Short Report

Congenital myasthenic syndrome associated with epidermolysis bullosa caused by homozygous mutations in \textit{PLEC1} and \textit{CHRNE}


Mutations in the plectin gene (\textit{PLEC1}) cause epidermolysis bullosa simplex (EBS), which may associate with muscular dystrophy (EBS–MD) or pyloric atresia (EBS–PA). The association of EBS with congenital myasthenic syndrome (CMS) is also suspected to result from \textit{PLEC1} mutations. We report here a consanguineous patient with EBS and CMS for whom mutational analysis of \textit{PLEC1} revealed a homozygous 36 nucleotide insertion (1506_1507ins36) that results in a reduced expression of \textit{PLEC1} mRNA and plectin in the patient muscle. In addition, mutational analysis of \textit{CHRNE} revealed a homozygous 1293insG, which is a well-known low-expressor receptor mutation. A skin biopsy revealed signs of EBS, and an anconeus muscle biopsy showed signs of a mild myopathy. Endplate studies showed fragmentation of endplates, postsynaptic simplification, and large collections of thread-like mitochondria. Amplitudes of miniature endplate potentials were diminished, but the endplate quantal content was actually increased. The complex phenotype presented here results from mutations in two separate genes. While the skin manifestations are because of the \textit{PLEC1} mutation, footprints of mutations in \textit{PLEC1} and \textit{CHRNE} are present at the neuromuscular junction of the patient indicating that abnormalities in both genes contribute to the CMS phenotype.

Conflict of interest
Nothing to declare.

Plectin is a large multidomain and ubiquitously expressed protein, which functions as a linker between the major components of the cytoskeleton (1–3). Mutations in \textit{PLEC1} cause epidermolysis bullosa simplex (EBS), which can occur in association with progressive muscular dystrophy (EBS–MD, OMIM 226670) or pyloric atresia (EBS–PA, OMIM 612138) (4, 5).

Congenital myasthenic syndromes (CMS) are a heterogeneous group of disorders of neuromuscular transmission resulting from mutations in many genes including those encoding the acetylcholine...
receptor (AChR) subunits (CHRNA1, CHRNB1, CHRNBD and CHRNE), rapsyn (RAPSN), agrin (AGRN), MuSK (MUSK), Dok-7 (DOK7), choline acetyltransferase (CHAT), the skeletal muscle sodium channel (SCN4A), the collagenic tail subunit of the acetylcholinesterase (COLQ) and the laminin β2 subunit (LAMB2) (6–11). One of the most common types of CMS is congenital AChR deficiency, which often results from mutations in CHRNE. A single CHRNE mutation, 1293insG, has been encountered in patients from America (12), eastern Europe (13), and North Africa (14), and it has been suggested that it may derive from a common founder.

While the relationship between EBS and myopathy is well established, the association between EBS and CMS is less clear and largely based on a single clinical report by Fine et al. (15) describing a family in which members were found to have either EBS, CMS, or both. In one publication, the muscle of a patient clinically affected by EBS and myopathy was found to have postsynaptic failure of neuromuscular transmission and no expression of plectin; however, the majority of the endplates examined by electron microscopy in this patient appeared structurally normal (16). In a more recent publication, clinical and electrophysiological features consistent with failure of neuromuscular transmission were reported in an 8-year-old boy with EBS–MD, who was compound heterozygous for an early-termination codon and a splice junction mutation in PLEC1 (17).

Here, we describe the clinical features, the endplate studies, and the molecular genetic analysis in a patient affected with EBS and CMS from the family originally reported by Fine et al. Our research shows that mutations in two separate genes (PLEC1 and CHRNE) account for this peculiar pedigree and that evidence for mutations in both genes is present at the neuromuscular junction (NMJ) of the affected patient.

Materials and methods

Muscle biopsy

A biopsy of the anconeus muscle, intracellular recordings, electron microscopy studies and morphometric analysis of the NMJ were performed as previously described (18).

Immunohistochemical analysis

Frozen cryostat tissue sections of 8 mm thickness were fixed on ice with cold acetone for 10 min. The tissue was incubated overnight at 4°C with a mouse antibody directed against plectin (1:50; Santa Cruz Biotechnology, Santa Cruz, CA). The following morning, the tissue was labeled for 1.5 h at room temperature with a goat anti-mouse FITC secondary antibody (Santa Cruz Biotechnology) and a rhodamine-conjugated alpha-Bungarotoxin (BGT) counterstain (125 nM; Sigma, St. Louis, MO). The slides were visualized using a Nikon E-600 fluorescent microscope (Nikon Instruments Inc., Melville, NY).

Array CGH studies

DNA extracted from blood was analyzed using an Agilent 244K human comparative genomic hybridization (CGH) microarray (Agilent Technologies, Santa Clara, CA) as previously described (19).

Mutational analysis

DNA amplification and sequencing

DNA was extracted from patient blood using the QIAmp DNA Blood Mini Kit (Qiagen, Valencia, CA). PCR products were sequenced on an ABI 3730 DNA Analyzer (Applied Biosystems, Foster City, CA). This study was approved by the institutional review board of the University of California, Davis. The patient and her cousin both signed an informed consent form.

Allele-specific PCR

Allele-specific PCR was performed as previously described (20). Primers for allele-specific PCR of 1293insG are provided elsewhere (12).

Expression studies

Construction of minigene

To evaluate the effects of the exon 14 duplication on pre-mRNA splicing, we constructed a plectin minigene spanning part of intron 12, exon 13, intron 13, exon 14, and part of intron 14. Human genomic DNA from the patient and a control were amplified with intron 12 forward and intron 14 reverse primers. The PCR products were analyzed on a 2% agarose gel and purified with a QIAquick gel extraction kit (Qiagen). The purified PCR products, 603 base pair (bp) and 639 bp for wild type (WT) and patient respectively, were sequenced, and cloned into a pcDNA4/HisMax TOPO expression vector (Invitrogen, Carlsbad, CA).

Transfection with plectin minigene

HEK 293 cells were transfected with the WT and mutant minigene constructs as previously described (21).
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RT-PCR assay
Total RNA was extracted from HEK 293 transfected with the minigene constructs, using RNeasy Mini Kit (Qiagen) as previously described (22). The reverse transcriptase (RT)-PCR in muscle mRNA was performed using SYBR Green PCR Master Mix (Applied Biosystems) to increase the DNA intensity of the amplicon.

Isolation of total RNA from muscle
Patient and control muscle was homogenized using a PowerGen Homogenizer (Fisher Scientific, Pittsburgh, PA, USA), and total RNA isolation was performed using an RNeasy Mini Kit (Qiagen).

Western blotting analysis of muscle
Proteins were isolated from homogenized control and patient muscle, and analyzed as previously described (23).

Real time quantitative RT-PCR
Quantitative PCR (qPCR) was performed using an ABI Prism 7800 Sequence Detection System (Applied Biosystems) and the TaqMan® Universal Master Mix reagent (Applied Biosystems) as previously described (24). Primers were designed with the assistance of the PRIMER EXPRESS software version 2.0 computer program (Applied Biosystems) and the service, Assays-on-Demand, provided by Applied Biosystems. We purchased the following TaqMan Gene Expression primers: Hs00356977_m1 for plectin with an amplicon length of 107 bp and exon boundary 19–20, and Hs03929097_g1 for GAPDH (Applied Biosystems). To correct for minor variations in mRNA extraction and reverse transcription, the gene expression values were normalized using the housekeeping gene GAPDH. The data from triplicate samples were analyzed with a sequence detector software (Applied Biosystems) and expressed as mean ± standard deviation of plectin mRNA relative to that of the control.

Results
Clinical report
A 63-year-old woman, born to a first-cousin couple of Russian heritage, was hypotonic with weak crying and sucking at birth. During infancy and childhood she developed bilateral ptosis and she fatigued very easily, although she never experienced swallowing or respiratory difficulties. Throughout her life, she responded moderately to anticholinesterase medication and Ephedrine. She and her twin brother, who never developed weakness, had blisters and erosions of the skin and palate since birth. Dystrophic nails and alopecia of the scalp and eyebrows also have been present in the patient. Her older brother and a maternal cousin were also affected with myasthenia since early childhood (Fig. 1a). At the age of 50, a skin biopsy showed findings consistent with EBS. A skin biopsy performed in the patient’s twin brother was also consistent with EBS and was reported elsewhere (15). A recent physical examination showed generalized skin blisters, especially in the legs and feet, alopecia of the scalp and eyebrows, and dystrophic nails (Fig. S1, supporting information online). The neurologic examination revealed bilateral ptosis and absence of external ocular movements, except for 20° of downward gaze. She had moderate facial and proximal limb weakness.

Fig. 1. Pedigree of the family and the results from PCR amplification of PLEC1 exon 14 and CHRNE exon 12. (a) Family tree showing members with epidermolysis bullosa in black and individuals with myasthenic symptoms with diagonal shading. (b) PCR amplification of PLEC1 exon 14 showed the expected single 423 base pair (bp) band in a control and a single 459 bp band in the patient, a finding consistent with homozygosity in the patient for the 36 bp insertion. The presence of both 423 and 459 bp bands in the cousin indicates heterozygosity for the 36 bp insertion. (c) Allele-specific PCR for CHRNE 1293insG in the patient showed no amplification of exon 12 with the wild-type (WT) forward primer, but clear amplification of the expected 179 bp band using a mutant (MT) forward primer. The opposite is seen in the control; amplification with the WT primer but not with the MT primer. The amplification with both WT and MT primers in the patient’s cousin is consistent with heterozygosity for 1293insG mutation.
with intact deep tendon reflexes. An electrodiagnostic evaluation showed no evidence of denervation, but repetitive stimulation of the left spinal accessory nerve at 2 Hz resulted in a 20% decrement of compound muscle action potential amplitudes, which did not change with faster frequencies of stimulation (Fig. S2, supporting information).

Muscle biopsy
To investigate the nature of the failure of neuromuscular transmission, we performed a biopsy of the anconeus muscle. The light microscopy of the anconeus muscle revealed widely scattered small necrotic fibers, endomysial fibrosis, variation in myofibers and splitting of hypertrophied fibers, consistent with a mild chronic myopathy. The acetylcholinesterase reaction revealed marked dispersion and fragmentation of the endplate area along individual fibers, with some fibers showing numerous chains of minute junctional segments (Fig. S3, supporting information).

The microelectrode recordings showed that the mean miniature endplate potential (MEPP) amplitude of 0.42 ± 0.07 mV (n = 5) in the patient was diminished in comparison with the mean MEPP amplitude of 0.95 ± 0.06 mV (n = 29) in controls (values reported as mean ± SEM; p < 0.001, Student t-test). However, with nerve stimulation at 1 Hz, the mean quantal content of endplate potentials (EPPs) in our patient 38.24 ± 8.34 (n = 5) was actually increased in comparison with the mean EPP quantal content of 12.30 ± 1.2 (n = 18) in controls (p < 0.05).

The most conspicuous abnormalities of the NMJ shown by electron microscopy were (i) fragmentation of endplate segments with numerous small nerve terminals scattered over long distances on a single fiber, (ii) simplification of postsynaptic folds, and (iii) collection of thread-like mitochondria in subsarcolemmal regions and at nerve terminals. No abnormalities of Z-discs or prominent subsarcolemmal vacuoles were seen (Fig. 2). The morphometric analysis of the NMJ revealed that, relative to controls, the patient showed marked

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![Fig. 2](image_url). Electron microscopy findings at the neuromuscular junction (NMJ). (a) A low magnification photograph of the patient’s NMJ showing a fragmented endplate with simplified postsynaptic membrane (arrow heads) innervated by several nerve terminals (arrows) scattered over a long distance on a single fiber. (b) High magnification photograph of a portion of the NMJ displayed in (a) showing severe simplification of postsynaptic folds and marked reduction of secondary synaptic folds (arrow heads). The nerve terminal has normal size, but is filled with a large number of thread-like mitochondria (arrows). (c) An example of a NMJ from a control showing normal postsynaptic folds, secondary synaptic clefts (arrow heads), mitochondria (arrows) and nerve terminal size. (d) Bar graph showing that the density of mitochondria per area of nerve of 4.31 ± 0.63 (n = 9) in the patient was increased in comparison with 2.30 ± 0.39 (n = 10) in controls (p < 0.02, Student t-test). (e) Bar graph showing that the average mitochondrial index of 6.14 ± 0.26 (n = 61) in the patient was markedly increased in comparison with 1.82 ± 0.13 (n = 72) in controls (p < 0.001, Student t-test). Calibration mark represents 1 μm in (b) and (c), and 5.4 μm in (a).
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reduction of the endplate index (ratio of postsynaptic membrane length/presynaptic membrane length) and a diminished average number of secondary folds per micrometer of primary cleft (Table S1, supporting information). At the nerve terminal, the number of synaptic vesicles was diminished but the density of mitochondria was increased, and the mitochondrial index, obtained by dividing the long axis by the short axis of the mitochondria was markedly increased (Fig. 2).

Immunofluorescent assessment showed that expression of plectin was reduced at the muscle membrane and NMJ of the patient (Fig. S4, supporting information).

Mutational analysis

As a result of the clinical and pathological evidence of congenital anomalies in more than one system, the patient’s DNA was studied with a CGH array. The CGH analysis of our patient’s DNA showed no major deletions, duplications or other chromosomal imbalances. Next, we sequenced the 32 exons and corresponding splice junctions of PLEC1 (NM_000445.2) using primers provided in Table S2 (supporting information), and found a homozygous in-frame 36 bp insertion in exon 14 (1506_1507ins36), not present in the DNA of 100 healthy controls. This 36 bp insertion duplicates the 1507-1542 WT sequence with the exception of two bp changes, 31A>G and 34C>T (Fig. 3). As our patient has both EBS and CMS, and three of her family members had either EBS (one member) or CMS (two members), it is unlikely that this pattern could be explained by a single gene defect. Because of the severe limitation of external ocular movements with mild involvement of limb muscles, the primary candidate gene for a second mutation was CHRNE. Indeed, direct sequencing of the promoter and the 12 exons of CHRNE (NM_000080.2) revealed the presence of a homozygous frame-shift insertion of G after

Fig. 3. Results of a PLEC1 minigene transfection into HEK cells and muscle western blot for plectin. (a) PCR amplification of genomic and plasmid DNA, and RT-PCR of RNA extracted from HEK cells transfected with the PLEC1 minigene shows a 603 base pair (bp) band in the wild-type (WT) DNA and RNA and 639 bp (603 + 36 bp) in the patient (Pt) DNA and RNA. In addition, the RT-PCR of the patient’s RNA shows a 438 bp band which includes the first part of exon 13, the last part of intron 13, exon 14 and the 36 bp insertion. The insertion starts after the first nucleotide of exon 14 indicated with a star (*) underneath. The insertion duplicates the 1507-1542 WT sequence indicated by the arrow heads with the exception of two bp changes, 31A>G and 34C>T. The position of these two bp changes is shown below the sequence of the insertion. Note that as the primers used for the amplification of the WT and mutant minigene constructs targeted intron regions flanking exons 13 and 14, the amplified cDNA products represent unspliced transcripts. (b) RT-PCR in muscle shows the expected 193 bp band in the WT and 229 bp band in the patient. The cDNA from cells transfected with the WT minigene showed both an unspliced 277 bp band and a spliced 193 band, however, the cDNA from cells transfected with the mutant minigene yielded only an unspliced 313 bp band. (c) A western blot using frozen tissue from muscle biopsies of the patient and control, and an antibody directed against plectin (Santa Cruz Biotechnology) shows a reduction of plectin expression in the tissue from the patient.
nucleotide 1293 (1293insG) in exon 12, which leads to a nonsense codon, four codons downstream from the mutation. The first cousin of the patient, affected only with CMS, is heterozygous for the same PLEC1 and CHRNE mutations of the proband (Fig. 1b,c), but in addition he is homozygous for a second novel mutation, CHRNE 1046_1072del27, which results in the elimination of amino acids LLPRLLGSP within the intracellular loop of the AChR epsilon subunit gene. This mutation was not found in the DNA of 100 healthy controls. No biological material was available from the two deceased brothers and parents of the patient to carry out further mutational analysis in the affected family.

Expression studies

To analyze the effects of PLEC1 1506_1507ins36 on gene splicing, we transfected HEK cells with a PLEC1 minigene construct harboring either the WT sequence encompassing exon 13, intron 13 and exon 14, or the corresponding mutant sequence containing the 36 bp insertion. RT-PCR of RNA isolated from HEK cells transfected with the WT minigene using a forward primer located in intron 12 and a reverse primer in intron 14, showed a 603 bp band, which included 26 bp of intron 12 (flanking exon 13), 155 bp of exon 13, 84 bp of intron 13, 319 bp of exon 14, and 19 bp of intron 14 (flanking exon 14). In contrast, RT-PCR in cells transfected with the mutant minigene resulted in a 639 bp band (603 bp + 36 bp insertion) and an aberrant 438 bp band. Direct sequencing of the 438 bp band showed that it comprised 26 bp of intron 12, the first 20 bp of exon 13, the last 18 bp of intron 13, all 319 bp of exon 14 with the 36 bp insertion and the 19 bp of intron 14 flanking exon 14 (Fig. 3a). We next performed RT-PCR on RNA from muscle and HEK cells transfected with the minigene using internal primers described in Fig. 3b. DNA sequencing revealed that the 201 bp band included the last 62 bp of exon 13, the 36 bp insertion and the first 103 bp of exon 14. Finally, we measured the relative amount of correctly spliced PLEC1 mRNA in the patient and control muscle using RT qRT-PCR (Fig. S6, supporting information). This analysis revealed that, in comparison with WT RNA, the patient’s RNA, showed a 28% reduction in the correctly spliced PLEC1 mRNA (p < 0.05, Student t-test).

In summary, the findings of the minigene study shows that the homozygous 36 bp insertion in PLEC1 generates an aberrant transcript that leads to a premature termination codon and reduced expression of plectin. Although the mutant minigene transfected into HEK cells failed to splice, RT-PCR and RT qRT-PCR showed that spliced transcripts of the gene were present, but decreased in the muscle of the patient; a finding that explains the reduction, but not complete elimination, of plectin expression.

Discussion

Clinical signs and pathologic findings of myopathy in our patient were mild, and symptoms of myopathy were not even present at the time of death in her twin brother, who had the same skin manifestations, and probably the same PLEC1 defect, as the patient. The oldest sibling had typical symptoms of myasthenia, including ptosis and muscle fatigability, but none of the skin manifestations present in the patient and her twin brother. Presumably, this individual carried the homozygous CHRNE 1293insG mutation without the homozygous PLEC1 insertion, but this assumption could not be verified as there was no available DNA from this sibling. We initially assumed that the lifetime symptoms of myasthenia in the patient’s cousin were also because of CHRNE 1293insG. However,
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this assumption was not borne out by the restriction analysis (Fig. 1c) and direct DNA sequencing, which showed that he was heterozygous for the CHRN
e 1293insG mutation, and homozygous for a novel 27 bp deletion in the same gene. The latter mutation involves highly conserved amino acids, which play a crucial role in cell surface expression of the AChR (25). Thus, the CMS phenotype of the patient’s cousin probably results from the homozygous deletion LLPRLLGSP.

Most cases of EBS–MD are because of nonsense mutations, out-of-frame insertions or deletions within exon 31 and 32 that lead to a premature termination codon. Protein-truncating mutations are predicted to induce degradation of unstable mRNA through nonsense-mediated mRNA decay (26), and this may explain why in our studies RT-PCR failed to detect the aberrant transcript shown by the minigene experiment. Patients carrying truncating mutations show early onset and severe signs of MD and immunohistochemical staining for plectin in tissue from these patients is often negative. Nevertheless, in some families with EBS–MD, the onset of weakness is considerably delayed and the progression of the muscle disease is slow. The majority of these cases with mild forms of EBS–MD result from in-frame insertion or deletion mutations within the globular domain of plectin, which do not completely abrogate plectin expression.

With exception to the mitochondrial changes, most of the findings of the patient’s muscle biopsy findings, including reduction of MEPP amplitudes, increment of quantal content and simplification of postsynaptic membranes, can be explained by the homozygous CHRN
e 1293insG mutation (12).

Altered mitochondrial morphology and distribution, similar to that observed in our patient, has also been observed in some patients with EBS–MD (16, 27) but not in others (28). Although, mitochondrial abnormalities per se are unlikely to alter neuromuscular transmission, they indicate an underlying disruption of the cellular cytoskeleton – a system that is required for several fundamental processes of synaptic organization, including clustering and anchoring of receptors at the NMJ (29, 30). Hence, the PLEC1 defect in our patient seemingly contributes to the impairment of neuromuscular transmission caused by the CHRN
e mutation. As in-frame mutations in PLEC1 associate with only mild weakness, and premature termination codon mutations in PLEC1 usually result in severe weakness because of the underlying myopathy, it is unclear whether PLEC1 mutations alone can result in a phenotype of EBS and CMS. A careful analysis of the function and structure of the NMJ in patients with EBS–MD will be required to define the role of neuromuscular transmission failure in the muscle dysfunction of these patients. In this report, we underscore the need to search additional genetic defects in consanguineous families in which EBS associates with symptoms of CMS, particularly when the signs of myopathy are mild.

Supporting Information

The following Supporting information is available for this article:

Fig. S1. Clinical findings.
Fig. S2. Electrodiagnostic findings.
Fig. S3. Light microscopy findings.
Fig. S4. Immunohistochemistry analysis.
Fig. S5. Analysis of muscle RNA using RT-PCR.
Fig. S6. Analysis of PLEC1 mRNA expression.
Table S1. Morphametric data.
Table S2. PLEC1 primer sequences.

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

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