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Dissecting the Molecular Basis of the Role of the O-Mannosylation Pathway in Disease: α -Dystroglycan and Forms of Muscular Dystrophy

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Abstract

Dystroglycanopathies are a subgroup of muscular dystrophies that arise from defects in the enzymes implicated in the recently elucidated *O*-mannosylation pathway, resulting in underglycosylation of α -dystroglycan. The emerging identification of additional brain proteins modified by *O*-mannosylation provides a broader context for interpreting the range of neurological consequences associated with dystroglycanopathies. This form of glycosylation is associated with protein mucin-like domains which present numerous serine and threonine residues as possible sites for modification. Further, the *O*-Man glycans coexist in this region with *O*-GalNAc glycans, conventionally associated with such protein sequences, resulting in a complex glycoconjugate landscape. Sorting out the relationships between the various molecular defects in glycosylation and the modes of disease presentation, as well as the regulatory interplay among the *O*-Man glycans, and the effects on other modes of glycosylation in the same domain is challenging. Here we provide a perspective on chemical biology approaches employing synthetic and analytical methods to address these questions.

Keywords

Glycopeptides; Carbohydrates; Muscular Dystrophy; α -Dystroglycan; Dystroglycanopathy; Protein *O*-Mannosylation

Introduction

Congenital Muscular Dystrophies (CMD) comprise a heterogeneous group of inherited disorders associated with muscle weakness and wasting, resulting in loss of ambulation.^[1–2] Most types of CMD are multi-system disorders with clinical manifestations in the heart, gastrointestinal system, nervous system, endocrine glands, eyes and brain. It is often associated with cognitive impairment and may result in premature death.^[3] Many of these disorders involve loss of function of proteins that are involved in the maintenance of the structural integrity of the cardiac and/or skeletal muscle sarcolemmal membrane. In particular, it is often associated with defects in proteins of the dystrophin-associated glycoprotein (DAG) complex (Figure 1).

DAG is a multimeric transmembrane protein complex that links the intracellular cytoskeleton to the extracellular matrix^[4] and confers structural stability to the sarcolemma

during muscle contraction.^[5] Dystroglycan is an important component of the DAG that spans the sarcolemma and interacts directly with cytoskeleton proteins and components of the extracellular matrix, including laminins, agrin, and perlecan.^[6] It consists of two tightly bound subunits, which are referred to as α - and β -dystroglycan. These proteins are translated from a single mRNA and then cleaved into the two polypeptide chains. β -Dystroglycan is an integral membrane protein having a potential *N*-linked glycosylation site, and a C-terminal cytoplasmic tail.^[4] α -Dystroglycan (α -DG) is an extracellular protein that, in addition to its cleavage from the α/β dystroglycan precursor, undergoes scission of an N-terminal segment.^[4] It is post-translationally modified by *N*- and *O*-glycans and recent studies have shown that aberrant glycosylation of α -dystroglycan is the primary cause of a subset of Congenital Muscular Dystrophies, which are often referred to as dystroglycanopathies.^[4, 7–9] α -Dystroglycan is also expressed in a number of other cell types in addition to muscle.^[10] It has been identified as a receptor for infection by the Lassa fever virus and other Old World arenaviruses, several of which cause viral hemorrhagic fever.^[11] It appears to have a role in metastatic cancer, probably through modulating interactions of the cell with the extracellular matrix.^[12–14] It impacts early mouse development,^[15] structure and function of the central nervous system, myelination and nodal architecture of peripheral nerves, and synaptogenesis.^[16] In the following sections, the emerging understanding of glycosylation of α -dystroglycan and its involvement in disease is described, as well as a number of chemical biology approaches that have provided a more detailed understanding of the biosynthesis of the glycans of α -dystroglycan and its relationship to disease.

The Glycans of α -Dystroglycan

Almost all naturally occurring protein glycosylation can be classified as either *O*-glycans whereby a saccharide is linked to the hydroxyl group on the side chain of an amino acid, or as *N*-glycans in which *N*-acetyl glucosamine (GlcNAc) is linked to the amide of the side chain of asparagine (Scheme 1). The biosynthesis of *N*-linked oligosaccharides is initiated in the endoplasmic reticulum (ER), where a dolichol-linked Glc₃Man₉GlcNAc₂ oligosaccharide precursor is transferred *en bloc* to an Asn-X-Ser/Thr sequon on newly synthesized polypeptides.^[18] Subsequent trimming and processing of the oligosaccharide results in GlcNAcMan₃GlcNAc₂. After translocation of the glycoprotein to the medial stack of the Golgi complex, further maturation of the oligosaccharide occurs, giving rise to wide structural diversity.^[18–20]

The most common form of secretory *O*-linked glycosylation involves the modification of the side chain of serine or threonine by *N*-acetyl- α -D-galactosamine (GalNAc) (Scheme 1).^[18] The biosynthesis of these glycans occurs in the Golgi apparatus where the GalNAc moiety of UDP-GalNAc is transferred to the hydroxyl of serine or threonine catalyzed by polypeptide GalNAc transferases, although under unusual conditions, particularly in cancer, this activity has been found in the ER.^[21–22] *O*-glycans are extended in a stepwise fashion on the glycoprotein, and many display bi-antennary chains having variable termini. Glycoproteins that are modified by dense clusters of *O*-glycans are often referred to as mucins.^[18]

α -Dystroglycan contains three potential *N*-glycosylation sites and has a mucin-like domain that connects the globular N- and C-terminal domains.^[4] The mucin-like domain contains, in addition to the classical *O*-GalNAc initiating glycans, rather unusual mannosylated serine and threonine (*O*-Man) residues (Scheme 2). Glycans that extend from these *O*-Man residues can possess the laminin binding property that is important to the physiological function of α -dystroglycan in its interactions with the extracellular matrix.^[4, 23]

Dystroglycanopathies, with one exception,^[24] have been associated with mutations in the enzymes that assemble these glycans.^[4, 7–9]

O-mannosylation of serine and threonine was first described in yeast, however, it is now known to be wide spread in nature, having been observed from unicellular eukaryotes to primates.^[25] An increased appreciation for the prominence of *O*-mannosylation of proteins came from recent glycomic analyses of mouse brain, which showed that approximately one third of the *O*-glycans in the brain are of the *O*-Man type, with a more detailed picture now emerging of other brain proteins having this attribute.^{[26] [27–29]} Only a minority of the brain *O*-Man glycans are associated with α -dystroglycan.^[27] Comprehensive identification of the proteins that have undergone this type of post-translational modification remains, however, a considerable challenge.

Multiple sites of *O*-mannosylation have been established on α -dystroglycan.^[27, 30–32] The *O*-mannosides are usually extended and the reported structures are often variations of the tetrasaccharide NeuAc α (2,3)-Gal β -(1,4)-GlcNAc β (1,2)-Man α 1-Ser/Thr (Compound **1**, Scheme 2). Common modifications include the asialo-, or asialo and agalacto-, or fucosylated forms of the tetrasaccharide. In addition, sulfation addition has been observed that is likely in the form of sulfoglucuronic acid moiety in place of sialic acid, or in the repeating α (1,3)-GlcUA- β (1,3)-Xyl (GlcUA= glucuronic acid, and Xyl = xylose) disaccharide moiety (see below).^[13, 33–35] Small quantities of branched *O*-mannosylated structures have been found in brain and testes, and the biosynthesis of such compounds is probably mediated by β (1,6)-*N*-acetylglucosaminyltransferase Vb (GlcNAcT-Vb or GlcNAcT-IX) which adds a β (1,6)-linked GlcNAc residue to *O*-linked mannosides that also express β (1,2)-linked GlcNAc.^[36–37]

An additional and partially characterized *O*-mannosyl initiating structure that is essential for laminin and arenavirus binding has recently been found on a limited number of α -dystroglycan sites.^[23] Its core is composed of a GalNAc β (1,3)-GlcNAc β (1,4)-[6-phosphoryl]-Man to which the laminin-binding element is attached (Compounds **2** and **3**, Scheme 2). Along with the phosphate, an important distinction between the two structures is the GlcNAc β (1,2)-Man linkage in the compound **1**, and the GlcNAc β (1,4)-Man moiety in derivatives **2** and **3**. Depending on the tissue,^[38] the phosphate is further extended with a linker of unknown chemical structure forming a phosphodiester. A polysaccharide composed of the repeating disaccharide α (1,3)-GlcUA- β (1,3)-Xyl in which the laminin binding property seems to reside is incorporated on the distal side of the linker.^[34] It is unknown which of the two residues initiates this polysaccharide, or whether it has an additional terminal modification. So far this subset of *O*-Man glycans has only been found on a restricted number of sites.^[23, 39]

Relationship of Specific Enzyme Mediated Glycosylations to Dystroglycanopathies

In combination, the mannosyltransferases POMT1 and POMT2, which are localized in the ER,^[4] initiate the attachment of a mannosyl residue to Ser/Thr (Scheme 3) by employing dolichol phosphate-activated mannose (Dol-P-Man) as the glycosyl donor.^[40] Walker-Warburg Syndrome (WWS), the most severe form of the dystroglycanopathies,^[7] results when the functions of these enzymes are abolished.^[41–43] Three cases of mutations in genes for enzymes of the dolichol-P-mannose pathway have also been implicated in dystroglycanopathies, presumably by interfering with the production of the substrate of POMT1/POMT2.^[40, 44–45] More recent work has identified mutations in a gene *ISPD* (isoprenoid synthase domain containing), encoding a protein of unknown function that also

impinges on the ability of POMT1/POMT2 to transfer mannosyl groups to the protein and has been associated with WWS patients.^[46–47] [48]

Glycoproteomic analyses of α -dystroglycan by mass spectrometry have uncovered a specific distribution of particular *O*-glycans across its mucin like region, and it was found that *O*-mannosylation is concentrated in the N-terminus of this region.^[49–52] There is evidence that local as well as more remote sequence motifs regulate the selection of the sites of *O*-mannosylation.^[53–54] Since POMT1 and POMT2 are localized in the ER, *O*-mannosylation normally occurs without competing for available Ser/Thr sites with polypeptide *O*-GalNAc glycosyltransferases that are localized in the cis Golgi apparatus.^[55] This aspect has ramifications for the segregation of *O*-Man and *O*-GalNAc glycans noted in the glycoproteomic analysis.^[49–52] Specifically, loss of *O*-mannosylation is expected to perturb the subsequent *O*-GalNAc modification pattern,^[56] which in turn may have functional consequences (*i.e.* potential *O*-GalNAc-dependent gain of function in dystroglycanopathies). Studies on the impact of *O*-Man modification on the location of *O*-GalNAc glycans are discussed below. Further, it has been noted that under conditions of Src activation, particularly in cancer, polypeptide GalNAc transferases may be mislocalized to the ER.^[21–22] This can lead to competition of this enzyme with POMT1/POMT2 for modification of Ser/Thr sites, resulting in a disruption of the proper segregation of *O*-Man and *O*-GalNAc sites, which in turn may cause detrimental effect on the functional activity of α -dystroglycan.

As recently shown, while still in the ER, selected *O*-Man sites on nascent α -dystroglycan can be modified by an enzyme designated glycotransferase-like domain containing 2 (GTDC2),^[57,58] that mediates the formation of the GlcNAc β (1,4)-Man linkage of compound, **2** and **3** (Schemes 2 and 3). This disaccharide can subsequently be elaborated to incorporate the laminin-binding element. It has been suggested that this enzyme be renamed POMGNT2,^[58] for protein *O*-mannose *N*-acetylglucosaminyltransferase 2. Mutations in the gene for this enzyme are associated with the WWS phenotype.

β 3-*N*-acetylgalactosaminyltransferase2 (B3GALNT2) can add a β (1,3)-GalNAc residue to the GlcNAc β (1,4)-Man moiety in the ER.^[58] CMD associated mutations in this protein can lead to mislocalization, and are associated with WWS as well as less severe forms of disease.^[59]

The phosphorylation of C-6 position of the mannosyl residue, which appears to be restricted to only those sites carrying GalNAc β (1,3)-GlcNAc β (1,4)-Man,^[58] has recently been shown to be mediated by a kinase, SGK196 (Scheme 3). The identification of this enzyme came from a series of elegant experiments using elaborate genetic screening methods on a novel haploid cell line to reveal candidates believed to impact the functional glycosylation of α -dystroglycan.^[60] In the screen, the gene for this enzyme was identified by the loss of the ability of modified cells to bind the IIH6 antibody that recognizes functional α -dystroglycan, and on the loss of infectivity of Lassa virus that also depends on functional α -dystroglycan.^[60] Based on sequence homology, SGK196 appeared to be kinase-like, although there is some discrepancy between the kinase consensus sequence and that in SGK196, initially raising questions about its activity. Thus, it is suggested that the operative mechanism for this phosphorylation likely has novel aspects.^[58]

After translocation to the Golgi, the majority of the remaining *O*-mannosides of α -dystroglycan are elongated by the *O*-linked mannose β 1,2-*N*-acetylglucosaminyltransferase 1 (POMGNT1),^[61–62] which attaches a β (1,2)-linked GlcNAc residue (compound **1** Schemes 2 and 3). Defects in POMGNT1 activity, which result in the elimination or reduction of *O*-Man extension, are associated with Muscle-Eye-Brain (MEB) disease.^[63–64]

MEB displays a range of severities due to differences in residual enzyme activity,^[65–66] and in some instances has been correlated with particular point mutations in POMGNT1.^[67] Further examination of the impacts of these specific mutations on the glycosylation pattern of α -dystroglycan may reveal the relative importance of the sequential locations at which particular *O*-Man glycans are displayed. Interestingly, the reduced molecular weight of α -dystroglycan in MEB is consistent with the absence of the laminin binding structure, even though this entity is not associated with the GlcNAc β (1,2)-Man linkage.^[23] A clinical assay for MEB has been described based on the ability of cell extracts from patient fibroblasts to facilitate incorporation of GlcNAc into the model substrate benzyl α -mannoside.^[68] As neuromuscular symptoms can be evident at birth, this assay provides a means of early diagnosis.

The likely enzyme candidate for adding the Gal to the GlcNAc β (1,2)-Man disaccharide structure is β 1,4-galactosyltransferase-II (β 4GALT-II).^[69] Further sialylation leads to the prevalent *O*-Man tetrasaccharide glycans (compounds such as **1**, Scheme 2).

The function of LARGE, an enzyme affecting *O*-Man glycan elaboration, and particularly in conferring laminin binding ability of α -dystroglycan, has recently been established.^[34] It was initially identified in a mouse model (*Large^{myd}*) where the gene for the protein LARGE was deleted, resulting in a range of dystroglycan related disease phenotypes.^[70–71] In humans, mutations in this gene have been associated with WWS,^[72] and with a form of congenital muscular dystrophy known as MDC1D.^[73] Gene transfer of LARGE into fibroblasts or myoblasts from WWS, MEB, and FCMD (Fukuyama congenital muscular dystrophy) patients led to a significant enhancement of the laminin-binding activity of α -dystroglycan, indicating that LARGE is significantly involved in forming the laminin binding receptor.^[74] LARGE is composed of two domains that share homology with glycosyltransferases and in particular contain conserved DXD motifs.^[75] DXD is a critical sequence for catalytic activities of many glycosyltransferases. Mutations in these motifs diminished LARGE-dependent modification of α -dystroglycan,^[75] suggesting a bi-functional enzyme. Recent studies using chemical approaches, have shown that LARGE assembles the high molecular weight oligosaccharide based on Xyl α (1,3)-GlcUA β (1,3) repeating disaccharide units (**3**, Scheme 2) as the distal part of the phosphodiester structure.^[34] Though this structure has been associated with the *O*-Man(6-P) glycan (**2**), there are indications in overexpression studies that LARGE can also functionally modify other *O*-or *N*-linked glycans of α -dystroglycan and other proteins.^[75–77] Experiments that led to the identification of the activity of LARGE are described below. Data have now been presented indicating that the enzyme human natural killer-1 sulfotransferase (HNK-1ST) has the ability to modulate the extent to which LARGE can fully elaborate the structure with laminin binding activity, possibly through sulfation of the GlcUA in the repeating polymer,^[35] or by some other means, but apparently after the phosphorylation of the mannose has taken place. These experiments also further highlight the significance of T379 as a site associated with the laminin binding structure.^[35]

Mutations in the genes for fukutin, and fukutin related protein (FKRP), which also impact α -dystroglycan glycosylation,^[4] have been associated with various forms of muscular dystrophies.^[7] The explicit functions of fukutin and FKRP have not been established, although it has been speculated that they exhibit glycosyltransferase activities.^[78] For example, a report linked FKRP to the post-phosphorylation modification of α -dystroglycan.^[38] Recent results with a fukutin knock-out mouse model that recapitulated disease, suggests that ablation of this protein inhibits or limits the extension on the *O*-Man phosphate moiety of compound **2**.^[79] Mutations in fukutin appear to result in both misfolding of the protein and its mislocalization along with that of POMGNT1,^[80] supporting the hypothesis that it may function as a chaperon for the latter.

The observation that functional defects in either LARGE, POMGNT1, POMT1 or fukutin result in a lower molecular weight of α -dystroglycan and a loss in laminin binding^[4, 6, 23] suggests that these proteins affect the assembly of the postulated highly negatively charged, high molecular weight laminin binding oligosaccharide.^[38] Unraveling their individual contributions to the formation of this structure is essential for fully understanding the various forms of dystroglycanopathies at a molecular level and to design effective therapeutic intervention. An important outstanding question is how defects in POMGNT1 which does not appear to have a direct involvement in the elaboration of the laminin binding glycan **3** (Scheme 2),^[81] but rather in the tetrasaccharide structure **1**, can inhibit the formation of the former.^[23]

A challenge in studies dealing with the glycosylation of α -dystroglycan is the considerable complexity and heterogeneity in the distribution of glycosylation sites and the multiple possible structures of the *O*-Man glycans. This is compounded by the coexistence of *O*-GalNAc and *O*-Man modifications in the mucin-like region.^[49–51, 82] The heterogeneity in glycosylation very likely contributes to the diversity of clinical presentation of dystroglycanopathies and their varying degrees of neurological involvement. The majority of investigations of α -dystroglycan have exploited transgenic or mutant animal models, or genetically manipulated cell lines. While these studies have provided important insights into the processing of α -dystroglycan and have pointed to molecular origins of disease, ambiguities in their interpretation persist due to the inherent heterogeneity of the glycosylation processes *in vivo*, and the multiple enzymatic transformations involved directly or indirectly in the ultimate formation of fully functional α -dystroglycan.

Chemical Biology Approaches to Examine O-Mannosylation

Chemical synthesis can provide homogeneous, well-characterized glycoconjugate substrates,^[83–84] which along with the application of advanced mass spectrometry and NMR studies to characterize the location and structure of modifications, has provided valuable insight in enzyme specificities. Furthermore, synthetic compounds make it possible to interrogate processes acting on intermediate structures that are not readily obtained from natural sources. With the rapid expansion of information of possible enzyme activities that may affect α -dystroglycan glycosylation,^[60] a synthetic approach will advance efforts to characterize the factors regulating the elaboration of the glycans.

The analysis of protein glycosylation has improved considerably with the recent developments in mass spectral methods that preserve side chain modifications even when there is fragmentation in the peptide backbone. These methods have been exemplified in the glycomic analysis of tissues from various mouse models.^[27, 49–51] With the continuing advances in the area of glycomic/glycoproteomic analysis, such as HCD (higher-energy collisionally activated dissociation) -triggered ETD (electron transfer dissociation), future research should be able to elucidate the repertoire of *O*-Man-initiated glycans as well as the proteins modified with these types of structures.^[85–87]

The sequence preferences of the enzyme pair POMT1 and POMT2 that initiates *O*-mannosylation of Ser and Thr has been investigated with the aid of synthetic 20-mer peptides, which reflected native α -dystroglycan sequences.^[53] Replacement of individual acceptor sites allowed the ranking of the preference of particular positions in the peptides for initial *O*-mannosylation. This study also indicated that subsequent *O*-mannosylation on other sites can be enhanced after a primary site has been modified. Based on a comparison of the two regions investigated, (336–355 and 401–420), a consensus sequence IXPT(P/X)TXPXXXXPTX(T/X)XX was proposed.^[53] However, mapping of endogenous sites of *O*-mannosylation on α -dystroglycan by multiple groups show deviations from this

consensus sequence in many cases, suggesting additional considerations.^[49–51] It has also been suggested that sites flanked by basic amino acid residues exhibit enhanced *O*-mannosylation.^[54]

The pathway for the biosynthesis of the phosphoryl trisaccharide **2** starting from the *O*-mannosylated α -dystroglycan has just been reported using chemically synthesized substrates and recombinant enzymes.^[58] The formation of the GlcNAc β (1,4) linkage was observed when GTDC2 (POMGNT2) was presented with a glycopeptide based on α -dystroglycan 316–329 having a His tag (AT(Man)PAPVAAIGPPAAHHHHHH) and UDP-GlcNAc. The linkage pattern and stereochemistry of the newly formed glycosidic bond was determined by NMR analysis of the product (**5**) formed by incubation 4-methylumbelliferyl (MU) α -D-mannoside (**4**) with the enzyme (Scheme 4).

Compound **5** was then used to demonstrate that B3GALNT2 can attach the terminal β (1,3)GalNAc moiety. The resulting trisaccharide **6** was used as a substrate for the putative kinase SGK196, which resulted in phosphorylation to provide compound **7**. The attachment of the phosphate at the C-6 position of the mannoside of **7** was established by NMR.^[58] The kinase operated only on the trisaccharide, and not shorter oligosaccharides. Thus, the activity of three important α -dystroglycan processing enzymes, all associated with the ER, have now been established.^[58] It appears that, *in vivo*, this glycan structure is found on a limited number of specific sites.^[23, 39] The origin of this specificity is not resolved with the studies starting from the Man- α -MU derivative. Thus, further studies on glycopeptide substrates in the relevant regions would be beneficial in elucidating the additional contextual features of the protein that dictate the location of this important structure.

Initial confirmation of the activity of POMGNT1 for attachment of GlcNAc to an *O*-Man Ser/Thr residue, was obtained by employing synthetic mannosylated glycopeptide substrates.^[62, 88] The formation of the β (1,2)-linkage was definitively shown by NMR.^[27] The observation that severity of disease varies depending on the POMGNT1 mutation,^[89] has prompted the analysis of specific mutations on enzyme activity.^[67] This effort was accomplished by assaying recombinant forms of the mutant enzyme with a series of synthetic *O*-mannosyl containing glycopeptide substrates derived from α -dystroglycan sequences.

The extension of *O*-Man by a β (1-2)- or β (1-4)-linked GlcNAc moiety determines whether a conventional tetrasaccharide (compound **1** or derivatives thereof) or the unusual *O*-Man-6-P glycan (compounds **2** and **3**) are produced. Glycan mapping has shown that the *O*-Man-6-P glycan is present at sites nearby the more common *O*-Man extension.^[23, 49] The proximity of these two types of glycans raises questions about the molecular mechanisms that control the observed regioselectivity, and it is possible that the formation of one class of *O*-Man influences the other. Such a phenomenon may provide a basis for connecting the hypoglycosylation of the common tetrasaccharide with disease (see above). In this respect, chemical synthesis made it possible to determine consequences of specific glycosylation patterns. Thus, to establish substrate preferences, we prepared a set of glycopeptides based on a fragment that was known to contain both types of *O*-Man glycans, and examined their acceptor behavior for the enzyme POMGNT1 that forms the common GlcNAc β (1-2)-Man linkage (Scheme 5).^[81] It was envisaged this approach would provide insight into how the POMGNT1 enzyme, whose mutations are associated with MEB, indirectly impacts laminin binding. One site for the laminin binding entity is T379, and POMGNT1 could append a GlcNAc β (1-2) moiety to *O*-Man at T379 of glycopeptide A (Scheme 5A).^[81] This finding implied that modification of the *O*-Man at this site must occur before α -dystroglycan encounters POMGNT1 to preclude diversion away from the biosynthetic path culminating in the laminin binding structure. This was born out by the recent finding that formation of the

trisaccharide core of the laminin binding structure occurs in the ER,^[58] before the Golgi resident POMGNT1^[4] can interact with the glycoprotein. The nearby T381 also carries an *O*-Man residue that can be extended by a common GlcNAc β (1,2) moiety as shown in fragments isolated from native α -dystroglycan.^[23] Interestingly, when presented with glycopeptide B, (Scheme 5B) having only this site modified by *O*-Man, no reaction involving POMGNT1 was detected. However, when both T379 and T381 were modified with an *O*-Man residue as in glycopeptide C, it was found that this site could be extended. Although the influence of another nearby mannosylated T residue 388 still needs to be examined, the results indicate a dependence of POMGNT1 preferences on neighboring glycans. Interestingly, α -dystroglycan isolated from the fukutin knock-out mouse model, which was subjected to treatment with HF to remove the component linked by the phosphodiester, showed a higher molecular weight than α -dystroglycan from wild type animals treated in the same way.^[79] Our *in vitro* results highlights the interrelationships of the *O*-Man glycan elaboration, leaving open the possibility that in the absence of the fully extended phosphodiester component, as in the knockout model, the non-phosphorylated *O*-Man residues in the vicinity may be extended to a greater degree leading to higher molecular weight structures. There have been several recent reported studies using synthetic glycopeptides demonstrating a dependence of POMGNT1 on the peptide sequence surrounding an isolated *O*-mannosylated site.^[67, 90] With the temporal relationship of the formation of the Man(6-P) core trisaccharide now elucidated, it is now possible to design substrates to further investigate how the two classes of *O*-Man glycans interact with one another.

Chemical approaches have contributed significantly to the identification of the function of the protein LARGE. As indicated above, it was hypothesized that LARGE may participate in the incorporation of xylose and glucuronic acid moieties on α -dystroglycan.^[34] To test this hypothesis, a secreted form of LARGE was presented with the putative synthetic substrate acceptor *p*-nitrophenyl α -xyloside (**8**, Scheme 6) in the presence of UDP-GlcA, and interestingly, formation of disaccharide **9** was observed.^[34] Additionally, when LARGE was presented with the synthetic 4-methylumbelliferyl β -D-glucuronide (**10**, Scheme 6) as an acceptor and UDP-Xyl as a donor, the Xyl-GlcUA adduct (**11**) was formed. Incubation of LARGE with the latter acceptor and both UDP sugars produced a polymer composed of repeating disaccharide blocks [-Xyl α (1,3)-GlcUA β (1,3)-] (compound **3**, Scheme 2).^[34] These studies have resolved ongoing speculation that LARGE might be a bifunctional glycosyltransferase as postulated from sequence homologies, and greatly clarifies its central role mediating the formation of functional α -dystroglycan. However, the exact nature of the intermediate α -dystroglycan glycan structure which LARGE initially modifies has yet to be determined. The same approach has now been extended to a paralog, LARGE2, showing that it too has similar enzymatic functions to LARGE, but displays differences in the pH optimum for reactivity.^[91]

Interplay between *O*-Mannosylation and *O*-GalNAcylation

In addition to *O*-mannosylation, α -dystroglycan also contains multiple *O*-GalNAc initiated glycans.^[82] Recent mapping studies have revealed interesting details about their prevalence and localization within the mucin-like region of the glycoprotein.^[49–52] These structures have received less attention because they have not been directly associated with profound pathologies. However, there is evidence from studies of α -dystroglycan from LARGE expressing Chinese hamster ovary (CHO) cell lines that under some circumstances, the *O*-GalNAc glycans can carry a laminin binding element.^[75–76] They also may play a structural role in maintaining the global organization of the protein, where two apparently globular ends are linked by an extended region.^[17, 92] The latter type of structure is typical of conventional mucins, where an extended organization is induced by *O*-GalNAc

glycosylation.^[93–94] Initial studies on model *O*-Man and *O*-GalNAc glycopeptides indicate that the former has less impact on promoting an extended structure than the latter, highlighting that the two forms of glycosylation have differential conformational effects.^[81, 95] A distinct pattern of distribution for these two types of glycan has emerged from the site mapping. The N-terminal region the mucin-like region is largely modified by *O*-Man glycans, which is followed by a region primarily modified by *O*-GalNAc glycans.^[49–52] Considering the highly conserved sequence of α -dystroglycan, this is an inviting and significant system for elucidating the factors that control the distribution of glycosylation, thereby furthering the understanding of the factors that govern the site selectivity of *O*-glycosylation.

Synthetic glycopeptide derived from two segments of α -dystroglycan were designed to study the impact of the *O*-Man sites on loci modified by polypeptide GalNAc transferase enzymes.^[56] In each case, there was a cluster of four adjacent possible sites of glycosylation. Interestingly, only three out of a number of polypeptide GalNAc transferase isoforms examined showed noticeable activity for these glycopeptides, with one, the T1 isoform found to be expressed at a reasonable level in muscle cells (Figure 2).^[56] The first sequence, P₄₁₉PTTTTKKP came from a region of α -dystroglycan where only *O*-Man glycans are present,^[51] even though not all potential sites for glycosylation are occupied. We demonstrated that the ability of the enzymes to add *O*-GalNAc was dependent on a particular pattern of preexisting *O*-Man substitution (Figure 2A).^[56] It therefore appears that the factors regulating the loci of modifications by POMT1 and POMT2 are important for ultimately defining the pattern of *O*-mannosylation such that GalNAc modifications are excluded in certain areas. It is reasonable to assume that in native α -dystroglycan the restricted loci of the two types of *O*-glycosylation are significant. In instances of disease arising from POMT1 or POMT2 mutations, such as Walker-Warburg syndrome with hypoglycosylation of the *O*-Man glycans, the results suggest that these conditions might allow for the appearance of *O*-GalNAc modifications ectopically in this region, possibly further compounding the severity of disease. This could also occur under disease conditions where GalNAc transferases are mislocalized to the ER.^[21–22] In the other sequence examined, R₄₇₉IRTTTSGVPR (Figure 2B), where *O*-Man and *O*-GalNAc sites coexist *in vivo*,^[51] we were able to emulate the native glycoforms with both *O*-Man and *O*-GalNAc modifications within the same cluster with recombinant polypeptide GalNAc transferases (Figure 2).^[56] The glycoform with an *O*-Man on T483 is devoid of other modifications including GalNAc both in our *in vitro* experiments and in material isolated from α -dystroglycan.^[51, 56] The results with these two sequences recapitulate the patterns found *in vivo*, and highlights the multifaceted influences of *O*-mannosylation on the action of the ppGalNAc transferases. This observation demonstrates the value of exploiting synthetic glycopeptides and recombinant enzymes in furthering our understanding how features of the glycoprotein affect the ultimate post-translational processing. It is anticipated that similar future studies will contribute to therapeutic developments through providing a molecular understanding of the process of *O*-glycosylation. The recent chemoenzymatic synthesis of the full tetrasaccharide structure starting from a mannosylated glycopeptide,^[96] suggests that this can be applied to examine more elaborate glycoforms.

Novel Therapeutic Approaches

Small molecule screening^[97] and glycosyltransferase overexpression^[74, 98] have been used to improve laminin binding. For example, Martin and colleagues established that overexpression of cytotoxic T cell-GalNAc transferase improves laminin binding.^[98–100] While one could speculate that this might compensate for, or rescue, deficient activity of the glycosyltransferase B3GALNT2 in adding the terminal GalNAc and facilitate phosphorylation, the cytotoxic T cell-GalNAc transferase is in the Golgi, and the

phosphorylation of the mannose, which requires the preformed trisaccharide structure, is normally completed in the ER.^[58] Alternatively, it might otherwise increase GalNAc sites which have been suggested as sometimes being able to serve as sites for LARGE action in rescuing α -DG laminin binding.^[75] Overexpression of LARGE also offers promise in addressing disease.^[74] A recent small molecule screen identified lobeline^[97] as promoting increased WFA (Wisteria floribunda lectin)-binding. This lectin binds terminal GalNAc, and such a binding correlates with improved laminin binding of cells in mouse myoblasts. Interestingly, lobeline-induced increases in WFA binding appears to be dependent on *N*-linked, and not *O*-linked, glycosylation.^[97] This intriguing finding is consistent with data from the Stanley laboratory that demonstrated the LARGE-dependent binding of laminin in cells could result from modification of *O*-Man- or *O*-GalNAc-initiated glycans as well as of *N*-linked structures.^[75–76] Common antenna termini from different classes of glycan structures may then present substrates for the addition of the laminin-binding structure, and provide compensatory mechanisms, albeit incomplete, that may offer the prospect of being therapeutically relevant.

Summary and Outlook

The pattern of disease presentation by dystroglycanopathies is complex due to the intricacies of the posttranslational glycosylation of α -dystroglycan. This feature complicates the task of identifying disease markers. It is clear that a more comprehensive knowledge of the roles of specific glycosyltransferases, and the products they form is required. The use of synthetic substrates provides a means to resolve these issues and identify partners with which the *O*-Man glycans can interact. A synthetic approach is uniquely suited to interrogate intermediate biosynthetic steps, and define how normal and aberrant structures impact the glycosylation pathway. It also offers the prospect for developing superior substrates for clinical assays,^[68] which in connection with patient cell culture extracts, would allow a more facile quantification of abnormal enzyme activity. The approach can also lead to the identification of key glycoconjugate structures that could be exploited in generating antibodies for use as diagnostic immunohistochemical reagents. It is to be expected that effective disease markers will ultimately provide tools for evaluating the effectiveness of emerging therapies.

With the identification of additional proteins impacting functional glycosylation of α -dystroglycan,^[60] and presumably other *O*-mannosylated glycoproteins, application of a synthetic strategy will continue to be important in characterizing specific activity of these enzymes. Efforts to generate relevant recombinant enzymes, currently under way at the Repository for Glyco-Enzyme Expression Constructs (<http://glycoenzymes.ccr.crc.uga.edu>), as well as the availability of synthetic glycopeptide substrates provides an opportunity to establish roles for LARGE, fukutin, FKRP, and other enzymes involved in building the laminin-binding structure. Further, the enzymes, in conjunction with azido-modified sugar nucleotides, will likely aid in the identification of the *O*-mannome, which is the set of proteins carrying an *O*-Man modification. This is particularly important in the brain where the presence of additional members of this class is evident.^[27, 29] Disruption of the dystrophin-glycoprotein complex is not sufficient to explain the neurological phenotype in severe CMDs, given that Duchenne muscular dystrophy patients usually only display mild cognitive impairment if any. Overall, the implementation of chemical synthetic and analytical strategies augments the genetic approaches largely used until now in sorting out the steps in proper processing of the glycoprotein, and the aberrations leading to disease. These data will contribute to better defining approaches to therapeutic intervention, and may help reveal the origins of the number of dystroglycanopathies that have remained cryptic.

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Biographies

David Live received a Ph.D. in Chemistry from the California Institute of Technology in 1974. His research has exploited the tools of chemistry, biochemistry and NMR in the study of peptides, proteins, glycoproteins and nucleic acids towards developing a better appreciation of the structure and function of biomolecules at a molecular level. He joined the Complex Carbohydrate Research Center at the University of Georgia in 2006. His major current interest is in understanding the impact of various forms of protein *O*-glycosylation on molecular structure and recognition.

Lance Wells received a Ph.D. in Biochemistry and Molecular Biology in 1998 from the Emory University School of Medicine. A postdoctoral research fellowship in the research group of Prof. Gerald Hart at the Johns Hopkins School of Medicine in Biological Chemistry followed, which was supported by a National Research Service Award from the National Cancer Institute of the NIH. Dr. Wells joined the Complex Carbohydrate Research Center of the University of Georgia in August of 2003 where he is currently the Georgia Research Alliance Lars G. Ljungdahl Distinguished Investigator and Associate Professor of Biochemistry and Molecular Biology. His research focuses on the roles post-translational modifications play in increasing protein functional diversity with an emphasis on *O*-glycoproteins involved in human diseases such as diabetes, cancer, intellectual disability and muscular dystrophy.

Geert-Jan Boons received a Ph.D. in Chemistry in 1991 from the University of Leiden (Prof. Jacques van Boom). Prior to joining the faculty of the Complex Carbohydrate Research Center of the University of Georgia (USA), he was a postdoctoral fellow at Imperial College and the University of Cambridge (Prof. Steven Ley), and then a lecturer and professor at the University of Birmingham (UK). Currently, he is the University of Georgia Foundation Distinguished Professor in Biochemical Science. He has received the Carbohydrate Research Award for Creativity in Carbohydrate Science, the Horace Isbell Award, and the Roy L. Whistler International Award. His research program focuses on the development of new approaches to synthesize complex glycoconjugates and employ the resulting compounds for biological and biomedical explorations.

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Figure 1.

A schematic representation of the dystrophin glycoprotein complex. The shape of α -dystroglycan is deduced from electron micrographs of fully processed mature chicken α -dystroglycan.^[17] Filled circles indicate *O*-glycans, with the laminin binding glycan structure believed to be associated with some of those on the *N*-terminal lobe.

Figure 2.

Summary of the sites of *O*-GalNAc addition by ppGalNAc-T1, -T3 and -T5 on peptide and *O*-mannosylated glycopeptides from regions of α -dystroglycan. Sites of addition were detected by mass spectrometry. Sites of pre-installed *O*-Man are indicated by filled green circles and sites of *O*-GalNAc addition by the ppGalNAc-T enzymes are denoted by filled yellow squares. **A)** Results for the PPTTTTKKP-derived peptides and glycopeptides, and **B)** Results for the RIRTTTSGVPR-derived peptides and glycopeptide. Reproduced from reference 56.

Scheme 1.
Common forms of protein glycosylation.

R= H or CH₃
O-GalNAc-Ser/Thr

R= H or CH₃
O-Man-Ser/Thr

Scheme 2.

O-Mannosyl glycan 1 is a common structural motif of glycoproteins of muscle and brain tissue. Glycans in this class may sometimes have branches at the C-6 of mannose. The unusual phosphoglycopeptide 2 has been identified on the mucin-like domain of α -Dystroglycan.^[23] Extension of the phosphodiester in 3, is required for laminin binding but may not occur in all tissues.^[38] The linking structure, XX, is not fully known, but includes a polysaccharide based on a repeat of xylose and glucuronic acid.^[34] It is not clear whether Xyl or GlcUA initiates the polymer, or whether there is any terminal modification.

Scheme 3.

Schematic illustration of the initial enzyme mediated steps in the two major pathways of *O*-Man glycan elongation.^[58] Sugar residues are depicted with the symbols widely used,^[18] green circle Man, blue square GlcNAc, yellow circle Gal, yellow square GalNAc and pink diamond sialic acid.

Scheme 4.
Enzymatic elongation of a mannoside derivative.^[58]

Scheme 5.

Products of POMGNT1 action on several *O*-mannosylated glycopeptide substrates of GAIQTPTLG, residues 374 to 383 of α -dystroglycan. T₃₇₉ is identified as one of the sites with a phosphorylated mannoside. Green circles indicate mannose, and blue squares *N*-acetylglucosamine.^[18] Positions of GlcNAc addition determined from mass spectrometry.^[81]

Scheme 6.
Reactions mediated by the enzyme LARGE on derivatives of xylose and glucuronic acid.^[34]