Aberrant glycosylation of α-dystroglycan causes defective binding of laminin in the muscle of chicken muscular dystrophy

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Abstract Dystroglycan is a central component of dystrophin–glycoprotein complex that links extracellular matrix and cytoskeleton in skeletal muscle. Although dystrophic chicken is well established as an animal model of human muscular dystrophy, the pathomechanism leading to muscular degeneration remains unknown. We show here that glycosylation and laminin-binding activity of α-dystroglycan (α-DG) are defective in dystrophic chicken. Extensive glycan structural analysis reveals that Galβ1-3GalNAc and GalNAc residues are increased while Siaα2-3Gal structure is reduced in α-DG of dystrophic chicken. These results implicate aberrant glycosylation of α-DG in the pathogenesis of muscular degeneration in this model animal of muscular dystrophy.
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1. Introduction

The dystroglycan complex is composed of two proteins, α- and β-dystroglycan (α- and β-DG), which are encoded by a single gene and cleaved by posttranslational processing [1]. α-DG is a highly glycosylated extracellular peripheral membrane protein and binds to several extracellular matrix (ECM) proteins including laminin, agrin, and perlecan [2–4]. In turn, the transmembrane protein β-DG anchors α-DG at the extracellular surface of the plasma membrane, while its cytoplasmic domain interacts with dystrophin, a large cytoplasmic protein that binds to F-actin [5]. Thus, the DG complex plays a crucial role to stabilize the plasma membrane by acting as an axis through which the ECM is tightly linked to the cytoskeleton.

Recently, primary mutations in the genes encoding putative glycosyltransferases have been identified in several types of congenital muscular dystrophies including Fukuyama-type congenital muscular dystrophy, muscle–eye–brain disease, Walker–Warburg syndrome, congenital muscular dystrophy 1C (MDC1C) and 1D (MDC1D) [6–10]. Because glycosylation and laminin-binding activity of α-DG are defective in these diseases [11], they are collectively called α-dystroglycanopathy [12]. However, the precise oligosaccharide structures defective in α-dystroglycanopathy have not been elucidated.

Muscular dystrophy in chicken was first described in 1956 [13]. Although dystrophic chicken has been established as an animal model of muscular dystrophy, the primary mutation has not yet been identified [14] and the pathomechanism leading to muscle cell degeneration remains unknown. We demonstrate here that glycosylation and laminin-binding activity of α-DG are defective in the skeletal muscle of dystrophic chicken. Extensive glycan structural analysis reveals that, compared to control chicken, the amount of Galβ1-3GalNAc and GalNAc residues are increased, whereas Siaα2-3Gal structure is reduced in α-DG of dystrophic chicken.

2. Materials and methods

2.1. Antibodies

Mouse monoclonal antibody against sugar chain moiety of α-DG (IIH6) and sheep polyclonal antibody against core protein of α-DG (sheep anti-α-DG) were described previously [2,15]. Mouse monoclonal antibody against sugar chain moiety of α-DG (IVA4-1) was obtained from Upstate Biotechnology. Mouse monoclonal antibody against β-DG (8D5), β-sarcoglycan (5B1) and γ-sarcoglycan (21B5) were kind gifts from Dr. L.V.B. Anderson (Newcastle General Hospital). Mouse monoclonal anti-dystrophin (MANDRA 1) and affinity isolated rabbit anti-laminin were obtained from Sigma. Mouse monoclonal anti-dystrobrevin was purchased from BD Biosciences.

2.2. Lectin chromatography

Dystrophic chicken used in this study is New Hampshire, line 413, the colony of which is maintained homozygously. Line GSN/1, was used as a control. Pectoralis muscle of dystrophic and control chicken of 3 months of age were used. Skeletal muscle was disrupted with a polytron followed by Daunce homogenization and incubation in 50 mM Tris–HCl, pH 7.4, 500 mM NaCl, 1% Triton X-100, 0.6 μg/ml pepstatin A, 0.5 μg/ml leupeptin, 0.5 μg/ml aprotinin, 0.75 mM benzamidine, and 0.1 mM PMSF. The extract was incubated with lectin

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Abbreviations: DG, dystroglycan; DGC, dystrophin–glycoprotein complex

agarose, including wheat germ agglutinin (WGA), concanavalin A (Con A), peanut agglutinin (PNA), *Vicia villosa* agglutinin isolectin B$_4$ (VVA-B$_4$), *Maackia amurensis* lectin (MAM) and lentil lectin (LCA). Bound proteins were eluted by boiling the beads in sample buffer (65 mM Tris–HCl, pH 6.9, 3% SDS, 1% β-mercaptoethanol, 115 mM sucrose, and 0.0004% bromophenol blue) and the eluates were analyzed by Western blotting using sheep anti-α-DG.

2.3. Miscellaneous

Chemical deglycosylation was described previously [2]. Sialidase digestion was performed using sialidase from *Clostridium perfringens* (Roche) according to the procedure described elsewhere [16]. Immunofluorescent microscopic analysis, Western blotting and blot overlay assay were performed as described elsewhere [11]. The amount of glycosidically bound sialic acid was compared by periodate–resorcinol method [17] and statistical significance was evaluated by *t* test. Solid-phase assay was performed as previously mentioned [11] except that WGA eluates were coated on 96 wells EIA/RIA plates (Coaster) after measuring the band intensity of α-DG on Western blots so that each well contained the same amount of α-DG.

3. Results

3.1. Decreased immunoreactivity of α-DG in the skeletal muscle of dystrophic chicken

We first performed immunofluorescent microscopic analysis. The immunoreactivity of α-DG revealed by antibody against sugar chain moiety of α-DG was significantly decreased in dystrophic chicken, whereas the immunoreactivity of α-DG was indistinguishable between control and dystrophic chicken when detected by antibody against core protein of α-DG. The other components of dystrophin–glycoprotein complex (DGC) were normally expressed in dystrophic chicken (Fig. 1). Consistent with the immunofluorescent analysis, Western

blotting with antibody against sugar chain moiety of α-DG demonstrated reduced immunoreactivity of α-DG in dystrophic chicken (Fig. 2). In addition, α-DG of dystrophic chicken migrated at 160 kD, faster than that of control which migrated at 200 kD (Fig. 2). The expression and molecular mass of the other components of the DGC were not altered (Fig. 2).

3.2. Altered glycosylation of α-DG in the skeletal muscle of dystrophic chicken

The results described above raise the possibility that the glycosylation, rather than expression, of α-DG in dystrophic chicken may be altered. In order to test this possibility, α-DG was enriched by WGA chromatography and chemically deglycosylated with trifluoromethanesulfonic acid. Similar to the antibody against sugar chain moiety of α-DG, antibody against core protein of α-DG recognized α-DG species migrating around 200 and 160 kD in control and dystrophic chicken, respectively (Fig. 3, deglycosylation −). In addition, however, the anti-core protein antibody also detected α-DG species with a lower molecular mass of 110 kD in control and 70–120 kD in dystrophic chicken (Fig. 3, deglycosylation −). In this report, we tentatively call the larger and smaller α-DG species as L-α-dystroglycan (L-α-DG) and S-α-dystroglycan (S-α-DG), respectively. Upon chemical deglycosylation, the molecular mass of α-DG was reduced to 55 kD both in control and dystrophic chicken equally, eliminating the difference in molecular mass (Fig. 3, deglycosylation +). These data indicate that α-DG is aberrantly glycosylated in the skeletal muscle of dystrophic chicken. We also examined various tissues of dystrophic chicken to see if defective glycosylation of α-DG was present. Western blot analysis using antibody against core protein of α-DG demonstrated a

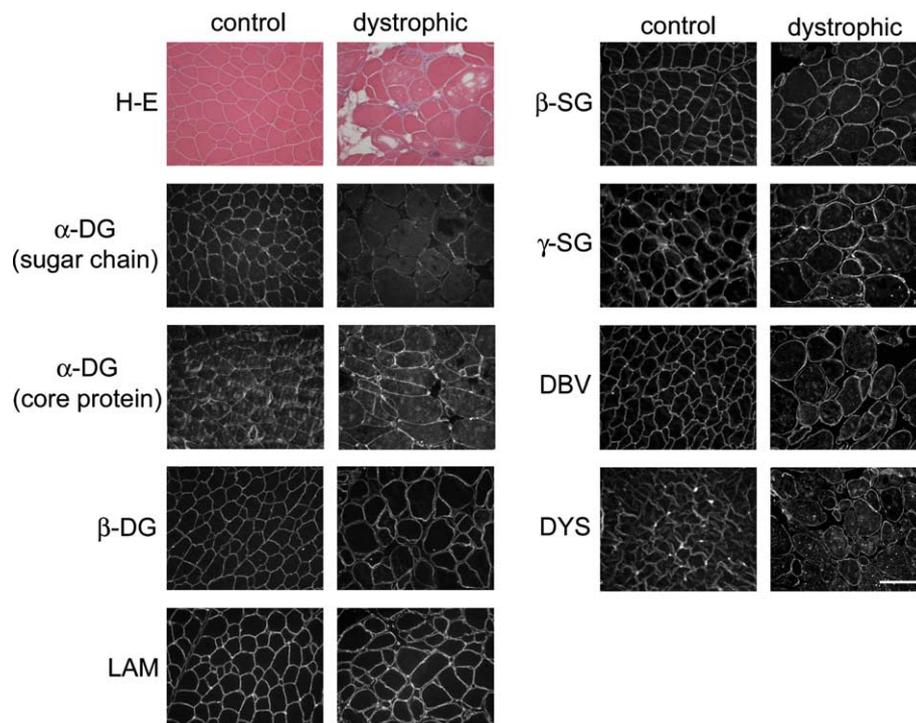Fig. 1. Immunoreactivity of α-dystroglycan is reduced in the skeletal muscle of dystrophic chicken when probed by antibody against sugar chain moiety. Expression and localization of each component of the DGC were analyzed by immunofluorescent microscopy. The immunoreactivity of α-DG, as revealed by antibody against sugar chain moiety of α-DG (IIH6), is reduced in dystrophic chicken. However, the expression of α-DG core protein is not altered. DG, dystroglycan; LAM, laminin; SG, sarcoglycan; DBV, dystrobrevin; DYS, dystrophin. Bar indicates 100 μm.
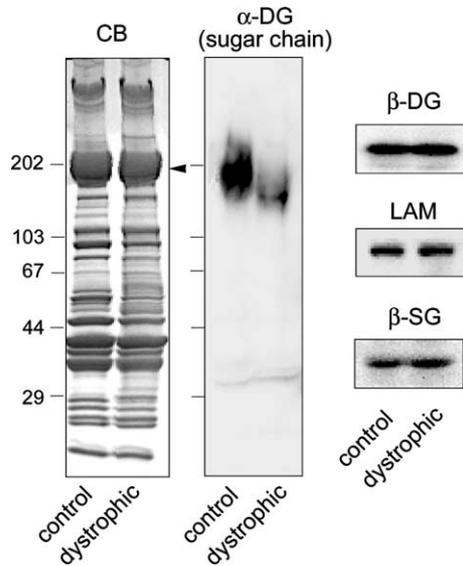
Fig. 2. The molecular mass of α-DG is decreased in the skeletal muscle of dystrophic chicken. Western blotting was performed to examine the expression of α-DG using whole skeletal muscle homogenates. The amount of protein loaded for each lane was normalized using myosin heavy chain as internal standard (arrowhead in the panel CB). α-DG in dystrophic chicken migrates faster than that in control and the immunoreactivity of α-DG is decreased in dystrophic chicken using antibody against sugar chain moiety of α-DG (IIH6). The expression of other components of the DGC is not altered. CB, Coomassie blue staining; DG, dystroglycan; LAM, laminin; SG, sarcoglycan.

downward shift in the molecular mass of α-DG in cardiac muscle, but not in other tissues including brain, peripheral nerve, kidney, spleen and liver (data not shown), indicating
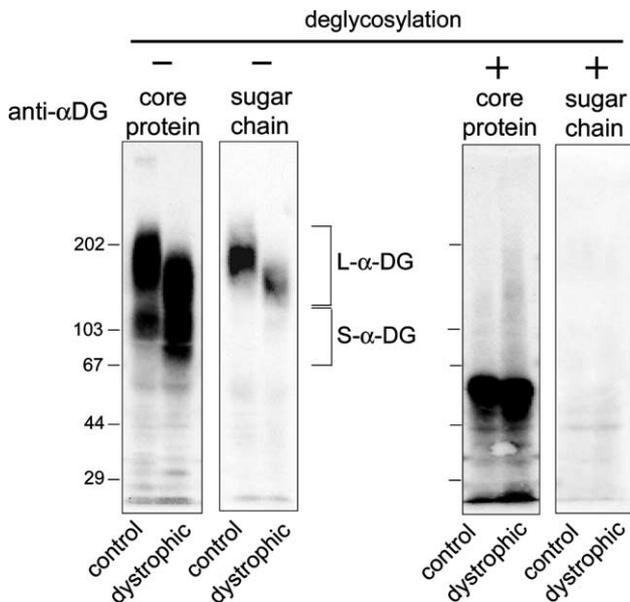
that glycosylation of α-DG was also altered in the cardiac muscle of dystrophic chicken.

3.3. Laminin-binding activity of α-DG is decreased in the skeletal muscle of dystrophic chicken

Blot overlay assays demonstrated that the binding of laminin 1 and 2 to α-DG was greatly reduced in dystrophic chicken (Fig. 4A). Notably, both laminin 1 and 2 bound to L-α-DG, but not S-α-DG (Fig. 4A). The band intensity of S-α-DG and L-α-DG was measured and the ratio of S-α-DG against total α-DG (intensity of S-α-DG/intensity of S-α-DG + L-α-DG) was calculated. The ratio of S-α-DG was $16.8 \pm 4.5\%$ in control versus $40.9 \pm 4.1\%$ in dystrophic chicken (Fig. 4B), indicating that many more α-DG molecules in dystrophic chicken lack the laminin-binding activity than control. Next, we performed quantitative solid-phase assay. The total laminin-binding activity was significantly decreased in the skeletal muscle of dystrophic chicken (Fig. 4C).

3.4. Glycosylation defects of dystrophic chicken α-DG analyzed by lectin chromatography

To investigate the change in glycan structure of α-DG in dystrophic chicken, we performed a set of lectin chromatographies.
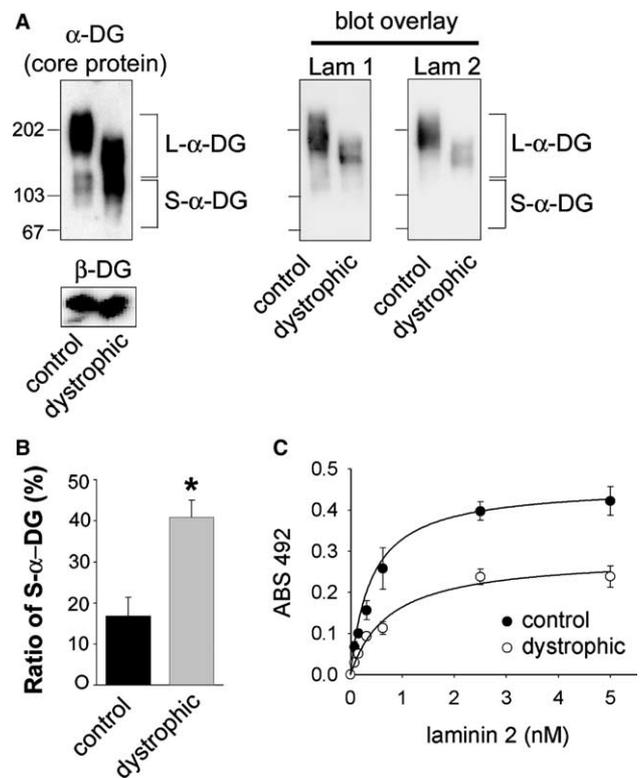

Fig. 4. Laminin-binding activity of α-DG is decreased in the skeletal muscle of dystrophic chicken. (A) Equal amount of DG was transferred to PVDF membranes as revealed by Western blotting for α-DG and β-DG. Blot overlay assays demonstrate that the binding of both laminin 1 and 2 to α-DG is substantially decreased in dystrophic chicken. Both laminin 1 and 2 bind to L-α-DG, but not S-α-DG. Lam 1, laminin 1; Lam 2, laminin 2. (B) The band intensity of L-α-DG and S-α-DG was measured and the ratio of S-α-DG against total α-DG was calculated. The ratio of S-α-DG is significantly higher in dystrophic chicken. $^*P < 0.003$. (C) Solid-phase assay reveals that laminin-binding activity is significantly reduced in the skeletal muscle of dystrophic chicken.

Fig. 3. Deglycosylation eliminates the difference in molecular mass of α-DG between control and dystrophic chicken. α-DG was enriched by WGA chromatography and chemically deglycosylated with trifluoromethanesulfonic acid. Antibody against core protein of α-DG recognizes α-DG species with higher molecular mass (L-α-DG), which are also detected by antibody against sugar chain moiety of α-DG (VIA4-1). In addition, the anti-core protein of α-DG recognizes α-DG species with lower molecular mass (S-α-DG). After deglycosylation, the molecular mass of α-DG decreases to 55 kD in both control and dystrophic chicken equally (deglycosylation +).

As shown in Fig. 5A, Con A bound most of the α-DG species, whereas LCA had no significant interaction with any α-DG species (Fig. 5A). In sharp contrast, MAM bound L-α-DG in control, while it interacted only weakly with α-DG in dystrophic chicken (Fig. 5A), indicating that Siaα2-3Gal moieties are profoundly reduced in α-DG of dystrophic chicken. Interestingly, PNA bound to a fraction of S-α-DG in dystrophic chicken, while no binding to α-DG occurred in control (Fig. 5B, sialidase −). VVA-B$_4$ bound weakly to S-α-DG in control, whereas it strongly interacted with L-α-DG and S-α-DG in dys-

trophic chicken. Because the reactivity of these lectins are known to be severely decreased when sialic acids are attached to non-reducing termini of their binding sugar chain moieties [18], we enzymatically removed sialic acids by sialidase and repeated the experiments. After sialidase digestion, both S-α-DG and L-α-DG extensively interacted with PNA in dystrophic chicken, whereas only a small amount of S-α-DG was recovered in control. These results indicate that Galβ1-3GalNAc moieties are much more abundant on α-DG in dystrophic chicken than that in control (Fig. 5B, sialidase +). Similar result was obtained with VVA-B$_4$, indicating that GalNAc structures are much more abundant on α-DG of dystrophic chicken (Fig. 5B, sialidase +). The amount of glycosidically bound sialic acids quantified by periodate–resorcinol method was substantially reduced in dystrophic chicken (Fig. 5C), which is consistent with the result of MAM lectin chromatography.

4. Discussion

The mucin-like domain of α-DG is heavily glycosylated by O-linked glycans [19], with the sugar chain moieties constituting up to two-thirds of its total molecular mass [1,2]. The antibody against sugar chain moiety of α-DG detected only L-α-DG, while anti-α-DG core protein detected both L-α-DG and S-α-DG (Figs. 2 and 3), indicating diverse glycosylation of α-DG in vivo. Notably, laminin bound to L-α-DG, but not to S-α-DG, in both control and dystrophic chicken (Fig. 4), indicating that the interaction of laminin with α-DG is strictly regulated through glycosylation of α-DG and that a fraction of α-DG does not possess the sugar chain moieties necessary for the binding of laminin in vivo. Furthermore, the ratio of non-laminin-binding α-DG (S-α-DG) is greatly increased in dystrophic chicken compared to control (Fig. 4). It would be intriguing to postulate that the increase of non-laminin-binding α-DG may contribute to the dystrophic phenotype by exerting a dominant negative effect in dystrophic chicken, where non-laminin-binding α-DG competes with laminin-binding α-DG for the cytoskeletal linkage via dystrophin. Consistent with this hypothesis, we have observed that adenovirus mediated gene transfer of non-laminin-binding α-DG constructs results in the degeneration of skeletal muscle in mice (Saito and Campbell, unpublished observation).

The results of lectin chromatography indicate that, compared to control chicken, the amount of Galβ1-3GalNAc and GalNAc residues are increased significantly while Siaα2-3Gal structure is severely decreased in α-DG of dystrophic chicken (Fig. 5). The reduction in the amount of sialic acids was confirmed by periodate–resorcinol sialic acid assay (Fig. 5). However, α-DG appears to be hyposialylated rather than asialylated (Fig. 5C). We have reported recently that hyposialylation of α-DG alone is not enough to abolish its laminin-binding activity in vivo [20]. It remains to be determined if hyposialylation in dystrophic chicken reflects the reduction of the sialyl O-mannosyl glycan, Sia2-3Galβ1-4GlcNAcβ1-2Man-Ser/Thr, implicated in the binding of laminin [21,22].

Pavoni et al. [23] reported recently that antibody against C-terminal portion of α-DG core protein detected α-DG with molecular mass of 109 kD in the skeletal muscle of normal chicken. Our S-α-DG may correspond to this small α-DG, as judged by molecular mass. Pavoni et al. further postulated that this 109 kD α-DG might be a partially glycosylated form of
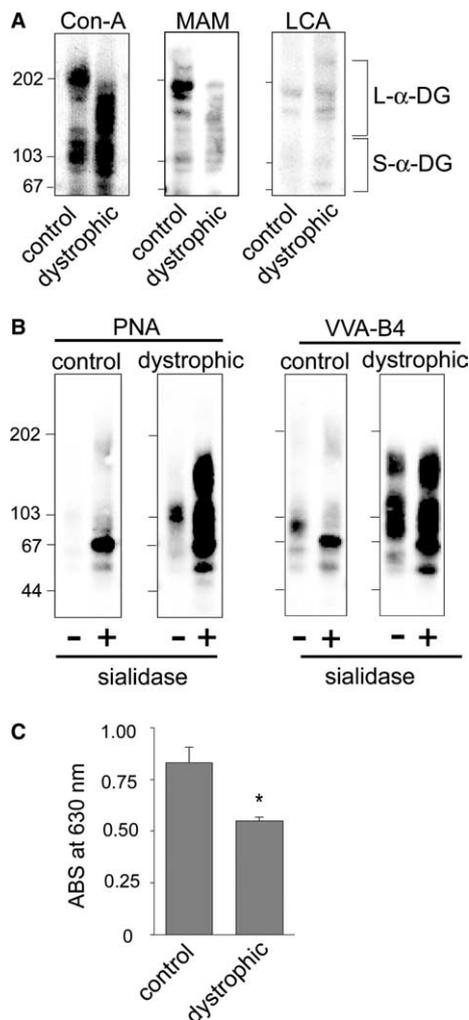
Fig. 5. Glycosylation of α-DG is altered in the skeletal muscle of dystrophic chicken. (A) α-DG was extracted using Triton X-100 and applied to lectin chromatography. The bound α-DG was visualized by Western blotting using antibody against core protein of α-DG. Con-A binds most of the α-DG species, while LCA does not interact with any α-DG species significantly. MAM strongly binds only to L-α-DG in control. (B) The Triton X-100 extracts were applied to PNA or VVA-B$_4$ chromatography with or without prior digestion by sialidase. Without sialidase treatment, PNA binds S-α-DG in dystrophic chicken, while it does not interact with α-DG in control (sialidase −). VVA-B$_4$ binds S-α-DG in control only weakly, while it interacts strongly with both L- and S-α-DG in dystrophic chicken (sialidase −). With sialidase digestion, both PNA and VVA-B$_4$ bind extensively to L-α-DG and S-α-DG in dystrophic chicken compared to control (sialidase +). (C) Quantification of sialic acid by periodate–resorcinol method reveals that the amount of glycosidically bound sialic acids in the skeletal muscle of dystrophic chicken is significantly less than that of control chicken. $^*P < 0.001$.

α-DG. In the present study, we provided clear evidence of actual alteration of glycosylation of this small α-DG molecule (Figs. 3 and 5). The molecular mass of α-DG in the skeletal muscle of normal chicken was reported to change during development [24]. It would be thus interesting to see if the molecular mass of α-DG in the skeletal muscle of dystrophic chicken also changes during development by future studies.

In conclusion, we have demonstrated altered glycosylation and decreased laminin-binding activity of α-DG in chicken muscular dystrophy. Furthermore, we have demonstrated that Siaα2-3Gal structure is reduced, while Galβ1-3GalNAc and GalNAc moieties are increased on α-DG of this animal model of muscular dystrophy. These data would contribute to further understand the molecular mechanism of muscular degeneration caused by disturbed glycosylation of α-DG in human muscular dystrophies.

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