

al., 1991). Various types of plectin transcripts, including rodless ones, that do not encode for a central rod domain have been reported (Elliott, et al., 1997).

Epidermolysis bullosa (EB) comprises a group of heterogeneous congenital disorders characterized by dermal-epidermal junction separation. EB is subdivided into the three major groups of EB simplex (EBS), junctional EB and dystrophic EB, and the one minor group of Kindler syndrome, based on the level of blister formation (Fine, et al., 2008). So far, mutations in 14 different genes have been identified as underlying the EB subtypes (Fine, et al., 2008; Groves, et al., 2010). Among them, mutations in the gene encoding plectin, *PLEC* (MIM# 601282), have been known to be causal for two subtypes of autosomal recessive EBS (EBS with muscular dystrophy (EBS-MD) and EBS with pyloric atresia (EBS-PA)) and for one subtype of autosomal dominant EBS (EBS-Ogna) (Fine, et al., 2008).

Characteristic manifestations of EBS-MD are generalized skin blistering and late onset muscle weakness. Previous studies revealed defective expression of plectin in EBS-MD skin samples (Gache, et al., 1996; Shimizu, et al., 1999a; Shimizu, et al., 1999b) and mutations in *PLEC* in EBS-MD patients (McLean, et al., 1996; Pulkkinen, et al., 1996; Smith, et al., 1996; Takizawa, et al., 1999). The *PLEC* mutations detected in EBS-MD patients are mainly within exon 31, which encodes the large-rod domain of plectin (Natsuga, et al., 2010; Pfindner, et al., 2005; Sawamura, et al., 2007).

In contrast to patients with EBS-MD, those with EBS-PA typically develop a more severe phenotype that includes more generalized blistering and pyloric atresia (PA) (Nakamura, et al., 2005). The prognosis of EBS-PA is very poor, and affected patients usually die within months after birth (Nakamura, et al., 2005; Pfindner, et al., 2005; Pfindner and Uitto, 2005). *PLEC* mutations of EBS-PA were mostly located outside of exon 31 (Natsuga, et al., 2010).

Although both EBS-MD and EBS-PA are autosomal recessive EBS caused by *PLEC* mutations, the pathomechanisms distinguishing two subtypes were unclear. Recently, our group and others demonstrated that EBS-MD patients typically express a rodless plectin isoform, although the full-length plectin is absent (Koster, et al., 2004; Natsuga, et al., 2010). In contrast, both full-length and rodless plectin isoforms are deficient in EBS-PA patients, leading to the more severe disease phenotype (Natsuga, et al., 2010). In light of these findings, it has been postulated that EBS-PA patients could develop muscular dystrophy (MD) if they survived longer (Natsuga, et al., 2010). However, to our knowledge, there have been no EBS patients who suffered from both MD and PA.

Here, we report the first patient with EBS who developed both PA and MD. Both of the mutations identified in the patient were within the last exon (exon 32) of *PLEC*. Immunofluorescence and immunoblot analysis confirmed diminished and truncated plectin expression, using several antibodies against different domains of plectin. This study gives further insight toward improving our understanding of the genotype-phenotype correlation in EBS patients with *PLEC* mutations.

## MATERIALS AND METHODS

### Electron Microscopy

Skin biopsy samples were fixed in 2% glutaraldehyde solution, post-fixed in 1% OsO<sub>4</sub>, dehydrated, and embedded in Epon 812. The samples were sectioned at 1 μm thickness for light microscopy and thin-sectioned for electron microscopy (70 nm thick). The thin sections were stained with uranyl acetate and lead citrate, and examined by transmission electron microscopy.

### Mutation Detection

Genomic DNA (gDNA) was isolated from peripheral blood leukocytes of the proband and her parents. The mutation detection was performed after polymerase chain reaction (PCR) amplification of all *PLEC* exons and intron-exon borders, followed by direct automated sequencing using an ABI PRISM 3100 genetic analyzer (Applied Biosystems, Foster City, CA). The oligonucleotide primers and PCR conditions used in this study were derived from a previous report (Nakamura, et al., 2005). The gDNA nucleotides, the complementary DNA (cDNA)

nucleotides and the amino acids of the protein, were numbered based on the GenBank sequence information (accession no. NM\_000445.3). PCR amplification of two parts of exon 32 was performed using the following primers. Primers 5'-GTGGAGACCACGCAGGTGTAC-3' and 5'-GGAGCCCGTGCCATAGAGG-3' for a single part of exon 32 synthesized a 420-bp fragment including c.10735 to c.11154. Primers 5'-AGCGGCTGACTGTGGATGAGG-3' and 5'-TGCGTGCCTTGTTGAGGT-3' for another single part of exon 32 synthesized a 283-bp fragment including c.11230 to c.11512. Both of the mutations in the proband were confirmed by restriction digestion of PCR products. c.10984C>T and c.11453\_11462del caused the generation of new restriction enzyme sites for *BsrI* and *BbvCI*, respectively.

The mutation nomenclature follows the journal's guidelines ([www.hgvs.org/mutnomen](http://www.hgvs.org/mutnomen)) according to the reference sequence NM\_000445.3, with +1 as the A of the ATG initiation codon.

### Haplotype analysis

Genotype analysis of this family to establish the *de novo* nature of c.11453\_11463del in the proband was performed using three chromosome 8 markers (D8S272, D8S264, D8S270) and six non-chromosome 8 markers (D1S468, D1S252, D1S2842, D3S1297, D3S1566 and D3S1311). All microsatellite markers (ABI Prism Linkage Mapping Set Version 2.5; Applied Biosystems, Warrington, UK) were amplified with fluorescently labeled oligonucleotides and used under conditions recommended by the manufacturer. Electrophoretic analysis was performed on an ABI Prism 310 Genetic Analyzer with Performance Optimized Polymer 4 (POP4) using GeneScan software (Applied Biosystems). The allele sizes were analyzed using Genotyper software (Applied Biosystems).

### Immunofluorescence Studies

Immunofluorescence analysis was performed using skin specimens from the proband as previously described (Natsuga, et al., 2010). Briefly, fresh-frozen skin specimens were embedded in optimal cutting temperature (OCT) compound and quickly frozen in isopentane cooled over liquid nitrogen. 5- $\mu$ m cryostat sections were incubated with primary antibodies. After washing in phosphate-buffered saline, the sections were incubated with secondary antibodies conjugated with fluorescein-isothiocyanate.

### Antibodies

The following antibodies against basement membrane zone (BMZ) components were used: monoclonal antibody (mAb) PN643 against the N-terminal actin-binding domain of plectin; mAb HD1-121 against the rod domain of plectin; C20 and mAb PC-815 against the C-terminal globular domain of plectin (Fig. 1A); mAbs GoH3 and 3E1 (Chemicon International, CA) against  $\alpha$ 6 and  $\beta$ 4 integrins, respectively; mAb GB3 (Sera-lab, Cambridge, UK) against laminin 332; mAb LH7.2 (Sigma, St. Louis, MO) against type VII collagen; mAb PHM-12<sup>+</sup>CIV22 against type IV collagen (NeoMarkers, Fremont, CA); and S1193 and mAb HDD20 against BP230 and type XVII collagen, respectively. mAbs PN643, HD1-121 and PC815 were generously donated by Prof. K. Owaribe of Nagoya University, and antibody S1193 by Prof. J. R. Stanley of the University of Pennsylvania. C20, a goat polyclonal antibody against the C-terminus of plectin, was purchased from Santa Cruz. Anti-beta-actin mAb (AC15, Sigma, St. Louis, MO) was used to confirm equal protein loading.

### Cell Culture and Immunoblot Analysis

Cell culture and immunoblot analysis was performed as previously described (Natsuga, et al., 2010). Cultured fibroblasts were obtained from skin biopsies of a normal human volunteer and the proband. Cultured fibroblasts were maintained in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) fetal bovine serum. For sample preparation, cultured cells were lysed in Nonidet-40 (NP-40) containing buffer (1% NP-40, 25mM Tris-HCl (pH 7.6), 4mM EDTA, 100mM NaCl, 1mM phenylmethylsulfonyl fluoride (PMSF), and proteinase inhibitor

cocktail (Sigma, St. Louis, MO)); cell debris was removed by centrifugation; and the supernatant was collected. Supernatants were boiled in Laemmli's sample buffer (Laemmli, 1970), applied to a 4–12% gradient Bis-Tris gel (Invitrogen, Carlsbad, CA), and transferred to a PVDF membrane. The membrane was incubated with PN643, HD1-121, C20 and AC15 followed by incubation with horseradish peroxidase (HRP) conjugated anti-mouse IgG (for PN643, HD1-121 and AC15) and HRP-conjugated anti-gout IgG (for C20). The blots were detected using ECL Plus Detection Kit (GE Healthcare, Fairfield, CT).

### Semi-quantitative RT-PCR Analysis

Semi-quantitative reverse transcription PCR (RT-PCR) analysis was performed as previously described (Natsuga, et al., 2010). Total RNA was isolated from cultured fibroblasts (from normal human volunteers and the proband, using RNeasy kit (Qiagen, Valencia, CA)), and first-strand cDNA was made using Superscript III reverse transcriptase (Invitrogen, Carlsbad, CA). First-strand cDNA was then amplified by PCR with primers specific for the exon boundaries flanking the rod domain of plectin as described previously (Koster, et al., 2004; Natsuga, et al., 2010). The following primers were used (Fig. 1B): 30F, 5'-CATCAGCGAGACTCTGCGGC-3'; 31R, 5'-TGCGCCTGTCGCTTTTGTGC-3'; 31F, 5'-AGCTGGAGATGAGCGCTGA-3'; 32R, 5'-TGCTGCAGCTCCTCCTGC-3'. To ensure equal loading, a housekeeping gene (GAPDH) was simultaneously amplified. The PCR products were assessed on a 2% agarose gel. The images were obtained with LAS-4000 mini (Fujifilm, Tokyo, Japan).

The medical ethics committee of Hokkaido University Graduate School of Medicine approved all of the described studies. The study was conducted according to The Declaration of Helsinki Principles. Participants gave their written informed consent.

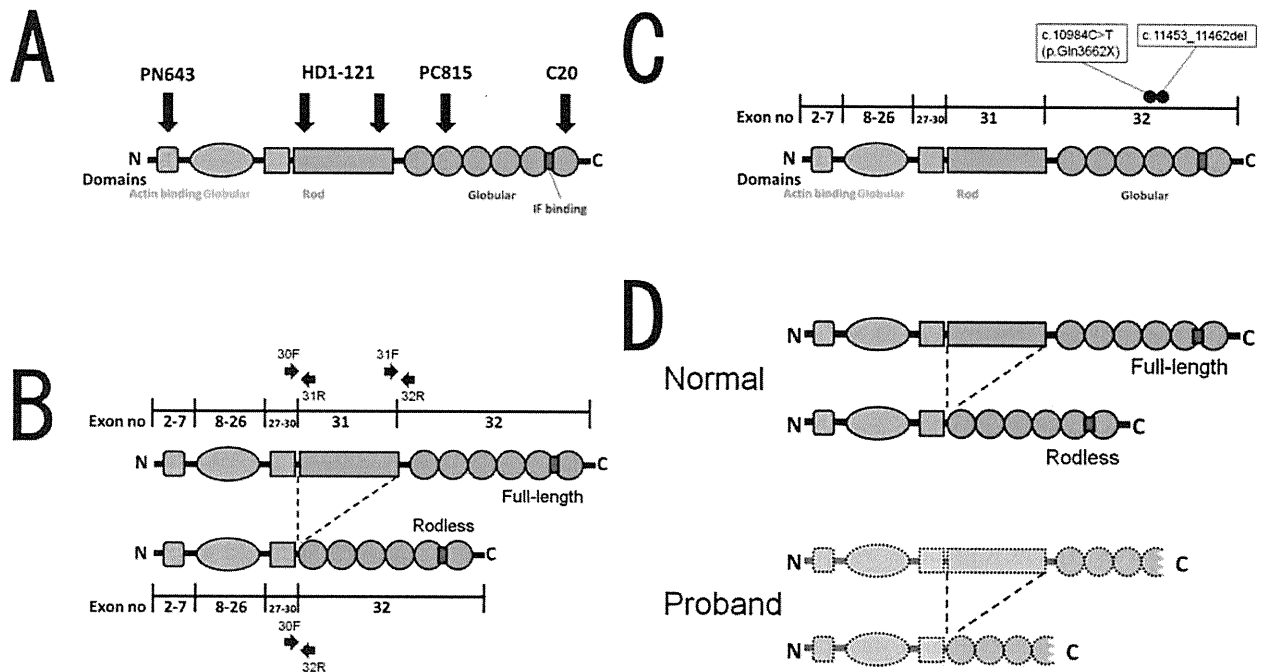
## RESULTS

### Case Description

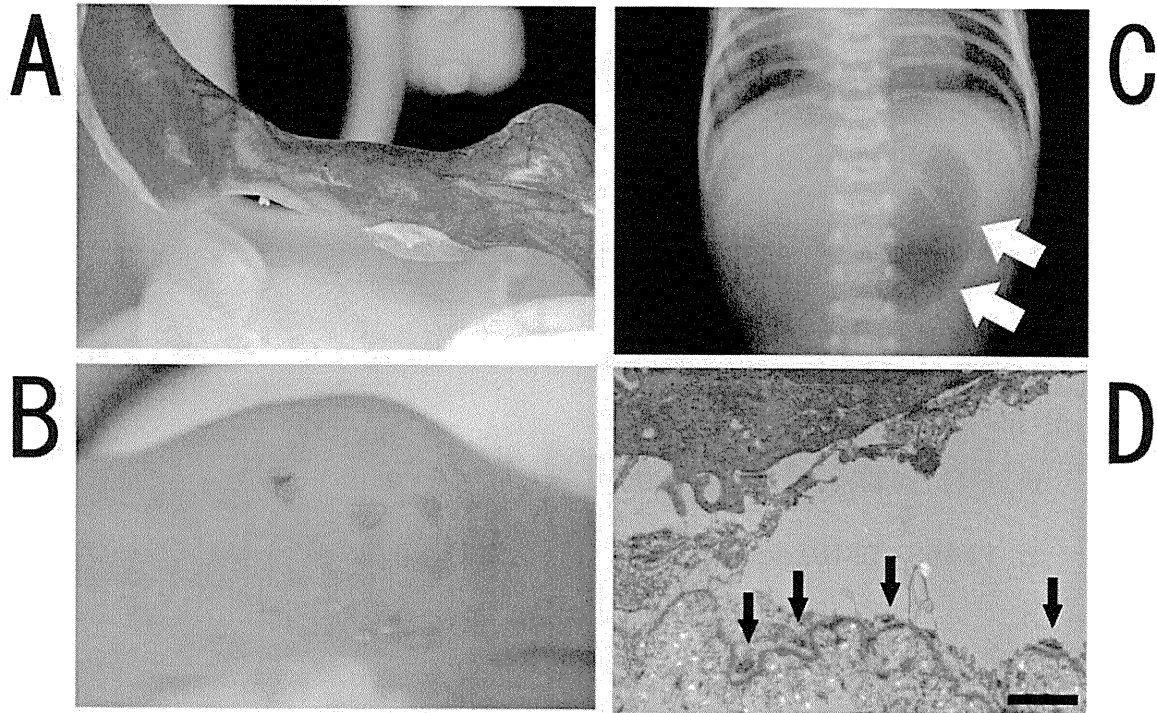
The proband was a first child of non-consanguineous Japanese parents. There was no family history of bullous diseases. He was born by cesarean section after a 39-week gestation because of non-reassuring fetal status. Clinically the proband showed extensive blistering and aplasia cutis on the extremities (Fig. 2A, B). Routine abdominal X-ray revealed a single bubble sign, suggesting the presence of PA (Fig. 2C). Generalized muscle hypotonia, dysphagia and difficulty in breathing were also observed from birth. Laboratory examination at birth revealed markedly elevated levels of creatine kinase (CK) (11,852U/L, normal value; 60-400U/L). The skeletal muscle isoform of CK (CK-MM) was 84% of total CK (CK, 2058U/L at age 12 days). Elevated levels of muscle enzymes including CK and aldolase (normal value; 1.7-5.7U/L) persisted over the course of his life (CK, 1924U/L; aldolase, 40.0U/L at age 25 days). Based on the clinical features and laboratory data, the presence of MD was confirmed. Muscle biopsy and reconstructive surgery for PA was not performed because the parents did not consent. The proband died 3 months after birth. Permission for autopsy was refused.

### Skin Separation in Basal Keratinocytes

Electron microscopy of the skin samples from the proband showed that the skin separation localized to the base of the basal keratinocytes (Fig. 2D). Hemidesmosomes were hypoplastic and found at the base of the intraepidermal split (Fig. 2D). Keratin clumps were not observed.



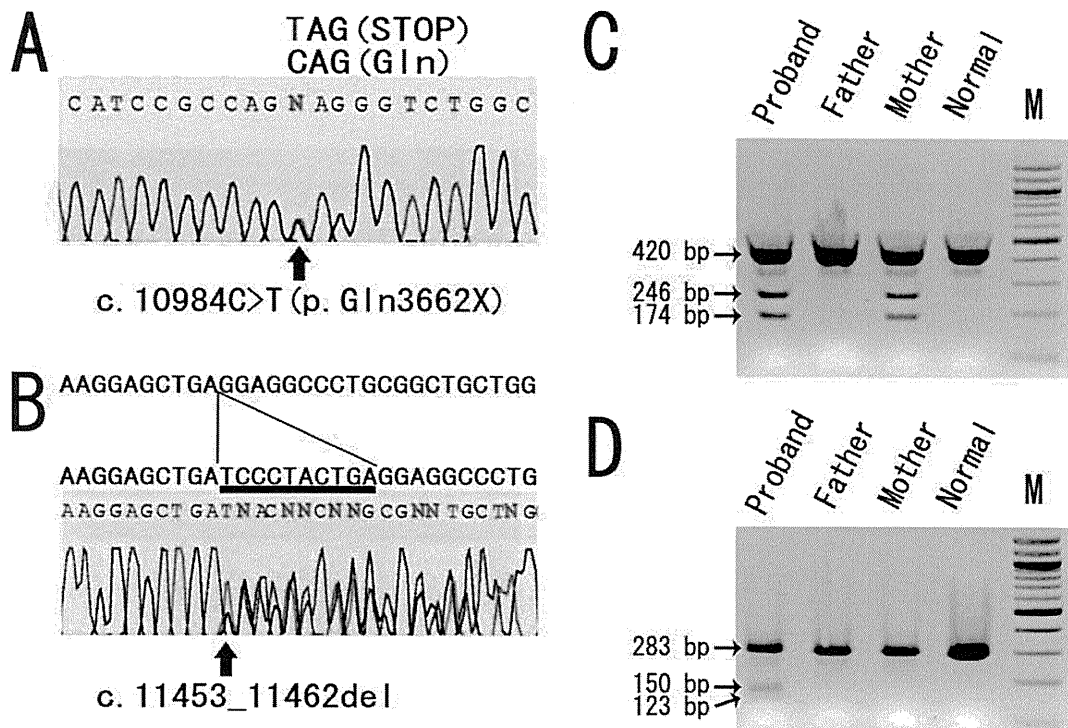
**Figure 1.** Plectin structure, antibodies against plectin, specific primers to amplify the full-length and the rodless plectin transcripts and *PLEC* mutations of the proband. (A) Plectin protein is composed of an actin-binding domain, N- and C-terminal globular domains, an intermediate filament (IF)-binding domain and a central rod domain. The C-terminal globular domain has 6 plectin repeat domains. The IF-binding domain is located between C-terminal repeats 5 and 6. PN643 is a monoclonal antibody (mAb) against the N-terminal actin-binding domain of plectin. HD1-121 is a mAb against the rod domain of plectin. PC815 is a mAb and C20 is a polyclonal antibody against the C-terminal globular domain of plectin. (B) The specific primers used to detect the presence of transcripts for full-length (30F/31R and 31F/32R) and rodless plectin (30F/32R) on cDNA synthesized from mRNA of normal humans and the proband's fibroblasts. (C) c.10984C>T and c.11453\_11462del are located in the *PLEC* encoding C-terminal plectin repeat 4. (D) Normal humans express both full-length and rodless plectin. In our case, the *PLEC* mutations produced diminished and truncated plectin protein without the IF-binding domain.



**Figure 2.** Clinical and ultrastructural features of the proband. (A) Aplasia cutis is observed on the left lower leg at birth. (B) Vesicles and erosions are scattered on the right knee. (C) Abdominal X-ray reveal single bubble sign (arrows), which indicated pyloric atresia. (D) Electron microscopy of the skin specimens from the proband reveals skin detachment within basal keratinocytes. Hemidesmosomes are hypoplastic and are observed at the base of the blisters (arrows) (Bar=1 $\mu$ m).

#### ***PLEC* Mutations in Exon 32**

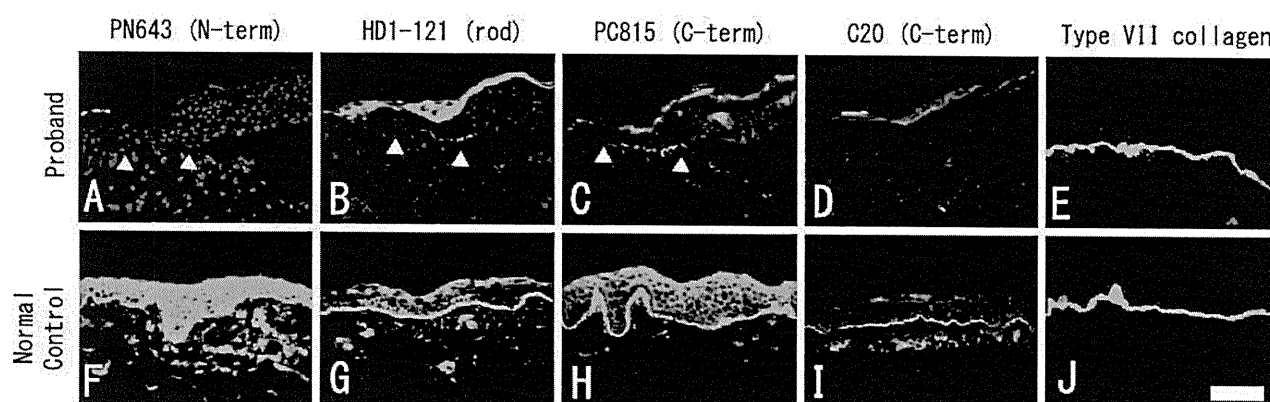
*PLEC* mutational analysis demonstrated that the proband was compound heterozygous for maternal c.10984C>T (p.Glu3662X) and de novo c.11453\_11462del in exon 32, the last exon of *PLEC* (Fig. 3A, 3B, 1C). The latter mutation is predicted to result in a frameshift that causes 88-amino-acid missense sequences followed by a premature termination codon (PTC). Both of the mutations were novel. c.10984C>T was confirmed by *BsrI* restriction enzyme digestion (Fig. 3C). c.11453\_11462del was also confirmed by *BbvCI* restriction enzyme digestion (Fig. 3D) and TA-cloning (data not shown). Haplotype analysis of this family using microsatellite markers excluded false paternity as well as false maternity (data not shown) to establish the de novo nature of c.11453\_11463del. The father's sperm has not been tested, although it might be beneficial to exclude the small possibility of paternal germ-line mosaicism through analyzing the father's sperm for any future prenatal diagnosis. In addition, c.7587G>A (p. =) transition in exon 32 was also detected in one allele of the proband and his father. This c.7587G>A transition was found in 3 of 100 normal unrelated alleles (50 healthy Japanese individuals), and was likely a polymorphism.



**Figure 3.** The novel *PLEC* mutations detected in the study. Maternal c.10984C>T (p.Gln3662X) (A) and de novo c.11453\_11462del (B) in exon 32 were found in genomic DNA derived from the proband. A thymine substituted for a cytosine in the former mutation is indicated by the red character (A). Deleted nucleotides in the latter mutation are underlined (B). (C) c.10984C>T mutation caused the generation of a site for *BsrI* restriction enzyme. *BsrI* digestion of the 420-bp PCR product with and without the mutation resulted in a single band of 420-bp and in double bands of 246-bp and 174-bp, respectively. c.10984C>T was a maternal mutation. (D) c.11453\_11462del caused the generation of a site for *BbvCI*. The 283-bp PCR product without the mutation was not digested by *BbvCI*. *BbvCI* digestion of the 273-bp PCR product with the deletion mutation showed two bands of 150 and 123-bp. c.11453\_11462del was not detected in the parents' gDNA.

#### Diminished and Truncated Plectin Expression in Skin

We performed immunofluorescence analysis of the skin specimens from the proband using several antibodies that react with molecules of the dermo-epidermal junction (DEJ). To check plectin expression patterns in the skin specimens from the proband, we used four antibodies: PN643 (N-terminal globular domain), HD1-121 (rod domain), PC815 (C-terminal globular domain) and C20 (C-terminal globular domain) (Fig. 1A). Normal human control shows bright DEJ staining of all the antibodies tested (Fig. 4F-I). DEJ labeling of PN643, HD1-121 and PC815 was markedly diminished in the skin specimens from the proband (Fig. 4A-C). Staining of C20 was absent in the proband's skin (Fig. 4D). Immunostaining for type VII collagen (Fig. 4E), laminin 332, type IV collagen, type XVII collagen,  $\alpha 6$  and  $\beta 4$  integrin, and BP230 revealed normal DEJ labeling patterns (data not shown).



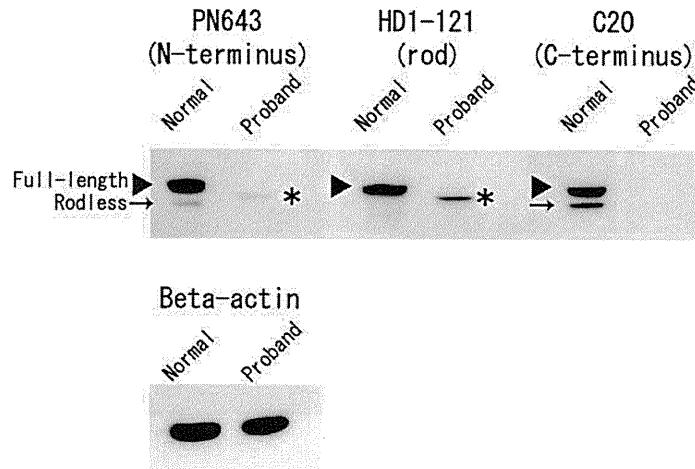
**Figure 4.** Immunofluorescence analysis of the proband's skin sample. In normal human skin, immunofluorescence shows that all of the antibodies against plectin (PN643, HD1-121, PC815 and C20) tested in this study bound to the dermal epidermal junction (DEJ) (F - I). DEJ labeling of PN643, HD1-121 and PC815 are weakly positive in the proband (A - C). In contrast, staining with C20 is negative in the proband's skin sample (D). Type VII collagen shows normal linear labeling in the proband and in the normal control (E, J). Weak labeling is indicated by arrowheads (Bar=100 $\mu$ m).

#### Diminished and Truncated Plectin in Cultured Fibroblasts

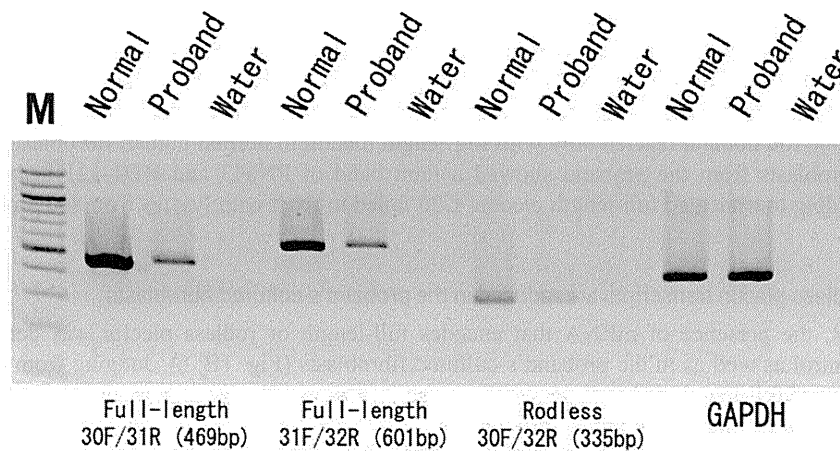
Immunoblot analysis of lysates from normal human cultured fibroblasts revealed that two closely spaced bands, corresponding to two forms of plectin (500kDa full-length and 390kDa rodless), reacted with PN643 and C20 antibodies recognizing the N- and C-termini of plectin (Fig. 5), as previously described (Natsuga, et al., 2010). HD1-121 against the rod domain reacted only with full-length plectin in normal human fibroblasts (Fig. 5). Lysates from cultured fibroblasts from the proband showed a faint band of PN643 and HD1-121 between 500kDa and 390kD, corresponding to truncated full-length plectin. C20 failed to react with lysates from the proband's cells (Fig. 5).

#### Full-length and rodless plectin transcripts are reduced in the proband's cultured fibroblasts

Using RT-PCR, the presence of mRNA that encodes full-length or rodless plectin was demonstrated in the normal human control as well as in the proband's cultured fibroblasts (Fig. 1B, 6). Judging from the PCR analysis results, the quantity of full-length and rodless plectin transcripts was markedly reduced in the proband's fibroblasts compared with those of the normal human control (Fig. 6).



**Figure 5.** Immunoblot analysis of cultured fibroblasts from the normal human control and the proband. Immunoblot analysis of extracts from fibroblasts of the normal control and the proband by using PN643 against the N-terminal actin-binding domain, HD1-121 against the rod domain and C20 against the C-terminal plectin repeats. Rodless plectin (arrows), detected with PN643 and C20, migrates just below full-length plectin (arrowheads) in normal human fibroblasts. Using HD1-121, only full-length plectin is observed in the normal control. In contrast, fibroblasts of the proband contained smaller proteins than 500-kDa full-length plectin, the putatively truncated full-length plectin (asterisks), which was detected with PN643 and HD1-121. C20 did not react with lysates of the proband's fibroblasts. Equal protein loading was confirmed by reprobing with AC15 (anti-beta-actin antibody).



**Figure 6.** Semi-quantitative RT-PCR for full-length and rodless plectin transcripts. The quantity of full-length (30F/31R and 31F/32R) and rodless (30F/32R) plectin transcripts in the proband's cultured fibroblasts is reduced in comparison to those of the normal control. GAPDH mRNA expression was used as the loading control in these experiments. The negative control reaction (DNA-free water instead of cDNA) shows no PCR products. The molecular weight standard (lane M) is a 100-bp ladder.



## DISCUSSION

This is the first report of EB complicated with both MD and PA. Skin detachment within basal keratinocytes was demonstrated by electron microscopy, which indicated the simplex subtype of EB. The proband's skin sample and cultured fibroblasts showed reduced and truncated plectin expression (Fig. 1D).

Both of the premature termination codon (PTC)-causing mutations detected in our case are within exon 32, the last exon of *PLEC*. Nonsense-mediated mRNA decay (NMD) is a quality-control mechanism that selectively degrades mRNAs with PTCs (Holbrook, et al., 2004). When the mRNA has a PTC more than a certain range upstream of one exon-exon junction, the transcript is down-regulated by NMD. In contrast, when PTCs are located in the last exon, NMD does not generally occur and abnormal mRNA is translated into truncated protein. However, some exceptions were described in a study in which transcripts underwent NMD despite having a PTC in the last exon (Chan, et al., 1998). Reduced amounts of full-length and rodless plectin transcripts in the proband's cells are explained by NMD, even though the PTCs of the proband are in the last exon.

One reported EBS-MD case was homozygous for a PTC-causing mutation (c.13458\_13473dup) in exon 32 (Schroder, et al., 2002). c.13458\_13473dup is at the downstream of the 6<sup>th</sup> plectin repeat and is predicted to cause a frameshift followed by a premature termination codon. The age of onset for MD in the patient with c.13458\_13473dup was 4 years (Schroder, et al., 2002); in our case, severe muscle weakness was observed immediately after birth. This clinical difference might be explained by the length of truncated proteins identified in each patient. Compound heterozygous mutations of c.10984C>T and c.11453\_11462del encode truncated plectin protein that does not include the intermediate filament (IF) binding site that was mapped to an approximately 50-amino-acid sequence between the 5<sup>th</sup> and 6<sup>th</sup> plectin repeat (Nikolic, et al., 1996; Rezniczek, et al., 2010). Therefore, the truncated plectin in our case might not have bound to IF including desmin in muscle tissues, which might account for the congenital muscle weakness. In contrast, the truncated plectin produced by c.13458\_13473dup harbors the IF-binding site described above. Although the amount of plectin protein was slightly diminished, it may be that substantial amounts of truncated plectin with the residual IF-binding site delayed the development of muscular dystrophy and prevented pyloric atresia in the previous patient (Schroder, et al., 2002).

EBS-MD patients do not have muscular symptoms at birth, but muscle weakness appears later in their life. The type of *PLEC* mutations (PTC-causing mutations or in-frame insertions/deletions) influences the timing of MD onset (Chiaverini, et al., 2010). Also, it may be that, in most EBS-MD cases, the presence of residual rodless plectin resulting from PTC-causing mutations in exon 31 delays the onset of MD because of the remaining IF-binding site in rodless plectin.

It has been postulated that two pathologic elements are involved in the development of PA in EB patients: 1) the integrity of basement membrane and hemidesmosomes, and 2) the control of the normal process of fibrosis in the course of wound healing (Maman, et al., 1998). The sequence of events might be initiated by the separation of the intestinal mucosal layer as a result of disintegration of basement membrane and hemidesmosomes. Inflammatory responses cause massive fibrosis, which might lead to the obstruction of the intestinal lumina, especially in anatomically narrow passages, such as pylorus (Maman, et al., 1998). Previously described EBS-MD patients do not suffer from PA, which suggests that residual rodless plectin can prevent the development of PA. In our case, both full-length and rodless plectin proteins are quantitatively reduced and the shortened plectin might not have functioned normally, which might have lead to the PA phenotype.

It had been predicted that some cases of EBS-PA would develop MD, although no such case had been reported in the literature (Natsuga, et al., 2010). One possible explanation is that the poor systemic condition of EBS-PA and the limited observation period due to the patient's very short lifespan prevented the diagnosis of MD. Our data suggests that surgical correction of PA is insufficient to treat EBS-PA patients because they would most likely go on to develop MD even if they survive surgery. Therefore, we should look to develop more fundamental therapeutic options for those patients.

In summary, this study clearly shows that plectin mutations lead to both MD and PA phenotypes in an individual EBS patient.

## ACKNOWLEDGMENTS

We thank Ms. Yuko Hayakawa and Ms. Yuki Miyamura for their technical assistance. This work was supported by Health and Labor Sciences Research grants for Research on Measures for Intractable Diseases from the Ministry of Health, Labor and Welfare of Japan (to H.S.).

Contract grant sponsor: The Ministry of Health, Labour and Welfare; Contract grant number: H20-Nanchi-Ippan-028.

## REFERENCES

- Chan D, Weng YM, Graham HK, Sillence DO, Bateman JF. 1998. A nonsense mutation in the carboxyl-terminal domain of type X collagen causes haploinsufficiency in schmid metaphyseal chondrodysplasia. *J Clin Invest* 101:1490-9.
- Chiaverini C, Charlesworth A, Meneguzzi G, Lacour JP, Ortonne JP. 2010. Epidermolysis bullosa simplex with muscular dystrophy. *Dermatol Clin* 28:245-55, viii.
- Elliott CE, Becker B, Oehler S, Castanon MJ, Hauptmann R, Wiche G. 1997. Plectin transcript diversity: identification and tissue distribution of variants with distinct first coding exons and rodless isoforms. *Genomics* 42:115-25.
- Fine JD, Eady RA, Bauer EA, Bauer JW, Bruckner-Tuderman L, Heagerty A, Hintner H, Hovnanian A, Jonkman MF, Leigh I and others. 2008. The classification of inherited epidermolysis bullosa (EB): Report of the Third International Consensus Meeting on Diagnosis and Classification of EB. *J Am Acad Dermatol* 58:931-50.
- Gache Y, Chavanas S, Lacour JP, Wiche G, Owaribe K, Meneguzzi G, Ortonne JP. 1996. Defective expression of plectin/HD1 in epidermolysis bullosa simplex with muscular dystrophy. *J Clin Invest* 97:2289-98.
- Groves RW, Liu L, Dopping-Hepenstal PJ, Markus HS, Lovell PA, Ozoemena L, Lai-Cheong JE, Gawler J, Owaribe K, Hashimoto T and others. 2010. A Homozygous Nonsense Mutation within the Dystonin Gene Coding for the Coiled-Coil Domain of the Epithelial Isoform of BPAG1 Underlies a New Subtype of Autosomal Recessive Epidermolysis Bullosa Simplex. *J Invest Dermatol* 130:1551-7.
- Holbrook JA, Neu-Yilik G, Hentze MW, Kulozik AE. 2004. Nonsense-mediated decay approaches the clinic. *Nat Genet* 36:801-8.
- Koster J, van Wilpe S, Kuikman I, Litjens SH, Sonnenberg A. 2004. Role of binding of plectin to the integrin beta4 subunit in the assembly of hemidesmosomes. *Mol Biol Cell* 15:1211-23.
- Laemmli UK. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227:680-5.
- Maman E, Maor E, Kachko L, Carmi R. 1998. Epidermolysis bullosa, pyloric atresia, aplasia cutis congenita: histopathological delineation of an autosomal recessive disease. *Am J Med Genet* 78:127-33.
- McLean WH, Pulkkinen L, Smith FJ, Rugg EL, Lane EB, Bullrich F, Burgeson RE, Amano S, Hudson DL, Owaribe K and others. 1996. Loss of plectin causes epidermolysis bullosa with muscular dystrophy: cDNA cloning and genomic organization. *Genes Dev* 10:1724-35.
- Nakamura H, Sawamura D, Goto M, Nakamura H, McMillan JR, Park S, Kono S, Hasegawa S, Paku S, Nakamura T and others. 2005. Epidermolysis bullosa simplex associated with pyloric atresia is a novel clinical subtype caused by mutations in the plectin gene (PLEC1). *J Mol Diagn* 7:28-35.
- Natsuga K, Nishie W, Akiyama M, Nakamura H, Shinkuma S, McMillan JR, Nagasaki A, Has C, Ouchi T, Ishiko A and others. 2010. Plectin expression patterns determine two distinct subtypes of epidermolysis bullosa simplex. *Hum Mutat* 31:308-16.
- Nikolic B, Mac Nulty E, Mir B, Wiche G. 1996. Basic amino acid residue cluster within nuclear targeting sequence motif is essential for cytoplasmic plectin-vimentin network junctions. *J Cell Biol* 134:1455-67.
- Pfendner E, Rouan F, Uitto J. 2005. Progress in epidermolysis bullosa: the phenotypic spectrum of plectin mutations. *Exp Dermatol* 14:241-9.
- Pfendner E, Uitto J. 2005. Plectin gene mutations can cause epidermolysis bullosa with pyloric atresia. *J Invest Dermatol* 124:111-5.

- Pulkkinen L, Smith FJ, Shimizu H, Murata S, Yaoita H, Hachisuka H, Nishikawa T, McLean WH, Uitto J. 1996. Homozygous deletion mutations in the plectin gene (PLEC1) in patients with epidermolysis bullosa simplex associated with late-onset muscular dystrophy. *Hum Mol Genet* 5:1539-46.
- Rezniczek GA, Walko G, Wiche G. 2010. Plectin gene defects lead to various forms of epidermolysis bullosa simplex. *Dermatol Clin* 28:33-41.
- Sawamura D, Goto M, Sakai K, Nakamura H, McMillan JR, Akiyama M, Shirado O, Oyama N, Satoh M, Kaneko F and others. 2007. Possible involvement of exon 31 alternative splicing in phenotype and severity of epidermolysis bullosa caused by mutations in PLEC1. *J Invest Dermatol* 127:1537-40.
- Schroder R, Kunz WS, Rouan F, Pfindner E, Tolksdorf K, Kappes-Horn K, Altenschmidt-Mehring M, Knoblich R, van der Ven PF, Reimann J and others. 2002. Disorganization of the desmin cytoskeleton and mitochondrial dysfunction in plectin-related epidermolysis bullosa simplex with muscular dystrophy. *J Neuropathol Exp Neurol* 61:520-30.
- Shimizu H, Masunaga T, Kurihara Y, Owaribe K, Wiche G, Pulkkinen L, Uitto J, Nishikawa T. 1999a. Expression of plectin and HD1 epitopes in patients with epidermolysis bullosa simplex associated with muscular dystrophy. *Arch Dermatol Res* 291:531-7.
- Shimizu H, Takizawa Y, Pulkkinen L, Murata S, Kawai M, Hachisuka H, Uono M, Uitto J, Nishikawa T. 1999b. Epidermolysis bullosa simplex associated with muscular dystrophy: phenotype-genotype correlations and review of the literature. *J Am Acad Dermatol* 41:950-6.
- Smith FJ, Eady RA, Leigh IM, McMillan JR, Rugg EL, Kelsell DP, Bryant SP, Spurr NK, Geddes JF, Kirtschig G and others. 1996. Plectin deficiency results in muscular dystrophy with epidermolysis bullosa. *Nat Genet* 13:450-7.
- Takizawa Y, Shimizu H, Rouan F, Kawai M, Uono M, Pulkkinen L, Nishikawa T, Uitto J. 1999. Four novel plectin gene mutations in Japanese patients with epidermolysis bullosa with muscular dystrophy disclosed by heteroduplex scanning and protein truncation tests. *J Invest Dermatol* 112:109-12.
- Wiche G, Becker B, Lubert K, Weitzer G, Castanon MJ, Hauptmann R, Stratowa C, Stewart M. 1991. Cloning and sequencing of rat plectin indicates a 466-kD polypeptide chain with a three-domain structure based on a central alpha-helical coiled coil. *J Cell Biol* 114:83-99.

Department of Health (AF0301). We thank Dr Michele Weiss for the figure illustration.

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#### REFERENCES

- Davies H, Bignell GR, Cox C *et al.* (2002) Mutations of the BRAF gene in human cancer. *Nature* 417:949–54
- Flaherty K, Puzanov I, Sosman J *et al.* (2009) Phase I study of PLX4032: proof of concept for V600E BRAF mutation as a therapeutic target in human cancer. *J Clin Oncol* 27: 155 (Abstract)
- Halaban R, Zhang W, Bacchiocchi A *et al.* (2010) PLX4032, a selective BRAF V600E kinase inhibitor, activates the ERK pathway and enhances cell migration and proliferation of BRAF WT melanoma cells. *Pigment Cell Melanoma Res* 23: 190–200
- Hall-Jackson CA, Evers PA, Cohen P *et al.* (1999) Paradoxical activation of Raf by a novel Raf inhibitor. *Chem Biol* 6:559–68
- Hatzivassiliou G, Song K, Yen I *et al.* (2010) RAF inhibitors prime wild-type RAF to activate the MAPK pathway and enhance growth. *Nature* 464:431–5
- Heidorn SJ, Milagre C, Whittaker S *et al.* (2010) Kinase-dead BRAF and oncogenic RAS cooperate to drive tumor progression through CRAF. *Cell* 140:209–21
- King AJ, Patrick DR, Batorsky RS *et al.* (2006) Demonstration of a genetic therapeutic index for tumors expressing oncogenic BRAF by the kinase inhibitor SB-590885. *Cancer Res* 66:11100–5
- Michaloglou C, Vredeveld LC, Mooi WJ *et al.* (2008) BRAF(E600) in benign and malignant human tumours. *Oncogene* 27:877–95
- Poulikakos PI, Zhang C, Bollag G *et al.* (2010) RAF inhibitors transactivate RAF dimers and ERK signalling in cells with wild-type BRAF. *Nature* 464:427–30
- Ritt DA, Monson DM, Specht SI *et al.* (2010) Impact of feedback phosphorylation and Raf heterodimerization on normal and mutant B-Raf signaling. *Mol Cell Biol* 30:806–19
- Rushworth LK, Hindley AD, O'Neill E *et al.* (2006) Regulation and role of Raf-1/B-Raf heterodimerization. *Mol Cell Biol* 26:2262–72
- Tsai J, Lee JT, Wang W *et al.* (2008) Discovery of a selective inhibitor of oncogenic B-Raf kinase with potent antimelanoma activity. *Proc Natl Acad Sci USA* 105:3041–6

## Complete Paternal Isodisomy of Chromosome 17 in Junctional Epidermolysis Bullosa with Pyloric Atresia

*Journal of Investigative Dermatology* (2010) 130, 2671–2674; doi:10.1038/jid.2010.182; published online 1 July 2010

#### TO THE EDITOR

Uniparental disomy (UPD) is a condition in which two chromosomes of the same pair are inherited in whole or in part from only one parent. There are two types of UPD: uniparental isodisomy and uniparental heterodisomy. The former refers to two identical copies of a single homolog of a chromosome from one parent, and the latter indicates two different chromosome homologs from one parent. UPD can lead to an abnormal phenotype when isodisomy for a chromosome carrying a mutation for an autosomal-recessive disease gene results in homozygosity for the mutation.

Epidermolysis bullosa (EB) is a collection of heterogeneous disorders, in which congenital skin fragility leads to separation of the dermo-epidermal junction. EB has been subdivided into three major groups and one minor group, based on the level of blister formation:

EB simplex, junctional EB, (JEB), dystrophic EB, and Kindler syndrome (Fine *et al.*, 2008). Mutations in 14 different genes have been identified as underlying EB subtypes (Fine *et al.*, 2008; Groves *et al.*, 2010). Among them, mutations in the gene encoding  $\alpha 6$  integrin subunit (*ITGA6*) or  $\beta 4$  integrin subunit (*ITGB4*) are responsible for one rare subtype of JEB: JEB associated with pyloric atresia (JEB-PA). JEB-PA is inherited autosomal recessively and is characterized by generalized blistering and occlusion of the pylorus at birth, which usually leads to early demise. Most patients with JEB-PA have mutations in *ITGB4*, and more than 60 *ITGB4* mutations have been identified in JEB-PA cases.

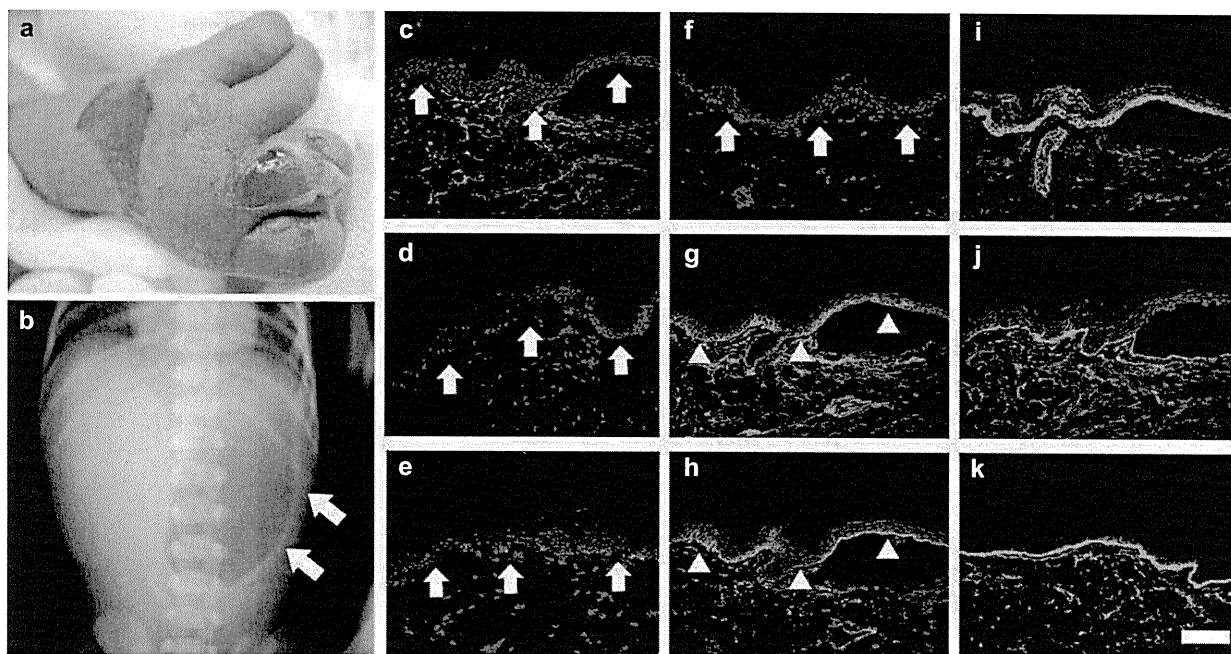
The proband was the first child of nonconsanguineous healthy parents. There was no family history of bullous diseases. The child was born by cesarean section after a 35-week gestation

because of polyhydramnion and had a birth weight of 1916 g and a birth length of 46.5 cm. Clinical manifestations of the proband included extensive blistering, especially on the extremities (Figure 1a). Routine abdominal X-ray demonstrated pyloric atresia (PA) (Figure 1b). No abnormalities other than skin fragility and PA were apparent. The proband died of sepsis 2 months after birth.

Immunofluorescence analysis revealed an absence of the  $\beta 4$  integrin subunit in skin specimens from the proband (Figure 1c–f). Expression of  $\alpha 6$  integrin subunit and plectin was reduced in proband skin samples (Figure 1g and h). Immunostaining for BP230, laminin 332, and type VII collagen revealed normal linear-labeling patterns (Figure 1i–k).

Mutational analysis of all coding exons (exons 2–41) including the exon–intron boundaries of the *ITGB4* revealed that the proband was homozygous for c.953\_955del in exon 8 (Figure 2a). The genomic DNA nucleotides, the

Abbreviations: EB, epidermolysis bullosa; JEB, junctional EB; JEB-PA, JEB with pyloric atresia; *ITGA6*,  $\alpha 6$  integrin subunit; *ITGB4*,  $\beta 4$  integrin subunit; UPD, uniparental disomy



**Figure 1. Clinical features of the proband and immunofluorescence analysis.** (a) Extensive blistering is seen on the extremities at birth. (b) Abdominal X-ray reveals a single-bubble sign (arrows).  $\beta 4$  integrin subunit (3E1 (c), 113C (d), 450-9D (e), and 450-11A (f)) is not detected in the proband's skin (arrows). The expression of  $\alpha 6$  integrin subunit (GoH3, g) and plectin (HD1-121, h) is diminished (arrowheads). BP230 (S1193, i), laminin-332 (GB3, j), and type VII collagen (LH7.2, k) show a normal linear staining pattern (scale bar = 100  $\mu$ m). HD1-121 was donated by Professor Owaribe of Nagoya University, 113C by Professor Sonnenberg of the Netherlands Cancer Institute, 450-9D and 450-11A by Professor Lankford of the Oak Ridge National Laboratory, and S1193 by Professor Stanley of the University of Pennsylvania.

complementary DNA nucleotides and the amino acids of the protein were numbered based on the following sequence information (GenBank accession no. NM\_000213). Mutation c.953\_955del is predicted to result in the loss of asparagine at amino acid position 318 (p.Asn318del, Figure 2b), and this deletion mutation is not expected to cause subsequent frame-shift followed by a premature termination codon. Mutation c.953\_955del was previously described in two JEB-PA cases (Iacovacci *et al.*, 2003; Varki *et al.*, 2006). The proband's father was heterozygous for this mutation (Figure 2a), although her mother revealed only normal sequences (Figure 2a).

To elucidate the origin of c.953\_955del homozygosity in the proband, haplotype analysis of the entire chromosome 17 using 15 microsatellite markers (the ABI Prism Linkage Mapping Set Version 2.5 (Applied Biosystems, Warrington, UK)) was performed. The proband was found to be homozygous for all 15 microsatellite markers (Figure 2d). Ten of the 15 markers were

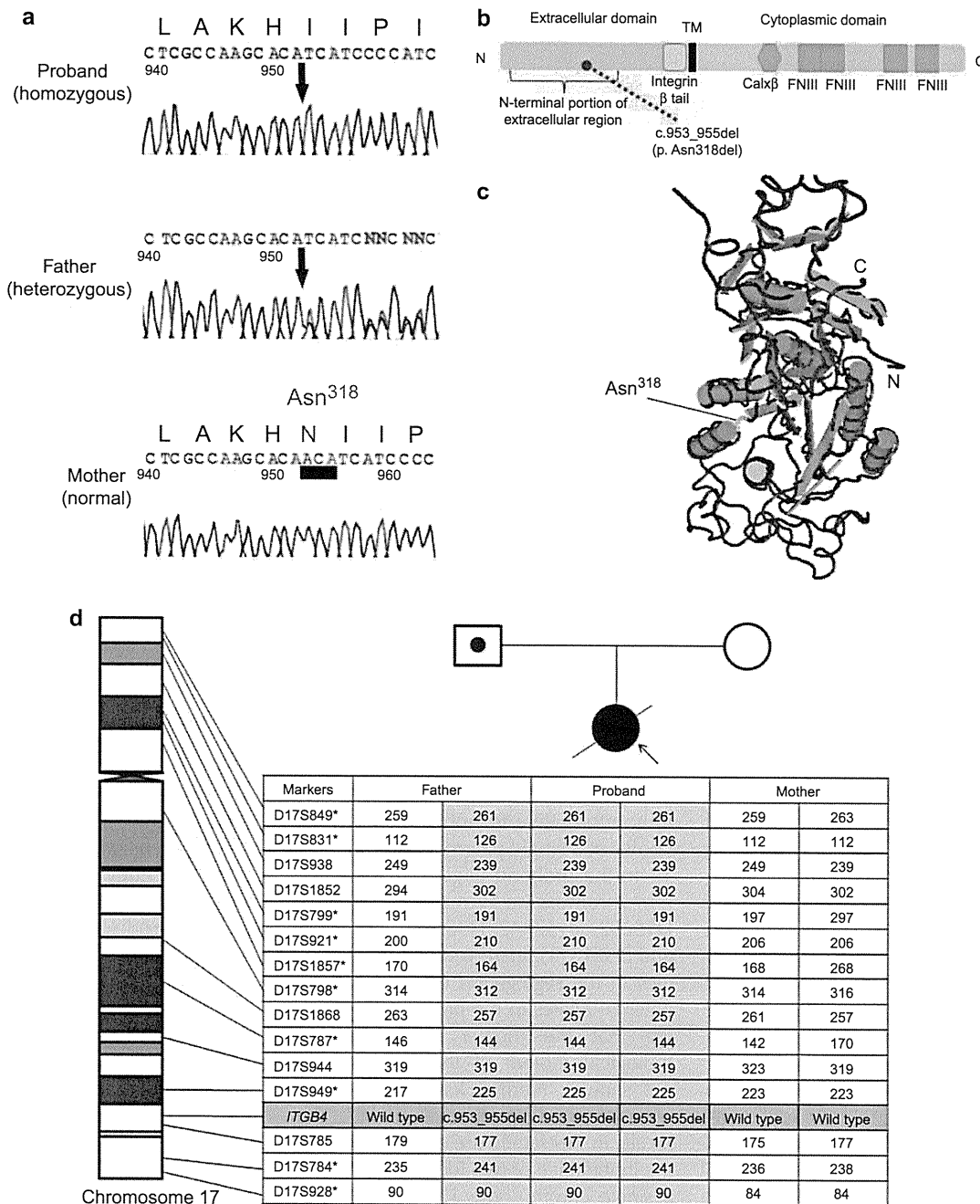
fully informative for inheritance of two copies of a single paternal chromosome 17 in the proband (Figure 2d). For the non-chromosome 17 markers (D1S468, D1S252, D1S2842, D3S1297, D3S1566 and D3S1311), there were no discrepancies in the segregation of maternal and paternal alleles to the proband, confirming that the mother is indeed the biological mother of the patient (data not shown). Normal karyotyping ruled out monosomy of chromosome 17. The results in this family are compatible with the inheritance of two identical copies of a single chromosome 17 from the proband's father, which indicates complete paternal isodisomy of chromosome 17 in the proband. The medical ethical committee of Hokkaido University approved all the described studies. The study was conducted according to the Declaration of Helsinki Principles. Participants gave their written informed consent.

A recent review of the literature on UPD summarized 197 maternal and 68 paternal cases of UPD (Kotzot and Utermann, 2005). For UPDs of chromosome 17, only a few cases of maternal

heterodisomy have been described (Genuardi *et al.*, 1999; Rio *et al.*, 2001). Recently, UPD of the whole chromosome 17 was reported as maternal heterodisomy of 17q and proximal 17p, and isodisomy of distal 17p (Lebre *et al.*, 2009). As far as we know, uniparental isodisomy of the whole chromosome 17 has not been described in the literature.

More than 35 cases of recessive-inherited disease have been reported as being caused by UPD (Kotzot and Utermann, 2005). UPD has been reported to be responsible for several EB subtypes, including Herlitz JEB (Castori *et al.*, 2008; Fassih *et al.*, 2005; Pulkkinen *et al.*, 1997; Takizawa *et al.*, 2000; Takizawa *et al.*, 1998), EB simplex with pyloric atresia (Nakamura *et al.*, 2005) and recessive dystrophic EB (Fassih *et al.*, 2006). So far, JEB-PA has not been described to result from UPD.

p.Asn318del (c.953\_955del) has been identified as responsible for JEB-PA in two reports (Iacovacci *et al.*, 2003; Varki *et al.*, 2006). Asn<sup>318</sup> in  $\beta 4$  integrin subunit resides in the extracellular



**Figure 2. *ITGB4* mutation analysis.** (a) The proband is homozygous for c.953\_955 del (arrow). The proband's father is heterozygous for c.953\_955 del (arrow). In contrast, the mother's gDNA shows only wild-type sequences. Deleted nucleotides are underlined. (b) Schematic arrangement of  $\beta 4$  integrin subunit and the positions of the mutation in the proband. (c) 3D imaging of the N-terminal extracellular domain of the integrin  $\beta$  chain (NCBI Conserved Domain Database (code: smart00187)) was done using Cn3D-4.1 software. Asn<sup>318</sup> is in the linking loop between the  $\alpha$  helix and the  $\beta$  sheet (arrow). (d) The proband was homozygous for all 15 microsatellite markers spanning all of chromosome 17. Ten of the 15 markers (\*) suggest that the proband's alleles originated from one homolog of paternal chromosome 17.

domain of the protein (Figure 2b). This asparagine residue is an amino acid that is conserved not only in the  $\beta 4$  integrin subunit of vertebrates but in all the human integrin- $\beta$  chains (Iacovacci *et al.*, 2003).

The 3D structure of the N-terminal portion of the extracellular domain indicates that Asn<sup>318</sup> consists of a linking loop between the  $\alpha$  helix and the  $\beta$  sheet (Figure 2c). It is possible that the loss of

this asparagine in the extracellular domain leads to significant conformational change and protein instability.

In summary, to our knowledge, we have reported the first case of complete

isodisomy of chromosome 17 and the first example of UPD underlying JEB-PA.

#### CONFLICT OF INTEREST

The authors state no conflict of interest.

#### ACKNOWLEDGMENTS

We thank Professor Jouni Uitto for his critical comments on the present case, Ms Yuko Hayakawa and Ms Yuki Miyamura for their technical assistance, and Mr Michael O'Connell for his proofreading. This work was supported in part by grants-in-aid from the Ministry of Education, Culture, Sports, Science and Technology to H. Shimizu (Kiban A 21249063).

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#### REFERENCES

- Castori M, Floriddia G, Pisaneschi E *et al.* (2008) Complete maternal isodisomy causing reduction to homozygosity for a novel LAMB3 mutation in Herlitz junctional epidermolysis bullosa. *J Dermatol Sci* 51:58–61
- Fassih H, Lu L, Wessagowit V *et al.* (2006) Complete maternal isodisomy of chromosome 3 in a child with recessive dystrophic epidermolysis bullosa but no other phenotypic abnormalities. *J Invest Dermatol* 126: 2039–43
- Fassih H, Wessagowit V, Ashton GH *et al.* (2005) Complete paternal uniparental isodisomy of chromosome 1 resulting in Herlitz junctional epidermolysis bullosa. *Clin Exp Dermatol* 30:71–4
- Fine JD, Eady RA, Bauer EA *et al.* (2008) The classification of inherited epidermolysis bullosa (EB): Report of the Third International Consensus Meeting on Diagnosis and Classification of EB. *J Am Acad Dermatol* 58:931–50
- Genuardi M, Tozzi C, Pomponi MG *et al.* (1999) Mosaic trisomy 17 in amniocytes: phenotypic outcome, tissue distribution, and uniparental disomy studies. *Eur J Hum Genet* 7:421–6
- Groves RW, Liu L, Dopping-Hepenstal PJ *et al.* (2010) A homozygous nonsense mutation within the dystonin gene coding for the coiled-coil domain of the epithelial isoform of BPAG1 underlies a new subtype of autosomal recessive epidermolysis bullosa simplex. *J Invest Dermatol* 130:1551–7
- Iacovacci S, Cicuzza S, Odorisio T *et al.* (2003) Novel and recurrent mutations in the integrin beta 4 subunit gene causing lethal junctional epidermolysis bullosa with pyloric atresia. *Exp Dermatol* 12:716–20
- Kotzot D, Utermann G (2005) Uniparental disomy (UPD) other than 15: phenotypes and bibliography updated. *Am J Med Genet A* 136:287–305
- Lebre AS, Moriniere V, Dunand O *et al.* (2009) Maternal uniparental heterodisomy of chromosome 17 in a patient with nephropathic cystinosis. *Eur J Hum Genet* 17: 1019–23
- Nakamura H, Sawamura D, Goto M *et al.* (2005) Epidermolysis bullosa simplex associated with pyloric atresia is a novel clinical subtype caused by mutations in the plectin gene (PLEC1). *J Mol Diagn* 7:28–35
- Pulkkinen L, Bullrich F, Czarnecki P *et al.* (1997) Maternal uniparental disomy of chromosome 1 with reduction to homozygosity of the LAMB3 locus in a patient with Herlitz junctional epidermolysis bullosa. *Am J Hum Genet* 61:611–9
- Rio M, Ozilou C, Cormier-Daire V *et al.* (2001) Partial maternal heterodisomy of chromosome 17q25 in a case of severe mental retardation. *Hum Genet* 108:511–5
- Takizawa Y, Pulkkinen L, Chao SC *et al.* (2000) Mutation report: complete paternal uniparental isodisomy of chromosome 1: a novel mechanism for Herlitz junctional epidermolysis bullosa. *J Invest Dermatol* 115: 307–11
- Takizawa Y, Pulkkinen L, Shimizu H *et al.* (1998) Maternal uniparental merodisomy in the LAMB3 region of chromosome 1 results in lethal junctional epidermolysis bullosa. *J Invest Dermatol* 110:828–31
- Varki R, Sadowski S, Pfendner E *et al.* (2006) Epidermolysis bullosa. I. Molecular genetics of the junctional and hemidesmosomal variants. *J Med Genet* 43:641–52

## CD44-Deficient Mice Do Not Exhibit Impairment of Epidermal Langerhans Cell Migration to Lymph Nodes after Epicutaneous Sensitization with Protein

*Journal of Investigative Dermatology* (2010) 130, 2674–2677; doi:10.1038/jid.2010.170; published online 24 June 2010

#### TO THE EDITOR

CD44 is a type I transmembrane protein that binds extracellular matrix nonsulfated glycosaminoglycan hyaluronan and has an important role in cell adhesion and migration (Isacke, 2002). Thus, CD44 is involved with leukocyte egress, tumor invasiveness, and metastasis (Isacke, 2002).

The role of CD44 in epidermal Langerhans cell (LC) migration to drain-

ing lymph nodes (LNs) was first evaluated by an antibody blocking system. Antibodies against CD44 epitopes inhibited emigration of LCs from the epidermis and prevented cultured LC binding to T-cell zones in LN-frozen sections (Weiss *et al.*, 1997). In a CD44-deficient mouse system, CD44 deficiency did not impair LC emigration from the epidermis, but significantly influenced their LN homing (Mummert

*et al.*, 2004). In recent years, there has been significant progress in understanding the characteristics and kinetics of LCs. It is known that there are two kinds of Langerin<sup>+</sup> dendritic cells (DCs) (definition of LCs): one resides in the epidermis and another resides in the dermis (Bursch *et al.*, 2007). They show different migration patterns to draining LNs after immunization. Dermal Langerin<sup>+</sup> DC migration peaks early at 24 hours, whereas peak migration of epidermal LC is delayed until

Abbreviations: DC, dendritic cell; LC, Langerhans cell; LN, lymph node; Th, T helper

## Plectin Expression Patterns Determine Two Distinct Subtypes of Epidermolysis Bullosa Simplex

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Communicated by Peter H. Byers

Received 30 June 2009; accepted revised manuscript 8 December 2009.

Published online 5 January 2010 in Wiley InterScience (www.interscience.wiley.com). DOI 10.1002/humu.21189

**ABSTRACT:** Plectin is a cytoskeletal linker protein that has a dumbbell-like structure with a long central rod and N- and C-terminal globular domains. Mutations in the gene encoding plectin (*PLEC1*) cause two distinct autosomal recessive subtypes of epidermolysis bullosa (EB): EB simplex with muscular dystrophy (EBS-MD), and EB simplex with pyloric atresia (EBS-PA). Here, we demonstrate that normal human fibroblasts express two different plectin isoforms including full-length and rodless forms of plectin. We performed detailed analysis of plectin expression patterns in six EBS-MD and three EBS-PA patients. In EBS-PA, expression of all plectin domains was found to be markedly attenuated or completely lost; in EBS-MD, the expression of the N- and C-terminal domains of plectin remained detectable, although the expression of rod domains was absent or markedly reduced. Our data suggest that loss of the full-length plectin isoform with residual expression of the rodless plectin isoform leads to EBS-MD, and that complete loss or marked attenuation of full-length and rodless plectin expression underlies the more severe EBS-PA phenotype. These results also clearly account for the majority of EBS-MD *PLEC1* mutation restriction within the large exon 31 that encodes the plectin rod domain, whereas EBS-PA *PLEC1* mutations are generally outside exon 31.

Hum Mutat 31:308–316, 2010. © 2010 Wiley-Liss, Inc.

**KEY WORDS:** *PLEC1*; basement membrane zone; skeletal muscle; mRNA decay; truncation

### Introduction

Plectin is a 500-kDa intermediate filament-binding protein that serves as a crosslinking element of the cytoskeleton to supply mechanical strength to cells and tissues [Wiche, 1998]. Plectin is expressed in a wide variety of tissues, including skin, striated

muscle, and gastrointestinal tract epithelia. Within the cutaneous epithelium, plectin is especially concentrated along the basal pole of basal keratinocytes, where it functions as a linker between the keratin intermediate filament cytoskeleton, hemidesmosomes, and the underlying basement membrane zone (BMZ) [Borradori and Sonnenberg, 1999]. Plectin interacts with  $\alpha 6$  and  $\beta 4$  integrins [Geerts et al., 1999; Litjens et al., 2003, 2005; Niessen et al., 1997a,b; Rezniczek et al., 1998; Schaapveld et al., 1998], BPAG2 [Koster et al., 2003], and periplakin [Boczonadi et al., 2007].

Epidermolysis bullosa (EB) comprises a group of heterogeneous disorders in which congenital skin fragility leads to dermal–epidermal junction separation. EB is subdivided into the three major groups of EB simplex, junctional EB, dystrophic EB, and the one minor group of Kindler syndrome, based on the level of blister formation [Fine et al., 2008]. So far, mutations in 13 different genes have been identified as underlying EB subtypes [Fine et al., 2000, 2008]. Among them, mutations in the gene encoding plectin, *PLEC1* (MIM# 601282), are responsible for two distinct types of autosomal recessive EBS (EBS with muscular dystrophy [EBS-MD] and EBS with pyloric atresia [EBS-PA]) and one subtype of autosomal dominant EBS (EBS-Ogna) [Fine et al., 2008]. Patients with EBS-Ogna are heterozygous for one amino acid substitution in the rod domain of plectin [Koss-Harnes et al., 2002]. EBS-Ogna is thought to be caused by plectin perturbation that results from dominant negative interference [Pfundner et al., 2005]. In contrast, homozygous or compound heterozygous loss-of-function mutations in *PLEC1* lead to EBS-MD or EBS-PA.

EBS-MD is characterized by generalized blistering and delayed onset of muscular dystrophy. Defective expression of plectin was found in patients with EBS-MD [Gache et al., 1996] and mutations in *PLEC1* were found to be responsible for the EBS-MD phenotype [McLean et al., 1996; Smith et al., 1996]. To date, more than 30 EBS-MD patients have been reported to have *PLEC1* mutations [Bauer et al., 2001; Chavanas et al., 1996; Dang et al., 1998; Koss-Harnes et al., 2004; Kunz et al., 2000; McMillan et al., 2007; Mellerio et al., 1997; Pfundner et al., 2005; Pulkkinen et al., 1996; Rouan et al., 2000; Sawamura et al., 2007; Takahashi et al., 2005; Takizawa et al., 1999]. Most reported *PLEC1* mutations in EBS-MD patients are located within exon 31 encoding the large rod domain of plectin [Pfundner et al., 2005; Sawamura et al., 2007]. In contrast to the phenotype seen in EBS-MD, clinical manifestations of EBS-PA are more severe and are characterized by more generalized blistering and pyloric atresia,

Additional Supporting Information may be found in the online version of this article.

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which frequently leads to early death in affected patients. Similarly, junctional EB with pyloric atresia (JEB-PA) has been known to be caused by the mutation in the gene encoding  $\alpha 6/\beta 4$  integrin (*ITGA6*; MIM# 147556; *ITGB4*; MIM# 147557), and about 60 *ITGA6* or *ITGB4* mutations have been described [Fine et al., 2008; Varki et al., 2006]. Recently, our group and others identified *PLEC1* mutations in eight patients with EBS-PA [Nakamura et al., 2005; Pfindner et al., 2005; Pfindner and Uitto, 2005; Sawamura et al., 2007]. EBS-MD and EBS-PA represent distinct clinical phenotypes, although both are caused by *PLEC1* mutations. The exact mechanisms that produce the clinical differences between EBS-MD and EBS-PA subtypes have not been elucidated, although it has been postulated that the severity of EBS patients with *PLEC1* mutations could be associated with alternative splicing of plectin [Sawamura et al., 2007; Sonnenberg and Liem, 2007].

The present study demonstrates that normal human fibroblasts express two different plectin isoforms: full-length plectin, and a shorter rodless plectin. In light of this finding, we collected skin samples and cultured cells from patients with EBS-MD and EBS-PA in which we precisely analyzed their expression levels of plectin using immunoblotting, immunofluorescence, and semiquantitative RT-PCR to determine the different pathogenic mechanisms underlying *PLEC1* mutations. Our data suggest that EBS-MD and EBS-PA exhibit different plectin expression patterns, and this study gives further insight toward improving our understanding of genotype-phenotype correlation in EBS patients with *PLEC1* mutations.

## Materials and Methods

### Patients and Mutation Detection

Nine EBS patients in whom *PLEC1* mutations had been confirmed were analyzed: six EBS-MD and three EBS-PA unrelated individuals (Table 1). *PLEC1* mutations in four EBS-MD and three EBS-PA cases were previously described in the literature [Kunz et al., 2000; Nakamura et al., 2005; Pulkkinen et al., 1996; Sawamura et al., 2007; Takizawa et al., 1999]. Patients EBS-MD1 and EBS-MD5 were newly identified cases in the present study.

EBS-MD1 was a 24-year-old Japanese female. She was the first child of nonconsanguineous, healthy parents. Generalized blistering and erosions of the skin were noted at birth, together with nail dystrophy. She had no history of pyloric atresia. At the age of 10, she developed muscular dystrophy. EBS-MD5 was a 7-year-old Croatian male. He was the second child of nonconsanguineous, healthy parents. His elder brother was healthy. He developed generalized blistering, including of the oral mucosal, and laryngeal

stridor, immediately after birth. Pyloric atresia was not observed. To date, he has not developed muscular dystrophy.

Genomic DNA (gDNA) was isolated from peripheral blood leukocytes (EBS-MD1 and her parents) or cultured fibroblasts (EBS-MD5). The mutation detection was performed after polymerase chain reaction (PCR) amplification of all *PLEC1* exons and intron-exon borders, followed by direct automated sequencing using an ABI PRISM 3100 genetic analyzer (Applied Biosystems, Foster City, CA). Oligonucleotide primers and PCR conditions used in this study were derived from a previous report [Nakamura et al., 2005]. The gDNA nucleotides, the complementary DNA (cDNA) nucleotides, and the amino acids of the protein, were numbered based on the previous sequence information (GenBank accession no. AH003623) [McLean et al., 1996].

The medical ethical committees of Hokkaido University, Keio University, and University Medical Center Freiburg approved all described studies. The study was conducted according to The Declaration of Helsinki Principles. Participants gave their written informed consent.

A schematic of plectin structure and *PLEC1* mutations detected in EBS patients in this study is shown in Figure 1A.

### Antibodies

The plectin domains where the antibodies used in this study react are summarized in Figure 1B. Mouse monoclonal antibodies (mAbs), PN643 against the actin-binding domain of plectin and PC815 against the C-terminal plectin repeats were prepared by immunizing mice with recombinant His-tagged fusion proteins. To produce recombinant proteins, the cDNAs that encode the actin-binding domain of plectin and C-terminal plectin repeats comprising amino acids 171–595 and 2,930–3,153 (GenBank accession no. AAB05428.1), respectively, were cloned into a pET32c vector. The resultant recombinant proteins were expressed in the *Escherichia coli* expression host BL21(DE3)pLysS and purified using a His-Bind column (Novagen, Madison, WI). Spleen cells derived from immunized mice were fused with mouse myeloma cells. Hybridomas producing antibodies against plectin were selected by immunofluorescent microscopy screening using normal human skin. Immunoblotting using cytoplasmic extracts from DJM-1 cells confirmed that both of the antibodies reacted with a 500-kDa protein.

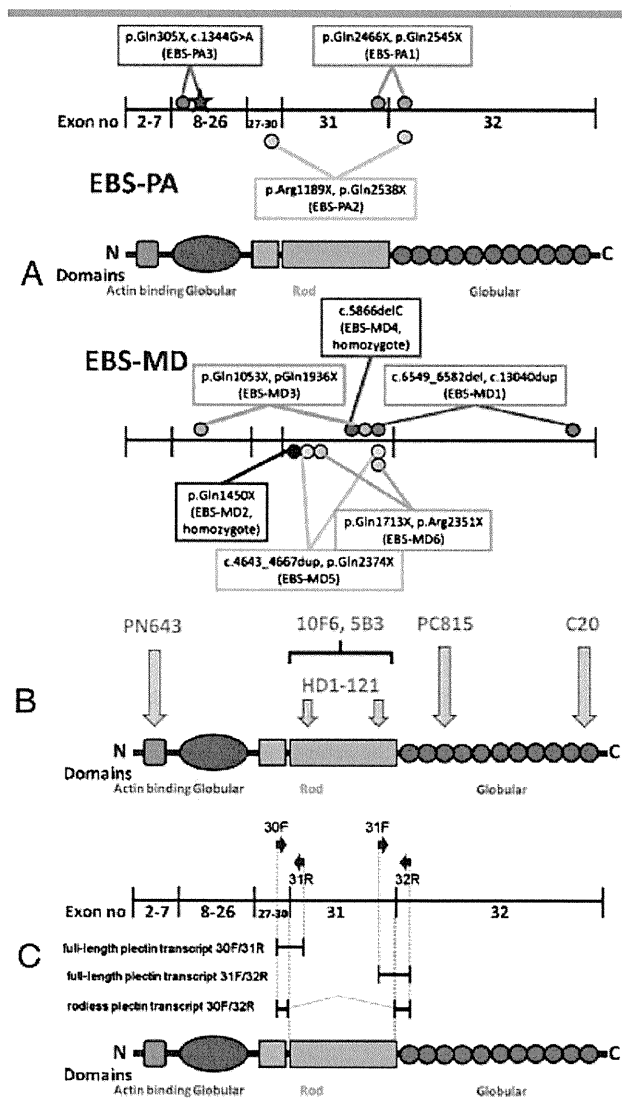
In addition to PN643 and PC815, the following mAbs against BMZ components were used: mAbs HD1-121 [Hieda et al., 1992; Okumura et al., 1999], 10F6 [Foisner et al., 1991], and 5B3 [Foisner et al., 1994] against the rod domain of plectin; mAb LH7.2 (Sigma, St. Louis, MO) against type VII collagen. mAb 10F6 and 5B3 were kind gifts from Dr. G. Wiche of the University of Vienna. C20, a goat polyclonal antibody against the C-terminus

**Table 1. EBS Patients and *PLEC1* Mutations**

Patient	Mutation 1 (predicted consequence)	Exon	Mutation 2 (predicted consequence)	Exon	Reference
EBS-MD1	<b>c.6549_6582del</b> (p. Ala2183fs)	31	<b>c.13040dup</b> (p. Gly4347fs)	32	Present case
EBS-MD2	c.4348C>T (p. Gln1450X)	31	c.4348C>T (p. Gln1450X)	31	Sawamura et al., 2007
EBS-MD3	c.3157C>T (p. Gln1053X)	24	c.5806C>T (p. Gln1936X)	31	Takizawa et al., 1999
EBS-MD4	c.5866del (p. Leu1956fs)	31	c.5866del (p. Leu1956fs)	31	Pulkkinen et al., 1996
EBS-MD5	<b>c.4643_4667dup</b> (p. Arg1556fs)	31	<b>c.7120C&gt;T</b> (p. Gln2374X)	31	Present case
EBS-MD6	c.5188C>T (p. Gln1713X)	31	c.7102C>T (p. Arg2351X)	31	Kunz et al., 2000
EBS-PA1	c.7396C>T (p. Gln2466X)	31	c.7633C>T (p. Gln2545X)	32	Sawamura et al., 2007
EBS-PA2	c.3565C>T (p. Arg1189X)	27	c.7612C>T (p. Gln2538X)	32	Nakamura et al., 2005
EBS-PA3	c.913C>T (p. Gln305X)	9	c.1344G>A (p. Gln447fs; =) <sup>a</sup>	12	Nakamura et al., 2005

The novel *PLEC1* mutations detected in this study are in bold. MD, Muscular dystrophy; del, deletion; dup, duplication; fs, frameshift.

<sup>a</sup>The mutation c.1344G>A is at the 3' end of exon12. Predicted consequences resulting from this mutation are discussed in the Results.



**Figure 1.** Scheme of plectin structure, PLEC1 mutations, antibodies against plectin and specific primers to detect the rodless transcript form of plectin. **A:** Plectin molecules consist of a central rod flanked by amino-terminal and carboxy-terminal globular domains. PLEC1 mutations observed in EBS patients of this study are indicated. Dots represent truncated mutations. The star indicates a splice-site mutation (c.1344G>A). **B:** PN643 is a monoclonal antibody (mAb) against the N-terminal actin-binding domain of plectin. HD1-121, 10F6, and 5B3 are mAbs against the rod domain of plectin. PC815 is a mAb and C20 is a polyclonal antibody against the C-terminal globular domain of plectin. **C:** Specific primers used to detect the presence of transcripts for full-length (30F/31R and 31F/32R) and rodless plectin (30F/32R) on cDNA synthesized from the mRNA of normal human, EBS-MD5 and EBS-PA3F cells.

of plectin, was purchased from Santa Cruz (Santa Cruz, CA). Anti-beta-actin mAb (AC15, Sigma) was used to confirm equal protein loading.

### Immunofluorescence Studies

Immunofluorescence analysis was performed using skin specimens from the patients (Table 1). Fresh-frozen skin specimens were embedded in optimal cutting temperature (OCT) compound and quickly frozen in isopentane cooled over liquid nitrogen.

5- $\mu$ m cryostat-cut sections were incubated overnight at 4°C with primary antibodies including the following mAbs: PN643 (working dilution of 1:160), HD1-121 (1:100), 10F6 (1:10), 5B3 (1:20), PC815 (1:20), and LH7.2 (1:10). After washing in phosphate-buffered saline, the sections were incubated with secondary antibodies conjugated with fluorescein-isothiocyanate.

### Cell Culture and Immunoblot Analysis

Cultured fibroblasts were obtained from skin biopsies of a normal human volunteer and of patient EBS-MD5. Cultured amniocytes were derived from an aborted fetus who was a sibling of EBS-PA3 (EBS-PA3F). Prenatal diagnosis of EBS-PA3F revealed that the fetus had the same *PLEC1* mutations as were detected in EBS-PA3 (data not shown). Cultured fibroblasts and amniocytes were maintained in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) fetal bovine serum. Cultured oral keratinocytes were obtained from biopsies of a normal human volunteer and were maintained in CnT-57 medium (CELLnTEC). Whole-cell lysates of human skeletal muscle were purchased from Abcam (ab82589). For sample preparation, cultured cells were lysed in Nonidet-40 (NP-40) containing buffer (1% NP-40, 25 mM Tris-HCl [pH 7.6], 4 mM EDTA, 100 mM NaCl, 1 mM phenylmethylsulfonyl fluoride [PMSF], and protease inhibitor cocktail [Sigma]) on ice for 30 min; cell debris was removed by centrifugation at 14,000 rpm for 15 min; and supernatant was collected. Supernatants were solubilized in Laemmli's sample buffer [Laemmli, 1970], applied to SDS-polyacrylamide gels, and transferred to a PVDF membrane. The membrane was incubated with PN643, HD1-121, C20, and AC15 overnight at 4°C followed by incubation with horseradish peroxidase (HRP) conjugated anti-mouse IgG (for PN643, HD1-121, and AC15) and HRP-conjugated anti-goat IgG (for C20) for 1 hr at room temperature. The blots were detected using ECL Plus Detection Kit (GE Healthcare, Fairfield, CT). The images were obtained with LAS-4000 mini (Fujifilm, Tokyo, Japan). To elucidate the quantitative ratio of full-length/rodless plectin, immunoblotting of lysates from normal human fibroblasts, keratinocytes, and skeletal muscle was performed in triplicate. Band intensities were analyzed by densitometry (ImageJ).

### Semiquantitative RT-PCR Analysis

Total RNA was isolated from cultured fibroblasts (normal human volunteers and EBS-MD5) or amniocytes (EBS-PA3F) using RNeasy kit (Qiagen, Valencia, CA), and first-strand cDNA was made using Superscript II reverse transcriptase (Invitrogen, Carlsbad, CA). First-strand cDNA was then amplified by PCR with primers specific for the exon boundaries flanking the rod domain of plectin as described previously [Koster et al., 2004] (Fig. 1C). The following primers were used in this study: 30F, 5'-CATCAGCGAGACTCTGCGGC-3'; 31R, 5'-TGCGCCTGTCGCTTTTGTGC-3'; 31F, 5'-AGCTGGAGATGAGCGCTGA-3'; 32R, 5'-TGCTGCAGCTCCTCCTGC-3'. PCR conditions were as follows: 94°C for 5 min, followed by 30 cycles (31F/32R, 30F/32R) and 35 cycles (30F/31R) at 94°C for 1 min, 62°C for 1 min, and 72°C for 1 min, and extension at 72°C for 7 min. To ensure equal loading, a housekeeping gene (GAPDH) was simultaneously amplified. The PCR products were assessed on a 2% agarose gel. The images were obtained with LAS-4000 mini (Fujifilm). To confirm the skipping of exon 31 in rodless transcript, direct sequencing was performed for the PCR products (30F/32R). To analyze transcripts derived from the c.1344G>A mutant allele,

PCR amplification of synthesized EBS-PA3F cDNA from exon 9 to exon 14 was performed using the following primers: 5'-GATTGAGATCTGTGGTCTC-3' and 5'-CTCTGCACACTCTGCAGAGT-3'. PCR products were cloned in the TA cloning vector pCRII (Invitrogen) and then sequenced.

## Results

### *PLEC1* Mutation Detection

*PLEC1* mutational analysis in case EBS-MD1 demonstrated that the affected patient was a compound heterozygote for the maternal c.13040dup mutation in exon 32 and the paternal c.6549\_6582del mutation in exon 31 (Supp. Fig. 1A). Both of the mutations resulted in a frameshift that caused 8- and 21-amino-acid missense sequences, respectively, followed by a premature termination codon (PTC). These mutations were novel, and they were confirmed by *MwoI* restriction enzyme digestion and TA-cloning, respectively (data not shown). In addition, the c.10453C>T (p.Arg3485Trp) transition in exon 32 was also detected in one allele of the patient and her mother. This c.10453C>T transition was not found by sequence analysis in 100 normal unrelated Japanese alleles (50 healthy unrelated Japanese individuals), and it was unlikely to be polymorphism, although the contribution of this missense mutation to the EB phenotype remains unclear.

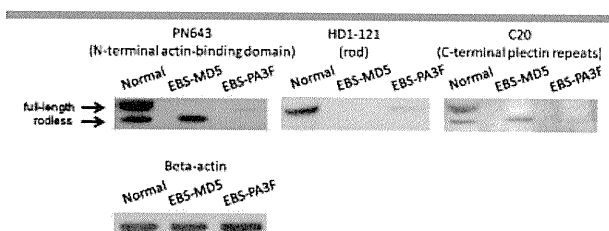
EBS-MD5 was a compound heterozygote for the c.4643\_4667dup and c.7120C>T (p.Gln2374X) mutations in exon 31 (Supp. Fig. 1B). The c.4643\_4667dup resulted in a frameshift that caused a 90-amino-acid missense sequence, followed by a PTC. These mutations were also novel and were confirmed by TA-cloning and *PstI* restriction enzyme digestion respectively (data not shown).

### Differential plectin isoform expression by immunoblotting in normal human fibroblasts

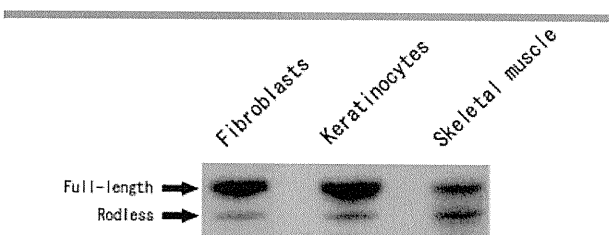
Immunoblot analysis of lysates from normal human cultured fibroblasts revealed that two closely spaced bands, putatively corresponding to two forms of plectin (500 kDa full-length and 390 kDa rodless form) reacted with PN643 and C20, antibodies recognizing the N- and C-termini of plectin (Fig. 2). Using HD1-121, an antibody against the rod domain of plectin, lysates from normal human fibroblasts reacted only with full-length plectin (Fig. 2). These results showed that normal human fibroblasts expressed two different *PLEC1* isoforms: full length and a shorter rodless plectin isoforms.

### The Quantitative Ratio of Full-length/Rodless Plectin in Normal Human Fibroblasts, Keratinocytes, and Skeletal Muscle

To elucidate the relative amount of full-length and rodless plectin in normal human fibroblasts, keratinocytes, and skeletal muscle, we performed immunoblot analysis of lysates from each sample using PN643, an antibody against the N-terminus of plectin. Both full-length and rodless plectin were detected in each sample (Fig. 3). Band intensities were measured in triplicate  $\pm$  SD. The quantitative ratio of full-length/rodless plectin was  $14.2 \pm 4.2$  in fibroblasts,  $21.3 \pm 6.4$  in keratinocytes, and  $1.37 \pm 0.23$  in skeletal muscle.



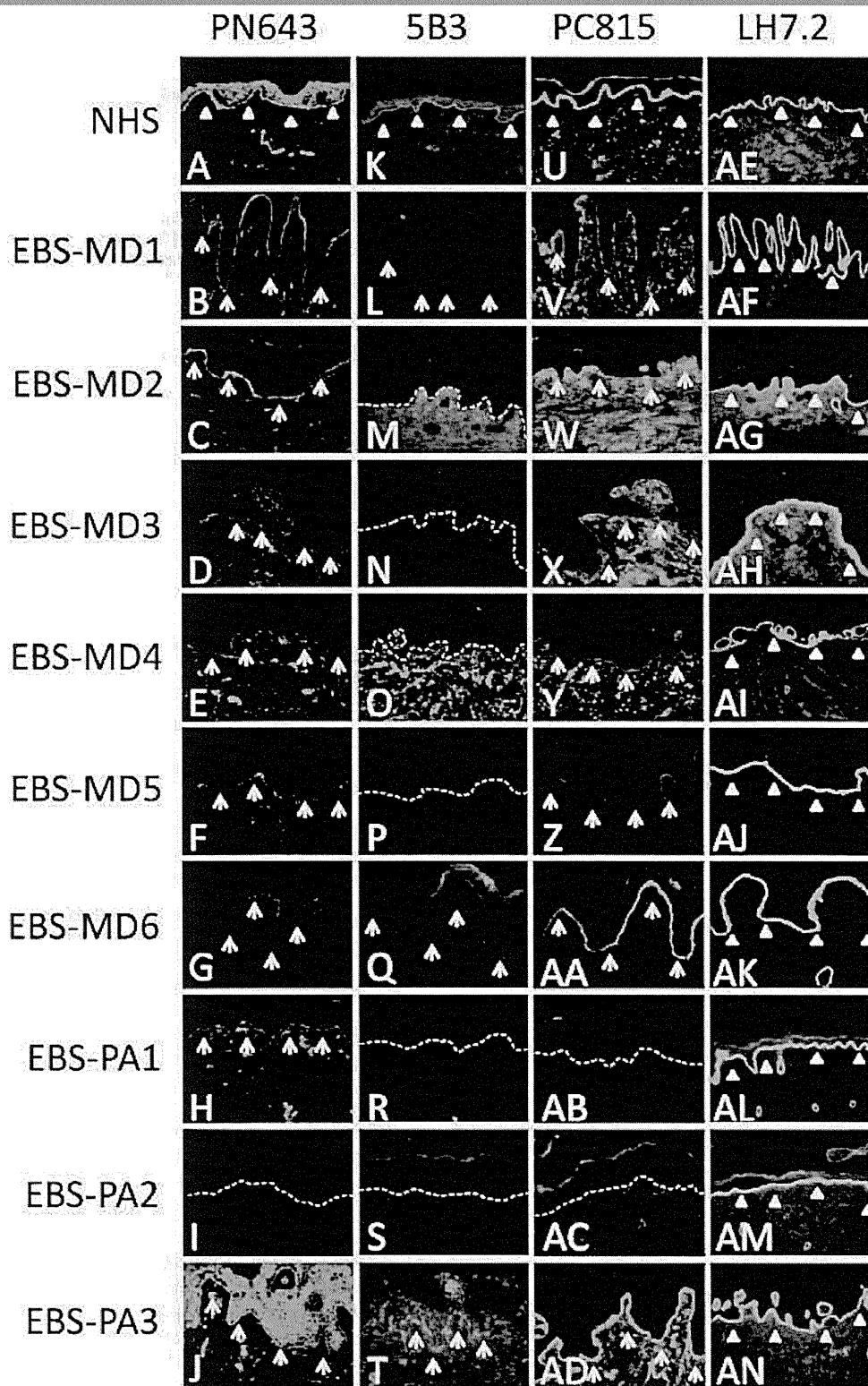
**Figure 2.** Immunoblot analysis of cultured fibroblasts from normal human control and EBS-MD and amniocytes from EBS-PA. Immunoblot analysis of extracts from cells of normal control, EBS-MD5, and an aborted sibling of EBS-PA3 (EBS-PA3F). Analysis used PN643 against the N-terminal actin-binding domain, HD1-121 against the rod domain and C20 against the C-terminal plectin repeats. Rodless plectin, detected with PN643 and C20, migrates just below full-length plectin in normal human fibroblasts. Using HD1-121, only full-length plectin was demonstrated in the normal control. In contrast, EBS-MD5 fibroblasts contained only rodless plectin, which was detected with PN643 and C20. Full-length plectin did not appear in EBS-MD5 using any antibody. EBS-PA3F amniocytes contained a greatly reduced amount of full-length plectin. Equal protein loading was confirmed by reprobing with AC15 (anti-beta-actin antibody). [Color figure can be viewed in the online issue, which is available at [www.interscience.wiley.com](http://www.interscience.wiley.com).]



**Figure 3.** Relative amounts of full-length and rodless plectin in normal human fibroblasts, keratinocytes and skeletal muscle. Immunoblot analysis of lysates from normal human fibroblasts, oral keratinocytes, and skeletal muscle using PN643 against the N-terminal actin-binding domain of plectin was performed. Both full-length and rodless plectin were detected in each sample. The quantitative ratio of the two isoforms was calculated, using Image J software, as follows:  $14.2 \pm 4.2$  in fibroblasts,  $21.3 \pm 6.4$  in keratinocytes, and  $1.37 \pm 0.23$  in skeletal muscle. [Color figure can be viewed in the online issue, which is available at [www.interscience.wiley.com](http://www.interscience.wiley.com).]

### Characterization of cutaneous plectin expression patterns in EBS-MD and EBS-PA patients by immunofluorescence analysis

To assess whether expression patterns of plectin in the skin differ between EBS-MD and EBS-PA, we performed immunofluorescence analysis using five different antibodies (Fig. 1B). PN643 weakly reacted with skin specimens from all EBS-MD patients and two out of three EBS-PA patients (EBS-PA1, 3), but failed to react with specimens from EBS-PA2 (Fig. 4A–J). HD1-121 showed weakly reactivity in three EBS-MD patients (EBS-MD1, 4, 6) and one EBS-PA patient (EBS-PA3), but was negative in the other patients (EBS-MD2, 3, 5, EBS-PA1, 2) (data not shown). 5B3, the mAb against the rod domain of plectin, was faint but identifiable in two EBS-MD patients (EBS-MD1, 6) and one EBS-PA patient (EBS-PA3), but was negative in the other patients (EBS-MD2-5, EBS-PA1, 2) (Fig. 4L–T). No skin specimens reacted with 10F6, a monoclonal antibody against the rod domain, except EBS-PA3 (data not shown). PC815 recognized the C-terminus of plectin weakly but detectably in all EBS-MD patients and EBS-PA3, but not in EBS-PA patients 1 and 2 (Fig. 4V–AD). These results



**Figure 4.** Immunofluorescence analysis of cutaneous plectin expression in EBS-MD and EBS-PA. In normal human skin (NHS), immunofluorescence shows that all mAbs against plectin (PN643, 5B3, and PC815) and type VII collagen (LH7.2) tested in the study bind to the dermal epidermal junction (DEJ) (A, K, U, AE). DEJ labeling of PN643 is weakly positive in all EBS-MD cases (B–G) and EBS-PA1, 3 (H, J), but negative in EBS-PA2 (I). DEJ labeling of 5B3 show faintly positive in all EBS-MD cases (B–G) and EBS-PA3 (L, Q, T), and negative in EBS-MD2–5 and EBS1, 2 (M–P, R, S). DEJ labeling using PC815 is weakly positive in all EBS-MD cases and EBS-PA3 (V–AA, AD), but negative in EBS-PA1, 2 (AB, AC). Type VII collagen shows normal linear labeling in all EBS cases (AF–AN). Strong staining is indicated by arrowheads. Weak labeling is indicated by arrows. Negative labeling is indicated by dotted lines.