Alterations of dystrophin associated glycoproteins in the heart lacking dystrophin or dystrophin and utrophin

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Abstract

Heart disease is a leading cause of death in patients with Duchenne muscular dystrophy (DMD). Patients with DMD lack the protein dystrophin, which is widely expressed in striated muscle. In skeletal muscle, the loss of dystrophin results in dramatically decreased expression of the dystrophin associated glycoprotein complex (DGC). Interestingly, in the heart the DGC is normally expressed without dystrophin; this has been attributed to presence of the dystrophin homologue utrophin. We demonstrate here that neither utrophin nor dystrophin are required for the expression of the cardiac DGC. However, alpha-dystroglycan (α-DG), a major component of the DGC, is differentially glycosylated in dystrophin-(*mdx*) and dystrophin–/utrophin– (dko) deficient mouse hearts. In both models the altered α-DG retains laminin binding activity, but has an altered localization at the sarcolemma. In hearts lacking both dystrophin and utrophin, the alterations in α-DG glycosylation are even more dramatic with changes in gel migration equivalent to 24 ± 3 kDa. These data show that the absence of dystrophin and utrophin alters the processing of α-DG; however it is not clear if these alterations are a consequence of the loss of a direct interaction with dystrophin/utrophin, or results from an indirect response to the presence of severe pathology. Recently there have been great advances in our understanding of the glycosylation of α-DG regarding its role as a laminin receptor. Here we present data that alterations in glycosylation occurs in the hearts of animal models of DMD, but these changes do not affect laminin binding. The physiological consequences of these alterations remain to be determined, but may have significant implications for the development of therapies for DMD.

Keywords

Dystrophic cardiomyopathy; dystrophin; utrophin; alpha-dystroglycan; glycosylation

INTRODUCTION

DMD and Becker muscular dystrophy (BMD) are caused by mutations in the DMD gene, which encodes the cytoskeletal protein dystrophin (Hoffman et al. 1987). Dystrophin is a large protein that facilitates the connection between the extracellular matrix and the cytoskeleton (Ervasti and Campbell 1991, 1993b; Ohlendieck et al. 1991). Critical to this connection is dystrophin's interaction with the glycosylated sarcolemmal proteins α- and β-dystroglycan (DG), the sarcoglycan complex (α-, β-, γ-, δ-SG), and sarcospan. Together, these proteins make up the DGC. It is important to note, however, that assembly of this complex does not require dystrophin to be present. For example, the DGC is fully retained in the dystrophin deficient myocardium (Bies et al. 1997; Matsumura et al. 1992; Townsend et

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al. 2007). It is hypothesized that the linkage between the extracellular matrix (ECM) and the cytoskeleton is critical for the maintaining the integrity of the sarcolemma and cellular calcium gradient (Ervasti and Campbell 1991; Hutter et al. 1991; Menke and Jockusch 1991; Ervasti and Campbell 1993a; Petrof et al. 1993). Reductions in dystrophin and associated proteins renders the myocyte susceptible to increased cell damage and necrosis, contributing to the muscular dystrophy phenotype (Ervasti and Campbell 1993b; Ohlendieck and Campbell 1991). Mice lacking dystrophin (*mdx*) show markedly reduced levels of all DGC proteins in skeletal muscle (Ohlendieck and Campbell 1991). Without dystrophin, skeletal muscle DGC proteins are degraded by the proteosome (Bonuccelli et al. 2003; Bonuccelli et al. 2007). Several components of the DGC also are down-regulated at the transcriptional level in *mdx* compared to wild type (WT) skeletal muscle (Turk et al. 2005). It therefore seems likely that the marked decrease in the DGC observed in *mdx* skeletal muscle results from a combination of increased degradation and decreased production. The role of these processes in the dystrophic heart is unknown.

Understanding how the functions of dystrophin differ between skeletal muscle and the heart provides unique insight into the function of dystrophin. Furthermore, understanding cardiac dystrophin has therapeutic relevance given the clinical importance of heart disease in the management of DMD (Bushby et al. 2010b, a). The *mdx* mouse, lacking dystrophin, is a genetic model of DMD that displays a subtle cardiac phenotype at baseline and becomes highly apparent with stress testing (Yasuda et al. 2005; Townsend et al. 2007; Bostick et al. 2008). In contrast to skeletal muscle, the components of the DGC are present in the dystrophin-deficient heart (Matsumura et al. 1992; Townsend et al. 2007; Yang et al. 1994), although their function in the absence of dystrophin is not clear. Increased expression of dystrophin's autosomal homologue, utrophin, has been suggested to functionally replace dystrophin in both skeletal muscle and the heart (Matsumura et al. 1992; Tinsley et al. 1998). Furthermore, mice lacking both dystrophin and utrophin (dko) have a very severe disease (Grady et al. 1997; Connolly et al. 2001; Janssen et al. 2005; Deconinck et al. 1997). It is hypothesized that utrophin expression in the *mdx* heart is responsible for the continued expression of the DGC without dystrophin. Evaluating the status of the DGC in hearts with neither dystrophin nor utrophin is an important objective of the studies reported here.

The presence of the DGC in the dystrophin-deficient heart permits the unique ability to examine the post-translational processing and trafficking of the DGC without the presence of dystrophin. This is of particular interest for α-DG, which requires glycosylation to perform its primary function of binding laminin (Ibraghimov-Beskrovnaya et al. 1992; Ervasti and Campbell 1993b; Ibraghimov-Beskrovnaya et al. 1993). The core α-DG backbone alone has a predicted mass of 40 kDa, however additional post-translational glycosylation of α-DG in skeletal muscle creates a final protein that migrates equivalent to a 156 kDa protein (Ibraghimov-Beskrovnaya et al. 1992). This glycosylation occurs in a tissue-specific manner, with cardiac α-DG migrating slightly faster than the skeletal muscle form, while brain α-DG migrates equivalent to a 120 kDa protein (Gee et al. 1993; Ibraghimov-Beskrovnaya et al. 1992; Ervasti et al. 1997). Because glycosylation greatly alters the structure, localization, and binding characteristics of a protein, these tissue-specific differences in glycosylation likely reflect necessary modifications in α-DG function for each tissue and stage of development. α-DG is composed of three domains: two globular *N*-glycosylated domains at the N- and C-terminus and a central heavily *O*-glycosylated mucin domain (Brancaccio et al. 1995, 1997). Within this mucin domain are a large number of *O*-glycan structures, including both N-acetyl-galactosamine and mannose initiated O-glycans (Stalnaker et al. 2010). The O-mannose glycans are of particular importance to the laminin binding function of α-DG, as loss of the enzymes that synthesize the O-mannose result in the complete loss of laminin binding (Yoshida et al. 2001; Willer et al. 2002; Beltran-Valero de Bernabe et al. 2002). A subset of these O-mannose initiated structures are further

modified by the addition of xylose-glucuronic acid moieties which have an important role in binding to laminin (Yoshida-Moriguchi et al. 2010; Inamori et al. 2012). Defects in the synthetic pathway of these O-mannose initiated glycans constitute an entire class of muscular dystrophies, characterized by muscle degeneration and a high prevalence of neurological deficits. The role of either dystrophin and/or utrophin in regulating the post-translational modification of α-DG is unknown and understanding this is an important focus of these studies.

EXPERIMENTAL PROCEDURES

Animal Care and Use

All animal experiments described here were reviewed and approved by the University of Minnesota's Institutional Animal Care and Use Committee. C57BL/10 and *mdx* mice were obtained from locally maintained SPF colonies replenished every four generations with breeders from Jackson Labs (Bar Harbor, ME). Utrophin and dystrophin double knockout (dko) mice were provided by a colony maintained by the Muscular Dystrophy Center at the University of Minnesota (Landisch et al. 2008).

Immunohistochemistry

Excised heart tissue was embedded in Tissue-Tek O.C.T. Compound (Andwin Scientific, Woodland Hills, CA) and snap-frozen in liquid nitrogen-cooled isopentane. Frozen tissues were cut into 7μm sections and placed on glass slides and stored at −80°C. Slides stained with Cathepsin D required fixation (4% PFA for 10 minutes) and denaturing (1% SDS for 5 minutes) prior to the initial blocking step. At the time of staining, slides were removed from the freezer and allowed to warm to room temperature (RT); sections were washed with PBS and blocked for 30 minutes with 5% bovine serum albumin (BSA) and 0.5% Triton X-100 in PBS. Primary antibodies were diluted in PBS+0.5% Triton X-100+5% BSA and incubated for 1 hour at RT. Primary antibodies were diluted as follows: α-DG 1:100 (IIH6C4, EMD Millipore, Ballerica, MA), β-SG 1:200 (bSarc/5B1, Vector Laboratories, Burlingame, CA), Laminin 1:1000 (L9393, Sigma-Aldrich, St. Louis, MO), Cathepsin D 1:500 (ab75852, Abcam, Cambridge, MA). Slides were washed with 0.5% Triton X-100 in PBS. Goat-anti-mouse (GAM) and/or goat-anti-rabbit (GAR) secondary antibodies conjugated to either Alexa-488 or Alexa-594 were diluted to 1:500 in PBS+0.5% Triton X-100+5% BSA and incubated for 1 hour at RT. Slides were washed and mounted using ProLong Gold Antifade Reagent with DAPI (Invitrogen, Carlsbad, CA). The slide was allowed to cure 24 hours before imaging on the Zeiss Axio Imager M1 epifluorescent and/or Zeizz LSM 510 Meta confocal microscope (Carl Zeiss, Oberkochen, Germany). Laminin and α-DG membrane distributions were measured using a custom line scanning macro in ImageJ and Excel (NIH, Bethesda, MD and Microsoft, Redmond, WA). Briefly, line scans were taken across cellular borders, pixel intensities were normalized and the distance between 50% of peak height was determined, with linear approximations of inter-pixel intensities. Statistical analysis of data was performed by ANOVA with a Dunnett's post-hoc test using Prism (GraphPad, La Jolla, CA); data from 419–596 individual line scans, from 21–30 images from 2–3 mice.

KCl Washed Membranes

KCl washed membranes were prepared as described previously (Ohlendieck and Campbell 1991). Briefly, excised tissues were snap-frozen and pulverized in liquid nitrogen. Tissues were homogenized and centrifuged at 20,000g for 20 minutes at 4°C. The supernatant was centrifuged again at 4°C at 100,000*g* for 35 minutes. The resulting pellet was incubated in buffer containing 0.6 M KCl for 1 hour at 4°C. KCl washed membranes were sedimented by a 150,000*g* spin at 4°C for 37 minutes. Samples were stored at −80°C prior to use. The

protein content of the sample was determined using the BCA Protein Assay Kit (Thermo Scientific Pierce, Rockford, IL).

Gel Electrophoresis and Western Blotting

SDS-PAGE was performed on 4–20% gradient gels (Bio-Rad, Hercules, CA) using standard procedures (Towbin et al. 1979; Laemmli 1970). Nitrocellulose membranes were blocked with 5% nonfat milk in Tris-buffered saline (TBS, 50mM Tris, 150mM NaCl)+0.5% Tween-20 (TBS-T) at room temperature for 1 hour. Primary antibodies were diluted in PBS-T; α-DG 1:1000 (IIH6C4, Millipore), β-DG 1:100 (43DAG1/8D5, Vector Laboratories), γ-SG 1:100 (35DAG/21B5, Vector Laboratories), NaKATPase 1:10,000 (α6F Concentrate, Developmental Studies Hybridoma Bank, Iowa City, IA). Following a 1 hour incubation at RT, the membrane was washed with TBS-T and incubated with 1:5000 diluted GAM or GAR secondary antibody conjugated to a Alexa-688 (Invitrogen, Carlsbad, CA) or IR-800nm (Rockland Immunochemicals, Gilbertville, PA) for one hour at RT. After a final wash the membrane was visualized and quantified using the Odyssey Imaging System (Li-Cor, Lincoln, NE).

N-glycosidase F and Neuraminidase Treatments

Our protocols for N-glycosidase F and Neuraminidase treatments were performed as described previously (Ervasti and Campbell 1991). Briefly, 50 μg of protein were boiled in the presence of 1% SDS for five minutes. The sample was allowed to cool and 3 units of PNGase F (New England Biolabs, Ipswich, MA) or 0.0025 units of V. cholerae Neuraminidase (Sigma-Aldrich, St. Louis, MO) were added to the reaction. An equivalent amount of water was added for negative control reactions. The reactions were allowed to incubate at 37°C for 2 hours. 5X sucrose buffer that did not contain SDS was used to load each sample for SDS-PAGE. Migration measurements were calculated from the densest point in each band.

Lectin Binding Assays

Tissues were pulverized and homogenized as described for the KCl-washed membranes. An equal volume of 2X Solubilization Buffer (Tris-HCl 100mM, NaCl 1M, Digitonin 1mM, Pepstatin 0.6mM, Aprotinin 0.5mM, Leupeptin 0.5mM, PMSF 0.1mM, Benzamidine 0.75mM) was added to the tissue homogenate and allowed to incubate in a rotisserie at 4°C for 1 hour. Following this incubation, the homogenate was added to washed lectin beads (wheat germ agglutinin/WGA or Jacalin, Vector Labs, Burlingame, CA) and mixed with constant agitation for 2 hours at 4°C. The samples were spun at 225g for 1 minute and the supernatant was discarded. The samples were washed with Wash Buffer (Tris-HCl 50mM, NaCl 0.5M, Digitonin 0.1mM, and protease inhibitors) 3 times for 5 minutes at 4°C with constant agitation. Bound proteins were eluted by boiling 5 minutes in sample buffer. Beads were removed by centrifugation and eluted proteins were stored at −80°C until use.

Laminin Overlay Assays

Laminin overlay binding assays were preformed as previously described (Michele et al. 2002). Briefly, KCl washed microsome samples were subjected to SDS-PAGE and transferred to nitrocellulose membranes. The blots were hydrated with water and rinsed well with Tris buffered saline+0.05% Triton X-100+Ca^{2+} (TBS-T+Ca^{2+}, 20mM Tris pH 7.5, 100mM NaCl, 1mM $CaCl_2$, 0.05% Tween-20). The blot was blocked for 1 hour at RT in laminin blocking buffer (laminin binding buffer (LBB; 10mM triethanolamine, 100mM NaCl, 1mM $MgCl_2$, 1mM $CaCl_2$, pH 7.6) + 5% nonfat dry milk, pH 7.4). The blot was washed 3 times for 10 minutes at RT with TBS-T+Ca^{2+}. Engelbreth-Holm-Swarm (EHS) laminin (Invitrogen, Carlsbad, CA) was diluted 1μg/ml in laminin binding buffer and added

to the blot which was incubated overnight at 4°C. Following 3 washes of 10 minutes each with TBS-T+Ca^{2+}, anti-laminin primary antibody (L9393, Sigma) was diluted 1:1000 in LBB and added to cover the blot. The blot was incubated at RT for 1 hour, then washed with TBS-T+Ca^{2+} and incubated with the GAR secondary antibody (diluted 1:5000 in laminin blocking buffer) for 1 hour at RT. Following washes with TBS-T+Ca^{2+}, blots were imaged on the Odyssey Imaging System (Licor, Lincoln, NE).

Solid-Phase Laminin Binding Assay

Solid phase binding assays were performed as previously described (Michele et al. 2002). Briefly, cleared heart homogenates were allowed to adhere to 96 well plates for 16 hours at 4°C. After washing, laminin was added to the wells at varying concentrations for 2 hours at room temperature. Following washing, 1:10,000 anti-laminin antibody (L9393, Sigma) overnight at 4°C. Plates were washed and 1:1000 secondary antibody was added for 30 minutes at room temperature. Signal intensity quantified using the Odyssey Imaging System (Licor).

RESULTS

Maintenance of the DGC in the Absences of Both Dystrophin and Utrophin

In contrast to skeletal muscle, α-DG and β-SG were expressed at the sarcolemma in the dystrophin-deficient heart (Fig. 1). Furthermore, these proteins in the DGC complex were also present at the sarcolemma in the absence of both dystrophin and utrophin. Quantitative western blotting of KCl-washed microsomal membranes isolated from the hearts of WT, *mdx*, dko, and utrophin-null (utr) mice demonstrated that α-DG, β-DG and γ-SG were detected at normal levels in the presence and absence of dystrophin and/or utrophin (Fig 2a). Interestingly, there were differences in the migration pattern of the α-DG from dko hearts, suggesting an alteration in the glycosylation of this protein. To begin assessing changes in α-DG glycosylation the differences in the overall migration of α-DG in WT, *mdx*, dko and utr mice were quantified. Migration of α-DG from *mdx* and utr hearts was not different from WT, however, dko α-DG migrated 24 ± 3 kDa faster (p<0.0001) than WT α-DG (Mean ± SEM) (Fig 2a). α-DG migration differences results are reported as a change in kDa relative to WT α-DG. However, it should be noted that these differences reflect changes in the overall size of the protein and/or its ability to migrate through a polyacrylamide gel.

Glycosidases Reveal Altered α-DG Glycosylation

Given that α-DG is heavily glycosylated, alterations in glycosylation processing seemed a potential mechanism for the increased mobility of α-DG observed in dko mice. To assess the level of *N*-linked glycosylation present on α-DG, we enzymatically removed *N*-linked carbohydrates via incubation with *N*-glycosidase F (PNGase F). No changes in α-DG mobility were observed between KCl washed microsome samples treated with or without PNGase F in WT and dko mice (Fig 2b). In contrast, incubation of samples with neuraminidase (NA), which removes sialic acid, resulted in a marked shift in α-DG mobility (Fig 2c). This shift was approximately equal for WT, *mdx* and utr α-DG (WT=42 ± 2), but was significantly lower for dko (26 ± 8) α-DG (p<0.05).

Altered α-DG Lectin Binding in mdx and dko Hearts

The binding of lectins are often used to characterize the properties of glycosylated proteins. To further assess the glycosylation properties of cardiac α-DG from WT, *mdx*, and dko mice, α-DG binding to wheat germ agglutinin-(WGA) and jacalin-conjugated agarose beads was determined in pull-down assays. These lectins preferentially bind to distinct carbohydrate moieties; jacalin binds to N-acetyl galactosamine in which the C-6 is free

(Tachibana et al. 2006) and WGA has affinity for both N-acetyl-D-glucosamine and sialic acid moieties (Gallagher et al. 1985). In hearts without dystrophin or utrophin, significantly less α-DG bound to either WGA or jacalin compared to that in WT samples (p<0.0001, Fig 3). Interestingly, α-DG from *mdx* mice showed significantly more binding to jacalin compared to WT (p<0.001, Fig 3) indicating that the loss of dystrophin alone alters α-DG glycosylation in the heart.

Laminin Binding Unaffected by Altered Glycosylation of α-DG

Glycosylation is critically important for α-DG ligand binding, thus alterations in glycosylation may effect the affinity of its laminin binding and perturb the connection to the extracellular matrix (Michele et al. 2002; Yoshida-Moriguchi et al. 2010; Inamori et al. 2012). We investigated this possibility by performing a laminin overlay binding assay in WT, *mdx*, dko and utr mice. Despite the significant differences in the gel migration and lectin binding α-DG in dko hearts, the affinity and levels of laminin binding to α-DG was not significantly different in any of the mouse genotypes examined here (Fig 4). Specific laminin binding affinities of 21.2 ± 7.6 nM, 33.0 ± 12.5 nM, and 18.8 ± 8.2 nM were observed in WT, *mdx*, and dko homogenates respectively. These data indicate that the changes in glycosylation observed in dko α-DG do not affect laminin binding.

Trafficking of α-DG in mdx and dko Hearts

To test the possibility that the absence of dystrophin, with or without utrophin, may result in an unstable α-DG, the potential lysosomal degradation of α-DG was assessed via immunofluorescent label co-localization with Cathepsin D. These studies revealed that lysosomal content is increased in *mdx* hearts compared to WT hearts and is further increased in dko hearts; however, the majority of α-DG is not co-localized with Cathepsin D in the heart (Supplemental Fig 1). Using confocal microscopy, the distribution of laminin and α-DG was measured across cellular interface (Fig 5a). The width at 50% of the normalized height was determined for each line-scan for both α-DG and laminin for WT, *mdx*, and dko (Fig 5b and c). Interestingly, both *mdx* and dko hearts demonstrated significantly wider distributions of immunoreactivity for laminin and α-DG compared to WT values. Furthermore, the calculated difference between laminin and α-DG distributions is also significantly greater than that observed in WT hearts.

DISCUSSION

We have shown normal localization of α-DG and β-SG at the cardiac sarcolemma in dystrophin-deficient *mdx* mice and demonstrate that α-DG, β-DG and γ-SG are expressed at normal levels in KCl-washed microsomes (Figs. 1 and 2a). These findings are in contrast to skeletal muscle, where the absence of dystrophin results in the nearly complete loss of the DGC (Ohlendieck and Campbell 1991; Ervasti et al. 1990). Interestingly, the expression of these proteins is reintroduced in skeletal muscle by over-expression of the dystrophin homologue utrophin (Matsumura et al. 1992). This observation has led to a potential therapeutic approach using utrophin over-expression to compensate for the loss of dystrophin (Rafael et al. 1998; Tinsley et al. 1996; Tinsley et al. 1998; Sonnemann et al. 2009; Matsumura et al. 1992). The up-regulation of utrophin in the *mdx* heart has been proposed as a mechanism by which the DGC is maintained in this tissue (Matsumura et al. 1992). The current study and several others (Matsumura et al. 1992; Yang et al. 1994; Townsend et al. 2007) provide evidence that the cardiac DGC is maintained in the absence of both dystrophin and utrophin, indicating that neither of these cytoskeletal proteins are required for sarcolemmal localization of DGC proteins in the heart. However, several studies have reported decreases in α-DG content from dystrophin deficient hearts (Bies et al. 1997; Lohan et al. 2005).

It is possible that the α-DG may be present in a mislocalized form. Our data demonstrates that α-DG is not colocalized with the lysosomal marker Cathepsin D in WT, *mdx* or dko mouse cardiac cells, although cathepsin D content is increased in both *mdx* and dko hearts, similar to that seen in DMD patient (Whitaker et al. 1983) and *mdx* (Aoyagi et al. 1981) skeletal muscle. Confocal immunofluorescence studies demonstrate that the distribution of α-DG immunoreactivity is significantly greater in hearts lacking dystrophin; furthermore, the calculated difference between the laminin and α-DG distributions is significantly greater in hearts lacking dystrophin. These results are consistent with the hypothesis that the localization of α-DG is different without dystrophin, although the nature and functional consequences of this diffuse localization are unclear. It is important to recognize that the direct detection of α-DG in these studies uses the IIH6 monoclonal antibody, which has an epitope that is very tightly associated with the laminin binding function of α-DG (Ervasti and Campbell 1993b). While the overall levels of IIH6 immunoreactivity are the same as that seen in WT mice, it is possible that other, non-IIH6 binding forms of α-DG may be present in these dystrophic hearts.

The nature of the altered gel migration of α-DG observed in the dko hearts is of great interest. Changes in gel migration can result from changes in protein mass, structural stiffness, and/or net charge. The results presented here indicate that a combination of effects are likely responsible for the shift in migration observed in α-DG from dko hearts. Treatment with NA results in significant shifts in the migration of α-DG from all genotypes of mice examined here, but the change is markedly smaller in the dko. Interestingly, the final NA-treated α-DG has essentially equivalent migration in all genotypes. This observation indicates that the increased gel migration seen in the dko α-DG results from changes in the glycosylation, rather than proteolysis of the core peptide. Previous data has indicated that nearly $\frac{2}{3}$ of the glycosylation sites of α-DG contain a sialic acid moiety (Stalnaker et al. 2010), the sugar removed by NA. This observation suggests that α-DG from dko hearts does not have the full complement of sialic acid moieties. The decreased change in gel migration following NA treatment indicates a reduced sialic acid content in the dko α-DG, which contributes to increased α-DG migration through reduction in size, charge, and protein stiffness. Modification of the sialic acids of α-DG has been shown to significantly modulate the severity of disease in the dystrophin-deficient mouse. Loss of a single hydroxyl group on a subset of α-DG carbohydrate chains significantly worsens the pathology of both skeletal and cardiac muscle (Chandrasekharan et al. 2010). In contrast, the addition of a N-acetyl-galactosamine, through the over-expression of Galgt2, provides significant protection to skeletal muscle of the *mdx* mouse (Martin et al. 2009).

The lectin WGA binds to sialic acid moieties (Combs and Ervasti 2005). The observation that WGA binds very poorly to α-DG from dko hearts is consistent with the hypothesis that sialic acid addition is disrupted without dystrophin and utrophin. However, WGA also binds to N-acetyl-glucosamine (Nagata and Burger 1974), allowing for the possibility that alterations in glycosylation extend beyond changes in sialic acid content. To explore the status of other forms of glycosylation, we examined the binding of α-DG to the lectin jacalin, which has an affinity for a subset of N-acetyl-galactosamine-containing carbohydrates (Tachibana et al. 2006). The marked reduction in jacalin binding to α-DG from dko hearts indicates that several forms of α-DG glycosylation are altered without dystrophin and utrophin. Interestingly, there is significantly greater binding of jacalin to α-DG from *mdx* hearts, suggesting that excess N-acetyl-galactosamine is present on the α-DG in the absence of dystrophin. Together these data strongly suggest that the glycosylation of the α-DG mucin domain is altered in these dystrophic hearts.

The main function of α-DG is to bind to the extracellular matrix proteins, such as laminin, an activity that is dependent on glycosylation (Combs and Ervasti 2005; Michele et al.

2002). Despite the changes in glycosylation, no significant differences in EHS mouse laminin binding were observed between WT, *mdx*, dko and utr α-DG in the laminin overlay assay. Similarly, no differences were observed between in the solid-state laminin binding assays, although the laminin binding affinities of the homogenates used in the current study were much lower than that observed with WGA-purified α-DG (Michele et al. 2009). These observations suggest that neither dystrophin nor utrophin are critically important for the addition of the carbohydrate structures that are essential for the binding of laminin (Inamori et al. 2012). This raises fundamental questions regarding the function of the α-DG glycosylation not participating in laminin binding. The high concentrations of serine, threonine, and proline in the central domain of α-DG are similar to the *O*-glycosylation domains of the mucin proteins (Ibraghimov-Beskrovnaya et al. 1992). In mucin proteins *O*-glycosylation has been proposed to function as a structural modifier. The extensive glycosylation of the central domain of α-DG would be expected to both stiffen and extend this domain, creating a rod like structure protruding ≈25 nm from the surface of the cell (Jentoft 1990; Hilkens et al. 1992). LARGE-modified glycosylation has been proposed to attach to the α-DG core protein at the end of this rod-like mucin domain (Yoshida-Moriguchi et al. 2010; Hara et al. 2011). The consequences of changes in the extent or nature of α-DG glycosylation is unknown, but changes to the mechanical properties of the protein would be expected to alter its function and may contribute to the severity of the dystrophic phenotype in the dko mouse.

The underlying mechanism by which the loss of dystrophin or dystrophin and utrophin result in altered α-DG glycosylation is not clear. One possibility is that the dystrophic disease process fundamentally alters protein processing in the cardiomyocyte. In heart failure there is evidence of disruptions of protein glycosylation and trafficking (Kiarash et al. 2004), although cardiac α-DG migrates normally in our studies of failing human hearts (data not shown). In skeletal muscle, the loss of dystrophin results in significant disruption of both the Golgi complex (Percival et al. 2010) and the microtubule network (Prins et al. 2009). If similar disruptions occur in the heart, they could contribute to the perturbations in α-DG processing observed in the current study. Alterations in glycosylation and trafficking of the DGC may be explained by a necessity for a direct interaction between the DGC and dystrophin or utrophin before insertion into the sarcolemma, however the failure to observe dystrophin association with any internal membranes argues against this hypothesis (Watkins et al. 1988; Byers et al. 1991). Although given the long half-life of dystrophin (Lu et al. 2005), the probability of observing such a transient complex would be low making it difficult to exclude a direct interaction of the DGC with dystrophin or utrophin in the internal membranes. Another possibility is that a portion of the post-translational processing of α-DG is completed following insertion in the surface membrane. This is supported by the observation that many glycosyltransferases can be found expressed on the surface membrane (Berger 2002), including enzymes implicated in α-DG processing (Beedle et al. 2007). A shift in gel migration of α-DG similar to that observed in the utrophin/dystrophin-null mice is observed in a subset of patients with mutations in the fukutin-related protein (FKRP) gene (Brockington et al. 2001). In the intact skeletal muscle, this protein is localized to the same surface membrane domain as the rest of the DGC (Beedle et al. 2007). Which, with the data presented here, supports a model by which the final maturation of α-DG occurs at the cell surface and this process is partially coordinated by dystrophin or utrophin. In summary, we demonstrate novel findings regarding the processing of dystrophin-associated proteins in the hearts of several relevant models of dystrophic cardiomyopathy. Specifically, the alterations in glycosylation of α-DG in the heart of the dko mouse may significantly contribute to the severity of the disease in this model of muscular dystrophy.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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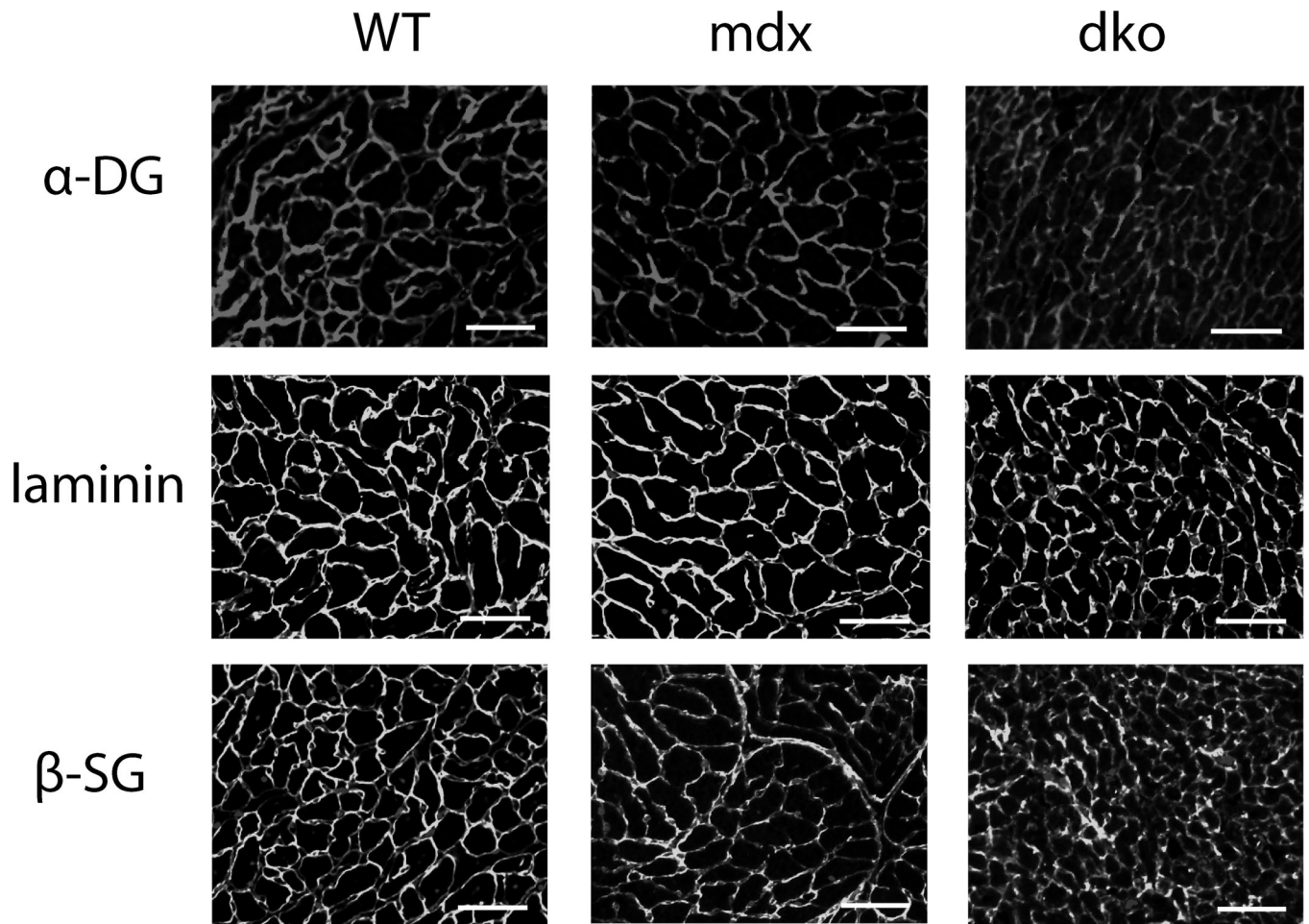
Figure 1. Immunohistochemistry of mouse cardiac tissue
Antibodies bound to α-DG, laminin and β-SG are observable in cardiac tissue of WT, *mdx* and dko mice. Line represents 100µm. *n* = 3
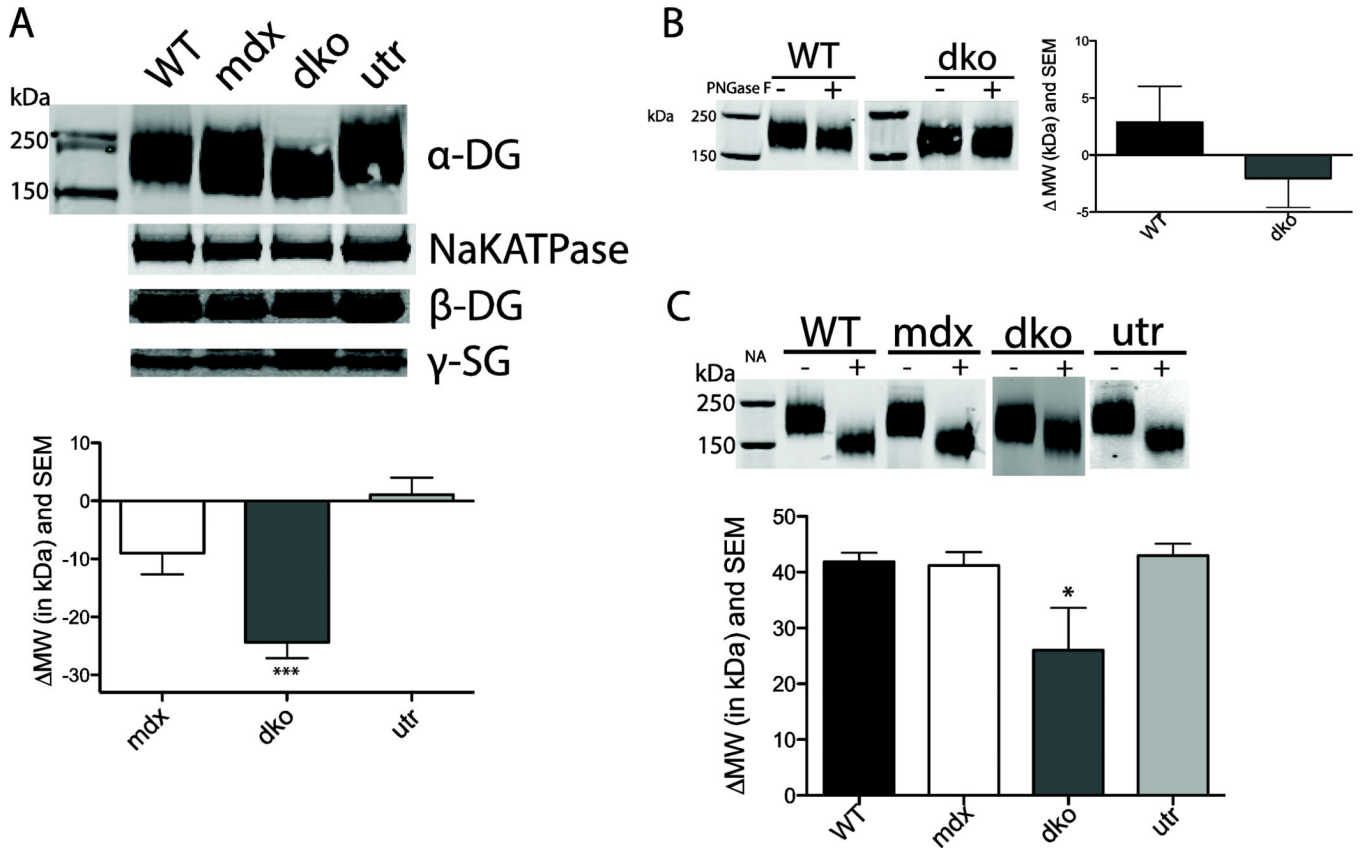
Figure 2. Western blots of mouse KCl washed cardiac microsomes and α-DG glycosylation
a) Representative western blot results of KCl washed cardiac microsomes probed with α-DG, sodium-potassium ATPase (NaKATPase), β-DG and γ-SG antibodies for WT, *mdx*, dko and utr mice. Differences in α-DG migration are quantified based on the average WT molecular weight (MW) value. KCl migration WT *n* = 12, *mdx n* = 14, dko *n* = 14, utr *n* = 12. *** p < 0.0001. b) N-glycosidase-F (PNGase F), an enzyme that removes most types of *N*-linked glycosylation, does not significantly shift MW for WT or dko α-DG compared to untreated control samples (n = 4–5 sample). c) Treatment with neuraminidase (NA), which removes sialic acid and some glucosamines, shifts dko α-DG gel mobility significantly less than WT (n=3–4). * p < 0.05
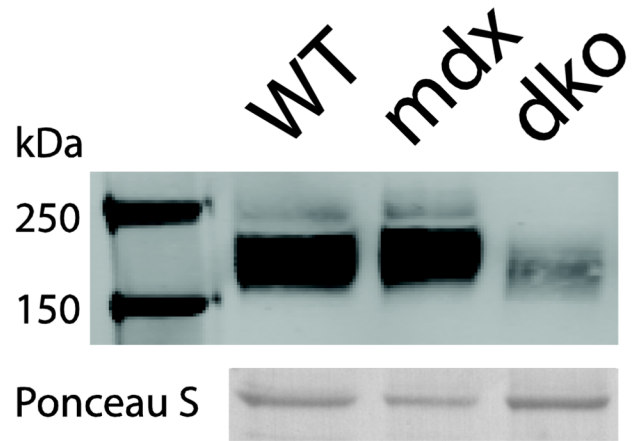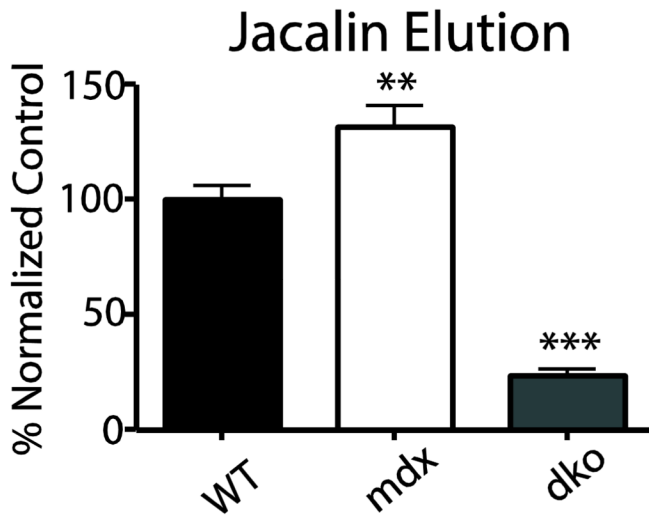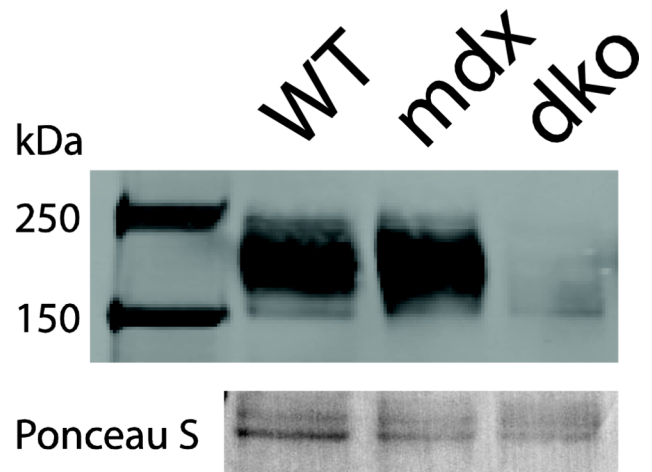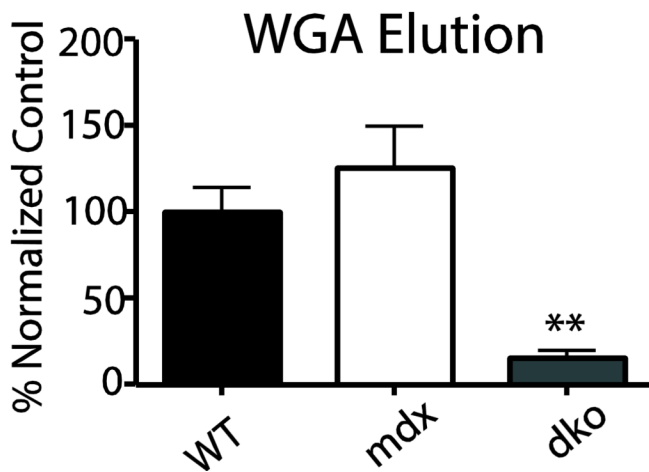
Figure 3. Lectin binding assays
Mouse cardiac homogenate proteins bound to jacalin- (a) or WGA- (b) conjugated agarose beads. Proteins were eluted, subjected to SDS-PAGE, and probed for α-DG on a western blot. α-DG expression is reported as a normalized percentage of WT α-DG signal +/− SEM. Significantly less dko α-DG was eluted from both WGA and jacalin lectins compared to WT, whereas *mdx* α-DG eluted more α-DG from the jacalin lectin compared to WT α-DG. Ponceau S stained band (~70 kDa) provided as a loading control. ($n = 4$–9) ** $p < 0.001$, *** $p < 0.0001$
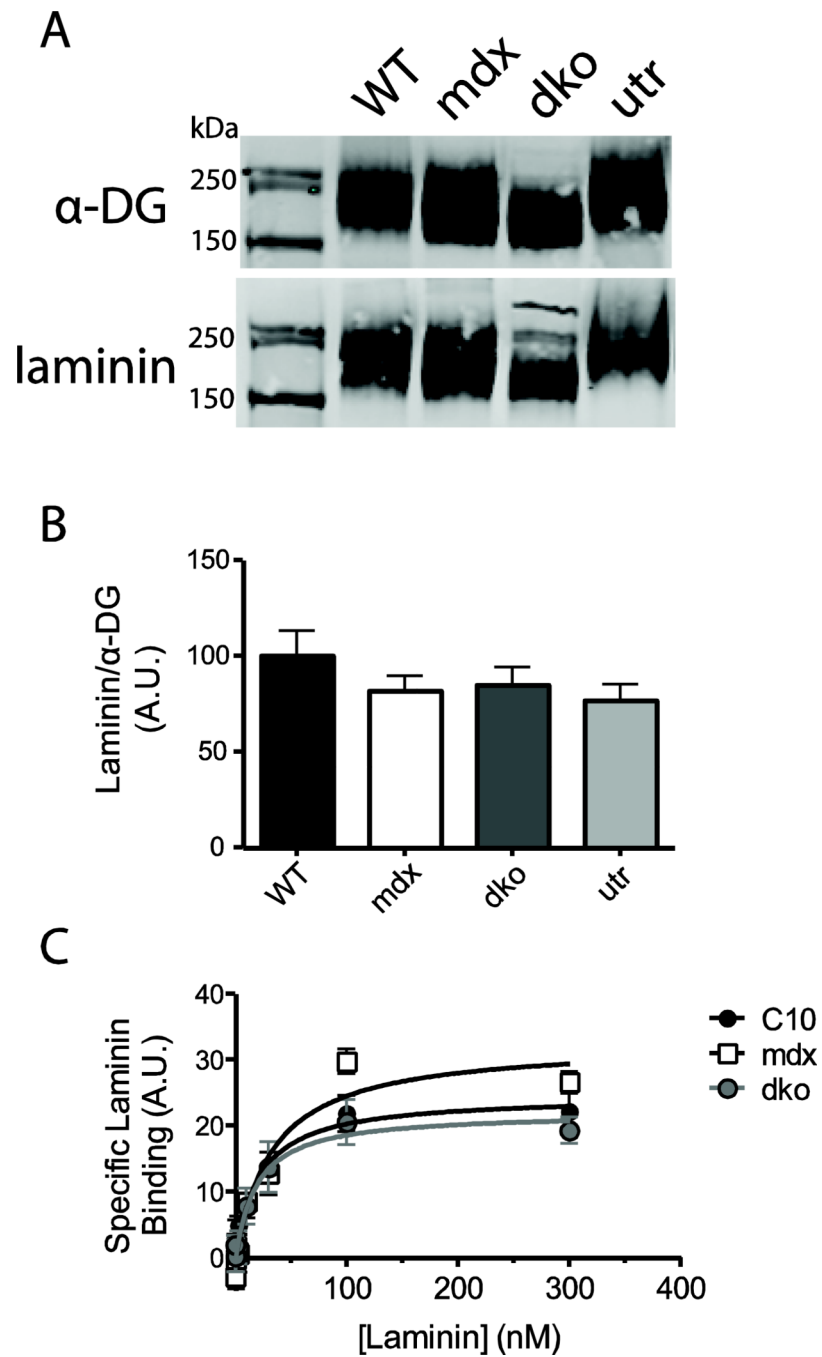
Figure 4. Laminin overlay binding assay
The laminin binding capacity of α-DG was assessed using a far-western technique (a), and results were quantified by calculating the raw laminin signal over the raw α-DG signal (b). A solid-state binding assay was used to determine the affinity the laminin binding sites in cardiac homogenates (c). No significant difference in laminin binding capacity or affinity was observed between WT, *mdx*, dko and utr α-DG. (n=7–8)

Figure 5. Line scanning of laminin and α-DG immunoreactivity

Immunofluorescent colocalization of laminin and α-DG was measured by confocal microscopy. Line scans of membrane interfaces expressing both laminin and α-DG were collected and analyzed. (a) Representative images demonstrating example line scans. (b) Representative normalized line scans from WT, *mdx*, and dko sections. The dashed line represents 50%, used to calculate the distribution of immunofluorescence. (c) Summary of the distribution of immunoreactivity for laminin and α-DG. n= 419–596 individual line scans. * P<0.0001 versus WT α-DG distribution, † P< 0.0001 versus WT laminin distribution