Differential glycosylation of α-dystroglycan and proteins other than α-dystroglycan by like-glycosyltransferase

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Genetic defects in like-glycosyltransferase (LARGE) cause congenital muscular dystrophy with central nervous system manifestations. The underlying molecular pathomechanism is the hypoglycosylation of α-dystroglycan (α-DG), which is evidenced by diminished immunoreactivity to IIH6C4 and VIA4-1, antibodies that recognize carbohydrate epitopes. Previous studies indicate that LARGE participates in the formation of a phosphoryl glycan branch on O-linked mannose or it modifies complex N- and mucin O-glycans. In this study, we overexpressed LARGE in neural stem cells deficient in protein O-mannosyltransferase 2 (POMT2), an enzyme required for O-mannosyl glycosylation. The results showed that overexpressing LARGE did not lead to hyperglycosylation of α-DG in POMT2 knock-out (KO) cells but did generate IIH6C4 and VIA4-1 immunoreactivity and laminin-binding activity. Additionally, overexpressing LARGE in cells deficient in both POMT2 and α-DG generated laminin-binding IIH6C4 immunoreactivity. These results indicate that LARGE expression resulted in the glycosylation of proteins other than α-DG in the absence of O-mannosyl glycosylation. The IIH6C4 immunoreactivity generated in double-KO cells was largely removed by treatment either with peptide N-glycosidase F or with cold aqueous hydrofluoric acid, suggesting that LARGE expression caused phosphorylated glycosylation of N-glycans. However, the glycosylation of α-DG by LARGE is dependent on POMT2, indicating that LARGE expression only modifies O-linked mannose glycan of α-DG. Thus, LARGE expression mediates the phosphoryl glycosylation of not only O-mannosyl glycan including those on α-DG but also N-glycans on proteins other than α-DG.

Keywords: congenital muscular dystrophy / dystroglycan / dystroglycanopathy / like-glycosyltransferase / neural stem cells

Introduction

Mutations in genes encoding glycosyltransferases (or putative glycosyltransferases), including POMT1 (protein O-mannosyltransferase), including POMT1 (protein O-mannosyltransferase), including POMT2 (van Reeuwijk et al. 2005), POMT2 (van Reeuwijk et al. 2005), POMT2 (van Reeuwijk et al. 2005), POMT2 (van Reeuwijk et al. 2005), POMT2 (van Reeuwijk et al. 2005), POMT2 (van Reeuwijk et al. 2005), POMT2 (van Reeuwijk et al. 2005), POMT2 (van Reeuwijk et al. 2005), POMT2 (van Reeuwijk et al. 2005), LARGE (long垃圾eloseglycosyltransferase), Longman et al. 2003; van Reeuwijk et al. 2007; Clarke et al. 2011; Vuilamier-Barrot et al. 2011), FKTN (fukutin; Kobayashi et al. 1998; de Bernabe et al. 2003) and FKRP (fukutin-related protein; Brockington et al. 2001; de Beltran-Valero et al. 2004), cause congenital muscular dystrophies (CMDs) with manifestations in the central nervous system and the eye, such as Walker–Warburg syndrome (WWS), muscle–eye–brain disease (MEB), Fukuyama CMD (FCMD) and CMD type 1D. Some of these genes are involved in the synthesis of O-linked mannose glycans, e.g. Sias2,3Galβ1,4GlcNAcβ1,2Man-Ser/Thr (Chiba et al. 1997; Sasaki et al. 1998; Smalheiser et al. 1998), which account for one-third of O-linked glycans in the medicinal brain (Finne et al. 1979; Krusius et al. 1986; Chai et al. 1999; Kogelberg et al. 2001). POMT1 and POMT2 form a mutually indispensable enzyme complex and catalyze the initiation step of the O-mannosyl glycosylation pathway to form O-linked mannose (Manya et al. 2004; Akasaka-Manya et al. 2006). POMGnT1 transfers N-acetylgalcosamine (GlcNAc) to O-linked mannose forming a β1,2 linkage (Yoshida et al. 2001; Zhang et al. 2002). The biochemical functions of fukutin, FKRP and LARGE are not yet fully elucidated.

α-Dystroglycan (α-DG) is a functional target of O-mannosyl glycosylation. It is an extracellular matrix receptor that binds with high affinity to several extracellular matrix components, including laminin (Ervasti and Campbell 1993; Gee et al. 1993; Yamada et al. 1994; Montanaro et al. 1999; Smalheiser and Kim 1995), agrin (Gee et al. 1994; Yamada et al. 1996), perlecan (Peng et al. 1998; Talts et al. 1999), neurexin (Sugita et al. 2001) and pikachurin (Sato et al. 2008). α-DG interacts with the transmembrane β-DG (Ervasti and Campbell 1991; Ibraghimov-Beskrovnaya et al. 1992), which in turn binds to the cytoskeleton (Ervasti and Campbell 1991; Winder 2001). Functional glycosylation of α-DG is essential in its interaction with the extracellular matrix. To evaluate the glycosylation status of α-DG, the monoclonal antibodies IIH6C4 and VIA4-1 have been widely used because they recognize carbohydrate epitopes (Ervasti and Campbell 1991, 1993). Hypoglycosylation is revealed by loss...
(or reduction) of IIH6C4 (or VIA4-1) immunoreactivity (Kano et al. 2002; Michele et al. 2002; Kim et al. 2004; Liu et al. 2006). Mutations in CMD-causing glycosyltransferases lead to the hypoglycosylation of α-DG with markedly reduced IIH6C4 (or VIA4-1) immunoreactivity and reduced laminin-binding activity (Grewal et al. 2001; Kano et al. 2002; Michele et al. 2002; Takeda et al. 2003; Kim et al. 2004; Liu et al. 2006) and pikachurin-binding activity (Kanagawa et al. 2010; Hu, Li, Zhang, et al. 2011) by α-DG.

LARGE is a putative glycosyltransferase (Peyrard et al. 1999). It contains two transferase-like domains (Grewal et al. 2001). The Large heartbeat mice bear a spontaneous deletion in the Large gene and exhibit phenotypes similar to CMD in humans (Grewal et al. 2001; Holzfeind et al. 2002). Site-directed mutagenesis of the transferase-like domains abolishes its glycosylation capability, suggesting that LARGE may indeed function as a glycosyltransferase (Brockington et al. 2005; Aguilan et al. 2009). Overexpressing LARGE or its homolog LARGE2 dramatically increases IIH6C4 immunoreactivity and the apparent molecular weight of α-DG on sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) gels (Barresi et al. 2004; Brockington et al. 2005; Fujimura et al. 2005; Grewal et al. 2005). The IIH6C4 immunoreactive protein(s) confer laminin binding. The only known IIH6C4 immunoreactive protein is α-DG. However, we recently showed that LARGE expression can glycosylate proteins other than α-DG to confer IIH6C4 immunoreactivity and laminin-binding activity (Zhang et al. 2011). The increased glycosylation caused by LARGE overexpression occurs in cells isolated from not only Large heart mice, but also patients with WWS, MEB and FCMD (Barresi et al. 2004). These studies raise the hope of using LARGE in gene therapy for all CMDs caused by defective α-DG glycosylation.

The biochemical function of LARGE becomes elusive. Early evidence suggested that LARGE modifies O-linked mannosylglycans, complex N-glycans, and mucin-type O-glycans (Patnaik and Stanley 2005; Aguilan et al. 2009). More recent data indicate that LARGE is involved in the extension of an unidentified phosphoryl glycosylation branch on O-linked mannose (Yoshida-Moriguchi et al. 2010). In this report, we overexpressed LARGE in POMT2-deficient and in POMT2/DG double-deficient cells and analyzed LARGE-mediated glycosylation by immunoblot with the IIH6C4 antibody. Our results indicate that LARGE can mediate phosphoryl glycosylation on N-linked glycans of non-α-DG proteins to generate IIH6C4 immunoreactive epitopes capable of laminin binding. However, the glycosylation of α-DG by LARGE is dependent on POMT2-mediated O-mannosyl glycosylation.

Results

Establishment and characterization of POMGnT1, POMT2-deficient and POMT2/DG double-deficient neural stem cells

Neural stem cells were extracted from Dag1EMX1-CrePOMT2P0f/f fetuses obtained from crosses of Dag1EMX1-CrePOMT2P0f/f animals with Dag1EMX1-CreP0f/f animals. From these cultures, 22 clones were isolated. Genotyping for POMT2 and DG knockout (KO) primers showed that nine clones were double KO. POMT2-deficient (Hu, Li, Gagen, et al. 2011) and DG-deficient (Zhang et al. 2011) neural stem cells were described previously. POMGnT1-deficient neural stem cells were isolated from fetuses of POMGnT1 KO mice similarly (Liu et al. 2006). To confirm the non-expression of POMT2 in POMT2 and DG/POMT2 double-KO clones, reverse transcription (RT)–polymerase chain reaction (PCR) with POMT2-specific primers that flank the deleted exons were carried out (Figure 1A). The expected RT–PCR fragments for wild-type POMT2 mRNA (413 bp) were detected in wild-type neural stem cells but not in POMT2 KO or in double-KO clones. Instead, the expected RT–PCR fragments for POMT2 KO mRNA (113 bp) were detected. To confirm the non-expression of DG in DG/POMT2 double-KO cells, RT–PCR with forward primer from exon 1 and reverse primer from floxed exon 2 was carried out. Although an expected RT–PCR fragment (561 bp) was detected from the wild type, it was not detected in the double-KO clones. To confirm the non-expression of POMGnT1 mRNA in POMGnT1 KO neural stem cells, RT–PCR was performed as described previously (Liu et al. 2006). The expected fragment for wild-type POMGnT1 mRNA was observed only in the wild-type but not in POMGnT1 KO neural stem cells (Figure 1B). Additionally, western blot analysis with the β-DG antibody did not detect β-DG in DG KO and in double-KO cells (Figure 1C). Similarly, western blot with the IIH6C4 antibody did not detect glycosylated α-DG in either DG or double-KO cells. As a loading control, endogenous laminin was comparably detected in all samples. These results indicate that POMT2 KO and DG/POMT2 double-KO cells were completely deficient in POMT2 and in DG and POMT2, respectively.

Mutually indispensable nature of POMT1 and POMT2 for protein O-mannosyl transferase activity (Manya et al. 2004) suggests that deficiency of POMT1 or POMT2 alone would result in diminished O-mannose. To evaluate O-mannose levels in POMT2-deficient neural stem cells, we released O-linked carbohydrate by reductive β-elimination and analyzed the reduced sugar alcohols by high-performance anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD). Although mannitol was readily detected in wild-type neural stem cells, it was not detectable in POMT2-deficient neural stem cells (Figure 1D). These results suggest that protein O-mannosylation is abolished in POMT2-deficient cells.

LARGE overexpression in POMT2-deficient cells generated glycoproteins that bind to laminin and are recognized by IIH6C4 antibody

Previous studies suggest that LARGE modifies O-linked mannosyl glycans, complex N-glycans, mucin-type O-glycans of α-DG (Patnaik and Stanley 2005; Aguilan et al. 2009) or participate in the formation of a phosphoryl glycosylation branch on O-linked mannose (Yoshida-Moriguchi et al. 2010). To determine whether LARGE modifies glycans in addition to O-mannosyl glycans in mouse neural stem cells, we overexpressed LARGE in POMT2-deficient neural stem cells. Glycoproteins were isolated by wheat germ agglutinin (WGA)-gel and immunoblotted with IIH6C4 antibody, an
antibody that recognizes a carbohydrate epitope on α-DG (Figure 2). In wild-type neural stem cells, IIH6C4 immunoreactivity was detected at \(\approx 120\) kDa (lanes A and E). Laminin overlay assay indicated laminin binding at the same location. Overexpressing LARGE in wild-type neural stem cells markedly increased IIH6C4 immunoreactivity and laminin-binding activity (lanes B and F). In POMT2 KO neural stem cells, IIH6C4 immunoreactivity was not detectable (lane C). The laminin overlay assay did not show laminin-binding activity. However, overexpressing LARGE in POMT2 KO neural stem cells resulted in IIH6C4 immunoreactivity (lane D). The laminin overlay assay indicated that these IIH6C4 immunoreactive species bound to laminin. Protein O-mannose should be present in POMGnT1 KO cells that can serve as acceptors of LARGE modification. Indeed, LARGE overexpression in POMGnT1 KO cells generated IIH6C4 immunoreactivity as well as laminin binding (lane H). As expected, β-DG immunoreactivity was similar in all samples and c-Myc-tagged

Fig. 1. Characterization of POMGnT1, DG, POMT2 and DG/POMT2 double-KO neural stem cells. RT–PCR (A and B) and western blot analysis (C) were carried out to confirm the loss of DG, POMGnT1 and POMT2 expression in respective clones. Mannitol analysis was carried out by HPAEC-PAD on proteins isolated from wild-type and POMT2 KO neural stem cells after reductive β-elimination (D). (A) RT–PCR with DG or POMT2 primers. For POMT2, the 413 bp amplicon expected for wild-type mRNA was only observed for wild-type and DG KO clones. In contrast, the 113 bp amplicon expected for the KO mRNA was only observed for POMT2 KO and double-KO clones. For DG, the 561 bp amplicon expected for the wild-type mRNA was detected only in wild-type and POMT2 KO cells but not in DG KO and double-KO cells. (B) POMGnT1 RT–PCR. The 217 bp amplicon expected for the wild-type mRNA was present in wild-type but not in POMGnT1 KO cells. (C) Western blot analysis with IIH6C4, β-DG and laminin antibodies. IIH6C4 immunoreactivity was detected at 120 kDa in wild-type neural stem cells, but not in any other KO cell lines. β-DG immunoreactivity was detected only in wild-type, POMGnT1 KO and POMT2 KO neural stem cells, but not in DG KO or DG/POMT2 double-KO cells. As a loading control, laminin expressed by neural stem cells was readily detected in all cells. (D) Mannitol analysis. Mannitol peak was present in wild-type neural stem cells but only background was observed for POMT2 KO neural stem cells.
LARGE was detected in all samples infected by the Ad-LARGE virus. These results indicate that LARGE overexpression can lead to glycosylation of some proteins in the absence of POMT2-mediated O-mannosyl glycosylation.

LARGE overexpression rescued laminin-binding activity and IIH6C4 immunoreactivity independent of α-DG in POMT2-deficient cells

To determine whether the hyperglycosylated proteins in LARGE-overexpressing POMT2 KO neural stem cells are α-DG, we carried out immunoprecipitation assays with VIA4-1, an antibody that recognizes the glycosylated form of α-DG. VIA4-1 immunoprecipitates were blotted with the IIH6C4 antibody, and a β-DG antibody, and overlayed with laminin (Figure 3A). Interestingly, immunoblotting with anti-β-DG showed that β-DG was present in VIA4-1 immunoprecipitates of wild-type samples with and without LARGE overexpression. In contrast, it was not present in VIA4-1 immunoprecipitates of POMT2 KO neural stem cells, it was detected in LARGE-overexpressing POMT2 KO cells. Laminin overlay experiments indicated that IIH6C4 immunoreactive species generated by LARGE overexpression in POMT2 KO cells also bound laminin. These results indicate that LARGE overexpression did not result in addition of carbohydrate epitope that is recognized by IIH6C4 and VIA4-1 to α-DG in POMT2 KO cells.

Next, we overexpressed LARGE in wild-type, POMT2 null, and POMGnT1 null neural stem cells and carried out immunoprecipitation with anti-β-DG followed by IIH6C4 immunoblot and laminin overlay assays (Figure 3B). LARGE overexpression increased IIH6C4 immunoreactivity and laminin-binding capacity in both wild-type (lane 2) and POMGnT1 null neural stem cells (lane 6). However, no IIH6C4 immunoreactivity and laminin binding were detected for POMT2 null cells with or without overexpressing LARGE (lanes 3 and 4). As a negative control, no β-DG, IIH6C4 immunoreactivity and laminin-binding activity were detected in immunoprecipitate from DG KO neural stem cells with or without LARGE overexpression (lanes 7 and 8). Thus, the hyperglycosylation of α-DG by LARGE was not observed in POMT2-deficient cells. These results indicate that while LARGE overexpression led to the glycosylation of α-DG in the wild-type and POMGnT1 KO neural stem cells, it did not glycosylate α-DG in POMT2 KO neural stem cells. The IIH6C4 immunoreactive protein species in LARGE-overexpressing POMT2 KO neural stem cells likely represents non-α-DG proteins.

**Fig. 2.** LARGE overexpression in POMT2 KO neural stem cells generated IIH6C4 immunoreactive proteins capable of laminin binding. POMT2 KO neural stem cells were infected with Ad-LARGE and cultured for 2 days. Glycoproteins were isolated from the cell lysates by WGA-gel and analyzed by western blot with the IIH6C4 antibody and the laminin overlay assay. (A and E) Wild-type neural stem cells show IIH6C4 immunoreactivity and laminin binding at 125 kDa location. (B and F) Overexpression of LARGE in wild-type neural stem cells increased IIH6C4 immunoreactivity and laminin-binding activity at molecular weight ranges higher than 120 kDa. (C) In POMT2 KO neural stem cells, IIH6C4 immunoreactivity and laminin-binding activity could not be detected. (D) LARGE overexpression in POMT2 KO neural stem cells generated IIH6C4 immunoreactivity and laminin-binding activity. (G) POMGnT1 KO exhibit diminished IIH6C4 immunoreactivity and laminin binding. (H) LARGE overexpression in POMGnT1 KO neural stem cells generated IIH6C4 immunoreactivity and laminin-binding activity. Asterisks indicate endogenous laminin that is sometimes detected in the laminin overlay assay. The expected molecular mass of LARGE is 90 kDa. On western blot, a band corresponding to the dimer of LARGE was detected frequently.
and double KO with LARGE overexpression, indicating an equivalent amount of glycoproteins isolated by WGA-gel. β-DG immunoblot detected β-DG in wild-type and LARGE-overexpressing wild-type cells, but not in double-KO cells. Thus, overexpressing LARGE in Dag1/POMT2 double-KO cells glycosylated non-α-DG glycoproteins, indicating that LARGE-mediated glycosylation can occur in the absence of O-linked mannose and that this glycosylation can confer both IIH6C4 immunoreactivity and laminin binding to a protein(s) other than α-DG.

**LARGE overexpression modified N-glycans of non-α-DG glycoproteins and conferred laminin-binding activity**

To identify a possible acceptor of LARGE-mediated glycosylation on non-α-DG glycoproteins, we initially considered N-linked glycans. VIA4-1 immunoprecipitate isolated from lysates of LARGE-overexpressing double-KO cells was treated with peptide N-glycosidase F (PNGase F), a glycosidase that cleaves N-linked glycans (Figure 5, lane C). The activity of PNGase F was evident in the reduced apparent molecular weight of Coomassie blue-stained VIA4-1 heavy chain in all treated samples (compare Figure 5 lanes C with B, E with D, C' with B' and E' with D', arrow indicates the location of heavy chain). PNGase F treatment significantly reduced IIH6C4 immunoreactivity at ~70 kDa with very weak laminin-binding activity in this assay. Overexpressing LARGE in POMGnT1 KO cells increased IIH6C4 immunoreactivity and laminin binding in anti-β-DG immunoprecipitate (B, lanes 7 and 8). As a control, anti-β-DG did not precipitate any IIH6C4 immunoreactivity and laminin-binding activity in DG KO cells with or without LARGE overexpression. No β-DG was detected in DG KO cells as expected.

![Fig. 3. LARGE overexpression in POMT2 KO neural stem cells did not functionally glycosylate α-DG. POMT2 KO neural stem cells were infected with Ad-LARGE and cultured for 2 days. Immunoprecipitation with VIA4-1 (A) and anti-β-DG (B) antibodies was carried out, followed by immunoblot with anti-β-DG and IIH6C4 antibodies and bound to laminin. (A, lane 1) VIA4-1 immunoprecipitate of wild-type cells showed immunoreactivity to anti-β-DG (asterisk) and IIH6C4 antibodies and bound to laminin. (A, lane 2) VIA4-1 immunoprecipitate of wild-type cells overexpressing LARGE showed immunoreactivity to anti-β-DG (asterisk) and dramatically increased immunoreactivity to IIH6C4 and laminin-binding activity. (A, lane 3) VIA4-1 immunoprecipitate from POMT2 KO cells showed no immunoreactivity to anti-β-DG and IIH6C4 with no laminin-binding activity. (A, lane 4) Overexpressing LARGE in POMT2 KO cells generated IIH6C4 immunoreactivity at higher molecular weight and laminin-binding activity but showed no β-DG in VIA4-1 immunoprecipitate. (B, lane 1) Anti-β-DG immunoprecipitate from wild-type cells showed IIH6C4 immunoreactivity and laminin-binding activity. (B, lane 2) Overexpressing LARGE in wild-type cells increased IIH6C4 immunoreactivity and laminin-binding activity in anti-β-DG immunoprecipitate. (B, lanes 3 and 4) IIH6C4 immunoreactivity and laminin-binding activity were not detected in anti-β-DG immunoprecipitate from POMT2 KO cells with or without LARGE overexpression. (B, lane 5) Anti-β-DG immunoprecipitate from POMGnT1 KO cells showed very weak IIH6C4 immunoreactivity at ~70 kDa with very weak laminin-binding activity in this assay. (B, lane 6) Overexpressing LARGE in POMGnT1 KO cells increased IIH6C4 immunoreactivity and laminin binding in anti-β-DG immunoprecipitate. (B, lanes 7 and 8) As a control, anti-β-DG did not precipitate any IIH6C4 immunoreactivity and laminin-binding activity in DG KO cells with or without LARGE overexpression. No β-DG was detected in DG KO cells as expected.](http://glycob.oxfordjournals.org/)

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Together, these results indicate that LARGE can modify N-linked glycans. They also indicate that O-mannosyl glycosylation is present and contributes significantly to LARGE modifications on proteins other than α-DG. At least some LARGE modification on non-α-DG glycoproteins belongs to phosphoryl glycosylation. LARGE participates in the formation of an unidentified phosphoryl glycan onto O-linked mannose of α-DG, which is specifically removed by aqueous hydrofluoric acid (HFaq) treatment (Yoshida-Moriguchi et al. 2010). To determine whether LARGE-mediated glycosylation on non-α-DG glycoproteins also contained phosphoryl glycosylation, we treated VIA4-1 immunoprecipitates isolated from...
LARGE-overexpressing cells that were blotted onto polyvinylidene fluoride (PVDF) membrane (duplicate of Figure 5 lanes A–F) with HFAq that hydrolyzes a phosphoester bond (Yoshida-Moriguchi et al. 2010; Figure 5, lanes A′–F). HFAq treatment did not affect the amount of proteins blotted onto the membrane as indicated by the apparent equal intensity of bovine serum albumin (BSA) and heavy and light chains of the antibody by Coomassie blue staining. As a control, HFAq treatment of β-DG immunoprecipitate isolated from wild-type neural stem cells completely abolished IIH6C4 immunoreactivity (compare Figure 5 lanes A with A′) consistent with previous finding that the phosphory glycosylation of α-DG was sensitive to HFAq (Yoshida-Moriguchi et al. 2010). VIA4-1 immunoprecipitate isolated from LARGE-overexpressing double-KO cells showed significantly reduced IIH6C4 immunoreactivity when treated with HFAq (77.8% reduction, 95% confidence interval 67.8–87.7%; compare Figure 5 lanes B with B′). However, residual IIH6C4 immunoreactivity remained (Figure 5, lane B′). PNGase F treatment followed by HFAq treatment further reduced IIH6C4 immunoreactivity (compare Figure 5 lanes C′ with C and B′). The IIH6C4 immunoreactivity was barely detectable even after overexpression (5 min of exposure; Figure 5, middle panel). These results suggested that there were N-linked glycans modified by LARGE, which were recognized by IIH6C4 immunoreactivity, some of which were sensitive and some of which were insensitive to HFAq treatment.

HFAq treatment of VIA4-1 immunoprecipitate from LARGE-overexpressing DG-deficient cells significantly reduced IIH6C4 immunoreactivity (91.0% reduction, 95% confidence interval 86.2–95.8%; compare Figure 5 lanes D with D). The PNGase F removal of N-linked glycans followed by HFAq treatment further reduced IIH6C4 immunoreactivity (compare Figure 5 lanes E′ with E and D′). The reduction in IIH6C4 immunoreactivity from HFAq treatment on DG-deficient cells was more pronounced than HFAq treatment of double-KO cells (compare Figure 5 lanes D and D′ with B and B′). Further, HFAq treatment reduced IIH6C4 immunoreactivity more strongly than PNGase F treatment (compare Figure 5 lanes D and D′ with D and E). These results indicate that when POMT2 is present, non-α-DG glycoproteins are modified by LARGE acting mostly on O-mannosyl phosphorylated glycans.

N-linked glycans are not required for LARGE overexpression to generate more IIH6C4 immunoreactivity on α-DG

N-linked glycans do not contribute to the laminin-binding activity of α-DG (Ervasti and Campbell 1993). However, PNGase F treatment reduced laminin-binding activity in Chinese hamster ovary (CHO) cells overexpressing LARGE (Patnaik and Stanley 2005), raising the possibility that overexpressing LARGE may generate laminin-binding motifs on N-linked glycans of α-DG. Results shown in Figure 3 indicated that IIH6C4 immunoreactivity and laminin binding were not restored to α-DG in POMT2-KO cells. To further determine whether N-linked glycans of α-DG contribute to its glycosylation by LARGE, we overexpressed LARGE in wild-type and POMGnT1 KO cells and immunoprecipitated DGs with the anti-β-DG antibody. The immunoprecipitates were treated with PNGase F to remove N-linked glycans and immunoblotted with the IIH6C4 antibody (Figure 6). After PNGase F treatment, the molecular weight of β-DG and the heavy chain of mouse IgG were reduced, indicating the effectiveness of N-glycan removal by PNGase F. There is no N-linked glycosylation on the light chain of mouse IgG and thus no change in its migration. In the wild-type, equal IIH6C4 immunoreactivity was observed at ~120 kDa for both untreated and PNGase F treated. IIH6C4 immunoreactivity was not readily detected in POMGnT1 KO as expected. LARGE overexpression increased IIH6C4 immunoreactivity in both the wild-type and the POMGnT1 KO samples. Similarly, PNGase F treatment did not show noticeable reduction in their IIH6C4 immunoreactivity. These results indicate that IIH6C4 immunoreactivity on α-DG generated by LARGE overexpression is not on N-linked glycans.

HFAq treatment completely removed IIH6C4 immunoreactivity from α-DG in LARGE-overexpressing cells

To examine whether all IIH6C4 immunoreactivity on α-DG caused by LARGE overexpression was from phosphoryl glycans, β-DG immunoprecipitates isolated from LARGE-overexpressing wild-type neural stem cells were treated with HFAq (Figure 7). Without LARGE overexpression, IIH6C4 immunoreactivity was completely removed by HFAq treatment (compare Figure 7 lanes A with A′). LARGE overexpression increased α-DG glycosylation as evidenced by increased apparent molecular mass. After HFAq treatment, IIH6C4 immunoreactivity became undetectable (Figure 7, lanes B and B′). However, HFAq treatment did not completely remove all IIH6C4 immunoreactivity from VIA4-1 immunoprecipitate isolated from LARGE-overexpressing wild-type
To determine whether LARGE differentially glycosylates α-DG and proteins other than α-DG, we overexpressed LARGE in POMT2 KO cells that were deficient in O-mannosyl glycosylation. Our results (summarized in Table I) showed that LARGE expression did not cause functional glycosylation of α-DG to confer laminin binding in the absence of O-mannose. However, IIH6C4 immunoreactivity and laminin-binding activity were readily observed on proteins other than α-DG, suggesting that LARGE-modified glycans other than O-mannosyl glycans on non-α-DG protein species. Additionally, overexpressing LARGE in POMT2 and DG double-deficient neural stem cells resulted in readily detectable IIH6C4 immunoreactivity and laminin-binding activity. These results indicate that LARGE mediates the glycosylation of α-DG and proteins other than α-DG to confer IIH6C4 immunoreactivity and laminin binding by different mechanisms. Although O-mannose is required for α-DG glycosylation by LARGE, it is not required for at least some non-α-DG glycoproteins to be glycosylated by LARGE.

LARGE has two glycosyltransferase domains, one similar to β-1,3-N-acetylglucosaminyltransferase and the other similar to UDP-glucose:glycoprotein glucosyltransferase (Grewal et al. 2001; Patnaik and Stanley 2005; Aguilan et al. 2009). The precise biochemical activity of LARGE, however, is unknown. Interestingly, overexpressing LARGE generated hyperglycosylated IIH6C4 immunoreactive protein species that binds laminin (Barresi et al. 2004) in cells isolated from not only Large<sup>myd</sup> mice but also patients with WWS, MEB and FCMD bearing mutations in POMT1, POMGnT1 and FKTN, respectively. It has been assumed that all the hyperglycosylated protein species in LARGE-overexpressing cells are α-DG (Barresi et al. 2004; Brockington et al. 2005; Fujimura et al. 2005; Patnaik and Stanley 2005; Bao et al. 2009; Hu, Li, Wu, et al. 2011). Since LARGE overexpression in α-DG-deficient cells generates IIH6C4 immunoreactivity with laminin-binding activity (Zhang et al. 2011), the IIH6C4 immunoreactivity detected in LARGE-overexpressing patient cells likely includes glycoproteins other than α-DG.

Three types of glycans have been found on α-DG, O-linked mannosyl glycans, mucin-type O-linked N-acetylgalactosaminy glycan and N-linked glycans. O-linked mannosyl can have several different branches: GlcNAc linked through β1,2 linkage catalyzed by POMGnT1 (Yoshida et al. 2001), GlcNAc linked through β1,6 linkages catalyzed by β1,6-N-acetylglucosaminyltransferase IX (Inamori et al. 2003, Table I). IIH6C4 immunoreactivity of anti-β-DG and VIA4-1 immunoprecipitates upon PNGase F or HFaq treatments

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<tr>
<td>Wild type + LARGE</td>
<td>Strong</td>
<td>ND</td>
<td>Significant reduction</td>
</tr>
<tr>
<td>DG KO + LARGE</td>
<td>Strong</td>
<td>Mild reduction</td>
<td>Significant reduction</td>
</tr>
<tr>
<td>Double KO + LARGE</td>
<td>Detected</td>
<td>Significant reduction</td>
<td></td>
</tr>
</tbody>
</table>

KO, knockout; ND, not done.
and an unidentified phospho-glycan linked to carbon-6 via a phosphodiester bond (Yoshida-Moriguchi et al. 2010). The extension of the phospho-glycan branch of O-linked mannose on α-DG requires the activity of LARGE (Yoshida-Moriguchi et al. 2010). In the present study, IIIH6C4 immunoblot analysis did not reveal any signal in β-DG immunoprecipitates from POMT2 KO cells with or without LARGE overexpression (Figure 3B). Meanwhile, β-DG could not be detected from VIA4-1 immunoprecipitates from POMT2 KO cells with or without LARGE overexpression (Figure 3A). These results indicate that LARGE overexpression in POMT2-deficient neural stem cells does not glycosylate α-DG, supporting that α-DG glycosylation by LARGE requires O-linked mannose. This finding implies that N-linked and mucin O-linked glycans are not sufficient for LARGE to glycosylate α-DG to generate IIIH6C4 immunoreactivity. Our finding that PNGase F treatment did not affect the IIIH6C4 immunoreactivity and laminin-binding activity of α-DG in LARGE-overexpressing wild-type cells supports the notion that N-linked glycans of α-DG are not involved in LARGE-mediated modification.

Overexpressing LARGE in CHO cells with specific glycosylation mutations suggested that LARGE can modify complex N- and mucin-type O-linked glycans of α-DG, using IIIH6C4 immunoreactivity as evidence (Patnaik and Stanley 2005; Aguilan et al. 2009; Hu, Li, Wu, et al. 2011). Given the caveat of IIIH6C4 immunoreactivity as a marker of glycosylated α-DG, it is possible that IIIH6C4 immunoreactive species upon LARGE overexpression in CHO cells include non-α-DG glycoproteins as well. It is also possible that PNGase F sensitive IIIH6C4 immunoreactive species may be of non-α-DG. In this study, IIIH6C4 immunoreactivities from LARGE-overexpressing cells deficient in both α-DG and POMT2 were markedly reduced by PNGase F treatment (compare Figure 5 lanes C with B), indicating that at least some of the LARGE-mediated glycosylation on non-α-DG proteins are N-linked. Similarly, since HFAq treatment significantly reduced IIIH6C4 immunoreactivity (compare Figure 5 lanes B’ with B and D’ with D), at least some of the LARGE-modified glycosylation on non-α-DG proteins are phosphoryl glycan. However, since residual IIIH6C4 immunoreactivity remains after HFAq treatment of LARGE-overexpressing double-KO and DG KO cells (Figure 5, lanes B’ and D’), it is possible that some LARGE-mediated modifications are not phosphoryl glycosylations on non-α-DG glycoproteins. Interestingly, we found that combined treatments of HFAq and PNGase F further reduced IIIH6C4 immunoreactivity when compared with single treatments suggesting that some non-phosphoryl glycans may be on N-glycans of non-α-DG glycoproteins (compare C’ with C and B’ and E’ with E and D’). The presence of the residual IIIH6C4 immunoreactivity after double treatment suggests that some LARGE modifications could be on other glycans such as mucin-type O-glycans (Patnaik and Stanley 2005; Aguilan et al. 2009; Hu, Li, Wu, et al. 2011).

Our results are consistent with a model that hyperglycosylation of α-DG and non-α-DG by LARGE overexpression to generate ligand-binding functional glycan (LARGE-dependent modification) is via different mechanisms (Figure 8). For α-DG, LARGE expression causes the functional phosphoryl glycosylation of only O-linked mannose but not other types of sugar substitutions. With respect to proteins other than α-DG, LARGE expression causes the functional phosphoryl glycosylation of not only O-linked mannose but also N-glycans. Additionally, LARGE may also cause a modification on N-linked glycans of non-α-DG target that is not phosphoesther linked. Our study does not address the LARGE glycosylation of mucin-type GalNAc glycosylation which likely occurs on proteins other than α-DG since IIIH6C4 immunoreactivity is not recovered on α-DG in LARGE-overexpressing POMT2 KO cells.

In addition to being associated with CMDs, epigenetic silencing of LARGE expression causes loss of laminin-α-DG binding in epithelium-derived cancers (de Bernabe et al. 2009). LARGE forms a complex with β3-N-acetylglucosaminyltransferase 1 and its glycosylation has tumor suppression activity (Bao et al. 2009). Thus, hyperglycosylation by LARGE may have therapeutic potential. It is not known whether the non-α-DG targets of LARGE are of a single protein species or of several proteins. Future studies should be directed at the identification of non-α-DG targets, carbohydrate structure and physiological effects of LARGE-mediated glycosylation.

Materials and methods

Animals

Dag1−foxed mice (Moore et al. 2002; Jackson Laboratories, Bar Harbor, ME) were crossed with Nestin-Cre transgenic mice (Tronche et al. 1999; Jackson Laboratories) to obtain central nervous-specific KO mice of DG (Dag1f/f; Nestin-Cre−).
**Neural stem cell cultures**

To establish the neural stem cell cultures, the neocortical wall of fetuses at embryonic day 13.5 were excised, meninges-removed, cut into small pieces, treated with 0.5% trypsin for 2 min at 37°C and triturated with fire-polished Pasteur pipette. After filtration with a cell strainer, the cells were then grown as neural spheres in neural basal medium (Invitrogen, Carlsbad, CA) supplemented with B27 (minus vitamin A), 20 ng/mL fibroblast growth factor (FGF)-2, 20 ng/mL epidermal growth factor (EGF) and 2 ng/mL heparin.

To isolate clones, the neural stem cell cultures were trypsinized and triturated into individual cell suspensions and diluted to 6 cells/mL in the neural stem cell growth medium. Ten milliliters were seeded into 100 mm tissue culture dishes. Fresh FGF-2 and EGF were added once every 3 days. Colonies appeared in about a week and individual colonies were picked with a pipettor and subcultured in a 12-well plate with 1 mL of culture medium for an additional week. The cloned cells were trypsinized and expanded as neural spheres.

To overexpress LARGE in neural stem cells, an adenoviral vector for c-Myc-tagged human LARGE (Ad-LARGE) was constructed at Vector Biolabs (Philadelphia, PA). Twenty vector for c-Myc-tagged human LARGE (Ad-LARGE) was transduced into individual cell suspensions and diluted to 6 cells/mL in the neural stem cell growth medium. Ten milliliters were seeded into 100 mm tissue culture dishes. Fresh FGF-2 and EGF were added once every 3 days. Colonies appeared in about a week and individual colonies were picked with a pipettor and subcultured in a 12-well plate with 1 mL of culture medium for an additional week. The cloned cells were trypsinized and expanded as neural spheres.

**Genotyping of clonally expanded neural stem cells**

DNA was extracted from each clone. PCR was carried out to confirm the genotypes of the clones. For **Dagl** locus, the wild-type primers were GCCTTCTCTGTACACTGA (forward) and GGACAGTCGCTGCTCATCA (reverse), whereas the KO primers were CGAACACTGAGTTCA TTC (forward) and GGACAGTCACTGGCTCA TCA (reverse), which generate a 106 bp amplicon only for the wild-type allele and a 217 bp amplicon for the knockout allele. For POMT2 genotype, the forward primer was CTCAGATGCTATCGGTCTC and the reverse primer was TAGCTGTGG, which generate a 106 bp amplicon only from wild type but not the truncated POMT2 allele.

**RT–PCR analysis**

RT–PCR was carried out to determine the expression of DG and POMT2 mRNA in the cloned neural progenitor cells. Total RNA was isolated from 5 x 10^5 neural stem cells with an RNeasy Micro kit (Qiagen, Valencia, CA). A total of 500 ng of total RNA from each sample was reverse transcribed into cDNA with Quantitech RT kit (Qiagen). RT–PCRs for POMGnT1 (Liu et al. 2006), DG (Moore et al. 2002) or POMT2 KO (Hu, Li, Gagen, et al. 2011) neural stem cells were described elsewhere.

**Antibodies**

Antibodies were obtained as follows: IIH6C4 and VIA4-1 (Ervasti and Campbell 1993) from Millipore Corporation (Billerica, MA); anti-β-DG (MANDAG2-7D11) from Developmental Studies Hybridoma Bank (Department of Biology, University of Iowa, Iowa City, IO); Rabbit polyclonal antibody against laminin-1 and monoclonal antibodies against c-Myc from Sigma-Aldrich (St Louis, MO).

**Western blot analysis and laminin overlay**

Cells were washed three times with ice-cold Hank’s balanced salt solution and lysed with pre-chilled cell lysis buffer (50 mM Tris–HCl, pH 7.4, 150 mM NaCl and 1% Triton X-100) with proteinase inhibitor cocktail (Roche). The lysates were centrifuged at 16,000 x g at 4°C for 20 min. The supernatant was collected.

For enrichment of glycoproteins by WGA-agarose, 3 mg of proteins of the total lysate was mixed with 50 μL of WGA-agarose (EY Laboratories, San Mateo, CA). After binding for 16 h, the WGA-agar was precipitated and washed three times with cold washing buffer (50 mM Tris–HCl, pH 7.4, 150 mM NaCl and 0.1% Triton X-100) containing proteinase inhibitor cocktail. Bound glycoproteins were eluted by SDS–PAGE loading dye and separated on SDS–PAGE and electro transferred onto PVDF membranes.

For immunoprecipitation with anti-β-DG or VIA4-1, 1.5 mg of total supernatant protein was mixed with affinity purified anti-β-DG (8 μg) or VIA4-1 (5 μg) and incubated for 2 h by rotation at 4°C. Protein G beads (10 μL, Pierce, Rockford, IL) were added and the tube rotated overnight at 4°C. Beads were collected by centrifugation at 500 x g for 4 min at 4°C and washed three times with ice-cold cell washing buffer (50 mM Tris–HCl, pH 7.4, 150 mM NaCl and 0.1% Triton X-100) containing proteinase inhibitor cocktail. The beads were further mixed with 5% gel loading dye and incubated at 95°C for 5 min. The soluble proteins were separated on 8% SDS–PAGE gels and transferred onto PVDF membranes.

For western blot with IIH6C4-4 and anti-β-DG antibodies, standard western blot procedures were carried out. Briefly, the PVDF membrane was incubated with 3% BSA in Tris-buffered saline with Tween-20 (TBST; 50 mM Tris, pH 7.4, 150 mM NaCl, 0.05% Tween-20) for 1 h to block non-specific binding. The membranes were incubated with the primary antibodies in TBST containing 3% BSA overnight. After washing three times with TBST, the membranes were incubated with Goat anti-mouse IgM or IgG conjugated with horseradish peroxidase (1:3000) for 45 min. After extensive washing with TBST, the signal was visualized with SuperSignal west pico chemiluminescent substrate (Thermo Scientific, Rockford, IL).

For laminin overlay assay, the PVDF membrane was incubated with TBS (50 mM Tris, pH 7.4, 150 mM NaCl) containing 3% BSA, 1 mM CaCl2 and 1 mM MgCl2 for an hour to block non-specific binding. The membrane was then probed with goat anti-laminin-1 IgG (1:200) for 1 h. After extensive washing with TBST, the membrane was incubated with anti-goat-HRP IgG (1:2000) for 45 min. The signal was visualized with SuperSignal west pico chemiluminescent substrate (Thermo Scientific, Rockford, IL).
incubated with 1.25 μg/mL of laminin-1 (Invitrogen) in TBST containing 1 mM CaCl2 and 1 mM MgCl2 overnight at 4°C. After washing, the detection of bound laminin was by standard western blot procedures in buffers containing 1 mM CaCl2 and 1 mM MgCl2.

### Semi-quantitative analysis of western blot

X-ray films from western blot analysis that were not overexposed were scanned with a HP Scanjet 8300 scanner (Hewlett-Packard Company, Palo Alto, CA). A rectangular frame of same size was selected for all samples. The intensity within the selected areas was measured with ImageJ 1.42q (rsb.info.nih.gov/ij/download/). For every experiment, 3–5 repeats were analyzed. Statistical comparison of different samples was carried out by Student’s t-test.

### Treatment with cold HFaq

To hydrolyze phosphoester linkages, proteins blotted onto PVDF membranes were incubated with ice-cold 48% HFaq (Sigma-Aldrich) at 0°C for 20 h. The membranes were then washed with ice-cold water three times to remove residual HF. Controls were the same glycoprotein preparations that were separated by the same SDS–PAGE gel, electro-transferred to the same piece of PVDF membrane, cut into the middle of the membrane and were then treated with ice cold water instead. Both halves of membrane were then stapled together, stained with Coomassie blue and processed for IIH6C4 immunoblot analysis.

### Removal of N-glycans

Removal of N-glycans on glycoproteins bound on WGA- or protein G-agarose beads was carried out by PNGase F treatment (New England Biolabs, Inc., Ipswich, MA) according to the manufacturer’s suggestions. Briefly, proteins bound on the agarose beads were denatured with 1× glycoprotein denature buffer by incubation at 95°C for 10 min and quickly chilled on ice. Reaction buffer containing protease inhibitor cocktail (Roche) was added. PNGase F (50 units) was added to the mixture and incubated at 37°C for 16 h. Heat-inactivated PNGase F was added to the control samples and incubated likewise.

### Mannitol analysis with HPAEC-PAD

Cell lysates were resolved by 10% SDS–PAGE. Gels were then placed in a dialysis bag with a minimum amount of elution buffer (25 mM Tris base, 250 mM glycine, pH 8.3). Proteins were electroeluted from the gel into the elution buffer by at 13.3 V/cm for 90 min. Eluted proteins were concentrated in an Amicon Ultra-15 Centrifugal Filter unit (Millipore Corporation) and buffer-changed to TE (10 mM Tris, pH 8.0, 1 mM ethylenediaminetetraacetic acid). O-linked mannos was analyzed at the Glycobioengineering Core Resource Center at University of California at San Diego. O-glycans from the proteins were released by incubating the sample with 0.1 M NaOH/1 M NaBH4 at 45°C overnight. To remove peptide, the sample was passed through a Dowex 50 (H+ form) cartridge. The sample was then lyophilized and treated by several rounds of methanol/acetic acid addition followed by drying under a stream of nitrogen. Mannitol was analyzed by HPAEC-PAD with an MA-1 column.

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### Conflict of interest

None declared.

### Abbreviations

BSA, bovine serum albumin; CHO, Chinese hamster ovary; CMD, congenital muscular dystrophy; DG, dystroglycan; EGF, epidermal growth factor; FCMD, Fukuyama congenital muscular dystrophy; FGF, fibroblast growth factor; FKRP, fukutin-related protein; Gal, galactose; GlcNAc, N-acetylgalactosamine; HFaq, aqueous hydrofluoric acid; HPAEC-PAD, high-performance anion-exchange chromatography with pulsed amperometric detection; KO, knockout; LARGE, like-glycosyltransferase; MEB, muscle–eye–brain disease; PCR, polymerase chain reaction; PNGase F, peptide N-glycosidase F; POMGnT1, protein O-mannose β1,2 N-acetylgalactosaminyltransferase1; POMT, protein O-mannosyltransferase; PVDF, polyvinylidene fluoride; RT, reverse transcription; SDS–PAGE, sodium dodeyl sulfate polyacrylamide gel electrophoresis; Sia, sialic acid; TBST, Tris-buffered saline with Tween-20; WGA, wheat germ agglutinin; WWS, Walker–West Syndrome.

### References


