ARTICLE

Homozygous deletion mutations in the plectin gene (PLEC1) in patients with epidermolysis bullosa simplex associated with late-onset muscular dystrophy

Leena Pulkkinen+, Frances J. D. Smith1, Hiroshi Shimizu2, Satoru Murata3, Hideo Yaoita3, Hiroshi Hachisuka4, Takeji Nishikawa2, W. H. Irwin McLean1 and Jouni Uitto*

Departments of Dermatology and Cutaneous Biology, and Biochemistry and Molecular Pharmacology, Jefferson Medical College, 233 South 10th Street, Suite 450, Philadelphia, PA 19107-5541, USA, 1CRC Cell Structure Research Group, University of Dundee, Dundee, UK, 2Department of Dermatology, Keio University School of Medicine, Tokyo, Japan, 3Department of Dermatology, Jichi Medical School, Minamikawach-Machi, Japan and 4Department of Dermatology, Kurume University School of Medicine, Kurume, Japan

Received May 23, 1996; Revised and Accepted July 8, 1996 GenBank accession nos U53204 and U53834

In a distinct autosomal recessive variant of epidermolysis bullosa, EB-MD, life-long skin blistering is associated with late-onset muscular dystrophy of unknown etiology. Electron microscopy of these patients’ skin suggests that tissue separation occurs intracellularly at the level of the hemidesmosomal inner plaque, which contains plectin, a high molecular weight cytoskeletal associated protein, also expressed in the sarcolemma of the muscle. In this study, we report two patients with EB-MD, each with a homozygous deletion mutation in the plectin gene, PLEC1. In the first case, the proband and her similarly affected sister had a homozygous 9 bp deletion mutation, designated as 2719del9, which resulted in elimination of three amino acids, QEA, in a sequence of 23 amino acids entirely conserved between the mouse and human sequences. The proband in the second family demonstrated a single nucleotide deletion at position 5866, designated as 5866delC, which resulted in frameshift and a premature termination codon for translation 16 bp downstream from the site of deletion. The absence of plectin in the hemidesmosomes, as reflected by negative immunofluorescence with an anti-plectin antibody (HD-1), associated with fragility of basal keratinocytes, implicates plectin as critical for binding of intermediate keratin filament network to hemidesmosomal complexes. The function of plectin as a putative attachment protein also in the muscle would explain the clinical phenotype consisting of cutaneous fragility and muscular dystrophy in EB-MD.

INTRODUCTION

Epidermolysis bullosa (EB) is a group of heritable blistering disorders characterized by fragility of the skin within the dermal–epidermal basement membrane zone (BMZ) (1,2). EB has been divided into three broad categories on the basis of the level of tissue separation within the dermal–epidermal junction, and underlying molecular defects in each of the three categories have been recently disclosed. Specifically, the dominantly inherited simplex forms of EB (EBS) result from mutations in the basal keratinocyte-specific genes, KRT5 and KRT14 (3–5). In the classic junctional forms of EB (lethal, Herlitz type), tissue separation occurs within the lamina lucida of the BMZ, and mutations in the genes (LAMA3, LAMB3 and LAMC2) encoding the subunit polypeptides of the anchoring filament protein laminin 5 have been disclosed in a number of patients (4–9). The dystrophic, severely scarring forms of EB are due to mutations in the gene (COL7A1) encoding the anchoring fibril protein, type VII collagen (10,11).

In all three forms of EB, the blistering tendency, which affects not only the skin but also the epithelia of various mucous membranes and the cornea of the eye, is usually present at birth and is associated with a number of extracutaneous manifestations. In a rare variant of EB (EB-MD), inherited in an autosomal recessive pattern, life-long blistering, noted usually at birth, is associated with late-onset muscular dystrophy of unknown etiology (12–15). Initially, ultrastructural evaluation of the skin lesions in these patients yielded conflicting results, and both simplex and junctional classifications were suggested, and the designation ‘pseudo-junctional’ was proposed (12–16). However, careful examination of ultrastructural features in these...
Figure 1. Pedigree and clinical features of the proband with epidermolysis bullosa and muscular dystrophy in Family A. The proband (arrow), a 46-year-old female, has an older sister with similar clinical manifestations. The clinically unaffected parents were first cousins. The proband also had an older brother and two younger sisters who are clinically unaffected (upper panel). The proband manifested with fragility of skin leading to blisters and erosions, and nail dystrophy, as well as with severe muscle weakness (lower panel).

patients, as recently reported (17), suggests that the tissue separation occurs within the basal keratinocytes at the level of the hemidesmosomal inner plaque, which is known to consist of two distinct proteins: (i) the 230 kDa bullous pemphigoid antigen, a protein serving as an autoantigen in an acquired autoimmune disease, bullous pemphigoid (18,19); and (ii) plectin, a high molecular weight cytomatrix protein, also expressed in the sarcolemma of the muscle, as detected by a monoclonal antibody HD-1 (17,20–23). In this study we report on two apparently unrelated Japanese families with EB-MD, each with a homozygous deletion mutation in the gene, PLEC1, encoding the plectin polypeptide.

RESULTS

Immunofluorescence, ultrastructural findings and demonstration of a 9 bp deletion mutation in Family A

In both families, the probands, offspring of consanguineous unions, had a history of blistering first noted during the neonatal period, with development of muscular dystrophy in their early 30s. In the case of one of the families, Family A, the proband was the third child of parents who were first cousins (Fig. 1). The patient also had an older sister who was similarly affected, while three other siblings, as well as the parents, were clinically normal. Immunofluorescence staining of the skin with a monoclonal anti-plectin antibody (HD-1), which recognizes a hemidesmosomal plectin epitope in the normal skin, revealed that plectin expression was markedly attenuated in the proband’s skin (Fig. 2A and B). Staining of her skin with a monoclonal antibody HD-2 recognizing the 230 kDa bullous pemphigoid antigen was also reduced (Fig. 2C and D), while staining for other BMZ components, i.e. for β4 integrin subunit, laminin 5, the 180 kDa bullous pemphigoid antigen, and type VII collagen, was essentially normal (Fig. 2E–H). Histopathology indicated dermal–epidermal blister formation, and transmission electron microscopy revealed tissue separation low in the basal cell compartment at the level of the hemidesmosomal inner plaque, the hemidesmosomal attachment plaque being on the floor of the blister (Fig. 3).
Figure 2. Immunofluorescence staining of the skin of the proband in Family A. Staining with a monoclonal anti-plectin antibody (HD-1), which recognizes a hemidesmosomal plectin epitope on the basement membrane zone in normal skin (A), was essentially negative in the proband (B). Staining with a polyclonal antibody recognizing the 230 kDa bullous pemphigoid antigen, which yields a linear staining pattern at the BMZ of normal skin (C), was markedly reduced and patchy in the proband’s skin (D). Staining of the skin with antibodies recognizing other BMZ components, including β4 integrin (E), the 180 kDa bullous pemphigoid antigen (F), laminin 5 (G) and type VII collagen (H), was normal. For description of the antibodies, see Materials and Methods.
Figure 3. Histopathology and transmission electron microscopy of the skin from the proband in Family A. Histopathology demonstrated dermal–epidermal tissue separation (A) [hematoxylin–eosin stain]. Transmission electron microscopy of the skin at low magnification revealed BMZ separation (B), which at higher magnification was noted to be above the lamina lucida (C). Specifically, the lamina densa was intact (arrow), and in some areas electron dense, apparently intracellular material was found in the floor of the blister attached to the underlying basement membrane (arrowhead), indicating that tissue separation occurred intracellularly within the basal keratinocytes.

We have recently cloned cDNAs which correspond to the full-length human plectin sequences (GenBank accession no. U53204) (24). We have also elucidated the intron–exon organization of the corresponding gene, PLEC1 (GenBank accession no. U53834), which has been mapped to human chromosomal location 8q24 (24,25). Based on this information, we have developed a mutation detection strategy which employs PCR amplification of exonic sequences directly from genomic DNA, and the PCR products are screened for sequence variations by heteroduplex analysis, the conformation-sensitive gel electrophoresis (CSGE), followed by automated nucleotide sequencing (26,27). Examination of the proband’s and her similarly affected sister’s DNA in Family A revealed a single band which had a mobility faster than the normal homoduplex present in control individuals, when the PCR product corresponding to nucleotides 2590–2744 (GenBank accession no. U53204) of exon 22 and flanking intronic sequences was analyzed. Parallel examination of the mother’s DNA revealed the presence of a normal homoduplex band, as well as the faster moving band (Fig. 4A). Direct nucleotide sequencing of the proband’s PCR product demonstrated a homozygous 9 bp deletion mutation, designated as a 2719del9, while the mother was heterozygous for the mutant and normal sequence (Fig. 4B). This genetic lesion resulted in loss of a BglI site, which confirmed that the mother was indeed a heterozygous carrier, while both affected individuals were homozygous for this mutation and both unaffected individuals were homozygous for the normal allele (Fig. 4C). Thus, the two affected individuals in this family were homozygous for a 9 bp deletion which resulted in elimination of three amino acids, QEA. This deletion, which occurred in a sequence where the 9 bp segment, CAGGAGGCC, was tandemly repeated, resulted potentially from slipped mis-pairing of DNA (28).

Three lines of evidence suggested that this mutation was pathogenetic. First, examination of 135 unrelated healthy control individuals, 18 of them being ethnically matched, did not reveal the presence of this genetic lesion, suggesting that 2719del9 is not a polymorphism. Secondly, scanning of the rest of the plectin gene by CSGE did not reveal any other, potentially pathogenic sequence variants in the proband. In this context, it should be noted that during cloning of the plectin gene we have encountered seven exonic and two intronic single-base polymorphisms (Table 1). However, none of them were present in this proband. Finally, comparison of the amino acid sequence encoded by exon 22 in the human and recently cloned mouse gene revealed 96.2% similarity and 90.4% identity, and the deleted amino acids QEA were within a stretch of 23 identical amino acids (unpublished).

Table 1. Polymorphisms in the plectin gene (PLEC1)

<table>
<thead>
<tr>
<th>Nucleotide*</th>
<th>Polymorphism b</th>
<th>Detection c</th>
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<tr>
<td>4046</td>
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<tr>
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<tr>
<td>21278</td>
<td>T(2)T(F)</td>
<td>T(2)T(F)</td>
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*The numbers refer to genomic sequence (GenBank accession no. U53834).

bThe intronic polymorphisms are indicated by a single nucleotide; the exonic polymorphisms are indicated by underlying the nucleotide within a codon, followed by the corresponding amino acid in parenthesis.

cThe sequence variants change a restriction site for endonucleases indicated.
Figure 4. Mutation detection in Family A. Heteroduplex analysis by conformation sensitive gel electrophoresis (CSGE) revealed a single band with DNA from the two affected individuals (III-1 and III-3), which moved faster than the normal homoduplex present in unaffected individuals (III-4 and III-5) or in unrelated controls (not shown). The mother (II-1) was noted to have both a normal homoduplex band and a faster moving band similar to that noted in the affected individuals (A, arrows). Direct sequencing of the proband’s DNA revealed a homozygous 9 bp deletion mutation, designated as 2719del9 (B, upper panel). The mother’s DNA contained both the mutant allele and normal allele (B, center panel), by comparison with the normal sequence (B, lower panel). The 2719del9 mutation resulted in loss of a restriction enzyme site for BglI. Amplification of the normal and mutant alleles resulted in 330 and 321 bp PCR products, respectively, and the normal allele was digested to 240 and 90 bp fragments while the mutated allele remained undigested (C). The two affected individuals were shown to be homozygous for the mutation, while the mother was heterozygous.

Demonstration of a single base pair deletion in Family B

The proband in the second family, Family B, was the first child of clinically unaffected parents who were deceased (Fig. 5). Immunofluorescence with the HD-1 antibody demonstrated absence of staining in her skin, while staining for the 230 kDa bullous pemphigoid antigen was positive but attenuated, and staining for type VII collagen was entirely normal (Fig. 6). Electron microscopy, similar to the proband in Family A, demonstrated intraepidermal tissue separation at the level of hemidesmosomal inner plaque (Fig. 6D). Initial scanning of PLEC1 exonic sequences (nucleotides 5786–6191) within the 3.4 kb exon 32 revealed heteroduplex bands in several control individuals (Fig. 7A, lanes 1, 4 and 5) while the proband’s DNA revealed a homoduplex band only (lane 2). However, mixing of proband’s PCR product with that of the control DNA shown on lane 1 resulted in two additional heteroduplex bands (Fig. 7A, lane 3). Direct sequencing of the proband’s PCR product revealed a homozygous single nucleotide deletion, C at position 5866 (GenBank accession no. U53204) (Fig. 7B, upper panel). This mutation, designated as 5866delC, resulted in a frameshift and a premature termination codon which predicts the synthesis of a truncated plectin polypeptide as well as reduced levels of the corresponding mRNA due to nonsense mediated decay of the transcript containing the mutation (29,30). In addition, the proband was homozygous for a silent polymorphism, T at nucleotide position 5869, by comparison with the control shown in lane 1 who was homozygous for a G in the same position (Fig. 7B, lower panel). The heteroduplex band in the controls shown on lanes 1 and 4 reflects a neutral polymorphism, A/G at nucleotide position 6060, while the heteroduplex band in the control individual shown in lane 5 results from another neutral polymorphism, G/A at position 5997 (not shown). The presence of heteroduplexes when the 406 bp PCR products were examined illustrates the sensitivity of the mutation detection strategy for 1 bp mismatches, and also reflects the polymorphic nature of the PLEC1 gene.

DISCUSSION

In this study we report two families with a proband demonstrating life-long blistering of the skin, first noted during the early neonatal period, and ultrastructural evidence revealing intraepidermal blister formation, thus confirming that these patients represent a form of EBS. Since the mother of the proband in Family A was clinically unaffected carrier of the mutation found in homozygous state in the proband, this disease is apparently inherited in a recessive manner. However, the fragility of the epidermal basal keratinocytes is distinctly different from that noted in classic, dominantly inherited EBS due to mutations in KRT5 or KRT14 genes (3,4). Specifically, the fragility in EB-MD is limited to the lower portion of the basal cells, tissue separation being at the level of the hemidesmosomal inner plaque. Previously,
the role of hemidesmosomal inner plaque in maintaining intermediate filament–plasma membrane association has been highlighted by development of ‘knockout’ mice with the ablation of the gene BPAG1 which encodes the 230 kDa bullous pemphigoid antigen (31). In this context, it was of interest that markedly reduced or absent expression of plectin within the skin was associated with reduced staining for the 230 kDa bullous pemphigoid antigen, suggesting that the pathology of the skin may be, at least in part, a reflection of the reduction of this protein, or that plectin and the 230 kDa bullous pemphigoid antigen play similar but non-redundant roles. It was also of interest to note that in the patient with an in-frame 9 bp deletion, staining with HD-1 antibody was slightly positive, while in the patient with the 1 bp deletion resulting in frameshift and premature stop codon, the staining was entirely absent, potentially as a result of nonsense mediated mRNA decay (29,30). Since the clinical picture of the affected individuals in these two families was similar, it appears that the deleted 3 amino acid sequence, QEA, plays a critical role in the function of plectin.

EBS was first observed in these patients at birth with continued blistering tendency throughout the life, while a progressive muscular weakness was noted beginning in their 30s. This clinical association has been previously noted in several patients, indicating that this phenotype, inherited in an autosomal recessive manner, is likely to reflect a common pathogenetic mutation, rather than being a coincidental occurrence of these two relatively rare clinical conditions (12–16). In addition to the hemidesmosomes in the skin, plectin is expressed in sarcolemma of the muscle, and recent immunofluorescence staining of both skin and muscle in patients with EB-MD has revealed absent expression of the HD-1 epitope (17). Thus, plectin could function as a putative cytoskeletal–membrane attachment protein also in the muscle, mediating binding of the muscle proteins, such as actin, to membrane complexes possibly through attachment junctions different from those found in the skin (17,32). Collectively, our results demonstrating skin fragility and muscular dystrophy in patients with abnormal plectin expression provides novel insights into the function of this large multifunctional cytoskeleton associated adhesion protein.

MATERIALS AND METHODS

Detection and verification of mutations in the PLEC1 gene

Total genomic DNA (200 ng) was used as a template for PCR amplification of exonic sequences of the PLEC1 gene. The primers used for PCR amplification were placed on the adjacent introns, or in the case of two last exons, the primers were chosen to produce overlapping PCR products. The PCR products were then subjected to heteroduplex analysis by conformation sensitive gel electrophoresis (CSGE) (26), followed by direct automated sequencing of the PCR products containing heteroduplexes. Specifically, for identification of the mutations 2179del9 and 5866delC, the following PCR primer pairs were used. Amplification of exon 22 and flanking intronic sequences: L-primer 5′-TAAGCAGGTGGAGGTGAGTG-3′, R-primer 5′-CAGGT-CAGGGCTACAGTCAG-3′. The amplification conditions were: denaturation for 5 min at 95°C, followed by 35 cycles of 45 s at 95°C, 45 s at 60°C and 45 s at 68°C. The enzyme and buffer used for this PCR reaction were in a kit Expand High Fidelity PCR system (Boehringer Mannheim). The 2719del9 mutation resulted in the loss of the recognition site for restriction endonuclease BglI, which was used to verify the presence of this mutation in genomic PCR products encompassing the exon 22. The enzyme digestion
Figure 6. Immunofluorescence staining and transmission electron microscopy of the skin from the proband in Family B. Staining with the monoclonal antibody HD-1 yielded entirely negative staining pattern (A). Staining with an antibody recognizing the 230 kDa bullous pemphigoid antigen was positive, yet markedly attenuated (B), while staining for type VII collagen was entirely normal (C). Transmission electron microscopy revealed normal lamina densa and lamina lucida with rudimentary hemidesmosomes (arrow). Tissue separation was at the level of the inner hemidesmosomal plaque (arrowhead).

Figure 7. Mutation detection in the proband in Family B. Initial scanning of control individuals’ DNA corresponding to nucleotides 5786–6191 (lanes 1, 4 and 5) by CSGE revealed the presence of heteroduplex bands (He), while the patient’s PCR product yielded a homoduplex band only (lane 2). However, mixing the patient’s PCR product with that from the control individual shown on lane 1 resulted in two additional heteroduplex bands (lane 3) (A). Direct sequencing of the PCR product indicated that the patient was homozygous for a single nucleotide deletion, 5866delC (B, arrow in upper panel). In addition, the patient was shown to be homozygous for a T at position 5869 (*), while the control individual shown on lane 1 was homozygous for a G (B, lower panel). The heteroduplex band noted on lane 1 reflects the presence of a neutral polymorphism, A/G at position 6060, downstream from the sequence shown in (B). The heteroduplex noted on lane 5 in a control individual reflects the presence of another neutral polymorphism, G/A at position 5997.

was performed under the conditions recommended by the manufacturer (New England Biolabs).

For amplification of the DNA segment within exon 32, corresponding to nucleotides 5786–6191 (GenBank accession no. U53204), the following primers were used: L-primer 5'-CGCAACA-CAAGCTGACATC-3', R-primer - 5'-TCCTCCACGTGGCT- TTCAG-3'. Amplification was performed using AmpliTaq enzyme and buffer (Perkin-Elmer Cetus) under the following conditions: denaturation for 5 min at 95°C, followed by 35 cycles of 45 s at 95°C, 45 s at 60°C and 45 s at 72°C. The mutation was verified using direct sequencing of the PCR product.

Immunofluorescence

The following antibodies were used for immunofluorescence of the skin. For plectin, a monoclonal antibody 121 (HD-1) (21); for the 230 kDa bullous pemphigoid antigen, a polyclonal antibody S193 (33); for the 180 kDa bullous pemphigoid antigen, a monoclonal antibody D20 (34); for β4 integrin, a monoclonal antibody 3E1 (Chemicon International, Temecula, CA); for laminin 5, a monoclonal antibody GB3 (Sera-lab, Cambridge, UK) (35); for type VII collagen, a monoclonal antibody LH7.2 (Sigma, St Louis, USA) (36).
ACKNOWLEDGEMENTS

We thank Yili Xu for excellent technical assistance; Hans-Jürg Alder for providing nucleotide sequencing and oligonucleotide synthesis services; K. Owaribe and J. Stanley for providing antibodies; J.-P. Ortonne and G. Meneguzzi for sharing their unpublished data; E. B. Lane for her interest and support. This study was supported by the USPHS, NIH grant PO1AR38923 (J.U.), the Dermatology Foundation (L.P.), the Dystrophic Epidermolysis Bullosa Research Association of UK (F.J.D.S.) and the Wellcome Trust (W.H.I.M.).

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