Mutational and functional analysis of Large in a novel CHO glycosylation mutant

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Inactivating mutations of Large reduce the functional glycosylation of α-dystroglycan (α-DG) and lead to muscular dystrophy in mouse and humans. The N-terminal domain of Large is most similar to UDP-glucose glucosyltransferases (UGGT), and the C-terminal domain is related to the human blood group transferase β1,3GlcNAcT-I. The amino acids at conserved motifs DQD+1 and DQD+3 in the UGGT domain are necessary for mammalian UGGT activity. When the corresponding residues were mutated to Ala in mouse Large, α-DG was not functionally glycosylated. A similar result was obtained when a DXD motif in the β1,3GlcNAcT-I domain was mutated to AIA. Therefore, the first putative glucosyltransferase domain of Large has properties of a UGGT and the second of a typical glycosyltransferase. Co-transfection of Large mutants affected in the different glycosyltransferase domains did not lead to complementation. While Large mutants were more localized to the endoplasmic reticulum than wild-type Large or revertants, all mutants were in the Golgi, and only very low levels of Golgi-localized Large were necessary to generate functional α-DG. When Large was overexpressed in idID.Lec1 mutant Chinese hamster ovary (CHO) cells which synthesize few, if any, mucin O-GalNAc glycans and no complex N-glycans, functional α-DG was produced, presumably by modifying O-mannose glycans. To investigate mucin O-GalNAc glycans as substrates of Large, a new CHO mutant Lec15.Lec1 that lacked O-mannose and complex N-glycans was isolated and characterized. Following transfection with Large, Lec15.Lec1 cells also generated functionally glycosylated α-DG. Thus, Large may act on the O-mannose, complex N-glycans and mucin O-GalNAc glycans of α-DG.

Keywords: α-dystroglycan/CHO mutants/DXD/laminin/
Large/mutagenesis

Introduction

α-Dystroglycan (α-DG) is the component of the dystrophin glycoprotein complex that links extracellular matrix proteins including laminin to the intracellular cytoskeleton to maintain skeletal muscle function (Ibraghimov-Beskrovnaya et al. 1992; Barresi and Campbell 2006). Abnormal glycosylation of α-DG disrupts binding to laminin and other proteins of the extracellular matrix resulting in congenital muscular dystrophies termed dystroglycanopathies (Martin and Freeze 2003; Endo 2007; Moore and Hewitt 2008). Mutations in glycosyltransferase, putative glycosyltransferase, or chaperone genes that mediate glycosylation of α-DG lead to the dystroglycanopathies termed Walker-Warburg syndrome (Kanoff et al. 1998; Beltran-Valero de Bernabe et al. 2002; Jimenez-Mallebrera et al. 2003; Schachter et al. 2004; van Reeuwijk et al. 2007), muscle–eye–brain disease (Yoshida et al. 2001; Manya et al. 2003), and Fukuyama congenital muscular dystrophy (Toda et al. 2003). Mutations in the human LARGE gene are the basis of the dystroglycanopathy termed MDC1D (Longman et al. 2003; Brockington et al. 2005). The mouse Large ortholog is 97.8% identical to human LARGE, and a deletion in mouse Large is the basis of the myd mouse (Grewal et al. 2001; Grewal and Hewitt 2002) that develops a muscular dystrophy similar to MDC1D (Grewal et al. 2005).

The O-mannose glycans implicated in the binding of α-DG to extracellular matrix proteins including laminin (Ervasti and Campbell 1993; Seifert et al. 2000; McDearmon et al. 2006) are absent or altered in the dystroglycanopathies. The minimal glycan unit is a tetrasaccharide NeuAcα2,3Galβ1,4GlcNAcβ1,2Man linked to Ser or Thr in the mucin domain of α-DG (Smalheiser and Kim 1995; Chiba et al. 1997; Smalheiser et al. 1998; Breloy et al. 2008). The O-linked Man is transferred from Dol-P-Man by protein O-mannosyltransferases POMT1 and POMT2 in the endoplasmic reticulum (ER), and then β1,2GlcNAc is transferred from UDP-GlcNAc by POMGnT1 in the Golgi compartment (Endo 1999, 2003; Manya et al. 2004). There are six β1,4GalTαs and several α2,3SiaTs that may transfer Gal and sialic acid, respectively, but to date mutations in these genes have not been causally related to muscular dystrophy. The simple O-mannose glycan is thought to be further extended by LARGE, a putative glycosyltransferase, because the absence of LARGE leads to hypoglycosylation of α-DG and loss of α-DG functions in a manner similar to deficiencies of POMT1, POMT2, or POMGnT1. In the myd mouse which has a deletion in the Large gene, α-DG is hypoglycosylated and does not bind to laminin or the monoclonal antibodies (mAbs) IIH6 and VIA4.1 (Ibraghimov-Beskrovnaya et al. 1992; Grewal et al. 2001; Grewal and Hewitt 2002). Similarly, in the human disease MDC1D, α-DG is hypoglycosylated and does not bind to laminin (Longman et al. 2003). LARGE is a glycoprotein of 756 amino acids and is proposed to be a dual-domain glycosyltransferase. It is a type II transmembrane protein that is a resident of the Golgi complex (Brockington et al. 2005; Grewal et al. 2005) and contains four DXD motifs. Based on sequence similarities in the CAZY database, CAZy family 8 is related to the N-terminal domain

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of LARGE and CAZy family 49 is related to the C-terminal domain. The family 8 domain of LARGE is most similar to the C-terminal portion of the catalytic domain of mammalian UDP-glucose:glycoprotein glucosyltransferases (UGGT) (Patnaik and Stanley 2005) that belong to CAZy family GT24 which is related to family GT8. The sequences of UGGTs from human and rat are 60–70% identical over a 1500-aa-long stretch, with significant homology in the ∼300-aa-long C-terminal region (Arnold et al. 2000; Tessier et al. 2000). The N-terminal portion of mouse LARGE is ∼25% identical and 40% similar to UGGTs. Moreover, the second DXD domain of mouse LARGE (DQD at aa 334–336) and the amino acids at DQD+1 and DQD+3 are conserved, and mutational studies have shown that the latter two amino acids are necessary for UGGT glucosyltransferase activity (Arnold et al. 2000; Tessier et al. 2000). Mutation of either of the DXD motifs in the UGGT domain to NNN inactivates LARGE, although it remains localized to the Golgi (Brockington et al. 2005). The C-terminal glycosyltransferase domain of LARGE is similar to β1,3GlcNAcT-1 (sometimes termed β3GlcNAcT-6; Peyrard et al. 1999; Patnaik and Stanley 2005) which transfers GlcNAc to form polylactosamine units (Sasaki et al. 1997). The fourth DXD domain of mouse LARGE (DID at aa 563–565) has sequence similarity to, and aligns with, the DVD motif conserved in the family of β3GlcNAcTs. Mutation of this DXD to NNN caused LARGE to localize to the ER, and functional analysis was not pursued (Brockington et al. 2005).

Sugars not required for LARGE to generate functional α-DG that binds to laminin and to the monoclonal antibodies (mAb) IIH6 and VIA4.1 have been identified by exoglycosidase studies (Combs and Ervasti 2005) and experiments in Chinese hamster ovary (CHO) cell glycosylation mutants. CHO mutants that do not transfer CMP-sialic acid (Lec2) or UDP-Gal (Lec8) into the Golgi, or that synthesize little GDP-Fuc (Lec13) (Patnaik and Stanley 2006) nevertheless generate good levels of functional α-DG in the presence of LARGE (Patnaik and Stanley 2005). Therefore, none of these is a major sugar transferred by LARGE nor is any one of them required as a major substrate of LARGE. Sequential digestion of α-DG from various sources with exoglycosidases has shown that the binding of mAbs IIH6 and VIA4.1 and laminin-1 is enhanced after removal of sialic acid, β-linked Gal or β-linked GlcNAc (Chiba et al. 1997; Combs and Ervasti 2005), and therefore these sugars are not required in the glycans of functional α-DG.

Determining the biochemical activities of LARGE is important because overexpression of LARGE in cultured fibroblasts from patients with different dystroglycanopathies generates functional α-DG, even in cells from dystroglycanopathies caused by mutations in genes other than LARGE (Barresi et al. 2004). Thus, understanding the properties of LARGE is key not only to understanding the basis of MDC1D, but also for designing therapeutic approaches to ameliorating the effects of the dystroglycanopathies in general. CHO mutants have shown that the latter two amino acids are necessary for UGGT enzymatic activity (Arnold et al. 2000; Tessier et al. 2000; Arnold and Kaufman 2003). Of the four DXD motifs that bind to laminin and to the monoclonal antibodies (mAb) IIH6 and VIA4.1, only the second and the fourth are predicted to be spaced similarly in LARGE and UGGTs. The DID at aa 563–565 is conserved in mouse and human β3GlcNAcT-1 (or iGnT1), which initiates extension of polylactosamine units. (B) Constructs of Large and Large mutants with MycHis tag (●) in the pIRES-GFP vector.

**Results**

**Mutational analysis of Large**

There are two domains with similarities to glycosyltransferases in human and mouse LARGE (Figure 1A). Of the four DXD motifs in LARGE, only the second and the fourth are predicted to be within a catalytic domain involved in binding a nucleotide sugar donor. Amino acids at +1 and +3 from the second DXD domain (DQD, aa 334–336) are conserved in LARGE and UGGTs and are known to be important for UGGT enzymatic activity (Arnold et al. 2000; Tessier et al. 2000; Arnold and Kaufman 2003). The fourth DXD domain (DID, amino acids 563–565) aligns with a DVD motif in the family of β3GlcNAcTs. Site-directed cosylated α-DG in ldlD.Lec1 CHO cells that express few, if any, mucin O-GalNAc glycans and no complex N-glycans, and in a new CHO mutant termed Lec15.Lec1, that has few, if any, O-mannose glycans and no complex N-glycans.

![Diagram of Large and mutant constructs](image-url)

**Fig. 1.** Diagram of LARGE and mutant constructs. (A) Mouse LARGE is 756 amino acids including a transmembrane domain (TM, aa 12–34), coiled coil domain 1 (CC1, aa 55–90), coiled coil domain 2 (CC2, aa 483–496), and five putative N-glycosylation sites (*). There are four DXD motifs at aa 242–244 (DTD, not shown), aa 334–336 (DQD), aa 438–440 (DED, not shown), and aa 563–565 (DID). The DQD, DQD+1(I), and DQD+3(N) residues are conserved in the C-terminal domain of UGGTs. The DTD and DQD are also spaced similarly in LARGE and UGGTs. The DID at aa 563–565 is conserved in mouse and human β3GlcNAcT-1 (or iGnT1), which initiates extension of polylactosamine units. (B) Constructs of Large and Large mutants with MycHis tag (●) in the pIRES-GFP vector.
Fig. 2. Mutations that inactivate Large. (A) Lec8 cells were transiently transfected with the pIRES-eGFP vector (GFP) or wild-type (WT) Large, Large mutants, or revertants (R). Lysates were subjected to Western analysis, and blots were cut and probed separately with mAb IIH6, or anti-Myc mAb, or anti-β-DG. The laminin overlay assay was performed on the same amount of the same cell lysates. (B) Co-transfection of two Large constructs together was performed as shown and lysates analyzed as in (A).

Mutational analysis and substrates of Large

Sub-cellular localization of Large mutants

Since the Western analysis of Large mutants showed a reduction (AID and DIA) or loss (AIA, DQD+1, and DQD+3) of Large function, it was important to determine their location in the cell. Constructs of wild-type Large or Large mutants were transiently transfected into parent CHO cells, and co-localization experiments were performed by immunofluorescence microscopy (Figure 3A). Since all constructs were made using the bicistronic pIRES-eGFP vector, diffuse GFP background signals were observed in the green channel but did not interfere with identifying cells binding Alexafluor-conjugated antibodies (Figure 3A).

Using secondary antibodies conjugated to the red AlexaFluor 568 dye against Myc-tagged Large, and the nuclear stain DAPI, transfected cells could be clearly distinguished. Rabbit polyclonal antibodies to TGN 38, a Golgi marker, and protein disulfide isomerase (PDI), a marker of endoplasmic reticulum (ER), were controls. Immunofluorescent images of ~100 independent transfected cells were visually assessed to determine the sub-cellular localization of Large proteins (Figure 3B).
Wild-type Large was localized primarily to the Golgi or both Golgi and ER (Golgi/ER) (Figure 3A and B), as reported previously (Brockington et al. 2005). All the Large mutants were expressed in the Golgi complex, but to a lesser extent than wild-type Large or Large revertants. Less than 20% of wild-type Large was mainly in the ER, whereas for Large mutants, >20% of cells exhibited ER localization (Figure 3A and B). However, all mutant Large proteins showed substantial Golgi and Golgi/ER staining. In the β1,3GlcNAcT-1 domain Large mutants, about 24% of AIA, 57% of AID, or 79% of DIA transfectants exhibited Golgi or Golgi/ER localization. For the UGGT domain mutants, about 40% of DQD+1 and DQD+3 mutants were localized to the Golgi or Golgi/ER (Figure 3A and B). A titration experiment showed that this amount of Large should be sufficient to functionally glycosylate α-DG (see below). Importantly, all UGGT domain revertants were localized in the Golgi, similar to wild-type Large (Figure 3B). Thus, the lack of activity of the DQD+1 and DQD+3 mutants suggests that the first glycosyltransferase domain of Large indeed has a UGGT-like catalytic activity.

To investigate the amount of wild-type Large necessary to functionally glycosylate α-DG, Large cDNA of the usual amount (4 μg) was titrated to 0.1 μg and transfected into CHO or Lec8 cells (Figure 4A and B). Functional glycosylation of α-DG was observed even when only 0.1 μg Large cDNA was transfected. Similar results were obtained for the laminin-1 overlay assay. Therefore, Large mutants introduced using 4 μg cDNA and localized to Golgi or Golgi/ER in about 24% cells would give easily observable IIH6 and laminin signals, if they were indeed functionally active.

Glycosylation of α-DG in ldlD.Lec1 CHO cells expressing Large
It is known that Large modifies complex N-glycans when overexpressed in CHO cells (Patnaik and Stanley 2005) and
Mutational analysis and substrates of Large

Fig. 5. Effects of Large on α-DG in ldlD.Lec1 cells. (A) VVL binding to blots of cell lysates of ldlD.Lec1 grown in alpha-MEM with 10% dialyzed fetal bovine serum without exogenous sugars for 4 days and Lec8 and CHO grown in alpha-MEM with 10% fetal bovine serum. (B) DSA lectin binding to cell lysates from CHO, Lec8, and ldlD.Lec1 grown as in (A). (C) PNA binding to cell lysates from Lec2 and ldlD.Lec1 grown in serum-free medium (30 min exposure). (D) Western analysis of cell lysates from ldlD.Lec1 and Lec2 cells transfected with Large or pIRES-GFP vector and grown in serum-free medium. Blots were cut and probed separately with mAbs IIH6, anti-Myc, and anti-β-DG. The laminin overlay assay was performed using another portion of the same cell lysates.

suggestive evidence was obtained that Large modifies O-mannose glycans missing from Lec15 cells (Rojek et al. 2007; Patnaik and Stanley 2005). To investigate further, we examined the generation of functionally glycosylated α-DG by Large in the CHO mutant ldlD.Lec1. The ldlD.Lec1 mutant has a UDP-Gal/UDP-GalNAc-4-epimerase deficiency and consequently does not synthesize mucin O-glycans unless galactose and N-acetylgalactosamine are available from the medium (Kingsley and Krieger 1984; Kingsley, Kozarsky, Hobbie, et al. 1986; Kingsley, Kozarsky, Segal, et al. 1986; Kozarsky et al. 1986, 1988). ldlD.Lec1 double mutant cells are also defective in the Mga1 gene (Ravdin et al. 1989). It was reported previously that Large does not modify the oligomannosyl N-glycans synthesized in the absence of Mga1 (Patnaik and Stanley 2005), and ldlD.Lec1 cells synthesize few, if any, mucin O-glycans when grown in the ITS medium with low concentrations of dialyzed fetal bovine serum (Ravdin et al. 1989) or serum-free medium (Rojek et al. 2006). Under these circumstances, if Large causes functional glycosylation of α-DG in ldlD.Lec1 cells, it should be modifying mainly, if not solely, the O-mannose glycans of α-DG.

IdlD.Lec1 cells were grown in the serum-free (CHO-S-SFMII) medium for 3 days prior to transfection with Large or pIRES-EGFP control. To confirm the glycans expressed by ldlD.Lec1 cells under these conditions, lectin blot analyses were performed using Datura stramonium (DSA) or Vicia villosa (VVL) or peanut agglutinin (PNA) (Figure 5A–C). CHO cells that express polylactosamine served as a positive control for DSA binding, while Lec8 cells that do not transfer Gal served as a negative control. On the other hand, Lec8 cells served as a positive control for VVL binding to terminal GalNAc residues that are essentially missing from CHO cells since the addition of Gal to GalNAc blocks VVL binding. In the case of PNA, Lec2 cells provided a positive control since they do not add sialic acid to glycoconjugates. ldlD.Lec1 cells showed very low or no binding to VVL (Figure 5A) or DSA (Figure 5B) or PNA (Figure 5C) as expected. The low signal detected in the PNA blot was after a >30 min exposure and may reflect nonspecific background binding or O-glycans synthesized from the generation of UDP-Gal and UDP-GalNAc by scavenger pathways. The ldlD mutation is not known but in vitro assays revealed no UDP-Gal/UDP-GalNAc-4-epimerase activity (Kingsley, Kozarsky, Hobbie, et al. 1986). Therefore, IdlD.Lec1 cells synthesized very few, if any, core 1 mucin O-GalNAc glycans (T-antigen), or polylactosamine-containing N-glycans. Nevertheless, IdlD.Lec1 cells expressed functionally glycosylated α-DG in the absence of exogenous Gal and GalNAc, suggesting that the modification is on O-mannose glycans (Figure 5D). Proteoglycans are not modified by Large (Smalheiser and Kim 1995) but an as yet unidentified glycan acceptor cannot be ruled out.

Isolation of Lec15.Lec1 CHO cells

Indirect evidence has implicated mucin-type O-GalNAc glycans as substrates of Large (Patnaik and Stanley 2005). To obtain further evidence, we developed Lec15.Lec1 CHO cells with few, if any, O-mannose glycans. Lec15 cells have a mutation in the Dpm2 gene that largely prevents Dol-P-Man synthesis (Stoll et al. 1992; Maeda et al. 1998, 2000). C-Mannosylation is greatly
reduced while O-mannosylation is abrogated in Lec15 cells (Maeda and Kinoshita 2008). The Lec15.Lec1 double mutant can synthesize only oligomannosyl N-glycans due to a mutation in the MgaT1 gene. To obtain this double mutant, Lec15.1 CHO cells (Pu et al. 2003) were selected for resistance to 30 μg/mL Phaseolus vulgaris agglutinin (L-PHA). Resistant colonies that had also acquired hypersensitivity to concanavalin A (Con A) were analyzed for resistance to plant lectins in comparison with Lec1 and Lec15 CHO cells. Colonies 1A, 1F, and 1O were most similar to Lec1 in their pattern of lectin resistance (Table I), as predicted for a Lec15.Lec1 phenotype.

To determine if these isolates possessed a mutation in the MgaT1 gene by genetic complementation analysis, colonies 1A, 1O, and 1F (all proline auxotrophs) were fused with Gat- Ler 1 CHO cells (glycine, adenosine, and thymidine auxotroph) using polyethylene glycol, and hybrids were tested for resistance to glycine by genetic complementation analysis, colonies 1A, 1F, and 1O were most similar to Lec1 in their pattern of lectin resistance (Table I), as predicted for a Lec15.Lec1 phenotype.

Table I. Lectin resistance of new isolates

<table>
<thead>
<tr>
<th></th>
<th>L-PHA</th>
<th>ConA</th>
<th>WGA</th>
<th>Ricin</th>
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</thead>
<tbody>
<tr>
<td>Lec1.3c</td>
<td>&gt;200</td>
<td>3</td>
<td>30</td>
<td>0.4</td>
</tr>
<tr>
<td>Lec15.B4</td>
<td>5</td>
<td>16</td>
<td>2</td>
<td>0.05</td>
</tr>
<tr>
<td>Colony 1A</td>
<td>&gt;200</td>
<td>3</td>
<td>40</td>
<td>0.5</td>
</tr>
<tr>
<td>Colony 1F</td>
<td>&gt;200</td>
<td>3</td>
<td>40</td>
<td>0.4</td>
</tr>
<tr>
<td>Colony 1O</td>
<td>&gt;200</td>
<td>3</td>
<td>40</td>
<td>0.5</td>
</tr>
<tr>
<td>Colony 2A</td>
<td>&gt;200</td>
<td>3</td>
<td>8</td>
<td>0.1</td>
</tr>
<tr>
<td>Colony 2D</td>
<td>&gt;200</td>
<td>3</td>
<td>20</td>
<td>0.2</td>
</tr>
<tr>
<td>Colony 2J</td>
<td>&gt;200</td>
<td>3</td>
<td>20</td>
<td>0.2</td>
</tr>
</tbody>
</table>

*Surviving colonies per 10 cm dish in 0, 2, or 10 μg/mL WGA.

Table II. Complementation analysis

<table>
<thead>
<tr>
<th>Cell lines fused</th>
<th>No. WGA-resistant colonies*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lec1 × Lec15</td>
<td>808</td>
</tr>
<tr>
<td>Lec1 × Lec15.1A</td>
<td>928</td>
</tr>
<tr>
<td>Lec1 × Lec15.1F</td>
<td>1056</td>
</tr>
<tr>
<td>Lec1 × Lec15.1O</td>
<td>1360</td>
</tr>
</tbody>
</table>

In conclusion, a truncated, inactive form of GlcNAcT-I comprising 44 N-terminal amino acids is predicted to be translated in Lec15.Lec1.1A cells. For simplicity, Lec15.Lec1.1A is termed Lec15.Lec1 in this paper.

N- and O-glycans of Lec15.Lec1 CHO cells

The N- and O-glycans released from Lec1 and Lec15.Lec1 glycoproteins by N-glycanase (PNGase F) and chemical β-elimination, respectively, were permethylated and analyzed by MALDI-TOF mass spectrometry. Lec15.Lec1 and Lec1 had similar N-glycan profiles consistent with oligomannosyl N-glycans being predominant, and Man3GlcNAc2 being the major N-glycan (supplementary Figure S1). Core 1 O-glycans corresponding to NeuAcHexNAc-ol (m/z 895) consistent with SA-Gal-GalNAc-ol and the desialylated form HexNAc-ol (m/z 534) consistent with Gal-GalNAc-ol were detected in both Lec15.Lec1 and Lec1 O-glycans (supplementary Figure S1). Interestingly, other possible O-glycan structures (Kang et al. 2008) such as Hex3HexNAc-ol (m/z 942), Hex2HexNAc-ol (m/z 1147), Hex2HexNAcHexNAc-ol (m/z 1187) were also detected. The tetrasaccharide, NeuAcHexHexNAcHexNAc-ol (m/z 1099), consistent with SA-Gal-GlcNAc-Man-ol (Smalheiser et al. 1998) was not detected. However, an ion with m/z 738 which may correspond to Hex3HexNAc-ol (Gal-Gal-GalNAc-ol) mucin O-glycan, or an isobaric structure HexHexNAcHexNAc-ol (Gal-GlcNAc-Man-ol) (Smalheiser et al. 1998) was detected in both Lec15.Lec1 and Lec1 O-glycans. The latter may arise from the loss of sialic acid from NeuAcHexHexNAcHexNAc-ol (SA-Gal-GlcNAc-Man-ol) (Smalheiser et al. 1998; Jang-Lee et al. 2007; Breloy et al. 2008). These possibilities were further investigated by MS/MS and MS^n analyses. Mass spectra were interpreted and ions were assigned using the Glycoworkbench program (Ceroni et al. 2008).

MS/MS analysis of the precursor ion m/z 738 generated B, Y, and C product ions and cross-ring cleavages of X ions. The O-glycans corresponding to product ions m/z 463.2, 445.2, 357.1, 316.1, 227.1 were detected in Lec15.Lec1 O-glycans, consistent with the mucin O-glycan HexHexHexNAc-ol (Figure 6A). Importantly, B and Z ions of m/z 486.2 and 275.1, arising from fragmentation of HexHexNAcHex-ol (m/z 738), were not detected in Lec15.Lec1 O-glycans (Figure 6A). By contrast, m/z 738 from Lec1 O-glycans showed the expected mixture of the two isobaric structures with product ions of m/z 621.0, 592.1, 520.2, 486.2, 463.2, 445.1, 430.1, 316.1, and 275.1 detected (Figure 6B).

The precursor ion, m/z 738, from Lec15.Lec1 O-glycans was further analyzed using LTQ-ESI-MS^n which gave daughter ions detectable up to MS^4 (supplementary Figure S2). However, fragment ions which gave significant structural information was also found in Lec1.3C previously (Chen and Stanley 2003).
Mutational analysis and substrates of Large

Fig. 6. MS/MS analysis of precursor ion m/z 738 from Lec15.Lec1 and Lec1. (A) MALDI-TOF/TOF analysis of the precursor ion m/z 738 from the MALDI-TOF spectrum of Lec15.Lec1 permethylated O-glycans (see supplementary Figure S1) with fragment ions assigned using Glycoworkbench. (B) MALDI-TOF/TOF analysis of the precursor ion m/z 738 from the MALDI-TOF spectrum of Lec1 permethylated O-glycans (see supplementary Figure S1). Fragment ions show a mixture of isobaric structures corresponding to Hex2HexNAc-ol (e.g., Gal-GalNAc-ol) mucin O-glycans and HexHexNAcHex-ol (e.g., Gal-GlcNAc-Man-ol). Sialic acid (♦); Gal (○); GlcNAc (■); Man (●).

were detected only up to MS^4. Precursor ion, m/z 738, subjected to MS^2 generated m/z 520.27, 463.27, 445.27, 316.27, 284.18 product ions with the assignments shown in supplementary Figure S2. Since m/z 520 ion may correspond to either HexHexNAc-ol (Gal-GalNAc-ol) or HexNAcHex-ol (GlcNAc-Man-ol), m/z 520 was selected for MS^3. Fragmentation of the m/z 520 precursor ion showed more product ions corresponding to a mucin O-GalNAc glycan, including m/z 316 which gave rise to m/z 298 when selected for MS^4. In addition, when m/z 463 detected from MS^2 was subjected to a collision energy of 33%, product ions consistent with mucin O-glycans were obtained (supplementary Figure S2). The combined data suggest that the predominant O-glycans present in Lec15.Lec1 glycoproteins are mucin O-glycans. No product ions consistent with m/z 738 from Lec15.Lec1 O-glycans being Gal-GlcNAc-Man-ol were detected (supplementary Figure S2).

Cell surface expression of α-DG in Lec15.Lec1 cells

To determine whether the glycosylation changes expressed by Lec15.Lec1 CHO cells affected α-DG trafficking, cell surface expression of endogenous α-DG was examined using the anti-α-DG mAb 6C1 (Figure 7A). Lec15.Lec1 cells bound 6C1 similarly to parent CHO and Lec15 cells. Interestingly, Lec1 and

![Image](image_url)

Fig. 7. Cell surface expression of endogenous α-DG and effects of Large on α-DG in Lec15.Lec1 cells. (A) Flow cytometry using primary antibody 6C1 anti-cranin (α-DG) and secondary antibody FITC-conjugated to anti-mouse IgG+M (black line), secondary Ab alone (gray profile), and primary antibody alone (dark gray line). (B) Cell lysates of Lec8, Lec15, Lec1, and Lec15.Lec1 cells expressing Large or pIRES-GFP vector control and probed using primary mAb IIH6, anti-Myc, or anti-β-DG followed by HRP-conjugated goat anti-mouse IgG+M Ab. The laminin overlay assay was performed using another portion of the same cell lysates. (C) Another portion of the same cell lysates was analyzed before and after treatment with PNGase F. PNGase F digestion was confirmed by stripping the membrane and probing with anti-NCAM, and expression levels of Large by stripping the same membrane again and probing with anti-Myc mAb.
ldL.Lec1 cells bound slightly more, and Lec8 cells bound significantly more 6C1 mAb than parent CHO. This suggests that either the latter mutants express more α-DG at the cell surface or that complex N-glycans and mucin O-glycans inhibit 6C1 mAb binding. Nevertheless, it was apparent that any differences in functional glycosylation of α-DG between Lec15 and Lec15.Lec1 cells would not be due to their differential cell surface expression of α-DG.

**Glycosylation of α-DG in Lec15.Lec1 CHO cells expressing Large**

Lec15.Lec1 cells were transfected with Large or pIRES-GFP as the vector control, and lysates were analyzed by Western blotting with mAb IIH6 for comparison with Lec1, Lec15, and Lec8 lysates (Figure 7B). Laminin-1 overlay assays showed that IIH6-positive samples also bound laminin. It is apparent that Large modified α-DG in Lec15.Lec1 cells. Expression levels of Large detected by anti-Myc mAb varied somewhat despite equal loading of the protein based on β-DG levels. This is probably due to small differences in transfection efficiencies. Functionally glycosylated α-DG from Lec15.Lec1 was not susceptible to PNGase F treatment, similar to Lec1 (Figure 7C), providing evidence that Large modified mainly O-glycans (or potentially some unidentified glycan) of Lec15.Lec1 cells. By contrast, α-DG from Lec15 cells was highly susceptible to PNGase F, indicating that Large modified N-glycans to a greater extent than O-glycans on α-DG in Lec15 cells. This was similar to α-DG from Lec8 cells which lost most IIH6 binding activity after PNGase F treatment (Figure 7C). PNGase F activity was confirmed by re-probing with an anti-NCAM mAb. The shift in molecular weight after PNGase F was more pronounced in N-CAM from Lec15 because the immature N-glycan Man3GlcNAc2-PP-dolichol that accumulates in Lec15 cells is converted to complex N-glycans (Maeda and Kinoshita 2008) which serve as substrates for polysialic acid transfer.

Further evidence that Large modified mucin O-GalNAc glycans on α-DG in Lec15.Lec1 cells was obtained by overexpressing Large in the presence of benzyl-α-GalNAc (BzOGalNAc), a competitive inhibitor of mucin O-glycan synthesis. Following treatment with 10 mM BzOGalNAc beginning 24 h before transfection, a reduction in IIH6 signal was observed in Lec15.Lec1 cells. This was similar to α-DG from Lec8 cells which lost most IIH6 binding activity after PNGase F treatment (Figure 7C). PNGase F activity was confirmed by re-probing with an anti-NCAM mAb. The shift in molecular weight after PNGase F was more pronounced in N-CAM from Lec15 because the immature N-glycan Man3GlcNAc2-PP-dolichol that accumulates in Lec15 cells is converted to complex N-glycans (Maeda and Kinoshita 2008) which serve as substrates for polysialic acid transfer.

**Discussion**

In this paper, we provide new insights into the properties of the putative glycosyltransferase Large by mutational analyses and by analyzing Large functions in ldLd.Lec1 CHO cells and a novel CHO mutant Lec15.Lec1. Brockington et al. (2005) previously showed that changing either of two DXD motifs in the UGGT domain of human LARGE to NNN destroyed the ability of LARGE to functionally glycosylate α-DG. Interestingly, the introduction of three Asn residues did not affect the localization of either LARGE mutant to the Golgi complex. These observations are consistent with both DXD motifs in the N-terminal domain of LARGE being potential sites of nucleotide sugar binding (Boure and Henrissat 2001). Our previous analysis revealed that the N-terminal portion of mouse Large is similar to the C-terminal region of ER-resident UGGTs (Patnaik and Stanley 2005) and contains the conserved DQD as well as amino acids at positions DQD+1 and DQD+3 known to be necessary for UGGT glucosyltransferase activity. In human UGGT, mutation of DQD+1 (L) or DQD+3 (N) to Ala reduces activity to background levels, despite appropriate localization to the ER (Arnold et al. 2000; Tessier et al. 2000). Here, we demonstrated the corresponding mutations in Large and compared their activity to revertants derived from the respective mutant.

Both the Large DQD+1 and DQD+3 mutants were well expressed but IIH6 immunoreactivity and laminin-1 binding to α-DG were dramatically reduced. Revertants had similar activity to wild-type Large and were also predominantly localized to the Golgi. By contrast, both DQD+1 and DQD+3 mutant Large were localized more to the ER. Nevertheless, immunofluorescence microscopy showed that ~40% of the DQD+1 and >30% of the DQD+3 mutant-expressing cells had Large in the Golgi. This amount of Golgi-localized Large would give a robust IIH6 signal if a mutant was active because even as...
little as 100 ng (or 2.5%) of the 4 \(\alpha\) DG. Thus, the first glycosyltransferase domain of Large has properties related to UGGTs and is predicted to transfer \(\alpha_1,3\)Glc, \(\alpha_1,3\)GlcNAc, or \(\alpha_1,3\)GlcUA to \(\alpha\)-DG. While members of the UGGT family may also catalyze the transfer of \(\alpha_1,3\)Gal, Large is predicted not to transfer Gal because it functionally glycosylates \(\alpha\)-DG in Lec8 CHO cells that have no Gal in their glycoconjugates (Patnaik and Stanley 2005). The Large UGGT-like activity may act on \(\alpha\)-DG in the ER.

We also mutated the DID at amino acids 563–565 in the \(\beta_1,3\)GlcNAcT-1 domain. Mutation of this domain to NNN in human LARGE causes localization to the ER and further analysis was not pursued (Brocksigton et al. 2005). We found that mutation of DID to AID or DIA in mouse Large gave IIH6 immunoreactivity and laminin-1 binding of \(\alpha\)-DG. Compared to wild-type and revertants, the AID and DIA Large mutants were \(\sim 55\%\) localized to the Golgi or Golgi/ER. A much more dramatic effect was achieved by mutating DID to AIA. Large with the AIA mutations was similar to DQQ+1 or DQQ+3 mutants and essentially lacked the ability to functionally glycosylate \(\alpha\)-DG. Large/AIA was localized to the Golgi or Golgi/ER in \(\sim 25\%\) of transfected cells and thus was well positioned to act on \(\alpha\)-DG. The fact that it did not provide good evidence that disruption of the DID at aa 563–565 leads to the inactivation of Large and is consistent with this DID being required for nucleotide sugar binding.

Wiggins and Munro (1998) identified a consensus sequence in which the pair of aspartate residues in a functional DXD is flanked by four hydrophobic residues (h) on the N-terminal side with the third being an aromatic residue and a hydrophobic residue on the C-terminal side (hdddDxdh). Motifs are found in numerous glycosyltransferases (Busch et al. 1998; Wiggins and Munro 1998). In mouse, human, and dog Large, the flanking aa for DQQ at amino acids 334–336 are STLADQDDIF and for DID at amino acids 563–565 are MFLSDDIFW, with bold residues conforming to the consensus. DXD motifs may be required for nucleotide sugar binding and/or for catalysis (Gulberti et al. 2003). For example, the DDD motif in GM2 synthase (Li et al. 2001) is required for enzyme activity but is not necessary for UDP-GalNAc binding, unlike the DVD in Clostridial cytotoxin which is essential for both donor and substrate binding (Busch et al. 1998). Mutation of the spaced pair of invariant Asp residues is required to completely block glycosyltransferase activity of the Clostridial cytotoxins unlike for the \(\alpha_1,3\)mannosyltransferase of yeast or Fringe which requires mutation of only one Asp to block enzyme activity (Busch et al. 1998; Wiggins and Munro 1998; Munro and Freeman 2000).

It is also interesting to note that although glycosaminoglycans (GAGs) are not the primary glycans involved in laminin binding by \(\alpha\)-DG (Smaleheiser and Kim 1995), we found that the DDD motif of GlcAT1 aligns with the DID motif of the \(\beta_1,3\)GlcNAcT-1 domain of Large. When the DDD at amino acids 194–196 of GlcAT1 were mutated one at a time (Gulberti et al. 2003), the ADD and EDD mutants produced only \(\sim 15\%\) reduction in activity whereas DAD, DDA, and double mutants gave \(\sim 85\%\) reduction, suggesting that the Asp at position 194 is not so important for transferase activity. This may also be the case in Large as the AID and DIA mutants retained substantial activity. It took removal of both Asp residues in the AIA mutant to inactivate Large.

The mutational analyses reported here and those of Brocksigton et al. (2005) targeted only one glycosyltransferase domain of Large at a time. We investigated whether there could be intermolecular complementation if two mutants of Large mutated in separate glycosyltransferase domains were co-expressed. When the Large mutants DQQ+3 (UGGT domain) and AIA (\(\beta_1,3\)GlcNAcT-1 domain) were co-transfected, no functional glycosylation of \(\alpha\)-DG was observed. Similarly, when the AID (slightly active) and DQQ+3 (inactive) Large cDNA were co-transfected, no enhanced IIH6 immunoreactivity or laminin binding were observed. Only very weak complementation was observed when the mutants DQQ+1 and AIA were co-transfected. Therefore, it would appear that the two glycosyltransferase activities of Large must be present in the same molecule for endogenous Large to be active. In addition, no dominant negative effect was observed when the inactive mutants were co-transfected with wild-type Large.

The second goal of this analysis was to further investigate the range of substrates that may be acted on by Large using CHO glycosylation mutants. These mutants provide a qualitative but not quantitative approach to identifying glycans that may be used by Large. We showed previously (Patnaik and Stanley 2005), and in this paper, that Large functionally glycosylates the complex N-glycans of \(\alpha\)-DG. To investigate the extent to which Large modifies O-mannose glycans on \(\alpha\)-DG, Large was overexpressed in the ldlD.Lec1 CHO mutant cell line. This double mutant expresses oligomannosyl N-glycans and O-mannose glycans. It appears to lack mucin glycans due to the loss of UDP-Gal/UDP-GalNAc 4-epimerase activity (Kingsley, Kozarsky, Hobbie, et al. 1986) but it is not established that this stems from a null mutation. In fact, there may be a very low level of O-GalNAc glycans in ldlD.Lec1 if the mutation is hypomorphic or circumvented in other ways. Nevertheless, Large was expected to modify mainly the O-mannose glycans on \(\alpha\)-DG in ldlD.Lec1 cells. When Large was overexpressed in ldlD.Lec1, \(\alpha\)-DG was functionally glycosylated albeit at a rather low level. These data suggest that the O-mannose glycans of \(\alpha\)-DG may not be a preferred substrate of overexpressed Large. While the presence of potentially novel glycan substrates as substrates for Large cannot be ruled out, only O-mannose, N- and O-GalNAc glycans have been found to date on \(\alpha\)-DG in mammals (Manya et al. 2007) and implicated in mAb IIH6 and laminin-1 binding, which in turn require functional Large. In addition, it is of note that Large is a glycoprotein which carries \(N\)-glycans. Thus, changes in \(N\)-glycan structure may theoretically affect Large activity. However, Large expressed in ldlD.Lec1 versus Lec15.Lec1, both of which synthesize only oligomannosyl \(N\)-glycans and therefore should express Large with equivalent activity, gives rise to different levels of functionally glycosylated \(\alpha\)-DG (Figure 8), consistent with the mucin O-GalNAc glycans of Lec15.Lec1 being better substrates than the O-mannose glycans of ldlD.Lec1.

The O-mannose glycans modified by Large are also necessary for LCMV virus to bind to \(\alpha\)-DG (Imperiali et al. 2005; Rojek et al. 2007). Evidence for this was obtained using Lec15 CHO mutant cells mutated in the Dpm2 gene (Maeda et al. 1998).
Lec15 defect reduces the levels of Dol-P-Man and thus is a hypomorphic rather than a null mutation which nevertheless results in markedly reduced transfer of N-glycans to protein and inhibits O-mannose to a greater extent than C-mannose glycan synthesis (Maeda and Kinoshita 2008). In this paper, we used the Lec15.1 mutant (Pu et al. 2003) to generate a new mutant, Lec15.Lec1, that allowed us to investigate whether Large modifies mucin O-GalNAc glycans. In-depth characterization of Lec15.Lec1 cells revealed that the Mgtl gene is nonfunctional due to an insertion mutation. Based on MALDI-TOF and ESI mass spectrometry analyses, Lec15.Lec1 cells synthesize oligomannosyl N-glycans which are not modified by Large and mucin O-GalNAc glycans but no detectable O-mannose glycans. We have shown previously that increased expression of functionally glycosylated α-DG is observed when core 2 N-acetylglucosaminyltransferase 1 is overexpressed with Large in Lec15 CHO mutant cells (Patnaik and Stanley 2005). Lec15.Lec1 provided a more direct approach to determining if Large modifies O-GalNAc glycans. In fact, the laminin-binding glycans generated by Large on α-DG from Lec15.Lec1 cells were resistant to PNGase F digestion, and it is likely that O-GalNAc is the sugar acted on by Large since Gal and sialic acid are not necessary for Large to function (Patnaik and Stanley 2005). Consistent with this is the significant inhibition of functional glycosylation of α-DG by AcBzOGalNAc (Figure 8A).

Previous studies have identified glycoforms of α-DG with mucin O-GalNAc glycans that react with VVL and perform distinct functions in skeletal muscle (McDearmon et al. 2001). Sialylated core 1 oligosaccharides of α-DG in myotubes or muscle cells mediate laminin-induced AChR clustering (McDearmon et al. 2003). Mammalian brain α-DG affinity-purified on VVL was resolved into populations containing or lacking sulfated glucuronic acid epitopes which bind preferentially to laminin 10/11 unlike skeletal muscle α-DG, suggesting that tissue-specific glycosylation modifies the laminin binding specificity of α-DG (McDearmon et al. 2006). The fact that Large functions well in Lec1, Lec2, Lec8, Lec13, Id1D.Lec1, and Lec15.Lec1 CHO glycosylation mutants suggests that Large transfers a sugar (α1,3Glc, α1,3GlcNAc, α1,3GlcUA) by the UGGT-like domain and β1,3GlcNAc or β1,3GlcNAcT1-1-like domain as discussed above, to an N-acetylhexosamine residue (GlcNAc or GalNAc) at the 6 position to initiate the formation of a glycan polymer (Figure 9).

Lec15.Lec1 is a cell line well suited for investigating how Large bypasses defects in POMT1 or POMT2 or POMGnT1, as previously shown in myoblast and fibroblast cells from patients having the corresponding dystroglycanopathies (Barresi et al. 2003). In CHO glycosylation mutants suggests that Large transfers a sugar (α1,3Glc, α1,3GlcNAc, α1,3GlcUA) by the UGGT-like domain and β1,3GlcNAc or β1,3GlcNAcT1-1-like domain as discussed above, to an N-acetylhexosamine residue (GlcNAc or GalNAc) at the 6 position to initiate the formation of a glycan polymer (Figure 9).

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Material and methods

Mouse Large cDNA expression constructs and site-directed mutagenesis

The full-length Large mouse cDNA generated previously in pcDNA 3.1/Myc-His(-)B (Patnaik and Stanley 2005) was amplified by PCR using primers PS829 (5'-AGACTCAGG0CCACCATGTGGAAATCTGCAGAGGGAA A, forward, with an Xhol site) and PS830 (5'-CCGGGCCCGGTTCATGATGATGATGATGTGATGTC, reverse, with Xmal, SmaI, and Apal sites), purified by agarose gel electrophoresis, digested with Xhol and Xmal and cloned into pIRE2-EGFP (Clontech Laboratories, Mountain View, CA). This was the template used for subsequent site-directed mutagenesis by PCR using the QuickChange™ XL Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA). Point mutations were made in the second and fourth DXD domains of Large. Each mutant was subsequently reverted back to wild type by site-directed mutagenesis of the respective mutant. Mutations

Fig. 9. Large modifies different mammalian glycans. The diagram shows a model of α-DG with the range of glycans it is proposed to carry when synthesized in CHO cells or the mutants Lec8, Id1D.Lec1, or Lec15.Lec1. Large generates functionally glycosylated α-DG when produced in each of these cell lines. In CHO and Lec8 cells, Large-modified N-glycans are sensitive to treatment with PNGase F. Large-modified N-glycans are not sensitive to PNGase F in Id1D.Lec1 or Lec15.Lec1 and thus Large modifies complex N-glycans but not oligomannosyl N-glycans. In Id1D.Lec1 cells, the major substrate of Large is O-mannose glycans because O-GalNAc glycans are not synthesized and oligomannosyl N-glycans are not a substrate. In Lec15.Lec1 cells, the major substrate of Large is O-GalNAc glycans because O-mannose glycans are not synthesized, and oligomannosyl N-glycans are not a substrate. We propose that Large transfers either a Glc, GlcNAc, or GlcUA in α1,3-linkage to the hydroxyl at C6 of GlcNAc or GalNAc. The C6 hydroxyl is proposed because Large modifies glycans in which GlcNAc or GalNAc is substituted at the C2, C3, and/or C4 positions.
were confirmed by sequencing of each strand. The following primers were used to generate Large mutants and revertants:

- **DQD+1→A mutant (DQD+1)**
  - Forward 5'-GCTGACCAGGATGCCTGACTGCTTCTGAATGCTGTTTA TCAACCAAAACC3'
  - Reverse 5'-GGGGTGTGTTTTGATAACACATGGGAA ACTGCCTGGTCAGC3'
- **DQD+3→N mutant (DQD+3)**
  - Forward 5'-GCTGACCAGGATTTTTTAACACATGGGAA ACTGCCTGGTCAGC3'
  - Reverse 5'-GGGGTGTGTTTTGATAACACATGGGAA ACTGCCTGGTCAGC3'

**Immunofluorescence microscopy**

CHO and Lec8 cells growing in suspension in α-MEM containing 10% FBS were plated on poly-L-lysine-coated coverslips at 1 × 10^5 cells and incubated for 24 h at 37°C in 5% CO₂. Cells were transfected with Large-Myc cDNA constructs using Lipofectamine 2000. After 16 h at 37°C in 5% CO₂, cells were washed, fixed with 3% paraformaldehyde, blocked, and semi-permeabilized with bovine serum albumin and saponin (Sigma, St. Louis, MO) in phosphate buffered saline (PBS) with calcium and magnesium as described (Chiu et al., 2002; Mukherjee et al., 2007). One set of duplicate cover slips was double-stained with an anti-Myc mAb (1:5) and an anti-TGN38 rabbit polyclonal antibody (1:200; a gift from Dr. Dennis Shields, Albert Einstein College of Medicine). A second set of duplicate cover slips was stained with an anti-Myc mAb (1:5) and an anti-protein disulfide isomerase (PDI) rabbit polyclonal antibody (1:200; Stressgen, Ann Arbor, MI). The cells were then incubated with Alexa Fluxo 568- (1:150; Molecular Probes, Eugene, OR) or 488- (1:100; Invitrogen) conjugated secondary antibodies. DAPI (1:1000) was used for nuclear staining. Cells were mounted using Fluoromount™ antifade (Southern Biotech, Birmingham, AL). Immunofluorescent images were digitally acquired on an Eclipse™ TE300 microscope (Nikon) using a 60× objective.

**Isolation of the CHO double mutant Lec15.Lec1**

To obtain Lec15.Lec1 double mutant CHO cells, Lec15.1 CHO cells (Pu et al., 2003) were selected for resistance to 30 μg/mL *Phaseolus vulgaris* agglutinin (L-PHA). Resistant colonies that had also acquired sensitivity to Concanavalin A (Con A) arose at a frequency of ∼10⁻⁵. They were analyzed for resistance to plant lectins in comparison with Lec1 and Lec15 cells as described previously (Stanley et al., 1975; Stanley and Siminovitch, 1976, 1977). Three colonies with a lectin-resistance phenotype most resembling Lec1 were selected for complementation analysis and assay of GlcNAcT-I activity using Man₃GlCNac₂Asn as a substrate and β4GalT-I using GlcNAc as a substrate as described previously (Chen et al., 2001; Chen and Stanley, 2003).

For complementation analysis, Lec15 or Lec15.Lec1 cells which carried the Pro⁻ auxotrophic mutation were fused with Gat⁻Lec1 cells. Controls were co-culture of the respective cell lines without inducing fusion by polyethylene glycol. Hybrid cells were selected in medium lacking proline, glycine, adenine, and thymidine in the presence of 0, 1, 2, and 10 μg/mL wheat germ agglutinin (WGA). Colonies of survivors were counted after ∼10 days of monolayer culture.

**Mgt1 gene sequencing**

Total RNA from 10⁷ Lec15.Lec1.1A or Lec1.3C cells (Chen and Stanley, 2003) were prepared using 1 mL of Trizol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. The RNA was precipitated with 0.5 mL cold isopropanol on ice for 10 min and centrifuged at 12,000 × g for 10 min at 4°C. The RNA was washed with 0.5 mL cold 75% EtOH and centrifuged at 5000 × g for 10 min at 4°C. Aliquots of 1 μg total were treated with 1 U of Dnase (Invitrogen) in 10 μL of reaction buffer and nuclease-free water for 12 min at room temperature (RT); 1 μL 25 mM EDTA was added, and the mixture was incubated for 10 min at
65°C. After cooling, oligo(dT)12-18 (1 μL) and 10 mM dNTP mix (1 μL) were added; the mixture was heated to 65°C for 5 min and cooled on ice for at least 1 min. Reverse transcriptase Superscript III RT (1 μL; Invitrogen) was added to half the tubes in the 5× first strand buffer (4 μL), 0.1 M DTT (1 μL), RNaseOUT (1 μL) to a final volume of 20 μL. Control tubes contained nuclease-free water in place of RT. The samples were incubated at 25°C for 5 min, 50°C for 5 min, and 70°C for 15 min. Treatment with RNase H (1 μL; Invitrogen) was at 37°C for 20 min. For PCR amplification, degenerate primers (Chen and Stanley 2003) PS150, forward, 5′-GGC GCC AGG, and PS154, reverse, 5′-GGC RGA GCC CAG RAR GGA MAG GCA GGW GCT, were used. Reactions consisted of 1× High Fidelity buffer (Invitrogen), 2 mM MgSO4, 10 nmoles dNTP mix, 10 pmoles of each primer, 5 U High Fidelity Platinum Taq DNA Polymerase (Invitrogen), 2.5 U Pfu DNA Polymerase (Stratagene), cDNA (45 U High Fidelity Platinum Taq DNA Polymerase (Invitrogen), and DNAZOL (GibcoBRL Life Technologies, Carlsbad, CA) were maintained in monolayer culture using the same medium at 37°C in 10 cm plates using Lec15.B4 (Lec15.1 subtype; Beck et al. 1990; Maeda et al. 1998; Pu et al. 2003) and Lec15.Lec1 cells, in a 5% CO2 incubator. For transient transfection, cells were grown in monolayer using the conditions mentioned above except for ldlD.Lec1 cells which were grown in medium containing 10% dialyzed fetal bovine serum (Invitrogen) or serum-free medium (CHO-S-SFM II; (Gibco) Invitrogen) or alpha minimal Eagle’s medium supplemented with ITS (Invitrogen) for at least 48 h prior to transfection. Transient transfection was performed on cells grown either on 6-well dishes or 10 cm plates using Lipofectamine 2000 (Invitrogen) or Eugene 6 (Roche Applied Science, Indianapolis, IN) following manufacturer’s protocols.

**Electrophoresis of cell lysates and immunoblotting**

At 24–48 h after transfection, cell lysates were prepared using 50–110 μL of cell lysis buffer (1× Tris-buffered saline [TBS] pH 7.5, 1.5% Triton-X100 [Sigma], and Complete™ EDTA-free protease inhibitor cocktail [Roche, Indianapolis, IN]). For electrophoresis, approximately 40 μg of protein were loaded on either 4–20% or 7.5% Tris-HCl gradient polyacrylamide gels (BioRad or prepared fresh) at 10–30 mA for 2 h. Proteins were transferred to polyvinylidene difluoride (PVDF) membrane overnight at 50 mA in a transfer buffer containing 5% methanol. For immunoblotting, membranes were blocked with 3–5% nonfat dry milk (Carnation) in 1× Tris buffered saline with 0.05% Tween-20 (TBST), pH 7.5, and incubated in either the same solution or 3% bovine serum albumin (Fraction V, Sigma) solution containing primary antibody for 1–2 h. Membranes were washed with 1× TBST for 30 min and incubated in 3–5% nonfat dry milk in TBST containing an anti-mouse horseradish peroxidase (HRP)-conjugated secondary antibody (IgG+IgM; Pierce) for 1 h at 1:10,000–20,000 dilution. Membranes were washed thoroughly for 30 min, incubated in SuperSignal™ West Pico chemiluminiscence reagent (Pierce), and exposed to photographic film (Eastman Kodak or Denville Scientific). The mouse monoclonal antibody anti-α-dystroglycan IIH6 C4 (Upstate Biotechnology-Millipore, Billerica, MA) was used at a 1:1000 dilution. The mouse monoclonal antibody anti-Mye AB164 and AB166 (9E10) was used at 1:200 dilution. Mouse anti-NCAM OB11 (Sigma) was used at 1:500 dilution. The mouse monoclonal antibody, anti-β-DG (43DAG1/8DG) (Novoceastra Laboratories Ltd., Newcastle, UK), was used as a loading control at a 1:200 dilution.

**Laminin overlay assay**

Proteins transferred to PVDF membranes were blocked in 5% nonfat dry milk in the laminin binding buffer (LBB; 10 mM ethanolamine, 140 mM NaCl, 1 mM MgCl2, 1 mM CaCl2). After 2 h at room temperature, the membrane was incubated in LBB containing 5 μg/mL EHS Laminin I (Roche) (1:1000) at 4°C overnight. The membrane was washed repeatedly with LBB before incubation in rabbit polyclonal anti-Laminin I (Sigma) (1:1500) for 1 h at 4°C. The membrane was washed thoroughly in horseradish peroxidase (HRP)-conjugated anti-rabbit secondary antibody (Zymed) for 1 h at 4°C. The membrane was washed, incubated in SuperSignal™ West Pico (Pierce, Rockford, IL) chemiluminiscence reagent, and exposed to photographic film (Eastman Kodak or Denville Scientific, Matuchen, NJ).
**Lectin blot analysis**

To determine lectin binding, proteins transferred to PVDF membranes were blocked using 3–5% nonfat dry milk in TBST for 2 h at room temperature. For lectin binding, membrane was subsequently incubated in the blocking buffer containing lectin at 10–20 μg/mL. The following lectins were used: biotinylated *Datura stramonium* (DSA), *Vicia villosa* (VVL) lectins, and Peanut agglutinin (PNA) (Vector Laboratories, Burlingame, CA). For biotinylated lectins, the membrane was washed repeatedly with TBST and then incubated in a blocking buffer-containing HRP-conjugated streptavidin (Vector Laboratories) (1:10,000) for 1 h at room temperature. The membrane was washed well, incubated in Super Signal West Pico (Pierce) chemiluminescence reagent, and exposed to photographic film (Eastman Kodak or Denville Scientific).

**Release of N- and O-glycans**

Approximately 0.5 to $1 \times 10^8$ cells were lysed using 0.5% Triton-X 100, centrifuged at 8000 × g, and the supernatant collected. Approximately 2 mg lysate was reduced and denatured with β-mercaptoethanol and 10% SDS in 300 μL of sodium phosphate buffer, pH 7.2, at 95°C for 10 min. The N-glycans were released by adding 500 U of PNGase F (New England Biolabs, Ipswich, MA) and incubated at 37°C overnight. An additional 500 U was added and digestion was continued for 6–8 h. N-Deglycosylated proteins were precipitated by adding 900 μL of cold 67% ethanol, stored at −20°C for 16–18 h, and centrifuged at 14,000 rpm for 10 min. The pellet was washed twice with 300 μL of cold ethanol, and supernatant was collected, pooled, and evaporated in a speed vac. The dried material was resuspended in 13 mM acetic acid and incubated at room temperature for 2 h and evaporated in a speed-vac. To further remove detergent contaminants, 10 ethyl acetate extractions were performed (Yeung et al. 2008). The material was redissolved in 100 μL of 100 mM sodium phosphate buffer, pH 7.1, and loaded on a 400 μL bed volume column of Extracti-gel (Thermo-Scientific) pre-washed four times with the same buffer. The column was washed three times and eluted with 3 volumes (400 μL) of the same buffer and loaded onto a PGC column (Thermo-Scientific, Franklin, MA) pre-conditioned with 80% acetonitrile in 0.1% trifluoroacetic acid (TFA; Pierce) and water. The column was further washed with 20 mL of Chrommosolve (HPLC grade water, Sigma), eluted with 3 mL 50% acetonitrile in 0.1% TFA, and concentrated using a speed-vac.

To release O-glycans, the N-deglycosylated protein was re-suspended in 200 μL 0.05 M NaOH/1 M NaBH₄ and incubated at 45°C for 16 h. Processing of the O-glycans was performed according to the methods of Morelle and Michalski (2007) and further purified by the method described above for N-glycans. Released N- and O-glycans were permethylated according to the methods of Kang et al. (2005, 2007, 2008).

**Mass spectrometry analyses**

The permethylated N- and O-glycans were redissolved in 10 μL of 2.5 mM sodium acetate in 50% methanol, and 1 μL of sample was spotted on a MALDI-target plate (Applied Biosystems, Foster City, CA) followed by 1 μL of matrix and dried under vacuum. Matrices used were dihydrobenzoic acid (DHB; Fluka, St. Louis, MO) at 10 mg/mL in 1 mM sodium acetate in 50% methanol or D-arabinosazone (3 mg/mL) in 75% ethanol (Chen et al. 1997). Profiling of N- and O-glycans was performed using an Applied Biosystems Voyager DE-STR MALDI-TOF mass spectrometer, and mass spectra were acquired in the reflector and positive ion mode. For MS/MS analysis, samples were prepared in the same way, the Applied Biosystems 4800 Proteomics Analyzer (AB4800) MALDI-TOF/TOF instrument was used and mass spectra were acquired in the reflector and positive ion mode. Prior to MSⁿ analysis, samples were resuspended in 1 mM NaOH in 50% methanol and introduced by direct infusion into a linear ion trap mass spectrometer, LTQ-ESI-MS (Thermo Finnigan), at a flow rate of 0.75 μL per min. MSⁿ analysis was performed in the positive ion mode (Aoki et al. 2008). For MSⁿ analyses, 10%, 25%, and 33% collision energy (CE) were applied for fragmentation by collision-induced dissociation (CID) of the selected precursor ions. Interpretation of mass spectra was performed using Glycoworkbench (Ceroni et al. 2008).

**Flow cytometry analysis**

Cells were plated in 10 cm plates with alpha-modified minimal Eagle’s medium (Invitrogen) with 10% fetal bovine serum (Gemin) except for IdlD.Lec1 which was grown in serum-free (CHO-S-SFM II) medium (Gibco Invitrogen) and incubated at 37°C for 24 h. After washing with 10 mL phosphate buffer saline (PBS), cells were detached using 5 mL of enzyme free cell dissociation buffer, PBS-based (Chemicon, Temecula, CA). Cells were resuspended, counted, and 5 × 10⁵ cells used for flow cytometry analysis. After centrifugation, the cell pellet was washed twice with the FACS binding buffer (PBS with 1% bovine serum albumin (BSA) and 0.05% Na azide) and incubated with a 6C1 anti-cranin (dystroglycan) monoclonal antibody (Chemicon, Millipore) in 0.1 mL of PBS with 1% BSA in PBS for 20 min at 4°C. The cells were washed twice with the FACS binding buffer and incubated with 0.1 mL of FACS binding buffer containing FITC-conjugated anti-mouse IgG+Al+M (Zymed). After 20 min at 4°C, the cells were washed twice and 7-aminocytomycin (7-AAD; BD Pharmarmin, San Diego, CA) was added; the cells were resuspended in 0.5 mL PBS/1% BSA and subjected to flow cytometric analysis using a FACScan or FACSCalibur (BD Biosciences, Rockville, MD) instrument. Raw data were analyzed using FlowJo software (Tree Star Inc., Ashland, OR) after 7-AAD-positive cells were gated out.

**Treatment with benzyl-O-galNAc and acetylated benzyl-O-galNAc**

Cells were plated at $1.7 \times 10^6$ cells per 10 cm plate in α-MEM containing 10% FBS and incubated for 24 h at 37°C. BzO-GalNAc (2 mM or 10 mM) (Sigma) was added at least 3 h prior to transfection. In another experiment, 1 mM acetylated benzyl galNAc (Carbosynth, San Francisco, CA) dissolved in 0.5% dimethylsulfoxide (DMSO) was added 24 h before transfection. Transient transfection with Large cDNA (6 μg) was performed using FuGene 6™ (Roche Applied Sciences) for 16 h following the manufacturer’s protocol. Cells were lysed, protein concentration was determined, and cell lysates were analyzed by Western blot using the antibodies mentioned above. Alternatively, cells were washed and prepared for flow cytometry following the method described above. The primary antibody used was 6C1 monoclonal antibody (Chemicon, Millipore) and
the secondary antibody used was anti-mouse (R-Phycoerythrin) PE (Jackson Immuno Research, West Grove, PA).

Supplementary Data
Supplementary data for this article is available online at http://glycob.oxfordjournals.org/.

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Conflict of interest statement
None declared.

Abbreviations
α-DG, α-dystroglycan; BSA, bovine serum albumin; CE, collision energy; CHO, Chinese hamster ovary; Con A, Concanavalin A; DSA, Datura stramonium agglutinin; FBS, fetal bovine serum; GFP, green fluorescent protein; GlcAT1, glucuronic acid transferase; L-PHA, Phaseolus vulgaris; PBS, phosphate buffered saline; PDI, protein disulfide isomerase; PNGase F, N-glycanase or peptide-N-glycosidase F; PVDF, polyvinylidene difluoride; RT-PCR, reverse transcriptase polymerase chain reaction; TBS, tris buffer saline with Tween 20; VVL, Vicia villosa lectin; WGA, wheat germ agglutinin.

References


