A Compound Heterozygous One Amino-Acid Insertion/Nonsense Mutation in the Plectin Gene Causes Epidermolysis Bullosa Simplex with Plectin Deficiency

Johann W. Bauer,^{*,†} Fatima Rouan,[§]
Barbara Kofler,^{*,†} Günther A. Reznicek,[¶]
Iris Kornacker,[¶] Wolfgang Muss,[‡]
Rudolf Hametner,^{*} Alfred Klausegger,^{*}
Ariana Huber,^{*} Gabriele Pohla-Gubo,^{*}
Gerhard Wiche,[¶] Jouni Uitto,[§] and Helmut Hintner^{*}

From the Department of Dermatology,* Children's Hospital[†]
Salzburg, Austria; the Institute of Pathology,[‡] General Hospital
Salzburg, Salzburg, Austria; the Department of Dermatology and
Cutaneous Biology,[§] Thomas Jefferson University, Philadelphia,
Pennsylvania; and the Institute for Biochemistry and Molecular Cell
Biology,[¶] Vienna Biocenter, University of Vienna, Vienna, Austria

Plectin is a cytoskeleton linker protein expressed in a variety of tissues including skin, muscle, and nerves. Mutations in its gene are associated with epidermolysis bullosa simplex with late-onset muscular dystrophy. Whereas in most of these patients the pathogenic events are mediated by nonsense-mediated mRNA decay, the consequences of an in-frame mutation are less clear. We analyzed a patient with compound heterozygosity for a 3-bp insertion at position 1287 leading to the insertion of leucine as well as the missense mutation Q1518X leading to a stop codon. The presence of plectin mRNA was demonstrated by a RNase protection assay. However, a marked reduction of plectin protein was found using immunofluorescence microscopy of the patient's skin and Western blot analysis of the patient's cultured keratinocytes. The loss of plectin protein was associated with morphological alterations in plectin-containing structures of the dermo-epidermal junction, in skeletal muscle, and in nerves as detected by electron microscopy. In an *in vitro* overlay assay using recombinant plectin peptides spanning exons 2 to 15 the insertion of leucine resulted in markedly increased self-aggregation of plectin peptides. These results describe for the first time the functional consequences of an in-frame insertion mutation in humans. (Am J Pathol 2001, 158:617–625)

Plectin is a cytoskeleton linker protein expressed in a variety of tissues including skin, muscle, and nervous

tissue. Its gene PLEC1 has been localized to chromosome 8q24 coding for a 14.8-kb mRNA.^{1,2} Protein structure predictions have suggested that the N- and C-terminal sequences assume globular structures, which are connected by a central rod domain.³ Plectin's role as an essential intermediate filament-binding protein has recently been supported by the fact that mutations in its gene cause epidermolysis bullosa (EB) with late onset muscular dystrophy.⁴ Similar symptoms have been noticed in a plectin knock-out mouse model.⁵ Plectin is involved in the formation of hemidesmosomes of epithelial cells⁶ and links Z-disks to the intermediate filament network in skeletal muscle fibers.⁷ Using deletion studies and overlay assays the binding domains for vimentin/keratin filaments,⁸ integrin $\beta 4$,^{9,10} desmin,¹¹ and actin^{12,13} have been identified. No particular sequence has been identified for homodimer formation.³

EB is a term used for a heterogeneous group of mechanobullous disorders, in which minor trauma leads to blister formation on skin and mucous membranes. Three major groups of EB have been defined according to the plane of split formation within the skin or mucous membranes: intra-epidermal cleavage in EB simplex, cleavage in the lamina lucida in junctional EB, and below the lamina densa in dystrophic EB.¹⁴ In a total of 10 different genes mutations have been found.¹⁵ EB simplex with late onset muscular dystrophy (EBS-MD), an autosomal recessive disease, is caused by nonsense mutations, out-of-frame deletions, and insertions in the plectin gene in most of the published cases.¹⁶ In one case, an in-frame deletion of three amino acids has been reported¹⁷ whereas there are no reports on in-frame insertions. In case of nonsense and out-of-frame mutations the functional consequences of mutations are thought to be mediated by nonsense-mediated mRNA decay, in case of

Supported by the Medizinische Forschungsgesellschaft Salzburg (to A. K., A. H., and R. H.); a predoctoral fellowship from the Austrian Academy of Sciences (to G. A. R.); by grants from the Austrian Science Research Fund (to G. W.); the Austrian National Bank; and Verein zur Erforschung von Muskelkrankheiten bei Kindern.

Accepted for publication October 24, 2000.

Address reprint requests to Johann W. Bauer, M.D., Department of Dermatology, General Hospital Salzburg, Müllner Hauptstrasse 48, A-5020 Salzburg, Austria. E-mail: jo.bauer@lks.at.

in-frame mutations the pathophysiological consequences are less clear.

In this report we describe compound heterozygosity for a nonsense mutation and a 3-bp in-frame insertion in the plectin gene defining the smallest insertion mutation leading to a pathological phenotype in humans. This mutation leads to hemidesmosomal insufficiency in keratinocytes with blister formation and structural changes in skeletal muscle and nerves. Despite unaltered levels of plectin mRNA expression the amount of plectin protein is markedly reduced in skin and muscle. Protein overlay assays demonstrated increased self-aggregation of the mutated plectin molecules, thus providing a mechanistic explanation for the consequences of the insertion mutation.

Materials and Methods

Patients, Cells, and Antibodies

The nuclear family consisted of the index patient (II-1), his affected brother, his unaffected sister, and their parents, who are of Caucasian origin. At the time of analysis patient II-1 was a 4-year-old boy. Keratinocyte and fibroblast cultures were initiated from biopsies of patient's skin by sequential dispase and trypsin treatment and culturing in keratinocyte growth medium and fibroblast growth medium, respectively (BioWhittaker, Vervier, Belgium). Muscle biopsies were obtained from left quadriceps muscle of the patient II-1 (for patient numbering see Figure 4) after gaining consent by the parents. Anti-rat plectin antibodies 6C6 and 5B3 that cross-react with human tissues were described elsewhere.¹⁸ Other antibodies were from the following sources and were used with the following dilutions: anti-keratin 14 (Sigma, St. Louis, MO) dilution 1:100 in PBS; anti-keratin 5 (antibody AE14, kindly provided by H. Sun, New York, NY) dilution 1:100 in phosphate-buffered saline (PBS); anti-BPAG1 (antibody 233, kindly provided by J. Stanley, Philadelphia, PA), anti-integrin $\beta 4$ (Chemicon, Temecula, CA) dilution 1:20 in PBS, anti-BPAG2 (antibody HD 18, kindly provided by G. Giudice, Milwaukee, WI) was used undiluted, anti-laminin 5 (GB3, Sera-Lab; Vienna, Austria) dilution 1:20 in PBS, anti-type VII collagen (Chemicon) dilution 1:20 in PBS. Fluorescein-conjugated secondary antibodies were from Amersham Pharmacia Biotech (Little Chalfont, UK) and were used 1:40.

Immunofluorescence Microscopy

Immunofluorescence microscopy was performed on 5- μ m cryostat sections of skin from two control patients, nonlesional skin as well as musculus vastus lateralis of patient II-1. Specimens were incubated with first-step antibodies for 1 hour at room temperature. 5B3 was diluted 1:2 in PBS. After three washes with PBS the respective fluorescein isothiocyanate-conjugated second step antibodies (1:100 in PBS) were added for 30 minutes at room temperature. Then, the sections were washed again, mounted, and evaluated under an immunofluorescence microscope (Zeiss, Vienna, Austria).

Immunohistochemistry and Myofibrillar Typing of Muscular Tissue

Tissue specimens were snap-frozen in liquid nitrogen and stored at -70°C . Sections (7- μ m) were cut from each block and mounted. Protein expression was visualized with a three-step immunoperoxidase technique [streptavidin-biotin-peroxidase complex (S-ABC)]. Endogenous peroxidase activity was blocked by H_2O_2 methanol (0.3%) for 15 minutes. Sections were then rehydrated and rinsed with PBS (pH 7.2) throughout a 10-minute period. Nonspecific-binding sites were blocked with normal sheep serum (1:20 in PBS; DAKO, Glostrup, Denmark). All primary antibodies were incubated overnight at 4°C . The following primary antibodies were used: vimentin (dilution 1:200; DAKO); dystrophin 1-3 (dilution 1:4; Novocastra, Newcastle on Tyne, UK). The biotinylated second antibody (1:200, anti-mouse Ig from sheep; Amersham) was added and incubated for 30 minutes at room temperature. The S-ABC complex was visualized with aminoethylcarbazole in the presence of hydrogen peroxide. The reaction was stopped by immersion in PBS and finally sections were slightly counterstained with hematoxylin and mounted under coverslips with Kaiser's glycerin-gelatine (Merck, Darmstadt, Germany).

To type muscular fibers a myofibrillar ATPase reaction at pH 4.2 and 9.4 was used according to Hayashi and Freimann.¹⁹

Western Blot Analysis

Confluent cells were washed with PBS and scraped off the plate. Cell pellets were lysed in 50 mmol/L Tris-HCl, pH 7.5, 150 mmol/L NaCl, 1% (v/v) Triton-X 100, 0.1% (w/v) sodium dodecyl sulfate, 0.5 mmol/L ethylenediaminetetraacetic acid, 10 μ mol/L leupeptin, 100 μ mol/L phenylmethyl sulfonyl fluoride, 100 μ mol/L dithiothreitol. Twenty μ g of protein of control and patient II-1 keratinocytes were loaded on a 5% sodium dodecyl sulfate-polyacrylamide gel. After electrophoresis, proteins were transferred to nitrocellulose (Hybond C pure; Amersham Pharmacia Biotech) in 48 mmol/L Tris-HCl, 39 mmol/L glycine, 20% (v/v) MeOH, 0.037% (w/v) sodium dodecyl sulfate. The primary monoclonal antibody 5B3 was diluted 1:3 in blocking buffer (200 mmol/L Tris-HCl, pH 7.6, 137 mmol/L NaCl, 0.2% (w/v) I-Block, 0.1% (v/v) Tween 20). Immunodetection was monitored with the Western-Star chemiluminescent detection system (Tropix Inc., Bedford, MA) following the manufacturer's instructions.

Electron Microscopy

Specimens were prepared for conventional transmission electron microscopy using standard protocols. In brief, skin punch biopsies from blister sites were immediately placed in the prefixation solution and dissected in an oriented manner. Muscular tissue was also immersed in prefixative solution and dissected at room temperature. The prefixation solution consisted of a mixture of buffered formaldehyde: 0.5% (v/v) and electron microscopic

grade glutaraldehyde: 1.5% (v/v), in 0.1 mol/L phosphate buffer (pH 7.5). After trimming, all specimens were processed manually as follows: fixation in buffered glutaraldehyde (4% of 0.1 mol/L phosphate buffer, pH 7.5, at room temperature for 5 to 6 hours). Rinsing in phosphate buffer (0.13 mol/L, pH 7.5, 4 × 5, 30, 5, and 5 minutes; 30-minute wash containing 50 mmol/L NH₄Cl), postfixation in buffered 1% osmium tetroxide (0.13 mol/L phosphate buffer according to Millonig²⁰ room temperature for 1.5 hours); rinsing twice again in phosphate buffer (0.1 mol/L, 5 minutes each); dehydration in 50% ethanol (EtOH), followed by 1% para-phenylenediamine in 70% EtOH, rinsing specimens afterward in 70% EtOH; dehydration in a graded series of EtOH; as an intermedium acetonitrile (3× pure, minimally 15 minutes each) was used. Infiltration and subsequent embedding in the epoxy resin EPON 812 substitute (based on Glycidether 100; Serva, Germany); polymerization at 37°C, 45°C, and 65°C for ~24 hours each. Semithin sections (1-μm thick for localization purposes) were stained with a modified methylene azure II basic fuchsin sequence²¹ followed by ultrathin sectioning: 60 to 80 nm, mounting sections on 75-mesh Formvar-coated copper grids, conventional staining of ultrathin sections by a modified sequence, using a hydrous solution of 0.05% tannic acid (6 to 8 minutes at room temperature), 1% (w/v) methanolic uranylacetate containing 50 μl/100 ml (end-volume) of concentrated acetic acid (15 minutes at room temperature), followed by lead citrate²² (2 to 3 minutes at room temperature).

Genomic Analysis

Genomic DNA from leukocytes from peripheral blood of the probands was purified using a column method according to the manufacturer's protocol (Qiagen, Hilden, Germany). Primers for amplification of exon 9 (according to the sequence published by Smith and colleagues⁴ and McLean and colleagues,² GenBank accession numbers U53204, U53834, and U63610, this exon is exon 10, the maternal mutation located there is 1008ins3. The paternal mutation is termed Q1408X at the start of exon 32) (GenBank accession number z54367¹) were 5'-GGCAGAC-CAACCTGGAGAAC-3' (nucleotides 1046 to 1065; z54367) and 5'-GTGTCGCATCCACTGAAGCA-3' (nucleotides 1302 to 1283; z54367). Primers for amplification of the mutated region in exon 31: 5'-TGAGTGAAGTGTGC-CGGTGC-3' (nucleotides 11,814 to 11,833; U63610) and 5'-TTTGTGCCTCAGCCTCCTCC-3' (nucleotides 4876 to 4857; z54367). Polymerase chain reactions (PCRs) were performed at 95°C denaturing temperature, 60°C and 62°C annealing temperature, respectively, and 68°C extension temperature using Expand Long-Range PCR kit (Roche Molecular Biomedicals, Indianapolis, IN). Amplified DNA was analyzed by conformation-sensitive gel electrophoresis following a standard protocol.²³ Heteroduplex bands were automatically cycle-sequenced with a ABI Prism 377 (ABI, Foster City, CA) using the above-mentioned 5' primers to detect mutations in exon 9 and exon 31, respectively. Primers and PCR conditions for

sequencing were the same as used for initial PCR reactions. Restriction enzyme digests were performed according to the manufacturer's protocols (New England Biolabs, Beverly, MA). The DNA fragments were analyzed on a 4% agarose gel.

RNA Isolation and RNase Protection Assay

Total RNA was isolated from cultured fibroblasts of patient II-1 by the method of Chomczynski and Sacchi.²⁴ RNase protection analysis was performed as previously described²⁵ with the exception that hybridization was done at 60°C. Ten μg of total RNA was used per lane. Probes specific for exon E1 (human plectin cDNA nucleotides 34 to 141; z54367) and E32 (nucleotides 13,650 to 13,906; z54367) were subcloned from genomic DNA clone pCGL66 (containing nucleotides 1 to 141 of exon E1 and 600 nucleotides of upstream sequences) and cDNA clone pCGL53,¹ respectively. The probe specific for plectin's alternative first exon E1c (human plectin cDNA, nucleotides 73 to 220; NM000445) was subcloned from cDNA clone pLK7 (containing E1c-E3 of human plectin cDNA). The DNA fragments were inserted into the polylinker of the plasmids pSP64 or pSP65 (Promega, Madison, WI) in opposite orientation to the direction of SP6 transcription. After linearization at a suitable restriction site downstream of the plectin insert *in vitro* transcription with SP6 polymerase was performed to produce an antisense riboprobe. A glyceraldehyde-3-phosphate dehydrogenase (GAPDH)-specific probe was shortened to produce a signal short enough to serve as loading control. Quantification of transcript levels was performed with a PhosphorImager (Molecular Dynamics, Sunnyvale, CA), the values were normalized against the protected GAPDH fragment and corrected for the G content of the different riboprobes. A radioactively labeled *Hpa*II digested pUC18 DNA was run simultaneously to estimate the size of the protected fragments.

Cloning and Expression of Recombinant Proteins

A mouse plectin cDNA construct comprising exons 2 to 15 (GenBank accession number z54367) flanked by in-frame *Eco*RI restriction sites was generated by PCR using mouse cDNA not containing any of the alternative α-exons¹³ as template and primers mEx2fw (5'-cgg gaa ttc GAT GAA CGA GAC CGT GTG CAG-3', corresponding to nucleotides 523 to 543 in the human sequence; z54367) and mEx15rev (5'-aag gaa ttc ACC CCG GGT GGC AGG GGA G-3', nucleotides 2157 to 2175; z54367). To introduce the additional leucine into the sequence, we took advantage of the immediate proximity of a *Bam*HI restriction site to the insertion point and a unique *Bgl*II restriction site within exon 6. PCR mutagenesis using primers mEx6fw (5'-cgg gaa ttc CAG ATC TCA GAC ATT CAG-3', nucleotides 844 to 861; z54367) and mEx9leu ins (5'-ccg gaa ttc CCG GAT CCA TTG Cag cAG CAG CAG CAA CAC AAG CTC CCG-3', nucleotides 1264 to 1295; z54367) was performed. The amplified

mutagenized PCR product was subcloned, sequenced, and the *Bgl*II/*Bam*HI restriction fragment from the exon 2 to 9 wild-type construct replaced with the mutated fragment. Constructs were then extended to the beginning of exon 15 and subcloned into the unique *Eco*RI sites of the HIS-tag expression vector⁸ pBN120. Proteins (2 to 15 wild type and 2 to 15 mutated) were expressed in *E. coli* BL21(DE3) and affinity purified under nondenaturing conditions. The HIS-tagged cytoplasmic domain of the integrin $\beta 4$ subunit protein ($\beta 4$ cyt), starting with the first pair of fibronectin type III repeats, was described earlier.⁹ Rabbit skeletal muscle actin was from Sigma Chemical Co. (St. Louis, MO).

In Vitro Protein Interaction Assay

Influences of the mutation on the properties of plectin were assessed by a microtiter-plate format protein-protein interaction assay that had been successfully used to characterize interactions of N-terminal plectin fragments with the integrin subunit $\beta 4$ ⁹ and actin.¹³ Expressed proteins were labeled with Eu^{3+} -labeling reagent (Wallac, Turku, Finland) as previously described.⁹ Microtiter plates were coated with 100 μl of 100 nmol/L protein in 25 mmol/L sodium borate buffer, pH 9.2, overnight at 4°C. Blocking was performed with 4% (w/v) bovine serum albumin (BSA) in overlay buffer [50 mmol/L Tris-HCl, pH 7.5, 150 mmol/L NaCl, 1 mmol/L EGTA, 2 mmol/L MgCl_2 , 1 mmol/L dithiothreitol, and 0.1% (v/v) Tween 20] for 1 hour, followed by incubation with different concentrations of Eu^{3+} -labeled proteins (in 100 μl of overlay buffer) for 90 minutes at room temperature. After extensive washing with overlay buffer, the amount of bound proteins was determined by measuring Eu^{3+} -fluorescence with a Delfia time-resolved fluorometer (Wallac). The fluorescence values were converted to concentrations by comparison with an Eu^{3+} standard.

Results

The nuclear family consisted of two affected individuals with unaffected parents and an unaffected sister (Figure 4A). Patients II-1 and II-2 showed blistering since birth. Blisters tended to form erosions but were also present as blood blebs on fingers and toes (Figure 1, A and B). The blisters healed without scarring. In addition, nails were dystrophic, but mucous membranes, teeth, and hair were not affected. Clinical tests including neuropsychiatric evaluation, evaluation of muscle strength, and cranial CT did not reveal any abnormalities.

Cryostat sections of patient II-1 skin analyzed by indirect immunofluorescence microscopy gave negative staining with plectin antibody 5B3 compared to control skin (Figure 2a). Control antibodies including antibodies detecting BPAG1, BPAG2, integrin $\beta 4$, laminin 5, and type VII collagen did not reveal significant alterations. Immunohistochemical analysis of skeletal muscle revealed normal tissue distribution of ATPase, vimentin, and dystrophin in a biopsy of musculus vastus lateralis from patient II-1, whereas plectin mAb 5B3 binding was absent. In addition, an irregular distribution of nuclei,

Figure 1. Clinical findings on patient II-1 at the age of 3 years. **A:** Erosions, blister formation, and nail dystrophy on upper and lower extremities. **B:** Close-up of hemorrhagic blisters on the palm and fingers.

variation in size and shape, was seen in the muscle fibers as compared to a control muscle. Furthermore, in indirect immunofluorescence microscopy analysis of patient muscle mAb 5B3 staining was absent. Control muscle cells showed typical predominant staining of Z-lines in longitudinal sections and peripheral and sarcoplasmic staining in cross sections (data not shown). Western blot analysis of cultured patient keratinocytes with 5B3 gave only a very faint band (Figure 2b). In addition, several bands of 40 to 50 kd suggestive for degradation products were seen using antibody 6C6 recognizing an epitope in

Figure 2. Plectin protein expression in keratinocytes. **a:** Immunofluorescence microscopy. Sections of skin biopsies of patient II-1 were stained with mAb 5B3 and anti-mouse FITC-conjugated IgG. Loss of staining in patient's skin (P) compared to sections of normal skin (C) was observed. In patient's skin, the position of a blister roof and blister floor at the dermo-epidermal junction is indicated by **arrows**, the cutaneous basement membrane is marked by **arrowheads**. The apparent staining at the top of the epidermis in patient's skin is caused by autofluorescence of the stratum corneum. Original magnification, $\times 1:20$. **b:** Western blot analysis. Skin keratinocytes from patient II-1 were cultured and protein extracts were analyzed using mAb 5B3. A grossly reduced full-length protein was detected in patient keratinocytes (P) as compared to controls (C). The positions of molecular weight markers are indicated.

plectin's rod domain upstream of that of 5B3 (data not shown).

In the electron microscope analysis of skin sections the hemidesmosomes were only slightly reduced in number, but almost all of them seemed to be hypoplastic (Figure 3a). Keratin filaments were not inserting in the residual hemidesmosomes with a few exceptions. Also, desmosomes in the basal layer and in the higher layers of the epidermis were hypoplastic (not shown). Sections of skeletal muscle showed moderate pathological changes: Z- and I-band alterations and disoriented muscle fibers were seen (Figure 3b). Mitochondria were often misplaced and degenerating (Figure 3, c and d). Around muscle fibers muscular nerves showed axons with adaxonal microlamellar inclusions and myelin-like lamellar bodies (Figure 3, e–g). The axonal ending exhibited discrete signs of degeneration such as the basal lamina redundant foldings (not shown).

The plectin gene was analyzed by heteroduplex scanning, revealing heteroduplexes in fragments containing exons 9 and 31. Sequencing of the respective PCR fragment of exon 9 showed a heterozygous 3-bp insertion GCT at position 1287 (Figure 4B). The insertion of GCT is placed after four other GCTs and might have been caused by slipped mispairing. It leads to the insertion of another leucine in a row of four leucines. Another heteroduplex formation was observed at the beginning of exon 31. Here a C-to-T transition leads to a stop codon designated Q1518X. The paternal mutation Q1518X was verified by digestion of the respective exon 31 fragment with *BfaI* (Figure 4C–I). Because of the creation of a new *BfaI* restriction site by the mutation this digest leads to the heterozygous occurrence of two fragments of 325- and 185-bp length in the patients II-1, II-2, the unaffected sister II-3, and the father. These fragments were not observed in the mother and in 80 control alleles. The maternal mutation 1287ins3 was verified by *PvuII* digestion of a PCR fragment spanning exon 9 (Figure 4C–II). This digest leads to two fragments of 361 bp and 57 bp in length in the father, the unaffected sister and 80 control alleles. In the mother, patient II-1 and patient II-2 a heterozygous 60-bp fragment created by the 3-bp insertion was seen in addition. To determine the mRNA levels in fibroblasts from patient II-1 and unaffected controls RNase protection assays were performed using riboprobes specific for the alternative first N-terminal exons E1 and E1c and the C-terminal exon E32. This analysis showed similar levels of plectin E1c transcripts in patient and control cells (Figure 4D, left). Furthermore, the ratio between the levels of alternative plectin transcripts E1 and E1c was similar to that found in control fibroblasts (not shown). Also, no apparent reduction in the levels of exon 1- and exon 32-specific transcripts was noticed suggesting that in patients' fibroblasts full-length mRNA was produced (Figure 4D, right).

The insertion mutation of the N-terminal globular domain lies in close proximity to the multifunctional actin binding domain, encoded by exons 2 to 8. Therefore, it was of interest to analyze whether this mutation affected the binding of plectin to the established binding partners in this region, actin¹³ and integrin $\beta 4$.^{9,10} Wild-type and

Figure 3. Electron microscopy. **a:** Keratinocytes: High-power view of junctional aspect (lateral borders of two basal keratinocytes). The cell on the left showed one fairly normal hemidesmosome (HD) with correctly inserting tonofilament bundles (tf); the cell on the right showed some hypoplastic (**arrow**) as well as one fairly normal looking hemidesmosome. This cell lacked correctly inserting tonofilaments on its whole cellular base (right side not shown). Note small areas of dilated lamina lucida (**arrowheads**), which were seen frequently. Dilated intercellular space between lateral borders of keratinocytes is marked by an **asterisk**. Anchoring fibrils appeared to be normal. Scale bar, 0.5 μ m. **b:** Low-power view of patient's muscle tissue (musculus vastus lateralis). Note the circular arrangement of filament bundles. Some Z-bands are lacking. Scale bar, 5 μ m. **c:** Muscular tissue (musculus vastus lateralis). Note the disorientation of the filament bundle system (**asterisk**), the uneven diameter of filament bundles, discrete Z-band alterations (**arrows**) as well as accumulation of altered mitochondria. Scale bar, 2.5 μ m. **d:** Muscular tissue (musculus vastus lateralis). Altered, degenerating, or necrotizing mitochondria could be observed (cristolysis, left upper corner; disappearance of cristae and densification of inner mitochondrial matrix). Scale bar, 0.5 μ m. **e:** Intramuscular myelinated nerves (musculus vastus lateralis, low-power view). Slightly altered, at places vacuolized adaxonal bodies (v), and small axons (a, cross-section) were seen. Scale bar, 1 μ m. **f:** Intramuscular myelinated nerves (musculus vastus lateralis, high-power view). Slightly altered adaxonal bodies (**arrow**), one of them (**asterisk**) lipidous, displaying myelin-like lamellar material were observed; the myelin-sheath showed ordinary periodicity of lamellae. The axon was triangle shaped (a, cross-section). Scale bar, 0.5 μ m. **g:** Lateral part of neuromuscular junction (musculus vastus lateralis, high-power view). A slightly altered postsynaptic cleft system was observed. Basement membrane material, at places, had floccular appearance. Note calcospherite inclusion body in the upper left corner (incrustation was partially lost during sectioning because of poor resin penetration). Interstitial space is shown at the right side. Scale bar, 0.5 μ m.

mutated plectin proteins encoded by exons 2 to 15 were generated by PCR-mediated mutagenesis and expressed as N-terminal HIS-tag fusions in *Escherichia coli*. During the isolation and purification of the mutated recombinant protein from bacteria we found it to be much less soluble than its wild-type counterpart, with expression levels being equal. Wild-type and mutated proteins

were coated (BSA served as the negative control) and overlaid with Eu^{3+} -labeled recombinant integrin $\beta 4$ or skeletal muscle actin. Although no significant changes in binding to skeletal muscle actin could be observed (data not shown), binding of the mutated protein to integrin $\beta 4$ was $\sim 30\%$ reduced when compared to binding to the wild-type protein (Figure 5A). However, when integrin $\beta 4$

Figure 4. Genetic analysis. **A:** Pedigree of the patients with plectin deficiency. The parent generation is labeled I. The children are labeled II. Children II-1 and II-2 are compound heterozygous for the paternal and the maternal mutation. The older, clinically unaffected sister (#) is heterozygous for the paternal mutation. **B:** Identification of mutations 1287ins3 and Q1518X. Sequencing of a region of exon 9 carrying a heteroduplex identified by CSGE revealed a 3-bp insertion GCT at 1287 (**top**). Another heteroduplex formation was observed at the beginning of exon 31. Sequencing showed a heterozygous C-to-T change leading to a stop codon designated Q1518X (**bottom**). **C:** Verification of mutations by restriction enzyme digestion. **I:** The paternal mutation Q1518X creates a new *Bfa*I site that leads to the digestion of the normal allele of 510 bp to two fragments of 325 and 185 bp. These fragments were found in the father (F), patient II-1 (C), his brother II-2 (C1), and the unaffected sister II-3 (C2). **II:** The maternal mutation 1287ins3 leads to the extension of a *Pvu*II fragment of 57 bp for 3 bp creating a 60-bp fragment. This extension was seen in the mother (M), patient II-1 (C), and patient II-2 (C1). **D:** RNase protection analysis of plectin transcripts in fibroblasts of patient II-1 and a control. Autoradiography of RNase protected bands specific for plectin's exon E1 (108 nucleotides), E1c (148 nucleotides), and E32 (257 nucleotides) revealed that no apparent reduction in E1c transcript levels in patient's fibroblasts (**left**) and abundant expression of E32 and E1 mRNA was present (**right**). A GAPDH-specific probe protecting 53 nucleotides of GAPDH RNA was used as a control for RNA quantification.

Figure 5. Protein overlay assay. **A:** Influence of the 1287ins3 mutation on the binding of integrin $\beta 4$ to plectin. Increasing concentrations (10 to 1,000 nmol/L) of Eu^{3+} -labeled integrin $\beta 4$ were incubated with coated (100 nmol/L) wild-type (closed circles) and mutated (open circles) recombinant proteins, as well as BSA (closed triangles). All data are presented as the mean $\pm$ SD of triplicate determinations. **B:** Increased self-binding of recombinant plectin proteins containing the 1287ins3 mutation. Wild-type or mutated recombinant proteins, as well as BSA, were coated onto microtiter plates (100 nmol/L) and overlaid with increasing concentrations (10 to 500 nmol/L) of Eu^{3+} -labeled wild-type (circles) or mutated (triangles) recombinant proteins.

was coated and overlaid with Eu^{3+} -labeled recombinant plectin proteins, we found the mutated protein to bind significantly stronger (data not shown). Because the latter finding could be an artifact of solubility of the mutated plectin protein we speculated that the mutated protein had a potential for self-binding/aggregation. To test this possibility, we performed assays in which wild-type or mutated proteins were coated and overlaid with Eu^{3+} -labeled versions of the same kind. As shown in Figure 5B, no self-binding activity was observed for the wild-type protein, whereas the mutated protein exhibited strong self-binding activity. Both proteins gave the same low background levels when BSA was coated.

Discussion

We show in this study that the combination of a heterozygous stop-codon and a heterozygous one amino-acid insertion in the plectin gene leads to EB as well as muscle and nerve pathology on an ultrastructural level. These pathological changes suggest that clinical overt myopathy might occur in both affected children. The pathophysiological link between deficient expression of plectin and onset of muscular weakness is unknown. To solve this problem Rouan and colleagues¹⁶ analyzed all currently published cases of EBS-MD. They show that neither immunofluorescence microscopy nor mutational analyses are sufficient to predict the time of onset of the muscular weakness. A so far underestimated factor in the development of the myopathy might be neurological pathology. In a recent study on a plectin-deficient patient with signs of EB, myopathy, and myasthenic syndrome, myopathic features comparable to our patient involving nuclei, myofibrils and membranous organelles have been observed.²⁶ In addition, a considerable reduction of neuromuscular transmission was suggested by microelectrode detection of end-plate potential. We have observed degenerative signs such as axonal microlamellar inclusions and myelin-like lamellar bodies in nerves located around muscular fibers. Our results further support the notion that the muscular deficiency in these patients is also regulated by neuromuscular transmission making it, at least in part, a treatable symptom.

In-frame insertion mutations rarely cause human pathology. The combination of a stop codon and a three amino acid insertion in the insulin receptor gene led to severe insulin resistance.²⁷ On the other hand, the insertion of three and six amino acids in the pro-opiomelanocortin gene does not cause a pathological phenotype.²⁸ To date, we are not aware of other in-frame insertion mutations in EB. Together with a 3-bp insertion in the human phytyl-CoA hydroxylase gene causing Refsum's disease²⁹ the mutation 1287ins3 defines the smallest possible amino acid insertion causing a pathological phenotype.

The pathophysiological consequences of in-frame mutations are probably mediated by disruption of functional domains. However, to date no experimental evidence for this assumption has been published to our knowledge. The N-terminal globular domain of plectin harbors an actin binding domain¹² and regions for integrin $\beta 4$ binding.^{9,10} The importance of this region has also been demonstrated by a three amino-acid deletion in the N-terminal globular domain of plectin in a patient with EBS with muscular dystrophy.¹⁷ In that study the functional effects of the mutation were not analyzed. Using a protein overlay assay we have observed increased self-association of the recombinant plectin mutant protein mimicking the insertional mutation of the patient. A likely explanation for this observation is that in the mutated protein the insertion of an additional leucine into a stretch of four existing consecutive leucines leads to an increase in hydrophobicity of this region resulting in conformational changes in the molecule. This could lead to reduced integrin $\beta 4$ -binding and a possible surface exposure of

hydrophobic amino acids normally buried within the naturally folded proteins, which in turn could be responsible for the observed self-binding and decreased solubility. We speculate that these self-aggregated plectin molecules are degraded rapidly, explaining the greatly reduced amount of plectin protein in our patient cells. To our knowledge, these results demonstrate the functional consequences of an in-frame insertion mutation for the first time and suggest that the protein overlay assay is a valuable tool in defining the functional deficiencies caused by in-frame mutations.

In summary, we describe here that a combination of a stop codon in one allele and a single amino acid insertion in the other allele of the plectin gene can lead to loss of plectin protein possibly caused by increased self-aggregation and subsequent degradation. This leads to blister formation in the skin and ultrastructural pathology in muscles and nerves. A follow-up study on these patients including re-evaluation of muscle tissue when clinically overt muscular weakness starts might further help to reveal the pathophysiology of muscular disease in EB.

Acknowledgments

We thank M. Busslinger (Vienna, Austria) for providing the GAPDH-specific probe; H.-J. Alder (Philadelphia, Pennsylvania) for performing the sequence analysis; and R. Pilz (Salzburg, Austria) for performing the immunohistochemical analysis of muscle tissue.

References

- Liu CG, Maercker C, Castanon MJ, Hauptmann R, Wiche G: Human plectin: organization of the gene, sequence analysis, and chromosome localization (8q24). *Proc Natl Acad Sci USA* 1996, 93:4278–4283
- McLean WH, Pulkkinen L, Smith FJ, Rugg EL, Lane EB, Bullrich F, Burgeson RE, Amano S, Hudson DL, Owaribe K, McGrath JA, McMillan JR, Eady RA, Leigh IM, Christiano AM, Uitto J: Loss of plectin causes epidermolysis bullosa with muscular dystrophy: cDNA cloning and genomic organization. *Genes Dev* 1996, 10:1724–1735
- Wiche G: Role of plectin in cytoskeleton organization and dynamics. *J Cell Sci* 1998, 111:2477–2486
- Smith FJ, Eady RA, Leigh IM, McMillan JR, Rugg EL, Kelsell DP, Bryant SP, Spurr NK, Geddes JF, Kirtschig G, Milana G, de Bono AG, Owaribe K, Wiche G, Pulkkinen L, Uitto J, McLean WH, Lane EB: Plectin deficiency results in muscular dystrophy with epidermolysis bullosa. *Nat Genet* 1996, 13:450–457
- Andrá K, Lassmann H, Bittner R, Shorny S, Fässler R, Propst F, Wiche G: Targeted inactivation of plectin reveals essential function in maintaining the integrity of skin, muscle, and heart cytoarchitecture. *Genes Dev* 1997, 11:3143–3156
- Schaapveld RQ, Borradori L, Geerts D, van Leusden MR, Kuikman I, Nievers MG, Niessen CM, Steenbergen RD, Snijders PJ, Sonnenberg A: Hemidesmosome formation is initiated by the beta4 integrin subunit, requires complex formation of beta4 and HD1/plectin, and involves a direct interaction between beta4 and the bullous pemphigoid antigen 180. *J Cell Biol* 1998, 142:271–284
- Hijikata T, Murakami T, Imamura M, Fujimaki N, Ishikawa H: Plectin is a linker of intermediate filaments to Z-discs in skeletal muscle fibers. *J Cell Sci* 1999, 112:867–876
- Nikolic B, MacNulty E, Mir B, Wiche G: Basic amino acid residue cluster within nuclear targeting sequence motif is essential for cytoplasmic plectin-vimentin network junctions. *J Cell Biol* 1996, 134:1455–1467
- Reznicek GA, de Pereda JM, Reipert S, Wiche G: Linking integrin alpha6beta4-based cell adhesion to the intermediate filament cytoskeleton: direct interaction between the beta4 subunit and plectin at multiple molecular sites. *J Cell Biol* 1998, 141:209–225
- Geerts D, Fontao L, Nievers MG, Schaapveld RQ, Purkis PE, Wheeler GN, Lane EB, Leigh IM, Sonnenberg A: Binding of integrin alpha6beta4 to plectin prevents plectin association with F-actin but does not interfere with intermediate filament binding. *J Cell Biol* 1999, 147:417–434
- Reipert S, Steinbock F, Fischer I, Bittner RE, Zeold A, Wiche G: Association of mitochondria with plectin and desmin intermediate filaments. *Exp Cell Res* 1999, 252:479–491
- Andrá K, Nikolic B, Stöcher M, Drenckhahn D, Wiche G: Not just scaffolding: plectin regulates actin dynamics in cultured cells. *Genes Dev* 1998, 12:3442–3451
- Fuchs P, Zorer M, Reznicek GA, Spazierer D, Oehler S, Castanon MJ, Hauptmann R, Wiche G: Unusual 5' transcript complexity of plectin isoforms: novel tissue-specific exons modulate actin binding activity. *Hum Mol Genet* 1999, 8:2461–2472
- Fine J, Bauer E, McGuire J, Moshell A: Epidermolysis bullosa. Clinical, Epidemiologic, and Laboratory Advances and the Findings of the National Epidermolysis Bullosa Registry. Baltimore and London, The Johns Hopkins University Press, 1999
- Pulkkinen L, Uitto J: Mutations analysis and molecular genetics of epidermolysis bullosa. *Matrix Biol* 1999, 18:29–42
- Rouan F, Pulkkinen L, Meneguzzi G, LaForgia S, Hyde P, Kim D, Richard G, Uitto J: Epidermolysis bullosa: novel and de novo premature termination codon and deletion mutations in the plectin gene predict late-onset muscular dystrophy. *J Invest Dermatol* 2000, 114:381–387
- Pulkkinen L, Smith FJ, Shimizu H, Murata S, Yaoita H, Hachisuka H, Nishikawa T, McLean WH, Uitto J: Homozygous deletion mutations in the plectin gene (PLEC1) in patients with epidermolysis bullosa simplex associated with late-onset muscular dystrophy. *Hum Mol Genet* 1996, 5:1539–1546
- Foisner R, Feldman B, Sander L, Seifert G, Artlieb U, Wiche G: A panel of monoclonal antibodies to rat plectin: distinction by epitope mapping and immunoreactivity with different tissues and cell lines. *Acta Histochem* 1994, 96:421–438
- Hayaishi T, Freiman M: Typing of Muscular Fibers. Muscle Biopsy: A Laboratory Investigation. Edited by M Laughlin. Oxford, Butterworth-Heinemann Ltd., 1993, pp 78–80
- Millonig G: Advantages of a phosphate buffer for OsO₄ solutions in fixation. *J Appl Physiol* 1961, 32:1637
- Humphrey CD, Pittman FE: A simple methylene-azure II-basic fuchsin stain for epoxy-embedded tissue sections. *Stain Technol* 1974, 49:9–14
- Venable JM, Coggeshall R: A simple lead citrate stain for use in electron microscopy. *J Cell Biol* 1965, 25:407–408
- Ganguly A, Rock M, Prockop D: Conformation-sensitive gel electrophoresis for rapid detection of single-base differences in double stranded PCR products and DNA fragments: evidence for solvent-induced bends in DNA heteroduplexes. *Proc Natl Acad Sci USA* 1993, 90:10325–10329
- Chomczynski P, Sacchi N: Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal Biochem* 1987, 162:156–159
- Vitelli L, Kemler I, Lauber B: Developmental regulation of micro-injected histone genes in sea urchin embryos. *Dev Biol* 1988, 127:54–63
- Banwell B, Russel J, Fukudome T, Shen X, Stilling G, Engel A: Myopathy, myasthenic syndrome, and epidermolysis bullosa simplex due to plectin deficiency. *J Neuropathol Exp Neurol* 1999, 58:832–846
- Mueller-Wieland D, van der Vorm E, Streicher R, Krone W, Seemanova E, Dreyer M, Rüdiger H, Rosipal S, Maassen J: An in-frame insertion in exon 3 and a nonsense mutation in exon 2 of the insulin receptor gene associated with severe insulin resistance in a patient with Rabson-Mendenhall syndrome. *Diabetologica* 1993, 36:1168–1174
- Hinney A, Becker I, Heibult O, Notteboom K, Schmidt A, Ziegler A, Mayer H, Siegfried W, Blum W, Remschmidt H, Hebebrand J: Systematic mutation screening of the pro-opiomelanocortin gene: identification of several genetic variants including three different insertions, one nonsense and two missense point mutations in probands of different weight extremes. *J Clin Endocrinol Metab* 1998, 83:3737–3741
- Jansen GA, Hogenhout EM, Ferdinandusse S, Waterham HR, Ofman R, Jakobs C, Skjeldal OH, Wanders RJ: Human phytanoyl-CoA hydroxylase: resolution of the gene structure and the molecular basis of Refsum's disease. *Hum Mol Genet* 2000, 9:1195–1200