Absence of integrin α6 leads to epidermolysis bullosa and neonatal death in mice

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Cell-extracellular matrix interactions have important roles in many biological processes, including embryonic development, growth control and differentiation. Integrins are the principal receptors for extracellular matrix[1]. They are composed of non-covalently associated α and β chains[1]. Integrin α6 can associate with either β1 or β4 (refs 2,3). Both integrin complexes are receptors for laminins, major components of basement membranes[2,3]. The distribution of α6 (refs 4–10) as well as studies using function-blocking antibodies have suggested an essential role for this laminin receptor during embryogenesis, in processes such as endoderm migration[4,5] or kidney tubule formation[9]. Here we report that, surprisingly, mice lacking the α6 integrin chain develop to birth. However, they die at birth with severe blistering of the skin and other epithelia, a phenotype reminiscent of the human disorder epidermolysis bullosa[11]. Hemidesmosomes are absent in mutant tissue. This absence is likely to result from the lack of α6/β4, the only integrin in hemidesmosomes of stratified squamous and transitional epithelia[12–14]. Mutations in the genes encoding integrin β4 and chains of laminin-5 have been implicated in junctional epidermolysis bullosa[15–18]. Our study provides evidence that some forms of epidermolysis bullosa may originate from defects of the *α6* gene.

To target inactivation of the *α6* gene, we made a construct that had seven kilobases (kb) of genomic DNA containing three *α6* exons deleted and replaced with a pgk-neomycin cassette (Fig. 1*a*). We identified one embryonic stem (ES) cell clone that carried the targeted allele (Fig. 1*b*), and used it to generate chimaeric animals and to establish a heterozygous strain for the mutated allele (Fig. 1*c*). Northern analysis of total RNA extracted from mutant tissue showed the absence of normal *α6* mRNA (Fig.1*d*). A faint signal corresponding to a truncated mRNA was observed, which should yield a non-functional α6 protein (see Methods). We did not observe any α6 protein in tissue sections from six $\alpha6^{-/-}$ fetuses after staining with antibodies for α6 or for the cytoplasmic domain of α6A (data not shown). This mutation, therefore, corresponds to a loss-of-function of the integrin α6 chain.

Heterozygous animals appeared normal and fertile. After genotyping 62 weanlings from heterozygous intercrosses two weeks post-partum, no live $\alpha6^{-/-}$ animal were found, implying a recessive lethal mutation. When progeny was examined at birth, we found ten dead pups and one that survived a few hours, out of 48 examined. All 11 dead pups showed a very marked skin defect; skin was loosely attached and would detach easily, especially from the legs and tails (Fig. 2*a*,*b*). These animals were homozygous mutant. To verify that homozygous mutants were not dying at earlier stages, we followed the appearance of homozygous mutant animals from embryonic day 14.5 (E14.5) to E18.5. We observed expected mendelian ratios of $\alpha6^{-/-}$ (23/95 $\alpha6^{-/-}$ on a mixed 129Sv/C57 background, 43/156 $\alpha6^{-/-}$ after a single cross onto the CD1 background), thus there was no indication of homozygous mutant animal death earlier during embryogenesis.

Integrin α6, associated with the β1 subunit, is expressed very early in mouse development (two-to-

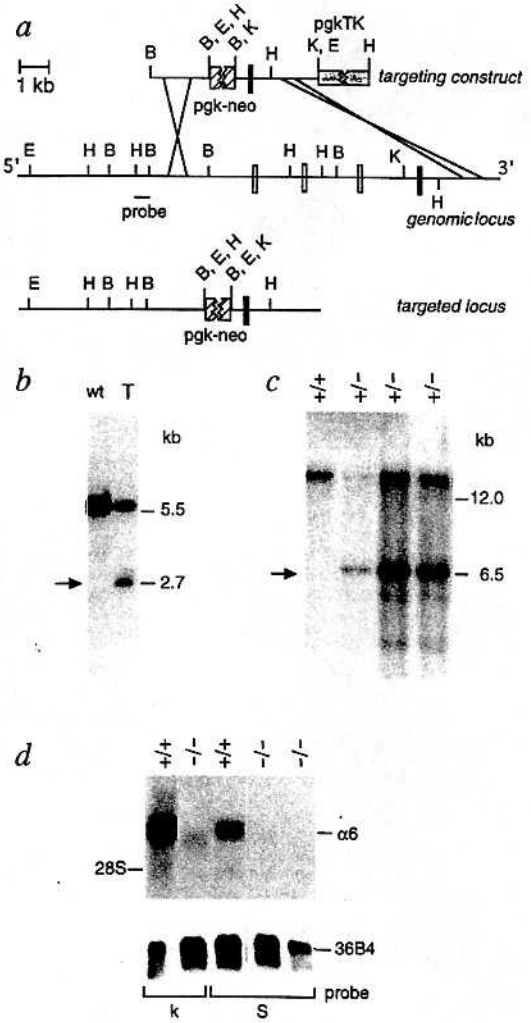

Fig. 1 *a*, Top: targeting construct to inactivate the *α6* gene; middle: parental genomic locus; bottom, resulting targeted locus. A region of 7 kb containing three exons (white boxes) was deleted and replaced by a pgk-neomycin cassette. After homologous recombination, the targeted locus contains a fragment of 2.7 kb after *Hin*dIII digestion or a 6.5-kb fragment after *Eco*RI digestion. The probe 5' of the deletion outside the construct used in Southern analysis is shown. E: *Eco*RI, B: *Bam*HI, H: *Hin*dIII, K: *Kpn*I. *b*, Southern analysis of transfected ES cells; wt: parental clone containing the normal 5.5-kb fragment, and (T) targeted clone, containing the targeted allele, as assessed by the presence of an additional 2.7-kb *Hin*dIII fragment (arrow). *c*, Southern analysis of tail biopsies of the first three heterozygous animals (+/–) with the additional 6.5-kb *Eco*RI-fragment corresponding to the targeted allele (arrow). *d*, Northern analysis of total RNAs extracted from kidney (k) or skin (S) of wild-type (+/+) and homozygous mutant (–/–) animals. Top: *α6* mRNA, bottom: control *36B4* mRNA.

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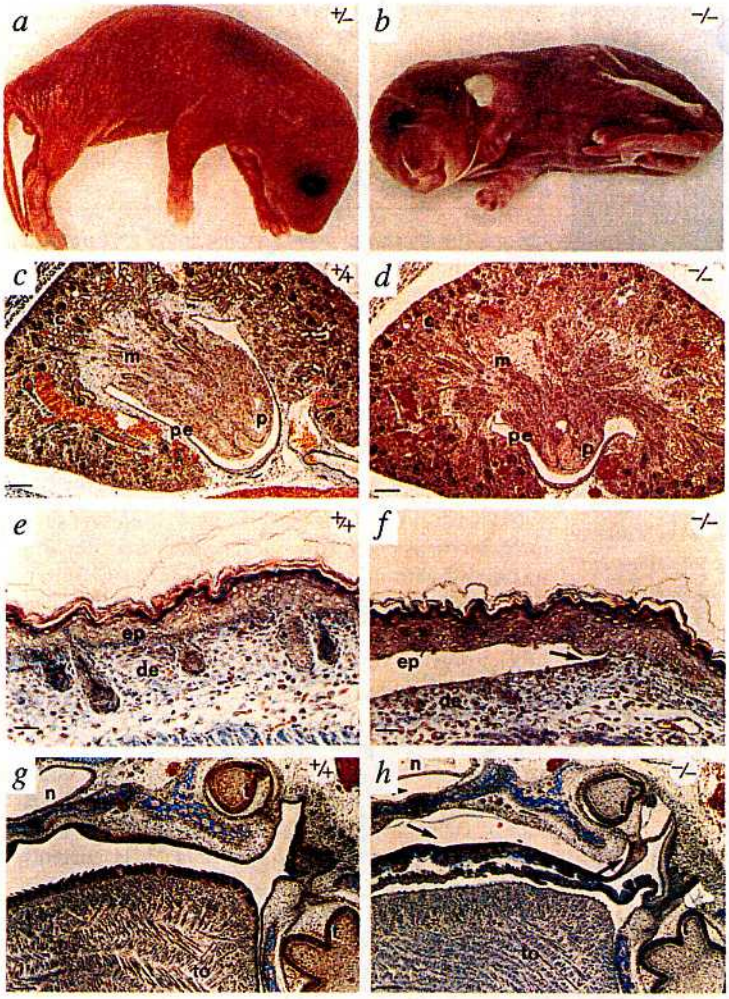

Fig. 2 *a*, Normal (+/–) animal from a heterozygous intercross soon after birth, *b*, $\alpha6^{-/-}$ animal, the skin is detached, note: one foreleg is underneath the skin; *c,d*, Sections through kidney at E18.5. Preservation of tissue organization shown in –/– (*d*) compared to +/+ animals (c); *e*, Coronal section through an +/+ fetus at 18.5 days, showing the epidermal and dermal layers of the skin. *f*, Same view from a $\alpha6^{-/-}$ animal at 18.5 days, showing detachment of epidermis from dermis. Note detached epidermis with nested cells and disorganized basal layer. *g, h*, Coronal sections of oral cavity and tongue of +/+ (*g*) and –/– animals (*h*). In *h*, epithelium is completely detached from the underlying connective tissue. Arrows point to the sites of detachment between the epithelium and the connective tissue. c: renal cortex, m: renal medulla, pe: renal pelvis, p: papilla, ep: epidermis, de: dermis, n: nasal epithelium, to: tongue, t: tooth bud. Bar = 30 μm (*e, f*) or 120 μm (*c, d, g, h*).

four cell stage), at stages when the first laminin-containing basement membrane is assembled[4–6]. Its expression remains throughout embryogenesis in a number of tissues[7–10]. Unlike other laminin receptors, such as α1β1, α2β1, α3β1, integrins α6β1 and α6β4 are known to associate only with laminins[2]. The recognition site of α6β1 has been localized to the laminin-1 E8 fragment, a fragment involved in cell growth and migration, kidney cell polarity development and lung alveolar formation (reviewed in 2). Blocking antibodies against α6 inhibit adhesion and migration on laminin of parietal endoderm in explant cultures[4,5], and conversion of mesenchymal cells to epithelial tubular cells in the developing kidney[9]. In addition, α6 cytoplasmic splice variants show a very dynamic expression regulation during cardiac development[7,8]. All these results suggested that α6-containing integrins are major laminin receptors *in vivo* and may have important roles during embryogenesis. It was thus very much unexpected that $\alpha6^{-/-}$ mice would be able to develop to birth. By histological analysis of $\alpha6^{-/-}$ mice at day 18.5 of gestation, we did not observe obvious abnormalities in heart (data not shown) or kidney (Fig. 2*c,d*). Similarly, analyses indicated tooth (Fig. 2*g,h*) and intestine (data not shown) morphogenesis had occurred. These data do not preclude that functional defects are present; however, they indicate that in these tissues, integrin α6 is dispensable for morphogenesis and cytodifferentiation.

Histological analyses on sections of animals dissected at day 18.5 showed that skin blistering corresponded to a detachment of the epidermal layer (Fig. 2*e,f*). Detachment also occurred at other sites, including the epithelium of the tongue, of the oral and nasal cavities (Fig. 2*g,h*) and of the larynx and esophagus (data not shown). The severity of detachment was variable, depending on the animal, suggesting that detachment is secondary to tissue traumas. All these symptoms are reminiscent of human epidermolysis bullosa[11]. At this level of analysis, the morphology or differentiation of the epidermal layer appeared normal in most places where epidermis was still attached. However, in some sites, detached epidermis showed alterations in cellular organization that may correspond to necrosis following detachment (Fig. 2*f* and data not shown).

Integrins α6/β4 and α6/β1 are receptors for several laminins[2,3,19], including laminin-1 and the recently characterized laminin-5 (also called kalinin, epiligrin and nicein) expressed in epithelial basement membranes[20–23]. α6/β4 is localized specifically at the basal layer. In contrast to β1, β4 associates only with the α6 subunit[2,19]. We examined β4 and laminin-5 localization in skin of homozygous mutant animals as both have been implicated in junctional epidermolysis bullosa[11,15–18] (Fig. 3). One of six homozygous mutant animals showed no expression of β4, whereas five still showed expression of β4 (Fig.3*a,b*). The β4 signal, however, was reduced in intensity, and, importantly, was not concentrated at the basal pole of the basal keratinocyte (Fig.3*a,b*). This suggests that the polarized expression of β4 is dependent upon the presence of an integrin α6 subunit. In all six $\alpha6^{-/-}$ animals, the signal for laminin-5 and laminin-1 was comparable to that of +/+ animals (Fig. 3*c–f*). In sites where the epithelium was detached, the laminin-5 and -1 staining was retained on the connective tissue side (Fig. 3*c–f*).

Integrin α6/β4 is a component of hemidesmosomes[12–14], which are dense cytoplasmic plaques at the basal pole of the basal keratinocyte that are linked intracellularly to keratin filaments and extracellularly to the basement membrane. Such structures contribute to the mechanical strength of the tissue. Strikingly, in areas where epidermis was still attached, no hemidesmosomes were observed (twelve $\alpha6^{-/-}$ fetuses examined from day 16.5 to 18.5 of gestation) (Fig. 4*b*). In a parallel study, a similar phenotype and an absence of hemidesmosomes were observed in tissues of integrin *β4* null mice[24]. These results all favour a direct role of integrin α6/β4 in the formation of hemidesmosomes. It should be noted that the presence of residual β4 alone is not sufficient to promote the assembly of hemidesmosomes, even though a truncated

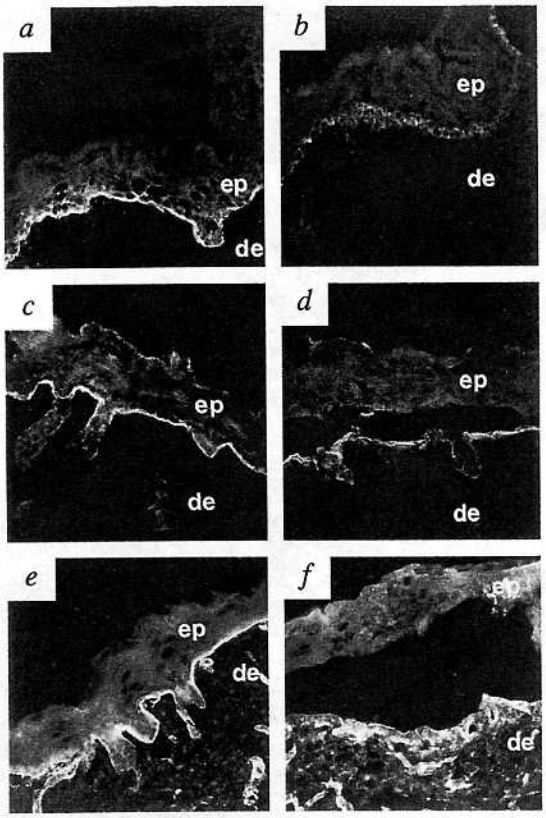

Fig. 3 *a, b*, β4 integrin detection in skin. *a*, β4 is present at the basal layer in +/+ tissue, concentrated at the basal pole. *b*, In the mutant tissue, β4 is present at a reduced level and not concentrated at the basal side. *c*, Laminin-5 is present at the basement membrane zone in +/+ skin section. *d*, Signal is retained in the $\alpha6^{-/-}$ skin, and is mostly distributed on the dermal side of the split. *e,f*, Laminin-1 in skin of a +/+ (*e*) and $\alpha6^{-/-}$ animals (*f*). In mutant tissue, staining is retained but stays on the side of connective tissue. ep: epidermis; de, dermis

monomeric β4 subunit can incorporate into preexisting hemidesmosomes[25].

No keratin filaments were found attached to the basal side of the basal keratinocyte, which could explain why, in detached areas (Fig. 4*c*), in addition to detachment at the basement membrane, ruptures were also observed above the basal pole of the basal keratinocyte (Fig. 4*d*). The complete absence of hemidesmosomes in α6 homozygous mutant mice reflects the increased phenotype severity as compared to one human patient with mutations in the *β4* gene[15]. In this patient, hemidesmosomes were present, although in reduced amounts and with altered structure[15]. Residual association with keratin filaments was also observed. Another contrasting phenotype, in mice where another component of hemidesmosomes, bullous pemphigoid antigen BP230, was recently inactivated[26], the hemidesmosomes were still present but the inner plate was missing[26].

In conclusion, we show here that the absence of integrin α6 is compatible with embryonic development, and with morphogenesis and differentiation of several organs such as heart, kidney or intestine. This suggests a functional redundancy or compensation by other integrins, or by non-integrin laminin receptors. However, the loss of α6 leads to epidermolysis bullosa in mice, suggesting that mutations in the *α6* gene (*ITGA6* on chromosome 2) could be involved in human epidermolysis bullosa. Whether other subtle defects are present in homozygous mutant mice in tissues or cells that express α6, such as the nervous system[8,19,27], blood or endothelial cells[2,19], is currently being investigated.

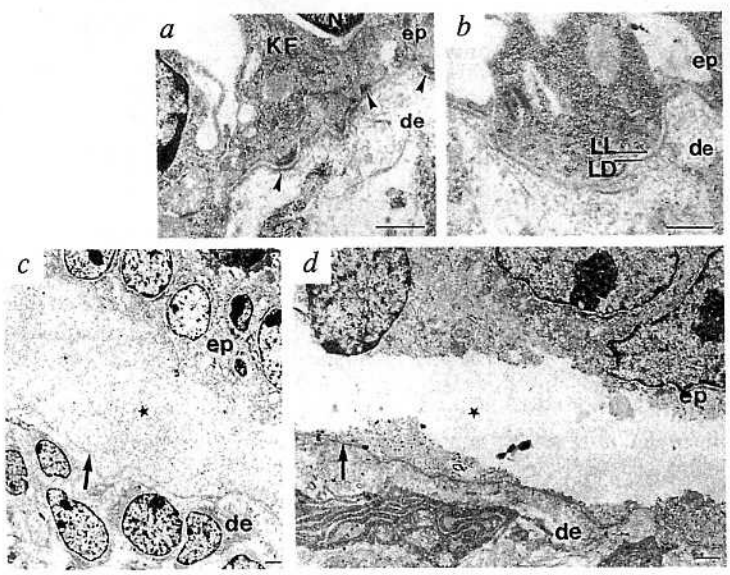

Fig. 4 *a, b*, Electron micrographs of skin biopsies from +/– animal (*a*) showing normal complement of hemidesmosomes (arrowheads) compared to $\alpha6^{-/-}$ animal (*b*). *c, d*, In blisters, (*), detachment occurs at the dermo-epidermal junction, at the basement membrane (*c*) or within the basal keratinocyte (*d*). Arrows indicate basement membrane LL: lamina lucida, LD: lamina densa, KF: keratin filaments, N: nucleus, ep: epidermis, de: dermis. Bar = 0.5 μm, (*a, b, d*), or 1 μm, (*c*).

Methods

Construction of targeting vector. A clone of 5' genomic DNA from the *α6* gene was isolated from a λEMBL3-recombinant 129Sv library (gift of J.M. Garnier at IGBMC). Four exons, excluding the first 61 amino-acids, were mapped by PCR and sequence comparisons with the published mouse α6 cDNA sequence[6]. A deleted version of the α6 genomic clone was constructed by removing three exons (corresponding to residues 62–194) within a 7-kb fragment. This was obtained by inserting a *Bam*HI 2.1-kb fragment upstream of the neomycine (neo) cassette of pPNT[28], and a 3-kb *Kpn*I fragment, distance of 7 kb from the previous fragment, on the other side (Fig. 1*a*). Sequence analysis indicated that splicing of the upstream exon to the exon downstream of *neo* produced a frameshift followed by a stop codon, 9 residues downstream.

ES cells and animals. Electroporation was as described[29] in the H1 line of ES cells established at IGBMC from the 129Sv mouse strain (A. Dierich, unpublished results). DNA was prepared from ES cell clones by lysis with proteinase K and isopropanol precipitation[30]. DNA was digested with *Hin*dIII restriction enzyme, and analysed by Southern hybridization with a ^{32}P-labelled 0.5-kb fragment outside the construct. Chimaeric animals were produced by microinjection of ES cells into C57Bl/6J blastocysts, as described[29]. Genotype analysis was performed on tail biopsies as described[30]. For analysis of embryos or fetuses, DNA was extracted from placentae by lysis followed by phenol/chloroform extractions. For genotyping, 10 μg of DNA from tail or placenta were digested with *Eco*RI and analysed by Southern hybridization.

Northern analysis. Total RNA from kidney or skin was prepared in guanidinium thiocyanate, as described[31]. For each sample, 10 μg of total RNA were separated by electrophoresis through a 1% agarose/formaldehyde gel, and transferred to

Nytran membrane in 10x SSC, as described by the supplier (Schleicher and Schüell). Membranes were hybridized with a probe corresponding to the transmembrane and B exons of α6. For loading control, membranes were hybridized with a probe corresponding to an ubiquitously expressed gene, *36B4* (ref. 32).

Histological procedures. Fetuses were fixed in Bouin's solution for 7 d, then processed for histological analysis as described[33]. After dehydration in 70%, 90% and 100% ethanol, fetuses were embedded in paraffin. Sections of 10 microns were cut and collected in a drop of gelatin 0.1% in water on glass slides. Sections were stained by the Mallory's tetrachrome method[33].

Immunofluorescence. Fetuses were frozen directly in isopentane cooled on dry-ice and stored at –80 °C until processing. Sections of 15 microns were cut with a Reichert-Jung cryostat and collected on gelatin/chrome-alun subbed slides. Antibody staining was performed as described[8]. Antibodies were anti-laminin-1 (SIGMA), anti-laminin-5 (gift from R.Burgeson[23]), and anti-β4 (346-11A, gift from S. Kennel[34]). Other antibodies used in this study were the monoclonal antibodies against α6 (GoH3, gift from A.Sonnenberg[35]), and the cytoplasmic domain of α6A (1A10, gift from A.Sonnenberg, 18C11, gift from J.C. Lissitsky). Briefly, after incubation with 10% normal goat serum (NGS), first antibody was diluted in NGS and applied for 1 h at 4 °C. After 3 washes in PBS at 4 °C, 2° antibody (FITC-conjugated goat anti-rabbit and goat anti-rat (SIGMA)) was applied for 1 h, at 4 °C. The sections were then washed 3 times in PBS, and fixed for 10 min in 2% paraformaldehyde in PBS. After 3 washes in PBS, sections were mounted in 50% glycerol, 25% Airvol (Air Products), 1.5% of 1,4-diazabicyclo2,2,2 octane (DABCO, SIGMA).

Electron microscopy. Tail or flank biopsies of control and mutant animals were fixed in a solution of 2.5% glutaraldehyde, 0.1 M sodium cacodylate, pH 7.2, for 24 h at 4 °C, washed in cacodylate buffer for 30 min and postfixed in 1% osmium tetroxide in cacodylate buffer for 1 h at 4 °C. After dehydration in alcohol and propylene oxide, samples were embedded in Epon 812. Thin sections were cut with a diamond knife on a Reichert Leica ultramicrotome. Semithin sections were stained with toluidine blue. Ultrathin sections (70 nm) were contrasted with uranyl acetate and lead citrate as described. Tissues were examined with a Phillips 208 electron microscope.

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