Epidermolysis Bullosa Simplex Associated with Severe Mucous Membrane Involvement and Novel Mutations in the Plectin Gene

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We report a novel case of epidermolysis bullosa simplex with severe mucous membrane involvement and mutations in the plectin gene (PLEC1). The patient suffered from extensive blistering of the skin and oral and laryngeal mucous membranes. Electron microscopy of a lesional skin biopsy showed cleft formation within the basal cell layer of the epidermis. Antigen mapping displayed entirely negative staining for plectin, a large (>500 kDa) multifunctional adhesion protein present in hemidesmosomes of the basal keratinocytes. Mutation analysis revealed compound heterozygous, previously undisclosed nonsense mutations, Q1713X and R2351X, of paternal and maternal origin, respectively, within exon 32 of PLEC1. Based on earlier reports, plectin deficiency is associated with late onset muscular dystrophy in patients with epidermolysis bullosa. No signs of muscle weakness have been observed during the 4 y follow-up of our patient. This case illustrates the fact that molecular pathological analyses have prognostic implications in identification and evaluation of patients who appear to be at risk for development of muscular dystrophy later in life. ***Key words: cytoskeleton/hemidesmosomes/muscular dystrophy/mutation analysis. J Invest Dermatol 114:376–380, 2000***

Hereditary epidermolysis bullosa (EB) comprises a group of clinically related conditions that may be differentiated by phenotypic appearance and genetic mode of inheritance (Fine *et al*, 1999). A common clinical feature of all EB subtypes is skin blistering, but on the basis of ultrastructural criteria EB has been traditionally divided into three major categories, based on the level of blistering within the skin (Uitto and Christiano, 1992; Eady *et al*, 1994). These include EB simplex (EBS), which demonstrates intraepidermal tissue separation; junctional EB with tissue separation occurring within the dermal-epidermal basement membrane; and dystrophic forms of EB with sublamina densa cleavage.

The simplex types of EB (EBS) are specifically characterized by blistering within the basal layer of the epidermis. The genetic defects in the majority of cases with EBS have been disclosed in the keratin 5 and 14 genes, which are expressed exclusively in the basal keratinocytes (Corden and McLean, 1996). These keratin mutations lead to disruption of the intracellular intermediate filament network, which extends from the nucleus to plasma membrane-associated attachment structures, hemidesmosomes and desmosomes. As a result, cytolysis of the basal keratinocytes ensues following minor trauma, and the clinical phenotype consists of blistering and erosions of the skin. In EBS, blistering is generally confined to the skin, and mucous membrane involvement is an uncommon finding, although mucous membrane lesions may be encountered in the relatively severe, Dowling-Meara subtype of EBS (Fine *et al*, 1999).

The majority of EBS cases are inherited in an autosomal dominant pattern with keratin 5 or 14 mutations being detectable in one of the alleles in affected individuals (Corden and McLean, 1996). The overall prognosis of the dominantly inherited EBS is relatively benign, and the lesions, which often become less frequent with advancing age, may heal without scarring and only with occasional hyperpigmentation. A more severe, recessively inherited EBS, however, is due to keratin 14 mutations, which result in null alleles (Hovnanian *et al*, 1993; Chan *et al*, 1994; Jonkman *et al*, 1996).

EB associated with late onset muscular dystrophy (EB-MD), an autosomal recessive disorder, is traditionally categorized as a variant of EBS, although it has also been considered as ''pseudojunctional'' (Niemi *et al*, 1988; Fine *et al*, 1989; Kletter *et al*, 1989), and most recently it has been classified as a hemidesmosomal variant (Pulkkinen and Uitto, 1998). In EB-MD patients, skin blistering is noted at birth or shortly thereafter, whereas the age of onset of muscle involvement is highly variable and has been reported to take place as early as at 2 y of age or not until the fourth decade of life. Recently, EB-MD has been shown to be associated with plectin deficiency, and a number of mutations have been characterized in the corresponding gene, PLEC1 (Uitto *et al*, 1996). Defective

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Abbreviations: EB-MD, epidermolysis bullosa associated with late onset muscular dystrophy; PTT, protein truncation test.

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expression of this protein has also been reported in EBS-Ogna (Koss-Harnes *et al*, 1997), a rare autosomal dominant form of EBS characterized by hemorrhagic blisters (Olaisen and Gedde-Dahl, 1973).

Plectin is a large (>500 kDa) multifunctional cytoskeleton-associated protein expressed in a number of tissues, including the skin and the muscle (Wiche *et al*, 1983, 1993; Nikolic *et al*, 1996; Uitto *et al*, 1996). A plectin-related protein, HD1, is detected by a monoclonal antibody HD121 and is predominantly recognized at the inner plaque of the hemidesmosome as well as in the sarcolemma of the muscle (Wiche *et al*, 1983; Hieda *et al*, 1992).

In this study, we report a case of severe EBS due to plectin deficiency, associated with extensive mucous membrane involvement.

MATERIALS AND METHODS

Light and electron microscopy and immunohistochemistry For routine histopathology, electron microscopy, and antigen mapping, biopsies were taken from gently rubbed skin at the lower limbs in close vicinity to existing blisters. Biopsies were fixed in formalin for routine histopathology, in glutaraldehyde for electron microscopy, or snap frozen in liquid nitrogen for immunohistochemistry. For antigen mapping, monoclonal antibodies recognizing the following antigens were employed: 5B3 to plectin (Foisner *et al*, 1994); to cytokeratins 5 and 14 (Dako, Hamburg, Germany); 1A8C to the intracellular and 1D1 to the extracellular domain of type XVII collagen (Kitajima *et al*, 1992; Nishizawa *et al*, 1993); HD11 to the extracellular domain of type XVII collagen (kind gift from Dr. G. J. Giudice, Medical College of Wisconsin, Milwaukee, WI); GOH3 and 3E1 to α6 and β4 integrin subunits (Gibco Life Technologies, Eggenstein, Germany); to type IV collagen (Dako); to laminin 1 (Dianova, Hamburg, Germany); GB3 to laminin 5 (kind gift from Dr. R.E. Burgeson, CBRC, Harvard Medical School, Cambridge, MA); and LH7.2 to type VII collagen (Bruckner-Tuderman *et al*, 1995).

Mutation analysis The strategy for mutation analysis in the plectin gene (PLEC1) utilized the protein truncation test (PTT) for the last two exons (32 and 33). PTT is designed to detect mutations that result in premature termination codons, whereas heteroduplex scanning can detect different types of sequence variants, including missense mutations, insertions and deletions, as well as normal polymorphisms in the gene.

Genomic DNA was isolated from peripheral blood of the patient, his immediate family members, and unrelated control individuals. Genomic DNA was used as a template for polymerase chain reaction (PCR) amplification of exons 32 and 33. In order to screen exon 32 by PTT we divided it into two overlapping segments, 32-I and 32-II, included in PCR products of 1623 bp and 2097 bp, respectively. For both PCR products, the forward primers (L) were designed to include 5′ sequence of the corresponding region preceded by T7 promoter sequence (underlined) and a eukaryotic translation initiation codon (ATG) (bold) (Dang *et al*, 1998). The reverse primers (R) were designed to include a downstream stop codon (TGA) (shown bold and in reverse). The primers were as follows:

32-I L 5′ GCTAATACGCATCACTATAGGAACAGACCAC-C**ATG**CTGAGTGAACTGTGCCGG 3′
R 5′ **TCA**CTTCTCCTTGAGCGCGATCT 3′
32-II L 5′ GCTAATACGCATCACTATAGGAACAGACCAC-C**ATG**TTCCGCGAGCTGGCCGAGG 3′
R 5′ **TCA**ACCCACCAAAGCAGATCC 3′

The DNA fragments were amplified by PCR using Expand Long Template PCR System polymerase enzyme and the corresponding buffer under standard conditions (Boehringer-Mannheim, Germany). The PCR conditions were: 94°C for 5 min; followed by 94°C, 45 s; 60°C for 32-I or 63°C for 32-II, 30 s; 68°C, 1.5 min (38 cycles).

For PTT, the PCR products were used as templates for coupled transcription/translation in reticulocyte lysate system reaction with [S^{35}] methionine, as instructed by the supplier's protocol (TnT/T7; Promega, Madison, WI). The radiolabeled protein fragments were examined by 10%–15% sodium dodecyl sulfate polyacrylamide gel electrophoresis. The gels were washed, dried under vacuum, and exposed to an X-OMAT AR film (Kodak), overnight at room temperature. When a truncated polypeptide was detected by PTT, the corresponding PCR product was subjected to direct nucleotide sequencing. The mutations were verified by restriction enzyme digestions of the PCR products, obtained with primers flanking the site of mutation, according to the manufacturer's recommendations (New England Biolabs).

RESULTS

Clinical features and diagnostic findings in the family The proband of this study was born at the thirty-eighth week of gestation after an uneventful pregnancy. He was an offspring of nonconsanguineous healthy parents and had two older siblings who were clinically unaffected. He was first noted at the age of 16 d to have blisters on hands, feet, and oral mucosa, accompanied by hoarseness. In addition, extensive nail dystrophy was observed (**Fig 1**). The patient also suffered from an axial herniation of the stomach resulting in a gastro-esophageal reflux that necessitated a corrective operation. Intraoperatively, no pyloric atresia or stenosis was detected. After surgery, the proband's developmental milestones were normal, but towards the end of the first year of life he started to suffer from recurrent infections of the upper respiratory tract requiring repeated intensive care treatments. With time, hoarseness became more prominent, and laryngoscopic examination revealed blisters, erosions, and strictures of laryngeal mucous membranes. Also, strictures and immobility of the arytenoid cartilage required repeated balloon dilatations of the larynx, with subsequent improvement of the respiratory distress. Currently, this 4-y-old patient suffers from recurrent blistering following mild local trauma and blistering of the oral mucosa, especially of the tongue (**Fig 1**). Healing occurred without scarring but occasionally with mild residual atrophy. No muscle weakness or signs of muscular dystrophy have been observed thus far, and no permanent developmental abnormalities have been noted.

Histopathology with routine hematoxylin and eosin staining revealed tissue separation at the dermal-epidermal junction (not shown). Transmission electron microscopy disclosed that the cleavage occurred within basal keratinocytes (**Fig 2**).

No immunoreactivity at the basement membrane zone was detected to plectin in two different biopsies from the patient's skin, whereas staining of a skin specimen from a normal control patient displayed a linear pattern along the basal layer (**Fig 3**). Antibodies to type IV, VII, and XVII collagens, laminins 1 and 5, and α6β4 integrin subunits stained the floor of the patient's blister. Cytokeratins 5 and 14 were detected both at the floor and the roof of the blister. These findings indicate that the cleavage plane is intracellular, consistent with the diagnosis of EBS.

Mutation analysis The mutation analysis was initiated with PTT designed to detect premature termination codon mutations in

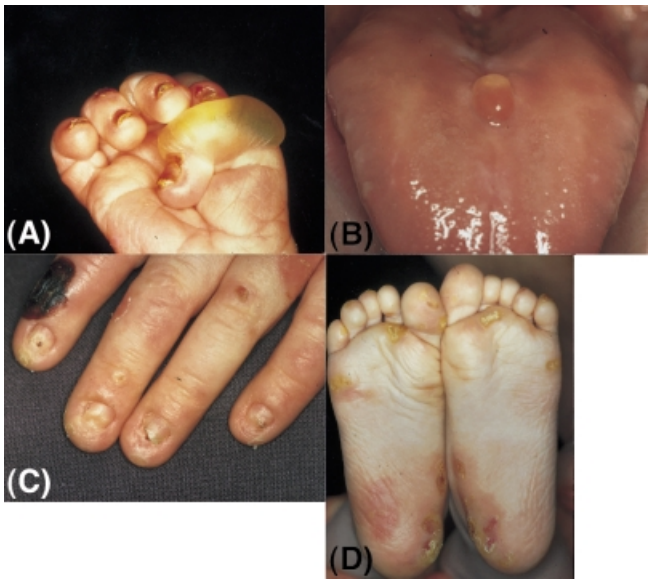

Figure 1. Clinical features of the patient with plectin-deficient EBS. (*A*) Age 16 d, (*B*, *C*, *D*) age 4 y. (*A*) Note a large blister on the right thumb; (*B*) tense blister on the tongue; (*C*) nail dystrophy and residues of blisters on the dorsum of the left hand; (*D*) crusted lesions on the plantar surface of both feet.

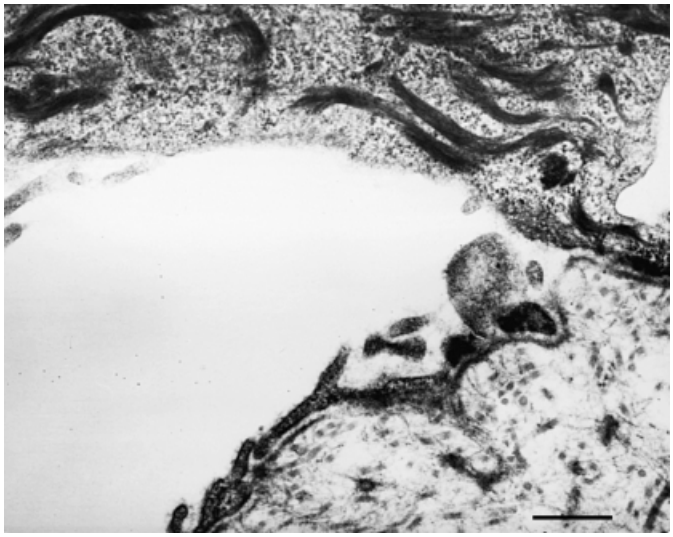

Figure 2. Electron microscopy of the dermo–epidermal junction showing low intraepidermal split formation at the level of basal keratinocytes. Note intact basement membrane at the floor of the blister and attachment of fragments of basal keratinocytes to the basement membrane. *Scale bar* = 150 nm.

exons 32 and 33. For this purpose, genomic DNA was amplified from the patient and a healthy control, as described in *Materials and Methods*, and PCR products were subjected to coupled *in vitro* transcription/translation reaction. Analysis on sodium dodecyl sulfate polyacrylamide gel electrophoresis revealed truncated polypeptides in the patient when the PCR products spanning exon 32 were examined. Specifically, the polypeptides obtained with the PCR product spanning the 5′-half of exon 32 (Ex32-I; 1.6 kb) revealed the presence of a distinct 43 kDa polypeptide in the proband, which was not present in the control (**Fig 4*B***, left lanes). In addition, the proband showed the presence of the expected 68 kDa band, which was also present in the control. Direct nucleotide sequencing revealed a heterozygous C-to-T transition at nucleotide position 5188, which changed a glutamine (CAG) to a stop codon (TAG) at amino acid position 1713 (a mutation designated Q1713X) (**Fig 4*C***). This mutation created a restriction enzyme site for BsaWI, which was used for verification of the mutation in family members (**Fig 4*D***). Specifically, the allele containing the mutation was PCR amplified to yield a 314 bp fragment, which was submitted to digestion with BsaWI. The presence of 211 and 103 bp bands, in addition to a 314 bp band, in the father (II-1) and in the proband (III-3) indicated that they were heterozygous for the mutation Q1713X, whereas the mother (II-2) and the older sister (III-2) did not have this mutation.

The polypeptides corresponding to the 3'-half of exon 32 (Ex32-II; 2.1 kb) revealed the presence of an 81 kDa band in the control as well as in the patient (**Fig 4*B***, right lanes). In addition, the patient showed a prominent polypeptide of 67 kDa, suggesting the presence of a premature termination codon. Direct nucleotide sequencing revealed a heterozygous C-to-T transition at nucleotide position 7102, which changed an arginine (CGA) to a stop codon (TGA) at amino acid position 2351 (a mutation designated R2351X). This mutation was recognized by *Pml*I restriction enzyme, which digested a 429 bp PCR product containing the mutation to 218 and 211 bp fragments. As shown in **Fig 4(*D*)**, the mother (II-2) and the patient (III-3) were heterozygous for the mutation R2351X, whereas the father (II-1) and the sister (III-2) were not carriers of this mutation.

In conclusion, the proband was a compound heterozygote for two novel nonsense mutations, Q1713X and R2351X, in exon 32 of the plectin gene, these mutations being inherited from the father and the mother, respectively. The older sister was both phenotypically and genotypically unaffected.

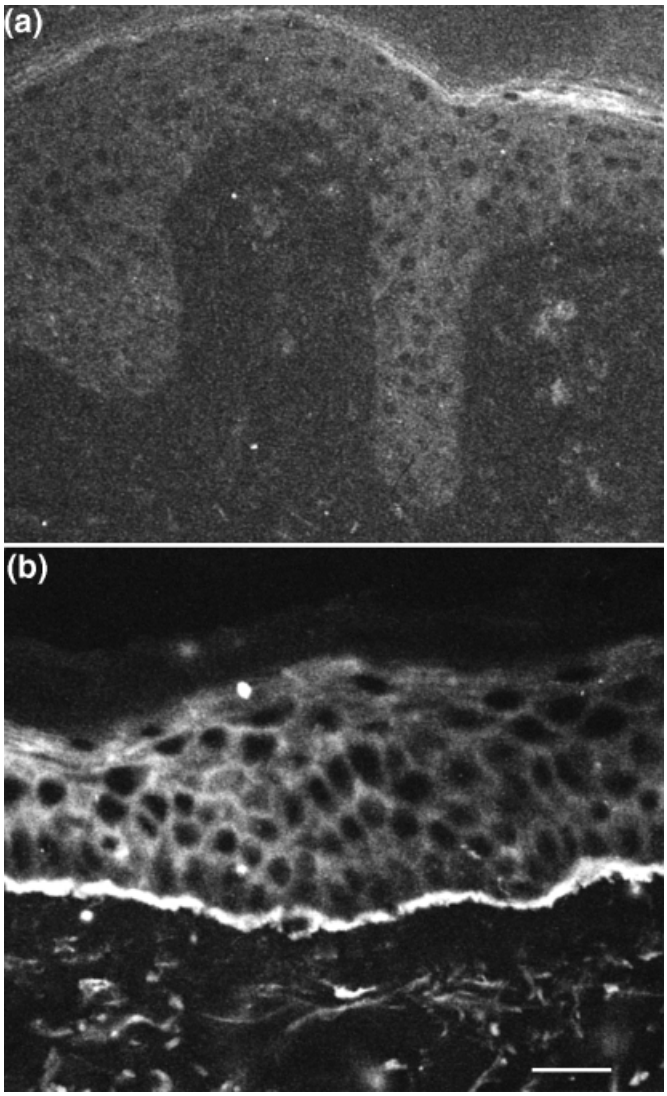

Figure 3. Immunofluorescence staining of skin with monoclonal antibody 5B3 to plectin. (*a*) The skin of the proband shows absent staining at the dermal-epidermal basement membrane zone. The linear staining at the top of the epidermis is nonspecific at the interphase of stratum corneum and stratum granulosum. (*b*) Bright linear staining at the dermal-epidermal junction in a skin biopsy from a healthy control. Note weakly positive intercellular staining at suprabasilar layers; this staining, as well as that at the dermal-epidermal junction, is absent in the patient's skin, as shown in (*a*). *Scale bar* = 10 μm.

DISCUSSION

We report an unusual case of EBS with severe mucous membrane involvement associated with novel nonsense mutations Q1713X and R2351X in the plectin gene. Mucosal involvement is an uncommon finding in simplex types of EB, and is mostly restricted to the EBS of the Dowling-Meara variant. Our patient suffered from severe complications of mucous membrane involvement, however, including strictures of the larynx and subsequent cartilage immobility, which resulted in a respiratory stridor and hoarseness. Balloon dilatations of the larynx were necessary to improve respiratory distress. Recurrent infections of the upper respiratory tract further aggravated respiratory function requiring repeated intensive care treatments. The severity of the cutaneous symptoms was somehow reminiscent of the lethal type of EBS described in a Sudanese family (Salih *et al*, 1985). In contrast to our patient, who displayed severe mucous membrane involvement and nail dystrophy immediately after birth, mucous membrane involvement was

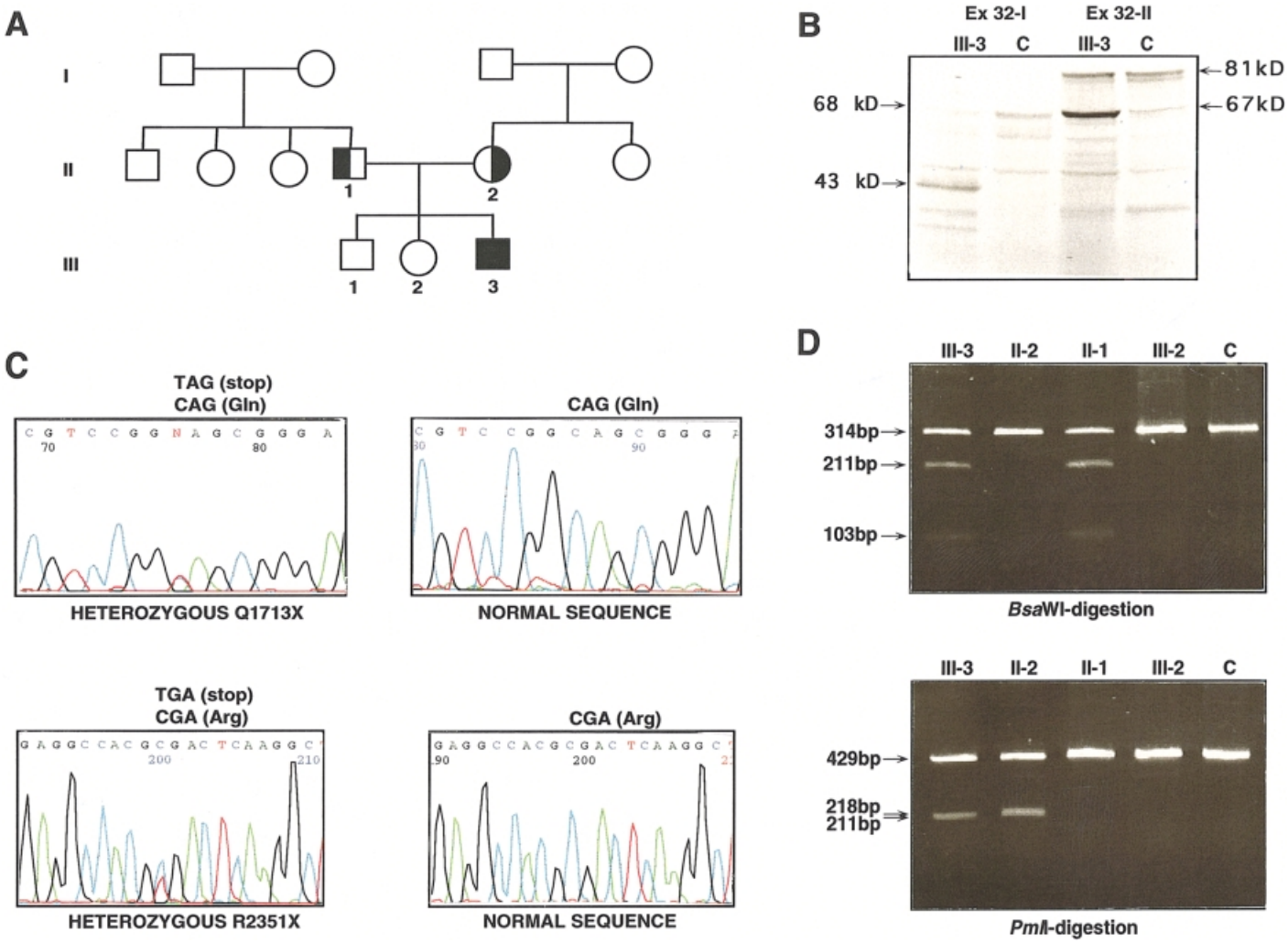

Figure 4. Identification and verification of mutations in the plectin gene. (*A*) Pedigree of the proband with plectin deficiency. (*B*) PTT analysis of exon 32 using the proband's (III-3) and an unrelated control (C) genomic DNA as templates. Autoradiogram of 10% polyacrylamide gel electrophoresis of the polypeptide products of the coupled transcription/translation reaction performed in the presence of [S^{35}] methionine. (*C*) Identification of the mutations Q1713X and R2351X. Direct nucleotide sequencing of the patient's 32-I DNA fragment (left upper panel) revealed a heterozygous C→T transition at nucleotide position 5188, compared with the normal sequence (right upper panel). This change resulted in substitution of a codon for glutamine (CAG) by a stop codon (TAG) at amino acid position 1713; the mutation is designated Q1713X. Direct nucleotide sequencing of the patient's 32-II DNA fragment (left lower panel) revealed a heterozygous C→T transition at nucleotide position 7102, compared with the normal sequence shown on the right. This change resulted in substitution of a codon for arginine (CGA) by a stop codon (TGA) at amino acid position 2351; the mutation is designated R2351X. (*D*) Verification of the mutation by restriction enzyme digestions. The mutation Q1713X creates a recognition site for *BsaWI* restriction endonuclease. In the normal allele the size of the PCR product is 314 bp whereas in the mutant allele the enzyme digests the 314 bp PCR product into 211 bp and 103 bp fragments (upper panel). The results indicate that the patient and the father are heterozygous for Q1713X mutation. The mutation R2351X creates a restriction site for *PmlI* endonuclease, which digests the normal allele 429 bp PCR product into 218 bp and 211 bp fragments in the mutant allele (lower panel). The patient and his mother were heterozygous for the mutation R2351X.

slight in the Sudanese family and the nails were not affected. Thus, these patients are likely to suffer from a disease different from the patient described in our study. Furthermore, the molecular basis of the blistering disease in the Sudanese family is unknown.

The initial findings of a gastro-esophageal reflux and severe mucous membrane involvement in the proband of our family were clinically suggestive of junctional EB with developmental gastrointestinal anomalies, as noted in a variant of EB with pyloric atresia. Further investigations revealed, however, that axial herniation of the stomach was the cause of the complaints. The underlying genetic defects of junctional EB with pyloric atresia have been shown to be mutations in the genes encoding the α6 and β4 integrin subunits (Pulkkinen and Uitto, 1998, 1999). Immunohistochemistry of skin biopsies in this case, however, demonstrated normal expression of α6 and β4 integrins, as well as of other basement membrane and cytoskeletal proteins. Interestingly, total absence of immunoreactivity to plectin was observed in the basement membrane zone of the skin of the proband, and subsequently the plectin gene was screened for mutations. These studies revealed that the patient harbored two novel nonsense mutations in exon 32 encoding the rod domain of plectin.

Prior to this study, mutations in the plectin gene have been shown to be associated with EB-MD in 10 families, most of the probands being homozygous for stop codon mutations (Pulkkinen and Uitto, 1998, 1999). This rare variant of EB generally leads to profound muscle weakness, but the onset of the muscle involvement is delayed, occurring in most patients in adulthood, beyond the age of 20 y, and in one family even during the fourth decade of life (Uitto *et al*, 1996). The demonstration of absent expression of plectin by immunofluorescence and the identification of plectin mutations in both alleles may therefore have prognostic value in predicting the future development of muscular dystrophy in patients with EBS, and forms a basis for appropriate genetic

counseling. Mucous airway disease, especially laryngeal involvement, has been previously described in four other cases of EB-MD (Smith *et al*, 1996; Mellerio *et al*, 1997; Dang *et al*, 1998). Two of these cases developed muscular dystrophy, one during childhood, the other beyond the age of 20 y (Smith *et al*, 1996; Dang *et al*, 1998). The two other cases are still below 10 y of age and predictions that they are at risk to develop muscular dystrophy in the future were made, similar to our study (Mellerio *et al*, 1997). Interestingly, muscle biopsy in one of the reported cases by Mellerio *et al* (1997) showed no evidence of muscle pathology, even though the immunofluorescence staining for plectin was reduced. This finding clearly agrees with the late onset of muscle weakness in these patients. Laryngeal involvement in these cases varied from isolated blisters to severe cases with stridor and hoarseness (Mellerio *et al*, 1997). These observations suggest that EB-MD should be included in differential diagnosis of inherited cutaneous blistering associated with mucous membrane involvement.

Mutation analysis in the proband of this study revealed compound heterozygosity for novel nonsense mutations in the plectin gene leading to a premature termination of translation in the central rod domain of the protein. This is predicted to result in synthesis of plectin polypeptides lacking part of the rod domain and the entire carboxyterminal globular region of the molecule. These deleted domains are important for intracellular dimerization of plectin polypeptides (Foisner *et al*, 1991) and for association of plectin with intermediate filaments within the epidermal keratinocytes, respectively (Wiche *et al*, 1993; Nikolic *et al*, 1996; Andra *et al*, 1997). Furthermore, these kinds of stop codon mutations frequently result in downregulation of the corresponding transcript due to nonsense-mediated mRNA decay (Cui *et al*, 1995), and the shortened polypeptides are synthesized at reduced levels or not at all. Through these combined mechanisms, the mutations characterized in this study destroy the functions of plectin, resulting in fragility of the basal keratinocytes and causing blister formation in the skin and mucous membrane as a result of minor trauma. As plectin is also expressed in the skeletal muscle, these skin findings are potentially associated with muscular dystrophy later in life.

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