Identification of a novel mutant transcript of laminin α2 chain gene responsible for muscular dystrophy and dysmyelination in dy^2J mice

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Murine dystrophia muscularis-2J (dy^2J) is an autosomal recessive disorder characterized by muscular dystrophy and dysmyelination of peripheral nerve. Biochemical characterization of dy^2J mice revealed the expression of a mutant laminin α2 chain with a smaller molecular weight in the basal lamina of striated muscle and peripheral nerve. DNA sequencing of the α2 chain cDNA amplified by RT–PCR from dy^2J mice identified a novel and predominant transcript with a 171 base in-frame deletion. We also confirmed an underlying splice donor site mutation in the α2 chain gene of the dy^2J mouse. Translation of this variant transcript would result in the expression of a truncated α2 chain having a 57 amino acid deletion (residues 34–90) and a substitution of Glu91Glu in the N-terminal domain VI, which is presumed to be involved in self-aggregation of laminin heterotrimers. Thus, the mutant α2 chain could disrupt the formation of the laminin network and lead to muscle cell degeneration. Our results provide a molecular basis of muscular dystrophy and dysmyelination of peripheral nerve.

INTRODUCTION

Laminin-2 (formerly merosin) is a tissue-specific laminin heterotrimer consisting of laminin α2 (M), β1 (B1), and γ1 (B2) chains, is expressed in the basal lamina of striated muscle and peripheral nerve (2–4). As a member of the laminin family, laminin-2 has various biological functions. These include cell attachment, neurite outgrowth promotion (5), Schwann cell migration (6) and formation of supramolecular structures by either self-aggregation or interaction with other components of the basal lamina. Laminin α2 is a homologue of the prototypical laminin α1 (A) chain of laminin-1 (EHS laminin). The complete primary structure of laminin α2 chain has been determined in human (7) and mouse (8) by cDNA cloning. The cDNA sequence for the mouse α2 chain predicts a molecular weight of approximately 390 kDa consisting of 3106 amino acids and 30 putative N-linked glycosylation sites (8). Laminin α2 chain has a domain structure similar to that of laminin α1 chain; domains VI, IVb and IVa of α2 chain are predicted to form globular structures. Domains V, IIIb and IIIa contain cysteine-rich EGF-like repeats and are predicted to form rigid rod-like structures. Domains I + II are involved in the formation of a triple-stranded coiled-coil structure that forms the long arm of the laminin heterotrimer molecule together with laminin β1 and γ1 chains. The C-terminal region contains five internal homologous repeats to form a large globular (G) domain (7,8).

Laminin α2 chain is a native ligand for α-dystroglycan (9), an extracellular component of the dystrophin–glycoprotein complex (DGC). This complex spans the sarcolemma, forming a link between the extracellular matrix and the subsarcolemmal cytoskeleton (10). A disruption of this linkage plays a critical part in the pathogenesis of muscular dystrophies such as Duchenne muscular dystrophy (DMD) and severe childhood autosomal recessive muscular dystrophy (SCARMN) (11). Laminin-2 may play an important role in maintaining the integrity of muscle cell function.

The mouse α2 chain gene, Lama2, has been mapped to a proximal region of Chromosome 10, close to the dystrophia muscularis (dy) locus (9). Murine dystrophia muscularis (dy) is an autosomal recessive disease characterized by muscle degeneration (12) and developmental dysmyelination of peripheral nerve (13,14). The dy mouse has an allelic phenotype referred to as dystrophia muscularis-2J (dy^2J). The dy^2J mouse appears to be milder, but similar clinically and histologically to the dy mouse (15,16).

Our group (9) and other investigators (17,18) have demonstrated a specific deficiency of α2 chain in the dy mouse, suggesting that mutations of the α2 chain gene may cause the dy or dy^2J phenotype. Furthermore, we have identified congenital muscular dystrophy (CMD) patients deficient in α2 chain (19). As the dy and dy^2J mice could be useful murine models providing a basis for understanding the pathogenesis of CMD, it is important to identify possible mutations in the α2 chain gene of dy or dy^2J mice. Recently, Xu and colleagues (20) reported the expression of a truncated α2 chain in the dy^2J mouse and identified a splice mutation in the α2 chain gene, which generates five different variant transcripts (M1–M5) with insertions and/or deletions. They concluded that the M5 transcript possesses a 165 base in-frame deletion and is the only possible transcript for a truncated α2 chain. We independently identified a truncated α2 chain in the dy^2J mouse and a novel, predominant transcript having a 171 base in-

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frame deletion. Translation of this variant transcript would result in the expression of a truncated α2 chain lacking a portion of domain VI. As the corresponding domain of α1 chain has been shown to be involved in laminin self-aggregation (21), this mutation in the α2 chain may disrupt the laminin network and lead to muscle cell degeneration.

RESULTS

**Immunohistochemical analysis of laminin α2 chain in dy²/² mice**

The immunofluorescence localization of laminin α2 chain in skeletal muscle from control +/+ and dystrophic dy/dy and dy²/² mice was examined using an affinity-purified sheep antibody against the α2 chain (Fig. 1). In the dy mouse, the immunostaining of the α2 chain at the basal lamina surrounding each muscle fiber was drastically reduced as described previously (9). In the dy²/² mouse skeletal muscle, reduction of the α2 chain immunostaining was less severe than that observed in the dy mouse. Furthermore, immunoblot analysis of EDTA extracts from skeletal muscle, cardiac muscle and peripheral nerve from the dy²/² mouse revealed that the size of the α2 chain was noticeably decreased concomitant with a moderate decrease (~50%) in abundance (Fig. 2). Under reducing conditions, the α2 chain migrates as an N-terminal fragment of ~300 kDa and a C-terminal fragment of ~80 kDa. A rabbit anti-α2 chain antibody recognizes the former as a 325 kDa band. Based on relative mobility on SDS–PAGE, we have estimated the molecular mass of the dy²/² mouse N-terminal fragment at 270 kDa in skeletal muscle, 290 kDa in cardiac muscle, and 300 kDa in peripheral nerve as compared with the 325 kDa normal fragment (Fig. 2B). Although this antibody does not recognize the C-terminal fragment of ~80 kDa, it does recognize the laminin-2 heterotrimer of ~700 kDa under non-reducing conditions and reveals a consistent reduction in molecular size of dy²/² laminin-2 (Fig. 2C). This finding suggests that the mutant α2 chain interacts with β1 and γ1 chains to form the laminin-2 heterotrimer. As α2 chain is glycosylated by post-translational modification, the reduction in size of dy²/² mouse α2 chain could be explained by an aberrant glycosylation. To check this possibility, we compared the molecular weight of the core protein of the α2 chain by enzymatic deglycosylation. Deglycosylation treatment of the
α2 chain by N-glycosidase F still showed a consistent difference in molecular size between control and dy2/2 mice (Fig. 2D). Thus, these results strongly suggest that the dy2/2 mouse may have a mutation in the α2 chain gene affecting a region encoding the 325 kDa N-terminal fragment and resulting in a truncated α2 chain polypeptide.

Northern blot analysis and RT–PCR of the dy2/2 mouse α2 chain

To determine whether a deletion in the α2 chain transcript may be involved in the dy2/2 mouse, we first investigated the α2 chain mRNA expression by northern blot analysis. Total RNA isolated from skeletal and cardiac muscle of dy2/2 and control +/+ mice, was subjected to northern blot analysis and probed with a 3.8 kb mouse α2 chain cDNA (pmm16) (9). Laminin α2 chain mRNA of ~9.5 kb was detected in normal mouse skeletal and cardiac muscle. However, in dy2/2 mouse skeletal and cardiac muscle, the size of α2 chain mRNA was slightly decreased as compared with control (Fig. 3).

To identify a possible deletion in the dy2/2 α2 chain transcript, we amplified α2 chain cDNA in eight overlapping pieces (PCR 1–8) by RT–PCR starting from poly(A)+ RNA isolated from skeletal muscle (Fig. 4A). PCR fragments 2–8 were identical in size for both control and dy2/2 mice. However, the PCR 1 (using primers MMfor1/MMrev1a) amplified a single band, although the PCR1 fragment in dy2/2 was approximately 200 bp smaller than the 1 kb PCR 1 fragment in control mice (Fig. 4B). Direct sequencing of the RT–PCR 1 fragment from dy2/2 mice revealed a single 171 base deletion at nucleotide positions 101–271. This deletion results in the removal of 57 amino acids corresponding to residues 34–90 with a substitution of glutamine for glutamic acid at residue 91 (Fig. 5B), and the normal reading frame is maintained after this point. The deletion occurs in domain VI near the N-terminus of the α2 chain and contains two potential N-linked glycosylation sites (Fig. 5A). The α2 chain transcript with α2 identical deletion was also identified in dy2/2 mouse cardiac muscle. This variant transcript, now designated as M0, was distinct from five variant transcripts (M1–M5) identified by Xu et al. (20).

M0 is a major transcript for a truncated α2 chain

M1–M4 transcripts have insertions and/or deletions which cause a shift in the reading frame. This shift produces a stop codon and results in aborted translation of these transcripts (20). Thus, Xu and colleagues concluded that the M5 transcript having a 165 base in-frame deletion (nucleotides 220–384; the number based on the mouse α2 chain cDNA; 8) is the only possible transcript for a truncated α2 chain. However, we have identified a distinct transcript M0 with a 171 base in-frame deletion (nucleotides 101–271). In order to determine which transcript, M0 or M5, is the predominant form in the dy2/2 mouse, we amplified cDNA fragments using a primer set flanking both deleted regions from total RNA as well as poly(A)+ RNA. Southern transfer membranes were hybridized with two different oligonucleotide probes; probe A is from a sequence deleted in M0 and probe B is from a sequence deleted in M5 (Fig. 6A). RT–PCR amplified a single band in the dy2/2/dy2/2 mouse as well as the control mouse. Probe A hybridized with only control RT–PCR product, not dy2/2 RT–PCR product, whereas probe B hybridized with both control and dy2/2 RT–PCR products (Fig. 6B). These results indicate that the transcript in dy2/2 mice must possess the sequence recognized by probe B while the sequence recognized by probe A is missing. Therefore, we conclude that M0 is the predominant transcript responsible for production of the truncated α2 chain in dy2/2 mice.

Status of laminin α2 chain expression in dy2/2/+ heterozygous mice

The dy2/2 phenotype is transmitted as an autosomal recessive trait. To clarify the molecular mechanism for the recessive inheritance, we examined the expression of the mutant α2 chain in the dy2/2/+ heterozygous mouse at both mRNA and protein levels. Both normal and deleted cDNA fragments were amplified from heterozygous mouse skeletal muscle RNA by RT–PCR. However, immunoblotting revealed an exclusive expression of a normal, 325 kDa α2 chain fragment in the heterozygous mouse skeletal muscle (data not shown). These results suggest that in the presence of normal laminin-2 network, the truncated α2 chain is not incorporated into the network and may be degraded rapidly.

DISCUSSION

We have identified a novel variant α2 chain transcript, M0, having a 171 base in-frame deletion in the dy2/2/dy2/2 mouse. The M0 transcript encodes an α2 chain polypeptide with a deletion. This deleted region (residues 34–90) contains two potential N-linked glycosylation sites. Additionally, this protein has a glutamine to glutamic acid substitution at residue 91. The deletion of 57 amino acids predicts a 7.2 kDa decrease in molecular weight, which is less than the actual difference observed on the immunoblot. As the deletion eliminates two potential N-linked glycosylation sites, the larger difference in molecular weight observed on immunoblots is consistent with one or both of the N-linked glycosylation sites in this region being glycosylated in vivo. Based on our immunoblotting data, the size of the truncated α2 chain differs among tissues,
although M0 is the predominant transcript in those tissues (data not shown). The elimination of two glycosylation sites and possible differential glycosylation in different tissues could explain the heterogeneity of the mutant α2 chain size.

Paradox of two possible transcripts for the truncated α2 chain

Curiously, the M0 transcript is distinct from previously identified M1–M5 transcripts. Among these transcripts, M5, which has a 165 base in-frame deletion is the only possible transcript for the truncated α2 chain consisting of a 55 amino acid deletion (residues 74–128) with a single potential N-linked glycosylation site (20). These variant transcripts result from a splice donor site mutation (20). We also confirmed a G to A transition at the first nucleotide of an intron existing between nucleotides 271 and 272. Based on our results and those from Xu et al. (20), we conclude that the splice site mutation leads to six variant transcripts (M0–M5) by aberrant splicing at different cryptic splice sites. However, a major difference in conclusions between the two groups is a variant transcript encoding the truncated α2 chain. The discrepancy came from a critical difference in the primers used for RT–PCR. The forward primer sequence used by Xu et al. was chosen within a deleted region of the M0 transcript. Thus, such RT–PCR was unable to amplify the M0 transcript. Furthermore, RT–PCR using a primer set, MMfor1/MMrev1a, flanking both M0

Figure 5. Sequence analysis of PCR 1 fragment of the d422 α2 chain cDNA. (A) Domain structures of a lamin α2 chain molecule are shown. A 171 base in-frame deletion was localized to the N-terminal domain VI. (B) DNA and amino acid sequence of mouse α2 chain cDNA corresponding to the 5' portion of RT–PCR 1 fragment. A shaded box indicates a 171 base deletion and corresponding 57-amino acid deletion in the d422 mouse. Glutamine at residue 91 (circled) is also substituted for glutamic acid (circled). Two potential N-linked glycosylation sites are boxed.
and M5 deleted regions amplified only a single band which hybridized with only probe B recognizing M0 but not M5. The M0 transcript was amplified using a sense primer MMfor1 which locates in the same exon as the initiator methionine codon. Moreover, RT–PCR using a second antisense primer MMrev1b (nucleotides 1485–1505) which locates at least one exon further 3′ than MMrev1a also detected the M0 transcript (data not shown). Taken together, we conclude that M0 is a major transcript from which a truncated α2 chain is translated and that M5 is only a minor transcript. Thus, the deletion of 57 amino acids accompanied with a Gln to Glu substitution in domain VI of α2 chain is essentially responsible for the dy/Cj phenotype.

Pathogenesis of dy/Cj muscular dystrophy

Laminin α2 chain has a domain structure similar to that of the α1 chain and constitutes laminin-2 heterotrimer by interacting with β1 and γ1 chains through the α helical domain I+II. Laminin heterotrimers self-aggregate to form an independent network via the N-terminal short arm domains, which include domain VI of α1 chain (21,22). The significant sequence homology of the N-terminal domains between α1 and α2 chains suggests that domain VI of the α2 chain is likely to have self-aggregating activity similar to that of α1 chain. On the other hand, α2 chain binds α-dystroglycan (9), an extracellular component of the DGC, at a putative binding site in the C-terminal G domain. Thus, the extracellular laminin network is linked to the subsarcolemmal actin cytoskeleton via the DGC. Previous studies suggest that a disruption of this link plays a critical role in the pathogenesis of muscular dystrophies such as DMD (23) and SCARMD (24,25). Interestingly, the M0 transcript is missing the N-terminal domain VI, which is involved in self-aggregation process of laminin heterotrimers (21) and interaction with extracellular matrix components (e.g. collagen type IV; 26). These interactions form a supramolecular structure in the basal lamina. Although the truncated α2 chain interacts with β1 and γ1 chains to form a laminin-2 heterotrimer, the defective laminin-2 may not be able to form a complete laminin network in the basal lamina. Exclusive expression of normal α2 chain in the heterozygous mouse also suggests that the truncated α2 chain failed to be incorporated into normal laminin-2 network (loss of function effect). Thus, the disrupted formation of laminin-2 network may disrupt the integrity of the linkage between the extracellular matrix and the DGC and lead to muscle cell degeneration in the dy/Cj mouse (Fig. 7).

Defective α2 chain and dysmyelination

The dy/Cj mouse also possesses developmental dysmyelination of peripheral nerve where the paucity of Schwann cells and a defective myelination process result in the presence of large bundles of naked axons (13,14). At birth, Schwann cells are
deficient in the dy<sup>2</sup> mouse spinal roots, but many undifferentiated cells capable of cell division are present. These cells may be uncommitted Schwann cells that can differentiate when transplanted to a normal environment (27). Thus, Schwann cells, responsible for wrapping and myelinating neuronal cells, appear to lack the ability to differentiate and migrate on the axonal surface of neuronal cells in the dy<sup>2</sup> mouse. Laminin-2 has been shown to promote neurite growth and Schwann cell migration (5,6), which are the two most critical cellular events during the normal development of the nervous system. These biological effects are mediated by specific interactions between structural domains of the laminin molecule and different cell surface laminin receptors. However, the precise mechanism of interaction between laminin-2 and Schwann cells remains unknown. In peripheral nerve, α2 chain binds α-dystroglycan (9,28), which is coexpressed with Dp116, the Schwann cell-specific DMD gene product (29). Thus, interaction of laminin-2 with Schwann cells may require normal laminin heterotrimer aggregation, which may be disrupted in dy<sup>2</sup> mice and may be mediated by the interaction of the laminin-2 network into the Dp116-dystroglycan complex. Alternatively, our data raise an intriguing possibility that the interaction site with Schwann cells may be in the N-terminal region of the α2 chain, which is deleted in the dy<sup>2</sup> mouse. Furthermore, sugar chain(s) could be involved in the interaction of the α2 chain with Schwann cells, as the dy<sup>2</sup> deletion contains two N-linked glycosylation sites. This model will be important for dissecting domain functions of the α2 chain and for understanding the molecular basis of neuron–Schwann cell interactions during development of the nervous system.

Possible model for congenital muscular dystrophy (CMD)

Besides providing a molecular genetic explanation of a murine inherited disorder, this finding may shed light on a related human muscular dystrophy. Some patients with CMD show a specific deficiency of laminin α2 chain (19). These CMD patients have an abnormal signal intensity of brain white matter detected by magnetic resonance imaging (MRI), suggesting abnormal myelination in the central nervous system. Interestingly, there is evidence for defective myelination, also in the central nervous system of the dy mouse (30), where the expression of laminin-2 has been recently demonstrated (7). Thus, the dy phenotype is homologous to CMD with α2 chain deficiency. Our data raise the possibility that some CMD patients may express a truncated α2 chain caused by an in-frame deletion as in the case of Becker muscular dystrophy where the expression of truncated dystrophin causes a milder phenotype of muscular dystrophy. Thus, the dy<sup>2</sup> mouse could be a useful murine model providing a basis for understanding the molecular pathogenesis of CMD.

MATeRIALS AND METHODS

Animals
dy<sup>2</sup> (dy<sup>2</sup>), or dy<sup>dy</sup> homozygous mice and heterozygous breeding pairs on strain C57BL/6J background were purchased from the Jackson Laboratories. The homozygous and wild-type mice were bred in the animal care unit at the University of Iowa.

Immunofluorescence and immunoblotting

Seven μm thick transverse cryosections from control, dy<sup>dy</sup>, and dy<sup>2</sup>/dy<sup>2</sup> mouse skeletal muscle were immunostained with an affinity-purified sheep polyclonal antibody specific for α2 chain as described previously (9). EDTA extracts were prepared from age-matched dy<sup>2</sup>/dy<sup>2</sup>, dy<sup>2</sup>+/+ and control α/+ mouse skeletal muscle, cardiac muscle and sciatic nerve as previously described (9). Samples containing 100 μg of protein were separated on a 3–12% gradient SDS–PAGE (31) in the presence of 1% 2-mercaptoethanol or in the presence of 10 mM N-ethylmaleimide and stained with Coomassie Blue or transferred to nitrocellulose (32). Nitrocellulose transfer was immunostained with a rabbit polyclonal antibody against a recombinant α2 chain as previously described (9).

Enzymatic deglycosylation

Forty percent ammonium sulfate precipitate of skeletal muscle EDTA extracts was resuspended in 50 mM Tris–HCl, pH 7.4, 0.15M NaCl, 2.5 mM EDTA, 0.5 mM PMSF, 0.5 mM NEM. After centrifugation, the supernatant containing α2 chain treated with Flavobacterium meningosepticum N-glycosidase F (Boehringer, Mannheim) was first made in 1% SDS and incubated at 100°C for 5 min, then diluted 10-fold to a final concentration of 50 mM sodium phosphate (pH 7.4), 1% Triton X-100, 0.1% SDS, and 12 U/ml N-glycosidase F. After incubation at 37°C for 2 h, samples were analyzed by SDS–PAGE.

Northern blot analysis, RT–PCR and DNA sequencing

Total RNA was extracted from skeletal muscle using RNAzol (Tel-Test, Friendswood, TX) according to the manufacturer’s directions. Total RNA (20 μg per lane) was fractionated by electrophoresis through a 1.25% agarose, 3% formaldehyde gel. The RNA was transferred overnight by capillary action to Biostreane membrane (BIOS Co.) and hybridized with a random-primed probe of 3.8 kb mouse merosin cDNA. Poly(A)+ RNA was prepared with the Oligotex-d<sup>+</sup> mRNA kit (Qiagen) and was subjected to reverse transcription with Stratascript (Stratagen) using an oligo(dT) primer. Overlapping primer sets were designed for PCR amplification of eight fragments that cover 99.5% of the full-length α2 chain cDNA. These primer pairs are based on the mouse α2 chain cDNA sequence (8); MMfor1: 5'-ATCTCTTGCGCTGCTTCT3' (nucleotides 22–43); MMrev1: 5'-TGATGCTTCCAACATCTC-3' (1034–53); MMrev1b: 5'-TTACCTTTAACTCTCCATTCTT3' (1485–1505); MMfor2: 5'-CTCGCAATAAAATACCCG-3' (919–938); MMrev2: 5'-GGTGCACTGCGCAACTTCT-3' (2184–2202); MMfor3: 5'-ATCTCTCTTGCTTCCTCTCT3'-3' (2138–2157); MMrev3: 5'-ACATTCTCTCCGTCTCACTC-3' (2989–3008); MMfor4: 5'-ACGAAGTGAAGGCCGGAAC-3'.
Southern blot analysis of RT-PCR products

RT-PCR products using a primer pair of Mm041/Mm041a amplified from control or dy2/dy2 mouse RNA were transferred to Hybond-N+ membranes (Amersham). Identical Southern transfer membranes were hybridized with two different oligonucleotide probes, probes A and B. Probe A sequence corresponds to nucleotides 101–150; probe B sequence corresponds to nucleotides 326–377.

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