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Mammalian O-Mannosylation: Unsolved Questions of Structure/Function

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Abstract

Post-translational modification of polypeptides with glycans increases the diversity of the structures of proteins and imparts increased functional diversity. Here, we review the current literature on a relatively new O-glycosylation pathway, the mammalian O-mannosylation pathway. The importance of O-mannosylation is illustrated by the fact that O-mannose glycan structures play roles in a variety of processes including viral entry into cells, metastasis, cell adhesion, and neuronal development. Furthermore, mutations in the enzymes of this pathway are causal for a variety of congenital muscular dystrophies. Here we highlight the protein substrates, glycan structures, and enzymes involved in O-mannosylation as well as our gaps in understanding structure/function relationships in this biosynthetic pathway.

Introduction

Glycosylation is arguably the most commonly observed post-translational modification found on mammalian proteins. The addition of various carbohydrate moieties to eukaryotic proteins and their extension is often regulatory and in many cases required for proteins to function properly as receptors for bacterial and viral infection, for modulating cell adhesion, to mediate inflammation and metastasis, and in dictating differentiation and development [1,2]. In the last couple of decades, it has become apparent that *O*-glycans can have a wide variety of sugars found at the reducing end beyond the most widely recognized mucin-like glycans that are initiated by *N*-acetylgalactosamine (GalNAc) [3]. Observed reducing-end glycans in mammals now also include *N*-acetylglucosamine (GlcNAc), fucose, xylose, galactose, glucose, and mannose [2]. In this review, we will focus solely on mammalian *O*-mannosylation [4,5] including the glycan structures and associated enzymes, the protein substrates, and the pathophysiologicals associated with disruption in this biosynthetic pathway. Furthermore, we highlight many of the unanswered questions in the field including the need to identify the proteins substrates, the functional glycan structures, and the enzymes catalyzing the biosynthetic steps of the pathway.

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O-Mannose-Initiated Glycan Structures

In mammals, O-linked glycans initiated by mannose were first isolated from an enriched mixture of unidentified brain proteoglycans more than 30 years ago [6]. Since that time, multiple groups have analyzed the repertoire of O-mannose-initiated glycans produced from mammalian tissues (Fig. 1), and it has been estimated that ~30% of all O-linked glycans in brain are O-mannose-initiated structures [7–11]. The structures reported are predominantly based on a common core of Sia α 3Gal β 4GlcNAc β 2Man α -Ser/Thr. Common modifications generate the asialo-, or asialo-agalacto-, or fucosylated forms of the core. In addition, a core form bearing sulfoglucuronic acid in place of sialic acid could yield the HNK-1 epitope that has been observed on the O-mannosylated protein α -dystroglycan [5]. A number of branched O-mannose-initiated structures have also been observed in the brain with a GlcNAc at the 6-position of the core mannose and are likely due to the almost exclusively brain-specific GnTV- β enzyme (Fig. 1, [7,12]). Recently, an additional O-mannose-initiated structure essential for protein function, laminin and arenavirus binding by α -dysroglycan, has been partially characterized that is GalNAc β 3GlcNAc β 4(6-P-X)Man, where at the 6-position of the initiating mannose there is a phosphate (P) group in a phosphodiester linkage (based on HF sensitivity) to an unknown moiety (X) [13]. The full elucidation of this novel structure, that appears key for function, has yet to be achieved nor have clear functional consequences been assigned to the classic or branched structures.

Protein Substrates

Through the work of Margolis and co-workers in the late 70's, O-mannosyl glycans were identified on proteoglycans isolated from mammalian brain tissue [6]. Several years later there were additional reports of O-mannosyl glycans found to modify mammalian proteins. For example, O-mannosyl glycans were identified on unassigned glycoproteins modified with the HNK-1 epitope [14], as well as α -dystroglycan isolated from bovine peripheral nerve [15], sheep brain [9], and rabbit skeletal muscle [16]. Overall, among the O-glycans, 30% are estimated to be O-Mannose-initiated [7–11]. However, the only well-characterized mammalian protein identified as being O-mannosylated is α -dystroglycan. In order to account for all of the O-mannosylated structures present in the mammalian brain, it has been proposed that there must be many other proteins that bear this modification. Consistent with this hypothesis, glycomic characterization of brains from mice with a conditional neural knockout of α -dystroglycan demonstrated that the prevalence of O-mannose-initiated glycan structures was indistinguishable from that observed in wild-type mouse brain proteins [10]. Therefore, the identification and characterization of the O-mannosylated proteome is currently an important challenge in the field.

α -Dystroglycan and the Dystrophin Glycoprotein Complex (Fig. 2)

Dystroglycan is a central component of the dystrophin-glycoprotein complex (DGC), and is produced by a single gene (DAG1), which is post-translationally cleaved into two subunits, α - and β -dystroglycan [4]. Upon cleavage, the two subunits remain associated and participate as components of the DGC complex to link the cytoskeleton to extracellular components [17]. α -dystroglycan binds a host of extracellular matrix proteins such as laminin [18], agrin [19], perlecan [20], pikachurin [21], and neurexin [22] in a glycosylation dependent manner (Fig. 2). Properly O-mannosylated α -dystroglycan also serves as the receptor for several arenaviruses to gain entry into the cell [23,24]. The O-glycan structures that decorate the mucin region of α -dystroglycan are a mixture of both O-GalNAc- and O-mannose-initiated glycans [8,11]. Multiple sites of attachment have recently been elucidated including at least one site that bears the phosphorylated O-mannose structure [8,11,13]. While a primary amino acid sequence for O-mannosylation attachment has been proposed

based on *in vitro* work [25], site-mapping studies on endogenous protein from rabbit and human α -dystroglycan have demonstrated a more complex and less straightforward mechanism for selecting the site of O-mannose attachment *in vivo* [8,11]. Of particular interest, mutations in genes encoding the enzymes/proteins involved in the synthesis of O-mannosyl glycan structures disrupt the function of the DGC and are causal for various forms of congenital muscular dystrophy [26] as well as being associated with cancer metastasis in the case of at least one processing enzyme [27].

Congenital Muscular Dystrophy

Muscular dystrophy is a heterogeneous group of inherited genetic diseases that cause progressive weakness and wasting of skeletal muscle [28,29]. Muscular dystrophies can arise from a plethora of genetic mutations in a wide-variety of muscle proteins [29]. In 1987, a major breakthrough was made in understanding the molecular mechanisms behind muscular dystrophy when the mutated target gene responsible for the most common form of X-linked muscular dystrophy, Duchenne muscular dystrophy (DMD), was identified [30]. For the majority of patients presenting with DMD, the underlying cause was determined to be a mutation in the gene encoding the protein dystrophin [31]. The absence of dystrophin from the DGC results in loss of stability of the sarcolemma making it more vulnerable to injury during muscle contraction [28]. An additional group of related disorders are the congenital muscular dystrophies (CMD) that also disrupt the DGC [29]. The so-called dystroglycanopathies result from genetic mutations in confirmed or putative glycosyltransferases involved in the O-mannosylation pathway and result in hypoglycosylated α -dystroglycan that is deficient in its ability to bind extracellular matrix components [29,32]. Of particular note, of the more than 40 different types of muscular dystrophy, it is predominantly only the dystrophies associated with O-mannose glycosylation defects that have a significant neurological component including cobblestone lissencephaly in many instances resulting in severe mental retardation of the affected individuals [32]. While this may in part be due to improper glycosylation of α -dystroglycan in the developing brain, the loss of α -dystroglycan in neural tissues of mice fails to fully recapitulate the entire phenotype and severity observed in mouse models incapable of O-mannosylation [33,34]. Furthermore, disruption of the DGC by disruption of dystrophin does not have a pronounced neurological phenotype [35]. This may suggest that improper O-mannosylation of other brain proteins are involved in the neurological pathophysiology and further warrants efforts towards their identification.

Enzymes of the O-Mannosylation Pathway

To date, mutations in seven glycosyltransferase or glycosyltransferase-like genes have been reported to affect the O-mannosylation pathway, alter the functional glycosylation of α -dystroglycan, and are causative for various forms of CMD [4]. These genes include: *Protein O-mannosyltransferase 1 and 2* (*POMT1* and *POMT2*)-Walker-Warburg Syndrome (WWS) [36], *Protein O-linked-mannose beta-1,2-N-acetylglucosaminyltransferase 1* (*POMGnT1*)-Muscle Eye Brain Disease (MEB) [37,38], *Fukutin*-Fukuyama Congenital Muscular Dystrophy [39], *Fukutin-Related Protein (FKRP)*- Congenital Muscular Dystrophy 1C MDC1C [40], Limb Girdle Congenital Muscular Dystrophy (LGMD) [41], Walker Warburg Syndrome (WWS), and MEB [42], *LARGE* and *LARGE2*- Congenital Muscular Dystrophy 1D (MDC1D) [43] [4]. While each of these genes was originally associated with a particular form of CMD, recent evidence suggests that different mutations in the same gene can generate phenotypes associated with different forms of CMD arguing for some mutations being hypomorphic, as opposed to null, alleles and makes a strong case for future efforts directed at elucidating genotype/phenotype relationships for this set of disease-causing genes. It should also be noted that approximately half of patients diagnosed with various

forms of dystroglycanopathy have no apparent mutations in the genes listed above suggesting that other disease-related enzymes in the O-mannosylation pathway remain to be discovered.

Protein O-mannosyltransferase 1 (POMT1) and 2 (POMT2) encode transmembrane ER proteins that are widely expressed in all mammalian tissues and catalyze the addition of an O-mannosyl residue from dolichol-phosphate-mannose onto Serine/Threonine residues of proteins in the secretory pathway [36,44]. The expression of both POMT1 and 2 are required for proper O-mannosyltransferase activity in mammalian cells [25,45]. Mutations in the genes of POMT1 and POMT2 were first identified in patients presenting with Walker-Warburg Syndrome (WWS), which is a severe, recessive form of CMD characterized by ocular abnormalities and severe brain malformations and a life expectancy typically less than one year [46]. However, mutations in these genes can also result in milder phenotypes of CMD such as muscle-eye-brain disease [47]. *POMGnT1* encodes the protein *O*-linked mannosyl β 2-*N*-acetylglucosaminyltransferase 1, which catalyzes the transfer of GlcNAc from UDP-GlcNAc onto O-mannosyl modified glycoproteins [38,48]. Mutations in *POMGnT1* are causal for muscle-eye-brain disease (MEB) [38], although some patients present with a WWS-like phenotype [37]. Recent glycomic analysis has revealed a complete absence of classically extended or branched O-mannose glycans in a mouse model of MEB lacking POMGnT1 suggesting there are no other compensatory glycosyltransferases exist for extension of O-mannose in the 2-position and confirming that extension in the 2-position is a prerequisite for extension on the 6-position with a GlcNAc ([10], Fig. 3). Mutations in the *fukutin* gene are causative for Fukuyama congenital muscular dystrophy (FCMD), which among the Japanese population is one of the most common autosomal recessive diseases [26]. FCMD is associated with brain malformations due to defective neuronal migration [49]. While the exact function of the Golgi-localized fukutin remains unclear, the putative enzyme has sequence similarities with other glycosyltransferases including the DXD motif common to glycosyltransferases [40]. Similar to *fukutin*, *Fukutin related protein (FKRP)* encodes a putative glycosyltransferase belonging to the fukutin protein family. Mutations in the *FKRP* gene are causative for both severe and mild phenotypes of CMD, MDC1C and LGMD2I respectively [40]. In skeletal muscle, FKRP is localized to the sarcolemma of muscle fibers in association with the DGC [50]. It should be noted that both fukutin and FKRP do not fit into any known CAZY family of glycosyltransferases and contain LicD domains that are usually associated with enzymes involved in phosphorylcholine metabolism [51]. The *LARGE* gene, aptly named due to its more than 660 kilobase size, encodes a type II transmembrane protein located to the Golgi apparatus [52]. LARGE is postulated to be a putative glycosyltransferase that is predicted to contain two catalytic DXD domains that are structurally similar to an α -glycosyltransferase and a β 3-*N*-acetylglucosaminyltransferase [53]. Mutations in *LARGE* result in a substantial decrease in the molecular weight of α -dystroglycan as measured by SDS-PAGE, the inability of α -dystroglycan to bind to extracellular ligands such as laminin and are causative for CMD in mice (myodystrophy-LARGE^{myd}) and humans (MDC1D) [43,53,54]. Although the transfer reactions catalyzed by LARGE remains unknown, reports have indicated that LARGE is involved in the synthesis of glycan structures that are both on O-mannose-initiated structures as well as other types of glycans [55,56]. Of exceptional interest, over expression of LARGE can rescue laminin binding not only in LARGE-deficient cell lines but also in cell lines with deficiencies in the other known O-mannose pathway enzymes except for POMT1/2 [54]. Recently, the necessary extension (that has yet to be characterized) of the novel, laminin/virus-binding phosphorylated *O*-mannosyl trisaccharide structure on α -dystroglycan was shown to be LARGE dependent [13]. In addition to LARGE, a similar enzyme, LARGE2 has been identified [57]. Although the exact function of LARGE2 remains unknown, this analogous enzyme is hypothesized to function similarly to LARGE and is involved in the *O*-mannosylation of α -dystroglycan [58]. Beyond the enzymes listed above, it is clear that

other enzymes remain to be discovered including the β 4-GlcNAc transferase and the β 3-GalNAc transferase necessary for generating the functionally important phosphorylated trisaccharide structure (Fig. 3). Along these lines, overexpression of cytoxic T cell GalNAc transferase has been demonstrated to repress the phenotype in mdx mice [59] and this may be related to the GalNAc-capped phosphotrisaccharide structure recently shown to be involved in functional O-mannosylation of α -dystroglycan [13].

Concluding Remarks

While substantial progress has been made by multiple investigators in the O-mannosylation field, a host of structure/function questions remain to be addressed in order to address the pathophysiologies associated with defective O-mannosylation. These include elucidating the O-mannose modified proteome, determining the structure(s) of the functional glycans and the enzymes required for their synthesis, developing an understanding for the complexity of O-mannose extension (Fig. 3) and characterizing the naturally occurring mutations in enzymes of the mammalian O-mannosylation pathway associated with disease.

Highlights

- O-Mannosylation regulates arenavirus entry, cancer metastasis, and cell adhesion
- Defects in enzymes of the O-mannosylation biosynthetic pathway are causal for congenital muscular dystrophy
- Approximately 1/3 of O-glycans in mammalian brain are O-mannose-initiated
- O-Mannose glycans are diverse and include a phosphodiester-containing structure
- Many of the protein substrates, functional glycan structures, and enzymes are unknown

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Figure 1. Diversity in Mammalian O-Mannose Structures

O-Mannose structures that have been observed included linear and bisected structures as well as a recently discovered phosphorylated mannose structure. Mannose (Green circle), GlcNAc (Blue square), Galactose (Yellow circle), Fucose (Red triangle), Sialic Acid (Purple diamond), Glucuronic Acid (Blue/White diamond), GalNAc (Yellow square).

Figure 2. The Dystrophin-Glycoprotein Complex and Pathophysiology

α-dystroglycan, in a glycan-dependent manner, serves to bind extracellular matrix proteins as part of the complex and defects in O-mannosylation of this protein are associated with cancer metastasis, congenital muscular dystrophy, and arenavirus entry.

Figure 3. Elaboration of Glycan Structures on O-Mannose modified proteins

O-Mannose can be extended with a GlcNAc in a β 2-linkage by POMGnT1 and then further elaborated into a variety of classical structures by non-O-Man pathway specific enzymes (grey) as well as bisected by an additional GlcNAc at the 6-position catalyzed by GnTVb. Recently a β 4-linked extended O-mannose structure that is phosphorylated at the 6-position has been described that is further elaborated in a phosphodiester linkage by an unknown moiety (X). What dictates whether an O-Man will be extended at the 2 and subsequently perhaps the 6 position with a GlcNAc or extended at the 4 position by a GlcNAc and a phosphate at the 6 position remains to be elucidated.