CHAPTER 1

Introduction

Chemical and Structural Diversity of Glycans

Glycans are carbohydrate biopolymers with important biological roles found in all organisms. These biopolymers, synthesized from eight possible monosaccharide building blocks, can range from single monosaccharide units to polysaccharides containing hundreds of sugars in branched or linear arrays (1). The structural and chemical complexity of glycans give rise to a diverse set of functions within the cell. The cellular "glycome," or the collective set of glycans within the cell, has roles from increasing the complexity of cellular signaling to expanding the ability of a cell to modulate protein function (2-6). Glycosylation is one of the most ubiquitous post translational modifications, with more than 50 percent of the human proteome estimated to be glycosylated (7). Glycolipids are another important class of glycans that serve critical structural roles in cellular membranes and can regulate signal transduction and cellular function (8). As the glycome is influenced by the genome, the transciptome, and the proteome, as well as environmental and nutrient cues, it can report on the physiological state of the cell (9). Specific changes in glycan profiles correlate with certain disease states such as cancer and inflammation (10), suggesting that glycans could be used in clinical diagnostics and perhaps as targets for developing therapeutics**.**

In vertebrates, glycans may be intracellular, membrane associated, or secreted (11, 12). Within the cell, glycans can direct protein folding and trafficking, and are critical for protein quality control (13-16). Membrane associated glycans can mediate molecular recognition events, serving as points of attachment for viruses, bacteria, and other cells and participating in many facets of the vertebrate immune system (17). Additionally, cell-surface glycans participate in cell-cell interactions involved in embryonic development (18), leukocyte homing (19), and cancer cell metastasis (20, 21).

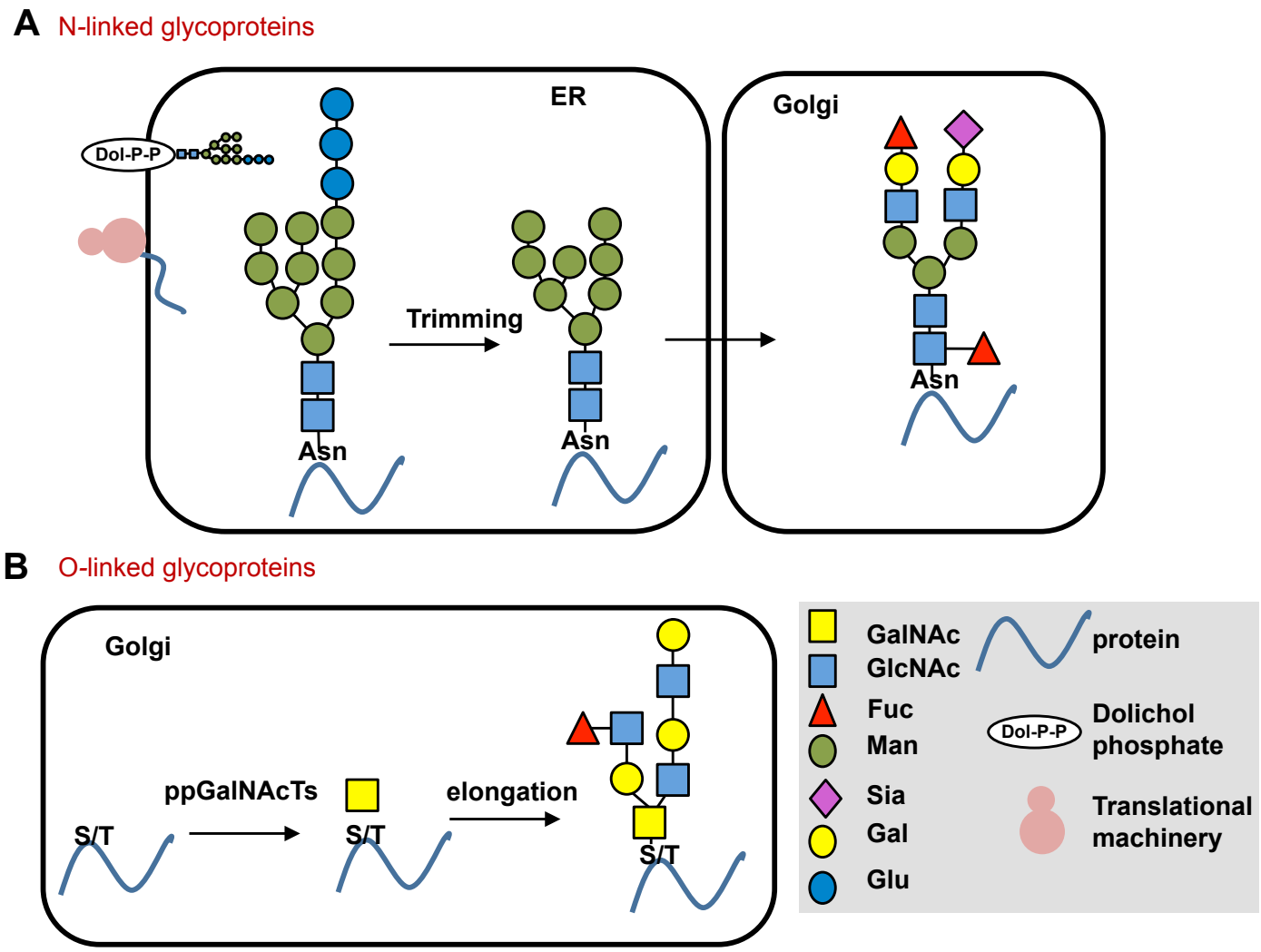


Figure 1.1 Glycoprotein Biosynthesis. (A) N-linked glycoproteins are synthesized in the ER and Golgi and modify Asn residues on the protein backbone. (B) O-linked glycoproteins are synthesized in the Golgi, modifying Ser or Thr residues on the protein backbone.

Biosynthesis of glycans occurs in the endoplasmic reticulum (ER) and the Golgi, to which various glycosyltransferases are localized. Glycosyltranferases accept specific monosaccaharides and transfer them to growing glycan chains via specific linkages. N-linked glycoproteins are formed by the addition of a core structure synthesized on the lipid dolichol in the ER (Figure 1.1A) (1). This core oligosaccharide is formed by the sequential addition of three glucosamine (Glc), nine mannose (Man),

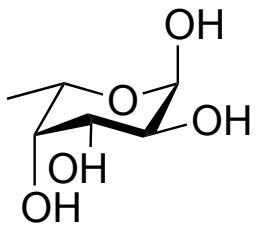


Figure 1.2 The structure of L-Fucose.

and two *N*-acetylglucosamine (GlcNAc) residues, which is transferred to the asparagine (Asn) residue on the nascent protein chain. The modified Asn is generally within a peptide consensus sequence of Asn-X-Ser/Thr, where X can be any amino acid residue except proline, which determines a likely site of N-glycosylation. The glycoprotein is trimmed of the Glc residues and one Man, and is then trafficked to the Golgi apparatus for terminal processing. The carbohydrate structure is subsequently trimmed and elongated by various glycosyltransferases, leading to substantial diversity in composition and chain length of N-linked glycans. N-linked glycans are generally of three types based on how they are processed; these include high-mannose (characterized by unsubstituted Man residues), complex (substituted Man residues), or hybrid glycans (with both substituted and unsubstituted Man residues). The majority of the *N*-glycans within vertebrate cells are of the complex type (22).

In contrast to N-linked glycosylation, O-linked glycosylation occurs in the Golgi apparatus with the attachment of either *N*-acetylgalactosamine (GalNAc) or Man to serine (Ser) or threonine (Thr) residues on nascent proteins (Figure 1.1B) (1). Unlike with N-glycosylation, there is no peptide consensus sequence determining O-glycosylation. The core monosaccharides are then elongated with GlcNAc, Gal, Fuc, or sialic acid (Neu5Ac) in different variations to create heterogeneity in glycan composition. O-linked oligosaccharides tend to be smaller than N-linked glycans. However, the structural diversity in both N- and O-linked glycans is enormous, due to the hundreds of possible combinations of chain length, composition, and monosaccharide linkages present in each glycan structure.

Biological Roles of Fucosylation

Among the monosaccharide building blocks that compose structurally diverse glycans is L-fucose (Figure 1.2). L-Fucose is a monosaccharide that is a common component of many N- and O- linked glycoproteins and glycolipids. It is structurally unique compared to other monosaccaharides in that it lacks a hydroxyl group on the carbon at the 6-position and it exists in the L- configuration. Fucose commonly exists as a terminal modification, and can therefore serve as a molecular recognition element (17). Important roles for fucosylated glycans have been demonstrated in a variety of biological systems (23, 24). Fucosylated glycans include the ABO blood group antigens and are crucial to the recruitment and adhesion of leukocyte antigens. Fucosylated antigens also play a key role in host-microbe interactions (25). Deficiencies in fucosylation are associated with human disease, such as the leukocyte adhesion disorder type II (LAD II). The disease causes an impairment of leukocyte-endothelium interactions and are characterized by immunodeficiency, developmental abnormalities, and deficiencies in cognitive development (26). Fucose is synthesized via one of two cellular biosynthetic pathways: the *de novo* pathway or the salvage pathway (Figure 1.3). In the *de novo* pathway, GDP-fucose is synthetized from GDP-mannose through a series of enzymatic reactions, carried out by two proteins: GDP-mannose 4,6-dehydratase (GMD) and GDP-keto-6-doeoxymanose 3,5-epimerase, 4-reducatase (FX protein) (27). GDP-fucose can also be synthetized via the salvage pathway from free fucose present in the cytosol from lysosomal or extracellular sources. In either case, the GDP-fucose is transported into the Golgi apparatus and made available to the cellular glycosylation machinery. Studies of fucose metabolism in HeLa cells report that more than 90% of GDP-fucose is synthesized from the *de novo* pathway, even in cells supplemented with radiolabeled fucose (28, 29). However, the salvage pathway still provides a mechanism to correct deficiencies in fucose metabolism, such as in LAD II, for which exogenous fucose can be administered for therapeutic purporses (30).

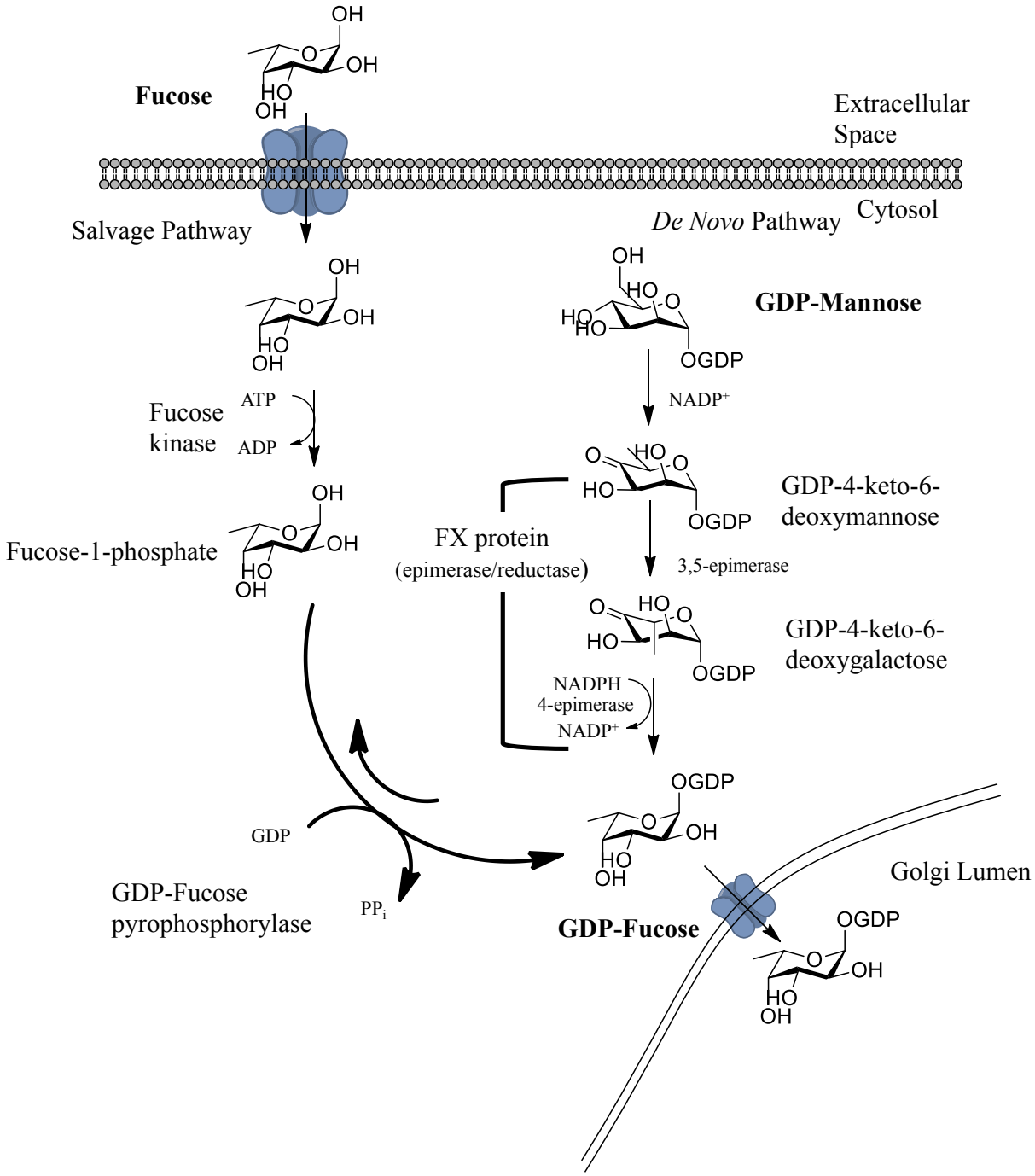


Figure 1.3 Biosynthesis of fucose. In eukaryotes, the biosynthesis of GDP-fucose occurs through two distinct pathways: the *de novo* pathway and the salvage pathway. The *de novo* pathway synthesizes GDP-fucose from GDP-mannose via enzymatic reactions carried out by two proteins, GMD and FX. The salvage pathway "salvages" free fucose found in the cytosol to synthesize GDP-fucose using the enzymes fucose kinase and GDP-fucose pyrophosphorylase. GDP-fucose is then transported into the Golgi lumen for glycosyltransferase reactions.

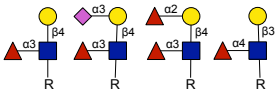
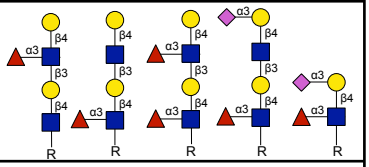
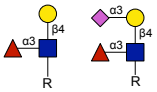
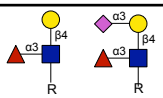
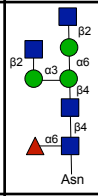
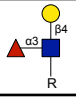
Enzyme	Representative Structure
FUT1	
FUT2	
FUT3	
FUT4	
FUT5	
FUT6	
FUT7	
FUT8	
FUT9	
FUT10	unknown
FUT11	unknown
POFUT1	O-fucosylation in EGF repeats
POFUT2	unknown

Figure 1.4 Human fucosyltransferases. List of human fucosyltransferases and representative structures synthesized by each fucosyltrasferase.

Fucosylated glycans are synthesized by fucosyltransferases (FUTs) that reside in the Golgi apparatus and ER. Thirteen FUT genes have so far been identified in the human genome, two of which transfer fucose directly to a polypeptide chain (O-fucose) (23). Fucose can be linked to C-2 or C-4 positions of the penultimate galactose in oligosaccharides, or to the C-3 or C-6 position of GlcNAc residue of N-linked glycans (Figure 1.4). O-Fucosylation is the direct modification of Ser or Thr residues by α-L-fucose, and is observed on epidermal growth factor (EFG) repeats of glycoproteins such as Notch (23). While fucose is not elongated in N-linked and O-linked glycans, O-linked fucose can be elongated by other sugars.

FUT1 and FUT2 are dedicated to the synthesis of Fucα(1-2)Gal glycans, the epitope found on the ABO blood group antigens, which determine blood and tissue type (Figure 1.5) (31, 32). FUT3 catalyzes the synthesis of both α(1-3) and α(1-4) fucosylated glycans and can transfer fucose to both a Gal and a GlcNAc, to synthesize structures such as the Lewis y and Lewis b antigens, thought to be critical for host-microbe interactions (Figure 1.5). FUT4-7 form only α(1-3) fucosylated glycans, such as within the sialylated Lewis^x structures, important to leukocyte adhesion

(32, 33). FUT8 and FUT9 generate Fucα(1-6)GlcNAc structures, with FUT8 generally catalyzing attachment of this structure to the core Asn residue of N-linked glycans, and FUT9 catalyzing its attachment to a distal GlcNAc of polylactosamine chains. Core fucosylation, a product of FUT8, may play a role in cancer progression as increased incidences of core fucosylation have been reported in many cancers (34). FUT9 is responsible for the synthesis of Lewis^x structures during embryogenesis, promoting cell-adhesion in early embryos. FUT10 and FUT11 are putative fucosyltransferases that are reported to synthesize α(1-3) structures based on sequence homology. POFUT 1 and 2 are *O*-fucosyltransferases 1 and 2 and catalyze the direct fucosylation of Ser and Thr residues (23).

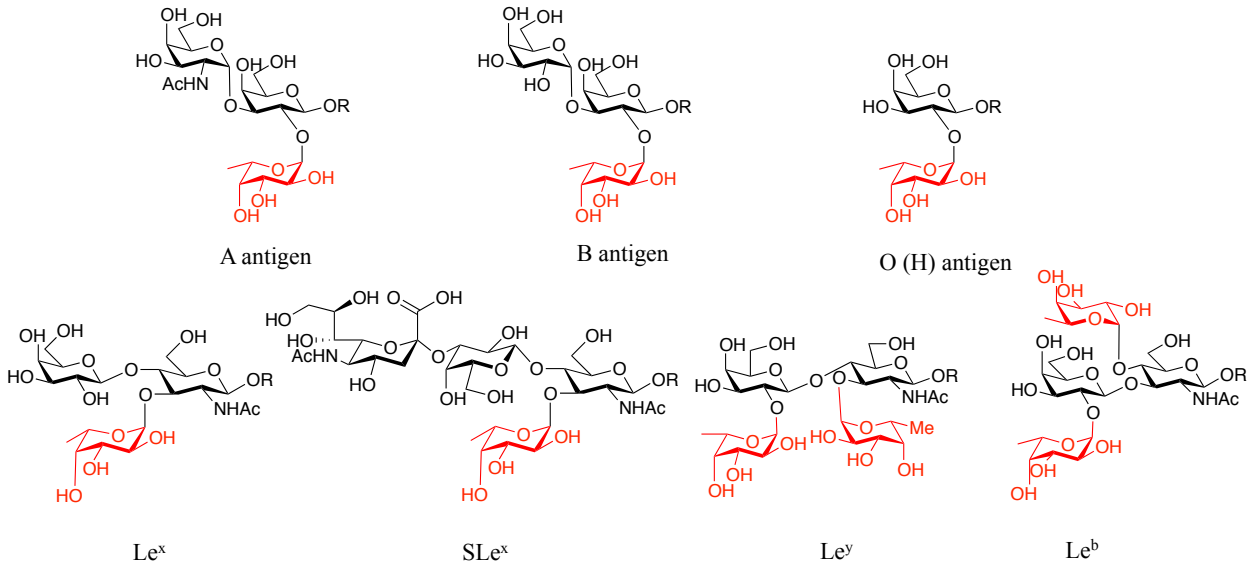


Figure 1.5 Biologically Relevant Fucosylated Structures. Fucosylated glycans include the blood group antigens A, B, and O (H), which determine blood and tissue type. Other biologically relevant fucosylated glycans include the Le^x, SLe^x, and Le^y antigens, which are important molecular determinants in processes such as embryogenesis and leukocyte adhesion.

The Role of Fucosylated Glycans in Neurons

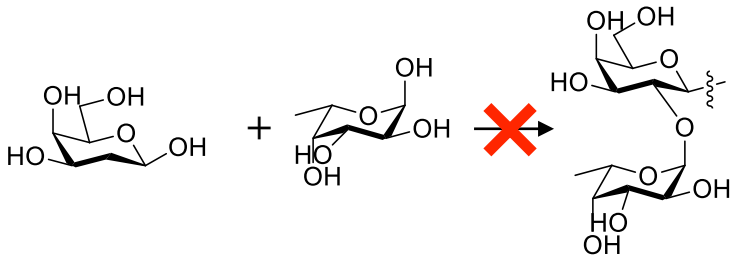
Increasing evidence suggests a critical role for fucosylated glycans in the nervous system (35). For example, fucose has been shown to play an important role in neural development. O-Fucosylation is essential for the activity of Notch, a receptor that controls many aspects of cell fate during development including neuronal progenitor maintenance and regulation of cell-fate decisions in neuronal and glial

lineages (36, 37). Previous studies have suggested that fucosylation modulates Notch signaling by either interacting directly with ligands, or inducing some sort of conformational change (38). In addition to neural development, Notch signaling may have a role in neuronal migration (39). Genetic deletion of POFUT1, the enzyme responsible for *O*-fucosylation, is embryonic lethal and causes developmental defects similar to that of a Notch deletion (40, 41). The Lewis x (Le^x) epitope, an α(1-3) fucosylated glycan, is also implicated in neurogenesis, as it is dynamically expressed at different embryonic stages (42). Recent work has also implicated the Le^x epitope in neurite outgrowth (43-45).

Fucosylated glycans are known to be important in learning and memory processes. Incorporation of fucose into neuronal glycoconjugates was significantly enhanced by task-dependent learning in both chicks and rats (46-49). When trained in a brightness discrimination task, rodents demonstrated an increase in [^{3}H]-fucose into forebrain glycoproteins (48). Moreover, the addition of fucose or 2'-fucosyllactose (a Fucα**(**1-2)Gal containing trisaccharide) enhanced long-term potentiation (LTP), an electrophysiological model of learning and memory, both in hippocampal slices and *in vivo* (50, 51). Fucosylated glycans have also been reported to be enriched in synapses (52-54), where the majority of fucosylated glycans exist as complex N-linked structures (55). Studies have indicated that the activity of fucosyltransferases increases both during synaptogenesis (56), and in response to passive-avoidance training (57). Moreover, the presence of dendritic Golgi (58, 59) raises the intriguing possibility of local protein synthesis and glycosylation in response to neuronal stimulation.

The Fucα(1-2)Gal structure in particular has been implicated in neuronal processes. The probe 2-deoxy-D-galactose (2dGal) has been utilized to inhibit Fucα(1-2)Gal formation by competitively inhibiting fucose incorporation (60). The lack of a C2 hydroxyl group prevents the incorporation of the terminal fucose structure (Figure 1.6). Treatment with 2dGal, but not other sugars, caused reversible amnesia in both chicks and rats, indicating the importance of the Fucα(1-2)Gal linkage (60-62). 2dGal has also been reported to interfere with the maintenance of LTP *in vitro* and *in vivo* (51, 63). Additionally, a monoclonal antibody specific for the Fucα(1-2)Gal epitope (64) inhibited memory

formation in animals, presumably by blocking the interaction of the Fucα(1-2)Gal epitope with its relevant binding partners (65). Hsieh-Wilson and coworkers investigated the effects of 2dGal treatment in cultured hippocampal neurons, observing that 2dGal treatment caused hippocampal neurons to retract their neurites (66). Other sugars had no effect, and treatment with natural galactose moderately rescued the neurite retraction, suggesting that *de novo* synthesis of Fucα(1-2)Gal glycans was necessary for regaining normal morphology.



2-dGal Fuc Fucα(1-2)Gal

Figure 1.6 Incorporation of 2-deoxy-D-galactose (2-dGal) inhibits formation of Fucα(1-2)Gal linkages.

Another relevant structure in the brain is the Le^x structure. Downregulation of FUT9 in human NT2N neurons led to significant decrease of Le^x, as well as GAP-43, a marker of neurite outgrowth. In parallel, there was a decrease in neurite outgrowth that was reversed with the overexpression of FUT9 (45). Moreover, Le^x glycans on glial CD24 glycoforms were shown to mediate CD24-induced effects on neurite outgrowth (44).

Despite their importance, only a handful of fucosylated proteins have been identified from the brain. Moreover, the functional relevance of the fucose epitope has only been characterized on one neuronal glycoprotein, synapsin I (52). Synapsin I, a synaptic-vesicle associated protein with critical roles in neurotransmitter release and synaptogenesis (67), was identified as a Fucα(1-2)Gal glycoprotein using a gel-based mass spectrometry approach. Importantly, fucosylation was found to protect synapsin I from proteolytic degradation, suggesting that fucosylation regulates the expression of synapsin I (52).

Additionally, studies using 2dGal and synapsin I deficient mice demonstrated that synapsin I fucosylation contributed to the effects of 2dgal on neurite outgrowth.

To further investigate whether Fucα(1-2)Gal binding receptors, or lectins, exist in neurons, Hsieh-Wilson and coworkers designed a biotinylated Fucα(1-2)Gal probe and assessed binding of the probe to lectins present on hippocampal neurons (66). The Fucα(1-2)Gal probe showed binding to the soma and neurites, suggesting the presence of fucose-specific lectins. Moreover, treating hippocampal neurons with multivalent Fucα(1-2)Gal probes promoted neurite outgrowth, while other sugars had no effect, suggesting the presence of a carbohydrate-regulated pathway mediating neuronal outgrowth.

The Fucα(1-2)Gal Biomarker

In addition to playing a role in neurochemical processes, fucosylation may also play an important role in several pathological processes, such as tumorigenesis (68). Changes in glycosylation are often a hallmark of disease states; cancer cells frequently display glycans at different levels or with fundamentally different structures than those observed on non-diseased cells (10, 21, 69-72). These structural changes may be due to changes in the expression levels of glycosyltransferases in the Golgi compartment of cancerous cells, which can lead to modifications in the core structure of N-linked and O-linked glycans. In addition to changes in the core structures of glycans, altered terminal structures are also associated with malignancy. Glycosyltransferases involved in linking terminating residues on glycans, such as sialyltransferases and fucosyltransferases, tend to be overexpressed in tumour tissue, leading to the overexpression of certain terminal glycans. Examples of terminal glycan epitopes commonly found on transformed cells include sialyl Lewis x (sLe^x), sialyl-Tn (sTn), Globo H, Lewis y (Le^y) and polysialic acid (PSA) (73, 74). Many of these epitopes are observed in malignant tissues throughout the body, including the brain, breast, colon and prostate. Although gross changes in glycosylation of tumor tissues are apparent, no single change seems to distinctly differentiate normal and malignant cells. Instead, each type of malignant tissue is characterized by a distinct set of changes in glycan expression, suggesting that glycans may serve as excellent biomarkers for cancer diagnosis.

One intriguing glycan biomarker is the Globo H antigen, a hexasaccharide with a terminal Fuca(1-2)Gal epitope (Figure 1.6), which is over expressed on a variety of epithelial cell tumors (75-77). Small cell lung carcinoma patients with Globo H positive tumors were shown to experience shorter survival compared to patients with Globo H negative tumors (68). In breast cancer, altered Globo H expression was observed on the majority of ductal lobular and tubular breast carcinomas and was found to be expressed in breast cancer stem cells (78-80). The serum of breast cancer patients contains high levels of antibodies against the Globo H epitope. Fucα(1-2)Gal glycan expression is also elevated in prostate cancer tissue and on the tumorigenic prostate-specific antigen (PSA) protein, when compared to normal epithelial tissue or PSA (77, 81). As such, the Fuca(1-2)Gal epitope is an attractive biomarker and potential therapeutic target for cancer (82-84). However, the extent to which this sugar epitope serves as a marker of disease progress and its precise contributions to cancer pathogenesis are not well understood.

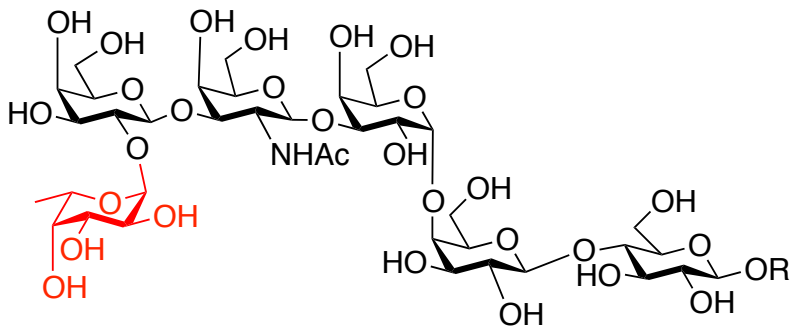


Figure 1.7 The Globo H hexasacharide. The Globo H hexasaccharide is overexpressed on a variety of epithelial cell tumors.

Motivation for Studies

Although glycans have long been known to have critical biological roles, the detection and manipulation of glycans has been challenging. One fundamental obstacle is that glycans have complex, branched structures and are intrinsically heterogeneous, making them inaccessible to common molecular

biological techniques. Traditional tools to study glycans include antibodies and lectins, which are carbohydrate binding proteins. However, lectins and antibodies are known to have weak affinity for their targets and poor specificity to small glycan structures (85). Cross-reactivity with other glycan epitopes may also be a problem (86, 87). For these reasons, the development of methods to chemically perturb and detect glycans would accelerate an understanding of their roles in vital biological processes and disease.

In this thesis, we describe two methods for the detection of fucosylated glycans. In Chapter 2 and 3, we exploit a metabolic labeling approach to identify and image fucosylated proteins in neuronal systems in order to gain insight into the roles of fucosylation in the brain. In Chapter 4, we describe the development of a new chemoenzymatic detection strategy for Fucα(1-2)Gal glycans, which we utilize to probe the surface Fucα(1-2)Gal expression on cancer cell lines. Both of these methods expand the technologies and applications available for the study of fucosylated glycans.

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