# The laminin α2 expressed by dystrophic *dy$^{2J}$* mice is defective in its ability to form polymers

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**Mutations in LAMA2 cause severe congenital muscular dystrophy accompanied by nervous system defects [1]. Mice homozygous for the *dy$^{2J}$* 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 *dy$^{2J}$* laminin assembled poorly and formed little polymer, in contrast to wild-type muscle laminin. Furthermore, dissolution of the collagen IV network caused *dy$^{2J}$* laminin to be released into solution, indicating that laminin polymers within the skeletal muscle basement membrane were defective. In addition to loss of polymerization, *dy$^{2J}$* laminin had a reduced affinity for heparin. Finally, recombinant laminin engineered with the *dy$^{2J}$* deletion was more sensitive to proteolysis and was readily cleaved near the junction of domains V and VI. Thus, the *dy$^{2J}$* 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 *dy$^{2J}$/dy$^{2J}$* muscle and nerve.**

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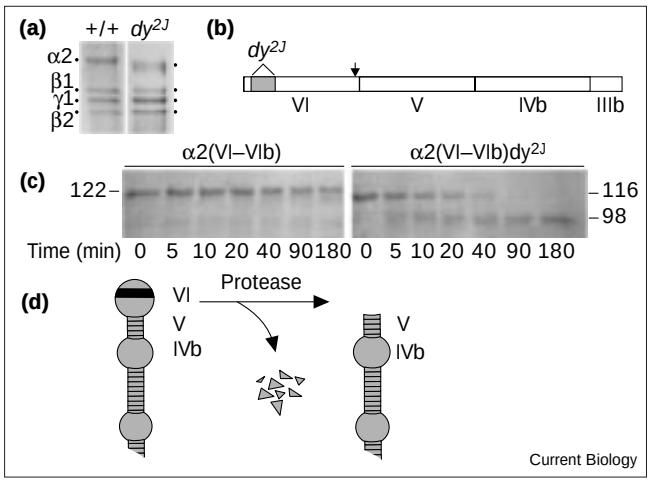
## Results and discussion

Mice homozygous for the *dy$^{2J}$* 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 *dy$^{2J}$* 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 *dy$^{2J}$* allele, α2(VI–IVb)Δdy$^{2J}$. This was compared with *dy$^{2J}$* 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 (α2β2γ1), was extracted from wild-type and *dy$^{2J}$/dy$^{2J}$* skeletal muscle and analyzed by SDS–PAGE (Figure 1a). The laminin α2 subunit had an apparent molecular weight of ~300 kDa, whereas the *dy$^{2J}$* α2 subunit was 25–30 kDa smaller. The engineered fragment α2(VI–IVb)Δdy$^{2J}$ 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)Δdy$^{2J}$, 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)Δdy$^{2J}$ 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 amino terminus of the truncated protein lies nine residues upstream from the predicted junction of domains VI and V [5]. These data suggest that the deletion destabilizes domain VI and exposes a proteolytically sensitive region just upstream of the junction of domains VI and V (Figure 1d). We propose that the mutant domain VI is unstable and is easily truncated *in vivo*, accounting for the apparent difference from the wild-type molecular weight of 25–30 kDa that was consistent in all preparations of *dy$^{2J}$/dy$^{2J}$* skeletal muscle. Other tissues of *dy$^{2J}$/dy$^{2J}$* mice are reported to show distinct, yet consistent, changes in the molecular weight of laminin α2 [3], so the degree of truncation that occurs *in vivo* may depend upon tissue-specific factors such as the local complement of proteases or post-translational modifications.

Several lines of evidence suggest that expression of *dy$^{2J}$* laminin leads to muscle pathology through a misregulation of basement membrane architecture rather than through loss of binding sites for laminin receptors. First, *dy$^{2J}$/dy$^{2J}$* sarcolemmal basement membranes appear abnormal, but do not appear to have a large reduction in laminin α2. Immunofluorescence studies suggest that the

**Figure 1**



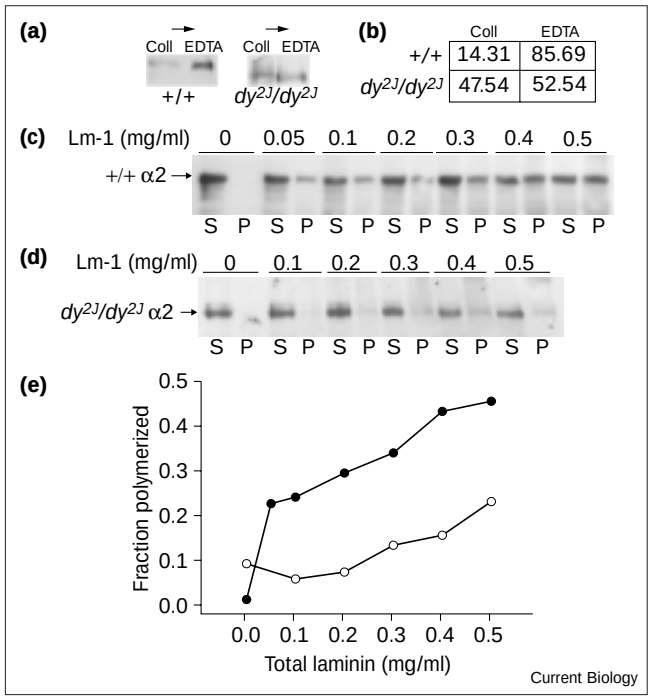

Limited proteolysis of wild-type and dystrophic α2(VI–IVb) shows that the *dy*$^{2J}$ domain VI is more sensitive to proteolysis and is truncated. (a) SDS–PAGE under reducing conditions of laminin-2/4 extracted from wild-type (+/+) and *dy*$^{2J}$/*dy*$^{2J}$ mice. Laminins were isolated using collagenase, 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)Δdy$^{2J}$ engineered laminin fragment. The amino-terminal region of the laminin α2 chain has a 57 amino acid deletion and Gln91Glu substitution (arrow) within domain VI. (c) Fragments α2(VI–IVb) and α2(VI–IVb)Δdy$^{2J}$ 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)Δdy$^{2J}$ 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 amino acids. (d) Model of the probable reason for the much smaller molecular weight of *dy*$^{2J}$/*dy*$^{2J}$ 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.

amount of laminin α2 in *dy*$^{2J}$/*dy*$^{2J}$ muscle is not significantly different than normal [2,3]. Using the ratio of myosin mass to total mass as a corrective factor for fibrosis, we consistently observed levels of laminin α2 that were ~70% of those in normal mice (data not shown). Second, the principal receptors for α2 laminins in mature skeletal muscle are thought to be α7β1 integrin and dystroglycan, both of which bind exclusively to the carboxy-terminal region of laminin-2/4 [6,7]. Direct binding was not detected between α2(VI–IVb) and myotubes and addition of laminin carboxy-terminal fragments to myotubes completely prevented binding of laminin-2/4 [8]. Finally, laminins-2 and -4 are known to form a three-dimensional, mesh-like polymer proposed to be mediated by interactions among the amino-terminal short arms of its α, β, and γ subunits [9,10].

We observed differences in the solubility of laminin-2/4 isolated from the skeletal muscle of wild type and *dy*$^{2J}$/*dy*$^{2J}$ 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 *dy*$^{2J}$/*dy*$^{2J}$ 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 *dy*$^{2J}$ 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 *dy*$^{2J}$ 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 *dy*$^{2J}$/*dy*$^{2J}$ mice remained in the soluble pool (Figure 2c–e). Thus, laminin-2/4 expressed by *dy*$^{2J}$/*dy*$^{2J}$ mice is defective in its ability to form a laminin polymer.

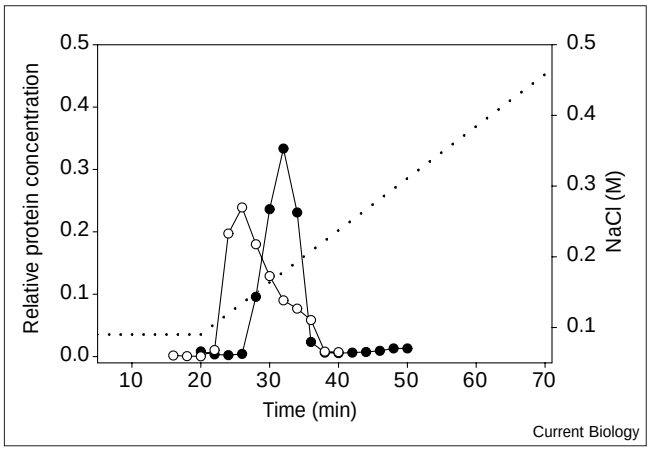
Next, the ability of *dy*$^{2J}$ laminin-2/4 to bind heparin was evaluated. Using high performance liquid chromatography (HPLC) heparin-affinity chromatography, we compared the elution positions of laminin-2/4 from normal and *dy*$^{2J}$/*dy*$^{2J}$ mice (Figure 3). Although *dy*$^{2J}$ laminin-2/4 bound 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 *dy*$^{2J}$/*dy*$^{2J}$ 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 *dy*$^{2J}$ 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 *dy*$^{2J}$ 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.

**Figure 2**




Polymerization of laminin-2/4 from wild type (+/+) and *dy*$^{2J}$/*dy*$^{2J}$ mice. (a,b) Mouse skeletal muscle was treated with bacterial collagenase (Coll) followed by 10 mM EDTA. (a) Solubilized protein was evaluated using SDS–PAGE and immunoblotted with an antibody against laminin α2 chain [20]. (b) The percentage of total protein released into solution at each step, determined by densitometry, is shown. (c,d) Copolymerization of laminin-2/4 from (c) wild-type and (d) *dy*$^{2J}$/*dy*$^{2J}$ mice. 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 laminin 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-1 and at higher laminin-1 concentrations was increasingly found in the polymer fraction, whereas *dy*$^{2J}$/*dy*$^{2J}$ laminin-2/4 remained largely in the supernatant fraction. (e) Graph of the fraction of laminin α2 subunit that appeared as polymer versus total laminin concentration. Filled circles, wild-type laminin-2/4; open circles, *dy*$^{2J}$/*dy*$^{2J}$ laminin-2/4.

**Figure 3**



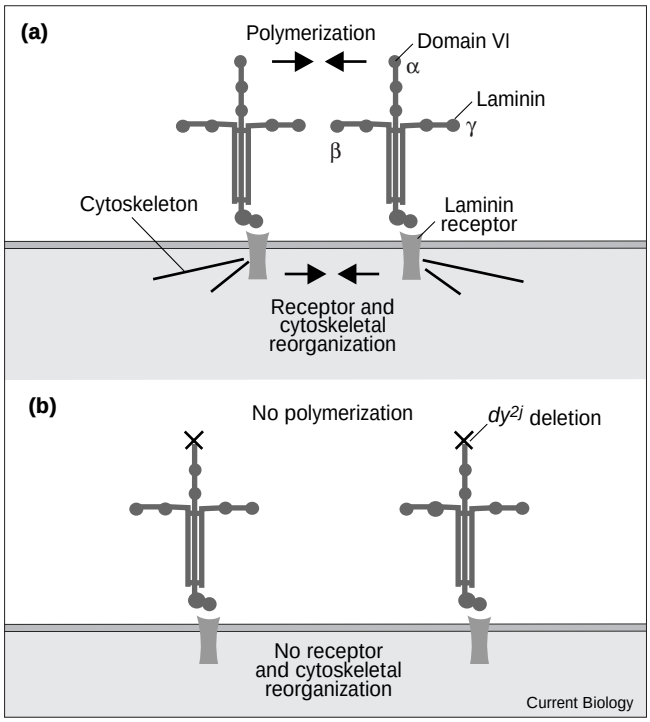

Heparin binding to *dy*$^{2J}$/*dy*$^{2J}$ laminin-2. Laminin-2/4 extracted from either wild-type (+/+) or *dy*$^{2J}$/*dy*$^{2J}$ mice was applied to a HPLC heparin-affinity column in 50 mM Tris pH 8.0 and 0.5 mM EDTA. Bound protein was eluted using a 0–1.0 M linear NaCl gradient. Both proteins bound to the heparin column, but *dy*$^{2J}$ 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 *dy*$^{2J}$/*dy*$^{2J}$ 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 *dy*$^{2J}$/*dy*$^{2J}$ 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 *dy*$^{2J}$/*dy*$^{2J}$ 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, *dy*$^{2J}$/*dy*$^{2J}$ 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

**Figure 4**




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 *dy$^{2J}$* 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.

α1β1 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.

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