Am. J. Hum. Genet. 65:1530–1537, 1999

Digenic Junctional Epidermolysis Bullosa: Mutations in COL17A1 and LAMB3 Genes

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Summary

Junctional epidermolysis bullosa (JEB), a genetically heterogeneous group of blistering skin diseases, can be caused by mutations in the genes encoding laminin 5 or collagen XVII, which are components of the hemidesmosome-anchoring filament complex in the skin. Here, a family with severe nonlethal JEB and with mutations in genes for both proteins was identified. The index patient was compound heterozygous for the COL17A1 mutations L855X and R1226X and was heterozygous for the LAMB3 mutation R635X. As a consequence, two functionally related proteins were affected. Absence of collagen XVII and attenuated laminin 5 expression resulted in rudimentary hemidesmosome structure and separation of the epidermis from the basement membrane, with severe skin blistering as the clinical manifestation. In contrast, single heterozygotes carrying either (1) one or the other of the COL17A1 null alleles or (2) a double heterozygote for a COL17A1 and a LAMB3 null allele did not have a pathological skin phenotype. These observations indicate that the known allelic heterogeneity in JEB is further complicated by interactions between unlinked mutations. They also demonstrate that identification of one mutation in one gene is not sufficient for determination of the genetic basis of JEB in a given family.

Received July 15, 1999; accepted September 8, 1999; electronically published November 8, 1999.

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0002-9297/1999/6506-0008$02.00

Introduction

Junctional epidermolysis bullosa (JEB) refers to a heterogeneous group of recessively inherited disorders with skin fragility, ultrastructurally abnormal hemidesmosomes (McMillan et al. 1998), and separation of the epidermis from the basement membrane. JEB includes several subtypes, varying from lethal JEB Herlitz (MIM 226700) to mild acral affection (Gedde-Dahl and Anton-Lamprecht 1996; Bruckner-Tuderman, in press), that are caused by mutations in at least six different genes encoding components of the hemidesmosome-anchoring filament complex in the skin, laminin 5, $\alpha 6\beta 4$ integrin, and collagen XVII (Pulkkinen and Uitto 1999).

JEB Herlitz is a severe condition that results in premature demise of the affected individuals within a few months of birth. Mutations in the three genes—LAMA3, LAMB3, and LAMC2—encoding the $\alpha 3$, $\beta 3$, and $\gamma 2$ polypeptide subunits of laminin 5 underlie this subtype (Pulkkinen and Uitto 1999). Characteristically, premature termination codons in both alleles of any of these three genes signify poor prognosis as a result of absence of laminin 5 in the skin of the affected individuals. In contrast, rare missense mutations in one or both alleles are associated with relatively mild phenotypes (McGrath et al. 1996*a*).

Generalized atrophic benign epidermolysis bullosa (GABEB [MIM 226650]) represents a nonlethal subtype with blistering, skin atrophy, alopecia, nail dystrophy, and dental anomalies. COL17A1 gene mutations underlie the majority of GABEB cases (McGrath et al. 1995, 1996*b*, 1996*c*; Chavanas et al. 1997; Gatalica et al. 1997; Jonkman et al. 1997; Schumann et al. 1997; Floeth et al. 1998; Darling et al. 1999). Most of these are homozygous or heterozygous mutations leading to a premature-termination codon and absence of collagen XVII in the skin, whereas missense mutations in one or both alleles are associated mainly with acral blistering and mild skin atrophy (Schumann et al. 1997).

Laminin 5 and collagen XVII are components of the hemidesmosome-anchoring filament complex at the dermal/epidermal junction (Rousselle et al. 1997;

Schäcke et al. 1998). This complex is pivotal for a stable association between the epidermis and the underlying dermis. It consists of (1) hemidesmosomes that extend from the intracellular space of basal keratinocytes to the basement membrane zone and (2) anchoring filaments—threadlike structures that extend from the hemidesmosomes to the underlying lamina densa of the basement membrane. Hemidesmosomes contain at least four proteins that display defined interactions with each other, as well as with other components of the basement membrane. These are the cytoskeleton-associated proteins BP230 and plectin and the transmembrane components collagen XVII and $\alpha 6\beta 4$ integrin (Nievers et al. 1999). The anchoring filaments contain laminin 5, a member of the laminin family of proteins, which consists of the $\alpha 3$, $\beta 3$, and $\gamma 2$ chains encoded by distinct genes LAMA3, LAMB3, and LAMC2 (Aumailley and Rousselle 1999). The extracellular domain of collagen XVII is likely to contribute to the structure of the anchoring filaments, since it colocalizes with laminin 5 (Masunaga et al. 1997) and binds to it in vitro (Reddy et al. 1998).

Here we describe a family with JEB that has abnormal expression of two functionally related proteins, collagen XVII and laminin 5, that is caused by mutations in two unlinked genes. The absence of collagen XVII and the reduction in the amount of laminin 5 in the skin lead to a severe blistering phenotype with features of both the lethal Herlitz and the nonlethal GABEB subtypes.

Material and Methods

Clinical and Diagnostic Features

The male proband, who is now 2 years old, is a product of a nonconsanguineous union. The parents and two half-sisters were unaffected, and there was no family history of skin or genetic diseases. The proband was born with acral skin blistering and with large erosions of the feet and shins. Within days, the fingertips were affected in a paronychia-like fashion, the mucous membranes were involved, and the pressure-prone areas in the back and buttocks were severely affected. Diagnostic electron microscopy of a postnatal skin-biopsy specimen revealed separation of the epidermis from the dermis, along the lamina lucida of the basement membrane and rudimentary hemidesmosomes. JEB Herlitz was suspected, and a subsequent mutation screening (T. Gedde-Dahl Jr., personal communication) revealed the presence of the heterozygous LAMB3 mutation R635X (Kivirikko et al. 1996). Lethal JEB Herlitz was diagnosed.

However, at the age of 7 mo, the proband had normal body weight and height, generalized skin blistering with emphasis in acral areas (fig. 1), mild affection of oral mucosa, and no involvement of internal organs. In the course of the disease, the symptoms remained stable, with the blistering being more severe at trauma-exposed and pressure-prone areas. The lesions resolved without scarring but with a mild skin atrophy, possibly as a consequence of secondary inflammatory processes. Dystrophy of all nails developed, whereas mucous membranes remained mildly affected. The teeth were dystrophic. A slight frontal alopecia was noted at the age of 18 mo. On examination of the family members, no skin blistering or nail dystrophy was observed, but the mother and both half-sisters presented with dental anomalies—that is, enamel hypoplasia and pitting.

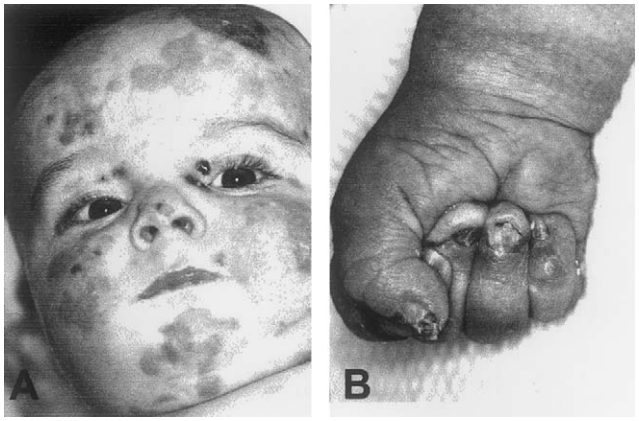

Figure 1 Phenotype of the proband. The clinical phenotype included features of both the GABEB and JEB Herlitz subtypes. *A*, Proband seen, at the age of 7 mo, with generalized skin blistering but with minimal involvement of the mucous membranes and with normal body height and weight. *B*, Paronychia-like affection of the fingers, as typically seen in JEB Herlitz.

Skin-biopsy specimens were obtained from the index patient and from both parents, and blood samples for mutation analysis were obtained from affected and unaffected family members. This study was approved by the institutional review board for human studies at the University of Münster, and informed consent was obtained from the family members before the tissue and blood samples were taken.

Mutation Detection

Genomic DNA was isolated from peripheral blood by use of the QIAAmp Blood Kit (QIAGEN), according to the manufacturer's instructions. PCR amplification of COL17A1 exons 37 and 51 was performed as described elsewhere (Gatalica et al. 1997; see also GenBank). The primers used for amplification of COL17A1 exon 37 were as follows: sense primer 5′-AGAGGTGAGAAGTGTGGCAG-3′ and antisense primer 5′-GCAGAAGAGAGCAAGGAAGA-3′. The size of the PCR product was 261 bp. Primers used for

amplification of COL17A1 exon 51 were as follows: sense primer 5′-TTTCTCTCCTCCCATCACCC-3′ and antisense primer 5′-TGTCCCTTTAAGTGCCT CCC-3′. The size of the PCR product was 374 bp. Heteroduplex analysis was performed as described by Ganguly et al. (1993), and heteroduplex-forming PCR products were sequenced by an automated sequencer (Genome Express). The mutation screening in LAMB3 exon 14 was performed as described elsewhere (Kivirikko et al. 1996). The primers used for amplification of the LAMB3 exon 14 were as follows: sense primer 5′-GCTGCGACTTCTGTTATTCT-3′ and antisense primer 5′-AAATGTAAGGAAGGACCAGC-3′. The size of the amplification product was 578 bp. The mutation was verified by both *Bgl*II digestion and dideoxynucleotide sequencing.

Northern Blotting

mRNA was isolated from cultured JEB and control keratinocytes, with the use of the Oligotex Direct mRNA Minikit (QIAGEN). For northern blotting, 1.5 μg mRNA was separated on formaldehyde-containing agarose gels and was then capillary transferred onto a positively charged nylon membrane. For hybridization, two probes were used—a collagen XVII cDNA (Floeth et al. 1998) and a LAMB3 amplification product—which were labeled by use of the digoxigenin DNA Labeling Kit (Roche Diagnostics). Detection was with alkaline phosphatase–labeled digoxigenin antibodies and with chlordioxetan phenyl phosphate CDP-Star chemiluminiscence substrate (Roche Diagnostics). Densitometric analysis of the bands was quantitated with the use of the BIO PROFIL software program (Vilber Lourmat France 96).

Immunodetection of Proteins

Indirect immunofluorescence staining of skin cryosections was performed by standard techniques and with the following primary antibodies: monoclonal antibodies 1A8C to the intracellular domain of collagen XVII and 1D1 to the extracellular domain of collagen XVII (generously provided by Dr. K. Owaribe, School of Informatics and Sciences, Nagoya, Japan), cytokeratins (Dako), BP230 (a high-titer autoantiserum from a pemphigoid patient), α6 and β4 integrins (Gibco Life Technologies), collagen IV (Dako), chain-specific antibodies to laminin 5 (α3- and β2-chain antibodies BM-165 and 6F12, respectively, which were kind gifts of Dr. R. Burgeson, Cutaneous Biology Research Center, Harvard Medical School, Cambridge, MA), γ2-chain antibody GB3 (kindly provided by Dr. J.-P. Ortonne, University of Nice, France), and collagen VII (Bruckner-Tuderman et al. 1995).

Results

Identification and Verification of Mutations

Since the proband's phenotype had features of both the JEB-Herlitz and GABEB subtypes, mutations in both LAMB3 and COL17A1 were analyzed. The LAMB3 mutation R635X was traced by PCR amplification of exon 14, followed by *Bgl*II digestion of the PCR products (Kivirikko et al. 1996). Dideoxynucleotide sequencing revealed, at nucleotide position 1903 of the LAMB3 cDNA, a C→T transition that caused a premature-termination codon. Verification by *Bgl*II digestion showed also that the mother was heterozygous for this mutation (fig. 2).

COL17A1 mutation screening included PCR amplification of genomic DNA and heteroduplex analysis of the PCR products (Gatalica et al. 1997). Broadened bands of exons 37 and 51 in conformation-sensitive gel electrophoresis suggested heteroduplex formation and, therefore, heterozygosity. Dideoxynucleotide sequencing showed that the index patient was heterozygous for a 2669T→G transversion in exon 37, a novel nonsense mutation designated "L855X" (fig. 2). Verification with *Asp*I digestion demonstrated that his father and paternal grandmother were heterozygous for this mutation. The proband was also heterozygous for a 3781C→T transition in exon 51, which was designated "R1226X" (McGrath et al. 1995; Jonkman et al. 1997; Schumann et al. 1997). Verification with *Taq*I digestion demonstrated that his mother and both half-sisters were heterozygous for this mutation. Both COL17A1 mutations caused a premature-termination codon and were not found in 150 chromosomes of asymptomatic unrelated individuals. The presence of the LAMB3 mutation R635X was investigated, by *Bgl*II digestion, in the genomic DNA of 16 unrelated patients with either GABEB or localized JEB and in that of 26 asymptomatic COL17A1 mutation carriers; however, it was not found in any of the samples.

Expression of COL17A1 and LAMB3 mRNA

Northern blots with normal keratinocyte mRNA showed a strong 6-kb collagen XVII mRNA signal (fig. 3). In contrast, mRNA from the patient's keratinocytes produced no signal, suggesting nonsense-mediated mRNA decay in these cells (Hentze and Kulozik 1999). Hybridization of the normal mRNA with a LAMB3 cDNA probe showed a strong 3.5-kb laminin 5 β3 signal, but mRNA from the patient's keratinocytes produced a clearly weaker band (fig. 3). Densitometric scanning of the LAMB3 bands, in relation to the glyceraldehyde-phosphate-dehydrogenase signals, indica-

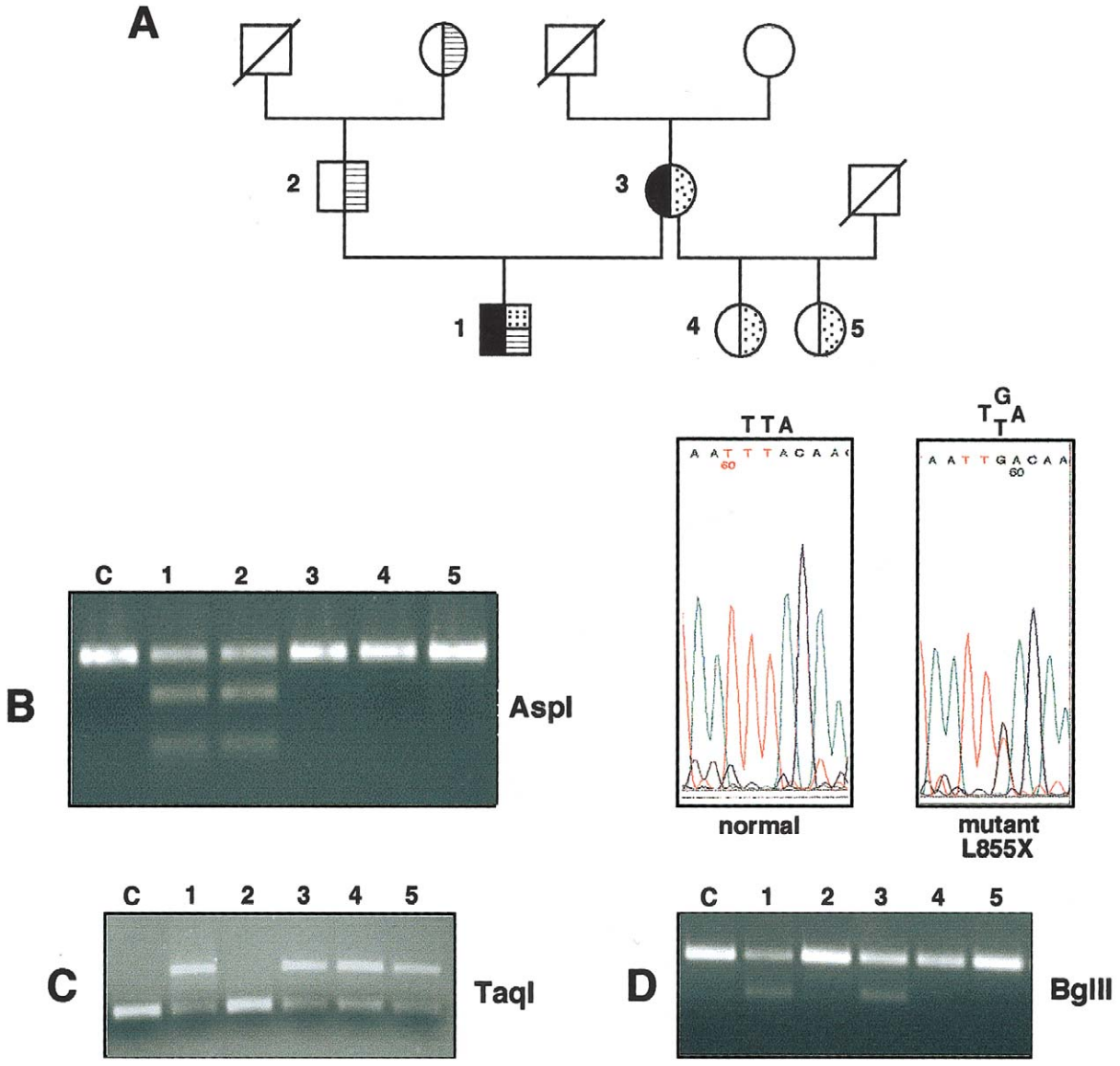

Figure 2 Pedigree and LAMB3 and COL17A1 mutations. *A,* Pedigree demonstrating recessive inheritance of the mutations. Areas with horizontal stripes indicate paternal COL17A1 mutations; dotted areas, maternal COL17A1 mutations; blackened areas, maternal LAMB3 mutations. *B,* Paternal COL17A1 mutation, which was novel—a heterozygous 2669 T→G transversion in exon 37—and was designated "L855X." Verification of the mutation by *Asp*I digestion demonstrated that the father and the paternal grandmother were heterozygous for this mutation. *C,* Maternal COL17A1 mutation, which was a heterozygous 3781C→T transition in exon 51 and was designated "R1226X" (McGrath et al. 1995; Jonkman et al. 1997; Schumann et al. 1997). The mutation was also found, by *Taq*I digestion, in the mother's and both the half-sisters' DNA, but it was not found in a control or in the father. *D,* LAMB3 mutation R635X (Kivirikko et al. 1996), which was verified by both *Bgl*II digestion of the PCR amplification products of exon 14 and by dideoxynucleotide sequencing (not shown). In addition to the proband, the mother was heterozygous for this mutation. In *B–D,* the numbers above the agarose-gel images denote the individuals in the pedigree, and "C" denotes the control.

ted that the JEB cells contained 55% of the LAMB3 mRNA of control keratinocytes.

Collagen XVII and Laminin 5 Protein in the Skin

Indirect immunofluorescence staining with collagen XVII and laminin 5 antibodies showed a linear signal along the basement-membrane zone of normal skin (fig. 4*A* and *B*). In the skin of the patient, staining with different domain-specific collagen XVII antibodies was negative (fig. 4*C*), whereas staining with chain-specific antibodies to the laminin 5 $\alpha 3$, $\beta 3$, and $\gamma 2$ chains showed a positive fluorescent signal—but

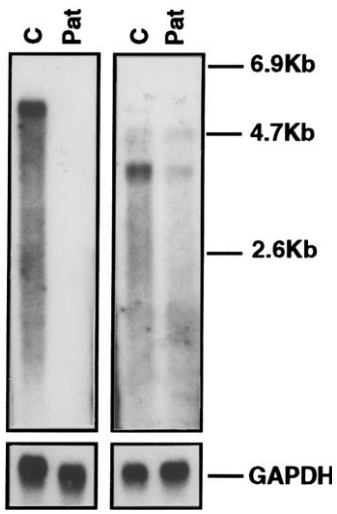

Figure 3 Northern blotting of collagen XVII and laminin 5 mRNA. A strong signal of the 6-kb collagen XVII mRNA was detected in control keratinocytes (*left panel*). In contrast, collagen XVII mRNA that was isolated from the JEB patient's keratinocytes (*left panel*) produced a negative signal, suggesting nonsense-mediated mRNA decay in these cells. The blot was intentionally overexposed to reveal potentially small amounts of mRNA in the patient's cells. When laminin 5 cDNA was used for hybridization, a strong 3.5-kb laminin 5 mRNA band was seen in normal keratinocytes (*right panel*). The keratinocytes of the patient with JEB produced a clearly weaker band (*right panel*). GAPDH cDNA was used as a reference probe to correct for minor differences in RNA loading. Densitometric analysis of the laminin 5 signals indicated that the patient's cells contained ~55% of the laminin 5 mRNA seen in controls. C = control; Pat = patient.

weaker than that seen in controls (fig. 4*D*). This finding, together with the northern blot signals, suggested that the JEB skin contained lower amounts of laminin 5 than did the control skin and that the synthesis of the $\beta 3$ chain was not compensatorily up-regulated. In spontaneously blistered skin of the proband, antibodies to cytokeratins, BP230, and $\alpha 6$ and $\beta 4$ integrins bound to the blister roof, and the antibodies to collagens IV and VII bound to the blister floor. This staining pattern was congruent with junctional-tissue separation (Hintner et al. 1981). Immunofluorescence staining patterns of the parents' skin samples were indistinguishable from those of controls.

Discussion

Digenic mutations have been reported in a few human diseases, such as retinitis pigmentosa (Kajiwara et al. 1994; Dryja et al. 1997), nonsyndromic hereditary hearing loss (Balciuniene et al 1998), and Waardenburg syndrome type 2 and autosomal recessive ocular albinism (Morell et al. 1997). The most extensively studied digenic disease is retinitis pigmentosa caused by mutations in the unlinked genes ROM1, on chromosomal locus 11q, and periferin/RDS, on locus 6p, both of which encode the polypeptide subunits of a photoreceptor protein complex. In Waardenburg syndrome type 2 in conjunction with ocular albinism, the phenotype has been suggested to result from digenic interactions between a gene for the transcription factor microphthalmia-associated transcription factor and a gene, TYR, that it regulates and that encodes tyrosinase (Morell et al. 1997).

We identified digenic mutations in two unlinked genes, LAMB3 and COL17A1, in subjects with JEB. The former gene is localized on chromosomal locus 1q32, the latter on 10q24.3. Homozygous or compound-heterozygous mutations in either gene have been known to be associated with JEB (Pulkkinen and Uitto 1999), but the present observations demonstrate that digenic interactions can cause additional heterogeneity. Similar to the ROM1 and the periferin/RDS gene products, which are polypeptide components of a transmembrane-protein complex in the retinal photoreceptors, the affected molecules in JEB are structural proteins of the hemidesmosome-anchoring filament complex, an oligomeric transmembrane-protein complex in the epidermis. Like rom-1 and periferin/rds, laminin 5 and collagen XVII have been postulated to be functionally related (Reddy et al. 1998), and our recent data indeed demonstrate

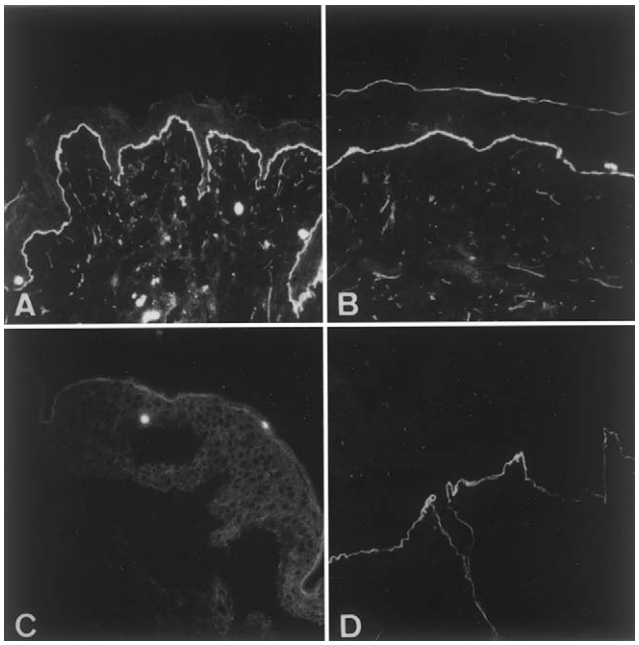

Figure 4 Collagen XVII and laminin 5 protein expression in situ. Indirect immunofluorescence staining of control (*A* and *B*) and JEB (*C* and *D*) skin biopsy specimens, with antibodies to collagen XVII (*A* and *C*) and laminin 5 (*B* and *D*). In normal skin, both collagen XVII (*A*) and laminin 5 (*B*) antibodies showed a linear fluorescence at the dermal-epidermal junction. In the proband's skin, collagen XVII was absent (*C*), and the laminin 5 staining was attenuated (*D*).

that these two trimeric proteins interact noncovalently in vitro (M. Aumailley, K. Tasanen, and L. Bruckner-Tuderman, unpublished observation). In the absence of collagen XVII from the skin, half-normal amounts of laminin 5 seem insufficient for formation of functional hemidesmosome-anchoring filament complexes. In analogy, the periferin/RDS mutant L185P cannot form nativelike homotetramers on its own (Goldberg and Molday 1996), but it can assemble with wild-type rom-1 to form structurally normal heterotetramers. However, if rom-1 is also defective, a pathological phenotype results.

The mutation R635X is one of two known recurrent LAMB3 mutations in JEB Herlitz; the other is R42X (Kivirikko et al. 1996). Identification of these two predominant gene defects has led to a mutation-detection strategy for JEB Herlitz that first analyzes, by restriction-enzyme digestions, the LAMB3 gene for these two common mutations and that then, if the results are negative, continues with heteroduplex scanning of the other LAMB3 exons and, finally, of the other laminin 5–encoding genes (Pulkkinen et al. 1997). This procedure was also followed in the case of the present proband with clinical and ultrastructural features suggestive of JEB Herlitz. The disclosure of the heterozygous R635X mutation endorsed the initial diagnosis of JEB Herlitz and led to counseling of the parents about poor prognosis and early demise of the proband. However, the course of the disease—that is, normal growth and lack of involvement of internal organs at the age of 7 mo—were not typical of JEB Herlitz. Subsequent antigen mapping of the skin basement-membrane zone revealed the absence of collagen XVII, thus identifying a second candidate gene.

Mutation screening of COL17A1 disclosed compound heterozygosity for nonsense mutations in this gene. Such genetic constellations are known in GABEB, but the clinical phenotype of the present proband was more extensive, showing features of both GABEB and JEB Herlitz. In general, GABEB phenotypes caused by COL17A1 mutations are milder and lack paronychia-like affection of the fingers and toes and severe affection of the buttocks. It seems that the severe phenotype and the JEB Herlitz–like features of the present proband resulted from digenic interactions. Biologically, lack of collagen XVII leads to weakened hemidesmosome-cytoskeleton and hemidesmosome-anchoring filament interactions. In this case, the extracellular interactions are additionally severed, since laminin 5 expression is reduced to half, as indicated by reduced mRNA levels in keratinocytes and by attenuated protein expression in the skin.

This family also reveals a novel aspect of skin biology. The mother of the proband is a double heterozygote for a null allele of both LAMB3 and of COL17A1. She shows that 50% reduction of the expression level of both gene products is tolerable and that it does not lead to skin fragility, if the other components of the hemidesmosome-anchoring complex are normal. So far, on the basis of the knowledge that single heterozygotes for nonsense mutations in JEB genes are unaffected, the hypothesis has been that 50% reduction in one protein of the complex does not attenuate epidermal adhesion. The mother now demonstrates that more-extensive biological safeguard measures exist for the protective functions of the epidermis than was previously assumed. In this context, the dental abnormalities of the mother and other heterozygous mutation carriers are also interesting. In the literature, enamel hypoplasia and pitting have been described in two siblings with the heterozygous COL17A1 mutation G627V (McGrath et al. 1996*c*), but no reports exist on dental abnormalities in heterozygotes with mutations in the genes encoding laminin 5, in spite of the fact that laminin 5 is expressed in the ameloblast basement membrane (Aberdam et al. 1994). Further dissection of the qualitative and quantitative role of the individual anchoring-complex proteins in epidermal or ameloblast adhesion obviously will not be possible in humans; however, mouse models for null alleles of the genes encoding these components—such as the laminin β3 chain (Kuster et al. 1997), the α6 (Georges-Labouesse et al. 1996) and β4 (van der Neut et al. 1996) integrins, collagen VII (Männikkö et al. 1999), or collagen XVII—will be useful for such studies.

This report provides a protein-level rationale for digenic interactions of COL17A1 and LAMB3 and extends the spectrum of genetic heterogeneity in JEB. Furthermore, it emphasizes that the identification of one mutation in one gene is not sufficient for determination of the genetic basis of JEB in a given family, a matter that is of utmost importance for the genetic counseling and prenatal diagnosis of affected individuals.

Acknowledgments

The authors thank Ms. M. Schubert and Ms. A. Wissel, for excellent technical assistance. Dr. Tobias Gedde-Dahl, Jr., University of Oslo, Norway, is gratefully acknowledged, for haplotyping the family and for identifying the laminin β3 mutation. This work was supported by Deutsche Forschungsgemeinschaft grants Br 1475/1-2 and SFB 293/B3 and by European Union contract BMH4-CT97-2062.

Electronic-Database Information

Accession numbers and URLs for data in this article are as follows:

GenBank, http://www.ncbi.nlm.nih.gov/Genbank/index.html
Online Mendelian Inheritance in Man (OMIM), http://www.ncbi.nlm.nih.gov/Omim/ (for JEB Herlitz [MIM 226700] and GABEB [MIM 226650])

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