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Research Article

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A Homozygous Nonsense Mutation in the PLEC1 Gene in Patients with Epidermolysis Bullosa Simplex with Muscular Dystrophy

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Abstract

Plectin is a widely expressed cytomatrix component involved in the attachment of the cytoskeleton to the plasma membrane. We have recently reported that the skin and muscles of three patients affected by epidermolysis bullosa simplex with muscular dystrophy (MD-EBS), a genetic disorder characterized by skin blistering associated with muscle involvement, are not reactive with antibodies specific to plectin. We demonstrated that in the skin, lack of plectin leads to failure of keratin filaments to connect to the plasma membrane via the hemidesmosomes, whereas in the muscle the deficient expression of the molecule correlates with an aberrant localization of desmin in the muscle fibers. In this study we demonstrate that in a MD-EBS kindred with two affected members, the disease results from a homozygous nonsense mutation in the plectin (PLEC1) gene leading to a premature stop codon (CGA to TGA) and decay of the aberrant plectin messenger RNA. The segregation of the mutated allele implicates the mutation in the pathology of the disorder. These results confirm the critical role of plectin in providing cell resistance to mechanical stresses both in the skin and the muscle. (*J. Clin. Invest.* 1996. 98:2196–2200.)
Key words: plectin • cytoskeleton • epidermolysis bullosa • muscular dystrophy • hemidesmosome

Introduction

Epidermolysis bullosa simplex (EBS)[1] comprises a group of inherited disorders of the epidermis characterized by a marked fragility of basal keratinocytes resulting in the development of blisters of the skin. In the majority of cases of EBS, the skin fragility is caused by mutations in keratins 5 and 14 that compromise the function of the intermediate filament cytoskeleton in resisting shear stresses (1–3). We have recently studied a recessively inherited form of epidermolysis bullosa simplex associated with late-onset muscular dystrophy (MD-EBS) (4). This disease is characterized by blistering of the skin as the primary clinical manifestation at birth. Nail dystrophy and muscle weakness progressively leading to disabling muscular atrophy and ptosis are other features observed in MD-EBS (5–7). In the patients affected by MD-EBS, skin blisters arise by a low level severing of the basal keratinocytes. The separation between dermis and epidermis occurs just above the hemidesmosomes that are dissociated from the keratin filaments and remain attached to the basal lamina of the dermal-epidermal junction at the floor of the blister. This observation suggests a defective anchorage of the cytoskeletal structures to the inner plaque of the hemidesmosome. In accordance with this possibility, immunohistochemical analysis of the skin and muscle of our MD-EBS patients demonstrated negative staining with monoclonal antibodies directed against plectin and the hemidesmosomal protein HD1, whereas all the other proteins of the dermal-epidermal junction tested were reactive (4).

Plectin is a widely expressed cytomatrix component involved in the attachment of the cytoskeleton to the plasma cell membrane (8). This high molecular weight protein interacts with a variety of intermediate filament proteins, fodrin, α-spectrin, microtubule-associated proteins, and nuclear lamins (9). In the skeletal muscle, plectin is found in the sarcolemma and in the Z-line structures, and colocalizes with the intermediate filament protein desmin (10). In the skin it localizes both in the desmosomes and the hemidesmosomes where it associates with cytoplasmic filaments (10, 11). At the dermal–epidermal junction, plectin, as detected by a hemidesmosome-specific antibody directed against the protein HD1, has been shown to localize to the periphery of the intracellular portion of the hemidesmosomal plaque, close to the region where the 230-kD bullous pemphigoid antigen (BPAG1) has been localized (12), but also in other tissues not possessing hemidesmosomes, such as the Schwann cells. Several lines of immunohistological evidence suggest that plectin and the hemidesmosomal component HD1 may be the same protein. The defective expression of plectin and HD1 we observed in the skin and muscles of our MD-EBS patients therefore substantiates this hypothesis (4).

The altered expression of this versatile cross-linking element of cytoskeleton correlated well with the altered labeling pattern of keratin intermediate filaments and desmin network

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1. *Abbreviations used in this paper:* ASO, allele-specific oligomer; CSGE, conformation sensitive gel electrophoresis; EBS, epidermolysis bullosa simplex; MD, muscular dystrophy.

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noticed in the MD-EBS keratinocytes and muscle fibers, respectively (4). The gene (PLEC1) encoding plectin was therefore a strong candidate gene for the site of mutations in our MD-EBS patients.

Since the human plectin cDNA has recently been isolated and sequenced, and the organization of the corresponding gene has been defined (13, 14), we investigated one of our MD-EBS families for genetic defects affecting the expression of this cytoskeletal protein.

Methods

Samples. Skin biopsies for cell culture were obtained from nonblistered skin of the MD-EBS patient and from healthy unrelated control subjects. The clinical, ultrastructural, and immunofluorescence findings in this patient (family C in reference 4) have been described previously (4). Keratinocytes were cultured on feeder layers of irradiated mouse 3T3 cells (15). DNA was extracted from peripheral blood or from secondary cultures of dermal fibroblasts (16).

Northern analysis. 20 μg of total RNA purified from cultured MD-EBS and normal keratinocytes (17), was fractionated by electrophoresis on a 0.8% agarose gel, blotted to a nylon membrane (Amersham International, Little Chalfont, UK) and hybridized with ^{32}P-random-labeled plectin and GAPDH probes (18). The plectin DNA probe (1.3 kb) was obtained by PCR amplification of genomic DNA sequences within exon 33 of the human *PLEC1* gene (for the *PLEC1* cDNA sequences, see GenBank accession no U53204) using control genomic DNA as a template and primers (L) 5′-AGGCGGAGCGGGTGCTTG-3′ and (R) 5′-GCCTTCAGCGCCAGGATCTC-3′. The PCR conditions were 95°C, 5 min; 94°C, 20 s; 58°C, 30 s; 72°C, 1 min 30 s (30 cycles), using 200 nM of each primer in a reaction mixture containing 200 μM dNTP, 1.5 mM $MgCl_2$, 4% (vol/vol) DMSO and 1.25 U of Taq polymerase (Perkin Elmer Cetus, San Francisco, CA). Quantitation of autoradiograms were performed by densitometric scanning with a Biocom Image Analysis computer model.

Mutation detection in the PLEC1 gene. Total genomic DNA was used as a template for PCR amplification of individual exons and the corresponding intron-exon boundaries in the *PLEC1* gene (GenBank accession no. U53834). PCR reactions (50 μl) were performed using 100 ng of genomic DNA and pairs of oligonucleotide primers synthesized on the basis of intronic sequences of the gene, and in the case of the two large exons 32 (3.3 kb) and 33 (7.2 kb), overlapping amplimers (300–500 bp) were synthesized on the basis of the exonic sequences. To analyze the mutation Q1910X, the oligonucleotide primers were: (L) 5′-AGGCGGAGCGGGTGCTTC-3′, (R) 5′-GCCTTCAGCGCCAGGATCTC-3′. The PCR conditions were: 95°C, 5 min; 94 s, 20 s; 58°C, 30 s; 72°C, 30 s (30 cycles), using 200 nM of each primer in a reaction mixture containing 200 μM dNTP, 1.5 mM MgCl2, 4% (vol/vol) DMSO and 1.25 U of Taq polymerase (Perkin Elmer Cetus). Aliquots of 5 μl were then checked on 2% agarose gels. For heteroduplex analysis, 20 ng of each PCR amplification product were diluted in a total volume of 8 μl, denatured for 5 min at 98°C and incubated 30 min at 68°C. The samples were analyzed on an 8% acrylamide-1,4 bis (acryl)piperazine (99:1) gel containing 15% (vol/vol) formamide, 10% (vol/vol) ethylene glycol in 0.5×GT buffer (45 mM Tris base, 15 mM Taurin, 0.25 mM EDTA) (United States Biochemicals, Cleveland, OH) and run at 700 V for 10 h in 0.5× GT buffer (19). After staining with ethidium bromide, the PCR products displaying bands of altered mobility were purified on Quiaquick columns (Quiagen Inc., Chatsworth, CA) and directly sequenced by the PRISM Ready Reaction Dye Deoxy Terminator Cycle Sequencing Kit (Applied Biosystems, Foster City, CA).

Verification and inheritance of the mutation. For detection of the mutation in the kindred, PCR reactions from genomic DNA (50 ng) were carried out using the same PCR conditions and primers as above. 20 μl of the PCR reaction were transferred onto a Zeta-probe membrane (BioRad Laboratories, Richmond, CA) using a dot blot apparatus. Two parallel membranes were prehybridized in a solution of 5× SSPE, 0.5% SDS, 5× Denhardt's, for 30 min at 37°C, and then hybridized for 1 h at 37°C with either the ^{32}P-labeled wild type allele-specific oligomer (ASO) (5×10^6 cpm/ml) with the sequence 5′-CTGGAGGAGCAGGCCGCGCAA-3′, and the mutated ASO 5′-CTGGAGGAGTAGGCCGCGCAA-3′. Filters were washed in 2× SSPE, 0.1% SDS for 15 min at room temperature followed by 15 min at the T_m of the oligonucleotides, and then exposed to x-ray film for autoradiography. The inheritance of the mutation in the family was also confirmed by comparative sequence analysis of the PCR products encompassing the mutation site in the different family members.

Results

Previous results indicated that the clinical phenotype of the two MD-EBS members of the family C is associated with an impaired expression of plectin (4). In fact, intact skin and muscle samples from the two probands stained negatively with a panel of monoclonal antibodies specific for plectin. Absence of plectin was confirmed by immunoprecipitation of cell extracts obtained from primary skin keratinocytes (4). To further assess the expression of PLEC1 in the MD-EBS keratinocytes, we determined the steady-state level of the mRNA encoding plectin by Northern analysis of RNA isolated from primary cultures of epidermal keratinocytes obtained from one of the probands. Hybridization with a radioactive cDNA probe for plectin resulted in a markedly reduced, by around 75%, signal in the MD-EBS patient as compared to a control, after correction of the signal by GAPDH mRNA levels (Fig. 1). We therefore concluded that PLEC1 was likely to be involved in the disease in this family.

Since no gross abnormalities were detected when genomic DNA was digested with a panel of restriction enzymes, blotted on nitrocellulose sheets (20), and hybridized with radioactive cDNA fragments spanning the full length plectin cDNA, we initiated a search for mutations in PLEC1. Taking advantage of the fact that the intron-exon organization of PLEC1 has recently been described (13, 14), we amplified by PCR all the 33 individual exons of the gene using genomic DNA as a template. The size of the amplified products was checked by electrophoresis on 2% agarose gels and compared with that obtained from healthy controls. Because no major differences were detected between the patient and controls, the PCR

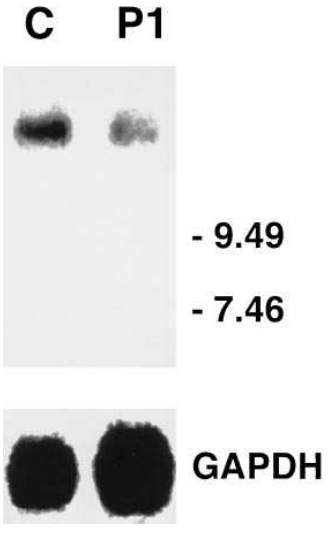

Figure 1. Reduced expression of plectin in MD-EBS keratinocytes. For Northern blot analysis, 20 μg of total RNA purified from cultures of primary keratinocytes was electrophoresed on agarose gel, blotted to a nylon membrane, and probed with a ^{32}P-labeled cDNA for plectin (*top*) and a ^{32}P-labeled GAPDH probe (*bottom*). Lane *C*, healthy control; lane *P1*, MD-EBS keratinocytes from proband 1. Bars on the right indicate the migration of RNA ladder markers (Life Technologies, Cergy-Pontoise).

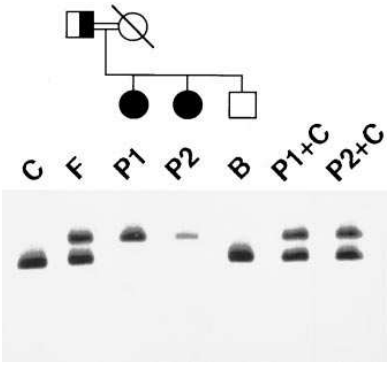

Figure 2. Nuclear pedigree of the MD-EBS family and genotypic analysis by CSGE. A 305-bp fragment, obtained by PCR amplification of exon 33 sequences of PLEC1 using genomic DNA as template, revealed a single band in the two probands (*P1* and *P2*) that migrated more slowly than the band detected in the clinically unaffected brother (*B*) and an unrelated healthy control (*C*). The patient's father (*F*) shows two homoduplex bands similar to those obtained when the PCR amplification products obtained from the probands and the healthy control were mixed, indicative of heterozygosity.

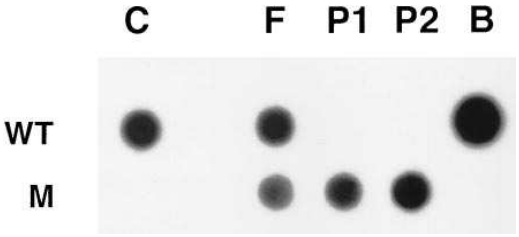

Figure 4. Inheritance of the Q1910X mutation in the MD-EBS family. A DNA fragment corresponding to the mutated region was amplified using genomic DNA from each family member. Hybridization with a wild type (*WT*) and mutated (*M*) ASO demonstrated the homozygosity of the probands for the mutated allele and the carrier status of the father. In the patients' brother and in an unaffected control no evidence for the mutated allele is found.

products were screened for sequence variations by heteroduplex analysis using conformation sensitive gel electrophoresis (CSGE) (19). CSGE analysis of the PCR product corresponding to nt 5621–nt 5944 of exon 32 revealed the presence of a homoduplex in the probands that was altered compared with the homoduplex found in the healthy control and the non affected patient's brother (Fig. 2). The two homoduplexes were detected in the proband's father and when the PCR-amplified products obtained with the patient's DNA and the control DNA were mixed together, suggesting the presence of a homozygous mutation in the patients' PLEC1 gene (Fig. 2). The brother's DNA showed the normal homoduplex band only. This amplified fragment corresponded to the repeat R_2C of the rod domain of the protein (14). Direct oligonucleotide sequencing detected a C-to-T transition at position 5728 leading to a nonsense mutation in which the termination codon TAG replaces a codon for glutamic acid (CAG) (Fig. 3). This mutation predicts truncation of the polypeptide at amino acid 1910 and is designated as Q1910X. Sequencing of the corresponding PCR products that in the father depicted the heteroduplexes revealed heterozygosity for the Q1910X mutation. Since the C-to-T transition did not alter any restriction enzyme site, the segregation of the mutation Q1910X and its linkage with the MD-EBS phenotype were confirmed by ASO on the DNA of four members of family. The amplified products were dot blotted on a nitrocellulose sheet and subsequently hybridized with two 21-mer ASO corresponding to the wild type and mutant Q1910X sequence, respectively (Fig. 4). The PCR-amplified samples from proband 1 and 2 hybridized only with the mutant ASO, whereas the healthy brother and the unrelated control showed strong hybridization signal only with the wild type ASO, indicating that occurrence of the MD-EBS phenotype correlates with homozygosity for the mutant allele. The sample obtained from the father hybridized to both the mutant and the wild type ASO, attesting to heterozygosity for the mutation. These results are therefore consistent with the notion that this nonsense mutation, 5728 C-to-T, is pathogenetic in this MD-EBS family.

Discussion

We have identified a homozygous point mutation in PLEC1 resulting in a premature termination codon of translation in a patient with MD-EBS. These findings, together with the identification of mutations in keratins 5 and 14 causing EBS, underscore the genetic heterogeneity of this group of genodermatoses.

The PLEC1 mutation is a C-to-T transition, possibly arising as a result of the hypermutability of 5-methyl-cytosine to

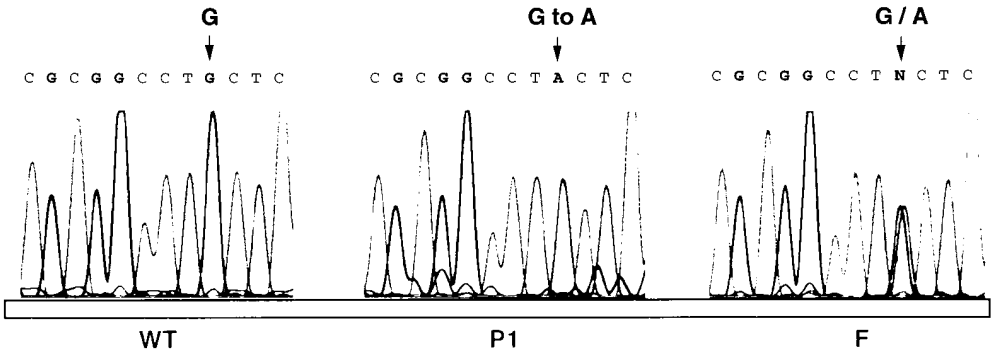

Figure 3. Nucleotide sequencing of PCR products demonstrating a homozygous nonsense mutation in the proband 1 (*P1*) and a heterozygous mutation in the probands' father (*F*). Antisense strand sequence is shown for these two individuals and a normal unrelated control. The G-to-A transversion shown in P1 results in a CAG to TAG/glutamic acid to premature stop codon substitution in the sense strand. This base change was present in one allele of the probands' father and was not detected in the control individual (*WT*).

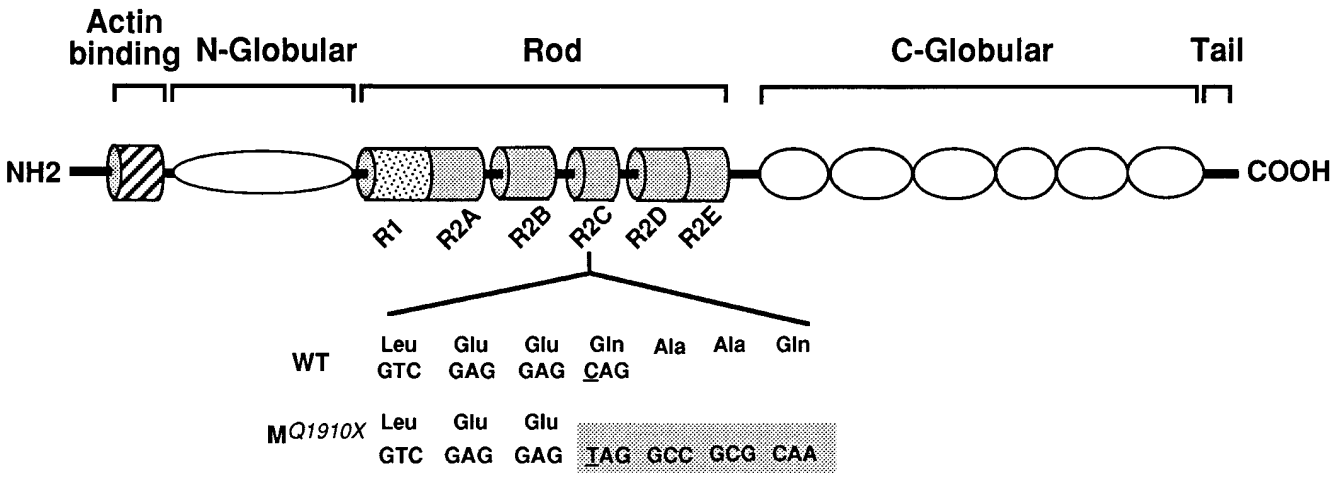

Figure 5. The molecular defect associated with the proband's PLEC1 alleles. Schematic representation of the domain structure of plectin (14, 30) showing the localization of the Q1910X mutation. The premature termination codon (TAG) and the deleted region of the rod subdomain R2A are boxed.

thymine at CpG nucleotides (21), that generates a nonsense (TAG) codon. It leads to truncated plectin molecules lacking the R2C, R2D and R2E regions of the α-helical coiled-coil rod domain and the globular COOH terminus (Fig. 5). Two different homozygous frame-shift mutations in the plectin mRNA have recently been identified in two unrelated MD-EBS patients (14, 22). These mutations, possibly arising as a result of slipped mispairing at the replication fork (21), are also predicted to cause premature termination of translation within the rod domain of plectin. Transient expression of plectin deletion mutants has identified the last two thirds of the globular COOH terminus of the protein as the region mediating the association with intermediate filaments (23). The aberrant plectin polypeptide is therefore expected to be unable to link the cytoskeletal structures that contribute to the strengthening of the cells toward mechanical stress, i.e., cytokeratins in basal keratinocytes and presumably desmin in myofibrils. Polymerization of the molecule, which is apparently mediated by the central rod domain, may also be compromised.

The nature of this mutation also explains the absence of immunoreactivity of the proband's dermal-epidermal basement membrane zone and muscle sarcolemma to monoclonal antibodies directed against plectin (4). Consistent with the observation that premature termination codons induce instability of the aberrant messenger RNA and reduced synthesis of the corresponding transcript due to alterations in RNA processing (24), the mutation Q1910X correlates with the low steady-state levels of the plectin mRNA detected by Northern analysis of the MD-EBS patient's keratinocytes. It also correlates with the absence of hybridization bands in Western analysis of the proband's skin and muscle cells performed with monoclonal antibodies specific to the central rod domain of plectin (4, 14, 25).

The role of plectin in the etiology of a disease with pleiotropic manifestations, i.e., EBS and muscular dystrophy, is now formally proven. In the skin, lack of plectin leads to failure of keratin filaments to connect to the plasma membrane via the hemidesmosomes, whereas in the muscle the deficient expression of the molecule results in an aberrant delocalization of desmin at the periphery of the muscle fibers, which suggests a defective anchorage of the cytoplasmic filaments to the sarcolemma (4). Considering the widespread cell distribution of plectin, its intracellular localization at the cytoskeleton-plasma membrane interface, and its presumed functional versatility, it has been hypothesized that defects in the PLEC1 gene may underlie a variety of diseases (13). Careful clinical examination of our MD-EBS patients did not disclose pathologic manifestations nor subtle defects, besides those affecting the integument and muscles, that could be considered a consequence of the mutation. It appears therefore that the aberrant expression of plectin correlates exclusively with defects caused by the impaired performance of cells in response to significant degree of mechanical stresses. However, considering the late-onset of muscular dystrophy affecting the MD-EBS patients, possible manifestation of additional clinical features along with ageing, for instance in the cardiac muscle and cells forming the blood-brain barrier where plectin is detected (25), cannot be excluded.

It has recently been demonstrated that BPAG1, a component of the inner plate of the hemidesmosome, and integrin α6β4 are essential for the anchorage of keratin intermediate filaments (26–29). These studies provide evidence that attachment of keratins to hemidesmosomes is crucial in endowing the basal pole of keratinocytes with mechanical resistance. The precise functions and the complex intermolecular interactions of each of the components of the hemidesmosomal plate remain to be elucidated. However, identification of genetic mutations underlying the epithelial-mesenchymal dysadhesion observed in epidermolysis bullosa will undoubtedly lead to a better understanding of the functions of hemidesmosomes.

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