The human LARGE gene encodes a protein with two putative glycosyltransferase domains and is required for the generation of functional α-dystroglycan (α-DG). Monoclonal antibodies IIIH6 and VIA4-1 recognize the functional glycan epitopes of α-DG that are necessary for binding to laminin and other ligands. Overexpression of full-length mouse LARGE generated functionally glycosylated α-DG in Pro-5 Chinese hamster ovary (CHO) cells, and the amount was increased by co-expression of protein:O-mannosyl N-acetylgalcosaminyltransferase 1. However, functional α-DG represented only a small fraction of the α-DG synthesized by CHO cells or expressed from an α-DG construct. To identify features of the glycan epitopes induced by LARGE, the production of functionally glycosylated α-DG was investigated in several CHO glycosylation mutants. Mutants with defective transfer of sialic acid (Lec2), galactose (Lec8), or fucose (Lec13) to glycoconjugates, and the Lec15 mutant that cannot synthesize O-mannose glycans, all produced functionally glycosylated α-DG upon overexpression of LARGE. Laminin binding and the α-DG glycan epitopes were enhanced in Lec2 and Lec8 cells. In Lec15 cells, functional α-DG was increased by co-expression of core 2 N-acetylgalcosaminyltransferase 1 with LARGE. Treatment with N-glycanase markedly reduced functionally glycosylated α-DG in Lec2 and Lec8 cells. The combined data provide evidence that LARGE does not transfer to Gal, Fuc, or sialic acid on α-DG nor induce the transfer of these sugars to α-DG. In addition, the data suggest that human LARGE may restore functional α-DG to muscle cells from patients with defective synthesis of O-mannose glycans via the modification of N-glycans and/or mucin O-glycans on α-DG.

Human LARGE was originally identified on chromosome 22 as a gene with deletions in meningioma tumors (1, 2). LARGE spans >650 kb and includes 16 exons. The cDNA of 4,326 bp encodes a protein of 756 amino acids, with a C-terminal domain similar to human 1 blood group β1,3,5-N-acetylgalactosaminyltransferase, and hence the gene was named the LARGE (like-acytelglucosaminyltransferase) gene (2). The mouse LARGE ortholog encodes a 756-aa protein that is 97.8% identical to human LARGE (2). A deletion in the mouse LARGE gene is the basis of the myd mouse that exhibits characteristic muscular dystrophy and neuronal cell migration defects (3, 4). The phenotype is similar to that in humans with muscle-eye-brain disease (MEB). MEB arises from mutations in protein:O-mannosyl N-acetylgalcosaminyltransferase 1 (POMGnT1), a glycosyltransferase that transfers GlcNac to O-linked Man on α-dystroglycan (α-DG) (5, 6).

Dystroglycan (DG) is synthesized as a protein of ~894 aa and cleaved post-translationally into two subunits (α-DG and β-DG) that remain noncovalently attached (7). The α-DG subunit of ~650 aa is extracellular and is attached to the cell surface through its association with β-DG, a 43-kDa transmembrane protein. α-DG interacts with various components of the extracellular matrix including laminin (8), agrin (9), neurexin (10), and perlecan (11). α-DG has a predicted molecular mass of ~72 kDa, but because of modification by glycosylation, it migrates at 120–250 kDa on SDS-PAGE (12). Human and mouse α-DG have ~44 serine and threonine residues predicted to carry O-Man and mucin O-glycans, three N-glycosylation sites, and five glycosaminoglycan attachment sites ((E/D)y~pXS/G/A) (13). Carbohydrate moieties on α-DG are essential for binding to laminin and other ligands (14, 15). These functional glycans are not, however, glycosaminoglycans (15, 16) and generally have been found to be resistant to sialidase, O-glycosidase, and N-glycanase treatments (14, 17–21).

Patients with defects in O-Man glycan synthesis do not express functional glycans on α-DG (22, 23). That glycosylation imparts functionality to α-DG has been confirmed in studies of muscle cells from patients with MEB, Fukuyama congenital muscular dystrophy, congenital muscular dystrophies MDC1C and MDC1D, limb girdle muscular dystrophy 2I (LGMD2I), and a subset of Walker-Warburg syndrome (WWS) (22, 23). In each of these muscle diseases, the binding of IIIH6 and VIA4-1 monoclonal antibodies (mAb) to α-DG is lost, and α-DG does not bind its ligands, including laminin, agrin, and neurexin (24). Consistent with the loss of α-DG glycosylation is the fact that genes mutated in the dystroglycanopathies encode known or putative glycosyltransferases. Protein:O-mannosyltransferase 1 (POMT1), in conjunction with POMT2, O-mannosylates α-DG (25) and is mutated in WWS (26). POMGnT1 that adds GlcNac in β1,2-linkage to O-Man on α-DG is mutated in MEB (5, 6). Fukutin and fukutin-related protein (FKRP) are mutated in Fukuyama congenital muscular dystrophy, LGMD2I, MDC1C, and MDC1D.

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1 The abbreviations used are: MEB, muscle-eye-brain disease; DG, dystroglycan; β3GnT, β1,3 N-acetylgalcosaminyltransferase; β4GalT, β1,4-galactosyltransferase; C2GnT, core 2 N-acetylgalcosaminyltransferase; CHO, Chinese hamster ovary; ER, endoplasmic reticulum; mAb, monoclonal antibody; NCAM, neuronal cell adhesion molecule; PDI, protein-disulfide isomerase; PNGase F, peptide N-glycosidase F; POMGnT, protein:O-mannosyl N-acetylgalcosaminyltransferase; POMT1, protein:O-mannosyltransferase; UGT, UDP-glucose glycoprotein glucosyltransferase; UTR, untranslated region; aa, amino acids; PBS, phosphate-buffered saline; WWS, Walker-Warburg syndrome; FKRP, fukutin-related protein.
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Type I collagen (27–32) and belong to a family of enzymes that imparting function are formed on GlcNAc_\beta_1,2Man-O-Ser/Thr in the mucin domain of α-DG. However, catalytic activities for fukutin, FKRP, and LARGE have not been demonstrated, although it has been shown that the interaction of LARGE with the N-terminal domain of α-DG is required for the functional glycosylation of α-DG (21). In addition, overexpression of LARGE in cultured cells causes the appearance of functional α-DG that binds to laminin, neurexin, and agrin and carries glycan epitopes recognized by the VIA4-1 and IIH6 monoclonal antibodies (17, 35). Most surprisingly, overexpression of LARGE, but not POMGnT1, “rescues” functional glycosylation of α-DG in dystrophic muscle cells derived from patients with WWS that cannot synthesize O-Man glycan (17).

In order to gain insight into the mechanism of this rescue by LARGE, as well as to better understand the nature of the sugars transferred by LARGE, we overexpressed mouse LARGE in a panel of CHO glycosylation mutants. Here we show that α-DG glycan epitopes and laminin binding are induced by mouse Large in mutants with a defect in the transfer of sialic acid, fucose, or galactose and in a mutant that cannot make O-Man glycan. In addition, we show that α-DG glycan epitopes are particularly sensitive to N-glycanase in Lec2 and Lec8 cells and are enhanced by core 2 N-acetylglucosaminyltransferase 1 (C2GnT1), indicating mechanisms by which LARGE may rescue muscle cells with dystroglycanopathies.

MATERIALS AND METHODS

Cell Culture and Transfection—Parent Pro 5 CHO cells, the loss-of-function CHO mutants derived from them termed Lec1 (clone 3C) (36), Lec2 (clone 6A) (37), Lec2.2 (clone 2B) (38), Lec8 (clone D3) (39), Lec13 (clone 6A) (40) and Lec15 (clone B4–2–1) (41), and the gain-of-function mutant LEC12 (clone 1B) (42) were isolated previously. All cells were grown and maintained in α-modified Eagle’s medium (Invitrogen) with 10% fetal bovine serum (Gemini) in suspension or on plates in 5% CO_2.

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aa) construct in the hIgG1Fc-pcDNA3 vector, was a kind gift of Dr. Michael Oldstone, The Scripps Research Institute (45). α-DG generated from this construct is fused at the C terminus to the hIgG1Fc domain and is secreted. CHO cells grown on a 10-cm dish were transiently transfected with 7.5 μg each of DGFc5 and full-length mouse Large or empty expression constructs using the FuGENE 6™ transfection reagent. Serum-free cell culture-conditioned Opti-MEM™ (Invitrogen) medium was collected and assayed for the presence of Myc-tagged Large (Myc) and PDI, an ER-resident protein. The panel on the right shows βGalT activity, a Golgi marker, in the same fractions.

Sequence Analyses—The protein sequence of mouse Large was analyzed using BLAST (www.ncbi.nlm.nih.gov/BLAST), Clustal W1.8 (www.ebi.ac.uk/Tools/st/protclust/), Transmembrane HMM 2.0 (www.cbs.dtu.dk/services/TMHMM/), COILS (www.ch.embnet.org/software/COILS_form.html), PSORT II (psort.iims.u-tokyo.ac.jp), and CD-search (ncbi.nih.gov/Structure/cdd/cdd.shtml). Potential N-glycan and O-GaINAc glycosylation sites were predicted using the NetNGlyc 0.1 (w Batesg.ch.embnet.org/software/NetNGlyc) and NetOGlyc (46) servers. Glycosaminoglycan attachment sites were predicted using the Eukaryotic Linear Motif server (elm.eu.org) (47).

RESULTS

α-DG Is Functionally Glycosylated by Overexpression of Large in Pro 5 CHO Cells—Mouse Large protein has four DXD motifs that are found in many families of glycosyltransferases and are essential for their catalytic activities (48). Previous sequence analyses showed that the N-terminal region of mouse Large and its human ortholog (142–386 aa in both) contain glycosyltransferase family 8 domain (GT8 in the CAZy data base, afmb.cnrs-mra.fr/CAZY.acc.html). Further comparisons revealed that the N-terminal region shares similarity with the C-terminal catalytic domain of UDP-glucose glycoprotein glucosyltransferase (UGGT). UGGT sequences from human, rat, Drosophila, Caenorhabditis elegans, and Schizosaccharomyces pombe are most similar in an ~300-aa C-terminal region that is the proposed catalytic domain (49, 50). Amino acids 104–356 of Large are related (25% identity, 40% similarity) to this catalytic domain of UGGTs. Moreover, the two DXD motifs contained in the GT8 domain of Large are spaced similarly to two DXD motifs that are essential for the catalytic function of UGGTs (49, 50). Positions +1 and +3 to the second DXD motif are occupied by leucine and asparagine, respectively, in both human and mouse Large proteins and in all UGGTs (49, 50) where they are required for enzymatic activity (49, 50). Unlike UGGTs, Large does not have an ER retrieval signal sequence. A signal peptide sequence predicted at the Large N terminus (1–24 aa) partially overlaps with a predicted transmembrane domain (12–34 aa). Two coiled-coil domains (55–90 and 483–496 aa) and five N-glycosylation sites are predicted. The sequence of the C-terminal region of mouse Large or its human ortholog (470–742 aa in both) is ~28% identical and ~43% similar to those of mouse and human β3GnT6 that generates polylactosamines and the i blood group antigen (51). These features of mouse Large are diagrammed in Fig. 1A.

A cDNA encoding full-length mouse Large was cloned from mouse liver cDNA as described under “Materials and Methods.” Parent Pro 5 CHO cells stably expressing the Large cDNA with a C-terminal Myc tag were selected with G418 and subjected to cell fractionation. Four membrane fractions were collected and assayed for the presence of Large, the Golgi marker PDI (Fig. 1B; Fractions 1 and 4 contained all the βGalT activity, and fractions 3 and 4 were greatly enriched for PDI. Large was found predominantly in the Golgi fractions with βGalT activity, consistent with a recent report on co-localization of human LARGE with the Golgi protein GM130 (35). Western analysis of parent CHO cells transiently transfected with the mouse Large expression construct gave a broad smear above 177 kDa that was detected with two mAbs that recognize functionally glycosylated α-DG (Fig. 2A). Transfection with vector alone gave no species reactive with the glycosylation-specific mAbs (Fig. 2A). The IIH6 (52) and VIA4-1 (18) mAbs and laminin-1 specifically bind to glycosylated forms of α-DG (14, 35). Furthermore, the binding sites on α-DG for IIH6 and laminin overlap (14), and IIH6 blocks laminin binding to α-DG (53). The ~81- and ~38-kDa bands present in equal amounts in all three samples in Fig. 2A were unrelated to Large expression.

FIG. 1. Mouse Large has homology to UGGT and is found in Golgi membranes. A, the GT8 domain (aa 142–386), predicted coiled-coil domains (TM, aa 12–34, hatched box), four DXD peptide motifs (aa 242–244, 334–336, 438–440, and 563–565, black boxes), and five N-glycosylation sites (N at aa 97, 122, 148, 272, and 754) are depicted. Also shown are regions that have sequence similarity with UGGT (aa 104–356, 40% similarity and 21% identity with mouse UGGT1, GenBank™ accession number NP_942602) and β3GnT6 enzymes (aa 470–742, 43% similarity and 28% identity with mouse β3GnT6, GenBank™ accession number NP_780592). B, mouse Large protein is localized to the Golgi apparatus in CHO cells. Four fractions containing endoplasmic reticulum or Golgi membranes were isolated from CHO cells stably expressing mouse Large by sucrose density gradient centrifugation (48). 15 μg of protein from each fraction were analyzed by immunoblotting after SDS-PAGE for the presence of Myc-tagged Large (Myc) and PDI, an ER-resident protein. The panel on the right shows βGalT activity, a Golgi marker, in the same fractions.
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and to each other as the ~81-kDa band was detected only in the VIA4-1 blot and the ~38-kDa band only in the IIH6 blot. Expression of Large at ~100 kDa was confirmed by using an anti-Myc mAb (Fig. 2A). Fig. 2B shows that α-DG from parent CHO cells expressing Large bound laminin-1. A strong smear at >100 kDa was detected in lysates from Large transfectants and was absent from vector control or when laminin-1 was not overlaid. When a cDNA encoding POMGnT1 was co-expressed with Large, increased binding of mAbs VIA4-1 (Fig. 2C) and IIH6 (not shown) was obtained, consistent with modification of O-Man glycans by Large.

Whereas VIA4-1, IIH6, and laminin-1 should bind only to functional α-DG, it was important to identify α-DG directly. CHO-K1 cells express DG (16), and the 6C1 mAb against mouse α-DG (54) bound to our CHO cells by using flow cytometry and cytochemistry, but this mAb could not be used in Western analysis. Binding of 6C1 mAb in flow cytometry was not affected by transfection of Large (data not shown), suggesting that the DG level was unaltered by Large. The confirmation that the species detected in Large transfectants by VIA4-1, IIH6, and laminin-1 indeed represented α-DG was therefore obtained by characterization of α-DG co-immunoprecipitated with β-DG. CHO transfectants expressing Large or vector alone were biotinylated, and cell extracts were incubated with anti-β-DG antibody. Immunoprecipitates were analyzed by Western analysis using horseradish peroxidase-streptavidin and anti-β-DG and IIH6 mAbs. Streptavidin detected equivalent levels of species with the molecular weight of various glycosylated α-DG and β-DG in both Large and vector transfectants (Fig. 3A, middle panel). However, functionally glycosylated α-DG detected by the IIH6 mAb was co-immunoprecipitated only from cells overexpressing Large (Fig. 3A, top panel). Most surprisingly, no signal in this region corresponding to the α-DG detected by IIH6 was detected by streptavidin in lysates from Large transfectants (Fig. 3A, middle panel). A similar result was obtained when a recombinant form of α-DG was produced from CHO cells and analyzed for staining with Coomassie Blue and Western analysis with IIH6 mAb and anti-Fc antibodies (Fig. 3B). Although the IIH6 mAb revealed a broad smear at >150 kDa, nothing was detected in this region with antibody to the Fc portion of α-DG-Fc (Fig. 3B, bottom panel) nor by Coomassie Blue staining (Fig. 3B, top panel) or silver staining (data not shown). Thus, only a small fraction of α-DG was modified by Large. A similar result was obtained for functional α-DG generated by human LARGE in TSA201 cells (21).

Functional Glycosylation of α-DG Induced by Large in CHO Glycosylation Mutants—To understand the nature of the glycan structures induced by Large, as well as to obtain insight into the sugar(s) it may transfer, functional glycosylation of α-DG by exogenous mouse Large was assayed in a variety of CHO glycosylation mutants. Fig. 4A shows the predicted structures of complex N-, mucin O-, and -mannosyl glycans synthesized by these mutants on the basis of previous structural and enzymatic analyses. Lec1 cells are defective in β1,2-N-acetylgalactosaminyltransferase I activity (39, 55) and do not synthesize complex or hybrid N-glycans. Lec2 cells are defective in CMP-sialic acid transporter activity and lack sialic acid on their glycans (56–58). Lec8 cells lack galactose on their glycans because of defective UDP-galactose transporter activity (37, 57, 59). LEC12 cells express α1,3-fucosyltransferase IX

Fig. 2. Generation of VIA4-1, IIH6, and laminin-1 binding species by overexpressing mouse Large in Pro-5 CHO cells. A. Large generates functional α-DG in CHO cells. Lysates of CHO cells transiently transfected with empty vector (−) or mouse Large (+) were analyzed by Western analysis using VIA4-1, IIH6, or anti-Myc mAbs. Asterisks indicate nonspecific bands. B, lysates of CHO cells transfected with the mouse Large gene bind laminin-1. A laminin overlay assay was performed on blot of lysates from CHO cells transfected with vector or Large, with (+laminin) and without (−laminin) as described under "Materials and Methods." C, lysates of CHO cells transfected with mouse Large with or without human POMGnT1 or vector control were analyzed by Western analysis using VIA4-1 or anti-Myc mAb.

Fig. 3. α-DG is modified by overexpression of Large in Pro-5 CHO cells. A. α-DG was co-immunoprecipitated with β-DG from surface-biotinylated CHO cells transiently transfected with Large (+) or empty vector (−). Immunoprecipitated proteins were analyzed by immunoblotting for glycosylated α-DG using mAb IIH6 (top panel). Heavy (HC) and light chains (LC) of the anti-β-DG mAb are visible. The same blot was stripped and probed with streptavidin-conjugated horseradish peroxidase (Avidin, middle panel) to detect biotinylated proteins. Both α- and β-DG are visible. An identically prepared blot was probed with an anti-β-DG mAb (bottom panel). B, recombinant, secreted rabbit α-DG, fused with the H1gG1 Fc domain at its C terminus, was purified from cell culture-conditioned medium using protein A/G beads. α-DG purified from CHO cells co-transfected with empty vector (−) or mouse Large (+) was analyzed by Coomassie Blue staining (top panel) or by immunoblotting using IIH6 mAb (middle panel) or an anti-Fc antibody (bottom panel).
that parent CHO cells do not (60), and Lec13 cells, deficient in the synthesis of GDP-fucose from GDP-mannose (40, 61, 62), have low levels of fucosylated glycans.

In Large transfectants of Lec2, Lec8, LEC12, and Lec13 cells, α-DG that bound laminin-1 and the IIH6 and VIA4-1 mAbs was generated (Fig. 4B and 5A). Large is predicted to modify only O-Man glycans (3, 34), and all of these mutants should synthesize the O-Man disaccharide GlcNAcβ1,2Manβ1,0-Ser/Thr, in some cases with additional Gal, sialic acid, or Fuc residues (63). However, Lec8 cells should not be able to add Gal to the disaccharide, and Lec2 cells should not be able to add sialic acid when Gal is present. The fact that Large causes the robust expression of functionally glycosylated α-DG in Lec2 and Lec8 mutants provides evidence that Gal or sialic acid is not required as part of the substrate recognized by Large and that neither sugar is transferred by Large. The same conclusions apply to Fuc because α-DG is also functionally glycosylated by Large in Lec13. Glycosylation of α-DG by Large also occurs in the absence of complex or hybrid N-glycans in Lec1 cells and in LEC12 cells, although to a reduced extent.

The spread of the α-DG smear detected by the two mAbs and by laminin-1 overlay generally appeared to be similar in the different cell lines. However, the intensity of the smear varied significantly. This variation does not appear to reflect transfection efficiency or gel loading differences. Thus all lysates had a similar signal for calnexin, and Lec2 and Lec8 transfectants had comparatively low levels of Myc-tagged Large but significantly stronger α-DG smears compared with α-DG from CHO, LEC12, or Lec13 cells (Fig. 4B, Myc panel). Fluorescence-activated cell sorter analyses with α-DG anti-peptide antibody 6C1 showed that CHO and Lec8 cells bound similar levels of antibody and slightly more than Lec1 and Lec2 cells (data not shown), so the differences did not correspond to α-DG expression levels. The increased binding of IIH6, VIA4-1, and laminin-1 by α-DG in Lec2 and Lec8 cells overexpressing Large appears to reflect an increase in the amount of functional α-DG induced by Large.

**Large Induces the Modification of N-Glycans on α-DG**—Previous experiments have found laminin binding of α-DG to be resistant to N-glycanase treatment (15, 17). However, after LARGE overexpression laminin binding appeared to diminish somewhat after N-glycanase treatment (17). To determine whether Large modifies N-glycans on α-DG from CHO, Lec1, Lec2, or Lec8, cell extracts were examined after treatment with PNGase F. Untreated Lec2 and Lec8 lysates had comparatively higher levels of glycosylated α-DG compared with CHO and Lec1 (Fig. 5A). However, most of the differences between cell lines were abolished by PNGase F treatment. Although the glycosylated α-DG signal for CHO and Lec1 was minimally affected by PNGase F treatment, consistent with Large modification of mainly O-Man glycans in these cell lines, the signal in Lec2 and Lec8 lysates was greatly reduced by PNGase F, almost to the intensity obtained for CHO and Lec1 α-DG. This suggests that Large modifies N-glycans in Lec2 and Lec8 cells. Immunoblotting using a mAb against NCAM that is known to be N-glycosylated in CHO cells (64, 65) showed that removal of
IIH6 mAb was used to detect glycosylated NCAM levels. Each lane has approximately the IIH6 mAb were performed on identically prepared blots. The blot anti-NCAM mAb. Each lane has an identically prepared blot was analyzed for NCAM using the OB11 glycan. As shown in Fig. 5

cosamine 2-epimerase that is required for CMP-sialic acid syn-

thase activity (41) because of a mutation in the

Large may modify mucin O-glycans in cells that functionally glycosylated O-DG occurs in Lec15 cells it must be on non-

O-mannosyl glycans (69). The intensity and spread of glycosylated N-DG by overexpression of Large. A, predicted structures of complex N- and O-mucin and O-mannosyl glycans synthesized in the Lec15 CHO glycosylation mutant are shown to illustrate its glycosylation defect. White square, N-acetylgalactosamine; black square, N-acetylgalcosamine; gray circle, mannose; white circle, galactose; triangle, fucose; diamond, sialic acid. B, Lec15 mutant cells were transiently transfected with 7.5 µg of plasmid DNA for expression of mouse Large and/or human C2GnT1; 7.5 µg of plasmid DNA for empty pZeo SV2+ or pcDNA3.1/Myc-His B(−) vector was added to make the final amount of transfected DNA 15 µg when only Large or C2GnT1 was transfected. Cell lysates were treated with PNGase F (+) to remove N-glycans. The enzyme was omitted from control reactions (−PNGase F). Glycosylated O-DG was detected using IIH6 mAb by Western analysis. The blot was stripped and assayed for the expression of NCAM. Identically prepared blots were analyzed for expression of Myc-tagged Large and for laminin-1 binding by overlay assay. Each lane had ~50 µg of protein. Similar results were obtained in two independent experiments.

N-glycans was efficiently achieved by the PNGase F treatment conditions used in this experiment. There was no effect of PNGase F on the ~38-kDa nonspecific band detected by IIH6. Because the laminin-1 and IIH6 mAb binding epitopes on α-DG overlap (Figs. 2–4) (14), PNGase F treatment was expected also to reduce laminin-1 binding. This was confirmed in the CHO mutant Lec3.2. These cells have null mutations in the CMP-sialic acid transporter (58) and in the UDP-N-acetylgalcosamine 2-epimerase that is required for CMP-sialic acid synthesis (65) and, like Lec2 cells, have little sialic acid on any glycan. As shown in Fig. 5B, Lec3.2 cells transiently transfected with Large but not the empty vector expressed glycosylated α-DG. The intensity and molecular weight range of the glycosylated α-DG smear was reduced by PNGase F treatment. Furthermore, an equivalent reduction was observed for laminin-1 binding (Fig. 5B). Similar results were obtained for glycosylated α-DG from Lec3.2.8 and Lec3.2.8.1 multiple glycosylation mutant cells (data not shown). The combined data show that functional glycosylation of α-DG occurs on PNGase F-sensitive N-glycans that represent major species in cells that cannot transfer sialic acid or Gal (and sialic acid) to their glycans.

Large Induces the Modification of Mucin O-Glycans on α-DG—Lec15 cells are deficient in dolichol-phosphate-mannose synthase activity (41) because of a mutation in the DPM2 gene (66). They consequently cannot express O-Man glycans because the human protein O-mannosyltransferase proteins POMT1 and POMT2 that O-mannosylate α-DG use dolichol-phosphate-mannose and not GDP-mannose as the sugar donor (25). Lec15 cells also do not synthesize glycosylphosphatidylinositol-anchored proteins and do not have N-glycans of the high mannose type (Manα2,6GlcNAc2-Asn), although they can synthesize complex N-glycans (41). Lec15 cells also cannot synthesize C-mannosylated proteins (67). Therefore, if functional glycosylation of α-DG occurs in Lec15 cells it must be on non-O-mannosyl glycans (Fig. 6A).

Lysates from Lec15 cells transiently transfected with mouse Large or vector were examined for IIH6 and laminin-1 binding. It can be seen in Fig. 6B that functionally glycosylated α-DG was produced in Lec15 cells. Removal of N-glycans by PNGase F treatment gave complete conversion of NCAM to a form lacking N-glycans and resulted in a reduction in functionally glycosylated α-DG. This suggests that Large may glycosylate both N-glycans and mucin O-glycans. Additional evidence that Large may modify mucin O-glycans was obtained by examining glycosylated α-DG in Lec15 cells co-transfected with both Large and a cDNA encoding human C2GnT1. C2GnT1 is expressed ubiquitously in the mouse (68) but is absent from CHO cells (69). The intensity and spread of glycosylated α-DG were increased in the co-transfected cells compared with cells transfected with Large alone (Fig. 6B, compare lanes 3 and 5). The increase in IIH6 and laminin binding of ~1.7-fold as measured by densitometric scanning was not because of a higher level of Large protein based on the Myc signal. In addition, PNGase F

![Figure 5. Functional modification of N-glycans of α-DG by overexpression of Large. A, predicted structures of complex N- and O-mucin and O-mannosyl glycans synthesized in the Lec15 CHO glycosylation mutant are shown to illustrate its glycosylation defect. White square, N-acetylgalactosamine; black square, N-acetylgalcosamine; gray circle, mannose; white circle, galactose; triangle, fucose; diamond, sialic acid. B, Lec15 mutant cells were transiently transfected with 7.5 µg of plasmid DNA for expression of mouse Large and/or human C2GnT1; 7.5 µg of plasmid DNA for empty pZeo SV2+ or pcDNA3.1/Myc-His B(−) vector was added to make the final amount of transfected DNA 15 µg when only Large or C2GnT1 was transfected. Cell lysates were treated with PNGase F (+) to remove N-glycans. The enzyme was omitted from control reactions (−PNGase F). Glycosylated α-DG was detected using IIH6 mAb by Western analysis. The blot was stripped and assayed for the expression of NCAM. Identically prepared blots were analyzed for expression of Myc-tagged Large and for laminin-1 binding by overlay assay. Each lane had ~50 µg of protein. Similar results were obtained in two independent experiments.](https://www.jbc.org/content/280/22/20856/F5)

![Figure 6. Functional modification of O-mucin glycans of α-DG by overexpression of Large. A, predicted structures of complex N- and O-mucin and O-mannosyl glycans synthesized in the Lec15 CHO glycosylation mutant are shown to illustrate its glycosylation defect. White square, N-acetylgalactosamine; black square, N-acetylgalcosamine; gray circle, mannose; white circle, galactose; triangle, fucose; diamond, sialic acid. B, Lec15 mutant cells were transiently transfected with 7.5 µg of plasmid DNA for expression of mouse Large and/or human C2GnT1; 7.5 µg of plasmid DNA for empty pZeo SV2+ or pcDNA3.1/Myc-His B(−) vector was added to make the final amount of transfected DNA 15 µg when only Large or C2GnT1 was transfected. Cell lysates were treated with PNGase F (+) to remove N-glycans. The enzyme was omitted from control reactions (−PNGase F). Glycosylated α-DG was detected using IIH6 mAb by Western analysis. The blot was stripped and assayed for the expression of NCAM. Identically prepared blots were analyzed for expression of Myc-tagged Large and for laminin-1 binding by overlay assay. Each lane had ~50 µg of protein. Similar results were obtained in two independent experiments.](https://www.jbc.org/content/280/22/20856/F6)
treatment of these lysates did not reduce glycosylated α-DG to the level seen in Large-only transfecteds (Fig. 6B, compare lanes 4 and 6). The combined data provide evidence that Large modifies N-glycans and mucin O-glycans on α-DG to a significant level when O-Man glycans are not present.

DISCUSSION

Dystroglycan (also known as cramin or dystrophin-associated glycoprotein 1) is an important component of the dystrophin glycoprotein complex that links the extracellular matrix with the intracellular cytoskeleton (70). The integrity of this complex is crucial for the maintenance and function of skeletal muscle and peripheral neurons. Mutations disrupting the DG complex result in muscle (71) and neuronal disorders (72). Dystroglycan is also expressed in other tissues, such as squamous epithelia, where it functions in a variety of processes such as polarization of epithelial cells (73) and modulation of signal transduction (74). Abnormalities in dystroglycan expression and processing have been correlated with the development and spread of certain cancers (75). Glycosylation of α-DG induced by POMT1, POMT2, POMGnT1, LARGE, Fukutin, and FKRP is essential for muscle development and function (22, 23). In this paper we provide insight into the nature of the sugars transferred to α-DG by the overexpression of mouse Large, characteristics of glycan substrates of Large, and the mechanism by which human LARGE rescues function to α-DG in muscle cells that cannot synthesize O-Man glycans (17).

We show here that mouse Large co-localizes in a sucrose gradient with Golgi membranes, consistent with immunolocalization of human LARGE to the Golgi compartment (35). Overexpression of mouse Large causes the functional glycosylation of endogenous α-DG in parent Pro-5 CHO cells and recognition of α-DG by the well characterized glycan-dependent IIH6 and VIA4-1 mAbs, as well as the α-DG ligand laminin-1. Similar effects of overexpressing a human LARGE cDNA have been reported in CHO-K1 cells (35) and TSA201 fibroblasts (21). However, only a small fraction of cellular or secreted α-DG is converted to the functionally glycosylated form (21), making it very difficult to obtain sufficient quantities for structural analyses. Most interestingly, glycosylation of NCAM with polysialic acid also occurs on only a small fraction of the total NCAM in CHO cells (64, 65). Although 3'-untranslated region-specific primers detected transcripts from the endogenous Large gene in CHO cells (data not shown), they do not express functionally glycosylated α-DG. This may be because CHO Large is inactive or transcript levels are inadequate.

Although there is no direct evidence that Large is itself a glycosyltransferase, it seems very likely that it is because it causes the synthesis of functionally glycosylated α-DG when overexpressed in a variety of cell types and because of the presence of two glycosyltransferase domains in Large. As noted in Fig. 1A, the N-terminal region (142–386 aa) in human and mouse Large contains a GTS domain (76), and aa 104–356 have ~40% similarity with the catalytic domain of UGGTs. Furthermore, it has the appropriately positioned DXXD motifs and two other residues known to be required for the catalytic activity of human UGGT1 (50). Mutation of these DXXD motifs to NNN in human LARGE prevents the generation of the IIH6 epitope on α-DG of CHO transfecteds (35). Therefore, a likely possibility is that Large transfers Glc to α-DG. Because Large has been shown to interact physically with α-DG (21), Large may transfer Glc to oligomannosyl N-glycans on α-DG during folding and maturation in the endoplasmic reticulum, as would be predicted for a UGGT (77). This Glc would be removed on the maturation of α-DG. However, Glc has been detected on α-DG purified from bovine peripheral nerve (78) and from rabbit skeletal muscle (79), suggesting that it may in fact be part of the functional glycan epitopes expressed on mature α-DG.

Functional glycosylation of α-DG by Large would seem likely to involve the generation of a glycan polymer giving rise to the broad molecular weight range observed for α-DG detected by mAbs VIA4-1 and IIH6. Because the C-terminal glycosyltransferase domain of human and mouse Large is most related to β3GnT6, which adds GlcNAc to Gal to generate linear polylactosamine sequences (51), the polymer formed by Large might be composed of GlcNAc and Glc. UGGT recognizes the core GlcNAc of oligomannosyl N-glycans and transfers Glc in α,1,3-linkage (80), and β3GnT6 transfers GlcNAc in β1,3-linkage (51). In UGGTs, the first DXXD motif in the catalytic domain binds UDP-Glc (42), whereas the second DXXD motif interacts with GlcNAc (43). Because functionally glycosylated α-DG is induced by Large in Lec2, Lec8, and Lec13 CHO glycosylation mutants, the polymer is not expected to include sialic acid, Gal, or Fuc. Although sialic acid has been implicated in the binding of laminin to bovine peripheral nerve α-DG (15), other studies have excluded a role for sialic acid in laminin or IIH6 binding to α-DG (14, 19–21). In addition, α-DG is functionally glycosylated to normal levels despite hyposialylation in patients having distal myopathy with rimmed vacuoles or hereditary inclusion body myopathy that is caused by mutations in the GNE gene that encodes UDP-N-acetylgalactosamine 2-epimerase/N-acetylmannosamine kinase (81, 82). However, although Gal and Fuc are known to be present on α-DG (20, 63), the results shown here are the first evidence that neither Gal nor Fuc appear to be involved in generating functionally glycosylated α-DG.

The combined data also suggest that the substrate of Large is not sialic acid, Gal, or Fuc. This leaves Man, GlcNAc, or GalNAc as potential acceptors. We propose GlcNAc as a key acceptor because GlcNAc becomes more accessible on Lec8 and Lec2 cells, and mucin O-glycans do not contain Man, but their modification is induced by Large. Moreover, Large transfecents of LEC12 cells had reduced levels of glycosylated α-DG (Fig. 3B). This could be because the modification of GlcNAc by Large is prevented by the addition of fucose at the C-3 position of GlcNAc in lactosamine by α,1,3-fucosyltransferase IX in LEC12 cells (60). α,1,3-Fucose linked to GlcNAc has been observed on O-Man glycans of α-DG (63) and may regulate ligand binding.

The amount of functionally glycosylated α-DG was markedly increased in Lec2 and Lec8 cells expressing Large, and the majority was sensitive to PNGase F digestion. Sialic acid on the N-glycans of CHO cells appears to preclude their modification by Large. Lec8 cells have GlcNAc-terminating N- and O-Man glycans allowing Large to generate a polymer of the type (GlcNAc\(\beta_1,3\)Glc\(\alpha_1,3\))\(\beta_1,2\)Man\(\beta_1,2\)Man. A Large-generated polymer could form a branch from the GlcNAc of O-Man, N-glycan, or core 2 mucin O-glycans. Although the majority of O-glycans of muscle and nerve α-DG are of the type SAc2,2,3⁺Gal\(\beta_1,4\)GlcNAc\(\beta_1,2\)Man-O (78, 79), O-Man glycans modified with a \(\beta_1,2\) or \(\beta_1,6\)-GlcNAc branch have been identified in brain glycopeptides (83). \(\beta_1,6\)-N-acetylgalactosaminyltransferase IX that adds a \(\beta_1,6\)-GlcNAc to N-glycans (84) also adds a branching GlcNAc in \(\beta_1,6\)-linkage to O-Man glycans (85). GlcNAc\(\beta_1,2\)-Man-O but not Man-O or Gal\(\beta_1,4\)-GlcNAc\(\beta_1,2\)-Man-O is a substrate for the enzyme (85). Of course, several other possibilities exist, including the involvement of fukutin and/or FKRP in the generation of Large substrate(s).

The findings reported here provide an explanation of the mechanism by which overexpression of human LARGE rescues the synthesis of functional α-DG in muscle cells from patients with WWS in which few if any O-mannose glycans should be produced (17). This is analogous to the situation in Lec15 cells in which Large generates functionally glycosylated α-DG in the...
absence of O-Man glycans (Fig. 6). Treatment with PNGase F showed that functional glycans on α-DG from Lec15 are N-linked as well as mucin O-glycans. Co-expression of C2GnT1 in Lec15 Large transfectants increased the amount of functionally glycosylated α-DG (Fig. 6), suggesting that modification of core 2 O-mucosyls may be achieved by Large. We propose that LARGE functionally glycosylates unsialylated branches of N-glycans and mucin O-glycans of α-DG to allow the bypass of glycosylation defects in WWS cells and cells from patients with several other dystroglycanopathies (17). It is of note that the human C2GnT1 gene is on chromosome 9p13 in a locus associated with a hereditary myopathy (MIM 605382) (86).

In conclusion, our findings provide insight into the potential substrate of Large and the sugars that may be transferred by Large, and our findings have revealed that the N-glycans of Lec2 or Lec6 modified by Large overexpression would be a source for determining the structure of the Large-generated glycan on α-DG, because in Lec2 and Lec6 this modification is present on truncated N-glycans. Finally, the data show that CHO glycosylation mutants provide hosts for characterizing mutations that cause MDC1D (34).

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