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Laminin α1 chain mediated reduction of laminin α2 chain deficient muscular dystrophy involves integrin α7β1 and dystroglycan

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M.D. would like to dedicate this work to the late Peter Ekblom (1952–2005).

Abstract **Transgenically introduced laminin (LN) α1 chain prevents muscular dystrophy in LNα2 chain deficient mice. We now report increased integrin α7Bβ1D synthesis in dystrophic LNα2 chain deficient muscle. Yet, immunofluorescence demonstrated a reduced expression of integrin α7B subunit at the sarcolemma. Transgenic expression of LNα1 chain reconstituted integrin α7B at the sarcolemma. Expression of α- and β-dystroglycan is enhanced in LNα2 chain deficient muscle and normalized by transgenic expression of LNα1 chain. We suggest that LNα1 chain in part ameliorates the development of LNα2 chain deficient muscular dystrophy by retaining the binding sites for integrin α7Bβ1D and α-dystroglycan, respectively.**

Keywords: Integrin; Dystroglycan; Laminin; Muscular dystrophy

1. Introduction

Laminins (LN) are large heterotrimeric glycoproteins composed of one α, one β and one γ chain [1]. The main LNα chain in muscle is LNα2 chain and mutations in the gene encoding this chain lead to LNα2 chain deficient congenital muscular dystrophy, MDC1A [2]. This disorder is characterized by severe muscle weakness, hypotonia and joint contractures with onset at birth or in young infancy [3]. Mouse models for the disease have been used by us and others to test novel therapeutic approaches for MDC1A [4–9]. We recently demonstrated that LNα1 chain significantly reduces muscular dystrophy [7] and restores fertility in LNα2 chain deficient dy^{3K}/dy^{3K} mice [9].

LNα2 chain is associated with muscle fibers by at least two distinct protein complexes; integrin α7β1 and dystroglycan [10,11]. Integrin subunits α7B and β1D are the major isoforms in skeletal muscle [12,13] and the importance of integrin α7 subunit in muscle is underscored by the fact that absence of integrin α7 in man and mouse causes muscular dystrophy [14,15]. Dystroglycan is a central element of the dystrophin-glycoprotein complex (DGC) and is composed of the α and β subunits, which are derived from a single gene by posttranslational cleavage [16]. α-Dystroglycan is an extracellular peripheral membrane protein that binds to LNα2 chain and β-dystroglycan is the transmembrane moiety that anchors α-dystroglycan to the sarcolemma [17]. Also, dystroglycan is crucial for normal muscle function as mice lacking dystroglycan in muscle, and humans and mice with hypoglycosylated α-dystroglycan develop muscular dystrophy [18–21]. LNα1 chain also binds integrin α7β1 and α-dystroglycan [22,23]. However, it remains to be determined if LNα1 chain mediated rescue of LNα2 chain deficient muscular dystrophy involves integrin α7β1 and dystroglycan. Here, we show that transgenic expression of LNα1 chain restores normal localization of integrin α7B subunit and normalizes dystroglycan expression in muscle. We conclude that LNα1 chain mediated correction of LNα2 chain deficient muscular dystrophy engages integrin α7Bβ1D and dystroglycan.

2. Materials and methods

2.1. Transgenic animals

Dy^{3K}/dy^{3K} and dy^{3K}LNα1TG mice were previously described [4,7,9]. Animals were maintained in the BMC animal facilities according to animal care guidelines.

2.2. Quantitative real time PCR

Total RNA was isolated independently from skeletal and cardiac muscles of three wild-type, three dy^{3K}/dy^{3K} and three dy^{3K}LNα1TG mice using TRIzol reagent (Invitrogen). First-strand cDNA synthesis and quantitative real time PCR was carried out as previously described [9] with the following primers pairs and probes: for integrin α7B, Fw-TCCATTAAgAACTTgTTgCTCAg, Rev-CTTgAAgAATCCCAgCTTCC, fluorescein-labelled-ATCCTCCTggCAgTgCTggC and Light-Cycler-Red-640-labelled-gggCTgTTAgTCCTggCCTTgC; for integrin β1D, Fw-gTggAgACTCCAgACTgTCCTACT, Rev-AACTCAgAgACCAgCTTTACgTC, fluorescein-TCAAATTTAgCAAATTCCCTTCTgTCATgAA and LC-Red-640-TATCATTAAAAgTTTCCAAATCAgCAgCAAgg; for dystroglycan, Fw-AgggACTggAAgAACCAgCTT, Rev-TATgACTgTgTgggTCCCAgTgTA, fluorescein-CCCgACAACAgCCgTACCgTC and LC-Red-640-ggAATgCCAACCACggTgggA; for GAPDH, Fw-TTgTCAgCAATg CATCCTgC, Rev-CCgTTCAgCTCTgggATgAC, fluorescein-CACCCAgAAgACTgTggATggCCCCT and LC-Red-640-TggAAAgCTgTggCgTgATggCCg. PCR was repeated one to three times per extraction. Statistical significance was examined by using Student's *t*-test.

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2.3. Protein isolation

Proteins were extracted from 150 mg skeletal muscle powder of two wild-type, two dy^{3K}/dy^{3K} and two dy^{3K}LNα1TG mice in 1% NP-40 in 50 mM Tris–HCl, pH 7.4; 2 mM PMSF, 1 mM $CaCl_2$, 1 mM $MgCl_2$ and 1:25 dilution of Protease Cocktail (Complete EDTA-free, Roche). Two independent extractions were performed. Protein concentration was determined by using BCA assay (Pierce).

Membrane proteins were isolated by using ProteoExtract Native Membrane Protein Extraction Kit (Calbiochem) according to the manufacturer's instructions. Two independent extractions from muscles of two wild-type, two dy^{3K}/dy^{3K} and two dy^{3K}LNα1TG mice were performed.

Dystroglycan enriched samples were independently extracted from 150 mg skeletal muscle powder of three wild-type, four dy^{3K}/dy^{3K} and three dy^{3K}LNα1TG mice in 1× TBS buffer containing 1% Triton X-100 and 1:25 dilution of Protease Cocktail (Complete EDTA-free, Roche) as previously described [21]. One to two extractions were performed per each set of animals. Protein concentration was determined by using DC assay (BioRad).

2.4. Western blot

Integrin and dystroglycan protein containing muscle samples, respectively, were separated on 7.5% polyacrylamide–SDS gels, blotted and hybridized as previously described [9]. The following primary antibodies were used: rabbit polyclonal antibodies against integrin α7B subunit [24], integrin β1D subunit [25], and β-dystroglycan [7] and mouse monoclonal antibody IIH6C4 against α-dystroglycan (Upstate). Experiments were repeated 1–4 times per protein extraction. Quantification of chemiluminescence signals was performed using a CCD camera (LAS 1000, Fujifilm) and the software program Image Gauge V4 (Fujifilm). Statistical significance was examined by using paired *t*-test.

2.5. Immunostaining

Immunofluorescence was performed as previously described [7,9]. Skeletal and cardiac muscles were collected and analyzed from three mice of each genotype. Sections were incubated with the same antibodies used in Western blot analyses except for the integrin β1D stainings, for which we used the mouse monoclonal antibody 2B1 [13]. For integrin β1D stainings we used the VECTOR M.O.M. Immunodetection kit (Vector Laboratories) according to the manufacturer's instructions.

3. Results

3.1. Integrin α7Bβ1D analyses

Quantitative real time PCR on cDNA obtained from total muscle RNA of 3–4-month-old wild-type, 4-week-old dy^{3K}/dy^{3K} and 3–4-month-old rescued (dy^{3K}LNα1TG) mice demonstrated an approximately 2.4-fold upregulation of integrin α7B mRNA and about 2.7-fold increase in β1D mRNA expression in muscles of dy^{3K}/dy^{3K} mice. A 2.6- and 2.9-fold increase in α7B and β1D mRNA expression, respectively, was noted in muscles of dy^{3K}LNα1TG animals (Table 1).

Table 1
Relative amounts of integrin α7B and β1D mRNAs in wild-type, dy^{3K}/dy^{3K} and dy^{3K}LNα1TG skeletal muscle

	α7B	β1D
WT	1.09 ± 0.22	5.27 ± 1.03
dy^{3K}/dy^{3K}	2.67 ± 0.57 ($P < 0.02$)	14.20 ± 3.71 ($P < 0.03$)
dy^{3K}LNα1TG	2.85 ± 0.81 ($P < 0.034$)	15.15 ± 4.04 ($P < 0.03$)

Quantitative RT-PCR with sequence-specific hybridization probes was performed. Integrin levels were normalized to GAPDH and expressed as relative mRNA amounts and are shown as mean values ± S.E.M. ($n = 4$–5). Significantly more integrin α7B and β1D mRNAs were seen in dy^{3K}/dy^{3K} and dy^{3K}LNα1TG skeletal muscle compared with wild-type muscle. No significant difference was seen between dy^{3K}/dy^{3K} and dy^{3K}LNα1TG skeletal muscle.

To analyze whether mRNA levels of integrin α7Bβ1D correlated with protein expression we performed Western blot analyses on muscle protein extracts from wild-type, dy^{3K}/dy^{3K} and dy^{3K}LNα1TG animals. Consistently with the mRNA data, immunoblotting experiments showed an approximately 1.5-fold upregulation of integrin α7B in dystrophic muscle and an approximately 1.4-fold upregulation in rescued muscle. Integrin β1D was upregulated about 4.8-fold in dystrophic muscle and about 4.5-fold in rescued muscles (Fig. 1). We also studied membrane enriched fractions (internal and cell surface membranes) from muscles of wild-type, dy^{3K}/dy^{3K} and dy^{3K}LNα1TG animals. Also this assay showed an upregulation of α7B (≈1.4-fold) and β1D (≈4–7-fold) integrin subunits, respectively, in both dystrophic and rescued muscles (Fig. 1 and data not shown).

By immunofluorescence we studied the distribution of integrin α7B subunit in different muscles from 4-month-old wild-type, 4-week-old dy^{3K}/dy^{3K} and 4-month-old dy^{3K}LNα1TG mice. Surprisingly, muscle cryosections prepared from dy^{3K}/dy^{3K} mice exhibited a severe reduction in sarcolemmal staining of integrin α7B subunit. Downregulation was most evident in quadriceps, triceps brachii, tibialis anterior and gluteus maximus but to a lesser extent in diaphragm (Fig. 2 and data not shown). The receptor was normally expressed in blood vessels, and staining was also maintained in myotendinous junctions (Fig. 2). Notably, in dy^{3K}LNα1TG mice the expression of integrin α7B subunit was restored at the sarcolemma of all examined muscles (Fig. 2 and data not shown).

Immunofluorescence revealed that expression of β1D integrin did not appear to be altered in dy^{3K}/dy^{3K} or dy^{3K}LNα1TG muscles as compared with wild-type muscles (Fig. 2).

Integrin α7B chain is also highly expressed in cardiac muscle [13] and to analyze whether absence of LNα2 chain leads to reduced integrin α7B expression at the heart sarcolemma we analyzed cardiac muscles from wild-type, dy^{3K}/dy^{3K} and dy^{3K}LNα1TG mice by immunofluorescence. Indeed, lack of LNα2 chain led to reduced levels of integrin α7B subunit in sarcolemma of dy^{3K}/dy^{3K} hearts. Transgenic expression of LNα1 chain restored integrin α7B expression (Fig. 3). Unlike skeletal muscle, however, no increase in integrin α7 mRNA expression was noted in dy^{3K}/dy^{3K} or dy^{3K}LNα1TG cardiac muscle (Table 2).

3.2. Dystroglycan analyses

To analyze whether absence of LNα2 chain alters dystroglycan expression in dy^{3K}/dy^{3K} muscle we performed quantitative RT-PCR on cDNA obtained from total muscle RNA of 3–4-month-old wild-type, 4-week-old dy^{3K}/dy^{3K} and 3–4-month-old dy^{3K}LNα1TG mice. This assay revealed an approximately 2.1-fold upregulation of dystroglycan mRNA in muscles of dy^{3K}/dy^{3K} mice, but no significant difference in dystroglycan mRNA expression between dy^{3K}LNα1TG and wild-type muscles (Table 3).

Western blot analysis demonstrated that both α- and β-dystroglycan were upregulated in dy^{3K}/dy^{3K} mice (about 1.7- and 2.0-fold, respectively). Notably, in muscles of animals rescued by the LNα1 chain transgene, expression of dystroglycans was normalized (Fig. 4).

By immunofluorescence, we assessed the expression of dystroglycans in muscles from 4-month-old wild-type, 4-week-old dy^{3K}/dy^{3K} and 4-month-old dy^{3K}LNα1TG mice. α- and β-Dystroglycan were clearly present in sarcolemma of all