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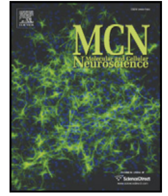

The synaptic CT carbohydrate modulates binding and expression of extracellular matrix proteins in skeletal muscle: Partial dependence on utrophin

Jung Hae Yoon, Kumaran Chandrasekharan, Rui Xu, Matthew Glass, Neha Singhal, Paul T. Martin *

Center for Gene Therapy, The Research Institute at Nationwide Children's Hospital, Department of Pediatrics, Ohio State University College of Medicine, 700 Children's Drive, Columbus, OH 43205, USA

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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.
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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, 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, 2000). α dystroglycan requires O-linked glycans, including Neu5Ac/Neu5Gcα2,3Galβ1,4GlcNAcβ1,2ManαO-linked structures in its mucin domain, to bind ECM proteins (Ervasti and Campbell, 1993; Michele et al., 2002). As such, it is likely that

* Corresponding author. Fax: +1 614 722 5893.
E-mail address: MartinPT@pediatrics.ohio-state.edu (P.T. Martin).

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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,b), 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 (Figs. 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, 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 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 CTαDG fraction.

In all cases, the presence of the CT carbohydrate on α dystroglycan increased the maximal amount of ECM binding (Figs. 1A–E). Binding of laminin α2 to the CT-glycoform was increased by 42 ± 3% relative to WT at 30 nM, the highest concentration used ($P = 0.002$). Laminin α4 showed a 100 ± 11% increase in binding to CT-α dystroglycan compared to WT at 300 nM, 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, 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 30 nM ($P = 0.003$, Fig. 1C), while muscle agrin was increased by 33 ± 6% ($P = 0.003$ at 30 nM, Fig. 1D) and neural agrin by 60 ± 2% ($P = 0.003$ at 50 nM, 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, 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-

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