

mutations were novel in EBS-K patients (428T → A, V143D; 558G → A, V186M; 573C → A, Q191P; 1550G → A, G517D). The V143D mutation (Patient 4) was in the V1 head domain and the G517D mutation (Patient 5) was in the non-helical tail V2 domain of K5. The other mutations (Patients 6 and 7) were in the 1A rod domain. We could not find any mutations in five EBS-K patients (Patients 15–19) who did not have family history of disease. All mutations found in EBS-DM in this study have been previously reported.<sup>7–12</sup> Patient 14 was given the diagnosis of EBS with mottled pigmentation (EBS-MP) that is characterized by mottled pigmentation, punctate or warty keratoses in addition to an EBS-WC type blistering pattern.<sup>12</sup> The P25L mutation in KRT5, which was specific for EBS-MP, was again confirmed in Patient 14.

**Discussion**

The ethnic and geographical features in this study of EBS patients are important. A recent report revealed EBS types in Israel that have a unique mutation spectrum and different patterns of inheritance including a high incidence of recessive cases compared with European or U.S.A. associated families.<sup>13</sup> Six Japanese EBS mutations have been previously reported,<sup>14–19</sup> which are not enough to reveal the full extent of the mutation spectrum. To assess the possibility that EBS may present with certain specific features in Japanese and Koreans, which also comprise a closed ethnic group, 17 Japanese and two Korean patients with EBS were examined in this study. We detected 14 mutations, six of which are novel, and have

summarized them together with all previously reported KRT5 and KRT14 mutations in the English literature (Fig. 1).

Japanese and Korean patients with EBS showed very similar phenotype/genotype correlations as patients from Western countries. All EBS-DM mutations in this study were the same as in previous reports, and mutations lay within the highly conserved ends of the rod domain [helix initiation peptide (HIP) and helix termination peptide (HTP)] that are critical for K5 and K14 filament assembly.<sup>5</sup> The E477K mutation in KRT5 and R125C or H mutation in KRT14, which accounts for about 70% of EBS-DM mutations,<sup>8</sup> were also detected in Japanese and Korean patients with EBS-DM. EBS-K and EBS-WC mutations found in this study and reported previously were located more centrally within the rod domain or within the nonhelical regions. It is remarkable that the novel R352S mutation in KRT5 is the first mutation found in the 2A rod domain. Two novel KRT5 mutations associated with EBS-K lay within the head or tail domains where EBS-K mutations are rarely identified. We could not find any clear distinctions between the EBS-WC and EBS-K mutation positions. The fact that the M119V mutation in KRT14 was detected in both EBS-K<sup>9</sup> and EBS-WC (Patient 3) supports the fact that the clinical manifestations of EBS-K and EBS-WC may overlap and that the disease severity is likely to be affected by other factors.

Six novel KRT5 missense mutations were identified. The V143D and D158V mutation are located within the H1 region known to be a subdomain with a high degree of sequence homology within all IF and located immediately adjacent to

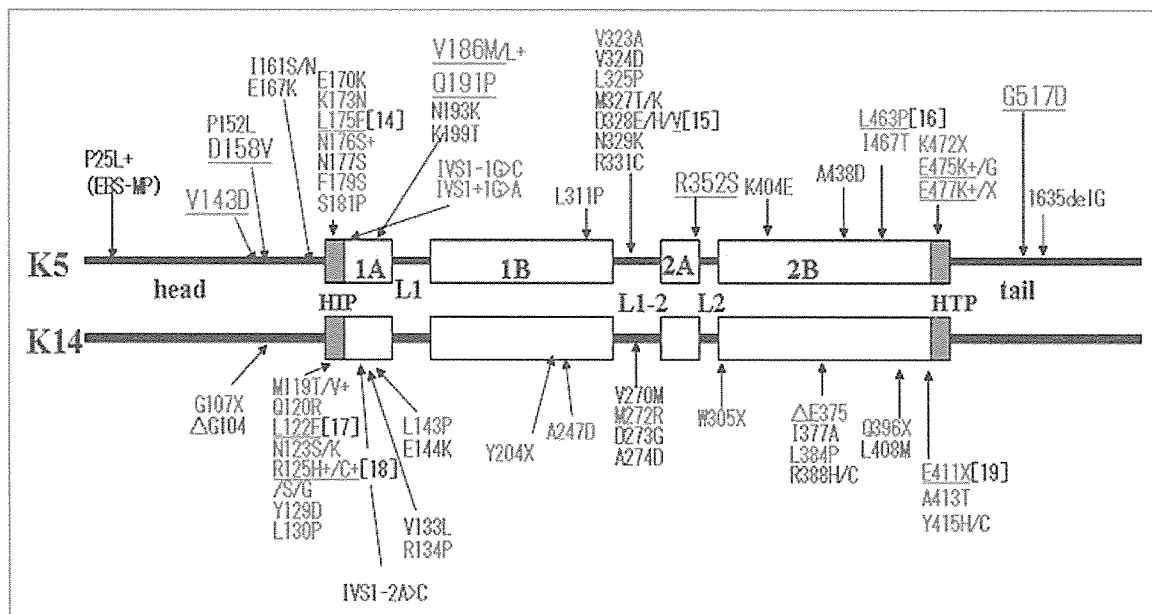


Fig 1. Correlation between previously reported and novel epidermolysis bullosa simplex (EBS) mutation locations and disease severity. Mutations identified in EBS-Weber-Cockayne were indicated in green, in EBS-Koebner by a blue colour and in EBS-Dowling-Meara by red. Novel mutations are shown by a large text font size. Mutations detected in Japanese and Koreans are underlined ([ ] is reference number) and a cross means mutations that were not novel but were detected in this study.

	Head (H1)		1A	2A
Type II	143	158	186 191	352
K5	IQEVTVNQSLLTPLNLQIDFSIQVRTEE	REQIKTLNNEKFAFIDKVFLEQQNK	YEEIANRSRTE	
K1	IQEVTVNQSLLTPLNVEIDPEIQRVKSE	REQIKSLNNEKFAFIDKVFLEQQNQ	NEDIAQRSKAE	
K2e	IHEVSVNQSLLTPLNVEIDPEIQNVKAE	REQIKTLNNEKFAFIDKVFLEQQNQ	YEEIAQRSKEE	
K3	IQEVTVNQSLLTPLNVEIDPEIQVKAQE	REQIKTLNNEKFAFIDKVFLEQQNK	YEDIAQRSKAE	
K4	IQEVTVNQSLLTPLNVEIDPEIQRVTEE	REQIKTLNNEKFAFIDKVFLEQQNK	YEEIAQRSKAE	
K6a	IQEVTVNQSLLTPLNLQIDPTIQVRRAE	REQIKTLNNEKFAFIDKVFLEQQNK	YEEIAQRSRAE	
Type I				
K14		KVTMQNLNDRLASYLKVRALAEANA	YEMAEKRNKD	
Type III				
Vimentin		KVEIQELNDFEANYIDKVRFL EQQNK	LSEAAHRRNDA	
Type IV				
NF-M		REQIQGLNDRFAGYIEKVVHYLEQQNK	LTEAAARNTDA	
Type V				
Lamin		KEDIQELNDRLAVYIDRVRSLEETENA	ARQSAAERNSNL	

Fig 2. Schematic demonstration of the V143, D158, V186, Q191 and R352 residues in K5. All of them are highly conserved among other type II keratins or intermediate filaments (IF). Shown are amino acid sequences of the H1 region, the 1A and 2A rod domain segment along with a selected group of other type II IF keratins, type III IF vimentin, type IV IF neurofilament subunit M (NF-M), type V IF lamin. Sequences were obtained from Genbank. GenBank accession numbers are as follows: K1, AF237621; K2e, AF019084; K3, X05418 X05421; K4, X61028; K5, NM000424; K6a, L42583; vimentin, BC000163; NF-M, Y00067; lamin, M13451.

the end of the 1A domain. Because the length of the H1 domain is different between individual IF,<sup>14,15</sup> we compared the corresponding amino acids of residues 143 and 158 with other type II keratins. They were highly conserved (Fig. 2). The positions of other mutations (valine at residue 186, glutamine at residue 191 in the 1A domain and arginine at residue 352 in the 2A domain) were also well conserved among not only type II keratins but also all other types of IF (Fig. 2).<sup>20,21</sup> We could not determine whether the glycine residue at 517 was evolutionarily conserved because of the diversity of tail domains in other type II keratins.

All mutations except the V186M mutation resulted in the alteration of the polarity of the affected amino acids. However, an alteration at residue 186 in KRT5 (V186L) has been previously described in association with EBS-K.<sup>9</sup> These findings suggest that all the novel mutations affect the important, conserved IF amino acids, suggesting that they may interfere with the keratin heterodimer formation and protein-protein interactions and are therefore likely to be pathogenic.

Mutation detection rates in patients with EBS were reported to be approximately 85–90%.<sup>3</sup> We also failed to detect any mutations in five *de novo* EBS-K cases. They might have mutations in the regulatory regions, including promoter regions, where we did not examine, or in genes other than KRT5 and KRT14.

The remarkable finding is that the proportion of KRT5 mutations (11 of 14; 78%) in Japanese and Korean patients with EBS is higher than that of the KRT14 mutation rate (3 of 14; 21%) in this study. In general, the ratio of KRT5 and KRT14 mutations has been reported to be approximately equal.<sup>3,7</sup> Whether this higher proportion of KRT5 mutations is a definite characteristic of Japanese and Korean patients with EBS or not, requires further research into mutations in Japanese and Korean patients.

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## *N*-Linked neutral oligosaccharides in the stratum corneum of normal and ichthyotic skin

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**Abstract** *N*-Glycan oligosaccharides are thought to play multiple, important roles in a variety of biological events. However, *N*-glycan profiles in the stratum corneum of human skin have not yet been studied in detail. To clarify the *N*-glycan profiles in the stratum corneum of normal and ichthyotic epidermis, *N*-glycan profiles were studied by high-performance liquid chromatography using normal human epidermal samples and scales from hyperkeratotic skin of ichthyosis patients. Chromatograms of patient scale samples showed unique alterations in three peaks eluted at 15.8, 18.8 and 26.9 min. The *N*-glycan profiles were significantly altered in ichthyotic hyperkeratotic skin compared with normal non-hyperkeratotic controls. These findings indicate the reduction of *N*-acetylglu-

cosaminyltransferase II and fucosyltransferase 8 activities. Alteration of *N*-glycan structures in hyperkeratotic skin suggests the biological role of *N*-glycans in keratinization.

**Keywords** High-performance liquid chromatography · Hyperkeratosis · Neutral oligosaccharide · *N*-Glycan · Oligosaccharide

Oligosaccharides are known to play important roles in a variety of biological events including cell–cell interactions and undergo modifications during cell differentiation [6]. *N*-Glycan oligosaccharides, which bind to asparagine, are a frequent post-translational modification of proteins. *N*-Glycan oligosaccharides exhibit diversity created by sugar linkages and components including: mannose, galactose, *N*-acetylglucosamine, *N*-acetylneuraminic acid. *N*-Glycan oligosaccharides modify specific protein properties, especially glycoprotein localization, secretion and degradation rates of proteins. Alterations in *N*-glycan composition have been reported in many human diseases [6, 12]. Oligosaccharide analyses using oligosaccharide-specific lectins and antibodies have suggested the biological importance of oligosaccharides in the epidermis [3, 5, 7, 19–21, 25, 28]. However, oligosaccharide-specific lectins and antibodies recognize only two or three sugar residues at most and little information has been obtained from studies with lectins or antibodies concerning the entire range of glycan structures or the *N*-glycan and *O*-glycan composition on glycoproteins and glycolipids. The cellular biosynthesis of *N*-glycan is highly regulated and glycan structures significantly affect protein properties [6, 15]. Alteration of *N*-glycan profiles in diseases have

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been reported in many human disorders, and these have been used for disease diagnosis and for the elucidation of disease pathomechanisms [4, 8]. Focusing on glycoproteins *N*-glycans, it was demonstrated that the mouse epidermis had abundant high-mannose oligosaccharides [24]. In addition, the oligosaccharide-binding proteins were also determined [24]. However, there are no reports of studies on *N*-glycans in human epidermis. In the present study, we studied *N*-glycan profiles in normal human epidermis and the stratum corneum of ichthyotic, hyperkeratotic skin. *N*-Glycans were specifically released by *N*-glycosidase F and were labeled by pyridylation with a fluorescence tag. Oligosaccharide profiles were analyzed using high-performance liquid chromatography (HPLC) [10, 22].

Skin samples of normal, non-hyperkeratotic epidermis were collected from control non-palmoplantar skin of three patients with benign skin tumors at resection operations. Skin scale samples were collected from hyperkeratotic skin from the legs of five ichthyosis patients, three non-bullous congenital ichthyosiform erythroderma patients and two lamellar ichthyosis patients. The medical ethical committee at Hokkaido University approved all the described studies. All the participants or parents of the participants gave their written informed consent.

Skin scales were collected by gently scraping the patients' legs with a dull scalpel and stored at  $-70^{\circ}\text{C}$ . Normal epidermal samples were obtained at resection operations of benign subcutaneous tumors after obtaining fully informed consent from the patients. Samples from both the ichthyosis patients and normal healthy controls were stored at  $-70^{\circ}\text{C}$ .

Normal skin samples were heated to  $60^{\circ}\text{C}$  for 1 min in phosphate buffered saline (PBS) and then cooled on ice. The epidermis was stripped off with a scalpel and was used for further analysis. The epidermis of normal skin samples was washed three times in PBS. Both the scales and the separated epidermis were denatured at  $90^{\circ}\text{C}$  for 10 min in 100 mM  $\text{NH}_4\text{HCO}_3$  and then finely shredded with scissors.

To degrease the samples, each sample was put into a mixture of chloroform and methanol 2:1 (v/v) 600  $\mu\text{l}$ , mixed well and centrifuged at 3,500 rpm,  $4^{\circ}\text{C}$  for 10 min. Then, the lower layer was removed. This procedure was repeated twice to remove lipids. Organic solvents in the upper layer were vaporized with nitrogen flow at  $80^{\circ}\text{C}$  and the aqueous solution dried in a centrifugal evaporator.

For trypsin and chymotrypsin digestion, the dried samples were suspended in 5 mg per 100  $\mu\text{l}$  of 45 mM Tris-HCl buffer pH 8 containing 10 mM  $\text{CaCl}_2$ , and

0.1 mg trypsin and 0.1 mg chymotrypsin (Sigma-Aldrich Japan, Tokyo, Japan) were added in a 1.5-ml microcentrifuge tube (Eppendorf type). The pH of the solutions was confirmed to be between 7 and 8 by the pH test paper. Toluene was added to the surface of the sample solution and kept at  $37^{\circ}\text{C}$  overnight in an incubator.

For *N*-glycosidase F digestion, trypsin and chymotrypsin were deactivated by heating sample tubes at  $90^{\circ}\text{C}$  for 10 min in a heat block. *N*-Glycosidase F (Roche Diagnostics, Tokyo, Japan) was added, 10 U for each 5 mg sample. The pH of the sample solutions was again confirmed and toluene was added. The samples were kept at  $37^{\circ}\text{C}$  overnight.

Pronase (0.1 mg, Calbiochem, San Diego, CA, USA) was added to each 5 mg of the sample for pronase digestion. pH of the sample solutions was checked again and toluene was added. The samples were incubated at  $37^{\circ}\text{C}$  overnight. After incubation, pronase was deactivated at  $90^{\circ}\text{C}$  for 10 min.

The oligosaccharide fraction was purified by gel-filtration on Bio-gel P-4 (Bio-Rad Laboratories, Hercules, CA, USA) column (1.0 cm  $\times$  38 cm) using water as an eluate, and lyophilized completely. The carbohydrates were reductively aminated with 2-aminopyridine and sodium cyanoborohydride [10, 29]. Pyridylaminated (PA)-oligosaccharides were separated from excess reagents by another gel-filtration column step using Sephadex G-15 (GE Healthcare Bioscience Corp., Piscataway, NJ, USA) and 10 mM  $\text{NH}_4\text{HCO}_3$  and lyophilized again.

Each PA-oligosaccharide was dissolved in 100  $\mu\text{l}$  of 0.01 N HCl and the pH was adjusted to 2 with 0.1 N HCl checking by test paper. PA-oligosaccharide solutions were heated at  $90^{\circ}\text{C}$  for 60 min to remove sialic acids and neutralized with 100  $\mu\text{l}$  of 1 M  $\text{NH}_4\text{HCO}_3$ .

Each PA-oligosaccharide solution was purified by HPLC on an amide-silica column (TSKgel Amide-80 4.6 mm  $\times$  250 mm, Tosoh Corporation, Tokyo, Japan) using two solvents, A and B, at a flow rate of 1.0 ml/min at  $40^{\circ}\text{C}$ . Solvent A comprised 65% (v/v) of acetonitrile and 35% of 0.5 M acetic acid-triethylamine buffer pH 7.3 and solvent B was 35 and 65%, respectively. The column was equilibrated only with solvent A and 7 min after injection of each sample, the flow was changed to solvent B only. The *N*-glycan fraction that eluted between 8 and 12 min was collected and centrifugally evaporated.

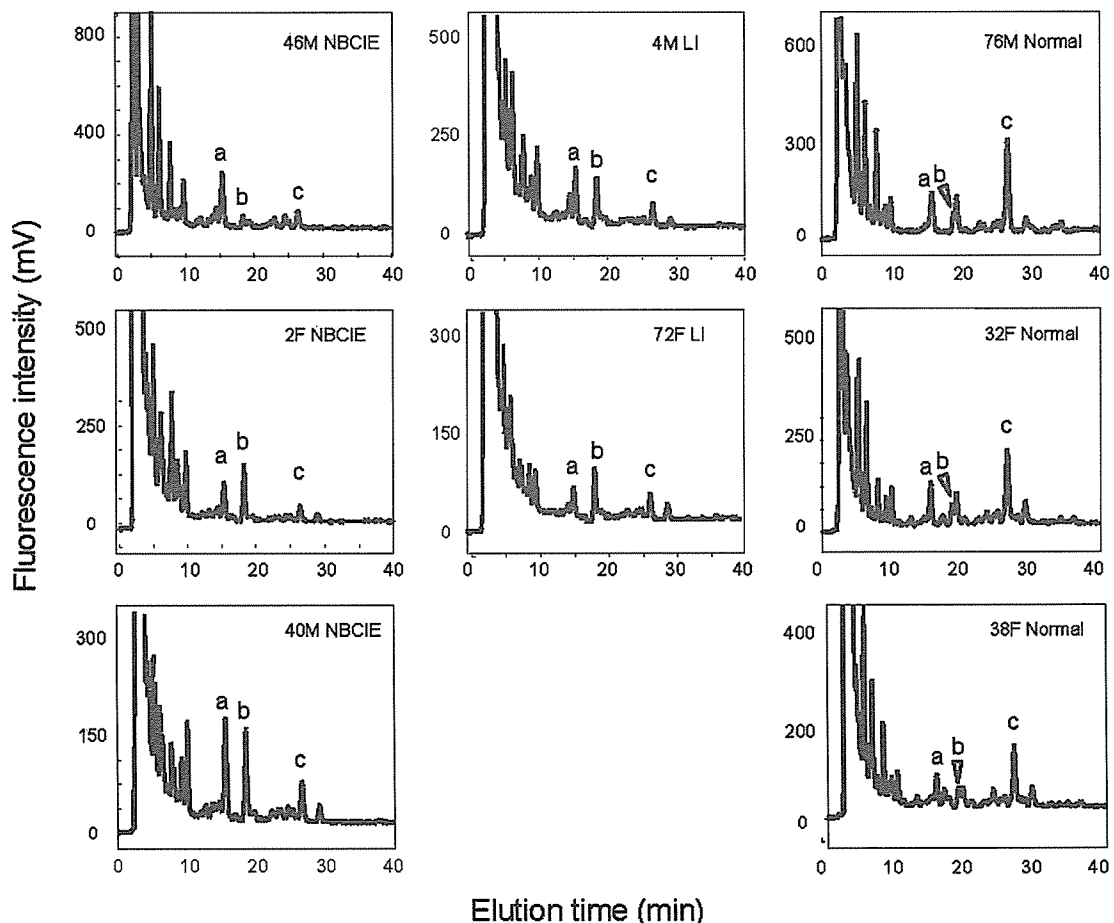
Each oligosaccharide mixture was dissolved in water and analyzed by HPLC using the previously described procedure [22]. The PA-oligosaccharide mixture was applied on an ODS column (HRC-ODS 6 mm  $\times$  150 mm, Shimadzu Corporation, Kyoto, Japan). The

column was equilibrated with 0.1% (v/v) 1-butanol in 10 mM sodium phosphate buffer pH 3.8 and concentration of 1-butanol was linearly increased to 0.25% in 60 min. The flow rate was 1.0 ml/min and the column was controlled at 55°C. PA-glycans were detected by fluorescence, excitation at 320 nm and emission at 400 nm.

*N*-Glycan profiles from the patients and normal control epidermis are shown in Fig. 1 as chromatograms on an ODS column. *N*-Glycan eluted after 5 min and few contamination peaks were detected in this region. High-mannose type *N*-glycans eluted from 5 to 10 min and complex-type and hybrid-type *N*-glycans appeared at 10 min or later in this analysis. Three peaks, a, b and c, eluted at 15.8, 18.8 and 26.9 min, respectively, and these profiles were significantly altered in ichthyotic skin. The rates of these peaks are shown in Fig. 2. Peak c significantly decreased and peak b increased in the

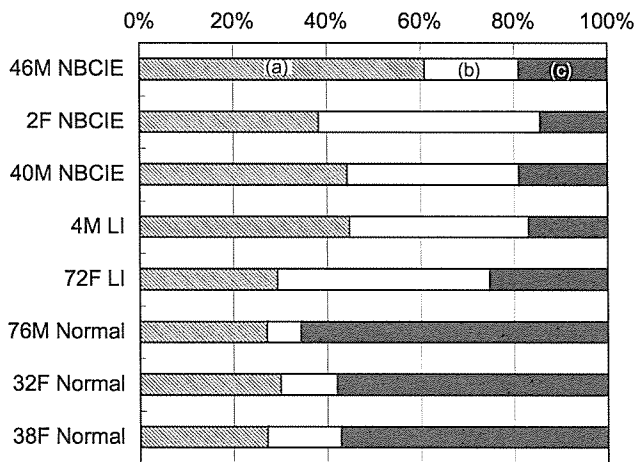
ichthyotic skin compared with normal controls. Peak a was also larger in the patients, but the statistical change in this level was not significant. Using two-dimensional mapping [22], peaks a, b and c corresponded to major serum *N*-glycans, Gal $\beta$ 1,4GlcNAc $\beta$ 1,2Man $\alpha$ 1,3(Gal $\beta$ 1,4GlcNAc $\beta$ 1,2Man $\alpha$ 1,6)Man $\beta$ 1,4GlcNAc $\beta$ 1,4GlcNAc, Gal $\beta$ 1,4GlcNAc $\beta$ 1,2Man $\alpha$ 1,3(Man $\alpha$ 1,6)Man $\beta$ 1,4GlcNAc $\beta$ 1,4(Fuc $\alpha$ 1,6)GlcNAc and Gal $\beta$ 1,4GlcNAc $\beta$ 1,2Man $\alpha$ 1,3(Gal $\beta$ 1,4GlcNAc $\beta$ 1,2Man $\alpha$ 1,6)Man $\beta$ 1,4GlcNAc $\beta$ 1,4(Fuc $\alpha$ 1,6)GlcNAc, respectively [17, 22].

Changes in *N*-glycan oligosaccharides have mainly been reported in carcinomas in the course of progression or during metastatic change [13, 14, 23]. In the epidermis, glycoconjugate profile changes were studied by lectin histochemistry in a variety of skin diseases [1, 2, 9]. In several autoimmune skin diseases, autoantibody targets have been shown to be *N*-linked oligosaccharides [18]. However, to our knowledge, this is the



**Fig. 1** Chromatograms of *N*-glycans from the epidermis. Fluorescence-tagged glycans released from the epidermis of patients and normal controls on an ODS column using HPLC. Peaks a, b and c correspond to *N*-glycans, Gal $\beta$ 1,4GlcNAc $\beta$ 1,2Man $\alpha$ 1,3 (Gal $\beta$ 1,4GlcNAc $\beta$ 1,2Man $\alpha$ 1,6)Man $\beta$ 1,4GlcNAc $\beta$ 1,4GlcNAc, Gal

$\beta$ 1,4GlcNAc $\beta$ 1,2Man $\alpha$ 1,3(Man $\alpha$ 1,6)Man $\beta$ 1,4GlcNAc $\beta$ 1,4(Fuc $\alpha$ 1,6)GlcNAc and Gal $\beta$ 1,4GlcNAc $\beta$ 1,2Man $\alpha$ 1,3(Gal $\beta$ 1,4GlcNAc $\beta$ 1,2Man $\alpha$ 1,6)Man $\beta$ 1,4GlcNAc $\beta$ 1,4(Fuc $\alpha$ 1,6)GlcNAc, respectively. *LI* lamellar ichthyosis, *NBCIE* non-bullous congenital ichthyosiform erythroderma



**Fig. 2** Rates of molecular compartments, *a*, *b* and *c*. Compartments *a* and *b* were increased and compartment *c* decreased in the hyperkeratotic diseased skin from patients. Colors of bar show following: slashed, *a*; white, *b*; black *c*; *LI* lamellar ichthyosis, *NBCIE* non-bullous congenital ichthyosiform erythroderma

first study that reported *N*-linked oligosaccharide profiles in normal and hyperkeratotic epidermis.

Glycosylation is the major post-translational modification of many proteins. The *N*-Glycan precursor, Glc3Man9GlcNAc2, is introduced to asparagine residues of Asn-X-Ser/Thr sequence while polypeptide synthesis occurs in the endoplasmic reticulum. The Glc residues are removed while protein holding occurs. High-mannose type *N*-glycans work as a tag in the transfer of a protein folded correctly to the Golgi apparatus or misfolded one to degradation system [11]. In the Golgi apparatus, several enzymes modify *N*-glycan oligosaccharides from the high-mannose type into a more complex type by trimming and repetitive addition of sugar residues [15]. The biosynthesis of *N*-glycans is highly conserved and these oligosaccharide residues on proteins have important roles in promoting protein regulation, folding, quality control, sorting and transport [6].

The *N*-glycan alterations seen in ichthyotic skin failed to suggest novel oligosaccharide structures, although the rates of known oligosaccharide structures were changed. Peak *a* is the most common *N*-glycan in the human serum. Peak *b* is formed due to insufficient activity of *N*-acetylglucosaminyltransferase II (GnT-II) and peak *c* is produced from peak *a* *N*-glycan by fucosyltransferase 8 (Fut8) activity [6]. The GnT-II knock-out mice were reported to die from serious defects of multiple organs in early postnatal development, mainly from abnormalities in the gastrointestinal tracts, hematopoietic systems and bones, although skin involvements were not apparent [26]. The Fut8 null mice were also lethal in early postnatal days

mainly from emphysema-like changes in the lung [27]. The Fut8 null mice were known to overexpress matrix metalloproteinases and to show function loss of TGF- $\beta$  receptor. TGF- $\beta$  mediates a variety of signaling pathways [16] and dysfunction of TGF- $\beta$  receptor may be associated with skin symptoms of ichthyosis in the present study. On the other hand, there is a possibility that disturbed differentiation of epidermal keratinocytes resulted in the altered *N*-glycan profiles in the ichthyotic lesions. In any case, the relationship between *N*-glycan alteration and hyperkeratosis is an interesting finding that may help to clarify the pathomechanism(s) of hyperkeratosis.

Epidermal proteins, which comprise high levels of mannose-type oligosaccharides, were analyzed in mouse [24]. These high-mannose type oligosaccharides are located in lysosomes, lamellar granules and cell-cell connection desmosome sites. Desmocollins and desmogleins are also assigned as carriers of high-mannose type oligosaccharides, and it was suggested that high-mannose type oligosaccharides control desquamation [24]. However, in the present samples of hyperkeratotic skin, the profile of the high-mannose type oligosaccharides was not changed. These results indicated that *N*-glycans bound to cell surface proteins were more seriously affected than those in the cytoplasm and desmosomes. Further studies into *N*-glycan structures and the respective proteins carrying the *N*-glycans in hyperkeratotic skin will provide further clues to determine the exact mechanisms that lead to hyperkeratosis in human skin.

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**ABSTRACT:** Epidermolysis bullosa simplex with muscular dystrophy (EBS-MD, MIM 226670) is caused by plectin defects. We performed mutational analysis and immunohistochemistry using EBS-MD (n = 3 cases) and control skeletal muscle to determine pathogenesis. Mutational analysis revealed a novel homozygous plectin-exon32 rod domain mutation (R2465X). All plectin/HD1-121 antibodies stained the control skeletal muscle membrane. However, plectin antibodies stained the cytoplasm of type II control muscle fibers (as confirmed by ATPase staining), whereas HD1-121 stained the cytoplasm of type I fibers. EBS-MD samples lacked membrane (n = 3) but retained cytoplasmic HD1-121 (n = 1) and plectin staining in type II fibers (n = 3). Ultrastructurally, EBS-MD demonstrated widening and vacuolization adjacent to the membrane and disorganization of Z-lines (n = 2 of 3) compared to controls (n = 5). Control muscle immunogold labeling colocalized plectin and desmin to filamentous bridges between Z-lines and the membrane that were disrupted in EBS-MD muscle. We conclude that fiber-specific plectin expression is associated with the desmin-cytoskeleton, Z-lines, and crucially myocyte membrane linkage, analogous to hemidesmosomes in skin.

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## PLECTIN DEFECTS IN EPIDERMOLYSIS BULLOSA SIMPLEX WITH MUSCULAR DYSTROPHY

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**G**enetic defects in the epidermal expression of plectin are known to form the basis of at least three disease subtypes: a rare, mild form of dominant epidermolysis bullosa simplex (EBS) with mottled pigmentation,<sup>19,20</sup> a severe recessive form of epidermolysis bullosa associated with pyloric atresia and severe loss of skin,<sup>5,30,33</sup> and a recessive form of EBS associated with muscular dystrophy (EBS-

MD).<sup>11,25,29,43,45,47</sup> In EBS-MD, plectin defects are thought to affect plasma membrane–cytoskeletal interactions in skin and muscle causing epidermal blistering and late-onset muscle weakness.<sup>11,25,29,45</sup>

Plectin is a large, 450–500 kDa, cytoplasmic protein that acts as a link between various cytoskeletal systems and cell junctions.<sup>50</sup> In skin, plectin links the basal cell keratin intermediate filaments to the transmembrane collagen XVII<sup>21</sup> and the  $\beta 4$  integrin subunit of the  $\alpha 6\beta 4$  integrin located in the hemidesmosome.<sup>22,23</sup> In many tissues and cultured cells, plectin interacts with several cytoskeletal systems: actin microfilaments, intermediate filaments, or microtubules.<sup>50</sup> Plectin in different cells and tissue has the ability to interact with multiple binding partners including intermediate filaments (keratin, desmin, and vimentin), actin microfilaments, microtubules, and specialized molecules at specific junctions such

**Abbreviations:** BSA, bovine serum albumen; CT, computerized tomography; EBS-MD, recessive epidermolysis bullosa simplex associated with muscular dystrophy; GEL, gelatin; MRI, magnetic resonance imaging; NGS, normal goat serum; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; TBS, Tris-buffered saline

**Key words:** plectin; intermediate filament; muscle; Z-line; epidermolysis bullosa

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as vinculin and  $\beta 1$  integrin in striated muscle costamere attachment sites<sup>17,39,40,48</sup> and Rack1.<sup>32,42</sup>

Multiple plectin isoforms are expressed in a tissue-specific manner in mammalian cells and tissues derived from a single gene, *PLEC1*.<sup>7,10,36</sup> In the mouse, plectin is expressed in at least 14 different isoforms, mostly resulting in different splice variants of the actin binding amino-terminal exon 2.<sup>10</sup> However, a form of plectin lacking all or part of the central rod domain (exons 31/32) has been proposed in the rat, similar to the system controlling dystrophin muscle expression.<sup>7</sup> More recently, distinct functions for different plectin isoforms have come to light after determining specific plectin isoform subcellular localization.<sup>36</sup>

Precise and complete isoform expression patterns in various human tissues including muscle have yet to be determined. However, intriguingly, muscle fiber type-specific expression patterns of plectin were reported in clinically normal and diseased human skeletal muscle<sup>11,39</sup> and at costamere membrane attachment sites.<sup>18</sup> Initial reports of control muscle staining with HD1-121 antibody suggested that HD1-121 stained Z-lines in type I muscle fibers, whereas other plectin antibodies (including the 10F6 and 5B3 antibodies) showed variable staining at the membrane.<sup>11</sup> EBS-MD muscle demonstrated a loss of membrane HD1-121 antibody staining with an unusual residual expression of sarcomere staining.<sup>11</sup> Further studies using control and diseased muscle have shown that plectin and desmin intermediate filaments closely encircle sarcomeres, as well as link to the plasma membrane.<sup>4,11,16,38,40,41,43</sup> More recently, in normal rat muscle plectin was suggested to associate with desmin at both vinculin- and dystrophin-containing plasma membrane junctions.<sup>17</sup>

In human skin plectin antibodies show two major epidermal staining patterns. First, a combined dermal-epidermal junction and suprabasal cytoplasmic pattern is seen with the majority of plectin antibodies (including 10F6, 5B3, 6C6,<sup>52</sup> and IFAP300, the hamster plectin homolog<sup>44</sup>). A second, dermal-epidermal junction restricted staining pattern is seen with the HD1-121 and plectin 7A8 antibodies.<sup>25,45</sup> Staining with all these antibodies is severely reduced or absent in EBS-MD patients' skin.<sup>25,45</sup> The binding sites of the majority of plectin antibodies have been identified as lying within the central rod domain including the HD1-121 antigen<sup>15</sup> and various rat plectin antibodies.<sup>8,9,51,52</sup> We now report the precise ultrastructural expression of plectin in normal human and EBS-MD muscle tissue using the most informative of the current battery of plectin antibodies. We have also examined the expression of

important integral muscle components such as dystrophin, desmin, and  $\alpha$  actinin in EBS-MD muscle. The aim of this study was to describe the plectin antibody expression patterns together with critical structural elements in striated muscle to determine the structure of plectin, its precise location, and likely function in striated muscle.

## MATERIALS AND METHODS

**Tissue Samples.** Muscle and skin biopsy samples were obtained from three EBS-MD patients whose cases have been previously described.<sup>34,45</sup> All samples were collected after the patient's consent was obtained, with the relevant institutional approval for experiments involving human material in accordance with the principles of the Helsinki Declaration of 1975.

The clinical, histopathological, ultrastructural, and immunohistochemical details of skin findings (excluding mutation data), limited muscle histology, and immunohistochemistry from Case 1 were previously included in the report by Smith et al. (patient D3).<sup>45</sup> Case 1 was a man aged 33 years who had suffered from widespread friction-induced blistering of his skin since just after birth. He subsequently developed progressive muscle weakness before the age of 2 years and then became confined to a wheelchair. The family was of Maltese origin and the parents were related. Blistering was noted around the hands, feet, scalp, and face as he grew, and he developed hoarseness due to scarring of the anterior commissure of the larynx. He developed early onset and progressive muscular dystrophy before the age of 2 years and computerized tomography (CT) and magnetic resonance imaging (MRI) showed him to have cerebellar and cerebral atrophy. This patient was previously shown to have absent muscle plectin staining as highlighted by the antibody HD1-121.<sup>6</sup> Mutational analysis for this patient was performed here and is reported below.

Case 2 was a 10-year-old, fourth female child of Italian parents who were first cousins. Previously, mutations in the plectin gene (5905del2/5905del2) together with skin immunohistochemistry and ultrastructural findings in this patient were reported by Mellerio et al.<sup>29</sup> Case 2 also presented with widespread trauma-induced blistering on her fingers, hands, feet, and face shortly after birth. She experienced stridor associated with supraglottic scarring and stenosis from the first few weeks of life and underwent a tracheostomy at the age of 20 months because of recurrent episodes of respiratory embarrassment. The two elder siblings in the family were

unaffected. A third child had suffered from blistering and apneic episodes from birth and had died at age 4 years from respiratory obstruction.

The third case was a 45-year-old Japanese wheelchair-bound man, previously described by Pulkkinen et al.,<sup>34,43</sup> including the homozygous 5866delC/5866delC mutation, clinical details, and skin immunohistochemical and ultrastructural findings.

Electron microscopy of the skin biopsy from each patient indicated that blistering arose as a result of rupture of the basal epidermal cells at a level that was just above the keratinocyte plasma membrane<sup>28,29,45</sup> and these changes were associated with previously reported hemidesmosome structural abnormalities.<sup>28</sup> Specifically, EBS-MD Cases 1 and 2 showed poor hemidesmosome inner plaque assembly and keratin intermediate filament linkage to the hemidesmosome plaque.<sup>36</sup>

Control muscle samples ( $n = 5$ , in total) from the flexor digitorum ( $n = 1$ ), gastrocnemius muscle ( $n = 2$ ), and from paraspinal column ( $n = 2$ ) were obtained from routine surgical procedures. Quadriceps muscle from patients with EBS-MD ( $n = 3$ ) were biopsied under anesthesia for diagnostic purposes. The biopsy samples were divided and fixed for electron microscopy; they were snap-frozen in Tissue-Tek OCT compound (Miles Diagnostic, Elkhart, IN) using isopentane cooled by liquid nitrogen for cryostat sectioning or cryofixed for immunoelectron microscopy.

**Mutation Detection.** Genomic DNA was isolated from peripheral blood cells from the patient (in Case 1) and his immediate family members, and DNA from unrelated healthy individuals was used as a control. DNA was subject to polymerase chain reaction (PCR) followed by automated nucleotide sequencing of the PCR products. PCR amplification of exon 32 that contained the mutation was performed with the following primer pairs as previously described by Pulkkinen et al.<sup>34</sup>: Ex32 II L: 5' GCTAAT-ACGCATCACTATAGGAACAGACCACCATGTTCCGCGAGCTGGCCGAGG 3'; Ex32 R: 5' TCACTTCTCCTTGAGCGCGATCT 3'.

The PCR products were subjected to automated sequencing using 12 overlapping primers covering the entire coding sequence of exon 32. The mutation was detected with Ex32 12/12 primers: sense, 5' CTC AAGGCTGAGGCGGA ACT 3'; antisense, 5' TCACCCACCAAAGCGATCC 3'.

**Light Microscopy and Immunohistochemistry.** Immunohistochemical staining was performed with modifications previously described by McMillan et al.<sup>26</sup>

Muscle cryostat sections (5  $\mu$ m) were collected and dried on 3-aminopropyltriethoxysilane (APES)-coated slides (Sigma, Poole, UK). Sections were fixed in acetone ( $-20^{\circ}\text{C}$ ) for 10 min before being washed in 0.1 M Dulbecco's phosphate-buffered saline (PBS). Primary antibodies were diluted in 0.3% w/v bovine serum albumen (BSA) (ICN, Poole, UK) in PBS and were incubated for 30 min at  $37^{\circ}\text{C}$  in a humidified chamber. The primary antibodies used included the anti-HD1 antigen HD1-121,<sup>15</sup> dilution 1 in 50, which recognizes two distinct plectin rod domain epitopes that are likely closely related in the folded, native protein<sup>31</sup>; 5B3 and 10F6 (neat), both mouse monoclonal antibodies raised against the rod domain of rat plectin (from G. Wiche, Vienna, Austria<sup>8</sup>); and 4B4 raised against the  $\beta$ 1 integrin (Beckman-Coulter, High Wycombe, UK), used at 1 in 200. Three antibodies recognizing three dystrophin antibodies were used: NCL-DYST1, NCL-DYST2, and NCL-DYST3 against the rod-, carboxyl-, amino-terminal domains (neat, 1 in 10, and 1 in 40, respectively; Novocastra, Newcastle upon Tyne, UK). Novocastra also supplied antibodies to  $\beta$  spectrin, (1 in 100), merosin (1 in 50), and  $\beta$  dystroglycan (1 in 50). Other antibodies and antiserum included were: desmin monoclonal D33 (Dako, High Wycombe, UK) 1 in 200; polyclonal desmin and vimentin (Sigma) used at 1 in 20, BM-75.2 against  $\alpha$  actinin (Sigma) used at 1 in 200; and mouse anti-vinculin IgG (Sigma) used at 1 in 100. The slides were washed in PBS for 10 min, dried, and blocked with normal rabbit or goat serum (Dako) for 5 min, depending on the secondary antibody.

**Immunoperoxidase.** Consecutive 5- $\mu$ m transverse frozen sections of normal and EBS-MD muscle were fixed in cold acetone, and endogenous biotin activity was blocked using 0.1% hydrogen peroxide. The immunohistochemical technique used streptavidin-biotin complexes coupled to horseradish peroxidase with diaminobenzidine visualization.<sup>2</sup> For primary antibody details, see the previous section. The secondary antibody was rabbit antimouse peroxidase conjugated and was diluted 1 in 500 (Dako) or biotin conjugated goat antimouse (1 in 500) followed by peroxidase conjugated streptavidin (diluted 1 in 1,000). Routine enzyme histochemistry fiber typing was performed by staining with NADPH and ATPase preparations at pH 9.4, 4.6, and 4.3. All experiments included appropriate positive and negative controls and were performed in duplicate.

**Immunofluorescence.** The secondary antibody dilution was 1 in 500 in 0.3% BSA in PBS. The secondary

**Table 1.** Details of the patients having epidermolysis bullosa simplex with muscular dystrophy.

	Case 1	Case 2	Case 3
Patient age, origin, muscle biopsy site, parental relationship	Male, 33 y, Maltese origin, quadriceps biopsy, consanguineous	Female, 10 y, Italian origin, quadriceps biopsy, consanguineous	Male, 45 y, Japanese origin, thigh muscle biopsy, consanguineous
<i>PLEC1</i> mutation/site	R2465X/R2465X* Exon 32	5905del2/5905del2 Exon 31	5866delC/5866delC Exon 31
References			
Mutation report	R2465X/R2465X*	Mellerio et al. <sup>29</sup>	Pulkkinen et al. <sup>34</sup>
Clinical report	Smith et al. <sup>45</sup>	Smith et al. <sup>45</sup>	Pulkkinen et al. <sup>34</sup>

\* Novel mutation detailed in this report.

antibodies used were rabbit antimouse FITC (Dako) or goat antimouse FITC (Dako) and donkey antirabbit Texas red (Amersham, Amersham, UK) and goat antimouse Texas red (Amersham). The muscle and skin sections were incubated for 30 min at 37°C in a dark chamber and then washed in PBS. Sections were dried in the dark and mounted in a 10% PBS/glycerol anti-fade mountant. All experiments included appropriate positive and negative controls and were performed in duplicate.

**Transmission Electron Microscopy.** Control muscle specimens (gastrocnemius and flexor digitorum, n = 2) and EBS-MD muscle (n = 3) were cut, attached to an immovable cork surface, fixed in half-strength Karnovsky's fixative containing 2% formaldehyde and 2.5% glutaraldehyde,<sup>18</sup> and routinely processed as described by McMillan and Eady<sup>27</sup> for electron microscopy. Briefly, samples were washed in 0.067 M cacodylate buffer; osmium postfixation was in 1.3% osmium for 2 h at room temperature. Specimens were stained en bloc in 5% uranyl acetate in 50% ethanol (1 h), dehydrated in a graded ethanol series (15 min each), and embedded in TAAB 812 or Araldite resin with hard hardener (TAAB, Aldermaston, UK) via propylene oxide (two washes). Semithin and ultrathin sections were cut on a Reichert OMU-4 ultramicrotome (Leica, Milton Keynes, UK). Semithin sections (0.5 μm thick) were stained with azure II and methylene blue.<sup>37</sup> Ultrathin sections (60–90 nm) were collected on copper grids, stained in 50% alcoholic uranyl acetate and lead citrate, and examined in either a JOEL 100CX or Hitachi H7100 electron microscope with an accelerating voltage of 75 kV.

**Immunoelectron Microscopy.** Muscle cryofixation and cryosubstitution was carried out as previously described.<sup>26</sup> Briefly, control muscle samples (calf leg muscle and control flexor digitorum muscle, n = 2) were cut, cryoprotected, and plunged into liquid propane cooled by liquid nitrogen in a Leica KF 80

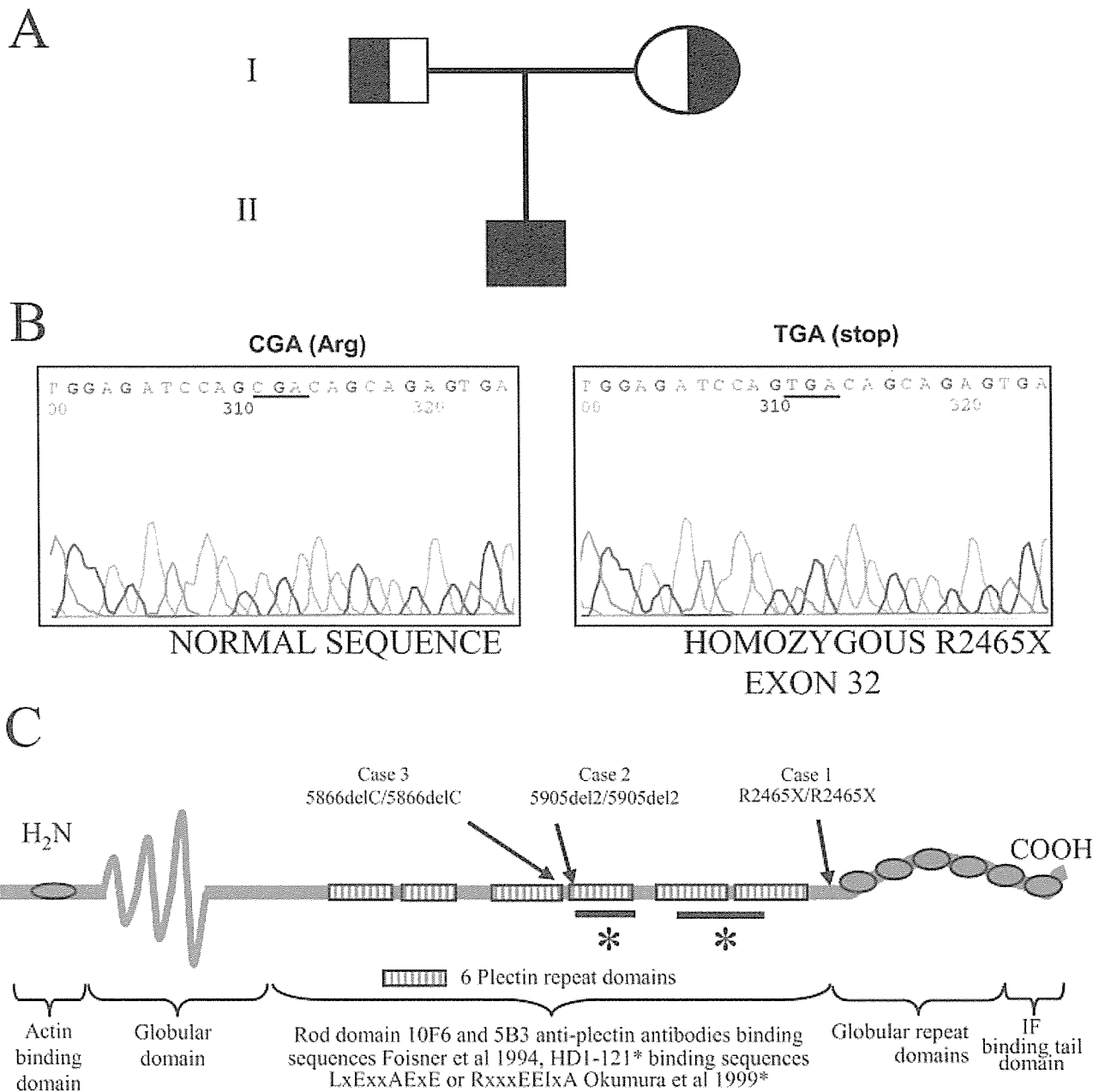
freeze plunging machine. The EBS-MD muscle sample was taken from the thigh leg muscle (Table 1).

Samples were then placed in methanol at –80°C for 3 days in either an AFS or a CS AUTO freeze substitution machine (Reichert, Depew, New York). The samples were embedded in Lowicryl K11M resin/methanol mixtures at –60°C for 4 days while the percentage of Lowicryl K11M resin mixture was increased. The samples were embedded in K11M resin while being polymerized under an ultraviolet light in a dry nitrogen atmosphere. The edges of the blocks were sectioned to check orientation and ultrastructure. Ultrathin sections of 90-nm thickness were collected on pioloform-coated nickel grids and stored for immunolabeling.

Labeling was carried out by floating the nickel grids on drops of buffer in a clean, humid chamber. The grids were incubated in PBS containing 5% normal goat serum (NGS), 1% gelatin (GEL), and 1% bovine serum albumin (BSA) for 30 min. Primary antibodies were diluted in 1% NGS, 1% GEL, 1% BSA in PBS for 1 h followed by four PBS washes. All gold-conjugated antibodies were diluted in 0.2 M Tris-buffered saline (TBS), pH 8.2, with 1% NGS, 1% GEL, and 1% BSA. The secondary antibodies were 5- or 15-nm gold-conjugated goat antimouse and 5- or 15-nm gold-conjugated goat antirabbit (Biocell, Cardiff, UK). The grids were washed twice in TBS and distilled water and stained with 2.5% uranyl acetate in 50% ethanol for 15 min and observed. For each control or EBS-MD sample a total of 7–10 blocks were examined.

## RESULTS

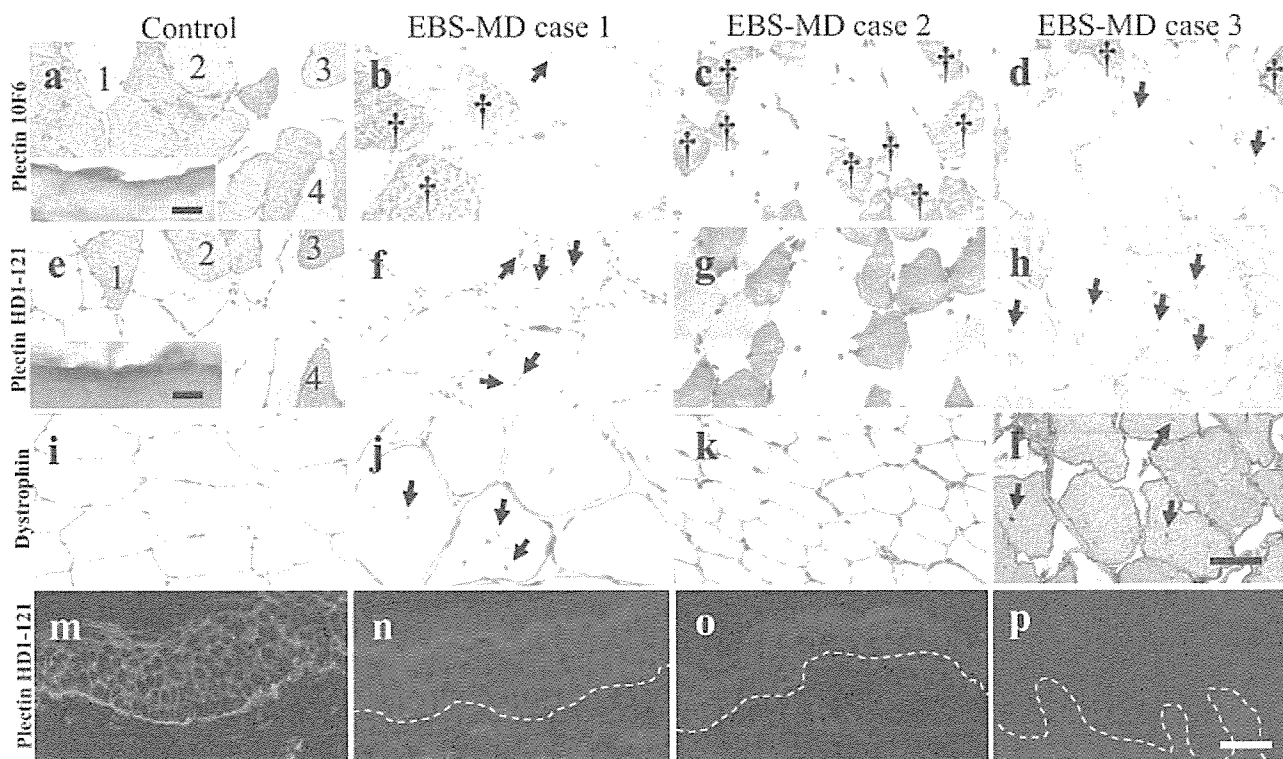
**Mutation Detection.** DNA isolated from peripheral blood cells of the patient (Case 1) with EBS-MD (Fig. 1A) and his parents was subjected to mutation detection analysis. Proband DNA underwent direct nucleotide sequencing for the entire intron–exon borders of the plectin gene. Direct nucleotide sequencing of exon 32 PCR products



**FIGURE 1.** Mutation detection by automated nucleotide sequencing in a Maltese family with epidermolysis bullosa simplex and muscular dystrophy (EBS-MD). **(A)** Pedigree of the family from Case 1 with the proband suffering from EBS-MD. **(B)** Direct nucleotide sequencing of Ex32 PCR products revealed that the proband is homozygous for a C–T transition in nucleotide position 7444. This nucleotide substitution results in the change of an arginine residue (CAG) to a stop codon (TAG), and this mutation was designated R2465X. Predictions suggest that this mutation should lead to nonsense-mediated mRNA decay and a severe reduction or absence of plectin expression. **(C)** Summary of mutations in the three EBS-MD cases whose muscle findings are included in this report. Below, a schematic highlighting plectin domains and the binding sites of the three plectin antibodies (10F6/5B3/HD1-121) used in this study. The precise binding site in the rod domain of 5B3 and 10F6 antibodies is unknown (G. Wiche, personal communication).

revealed that the proband was homozygous for a C–T transition in nucleotide position 7444 when compared to unrelated, healthy individuals ( $n = 50$ ). This nucleotide substitution resulted in a change of an arginine residue (CAG) to a stop

codon (TAG), and this mutation was designated R2465X. We predict that this mutation will lead to nonsense-mediated mRNA decay and to a severe reduction or absence of plectin expression. The site of this mutation is at the very terminal, car-



**FIGURE 2.** Loss of plectin expression at the periphery of all EBS-MD muscle fibers compared to control muscle myocytes. Plectin stained the membrane periphery of all control muscle fibers using all antibodies but only the center of selected fibers with the 10F6 antibody (a and inset) and the HD1-121 antibody (e and inset). However, plectin antibodies 10F6 (b–d) and HD1 (f–h) showed no staining at the cell periphery in all three EBS-MD patient muscle samples. In control type II muscle fibers, plectin (10F6) is expressed at the sarcolemma and within the cytoplasm but is restricted to the periphery of type I fibers. Conversely, plectin-HD1 is expressed at the periphery of all (type I and II) control muscle fibers but is restricted to the central cytoplasm of type I fibers. In EBS-MD muscle, plectin (b–d) and HD1 (f–h) expression is absent at the periphery of all muscle fibers. However, plectin expression is retained within the center of type II fibers in all EBS-MD cases together with multiple internalized nuclei (arrows). In comparison, HD1 was expressed in the center of type II fibers in only one case of EBS-MD muscle (Case 2, g). Thus, there is a profound reduction or loss of both plectin 10F6 and HD1-121 staining at the plasma membrane in EBS-MD tissue. In the youngest EBS-MD case (g) HD1 shows some cytoplasmic staining, but no staining occurs in the other cases (f, h). Control muscle (i) and EBS-MD muscle (j–l), showed normal peripheral dystrophin staining. Control skin showed linear staining (m) for plectin HD1-121, but in EBS-MD patients skin this staining was absent (n–p). Inset (a, e) scale bar, 20  $\mu\text{m}$ . Scale bar, 100  $\mu\text{m}$ .

boxyl end of the rod domain that is encoded for by exon 32. Three different EBS-MD mutations in our patients' R2465X/R2465X (reported here for the first time, to our knowledge), 5866delC/5866delC, and 5905del2/5905del2 were located within the same plectin rod domain (Fig. 1C) and were all predicted to result in a severe reduction or ablation of full-length plectin expression in skin and skeletal muscle through nonsense-mediated mRNA decay. Any exon-specific plectin splicing involving the rod domain (as previously suggested<sup>7,10,36</sup>) could theoretically lead to some persistent plectin isoforms expression within certain organs and tissues.

**Light Microscopy and Immunohistochemistry.** Light microscope findings in Case 1 were previously reported briefly by us.<sup>45</sup> The appearances of the mus-

cle biopsies particularly from the two oldest cases (Cases 1 and 3) were that of a chronic myopathy (Fig. 2b,d,f,h,j,l) sharing many of the histological features previously described.<sup>3,45</sup> There was wide variation in striated muscle-fiber size, the largest being hypertrophied, measuring more than 150–200  $\mu\text{m}$ . There were both individual and grouped atrophic fibers. There were also increases in the amount of endomysial and perimysial connective tissue. Pyknotic nuclear clumps were present, and several fibers displayed multiple, internal nuclei (Fig. 2), as previously described.<sup>45</sup> There was evidence of continuing muscle-fiber necrosis: scattered fibers undergoing phagocytosis were observed and there were several hyaline (segmentally necrotic) myocytes. A few regenerating fibers were also observed. Case 3 also showed the formation of very small vacuoles at

the edge of occasional fibers that were not observed in Cases 1 or 2. Case 2, the youngest case included in this study, showed a very mild variation in myocyte fiber size but was otherwise unremarkable.

**Immunoperoxidase.** Plectin was localized in control muscle (using both 10F6 and 5B3 antibodies) to the membranes of both type I and type II fibers (Fig. 2a). Plectin was also expressed throughout the center of type II muscle fibers (see Fig. 2a), as defined by ATPase staining at pH 9.4, 4.6, and 4.3 (data not shown). This HD1-121 fiber type-specific finding was in agreement with the report of Gache et al.<sup>11</sup> In control muscle the majority (60%) of all muscle fibers exhibited staining for plectin within the center of the fibers. In EBS-MD Cases 1 and 3, plectin expression (using the same two antibodies) was completely absent from the plasma membrane and expressed in a speckled pattern within the center of 50%–60% of type II muscle fibers. Case 2 showed a similar loss of plectin immunoreactivity at the plasma membrane but a normal persistence of plectin expression within type II fibers (Fig. 2c).

Conversely, in control muscle the HD1 plectin antigen (recognized by the HD1-121 antibody) was expressed at the plasma membrane (Fig. 2e) of both type I and II muscle fibers. This showed similar staining patterns to previously reported plectin in muscle.<sup>11</sup> However, HD1 showed restricted expression within the center of type I fibers (see fibers with asterisks in Fig. 2e). HD1 antigen expression in the center of type II muscle cells was complementary (i.e., the exact reverse) of the plectin (10F5 and 5B3) staining observed in type I muscle fibers. Less than half of all control muscle fibers examined (between 30%–40%) exhibited this central HD1-121 staining pattern.

In EBS-MD muscle there was a loss of plasma membrane plectin and HD1 expression (in all plectin-deficient cases, Fig. 2f–h). In the majority of EBS-MD muscle (Cases 1 and 3, Figs. 2f,h) there was no HD1 expression within any of the muscle fibers except in Case 2 (Fig. 2g), where there was residual staining within the center of type I fibers. In control (Fig. 2i) and EBS-MD muscle (Fig. 2j–l), dystrophin stained the periphery of both types I and II muscle fibers. In all EBS-MD cases there was also a normal staining pattern for the integrin  $\beta 1$  subunit,  $\beta$  spectrin, merosin,  $\beta$  dystroglycan, and vinculin similar to control muscle. Vimentin was expressed in selected muscle-fibers from EBS-MD cases 1 and 3 but not in control muscle.

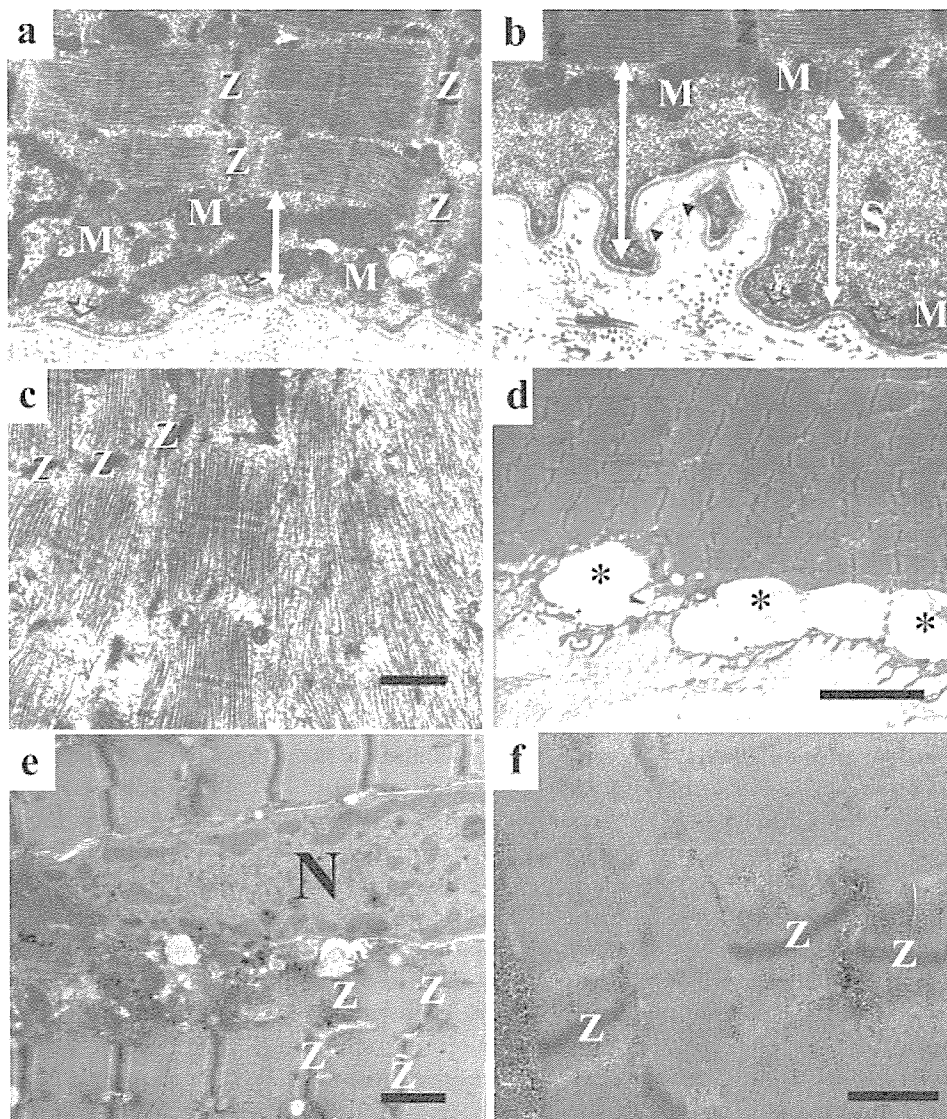
Longitudinal muscle sections of  $\alpha$  actinin and desmin staining (both Z-line-associated proteins)

produced a regular banded pattern of staining in control muscle, but in EBS-MD muscle from Cases 1 and 3 this pattern was weak or severely, focally disrupted. An abnormal increase or clumping of  $\alpha$  actinin and desmin staining was observed at the periphery of EBS-MD fibers in Case 1. Thus, the normal, striated pattern of the intermediate filament protein desmin (linking adjacent Z-lines and Z-lines to the plasma membrane) and  $\alpha$  actinin, a structural protein and constituent of the Z-line, were disrupted in EBS-MD compared to control muscle.

**Immunofluorescence.** In control skin, bright, linear fluorescence was noted along the dermal–epidermal junction and within the cytoplasm of suprabasal keratinocytes (Fig. 2m) for plectin HD1-121, but in EBS-MD patients' skin this staining was completely absent (Fig. 2n–p). All other plectin antibodies including HD1-121, 10F6, 5B3, and 7A8<sup>9</sup> showed similar or identical skin staining patterns. This expression of plectin in EBS-MD skin was reported previously.<sup>25,34,45</sup>

**Transmission Electron Microscopy.** The most striking ultrastructural change in EBS-MD muscle was the increased space between the plasma membrane and the muscle sarcomere, seen in EBS-MD muscle (Fig. 3b,d) but not in controls (Fig. 3a). Case 1 (Fig. 3b) contained enlarged spaces between the folded plasma membrane and contractile units. This was a consistent finding in all five biopsy specimens. In addition, there was extensive reduplication of the basement membrane (Fig. 3b) caused by successive fiber necrosis and regeneration events. Small “focal densities” on the plasma membrane, associated with cytoskeletal filaments, were seen at regular intervals on the cytoplasmic surface of the plasma membrane in both control and EBS-MD muscle fibers (Fig. 3a, vs. 3b,d, respectively). The structure of these densities was unaffected by the muscle fiber pathology. Ultrastructural investigation of EBS-MD muscle from the youngest patient (Case 2, four samples) showed relatively mild myocyte disorganization and only very limited enlargement of the space between the membrane and the plasma membrane.

Comparison of control muscle (Fig. 3a) and EBS-MD muscle (Case 1, Fig. 3b,c; Case 3, Fig. 3d,e) revealed a focal loss of normal sarcomere organization in selected muscle fibers that appeared to resemble a fiber type-specific grouped pattern. However, the severe pathological changes in the EBS-MD muscle tissue made accurate ultrastructural fiber typing difficult (Fig. 3b–f). A further striking ultrastructural change in EBS-MD Cases 1 and 3 were the areas

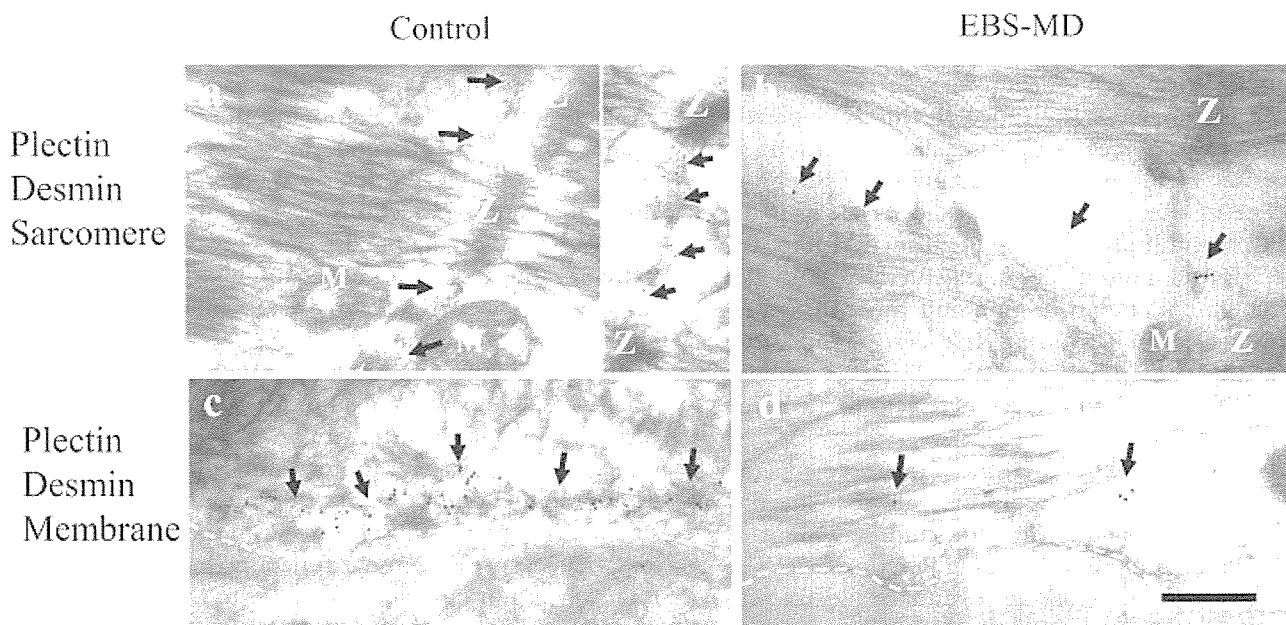


**FIGURE 3.** Muscle of patients with EBS-MD shows a severe loss of normal sarcomere organization and Z-line-plasma membrane anchorage. Transmission electron microscopy of muscle from normal control (**a**) and EBS-MD patients' muscle (**b–e**). Compared to control muscle tissue (**a**), EBS-MD muscle (**b**) shows a variably enlarged space (S, white arrow) between the plasma membrane and the muscle contractile units (sarcomeres). In both normal muscle (**a**) and EBS-MD Case 1 (**b**), small electron densities were seen on the cytoplasmic surface of the plasma membrane (open arrows) (**a,b,d**). Mitochondrial distributions within the normal parameter limits (M) were seen in the spaces beneath the plasma membrane both in normal and EBS-MD muscle. EBS-MD Case 1 also showed extensive basement membrane reduplication, suggesting that repetitive fiber atrophy and regeneration had taken place (**b**, arrowheads). In a subset of myocytes there was focal loss of normal sarcomere organization (**c**) including the presence of small or disorganized Z-lines (Z) or a loss of Z-lines (asterisk, in **c**). In muscle from Case 3 (**d–f**), similar sarcomere changes were present but in addition there were large vacuolated areas adjacent to the plasma membrane (asterisks in **d**). Within the center of muscle fibers in Case 3 there was a mild disorganized misalignment of sarcomeres and focal variability in Z-line size (Z in **e,f**). Abnormal, centrally located myocyte nuclei (N) were also observed (**e**). Biopsies from Cases 1 and 3 both showed enlarged spaces between the myofibrillar apparatus and the myocyte plasma membrane and contained thin filaments that were possibly lost from the edge of the sarcomeres (**b,d**). Scale bars: 1  $\mu\text{m}$  (**a–c,e**); 5  $\mu\text{m}$  (**d**); 0.5  $\mu\text{m}$  (**f**).

of focal myofibrillar disorganization. The loss of normal sarcomere organization was chiefly characterized by focal loss of or variation in normal Z-line size and alignment (Fig. 3). The width of the sarcomere unit and length of Z-lines were often thinner and

smaller than controls (Fig. 3). This variation in Z-line size was beyond the normal variation observed in either type I or II fibers.<sup>12,13</sup> Where sarcomeres remained intact, they were incomplete or misaligned with their neighboring sarcomeres (Fig. 3b–f).





**FIGURE 4.** Plectin is coexpressed in control muscle with the intermediate filament protein desmin between adjacent Z-lines and between peripheral Z-lines and the plasma membrane, but this pattern is disrupted in plectin-deficient muscle. Double-labeling using immunogold electron microscopy in control muscle shows numerous plectin (5B3, small 5-nm gold particles) and desmin (larger 15-nm particles) labeling (arrows in **a**, and inset) adjacent to Z-lines (Z) but sparse labeling between Z-lines in EBS-MD muscle (arrows in **b**). In control muscle (**a**), mitochondria (M) were regularly seen close to or adjacent to Z-lines (Z). In EBS-MD muscle (Case 3) Z-line plectin and desmin labeling (**b**) was severely reduced and disrupted compared to controls (see inset **b**). Furthermore, in control muscle significantly greater densities of plectin and desmin labeling were observed adjacent to the plasma membrane (arrows in **c**). In contrast, the periphery and plasma membrane of EBS-MD muscle showed significantly reduced plectin and desmin staining (arrows in **d**). Scale bar, 100 nm.

There was little uniformity between sarcomeres; thick and thin filaments could regularly be seen running at a tangent to the plasma membrane. The thick and thin filaments were observed at the edge of the sarcomere and often became extruded into the spaces between the sarcomeres (Fig. 3c). Z-line smearing was apparent in focal areas of sarcomere disorganization (Fig. 3c) but was not associated with any abnormal storage material within the myocyte. The loss of myofibrils was replaced by amorphous material, glycogen and swollen sarcoplasmic reticulum cisternae. The number and position of mitochondria at the cell periphery adjacent to the sarcomeres was within normal limits, occasionally showing a mild disruption in distribution in Cases 1 and 3.

**Immunoelectron Microscopy.** Double-labeling immunogold electron microscopy of control muscle fibers colocalized plectin (5B3) and desmin to between Z-lines (Fig. 4a,b). The muscle intermediate filament protein desmin showed a similar colocalization with HD1 in all fiber types. Mitochondria could be seen close to the Z-lines in control muscle (Fig. 4a). In EBS-MD muscle there was a severe loss or reduction and disruption in both plectin and desmin staining (Fig. 4b) compared to control muscle (Fig.

4a vs. 4b). Plectin and desmin colocalized to beneath the cytoplasmic face of small electron densities underlying the sarcolemma in control muscle (Fig. 4c), but in EBS-MD muscle there was a severe loss or reduction in this staining (Fig. 4d).

EBS-MD muscle also showed normal expression of all dystrophin antibody staining (including N- and C-terminal epitopes) in a regular fashion over small, discrete densities along the plasma membrane in a similar fashion to that observed in control muscle. Immunogold particle (5 nm) distribution along the plasma membrane demonstrated similar expression of the integrin  $\beta 1$  subunit in control and EBS-MD muscle.

#### DISCUSSION

There are several major findings in this report. First, plectin (using all the plectin antibodies in this study) is expressed adjacent to the myocyte membrane in control type I and type II muscle fibers. However, multiple plectin antibodies show distinct fiber type-specific expression patterns within the center of normal type II muscle fibers. Conversely, staining for plectin with the HD1-121 antibody was restricted within the center of control type I muscle fibers.

From our data, we suggest that plectin or a closely related molecule is expressed in multiple fiber-specific isoforms comprising different rod domain sequences.<sup>7,10</sup> The significance of different plectin isoforms in at least these two subcellular locations, in the center of the muscle fiber between Z-lines and adjacent to the plasma membrane, is likely to be related to multiple crosslinking functions. In the center of the muscle fiber, plectin crosslinks the intermediate filaments desmin and vimentin (in regenerating fibers) running between Z-lines, and therefore might indirectly link Z-lines via interactions with components such as  $\alpha$  actinin (a Z-line component).

At the muscle-fiber periphery, possible plectin-binding partners include desmin intermediate filaments, but also actin, vinculin, and  $\alpha$  actinin at costamere  $\beta$ 1 integrin-containing junctions.<sup>38,40</sup> The previously reported close association of plectin with costameres does not preclude it from association with dystrophin complex components; however, our data show no alterations in dystrophin proteins in EBS-MD muscle. In EBS-MD muscle there was a severe disruption in normal plectin,  $\alpha$  actinin (a Z-line component), and desmin organization, suggesting that plectin is critical for normal sarcomere arrangement. Vimentin is an intermediate filament protein expressed in regenerating muscle, and was frequently observed in EBS-MD cases 1 and 3. Myocyte damage in plectin-deficient muscle may result from an uneven transfer of contractile energy between Z-lines and from Z-line to the myocyte costamere-plasma membrane via the desmin cytoskeleton. However, plectin may also play an additional role in supporting the normal vimentin-cytoskeleton during muscle-fiber regeneration. Plectin therefore might play multiple roles in EBS-MD pathogenesis.

In addition, plectin is thought to be responsible for maintaining the shape and mechanical stability of the myocyte between the Z-line and the myocyte plasma membrane.<sup>14,24</sup> Ultrastructurally, plectin- and desmin-deficient muscle share many similar features.<sup>14,24</sup> However, the loss of desmin was reported to cause more severe Z-line and sarcomere disruption than was seen in our plectin-deficient samples.<sup>16</sup>

A further significant finding was the loss of plectin staining from all EBS-MD patient tissue at the periphery of muscle fibers. Previously, plectin and HD1-121 had been reported present just within the myocyte membrane, and reduced or absent staining was reported in EBS-MD.<sup>11</sup> The colocalization of plectin and desmin intermediate filaments parallel to Z-lines suggests a role in the attachment and organizing of these structures. In addition, our stud-

ies colocalized plectin and desmin to the region immediately beneath the myocyte plasma membrane, which underlines the importance of anchorage between the cytoskeleton and the plasma membrane, analogous to hemidesmosomes in epithelia. In EBS-MD muscle the loss of membrane-intermediate filament connections via plectin caused increases in subsarcolemmal space and frequent juxtamembrane vacuolization, again highlighting the importance of cytoskeletal-plasma membrane anchorage in normal muscle.

A recently proposed role for desmin-mitochondrial interactions was not supported by a significant displacement of mitochondria in our EBS-MD muscle samples.<sup>1,6,35</sup> Proper uniform subcellular localization of mitochondria within the muscle fiber is essential for efficient energy transfer to the sarcomere, and hence correct muscle function, but may be a relatively minor contributing factor to the late-onset muscle weakness seen in EBS-MD.<sup>35,36,46,49</sup> However, we cannot rule out the possibility that a subtle redistribution of mitochondria may result in some loss of contractile capability in EBS-MD muscle. Further specific mitochondria staining and oxidative phosphorylation studies are required to investigate this.

From our data, it seems that plectin is vital in linking multiple proteins at different sites within muscle fibers to the cytoskeleton. Plectin deficiency in muscle has serious deleterious effects on the transmission of contractile forces from the adjacent sarcomeres via the desmin cytoskeleton to the plasma membrane and leads to the progressive pathological changes seen in this disorder. Elucidation of the full range of molecular interactions between plectin isoforms and the myocyte cytoskeleton will pave the way for a better understanding of the pathogenesis of EBS-MD and other muscular dystrophies.

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