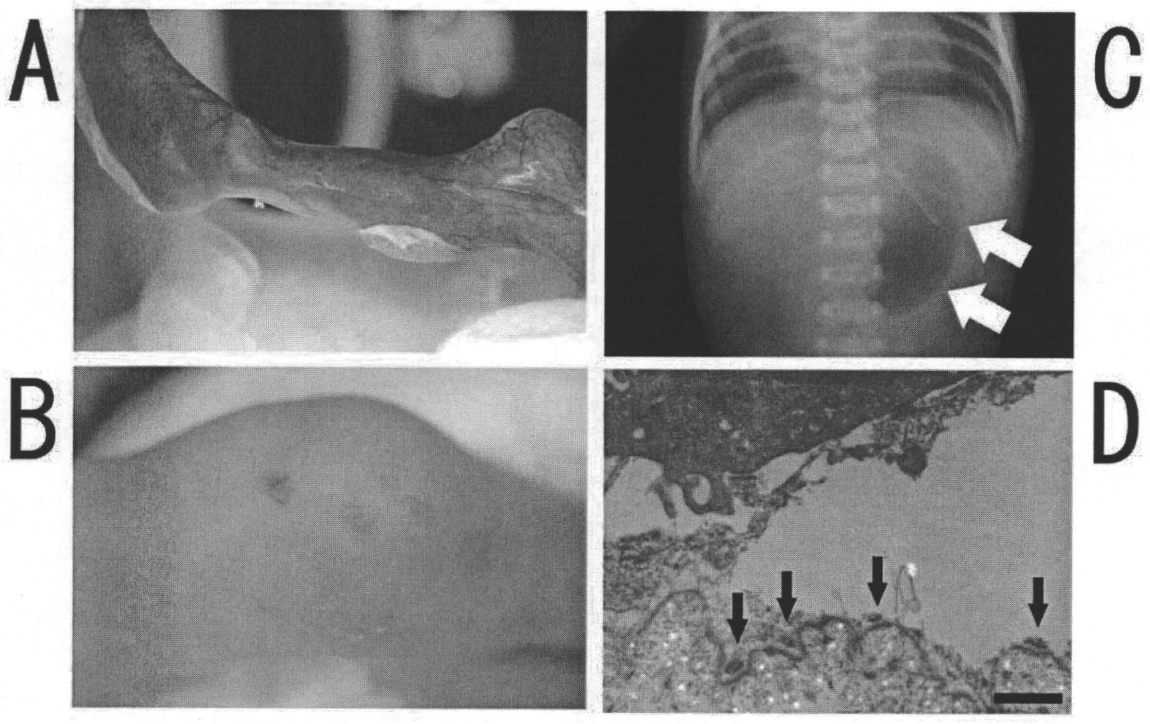

Figure 2. Clinical and ultrastructural features of the proband. (A) Aplasia cutis is observed on the left lower leg at birth. (B) Vesicles and erosions are scattered on the right knee. (C) Abdominal X-ray reveal single bubble sign (arrows), which indicated pyloric atresia. (D) Electron microscopy of 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=1μm).

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.

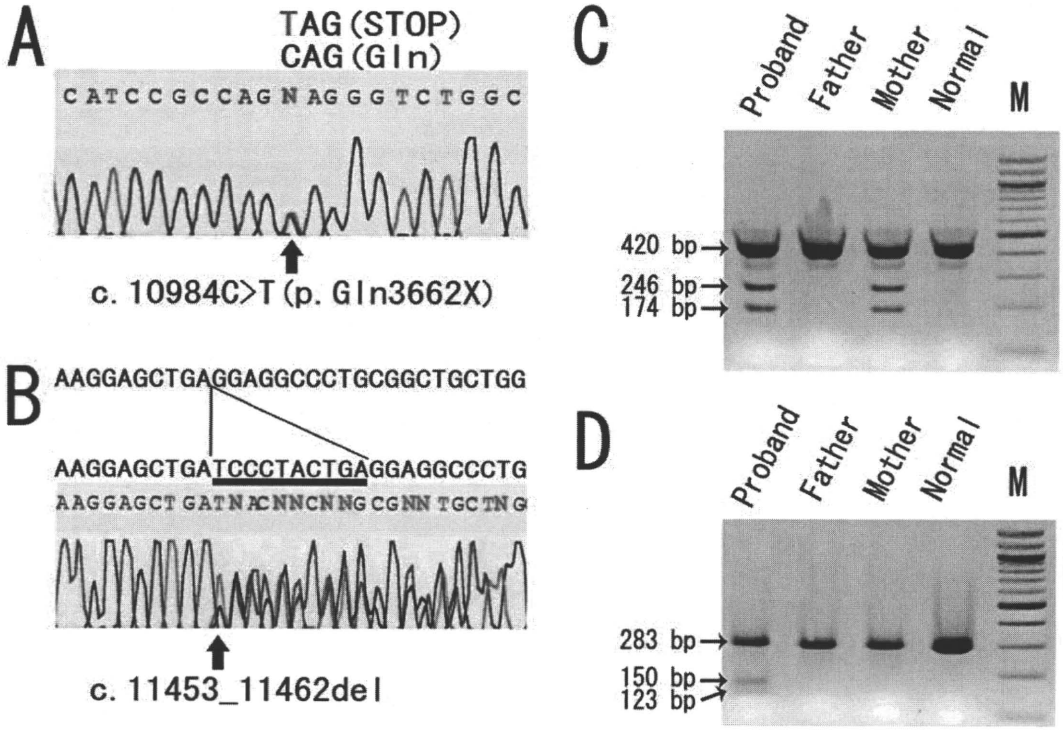

Figure 3. The novel *PLEC* mutations detected in the study. Maternal c.10984C>T (p.Gln3662X) (A) and de novo c.11453_11462del (B) in exon 32 were found in genomic DNA derived from the proband. A thymine substituted for a cytosine in the former mutation is indicated by the red character (A). Deleted nucleotides 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, α6 and β4 integrin, and BP230 revealed normal DEJ labeling patterns (data not shown).

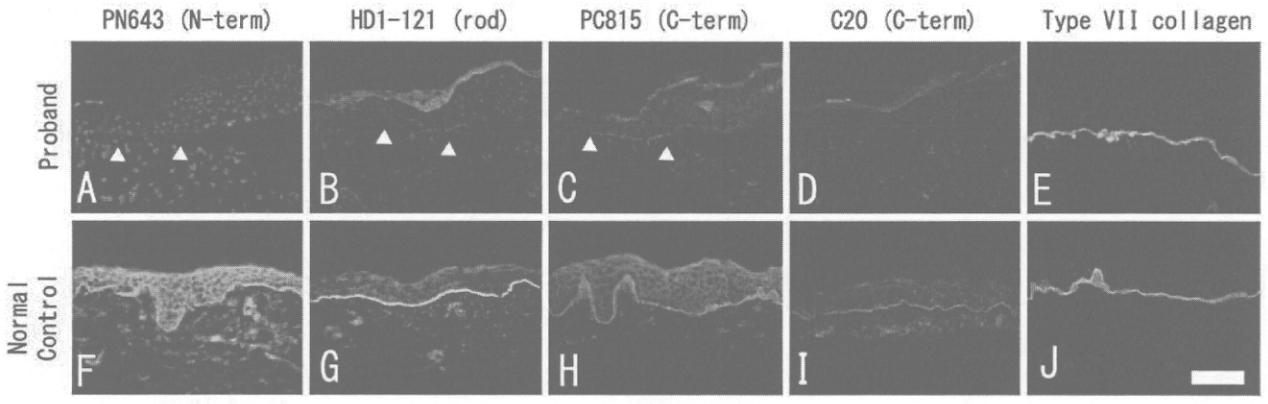

Figure 4. Immunofluorescence analysis of the proband's skin sample. In normal human skin, immunofluorescence shows that all of the antibodies against plectin (PN643, HD1-121, PC815 and C20) tested in this study bound to the dermal epidermal junction (DEJ) (F - I). DEJ labeling of PN643, HD1-121 and PC815 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=100μm).

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).

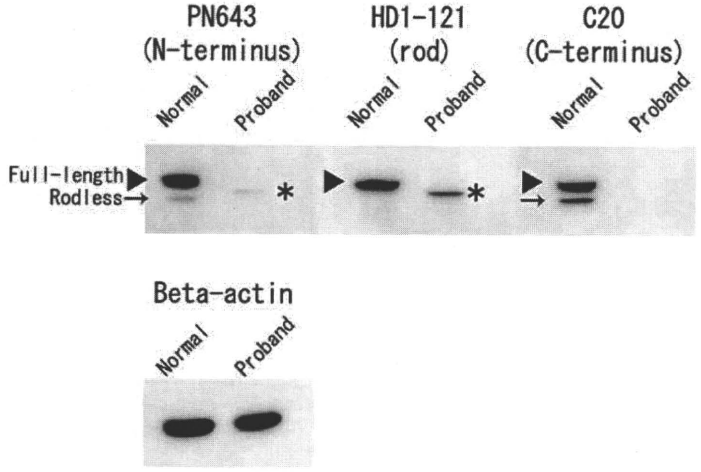

Figure 5. Immunoblot analysis of cultured fibroblasts from the normal human control and the proband. Immunoblot analysis of extracts from fibroblasts of the normal control and the proband by using PN643 against the N-terminal actin-binding domain, HD1-121 against the rod domain and C20 against the C-terminal 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).

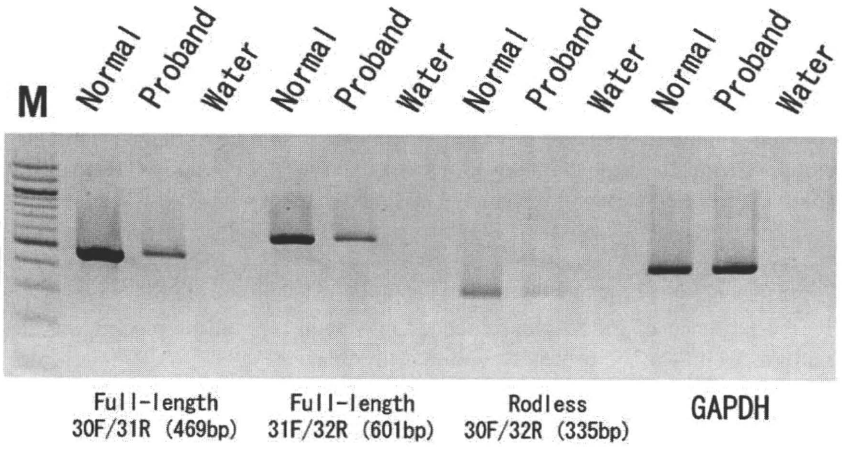

Figure 6. Semi-quantitative RT-PCR for full-length and rodless plectin transcripts. The quantity of full-length (30F/31R and 31F/32R) and rodless (30F/32R) plectin transcripts in the proband's cultured fibroblasts is reduced in comparison to those of the normal control. GAPDH mRNA expression was used 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 6th 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 5th and 6th 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.

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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 α6 integrin subunit (*ITGA6*) or β4 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 β4 integrin subunit in skin specimens from the proband (Figure 1c–f). Expression of α6 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, α6 integrin subunit; ITGB4, β4 integrin subunit; UPD, uniparental disomy

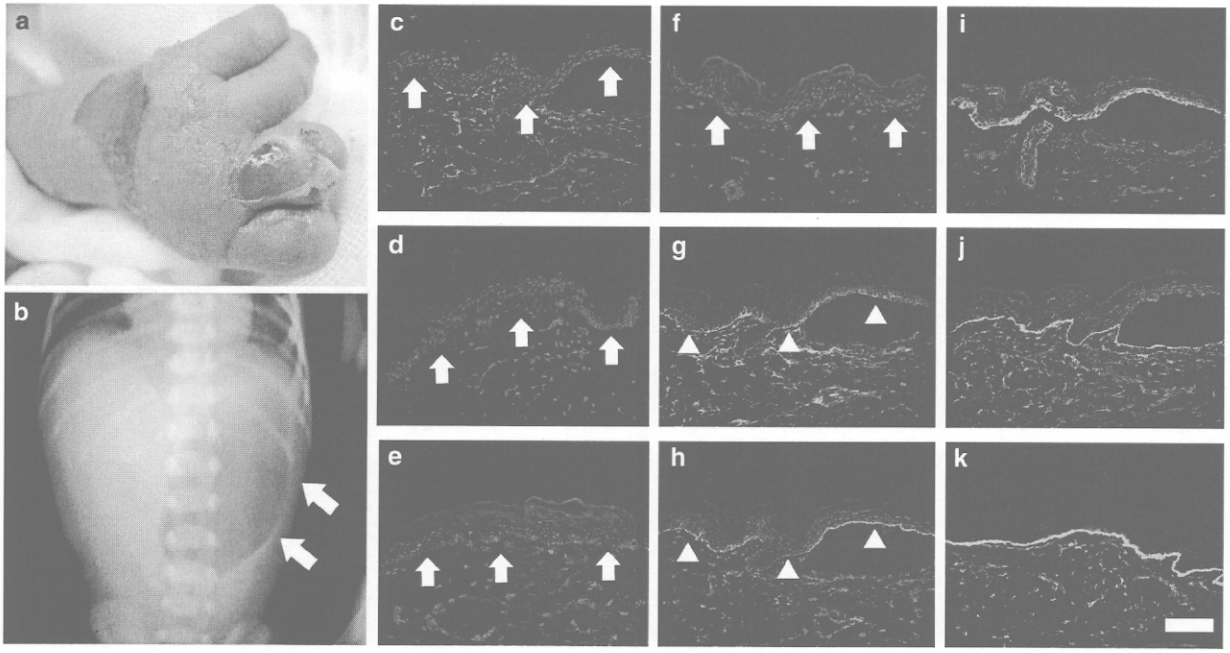


Figure 1. Clinical features of the proband and immunofluorescence analysis. (**a**) Extensive blistering is seen on the extremities at birth. (**b**) Abdominal X-ray reveals a single-bubble sign (arrows). β4 integrin subunit (3E1 (**c**), 113C (**d**), 450-9D (**e**), and 450-11A (**f**)) is not detected in the proband's skin (arrows). The expression of α6 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 μm). 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.

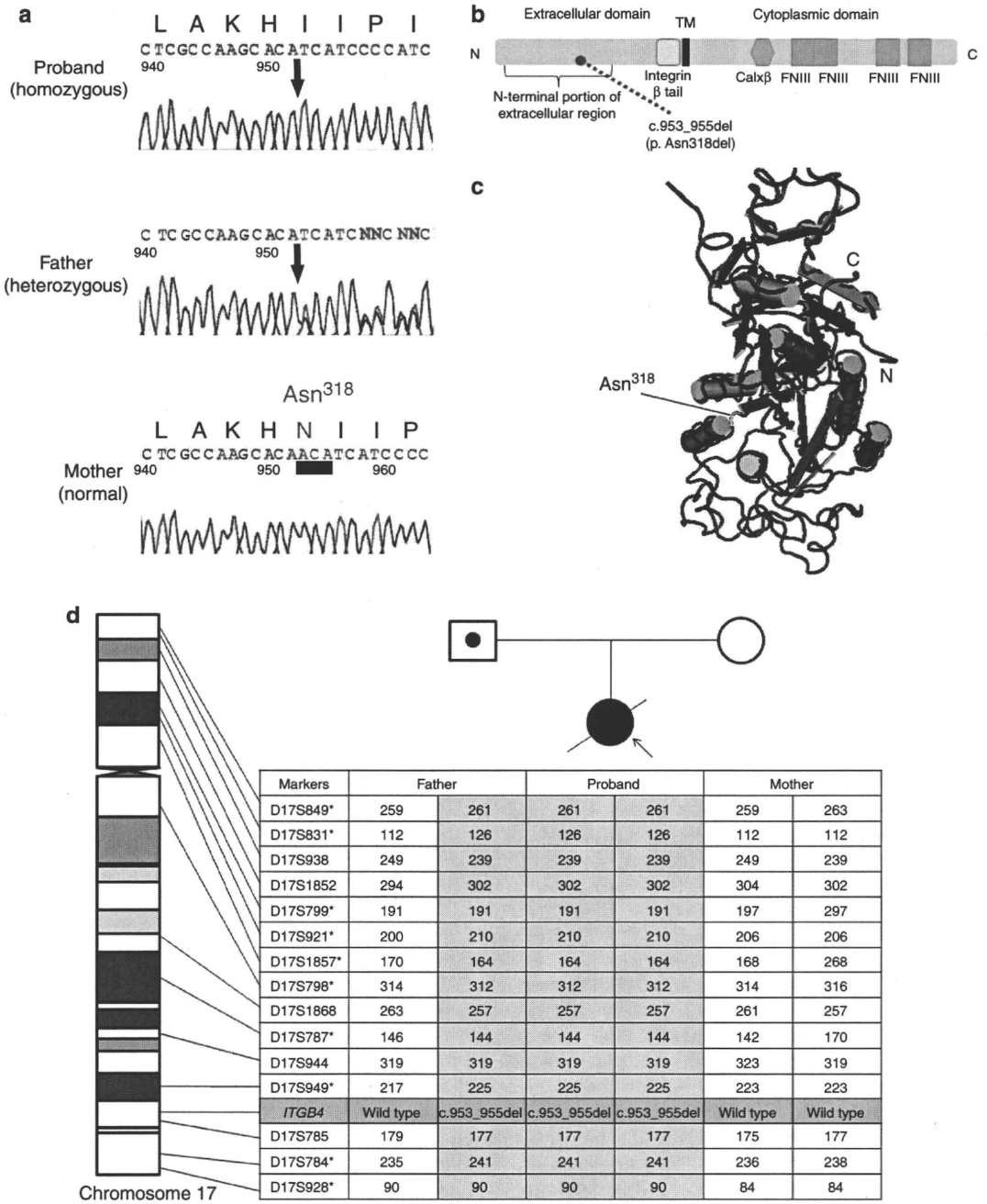
complementary DNA nucleotides and the amino acids of the protein were numbered based on the following sequence information (GenBank accession no. NM_000213). Mutation c.953_955del is predicted to result in the loss of asparagine at amino acid position 318 (p.Asn318del, Figure 2b), and this deletion mutation 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; Fassihi *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 (Fassihi *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^{318} in β4 integrin subunit resides in the extracellular



Markers	Father		Proband		Mother	
D17S849*	259	261	261	261	259	263
D17S831*	112	126	126	126	112	112
D17S938	249	239	239	239	249	239
D17S1852	294	302	302	302	304	302
D17S799*	191	191	191	191	197	297
D17S921*	200	210	210	210	206	206
D17S1857*	170	164	164	164	168	268
D17S798*	314	312	312	312	314	316
D17S1868	263	257	257	257	261	257
D17S787*	146	144	144	144	142	170
D17S944	319	319	319	319	323	319
D17S949*	217	225	225	225	223	223
ITGB4	Wild type	c.953_955del	c.953_955del	c.953_955del	Wild type	Wild type
D17S785	179	177	177	177	175	177
D17S784*	235	241	241	241	236	238
D17S928*	90	90	90	90	84	84

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 β4 integrin subunit and the positions of the mutation in the proband. (**c**) 3D imaging of the N-terminal extracellular domain of the integrin β chain (NCBI Conserved Domain Database (code: smart00187)) was done using Cn3D-4.1 software. Asn^{318} is in the linking loop between the α helix and the β 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 β4 integrin subunit of vertebrates but in all the human integrin-β chains (Iacovacci *et al.*, 2003). The 3D structure of the N-terminal portion of the extracellular domain indicates that Asn^{318} consists of a linking loop between the α helix and the β 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.

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

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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 draining 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$^+$ 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$^+$ 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

RESEARCH ARTICLE

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Plectin Expression Patterns Determine Two Distinct Subtypes of Epidermolysis Bullosa Simplex

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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.
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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 α6 and β4 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 [Pfendner 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; Pfendner 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 [Pfendner 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 α6/β4 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; Pfendner et al., 2005; Pfendner 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	**c.6549_6582del (p. Ala2183fs)**	31	**c.13040dup (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	**c.4643_4667dup (p.Arg1556fs)**	31	**c.7120C>T (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; =)[a]	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.
[a]The mutation c.1344G>A is at the 3′ end of exon12. Predicted consequences resulting from this mutation are discussed in the Results.

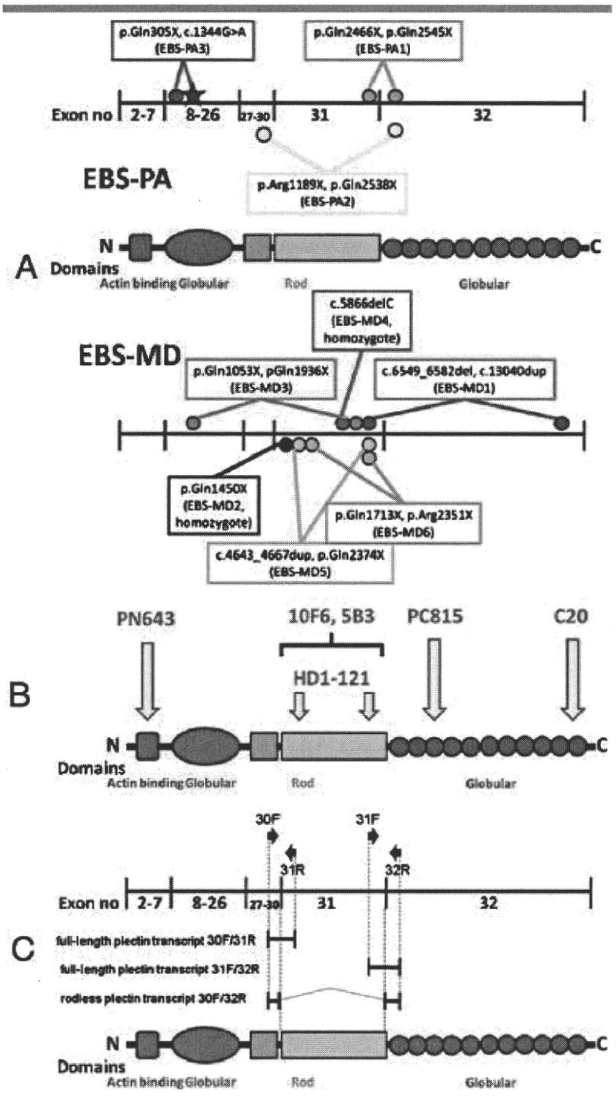

Figure 1. Scheme of plectin structure, PLEC1 mutations, antibodies against plectin and specific primers to detect the rodless transcript form of plectin. **A:** Plectin molecules consist of a central rod flanked by amino-terminal and carboxy-terminal globular domains. PLEC1 mutations observed in EBS 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-μm 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 proteinase 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 antigout 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′-TGCGCCTGTCG-CTTTTGTGC-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′-GATTGAGATCCTGTGGTCTC-3′ and 5′-CTCTGCACACTCT-GCAGAGT-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 *Mwo*I 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 *Pst*I 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 ±SD. The quantitative ratio of full-length/rodless plectin was 14.2±4.2 in fibroblasts, 21.3±6.4 in keratinocytes, and 1.37±0.23 in skeletal muscle.

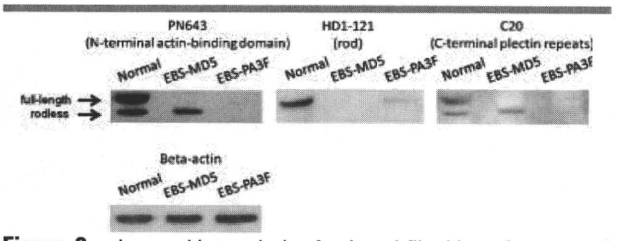

Figure 2. Immunoblot analysis of cultured fibroblasts from normal human control and EBS-MD and amniocytes from EBS-PA. Immunoblot analysis of extracts from cells of normal control, EBS-MD5, and an aborted sibling of EBS-PA3 (EBS-PA3F). Analysis used PN643 against the N-terminal actin-binding domain, 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.]

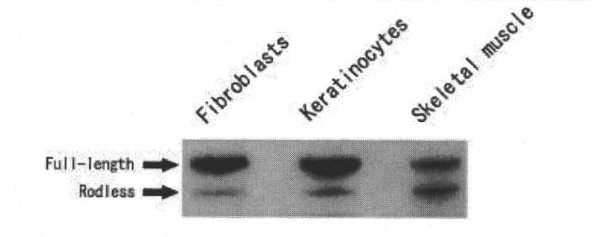

Figure 3. Relative amounts of full-length and rodless plectin in normal human fibroblasts, keratinocytes and skeletal muscle. Immunoblot analysis of lysates from normal human fibroblasts, oral keratinocytes, and skeletal muscle using PN643 against the N-terminal actin-binding domain of plectin was performed. 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±4.2 in fibroblasts, 21.3±6.4 in keratinocytes, and 1.37±0.23 in skeletal muscle. [Color figure can be viewed in the online issue, which is available at 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

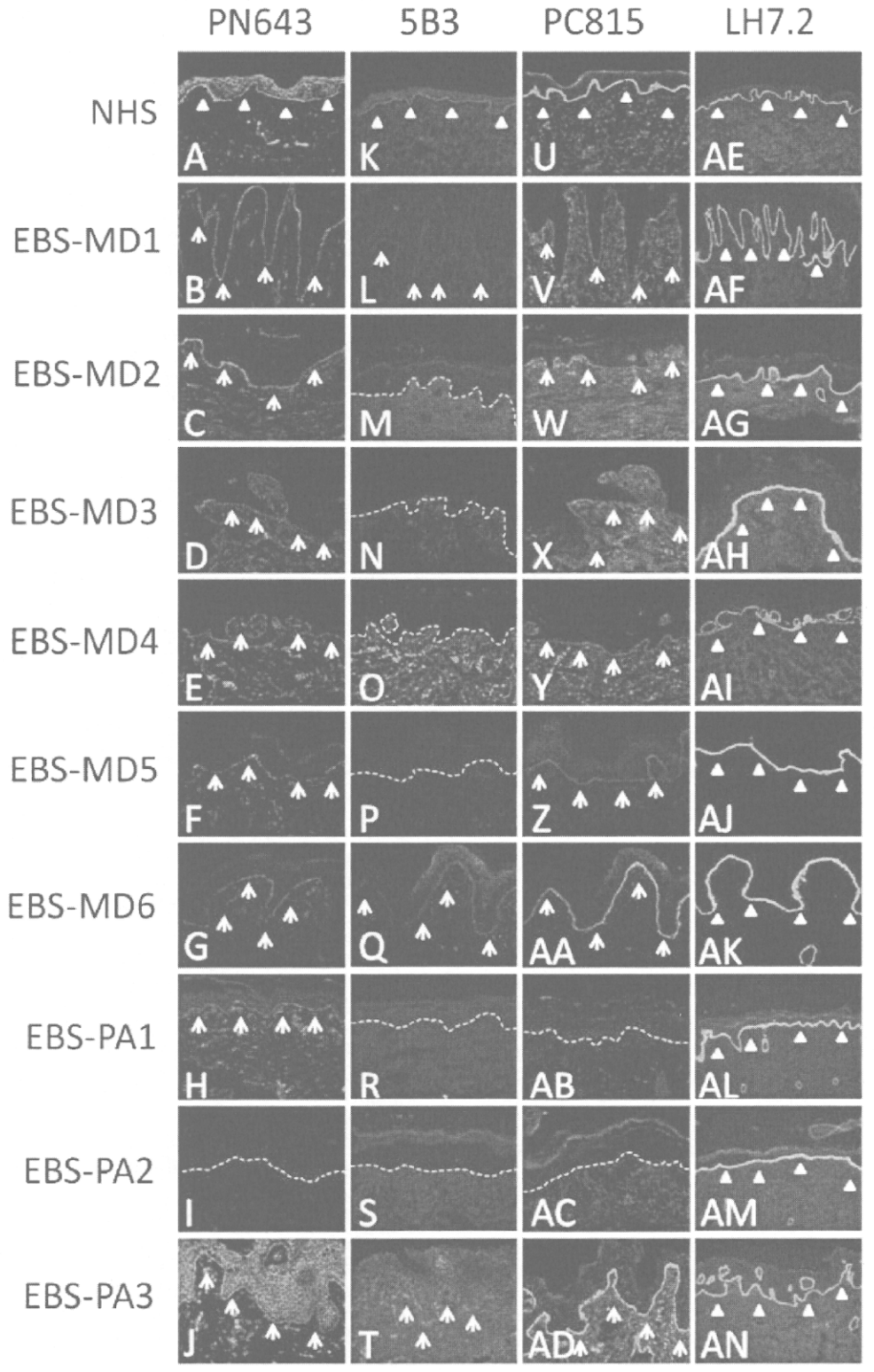

Figure 4. Immunofluorescence analysis of cutaneous plectin expression in EBS-MD and EBS-PA. In normal human skin (NHS), immunofluorescence shows that all mAbs against plectin (PN643, 5B3, and PC815) and type VII collagen (LH7.2) tested in the study bind to the dermal epidermal junction (DEJ) (**A, K, 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 EBS-MD1, 6 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.

revealed loss of full-length plectin with the maintenance of a rodless plectin isoform in EBS-MD. EBS-PA skin specimens harbored greatly reduced amounts of both full-length and rodless plectin.

Protein and mRNA expression patterns of plectin in cultured cells from EBS-MD and EBS-PA patients

Plectin expression patterns of EBS-MD and EBS-PA cultured cells were assessed at both the protein and mRNA levels to confirm the comparative immunofluorescence analysis using skin biopsy specimens showing that the majority of EBS-MD patients expressed a rodless plectin variant, but not full-length plectin and that expression of both full-length and rodless-plectin variant peptides was remarkably reduced or completely abolished in EBS-PA patients. Immunoblot analysis of lysates from fibroblasts of patient EBS-MD5 failed to show any HD1-121 bands, although a band corresponding to rodless plectin was observed by using PN643 and C20 (Fig. 2). Lysates from cultured amniocytes from an aborted sibling of EBS-PA3 (EBS-PA3F) showed that a diminished amount of full-length plectin reacted with PN643, HD1-121, and C20 (Fig. 2).

Using RT-PCR, the presence of an RNA message that does not encode the rod domain was demonstrated in the normal human control as well as the EBS-MD5 and EBS-PA3F cells (Figs. 1C and 5A) (30F/32R). Direct sequencing confirmed the skipping of exon31 in the PCR products (30F/32R) (Fig. 5B). mRNA encoding full-length plectin containing the rod domain was also detected in normal human control, EBS-MD5, and EBS-PA3F cells (Figs. 1C and 5A) (30F/31R and 31F/32R). Judging from the PCR analysis results, the quantity of full-length plectin transcript was greatly reduced in EBS-MD5 and EBS-PA3F compared with those in the normal human controls. In addition, the rodless plectin transcripts were markedly diminished in quantity in EBS-PA3F compared with those of the normal human controls, although expression of the rodless plectin transcripts was maintained in EBS-MD5.

These data suggest that EBS-MD5 fibroblasts express only rodless truncated forms of plectin without the full-length isoform, presumably because of nonsense-mediated mRNA decay (NMD) of the full-length plectin transcript induced by the mutations within *PLEC1* exon 31 (Table 1 and Fig. 1A). Conversely, EBS-PA3F amniocytes expressed a much lower level of plectin than normal human fibroblasts due to NMD of both full-length and rodless plectin transcripts induced by mutations within exons encoding the N-terminal globular domain.

The expression of a small amount of plectin in EBS-PA3 and EBS-PA3F is explained by the splice donor site mutation, c.1344G>A (Table 1 and Fig. 1A). The *PLEC1* cDNA corresponding to exons 9–14 was amplified by PCR using synthesized first-strand cDNA from EBS-PA3F and was cloned into a TA vector. Sequence analysis of the cloned PCR products revealed three different splicing patterns, one of which was a normal transcript from the wild-type allele without c.1344G>A (Fig. 6A and B). In addition to the normal transcript, most of the transcripts derived from the c.1344G>A mutant allele exhibited a 4-bp deletion at nucleotide position 1341–1344 in cDNA (Fig. 6C). This led to a frameshift followed by a PTC at amino acid position 475 (Fig. 6D), whereas small amounts of mRNA exhibiting a

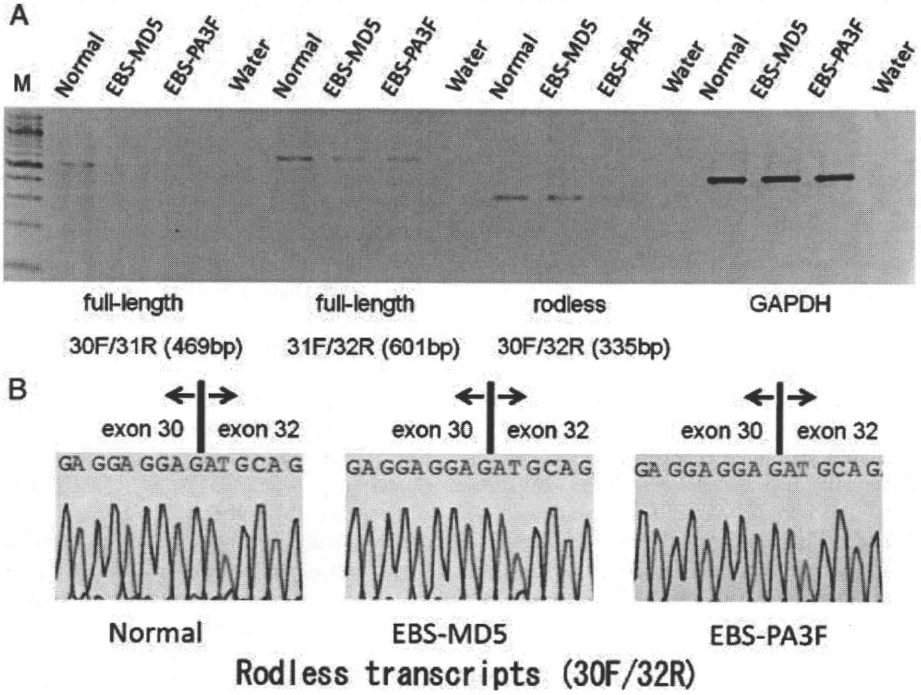

Figure 5. Semiquantitative RT-PCR on full-length and rodless plectin transcripts. **A:** Compared with the normal human control, the EBS-MD5 and EBS-PA3F cells revealed a reduced mRNA level of full-length plectin (30F/31R and 31F/32R). mRNA levels of rodless plectin in EBS-PA3F cells are reduced compared with EBS-MD5 and the normal human control (30F/32R). GAPDH mRNA expression was used as a 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. **B:** Direct sequencing demonstrates skipping of exon 31 in PCR products (30F/32R) from normal human, EBS-MD5, and EBS-PA3F.

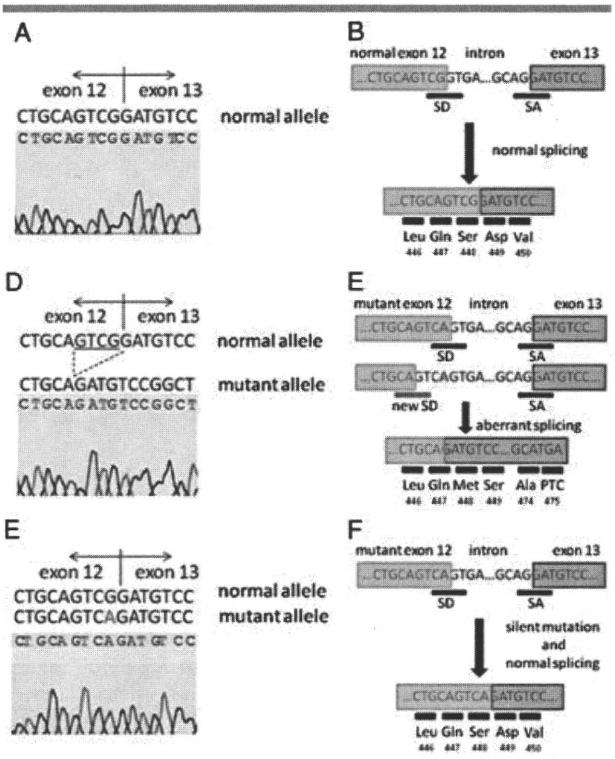

Figure 6. Abnormal splicing due to c.1344G>A mutation in EBS-PA3F, and its consequences **A:** Normal transcripts of the exon 12–exon 13 junction derived from EBS-PA3F cells. **B:** Normal splicing at the exon 12–exon 13 junction. Boxes represent exons, blue underlines are splice sites (SD: splice donor site; SA: splice acceptor site) and black underlined regions are amino acids. **C:** Mutant transcripts with deletion of four nucleotides from exon 12. Deleted nucleotides are underlined. **D:** c.1344G>A mutation altered the G nucleotide of the original splice donor site at the end of exon 12 and activated a cryptic splice donor site (red underline) four nucleotides upstream, leading to aberrant splicing with 4-bp deletion and subsequent frameshift, resulting in a premature termination codon at the amino acid position 475 in the N-terminal globular domain. **E:** Mutant transcripts with c.1344G>A. **F:** A small amount of mRNA carrying a silent nucleotide alteration c.1344G>A at amino acid position 448 Ser was also expressed by the original wild-type splicing.

normal splicing pattern with a silent mutation c.1344G>A at amino acid position 448 Ser were expressed (Fig. 6E and F).

Discussion

This study has demonstrated that two distinct plectin isoforms function in the skin, and that their truncation by *PLEC1* mutations causes the distinct EBS subtypes of EBS-MD and EBS-PA, depending on the pattern of remaining plectin peptide expression.

Plectin has a large rod domain encoded by *PLEC1*exon 31. Alternative splicing of transcripts lacking exon 31 results in a rodless plectin isoform, and it has been demonstrated that the rodless variant is expressed in various rat tissues, including skin, heart, brain, muscle, testis, and liver [Elliott et al., 1997; Fuchs et al., 2005; Steinboeck and Kristufek, 2005]. In addition, the rodless plectin isoform has been found in human muscle cells and keratinocytes [Koster et al., 2004; Schroder et al., 2000]. The significance of this rodless plectin splice variant in the skin remains unclear, but accumulation of *PLEC1* mutational data has revealed that most EBS-MD patients have mutations in exon 31 encoding the large rod domain of plectin, suggesting that conserved expression of the rodless variant plectin could be related to the pathogenesis of EBS-MD in patients with mutations in exon 31 [Pfendner et al., 2005; Sawamura et al., 2007]. However, little data that clarify this hypothesis has been reported, and only one report noted that cultured keratinocytes from one EBS-MD patient were able to express both N- and C-termini plectin epitopes without the expression of rod domain [Koster et al., 2004]. Our data including plectin isoform expression patterns in six EBS-MD patients clearly demonstrate that loss of full-length plectin with conserved rodless plectin isoform expression leads to an EBS-MD phenotype, which is consistent with accumulated clinical and genetic data. We also analyzed the relative amounts of two isoforms of plectin in normal human fibroblasts, keratinocytes, and skeletal muscle (Fig. 3). Our data revealed that the amount of full-length plectin is much greater than that of rodless plectin in fibroblasts and keratinocytes. In contrast, the full-length/rodless ratio in skeletal muscle is a little more than 1. These data are compatible with the fact that EBS-MD patients have skin fragility at birth and develop muscular dystrophy later in life. These data suggests that substantial amounts of rodless plectin in skeletal muscle might delay muscular symptoms while EBS-MD patients are in infancy.

In contrast to the EBS-MD patients, EBS-PA patients are significantly more likely to have mutations in domains outside exon 31 [Pfendner et al., 2005; Sawamura et al., 2007]. The majority of EBS-PA patients included in this study also exhibited *PLEC1* mutations in the gene outside exon 31(Table 1 and Fig. 1A). In the EBS-PA patients in this study, at least one allele is expected to have a stable product (the normal splicing variant from c.1344G>A in EBS-PA1; p.Gln2538X in EBS-PA2, and p.Gln2545X in EBS-PA3). There are three examples in which there are nulls in both alleles that have the PTC outside exon 31 but not in the terminal exon: (1) c.[2727_2740del]+c.[2727_2740del] (exon 22) [Charlesworth et al., 2003], (2) c.[1567_1570del]+c.[1567_1570del] (exon 14) [Pfendner and Uitto, 2005], and (3) p.[Gln305Term]+p.[Gln305Term] (exon 9) [Pfendner and Uitto, 2005]. All three patients had early deaths. Patients (2) and (3) had the EBS-PA phenotype [Pfendner and Uitto, 2005]. Patient (1) had the EBS phenotype, but the occurrence of PA was not substantiated [Charlesworth et al., 2003]. Due to the limited number of EBS-PA patients available, detailed expression patterns of plectin in the skin of EBS-PA patients has not been performed. In addition, comparative analysis of EBS-MD and EBS-PA skin specimens has not been performed. To our knowledge, the present report is the first to compare cutaneous plectin expression in EBS-MD and EBS-PA subtypes using multiple tissues and cells with antibodies that span a range of plectin domains including the N-terminus, rod domain, and C-terminus of plectin. This comparison between EBS-MD and EBS-PA enabled us to identify the differences in these EBS subtypes and to gain a better understanding of the consequences that complete loss or markedly attenuated expression of plectin has. These data are also consistent with the fact that EBS-PA patients generally show more severe skin symptoms than EBS-MD cases, in which expression of a rodless plectin isoform is maintained at least in the skin, although one EBS-PA patient (EBS-PA1) showed a relatively mild skin phenotype [Sawamura et al., 2007]. Also, in some cases of JEB-PA, another subtype of EB with pyloric atresia, the skin manifestations have been reported to be relatively mild and to improve with age, and surgical correction of the PA allowed growth of the patients [Pulkkinen et al., 1998]. It is possible that EBS-PA patients could develop muscular dystrophy if they survived longer. However, to our knowledge, such EBS-PA patients have not been reported in the literature. Figure 7A–C

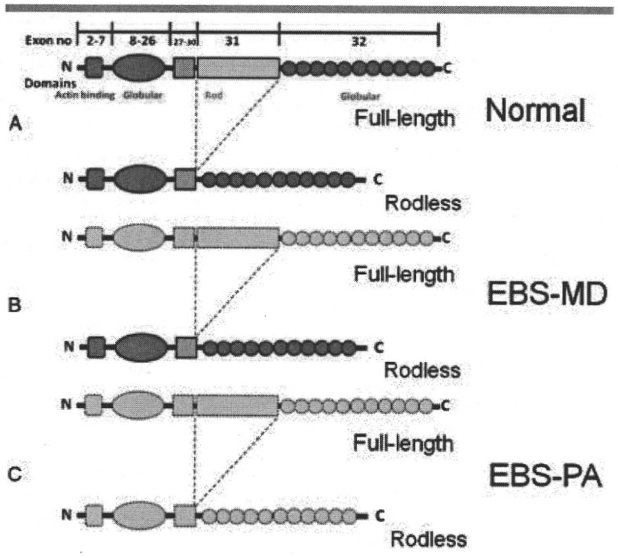

Figure 7. Schematic diagram of cutaneous plectin expression patterns in normal human skin and in skin from EBS-MD and EBS-PA patients. **A:** Two distinct isoforms of plectin—full-length and rodless—are expressed in the normal human control. **B:** Only rodless plectin is expressed in EBS-MD. **C:** Both the full-length and rodless plectin isoforms are greatly diminished or completely lost in EBS-PA. The peptides in light gray are not expressed or are markedly diminished in the patients.

depicts a schematic diagram of the predicted plectin expression pattern among the normal human control, EBS-MD, and EBS-PA.

As described above, almost all EBS-MD patients have one or two truncated mutations in exon 31 encoding the large rod domain of plectin, whereas most *PLEC1* mutations detected in EBS-PA are outside exon 31. To our knowledge, we have three cases of EBS-MD and one case of EBS-PA in the literature whose mutations are not explained by our data: (1) c.[2719_2727del] (exon 21)+c.[2719_2727del] (exon 21) (EBS-MD) [Pulkkinen et al., 1996], (2) c.[1541_1576del] (exon 14)+c.[2677_2685del] (exon 21) (EBS-MD) [Uitto and Pfendner, 2004], (3) c.[2769_2789del] (exon 21)+c.[2769_2789del] (exon 21) (EBS-PA) [Uitto and Pfender, 2005], and (4) c.[13803_13804ins16] (exon 32)+c.[13803_13804ins16] (exon 32) (EBS-MD) [Schroder et al., 2002]. The former three EBS patients had in-frame *PLEC1* deletion mutations outside exon 31 but not in the terminal exon. The last EBS-MD patient was homozygous for out-of-frame mutation in the terminal exon predicting a premature stop-codon within the exon. c.[2719_2727del] was in the nucleotide sequence where CAGGAGGCC was tandemly repeated. Therefore, this in-frame deletion was predicted to result in slipped misparing of DNA [Krawczack and Cooper, 1991; Pfendner and Uitto, 2005]. It is hard to figure out how altered plectin is synthesized from c.[1541_1576del]+c.[2677_2685del] and c.[2769_2789del]+c.[2769_2789del]. It is noteworthy that the phenotype of the EBS-MD patient with c.[1541_1576del]+c.[2677_2685del] was relatively mild, and that muscular dystrophy did not develop until the age of 42 [Uitto and Pfendner, 2004].

In previous studies, the expression of plectin was mainly evaluated by monoclonal antibodies raised against the rod domain. However, several splicing variants had previously prevented us from identifying whether plectin is completely lost or expressed in a truncated protein form in EBS patients with *PLEC1* mutations. Antibodies including those raised against both the plectin N- and C-termini are required to distinguish the expression of rodless splicing variants from a complete protein loss. Nevertheless, we have now elucidated how differences in plectin expression can lead to the two distinct skin blistering-associated phenotypes of muscular dystrophy and pyloric atresia.

Our former study on an EBS-PA3 patient [Nakamura et al., 2005] described different predicted transcripts of the c.1344G>A splice-site mutation from those of the present study. Our previous report employed an exon-trapping system, which is a tool to predict the transcripts that arise from a splice-site mutation when mRNA samples from patient tissues or cells are not available [Buckler et al., 1991]. In that system, the gDNA that is to be screened is subcloned into the exon trapping vector. The subcloned vector is transfected into cells, and mRNA is extracted from the cells to elucidate the splicing consequences. The system is useful, but it is such an artificial way of predicting the splicing products that the induced splicing patterns in the cell culture system are not necessarily correct nor are they the same as those in patient tissues or cultured cells [Schneider et al., 2007]. Because we used cultured amniocytes from EBS-PA3F in the present study, the results shown in Figure 6 supersede the results that were obtained by using an exon-trapping system in the previous report.

To summarize, EBS-MD patients typically express a rodless plectin isoform, although the full-length plectin is lost. In contrast, both full-length and rodless plectin isoforms are deficient in the EBS-PA patients, leading to a more severe disease phenotype. These findings demonstrate that deficiency of both plectin isoforms—full-length and rodless—leads to the severe phenotype of EBS-PA, and in contrast, conserved expression of the rodless isoform results in muscular dystrophy without pyloric atresia. The present results provide important insights toward further understanding the pathomechanisms of muscular dystrophy and pyloric atresia in plectin-deficient patients.

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