

Review

Matriglycan: a novel polysaccharide that links dystroglycan to the basement membrane

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Abstract

Associations between cells and the basement membrane are critical for a variety of biological events including cell proliferation, cell migration, cell differentiation and the maintenance of tissue integrity. Dystroglycan is a highly glycosylated basement membrane receptor, and is involved in physiological processes that maintain integrity of the skeletal muscle, as well as development and function of the central nervous system. Aberrant O-glycosylation of the α subunit of this protein, and a concomitant loss of dystroglycan's ability to function as a receptor for extracellular matrix (ECM) ligands that bear laminin globular (LG) domains, occurs in several congenital/limb-girdle muscular dystrophies (also referred to as dystroglycanopathies). Recent genetic studies revealed that mutations in *DAG1* (which encodes dystroglycan) and at least 17 other genes disrupt the ECM receptor function of dystroglycan and cause disease. Here, we summarize recent advances in our understanding of the enzymatic functions of two of these disease genes: the like-glycosyltransferase (LARGE) and protein O-mannose kinase (POMK, previously referred to as SGK196). In addition, we discuss the structure of the glycan that directly binds the ECM ligands and the mechanisms by which this functional motif is linked to dystroglycan. In light of the fact that dystroglycan functions as a matrix receptor and the polysaccharide synthesized by LARGE is the binding motif for matrix proteins, we propose to name this novel polysaccharide structure matriglycan.

Key words: dystroglycan, LARGE, muscular dystrophy, O-mannosyl glycan, POMK

Introduction

Cells are associated with the surrounding basement membrane through various cell-surface receptors including dystroglycan, integrins and sulfatides (Roberts et al. 1985; Sonnenberg et al. 1988; Ibraghimov-Beskrovnaya et al. 1992). In muscle cells, such associations maintain the integrity of the cell membrane (sarcolemma); their disruption renders the sarcolemma sensitive to the mechanical stress imposed by cycles of contraction, thus leading to muscle degeneration (Hayashi et al. 1998; Michele et al. 2002). Phenotypes caused by defects in the receptor function of dystroglycan, in both patients with muscular dystrophy and animal models, suggest that this protein is required not only for muscle maintenance but also peripheral-nerve myelination, neuromuscular-junction formation, neuronal migration

in the brain, axon guidance and development of eye, brain and other tissues (Moore et al. 2002; Saito et al. 2003; Wright et al. 2012; Meilleur et al. 2014). Although these findings underscore the importance of dystroglycan in various biological processes, the molecular mechanism that renders this receptor capable of ligand binding was elusive until recently. Soon after dystroglycan was identified in skeletal muscle as 156 K dystrophin-associated glycoprotein (156-DAG), treatment with trifluoromethanesulfonic acid revealed that its glycans confer the ability to bind ligands within the extracellular matrix (ECM; Ervasti and Campbell 1993). However, gaining a structural understanding of this “ligand-binding” glycan moiety was a challenge because dystroglycan, which to date is the only protein known to receive this particular modification in native tissues, is

heavily O-glycosylated and the O-glycan population that decorates it is heterogeneous across tissues and cell types.

Recent genetic studies on muscular dystrophies identified genes whose products are required for the maturation of dystroglycan into a functional receptor. Molecular studies on these gene products led to significant advances in our understanding of the chemical structure of the ECM ligand-binding moiety and its biosynthetic pathway. In this review, we summarize recent discoveries on the glycosylation steps that confer ECM-binding properties to dystroglycan. First, we provide an overview of the receptor function of dystroglycan and of the enzymes that are required for its posttranslational processing. Second, we discuss the ECM-binding structure—a novel glycosaminoglycan (GAG) subspecies—and the biosynthetic pathways that contribute to its production. The key components of the latter are the bifunctional glycosyltransferase LARGE and several O-mannosyl glycans, in particular a phosphorylated structure that links the ECM-binding motif to dystroglycan (platform structure).

α -Dystroglycan: an ECM receptor in skeletal muscle

Dystroglycan was originally identified as a component of the dystrophin–glycoprotein complex (DGC) of skeletal muscle (Ervasti et al. 1990). The DGC plays an important role in muscle maintenance, since mutations in multiple components, the dystrophin and sarcoglycans, cause Duchenne/Becker muscular dystrophy and limb-girdle muscular dystrophies (LGMDs), respectively (see more in reviews; Ozawa et al. 1995; Cohn and Campbell 2000; Nowak and Davies 2004). Biochemical analyses revealed that a single polypeptide encoded by *DAG1* is cleaved into α - and β -dystroglycan subunits by autoproteolysis of its SEA (sea urchin sperm protein, enterokinase and agrin) module (Ibraghimov-Beskrovnaya et al. 1992; Deyst et al. 1995; Smalheiser and Kim 1995; Akhavan et al. 2008). Within the extracellular space, α -dystroglycan binds directly to several non-collagenous proteins including laminin, perlecan and agrin—components of the basement membrane that are essential for its formation. β -Dystroglycan is the transmembrane subunit and its cytoplasmic C-terminal region associates with dystrophin, which in turn binds to the F-actin cytoskeleton. Its N-terminal region, on the other hand, associates with the C-terminal region of α -dystroglycan, securing this soluble subunit to the outer surface of the cell. By bridging the cell membrane and binding to both dystrophin and extracellular proteins, dystroglycan physically links the cytoskeleton to the basement membrane.

Among the ECM proteins so far found to bind to α -dystroglycan, the laminins have been characterized most extensively. These ligands contain multiple domains to which other laminins, entactin/nidogen, integrins, α -dystroglycan, heparin, heparan sulfate and sulfatides can bind. Thus, laminins play a central role in establishing networks among ECM proteins in the basement membrane, and connect this membrane to the surfaces of adjacent cells. Five laminin globular (LG) domains at the C-termini of the α -chain of laminin-111 are the sites of interaction with cell-surface receptors of various types. In particular, the LG4–5 domains interact with α -dystroglycan, heparin and galactosyl sulfatide (Tarabozetti et al. 1990), and the LG1–3 domains interact with integrins (Sung et al. 1993). The crystal structure of LG4–5 within the laminin α 1 chain revealed that these two domains fold into curved β -sandwiches, each of which is built from two anti-parallel sheets and contains a metal ion, likely a calcium ion under physiological conditions (Harrison et al. 2007). A combination of site-directed mutagenesis and *in vitro* binding analysis further demonstrated that α -dystroglycan and heparin bind to partially overlapping

basic amino acid patches near the Ca^{2+} -folding region on the LG4–5 domain surface. These data may explain why the dystroglycan–laminin interaction is sensitive to EDTA and heparin. Laminin-211 (Pall et al. 1996), laminin-511, laminin-521 (Yu and Talts 2003), agrin (Bowe et al. 1994), neurexin (Sugita et al. 2001), perlecan (Peng et al. 1998), pikachurin (Sato et al. 2008) and Slit (Wright et al. 2012) also have highly conserved LG-like domains and are known to bind α -dystroglycan in a Ca^{2+} -dependent manner.

The ability of α -dystroglycan to function as a receptor relies on post-translational modifications, especially glycosylation. α -Dystroglycan is comprised of three domains: the α -dystroglycan N-terminal (DGN) domain (1–312 amino acid: aa), a serine–threonine-rich mucin-like domain (313–485 aa) and a C-terminal domain (486–653 aa) (Figure 1). Analyses by mass spectrometry have shown that the N- and C-terminal domains have several N-glycosylation sites, and that the mucin-like domain contains at least 21 O-glycosylation sites (Nilsson et al. 2010; Stalnakker et al. 2010). O-Glycosylation at residues Thr-317 and Thr-319, which fall within the highly conserved first 18 amino acids of the mucin-like domain, is thought to be crucial for α -dystroglycan's ability to function as a receptor (Hara, Kanagawa, et al. 2011), although the complete structure of the LG domain-binding glycan that is linked to these sites has not yet been determined. It is clear that synthesis of this functional moiety depends on the presence of the DGN domain, which is cleaved by a furin-like proprotein convertase during the posttranslational processing of α -dystroglycan (Kanagawa et al. 2004). However, other than contributing to glycosylation of the neighboring mucin-like domain, the physiological roles of the DGN domain remain largely unknown.

The glycosylation status of α -dystroglycan is strictly regulated with respect to both developmental stage and tissue, with its apparent molecular weight (MW; as determined by western blotting) varying from 100 to 200 kDa across tissues. In the brain, heart, skeletal muscle and kidney, α -dystroglycan is modified such that it can function as an ECM-receptor. Monoclonal antibody (mAb) IIIH6 (available from the Developmental Studies Hybridoma Bank at the University of Iowa) is widely accepted to recognize specifically an LG domain-binding modification on α -dystroglycan because it can inhibit the binding of laminin (Ervasti and Campbell 1993). Another glyco-specific α -dystroglycan mAb (2238) of potential importance was recently developed; it binds to the brain, but not muscle, form of α -dystroglycan (McDearmon et al. 2006). Notably, the brain form of α -dystroglycan has a higher affinity for laminin-511/521 than its muscle counterpart, and the brain-specific mAb is thought to recognize the modification that binds to the LG domains of the laminin α 5 chain. The development of this antibody suggests that the glycan modification on α -dystroglycan is heterogeneous among tissues, and that the tissue-dependent glycosylation fine-tunes the protein's affinity for the ECM ligands that are most relevant in that context.

Genes that are disrupted in cells defective for α -dystroglycan receptor function

The discovery of a group of genetic disorders in which the receptor function of α -dystroglycan is disrupted has greatly accelerated our knowledge of the posttranslational processing pathway that enables α -dystroglycan to bind LG domains. Collectively termed dystroglycanopathies, these disorders include various forms of LGMD and congenital muscular dystrophy (CMD), with or without ocular and brain abnormalities. The CMDs include Fukuyama CMD (FCMD), muscle-eye-brain disease (MEB) and Walker–Warburg syndrome (WWS). Hallmarks include: a reduction, or complete loss, of the

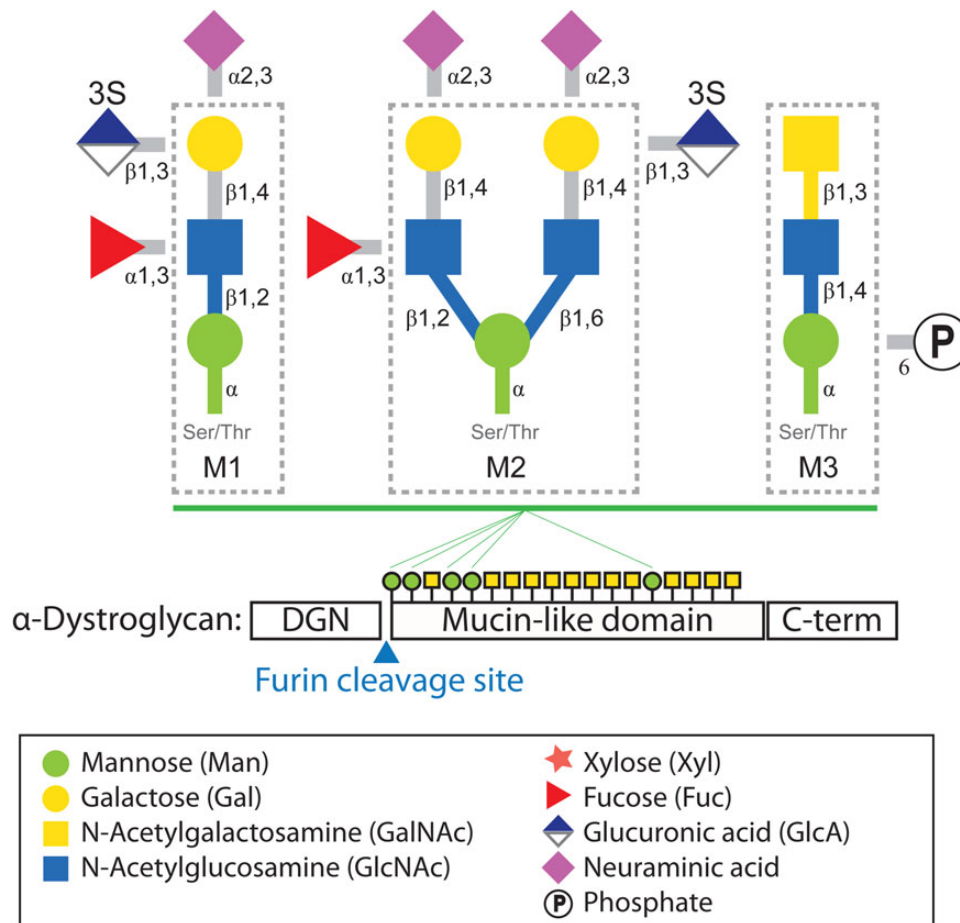


Fig. 1. *O*-Mannosyl glycans identified on α -dystroglycan. The *O*-mannosyl glycan structures designated as cores M1, M2 and M3 are shown surrounded by dotted lines. The modifications outside each box are those known to enable extension of the respective core glycan. 3S represents 3-O-sulfation. The three domains of α -dystroglycan are indicated below the glycan structures. Green circles and yellow squares indicate *O*-mannose and *O*-GalNAc-initiated glycans on the mucin-like domains, respectively (the numbers and the order of glycosylation sites do not follow the published mapping studies precisely). Symbolic representations of monosaccharides and other molecules are described in the box at the bottom. DGN stands for the N-terminus of α -dystroglycan.

laminin-binding ability of α -dystroglycan in skeletal muscle; a loss of α -dystroglycan immunoreactivity to mAb I1H6; and a reduction in MW of the α -dystroglycan produced by skin fibroblasts and tissues derived from the patients (Willer et al. 2012). The brain abnormalities associated with these disorders include hydrocephalus, cobblestone lissencephaly, complete or partial absence of the corpus callosum, cerebellar hypoplasia, flattening of the brainstem, the presence of cerebellar cysts and/or abnormalities in white matter signal (as assessed by MRI; for more detail, see Topaloglu and Talim 2007). Eye abnormalities can include congenital glaucoma, microphthalmia, buphthalmos, congenital cataracts, retinal malformations, myopia and anterior-chamber defects. These abnormalities underscore the importance of dystroglycan's ability to function as a receptor during development of the brain and eye, as well as in the context of muscle maintenance.

To date, 17 genes have been identified as causative in "secondary dystroglycanopathies" (so named because the affected gene is not *DAG1*, which encodes dystroglycan) (Table I). Numerous genetic studies of these secondary disorders have revealed that clinical severity does not necessarily correlate with genotype. For example, defects in the *FKTN* gene, which was originally discovered in FCMD patients, can also cause WWS or a mild LGMD phenotype (LGMD2M). Early studies suggested that the severity of the phenotype was inversely

associated with the degree of functional glycosylation on α -dystroglycan (Kanagawa et al. 2009). This notion was supported by a correlation between clinical severity and the extent to which glycosyltransferase activity was reduced in cells from patients with *POMT1* mutation-associated LGMD and CMD (Lommel et al. 2010). Furthermore, flow cytometry analysis of skin fibroblasts from 21 dystroglycanopathy patients revealed an inverse correlation between clinical severity and the amount of I1H6-reactive glycan at the cell surface (Stevens et al. 2012). Whereas an attempt to correlate the genotype to the pathological phenotype among a cohort of 24 patients carrying mutations in *POMT1*, *POMT2*, *POMGNT1*, *FKTN* or *FKRP* demonstrated a good correlation between overall clinical severity and the glycosylation status of α -dystroglycan in patients with mutations in the first three genes, this was not the case in patients with *FKTN* or *FKRP* mutations (Jimenez-Mallebrera et al. 2009). However, it is possible that some of the proteins whose defects cause secondary dystroglycanopathies have additional substrates besides α -dystroglycan (discussed in greater detail in section: Significance of *POMGNT1*-mediated *O*-mannosyl glycan synthesis). The aberrant function of proteins with such pleiotropic roles may contribute to the observed ambiguities in the correlation between clinical severity and the glycosylation status of α -dystroglycan.

Table 1. Secondary dystroglycanopathy genes

Gene name	OMIM	Protein function(s)
<i>Protein-O-mannosyl transferase 1 (POMT1)</i> (Beltran-Valero de Bernabe et al. 2002)	607423	Protein-O-mannosyl transferase (Manya et al. 2004)
<i>Protein-O-mannosyl transferase 2 (POMT2)</i> (van Reeuwijk et al. 2005)	607439	Protein-O-mannosyl transferase (Manya et al. 2004)
<i>Protein-O-mannose β2-N-acetylglucosaminyltransferase 1 (POMGNT1)</i> (Manya et al. 2003)	606822	Protein-O-mannose β 2-N-acetylglucosaminyltransferase (Manya et al. 2003)
<i>Fukutin (FKTN)</i> (Kobayashi et al. 1998)	607440	Not determined
<i>Fukutin-related protein (FKRP)</i> (Brockington et al. 2001)	606596	Not determined
<i>Protein O-linked mannanose N-acetylglucosaminyltransferase 2 (POMGNT2)</i> (Manzini et al. 2012)	614828	Protein O-linked mannanose β 4-N-acetylglucosaminyltransferase (Yoshida-Moriguchi et al. 2013)
<i>Transmembrane protein 5 (TMEM5)</i> (Vuillaumier-Barrot et al. 2012)	605862	Not determined
<i>Like-acetylglucosaminyltransferase (LARGE)</i> (Longman et al. 2003)	603590	β 3-Glucuronyltransferase (Inamori et al. 2012); α 3-xylosyltransferase (Inamori et al. 2012)
<i>β3-N-Acetylgalactosaminyltransferase 2 (B3GALNT2)</i> (Stevens et al. 2013)	610194	β 3-N-Acetylgalactosaminyltransferase (Hiruma et al. 2004)
<i>β3-N-Acetylglucosaminyltransferase 1 (B3GNT1)</i> (Buysse et al. 2013); <i>β4-glucuronyltransferase 1 (B4GAT1)</i> (Willer et al. 2014; Praissman et al. 2014)	605517	β 3-N-Acetylglucosaminyltransferase (Sasaki et al. 1997); β 4-glucuronyltransferase (Willer et al. 2014; Praissman et al. 2014)
<i>Protein-O-mannose kinase (POMK)</i> (Jae et al. 2013)	615247	Protein O-linked mannanose kinase (Yoshida-Moriguchi et al. 2013)
<i>Isoprenoid synthase domain containing (ISPD)</i> (Roscioli et al. 2012, Willer et al. 2012)	614631	Not determined
<i>GDP-mannose pyrophosphorylase B (GMPPB)</i> (Carss et al. 2013)	615320	GDP-mannose pyrophosphorylase (Ning and Elbein 2000)
<i>Dolichol kinase (DOLK)</i> (Lefebvre et al. 2011)	610746	Dolichol kinase (Fernandez et al. 2002)
<i>Dolichyl-phosphate mannosyltransferase polypeptide 1 (DPM1)</i> (Yang et al. 2013)	603503	Dolichyl-phosphate mannosyltransferase (Maeda et al. 2000)
<i>Dolichyl-phosphate mannosyltransferase polypeptide 2 (DPM2)</i> (Barone et al. 2012)	603564	Dolichyl-phosphate mannosyltransferase (Maeda et al. 2000)
<i>Dolichyl-phosphate mannosyltransferase polypeptide 3 (DPM3)</i> (Lefebvre et al. 2009)	605951	Dolichyl-phosphate mannosyltransferase (Maeda et al. 2000)

Thus far, two cases of primary dystroglycanopathy have been reported. One is a patient with LGMD accompanied by cognitive impairment. In this case, a homozygous C-to-T missense mutation at position c.575 of *DAG1* causes a threonine-to-methionine substitution at amino acid residue 192 (T192M) within the DGN domain (Dincer et al. 2003; Hara, Balci-Hayta, et al. 2011). As noted above, this domain is not present in the mature form of α -dystroglycan, due to processing by a furin-like protease (Figure 1). However, this prodomain binds directly to LARGE, an event that is a prerequisite for functional modification of the mucin-like domain of α -dystroglycan (Kanagawa et al. 2004). Studies using knock-in mice and in vitro binding assays demonstrated that the T192M mutation affects the LG-binding modification of α -dystroglycan by disrupting its interaction with LARGE (i.e., formation of the enzyme-substrate complex). In the second case of a primary dystroglycanopathy, a homozygous missense mutation (c.2006G>T) was found in two siblings with an MEB-like phenotype and multicystic leukodystrophy (Geis et al. 2013). This mutation, which results in a cysteine-to-phenylalanine substitution at amino acid residue 669 (C669P) in the extracellular portion of β -dystroglycan, is predicted to disrupt a disulfide bond between this cysteine and another at amino acid residue 713, and to concomitantly alter the tertiary structure of the β -dystroglycan ectodomain. Currently, it is not clear if the primary α -dystroglycan defect caused by this mutation is a deficiency in its association with the plasma membrane or in its posttranslational modification.

Dystroglycan also serves as a primary receptor for the prototypic arenavirus lymphocytic choriomeningitis virus (LCMV), the highly pathogenic Lassa virus (LASV), the African arenaviruses Mopeia and Mobala, and the Clade C New World arenaviruses (Cao et al. 1998; Spiropoulou et al. 2002; Oldstone and Campbell 2011). Initial

characterization of the virus-receptor interaction revealed that high-affinity binding of the envelope glycoproteins (GPs) of all known arenaviruses to α -dystroglycan depends on modification by LARGE (Kunz et al. 2005; Rojek et al. 2007) and that this interaction is essential for virus entry into the cell. The similarity in the recognition of α -dystroglycan by arenavirus GPs and ECM proteins suggested that the molecular mechanisms used by the pathogens for binding closely mimic those whereby the host-derived ligands are recognized by the receptor. A recent study employed recombinant vesicular stomatitis virus (VSV) expressing LASV-derived GP to perform a haploid screen for factors required for LASV entry (Jae et al. 2013). In addition to *DAG1* and *LARGE*, this robust screen implicated 10 genes already known to cause dystroglycanopathies in LASV entry into the host cell (*ISPD*, *FKTN*, *FKRP*, *POMT1*, *POMT2*, *DPM3*, *GTDC2*, *TMEM5*, *B3GNT1* and *B3GALNT2*). Moreover, using LASV GP as a molecular probe for α -DG glycosylation, this study revealed a new causative gene (called *SGK196* at the time) for the disease, emphasizing the striking similarities between the virus and ECM binding to DG. Additional genes isolated by this screen encode proteins required to synthesize UDP-xylose, UDP-glucuronic acid and dolichol-phosphate mannanose (e.g., UDP-glucose 6-dehydrogenase, UDP-glucuronate decarboxylase 1 and DPM1) and proteins that maintain function of the Golgi apparatus (e.g., subunits of the conserved oligomeric Golgi complex). Interestingly, genome-wide screens for positive selection in human populations revealed strong positive selection for specific *LARGE* alleles in Western African populations from regions where 20–50% of the population is seropositive for LASV (Sabeti et al. 2007; Andersen et al. 2012). Although correlative at the moment, these population genetics data likely reflect the importance of DG and its *LARGE*-dependent modification for human LASV infection.

Posttranslational modifications that confer adhesive properties to α -dystroglycan

Protein O-mannosylation: building a platform for addition of the laminin-binding moiety

Protein O-mannosylation is a modification that is highly conserved among many species, ranging from fungi to mammals (Lommel and Strahl 2009; Panin and Wells 2014). In the case of mammals, α -dystroglycan was the first protein confirmed to receive this modification (Chiba et al. 1997). In Figure 1, we depict the O-mannosyl glycans that have been characterized on α -dystroglycan isolated from various tissues including peripheral nerve (bovine) (Chiba et al. 1997), brain (sheep) (Smalheiser et al. 1998) and skeletal muscle (rabbit and human) (Sasaki et al. 1998; Nilsson et al. 2010; Stalnakar et al. 2010), as well as from cultured cells (Yoshida-Moriguchi et al. 2010). To simplify the description of the individual O-mannosyl glycans in this review, we have designated the three subtypes cores M1, M2 and M3 (Figure 1) (Yoshida-Moriguchi et al. 2013).

Although the sialylated mucin type O-glycan (O-GalNAc) is the most prevalent O-glycan detected on α -dystroglycan, it is the O-mannosyl glycans that appear to be essential for ECM binding given that mutations in the genes encoding POMT1, POMT2 and POMGNT1 are those that cause dystroglycanopathies (Yoshida et al. 2001; Beltran-Valero de Bernabe et al. 2002; van Reeuwijk et al. 2005). POMT1 and POMT2 form a complex within the ER, where they initiate O-mannosyl glycan synthesis by adding mannose to Ser and Thr residues (Figure 2) (Manya et al. 2004). POMGNT1 acts in the Golgi, where it transfers β 2-linked GlcNAc residues during synthesis of the core M1 and M2 glycans from the mannose residues added by POMT1 and POMT2 (Figure 2) (Yoshida et al. 2001). However, the sialylated core M1 glycan (Figure 1) appears not to be the moiety to which the ECM ligands

bind directly, because removing the Siao2-3Gal β 1-4GlcNAc β 1-terminus from this structure by applying a combination of glycosidases does not reduce the affinity of muscle α -dystroglycan for its ligands (Combs and Ervasti 2005). Rather, as demonstrated recently, the LG-domain-binding modification requires the core M3 glycan with a phosphate at the C6 position of its O-mannose (Yoshida-Moriguchi et al. 2010). This structure was originally found on α -dystroglycan produced in HEK293 cells, as an incomplete form of the LG-binding moiety. Subsequent analyses revealed that GTDC2 (also known as AGO61) and B3GALNT2—deficiencies in which have been implicated in dystroglycanopathies—contribute to synthesis of the moiety by catalyzing the transfer of GlcNAc and GalNAc, respectively (Figure 2) (Yoshida-Moriguchi et al. 2013). Moreover, labeling studies using patient fibroblasts and [32 P]orthophosphate indicated that mutations in GTDC2 and B3GALNT2 prevent generation of the phosphorylated structure on α -dystroglycan. This finding led to identification of SGK196 as a kinase capable of phosphorylating the 6-OH residue of an O-mannose that is modified by GTDC2 and B3GALNT2 prior to the reaction (Figure 2). Based on these assignments of enzymatic properties, from herein we refer to GTDC2 as protein O-linked mannose N-acetylglucosaminyltransferase 2 (POMGNT2) and to SGK196 as protein O-mannose kinase (POMK).

POMK is an unusual kinase in that it shares homology with protein kinases, yet it does not contain certain amino acids, within three highly conserved motifs, that are considered critical for kinase activity: the Lys within VALK, which positions the α - and β -phosphates of ATP; the Asp of HRD, which lies in the catalytic loop; and the Asp within DFG, which stabilizes the bound ATP (Manning et al. 2002). These findings had prompted speculation that POMK is a catalytically inactive protein kinase (pseudokinase). However, the above-described demonstration that POMK is required for phosphorylation of α -dystroglycan indicates that this protein is, in fact, catalytically active

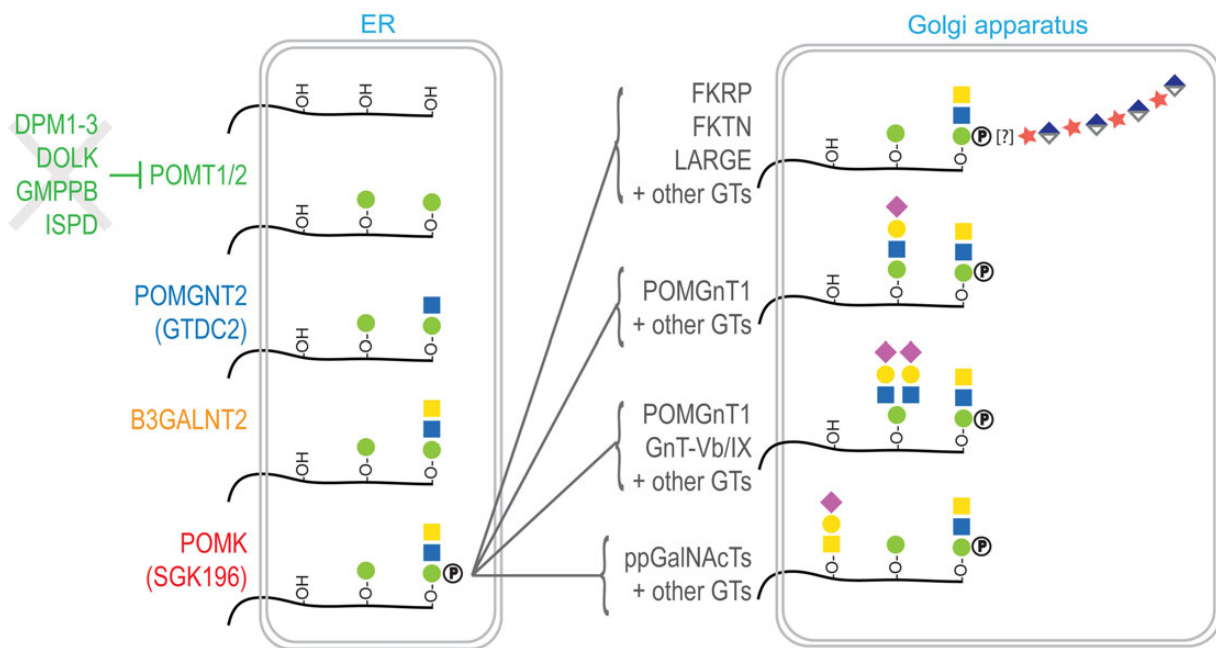


Fig. 2. O-Linked glycosylation of α -dystroglycan's mucin-like domain, and enzymes in the protein processing pathway that are known or predicted to contribute. The left-hand box indicates sequential O-glycosylation steps that occur in the ER. The right-hand box indicates how the nascent dystroglycan can be modified by various glycosyltransferases (GTs) in the Golgi after being processed in the ER. Currently, it is unclear how a defect in POMGNT1 perturbs the post-phosphoryl modification on the phosphorylated core M3 glycan in the Golgi. Defects in DOLK, GMPPB, ISPD and DPM1, 2 and 3 are predicted to perturb POMT activity by reducing the availability of the donor substrate (Table I) or co-factor(s) (Willer et al. 2012). The structure of the moiety that links the phosphorylated core M3 glycan to matriglycan (indicated as [?]) has not yet been solved. Symbols are colored as in Figure 1.

but uses an unusual mechanism for phosphotransfer. This finding raises the exciting possibility that other kinase-like proteins that lack the known consensus domains are perhaps likewise capable of phosphotransfer—to as yet unknown substrates.

Matriglycan: the ECM-binding motif of dystroglycan

Treatment with cold aqueous hydrofluoric acid (which hydrolyzes phosphoester linkages irrespective of whether they are the monoester or the diester form) but not alkaline phosphatase (hydrolyzes only monoester-linked phosphate) significantly reduces the MW of skeletal muscle-derived α -dystroglycan. This suggests that the distal hydroxyl residue of the phosphate at the six position of O-linked mannose is further modified and therefore forms a diester linkage during the production of α -dystroglycan with receptor function. Although the moiety that is attached directly to the phosphate is not yet known, the results of an experiment in which patient cells were labeled with [32 P]orthophosphate indicate that at least FKTN and LARGE are involved in this post-phosphoryl modification (Figure 2) (Yoshida-Moriguchi et al. 2010).

Multiple studies, carried out in cultured cells and mouse skeletal muscle, have demonstrated that forced expression of LARGE increases the MW of α -dystroglycan and its binding ability to LG domains (Barresi et al. 2004; Patnaik and Stanley 2005). LARGE is a type II transmembrane protein that contains two distinct domains: one with homology to β 3GNT1, and another with homology to proteins belonging to glycosyltransferase family 8. Consistent with the implications of this structure, a recent in vitro study demonstrated that LARGE possesses two glycosyltransferase activities: an α 3-xylosyltransferase activity and a β 3-glucuronyltransferase activity (Inamori et al. 2012). Furthermore, this study demonstrated that LARGE can synthesize a polysaccharide composed of the repeating disaccharide [Xyl α 1-3GlcA β 1-3] (Figure 3A) by alternating between transfers of these two monosaccharides. Earlier studies on LARGE had suggested that N- and O-glycans on non-dystroglycan proteins receive the laminin-binding modification when LARGE is overexpressed (Patnaik and Stanley 2005; Zhang and Hu 2012). Because LARGE can use both β -linked

GlcA and α -linked Xyl as acceptors, it may extend the non-reducing ends of certain glycans with these two monosaccharides when it is overexpressed. Notably, the LARGE paralog, LARGE2, possesses the same enzymatic function, although its optimal pH and pattern of expression differ from those of LARGE (Ashikov et al. 2013; Inamori et al. 2013).

The extent to which LARGE modifies individual α -dystroglycan molecules seems to have functional consequences. This notion was supported by a solid-phase binding assay using laminin-111, which demonstrated that ligand binding is proportional to polysaccharide-chain length (Figure 3B) (Goddeeris et al. 2013). However, it is not yet clear how many repeating units are needed to bind one molecule of laminin. Harrison et al. (2007) previously showed, by point mutagenesis and crystallography approaches, that three surface-exposed basic patches in the LG4–5 domains of laminin-111 contribute to its interaction with α -dystroglycan. It is likely that the negatively charged polysaccharide synthesized by LARGE binds to these basic patches through electrostatic associations when α -dystroglycan approaches the ligand. This notion is supported by the fact that one of the patches also contributes to binding with heparin, which contains GlcA as well and is able to compete with α -dystroglycan for binding to the ligand (Harrison et al. 2007). Considering the linear structure of the LARGE modification, its incorporation of GlcA, and the fact that it is synthesized by a polymerizing enzyme that possesses dual glycosyltransferase activities, this novel polysaccharide may fit into the category of GAGs. In light of confirmation by ELISA that both mAb I1H6 and laminin-111 directly recognize this GAG-like structure (Figure 3B and C) (Goddeeris et al. 2013), we propose to assign the name “matriglycan” to the polysaccharide composed of the repeating disaccharide [Xyl α 1-3GlcA β 1-3].

Currently, it is not known if, like most GAGs, LARGE glycan is further modified, for example by sulfation and epimerization. However, an in vitro study using human natural killer (HNK)-1ST, the enzyme responsible for synthesizing the HNK-1 glyco-epitope in brain, indicated that this enzyme can transfer a sulfate to a GlcA at the non-reducing end of the post-phosphoryl modification on α -dystroglycan (Nakagawa et al. 2012, 2013). The fact that both HNK-1ST and LARGE (via its xylosyltransferase activity) modify the 3-OH position of β -linked GlcA suggests that competition between these two enzymatic activities may determine the length of the LARGE glycan chain on α -dystroglycan in certain tissues. This may be the case in brain, which is known to produce an α -dystroglycan form that is recognized by the HNK-1 antibody. Since the degree of glycosylation of α -dystroglycan and the ability of this protein to bind LG domains appear to be regulated very precisely over time and across tissues, it is likely that specialized mechanisms determine the length of matriglycan in a given context. Notably, during myogenesis the MW of α -dystroglycan and the expression levels of DAG1 and LARGE increase simultaneously (Brockington et al. 2010). Also, in both cultured cells and mice, ectopic expression of LARGE leads to significant increases in the degree of glycosylation of α -dystroglycan and its ability to bind ligands within the ECM (Barresi et al. 2004; Patnaik and Stanley 2005; Brockington et al. 2010). These findings support the notion that the level of LARGE expression may be a determinant of the length of the LARGE glycan chain.

Currently, the most important unresolved question in the field of dystroglycan biology is how the LARGE glycan is linked to its platform (i.e., the phosphorylated core M3 glycan). An enzymatic assay using this phosphorylated glycan as the acceptor demonstrated that LARGE alone is not sufficient to transfer either Xyl or GlcA to this moiety (Yoshida-Moriguchi et al. 2013), suggesting that either (i) LARGE requires another enzyme(s) to modify the phosphate before

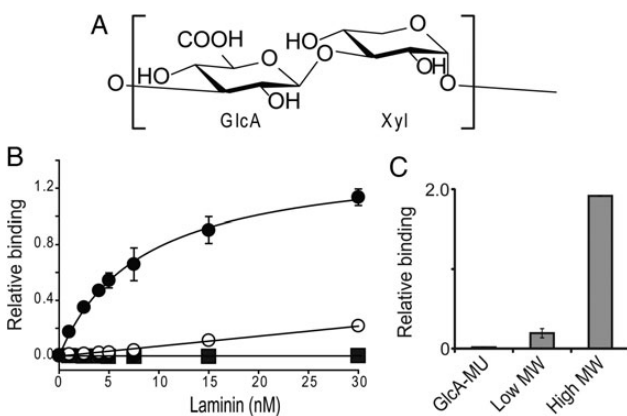


Fig. 3. The polysaccharide (matriglycan) synthesized by LARGE. (A) The chemical structure of matriglycan. (B) Solid-phase assay testing binding to laminin-111. ELISA plates were coated with biotinylated high-MW matriglycan (>13 disaccharide repeats, closed circles), low-MW matriglycan (<13 disaccharide repeats, open circles) or GlcA (negative control, closed square). (C) Quantitation of data from a solid-phase assay testing the binding of sugars that are described in (B) by mAb I1H6. (B) and (C) are modified from Figure 3 and extended data from Figure 7, respectively, in Goddeeris et al. (2013), and the error bars indicate s.e.m. ($n=3$).

it can add sugars to it or (ii) LARGE requires a chaperone(s) or co-factor(s) to recognize this structure as an acceptor. In support of the former notion, two recent *in vitro* studies showed that β 3GnT1, which had previously been implicated in synthesis of a linear poly-*N*-acetylglucosamine *in vitro* (Sasaki et al. 1997), instead has β 4-glucuroniltransferase activity toward xylose. This led to the proposal to rename this enzyme B4GAT1 (Praisman et al. 2014; Willer et al. 2014). The fact that LARGE can extend fluorescently tagged products of B4GAT1 (GlcA- β 4-Xyl- α -pNP and GlcA- β 4-Xyl- β -MU) with matriglycan *in vitro* strongly indicates that this enzyme synthesizes a part of the structure that links the phosphorylated core M3 glycan to matriglycan. This finding further indicates that matriglycan synthesis has similarity to the synthesis of GAG, which requires a polymerizing enzyme with dual glycosyltransferase activities like those of LARGE and a priming glycosyltransferase(s) that adds one of the monosaccharides found in the repeating structure (e.g. EXTs (exostosins) and EXTLs (exostosin-likes) in the case of heparan sulfate synthesis). The roles of three dystroglycanopathy-causing proteins that contribute to synthesis of the LG-binding moiety (FKRP, FKTN and TMEM5) are largely unknown. Notably, *TMEM5*, which was originally identified as a gene mutated in a patient with cobblestone lissencephaly (Vuillaumier-Barrot et al. 2012), encodes a protein that shares homology with an exostosin 1 domain (218–353 aa) and also contains a DxD motif. This structure is consistent with its participation in the synthesis of a glycan that links matriglycan to the phosphate on core M3, in cooperation with B4GAT1.

It should also be noted that both FKTN and FKRP feature an LicD domain. This domain is also present in the bacterial cholinephosphotransferase, an enzyme that transfers phosphocholine to hexose residues of teichoic acid and glycerophospholipids, using CDP-choline as substrate (Ishida et al. 2009). Insight into the molecular functions of FKRP and FKTN may be gained from studies on the synthetic pathway of mannose-6-phosphate in yeast. This pathway requires a protein known as *Mnn4p*, which also features an LicD domain and is believed to facilitate the addition, by a mannosylphosphate transferase (*Mnn6p*), of a phosphate residue to the C6-position of mannose on yeast *N*- and *O*-glycans (Jigami and Odani 1999). Although the mechanism underlying this facilitatory role is unknown, these studies suggest that FKTN and FKRP may act as regulatory units rather than glycosyltransferases, possibly protecting the phosphate linked to the core M3 glycan from phosphatase-mediated hydrolysis or presenting it to modifiers. A better understanding of the role of *Mnn4p* is expected to help to pinpoint the molecular functions of FKTN and FKRP with respect to the post-phosphoryl modification on α -dystroglycan.

Significance of POMGNT1-mediated *O*-mannosyl glycan synthesis

Discovery of the phosphorylated core M3 glycan as a platform that is ultimately extended by the LARGE-dependent matriglycan structure raised questions about how the activity of POMGNT1, which is specific for synthesis of the core M1 and M2 glycans, contributes to production of an α -dystroglycan form that is capable of functioning as a receptor. The available data are consistent with a number of explanations. One is that POMGNT1 activity is not limited to M1 and M2, but additionally modifies the phosphorylated core M3 glycan synthesized in the ER, and that the resulting highly branched moiety is required as a platform for addition and extension of the LG-binding polysaccharide. Thus far, we know that mannose-6-phosphate attached to α -dystroglycan-derived peptide (aa 374–383) does not

function as an acceptor for POMGNT1 *in vitro* (Mo et al. 2011), but this remains to be tested in the context of the phosphorylated core M3 glycan. A second potential explanation is supported by the observation that certain POMGNT1 mutations in dystroglycanopathy patients cause the protein to localize to the ER (vs. the Golgi) without significantly affecting its *in vitro* catalytic activity (Voglmeir et al. 2011; Pereira et al. 2014). Considering that POMGNT2 acts within the ER to add GlcNAc to an *O*-mannose during synthesis of the core M3 structure, it is possible that POMGNT1 mis-localized to the ER competes with POMGNT2 for the acceptor substrate (i.e. *O*-mannose), and thus interferes with proper modification of α -dystroglycan. A third possibility is supported by binding assays carried out in skeletal-muscle lysates using immobilized metal affinity chromatography beads. The latter can capture non-functional α -dystroglycan carrying the phosphorylated core M3 but lacking the post-phosphoryl modification, but cannot capture the functional protein whose phosphate is extended with the LG domain-binding motif (Yoshida-Moriguchi et al. 2010). These studies revealed that, in patients with *POMGNT1* mutations, α -dystroglycan is heterogeneous, with only a subpopulation of the protein possessing laminin-binding activity and the post-phosphoryl modification. This result suggests that lack of the core M1 structure on the mucin-like domain hinders processing of the phosphorylated core M3 within the Golgi.

The question of why a POMGNT1-dependent modification is required for α -dystroglycan to become a receptor for basement membrane proteins may be illuminated by identification of the sites that are decorated with the core M1 and M3 glycans. Mapping studies based on mass spectrometry analysis of skeletal muscle-derived α -dystroglycan have identified >20 Ser and Thr residues in the mucin-like domain as sites of *O*-GalNAc- or *O*-mannose-initiated glycosylation (Nilsson et al. 2010; Stalnakker et al. 2010). These studies failed to identify an obvious consensus recognition sequence for *O*-mannosylation. However, a study using deletion and point-mutation α -dystroglycan constructs indicated that functional modification, which promotes laminin clustering on the cell surface, requires a specific pair of Thr residues (317-TPT-319) located at the very N-terminus of the α -dystroglycan mucin-like domain, and that these residues are highly conserved (Hara, Kanagawa, et al. 2011). Although the ability to bind laminin was reduced when either Thr was replaced with Ala, recent mass spectrometry analysis using α -dystroglycan produced in COS7 cells revealed that the phosphorylated core M3 structure is produced only on Thr-317 (Yagi et al. 2013). These studies suggest that modifiers involved in the post-phosphoryl modification of α -dystroglycan may require the presence of a core M1 structure C-terminal to the acceptor moiety. In other words, the lack of a core M1 structure at Thr-319 may prevent post-phosphoryl modification of core M3 at Thr-317, which would account for detection of the incomplete form of the LG-binding moiety in this mapping analysis. This notion is supported by the results of another mass spectrometry-based analysis, which showed that in the case of another TPT motif (379-TPT-381) within the mucin-like domain of α -dystroglycan, the N-terminal Thr was modified with the phosphorylated core M3 glycan and the C-terminal side of Thr (381 aa) was decorated with a simple *O*-linked mannose (Yoshida-Moriguchi et al. 2010). Notably, a tryptic glycopeptide derived from the highly conserved N-terminal region of the mucin-like domain (313-QIHATPTPVTAGPPTTAIQEPPSR-337) has not been covered in most mapping analyses performed on α -dystroglycan. Identification of sites within this region that are modified by POMGNT1 and POMGNT2 might help to explain how defects in POMGNT1 perturb the maturation of α -dystroglycan into a functional receptor for ECM proteins.

The transfer of GlcNAc by POMGNT1 is a prerequisite for addition, by the β 1-6-*N*-acetylglucosaminyltransferase GnT-IX (also called GnT-Vb), of a GlcNAc residue during synthesis of core M2, which is found predominantly on α -dystroglycan in the brain (Inamori et al. 2004; Stalnakier et al. 2010). GnT-Vb/IX was cloned as a paralog of β 1-6-*N*-acetylglucosaminyltransferase V (GnT-V or Mgat5) and is expressed exclusively in neural tissues (Inamori et al. 2003; Kaneko et al. 2003). Although GnT-Vb/IX can transfer GlcNAc to an *N*-glycan in vitro, a comparison of GnT-V and GnT-Vb/IX null mice revealed that the function of the latter is limited to synthesis of the branched *O*-mannosyl glycan (i.e., core M2), and thus that it cannot compensate for GnT-V (Lee et al. 2012). The same study revealed that GnT-V, on the other hand, can compensate for a deficiency of GnT-Vb/IX in the brain. Since α -dystroglycan in the GnT-Vb/IX-null brain retains the ability to bind to laminin, defects in this enzyme are unlikely to interfere with the generation of an LG-binding moiety in the same way that defects in POMGNT1 do (Lee et al. 2012). In the brain, a 3-sulfoglucuronyl residue caps a small subpopulation of the core M1 and M2 glycans, as well as *N*-glycans, thereby forming the HNK-1 glyco-epitope mentioned above (Yuen et al. 1997). This epitope is present in several neuronal cell-adhesion molecules (including NCAM, P0 and L1), and the analysis of mice deficient for it suggests that it plays a role in synaptic plasticity and memory formation (Morita et al. 2008). Western blot analysis revealed that α -dystroglycan in the brain, but not muscle, features the HNK-1 epitope (McDearmon et al. 2006). A fucosylated *O*-mannose core M1 structure known as the Lewis X epitope has also been detected in brain α -dystroglycan (Smalheiser et al. 1998). This epitope has a high affinity for certain C-type lectins, including scavenger receptor C-type lectin and dendritic cell-specific intercellular adhesion molecule-3-Grabbing non-integrin (Taylor and Drickamer 2007). It is likely that in the nervous system, these adhesive *O*-mannosyl glycans enable α -dystroglycan to facilitate cell-cell and cell-substrate associations, in addition to anchoring the cell to the basement membrane via the LG-binding modification.

Is α -dystroglycan the only protein that contributes to the pathologies observed among dystroglycanopathy patients with defective *O*-mannosyl glycosylation? Mass spectrometry analysis revealed that the ratio of *O*-mannose glycans to *O*-GalNAc glycans among entire *O*-glycans prepared from brain is 1 : 3 (Chai et al. 1999). Given that *O*-GalNAc glycans are present in multiple mammalian proteins, these data indicate that α -dystroglycan may not be the only glycoprotein in the brain that is subject to *O*-mannosyl modification. In support of this notion, in brains from mice in which *DAG1* was knocked out using the Cre/loxP recombination system, the amounts of *O*-mannosyl glycan detected in the glycoprotein pool were similar to those in the brains of wild-type mice (Stalnakier et al. 2011). Also, mass spectrometry has shown that, within the brain, the cell-adhesion molecule CD24 is modified by core M1 (Bleckmann et al. 2009). The receptor tyrosine phosphatase β appears to be decorated with the HNK-1-positive core M2 glycan, because its recognition by mAb Cat-315 is reduced in GnT-Vb/IX-deficient mice (Kanekiyo et al. 2013). Finally, a robust proteomics study using human cultured cells in which POMGNT1 was deleted by a zinc finger nuclease recently revealed that proteins belonging to two families of membrane receptors (the cadherins and plexins), a disulfide-isomerase (PDIA3), and a membrane protein of unknown function (KIAA1549) are subject to *O*-mannosylation (Vester-Christensen et al. 2013). In addition, *O*-mannosylation was confirmed to be crucial for E-cadherin-mediated adhesion in cell aggregation assays (Lommel et al. 2013). Considering that cadherins and plexins mediate cell adhesion, these studies indicate that some of the abnormalities observed in patients with deficiencies

for POMT1, POMT2 and POMGNT1 may arise from defects in cell adhesion and cell-cell communication resulting from aberrant *O*-mannosylation of these adhesive proteins. Although dystroglycan-deficient mice recapitulate phenotypes that are present in the brain and muscle of dystroglycanopathy patients (Kanagawa et al. 2004; Satz et al. 2008; Nguyen et al. 2013), these findings suggest that the clinical manifestations observed among patients with mutations in POMT1, POMT2 and POMGNT1 may differ slightly from those observed in dystroglycanopathy patients with mutations that affect other glycosyltransferases. This notion is supported by the discovery of a patient who had a POMGNT1 mutation and exhibited profound cognitive deficiencies as well as structural defects in the brain and eyes, but had only mild myopathic changes without clear signs of dystrophy (Raducu et al. 2014).

Future directions

Identification of the genes that confer receptor function to α -dystroglycan was recently accelerated by several approaches: genetic studies of patients with CMD (Manzini et al. 2012; Roscioli et al. 2012; Willer et al. 2012; Buysse et al. 2013; Stevens et al. 2013) and severe cobblestone lissencephaly (Vuillaumier-Barrot et al. 2012); murine forward-genetics screens for factors that are involved in axon guidance (Wright et al. 2012) and responsible for congenital hydrocephalus (Vogel et al. 2012); and the implementation of a gene screening method using Lassa virus and haploid cells (Jae et al. 2013). The subsequent discoveries of the enzymatic functions of the disease-implicated proteins shed light on a novel pathway of mammalian posttranslational modification (Figure 2). This pathway is unique in that phosphorylation at the 6-OH of mannose is catalyzed directly by a kinase rather than being the consequence of two enzymatic reactions as is the case for Man-6-phosphate on *N*-glycans; in that case the phosphate is transferred as part of a GlcNAc-1-phosphate (a UDP-GlcNAc serves as the donor) and the GlcNAc moiety is subsequently removed (Varki and Kornfeld 1980; Reitman and Kornfeld 1981).

Which *O*-mannose-linked Ser/Thr will mature into the LG-binding moiety is determined within the ER, through the collective efforts of POMGNT2, B3GALNT2 and POMK. POMGNT2 and B3GALNT2 are required to synthesize the a GalNAc β 1-3GlcNAc β 1-terminus of core M3 (Figure 2, left), which serves as the substrate recognition motif for POMK, enabling it to phosphorylate the *O*-mannose to produce a platform that can ultimately be extended by LARGE within the Golgi (Figure 2, right). A better understanding of the determinants of this site will require knowledge of whether POMGNT2 recognizes the peptide region adjacent to the target *O*-mannose. It is likely that after certain *O*-mannose residues have been modified by these three enzymes within the ER, the hydroxyl residue on the distal side of the phosphate is further modified by Golgi-resident proteins such as FKRP, FKTN and B4GAT1, and ultimately extended with matriglycan. Given that forced expression of LARGE in cells led to a significant increase in the ability of α -dystroglycan to bind its ligands, and even ameliorated defects in receptor function of the α -dystroglycan produced by fibroblasts from dystroglycanopathy patients, this enzyme has received a great deal of attention. The feasibility of carrying out gene therapy for muscular dystrophy using the LARGE-encoding AAV9 vector was tested on mouse models in which LARGE, POMGNT1 or FKRP was defective (Yu et al. 2013; Vannoy et al. 2014). In all cases, gene delivery resulted in enhancement of both α -dystroglycan immunoreactivity to I1H6 and its ability to bind laminin. In the cases of both the LARGE- and POMGNT1-deficient mice, this treatment also led to improvements in the distance the mice were able to run and ameliorated fibrosis. In the

skeletal muscle of FKRP-mutated mice, the percentage of centrally nucleated fibers was reduced. However, a recent study demonstrated that crossing a transgenic mouse in which the LARGE gene is overexpressed (under control of the CAG promoter) with laminin α 2- and fukutin-deficient mice aggravated rather than ameliorated the dystrophic phenotype, and that this was because overexpression of LARGE suppresses muscle regeneration via downregulation of insulin-like growth factor 1 (Saito et al. 2014). Similarly, the muscle pathology in FKRP-deficient mice was exacerbated by crosses with a LARGE transgenic mouse (Whitmore et al. 2014). Goddeeris et al. (2013) demonstrated that the length of the matriglycan chain synthesized by LARGE varies over the course of muscle regeneration, suggesting that the structure to which the ECM ligands bind directly may have to be precisely regulated in vivo.

Recent progress in identifying the enzymatic functions of LARGE and other glycosyltransferases that have been implicated in the dystroglycanopathies has significantly improved our knowledge of how α -dystroglycan maintains tight connections between the muscle cell and the basement membrane. It also supports the potential of developing a therapeutic approach based on LARGE function. Finally, it underscores the need to understand the structure of the matriglycan present on α -dystroglycan in native tissues—with respect to chain length, the number and location of modification sites, and whether it is further modified by sulfation and/or epimerization.

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Conflict of interest statement

The authors have no conflict of interest.

Abbreviations

CMD, congenital muscular dystrophy; DGC, dystrophin–glycoprotein complex; DGN, α -dystroglycan N-terminal; ECM, extracellular matrix; FCMD, Fukuyama CMD; GAG, glycosaminoglycan; GPs, glycoproteins; HNK, human natural killer; LG, laminin globular; LGMDs, limb-girdle muscular dystrophies; mAb, monoclonal antibody; MW, molecular weight; POMK, protein O-mannose kinase; VSV, vesicular stomatitis virus; WWS, Walker–Warburg syndrome.

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