

## A Homozygous Mutation in the Integrin $\alpha 6$ Gene in Junctional Epidermolysis Bullosa with Pyloric Atresia

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### Abstract

The  $\alpha 6$  integrin subunit participates in the formation of both  $\alpha 6\beta 1$  and  $\alpha 6\beta 4$  laminin receptors, which have been reported to play an important role in cell adhesion and migration and in morphogenesis. In squamous epithelia, the  $\alpha 6\beta 4$  heterodimer is the crucial component for the assembly and stability of hemidesmosomes. These anchoring structures are ultrastructurally abnormal in patients affected with junctional epidermolysis bullosa with pyloric atresia (PA-JEB), a recessively inherited blistering disease of skin and mucosae characterized by an altered immunoreactivity with antibodies specific to integrin  $\alpha 6\beta 4$ . In this report, we describe the first mutation in the  $\alpha 6$  integrin gene in a PA-JEB patient presenting with generalized skin blistering, aplasia cutis, and defective expression of integrin  $\alpha 6\beta 4$ . The mutation (791delC) is a homozygous deletion of a single base (C) leading to a frameshift and a premature termination codon that results in a complete absence of  $\alpha 6$  polypeptide. We also describe the DNA-based prenatal exclusion of the disease in this family at risk for recurrence of PA-JEB. Our results demonstrate that, despite the widespread distribution of the  $\alpha 6$  integrin subunit, lack of expression of the  $\alpha 6$  integrin chain is compatible with fetal development, and results in a phenotype indistinguishable from that caused by mutations in the  $\beta 4$  chain, which is expressed in a more limited number of tissues. (*J. Clin. Invest.* 1997. 99:2826–2831.) **Key words:** inherited epidermolysis bullosa • keratinocyte • hemidesmosome • integrin  $\alpha 6$  gene • mutation

### Introduction

Integrins are  $\alpha/\beta$  heterodimeric transmembrane receptors that mediate cell–cell and cell–matrix interactions, and regulate cell growth, differentiation, and death through signal transduction

processes (1, 2). The  $\alpha 6$  integrin subunit associates with either  $\beta 1$  or  $\beta 4$  chains to form the laminin receptors  $\alpha 6\beta 1$  and  $\alpha 6\beta 4$  (1–4). The  $\alpha 6\beta 1$  heterodimer is expressed on platelets, lymphocytes, epithelial cells, and a variety of other cell types, and is involved in morphogenetic processes such as kidney tubule formation (5–8). The  $\alpha 6\beta 4$  complex is found predominantly in epithelia, and is the only integrin component of hemidesmosomes (9–11), the dense cytoplasmic devices that anchor basal cells of stratified and pseudostratified epithelia to the underlying basement membrane. Hemidesmosomes connect the cytoskeleton keratin intermediate filaments to the lamina densa of the basement membrane through anchoring filaments, thread-like structures which span the lamina lucida (12). The  $\alpha 6\beta 4$  integrin binds with high affinity to laminin 5, a putative component of anchoring filaments, and the major adhesion ligand of epithelial cells (13).

Defective expression of the  $\alpha 6\beta 4$  heterodimer has been described in a variant of junctional epidermolysis bullosa (JEB)<sup>1</sup> associated with congenital gastric outlet obstruction, pyloric atresia JEB, (PA-JEB) (MIM 226730) (14–16). These immunohistochemical findings have been supported by the identification of two distinct mutations in the  $\beta 4$  integrin gene (*ITGB4*, on chromosome 17q11-qter) in a patient affected with PA-JEB (17). JEB is a clinically and genetically heterogeneous group of autosomal recessively inherited blistering disorders of the skin and mucous membranes, characterized by mesenchymal–epithelial separation within the lamina lucida of the basement membrane zone (18). Typical ultrastructural findings in JEB are abnormalities in the hemidesmosomes. Mutations in genes encoding for laminin 5 and the transmembrane hemidesmosomal component collagen XVII, also known as the 180-kD bullous pemphigoid antigen (BPAG2), have been identified in different forms of JEB (19–21).

In this report, we describe the first mutation in the  $\alpha 6$  integrin gene (*ITGA6*, on chromosome 2q) in a patient with PA-JEB. We also describe the DNA-based prenatal exclusion of PA-JEB using allele-specific probes. Our results indicate that, despite the widespread distribution of the  $\alpha 6$  integrin subunit, lack of expression of the  $\alpha 6$  integrin chain is compatible with in utero development, and results in a phenotype indistinguishable from that caused by mutations in the  $\beta 4$  chain.

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1. *Abbreviations used in this paper:* ASO, allele-specific oligonucleotide; BPAG1, 230-kD bullous pemphigoid antigen; BPAG2, 180-kD bullous pemphigoid antigen; DEJ, dermal-epidermal junction; JEB, junctional epidermolysis bullosa; PA-JEB, junctional epidermolysis bullosa with pyloric atresia.

## Methods

**Immunofluorescence analysis.** Frozen 5- $\mu$ m-thick skin sections obtained from skin biopsies of the proband, of the fetus, of six patients affected with other JEB variants, and of healthy volunteers, were processed for immunofluorescence using a three-step biotin-streptavidin-fluorescein procedure, as described (22). The following mAb and polyclonal antisera were used: G0H3 (rat mAb to the extracellular domain of the  $\alpha 6$  integrin subunit, gift from A. Sonnenberg, the Netherlands Cancer Institute, Amsterdam, The Netherlands) (23), 450-30A1 (mouse mAb to  $\alpha 6$  subunit; obtained from the Fifth Workshop and Conference on Human Leukocyte Differentiation Antigens) (24), 3E1 (mouse mAb to  $\beta 4$ ; Telios Pharmaceuticals, San Diego, CA), 450-11A1 (mouse mAb against the cytoplasmic domain of  $\beta 4$  subunit; obtained from the Fifth Workshop and Conference on Human Leukocyte Differentiation Antigens) (24), GB3 (mouse mAb to laminin 5; from J.P. Ocotonne, U385 INSERM, Nice, France) (25), FP1 (rabbit antiserum to 230-kD bullous pemphigoid antigen [BPAG1], gift from J. Stanley, University of Pennsylvania, Philadelphia PA) (26), 1A8C (mouse mAb to cytoplasmic domain of BPAG2; gift from K. Owaribe, Nagoya University, Nagoya, Japan) (27), J143 (mouse mAb to  $\alpha 3$  integrin subunit; gift from L.J. Old, Ludwig Institute for Cancer Research, New York) (28), K20 (mouse mAb to  $\beta 1$  integrin subunit; Immunotech International, Marseille, France), LH7:2 (mouse mAb to type VII collagen; Cymbus Bioscience, Southampton, United Kingdom). The rabbit antiserum to type IV collagen was purchased from Institut Pasteur (Lyon, France). The following secondary sera and detection systems were employed: biotinylated horse anti-mouse IgG serum (Vector Laboratories, Burlingame, CA), biotinylated rabbit anti-rat IgG serum (Vector), biotinylated goat anti-rabbit IgG serum (Vector), and fluorescein-streptavidin (Amersham International, Little Chalfont, United Kingdom).

**Electron microscopy.** Skin specimens were fixed in 2% glutaraldehyde, postfixed in 1% osmium tetroxide, dehydrated in graded alcohols, embedded in Epon resin, and then sectioned on an ultramicrotome (Reichert Ultracut E; Leica, Wien, Austria). Ultrathin sections were stained with uranyl acetate and lead citrate and observed in a transmission electron microscope (CM100; Philips, Eindhoven, The Netherlands).

**Keratinocyte cultures.** Human epidermal keratinocytes were obtained from skin biopsies of the proband parents and healthy volunteers, and were cultivated on a feeder layer of lethally irradiated 3T3-J2 murine fibroblasts (a gift from H. Green, Harvard Medical School, Boston, MA), as described (29).

**Northern analysis.** Total RNA from primary cultures of epidermal keratinocytes was prepared in guanidine isothiocyanate, as described (30). For each sample, 20  $\mu$ g of total RNA was separated by electrophoresis through a 1% agarose/formaldehyde gel, and transferred to Hybond-N<sup>+</sup> nylon membrane in 20 $\times$  SSPE, as described by the supplier (Amersham International). Membranes were hybridized with <sup>32</sup>P-labeled probes  $\beta 4$  (31) and  $\alpha 6$  (4) to detect the mRNAs for the  $\beta 4$  and  $\alpha 6$  integrin subunits, respectively. For loading control, membranes were hybridized with a probe corresponding to the ubiquitously expressed gene glyceraldehyde-3-phosphate dehydrogenase.

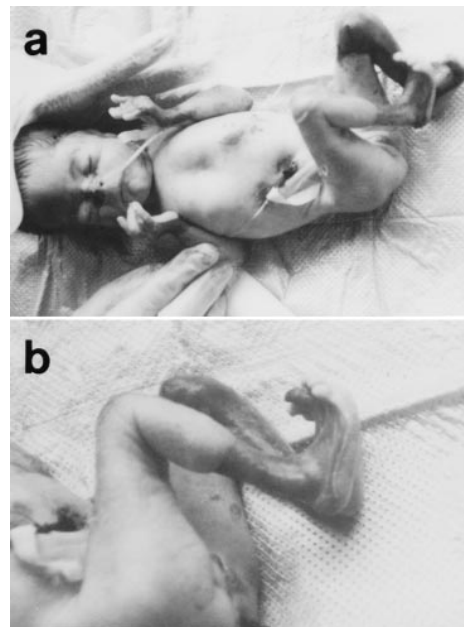
**Mutation detection.** Total RNA was prepared from 3-mm skin biopsies of the PA-JEB patient and healthy controls following the manufacturer's recommendations (Micro-Scale total RNA Separator Kit; Clontech Laboratories, Palo Alto, CA) and was reverse-transcribed in a volume of 20  $\mu$ l (SuperScript RNase H free reverse transcriptase; Life Technologies Italia, San Giuliano Milanese, Milan, Italy). The cDNA obtained was used in PCR reactions with specific primers producing nine overlapping fragments ( $\sim$  500 bp) covering the entire open reading frame of the  $\alpha 6$  cDNA. The product of each PCR reaction, analyzed by agarose gel, was subcloned into the PCR-TM II plasmid vector (Invitrogen, NV Leek, The Netherlands), following the manufacturer's instructions, and sequenced with the dideoxy termination method. The oligonucleotide primers used to identify the mutation 791delC were: (L) 5' TGTTATGTCCTGAGTCAGAA 3' and (R) 5' TCTCTCTTCAGCAAAACCAC 3', which amplify nucleotides

(nts) 442-866 of the  $\alpha 6$  integrin cDNA sequence. PCR cycling conditions were: 94°C for 5 min, followed by 94°C for 45 s; 58°C for 45 s; 72°C for 45 s (35 cycles), and extension at 72°C for 10 min.

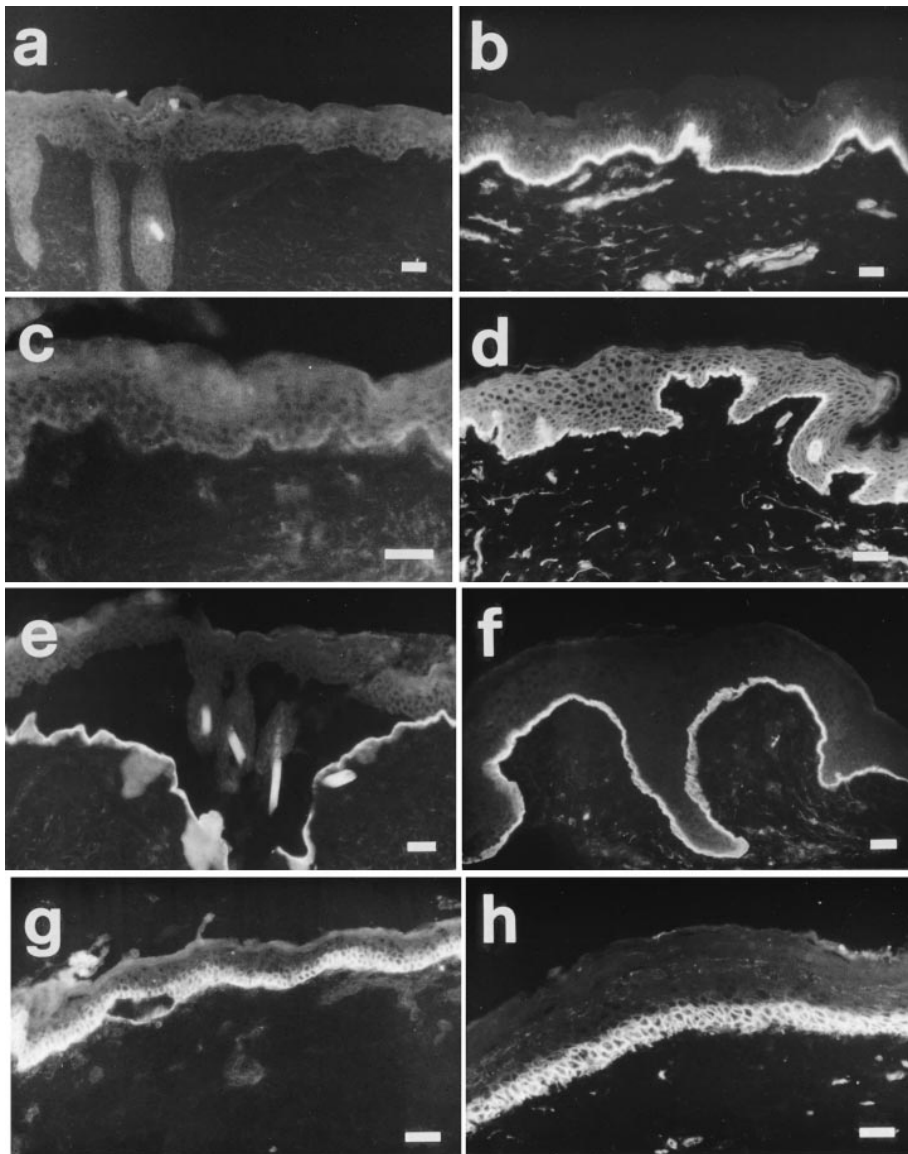
**Mutation verification.** The mutation detected in the proband's integrin  $\alpha 6$  cDNA was verified at the genomic level. Genomic DNA was extracted from peripheral blood by standard procedures. The forward primer, 5' GTTTTCTTTGGACTCAGGG 3', and the reverse primer, 5' CTCCACTGTGATTGGCTCT 3', were synthesized and used in a 25- $\mu$ l PCR reaction to amplify from 100 ng of total genomic DNA a 87-bp fragment extending from cDNA nts 758 to nts 844. PCR conditions were: 95°C for 7 min, followed by 94°C for 1 min, 56°C for 1 min, 72°C for 2 min (35 cycles), and extension at 72°C for 10 min. The amplified product was subcloned into the PCR-TM II plasmid vector. 10 independent clones were sequenced on both DNA strands using universal primers. Allele-specific oligonucleotide (ASO) analysis was carried out to confirm the homozygous state of the mutation in the proband, and to assess the inheritance in the kindred. Genomic DNA from each family member was amplified to produce the 87-bp PCR fragment as described above. 10  $\mu$ l of the PCR reaction was transferred onto a Hybond-N<sup>+</sup> membrane using a dot blot apparatus. The membranes were prehybridized in a solution of 5 $\times$  Denhardt's, 6 $\times$  SSC, 0.1% SDS for 10 min at 65°C, and then hybridized for 2 h at room temperature with either the <sup>32</sup>P-labeled wild-type ASO with the sequence 5' GTATTGTTTCTAAAGATGAG 3', or the mutated ASO 5' GGTATTGTTTAAAGATGAG 3'. Filters were washed twice in 6 $\times$  SSC, 0.1% SDS for 5 min at room temperature, and for 15 min at the T<sub>m</sub> of the oligonucleotides (52°C), and were exposed for autoradiography.

## Results

The proband was born from clinically normal Moroccan parents who denied consanguinity and referred to a previous, still-born infant with extensive skin defects. At birth, the patient presented cutaneous aplasia of both legs, feet, hands, and both



**Figure 1.** Clinical appearance of the newborn with junctional epidermolysis bullosa with pyloric atresia. (a) Blisters and erosions are present over the abdomen and the right knee. Extensive cutaneous aplasia affects the legs, feet and hands. (b) Closer view of the legs showing well-demarcated, depressed areas of aplasia cutis.



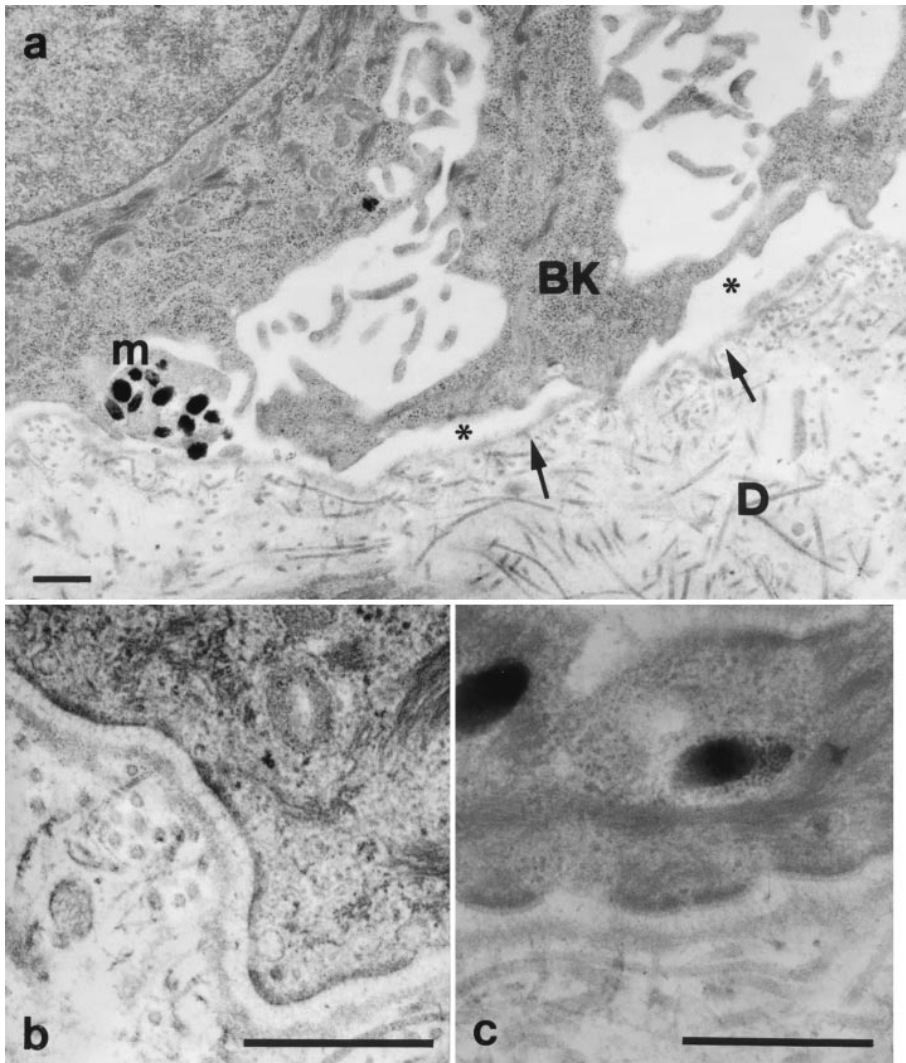
**Figure 2.** Immunofluorescence microscopy of the DEJ. Labeling of the PA-JEB skin with mAb G0H3 to  $\alpha 6$  integrin subunit (a) is completely negative, while normal skin (b) shows bright linear staining at the DEJ. Labeling of PA-JEB skin with mAb 3E1 against the  $\beta 4$  integrin chain (c) also appears strongly reduced compared to normal skin (d). In contrast, the labeling of the DEJ with mAb GB3 against laminin 5 is similar in normal (f) and PA-JEB skin (e), where it is localized at the blister floor. J143 mAb against the  $\alpha 3$  integrin subunit gives the expected staining of lateral and apical sides of basal keratinocytes in the skin of a patient affected from the generalized atrophic benign form of JEB (21) (h), but it also labels the basal pole of basal keratinocytes in PA-JEB skin (g). Bar, 20  $\mu\text{m}$ .

sides of the scalp, as well as several blisters and erosions distributed over the trunk and extremities (Fig. 1). The pinnae of both ears were small and malformed. The infant was also affected with pyloric atresia and severe esophageal stenosis. Eruption of new cutaneous and mucosal blisters and multisystem failure led to death 23 d after birth.

Immunofluorescence analysis of perilesional skin with mAb G0H3 and 450-30A1 against the  $\alpha 6$  integrin subunit revealed a complete absence of reactivity, whereas normal control skin showed the expected linear labeling of the dermal-epidermal junction (DEJ) (Fig. 2, a and b). With mAb 3E1 and 450-11A1 against  $\beta 4$  integrin, a strongly reduced staining of the DEJ was observed (Fig. 2, c and d). In contrast, antibodies recognizing type VII collagen, laminin 5 (Fig. 2, e and f), and the hemidesmosomal components BPAG1 (230-kD bullous pemphigoid antigen) and BPAG2 presented a labeling pattern comparable to controls. Interestingly, the  $\alpha 3$  and  $\beta 1$  integrin subunits, detected on the lateral sides of basal keratinocytes in normal skin and other JEB variants, were also expressed along the basal pole of basal keratinocytes in PA-JEB skin (Fig. 2, g and h).

Ultrastructural examination of PA-JEB skin showed the cleavage plane localized within the lamina lucida of the basement membrane (Fig. 3 a) and revealed the presence of hemidesmosomes. The hemidesmosomes were reduced in number and did not contain a sub-basal dense plate. In most of them, the inner cytoplasmic plaque was also markedly reduced or even absent, although a limited number of keratin filaments inserting on cytoplasmic electron-dense plaques were detected regularly (Fig. 3 b).

Given the paucity of the biopsy material available from the proband, we could not examine the level of expression of  $\alpha 6$  and  $\beta 4$  mRNA in PA-JEB skin. We therefore performed Northern blot analysis of total RNA from primary cultures of epidermal keratinocytes obtained from the proband's parents who were expected to be healthy carriers of the genetic defect. A reduced expression of  $\alpha 6$  mRNA was found in both parents, whereas the signal intensity obtained with a specific  $\beta 4$  probe was comparable to controls (not shown). Thus, we concluded that *ITGA6* was the candidate gene in this PA-JEB patient, and initiated the search for mutations.



**Figure 3.** Electron microscopy of PA-JEB skin. (a) In an initial cleavage area, the blister formation at the DEJ occurs within the lamina lucida (*star*), and the electron-dense lamina densa forms the blister floor (*arrow*) (BK, basal keratinocytes; D, dermis; m, melanosomes). Higher magnification of the DEJ of uninvolved PA-JEB skin (b) reveals the presence of rudimentary hemidesmosomes lacking the sub-basal dense plaque. The cytoplasmic plaque of hemidesmosomes with insertion of keratin filaments is clearly detectable, although markedly reduced compared to normal skin (c). Bar, 0.5  $\mu$ m.

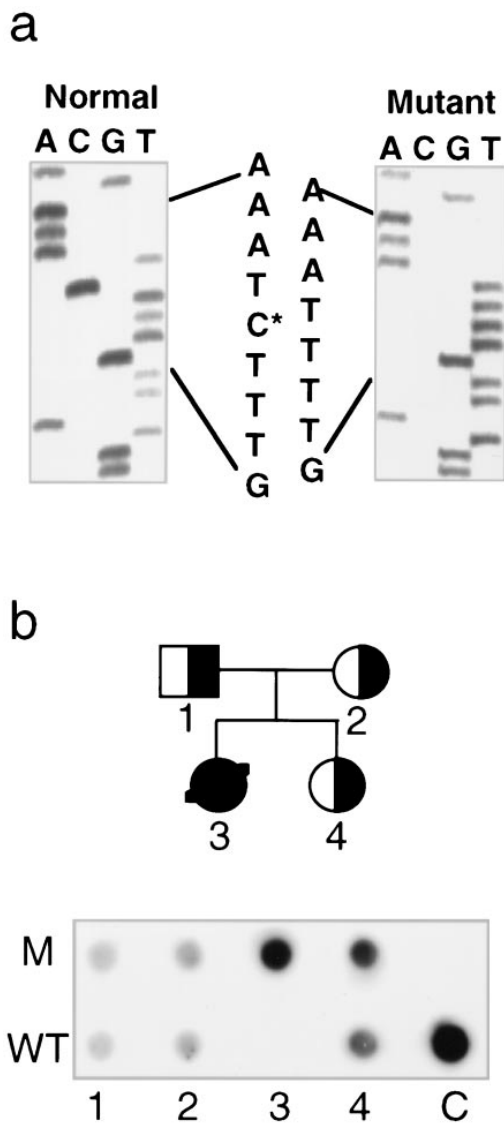
Overlapping cDNA fragments spanning the entire open reading frame for the  $\alpha 6$  polypeptide were obtained by RT-PCR amplification of total RNAs from the proband and healthy control skin biopsies using nine pairs of oligonucleotides. Sequence analysis of a 5' PCR product (nt 442–866) showed a deletion of a single base (C) at position 791, leading to a frameshift and a premature termination codon (TGA) 66 bp downstream from the deletion site. This mutation, designated 791delC, predicts a truncated  $\alpha 6$  polypeptide terminating at residue 268. Sequencing of the corresponding region of the proband genomic DNA demonstrated the presence of the C deletion in all the clones analyzed (Fig. 4 a), suggesting a homozygous mutation. ASO analysis of genomic DNA confirmed the homozygous state of the mutation in the proband and showed both parents to be heterozygous healthy carriers of this mutation (Fig. 4 b). Screening by the same technique of genomic DNA from 50 unrelated healthy subjects and from eight patients with different forms of JEB showed hybridization signals only with wild-type oligonucleotide, indicating that this mutation is neither a polymorphic variation in the population, nor the basis of JEB in the other patients studied.

The family requested a prenatal diagnosis for a new pregnancy. ASO analysis performed on genomic DNA extracted from a skin biopsy obtained at 16 wk gestation revealed that

the fetus was a heterozygous healthy carrier (Fig. 4 b), as confirmed by the clearly detectable expression of  $\alpha 6$  and  $\beta 4$  integrins in the fetus skin sections (not shown).

## Discussion

In this report, we describe the identification of the first mutation in the *ITGA6* gene which encodes for  $\alpha 6$  integrin in a patient presenting with PA-JEB. In the proband, the presence of a homozygous premature termination codon (PTC) predicts the synthesis of an  $\alpha 6$  polypeptide truncated at amino acid 268, and therefore lacking most of the extracellular domain and the whole transmembrane and intracellular domains. It has been shown that PTCs generated by genetic defects occurring in a variety of genes cause decay of the aberrant mRNA transcripts (32, 33). In the family described in this report, a degradation of the mutated  $\alpha 6$  mRNA would be consistent with the reduced steady state level of integrin  $\alpha 6$  transcripts detected in keratinocytes obtained from the proband's parents. Therefore, little if any truncated  $\alpha 6$  polypeptide would be synthesized by the patient's keratinocytes. Indeed, no staining for the  $\alpha 6$  integrin subunit was detected in the patient's skin. Absence of  $\alpha 6$  integrin subunit also explains the dramatic decline in the immunohistochemical expression of the  $\beta 4$  integrin subunit which asso-



**Figure 4.** Identification of the homozygous 791delC mutation in the proband's *ITGA6* gene and inheritance in the kindred. (a) Total genomic DNA was subjected to PCR reaction using primers which amplify an 87-bp fragment extending from cDNA nts 758 to nts 844. In comparison with the DNA of a normal control, nucleotide sequencing of the PA-JEB patient's DNA reveals a single base deletion (C\* at position 791) resulting in a frameshift and a premature termination codon (TGA) 66 bp downstream. The mutation is designated 791delC. (b) Pedigree of the PA-JEB family (top): proband died at 23 d (3), fetus of 16 wk gestation (4), clinically unaffected parents (1, 2). ASO analysis (bottom): hybridization of the 87-bp PCR product with a wild-type (WT) and mutated (M) ASO shows the homozygous state of the mutation in the proband, whereas both parents and the fetus are heterozygous carriers of the mutated allele. C, unaffected control individual.

ciates exclusively with the  $\alpha 6$  chain. Since  $\alpha 6\beta 4$  integrin plays a crucial role in hemidesmosome assembly and stability (11, 34, 35), the absence of this heterodimer correlates well with the reduction in the number and the rudimentary shape of hemidesmosomes in PA-JEB skin.

The recurrent blistering and erosions of the gastrointestinal, urinary, and respiratory tracts, which are additional clinical

hallmarks of PA-JEB (36), are also consistent with an altered expression of  $\alpha 6\beta 4$  in simple epithelia that do not possess hemidesmosomes (5, 6), as well as with in vitro data indicating that  $\alpha 6\beta 4$  mediates cell adhesion, even in the absence of formed hemidesmosomes (35). At present, it is still unclear whether esophageal and urethral stenosis and pyloric atresia, which are recurrent findings in PA-JEB patients, result from the fusion of the detached epithelial linings.

Mainiero et al. have shown recently that EGF, a growth factor crucial for the regulation of keratinocyte growth and migration, modifies the functions of  $\alpha 6\beta 4$  with a consequent disruption of hemidesmosomes and an upregulation of  $\alpha 6\beta 4$ -mediated cell migration towards laminins (37). The recurrent finding of extensive areas of aplasia cutis in PA-JEB patients (36) correlates well with a role for the  $\alpha 6\beta 4$  heterodimer in mediating epidermal cell migration in wound healing. The absence of  $\alpha 6\beta 4$  in PA-JEB skin might in fact impair the reepithelialization of skin blisters formed during embryonic and fetal life when the EGF receptor is expressed strongly in the epidermis (38).

The  $\alpha 6$  integrin is known to combine with both  $\beta 4$  and  $\beta 1$  chains. While the  $\alpha 6\beta 4$  heterodimer is restricted mainly to the basal pole of basal epithelial cells, the  $\alpha 6\beta 1$  laminin receptor is expressed on a variety of cell types, and, during morphogenesis, co-appears with laminin 1 at the four-cell stage, when the first basement membrane is assembled (3–11). Our findings demonstrate that, despite the widespread distribution of the  $\alpha 6$  chain, a genetic defect in the  $\alpha 6$  integrin leads to a PA-JEB phenotype similar to that associated with mutations in the *ITGB4* gene (17). Therefore, these observations suggest that during embryonic and fetal development, other laminin receptors, possibly of the  $\beta 1$  integrin family, can compensate for the absence of the  $\alpha 6$  integrin subunit. In this respect, the  $\alpha 3\beta 1$  integrin displays a basal polarization in the basal keratinocytes of our PA-JEB patient. Since this basal localization of  $\alpha 3\beta 1$  integrin is not observed in other JEB variants, nor in the skin of healthy controls, this integrin, which is also a laminin 5 receptor (39), may partially compensate for the lost functions of  $\alpha 6\beta 4$  integrin.

Our data are consistent with the recent demonstration that the inactivation of the genes for  $\alpha 6$  and  $\beta 4$  causes extensive detachment of epithelia in mice (40–42). Several distinct features, however, distinguish PA-JEB from the phenotype observed in  $\alpha 6$  and  $\beta 4$  null mice. Rudimentary and sparse hemidesmosomes are clearly detectable in the skin of PA-JEB patients, whereas no hemidesmosomal adhesion structure is observed in the knockout animals. In addition, pyloric atresia and aplasia cutis are clinical hallmarks specific to humans. These differences may imply a similar but not identical role of  $\alpha 6\beta 4$  integrin in epithelial adhesion and signal transduction in the human and in the mouse, and raise the question of the suitability of animal systems as models for the faithful reproduction of human diseases.

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