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Mammalian O-Mannosylation Pathway: Glycan Structures, Enzymes, and Protein Substrates

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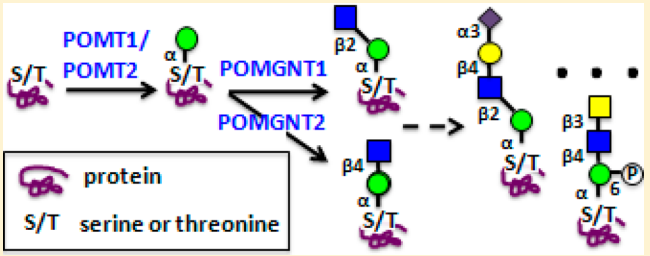
ABSTRACT: The mammalian O-mannosylation pathway for protein post-translational modification is intricately involved in modulating cell−matrix interactions in the musculature and nervous system. Defects in enzymes of this biosynthetic pathway are causative for multiple forms of congenital muscular dystophy. The application of advanced genetic and biochemical technologies has resulted in remarkable progress in this field over the past few years, culminating with the publication of three landmark papers in 2013 alone. In this review, we will highlight recent progress focusing on the dramatic expansion of the set of genes known to be involved in O-mannosylation and disease processes, the concurrent acceleration of the rate of O-mannosylation pathway protein functional assignments, the tremendous increase in the number of proteins now known to be modified by O-mannosylation, and the recent progress in protein O-mannose glycan quantification and site assignment. Also, we attempt to highlight key outstanding questions raised by this abundance of new information.



Throughout biology, the addition of carbohydrates, or glycans, to extracellular and membrane proteins is an important post-translational modification involved in protein stability, quality control, cell-surface retention, and ligand interactions.[1] Glycans modulate the biophysical properties of proteins and lipids and play particularly prominent roles in cellular interactions as the primary constituents of the often nanometer thick glycocalyces coating all mammalian cells. O-Linked mannose (O-mannose) glycans are initiated by covalent linkage of mannose to the hydroxyl oxygen of a serine or threonine amino acid residue. O-Mannose may then be extended by the addition of other monosaccharides and functional groups to form a variety of glycan structures.

In recent years, O-mannose glycans have been demonstrated to play critical roles in cellular interaction-based pathologies, including congenital muscular dystrophies (CMDs)[2−5] and cancers.[6−9] In particular, defects in the biosynthesis of O-mannose glycans often result in the hypoglycosylation of α-dystroglycan (α-DG), the most well characterized O-mannosylated mammalian protein. α-DG is a key part of the dystrophin−glycoprotein complex that links the extracellular matrix to the intracellular cytoskeleton. This linkage depends on the "functional glycosylation" of α-DG and its subsequent ability to bind to extracellular matrix proteins containing laminin globular (LG) domains.[10,11] Hypoglycosylation of α-DG thus results in compromised tissue structure and robustness, causing CMDs termed secondary dystroglycanopathies.[12]

In the past few years, glycomic advances have allowed the increasingly rapid characterization of O-mannose glycans, including further elucidation of the laminin-binding glycan structure.[13,14] These results in conjunction with results from other studies have brought the complement of observed O-mannose glycan structures to at least 23, some yet to be completely defined (Charts 1−3). Glycoproteomic advances have dramatically enhanced our knowledge of proteins

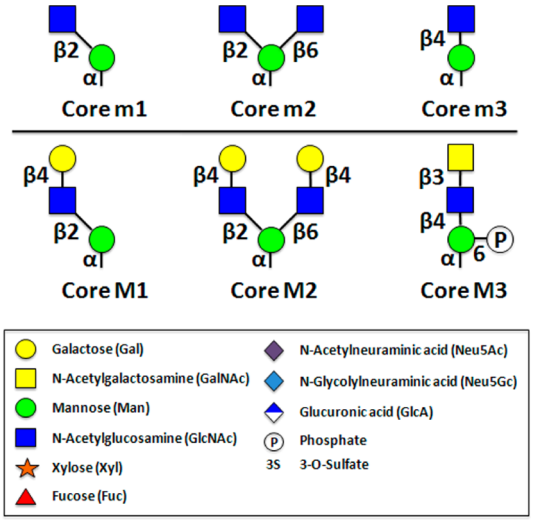
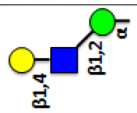
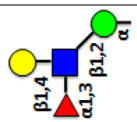
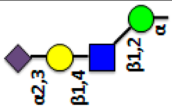
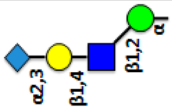
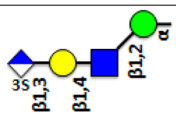


Figure 1. Core structures of O-mannose glycans. The naming scheme used in the bottom row was proposed in ref 22, while the top row includes our proposed naming scheme for substructures. Essentials of glycobiology symbolic representations of monosaccharides and other molecules are described in the box.[1]

Received: February 4, 2014
Revised: May 1, 2014
Published: May 1, 2014

Chart 1. Core m1 Glycans Found in Mammals[a]

#	Structure	References
1		[16, 19, 25, 113]
2		[16, 19, 25, 111, 113]
3		[19, 25, 111, 113]
4		[16, 19, 25, 111, 113]
5		[16, 19, 110]
6		[116]

[a]See Figure 1 for the symbol legend. Core m1 glycans account for more than 15% of protein-linked O-glycans in mouse brain.[19] Core m1 glycans are necessary for α-DG functional glycosylation and modulate O-GalNAcylation. Note that O-mannose without an extension has also been observed.[19]

modified by O-mannosylation with a 2013 publication from the Clausen laboratory expanding the number of known O-mannosylated proteins from approximately 10 to more than 50 O-mannosylated glycoproteins, modified at a minimum of 235 sites, including most prominently the cadherins and plexins, which further cements the role of O-mannosylation in cellular adhesion and interaction.[15] During the period from 2010 to mid-2013, the first papers mapping specific O-mannose glycans to distinct peptides and providing the first views of the actual sets of glycans cosynthesized *in vivo* were published.[13,16−18] Additionally, a complementary series of quantitative glycomic and glycoproteomic studies of O-mannosylation in mammalian systems and pathologies were also published.[16,19,20] In parallel, advances in gene-based technologies in the past three years have allowed increasingly rapid characterization of the biosynthetic pathways involved. This has led to an expansion in the number of genes encoding proteins known to be directly involved in O-mannose structure synthesis from roughly 9 to 17 (Tables 1 and 2). In particular, gene trap insertion in a haploid mammalian cell line coupled with flow cytometry allowed the Brummelkamp laboratory to locate nearly all genes known to play a role in mammalian pathologies related to O-mannosylation (the work of roughly 15 years of biochemistry) as well as to identify previously undiscovered genes in a single publication.[21] Information about the "α-DG glycosylome" published in this paper integrated with biochemical information from the Campbell laboratory mapping a significant portion of key outstanding O-mannosylation pathway enzymatic activities[22] will play a prominent role in this review as we highlight the most recent results and synthesize information from across the field.

However, our understanding of mammalian O-mannosylation is far from complete. Glycan structures remain to be completely elucidated; details of the α-DG functional glycan epitope recognized by LG domains and antibodies IIH6 and VIA4-1[23,24] remain to be conclusively resolved, and significant questions about the regulation and specificities of key enzyme activities and their functions across tissues and during development remain. Consequently, we will also attempt to highlight the most important outstanding questions in each section.

NOMENCLATURE

To organize and conveniently discuss O-mannose glycans and glycosylation, first let us briefly consider nomenclature. In a recent paper from the Campbell laboratory elucidating biosynthesis of the "core" glycan structure that is ultimately extended so as to serve as the only known normal physiological acceptor of α-DG functional glycosylation, a set of core O-mannose structures were proposed (Figure 1).[22] We will adopt this nomenclature when discussing the order-dependent biosynthesis of structures that represent base extensions beyond the N-acetylglucosamine (GlcNAc) residues directly attached to O-mannose. However, we also propose a corresponding set of named structures denoting O-mannose extended only by GlcNAc residues and will distinguish these smaller (sub)structures by the use of a lowercase m (Figure 1). This corresponding proposal more closely resembles the core structure naming convention adopted for O-GalNAc glycans.[1] Analogously, each core m structure is the most basic common core shared by a class of typically further extended O-mannose glycans. Consequently, this naming scheme allows for categorization of all of the known O-mannose structures observed to date (Charts 1−3) and highlights possible biosynthetic fates dependent only on monosaccharides attached directly to O-mannose. We will use the core m nomenclature when discussing categories of glycans sharing a given core O-mannose structure.

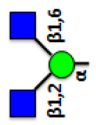
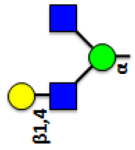
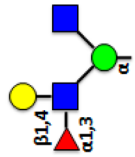
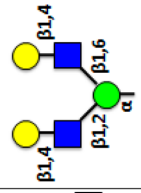
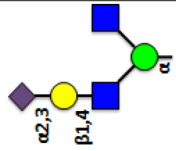
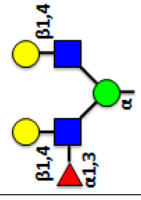
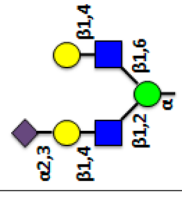
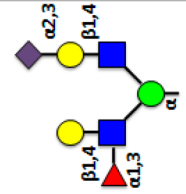
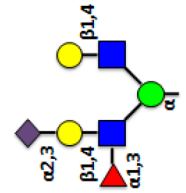
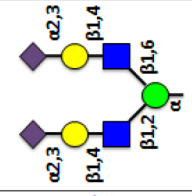
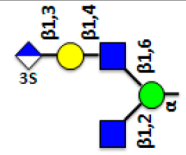
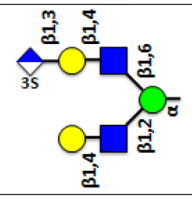
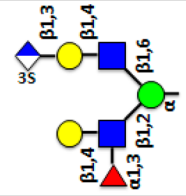
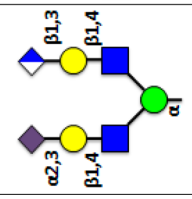
STRUCTURES

O-Mannose glycans account for up to 30% of all glycans O-linked to proteins in mammalian brain tissue.[19,25] It is therefore understandable that O-mannose glycans have been shown to be essential for normal nervous system development that is dependent on neuron migration[3,26,27] and axon path finding,[28] and to play a role in remyelination following myelin sheath damage.[29] Consequently, O-mannose glycans are tied to disorders causing cobblestone lissencephaly and mental retardation[30] as well as disorders such as multiple sclerosis in which myelin sheath destruction is a dominant factor.[29] O-Mannose glycans also play a critical role in muscle structure and function, and defects in O-mannosylation can cause congenital muscular dystrophies (CMDs) that may or may not exhibit neurological involvement.[30]

Protein-linked O-mannose glycans are initiated by the covalent attachment of mannose to serines and threonines via an α-linkage. Extended O-mannose glycans currently divide naturally into three categories based on known GlcNAc residue extensions of the initiating mannose (Figure 1, cores m1−m3). This set of core structures also captures the division in biological and biochemical roles ascribed to O-mannose structures (Charts 1−3). O-Mannose core structures may be extended in the Golgi by the addition of galactose (Gal)

dx.doi.org/10.1021/bi500153y | *Biochemistry* 2014, 53, 3066−3078

Chart 2. Core m2 Glycans Found in Mammals[a]

#	Structure	References
7		[42]
8		[42]
9		[42, 113]
10		[42, 113]
11		[42, 113]
12		[42, 113]
13		[19, 25, 42]
14		[19]
15		[113]
16		[16, 19, 25, 42]
17		[116]
18		[116]
19		[116]
20		[113]

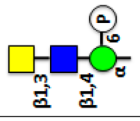
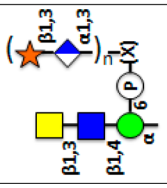
[a]See Figure 1 for the symbol legend. Core m2 glycans account for ~5% of protein-linked O-glycans in mouse brain.[19] Core m2 glycans are involved in the inhibition of RPTPβ activity, which causes an increased level of integrin-dependent cell migration.

residues, N-acetylgalactosamine (GalNAc) residues, sialic acid (SA) terminals, sulfated glucuronic acid terminals forming human natural killer-1 epitopes, α1,3-linked fucose residues forming Lewis x structures, and the rather elusive core m3-specific post-phosphoryl laminin globular (LG) domain-binding moiety containing xylose (Xyl) and glucuronic acid (GlcA) (Chart 3). To date, at least 23 distinct structures have been observed and characterized to varying degrees. Evidence indicates that O-mannose glycans show wide variation across tissue and cell types likely reflecting important differences in function.[5,12,16,18,19] For years, α-DG was among the few protein substrates known until more recent studies suggested and confirmed a wide distribution of O-mannosylation across proteins.[15,19,31,32]

Core m1. The class of core m1 glycans consists of all O-mannose glycans in which O-mannose is extended with β1,2-linked GlcNAc but not with β1,6-linked GlcNAc (Figures 1 and 2 and Chart 1). Core m1 glycans accounted for the largest fraction of O-mannose glycans released from mouse brain proteins, detected and quantified in a recent study, and constituted at least 15% of total brain protein O-glycans.[19] At least six different structures have been observed, including notably the "classical tetrasaccharide" originally thought to be directly involved in laminin binding (structure **4**, Chart 1) and a

dx.doi.org/10.1021/bi500153y | *Biochemistry* 2014, 53, 3066−3078

Chart 3. Core m3 Glycans Found in Mammals[a]

#	Structure	References
21		[13]
22		[44]

[a]See Figure 1 for the symbol legend. Core m3 glycans account for an unknown but probably small fraction of protein-linked O-glycans in mouse brain. Core m3 glycans include the α-DG functional glycan (structure **22**), are involved in LG domain binding and in the entry of viruses into cells. (X) depicts unknown elements, and a subscript n indicates polymer repeats.

Table 1. Genes Encoding Established O-Mannose Glycan Synthesis Activities

gene(s)	protein function	core glycans
POMT1 and POMT2	transfer of mannose from DPM to Ser/Thr	M1, M2, M3
POMGNT1	transfer of β1,2-GlcNAc to O-mannose	M1, M2
POMGNT2 (GTDC2)	transfer of β1,4-GlcNAc to O-mannose	M3
GNT-VB (GNT-IX)	transfer of β1,6-GlcNAc to O-mannose	M2
B3GALNT2	transfer of β1,3-GalNAc to GlcNAc-β1,4-Man-α	M3
POMK (SGK196)	6-phosphorylation of O-mannose	M3
LARGE	Xyl-GlcA repeat polymerization	M3
LARGE2	Xyl-GlcA repeat polymerization	M3
HNK-1ST	GlcA sulfation, including core M3 glycan Xyl-GlcA polymer sulfation	M1, M2, M3
FUT9	α1,3-fucose transfer	M1, M2
B3GAT1	β1,3-glucuronyl transfer	M1, M2

Table 2. Genes Encoding Hypothesized O-Mannose Glycan Synthesis Activities

gene(s)	protein function	core glycans
ISPD	role in O-mannose transfer activity	M1, M2, M3
FKTN	protein maturation?	M3
FKRP	protein maturation?	M3
B3GNT1	β1,3-GlcNAc transfer?	M3
TMEM5	unknown	M3

human natural killer-1 (HNK-1) epitope extended structure (structure **6**, Chart 1). The current literature suggests that core m1 glycans do not directly bind key extracellular factors[19,33] and that they play a less direct role at the cell surface in mammalian pathologies such as CMDs. Nevertheless, core m1 glycans are biologically essential because core m1 itself is the precursor for core m2 and core m1 structures appear to be highly important for the maturation of core m3 glycans on α-DG (functional glycosylation). Core m1 glycans are abundant on the mucin domain of α-DG[16,18] and contribute to the extended conformation adopted by this domain,[34] a potential factor influencing core m3 glycan maturation. Loss of core m1 glycans correlates with a spectrum of CMDs[3,35−37] perhaps because of the resulting disruption of the maturation of core m3 glycans on α-DG shown to underlie the molecular mechanism of these pathologies.[13,22] Core m1 glycans are typically sialylated, contributing to the charge state of the mucin domain of α-DG.[18,19]

Core m2. The class of core m2 glycans is initiated by β1,6-linked GlcNAc extension of core m1 (Figures 1 and 2). These structures are primarily found in brain and prostate tissue[6] and accounted for no more than 5% of brain protein-linked O-glycans quantified in a recent study.[19] Evidence of at least 13 different core m2-based structures exists (Chart 2), including HNK-1 epitope-containing structures that are linked to neural cell adhesion and migration.[38] Early studies demonstrated that an increased level of core m2 glycan synthesis in neuroblastoma cells leads to an increased level of integrin-dependent cell migration on laminin-coated plates.[39,40] It was subsequently demonstrated that this effect depends on an increased level of tyrosine phosphorylation of β-catenin caused by core m2 glycan-based inhibition of receptor tyrosine phosphatase β (RPTPβ) activity. Further, the decrease in RPTPβ activity appears to depend on the increased level of HNK-1 epitope presentation caused by increased core m2 glycan levels.[41] Interestingly, however, a recent study of a mouse model lacking core m2 glycan synthesis revealed no obvious developmental nervous system defects[42] despite changes in integrin-dependent cell adhesion and migration noted in earlier *in vitro* studies.[41] In this study, it was also shown that the lack of core m2 glycans does not alter α-DG functional glycosylation. Core m2 glycans may however play a role in demyelination pathologies such as MS as demonstrated in a recent study showing inhibition of axon remyelination by core m2 glycans in model systems.[29] An increased level of core m2 glycosylation has also recently been correlated with increased prostate cancer tumor growth and metastasis.[6]

Core m3. The class of core m3 glycans is initiated by β1,4-linked GlcNAc extension of O-mannose (Figures 1 and 3). Apparently constituting a small and highly heterogeneous portion of the O-glycome, these structures have outsized biological effects as cell surface determinants of the binding of α-DG to its ECM partners.[19,43] In particular, defective core m3 glycosylation appears to be the common factor in secondary dystroglycanopathies,[5,12,13,44] in increased metastasis of carcinomas, including prostate and breast cancers,[7−9,45] and in various forms of aberrant neuronal migration[3,27] and axon guidance[28] in mammals. On the other hand, properly extended core m3 glycans may contribute to more aggressive forms of melanomas[46] and can promote the entry of certain arenaviruses into cells.[47] The mechanisms involved are mediated through synthesis of a post-phosphoryl LG domain-binding extension apparently unique to core m3 glycans *in vivo*[13] containing -α3-GlcA-β3-Xyl- repeats[44] that has been shown to be a "tunable scaffold" regulating the avidity of cell surface receptors for ECM proteins.[48] In muscular dystrophies, shortening of this scaffold has deleterious effects on basement membrane compactness and structure and on neuromuscular junction formation.[48] A definitive structure of the post-phosphoryl LG domain-binding epitope remains an important question, although the -α3-GlcA-β3-Xyl- repeating structure is similar to known acidic sugar-containing LG domain-binding epitopes observed on glycosaminoglycans (GAGs) such as heparin.[44] These acidic GAG epitopes bind to basic residues in LG domains via electrostatic interactions.[44] Because core m3 glycans without LG domain-

dx.doi.org/10.1021/bi500153y | *Biochemistry* 2014, 53, 3066−3078

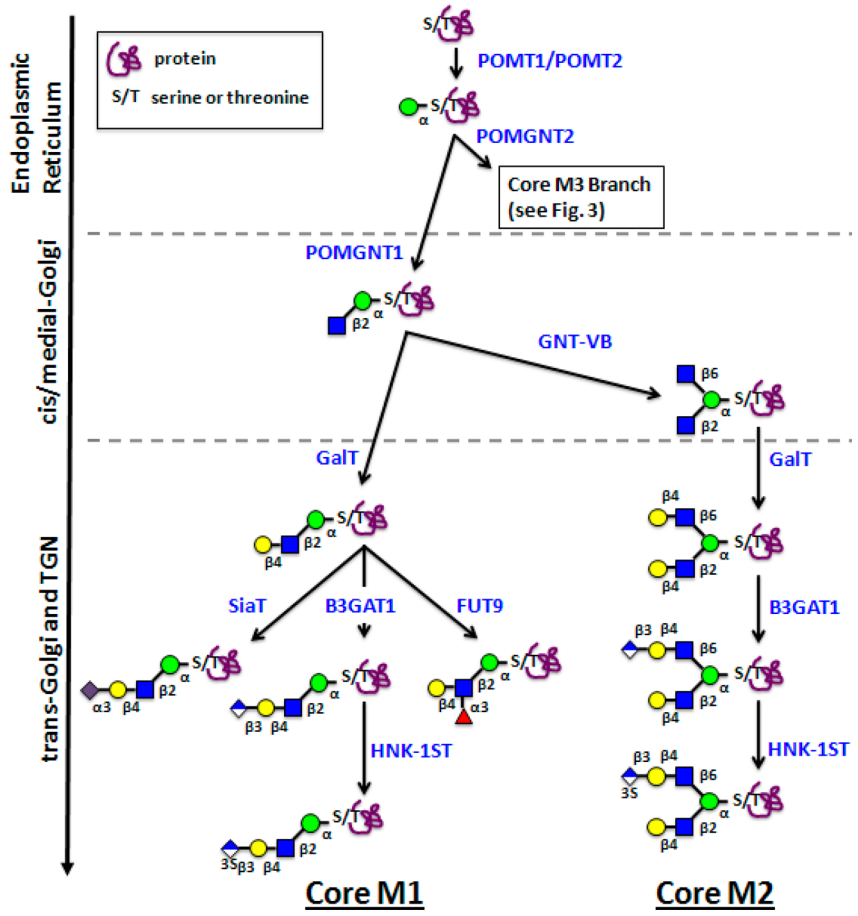


Figure 2. Synthesis of representative structures along the Golgi-centric core M1 and M2 glycan synthesis pathways. FUT9 and B3GAT1 (GlcAT-P) have been demonstrated in certain tissues and models to be the primary enzymes responsible for the steps indicated, although other fucosyl- and glucuronyltransferases may be present. Abbreviations: GalT, β1,4-galactosyltransferase; SiaT, α2,3-sialyltransferase. See Figure 1 for the monosaccharide code legend.

binding extensions are detected in other tissues, it is hypothesized that core m3 glycans lacking these extensions may play other roles.[5]

PATHWAY: GENES AND ENZYMES

O-Mannose glycan synthesis begins in the endoplasmic reticulum (ER) with addition of mannose to serine and threonine residues by the protein complex consisting of protein O-mannosyltransferase 1 (POMT1) and protein O-mannosyltransferase 2 (POMT2). Synthesis may then continue, producing various core structures and their elaborations (Figures 1−3). Current data suggest that addition of 6-phosphate to O-mannose in the ER (completing core M3) precludes the addition of β1,2-linked GlcNAc[49] in the cis-Golgi (Figure 1). As a consequence, β1,6-linked GlcNAc addition would also be prevented. However, the existence of other as yet undetected O-mannose glycan core structures cannot be ruled out. In this section, we focus on the synthesis and elaboration of core M3 glycans because of an abundance of recent discoveries, the remaining biochemical mysteries regarding their synthesis, and their centrality in human disease. We will also briefly discuss the synthesis of the other core structures and finally summarize information from the current literature pertaining to other genes that are involved in O-mannose glycan synthesis.

Protein O-Mannosylation (initiation). *Protein O-Mannosyltransferase 1 and 2 (POMT1 and POMT2, respectively).* POMT1 and POMT2 encode multipass membrane proteins that catalyze the transfer of mannose from dolichol-phosphate mannose (DPM) to serines and threonines in an O-linkage in the ER.[50] Mutations in these genes cause a spectrum of CMD phenotypes.[35,51−53] Recent studies have provided evidence that supports the hypothesis[54] that phenotype severity correlates with the predicted degree of gene disruption[36] and inversely correlates with measurable enzymatic activity.[55] Knockout of either gene is embryonic lethal,[56,57] while a significant loss of function results in the most severe CMD phenotype, Walker-Warburg syndrome. POMT1 and -2 are located early in the secretory pathway anterior to most other glycosyltransferases and have been shown to significantly influence patterns of O-GalNAcylation in *in vitro* studies,[58] increasing the level of interest in its specificity. This is due to the fact that O-GalNAcylation may greatly impact the biophysical properties of the α-DG mucin domain, particularly its conformational properties,[58,59] and thus processing of α-DGs by other enzymes during secretory pathway traversal. A recent study showed that a 40-amino acid peptide region upstream of α-DG O-mannose sites is involved in controlling specificity in EBNA-293 (human kidney) cells;[60] however, another study found that specificity may be controlled by different currently unknown elements in lectican O-mannosylation in the brain.[32] Additional recent

dx.doi.org/10.1021/bi500153y | *Biochemistry* 2014, 53, 3066−3078

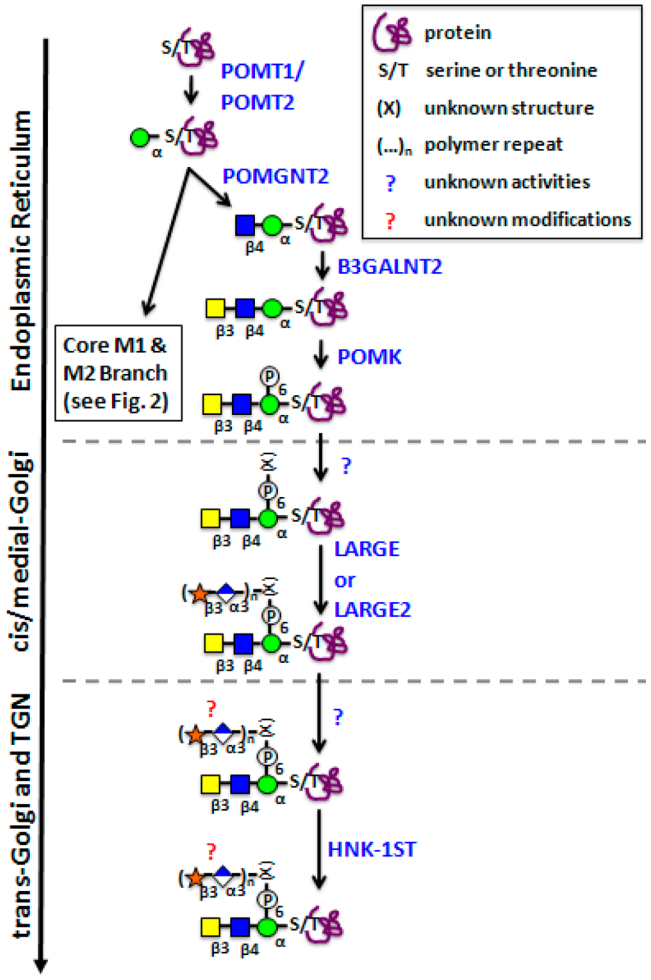


Figure 3. Synthesis of representative core M3 structures that are dependent on multiple initial activities based in the ER. Evidence suggests that phosphorylation of position 6 of O-mannose that is dependent on β1,4-GlcNAc extension precludes β1,2-GlcNAc addition in the Golgi.[49] Core M3 is the only core known to be modified with the α-DG functional glycan structure. Various steps as well as the structures ultimately built have not been fully elucidated. See Figure 1 for the monosaccharide code legend.

results of interest include the demonstration that N-glycosylation of POMT1 and -2 is necessary for activity[61] as well as the observation that POMT1 and -2 activity can be significantly reduced by defects in another gene, ISPD, encoding a putative nucleotidyltransferase.[62]

Core M3 Synthesis. Genes encoding proteins involved in core M3 synthesis (Figure 3) have been identified over the past few years primarily on the basis of genetic studies conducted on patients presenting with congenital muscular dystrophy (CMD) phenotypes. These genes also appear prominently in the recently published α-DG glycosylome[21] and comprise three of the eight genes shown to be involved in the mammalian O-mannosylation pathway in the past three years (Table 1). The three genes involved had been annotated as glycosyltransferase-like domain-containing protein 2 (GTDC2, now POMGNT2), UDP-GalNAc:β-1,3-N-acetylgalactosaminyltransferase 2 (B3GALNT2), and probable inactive protein kinase-like protein SgK196 (SGK196, now POMK). POMGNT2 was identified in a paper employing exome sequencing of Walker-Warburg syndrome (WWS) patients and validated in zebrafish models.[63] B3GALNT2 was identified and validated similarly[64] with the addition of the direct demonstration of α-DG hypoglycosylation accompanying B3GALNT2 deficiency. The *in vitro* activity of B3GALNT2 had been demonstrated previously, in 2004, but the *in vivo* function was not determined at that time.[65] Finally, POMK was identified as a cause of hydrocephalus and abnormal neuronal migration (WWS hallmarks) in genetically engineered mouse models in 2012[66] but does not appear to have been directly implicated in muscular dystrophy biology and O-mannosylation until the publication of the Brummelkamp laboratory α-DG glycosylome in 2013.[21] The biochemistry of these genes was elucidated in a 2013 paper from the Campbell laboratory.[22] As discussed in Structures, core M3 glycans are the only proven *in vivo* acceptors of the biologically critical LG domain-binding moiety of α-DG central to a number of disease processes including CMDs. This draws attention to the most important open question regarding core M3 glycan biosynthesis, the manner in which protein and site specificity is determined *in vivo*, which will also be discussed in this section.

Protein O-Linked Mannose N-Acetylglucosaminyltransferase 2 (POMGNT2 or GTDC2). Experiments conducted in the Campbell laboratory demonstrated that POMGNT2 was ER-localized and that it transfers GlcNAc from UDP-GlcNAc to a synthetic version of an α-DG O-mannose peptide in a β1,4-linkage *in vitro*[22] (Figure 3). Ogawa and colleagues also demonstrated ER localization and found that CTD110.6, an antibody raised against O-GlcNAc, can cross react with β1,4-GlcNAc-extended O-mannose.[67] Because POMGNT2 is localized earlier in the secretory pathway than other O-mannose-extending enzymes, it is noteworthy that prior studies of laminin binding reactivity, phosphorylation status,[5,12] and O-glycan sites of mammalian glycoproteins[13,16−18,68] suggest that core M3 structures are unique to a handful of sites of α-DG *in vivo*. In light of this, it is interesting that Yoshida-Moriguchi and colleagues demonstrated the transfer of β1,4-linked GlcNAc to 4-methylumbelliferyl-α-D-mannoside,[22] at least *in vitro*, and that Ogawa and colleagues demonstrated the CTD110.6 reactivity of a number of α-DG deletion and substitution mutants cotransfected with GTDC2 in HEK293T cells.[67] Consequently, it is currently unclear what structural or other biochemical determinants result in β1,4-GlcNAc modification of such a limited set of sites *in vivo* at this key biosynthetic point in the pathway.

UDP-GalNAc:β-1,3-N-Acetylgalactosaminyltransferase 2 (B3GALNT2). In 2013, B3GALNT2 was shown to be defective in some cases of congenital muscular dystrophy and to localize to the ER and was hypothesized to transfer GalNAc in a β1,3-linkage to GlcNAc-β1,4-Man-α on α-DG.[64] Significantly, B3GALNT2 is the first GalNAc-transferase that has been shown to localize primarily to the ER as opposed to relocating to the ER as a regulatory mechanism.[69] Yoshida-Moriguchi and colleagues demonstrated the apparent *in vivo* acceptor of B3GALNT2 activity for the first time in a paper published shortly thereafter.[22] B3GALNT2 transfers GalNAc in a β1,3-linkage to GlcNAc-β1,4-Man-α-R, where R may be a peptide from α-DG or 4-methylumbelliferyl. Given the early position of B3GALNT2 activity in ER and Golgi trafficking, O-mannose glycans may provide the only acceptors for this enzyme *in vivo*.

Protein O-Mannose Kinase (POMK or SGK196). In 2013, Yoshida-Moriguchi and colleagues established, through *in vitro* enzyme assays and high-performance liquid chromatography separation of fluorescently labeled substrates and products, that

dx.doi.org/10.1021/bi500153y | *Biochemistry* 2014, 53, 3066−3078

POMK is the kinase responsible for phosphorylating O-mannose at position 6, completing the core M3 structure[22] (Figure 3). In contrast to the seeming lack of specificity *in vitro* of the previously discussed core M3 synthesizing enzymes, POMK could be shown to phosphorylate α-linked mannose only after the addition of GalNAc-β1,3-GlcNAcβ1,4.[22] The discovery that POMK is a kinase is significant for two reasons. First, POMK lacks key catalytic residues found in other kinases characterized to date[22] and may therefore be the first discovered member of a new class of kinases. Second, extension of phosphate groups attached to sugars is an unusual, more difficult, chemistry for various reasons.[70] Therefore, the enzymatic activity stably extending the phosphate at position 6 is of clear interest.

Core M3 Elaboration and LG Domain-Binding Related Genes. Core M3 glycans have been observed only on α-DG and were shown in 2010 to be the only glycans extended with the LG domain-binding moiety (functional glycan) of α-DG *in vivo*.[13] Because the Brummelkamp laboratory gene disruption screening methodology is based on the functional glycan status of α-DG, their screen should detect positive regulators of functional glycosylation.[21] Negative regulators are unlikely to be detected by this screen; however, one was discovered by another group recently using different methods.[46] The genes from these studies encoding proteins in the secretory pathway are likely to play direct roles in core M3 elaboration. The genes in Tables 1−3 meeting these criteria are like-glycosyltransferase

Table 3. Genes Encoding Proteins Involved in Precursor Supply

gene(s)	protein function	core glycans
MPDU1	mannose supply	M1, M2, M3
PMM2	mannose supply	M1, M2, M3
GPMBB	Ssynthesis of GDP-Man	M1, M2, M3
DPM1, DPM2, and DPM3	GDP-Man to DPM transfer	M1, M2, M3
UGDH	UDP-Glc to UDP-GlcA conversion	M1, M2, M3
UXS1	UDP-GlcA to UDP-Xyl conversion	M3
SLC35A1	Golgi CMP-sialic acid antiporter	M1, M2, M3

(LARGE), glycosyltransferase-like 1B (GYLTL1B or LARGE2), UDP-GlcNAc:βGalβ-1,3-N-acetylglucosaminyltransferase (B3GNT1), human natural killer-1 sulfotransferase (HNK-1ST), solute carrier family 35 (CMP-sialic acid transporter), member A1 (SLC35A1), fukutin (FKTN), fukutin-related protein (FKRP), transmembrane protein 5 (TMEM5), and base core M3 synthesis genes and POMGNT1 discussed in other sections. LARGE, FKTN, and FKRP have been extensively studied since the late 1990s, while B3GNT1, HNK-1ST, SLC35A1, and TMEM5 have only recently been implicated in the modification of α-DG. Recent major results include the elucidation of the enzymatic reactions conducted by LARGE and LARGE2,[44,71,72] the determination that B3GNT1 forms complexes with LARGE and LARGE2 critical to their activity,[7] and the discovery that HNK-1ST negatively regulates LG domain-binding glycan synthesis.[43,46] Further, all of these results have been tied either directly[43] or indirectly[13,71−73] to the post-phosphoryl moiety of core M3 glycans. The roles of FKTN, FKRP, and TMEM5 remain less clear.

Like-glycosyltransferase (LARGE), Glycosyltransferase-like 1B (LARGE2), and N-Acetyllactosamide β-1,3-N-Acetylglucosaminyltransferase (B3GNT1). LARGE and LARGE2 encode proteins containing both a GT8 glycosyltransferase domain and a GT49 glycosyltransferase domain[74] and have been extensively studied using overexpression[2,7,43,73,75,76] and glycosylation deficient cell lines.[77−79] Native LARGE modification of α-DG is regulated directly and/or indirectly by the activities of a number of other enzymes, including HNK-1ST, B3GNT1, and possibly SLC35A1 and POMGNT1 (see below). Overexpression of LARGE has been shown to lead to LARGE modification of non-native acceptors, including N-glycans and O-GalNAc glycans,[73,77] and, potentially as a consequence, to partially rescue functional glycosylation in cells derived from patients deficient in other O-mannosylation pathway activities.[2,75] This has led to the suggestion that LARGE may be a particularly good target for gene therapy treatment strategies given that it can compensate for a variety of deficiencies.[75]

In 2012, Inamori and colleagues determined by compositional sugar analysis that recombinant α-DG co-expressed with LARGE in HEK293 cells is modified by substantial quantities of xylose and glucuronic acid.[44] Competition assays and experiments conducted in UDP-Xyl synthesis deficient cell lines demonstrated that functional glycosylation of α-DG depended on xylose. Subsequent *in vitro* enzyme assays using tagged xylose and glucuronic acid acceptors established the reactions catalyzed by LARGE. Specifically, it was shown that the GT8 domain catalyzes the transfer of xylose (Xyl) in an α1,3-linkage to β1,3-linked glucuronic acid (GlcA) from UDP-Xyl and that the GT49 domain catalyzes the transfer of GlcA in a β1,3-linkage to α1,3-linked Xyl from UDP-GlcA.[44] Furthermore, Inamori and colleagues demonstrated that LARGE can build polymers consisting of -α3-GlcA-β3-Xyl- repeats without the presence or action of other proteins. LARGE2 was subsequently shown to catalyze the same reaction,[71,72] although none of the currently published studies have established the initial acceptor for these activities *in vivo*. For example, Yoshida-Moriguchi and colleagues found that LARGE indeed appears to be unable to transfer directly to the phosphate at position 6 on the core M3 structure.[22] The role of B3GNT1 is currently unknown, but it is interesting to note that it is the only B3GNT to cluster into CAZy family GT49.

Human Natural Killer-1 Sulfotransferase (HNK-1ST). HNK-1ST encodes a sulfotransferase responsible for the sulfation of GlcA residues at position 3. In 2012, Nakagawa and colleagues observed an upregulation of HNK-1ST in S91 melanoma cells treated with the antitumor agent retinoic acid (RA) and demonstrated that the suppression of melanoma cell migration by RA depended on a reduction in the level of functional glycosylation of α-DG.[46] Furthermore, they demonstrated that the interaction between LARGE and α-DG was not disrupted by HNK-1ST and that the sulfo-transfer activity of HNK-1ST was the mechanism by which this enzyme modulates functional glycosylation of α-DG. In 2013, Nakagawa and colleagues demonstrated that HNK-1ST transfers sulfates to core M3 glycans in the post-phosphoryl moiety and that it is the activity of HNK-1ST in this post-phosphoryl moiety that is responsible for abolishing the functional glycosylation of α-DG.[43] Experiments utilizing the LARGE *in vitro* assay system established by Inamori and colleagues[44] coupled with HNK-1ST-based "pretransfer" of sulfates to α-DG suggest that the primary effect of sulfate transfer is the disruption of the ability of LARGE to build the repeating disaccharide.[43] These results taken together suggest a mechanism in which the relative activities between LARGE and HNK-1ST in a given cell

compete to mediate the length of the disaccharide repeat and thus its affinity for ECM ligands. These results also strengthen the glycosaminoglycan analogy because HNK-1ST has also been shown to negatively regulate GAG chain length by sulfate transfer.[80] An important distinction is the lack of HNK-1 reactivity of the resulting glycan on α-DG, indicating that GlcA is not β1,3-linked to Gal.

Solute Carrier Family 35 (CMP-sialic acid transporter), Member A1 (SLC35A1). SLC35A1 encodes the well-characterized Golgi CMP-sialic acid transporter in mammals.[81,82] Mucin domains are heavily sialylated with significant consequences for protein conformation during and after trafficking (Table 4).[34] Synthesis of LG domain-binding glycans

Table 4. Genes Encoding Proteins Involved in ER and Golgi Trafficking

gene(s)	protein function	core glycans
COG4	vesicle trafficking	?
COG5	vesicle trafficking	?
COG7	vesicle trafficking	?
COG8	vesicle trafficking	?
PTAR1	protein trafficking	?

on the mucin domain of α-DG clearly depends on structural features.[83,84] Furthermore, because sialidase treatment after synthesis of the functional glycans on α-DG results in increased laminin and IIH6 binding activity,[33] a lack of concurrent sialylation of O-mannose and O-GalNAc glycans during DAG1 traversal of the compartments of the Golgi that contain LARGE may result in an unfavorable protein state for effective LARGE modification. Regardless of the exact mechanism, the lack of appearance of specific sialyltransferases (SiaTs) in the published α-DG glycosylome[21] suggests that redundancy exists and that multiple SiaTs are involved. As with many of the defects found in α-DG processing, LARGE overexpression has been shown to mitigate sialic acid deficiency-based aberrant glycosylation.[77]

Fukutin (FKTN), Fukutin-Related Protein (FKRP), and Transmembrane Protein 5 (TMEM5). Genes FKTN, FKRP, and TMEM5 encode the remaining positive regulators of α-DG functional glycosylation. These proteins localize to the secretory pathway and are potentially directly involved in α-DG functional glycan synthesis (Table 2). While it is likely that at least one of these genes encodes an activity synthesizing a key part of the linker between core M3 itself and the LARGE modification, and there has been extensive speculation with regard to FKTN and FKRP activities in particular,[85−88] experiments to date have not revealed nucleotide−sugar transfer activities[89] or other activities.[90] Recent studies have shown that at least a fraction of α-DG from FKTN and FKRP deficient models is partially extended on core M3 glycans post-phosphate[5,12] and that mutation of the DXD motif of FKRP does not necessarily result in a loss of functional glycosylation of α-DG.[91] Furthermore, functional glycosylation of α-DG has been observed in cell lines in which FKRP transcripts were not detected.[7] It has also been shown that some mutants of FKTN fail to fold properly and are retained in the ER, causing POMGNT1 to be retained as well,[92] and that there may be direct interaction between FKTN and α-DG.[90] Thus, the role of FKTN and FKRP remains unclear, and they may not be directly involved in enzymatic synthesis. Recently, a study conducted in zebrafish has indicated that FKTN and FKRP may be required for appropriate folding and secretion of a set of proteins in a potentially non-glycosylation-dependent manner (viz. laminin-1).[93] This model may explain the hypoglycosylation of α-DG in FKTN and FKRP deficient patients given the importance of the α-DG and LARGE binding interaction that depends on protein conformation.[83] This model is also consistent with the lower degree of correlation between α-DG hypoglycosylation and CMD phenotype severity in such patients.[94] For example, studies including observations concerning a role for laminin-1 in muscular dystrophies have since been published.[95] Further evaluation of this model is needed. New animal models[12,75,96,97] and biochemical tools[98] developed in the past few years should help researchers to further resolve the roles of FKTN and FKRP, which generally occur at low levels, can be difficult to detect, and may be differentially required among cell types and during development.

TMEM5 has only recently been identified as a cause of dystroglycanopathies, and no biochemical characterization appears in the literature at present. It has been noted that TMEM5 defects can cause cobblestone lissencephaly A, a severe phenotype associated most closely with POMT1 defects,[99] placing TMEM5 centrally in α-DG functional glycan synthesis *in vivo*.

Core M1 and Core M2 Synthesis. Core M1 and core M2 glycans and their elaborated structures account for 20−30% of the O-glycans detected and quantified in various studies.[19,25] Core M1 glycan synthesis is initiated by POMGNT1, which has long been implicated in CMDs (see Structures). Core M2 glycan synthesis is initiated by GNT-VB (GNT-IX) and has been demonstrated to play a role in remyelination and potentially multiple sclerosis (see Structures).

Protein O-Linked Mannose N-Acetylglucosaminyltransferase 1 (POMGNT1). POMGNT1 encodes the enzyme that extends O-mannose with β1,2-linked GlcNAc in the cis-Golgi, an initial step in the synthesis of core M1-based and core M2-based structures (Charts 1 and 2). Although these structures are not directly involved in LG domain binding reactivity, POMGNT1 loss of function causes a loss of functional glycosylation of α-DG, a severe form of muscular dystrophy termed muscle eye brain disease, and a >10 kDa reduction in the molecular mass of α-DG in mouse skeletal muscle tissue.[5] This reduction is larger than that caused by deficiencies in other functional glycosylation genes and has potentially substantial structural consequences that may affect the activities of other enzymes implicated in CMDs. More directly, Nilsson et al. found strict core M1 glycan modification of the glycopeptide consisting of mucin domain residues 361−373 in human α-DG,[18] a peptide that is directly N-terminal to the peptide on which the core M3 phospho-O-mannose-trisaccharide was mapped by Yoshida-Moriguchi and colleagues.[13] The only detected heterogeneity was in sialylation, and Harrison et al. detected core M3 (minus phosphate) on the corresponding mouse α-DG site followed by a core M1 classical tetrasaccharide attached to the two C-terminal threonine residues.[17] These regions contain sequences that are significantly similar to the sequences of a region of α-DG most directly demonstrated to be highly important for laminin binding consisting of the first 24 residues of the mucin domain.[68] Specifically, paired threonine residues separated by a proline appear to be critical for LARGE-dependent functional glycosylation. Becauser enzymatic removal of core M1 glycans on α-DG from normal rabbit skeletal muscle causes an increase

in laminin binding reactivity,[33] one reasonable hypothesis is that specific sites of core M1 glycan modification could be a factor in the ability of LARGE to build the scaffold of the functional glycan structure of α-DG core M3 glycans. It has also been shown that CMD severity is inversely correlated with POMGNT1 activity.[100] Finally, one recent study found a correlation between POMGNT1 protein levels and glioma tissue grade,[101] but further studies are needed to refine this finding and possible mechanisms.

α-1,6-Mannosylglycoprotein 6-β-N-Acetylglucosaminyltransferase B (GNT-VB or GNT-IX). GNT-VB (GNT-IX) encodes the enzyme that extends O-mannose with β1,6-linked GlcNAc in the cis-Golgi that is dependent on prior β1,2-GlcNAc extension by POMGNT1. This activity is blocked when branch β2 is further extended.[102] GNT-VB is active primarily in brain and prostate tissue[6] and has been shown to negatively affect axon remyelination after neurotoxicant-induced myelin damage[29] and to promote prostate cancer metastasis.[6] These findings establish GNT-VB as a possible therapeutic target.

■ ADDITIONAL GENES IN O-MANNOSYLATION

Isoprenoid Synthase Domain-Containing Protein (ISPD). ISPD encodes a protein with an isoprenoid synthase domain most similar to 2-C-methyl-D-erythritol 4-phosphate cytidylyltransferase, an enzyme active in the non-mevalonate isoprenoid synthesis pathway found in bacteria that appears to be absent in mammals. The role of this gene in dystroglycanopathies was discovered in 2012 through genetic screening and complementation analyses.[62,85,99] Defects in ISPD appear to be a common cause of dystroglycanopathies accounting for 10% of dystroglycanopathies in studies to date[99,103] and 30% of the cases of Walker-Warburg syndrome examined in one study.[62] Willer and colleagues subsequently found that microsomal fractions from ISPD deficient Walker-Warburg syndrome patient fibroblasts showed significant reductions in the extents of transfer of O-mannose to proteins.[62] This is an intriguing result considering that ISPD does not appear to contain a signal sequence and may have a cytosolic or possibly nuclear localization, raising questions about how ISPD might affect the activity of the protein O-mannosyltransferase complex in a microsomal fraction supplied with exogenous DPM. Although ISPD appeared to associate most closely with more severe forms of CMD in previous studies, at least one study identified patients with mutations in ISPD and milder limb-girdle muscular dystrophy phenotypes.[104]

α-1,3-Fucosyltransferase 9 (FUT9). FUT9 encodes an α-1,3-fucosyltransferase that catalyzes synthesis of Lewis x glycans (Le^x). O-Mannose-initiated Le^x structures accounted for roughly 10% of the O-glycans released from mouse brain tissue and quantified in recent glycomic studies.[19] Studies have demonstrated that of the two fucosyltransferases capable of synthesizing Le^x glycans, FUT9 is substantially more active in such a synthesis and is expressed in mouse brain at levels far higher than those of FUT4.[105] Furthermore, $FUT9^{-/-}$ mice show a complete absence of detectable Le^x epitopes in brain tissue.[106] FUT9 is likely to encode the primary fucosyltransferase responsible for fucosylation of O-mannose glycans. Addition of fucose to GlcNAc has been shown in other glycan classes to block GlcA transfer and consequently HNK-1 epitope synthesis.[107] Given the prevalence of HNK-1 epitopes in brain tissue and their previously noted functions, the interplay between fucosylation and glucuronylation may have a regulatory role.

Galactosylgalactosylxylosylprotein 3-β-Glucuronosyltransferase 1 (B3GAT1). B3GAT1 encodes one of two primary HNK-1 epitope-synthesizing β1,3-glucuronyltransferases in mammals (GlcAT-P). In a recent paper, Morise et al. demonstrated that phosphacan is the major protein carrying the HNK-1 epitope in the developing mouse brain, that monoclonal antibody 6B4 specifically recognizes HNK-1-modified phosphacan, and that 6B4 reactivity is virtually completely abolished in B3GAT1 knockout mice.[108] Additionally, experiments involving cotransfection of phosphacan and GlcAT-P into COS-1 cells provided further evidence that GlcAT-P is required for HNK-1 epitope synthesis on O-linked glycan structures primarily attached to phosphacan.[108]

■ PROTEIN SUBSTRATES (O-MANNOSYLATED PROTEINS)

O-Mannose modification was first detected in mammalian tissue in 1979[109] and was subsequently shown to occur prominently on the protein α-DG from nervous[110,111] and skeletal muscle tissues.[112] Several additional proteins were identified in the 2000s, including RPTPβ,[41] cd24 from mouse brain,[113] and a human IgG2 light chain expressed in CHO cells,[114] primarily in the context of biochemical studies directed at characterizing specific proteins. Efforts directed specifically at finding O-mannosylated proteins have been undertaken more recently, beginning with a study based on a bioinformatic search for a previously identified cis-peptide determinant of O-mannosylation on α-DG that resulted in the demonstration that neurofascin 186 is O-mannosylated.[115] Further studies utilizing large-scale enrichment and fractionation-based strategies resulted in the identification of the four lecticans (aggrecan, brevican, neurocan, and versican) as an important class of O-mannosylated proteins,[32] and most recently, 37 cadherins and six plexins were shown to be O-mannosylated using a "SimpleCell" system and Concanavalin A chromatography.[15] One of the most consistent themes observed in O-mannosylation is the association of O-mannosylation with proteins involved in cell−cell and cell−matrix adhesion. For example, O-mannosylation of RPTPβ, which is not directly involved in cell−cell or cell−matrix interactions, has been shown to modulate cell−cell interactions and to result in an increased level of cell migration through an intracellular signaling mechanism.[41] Protein cd24 is likewise involved in cell adhesion in cancer biology, in immune system function, and in nervous system biology (potentially mediated in part through the presentation of the HNK-1 epitope on O-mannose glycans).[113] In cancer biology, increased levels of cd24 correlate with more aggressive metastatic carcinomas.[113] Finally, the newly demonstrated importance of O-mannosylation in cadherin-mediated cell−cell adhesion and its crucial role in development[31] as well as the discovery of the prevalence of O-mannose glycans linked to cadherins[15] further illustrate this theme and open many avenues of additional study.

■ CONCLUSIONS

Progress in the field of O-mannosylation within the past few years has been substantial, culminating in the publication of three high-impact papers in 2013, the Brummelkamp laboratory α-DG glycosylome, the Clausen laboratory O-mannose glycoproteome, and the Campbell laboratory core M3 enzymes.

Publication of the Brummelkamp laboratory α-DG glycosylome suggests that we may finally be able to define the borders of genetic causes of secondary dystroglycanopathies and may be close to determining genetic etiologies for most dystroglycanopathy patients without previously understood genetic defects. It will also allow for biochemical characterization of the proteins encoded by these genes, ultimately allowing further development of treatments for dystroglycanopathies and other diseases caused by defects in O-mannosylation. The Clausen laboratory O-mannose glycoproteome provides a significant addition to the set of known O-mannosylated proteins, helping to explain how O-mannose glycans can account for approximately 30% of O-glycans released and quantified from brain proteins even in mouse models lacking α-DG. Finally, the Campbell laboratory assigned three enzyme activities critical to functional glycosylation of α-DG that are dependent on O-mannose glycans. The field of mammalian O-mannosylation is at an exciting juncture with completion of such a solid framework upon which accelerated progress in attaining a deeper understanding, particularly clinically, may rest.

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Funding

This work was supported in part by Grants P01GM107012 and P41GM103490 (L.W., senior investigator) from the National Institutes of Health.

Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

We thank Christina M. Dobson for thoughtful comments and careful editing of the manuscript. We also thank the members of the Wells laboratory and CCRC colleagues for valuable discussions and for reviewing the manuscript. We apologize ahead of time to those in the field whose work we failed to cite because of focus and/or space limitations.

ABBREVIATIONS

α-DG, α-dystroglycan; CMDs, congenital muscular dystrophies; Cyt, cytoplasm; DPM, dolichol-phosphate mannose; ECM, extracellular matrix; ER, endoplasmic reticulum; FG, functional glycosylation; Fuc, fucose; FucT, fucosyltransferase; GAG, glycosaminoglycan; Gal, galactose; GalT, galactosyltransferase; GalNAc, N-acetylgalactosamine; GlcA, glucuronic acid; GlcAT, glucuronyltransferase; GlcNAc, N-acetylglucosamine; HNK-1, human natural killer-1; LG, laminin globular; Loc, localization; Man, mannose; N-glycan, N-linked glycan; Nuc, nucleus; RA, retinoic acid; RPTPβ, receptor tyrosine phosphatase β; SA, sialic acid; SiaTs, sialyltransferases; WWS, Walker-Warburg syndrome; Xyl, xylose.

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