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Substitution of a Conserved Cysteine-996 in a Cysteine-Rich Motif of the Laminin α2-Chain in Congenital Muscular Dystrophy with Partial Deficiency of the Protein

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Summary

Congenital muscular dystrophies (CMDs) are autosomal recessive muscle disorders of early onset. Approximately half of CMD patients present laminin α2-chain (merosin) deficiency in muscle biopsies, and the disease locus has been mapped to the region of the LAMA2 gene (6q22-23) in several families. Recently, two nonsense mutations in the laminin α2-chain gene were identified in CMD patients exhibiting complete deficiency of the laminin α2-chain in muscle biopsies. However, a subset of CMD patients with linkage to LAMA2 show only partial absence of the laminin α2-chain around muscle fibers, by immunocytochemical analysis. In the present study we have identified a homozygous missense mutation in the α2-chain gene of a consanguineous Turkish family with partial laminin α2-chain deficiency. The T→C transition at position 3035 in the cDNA sequence results in a Cys996→Arg substitution. The mutation that affects one of the conserved cysteine-rich repeats in the short arm of the laminin α2-chain should result in normal synthesis of the chain and in formation and secretion of a heterotrimeric laminin molecule. Muscular dysfunction is possibly caused either by abnormal disulfide cross-links and folding of the laminin repeat, leading to the disturbance of an as yet unknown binding function of the laminin α2-chain and to shorter half-life of the muscle-specific laminin-2 and laminin-4 isoforms, or by increased proteolytic sensitivity, leading to truncation of the short arm.

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Introduction

Congenital muscular dystrophies (CMDs) are autosomal recessive muscle diseases that are manifest at birth or in the first months of life. The symptoms consist of muscle weakness and hypotonia, delayed motor development, and severe and early contractures often associated with joint deformities (Fardeau 1992). Brain abnormalities such as white-matter changes, cortical anomaly, and polymicrogyria can also be associated with the disorder (Philpot et al. 1995; Sunada et al. 1995*b*). Muscle biopsies exhibit large variation in the size of muscle fibers, some necrotic and regenerating fibers, as well as marked increase in endomysial collagen. Diagnosis is based on the clinical features and morphological changes (Fardeau 1992; Dubowitz 1995; Dubowitz and Fardeau 1995). Components of the extracellular matrix have been considered candidate proteins to be involved in CMD, because of the large increase of connective tissue in the muscle (Stephens et al. 1982; Hantai et al. 1985), and recently deficiency in laminin α2-chain has been reported in CMD patients (Tomé et al. 1994).

Laminins are a family of basement membrane–specific glycoproteins composed of α (~400 kD), β (~200 kD), and γ (~200 kD) subunit chains that form cross-shaped heterotrimers (Engel 1992; Tryggvason 1993; Burgeson et al. 1994). The short arms of the cross contain globular domains separated by rodlike domains containing several unique laminin motifs each containing eight cysteines. The long arm is formed by a triple-coiled coil with a large α2-chain globule at the end. The subunit chains exist in several genetically distinct forms that assemble into numerous laminin isoforms with varying tissue distribution. A specific role for laminin in the interactions of muscle with the extracellular matrix was realized through the identification of the quite muscle-specific α2-chain, previously termed "merosin" (Leivo and Engvall 1988; Ehrig et al. 1990), a component of the laminin-2 and laminin-4 isoforms (see Burgeson et al. 1994). The association of this chain with muscle disorders was implied by immunohisto-

chemical studies revealing its decrease or even complete absence in the skeletal muscle of several patients with classical type CMD (Tomé et al. 1994). Homozygosity mapping and linkage analysis of merosin-deficient CMD families localized the CMD gene to the same chromosome 6q2 region (Hillaire et al. 1994; Helbling-Leclerc et al. 1995*a*) where the α2-chain gene (LAMA2) is located (Vuolteenaho et al. 1994). A mutation was found in the LAMA2 gene in dy^{2J}/dy^{2J} mice, an animal model for human autosomal recessive CMD, associated with a partial deficiency and abnormal transcripts (Xu et al. 1994; Sunada et al. 1995*a*). Furthermore, in two families with CMD, the complete lack of laminin α2-chain was explained by mutations in the LAMA2 gene, leading to the formation of truncated α2-chains, thus preventing the formation of trimeric laminin molecules containing this chain (Helbling-Leclerc et al. 1995*b*). The muscle laminins are believed to bind to α-dystroglycan, a component of the large sarcolemma protein-complex dystroglycan, which, in turn, binds to the intracellular dystrophin (Campbell 1995; Worton 1995). Mutations in all these components have now been shown to result in different forms of muscular dystrophy (Monaco et al. 1986; Roberds et al. 1994; Bönnemann et al. 1995; Helbling-Leclerc et al. 1995*b;* Lim et al. 1995; Noguchi et al. 1995; Piccolo et al. 1995). Thus, the muscle laminins play a crucial role for skeletal muscle function, presumably by connecting the muscle fibers to the extracellular matrix.

In the present study we used microsatellite markers and homozygosity mapping to link the disease, in a CMD patient with only partial deficiency of the laminin α2-chain in muscle tissue, to the LAMA2 gene. Furthermore, SSCP analysis of exons was performed to search for mutations in the gene. This resulted in the identification of a missense mutation causing the substitution of cysteine-996 by arginine in a cysteine-rich repeat of domain IIIb in the short arm of the laminin α2-chain.

Subjects and Methods

Subjects

This study was carried out on members of a consanguineous Turkish family (2666). The proband was a 5-year-old boy with normal intelligence and a clinicopathological diagnosis of CMD (Dubowitz 1994; Dubowitz and Fardeau 1995). Hypotonia was evident in the neonatal period, and the patient presented delayed motor development in that he was first able to hold his neck at age 12 mo and could first sit unsupported at age 2 years. His maximal motor capacity was the ability to stand with support. He had very mild contractures of knees and ankles and an increasing lumbar lordosis. Serum creatine kinase (CK) activity was slightly increased (387 U/liter) at age 2.5 years.

Immunocytochemical Analysis

The biopsy samples were obtained from the deltoid muscle of the patients. They were frozen in isopentane cooled in liquid nitrogen, were stored at −80°C, and were studied by conventional histology, histochemistry, and immunohistochemistry on frozen cryostat sections. For immunocytochemical analysis, monoclonal antibodies against human laminin α2- (Chemicon), human laminin α1-, β1-, and γ1-chains (Gibco BRL) were used, as described elsewhere (Tomé et al. 1994). The sections were examined under a Zeiss Axioplan fluorescence microscope. Photographs were taken under identical conditions, with the same exposure time.

Brain Magnetic-Resonance Imaging (MRI)

Magnetic-resonance imaging of the proband was performed on a 0.5T Philips machine (model SE3000/100) using T2-weighted spin-echo sequences. The locations and extent of the white-matter lesions could be observed.

Microsatellite Genotyping

Genomic DNA used in this study was extracted either from blood cells or from lymphoblastoid cell lines, by use of an automated 341 Applied Biosystem Nucleic Acid Purification system apparatus. All members of the Turkish kindred 2666 studied in the present work were genotyped with the $(CA)_n$ microsatellite markers (D6S1712, D6S1639, D6S1702, D6S1715, D6S407, D6S1620, D6S1705, D6S1572, D6S262, D6S457, D6S1656, D6S413, D6S472, D6S1722, D6S975, D6S976, D6S270, D6S1626, and D6S292) either provided by the 1993–94 Généthon human genetic linkage map or more recently developed at Généthon (Gyapay et al. 1994; Dib et al. 1996). The high consanguinity led to the use of homozygosity mapping (Lander and Botstein 1987) with the highly polymorphic markers used in this study. PCR amplifications with primer pairs for the markers were performed in 50 μl containing 40 ng of genomic DNA, with 1 mM of each primer, 50 mM KCl, 10 mM Tris HCl (pH 8.3), 1.5 mM $MgCl_2$, 100 mM of each dNTP, and 1 U of *Taq* polymerase (Boehringer Mannheim). PCR cycles were as follows: 35 cycles of 1 min at 94°C, 1 min at 53°C, and 1 min at 72°C, as well as an additional cycle of 3 min at 72°C (HYBAID; OmniGene). The PCR products were size separated on 6% denaturing polyacrylamide gels and were transferred to Hybond N+ membranes (Amersham). Oligonucleotides $(CA)_{15}$ were tailed with dNTP by use of terminal transferase (Boehringer Mannheim). The membranes were hybridized overnight at 42°C, with the tailed $(CA)_{15}$ probe labeled with horseradish peroxidase, and specific binding was visualized by use of an enhanced-chemiluminescence detection system (ECL kit RPN 2101; Amersham).

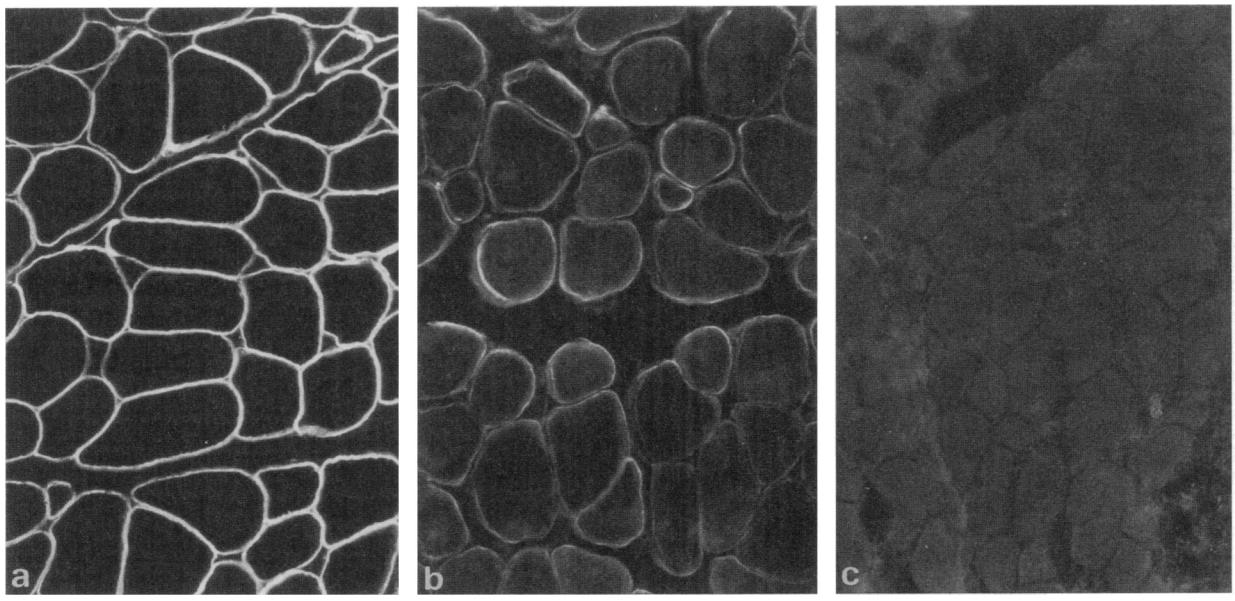

Figure 1 Immunocytochemical analysis of the laminin α2-chain in human skeletal muscle biopsies: staining of the laminin α2-chain in a control individual (*a*), family 2666 proband (1-4783) (*b*), and family 2657 affected patient (Helbling-Leclerc et al. 1995*b*) (*c*). Note diminution and considerable variability of the intensity of labeling in the proband 1-4783, as well as absence of labeling in the patient with the LAMA2 nonsense mutation (Q1241→X). (magnification 230×)

SSCP Analysis

Primer pairs were designed to amplify the regions around several known exons of the laminin α2-chain gene (Xu Zhang and Karl Tryggvason, unpublished data). To amplify exon 20, forward primer 5′-ACTTCGAGTTAACTGATT-3′ and reverse primer 5′-TATGTAGGACTTAAGTCAT-3′ were used with the following reaction conditions: 50 ng of genomic DNA, 1 mM of each primer, 10 mM Tris HCl (pH 8.3), 1.5 mM $MgCl_2$, 300 mM of each dNTP, and 1 U of *Taq* polymerase (Eurobio). The PCR amplifications were performed by use of the "touchdown" method (Don et al. 1991). The initial and final annealing temperatures were 44°C and 39°C, respectively, with the temperature decreased by 1°C after each two cycles and with 30 cycles at 39°C. At each cycle, the denaturation was performed at 94°C for 40 s and the extension was performed at 72°C for 1 min. Final extension was at 72°C for 2.5 min. An aliquot of the PCR product was analyzed after denaturation on a 10% polyacrylamide gel at 7°C and 20°C. A silver-staining kit (BioRad) was used to visualize the DNA bands on the gels.

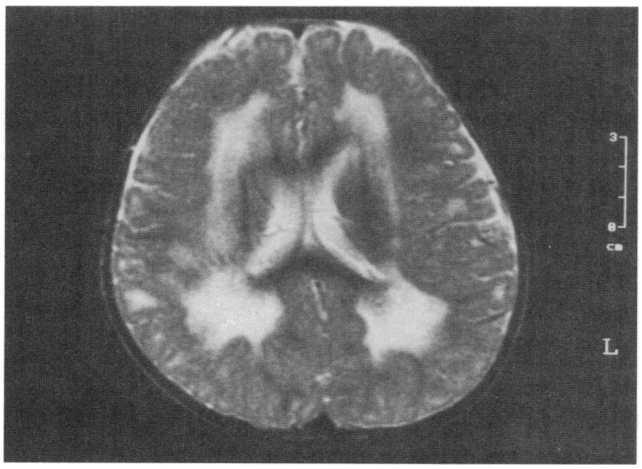

Figure 2 Magnetic resonance imaging. T2-weighted transverse scan of the proband shows mild symmetric hyperintense lesions in centra semiovale in the white matter, suggestive of dysmyelination.

Sequencing

Prior to the sequencing, the PCR products were purified through a Centricon 100 membrane (Amicon). A *Taq* polymerase cycle sequencing kit (Applied Biosystems) was used together with fluorescent dideoxynucleotides, and the sequencing was performed by use of an automated DNA sequencer (Applied Biosystems). All sequences were determined from both DNA strands.

Results

Immunocytochemical Analysis

The immunocytochemical studies of the laminin α2-chain (fig. 1) showed normal labeling in controls, faint staining of varying intensity in the family 2666 proband, and absence of labeling in a patient described elsewhere

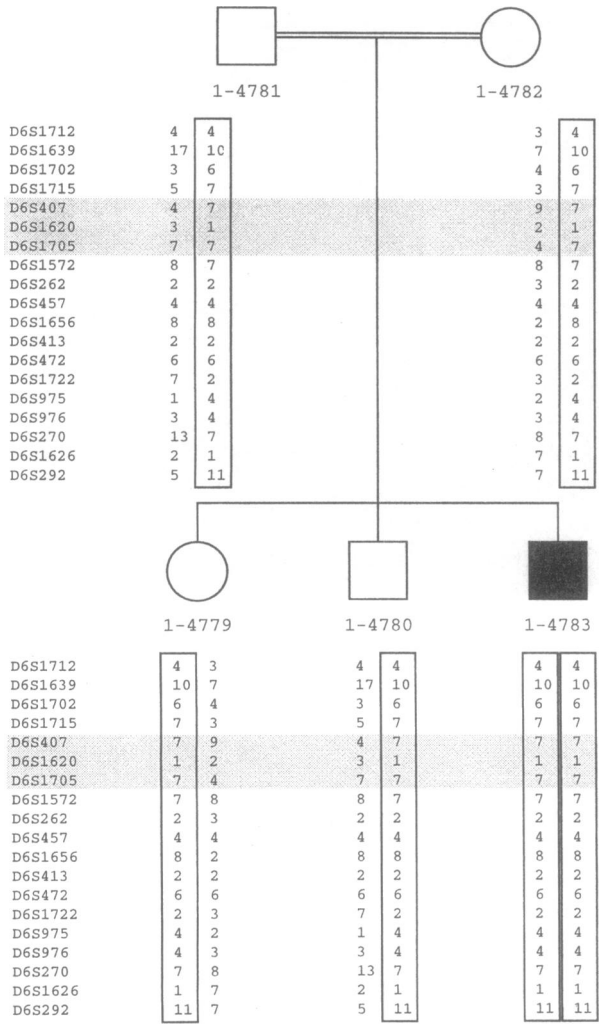

Figure 3 Pedigree of Turkish family 2666 presenting CMD boy patient with partial deficiency of the laminin α2-chain. Males and females are indicated by squares and circles, respectively; the affected boy is depicted by a blackened symbol, and unaffected subjects are depicted by unblackened symbols. Alleles from the homozygous haplotype cosegregating with the disease are boxed, and the three specific markers—D6S407, D6S1620, and D6S1705—corresponding to the disease locus are gray shadowed. The different alleles are numbered from the largest to the smallest, as observed on the film (Helbling-Leclerc et al. 1995*a*).

(Helbling-Leclerc et al. 1995*b*). In the latter case, a nonsense mutation in LAMA2 exon 24 (Q1241X), leading to truncated protein, explains the absence of the labeling. With anti-laminin α1-chain antibody, an overexpression of the labeling of the periphery of the proband muscle fibers was observed, as reported elsewhere (Tomé et al. 1994), in patients with complete laminin α2-chain deficiency (data not shown). The labeling with the antibodies against dystrophin and laminin β1- and γ1-chains was normal in the patients (data not shown), indicating that the expression of laminin molecules containing these chains is not significantly affected by the absence of the α2-chain.

MRI

MRI performed on the proband by use of a standard 0.5 Tesla unit showed mild hypointense (T1-weighted) and hyperintense (T2-weighted) signal abnormalities within the centra semiovale, as well as in the deep cerebrum, a result compatible with dysmyelination (fig. 2). No polymicrogyria or other brain abnormality was detected.

Microsatellite Genotyping

Genotyping of 19 microsatellite markers from chromosome 6q2 in family 2666 demonstrated that the proband (1-4783) was homozygous for all of them (fig. 3). These markers recently have been used to localize the laminin α2-chain–deficient CMD between D6S407 and D6S1705 in 19 consanguineous families (fig. 3, gray-shaded area). Marker D6S1620, which is located between these two, was the only one homozygous in all the families, and the location of LAMA2 gene was confirmed by radiation hybrid mapping (Helbling-Leclerc et al. 1995*a*). The haplotypes suggest that the disease gene is LAMA2. The disease is transmitted by the boxed haplotype; the parents and the two unaffected siblings are heterozygous carriers.

Identification of a Mutation in Exon 20

SSCP analysis of exon 20 PCR products amplified from family 2666 members and a control individual

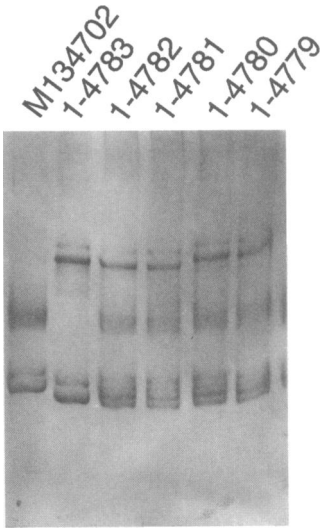

Figure 4 Identification of a mutation in exon 20 in Turkish family 2666: result of SSCP analysis of the PCR-amplified exon 20 region. Individuals are identified as follows: M134702, control DNA, showing the normal migration pattern; 1-4783, proband, showing a mutant pattern; 1-4781, 1-4783, and 1-4780, samples of other family members, displaying fused patterns of mutated and normal alleles.

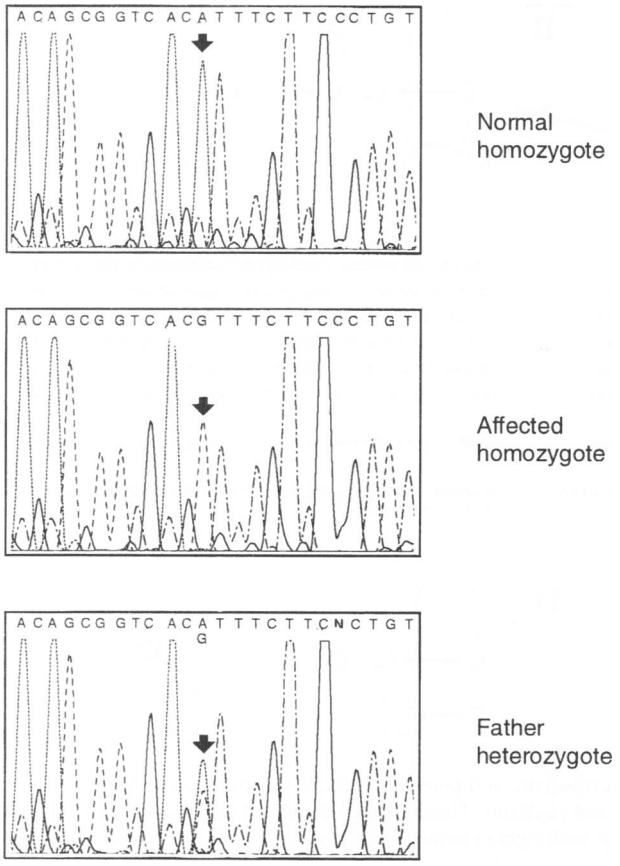

Figure 5 DNA sequence analysis. Direct sequencing of PCR-amplified exon 20 was performed in an ABI 373 automated sequencer. A T→C substitution was identified at position 3035 in the cDNA sequence (A→G substitution in the antisense strand is shown) in a heterozygous carrier and in the proband. The mutation changes the TGT codon of cysteine-996 in the sense strand to the CGT codon for arginine. Positions of the mutations are marked by arrows.

(M134702) revealed different abnormal conformers. As illustrated in figure 4, the proband pattern (1-4783) differs from that of the control, suggesting a sequence variation. Furthermore, the pattern observed in the four other family members is a mixture of those of the proband and the control, suggesting a heterozygous status. Direct sequence analysis of the normal and aberrant PCR products (fig. 5) revealed a T→C transition at position 3035 in the cDNA, as numbered by Vuolteenaho et al. (1994). This mutation changes the TGT codon of the highly conserved cysteine-996 to the CGT codon for arginine (Cys996→Arg). The proband is homozygous for the mutation, whereas the parents and the two unaffected children are heterozygous, in agreement with the haplotype analysis. This mutation creates a *Mae*II restriction site, thus shortening the 260-bp fragment found in normal individuals to a 180-bp fragment and a 80-bp fragment that are found in the mutation carriers (fig. 6). A total of 112 unrelated French and 54 unrelated Turkish individuals were analyzed for the *Mae*II restriction site, but the mutation was found only in members of family 2666 (data not shown).

Discussion

The present results demonstrated the association between a single amino acid substitution in the short arm of the laminin α2-chain and the development of CMD characterized by a decrease—but not absence—of this chain in muscle tissue. Specifically, we have identified a homozygous point mutation changing a conserved cysteine-996 located in domain IIIb of the short arm to arginine. This mutation is particularly interesting because it sheds new light on the role of the short arm of the α2-chain in muscle function. The nature of the present mutation differs substantially from that of two previously described nonsense mutations in the complete laminin α2-chain–deficient CMD families (Helbling-Leclerc et al. 1995*b*), in that those latter result in truncated α2-chains, lacking parts of domains I/II that together with the β- and γ-chains form the α-helical long arm of the molecule. Consequently, those two mutations result in complete lack, in muscle tissue, of laminin molecules containing the α2-chain. In contrast, the mutation described in this study should result in normal synthesis and incorporation of the mutated chain into a trimeric laminin molecule, since the short arm does not participate in trimer formation. This assumption is supported by the present histochemical findings showing presence—albeit decreased—of the laminin α2-chain in the CMD patient. The mutation reported in the laminin α2-

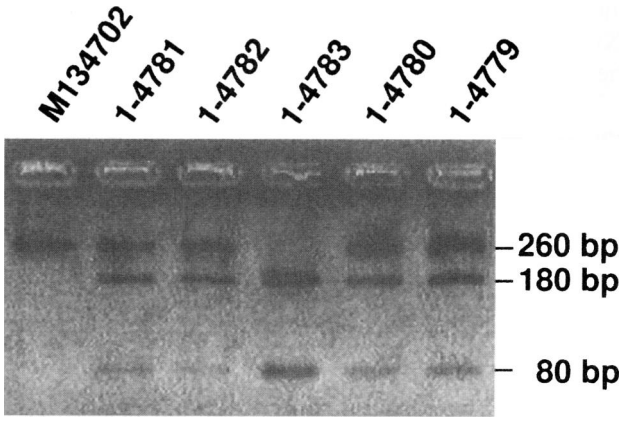

Figure 6 Genotype diagnosis by restriction-enzyme digestion in family 2666. Individuals are identified by their numbers. The creation of a *Mae*II site induces the presence of new fragments. An uncut 260-bp fragment was seen in control individuals (M134702); 180- and 80-bp fragments were observed in heterozygous family members, in addition to the normal 260-bp fragments (from 1-4779 to 1-4782); and the homozygous proband (1-4783) yields only the mutation-specific 180- and 80-bp fragments.

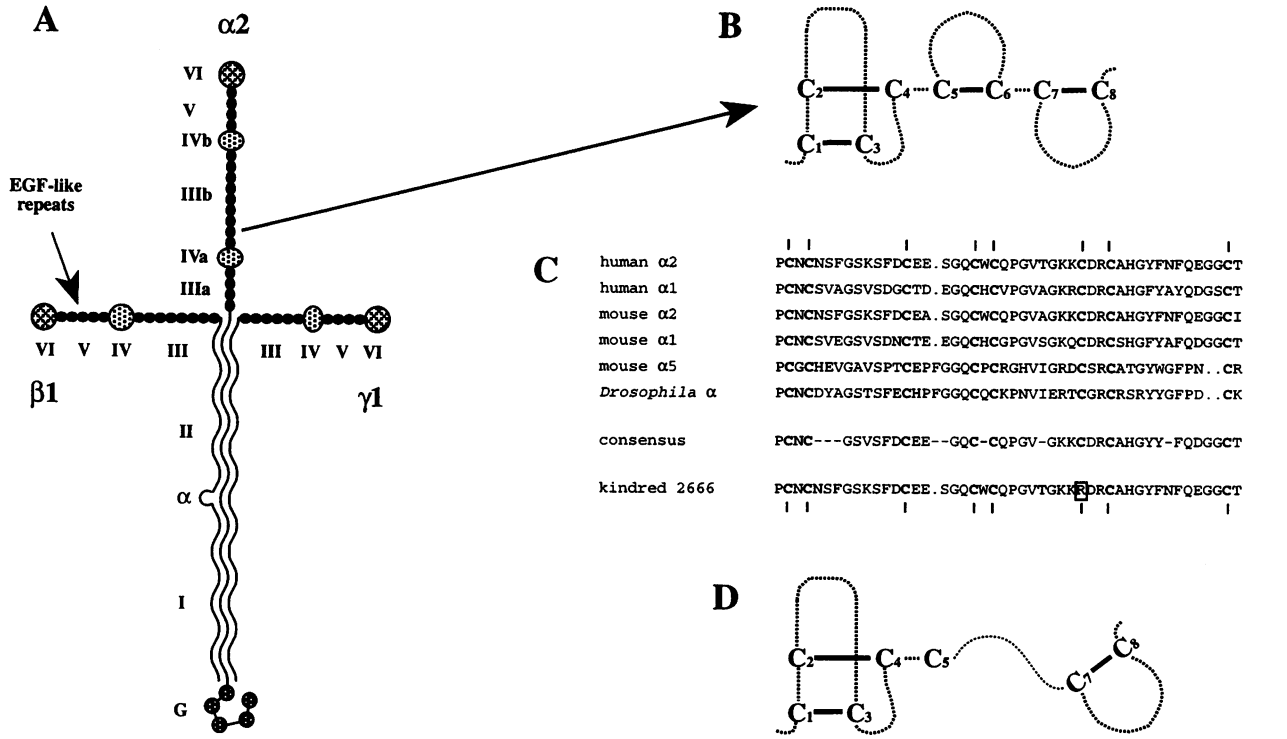

Figure 7 Schematic domain structure of the short arm of the laminin α2-chain, and potential effect of the mutation in Turkish kindred 2666. *A*, Domain structure of a laminin-2 heterotrimer containing α2-, β1-, and γ1-chains. The individual domains I–VI, α, and G are indicated (Engel 1992). *B*, Predicted disulfide bonding of a cysteine-rich laminin repeat with eight cysteines (Beck et al. 1992). *C*, Homology comparison of the laminin repeat IIIB6, between human and mouse α1- and α2-chains, mouse α5-chain, and *Drosophila* α-chain. The locations of the eight conserved cysteines are indicated by vertical bars and boldface letters. The repeat consensus sequence is also shown. The mutated sequence of kindred 2666, with the arginine substitution boxed, is shown. *D*, Putative consequence of the Cys996→Arg mutation in a cysteine-rich motif of domain IIIb of the human laminin α2-chain.

chain gene in dy^{2J}/dy^{2J} mice causes truncation within the amino-terminal domain VI, which presumably also allows the assembly of heterotrimeric laminin molecules (Xu et al. 1994; Sunada et al. 1995*a*), as is the case in the present study.

The reason for the development of muscular dysfunction as a result of the Cys996→Arg mutation is not clear. The mutated residue is the sixth of eight cysteine residues in the sixth of nine cysteine-rich repeats of domain IIIb, as counted from the amino-terminus (fig. 7). The laminin repeats are usually organized into one to three clusters in the short arm of the chains, each cluster containing between four and nine repeats (Beck et al. 1992). The repeat containing the Cys996→Arg substitution described here is very highly conserved between the α1- and α2-chains in human and mouse, the α-chain in *Drosophila*, and the α5-chain in mouse (fig. 7; Sasaki et al. 1988; Garrison et al. 1991; Nissinen et al. 1991; Vuolteenaho et al. 1994; Bernier et al. 1995; Miner et al. 1995).

The cysteine residues in the laminin repeats form the disulfide bonds C_1-C_3, C_2-C_4, C_5-C_6, and C_7-C_8 (Beck et al. 1992) as depicted in figure 7. Thus, the mutation should abolish a bond between C5 and C6, leaving one cysteine residue free. This, in turn, could cause abnormal folding of this particular domain and/or form abnormal disulfide bonds with other domains of the molecule—or even with other matrix molecules—because of the formation of a free cysteine.

The actual function of the cysteine-rich repeats for the α2-chain is not known. Similar repeats are present in clusterlike arrangements in a number of extracellular-matrix proteins other than laminin, such as nidogen, tenascin, and thrombospondin, but their biological function is still poorly understood (Engel 1991). Recently, it has been demonstrated that a single cysteine-rich motif in the laminin γ1-chain is responsible for the binding of that chain to nidogen (Mayer et al. 1993; Pöschl et al. 1994). This kind of specific binding to nidogen or to other proteins has not been demonstrated for any other such repeats in laminin chains. An indication of another protein-binding function of a laminin cysteine-rich motif comes from an in-frame deletion of such a repeat in domain III of the γ2-chain in a patient with epidermolysis bullosa (Pulkkinen et al. 1994). The present results may indicate that the repeat containing domain IIIb has

a binding function of an as yet unknown nature, since the mutation renders the muscle-specific isoform(s) laminin-2 and/or laminin-4 functionally abnormal, with malfunctional muscle fibers and accelerated degradation of the protein as a result. Another possible explanation is that the mutation renders the repeat more sensitive to proteolytic degradation, thus deleting the major portion of the short arm, including known sites for intermolecular assembly. The overexpression of the α1-chain in the patient's muscle, together with normal expression of the β1- and γ1-chains, indicates a replacement of the degraded muscle-specific laminins by the laminin-1 and laminin-3 isoforms (Tomé et al. 1994).

In conclusion, the present work describes the third example of a mutation in the LAMA2 gene in CMD and demonstrates the importance of α2-chain–containing laminin isoforms for the normal function and development of skeletal muscle and brain. The presently described missense mutation implies that the cysteine-rich repeat containing domain IIIb has a potential role in biological function of the molecule, possibly related to binding to other cellular or extracellular proteins; another alternative is that there is increased protease sensitivity as a result of the mutation.

Acknowledgments

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