Splicing modulation of integrin β4 pre-mRNA carrying a branch point mutation underlies epidermolysis bullosa with pyloric atresia undergoing spontaneous amelioration with ageing

S. Chavanas¹, Y. Gache¹, J. Vailly¹, J. Kanitakis², L. Pulkkinen³, J. Uitto², J.-P. Ortonne¹,⁴ and G. Meneguzzi¹,*

¹U385 INSERM, Faculté de Médecine, Avenue de Valombrose, 06107 Nice Cedex 2, France, ²Clinique Dermatologique, Hôpital Edouard Herriot, 69437 Lyon Cedex 3, France, ³Departments of Dermatology and Cutaneous Biology, and Biochemistry and Molecular Pharmacology, Jefferson Medical College, Philadelphia, PA, USA and ⁴Service de Dermatologie, Hôpital Pasteur, 06002 Nice Cedex 1, France

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A general improvement with ageing has been reported in a few cases of epidermolysis bullosa with pyloric atresia (PA-JEB), an autosomal recessive skin disease characterized by extensive adhesiveness of epithelia. In a patient who improved from severe to mild PA-JEB, a search for mutations in the integrin β4 gene (ITGB4) detected heterozygosity for a novel base substitution 3986–19T→A in the putative branchpoint sequence of intron 31, and a point mutation 3802+1G→A in the donor splice site of intron 30 previously associated with severe PA-JEB. Analysis of mRNA showed that the intronic mutation prevents legitimate splicing of the β4 pre-mRNA. Functional splicing can be restored in vitro by seeding the proband’s keratinocytes on feeders of irradiated fibroblasts. Study of mRNA in wild-type keratinocytes transfected with ITGB4 minigenes containing intron 31 with or without mutation 3986–19T→A, confirmed the causative role of the intronic mutation in PA-JEB, and highlighted the influence of feeders on the maturation process of the mutated β4 pre-mRNA. Our results show that in a context of overall reduction of the β4 mRNA levels, activation of the legitimate splice site in the aberrant β4 pre-mRNA underlies the transient severity of the condition. The results also point to the relevance which the interaction between epithelial and stromal cells may have in modulating expression of integrin receptors.

INTRODUCTION

Junctional epidermolysis bullosa (JEB) is a heterogeneous group of autosomal recessive bullous disorders of the skin and mucous membranes characterized by blister formation after trauma. In the blisters, the plane of the mesenchymal–epithelial separation lies within the lamina lucida of the basement membrane zone (BMZ). Based on morphology and body distribution of the lesions, the different forms of JEB have been classified into defined clinical subgroups (1). The severe forms of JEB manifest at birth with widespread blistering and erosions of the integument and oral mucosa. Involvement of the gastrointestinal, genitourinary and respiratory epithelia is also observed. Fluid loss, growth retardation and anaemia are also common. On the basis of clinical and molecular genetic findings, a variant of severe JEB associated with congenital pyloric atresia was designated PA-JEB (MIM 226730) (1,2). Congenital aplasia of the skin, and urethral occlusion, are clinical signs often observed in severe PA-JEB. PA-JEB may also manifest as a mild skin disorder in which aplasia cutis is absent and blister tendency is less pronounced than in the severe form of the disease (3,4). In mild PA-JEB, surgical treatment of pyloric atresia results in long-term survival.

In addition to the severe and mild forms of PA-JEB, rare cases have also been reported in which neonatal PA-JEB undergoes general improvement with ageing (3,5). In the transient forms of PA-JEB, remission of blistering is observed with increasing age, although skin fragility may persist in different degrees.

PA-JEB has been associated with mutations in the genes ITGA6 or ITGB4 that encode the α6 and β4 subunits of integrin α6β4, respectively (2,6). Integrin α6β4 is an adhesion cell receptor localized in the hemidesmosomes (HDs), specialized adhesion structures adjoining the basal cells to the underlying mesenchyme in stratified and transitional epithelia (7). The HDs associate intracellularly with the cytoskeleton intermediate filaments and extracellularly with the anchoring filaments of the BMZ. Integrin α6β4 plays a critical role in the assembly and stability of HDs, and abnormal expression of this protein results in malformation or absence of HDs (2,6,8).

β4 is unique among the integrin β subunits, because of its unusually long cytoplasmic domain (~1000 amino acids) which is connected to the cytoskeleton network and harbours the domains essential to the interaction of integrin α6β4 with the cellular components (collagen type XVII and plectin) of the HDs (9,10). Such interactions lead to the nucleation of the HD in

*To whom correspondence should be addressed. Tel: +33 4 93 37 77 79; Fax: +33 4 93 81 14 04; Email: meneguzz@unice.fr
Figure 1. Identification of the defective expression of integrin α6β4 in the PA-JEB patient. (A) Electron microscopy of traumatized mild PA-JEB skin shows slight widening of the lamina lucida (asterisk) between well-formed hemidesmosomes (hd), connected to the keratin intermediate filaments (ifi) and bridged to the lamina densa (ld) by anchoring filaments (af). Bar, 20 µm. (B) Immunofluorescence analysis of non-involved areas of the proband’s skin (a and c) using the anti-β4 pAb 439–9B (a and b) and the anti-α6 mAb GoH3 (c and d) shows reduced staining of the DEJ compared with the skin of a healthy control (b and d). Bar, 48 µm. (C) Northern analysis of control (lane 1) and mild PA-JEB (lane 2) keratinocytes using a 32P-labelled β4 cDNA probe shows a reduced expression of integrin β4 mRNA in the proband. G, hybridization signal of a GAPDH 32P-labelled probe. (D) Western analysis of extracts obtained from control (lane 1) and mild PA-JEB (lane 2) keratinocytes using an anti-β4 pAb and the anti-tubulin mAb T-4026 shows a reduced expression of the integrin β4 in proband’s cells.

vivo and the formation of the HD-like adhesion structures (SACs) that are assembled in vitro by keratinocytes. Integrin α6β4 is also detected in epithelia that do not possess HDs, which may reflect a multifunctional role of the protein (7). In this respect, five distinct and tissue-specific variants of β4 have been identified, but the functional role of these polypeptides, all generated by alternative splicing of the ITGB4 RNA transcript, has not yet been elucidated. The mechanisms regulating alternative splicing of the β4 pre-mRNA are also obscure.

In this study, we demonstrate that compound heterozygosity for integrin β4 gene mutations underlie PA-JEB in a patient presenting dramatic amelioration of the neonatal disorder. Our results suggest that cellular factors may influence the effect of splice site mutations in genes associated with severe EB.

RESULTS

Clinical observations

The proband was a 14-year-old boy, the child of the non-consanguineous union of clinically non-affected parents with an older healthy son. At birth, the patient presented with the hallmarks of severe PA-JEB including extensive skin blistering, pyloric atresia and urethrovaginal occlusion (5). Immunohistological analysis of lesional skin located the cleavage plane of the blisters within the lamina lucida of the BMZ. As the child grew, the blistering tendency markedly decreased and the proband’s skin and epithelia acquired resistance to trauma.

At the age of 14, induction of blisters required prolonged rubbing of the skin. Ultrastructural examination of the skin showed mature HDs connected to the cytokeratin intermediate filaments and presenting poorly formed sub-basal dense plates. The anchoring filaments of the lamina lucida were clearly visible (Fig. 1A). Immunostaining of non-involved skin, performed with a panel of antibodies directed against the antigens of the dermal–epidermal junction, revealed a slightly reduced immunoreactivity of integrin α6 and a marked decrease in integrin β4 immunostaining (Fig. 1B). The reactivity of all the other known components of the BMZ was comparable with that observed with normal control skin (data not shown).

Expression of integrin α6β4 in the proband’s keratinocytes

The expression of integrins α6 and β4 was assessed by northern blot analysis of total RNA purified from cultures of the proband’s and control keratinocytes. Hybridization with a probe for integrin α6 resulted in a signal of normal intensity (data not shown), whereas hybridization with a cDNA encoding integrin β4 was markedly reduced in the proband (Fig. 1C). Consistent with these observations, immunoblotting analysis of the PA-JEB keratino-
Identification of a point mutation in the maternal \( \beta_4 \) allele

Because the heteroduplex analysis of the proband’s mRNA failed to detect sequence variations in the maternal transcripts, we hypothesized that the maternal \( \beta_4 \) transcripts were underrepresented. We therefore specifically synthesized two overlapping cDNA fragments homologous to the full-length transcripts of the maternal allele using a long-range allele-specific RT–PCR procedure (see Materials and Methods). The resulting 1.7 kb cDNA fragment spanning the 3' sequence (nt 3754–5495) of the \( \beta_4 \) cDNA was cloned in the bacterial vector pTAg. Sequencing of 10 distinct clones detected a wild-type cDNA sequence in six of them, and a 38 bp out-of-frame deletion in the 5’ region of exon 32 (nt 3986–4023) in the remaining samples (Fig. 3A). The deletion results in a downstream premature stop codon (TGA) at nt 4061–4063 and predicts a truncated \( \beta_4 \) polypeptide terminating at residue 1324. PCR amplification of the genomic DNA of the proband using primers selected on the basis of the cDNA sequence flanking the 38 bp deletion followed by sequence analysis identified a heterozygous T→A transversion (3986–19T→A) located at position –19 of exon 32 (11) (Fig. 3B). Allele-specific oligonucleotide (ASO) hybridization analysis confirmed the heterozygous status of the proband. The proband’s mother, the maternal uncle and grandmother were confirmed the heterozygous status of the proband. The possibility that the T→A transversion represents a polymorphic variation in the population was tested by screening of PCR-amplified genomic DNA from 100 unrelated healthy individuals. The mutation was not found in any of these samples.

The branchpoint site mutation 3986–19T→A causes illegitimate splicing of \( \beta_4 \) pre-mRNA

To confirm the disruptive effect of mutation 3986–19T→A on maturation of the mRNA coding for integrin \( \beta_4 \), we constructed two recombinant microgenes expressing the mRNA transcript encoded by exons 31 and 32 of ITGB4 (Fig. 4A). Genomic DNA fragments (401 bp) encompassing exon 31 (182 bp), intron 31 (88 bp) and exon 32 (131 bp) of ITGB4 were specifically PCR-amplified using the proband’s genomic DNA as a template. The amplimers were inserted in the eucaryotic expression vector pcDNA3 to obtain the microgene construct \( \beta_4 \)-M, carrying mutation 3986–19T→A, and the wild-type counterpart \( \beta_4 \)-WT. The two constructs were transfected into normal human keratinocytes seeded on plastic dishes. RT–PCR analysis of the microgene transcripts isolated from the cell cultures was performed using primers I31L and Sp6R (Fig. 4A). In the cells transfected with plasmid \( \beta_4 \)-WT, a 402 bp cDNA band was observed that corresponded to the microgene mRNA transcribed from exons 31 and 32 (Fig. 4A, lane 1). A faint 490 bp band was also detected, which corresponded to the pre-mRNA transcripts containing intron 31. In the case of keratinocytes expressing plasmid \( \beta_4 \)-M, a unique 490 bp band was found, that resulted from the retention of the mutated intron 31 of IGTB4 (Fig. 4A, lane 2). The microgene cDNAs were further characterized by clonal analysis. cDNAs amplified from the keratinocyte cultures were reverse-transcribed from the total RNA purified from cultures of keratinocytes expressing \( \beta_4 \)-M or \( \beta_4 \)-WT. The resulting RT–PCR amplimer was inserted in the bacterial expression vector pTAg. Sequencing of 10 distinct clones detected a wild-type cDNA sequence in six of them, and a 38 bp out-of-frame deletion in the 5’ region of exon 32 (nt 3986–4023) in the remaining samples (Fig. 3A). The deletion results in a downstream premature stop codon (TGA) at nt 4061–4063 and predicts a truncated \( \beta_4 \) polypeptide terminating at residue 1324. PCR amplification of the genomic DNA of the proband using primers selected on the basis of the cDNA sequence flanking the 38 bp deletion followed by sequence analysis identified a heterozygous T→A transversion (3986–19T→A) located at position –19 of exon 32 (11) (Fig. 3B). Allele-specific oligonucleotide (ASO) hybridization analysis confirmed the heterozygous status of the proband. The proband’s mother, the maternal uncle and grandmother were confirmed the heterozygous carriers of the mutation (Fig. 3C). The possibility that the T→A transversion represents a polymorphic variation in the population was tested by screening of PCR-amplified genomic DNA from 100 unrelated healthy individuals. The mutation was not found in any of these samples.
intron 31, while two of them contained cDNAs carrying the 38 bp deletion corresponding to the deletion detected in the proband’s keratinocytes (data not shown). These results confirm that besides intron retention, mutation 3986–19T → A induces illegitimate splicing of the β4 pre-mRNA by activation of a cryptic splice site present in exon 32.

**Epigenetic factors modulate splicing of the β4 pre-mRNA with mutation 3986–19T→A**

Mouse 3T3-J2 keratinocytes constitute the optimal support of keratinocyte growth *in vitro* (13). Irradiated J2 fibroblasts secrete both extracellular matrix proteins that favour keratinocyte attachment and growth factors that stimulate proliferation (14 and references therein). The patient’s primary keratinocytes cultured on J2 feeders were immunoreactive to the antibodies directed against integrin α6β4. The fluorescence presented the basal ‘Swiss cheese-like’ pattern characteristic of keratinocytes assembling SACs (15) (Fig. 4B, a and c). Conversely, the patient’s keratinocytes grown on plastic did not concentrate into SACs (Fig. 4B, b and d). Indeed, this staining was similar to the labelling pattern observed in keratinocytes of an unrelated patient with severe PA-JEB which produced low amounts of abnormal β4 polypeptides (Δ17–β4) unable to nucleate into SAC (2,16) (Fig. 4B, e and f). Transfer of the proband’s keratinocytes from a feeder layer to

Figure 4. Expression of integrin β4 in the patient’s keratinocytes is modulated by epigenetic factors. (A) Transfection of wild-type human keratinocytes using an artificial β4 microgene carrying mutation 3986–19T→A. (Top) Schematic representation of the β4 microgenes. Genomic DNA fragments spanning exons 31–32 were cloned into a eucaryote expression vector to obtain microgenes pββ4-M, carrying mutation 3986–19T→A, and the wild-type counterpart pββ4-WT. Positions of primers Ex31L and SP6R are indicated. The transcription products of pββ4-WT (402 bp) and pββ4-M (490 bp) are represented. (Bottom) RT–PCR analysis of the mRNAs transcribed from pββ4-WT (lanes 1, 3 and 5) and pββ4-M (lanes 2, 4 and 6) by transfected human keratinocytes grown on plastic (lanes 1 and 2) or on feeders (lanes 3 and 4) and by irradiated 3T3-J2 fibroblasts transfected with pββ4-WT (lane 5) and pββ4-M (lane 6). C, RT–PCR amplification in the absence of RNA template. The migration position of the spliced (402 bp) and unspliced (490 bp) cDNAs are indicated on the right. The bottom frame shows the results of RT–PCR amplification of GAPDH mRNA from the cell culture analysed. (B) Immunofluorescence of normal (a and b), mild PA-JEB (c and d) and severe (e and f) PA-JEB keratinocytes grown on feeders (a, c and e) and on plastic (b, d and f) using pAb 450-11A. The mild PA-JEB keratinocytes grown on a feeder display the dotty staining characteristic of cells assembling SACs, whereas the keratinocytes obtained from an unrelated severe PA-JEB patient expressing the Δ17–β4 polypeptide do not assemble SACs. Bar, 24 μm. (C) Ribonuclease protection mapping of the β4 mRNAs expressed by the proband’s keratinocytes. Total RNA purified from wild-type (lane 1) and mild (lane 2) PA-JEB keratinocytes grown on a feeder were hybridized with an antisense β4 riboprobe spanning the region of the β4 mRNA affected by the paternal (Δ51) and the maternal (Δ38) mutations. The presence of a protected 359 bp band both in the control and the patient’s keratinocytes denoted the synthesis of a wild-type β4 transcript. No protected band is detected with the Δ51–β4 transcript synthesized by an unrelated PA-JEB patient (2) (lane 3). Yeast tRNA was used as negative control (lane 4). Migration of undigested β4 riboprobe (lane 5) and that of molecular weight markers (lane M) are shown. (Bottom) mRNA protected by a 255 bp β-actin riboprobe. (D) Mutation 3986–19T→A affects the legitimate branchpoint sequence of intron 31. Activation of a putative cryptic branchpoint (GGCTCAC) and acceptor splice site (CAG) within exon 32 results in an out-of-frame deletion of 38 bp affecting the 5′ end of the same exon.
plastic, or to extracellular matrix (ECM) produced by wild-type human keratinocytes grown on feeder layers, induced loss of the ‘Swiss cheese-like’ pattern. Feeding of these cells with culture medium conditioned by wild-type human keratinocytes grown on feeder layers did not restore the fluorescence (data not shown). The staining pattern was not influenced by the temperature at which the cells were grown (data not shown). In all the cell cultures, immunoreactivity of laminin-5 was as strong as in wild-type human keratinocytes (data not shown).

To assess whether the presence of SACs correlated with synthesis of wild-type β4 transcripts, total RNA isolated from the proband’s keratinocytes grown on a feeder layer was analysed by RNase protection assay. The antisense riboprobe (359 nt) spanned exons 30–32 (nt 3721–4080) and encompassed the regions of 51 and 38 nt deleted in the paternal and maternal β4 mRNAs, respectively. In control and PA-JEB mRNAs, the riboprobe protected a unique cDNA fragment of 359 nt, which revealed the presence of wild-type β4 mRNA transcripts in the patient cells (Fig. 4C, lanes 1 and 2). No protected β4 mRNA was observed in the case of the PA-JEB keratinocytes grown on plastic (data not shown). These data, therefore, demonstrated that the mutated IGTB4 allele carrying the transversion 3986–19T→A can generate wild-type RNA transcripts in cells grown on a feeder layer.

Since homozygosity for the paternal mutation 3802+1G→A is associated with lethal PA-JEB (12) it is conceivable that the wild-type mRNA detected in the patient’s cultured keratinocytes is synthesized from the maternal allele, and that the culture conditions influence the steady-state level of this transcript. To verify this possibility, µβ4-WT and µβ4-M were transiently transfected into wild-type human keratinocytes seeded on feeders. The microgene cDNAs produced were analysed by RT–PCR amplification of total RNA isolated from the cell cultures and by electrophoresis on an agarose gel. With the keratinocytes transfected with plasmid µβ4-M, two bands of 402 and 490 bp of comparable intensity were detected (Fig. 4A, lane 4) instead of the single 490 bp cDNA found in cells grown on plastic (Fig. 4A, lane 2). Conversely, in the cells expressing plasmid µβ4-WT, growth on feeders did not influence the size or intensity of the microgene cDNAs (Fig. 4A, lanes 1 and 4). These results demonstrated that the culture conditions modulate the rate of illegitimate splicing driven by the 3986–19T→A mutation in the branching-point sequence of intron 31 of IGTB4.

DISCUSSION

Genetic mutations of integrin α6β4 cause PA-JEB, a recessively inherited genodermatosis characterized by disadhesion of the integument and pyloric atresia. Mutations in β4 underlie most of the PA-JEB cases elucidated so far. Premature termination codons are associated with the lethal variants of the disease, while missense and splice site mutations enabling synthesis of abnormal β4 polypeptides cause the non-lethal forms (17,18).

In this study, we describe a patient presenting severe PA-JEB at birth, followed by an amelioration of the symptoms during childhood. The mild skin fragility correlated with impaired functions of integrin α6β4, following the downregulation of β4 expression at the mRNA and protein level. Mutation analysis disclosed compound heterozygosity for IGTB4 gene mutations affecting splicing of the β4 pre-mRNA and underlying this rare form of PA-JEB.

The mutation 3802+1G→A, affecting the paternal allele, is known to cause lethal PA-JEB (12). This genetic defect disrupts the 5′ donor splice site of intron 30 in the IGTB4 gene and activates a putative cryptic splice site (GTGAG) within exon 30 (Fig. 3D). This leads to the production of an aberrant β4 mRNA with an in-frame 51 nt deletion at the 3′ end of exon 30, and results in the synthesis of an abnormal β4 polypeptide (Δ17-β4) with a 17 amino acid deletion in the second fibronectin-like repeat of the cytoplasmic tail (2). In a recent study, the aberrant polypeptides Δ17-β4 were shown to associate with integrin α6 into α6β4 heterodimers that fail to nucleate functional HDs and cause defective keratinocyte adhesion which results in severe PA-JEB (16).

The transversion 3986–19T→A is a novel mutation affecting the putative branchpoint site (GGCT&CAC) of intron 31 of the maternal IGTB4 allele. This thymidine substitution results in the maturation of aberrant mRNAs generated by activation of putative cryptic branchpoint (CCTGAC) and acceptor splice sites (cag) located within exon 32. The abnormal mRNA shows an out-of-frame deletion of 38 bp in the 5′ end of exon 32 leading to a downstream premature termination codon (PTC) and rapid decay of the mutated RNA transcripts. Because the paternal mutation 3802+1G→A in IGTB4 causes severe PA-JEB, we hypothesized that the mild clinical phenotype of the proband is determined by the nature of the branchpoint mutation 3986–19T→A affecting the maternal allele. Substitution of the conserved thymidine in the branchpoint sequence is predicted to hamper the Watson–Crick pairing between the abnormal β4 pre-mRNA and the 5′ end of the U2 small nuclear RNA (snRNA) (19). Further interaction between U2 and the U6 snRNA that constitutes a critical step for exon definition and lariat formation is hindered (20). As a result, assembly of the spliceosome and scrutiny of the coding sequences of the maternal β4 pre-mRNA are likely to be altered.

Indeed, RNase protection assays of the RNA transcripts isolated from primary cultures of the proband’s keratinocytes demonstrated that a fraction of the mutated β4 pre-mRNA transcribed from the maternal allele escapes incorrect splicing and generates a wild-type mRNA. Production of wild-type β4 polypeptide and synthesis of functional α6β4 heterodimers is consistent with the immunoreactivity of the proband’s skin to anti-integrin α6β4 antibodies and the assembly of mature HDs connected to the intermediate filaments of the cytoskeleton. The low expression level of functional α6β4 molecules also correlates well with the residual fragility of the integument.

A limited number of human diseases have thus far been associated with genetic mutations affecting branchpoint sites, possibly because the branchpoint consensus sequence (YNNYTRAY) is loosely conserved in eucaryotes (21). Indeed, only the thymidine at position 4 and the adenosine at position 6 are preserved during evolution (22). Recently, it has been reported that substitution of the conserved thymidine in the branchpoint sequence of intron 4 of the lecithin:cholesterol acyltransferase (LCAT) gene induces intron retention and causes LCAT deficiency (23). In our patient, mutation 3986–19T→A did not induce retention of intron 31 in the β4 mRNA. Transient transfection of IGTB4 minigenes that contained intron 31 with or without mutation 3986–19T→A in normal human
keratinocytes confirmed the causative role of this intron mutation in PA-JEB. In fact, intron retention was observed in vivo in the majority of the mutated minigene RNA transcripts, while out-of-frame deletion of 38 bp in exon 32 was detected in a residual fraction of mRNAs. In remains unclear why in the cells transfected with the mutant minigene, intron retention rather than activation of illegitimate splicing constitutes the main effect of the intronic T→A transition. It is likely that distal regulatory sequences that favour activation of the cryptic splicing of the β4 mRNA are deleted in the short minigene construct (24). Nevertheless, the production of low amounts of the internally deleted mutated mRNA mirrors the effect that mutation 3986–19T→A exerts in vivo on maturation of the β4 pre-mRNA, and confirms the role of this intronic mutation in the aberrant expression of the maternal ITGB4 allele. These ex vivo observations also demonstrate that substitution of the thymidine in the branchpoint consensus sequence can activate alternative cryptic splice sites and promote differential splicing when alternative branchpoint sequences are present in the downstream exonic sequences. These results, therefore, are consistent with previous reports describing use of alternative splice sites in genes with mutations affecting putative branchpoint sites of human pre-mRNA (25,26).

The transfection experiments also helped us to interpret the molecular events underlying the benign outcome of PA-JEB in the proband. Analysis of the proband’s keratinocytes revealed that epigenetic factors could bias the effect of the β4 intronic mutation by acting on the splicing machinery, because the cell cultures expanded on feeders activated synthesis of wild-type β4 mRNA and nucleated SACs. The finding that legitimate splicing of the mutated minigene transcripts was restored by seeding the transfected keratinocytes on a feeder layer, correlated with the identification of functional β4 mRNA in the proband’s keratinocytes and with the presence of mature HDs in the skin.

Information on the expression of integrin β4 in the proband’s skin before clinical improvement and ultrastructural normalization of the BMZ observed with ageing was not available. However, it is tempting to speculate that in the context of an overall reduction of the β4 mRNA levels, activation with age of the legitimate splice site in the aberrant pre-mRNA transcribed from the maternal ITGB4 allele underlies the transition from severe to mild PA-JEB. This possibility would agree with the observation that in Nagase albunminogenic rats bearing a genetic mutation at a 5’ splice site of the albumin gene, ageing is associated with changes in the splicing pattern of the corresponding aberrant pre-mRNA (27). Moreover, a splice site mutation abolishing the obligatory consensus 3’ acceptor splice site in COL7A1 has recently been associated with the a case of transient bullous dermolysis of the newborn, a skin blistering disorder that rapidly ameliorates in infancy (28). Taken together, these observations suggest that subsets of splice mutations underlie the clinically transient phenotypes observed in inherited pathologies. In light of our results, we propose that improved splicing of specific mutant genes may depend on the production/activation of cellular factors along with ageing. At present, the nature of the factor(s) produced by the irradiated feeders that modify splicing of the mutated β4 allele in our PA-JEB patient remains to be elucidated. However, the observation that splicing activation requires a close interaction between the mutated keratinocytes and the J2 fibroblasts excludes a role of the ECM, and indicates that the possible candidates are cell surface proteins or labile cytokines exerting a labile paracrine effect.

With respect to the clinical heterogeneity, diagnosis, pathogenesis and therapy of epidermolysis bullosa, our data contribute to the establishment of a correlation between specific mutations and phenotypic characteristics. They also highlight the relevance that interactions between epithelial and stromal cells may have in modulating the expression of cell surface receptors.

**MATERIALS AND METHODS**

**Cells**

Primary human keratinocytes were isolated from skin biopsies. The cells were cultured on feeder layers of irradiated mouse 3T3-J2 fibroblasts, on keratinocyte ECM, or directly on plastic dishes in the presence of DMEM/F12 (2:1) medium (Life Technologies, Cergy-Pontoise, France) supplemented with 10% fetal calf serum, 4 mM glutamine, 5 μg/ml insulin, 0.4 μg/ml hydrocortisone, 2 mM cholaria, 10 ng/ml epidermal growth factor, 2 nM triiodothyronine and 0.18 mM adenine (13). The ECM was prepared by treating keratinocyte cultures grown on 3T3-J2 feeders with phosphate-buffered saline (PBS), pH 7.4, 10 mM EDTA for 10 min at 37°C. After detachment of the cells, the ECM was rinsed three times with PBS containing 1 mM CaCl2 and 1 mM MgCl2.

**Electron microscopy and immunohistochemistry**

Utrastructural examination of involved and non-involved PA-JEB skin was performed as described elsewhere (2). Immunofluorescence analysis of skin biopsies and keratinocyte cultures has been detailed previously (29). Expression of integrin β4 was monitored using monoclonal antibodies (mAb) 439-9B and 450-11A (30). Immunomapping of the basement membrane components was performed using mAb GB3 raised against native laminin-5 (31), mAb HD121, directed against HD1 (32), mAb FP1 and 1/8C, specific to bullous pemphigoid antigens of 230 kDa (33) and 180 kDa (34), respectively. Anti-integrin α6 was mAb GoH3 (35).

**Western blot analysis**

SDS–PAGE was performed using 7.5% polyacrylamide gels (36). Blotting onto nitrocellulose membranes and immunostaining with an anti-β4 polyclonal antibody (37) and mAb T-4020 (Sigma, St Louis, MO) specific to tubulin was achieved following standard procedures using the ECL detection system (Amersham France, Issy les Ulis, France).

**Northern blot analysis**

Total RNA was extracted from cultured keratinocytes using the RNable extraction kit according to the manufacturer’s recommendations (Eurobio, Les Ulis, France). Twenty micrograms of total RNA was electrophoresed in a 1% agarose–formaldehyde gel, and blotted onto a nylon membrane (Amersham France). The membranes were hybridized with a 1.9 kb integrin β4 and a 983 bp GAPDH 32P-random-labelled cDNA probes obtained by RT–PCR amplification of total RNA purified from wild-type human
keratinocytes. For amplification of the β4 cDNA (GenBank accession no. X52186) (11), the primer pair was (L) 5’-GGCAGATATGAAGGTTG-3’ (5’-AGGATGGAGTAGCTGAGGAG-3’). The PCR cycling conditions: 94°C, 3 min; 94°C, 20 s; 58°C, 45 s; 72°C, 2 min (30 cycles). For the GAPDH cDNA probe (GenBank accession no. M33197), primers were (L) 5’-AGATTCCTCCTAAAATCAAGT-3’ (5’-TAGGGCCCCTCCCTCCTTTA-3’).

RT–PCR amplification of β4 integrin mRNA

To search for mutations, total RNA was extracted from PA-JEB and control keratinocytes grown on a plastic support. Ten micrograms of total RNA was reverse-transcribed in the presence of oligodeoxynucleotide primers as recommended by the manufacturer (Promega, Charbonnière, France). To detect the PCR transcript of the paternal ITGB4 allele, PCR reactions were performed in the presence of Taq DNA polymerase (Life Technologies), using 0.1 µg of reverse transcription product, and 11 pairs of oligonucleotide primers synthesized on the basis of the integrin β4 cDNA sequence (11). Heteroduplex analysis was performed using the conformation-sensitive gel electrophoresis method (38). The specific primers used to detect the 3802+1G→A mutation were: (L) 5’-AACCCTAATGCTAAGGCCG-3’ (R) 5’-TCAGTTGTCATCGGAGACG-3’. The PCR cycling conditions were: 94°C, 3 min; 94°C, 20 s; 58°C, 45 s; 72°C, 30 s (30 cycles). Direct sequencing of the PCR products was performed using an ABI PRISM Dye Terminator Cycle Sequencing kit (Perkin Elmer, Foster City, CA). To detect the maternal mutation, 1 µl of the reverse transcription product was submitted to two long-range allele-specific PCR amplifications spanning the entire reading frame of the β4 cDNA, using primers homologous to nucleotide sequences comprised within the 51 bp deletion found in the transcripts of the paternal β4 allele. Amplifications were performed using the Expand Long Template PCR System (Boehringer Mannheim, Meylan, France). To amplify the β4 cDNA fragment bearing the maternal 38 bp deletion, the following oligonucleotides were used: (L) 5’-TGAGATCAGCCTACGTTACGAG-3’ (R) 5’-AGGATGGAGTAGCTGAGGAG-3’. The PCR cycling conditions were: 94°C, 3 min; 94°C, 20 s; 58°C, 45 s; 68°C, 1.5 min (10 cycles), 94°C, 20 s; 58°C, 45 s; 68°C, 1.5 min (20 cycles), with a 20 s increment in extension time at each stage. The amplicon was then subcloned into a pTAg vector according to the manufacturer’s recommendations (R&D Systems, Abingdon, UK) and sequenced.

Detection, verification and inheritance of the genetic mutations

PCR reactions of genomic DNA (100 ng) purified from the consenting members of the PA-JEB kindred and of unrelated controls were performed using standard conditions (39). To detect mutation 3802+1G→A, the primers were: (L) 5’-CAGTGACAGCCTCTTCAG-3’ (R) 5’-CAGGAAGAGTTGCTTAGGAG-3’ (GenBank accession no. U66539), and to detect the mutation 3986→19T→A, the primers were: (L) 5’-GACCCAAGAGCCTTGTTGCCAAT-3’ (R) 5’-GTTGCGCCTTTCCTGTTTTG-3’ (GenBank accession no. U66538). To determine the segregation of these genetic mutations in the kindred, a standard ASO hybridization protocol was used (2). For mutation 3802+1G→A, the ASOs were 5’-TGACAACCTAAGAAC-3’ (wild-type) and 5’-TGACAACCTAAGAAC-3’ (mutant); and for the mutation 3986→19T→A, 5’-CCCTGCTCCTCC-3’ (wild-type) and 5’-CCCTGCTCCTCC-3’ (mutant).

Construction of ITGB4 microgenes

DNA fragments (401 bp) of the ITGB4 gene were obtained by PCR amplification of the region encompassing exon 31, intron 31 and exon 32, using the proband’s DNA as a template and a primer pair synthesized on the basis of the cDNA sequence of the gene (GenBank accession no. X52186). Primer (L) (designated as III1L) was: 5’-ATGCAGACTATGGAGGCCCATGAG-3’ (nt 3803–3821 in the cDNA) and comprised four bases (underlined) generating a methionine codon in-frame with the open reading frame of exon 31. Primer (R) was 5’-AGTGTCATCGGAGACGCGGCT-3’ (nt 4093–4117 in the cDNA). PCR amplification was performed using Pfu DNA polymerase following the supplier’s recommendations (Stratagene, La Jolla, CA), and the cycling conditions were: 94°C, 3 min; 94°C, 20 s; 55°C, 45 s; 72°C, 1 min. One hundred micrograms of amplification products were ligated to 50 ng of expression vector pcDNA3 (InVitrogen, Groningen, The Netherlands) digested with the restriction enzyme EcoRV (New England Biolabs, Hitchin, UK) using 2×105 U of T4 DNA ligase (New England Biolabs) and amplified in competent E.coli, strain XL1 blue (Stratagene). Screening of transformants was performed by PCR analysis of total bacterial DNA extracted from 30 isolated colonies using primer I31L and a pcDNA3 specific reverse primer Sp6R: 5’-GATTTTAGGTGACACATATAGAATAG-3’. PCR conditions were: 94°C, 10 min; 94°C, 45 s, 60°C, 45 s, 72°C, 45 s (30 cycles); 72°C, 2 min. Direct sequence analysis of the amplimers identified the pμβ4-WT microgene construct carrying the wild-type intron 31 of ITGB4 and microgene pμβ4-M carrying mutation 3986→19T→A.

DNA transfection and analysis of the ITGB4 mRNA transcripts

Plasmid DNA was purified on affinity columns (Qiagen, Courtaboeuf, France) and subconfluent secondary cultures of wild-type human keratinocytes seeded in six-well plates (2×105 cells/well), either directly on plastic or on a feeder of lethally irradiated mouse 3T3-J2 fibroblasts, were transected using 2.5 µg of plasmid DNA and the FuGene-6 transfection kit (Boehringer Mannheim). Twenty-four hours later, the cells were harvested. Total RNA was extracted and submitted to RT–PCR amplification of pμβ4-WT and pμβ4-M microgene transcripts using the primer pair (L) I31L and (R) Sp6R. The RT–PCR products were analysed by electrophoresis on a 2% agarose gel. Two microlitres of the PCR reactions was used to subclone the pμβ4-WT and pμβ4-M microgene cDNAs in the bacterial plasmid pTOPO 2.1 (InVitrogen). After transformation of competent E.coli XL1 blue, 50 white colonies were isolated and lysed, and the nucleic acids directly submitted to PCR amplification using primers I31L and Sp6R in a final volume of 25 µl. The size of the amplimers was checked by electrophoresis in a 2% agarose gel. The PCR products were submitted to direct nucleotide sequencing.
Ribonuclease protection mapping of the integrin β4 transcripts

A 359 bp cDNA probe (nt 3721–4080) (11) encompassing the deletions detected in the β4 transcripts of the PA-JEB patient, was synthesized by RT–PCR using total RNA extracted from control keratinocytes. The primers were: (L) 5′-CTGAGCTCTGCCCTGAGCCCGC-3′, (R) 5′-TGGAGAGCCTGAGACCTGTA-3′. The amplimer was subcloned into pCR Script vector (Stratagene). The α [39x658]µ [39x669]GAGCCGGC-3′ Service, National Institutes of Health. 

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