Molecular Pathogenesis of Genetic and Inherited Diseases

Overexpression of the Cytotoxic T Cell (CT) Carbohydrate Inhibits Muscular Dystrophy in the dy^w Mouse Model of Congenital Muscular Dystrophy 1A

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A number of recent studies have demonstrated therapeutic effects of transgenes on the development of muscle pathology in the mdx mouse model for Duchenne muscular dystrophy, but none have been shown also to be effective in mouse models for laminin α 2-deficient congenital muscular dystrophy (MDC1A). Here, we show that overexpression of the cytotoxic T cell (CT) GalNAc transferase (Galgt2) is effective in inhibiting the development of muscle pathology in the dy^w mouse model of MDC1A, much as we had previously shown in mdx animals. Embryonic overexpression of Galgt2 in skeletal muscles using transgenic mice or postnatal overexpression using adeno-associated virus both reduced the extent of muscle pathology in dy^w/dy^w skeletal muscle. As with mdx mice, embryonic overexpression of the Galgt2 transgene in dy^w/dy^w myofibers inhibited muscle growth, whereas postnatal overexpression did not. Both embryonic and postnatal overexpression of Galgt2 in dy^w/dy^w muscle increased the expression of agrin, a protein that, in recombinant form, has been shown to ameliorate disease, whereas laminin α 1, another disease modifier, was not expressed. Galgt2 overexpression also stimulated the glycosylation of a glycolipid with the CT carbohydrate, and glycolipids accounted for most of the CT-reactive material in postnatal overexpression experiments. These experiments demonstrate that Galgt2 overexpression is effective in altering disease progression in skeletal muscles of dy^w mice and should be considered as a therapeutic target in MDC1A. (*Am J Pathol* 2007, 171:181–199; DOI: 10.2353/ajpath.2007.060927)

A number of recent studies have suggested that expression of transgenes in skeletal muscle can ameliorate aspects of muscular dystrophy in mouse models of the disease.^{1–15} Most of this work centers on testing therapeutic strategies in the mdx mouse model for Duchenne muscular dystrophy (DMD). Muscles in mdx animals (and DMD patients) fail to express dystrophin.^{16–18} Dystrophin is a cytoplasmic protein that helps to link extracellular matrix proteins, including laminin, that surround the myofiber membrane to the actin cytoskeleton.^{16,19} Dystrophin accomplishes this, at least in part, via its interactions with β -dystroglycan, a transmembrane glycoprotein, which in turn binds to α -dystroglycan, a laminin-binding protein on the extracellular face of the muscle membrane. Although work by Chamberlain and colleagues,^{20,21} Xiao and colleagues,^{22,23} and others has shown that dystrophin replacement can inhibit the dystrophic process in mdx animals, overexpression of a surprising number of other genes that are not mutated in DMD also has been shown to have therapeutic benefit: transgenic overexpression of ADAM12,²⁴ neuronal nitric-oxide synthase,^{7,25,26} calpastatin,²⁷ utrophin,^{28–30} neuregulin,⁶ calcineurin,³¹ integrin α 7B,^{14,15} and CT GalNAc transferase³² in skeletal muscles of mdx animals all inhibit the development of aspects of muscle pathology or disease. In addition, inhibition³³ or elimination of myostatin³⁴ benefits muscle regeneration in mdx animals and increases muscle strength.

The relatively robust nature of some of these effects begs the question of whether their therapeutic potential would be applicable in other forms of muscular dystrophy. A logical place to begin to ask such questions is with mouse models of laminin α 2 (or merosin)-deficient muscular dystrophy (MDC1A),³⁵ the most common inherited autosomal congenital muscular dystrophy.³⁶ Recent

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studies, however, suggest that several approaches that were effective in mdx animals did not alter muscular dystrophy in the dy^W mouse, an MDC1A model made by homologous recombination of the laminin $\alpha 2$ gene (*Lama2*) locus.^{37,38} For example, loss of myostatin had no effect on muscle pathology in dy^W/dy^W animals despite increasing muscle regeneration.³⁹ Similarly, transgenic overexpression of ADAM12 in dy^W/dy^W animals did not significantly alter disease progression or muscle pathology despite stimulating muscle regeneration.⁴⁰ In contrast to myostatin and ADAM12, expression of a recombinant extracellular matrix protein, mini-agrin, has been shown to inhibit muscular dystrophy in dy^W/dy^W animals as well as in dy^{3K}/dy^{3K} mice, an MDC1A model that is null for laminin $\alpha 2$ expression.^{8,41} Overexpression of laminin $\alpha 1$ also can substitute for laminin $\alpha 2$ in certain tissues in dy mice, including skeletal muscles.^{42–44} Because muscular dystrophy in various dy animal models (and MDC1A patients) results from a defect in extracellular matrix expression,^{36,45,46} these data suggest that therapies that target extracellular matrix expression may be more effective than approaches that target muscle regeneration in MDC1A.

One therapy effective in mdx animals that alters extracellular matrix expression is overexpression of the cytoxic T cell (CT) GalNAc transferase.^{32,47} The CT GalNAc transferase (Galgt2) is a $\beta 1,4$ -N-acetylgalactosaminyltransferase that creates the CT carbohydrate antigen [GalNAc $\beta 1,4$ (Neu{Ac or Gc} $\alpha 2,3$)Gal $\beta 1,4$ GlcNAc β] on select glycoproteins and glycolipids.⁴⁸ In skeletal muscle, both Galgt2⁴⁷ and the CT carbohydrate it creates⁴⁹ are concentrated at the neuromuscular junction, whereas no terminal β GalNAc of any kind is present along the extrasynaptic membrane of mammalian skeletal myofibers.^{49,50} Overexpression of the Galgt2 specifically in skeletal muscles of transgenic mice stimulates the glycosylation of α -dystroglycan with the CT carbohydrate along extrasynaptic regions of the myofiber membrane.^{32,47,51} Other synaptic proteins that may associate with dystroglycan, including utrophin, laminin $\alpha 4$, and laminin $\alpha 5$ are also ectopically expressed in Galgt2 transgenic muscles.^{32,47}

Because α -dystroglycan requires proper glycosylation to bind to laminin,^{1,52} ectopic glycosylation of dystroglycan with the normally synaptic CT carbohydrate may stimulate its preferential association with synaptic laminins and utrophin in the extrasynaptic membrane, thereby allowing their overexpression. Utrophin is a synaptically localized homologue of dystrophin that can functionally substitute, at least in part, for loss of dystrophin in the extrasynaptic membranes of mdx muscles,^{28–30} whereas laminin $\alpha 4$ and laminin $\alpha 5$ are synaptic homologues of laminin $\alpha 2$, the α chain of laminin-2 ($\alpha 2, \beta 1, \gamma 1$ or laminin 211 in the new nomenclature⁵³), the extrasynaptic form of laminin in adult skeletal muscle.^{54,55} Some increased laminin expression, particularly laminin $\alpha 4$, is known to occur in dy skeletal muscles,⁵⁶ but whether this ameliorates the severity of muscle disease is unknown. Overexpression of a recombinant form

of agrin in which the C-terminal portion of the protein is linked to its N-terminal laminin-binding domain can significantly alter disease progression in dy animals.^{8,57} In contrast to recombinant agrin, native muscle agrin is a highly glycosylated heparan/chondroitin sulfate proteoglycan.^{58–60} Ruegg and colleagues⁶¹ have shown that muscle agrin protein can be present at increased levels in the extrasynaptic basal lamina of dy^W skeletal muscles.

In this study, we show that overexpression of Galgt2, both in transgenic mice and in adeno-associated virus (AAV)-infected animals, inhibits the extent of skeletal muscle pathology in dy^W/dy^W animals. Galgt2 accomplishes this, however, via a mechanism that does not require increased overexpression of utrophin or increased CT glycosylation of α -dystroglycan, much as we have recently also shown for mdx muscles.⁶² Galgt2 overexpression does correlate with increased endogenous expression of agrin, a protein that, in recombinant form, can ameliorate disease.^{8,41,57}

Materials and Methods

Materials

N-Acetylgalactosamine (GalNAc) and N-acetylglucosamine (GlcNAc) were obtained from Calbiochem (San Diego, CA). Agarose-bound lectins (*Wisteria floribunda* agglutinin, WFA; and wheat germ agglutinin, WGA) were purchased from EY Laboratories (San Mateo, CA). AAV1-Galgt2 was made and purified by Virapure (San Diego, CA). AAV8-like Galgt2 (rh.74-Galgt2) was made by the Viral Vector Core at Children's Research Institute. Monoclonal antibodies to dystrophin (Dy4/6D3), utrophin (DRP3/20C5), β -dystroglycan (43DAG1/8D5), α -sarcoglycan (Ad1/20A6), and β -sarcoglycan (β Sarc1/5B1) were obtained from Nova Castra (Newcastle On Tyne, UK). Antibody to actin was obtained from Sigma (St. Louis, MO). Antibodies to α -dystroglycan (VIA4-1 and IIH6) were obtained from Upstate Biotechnology (Lake Placid, NY). Antibodies β -dystroglycan, CT1, CT2, CT GalNAc transferase were produced in our laboratory. Polyclonal antibodies to integrin $\alpha 7B$, utrophin, dystrophin, α -sarcoglycan, and caveolin 3 were a generous gift from Ling Guo (University of California at San Diego, San Diego, CA) and Eva Engvall (Burnham Institute, La Jolla, CA). Antibodies to laminin $\alpha 4$ ^{54,63} were a gift from Bruce Patton (Oregon Health Sciences, Portland, OR). A polyclonal antibody to laminin $\alpha 5$ ⁶⁴ was a gift from Jeff Miner (Washington University, St. Louis, MO). Monoclonal antibodies to agrin (mAb33, mAb86) and laminin $\alpha 1$ (AL-1) and polyclonal antibody to agrin were obtained from Accurate Chemical (Westbury, NY) or Chemicon (Temecula, CA). Secondary antibodies conjugated to horseradish peroxidase, fluorescein isothiocyanate, or Cy2 were purchased from Jackson Immunochemicals (Seattle, WA).

Transgenic Mice

Transgenic mice bearing the CT GalNAc transferase (Galgt2) specifically in skeletal muscles via the human skeletal α -actin promoter⁶⁵ were described by us previously,⁴⁷ as were Galgt2 transgenic mdx mice.³² $dy^{w/+}$ mice were obtained from Eva Engvall (Burnham Institute). These mice have lacZ inserted into the *Lama2* gene and consequently have very reduced levels of laminin $\alpha 2$ protein.³⁵ The Galgt2 transgene (CT) was bred into the $dy^{w/+}$ background, and CT/ $dy^{w/+}$ and $dy^{w/+}$ were mated to produce dy^{w}/dy^{w} , CT/ dy^{w}/dy^{w} , $dy^{w/+}$, and CT/ $dy^{w/+}$ animals. Mice were maintained on a mixed (C57BL/6 $\times$ BALB/c) background, and all control animals were littermates derived from the same litters. Galgt2 transgenic mdx mice were maintained similarly on an F1 C57BL/10 $\times$ BALB/c background. $dy^{w/+}$ intercrosses were also set up to produce dy^{w}/dy^{w} animals for AAV infection with AAV-Galgt2. All control muscles for AAV-Galgt2 infection were taken from a mock-infected contralateral limb muscles in the same animal. AAV-Galgt2-infected mdx muscles were also used as controls for comparisons as indicated.⁶²

Histology

Muscles were dissected and snap-frozen in liquid nitrogen-cooled isopentane and sectioned at 8 to 10 μ m on a cryostat. Sections were either stained with hematoxylin and eosin (H&E) or immunostained with various antibodies as previously described.^{32,47,51,66} Quantitation of central nuclei and myofiber diameters were done as previously described.^{32,47,66} Determinations of the presence of central nuclei versus CT carbohydrate overexpression were done at or near the midsection of infected skeletal muscles, near their widest diameter. All myofibers were counted in each section analyzed, and all data obtained was used in determinations of significance. Averages of central nuclei represent analysis of individual myofibers where n is always a single muscle from a unique animal, with the exception of AAV experiments, in which the AAV-Galgt2 and mock-infected muscles were taken from the same animal (ipsilateral and contralateral muscles of the same type). All immunostaining was done as previously described.^{32,47,51,66} Glycolipids were extracted from sections as previously described.⁴⁹ Identical time exposures were used for all comparisons of immunostaining.

Serum Creatine Kinase Assays

Blood was collected from the tail vein and allowed to clot for 1 hour at 37°C. Clotted cells were centrifuged at 1500 $\times g$ for 3 minutes, and serum was collected and analyzed without freezing. Creatine kinase activity assays were done using an enzyme-coupled absorbance assay kit (CK-SL; Diagnostic Chemicals Limited; Charlottetown, PEI, Canada) according to the manufacturer's instruc-

tions. Absorbance was measured at 340 nm every 30 seconds for 4 minutes at 25°C to calculate enzyme activity. All measurements were done in triplicate.

Infection of Muscles with Adeno-Associated Virus Containing Galgt2 cDNA (AAV-Galgt2)

The tibialis anterior, gastrocnemius, or quadriceps muscle on the left side of 2-week-old dy^{w}/dy^{w} or wild-type ($dy^{w}/+$) mice were injected with 1×10^{10} vector genomes (vg) of AAV1-Galgt2 or rh.74-Galgt2. AAV vectors were produced and purified using the triple transfection method as previously described.⁶⁷ The mouse CT GalNAc transferase gene (Galgt2) was expressed using a cytomegalovirus promoter. Although not muscle-specific, the preferential uptake of AAV into skeletal myofibers led primarily to myotube-specific expression using the intramuscular injection protocol. Gastrocnemius and quadriceps muscles were injected in a volume of 50 μ l of sterile phosphate-buffered saline (PBS) using a 0.3-cc insulin syringe, whereas tibialis anterior muscles were injected in a 25- μ l volume. Muscles were always injected at the midpoint of the belly of the muscle. Contralateral muscles (on the right side) were injected with sterile PBS alone. Some control infections were also done with AAV-lacZ or AAV-GFP to confirm that no changes came from nonspecific effects of AAV infection (not shown). After 1, 2, 3, 4, or 8 weeks, mice were sacrificed and muscles dissected and either snap-frozen in liquid nitrogen-cooled isopentane or placed in RNALater (Ambion, Austin, TX) for total RNA extraction.

Immunoblotting and Lectin Precipitation

Immunoblotting and lectin precipitations were done as previously described,^{32,47,51} with the exception that multiple extraction protocols were compared. To do this, we extracted identical weights (50 mg) of transgenic, AAV-Galgt2-infected, or control $dy^{w}/+$ or dy^{w}/dy^{w} muscles with Nonidet P-40 buffer (1% Nonidet P-40, 75 mmol/L Tris-HCl, pH 6.8, 150 mmol/L NaCl, 2 mmol/L ethylenediaminetetraacetic acid, and 1:200 protease inhibitor cocktail; Sigma, St. Louis, MO) or sodium dodecyl sulfate (SDS)/urea buffer (2% SDS, 4 mol/L urea, 75 mmol/L Tris-HCl, pH 6.8, 2 mmol/L ethylenediaminetetraacetic acid, and 1:200 protease inhibitor cocktail). Extractions were performed at 4°C with light shaking for 4 days. Protein amounts were measured from each extract using the Bio-Rad DC protein assay (Bio-Rad, Hercules, CA) versus standard curve made in the appropriate buffer. In all cases protein extraction was on the order of 2 to 4 mg/ml in a total volume of 2 ml per sample (or 4 to 8 mg protein extracted from the original 50 mg of skeletal muscle). For lectin pull-downs, all samples were dialyzed against 10,000 MW dialysis tubing (Pierce, Rockford, IL) against Nonidet P-40 buffer at a minimum of four exchanges of 1000-fold excess volume. After dialysis, protein levels were measured again, and 150 μ g of protein per sample was used for lectin precipitations, as before.^{32,47,51}

Table 1. TaqMan Gene Expression Assays Used for Real-Time PCR

	Gene name	Gene symbol	Applied Biosystems TaqMan assay identification no.*
1	Aggrin	Aggrn	Mm01545840_m1
2	Dystroglycan 1	Dag1	Mm00802400_m1
3	Dystrophin, muscular dystrophy	Dmd	Mm01216926_m1
4	Laminin α 1	Lama1	Mm01226098_m1
5	Laminin α 2	Lama2	Mm01193202_m1
6	Laminin α 4	Lama4	Mm01190521_m1
7	Laminin α 5	Lama5	Mm01222011_m1
8	Sarcoglycan, α (dystrophin-associated glycoprotein)	Sgca	Mm00486068_m1
9	Sarcoglycan, β (dystrophin-associated glycoprotein)	Sgcb	Mm00449389_m1
10	Sarcoglycan, δ (dystrophin-associated glycoprotein)	Sgcd	Mm00449392_m1
11	Sarcoglycan, γ (dystrophin-associated glycoprotein)	Sgcy	Mm00488741_m1
12	Utrophin	Utrn	Mm01168861_m1

*_m, TaqMan probe spans an exon-exon junction of the associated genes and will not detect genomic DNA.

Enzyme-Linked Immunosorbent Assay Assays

Twenty μ g of protein extracted via different detergent methods and dialyzed against Nonidet P-40 buffer was immobilized on 96-well enzyme-linked immunosorbent assay plates (Nunc, Rochester, NY) by dilution into excess 50 mmol/L sodium bicarbonate, pH 9.5, and incubation overnight at 4°C. Some comparisons were done using nitrocellulose-coated plates, with similar results. Wells were washed with TBST (20 mmol/L Tris, pH 7.4, 100 mmol/L NaCl, and 0.02% Tween 20) and blocked in TBST with 3% bovine serum albumin. Wells were incubated with antibodies against α -dystroglycan (IIH6) or CT carbohydrate (CT2), washed in TBST, incubated with a horseradish peroxidase-conjugated goat anti-mouse IgM secondary antibody, washed, and developed in substrate buffer (50 mmol/L Na₂HPO₄, 25 mmol/L citric acid, 0.1% α -phenylenediamine dihydrochloride, and 0.03% H₂O₂). Absorbance was read at 450 nm and relative binding determined as before.⁶⁸ Addition of secondary antibody alone never gave a signal that exceeded 5% of that for the primary antibody, and this signal was subtracted in all instances.

Quantitative Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR) Measurements

Gastrocnemius or quadriceps muscles were dissected out under RNase-free conditions and stored overnight at 4°C in RNALater (Ambion). After decanting the RNALater, tissues were kept frozen at -80°C until RNA extraction. Total RNA was isolated using Trizol reagent (Invitrogen, Carlsbad, CA) and further purified on a silica-gel-based membrane (RNeasy-Mini; Qiagen, Valencia, CA). RNA integrity 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 analysis. This criterion excluded one sample from analysis.

A high capacity cDNA archive kit (Applied Biosystems, Foster City, CA) was used to reverse transcribe 3 μ g of total RNA following the instructions provided. Samples

were subjected to real-time PCR in triplicate, on a TaqMan ABI 7500 sequence detection system (Applied Biosystems) with 18S ribosomal RNA (product no. 4308329, Applied Biosystems) as internal control. Primers and probe against CT GalNAc transferase were custom-made by Applied Biosystems and provided as a 20 $\times$ reaction mix containing 18 μ mol/L each of primers (forward primer sequence: 5'-GATGTCTGGAGAAAACCGAACT-3'; reverse primer sequence: 5'-GCAGCCTGAAGTGGTAAGTATTCC-3') and 5 μ mol/L of probe (probe sequence: 5'-CCGCCACCATCC-3'). All other primers and probes were purchased as predeveloped 20 $\times$ TaqMan assay reagents from Applied Biosystems, and the details are provided in Table 1. 18S ribosomal RNA probe contained VIC dye as the reporter whereas all other probes had FAM reporter dye at the 5' end. Each 25- μ l PCR reaction mix consisted of 1 $\times$ primer-probe mix, 1 $\times$ TaqMan Universal PCR master mix with AmpliTaq Gold DNA polymerase, uracil-N-glycosylase (AmpErase), dNTPs with dUTP, and a passive reference to minimize background fluorescence fluctuations (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^{- $\Delta\Delta$ Ct} method,⁶⁹ and the data are presented as fold difference normalized to 18S ribosomal RNA. All measures were done in triplicate for each data point.

Lipid Extraction, High-Performance Thin Layer Chromatography (HP-TLC) Separation, and Antibody Overlay

Glycolipids were extracted twice from 500 mg of pooled skeletal muscle samples (gastrocnemius, tibialis, quadriceps, and triceps) of varying genotypes for Galgt2 transgenic mice and various dy^w controls. For AAV-Galgt2-infected and mock-infected muscles, only quadriceps and gastrocnemius muscles were used, in which each muscle was infected with 1 $\times$ 10¹⁰ vg for 8 weeks each. Muscle glycolipids were extracted in 10 volumes of

Figure 1. Galgt2 transgenic *dy^W/dy^W* skeletal muscles have reduced muscle size and reduced muscle pathology. Cross sections of gastrocnemius and tibialis anterior muscles from age-matched *dy^W/+*, Galgt2 transgenic (CT) *dy^W/+*, *dy^W/dy^W*, and *dy^W/dy^W/CT* mice were stained with H&E. Nuclei (darker spots, **arrows**) remain centrally localized in dystrophic *dy^W/dy^W* muscles that have undergone a cycle of degeneration and regeneration but are primarily absent from *dy^W/dy^W/CT* muscle. Scale bar = 50 μ m.

CHCl_3 :MeOH:H₂O (4:8:3, v/v/v) with vigorous agitation. Samples were centrifuged, and the supernatants were combined and re-extracted with CHCl_3 :MeOH:H₂O (4:8:5.6, v/v/v). The final volume was then adjusted by evaporating the upper phase. Glycolipid quantitation was performed by a resorcinol assay with GM₂ as a standard. Some lipid profiles were run after purification using anion exchange resin to confirm the presence of CT antigen on charged (presumably sialylated) glycolipids (not shown).

Lipid extracts (20 μ g) were spotted on the stacking phase of a HP-TLC plate (Silica 60 A; size, 10 $\times$ 10 cm; thickness, 200 μ m; Whatman, Florham Park, NJ) and chromatographed using a solvent system containing CHCl_3 :MeOH:0.05% CaCl₂ (50:40:10, v/v/v) with the gangliosides GM₁, GM₂, GM₃, GD_{1a}, and GT_{1b} (Calbiochem or Sigma) loaded on separate lanes as standards.⁷⁰ Lipids run on one HP-TLC plate were visualized with resorcinol-HCl reagent to identify all glycolipids, while lipids loaded on another HP-TLC plate were immunostained for CT carbohydrate. For immunostaining, chromatographed HP-TLC plates were dried and dipped in hexane, followed by 0.01% PIBM (polyiso-butyl-methacrylate). The plates were sprayed with PBS; blocked with 1% bovine serum albumin in PBS for 2 hours, and exposed overnight to anti-CT carbohydrate monoclonal antibody (CT2, 1:10 in 1% bovine serum albumin/PBS). Peroxidase-conjugated goat anti-mouse IgM (1:2000) in 1% bovine se-

rum albumin/PBS and the chromogenic VIP vector substrate kit (Vector Laboratories, Burlingame, CA) were used to visualize the lipid bands containing the CT carbohydrate.

Statistics

Determinations of significance were done using a paired Student's *t*-test; **P* < 0.05, ***P* < 0.01, and ****P* < 0.001.

Results

Overexpression of Galgt2 Inhibits the Extent of Muscle Pathology in Transgenic *dy^W/dy^W* Mice

We crossed CT GalNAc transferase (Galgt2) transgenic mice that we had previously shown inhibited muscular dystrophy in *mdx* mice,³² a model of DMD, to *dy^W/dy^W* mice to determine whether Galgt2 overexpression would be similarly effective in an animal model for laminin α 2-deficient muscular dystrophy (MDC1A). We analyzed cross sections of skeletal muscles by staining with H&E (Figure 1) and quantified muscle growth (Figure 2A) and muscle pathology (Figure 2B) by analyzing stained muscle sections. Much as we had seen with Galgt2 transgenic *mdx* mice,³² myofiber diameters were significantly reduced in Galgt2 transgenic *dy^W/dy^W* muscles when compared with age-matched nontransgenic *dy^W/dy^W* littermates (Figures 1 and 2A). Some hypertrophic myofibers were evident in *dy^W/dy^W* muscles, as were regions with smaller regenerating myofibers with centrally located nuclei, both evidence of dystrophic muscle pathology.⁷¹ By contrast, little to no muscle pathology was evident in Galgt2 transgenic *dy^W/dy^W* muscles (Figure 1). The level of reduction in myofiber diameter between Galgt2 transgenic *dy^W/+* and *dy^W/+* littermates and Galgt2 transgenic *dy^W/dy^W* and *dy^W/dy^W* littermates was approximately equivalent for the gastrocnemius, quadriceps, diaphragm, triceps, and tibialis anterior muscles (Figure 2A). The level of reduction in myofiber diameters in Galgt2 transgenic animals also correlated with reduced mouse weight. By 6 weeks of age, *dy^W/dy^W/CT* mice were reduced in weight by $43 \pm 3\%$ compared with *dy^W/dy^W* animals (*P* < 0.001, *n* = 6 to 13 animals), whereas *dy^W/+/CT* mice were reduced in weight by $37 \pm 2\%$ compared with *dy^W/+* mice (*P* < 0.001, *n* = 9 to 12 animals). As previously seen, *dy^W/dy^W* mice were reduced in growth as well compared with *dy^W/+* animals (by $45 \pm 2\%$, *P* < 0.001, *n* = 12 to 13 animals per condition). This, however, did not correlate with reduced muscle size (Figure 2A). *dy^W/dy^W/CT* animals weighed less than 6 g in weight at 6 weeks of age (5.8 ± 0.4 g compared with 18.6 ± 0.8 g for *dy^W/+*, *P* < 0.001). The extremely small size of these transgenic *dy^W/dy^W* animals made it impossible to compare issues related to longevity in this model; however, an assessment of the extent of muscle pathology was possible.

To assess pathology associated with muscular dystrophy, we quantified the percentage of myofibers with central nuclei in transgenic *dy^W/dy^W* animals and com-

Figure 2. Quantitation of muscle growth and muscular dystrophy in Galgt2 transgenic dy^W/dy^W skeletal muscles. **A:** Galgt2 transgenic mice (CT) had reduced myofiber diameters in both the $dy^W/+$ and dy^W/dy^W background at 5 weeks of age. P values are for $dy^W/+$ /CT versus $dy^W/+$ or for dy^W/dy^W /CT versus dy^W/dy^W . **B:** Galgt2 transgenic dy^W/dy^W myofibers had fewer myofibers with central nuclei at 5 weeks of age. P values are for dy^W/dy^W /CT versus dy^W/dy^W . TA, tibialis anterior. **C:** dy^W/dy^W mice had elevated creatine activity in their serum at 5 weeks of age, whereas Galgt2 transgenic dy^W/dy^W mice had significantly reduced levels compared with dy^W/dy^W littermates. P values are for dy^W/dy^W /CT versus dy^W/dy^W . Errors are SEM for 3 to 6 animals in which data were collected from 250 myofibers per measurement in **A** and **B** and SEM for $n = 4$ to 13 animals per genotype in **C**.

pared these to age-matched dy^W/dy^W littermates at 5 weeks of age (Figure 2B). Galgt2 transgenic $dy^W/+$ and nontransgenic $dy^W/+$ muscles were also assessed to determine the extent of pathology in nondystrophic muscle. In rodents, nuclei are present in the middle of the myofiber early in development and migrate to the periphery as the muscle matures, such that less than 5% of myofibers have central nuclei in the adult animal.^{72–74} When dystrophic rodent muscles are damaged and induced to regenerate, however, nuclei remain in the center of the myofiber in the regenerated muscle for almost the remainder of the lifetime of the animal, providing an essentially indelible marker of the event.⁷⁵ Therefore, the presence of increased numbers of myofibers with central nuclei in dystrophic mice is a robust indicator of cycles of muscle degeneration and regeneration that result from muscular dystrophy. By 5 weeks of age, all dy^W/dy^W muscles analyzed had a significant increase in central nuclei compared with their nondystrophic $dy^W/+$ littermates (Figure 2B). Galgt2 transgenic dy^W/dy^W muscles all had significantly reduced levels of central nuclei when compared with dy^W/dy^W littermates. Thus, Galgt2 transgene overexpression inhibited the development of central nuclei in dy^W/dy^W muscles, much as we had seen before in Galgt2 transgenic mdx muscles.³² Unlike transgenic mdx mice, however, the reduction in central nuclei, with the exception of the diaphragm, was not reduced to the level found in wild-type ($dy^W/+$) or transgenic wild-type ($dy^W/+$ /CT) animals.

Dystrophic muscles, when damaged, release muscle enzymes such as creatine kinase (CK) into the serum. Serum CK activity, therefore, is a good measure of global muscle damage in the animal. Galgt2 transgenic $dy^W/+$ mice, which are not dystrophic, had serum CK activity levels that were indistinguishable from their nontransgenic $dy^W/+$ littermates. dy^W/dy^W mice, by contrast, had serum CK levels that were approximately four times wild-type levels ($P < 0.001$). Galgt2 transgenic dy^W/dy^W mice had serum CK activity levels that were reduced by $54 \pm$

9% compared with dy^W/dy^W littermates ($P < 0.01$) (Figure 2C), and the level of increased CK activity (relative to $dy^W/+$) was reduced by $74 \pm 12\%$ ($P < 0.01$ for dy^W/dy^W /CT compared with dy^W/dy^W). As with measures of muscle pathology, however, the level of serum CK activity in dy^W/dy^W /CT animals remained higher than that of wild-type littermates, unlike mdx/CT animals, in which both measures did not differ from wild type.³²

AAV Delivery of the Galgt2 Transgene to Postnatal dy^W/dy^W Muscles Inhibits Muscle Pathology without Altering Muscle Growth

The effect of Galgt2 transgene expression on muscle growth (Figures 1 and 2A) complicated the interpretation of the muscular dystrophy findings. CT carbohydrate overexpression most likely impacts muscle growth via its effects on satellite cell biology, and there is an order of magnitude more satellite cells in Galgt2 transgenic muscles than in control animals.⁴⁷ Most of these cells normally would fuse into myotubes to contribute to their robust growth in the early postnatal period, but they fail to do so in Galgt2 transgenic animals, most likely attributable to embryonic overexpression of the transgene.⁴⁷ Because satellite cell fusion primarily occurs in the first 2 postnatal weeks, we decided to induce Galgt2 transgene expression at 2 weeks of age to bypass this period of development. We recently showed that this strategy could divorce the therapeutic effects of Galgt2 overexpression from its developmental effects in mdx animals.⁶²

We used AAV to deliver the Galgt2 transgene (AAV-Galgt2) to dy^W/dy^W skeletal muscles. Most experiments were done using AAV1 serotype, although some were reproduced using the AAV8-like rhesus 74 (rh.74) serotype. Because it takes approximately a week before single-stranded AAV vectors begin to induce transgene expression,⁷⁶ Galgt2 overexpression would not commence until 3 weeks of age. We first verified that this was the case by performing a time course for

Figure 3. Time course of Galgt2 overexpression and CT carbohydrate expression in AAV-Galgt2-infected dy^w/dy^w skeletal muscles. **A:** Real-time PCR measurements were made at 1, 2, 3, 4, and 8 weeks after AAV-Galgt2 infection of dy^w/dy^w skeletal muscle (using AAV1 serotype). Galgt2 overexpression was significant at 1 week after infection and peaked by 4 weeks after infection, remaining high thereafter. *P* values are all compared with the 0 time point. **B:** CT carbohydrate overexpression, identified by staining with the CT2 antibody, paralleled Galgt2 gene overexpression, being evident by 1 week and maximal by 3 to 4 weeks of age. Note that although CT carbohydrate is expressed at 0 weeks at the neuromuscular junction and in capillaries, it was not evident at the time exposures used to observe overexpression at this low-level magnification. Secondary only mAb control is shown for 4 weeks. **C:** Longitudinal section of skeletal muscle (tibialis anterior) at 8 weeks after infection demonstrates that CT carbohydrate overexpression was maintained in most myofibers along their length. Scale bars = 100 μ m.

Galgt2 overexpression (Figure 3). dy^w/dy^w animals injected at 2 weeks of age were analyzed at 1, 2, 3, 4, or 8 weeks after infection for levels of Galgt2 gene

overexpression (via TaqMan qRT-PCR, Figure 3A) and CT carbohydrate overexpression, as assessed by immunostaining (Figure 3, B and C). As expected, based on the work of Danos and colleagues,^{77,78} AAV1-Galgt2 infection of skeletal muscles resulted in significant Galgt2 gene expression and CT carbohydrate overexpression by 1 week of age, and this peaked by 3 to 4 weeks of age and remained high thereafter. At 3 to 4 weeks after infection, CT carbohydrate could be overexpressed in almost all myofibers (Figure 3B), although there was variability in the percentage of myofibers infected in some instances. When the percentage of myofibers with overexpression was high, CT carbohydrate was overexpressed along the entire longitudinal length of the infected myofibers in most instances (Figure 3C). This occurred despite the fact that skeletal muscles were infected at the midsection in the belly of the muscle. Analysis of muscles by serial cross-sectioning along the entire length from the midsection of AAV-Galgt2-infected tibialis anterior muscles showed that at least 90% of myofibers overexpressing CT carbohydrate at their midpoint maintained overexpression 450 μ m anteriorly or posteriorly along their longitudinal axis ($n = 3$ animals, not shown). Thus, although cross-sectional analysis was done using sections taken at or near the site of injection, similar results were obtained along the entire longitudinal length of the muscle.

We next analyzed the extent of muscular dystrophy in CT-overexpressing dy^w/dy^w myofibers and compared these to myofibers from the same muscle that were not overexpressing CT carbohydrate (Figures 4 and 5). CT carbohydrate overexpression was assessed with the CT2 monoclonal antibody and central nuclei by co-staining the same section in a different fluorescence channel (Figure 4). Identical results were found when we analyzed serially stained sections for CT2 immunofluorescence and analyzed central nuclei by H&E staining of adjacent sections (not shown). dy^w/dy^w myofibers that overexpressed the CT carbohydrate had significantly reduced numbers of central nuclei as compared with myofibers not overexpressing CT carbohydrate or with myofibers in the mock-infected contralateral limb (Figures 4 and 5A). The percentage of myofibers with central nuclei in CT-overexpressing myofibers approached the baseline level for mock-infected dy^w/dy^w myofibers at 3 weeks of age, the time at which Galgt2 overexpression began. By 10 weeks of age (8 weeks after infection), the level of central nuclei in CT-overexpressing myofibers was significantly reduced ($P < 0.001$ for tibialis and gastrocnemius) compared with nonoverexpressing myofibers (Figure 5A). Therefore, Galgt2 overexpression inhibits the development of muscular dystrophy in dy^w/dy^w muscles when expressed in the early postnatal period.

Unlike embryonic overexpression in Galgt2 transgenic dy^w/dy^w animals, postnatal overexpression of Galgt2 did not inhibit muscle growth (Figure 5B). In fact, CT-overexpressing myofibers were larger, on average, than nonoverexpressing myofibers (Figure 5B). This increase in myofiber size was attributable to the selective presence

Figure 4. Postnatal CT carbohydrate overexpression after AAV-Galgt2 infection inhibits muscle pathology but not muscle growth. AAV-Galgt2-infected dy^w/dy^w myofibers were analyzed for CT carbohydrate overexpression (using CT2 immunostaining, green) and for the presence of central nuclei (red). Several central localized myofiber nuclei are indicated with **white arrows** (F). Most CT-overexpressing myofibers did not have central nuclei. Scale bars: 100 μm (A–E); 50 μm (F).

of smaller regenerating myofibers in the nonoverexpressing pool, which are presumably enriched because of increased dystrophy in this population (eg, Figure 4). These AAV experiments show that the effects of Galgt2 overexpression on muscle growth can be divorced from its therapeutic effects regarding muscular dystrophy if the transgene is overexpressed in the early postnatal period.

We have found similar results in *mdx* muscles when AAV-Galgt2 was infected at similar times in postnatal

animals.⁶² In those experiments, however, Galgt2 overexpression had an absolute effect with regard to inhibition of muscular dystrophy.⁶² Galgt2 overexpression in dy^w/dy^w muscles, by contrast, although highly significant, did not reach baseline wild-type levels in either Galgt2 transgenic dy^w/dy^w muscles (Figure 2B) or AAV-Galgt2-infected dy^w/dy^w muscles (Figure 5A). One explanation for this difference would be that there was less Galgt2 overexpression in dy^w/dy^w muscles than in *mdx* muscles. To assess this, we compared

Figure 5. Quantitation of myofiber diameters and central nuclei in AAV-Galgt2-infected dy^W/dy^W skeletal muscles. dy^W/dy^W skeletal muscles were infected with AAV-Galgt2 at 2 weeks of age and analyzed at 10 weeks of age. **A:** The percentage of myofibers with central nuclei was significantly reduced in both the gastrocnemius and tibialis anterior (TA) muscles in CT-overexpressing myofibers. **B:** CT-overexpressing myofibers did not have reduced myofiber diameters. Errors are SEM measured from three to six animals per genotype in which data were collected from 250 myofibers per measurement. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$.

levels of endogenous Galgt2 expression in mdx and dy^W/dy^W muscle (Figure 6A) and relative levels of Galgt2 overexpression in Galgt2 transgenic and AAV-Galgt2-infected mdx and dy^W/dy^W muscle (Figure 6B). Interestingly, endogenous Galgt2 expression was significantly increased in both mdx and dy^W/dy^W muscle (3.2 ± 0.1 -fold, $P < 0.01$ and 36 ± 1 -fold, respectively, $P < 0.01$ for both), suggesting that endogenous Galgt2 expression in diseased muscle tissue may ameliorate the extent of muscular dystrophy in these two models to some degree. Endogenous levels of Galgt2 expression in transgenic control backgrounds for mdx or dy^W mice were the same; however, Galgt2 transgenic $dy^W/+$ muscle had slightly increased (approximately twofold) Galgt2 expression compared with Galgt2 transgenic C57BL/10 muscle, the mdx strain control (Figure 6B). By contrast, Galgt2 was expressed 60-fold less in Galgt2 transgenic dy^W/dy^W muscle than in transgenic mdx muscle and 32-fold less in Galgt2 transgenic dy^W/dy^W muscle than in Galgt2 transgenic $dy^W/+$ muscle (Figure 6B). Thus, the skeletal α -actin

Figure 6. Endogenous Galgt2 gene expression is increased in mdx and dy^W/dy^W muscle and the Galgt2 transgene is differentially overexpressed in the two disease models. **A:** Galgt2 gene expression was measured by Taq-Man RT-PCR in mdx and dy^W/dy^W skeletal muscle compared with strain-specific control littermates. Endogenous Galgt2 expression is increased in both muscular dystrophy models. Errors are SEM for $n = 12$ to 18 animals per condition. **B:** Galgt2 transgenic dy^W/dy^W skeletal muscle has 60-fold less Galgt2 gene expression than Galgt2 transgenic mdx skeletal muscle, and AAV-Galgt2-infected muscles are also reduced (4.7-fold). Errors are SEM for $n = 6$ to 18 animals per condition. All data are from gastrocnemius muscles of 6- to 8-week-old animals. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$.

promoter used to drive transgene expression may be less active in dy^W/dy^W muscle. Even with AAV-Galgt2 infection, in which Galgt2 transgene expression is driven by a cytomegalovirus promoter, Galgt2 expression was reduced (by 4.7-fold) in dy^W/dy^W muscle compared with mdx (Figure 6B). Thus, Galgt2 overexpression was lower in dy^W/dy^W muscle than in mdx muscle, and this may explain the difference in therapeutic effectiveness.

α -Dystroglycan Glycosylation with the CT Carbohydrate Is Stimulated in Galgt2 Transgenic dy^W/dy^W Skeletal Muscles but Not in AAV-Galgt2-Infected Muscles

Because we had determined that Galgt2 overexpression inhibited the development of muscle pathology in dy^W/dy^W

$dy^{w/w}$ animals, we next wished to determine whether the molecular changes we attributed to the transgene's effectiveness in mdx mice³² also occurred in transgenic $dy^{w/w}/dy^{w/w}$ skeletal muscles. In mdx mice and in wild-type mice, overexpression of Galgt2 stimulates glycosylation of α -dystroglycan in skeletal muscle with the CT carbohydrate, and α -dystroglycan is the predominant glycoprotein modified with the CT carbohydrate in transgenic mdx skeletal muscles.^{32,47} As before, we took advantage of the fact that carbohydrate-binding lectins can be used to distinguish CT-glycosylated and non-CT-glycosylated forms of α -dystroglycan in skeletal muscle. Wheat germ agglutinin (WGA) is a lectin that binds sialic acid/GlcNAc and can be used to precipitate endogenous α -dystroglycan from nontransgenic muscles.⁵² By contrast, *Wisteria floribunda* agglutinin (WFA) is a lectin that binds β GalNAc, including that present on the CT carbohydrate,⁷⁹ and does not precipitate non-CT glycoforms of α -dystroglycan.^{32,47} Although WFA binds β GalNAc structures in addition to the CT carbohydrate, it is a far more reliable reagent for identifying β GalNAc-containing glycoproteins than the anti-CT antibodies when the starting material is a complex protein mixture.⁴⁷ Therefore, we solubilized total muscle cell protein from $dy^{w/w}/+$, $dy^{w/w}/dy^{w/w}$, $dy^{w/w}/+/CT$, $dy^{w/w}/dy^{w/w}/CT$, and AAV-Galgt2-infected $dy^{w/w}/dy^{w/w}$ skeletal muscles in Nonidet P-40, a nonionic detergent (as before⁴⁷), and precipitated 150 μ g of protein lysate with WGA or WFA agarose. As with previous transgenic mdx experiments, we found that Galgt2 overexpression stimulated glycosylation of α -dystroglycan with the CT carbohydrate, although less glycosylation was evident in $dy^{w/w}/dy^{w/w}$ than in $dy^{w/w}/+$ muscles (Figure 7). This may be attributable to the 32-fold reduced level of Galgt2 gene expression in transgenic $dy^{w/w}/dy^{w/w}$ muscle as compared with transgenic $dy^{w/w}/+$ muscle (Figure 6B). Immunoblotting with anti-CT antibody showed that α -dystroglycan was the primary, if not exclusive, glycoprotein modified with the CT carbohydrate in both transgenic $dy^{w/w}/dy^{w/w}$ and $dy^{w/w}/+$ skeletal muscles (Figure 7).

Although embryonic overexpression of Galgt2 in transgenic $dy^{w/w}/dy^{w/w}$ muscles led to robust glycosylation of α -dystroglycan with the CT carbohydrate, postnatal overexpression after infection with AAV-Galgt2 did not (Figure 7). We compared proteins from a gastrocnemius muscle injected with 2×10^{10} vg AAV-Galgt2, where more than half of the myofibers overexpressed CT carbohydrate, to contralateral limb muscles where no overexpression had occurred. Although a small increase in α -dystroglycan was present in the AAV-Galgt2-infected WFA precipitate, this precipitated protein did not contain increased levels of the CT carbohydrate. Therefore, postnatal overexpression of Galgt2, although able to inhibit muscular dystrophy, did not increase glycosylation of α -dystroglycan with the CT carbohydrate. This result is similar to our recent experiments with AAV-Galgt2 overexpression in mdx skeletal muscles.⁶²

Were α -dystroglycan glycosylation with the CT antigen able to decrease its solubility in the membrane, it is possible that we may have failed to identify glycosylated proteins using a nonionic detergent such as Nonidet P-40. Therefore, we compared the extraction

Figure 7. α -Dystroglycan glycosylation with the CT carbohydrate is increased in Galgt2 transgenic $dy^{w/w}/dy^{w/w}$ muscles but not after postnatal infection with AAV-Galgt2. Identical amounts of skeletal muscle Nonidet P-40 protein homogenates were precipitated by WGA agarose, a lectin that binds the GlcNAc/sialic acid, or by *Wisteria floribunda* agglutinin (WFA) agarose, a GalNAc-binding lectin that recognizes the CT carbohydrate. Precipitates were blotted for α -dystroglycan (α DG), β -dystroglycan (β DG), or CT2, an anti-CT carbohydrate antibody. α -Dystroglycan glycosylation with the CT carbohydrate was increased in Galgt2 transgenic muscles but not in AAV-Galgt2-infected muscles. α -Dystroglycan appears broader than CT carbohydrate because it was separated on a 6% SDS-PAGE gel, to allow greater resolution of its glycosylation, whereas β -dystroglycan and CT2 blots were separated on a 12% SDS-PAGE gel, to allow visualization of greater numbers of proteins.

of muscle samples in nonionic detergent (1% Nonidet P-40) to a denaturing detergent solution (2% SDS with 4 mol/L urea) (Figure 8). Levels of extracted CT carbohydrate and α -dystroglycan were measured using an enzyme-linked immunosorbent assay (Figure 8, A and B). There was significantly more CT carbohydrate extracted with SDS/urea than with Nonidet P-40 alone in all muscles (Figure 8A), although the amount of α -dystroglycan extracted under the two detergent conditions was not significantly changed (Figure 8B). Other proteins known to be relatively inert to solubilization in nonionic detergent, such as dystrophin, were far more abundant in the SDS/urea samples (not shown). If SDS/urea samples were dialyzed against 1% Nonidet P-40 buffer and subjected to WFA lectin pull-downs, however, there was no substantial difference in the results compared with samples extracted in Nonidet P-40 alone (Figure 8C); α -dystroglycan was still the primary glycoprotein glycosylated in Galgt2 transgenic $dy^{w/w}/dy^{w/w}$ muscle, whereas no CT-glycosylated α -dystroglycan was found in AAV-Galgt2-infected $dy^{w/w}/dy^{w/w}$ muscle (Figure 8C). We did identify a second CT-positive

Figure 8. Ionic and nonionic detergent extraction yield similar results with respect to protein glycosylation in Galgt2 transgenic and AAV-Galgt2-infected dy^W/dy^W skeletal muscles. **A** and **B**: Enzyme-linked immunosorbent assay were done for CT carbohydrate (**A**, using CT2) or α -dystroglycan (**B**, using IIH6) using 20 μ g of skeletal muscle cell lysate made by extraction with nonionic detergent (1% Nonidet P-40) or with ionic denaturing detergent (2% SDS with 4 mol/L urea). Errors are SD for $n = 3$ to 6 animals. **C**: SDS-urea-extracted glycoproteins were dialyzed against 1% Nonidet P-40 and compared with Nonidet P-40-extracted samples (Figure 7) for the ability of the GalNAc-binding lectin WFA to precipitate CT-glycosylated glycoproteins. No significant change was observed compared with extraction in Nonidet P-40 alone.

band in all SDS/urea lysates at 95 to 100 kD that was not specific to transgenic muscles (Figure 8C). Because CT carbohydrate is present in capillaries,⁴⁷ this may represent a blood vessel protein. Similar results were also found for Galgt2 transgenic and AAV-Galgt2-infected mdx muscle (not shown).

Galgt2 Overexpression Stimulates CT Glycosylation of a Glycolipid

Although it seemed counterintuitive that CT antigen could be overexpressed in AAV-Galgt2-infected muscle yet not be present on any glycoproteins, Galgt2 overexpression has been reported to selectively glycosylate a glycolipid in a tumor cell line.⁸⁰ To assess if Galgt2 overexpression resulted in changes in glycolipid glycosylation in skeletal muscle, we extracted glycolipids from skeletal muscles and performed antibody overlays to detect CT carbohydrate (Figure 9A). In addition, we assessed the contribution of glycolipids to CT carbohydrate overexpression by comparing CT immunostaining before and after lipid extraction of muscle sections (Figure 9B). In both instances, we found evidence of increased glycosylation of a glycolipid with the CT carbohydrate in Galgt2 transgenic and AAV-Galgt2-infected skeletal muscle. Increased CT glycosylation was identified on a single glycolipid that migrated differently from ganglioside standards, including GM3, GM2, GM1, GD1a, and GT1b (Figure 9A). Of particular note, no increase was observed in GM2 ganglioside levels, which is consistent with previous reports that Galgt2 does not synthesize this glycolipid.^{48,80} Extraction of lipids from muscle sections showed that significant levels of CT immunostaining remained in Galgt2 transgenic dy^W/dy^W and mdx muscle (Figure 9, B and C, respectively). Thus, glycoproteins such as α -dystroglycan probably contribute to CT antibody staining in such muscles. By contrast, very little CT immunostaining was evident in AAV-Galgt2-infected dy^W/dy^W or mdx myofibers (Figure 9, B and C, respectively), suggest-

ing that postnatal Galgt2 overexpression occurs primarily on glycolipids. These data are consistent with the lack of identified glycoproteins in AAV-Galgt2-infected muscles (Figures 7 and 8C) and are the first demonstration that Galgt2 overexpression stimulates the glycosylation of a glycolipid in any tissue.

Expression of Utrophin, Agrin, and Laminin α Chains in Galgt2 Transgenic and AAV-Galgt2-Infected dy^W/dy^W Skeletal Muscles

Galgt2 overexpression stimulates the ectopic expression of utrophin in wild-type and in mdx muscle.^{32,47} Galgt2 transgenic mdx muscles also have increased expression of utrophin-associated glycoproteins, including dystroglycan and sarcoglycans,³² which are normally down-regulated along the mdx myofiber membrane.⁸¹ We therefore determined if utrophin and its associated glycoproteins would be increased in Galgt2 transgenic dy^W/dy^W muscles by immunostaining (Figure 10A) and immunoblotting (Figure 11). Utrophin immunostaining was increased along Galgt2 transgenic dy^W/dy^W myofibers. CT2 immunostaining also was highly overexpressed in transgenic muscles, as expected (Figure 10A). In non-transgenic dy^W/dy^W muscle, by contrast, utrophin expression remained confined primarily to synaptic regions of the muscle membrane, although its expression was higher on myofibers than in $dy^W/+$ muscle (Figure 10B). Similarly, in $dy^W/+$ muscles, CT2 primarily stained blood vessels and neuromuscular synapses (Figure 10B), both as previously observed.^{49,82} By contrast, in dy^W/dy^W muscles, CT2 expression was increased on some myofibers, much as for mdx,³² but was also increased in mononuclear cells near sites of inflammation (Figure 10B). Thus, the increase in endogenous Galgt2 gene expression in dy^W/dy^W muscle (Figure 6A) includes a significant component from nonmuscle cells. The total

amount of utrophin protein was also increased in transgenic dy^w/dy^w muscles (Figure 11). We also observed a slight increase in α - and β -dystroglycan expression (both by staining and blotting) in transgenic dy^w/dy^w muscle. Expression of laminin $\alpha 4$, laminin $\alpha 5$, and agrin were complicated by the fact that they are all increased, to some extent, in dy^w/dy^w muscle as compared with $dy^w/+$ (Figure 10B), much as previously seen.^{43,56,61} Agrin expression, however, was far more elevated in Galgt2 transgenic dy^w/dy^w muscles, whereas laminin $\alpha 4$ and $\alpha 5$ were more modestly changed (Figures 10A and 11). Other muscle proteins, including α - and β -sarcoglycan, integrin $\alpha 7B$, and caveolin 3 were unchanged in transgenic dy^w/dy^w when compared with dy^w/dy^w littermates (not shown), whereas laminin $\alpha 1$ was not expressed in any intramuscular structure in skeletal muscles of any genotype examined (Figures 10A and 11).

We also determined whether increased levels of utrophin and agrin protein correlated with increased transcription of their cognate genes (Figure 12). In wild-type ($dy^w/+$) muscles, the Galgt2 transgene did increase transcription of utrophin and agrin, as well as β - δ sarcoglycan, dystrophin, and laminin $\alpha 2$. No significant increase was observed for laminin $\alpha 4$ or laminin $\alpha 5$, whereas laminin $\alpha 1$ signal could not be measured in skeletal muscle. Many of these same genes were also increased when comparing dy^w/dy^w muscle to $dy^w/+$ muscle. Here, laminin $\alpha 4$ transcripts were the most highly increased, but agrin, β - δ sarcoglycan, dystrophin, and utrophin were again significantly increased. By comparison, Galgt2 transgenic and AAV-Galgt2-infected dy^w/dy^w muscles did not significantly increase the expression of any of these genes, when compared with dy^w/dy^w muscle. These results suggest that the increase in utrophin and agrin protein in Galgt2 dy^w/dy^w muscle is not the result of increased transcription, although transcription of these genes was significantly increased in dy^w/dy^w muscles.

Last, we assessed whether a similar increase in utrophin and agrin expression occurred after postnatal Galgt2 overexpression in AAV-Galgt2-infected dy^w/dy^w skeletal muscles (Figures 11 and 13). In contrast to embryonic Galgt2 overexpression, myofibers infected with AAV-Galgt2 at 2 weeks of age and analyzed at 10 weeks of age showed no increase in utrophin expression in myofibers when compared with serial sections with clear overexpression of the CT carbohydrate (Figure 13). Neuromuscular expression of utrophin was still evident, however, providing a positive staining control within infected muscle sections. Similarly, no dramatic increase in laminin $\alpha 4$ or $\alpha 5$ was evident in

Figure 9. Galgt2 overexpression in skeletal muscle increases the glycosylation of a glycolipid with the CT carbohydrate. **A:** Glycolipids were extracted from skeletal muscles and separated by high-performance thin layer chromatography, followed by CT2 antibody overlay to identify CT-glycosylated glycolipids. Galgt2 transgenic (Tg) skeletal muscles had a large increase in a single glycolipid (arrow) whose migration was distinct from that of control gangliosides. **B:** Cross sections of skeletal muscle were immunostained with CT carbohydrate antibody (CT2) before or after extraction of lipids from the section. Some CT staining remained in Galgt2 transgenic (Tg) dy^w/dy^w muscle after lipid extraction, whereas very little staining remained in AAV-Galgt2-infected dy^w/dy^w muscles. Arrows point to a few positively stained remaining myofibers. A, arteriole; V, vein. **C:** Similar results were obtained in Galgt2 transgenic and AAV-Galgt2-infected mdx muscle. Scale bars: 50 μ m [**B** (bottom) and **C**]; 25 μ m [**B** (top)].

Figure 10. Utrophin and agrin are overexpressed in Galgt2 transgenic dy^W/dy^W myofibers relative to dy^W/dy^W muscle. **A:** Cross sections of dy^W/dy^W skeletal muscle or Galgt2 transgenic (CT) dy^W/dy^W skeletal muscle were immunostained with antibodies to the indicated proteins. Galgt2 transgenic muscle had increased expression of utrophin and agrin along extrasynaptic regions of the sarcolemma, whereas laminin $\alpha 1$ was not expressed. **B:** Staining compares dy^W/dy^W and $dy^W/+$ muscles. **Arrows** point to aggregates of CT-stained mononuclear cells, which are probably immune cell infiltrates within dystrophic muscle. nmj labels neuromuscular junction, the CT-positive structure just to the left of the label. Scale bars = 50 μm (**A, B**).

AAV-Galgt2-infected myofibers (Figure 13), although some increase in protein expression appeared to be present (Figure 11). As before, laminin $\alpha 1$ was not expressed in any AAV-Galgt2-infected muscles (Figures 11 and 13). Agrin, by contrast, was highly expressed in AAV-Galgt2-infected myofibers (Figure 13), and levels of agrin protein were increased by immunoblot in these muscles as well (Figure 11). Thus, muscle agrin protein was increased in response to both embryonic and postnatal overexpression of the Galgt2 transgene.

Discussion

The experiments presented demonstrate that overexpression of the CT carbohydrate by the Galgt2 transgene, either from embryonic time points onward in transgenic mice or from postnatal time points onward using AAV, is effective in inhibiting the development of skeletal muscle pathology in the dy^W/dy^W model for congenital muscular dystrophy 1A (MDC1A). Galgt2 overexpression, therefore, is therapeutic in mouse models for two forms of muscular dystrophy, having previously been shown to inhibit the development of skeletal muscle pathology in the mdx mouse model for DMD.^{32,62} By overexpressing Galgt2 postnatally, we have also shown that the inhibition of muscle pathology can be divorced from its effects on muscle growth; myofibers overexpressing the CT carbohydrate postnatally were not reduced in

size, in contrast to embryonic overexpression, yet inhibition of pathology was as robust. This too is similar to our recent findings using AAV to overexpressing Galgt2 in postnatal mdx muscle.⁶² In AAV-Galgt2-infected myofibers where a great majority of myofibers overexpress CT carbohydrate, little to no evidence of dystrophy is present in either model, suggesting that gene therapy may indeed be a viable approach using this transgene.

We have been able to use the fact that both embryonic overexpression of Galgt2 in transgenic mice and postnatal overexpression in AAV-Galgt2-infected muscle inhibit the development of muscle pathology to define candidates that may participate in Galgt2's therapeutic mechanism. For example, overexpression of utrophin can inhibit muscular dystrophy when overexpressed in mdx animals.^{28–30,83} Postnatal overexpression of Galgt2, however, does not stimulate overexpression of utrophin in infected mdx myofibers. Moreover, Galgt2 overexpression inhibits muscular dystrophy in utrophin-deficient mdx myofibers.⁶² Postnatal overexpression of Galgt2 in dy^W/dy^W skeletal muscle also did not increase the expression of utrophin, and we therefore presume that utrophin is not likely to be involved in Galgt2's effects. A similar parallel occurred with regard to CT glycosylation of α -dystroglycan: glycosylation was increased with embryonic overexpression but not with postnatal overexpression. This too is similar to previous results in mdx muscle.⁶² Durbeej and colleagues^{43,44} have shown that transgenic overexpression of laminin $\alpha 1$ can inhibit

Figure 11. Expression of proteins in Galgt2 transgenic and AAV-Galgt2-infected dy^w/dy^w muscles by immunoblotting. Whole-cell muscle protein lysates (20 μ g) were immunoblotted with the indicated antibodies. Utrophin protein was increased in Galgt2 transgenic (Tg) dy^w/dy^w skeletal muscle but not in AAV-Galgt2-infected dy^w/dy^w skeletal muscle, whereas agrin protein was increased in both. Molecular masses are 400 d for utrophin, agrin, laminin α 1, and dystrophin, 200 d for laminin α 4, 350 d for laminin α 5, 43 d for β -dystroglycan, ~160 to 180 d for α -dystroglycan, 42 kd for actin.

skeletal muscle pathology in dy mice. Here, our results seem quite clear. We found no expression of laminin α 1 in any skeletal myofibers of any genotype, making laminin α 1 unlikely to be involved. Indeed, although transgenic overexpression of laminin α 1 can ameliorate disease, both in dy skeletal muscle⁴³ and in other dy tissues,^{42,84} there is no evidence that this protein is naturally expressed in adult skeletal muscle.^{43,55}

Figure 12. Transcription of laminin α 4 and agrin is increased in dy^w/dy^w skeletal muscle but is not further increased in Galgt2 transgenic or AAV-Galgt2-infected dy^w/dy^w muscle. RNA was extracted from skeletal muscles and subjected to semiquantitative TaqMan RT-PCR for the indicated genes. No ECM genes or utrophin were increased in Galgt2 transgenic dy^w/dy^w or AAV-Galgt2-infected dy^w/dy^w when compared with dy^w/dy^w skeletal muscle. Laminin α 1 transcription was not present in skeletal muscle. Errors are SEM for $n = 3$ to 6 animals. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$.

In contrast to utrophin and laminin α 1, our results clearly point toward agrin as a potential mediator of Galgt2's therapeutic effects; agrin protein is increased with both embryonic and postnatal Galgt2 overexpression at levels above dy^w/dy^w muscle. Overexpression of a recombinant form of agrin, which links the C-terminal region of the protein to its laminin-binding domain (from the N terminus), has been shown by Ruegg and colleagues^{8,57} to inhibit the development of muscle pathology in dy animals. Xiao and colleagues⁴¹ have shown this same construct to work when overexpressed in the skeletal muscles of postnatal dy animals using AAV gene therapy techniques. Unlike Galgt2, however, the recombinant agrin protein used in these studies is distinctly different from endogenous muscle agrin, a highly glycosylated proteoglycan.^{59,60,85} Although we observed extrasynaptic expression of agrin in dy^w/dy^w muscles, much as previously published,⁶¹ we found much more highly increased expression in Galgt2 transgenic dy^w/dy^w muscles. With postnatal Galgt2 overexpression, in which CT overexpression is heterogeneous, the increased expression of agrin seemed to spread beyond myofibers overexpressing the CT carbohydrate. This suggests that CT overexpression may increase agrin expression in trans even in nonoverexpressing myofibers. This certainly would be plausible, given that agrin is a secreted muscle protein. Whether this occurs or not, however, our data clearly show that CT overexpression is a cell autonomous phenomenon with regard to inhibition of muscular dystrophy. It only has a therapeutic effect in myofibers where the CT carbohydrate is overexpressed. Thus, for agrin to be involved, it would have to bind CT

Figure 13. Expression of utrophin and extracellular matrix proteins in CT-overexpressing dy^w/dy^w myofibers after AAV-Galgt2 infection. dy^w/dy^w muscles were infected with AAV-Galgt2 at 2 weeks of age and analyzed for CT carbohydrate overexpression at 10 weeks of age using CT2 immunostaining at low ($\times 20$, left) and high ($\times 40$, right) power. Staining of serial sections with antibodies to utrophin, agrin, laminin $\alpha 2$, laminin $\alpha 4$, or laminin $\alpha 5$ (panels below CT2 immunostains) showed high levels of agrin and laminin $\alpha 4$ in regions of AAV-Galgt2 infection. Laminin $\alpha 1$ was not expressed. Scale bars: 50 μm (left); 25 μm (right).

glycans or a CT-modified receptor in expressing cells to affect muscle function. Agrin does require proper glycosylation of α -dystroglycan to bind to this cell surface protein,⁸⁶ and therefore it is not a stretch to think that modifying the glycosylation of the muscle membrane might alter agrin function. Agrin can also be glycosylated, at least in recombinant form,⁸⁷ with the CT carbohydrate, again lending credence to an agrin-CT model of membrane stability.

It is also impossible for us to exclude a role for other laminin α chains. For example, we found that laminin α 4 transcription and protein expression were highly increased in dy^w/dy^w myofibers, much as previously reported.^{43,56} Although laminin α 4 staining was not significantly further increased in Galgt2 transgenic dy^w/dy^w muscle, the already present ectopic expression of laminin α 4 could participate, along with increased CT carbohydrate, to impact the disease process. Similarly, laminin α 5 was increased to some extent in dy^w/dy^w muscle (much as before⁵⁶), although less-so than laminin α 4. It too, therefore, may have some beneficial effect in concert with increased CT glycosylation, perhaps analogous to the posited role for laminin α 5 in miniagrin experiments.⁸

Several other approaches have proved effective in dy mouse models, including corticosteroids such as prednisolone,⁸⁸ apoptosis inhibitors such as BCL2,⁸⁹ and muscle growth mediators such as IGF1.¹⁰ At the moment, we have no evidence as to whether these mechanisms are involved in the Galgt2's therapeutic effect. Other mechanisms involving ECM-transmembrane-cytoskeletal protein complexes, such as those involving integrins, also merit further investigation. For example, overexpression of certain integrins can ameliorate muscular dystrophy in DMD mouse models,¹⁴ and some integrins have increased endogenous expression in dy muscle.⁹⁰ That CT carbohydrate overexpression inhibits skeletal muscle pathology in multiple muscular dystrophy models, however, does distinguish it from some other therapeutic approaches. For example, therapies including myostatin inhibition and ADAM12 overexpression were not effective in dy^w/dy^w muscles,^{39,40} despite the fact that they did have a positive impact on mdx muscle pathology.^{13,24,33,34,91}

Although beyond the scope of the current study, it will be of interest to determine the extent to which Galgt2 overexpression will affect other cells and tissues where pathology exists in dy animals. For example, laminin α 2-deficient mice have peripheral neuropathy,⁹² sensorineural hearing loss,⁹³ aberrant myelination,^{94,95} and defects in synaptic plasticity,⁹⁶ thymocyte,⁹⁷ and testicular development.⁴² These findings show laminin α 2 mediates many developmental processes in addition to maintenance of muscle membrane integrity.

Two of our findings point to a CT-glycosylated glycolipid as being important to the mechanism by which Galgt2 overexpression inhibits muscle pathology; First, postnatal overexpression of the CT carbohydrate in extrasynaptic regions of skeletal myofibers primarily occurs on glycolipids because this staining can be removed by lipid extraction. This was true both in dy^w/dy^w and in mdx skeletal muscle. Second, Galgt2 overexpression, either prenatally or postnatally, stimulates the glycosylation of a

single glycolipid with the CT carbohydrate. The identity of this glycolipid is currently being investigated, but its migration pattern on HP-TLC separation suggests that it is not a known CT-like ganglioside (for example, GM2⁴⁹). Rather, this migration pattern would be consistent with a more heavily glycosylated glycolipid structure. Such a glycolipid could affect any number of aspects of membrane biology that could serve to increase muscle membrane integrity. Although our studies point in this direction, they by no means exclude the involvement of glycoproteins; Galgt2 overexpression could, for example, glycosylate a membrane glycoprotein such that it becomes rapidly degraded, making it difficult to identify. Indeed, some CT reactive material does remain after lipid extraction, suggesting that one or more glycoproteins are glycosylated with the CT carbohydrate, but perhaps not at levels detectable in the current experiments. The fact that very few proteins or lipids have been identified as being glycosylated with the CT carbohydrate,^{32,47,62,80,98–100} however, supports the idea that only one or a few CT glycosylated molecules act as the primary mediators of Galgt2's therapeutic effects in dystrophic skeletal muscle.

Note

Ruegg and colleagues¹⁰¹ have now provided evidence that overexpression of the native muscle agrin can affect muscular dystrophy in the dy^w/dy^w mouse model.

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