The laminin α2 expressed by dystrophic dy2J mice is defective in its ability to form polymers
Holly Colognato and Peter D. Yurchenco

Mutations in LAMA2 cause severe congenital muscular dystrophy accompanied by nervous system defects [1]. Mice homozygous for the dy2J allele of LAMA2 express a laminin α2 subunit that has a deletion in the amino-terminal domain VI, providing an animal model for study of the molecular basis of congenital muscular dystrophy [2,3]. Domain VI is predicted to be involved in laminin polymerization, along with amino-terminal domains from laminin β and γ chains [4]. In a solution-polymerization assay, we found that purified dy2J laminin assembled poorly and formed little polymer, in contrast to wild-type muscle laminin. Furthermore, dissolution of the collagen IV network caused dy2J laminin to be released into solution, indicating that laminin polymers within the skeletal muscle basement membrane were defective. In addition to loss of polymerization, dy2J laminin had a reduced affinity for heparin. Finally, recombinant laminin engineered with the dy2J deletion was more sensitive to proteolysis and was readily cleaved near the junction of domains V and VI. Thus, the dy2J deletion selectively disrupts polymer formation, reduces affinity for heparin, and destabilizes domain VI. These are the first specific functional defects to be identified in a muscular dystrophy laminin, and it is likely that these defects contribute to the abnormalities seen in dy2J/dy2J muscle and nerve.

Address: Department of Pathology and Laboratory Medicine, Robert Wood Johnson Medical School, Piscataway, New Jersey 08854, USA.

Correspondence: Peter D. Yurchenco
E-mail: yurchenc@umdnj.edu

Received: 17 September 1999
Revised: 18 October 1999
Accepted: 18 October 1999
Published: 8 November 1999

Current Biology 1999, 9:1327-1330

0960-9822/99$ – see front matter
© 1999 Elsevier Science Ltd. All rights reserved.

Results and discussion
Mice homozygous for the dy2J allele of LAMA2 express a laminin α2 subunit that has a 57 amino acid deletion and a Gln91Glu substitution within domain VI [2,3]. The dy2J laminin α2 subunit was reported to have an apparent molecular mass that, depending on the tissue, ranged from 10–55 kDa smaller than normal, which is seemingly at odds with a 57 amino acid deletion [2,3]. To further investigate this discrepancy, we engineered a recombinant amino-terminal fragment of laminin α2 that had the same deletion as in the dy2J allele, α2(VI–IVb)Δdy2J. This was compared with dy2J α2 from skeletal muscle and with a fragment without the deletion, α2(VI–IVb). Laminin-2/4, a mixture of laminin-2 (α2β1γ1) and laminin-4 (α3β2γ1), was extracted from wild-type and dy2J/dy2J skeletal muscle and analyzed by SDS–PAGE (Figure 1a). The laminin α2 subunit had an apparent molecular weight of ~300 kDa, whereas the dy2J α2 subunit was 25–30 kDa smaller. The engineered fragment α2(VI–IVb)Δdy2J had an apparent molecular weight that was only 6 kDa smaller than that of α2(VI–IVb), however, a size difference more consistent with the removal of 57 amino acids (Figure 1c, zero time points). We also noted a faster-migrating band (~100 kDa) in some of our preparations of α2(VI–IVb)Δdy2J, which is likely to be a degradation product as it reacted with α2-specific antibodies (data not shown).

We compared the stability of α2(VI–IVb) and α2(VI–IVb)Δdy2J using limited proteolysis and found that the mutant protein was cleaved by elastase at approximately 50 times the rate of wild-type protein (Figure 1c). Furthermore, proteolysis resulted in a truncated protein with an apparent molecular weight of ~98 kDa. The truncated protein was sequenced from its amino terminus and found to have the sequence XVK(X/D)ISVGXM (in the single-letter amino acid code, where X is an unknown amino acid). This sequence corresponds to the last nine residues of domain VI, so the degree of truncation was likely to be a degradation product as it reacted with α2-specific antibodies (data not shown).

Several lines of evidence suggest that expression of dy2J laminin leads to muscle pathology through a misregulation of basement membrane architecture rather than through loss of binding sites for laminin receptors. First, dy2J/dy2J sarcolemmal basement membranes appear abnormal, but do not appear to have a large reduction in laminin α2. Immunofluorescence studies suggest that the
Limited proteolysis of wild-type and dystrophic α2(VI–IVb) shows that the dy2J domain VI is more sensitive to proteolysis and is truncated. (a) SDS–PAGE under reducing conditions of laminin-2/4 extracted from wild-type (+/+) and dy2J/dy2J mice. Laminins were isolated using collagenase, EDTA, and heparin-affinity chromatography. The laminin α2 chain migrates faster; its apparent molecular mass is ~25 kDa less than that of wild-type α2 (300 vs 275 kDa). (b) Schematic representation of the α2(VI–IVb)Δdy2J engineered laminin fragment. The amino-terminal region of the laminin α2 chain has a 57 amino acid deletion and six Gln18 Thr substitution (arrow) within domain VI. (c) Fragments α2(VI–IVb) and α2(VI–IVb)Δdy2J were found to migrate with relative molecular masses of 122 kDa and 116 kDa, respectively, a difference of ~6 kDa (see zero time points). Protein fragments were digested with elastase for the indicated times at 25°C (enzyme:substrate ratio 1:50) and evaluated under reducing conditions. The α2(VI–IVb) fragment was fairly resistant to elastase over time, whereas α2(VI–IVb)Δdy2J was readily degraded to a smaller apparent molecular mass of 98 kDa (~24 kDa smaller than α2(VI–IVb)). Amino-terminal sequencing of the 98 kDa fragment (data not shown) indicated that domain VI was completely removed, with the exception of nine terminal residues. (d) Model of the probable reason for the much smaller molecular weight of dy2J/dy2J laminin than predicted from its structure. Deletion of 57 amino acids in domain VI exposes a proteolytically sensitive region just upstream of the junction with domain V. Proteolysis therefore leads to degradation of nearly all of domain VI.

We observed differences in the solubility of laminin-2/4 isolated from the skeletal muscle of wild type and dy2J/dy2J mice. Collagenase was used to disrupt the type IV collagen network, which can link to the laminin network through interactions with entactin/nidogen [11]. Subsequent EDTA treatment was used to dissociate the calcium-dependent laminin polymer [12] and to disrupt laminin–receptor interactions. A large fraction (nearly 50%) of laminin-2/4 was released into solution from dy2J/dy2J skeletal muscle following collagenase treatment, whereas the majority of laminin-2/4 from wild-type skeletal muscle required additional treatment with EDTA to be solubilized (Figure 2a,b). This difference in solubility indicated that dy2J laminin was primarily associated with the basement membrane through surviving non-polymer bonds, such as linkage to the collagen IV network through entactin/nidogen. We next evaluated dy2J laminin-2/4 purified from skeletal muscle in a copolymerization assay, which could detect the polymerization capacity of small quantities of protein [10]. Laminin-2/4 from wild-type skeletal muscle increasingly sedimented with the polymer fraction as total laminin concentration increased, whereas the majority of laminin-2/4 from dy2J/dy2J mice remained in the soluble pool (Figure 2c–e). Thus, laminin-2/4 expressed by dy2J/dy2J mice is defective in its ability to form a laminin polymer.

Next, the ability of dy2J laminin-2/4 to bind heparin was evaluated. Using high performance liquid chromatography (HPLC) heparin-affinity chromatography, we compared the elution positions of laminin-2/4 from normal and dy2J/dy2J mice (Figure 3). Although dy2J laminin-2/4 bound heparin, it eluted at a lower salt concentration than normal laminin-2/4. Both proteins contain a heparin-binding region in the carboxy-terminal G domain that is known to mediate interactions with dystroglycan, a molecule that links the dystrophin–glycoprotein complex and the extracellular matrix [6,7]. This site is unaffected in dy2J/dy2J mice, so the difference in relative heparin affinity must reflect changes in the second heparin-binding site, mapped to domain VI [13]. Basic amino acids arginine and lysine are thought to be essential for mediating electrostatic interactions with the negatively charged sulfate and carboxylic acid groups of heparin [14]. If a charge of +1 is assigned to arginine and lysine and –1 to aspartic acid and glutamic acid, wild-type domain VI has a net positive charge of +2 and dy2J domain VI has a net loss of basic residues that reduces the net charge to zero. Furthermore, two N-glycosylation consensus sites are deleted in dy2J α2, which may contribute to changes in heparin binding as well as to decreased stability. It is possible that the loss of these positively charged residues disrupts a crucial interaction with molecules containing negatively charged groups such as heparan sulfate or polysialic acid, but an in vivo role for the heparin binding site in domain VI has yet to be established.
Mouse skeletal muscle was treated with bacterial collagenase and laminin using SDS–PAGE and immunoblotted with an antibody against dy2J/dy2J. The percentage of total protein released into solution at each step, determined by densitometry, is shown.

Laminin polymer formation was evaluated in a sedimentation assay in which the degree of polymer formation has been found to be independent of the concentration of test laminin [10]. Briefly, fixed amounts of laminin-2/4 extracted from skeletal muscle (test laminin) were added to aliquots containing various concentrations of laminin-1 (Lm-1) and incubated at 37°C for 3 h. Differential centrifugation was used to pellet the laminin polymer, and unpolymerized laminin remained in solution. Immunoblots of supernatant (S) and polymer (P) fractions were probed with an antibody that recognizes the α2 subunit. Bands were visualized using a DuraSuperSignal chemiluminescence detection kit (Pierce) and quantitated by PhosphoAnalysis (BioRad). Wild-type laminin-2/4 copolymerized with laminin-2. Laminin-2/4 extracted from either wild-type (+/+) or dy2J/dy2J mice was applied to a HPLC heparin-affinity column in 50 mM Tris pH 8.0 and 0.5 mM EDTA. Bound protein was eluted using a 0–1.0 M linear NaCl gradient. Both proteins bound to the heparin column, but dy2J laminin-2/4 eluted at a lower salt concentration, reflecting a lower affinity for heparin. Solid lines, protein concentrations; dotted line, NaCl concentration.

Laminin polymerization may be important for maintaining the structural integrity of the basement membrane, given the alterations seen in dy2J/dy2J mice. Several factors suggest this may not be the only role for laminin polymerization, however. For instance, the collagen IV network assembles through interactions of considerably higher affinity than laminin self-assembly interactions. Although the collagen IV network is present in the basement membranes of dy2J/dy2J mice, it clearly does not compensate for the functional deficits in laminin. Furthermore, one might expect a defect caused by structural weakness to be much more pronounced at points of attachment like the muscle–tendon junction, but there is no evidence for such a defect.

An additional role for laminin polymerization was described recently, in which polymerization was found to induce changes in the organization of muscle receptors and of components of the cortical cytoskeleton such as dystrophin and vinculin [8]. In agreement with this hypothesis, the sarcolemmal cortex of dy2J/dy2J skeletal muscle has a grossly irregular appearance in transmission electron micrographs (H.C. and P.D.Y., unpublished observations). Based on the data presented here, an emerging hypothesis is that laminin polymerization may be required for the development or maintenance of proper cortical architecture in the muscle sarcolemma (Figure 4). It is also realistic to assume that architectural changes propagated from basement membrane to cytoskeleton may have profound signaling consequences, as has been found for fibronectin fibrillogenesis [15–18]. The differential ability of various laminins to polymerize may represent a normal regulatory mechanism by which different laminins transmit different signals using common receptors.

As well as the muscle defect, dy2J/dy2J mice have poorly myelinated axons. Evaluation of this defect may prove to be more complex. In contrast to skeletal muscle, evidence suggests that Schwann cell receptors interact with domain VI of laminin α2. Schwann cells express high levels of the
Model depicting laminin polymerization in the muscle sarcolemmal basement membrane. (a) Wild-type laminin normally interacts with other laminin molecules to form a polymer. Laminin polymerization on the muscle cell surface can induce reorganization of receptors and cytoskeletal components (8) that may provide a needed signal for muscle stability or regeneration. (b) Dystrophic dy2J laminin is defective in polymer formation and therefore cannot induce the polymerization-dependent changes in cell architecture that may play a role in muscle survival.

αβ1 integrin [19], an integrin that interacts with domain VI of the laminin α2 chain [13]. Preliminary data suggest that interactions between primary Schwann cells and the amino-terminal region of laminin α2 may in part be mediated by additional receptor(s) (H.C. and P.D.Y., unpublished observations). Because of its heparin-binding characteristics, domain VI is also a good candidate to interact with negatively charged groups like sulfated glycosaminoglycan chains or polysialic acid. As sulfated proteoglycans and polysialic acid epitopes are highly expressed in the central nervous system, the brain abnormalities seen in many LAMA2 congenital muscular dystrophies may involve loss of such interactions.

Acknowledgements
We thank Yi-Shan Cheng for preparing human placental laminin-2. This project was supported by National Institutes of Health grant R01-DK36425.

References