, 417–430.

- DiPersio, C.M., Hodivala-Dilke, K.M., Jaenisch, R., Kreidberg, J.A. and Hynes, R.O. (1997) $\alpha 3 \beta 1$ integrin is required for normal development of the epidermal basement membrane. *J. Cell Biol.*, **137**, 729–742.
- Dowling, J., Yu, Q.-C. and Fuchs, E. (1996) $\beta 4$ integrin is required for hemidesmosome formation, cell adhesion and cell survival. *J. Cell Biol.*, **134**, 559–572.
- Einheber, S., Milner, T.A., Giancotti, F.G. and Salzer, J.L. (1993) Axonal regulation of Schwann cell integrin expression suggests a role for $\alpha 6 \beta 4$ in myelination. *J. Cell Biol.*, **123**, 1223–1236.
- Engvall, E., Davis, G.E., Dickerson, K., Ruoslahti, E., Varon, S. and Manthorpe, M. (1986) Mapping of domains in human laminin using monoclonal antibodies: localization of the neurite-promoting site. *J. Cell Biol.*, **103**, 2457–2465.
- Fang, F., Orend, G., Watanabe, N., Hunter, T. and Ruoslahti, E. (1996) Dependence of cyclin E-CDK 2 kinase activity on cell anchorage. *Science*, **271**, 499–502.
- Gandarillas, A. and Watt, F.M. (1997) c-Myc promotes differentiation of human epidermal stem cells. *Genes Dev.*, **11**, 2869–2882.
- Georges-Labouesse, E., Messaddeq, N., Yehia, G., Cadalbert, L., Dierich, A. and Le Meur, M. (1996) Absence of integrin $\alpha 6$ leads to epidermolysis bullosa and neonatal death in mice. *Nature Genet.*, **13**, 370–373.
- Giancotti, F.G. (1996) Signal transduction by the $\alpha 6 \beta 4$ integrin: charting the path between laminin binding and nuclear events. *J. Cell Sci.*, **109**, 1165–1172.
- Giancotti, F.G. (1997) Integrin signaling: specificity and control of cell survival and cell cycle progression. *Curr. Opin. Cell Biol.*, **9**, 691–700.
- Giancotti, F.G. and Mainiero, F. (1994) Integrin-mediated adhesion and signaling in tumorigenesis. *Biochim. Biophys. Acta*, **1198**, 47–64.
- Giancotti, F.G., Stepp, M.A., Suzuki, S., Engvall, E. and Ruoslahti, E. (1992) Proteolytic processing of endogenous and recombinant $\beta 4$ integrin subunit. *J. Cell Biol.*, **118**, 951–959.
- Gordon, J.I. and Hermiston, M.L. (1994) Differentiation and self-renewal in the mouse gastrointestinal epithelium. *Curr. Opin. Cell Biol.*, **6**, 795–803.
- Green, H. (1977) Terminal differentiation of cultured human epidermal cells. *Cell*, **11**, 405–416.
- Guo, L., Degenstein, L., Dowling, J., Yu, Q.-C., Wollmann, R., Perman, B. and Fuchs, E. (1995) Gene targeting of BPAG1: abnormalities in mechanical strength and cell migration in stratified epithelia and neurologic degeneration. *Cell*, **81**, 233–243.
- Hall, P.A. and Watt, F.M. (1989) Stem cells: the generation and maintenance of cellular diversity. *Development*, **106**, 619–633.
- Hemler, M.E. (1990) VLA proteins in the integrin family. *Annu. Rev. Immunol.*, **8**, 365–400.
- Hieda, Y., Nishizawa, Y., Uematsu, J. and Owaribe, K. (1992) Identification of a new hemidesmosomal protein, HD1: a major high molecular mass component of isolated hemidesmosomes. *J. Cell Biol.*, **116**, 1497–1506.
- Hogervorst, F., Kuikman, I., von dem Borne, A.E.G.Kr. and Sonnenberg, A. (1990) Cloning and sequence analysis of $\beta 4$ cDNA: an integrin subunit that contains a unique 118 kd cytoplasmic domain. *EMBO J.*, **9**, 745–770.
- Hynes, R.O. (1987) Integrins: a family of cell surface receptors. *Cell*, **48**, 549–554.
- Jones, P.H. and Watt, F.M. (1993) Separation of human epidermal stem cells from transit amplifying cells on the basis of differences in integrin function and expression. *Cell*, **73**, 713–724.
- Jones, P.H., Harper, S. and Watt, F.M. (1995) Stem cell patterning and fate in human epidermis. *Cell*, **80**, 83–93.
- Kajiji, S., Tamura, R.N. and Quaranta, V. (1989) A novel integrin ($\alpha E \beta 4$) from human epithelial cells suggests a fourth family of integrin adhesion receptors. *EMBO J.*, **8**, 673–680.
- Kennel, S.J., Foote, L.J., Falcioni, R., Sonnenberg, A., Stringer, C.D., Crouse, C. and Hemler, M.E. (1989) Analysis of the tumor-associated antigen TSP-180. Identity with alpha 6-beta-4 in the integrin superfamily. *J. Biol. Chem.*, **264**, 15515–15521.
- Kennel, S.J., Godfrey, V., Chang, L.Y., Lankford, T.K., Foote, L.J. and Makkinje, A. (1992) The $\beta 4$ subunit of the integrin family is displayed on a restricted subset of endothelium in mice. *J. Cell Biol.*, **101**, 145–150.
- Kimmel, K.A. and Carey, T.E. (1986) Altered expression in squamous carcinoma cells of an orientation restricted epithelial antigen detected by monoclonal antibody A9. *Cancer Res.*, **46**, 3614–3623.
- Klein, S., Giancotti, F.G., Presta, M., Albelda, S.M., Buck, C.A. and Rifkin, D.B. (1993) Basic fibroblast growth factor modulates integrin expression in microvascular endothelial cells. *Mol. Biol. Cell*, **4**, 973–982.
- Mainiero, F., Pepe, A., Wary, K.K., Spinardi, L., Mohammadi, M., Schlessinger, J. and Giancotti, F.G. (1995) Signal transduction by the $\alpha 6 \beta 4$ integrin: distinct $\beta 4$ subunit sites mediate recruitment of Shc/Grb2 and association with the cytoskeleton of hemidesmosomes. *EMBO J.*, **14**, 4470–4481.
- Mainiero, F., Murgia, C., Wary, K.K., Curatola, A.M., Pepe, A., Blumberg, M., Westwick, J.K., Der, C.J. and Giancotti, F.G. (1997) The coupling of $\alpha 6 \beta 4$ integrin to Ras-MAP kinase pathways mediated by Shc controls keratinocyte proliferation. *EMBO J.*, **16**, 2365–2375.
- Niessen, C.M., Hulsman, E.H.M., Rots, E.S., Sanchez-Aparicio, P. and Sonnenberg, A. (1997) Integrin $\alpha 6 \beta 4$ forms a complex with the cytoskeletal protein HD1 and induces its redistribution in transfected COS-7 cells. *Mol. Biol. Cell*, **8**, 555–566.
- Orian-Rousseau, V., Aberdam, D., Fontao, L., Chevalier, L., Meneguzzi, G., Keding, M. and Simon-Assmann, P. (1995) Developmental expression of laminin-5 and HD1 in the intestine: epithelial to mesenchymal shift for the laminin $\gamma 2$ chain subunit deposition. *Dev. Dynam.*, **206**, 12–23.
- Pulkkinen, L., Christiano, A.M., Airenne, T., Haakana, H., Tryggvason, K. and Uitto, J. (1994) Mutations in the $\gamma 2$ chain gene (LAMC2) of kalinin/laminin 5 in the junctional forms of epidermolysis bullosa. *Nature Genet.*, **6**, 293–298.
- Reznicek, G.A., de Pereda, J.M., Reipert, S. and Wiche, G. (1998) Linking integrin $\alpha 6 \beta 4$ -based cell adhesion to the intermediate filament cytoskeleton: direct interaction between the $\beta 4$ subunit and plectin at multiple molecular sites. *J. Cell Biol.*, **141**, 209–225.
- Rousselle, P., Lunstrum, G.P., Keene, D.R. and Burgeson, R.E. (1991) Kalinin: an epithelium-specific basement membrane adhesion molecule that is a component of anchoring filaments. *J. Cell Biol.*, **114**, 567–576.
- Ruoslahti, E. and Pierschbacher, D. (1987) New perspectives in cell adhesion: RGD and integrins. *Science*, **238**, 491–497.
- Ruzzi, L., Gagnoux-Palacios, L., Pinolaq, M., Belli, S., Meneguzzi, G., D'Alessio, M.D. and Zambruno, G.A. (1997) Homozygous mutation in the integrin $\alpha 6$ gene in junctional epidermolysis bullosa with pyloric atresia. *J. Clin. Invest.*, **99**, 2826–2831.
- Schlütter, C., Duchrow, M., Wohlenberg, C., Becker, M.H.G., Key, G., Flad, H.-D. and Gerdes, J. (1993) The cell proliferation-associated antigen of antibody Ki-67: a very large, ubiquitous nuclear protein with numerous repeated elements, representing a new kind of cell cycle-maintaining protein. *J. Cell Biol.*, **123**, 513–522.
- Shaw, L.M., Rabinovitz, I., Wang, H., Toker, A. and Mercurio, A.M. (1997) Activation of phosphoinositide 3-OH kinase by the $\alpha 6 \beta 4$ integrin promotes carcinoma invasion. *Cell*, **91**, 949–960.
- Shimizu, H., Hashimoto, T. and Eady, R.A. (1991) Human monoclonal anti basement membrane zone antibodies derived from virally transformed lymphocytes of a patient with bullous pemphigoid recognize epitopes associated with hemidesmosomes. *Br. J. Dermatol.*, **124**, 217–220.
- Simon-Assmann, P., Duclos, B., Orian-Rousseau, V., Arnold, C., Mathelin, C., Engvall, E. and Keding, M. (1994) Differential expression of laminin isoforms and $\alpha 6$ - $\beta 4$ integrin subunits in the developing human and mouse intestine. *Dev. Dynam.*, **201**, 71–85.
- Sonnenberg, A., Linders, C.J.T., Daams, J.H. and Kennel, S.J. (1990) The $\alpha 6 \beta 1$ (VLA-6) and $\alpha 6 \beta 4$ protein complexes: tissue distribution and biochemical properties. *J. Cell Sci.*, **96**, 207–217.
- Spinardi, L., Ren, Y.-L., Sanders, R. and Giancotti, F.G. (1993) The $\beta 4$ subunit cytoplasmic domain mediates the interaction of $\alpha 6 \beta 4$ integrin with the cytoskeleton of hemidesmosomes. *Mol. Biol. Cell*, **4**, 871–884.
- Spinardi, L., Einheber, S., Cullen, T., Milner, T.A. and Giancotti, F.G. (1995) A recombinant tail-less integrin $\beta 4$ subunit disrupts hemidesmosomes, but does not suppress $\alpha 6 \beta 4$ -mediated cell adhesion to laminins. *J. Cell Biol.*, **129**, 473–487.
- Stepp, M.A., Spurr-Michaud, S., Tisdale, A., Elwell, J. and Gipson, I.K. (1990) Alpha 6 beta 4 integrin heterodimer is a component of hemidesmosomes. *Proc. Natl Acad. Sci. USA*, **87**, 8970–8974.
- Stoward, P.J. and Everson, P.A. (1991) *Histochemistry*. Churchill Livingstone, Edinburgh, UK, Vol. 3, pp. 637–638.
- Suzuki, S. and Naitoh, Y. (1990) Amino acid sequence of a novel integrin $\beta 4$ subunit and primary expression of the mRNA in epithelial cells. *EMBO J.*, **9**, 757–763.
- Swiatek, P.J. and Gridley, T. (1993) Perinatal lethality and defects in hindbrain development in mice homozygous for a targeted mutation of the zinc finger protein Krox20. *Genes Dev.*, **7**, 2071–2084.
- Timpl, R. and Brown, J.C. (1996) Supramolecular assembly of basement membranes. *BioEssays*, **18**, 123–132.
- Tybulewicz, V.L., Crawford, C.E., Jackson, P.K., Bronson, R.T. and Mulligan, R.C. (1991) Neonatal lethality and lymphopenia in mice with a homozygous disruption of the *c-abl* proto-oncogene. *Cell*, **65**, 1153–1163.

- Vailly, J. *et al.* (1994) The 100-kDa chain of nicein/kalinin is a laminin B2 chain variant. *Eur. J. Biochem.*, **219**, 209–218.
- van der Neut, R., Krimpenfort, P., Calafat, J., Niessen, C.M. and Sonnenberg, A. (1996) Epithelial detachment due to absence of hemidesmosomes in integrin $\beta 4$ null mice. *Nature Genet.*, **13**, 366–369.
- Vidal, F., Aberdam, D., Miquel, C., Christiano, A.M., Pulkkinen, L., Uitto, J., Ortonne, J.P. and Meneguzzi, G. (1995) Integrin $\beta 4$ mutations associated with junctional epidermolysis bullosa with pyloric atresia. *Nature Genet.*, **10**, 229–234.
- Yurchenco, P.D. and O'Rear, J.J. (1994) Basal lamina assembly. *Curr. Opin. Cell Biol.*, **6**, 674–681.
- Wadsworth, S., Halvorson, M.J. and Coligan, J.E. (1992) Developmentally regulated expression of the $\beta 4$ integrin on immature mouse thymocytes. *J. Immunol.*, **149**, 421–428.
- Wary, K.K., Mainiero, F., Isakoff, S.J., Marcantonio, E.E. and Giancotti, F.G. (1996) The adaptor protein Shc couples a class of integrins to the control of cell cycle progression. *Cell*, **87**, 733–743.
- Wolf, G.T., Carey, T.E., Schmaltz, S.P., McClatchey, K.D., Poore, J., Glaser, L., Hayashida, D.J.S. and Hsu, S. (1990) Altered antigen expression predicts outcome in squamous cell carcinomas of the head and neck. *J. Natl Cancer Inst.*, **82**, 1566–1572.
- Zhu, X., Ohtsubo, M., Bohmer, R., Roberts, J.M. and Assoian, R.K. (1996) Adhesion dependent cell cycle progression linked to the expression of cyclin D1, activation of cyclin E-cdk2, and phosphorylation of the retinoblastoma protein. *J. Cell Biol.*, **133**, 391–403.

Received March 5, 1998; revised and accepted May 12, 1998

Note added in proof

A recent study provides further evidence that integrin-mediated Shc signalling regulates cell proliferation *in vivo* [Pozzi, A., Wary, K.K., Giancotti, F.G. and Gardner, H. (1998) Integrin $\alpha 1\beta 1$ mediates a unique collagen dependent proliferation pathway *in vivo*. *J. Cell Biol.*, in press].