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Review

The chemistry and biology of mucin-type O-linked glycosylation

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Abstract—Mucin-type O-linked glycosylation is a fundamental post-translational modification that is involved in a variety of important biological processes. However, the lack of chemical tools to study mucin-type O-linked glycosylation has hindered our molecular understanding of O-linked glycans in many biological contexts. The review discusses the significance of mucin-type O-linked glycosylation initiated by the polypeptide *N*-acetylgalactosaminyltransferases in biology and development of chemical tools to study these enzymes and their substrates.

Contents

1. Introduction

The surface of every cell is covered with a diverse array of carbohydrates that are poised to interact with extracellular environment.[1] These glycans not only serve as protective barriers, but also encode precise molecular details about the well being of the cell that dictates communication events with the surroundings. While it is well appreciated that glycans are involved in virtually every aspect of biology, from protein folding[2] to the immune response,[3] embryonic development[4] as well as host–pathogen interactions,[5] our understanding of how glycans influence these complex processes at the molecular level is still in its infancy. Herein, we review the most abundant form of O-linked glycosylation in higher eukaryotes, termed "mucin-type," which is characterized by α-*N*-acetylgalactosamine (GalNAc) attached to the hydroxyl group of Ser/Thr side chains (Fig. 1A).[6] In particular, we focus on the initiation of mucin-type O-linked glycosylation by the polypeptide *N*-acetyl-α-galactosaminyltransferases (ppGalNAcTs) (Fig. 1B)

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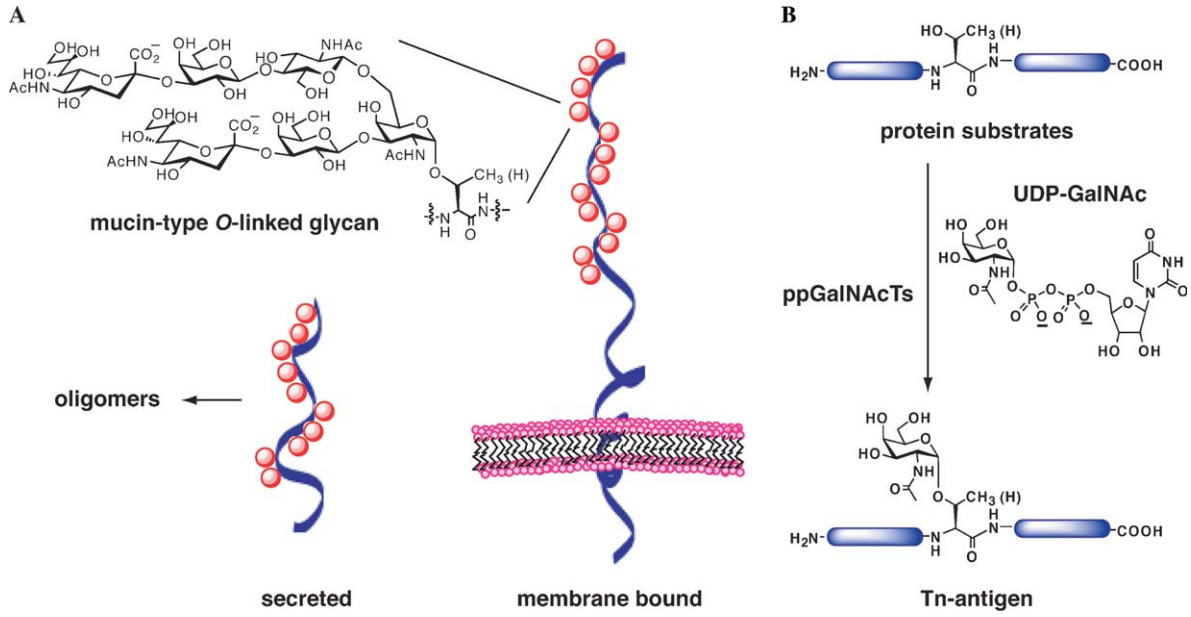

Figure 1. Mucin-type O-linked glycosylation. (A) Architecture of membrane-bound and secreted mucins that crosslink into oligomers. Blue ribbons represent protein backbones. Red spheres represent *O*-linked glycans. (B) Initiation of mucin-type O-linked glycosylation by the ppGalNAcTs, which utilize UDP-GalNAc as the nucleotide sugar donor and modify protein substrates.

and the development of chemical tools for understanding this family of glycosyltransferases in biology.

2. The discovery of mucin-type O-linked glycoproteins

The study of mucin-type O-linked glycosylation originates from the characterization of "mucoproteins" and "mucopolysaccharides" in the early 1800s.[7] The term "mucin" was first used by Nicolas Theodore de Saussure in 1835 to describe substances isolated from mucus[8] and then recognized by Eichwald in 1865 as a combination of protein and carbohydrate.[9] Thereafter, Olof Hammarsten performed many of the classical experiments in the late 1800s that established sugars as major constituents of mucins.[9] Despite these pioneering efforts to identify and classify mucins, there was little interest at that time in the roles mucins play in physiology.

In the early 1900s, two related discoveries on the "agglutination" of erythrocytes invigorated research on mucins. The first was the seminal discovery of the *ABO* blood groups in human serum by Karl Landsteiner in 1901[10] and the second was the characterization of influenza virus hemagglutination in 1942 by Hirst and Burnet.[11] In particular, the molecular basis for *ABO* blood group specificity sparked the structural analysis of erythrocyte extracts by the Morgan and Watkins and Kabat laboratories.[12] These landmark studies revealed the structures of the oligosaccharide antigens (*A*, GalNAcα1-3(Fucα1-2)Galβ1-4(3)GlcNAc; *B*, Galα1-3(Fucα1-2)Galβ1-4(3)GlcNAc; and *O*(*H*), Fucα1-2Galβ1-4(3)GlcNAc) that governed the *ABO* blood group specificities and also demonstrated these glycans were attached to mucin scaffolds through GalNAc residues. However, the alkaline conditions used to liberate the O-linked glycans from the proteins precluded the characterization of the glycopeptide linkage. The α-GalNAc linkage to Thr/Ser was ultimately determined by Weissman and Hinrichsen in 1969 with a purified α-*N*-acetylgalactosaminidase from bovine liver.[13]

3. Structural features of mucin-type O-linked glycoproteins

Mucin-type O-linked glycans typically occur clustered together in "mucin domains" that are found on membrane-bound or secreted mucins (Fig. 1A).[14,15] These dense clusters of O-linked glycans can occur 10–100 times within a given mucin and are often referred to as "tandem repeats." Consequently, the sugar component of mucins typically comprises greater than 50% of their molecular weight. Structural studies of mucins using atomic force microscopy (AFM),[16] light scattering,[17] and nuclear magnetic resonance (NMR) spectroscopy [18] have demonstrated that clustered O-linked glycosylation results in extended protein structures up to hundreds of nanometers in length.[19] Furthermore, NMR analysis of synthetic mucin-type O-linked glycopeptides derived from CD43 showed that α-GalNAc residues and not β-GalNAc residues confer unique secondary structure to the modified peptides.[20,21] These structural features of mucins are most likely the key determinants of their bulk physical properties.

4. The roles of mucin-type O-linked glycoproteins in physiology and disease

To date, over 150 mucin-type O-linked glycoproteins have been annotated in the SWISS-protein database,[22,23] which are involved in a variety of biological processes, some of which are highlighted in Table 1. The dense glycosylation in the tandem repeats of mucins

Table 1. Highlights of mucin-type O-linked glycoproteins in biology

Glycoprotein	Proposed function	Role of O-linked glycans	Reference
MUC1	Tumor antigen	Altered glycans on cancer cells	25
MUC2	Intestinal homeostasis	Lubrication	26,27
Interleukin-2	Cytokine	Regulation of serum half-life	29
Gonadotropin	Pituitary hormone	Apical targeting	30
CD45	Tyrosine phosphatase	Modulation of Lck signaling	31
GlyCAM-1	Leukocyte homing	Ligands for L-selectin	35
PSGL-1	Leukocyte homing	Ligands for P-selectin	36,37
Ebola GP	Viral glycoprotein	Required for cytotoxicity	40

enables them to function as protective barriers and provide lubrication in a variety of tissues due to their hydration capacity,[15,24] as exemplified by MUC1[25] and MUC2.[26] Targeted deletion of the MUC2 gene in mice resulted in the formation of colorectal tumors that were absent in wild-type mice,[27] which suggests a critical role for MUC2 in intestinal homeostasis.

Beyond the bulk properties exhibited by mucins, mucin-type O-linked glycosylation can modulate various aspects of protein function. Mucin-type O-linked glycans can block accessibility of the polypeptide backbone to proteases and confer resistance to degradation,[28] regulate the serum-half life of chemokines or hormones to attenuate their activity in vivo,[29] and modulate the intracellular trafficking of proteins[30] (Table 1). The presence of mucin domains can determine the aggregation state of membrane-bound glycoproteins and thus affect signaling properties or binding to receptors. For example, CD45 on the surface of T-cells exists either as a monomer (CD45 RABC) that includes three exons encoding mucin domains, or as homodimer (CD45 RO) in which the mucin domains have been removed by alternative splicing (Fig. 2).[31] In the monomeric form, CD45 RABC associates with the protein kinase Lck, enabling the intracellular tyrosine phosphatase domain of CD45 to dephosphorylate Lck and initiate a signaling cascade for T-cell activation. In the absence of the mucin domains, the CD45 RO homodimer precludes association with Lck and ultimately returns T-cells to their resting state.

In addition to modulating protein function, mucin-type O-linked glycans can serve as discrete ligands for cell surface receptors that mediate cell adhesion events.[32] Perhaps, the most prominent example is in the homing of leukocytes to sites of inflammation, which is dependent upon interactions of the selectins (E-, L-, and P-) with carbohydrate ligands presented on O-linked glycoprotein scaffolds.[33] For L-selectin, a variety of glycoproteins bearing sulfated O-linked glycans have been identified as ligands,[34] one of which is GlyCAM-1[35] (Table 1). Other cell surface O-linked glycoproteins such as P-selectin glycoprotein ligand-1 (PSGL-1) interact with P-selectin, but also require tyrosine sulfation for binding.[36,37] In addition, O-linked glycans are involved in cell-adhesion events during sperm–egg fertilization,[38] host–microbial interactions,[39] and viral infections.[40]

Mucin-type O-linked glycoproteins can serve as a source of antigens to the immune system and result in the presentation of glycopeptides by major histocompatibility complex proteins to T-cells.[41] In fact, a variety of tumor-associated antigens are O-linked glycans.[42] Consequently, synthetic mucin-type O-linked glycopeptides have been pursued as anti-tumor vaccines.[43,44]

5. Biosynthesis of mucin-type O-linked glycans

Mucin-type O-linked glycosylation is initiated by the family of ppGalNAcTs that utilize UDP-GalNAc as the nucleotide donor substrate to modify protein substrates (Fig. 1B).[45] The product of the reaction, α-Gal-

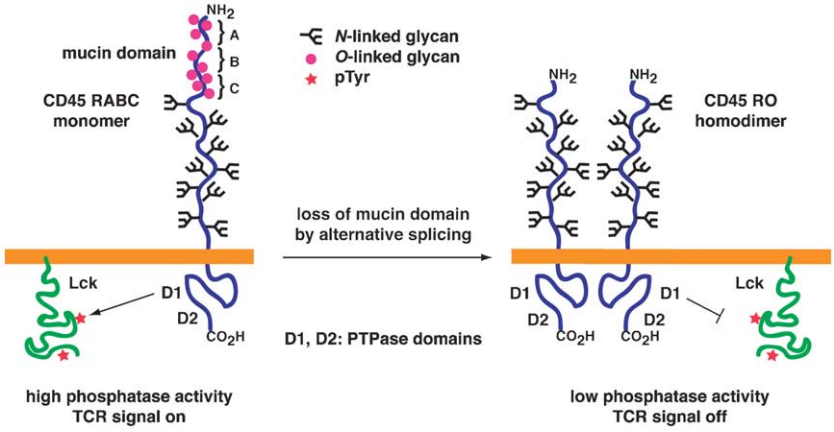

Figure 2. Mucin-type O-linked glycosylation determines the aggregation state of CD45, which modulates its tyrosine phosphatase activity that is critical for T-cell activation. The CD45 RABC isoform exists as monomer on surface of T-cells and efficiently dephosphorylates Lck, which initiates a cascade of events for T-cell activation. In contrast, the CD45 RO isoform lacks the mucin domain and forms a homodimer that exhibits low levels of protein tyrosine phosphatase (PTPase) activity towards Lck.

NAc on Ser/Thr residues, is termed the "Tn-antigen" and is further elaborated by downstream glycosyltransferases to generate a series of core O-linked glycans (Fig. 3).[46] These core structures are then further modified by other Golgi-resident glycosyltransferases to generate complex O-linked glycans that are involved in a variety of biological processes (Fig. 4). Changes in the elaboration of O-linked glycans are often correlated with a variety of diseases, such as Wiskott–Aldrich syndrome,[47] hematological disorders,[48] and cancer.[42,47]

6. Initiation of mucin-type O-linked glycosylation by the ppGalNAcTs

The first ppGalNAcT activity was reported in 1967 by McQuire and Roseman,[49] purified in 1986 by Elhammer and Kornfeld,[50] and subsequently cloned by the Tabak,[51] Elhammer,[52] and Clausen[53,54] laboratories. Analysis of the human genome has revealed ~24 putative ppGalNAcTs, with orthologs in higher eukaryotes that demonstrate 90–98% sequence homology across species. Genomic and functional analyses of ppGalNAcTs derived from *Caenorhabditis elegans*,[55] *Drosophila melanogaster*,[56–58] and the unicellular parasite *Toxoplasma gondii*[59] have revealed 9, 14, and 5 ppGalNAcT isoforms, respectively. To date, 21 unique ppGalNAcT isoforms have been cloned[51,52,54,56–64] and biochemically characterized with the exception of ppGalNAcT-8.[64]

The ppGalNAcT genes have a variable number of coding exons and disperse chromosomal organization.[65] Analysis of the intron/exon boundaries suggests that the ppGalNAcT family arose from gene duplication with subsequent divergence. Northern blot and real-time polymerase chain reaction (RT-PCR) analysis of the ppGalNAcT transcripts in human and rodent adult tissues revealed ubiquitous expression of some isoforms (ppGalNAcT-1 and -2) and more discrete tissue distribution of others (ppGalNAcTs 5, 7, and 10–12).[45,66] The patterns of ppGalNAcT expression in various tissues are further supported by analysis of protein levels with antibodies to ppGalNAcTs 1–4, 6, and 11.[57,62,67,68] These differences are underscored by transcriptional analysis of ppGalNAcTs during mouse embryonic development, which demonstrated spatial and temporal regulation of ppGalNAcT expression in a variety of tissues.[69] Evaluation of colon cancer cells has shown elevated expression and activity of a few ppGalNAcTs.[70] Recently, two rare autosomal recessive metabolic disorders have been mapped to mutations in the human gene GALNT3 that abrogate ppGalNAcT-3 activity.[71]

All of the ppGalNAcTs show considerable conservation of protein architecture, characterized by an N-terminal cytosolic tail, a type II transmembrane domain, a variable stem region, a catalytic domain (GT1), a Gal/GalNAc recognition domain, and a C-terminal lectin

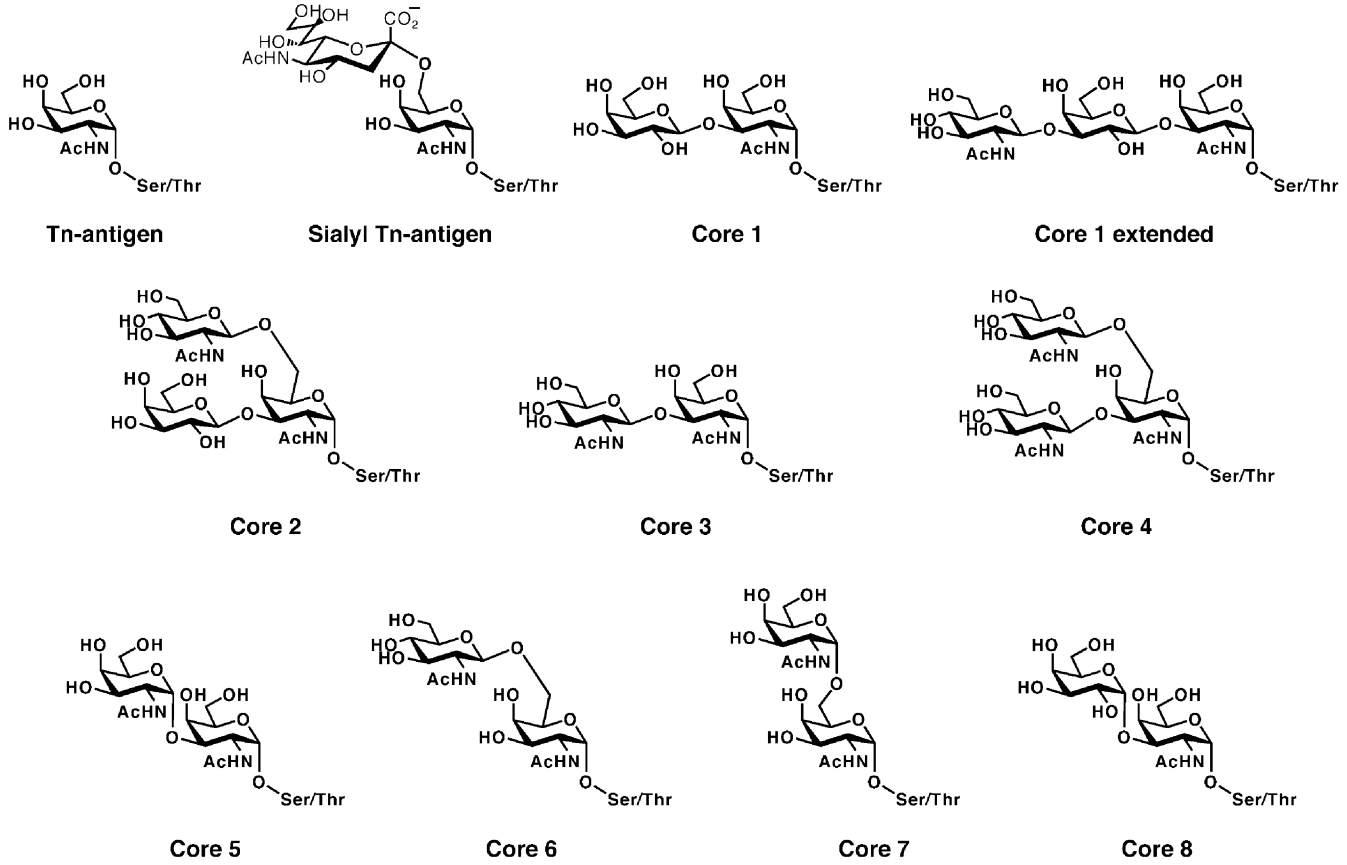

Figure 3. Structure of core O-linked glycans; Tn-antigen, Sialyl Tn-antigen, core 1: Galβ1-3GalNAc, core 1 extended: GlcNAcβ1-4Galβ1-3GalNAc, core 2: Galβ1-3(GlcNAcβ1-6)GalNAc, core 3: GlcNAcβ1-3GalNAc, core 4: GlcNAcβ1-3(GlcNAcβ1-6)GalNAc, core 5: GalNAcα1-3GalNAc, core 6: GlcNAcβ1-6GalNAc, core 7: GalNAcα1-6GalNAc and core 8: Galα1-3GalNAc.

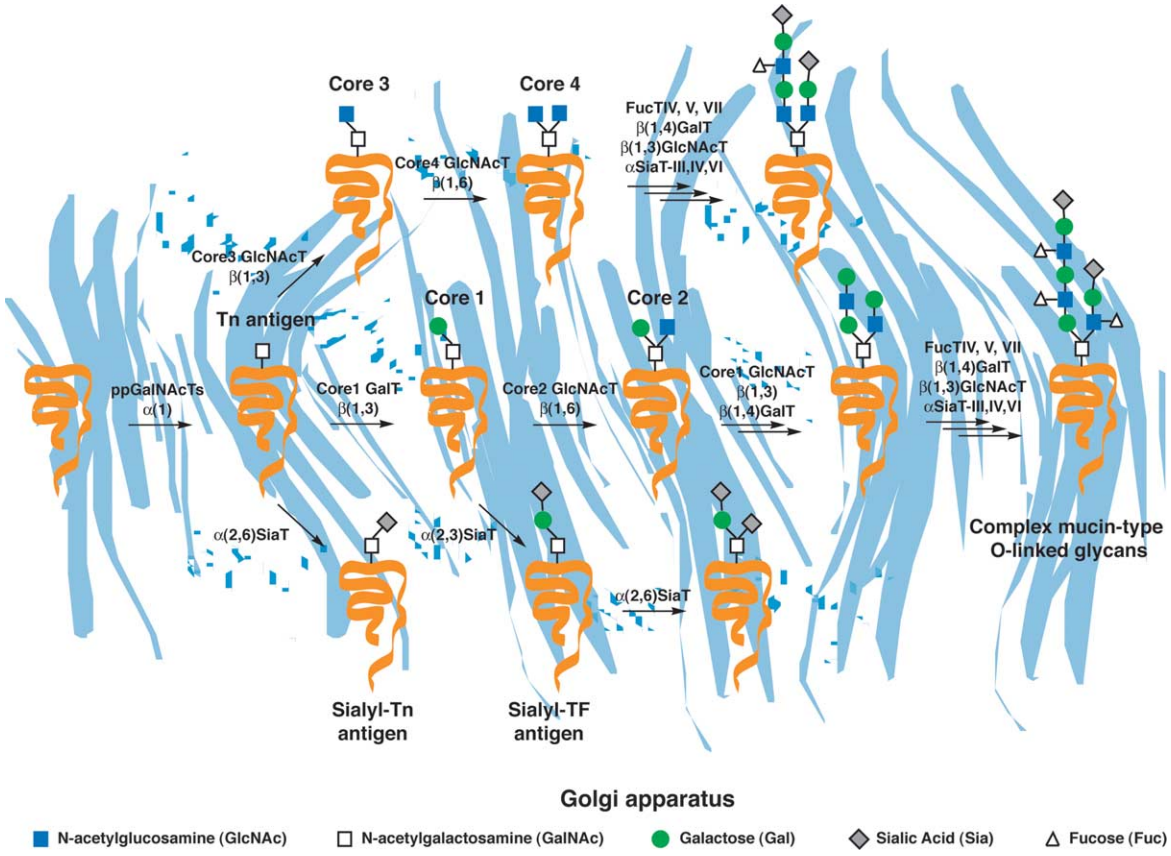

Figure 4. Biosynthesis of mucin-type O-linked glycoproteins in the Golgi apparatus.

domain that is thought to bind acceptor substrates (Fig. 5).[72,73] The GT1 and Gal/GalNAc recognition domains have approximately 60% amino acid sequence similarity among the different ppGalNAcT isoforms (Fig. 5). The lectin domain was identified by homology to ricin and has approximately 40% amino acid similarity among the ppGalNAcTs,[74] classifying them as R-type lectins.[75]

Antibody staining for the Tn-antigen and electron microscopy analysis has suggested that the ppGalNAcTs function in the Golgi apparatus and not in the ER.[76] Fluorescence and electron microscopy studies with green fluorescent protein (GFP) fusions of ppGalNAcTs (1–3) demonstrated that ppGalNAcT-1 is widely distributed throughout the Golgi apparatus, while ppGalNAcT-2 and -3 resided primarily in the *trans* and *medial* cisternae of the Golgi.[77] These results, taken together with variability in the stem region of the ppGalNAcTs, suggest that the precise localization of these glycosyltransferases within the Golgi apparatus may also govern their substrate specificity in cells, as observed for other Golgi-resident enzymes.[78]

7. Structural and mechanistic studies of ppGalNAcTs

Mechanistically, glycosyltransferases can be divided into two classes, inverting or retaining, based upon the relative stereochemistry of the anomeric glycosidic linkage in the nucleotide sugar donor substrate and the glycoside product.[79] The ppGalNAcTs are retaining glycosyltransferases and are categorized in the family 29 group of enzymes according to the classification system developed by Henrissat and co-workers.[80] Furthermore, glycosyltransferases can be classified based upon topology in order to group them with other en-

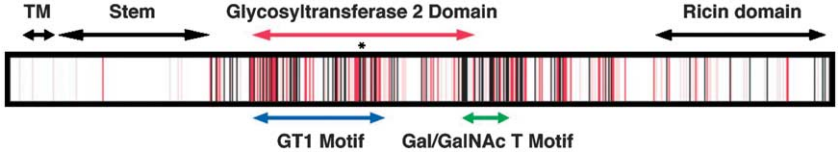

Figure 5. Domain architecture of ppGalNAcT family taken from reference 62 with permission from authors. Consensus sequence diagram of several ppGalNAcTs with demonstrated activity. Regions in white, pink, red, and black represent, respectively, 0–29%, 30–69%, 70–99%, and 100% sequence identity. The positions of the transmembrane (TM), stem, catalytic (glycosyltransferase 2 domain, GT1, and Gal/GalNAc T motifs) and ricin domains are indicated by the colored arrows. The glycosyltransferase 2 domain was identified from the Pfam database, the ricin domain was identified from the SMART (Simple Modular Architecture Research Tool) database, and the GT1 and Gal/GalNAc T motifs were described in Hagen et al.[72] The asterisk shows the location of the conserved DXH motif likely to be involved in sugar nucleotide binding.

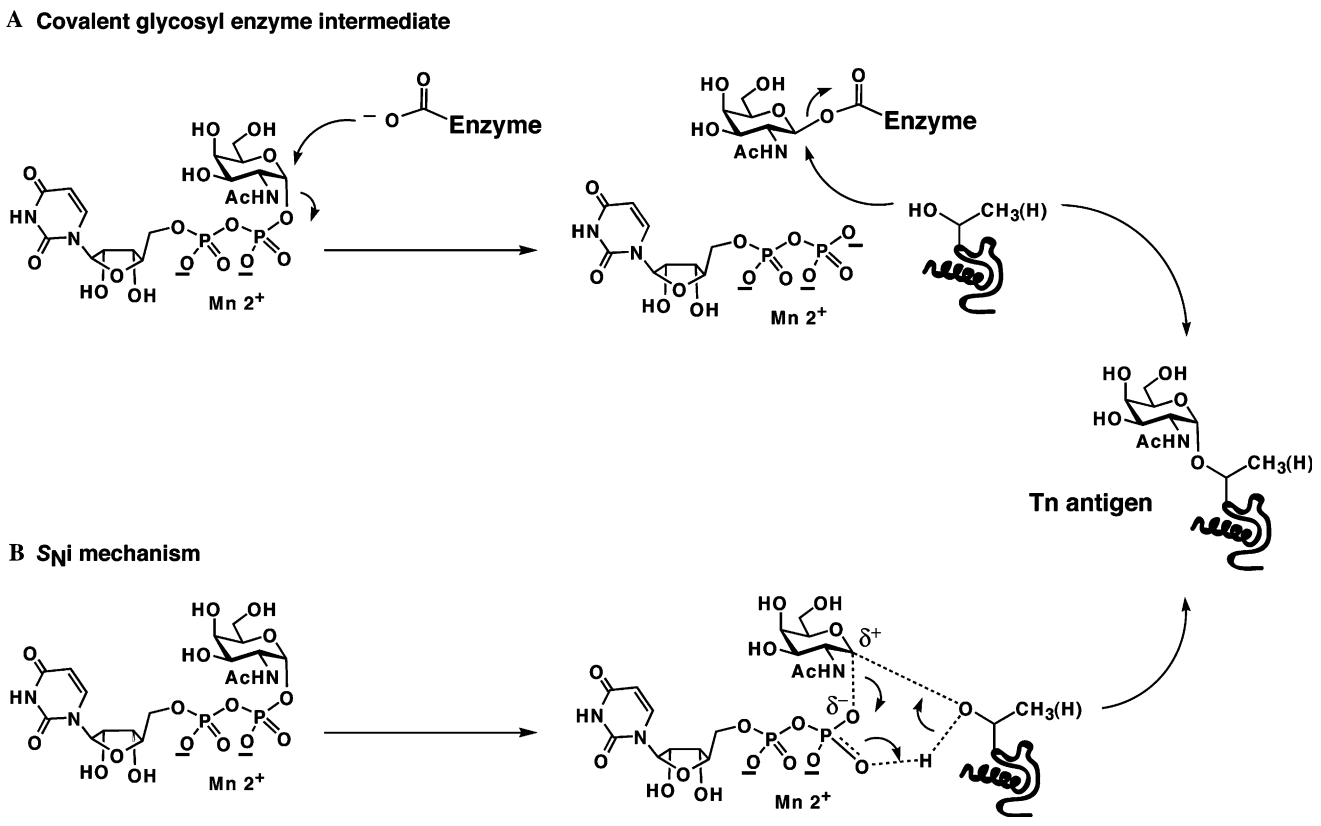

Figure 6. Proposed mechanisms of glycosyl transfer by retaining ppGalNAcTs. (A) Covalent glycosyl-enzyme intermediate analogous to retaining glycosidases.[83] (B) Nucleophilic substitution by internal return mechanism (S_Ni) suggested by the conformation of nucleotide sugar donor bound in the active site of retaining glycosyltransferases, asynchronous steps.[87]

zymes that share similar folds, which may also be mechanistically related.[81] By analogy to the studies on retaining glycosidases,[82] retaining glycosyltransferases are proposed to act through a ping-pong mechanism with a covalent glycosyl-enzyme intermediate (Fig. 6A).[83] However, mechanistic studies of a retaining α1-4galactosyltransferase failed to detect the accumulation of any covalent glycosyl-enzyme intermediate.[84] Alternatively, the recent crystallographic analysis of a few retaining glycosyltransferases has demonstrated a distorted conformation of the bound nucleotide sugar donor,[85,86] which is suggestive of a nucleophilic substitution by internal return mechanism that involves the transfer of sugar without a covalent glycosyl-enzyme intermediate (S_Ni, Fig. 6B).[87] More recent studies of a mutant retaining galactosyltransferase trapped a glycosyl-enzyme intermediate, but the identified Asp residue was positioned 8.9 Å away from the nucleotide sugar donor in the crystal structure.[88] In the absence of any definitive experimental evidence for either proposed mechanism, the details of glycosyl transfer by retaining glycosyltransferases remain a mystery.

Kinetic analysis of a recombinant ppGalNAcT demonstrated random-order sequential binding of substrates prior to catalysis,[89] in contrast to other glycosyltransferases which bind the nucleotide sugar donor first.[85] Mutagenesis studies of the ppGalNAcTs were originally guided by sequence homology and topology mapping to other glycosytransferases, which confirmed in silico predictions of domain functions and identified essential residues for Mn^{2+} and substrate binding as well as catalysis.[72,90,91] The recent crystal structure of mppGalNAcT-1 has solidified these observations and suggests a model for acceptor substrate recognition.[92] Unfortunately, the lack of electron density between UDP-GalNAc and mppGalNAcT-1 precluded any further mechanistic insight into the catalysis.

8. Nucleotide sugar donor substrate specificity of the ppGalNAcTs

The universal nucleotide sugar donor for the ppGalNAcTs is UDP-GalNAc, with K_M values that range from 10 to 50 μM.[61,93] Synthetic UDP-GalNAc analogs have been tested as in vitro substrates for ppGalNAcT-1. 3-, 4- or 6-methoxy,[94] 6-biotinyl,[95] and 6-azido GalNAc derivatives[96] were not recognized as substrates. However, the ppGalNAcTs appear to tolerate subtle modifications at the 2-position of the pyranose, as UDP-Gal is a substrate for ppGalNAcT-2 in vitro.[93] In addition, a ketone-isostere of GalNAc was incorporated into mucin-type O-linked glycans in Chinese hamster ovary (CHO) cells.[97] Lastly, *N*-azidoacetylgalactosamine (GalNAz) was efficiently incorporated into mucin-type O-linked glycans in a variety of mammalian cell lines[98] and in vivo.[99] Moreover, ppGalNAcTs (1–5, 7, 10, and 11) utilized UDP-GalNAz as a substrate in vitro, with a relative k_{cat}/K_M value of 0.2 compared to UDP-GalNAc.[100]

9. (Glyco)peptide substrate specificity of ppGalNAcTs

Unlike N-linked glycosylation, which occurs at a defined consensus sequence (AsnXaaSer/Thr) by the action of a single oligosaccharyl transferase (OT),[101] no primary amino acid consensus sequence has emerged for mucin-type O-linked glycosylation, despite considerable effort by a number of groups.[45,102,103] Biochemical studies of ppGalNAcTs derived from tissue extracts[104] and recombinant expression[93,102,105] with a variety of peptide substrates have demonstrated: (1) a preference for Pro at the P3′ position with respect to the site of modification, (2) charged residues at P1 and P3′ were disfavored, and (3) glycosylation of Thr was preferred over Ser. These results in vitro were supported by the analysis of reporter peptide and protein substrates expressed in a variety of cell lines.[106] From these studies, some isoform-specific substrates have emerged,[62,68,93,107] but these conclusions may be premature since all the ppGalNAcTs have not yet be characterized. NMR analysis of short peptide substrates suggests a propensity for β-turn conformations,[108] which is consistent with the observed glycosylation of many proline-rich sequences within proteins.[109] Thus, ppGalNAcTs may recognize peptide substrates based on secondary structural motifs, rather than primary amino acid sequence alone. Based upon empirical data, computational algorithms have been developed for predicting the likelihood of O-linked glycosylation from primary amino acid sequences.[23,110] While these programs are useful for predicting mucin domains, precise sites of glycosylation still require experimental confirmation.

The density of O-linked glycans within mucin domains suggests that glycosylation of neighboring residues could also affect the activity of ppGalNAcTs. Indeed, several studies have demonstrated that the density and glycosylation pattern of acceptor substrates affect the ability of ppGalNAcTs to modify Thr/Ser residues within mucin domains.[111,112] The cloning and biochemical characterization of mammalian ppGalNAcT-7 and -10 as well as *T. gondii* ppGalNAcT-1 have identified ppGalNAcTs that require glycopeptides rather than peptides as acceptor substrates.[59,63] Preliminary mutagenesis studies of ppGalNAcT-1 and -4 support the notion that the lectin domains of the ppGalNAcTs are responsible for determining the acceptor (glyco)peptide substrate specificities.[91,113] However, no quantitative analysis of ppGalNAcT mutants has been performed with glycopeptide substrates.

The elaboration of GalNAcα-Thr/Ser into core 1 O-linked glycans can also affect the extent of ppGalNAcT activity on neighboring sites.[111,114] In fact, the evaluation of ppGalNAcTs (1–5, 7, 10, and 11) with a library of EA2 (PTTDSTTPAPTTK) glycopeptides has demonstrated that all ppGalNAcTs, except ppGalNAcT-1, prefer glycosylated EA2 peptides with distinct profiles.[115] Collectively, these studies suggest that the family of ppGalNAcTs could be further classified into subfamilies of polypeptide and glycopolypeptide *N*-acetylgalactosaminyltransferases (gppGalNAcTs), and those with dual activities.[58] While the in vitro characterization of the (g)ppGalNAcTs has identified distinct glycopeptide and peptide transferases, these studies also demonstrated that many of the (g)ppGalNAcTs have overlapping acceptor substrate specificities.

10. Genetic approaches toward understanding mucin-type O-linked glycosylation

Cell lines with mutations in glycosylation pathways have provided insight into N-linked glycan[116] and proteoglycan biosynthesis.[117] However, only one mutant CHO cell line (termed "*ldlD*") exists that contains a defined defect in O-linked glycosylation.[118] *ldlD* CHO cells lack UDP-Glc/GlcNAc C_4-epimerase activity, which renders this cell line a UDP-GalNAc auxotroph, unable to generate the nucleotide sugar donor required for O-linked glycosylation in the absence of exogenous sources of GalNAc. This cell line has been useful for generating proteins deficient in O-linked glycans, but has a limited spectrum of biological activities pertinent to human health and disease.

Genetic approaches have provided significant insight into the study of glycosylation.[119] Unfortunately, targeted gene disruption of single ppGalNAcTs (1, 4, 5, and 13) in mice has produced no apparent phenotype with respect to development, reproduction or immunological response,[45,120] suggesting functional redundancy by other ppGalNAcT isoforms.[121] In hindsight, these results are not surprising given the overlapping acceptor substrate specificities of the ppGalNAcTs that have been observed in vitro and in vivo. Analyses of lethal *D. melanogaster* mutants demonstrated that one ppGalNAcT isoform, pgant35A, is essential for development.[56,57] Therefore, the generation of mice deficient in other ppGalNAcTs (i.e., T-3 or T-11) or multiple ppGalNAcTs with similar activities may still unveil novel functions of the ppGalNAcTs in vivo.

11. Synthesis of homogeneous mucin-type O-linked glycopeptides and glycoproteins

The chemical synthesis of glycoconjugates has provided homogeneous substrates for characterizing enzymes and provided useful reagents to probe protein–carbohydrate interactions.[122,123] The synthesis of mucin-type O-linked glycopeptides is generally accomplished by fluorenyl-methoxy carbonyl (Fmoc)-based solid-phase peptide synthesis (SPPS) using the appropriately protected glycosyl amino acid building block (i.e., for Tn-antigen: FmocHN-(α-Ac_3GalNAc)Thr/Ser-CO_2H) (Fig. 7).[124] Traditionally, the glycosyl amino acid building blocks were synthesized from the 2-azido-glycosyl bromide donors and coupled with a protected amino acid under Lewis acid catalyzed conditions (Fig. 7). This method enabled the synthesis of a glycopeptide library to characterize the acceptor substrate specificity of several ppGalNAcTs[115] as well as mu-

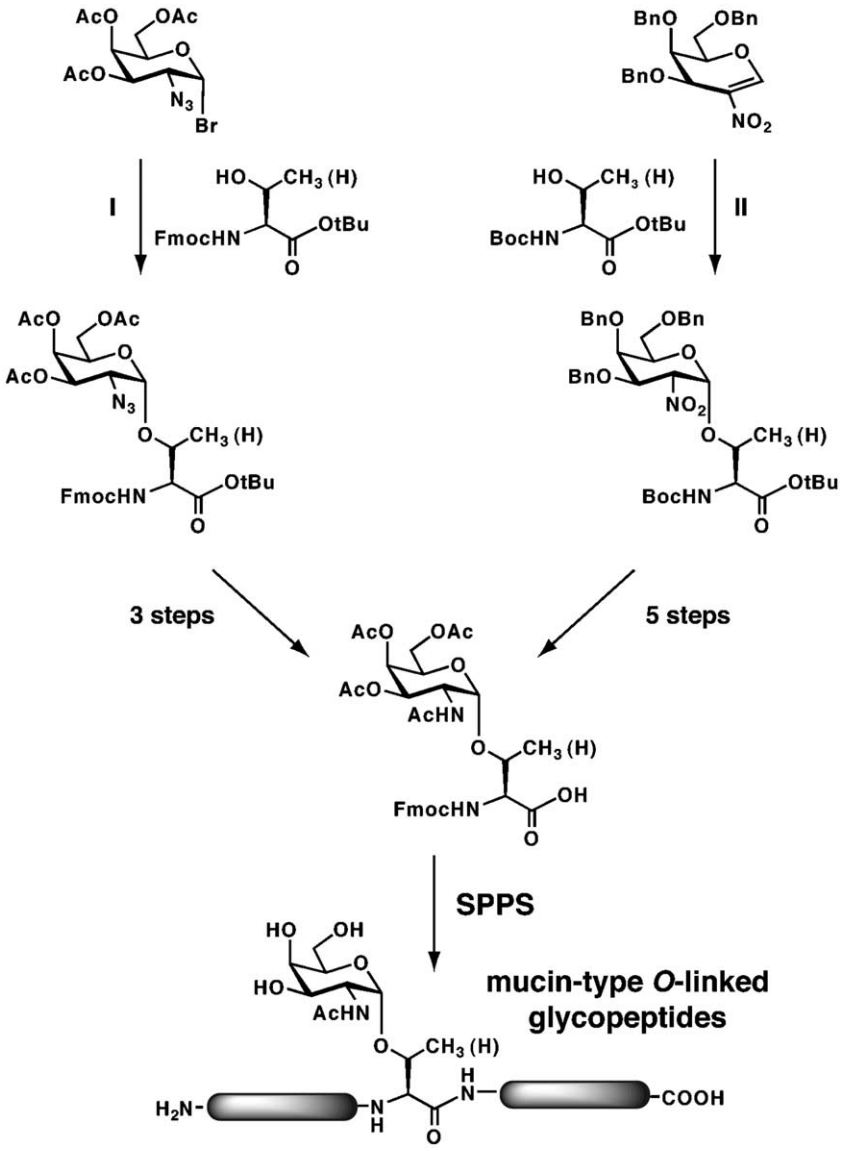

Figure 7. Methods for the construction of glycosyl amino acid building blocks for solid-phase synthesis of mucin-type O-linked glycopeptides.[124] I: Lewis-acid mediated glycosylation. II: Nitroglycal cancatenation.[126]

cin-type glycopeptides for anti-tumor vaccines[43,125] and structural studies[20]. Alternatively, glycosyl amino acid building blocks can also be generated efficiently with the ''nitroglycal cancatenation'' method (Fig. 7).[126] Importantly, both methods allow access to glycosyl amino acid building blocks with larger glycans. Furthermore, α-GalNAc-modified glycopeptides can be elaborated enzymatically to generate more complex structures for functional studies.[37]

The size of synthetic glycopeptides is generally limited by the efficiency of SPPS. To overcome these limitations, investigators have turned to native chemical ligation and expressed protein ligation methods[127] for the generation of homogenous glycoproteins from smaller glycopeptides and recombinant proteins.[122,124] The combination of these approaches has enabled the construction of a homogeneous mucin-type O-linked glycoprotein, GlyCAM-1, with multiple glycosylation sites in two different domains (Fig. 8A)[128] The development of unnatural amino acid mutagenesis in vivo has presented exciting opportunities for expression of recombinant glycoproteins with defined sites of glycosylation.[129] Indeed, a model protein has been expressed from bacteria with site-specific mucin-type O-linked glycosylation (Fig. 8B).[130] Collectively, these methods should provide sufficient quantities of homogeneous mucin-type O-linked glycoproteins for structural and functional analyses as well as glycoprotein-based therapeutics.

12. Development of small molecules for the study of mucin-type O-linked glycosylation in vivo

Small molecule inhibitors of enzymes involved in glycoconjugate biosynthesis have been instrumental for understanding glycobiology and development of new therapeutic strategies.[131] Unfortunately, very few chemical tools are available to address mucin-type O-linked

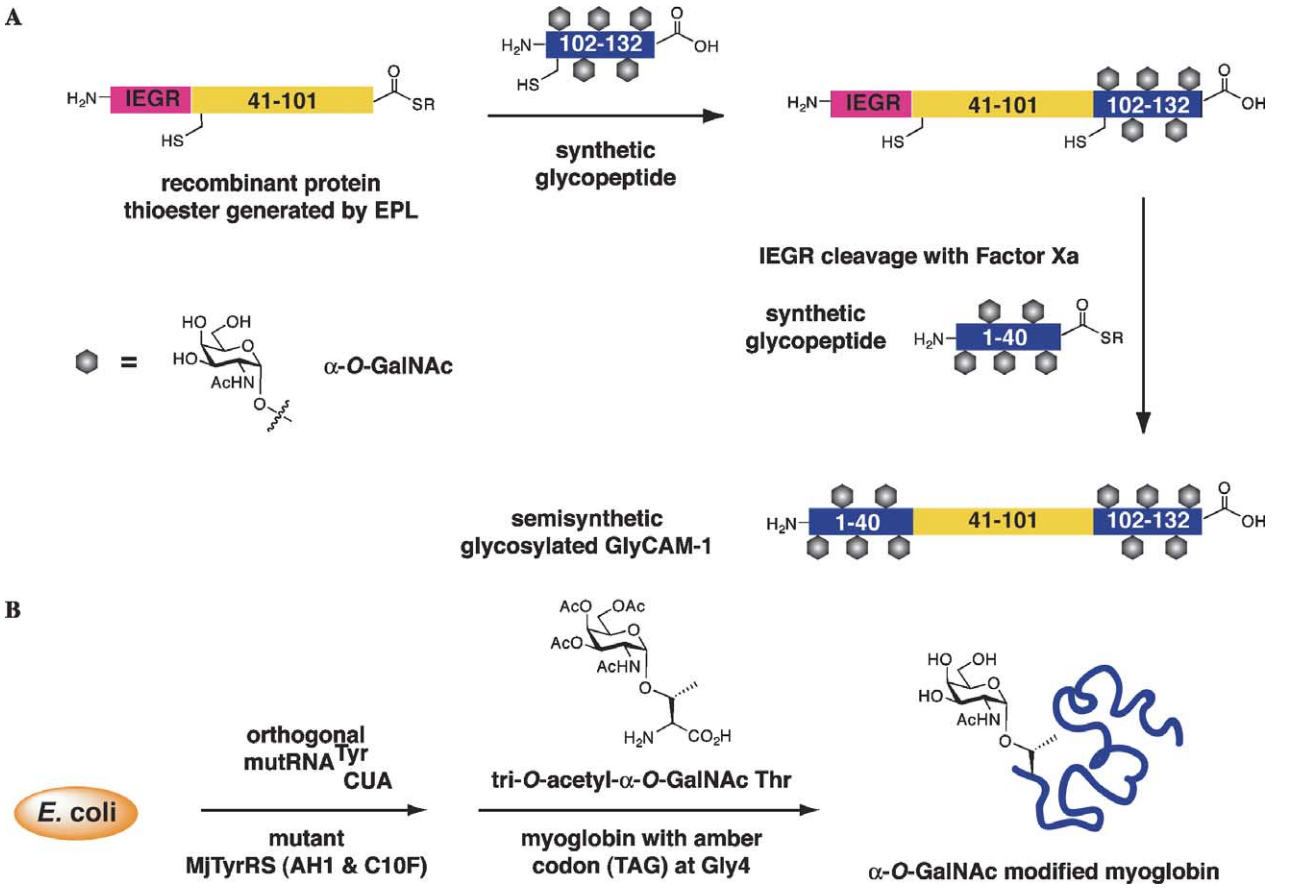

Figure 8. Semisynthetic methods for construction of homogeneous mucin-type O-linked glycoproteins. (A) Synthesis of GlyCAM by solid-phase glycopeptide synthesis and expressed protein ligation.[128] (B) Recombinant expression of mucin-type O-linked glycoproteins in bacteria with unnatural amino acid mutagenesis.[130]

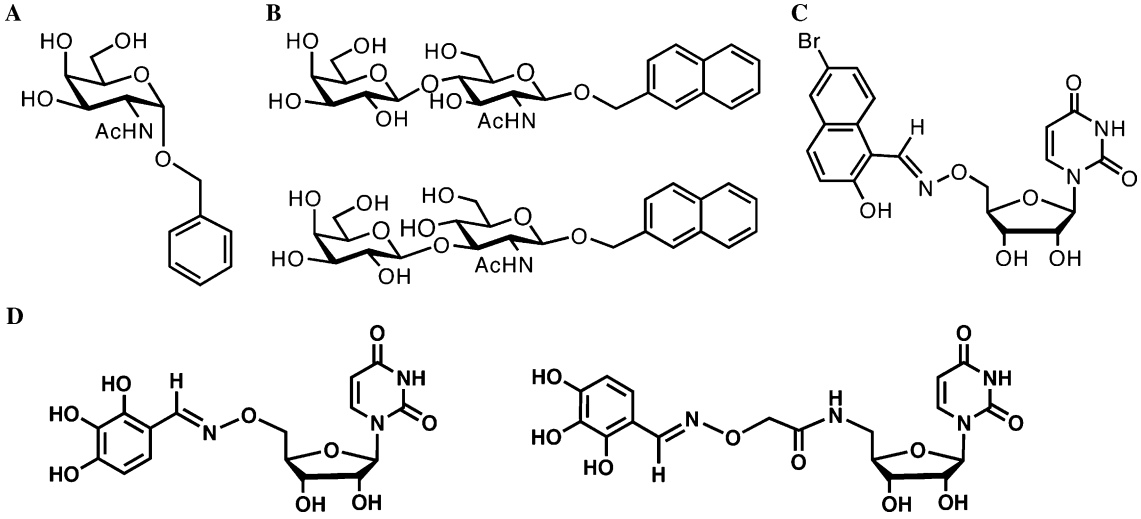

Figure 9. Compounds designed to interfere with the biosynthesis of mucin-type O-linked glycans. (A) α-benzyl-GalNAc (αBnGalNAc).[132] (B) Galβ1-4GlcNAcβ-*O*-NM[133] and GlcNAcβ1-3Galβ-*O*-NM.[134] (C) UDP-Glc/GlcNAc C_4-epimerase inhibitor identified from a uridine-based library.[136] (D) Broad-spectrum inhibitors of the ppGalNAcTs identified from a uridine-based library.[139]

glycosylation in cells. Competitive substrate-based primers such as α-benzyl-GalNAc,[132] Galβ1-4GlcNAcβ-*O*-naphthalenemethanol (NM),[133] and Galβ1-3 GlcNAcβ-*O*-NM[134] inhibit the elaboration of O-linked glycans, affording truncated O-linked glycans on cells[135] (Figs. 9A and B). However, these compounds do not affect the attachment of GalNAc to Thr/Ser residues and are often used at mM concentrations that are toxic to cells. In addition, the modification of these biosynthetic primers by glycosyltransferases in cells affords metabolites that can also affect the biosynthesis of other glycoconjugate classes.

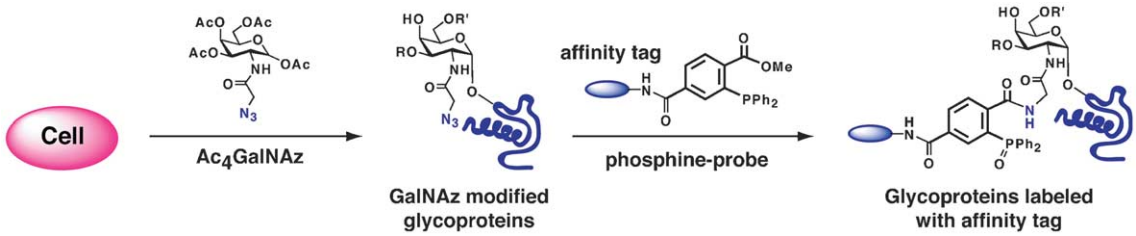

Figure 10. Metabolic labeling approach towards proteomic analysis of mucin-type O-linked glycosylation.[98] Treatment of cells with acetylated GalNAz affords mucin-type O-linked glycoproteins labeled with azides that can undergo Staudinger ligation with phosphine probes.

Alternatively, inhibition of O-linked glycosylation in cells can be achieved by interfering with the biosynthesis of UDP-GalNAc, the nucleotide donor utilized by the ppGalNAcTs. Indeed, the decrease in cell surface O-linked glycans exhibited by *ldlD* CHO cells grown in low concentrations of exogenous GalNAc suggested that UDP-Glc/GlcNAc C_4-epimerase is a viable target for disrupting O-linked glycosylation in cells. Towards this end, Winans et al. synthesized and screened a uridine-based library that afforded a selective UDP-Glc/GlcNAc C_4-epimerase inhibitor with a K_i value of 11 μM (Fig. 9C).[136] While the efficacy of this compound in cells has not been determined, it could emerge as a small molecule inhibitor of O-linked glycosylation analogous to tunicamycin for N-linked glycosylation.[137]

The complexity of ppGalNAcT activities in vivo underscores the need for general and isoform-specific inhibitors of the ppGalNAcTs. The design of glycosyltransferase inhibitors has been very challenging due to limited structural and mechanistic information. Thus, traditional approaches toward designing glycosyltransferase inhibitors have focused on substrate-based mimetics and transition-state analogs.[138] However, most of these compounds are charged and membrane-impermeable, precluding their use in living cells. In order to identify ppGalNAcT inhibitors, a high-throughput ppGalNAcT assay was developed for screening small molecule libraries.[139] Evaluation of the uridine-based library[136] resulted in two general inhibitors of the ppGalNAcTs with K_i values of ~8.0 μM (Fig. 9D).[139] Incubation of cells with these broad-spectrum ppGalNAcT inhibitors afforded a reduction in cell surface O-linked glycans and not N-linked glycans. More detailed analysis of these ppGalNAcT inhibitors demonstrated the abrogation of O-linked glycosylation induced apoptosis in cell culture and developing mandibular tissues, which suggests proper O-linked glycosylation is required for cell viability.[140] These compounds represent the first generation of ppGalNAcT inhibitors that function in cells and provide a starting point for the development of isoform-specific inhibitors.

13. Mucin-type O-linked glycoproteomics

The interest in functional proteomics has motivated a number of approaches toward systems-wide analysis of post-translational modifications.[141] Unfortunately, the proteomic analysis of mucin-type O-linked glycosylation has been difficult due to the complexity and microheterogeneity of O-linked glycans and lack of general reagents for efficient enrichment and recovery. More specifically, no single lectin or antibody can bind the diverse array of O-linked glycans produced by mammalian cells. Thus, the use of lectins for enrichment of O-linked glycopeptides or glycoproteins for proteomic analysis requires extensive digestion by glycosidases and is not general.

The metabolic incorporation of unnatural monosaccharides that bear uniquely reactive chemical handles such as ketones[142] or azides[143] has enabled the selective labeling of glycoconjugates with a variety of reagents.[144] The application of this strategy using unnatural GalNAc derivatives has allowed the metabolic labeling of mucin-type O-linked glycans with ketones[97] and azides.[98] In particular, efficient metabolic incorporation of *N*-azidoacetylgalactosamine (GalNAz) into mucin-type O-linked glycans in a variety of mammalian cell lines[98] and in vivo[99] provides a general strategy for tagging mucin-type O-linked glycans with azides. These azide-modified glycoproteins could then be selectively reacted with phosphine reagents for proteomic analysis (Fig. 10).[98] This approach takes advantage of the bio-orthogonal reactivity of azides and the only conserved feature of mucin-type O-linked glycans, their core GalNAc residue, for proteomic analysis of mucin-type O-linked glycoproteins.

14. Summary and future outlook

In conclusion, mucin-type O-linked glycosylation is a critical post-translational modification that is involved in a variety of physiological events and diseases. However, the precise roles of the ppGalNAcTs in mucin-type O-linked glycosylation have been elusive due their complex spatial and temporal regulation and the lack of primary consensus sequence in their protein substrates. Consequently, identification of the physiological substrates for each of the ppGalNAcT isoforms has been extremely challenging.

The development of tools such as RNA interference for temporal silencing of genes in mammalian systems,[145] small molecule inhibitors of the ppGalNAcTs,[139] and unnatural substrates for metabolic labeling of mucin-type O-linked glycoproteins[98] sets the stage for unraveling the functions of ppGalNAcTs and mucin-type O-linked glycoproteins in biology. Furthermore, the implementation of chemical genetic strategies[146] for iso-

form-specific inhibitors and orthogonal enzyme/substrate pairs should accelerate the deconvolution of the individual ppGalNAcT functions.

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