The synaptic CT carbohydrate modulates binding and expression of extracellular matrix proteins in skeletal muscle: partial dependence on utrophin

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Abstract

The CT carbohydrate, Neu5Ac/Neu5Gcα2,3[GalNAcβ1,4]Galβ1,4GlcNAcβ–, is specifically expressed at the neuromuscular junction in skeletal myofibers of adult vertebrates. When Galgt2, the glycosyltransferase that creates the synaptic β1,4GalNAc portion of this glycan, is overexpressed in extrasynaptic regions of the myofiber membrane, α-dystroglycan becomes glycosylated with the CT carbohydrate and this coincides with the ectopic expression of synaptic dystroglycan-binding proteins, including laminin α4, laminin α5, and utrophin. Here we show that both synaptic and extrasynaptic forms of laminin and agrin have increased binding to the CT carbohydrate compared to sialyl-N-acetyllactosamine, its extrasynaptically expressed precursor. Muscle laminins also show increased binding to CT-glycosylated muscle α-dystroglycan relative to its non-CT-containing glycoforms. Overexpression of Galgt2 in transgenic mouse skeletal muscle increased the mRNA expression of extracellular matrix (ECM) genes, including agrin and laminin α5, as well as utrophin, integrin α7, and neuregulin. Increased expression of ECM proteins in Galgt2 transgenic skeletal muscles was partially dependent on utrophin, but utrophin was not required for Galgt2-induced changes in muscle growth or neuromuscular development. These experiments demonstrate that overexpression of a synaptic carbohydrate can increase both ECM binding to α-dystroglycan and ECM expression in skeletal muscle, and they suggest a mechanism by which Galgt2 overexpression may inhibit muscular dystrophy and affect neuromuscular development.

Introduction

Many synaptic proteins are differentially modified by posttranslational modifications, including glycosylation, such that they take on unique functions or properties (Martin, 2002). At the vertebrate neuromuscular junction, there are multiple glycan structures that are restricted in expression to the synaptic region formed between the nerve terminal of the motor neuron and the postsynaptic membrane of the skeletal myofiber (Martin, 2003b). These include synaptic isoforms of heparan sulfate (Dennissen et al., 2002; Jenniskens et al., 2000), synaptic glycolipids (Scott et al., 1988), and synaptic glycans on glycoproteins (Martin et al., 1999). The CT carbohydrate (Lefrancois and Bevan, 1985a), Neu5Ac (or Neu5Gc)α2,3[GalNAcβ1,4] Galβ1,4GlcNAcβ– (Conzelmann and Lefrancois, 1988), is a member of the latter two categories; transgenic overexpression Galgt2, the enzyme that creates the synaptic...
β1,4GalNAc linkage on the CT carbohydrate (Smith and Lowe, 1994), in skeletal muscle increases glycosylation of α dystroglycan (Xia et al., 2002), an important extracellular matrix (ECM) binding protein (Ervasti and Campbell, 1991), and an as yet defined glycolipid (Xu et al., 2007a; Xu et al., 2007b), with the CT carbohydrate.

The terminal β1,4GalNAc linkage on the CT carbohydrate is what defines its synaptic distribution (Martin et al., 1999). All terminal βGalNAc linkages in vertebrate skeletal muscle are localized to the neuromuscular synapse by adulthood (Martin et al., 1999; Sanes and Cheney, 1982). The synaptic β1,4GalNAc linkage on the CT carbohydrate is made by the CT GalNAc transferase (or Galgt2). Galgt2, a type II Golgi UDP-GalNAc:Neu5Ac/Neu5Gcα2,3Galβ1,4-R β1,4-N-acetylgalactosaminyltransferase (Smith and Lowe, 1994), is also highly localized to synaptic regions in adult skeletal myofibers (Xia et al., 2002). Transgenic overexpression of Galgt2 in skeletal muscle leads to increased extrasynaptic expression of the CT carbohydrate in adult muscle, aberrant neuromuscular development (including changed axonal migration and synaptic topography), and inhibition of muscle growth (Xia et al., 2002). Transgenic overexpression of Galgt2 also leads to increased expression of extracellular matrix (ECM) proteins that, like the CT carbohydrate, are normally confined to the synapse (Xia et al., 2002). Many of these proteins, including laminin α4, laminin α5, and utrophin are proteins that bind to dystroglycan on its α or β chain (Chung and Campanelli, 1999; Ervasti and Campbell, 1993; Talts et al., 1999; Talts et al., 2000). α dystroglycan requires O-linked glycans, including Neu5Ac/Neu5Gcα2,3Galβ1,4GlcNAcβ1,2ManO-linked structures in its mucin domain, to bind ECM proteins (Ervasti and Campbell, 1993; Michele et al., 2002). As such, it is likely that modification of such structures (or related ones) by Galgt2 to create the CT carbohydrate would alter ECM binding or expression. Such changes have a clear clinical significance. Transgenic overexpression of Galgt2 in myofibers of animals with muscular dystrophy can inhibit the development of disease (Nguyen et al., 2002; Xu et al., 2007a; Xu et al., 2007b), much as altered expression of utrophin (Deconinck et al., 1997c; Rafael et al., 1998; Tinsley et al., 1998), agrin, or laminin-1 (Gawlik et al., 2004; Moll et al., 2001) can. In this paper, we have undertaken a study to understand whether the presence of β1,4GalNAc on glycans or on α dystroglycan alters ECM binding in ways that might explain the increased extrasynaptic expression of synaptic ECM proteins in Galgt2 transgenic skeletal muscle.

Results

Increased binding of agrins and laminins to CT-glycosylated α dystroglycan

The first question we asked was whether the presence of the CT carbohydrate on α dystroglycan altered the binding of extracellular matrix (ECM) proteins (Fig. 1A-F). We tested this in two ways. First, we measured solid-phase binding of purified recombinant ECM proteins, including the G1-G5 domains of laminin α2 (Fig. 1A), laminin α4 (Fig. 1B), laminin α5 (Fig. 1C), and the G2-G3 domains of the muscle splice form (C45z0, Fig. 1D) or the neural splice form (C45z8, Fig. 1E) of agrin, to CT-glycosylated or non-CT glycosylated α dystroglycan purified from skeletal muscle. While all of these proteins are expressed at the neuromuscular junction, laminin α2 and the muscle splice form of agrin (z0) also have extrasynaptic expression in the muscle basal lamina of adult animals, while the other proteins do not (Chiu and Sanes, 1984; Eusebio et al., 2003; Hoch et al., 1993; Patton et al., 1997). The recombinant fragments of laminin and agrin used here have been shown in previous studies to be sufficient for high affinity binding to native α dystroglycan (Campanelli et al., 1996; Gesemann et al., 1996; Shimizu et al., 1999; Talts et al., 1999; Talts et al., 2000).

We purified CT-glycosylated α dystroglycan using a βGalNAc-binding lectin (Wisteria floribunda agglutinin, WFA) from Galgt2 transgenic skeletal muscle and non-CT glycosylated (wild type, WT) α dystroglycan using a sialic acid/GlcNAc-binding lectin (Wheat germ

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agglutinin, WGA) from wild type skeletal muscle, much as before (Hoyte et al., 2002). Purified proteins were assessed for the presence of the CT carbohydrate by immunoblotting with CT2, a monoclonal antibody that recognizes the CT carbohydrate (Conzelmann and Lefrancois, 1988), for the presence of α dystroglycan by immunoblotting with IIH6, a monoclonal antibody that recognizes the natively glycosylated α dystroglycan (Ervasti and Campbell, 1993), and for relative purity by silver staining of SDS-PAGE gels (Supplemental Fig. 1). Muscle α dystroglycan was very difficult to see by silver staining due to its very high degree of glycosylation, but immunoblotting with IIH6 showed that the protein was present and in fact abundant. Recombinant laminins and agrins were analyzed by immunoblotting using an anti-FLAG antibody to verify the presence of the epitope-tagged proteins and for relative purity by silver staining (Supplemental Fig. 1).

We immobilized CT-glycosylated α dystroglycan (CT) and non-CT glycosylated α dystroglycan (WT) on ELISA plates. Immunoblotting using IIH6, which recognizes both proteins equally well (Hoyte et al., 2002; Nguyen et al., 2002; Xia et al., 2002), verified equal protein coating, while binding of CT2 showed a greatly increased amount of CT carbohydrate in the CT fraction relative to the WT fraction (Supplemental Figs. 1 and 2). ECM proteins were added at varying concentrations until saturation binding was observed. Each experiment compared binding of a single ECM ligand to CT and WT α dystroglycan. The maximal binding within each experiment was identified and given a value of 1, with all data then normalized to this value, as previously described (Brinkman-Van der Linden and Varki, 2000), to determine relative binding. Repetitions of each normalized experiment were averaged to determine overall binding differences. In each experiment (Figs. 1A-E), the highest value was always that of the ECM ligand at its highest concentration binding to the CTDG fraction.

In all cases, the presence of the CT carbohydrate on α dystroglycan increased the maximal amount of ECM binding (Figs. 1A-1E). Binding of laminin α2 to the CT glycoform was increased by 42±3% relative to WT at 30nM, the highest concentration used (P=0.002). Laminin α4 showed a 100±11% increase in binding to CT-α dystroglycan compared to WT at 300nM, also the highest concentration used (P<0.001), (Fig. 1B). The lowered apparent binding affinity for laminin α4 compared to laminin α2 is similar, at least in its direction, to previous binding studies (Talts et al., 1999; Talts et al., 2000), however, we observed binding of LNα4 at lower concentrations than previously reported. This may be due to our use of a larger (G1-G5) recombinant laminin α4 protein than was used in prior studies. Binding of laminin α5 to CT-α dystroglycan was increased by 80±5% compared to WT at 30nM (P=0.003, Fig. 1C), while muscle agrin was increased by 33±6% (P=0.003 at 30nM, Fig. 1D) and neural agrin by 60±2% (P=0.003 at 50nM, Fig. 1E). In addition, the extent of increased binding (CT vs. WT) at the highest concentration for each experiment was significantly greater for synaptic laminin (α4) or agrin (neural, C45z8) than for extrasynaptic laminin (α2) or agrin (muscle, C45z0) (P<0.05 for both comparisons). Careful consideration was given to insuring that both CT and WT glycoforms of α dystroglycan were present in equal amounts within each experiment (Supplemental Fig. 2). All binding could be blocked by the addition of IIH6, an antibody that blocks laminin binding to α dystroglycan (Ervasti and Campbell, 1993), and all reactions were calcium dependent, as previously described (not shown) (Campanelli et al., 1996; Ervasti and Campbell, 1993; Gesemann et al., 1996; Talts et al., 1999; Talts et al., 2000).

In the second experiment, we assessed the binding of recombinant CT-glycosylated and non-CT-glycosylated α dystroglycan to native laminin-111, a protein expressed in embryonic skeletal muscle (Chiu and Sanes, 1984; Engvall et al., 1990; Sanes et al., 1990). Laminin-111, a trimeric protein composed of a β1, α1, and γ1 laminin chain, was isolated from Engelbreth-Holm-Swarm (EHS) tumor. To purify α dystroglycan for this experiment, we produced a FLAG-tagged cDNA for dystroglycan that encodes a stop codon after the last amino acid of α dystroglycan coding sequence so that the β chain was not expressed. Because α dystroglycan
is cleaved by furin at amino acid R312 (Singh et al., 2004), we deleted this cleavage site (αDGdelR312) to facilitate production of a full-length secreted α dystroglycan protein. We transfected this cDNA into HEK293 cells, which do not express appreciable levels of the CT carbohydrate (Parkhomovskiy et al., 2000), and into HEK293CT cells, which stably overexpress Galgt2 and the CT carbohydrate (Parkhomovskiy et al., 2000). Resultant purified proteins showed very different amounts of CT glycosylation, but equivalent relative purity by silver staining (Supplemental Fig. 1). Unlike skeletal muscle α dystroglycan, which is very difficult to identify on silver stained gels due to its high degree of glycosylation and cleavage of the N-terminal third of the polypeptide by furin, these recombinant α dystroglycan proteins, in which the N-terminal third of the protein was retained, were more easily stained (Supplemental Fig. 1). Using anti-peptide antibodies to α dystroglycan, we confirmed that full-length αDGdelR312 could be purified from transfected HEK293 cell supernatant (Supplemental Fig. 3A) and that this protein was a substrate for CT glycosylation by purified recombinant Galgt2 (Supplemental Fig. 3B). Using CHO cells stably overexpressing LARGE (CHO-LARGE), we also showed that Galgt2 overexpression could similarly increase CT glycosylation on more highly glycosylated forms of α dystroglycan (Supplemental Fig. 3C).

Having characterized the recombinant proteins, we measured binding of CT-glycosylated recombinant α dystroglycan and non-CT glycosylated recombinant α dystroglycan to native laminin-111 (Fig. 1F). CT-glycosylated α dystroglycan showed an 80±3% increase in binding (P<0.01) compared to non-CT α dystroglycan (WT) at the highest concentration used (30nM). Thus, changed binding of CT-glycosylated recombinant α dystroglycan to laminin-111 (Fig. 1F) was much as had been found for changed binding of recombinant laminins to native CT-glycosylated muscle α dystroglycan (Figs. 1A-1C).

Increased binding of the CT carbohydrate to laminins and agrin

The next question we wished to address was whether the CT carbohydrate, when devoid of a protein or lipid carrier, would be sufficient to bind laminins and agrins. Such results would show that these ECM proteins are in fact mammalian lectins. Endo and colleagues had previously demonstrated that exogenously added monovalent sialyl-N-acetyllactosamine (3′SNL(Neu5Ac)), which is present on the O-linked mannose chains of α dystroglycan (Chiba et al., 1997; Sasaki et al., 1998; Smalheiser et al., 1998), blocks laminin binding to α dystroglycan (Chiba et al., 1997). This occurs, however, only when millimolar concentrations of the saccharide are added. This is a concentration 5 orders of magnitude outside the binding range of laminins to native skeletal muscle α dystroglycan (Talts et al., 1999; Talts et al., 2000).

Because O-linked glycans on α dystroglycan are present in multiple copies in a mucin-like domain in the middle third of the protein (Ibraghimov-Beskrovnaya et al., 1992), we chose to study binding of glycans presented in a multivalent form in the hope that this might more closely mimic their presence on the native glycoprotein. To do this, we used glycans that were multivalently conjugated to polycrylamide (PAA) and also contained biotin. All glycans were conjugated to PAA in a 6:1 glycan to PAA ratio on 30kDa particles. We used glycans configured in this way to compare the binding of sialyl-N-acetyllactosamine, Neu5Acα2,3Galβ1,4GlcNAc-PAA-biotin (3′SNL(Neu5Ac)), a glycan present on α dystroglycan and previously shown to modulate ECM binding (Chiba et al., 1997), N-acetyllactosamine, Galβ1,4GlcNAc-PAA-biotin (NL), its non-sialylated precursor, and the CT carbohydrate, Neu5Acα2,3GalNAcβ1,4Galβ1,4GlcNAc-PAA-biotin (CT(Neu5Ac)), which differs from 3′SNL(Neu5Ac) only by the additional presence of the synaptic β1,4GalNAc linkage.

PAA-glycans were added in a 100μL volume in a range between 0.25μg/well and 3μg/well (yielding concentrations of 42nM to 500nM, respectively) to purified recombinant forms of the G1-G5 domains of laminin α2 (Fig. 2A), laminin α4 (Fig. 2B), or laminin α5 (Fig. 2C) or
the G2-G3 domains of the muscle-specific splice form (C45z0, Fig. 2D) or the neural-specific splice form (C45z8, Fig. 2E) of agrin immobilized on ELISA plates. Glycan concentrations were determined assuming that the 6 moles of glycan per mole PAA reflected a single mole of polymeric PAA-glycan. Immobilized FLAG-tagged ECM proteins were purified based on their epitope tag and assessed by immunoblotting, for expression, and silver staining, for relative purity (Supplemental Fig. 1). We verified addition of equivalent amounts of each glycan by immobilizing them on ELISA plates and probing with streptavidin-HRP, which binds to biotin present on the PAA-glycans (Supplemental Fig. 2). All three glycans were compared for binding to all five recombinant ECM proteins in each experiment. Data in each experiment was normalized to 1, with 1 being the signal for the maximal binding within the experiment, as done previously (Brinkman-Van der Linden and Varki, 2000). This value was always the signal for CT(Neu5Ac) binding to neural agrin at 500nM. After normalization, repeated experiments were averaged to obtain each binding curves. PAA-glycan binding in the absence of the ECM protein was not significant (less than 10% of signal with protein in all cases).

3'SNL(Neu5Ac) showed binding at above 250nM (2μg/well) to all three laminin proteins, and this was elevated relative to NL in some instances, however, CT(Neu5Ac) binding was significantly higher than 3'SNL(Neu5Ac) and NL in every instance (Figs. 2A-2C) (P<0.01 for all at 3μg/well, or 500nM). Half-maximal binding of CT(Neu5Ac) to laminin α4 and laminin α5 occurred at 69nM and 57nM, respectively, based on curve fits where the correlation coefficient was 0.99 (for each). These values are below those for half-maximal binding of laminins to native α dystroglycan or CT-α dystroglycan (which ranged from 10-20nM), but they are within one log of most of these values. Binding of CT(Neu5Ac) to laminin α4 and laminin α5 approached saturation at 500nM (3μg/well, Figs. 2B and 2C). By contrast, binding of CT (Neu5Ac) to laminin α2 did not (Fig. 2A).

Results for muscle agrin and neural agrin (Ferns et al., 1992; Hoch et al., 1993) were similar (Figs. 2D and 2E). CT(Neu5Ac) showed increased binding compared to 3'SNL(Neu5Ac) and NL to both agrin proteins (P<0.01 for both at 3μg/well). Binding of CT(Neu5Ac) to neural agrin approached saturation at 3μg/well (500nM), while the binding curve for muscle agrin did not. Sufficient amounts of PAA-glycans to perform saturation curves beyond these concentrations were not available. Addition of a 10-fold excess of monovalent saccharides (Neu5Ac, GalNAc, Gal, GlcNAc, 3'SNL(Neu5Ac) did not block PAA-glycan binding, supporting the importance of glycan multivalency for binding. Further, Galα1,3Galβ1,4GlcNAc–PAA–biotin did not bind appreciably to any of these recombinant ECM proteins when added at 500nM, supporting the importance of the GalNAc1β1,4 linkage for CT carbohydrate binding (not shown).

Galgt2 transgenic muscles have increased expression of synaptic genes

While we had shown that the CT carbohydrate could alter binding to ECM proteins, the extrasynaptic expression of these proteins in Galgt2 transgenic myofibers likely would also require increased gene expression. We therefore measured the mRNA expression of genes that had shown altered protein expression in Galgt2 transgenic muscles by Taq-Man semi-quantitative RT-PCR and compared levels of expression to those in wild type (non-transgenic) littermates. A number of genes, including agrin, laminin α2, laminin α5, collagen IV(α1), collagen IV(α2), dystroglycan, β-δ sarcoglycan, integrin α7, integrin β1, dystrophin and utrophin, showed increased expression in Galgt2 transgenic muscle (Fig. 3A), while two genes, laminin α4 and α sarcoglycan, did not (Fig. 3A).

As neuregulin can alter the expression of some of these genes (Schaeffer et al., 2001), we next determined if Galgt2 increased neuregulin mRNA expression (Fig. 3B). Indeed, neuregulin mRNA expression was increased 6.2-fold, as was the epsilon chain of the acetylcholine
receptor (AChRζ, 4-fold), a neuregulin-regulated gene (Burden, 2002) (Fig. 3B). Ncam1, a gene encoding another protein that is overexpressed in Galgt2 transgenic muscle (Xia et al., 2002), also had increased mRNA levels, while Cdk2, a control, did not (Fig. 3B). Thus, increased expression of proteins in Galgt2 transgenic muscles correlated with increased mRNA expression in most, but not all, cases. Moreover, increased neuregulin expression correlated with the increased expression of neuregulin-regulated genes (Figs. 3A and 3B).

**Utrophin and dystrophin are present in protein complexes containing CT-glycosylated dystroglycan**

Since utrophin is highly overexpressed in Galgt2 transgenic muscles (Nguyen et al., 2002; Xia et al., 2002), and since utrophin overexpression, like Galgt2, can inhibit the development of muscular dystrophy in dystrophin-deficient mice (Deconinck et al., 1997b; Deconinck et al., 1997c; Nguyen et al., 2002; Rafael et al., 1998; Tinsley et al., 1998; Xu et al., 2007a), we were particularly interested in understanding the potential role of utrophin in Galgt2’s molecular and biological effects. To begin to address this issue, we first determined whether dystrophin and utrophin, two intracellular proteins known to bind to the cytoplasmic face of β dystroglycan (Chung and Campanelli, 1999), would associate with the CT glycoform of the α/β dystroglycan complex (Fig. 4A). We used WGA and WFA lectin precipitation of non-ionic detergent skeletal muscle lysates, as we had done previously (Jayasinha et al., 2003; Nguyen et al., 2002; Xia et al., 2002), to precipitate the CT-glycoform and the non-CT glycoform of α dystroglycan (Fig. 4A). WFA and WGA precipitates contained the appropriate glycoforms of α dystroglycan, and also β dystroglycan, α, β, and, γ sarcoglycan and dystrophin. WFA and WGA precipitates also contained a 40kDa protein fragment of utrophin that could be recognized by an antibody to the C-terminal region of the utrophin (Fig. 4A), but neither contained native full-length protein (400kDa). WFA also precipitated a 52kDa C-terminal fragment of utrophin that was not present in the WGA fraction. Addition of exogenous GalNAc blocked precipitation of all proteins by WFA, demonstrating carbohydrate-binding specificity of the lectin. Unlike PAA-glycan binding to ECM, binding by these plant lectins can be blocked by excess monosaccharide. Thus, dystrophin and utrophin are both present in protein complexes with CT-glycosylated α/β dystroglycan, and this suggests that they both can associate with CT-glycosylated α/β dystroglycan in a protein complex.

**Creation of utrophin-deficient Galgt2 transgenic mice**

Since utrophin could associate with CT-glycosylated dystroglycan (Fig. 4A) and because utrophin, along with CT-α dystroglycan, is highly overexpressed along regions of the extrasynaptic membrane in Galgt2 transgenic mice (Nguyen et al., 2002; Xia et al., 2002), we next tested whether ectopic expression of other ECM proteins in these animals would require utrophin. Such a result would suggest the importance of a utrophin-glycoprotein complex for this aspect of CT-dystroglycan function. To do this, we made utrophin-deficient Galgt2 transgenic mice. The level of Galgt2 protein overexpression, assessed by immunoblotting, was the same in Uttrn+/- and Uttrn-/- backgrounds (Fig. 4B). CT2 staining in non-transgenic Uttrn +/- and Uttrn-/- muscle was confined to capillaries, blood vessels, and neuromuscular junctions, as seen previously (Martin et al., 1999), and was upregulated along extrasynaptic regions of the sarcolemmal membrane in both Galgt2 transgenic Uttrn+/- and Uttrn-/-muscles (Fig. 4C). Utrophin protein levels were dramatically increased in Galgt2 transgenic Uttrn+/- muscle (compared to non-transgenic Uttrn+/-), much previously described (Deconinck et al., 1997a; Grady et al., 1997; Nguyen et al., 2002; Xia et al., 2002), and utrophin protein was not detectable in the Uttrn-/-background (Figs. 4B and C). We also assessed CT-glycosylation of α dystroglycan in Galgt2 transgenic Uttrn+/- and Uttrn-/- muscles using WFA and WGA precipitation (Fig. 4D). There was no difference in the amount of α dystroglycan precipitated by WFA in Galgt2 transgenic Uttrn+/- and Uttrn-/- muscles or in the amount of CT carbohydrate on α dystroglycan. Thus, utrophin is not required for α dystroglycan glycosylation by Galgt2.

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Partial requirement of utrophin for increased ECM protein expression in Galgt2 transgenic muscle

Galgt2 overexpression in skeletal muscle increases both the overall expression and the extrasynaptic distribution of synaptic ECM proteins, including laminin α4 and laminin α5 (Xia et al., 2002). In Galgt2Utrn+/+ muscles, laminin α4 and laminin α5 protein levels were increased by 167% and 56%, respectively (compared to Utrn+/-, Fig. 5A). Galgt2-dependent increases in laminin α4 and laminin α5 proteins in Utrn-/- muscles (vs. Utrn+/-), by contrast, were reduced by 60% and 58%, respectively (compared to changes in Galgt2 transgenic Utrn+/- vs. Utrn+/-, Fig. 5A). This result was confirmed by immunostaining (Fig. 5B). Laminin α4 and laminin α5 expression, like the CT carbohydrate (Martin et al., 1999), is normally confined capillaries, blood vessels, and neuromuscular junctions in non-transgenic muscles (Patton et al., 1997), and this was the case in Utrn+/- and Utrn-/- muscle (Fig. 5B). Both proteins showed increased expression in the extrasynaptic basal lamina in Galgt2Utrn+/+ muscle, and this increased expression was reduced in Galgt2Utrn-/- muscle (Fig. 5B). Expression of laminin α2, which is normally found in the extrasynaptic ECM (Chiu and Sanes, 1984), was unchanged in all genotypes (Fig. 5B), as were the expression of other muscle proteins, including dystrophin, α, β, and γ sarcoglycan, α and β dystroglycan, and dystrobrevin (data not shown). These data show that increased expression of laminin α4 and laminin α5 in Galgt2 transgenic muscle is partially dependent on utrophin.

As neuregulin and agrin mRNA expression were increased in Galgt2 transgenic muscle (Fig. 3), we also assessed their protein expression (Fig. 5A). Agrin and a 53kDa neuregulin protein band were increased in Galgt2Utrn+/+ muscle (relative to Utrn+/-), and increased expression of both of these proteins was eliminated in Galgt2Utrn-/muscles (relative to Utrn-/-). Like synaptic laminins, agrin staining showed increased extrasynaptic distribution on Galgt2Utrn+/+ myofibers, and this was reduced in the absence of utrophin (Fig. 5B). Using an antibody to the C-terminal intracellular domain of neuregulin, we identified three other neuregulin protein bands in muscle extracts of all four genotypes (Fig. 5A). Consistent with the presence of neuregulin forms on immunoblots, neuregulin staining was present in all muscles, with staining enriched at the neuromuscular junction (Fig. 5B). These data show that utrophin is required for Galgt2-dependent overexpression of agrin and one, but not all, protein species of neuregulin in skeletal muscle.

Developmental changes in Galgt2 transgenic muscles do not require utrophin

We next determined whether utrophin would be required for Galgt2-induced changes in muscle growth and neuromuscular development (Figs. 6 and 7). Myofibers from Galgt2 transgenic Utrn+/+ and Utrn-/- mice were both severely reduced in diameter when compared to non-transgenic Utrn+/+ or Utrn-/- littermates, respectively, consistent with our previous findings in Galgt2Utrn+/+ animals (Xia et al., 2002). The reduction in myofiber diameter ranged between 20 and 50%, depending on the muscle, but was consistently reduced in the gastrocnemius, tibialis anterior, gluteus maximus, quadriceps, triceps, and trapezius muscles (Figs. 6A and 6B). The level of reduction in transgenic muscles did not differ, on average, between Utrn+/+ and Utrn-/- animals (P>0.05 for all). Galgt2Utrn+/+ and Galgt2Utrn-/- muscles showed no significant difference in the number of centrally located nuclei per muscle section (Fig. 6C) or in the number of myofibers per muscle group (data not shown), which was again consistent with our previous findings in Galgt2Utrn+/+ mice (Xia et al., 2002).

Galgt2 transgenic muscles have aberrant neuromuscular structure as well as altered neuromuscular topography (Xia et al., 2002). This can be shown by staining skeletal muscles with rhodamine-α-bungarotoxin, a high affinity probe for nicotinic acetylcholine receptors, which are highly concentrated in the postsynaptic membrane (Sanes and Lichtman, 2001). As expected, both Galgt2Utrn+/+ and Galgt2Utrn-/- muscles showed a discontinuous pattern of
α bungarotoxin staining (Fig. 7A), much as previously seen in Galgt2Utrn+/+ muscles (Xia et al., 2002). This was true in 57±4% of Galgt2Utrn+/+ myofibers and 40±6% of Galgt2Utrn-- myofibers. By contrast, only 1±1% of non-transgenic Utrn+/+ neuromuscular junctions showed this discontinuous staining pattern, while 18±7% of Utrn-- muscles did. The increase in Utrn-- animals was likely due to the previously reported dearth in secondary folds in their neuromuscular junctions (Deconinck et al., 1997a; Grady et al., 1997).

To analyze synaptic topography, we stained whole mounts of diaphragm muscle with rhodamine-α-bungarotoxin to determine the location of individual synapses along myofibers (Fig 7A). Galgt2 expression expands the endplate band by about 300% relative to wild type mice (Xia et al., 2002), and this was also true for Galgt2Utrn+/+ mice relative to Utrn+/+ mice and for Galgt2Utrn-- mice relative to Utrn-- (Fig. 7B). Utrn-- mice, however, had a 40% reduction in the size of the endplate band, on average, relative Utrn+/+ animals, and this was also true of Galgt2Utrn-- mice relative to Galgt2Utrn+/+ animals. Therefore, loss of utrophin narrowed the distribution of neuromuscular synapses along myofibers, while Galgt2 expanded the area of innervation in both Utrn+/+ and Utrn-- muscles to similar degree.

**Discussion**

We have provided four major findings in this study. First, and most importantly, glycosylation of α dystroglycan with the CT carbohydrate, a glycan normally concentrated at the neuromuscular synapse in skeletal muscle (Martin et al., 1999), increases the binding of extracellular matrix (ECM) proteins. Laminin and agrin proteins normally expressed in adult or embryonic skeletal muscle or at the neuromuscular junction, including laminin α2, laminin α4, laminin α5, neural (z8) agrin, muscle (z0) agrin, and laminin-111 (Chiu and Sanes, 1984; Engvall et al., 1990; Hoch et al., 1993; Patton et al., 1997), have increased binding to the CT-glycoform of muscle α dystroglycan when compared to non-CT glycoforms. Moreover, synaptically expressed laminin (α4) showed a greater maximal increase in binding to CT-α dystroglycan than its extrasynaptic counterpart (laminin α2). As such, creation of the CT carbohydrate by Galgt2 at the neuromuscular synapse may help dictate adhesion of the synaptic basal lamina to the postsynaptic muscle membrane. When Galgt2 is overexpressed in extrasynaptic regions of myofibers, as it is in Galgt2 transgenic muscles (Nguyen et al., 2002; Xia et al., 2002), increased expression of the CT-glycoform of α dystroglycan may increase overall adhesion to the ECM to the sarcolemmal membrane. The ability of Galgt2 to inhibit muscular dystrophy, which it does when overexpressed in mdx or in dyW/dyW mouse muscles (Xu et al., 2007a; Xu et al., 2007b), may be related in part to such changes. As all of the muscle ECM proteins we have studied are present in the basal lamina in multimeric protein complexes (Yurchenco et al., 2004; Yurchenco and O'Rear, 1994), altered binding capacity may be important in dictating the strength of ECM-membrane interactions, a finding consistent with our recent studies showing Galgt2 overexpression prevents muscle damage in mdx muscles (Martin et al., 2009). As laminin binding to α dystroglycan can also stimulate its polymerization into an ECM (Henry and Campbell, 1998), increased binding could alter ECM protein expression by this mechanism as well.

Our second result is that the CT carbohydrate has significant binding affinity for muscle ECM proteins even when it is not conjugated to a glycoprotein. Moreover, the CT carbohydrate shows higher binding to recombinant laminins and agrins than does sialyl-N-acetyllactosamine, the same glycan devoid of the synaptic β1,4GalNAc linkage. This result was much as we also observed for ECM binding to glycoforms of muscle α dystroglycan. These studies strongly suggest that laminins and agrins are in fact carbohydrate-binding lectins and that changes in ECM binding to the CT glycoform of α dystroglycan are mediated, at least in part, by direct ligand-glycan binding.
It has been known for some time that α dystroglycan requires proper O-linked glycosylation in order for laminin, agrin, and other ECM proteins to bind (Ervasti and Campbell, 1993; Michele and Campbell, 2003), but whether the glycans present in the mucin domain are directly involved in binding, or rather alter binding to the polypeptide by inducing changes in the protein's tertiary structure, has remained an open question. While work of Endo and colleagues showed sialyl-N-acetyllactosamine can block laminin binding to α dystroglycan, the amounts needed to see an effect were far greater than the affinity of these proteins for the native glycoprotein (Chiba et al., 1997). Our data suggest that this may be an issue of valency. Multivalent PAA-glycans, including sialyl-N-acetyllactosamine, bind to ECM proteins at concentrations where their monovalent forms do not. While this binding does not equal the affinity of the ECM for native α dystroglycan (Campanelli et al., 1996; Gesemann et al., 1998; Gesemann et al., 1996; Talts et al., 1999; Talts et al., 2000), such an equivalency of glycan and glycoprotein likely could be achieved if the glycans were conjugated in a way that better approximated the mucin domain of the native glycoprotein. There, they could be present at a valency greater than 6:1, the ratio we have used here or show a different spacing along the polypeptide (Ibraghimov-Beskrovnaya et al., 1992; Martin, 2003a). The glycans used in this study also do not contain O-linked mannose, the first saccharide present on α dystroglycan that is subsequently modified with sialyl-N-acetyllactosamine (Chiba et al., 1997; Sasaki et al., 1998; Smallheiser and Kim, 1995). The inclusion of O-mannose in PAA-linked glycans could also increase binding, as could the presence of other glycan structures not studied here.

While our data point to a primary role for the CT carbohydrate in modulating ECM adhesion to α dystroglycan, other mechanisms that we have not investigated may also account for such changes. For example, CT-dystroglycan may have altered glycan density or packing that in turn alters ECM affinity as a secondary consequence of Galgt2 glycosylation. It is also possible that while the extent of glycosylation appears similar in both dystroglycan glycoforms, this may result from an averaging of glycans on each protein that are in fact of different chain lengths, with some shorter and some longer. While our unpublished studies suggest that the CT glycan is present on O-linked chains, in fact we do not yet understand the stoichiometry of this type of glycosylation on the α dystroglycan protein or where exactly it is located.

Our third result is that overexpression of Galgt2 increases the expression of mRNAs for important muscle genes. Galgt2 induced the mRNA expression of laminin α2, laminin α5, agrin, collagen IV (α1, and α2), integrin α7, dystrophin, utrophin, and β, γ, and δ sarcoglycan. Thus, some Galgt2-dependent changes in skeletal muscle protein expression may result from increased gene expression. Galgt2 also induces expression of neuregulin, a gene that induces its own expression as well as the expression of utrophin and the epsilon chain of the acetylcholine receptor (Achrε) (Burden, 1993; Martin, 2003c; Schaeffer et al., 2001), two genes increased in Galgt2 transgenic muscle. Thus, neuregulin could have a central role in coordinating Galgt2-dependent changes in gene and protein expression as well as effects on muscle biology. With regard to protein expression, it is of interest to note that Galgt2 transgenic muscles deficient in utrophin not only have reduced expression of synaptic laminins but also in one of four forms of neuregulin. Because we do not know exactly how the four neuregulin protein forms identified relate to neuregulin's biological activities, it could be that the remaining forms, at least one of which shows increased expression even in Galgt2Utrn-/- muscle, account for residual increase in ECM expression in Galgt2Utrn-/- muscles or, alternatively, that neuregulin is not involved in Galgt2-dependent ECM changes. Some proteins that are overexpressed in Galgt2 transgenic skeletal muscle, for example laminin α4 and α sarcoglycan, do not have altered mRNA levels. Therefore, not all ECM changes can be accounted for by altered transcription, regardless of neuregulin's possible contributions. With regard to function, neuregulin overexpression can ameliorate muscular dystrophy (Krag et al., 2004), something Galgt2 can also do (Nguyen et al., 2002). Further studies will be required to determine whether these effects are interrelated.
Our fourth result is that utrophin is partially required for Galgt2-dependent increases in ECM protein expression. In skeletal muscle, there are two distinct dystrophin-associated glycoprotein complexes—one in the extrasynaptic membrane and also at the neuromuscular junction, going from laminin $\alpha 2$ (and muscle agrin) in the ECM via dystroglycan in the membrane to dystrophin in the cytoplasm, and another exclusively at the neuromuscular junction, likely going from laminin $\alpha 4$ and $\alpha 5$ (and neural agrin) in the ECM via a CT-glycosylated dystroglycan in the membrane to utrophin in the cytoplasm (Martin, 2003a).

Because Galgt2 overexpression leads to the ectopic expression of laminin $\alpha 4$, laminin $\alpha 5$, agrin, and utrophin in the extrasynaptic membrane, it stands to reason that Galgt2 overexpression leads to the extrasynaptic expression of a functional utrophin glycoprotein complex. Our studies support this notion. We show utrophin is present in CT-dystroglycan complexes and that it is partially required for Galgt2-dependent overexpression of laminin $\alpha 4$, laminin $\alpha 5$, and agrin. Thus, utrophin provides a component of Galgt2's molecular effects on ECM expression. Presumably, as both Galgt2 and utrophin are localized, along with synaptic laminins, normally at the neuromuscular junction, the ectopic expression of utrophin along with Galgt2 reflects the unique nature of this normally synaptic complex. We have begun to elucidate the unique nature of this complex by showing changed binding of CT-glycosylated $\alpha$ dystroglycan to ECM proteins, but we cannot yet explain how this might be translated through the muscle membrane preferentially to utrophin and not, for example, to dystrophin. Because $\alpha$ dystroglycan also interacts extracellularly with $\beta$ dystroglycan to mediate ECM-directed intracellular binding interactions, we presume this may involve the differential modification of the cytoplasmic domain of $\beta$ dystroglycan, perhaps by tyrosine phosphorylation, which is known to occur there (Sotgia et al., 2001) and also on syntrophins (Zhou et al., 2006), or via other altered protein-protein associations or conformation changes.

Because utrophin interacts with $\beta$ dystroglycan, a transmembrane protein that in turn interacts with $\alpha$ dystroglycan on the extracellular face of sarcolemmal membrane, it is also possible that such altered intracellular interactions could modulate extracellular carbohydrate-ECM interactions by a mechanism akin to inside-out signaling found with integrins (Hynes, 2002). There, ECM binding to the integrin extracellular domain requires proper association of the integrin's cytoplasmic domain with cytoskeletal-associated proteins (Arnaout, 2002; Arnaout et al., 2007; Kinashi, 2005). Of course, ECM-carbohydrate interactions could similarly have reciprocal effects on $\beta$ dystroglycan binding to utrophin or dystrophin. There is little to no data as yet that suggests dystroglycan behaves in this way (Chung and Campanelli, 1999; Huang et al., 2000; Isley et al., 2001; Ishikawa-Sakurai et al., 2004), however, such studies have yet to take synaptic glycosylation into account. The increased gene expression of integrin $\alpha 7$ shown here also could contribute to Galgt2's therapeutic effects on muscular dystrophy, as overexpression of this adhesion molecule can ameliorate aspects of dystrophy in some mouse models (Burkin et al., 2005; Burkin et al., 2001).

Clearly utrophin is not required for the biological effects of Galgt2 we have studied here. Galgt2 transgenic muscles lacking utrophin still showed reduced muscle growth and aberrant neuromuscular structure. As such, it would also appear that agrin and synaptic laminins are not involved in these phenotypes, as their levels were reduced in Galgt2Utrn-/- muscle. We recently showed that Galgt2-dependent changes in muscle growth involve increased expression of myostatin, decreased expression of the myostatin inhibitor follistatin, and increased myostatin signaling (Chandraskeharan and Martin, 2009). Thus, it is not surprising that muscle growth is not affected by the absence of utrophin, which does not appear to play a role in the myostatin pathway. The effects on neuromuscular structure are less clear. Absence of utrophin does have effects on synaptic ultrastructure (Deconinck et al., 1997a; Grady et al., 1997), and we show here that absence of utrophin also alters synaptic topography. The effect of Galgt2 on these same parameters, however, appears to be independent of utrophin.
Experimental methods

Materials

Monoclonal antibodies to α sarcoglycan (Ad1/20A6), β sarcoglycan (βSarc1/5B1), γ sarcoglycan (35DAG2/B5), β dystroglycan, (43DAG1/8D5), utrophin (DRP3/20C5), and dystrophin (Dy4/6D3) were obtained from Nova Castra (Newcastle Upon Tyne, UK). An additional polyclonal antiserum, DG67, was made against the C-terminal 15 amino acids of β dystroglycan in our laboratory and purified against an immunogenic peptide-coupled resin (Xia et al., 2002). Antibody to laminin α2 was purchased from Alexis Biochemicals (San Diego, CA). Monoclonal antibodies to α dystroglycan (VIA4-1 and IIH6) were purchased from Upstate Biotechnology (Lake Placid, NY). Some IIH6 monoclonal antibody was a gift from Kevin Campbell (HHMI, U. Iowa). DG2, a rabbit polyclonal antiserum against the HIANKKPLPKRVR peptide in the C-terminal region of α dystroglycan (Ibraghimov-Beskrovnaya et al., 1992), was produced and affinity purified in our laboratory using methods previously described (Xia et al., 2002). Hybridomas producing antibodies against the CT carbohydrate (CT1, CT2) were grown in our laboratory and mAbs purified as before (Martin et al., 1999). These hybridomas were originally made by Leo Lefrancois (Lefrancois and Bevan, 1985a, b). Antibodies to Galg2, were made and purified in our laboratory as previously described (Xia et al., 2002). Polyclonal antibody to laminin α4 (Patton et al., 1997) was a gift from Bruce Patton (Oregon Health Sciences University) and polyclonal antibody to laminin α5 (Miner et al., 1997) was a gift from Jeffery Miner (Washington University, St. Louis). Monoclonal antibody to NCAM (H28) was purchased from Developmental Studies Hybridoma Bank (U. Iowa). Polyclonal antibody to actin was obtained from Sigma (St. Louis, MO).

Production and purification of recombinant laminins and agrins

Partial cDNAs encoding recombinant, secreted, and FLAG-tagged muscle agrin (C45z(0)) and neural agrin (C45z(8)) were transfected into HEK293T cells and secreted recombinant FLAG-tagged proteins purified as previously described (Parkhomovskiy et al., 2000; Xia and Martin, 2002). These proteins contain the G2 and G3 domains of agrin, with the intervening EGF4 repeat. Both forms contain the y4 heparin binding exon and neural agrin contains the z8 exon, while muscle agrin does not (thus signified as z0) (Ferns et al., 1992). The C45 fragments of agrin are sufficient for high affinity binding to α dystroglycan (Campanelli et al., 1996; Gesemann et al., 1996; Gesemann et al., 1995).

cDNAs encoding secreted FLAG-tagged mouse sequences for the G1-G5 domains of laminin α2 (Bernier et al., 1995), laminin α4 (Frieser et al., 1997), or laminin α5 (Miner et al., 1995) were made by RT-PCR using C2C12 myotube RNA (for laminin α2 and α5) or mouse muscle RNA (for laminin α4) as follows. After reverse transcription primed by oligo dT, a FLAG-tagged cDNA encoding the G1-G5 domains of laminin α2 was amplified by PCR using Pfu polymerase
(Stratagene; La Jolla, CA) with 5′GAAGATCTTGGAAGCTACAATAACATCGTTGTC (forward) and 5′GCTCTAGATCAGTTCCAGGGCCTTGGC (reverse) primers. The amplified fragment was gel purified, digested with BglII and XbaI, and subcloned into these same restriction sites in the pFLAG.CMV1 (Sigma; St. Louis, MO) expression plasmid. A cDNA encoding the G1-G5 domains of laminin α4 was amplified using 5′TGACTCCATCGATACGCTGGAAGCTACAATAACATCGTTGTC (forward) and 5′GATTCCGGGTACCTCAGGCTGTGGGACAGGAGGTGA (reverse) primers. The amplified fragment was gel purified, digested with ClaI and KpnI, and subcloned into these sites in pFLAG.CMV1 expression plasmid. A cDNA encoding the G1-G5 domains of laminin α5 was amplified using 5′GAAGATCTTACTGCCCTCAAGTTCCACATTC (forward) and 5′GCTCTAGACTAGGTTCCTGAGGGGCATCC (reverse) primers. The amplified fragment was gel purified, digested with BglII and XbaI, and subcloned into pFLAG.CMV1 at the same sites. All subcloned cDNAs were confirmed by DNA sequencing.

Plasmids were transfected into HEK293T cells and production of recombinant epitope-tagged protein verified by immunoblotting, as before, using both antibodies to the native polypeptide sequence and antibodies to the epitope tag (Xia and Martin, 2002). Functional studies on heparin and α1 dystroglycan binding were also done, as before (Parkhomovskiy et al., 2000; Xia and Martin, 2002), to verify production of functional laminin proteins. Laminin α2 showed preferential cleavage into two proteins when secreted, much as described by Engvall and colleagues for the native protein (Ehrig et al., 1990a; Ehrig et al., 1990b). To control for this cleavage, experiments were done using proteins produced from cell lysates, where this proteolytic cleavage was less prevalent.

To produce proteins for binding experiments, cDNA expression vectors were transfected into HEK293T cells, as before (Xia and Martin, 2002). Secreted proteins were purified from the supernatant using anti-FLAG (M2) affinity chromatography in the presence of added protease inhibitors, as previously described (Xia and Martin, 2002). After washing the column in Tris-buffered saline (TBS), purified proteins were eluted with 3XFLAG peptide (MDYKDHDGDYKDHDIDYKDDDDK), also as previously described (Kim et al., 2008). Purified proteins were characterized by immunoblotting (for the presence of the recombinant protein) and by silver staining of SDS-PAGE gels (for overall purity) (Supplemental Fig. 1). Protein concentrations were also determined as previously described (Xia and Martin, 2002).

For purification of proteins from cell lysates, cells were harvested in lysis buffer containing 20mM Tris pH 7.4, 150mM NaCl, 1%NP40, and protease inhibitors (Xia and Martin, 2002) for 1hr at 4°C. Unless otherwise specified, protease inhibitors included 5mM EDTA, 10μg/ml aprotinin, 10μg/ml leupeptin, 1μM PMSF, 1μM benzamidine, 10μg/ml pepstatin, and 5μg/ml bestatin for cell lysates. Cell lysates were then centrifuged at 13000g and supernatants affinity purified over M2 agarose and eluted with 3X FLAG peptide as before (Xia and Martin, 2002). In all cases, purified proteins were washed over a 10K spin column to remove 3X FLAG peptide from the eluent prior to measurement of protein amounts.

**Solid phase binding of PAA-glycans to agrins and laminins**

Polyacrylamide (PAA)-linked glycans also containing biotin were obtained from the Consortium for Functional Glycomics (Core D; Scripps Research Institute; La Jolla, CA) or were purchased from Glycotech (Gaithersburg, MD). All glycans were purified to ca. 95% purity as analyzed by thin layer chromatography and 1H NMR and/or mass spectrometry (http://www.functionalglycomics.org/static/consortium/resources/resourcecored1.shtml). PAA-glycans used were 30kDa and contained 20% glycan and 5% biotin. As such, the valency of glycan to PAA was ca. 6:1 for each glycan used. All glycans were first verified to have equivalent levels of biotin by immobilizing them on ELISA plates, as before (Brinkman-Van der Linden and Varki, 2000), and probing with streptavidin-HRP.

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To study PAA-glycan binding to recombinant laminins or agrins, a monoclonal anti-FLAG antibody (M2) was immobilized on ELISA plates at a concentration of 500 ng/well in carbonate/bicarbonate buffer (50 mM, pH 9.5) overnight at 4°C. Wells were blocked with ELISA-buffer for 1 hr. Purified agrins (C45(z0) or C45(z8)) or laminins (G1-G5; α2, α4 or α5) were added to each well at 200 ng/well and incubated overnight at 4°C. PAA-glycan binding to all five proteins was done in each experiment. After washing, representative wells were incubated in SDS denaturing buffer, separated by SDS-PAGE, and immunoblotted with M2 antibody to verify equivalent amounts of protein were present and analyzed by silver staining to determine their relative purity. PAA-glycans were added in 100 μL binding buffer at concentrations ranging from 0.25 μg/well to 3 μg/well for 2 hrs (yielding an effective concentration range of 42-500 nM), followed after washing and incubation with peroxidase-conjugated streptavidin (1:1000, for 1 hr). After subsequent washes, plates were developed in SIGMA Fast™ OPD.

All binding was followed at 5-minute intervals for 30-60 minutes and only data representative of the linear range of response was used for analysis. OD signals varied between 0.1-1.0. All data points used represent triplicate measures of each condition. Binding of PAA-glycans to wells coated with M2 antibody but not with ECM protein was negligible (less than 10% of signal), as was binding of secondary reagents to wells containing immobilized laminin or agrin proteins. Aliquots of PAA-glycans added for the 3 μg/well concentration were immobilized on ELISA plates and probed with streptavidin-HRP to verify equivalent levels of biotin were present (Supplemental Fig. 2). Estimates of binding affinity were done by plotting binding curve using a standard receptor binding model (fractional ligand binding=[L]/(K_d+[L])) with XLfit software (ID Business Solutions, Guildford UK).

Cell culture and transfection

HEK293T and HEK293 cells were grown in Dulbecco's Modified Eagle's Media (DMEM) containing 10% fetal calf serum, 50 μg/ml streptomycin, and 50 U/ml penicillin. CHO cells stably overexpressing LARGE (CHO-LARGE), or HEK293 cells stably overexpressing Galgt2 (HEK293CT) were grown in the same media with 400 μg/ml G418 added. Cells were transfected using Effectene Reagent (Qiagen; Valencia, CA) according to the manufacturer's instructions.

Synthesis and purification of Galgt2 and α dystroglycan

A recombinant cDNA encoding a FLAG epitope-tagged luminal domain of human Galgt2 (GALGT2) obtained from Harduin-Lepers and colleagues (Montiel et al., 2003) was transfected into HEK293T cells and purified from cell supernatant as previously described (Parkhomovskiy et al., 2000). A FLAG-tagged cDNA encoding α dystroglycan (Jayasinha et al., 2003) with a deletion of the R312 furin cleavage site (delR312) was similarly produced and purified from transfected HEK293T supernatant using anti-FLAG antibody (M2) affinity chromatography (Parkhomovskiy et al., 2000).

In vitro glycosylation of α-dystroglycan by Galgt2

An enzyme assay mixture was formulated using UDP–N-acetyl-D-galactosamine at 1 mM as substrate donor and FLAG-tagged delR312 α-dystroglycan (purified from HEK293T cells) at 4 μM as the substrate acceptor in 10 μl of cacodylate buffer, pH 6.2, containing 10 mM MnCl_2, 0.2% CF-32, 4.5 mM ATP. Galgt2 catalytic domain was expressed and purified from HEK293 cell supernatant was included in the assay in a volume of 100 μL and a control reaction was set up without the enzyme but with UDP-GalNAc. The reaction was allowed to continue overnight at 37°C. To analyze glycosylation, 10 μL of the reaction mixture was heated to 100°C for 5 minutes in denaturing buffer, separated by SDS-PAGE, transferred to nitrocellulose, and probed with anti-FLAG M2 monoclonal antibody conjugated with HRP (1:1000) or with CT2 (1:10) followed by peroxidase–conjugated goat anti-mouse IgM (1:10000). Binding was
visualized by ECL chemiluminescence detection (Amersham; Piscataway, NJ) (Supplemental Fig. 3).

Solid phase binding of recombinant laminins and agrins to CT- and non-CT glycoforms of α dystroglycan

CT-glycosylated α dystroglycan was partially purified from Galgt2 transgenic skeletal muscle by WFA column chromatography. Briefly, ca. 100mg of lower leg muscles (gastroc and quad) from Galgt2 transgenic mice were solubilized in 10mL of NP40 lysis buffer overnight at 4°C (1%NP40, Tris-buffered saline pH 7.4, 2mM EDTA+protease inhibitors). Lysate was incubated with WFA agarose overnight at 4°C, washed extensively with lysis buffer, and eluted in lysis buffer containing 0.3M GalNAc. To purify non-CT containing α dystroglycan, ca. 100mg of lower leg muscle (gastroc+quad) from wild type mice was solubilized as above and incubated with WGA agarose, washed, and eluted with lysis buffer containing 0.3M GlcNAc. Monosaccharides were removed by washing proteins over a 10K spin column. Protein amounts were measured using a modified Bradford assay and identical amounts of protein were characterized for the presence of α dystroglycan (using immunoblotting with IIH6) and for relative purity by silver staining of SDS-PAGE gels (Supplemental Fig. 1).

Identical amounts (200ng/well) of α dystroglycan were immobilized in sodium bicarbonate buffer (50mM, pH 9.5) overnight at 4°C at 200ng protein per well. Equivalent immobilization was verified by probing some wells with antibody to IIH6 (Supplemental Fig. 2). VIA4-1 gave similar results (not shown). Increased glycosylation with the CT carbohydrate in the CταDG fraction was verified by probing some wells with CT2 (Supplemental Fig. 2). Wells were blocked with ELISA-buffer for 1hr and incubated with purified recombinant laminins or agrins purified as before (Supplemental Fig. 1) in binding buffer. After washing, wells were incubated with anti-FLAG antibody (M2-HRP conjugated: Sigma), washed, and developed using a peroxidase-based immunosorbent assay (SIGMA Fast™ OPD).

Solid phase binding of α-dystroglycan glycoforms to Laminin-111

Laminin-111 (β1,α1,γ1) from Engelbreth-Holm-Swarm (EHS) tumor (Sigma, St. Louis MO) was plated on ELISA plates in sodium bicarbonate buffer (50mM, pH 9.5) overnight at 4°C at 200ng protein per well. Wells were blocked with ELISA-buffer for 1hr and incubated with FLAG-tagged α-dystroglycan (delR312 furin cleavage mutant) either containing (αDGCT) or not containing (αDGWT) the CT carbohydrate overnight at 4°C. To make αDGWT and αDGdelR312 cDNA was respectively transfected into HEK293 cells, which have little or no Galgt2 expression (Parkhomovskiy et al., 2000), or HEK293CT cells, which stably overexpress Galgt2 (Parkhomovskiy et al., 2000). After purification of transfected cell supernatants using M2 agarose as before (Kim et al., 2008) and assessment of CT glycosylation and relative purity (Supplemental Fig. 1), α-dystroglycan binding was assessed by adding different concentrations of αDG(WT) or αDG(CT) for 1-2 hours in binding buffer to laminin 111-coated ELISA plates. Plates were washed, an anti-FLAG (M2-HRP: Sigma) added at 1:1000 for one hour, washed again, and developed using a HRP substrate (SIGMA Fast™ OPD). Binding of αDG(WT or CT) to wells not coated with laminin-111 never exceeded 10% of signal with laminin-111 present.

Measurement of Gene Expression by TaqMan RT-PCR

Total RNA was isolated using Trizol reagent (Invitrogen; Carlsbad, CA) from gastrocnemius muscle samples stabilized in RNALater (Ambion; Austin, TX). RNA was further purified on a silica-gel-based membrane (RNeasy-Mini; Qiagen, Valencia, CA) and the integrity of RNA was determined by capillary electrophoresis using 6000 Nano LabChip kit on a Bioanalyzer 2100 (Agilent; Foster City, CA). RNA content was measured using an ND-1000 spectrophotometer (Nanodrop; Wilmington, DE). Only samples with no evidence of RNA
degradation were used for TaqMan Gene Expression studies. A high capacity cDNA archive kit (Applied Biosystems; Foster City, CA) was used to reverse transcribe 3μg of total RNA as per the manufacturer's guidelines. Samples were subjected to real-time PCR in triplicate using TaqMan ABI 7500 sequence detection system (Applied Biosystems; Foster City, CA) with 18S ribosomal RNA (product no. 4308329, Applied Biosystems) as internal control. Primers and probe against CT GalNAc transferase (Galgt2) were custom-made and provided as a 20× reaction mix containing 18μmol/L of each of primers (forward primer sequence: 5′-GATGTCCTGGAGAAAACCGAACT-3′; reverse primer sequence: 5′-GCAGCCTGAACCTGGTTAGTATTCC-3′) and 5μmol/L of probe (probe sequence: 5′-CCGCCACCACATCC-3′) (Applied Biosystems). All other primers and probes were purchased as predeveloped 20× TaqMan assay reagents from Applied Biosystems (details are provided in Table 1). Each 25μl PCR reaction mix consisted of 1× primer-probe mix, 1× TaqMan Universal PCR master mix (product no. 4304437; Applied Biosystems). After an initial hold of 2 minutes at 50°C to allow activation of AmpErase and 10 minutes at 95°C to activate the AmpliTaq polymerase, the samples were cycled 40 times at 95°C for 15 seconds and 60°C for 1 minute. Gene expression was determined as relative changes by the 2−ΔΔCt method (Livak and Schmittgen, 2001). Data are presented as fold difference normalized to 18S ribosomal RNA. All measures were done in triplicate for each data point.

Animals

Transgenic mice that express the cytotoxic T cell (CT) GalNAc transferase, or Galgt2, in their skeletal muscles by virtue of the skeletal alpha actin promoter/intron have been described previously (Xia et al., 2002). Mice that fail to express utrophin due to a gene deletion have also been previously described (Deconinck et al., 1997a; Grady et al., 1997) and were a generous gift from Mark Grady (Washington University; St. Louis, MO). Galgt2 transgenic mice (line 2159) were mated to utrophin–deficient (Utrn-/-) animals, and crossed again to Utrn-/- animals to create utrophin-deficient Galgt2 transgenic mice (Galgt2Utrn-/-) and control littersmates. Genotyping for utrophin deletion and the presence of the Galgt2 transgene were done as previously described (Grady et al., 1997; Xia et al., 2002). Aged-matched Utrn+/+, Utrn+/-, and Utrn-/- littersmates containing or not containing the Galgt2 transgene were compared. All animals analyzed were 6-8 weeks of age. Experiments were done in accordance with NIH guidelines on the use of experimental animals and according to protocols approved by the Institutional Animal Care and Use Committee at Nationwide Children's Hospital.

SDS-PAGE gels and immunoblotting

Whole minced skeletal muscles were solubilized by homogenization in 75mM Tris, pH 6.8, 4% SDS, 4M Urea, 20% glycerol, and 1% β-mercaptoethanol. Samples were diluted and protein levels quantitated using the BioRad DC protein assay kit (BioRad, Richmond CA). Proteins were separated by on 6% or 12% SDS-PAGE and transferred to nitrocellulose. For blotting with anti-carbohydrate antibodies, blots were blocked in Tris-buffered saline (pH 7.4) with 0.1% Tween 20 (TBST) and 3mg/ml BSA. All other blots were blocked in TBST with 5% non-fat dry milk. After blocking, blots were incubated with primary antibody, washed, bound to the appropriate peroxidase-conjugated secondary antibody, washed and developed using ECL (Amersham, Piscataway, NJ). Blots shown are representative of at least three similar experiments. Silver staining of SDS-PAGE separated proteins was done using a silver stain kit (24597) from Pierce (Rockford, IL).

Lectin precipitations

Gastrocnemius muscles were dissected, minced, and solubilized in Tris-buffered saline (TBS) pH7.4 with NP-40 buffer with shaking overnight at 4°C. Solubilized proteins were assayed for protein concentration as described previously (Xia et al., 2002). 20μl of Wisteria floribunda
agglutinin (WFA) or Wheat germ agglutinin (WGA) conjugated agarose was incubated with 150μg of protein extract overnight at 4°C. Identical samples were also incubated with resins in the presence of 0.3M GalNAc (for WFA) or 0.3M GlcNAc (for WGA) to verify specificity of lectin binding. Pellets were spun at 1000g for 3 minutes, washed twice for 15 minutes in a large excess of NP40 lysis buffer, boiled in SDS sample buffer, separated by SDS-PAGE, and analyzed by immunoblotting with antibodies to α-dystroglycan (VIA4-1 or IIH6), CT carbohydrate (CT2), α, β or γ sarcoglycan, dystrophin, or utrophin.

**Histology**

For hematoxylin-eosin and immunofluorescence staining, muscles were snap frozen in liquid nitrogen-cooled isopentane and sectioned at 8μm on a cryostat. For whole mount preparations, diaphragm and gastrocnemius muscles were dissected, fixed in 2% paraformaldehyde at 4°C for 30 minutes, washed, and stained with rhodamine-α-bungarotoxin as previously described (Xia et al., 2002). For staining of with anti-carbohydrate antibodies, sections were blocked in Tris-buffered saline pH 7.4 in 3 mg/ml bovine serum albumin (BSA). CT1 or CT2 antibody (5μg/ml) was pre-complexed with goat anti-mouse IgM-FITC (2.5μg/ml) for one hour. The complex was then added with 50nM rhodamine-α-bungarotoxin for one hour. All other antibodies were added at 1μg/ml. Anti-peptide antibodies were added separately from secondary antibodies at 2μg/ml in TBS with 3mg/ml BSA. Sections were washed in TBS and incubated with 10μg/ml of the appropriate FITC-conjugated secondary antibody and 50nM rhodamine-α-bungarotoxin. Anti-peptide antibody specificity was confirmed by blocking with excess peptide. Slides were dried, mounted in glycerol with paraphenylenediamine to prevent quenching, and analyzed using a Nikon E800 epifluorescence microscope with fluorescein, rhodamine, or bright field optics. Staining with secondary antibody alone was always done as a control to verify that background staining was insignificant.

**Analysis of muscles and neuromuscular junctions**

Quantitation of percentage of myofibers with central nuclei and myofiber diameter were done using hematoxylin- and eosin-stained sections as previously described (Nguyen et al., 2002; Xia et al., 2002). The length of the endplate band was analyzed using whole mount stains of the diaphragm in which postsynaptic areas were visualized with rhodamine-α-bungarotoxin, as previously described (Xia et al., 2002). For neuromuscular junction analysis, 100-200 synapses were analyzed per animal. Neuromuscular structure was analyzed using 8μm cross-sections of gastrocnemius muscle labeled with rhodamine-α-bungarotoxin.

**Statistics**

Comparisons of significance were determined using a paired Student’s t test with equal weighting between samples. *P<0.05, **P<0.01, ***P<0.001.

**Supplementary Material**

Refer to Web version on PubMed Central for supplementary material.

**Acknowledgments**

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deficient mice were done by PTM with help from Bing Xia, Kwame Hoyte, and Vianney Jayasinha while at the University of California, San Diego.

References


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Zhou YW, Thomason DB, Gullberg D, Jarrett HW. Binding of laminin alpha1-chain LG4-5 domain to alpha-dystroglycan causes tyrosine phosphorylation of syntrophin to initiate Rac1 signaling. Biochemistry 2006;45:2042–2052. [PubMed: 16475793]
Fig. 1.
Laminins and agrins have increased binding to CT-glycosylated α dystroglycan compared to non-CT-glycoforms.
Recombinant laminin α2 (G1-G5) (A), laminin α4 (G1-G5) (B), laminin α5 (G1-G5) (C), muscle-specific agrin (G2-G3, C45(z0)) (D), or neural specific agrin (G2-G3, C45(z8)) (E) were added to CT-glycosylated α dystroglycan (CT) or non-CT-glycosylated (WT) α dystroglycan immobilized on ELISA plates (see Supplemental Figs. 1 and 2). (F) Recombinant α dystroglycan was produced with (CT) or without (WT) the CT carbohydrate (see Supplemental Fig.1). Proteins were added to laminin-111 immobilized on ELISA plates. Errors are SEM for n=6-12 experiments.

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The CT carbohydrate has increased binding to laminins and agrin compared to sialyl-N-acetyllactosamine. Glycans linked to polyacrylamide (PAA) and containing biotin were added to recombinant forms of laminin α2 (A), laminin α4 (B), laminin α5 (C), muscle-specific agrin (D), or neural specific agrin (E) immobilized on ELISA plates (See supplemental Figs. 1 and 2). Binding of sialyl-N-acetyllactosamine, Neu5Acα2,3Galβ1,4GlcNAc-PAA (3′SNL(Neu5Ac)), N-acetyllactosamine, Galβ1,4GlcNAc-PAA (NL), and the CT carbohydrate, Neu5Acα2,3GalNAcβ1,4Galβ1,4GlcNAc-PAA (CT(Neu5Ac)) were compared over a concentration range 0.25-3 μg/well (42nM-500nM of PAA-glycan). Errors are SEM for n=6-12 experiments.
Fig. 3. Transgenic overexpression of Galgt2 increases mRNA levels of extracellular matrix genes, utrophin, and neuregulin in skeletal muscle. mRNA levels were measured by semi-quantitative TaqMan Real-time PCR in Galgt2 transgenic (CT) and wild type (WT) skeletal muscle using the probes described in Table 1. (A) Expression of extracellular matrix genes and genes encoding proteins in the dystrophin- or utrophin-associated glycoprotein complexes were elevated in CT muscle. (B) Genes with elevated transcription in CT muscle included neuregulin, NCAM, and the epsilon chain of the acetylcholine receptor (AChε). Elevations in CT muscle, compared to WT, were significant P<0.01 for all but laminin α4, α sarcoglycan, and Cdk2. Errors are SEM for n=6-12 experiments.
Fig. 4. Association of utrophin with CT-glycosylated dystroglycan and creation of utrophin-deficient Galgt2 transgenic mice.

(A) 150μg of whole muscle NP40 detergent lysate from wild type muscle was precipitated by Wheat germ agglutinin (WGA), a GlcNAc/sialic acid-binding lectin that precipitates non-CT glycosylated (WT) dystroglycan, or from Galgt2 transgenic muscle by *Wisteria floribunda* agglutinin (WFA), a βGalNAc-binding lectin that binds CT-glycosylated dystroglycan. (B) Utrophin and Galgt2 protein expression were analyzed by Western blotting in Galgt2 transgenic (Tg) and non-transgenic mice that either express (Utrn +/-) or do not express (Utrn-/-) utrophin, with actin as a control for protein loading and transfer. (C) Immunostaining with antibodies to utrophin show increased extrasynaptic expression in Galgt2Utrn+/- muscles (relative to Utrn+/+) and loss of expression in Utrn-/- muscles. Galgt2 transgene expression increased the expression of the CT carbohydrate along myofibers in both Galgt2Utrn+/- and
Galgt2Utrn-/− muscles. Bar is 50μm. (D) WFA precipitated α dystroglycan (VIA4-1) containing the CT carbohydrate (CT2) from both Galgt2Utrn+/− and Galgt2Utrn-/− muscles. WGA precipitated α dystroglycan from non-transgenic muscle. No other bands of any molecular weight were present.
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Fig. 5.
Utrophin is partially required for Galgt2-dependent changes in extracellular matrix protein expression.
(A) 40 μg SDS/Urea muscle cell protein extract was separated by SDS-PAGE and analyzed by Western blot. Galgt2 increased the expression of laminin α4, laminin α5, agrin, and one protein isoform of neuregulin in Galgt2Utrn+/- muscle, but to a lesser degree in Galgt2Utrn-/- muscle.
(B) Immunostaining shows that laminin α2, a normally extrasynaptic laminin chain, was expressed equally well in Utrn+/-, Galgt2Utrn+/-, Utrn-/-, and Galgt2Utrn-/- muscles. Laminin α4, laminin α5, and agrin were overexpressed along myofibers in Galgt2utrn+/- transgenic muscle, but less so in Galgt2Utrn-/- muscle. Neuregulin was expressed in muscles of all genotypes, with concentration at neuromuscular junctions. Bar is 50 μm.
Fig. 6.
Utrophin is not required for Galgt2-dependent changes in muscle growth.
(A) Muscles were stained with hematoxylin and eosin. Galgt2 transgene (Tg) expression severely inhibited muscle growth in both Utrn+/+ and Utrn+/- mice. Arrows show muscle spindles. Bar is 50μm (upper 8 panels) or 100μm (lower 4 panels). (B) Average myofiber diameter was calculated for various muscles. Errors are SEM for 250 myofibers per experiment per genotype for n=6 experiments per condition. (C) The percentage of myofibers with centrally located nuclei was unchanged by Galgt2 (CT+) expression. Errors are SEM for 250 myofibers per experiment. Errors are SEM for n=6 experiments.
Utrophin is not required for Galgt2-dependent changes in synaptic structure and topography. (A) Whole muscles or muscle fibers were labeled with rhodamine-α-bungarotoxin to visualize nicotinic acetylcholine receptors in the postsynaptic membrane. Bar is 10 μm (upper panels) and 250 μm (lower panels). (B) Quantitation of changes in neuromuscular topography, as assessed by average length of the endplate band in the diaphragm. Errors are SEM for n=20 muscles per condition.

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Fig. 7.
Table 1
TaqMan gene expression assays used for Real-Time PCR

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