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"Stuck on Sugars – How Carbohydrates Regulate Cell Adhesion, Recognition, and Signaling"

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Abstract

We have explored the fundamental biological processes by which complex carbohydrates expressed on cellular glycoproteins and glycolipids and in secretions of cells promote cell adhesion and signaling. We have also explored processes by which animal pathogens, such as viruses, bacteria, and parasites adhere to glycans of animal cells and initiate disease. Glycans important in cell signaling and adhesion, such as key O-glycans, are essential for proper animal development and cellular differentiation, but they are also involved in many pathogenic processes, including inflammation, tumorigenesis and metastasis, and microbial and parasitic pathogenesis. The overall hypothesis guiding these studies is that glycoconjugates are recognized and bound by a growing class of proteins called glycan-binding proteins (GBPs or lectins) expressed by all types of cells. There is an incredible variety and diversity of GBPs in animal cells involved in binding N- and O-glycans, glycosphingolipids, and proteoglycan/glycosaminoglycans. We have specifically studied such molecular determinants recognized by selectins, galectins, and many other C-type lectins, involved in leukocyte recruitment to sites of inflammation in human tissues, lymphocyte trafficking, adhesion of human viruses to human cells, structure and immunogenicity of glycoproteins on the surfaces of human parasites. We have also explored the molecular basis of glycoconjugate biosynthesis by exploring the enzymes and molecular chaperones required for correct protein glycosylation. From these studies opportunities for translational biology have arisen, involving production of function-blocking antibodies, anti-glycan specific antibodies, and synthetic glycoconjugates, e.g. glycosulfopeptides, that specifically are recognized by GBPs. This invited short review is based in part on my presentation for the IGO Award 2019 given by the International Glycoconjugate Organization in Milan.

Introduction

The specific interactions of sugars on proteins with glycan-binding proteins (GBPs) including lectins, of human, animal, plant, and pathogen origin, are now widely appreciated[1–8]. Glycans on glycoproteins have at least two broad functions, those direct

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ones in which glycans are directly recognized by a GBP, and indirect ones, in which the glycans on a glycoprotein can indirectly influence its structure and conformation, stability, turnover, localization, and other types of protein interactions[9,10].

The mechanisms by which sugars are directly recognized and can indirectly influence many biological pathways, however, have been historically difficult to understand. But tremendous changes in technology and genetic and biochemical approaches over the past few decades have brought into focus a deeper appreciation of such mechanisms. Here in this review, I will focus on various approaches we have used to explore structure/function relationships of carbohydrates and how they help to regulate a variety of biological pathways, including cell adhesion, signaling, and recognition (Figure 1). In addition, we have studied the biosynthesis of key glycans, especially O-glycans, which requires a complex orchestration of glycosyltransferases and chaperones to generate a functional pathway.

Roles of Glycans in Adhesion of Leukocytes and Platelets

The discoveries of the selectins sparked a fresh interest in the physiological potential of glycans to be recognized and promote the adhesion of leukocytes to endothelial surfaces, both for leukocyte extravasation at sites of infection and inflammation, and in leukocyte trafficking through lymph nodes[11,12]. Such potential had been identified in the 1960's in original studies by Ginsburg and Gesner on the roles of lymphocyte carbohydrates in regulating lymphocyte movement into lymph nodes[13]. Our work largely through collaborations with McEver, was focused on the glycans and physiological ligands recognized by P-selectin, which were originally thought to be only sialyl Lewis x (SLe^x) or sulfated versions that might be expressed on multiple glycoproteins[14,15]. However, we found that engineered CHO cells expressing copious amounts of SLe^x were bound poorly by native platelet-derived P-selectin, compared to its binding to neutrophils[16]. Subsequently, we identified a specific P-selectin glycoprotein ligand (now termed PSGL-1) by direct affinity isolation using HL-60 cell extracts and native platelet-derived P-selectin[17]; that glycoprotein was also identified in human neutrophils[18]. PSGL-1 was cloned by expression cloning techniques using an HL-60 cDNA library[19].

The identification of PSGL-1, while a milestone in selectin research, however, did not resolve the mystery of how it could be specifically recognized by P-selectin amongst many other similar mucin-like glycoproteins and others that also express the SLe^x antigen. To aid in analyzing the minor amount of PSGL-1 glycosylation, we analyzed PSGL-1 that was metabolically labeled with radioactive sugar precursors [^{3}H]-glucosamine, [^{3}H]-mannose, and [^{35}S]-sulfate. Sequencing of the N- and O-glycans revealed that some O-glycans were core 2 O-glycans with the SLe^x antigen and others were fucosylated and sialylated poly-N-acetyllactosamine type glycans[20]. We showed that recombinant PSGL-1 was metabolically labeled with [^{35}S]-sulfate, at first suggesting that PSGL-1 might contain sulfated glycans, but further analysis demonstrated that the sulfate was not present in glycans but in tyrosine sulfate[21]. Indeed, the sulfate could be removed by bacterial aryl sulfatase, and this removal abrogated high affinity binding of PSGL-1 to P-selectin. Several studies demonstrated the need for tyrosine sulfation for recombinant PSGL-1 to interact with P-selectin[22–25]. Studies by us and others showed that for recombinant PSGL-1 to bind P-

selectin it had to be co-expressed in cells with specific glycosyltransferases that generated core 2 O-glycans and the SLe^x antigen[23]. Furthermore, PSGL-1 was found to be dimeric[26], and specific proteases that partly cleaved PSGL-1 could release its N-terminal domain, which also abrogated binding of cells to P-selectin[22,26], suggesting that the extreme N-terminus of PSGL-1 contained the key determinants recognized by P-selectin.

Synthetic Glycopeptides to Explore Glycan Recognition in Context of Peptide Determinants

To directly identify the N-terminal determinants of PSGL-1 important for P-selectin recognition, we approached the problem from a chemical biology perspective. Our ambitious goal was to synthesize a panel of glyco(sulfo)peptides or GSPs representing many potential structures of the extreme N-terminus of mature PSGL-1. In conjunction with this, two key discoveries made by us and our collaborators helped to make this project feasible. One was the identification and purification of the tyrosine sulfotransferase by Moore et al[27], which could be used to add sulfate to tyrosine residues in synthetic glycopeptides. The second was the purification of the core 1 β−1,3 galactosyltransferase, now called the T-synthase, by Ju et al[28,29], which allowed us to synthesize from the precursor Tn antigen GalNAcα1-Thr-R precursor, the T antigen Galβ13GalNAcα1-Thr-R (Figure 2).

Using a semi-synthetic chemoenzymatic approach, Leppänen et al directly addressed the key importance of the N-terminal peptide segment of PSGL-1 for binding to L-selectin by synthesizing a variety of GSPs with extended core 2 O-glycans containing SLe^x along with various tyrosine sulfates and peptide sequences[30]. These and other studies over several years and using dozens of different glycopeptides, some isomeric, demonstrated that a synthetic GSP containing a specific core 2 O-glycan on a specific threonine residue and expressing the SLe^x antigen along with multiple tyrosine sulfate residues N-terminal to the glycan could bind with high affinity to P-selectin[30–33] (Figure 2). Co-crystallization of the recombinant PSGL-1 N-terminal domain with P-selectin confirmed the unique and specific nature of P-selectin recognition of this highly complex and large glyco(sulfo)peptide determinant[34].

In more recent studies on this subject with Chaikof's group we identified a novel route for generating GSPs in which the tyrosine sulfates are replaced by more stable sulfonated tyrosines[35], along with other routes for synthesizing glycoamino acid precursors[36–38] to reduce the need for enzymatic involvement in generating materials. All of these studies have demonstrated the importance and unique interactions of the N-terminal domain of PSGL-1 with P-selectin and L-selectin and raise opportunities for developing glycomimetic drugs. Such drugs have been successfully developed by John Magnani and colleagues[39–44] and are showing great promise in treating human diseases involving selectin-mediated adhesion, including the vasoocclusive crises in Sickle Cell Disease. Complementary studies using a humanized antibody to P-selectin and prophylactic administration to patients have also shown promise in preventing pain crises in Sickle Cell Disease[45,46]. These represent key translational advances in glycoscience leading to major pharmaceutical companies taking the lead on further development of drugs in this area.

The ability to generate synthetic glycopeptides affords a tremendous opportunity to explore glycan recognition in terms of such presentations. It is becoming clear that glycan recognition can occur in the context of peptide determinants and in combination with other post-translational modifications (PTMs) of the glycopeptides (Figure 3). Such is the case with human and murine PSGL-1, in which peptide, tyrosine sulfate, and O-glycan determinants are all necessary for high affinity recognition[30,33,25]. Similar recognition of both tyrosine sulfate and O-glycans contribute to high affinity binding of chemokines to the NH_2-terminal domain of PSGL-1[47–49] and the CC chemokine receptor 5 (CCR5)[50]. Other examples of dual glycan and peptide recognition are podoplanin recognition by CLEC-2, which requires podoplanin O-glycans and peptide determinants[51], PILRα recognition of the Sialyl Tn-linked glycopeptide of herpes simplex virus 1 glycoprotein B[52,53], and recognition of O-GlcNAc-containing glycopeptides by 14–3-3 protein[54].

Interestingly, there are also several glycosyltransferases, such as glycopeptide transferase ppGalNAcT-10, which initiates α-O-GalNAc addition to Ser/Thr residues, where there is independent recognition of a peptide acceptor through its catalytic domain and recognition of a nearby α-O-GalNAc-containing peptide determinant through its lectin domain, both of which are required for efficient α-O-GalNAc addition[55]. Another example of a glycosyltransferase with dual recognition of peptide and glycans is the polybasic region (PBR) of the polysialyltransferase that binds to an acidic surface patch in the first fibronectin type III repeat (FN1) of the neural cell adhesion molecule, NCAM, to promote recognition of the sialic acid acceptor and addition of α2,8-linked sialic acid to initiate polysialylation[56]. Dual recognition of peptide determinants in the common α subunit of pituitary glycoprotein hormones, e.g. LH and FSH, and the N-glycan precursor regulates their interactions with the N-acetylgalactosaminyltransferase that initiates LDN formation[57] allowing subsequent 4-O-sulfation of the GalNAc residues. A similar recognition of specific peptide features also guides the GlcNAc-1-phosphotransferase subunits in their recognition of lysosomal hydrolases to initiate addition of GlcNAc-1-phosphate to the 6-OH group of mannose residues[58]. The obvious paradigm from these types of observations is that the glycan position within a peptide portion of a glycoprotein, the structure of the glycans, the peptide sequence itself, and other PTMs of a glycopeptide domain can create a functional recognition unit for GBPs, enzymes, and other molecules.

Roles of Glycans in Cell Signaling – Galectins and C-type Lectins

The unexpected ability of lectins and GBPs to signal cells by binding specific glycans arose from the original discovery in 1960 by Nowell that the plant lectin phytohemagglutinin, now termed L-PHA, from the red kidney bean *Phaseolus vulgaris*, is mitogenic toward resting lymphocytes[59]. In studying the types of carbohydrates bound by L-PHA, we subsequently discovered that L-PHA specifically binds to complex-type branched N-glycans containing LacNAc in β1–6-branched mannose residues, as in (R-Galβ1–4GlcNAcβ1–2(R-Galβ1–4GlcNAcβ1–6)Manα-R[60], consistent with studies by others[61]. This has been further confirmed by us and others using cells[62–64] and mice[65] missing the functional GNT-V or MGAT5 gene[66,67] responsible for GlcNAcβ1–6-branching of mannose residues, and by N-glycan microarray analyses[68]. These types of studies by us and others contrasted with the general concept that glycan recognition by GBPs and lectins was not highly

specific. But further discoveries such as these on the specificity of plant lectins for discrete glycan features, such as those by Rosalind Kornfeld[69,70], Goldstein and others[71–76], contributed to our early development, along with Kornfeld, of serial lectin affinity chromatography and the use of immobilized lectins to isolate and identify unique glycans from natural sources[77,60,78–82]. Additional lectins whose specificity we helped to define include those from *Datura stramonium* (common jimsonweed), specific for poly-N-acetyllactosamine and complex-type branched N-glycans, *Lycopersicon esculentum* agglutinin (tomato lectin), specific for poly-N-acetyllactosamine, and *Maackia amurensis* leucoagglutinin MAL, specific for α2,3-linked sialic acid on type 2 LacNAc. The impressive ability of lectins to discriminate among glycan structures and to affect cell viability was exploited by Stanley and colleagues who elegantly generated an incredibly useful panel of cell lines from Chinese hamster ovary cells that genetically differed in glycosylation capacities[83,84].

After discovery of the requirement of specific glycans on surface glycoproteins for cell signaling, as in mitosis, many began to study the potential ability of animal cell-derived glycan-binding proteins to also be mitogenic. This was first demonstrated by Novogrodsky and Ashwell[85] using the purified rabbit Ashwell-Morell Receptor or asialoglycoprotein receptor, who demonstrated that it has mitogenic activity toward human peripheral lymphocytes. Subsequent studies on the ubiquitous galectin family of β-galactoside-binding lectins, such as the chick embryo galectin[86], also demonstrated that it is mitogenic toward murine lymphocytes. In regard to galectins, Baum and colleagues[87,88] observed that galectins also had apoptosisinducing activity toward lymphocytes. In studies along this line, we then observed that galectin1 had a novel activity toward activated human neutrophils, and induced the reversible surface expression of phosphatidylserine (PS)[89], in a process we termed "*preaparesis*" (from the Latin *preaparare*), to indicate that the galectin prepares cells for phagocytic removal by causing PS exposure without accompanying apoptosis[90]. Macrophages express PS receptors used to phagocytose and clear such cells. The signaling pathway involves surface recognition of glycoprotein N-glycans and calcium signaling[91,92]. Other studies demonstrated that other galectins also had signaling activity toward activated but not resting neutrophils, and could trigger cytokine secretion, such as IL-10[90,93]. The ability of galectins to signal and attenuate cytokine secretion has been studied by many other investigators to other cell types and the impressive ongoing studies indicate that galectins have profound signaling activities in a wide variety of cells[94–97,5,98,99].

Most importantly, with Stowell et al we discovered that galectins have the direct ability to kill certain bacteria and probably are bacteriostatic to many[100–102]. This was observed for human galectin-4, galectin-8, and galectin-3 for certain microbes. Such interactions seem to involve recognition of ABO(H)-like glycans on microbial surfaces, i.e. glycans rich in galactose, fucose and GalNAc. This recognition and activity may provide innate immune protection against microbes expressing ABO(H)-like glycans independently of the blood group status of an individual, as well as perhaps providing some levels of generalized immunity against molecular mimicry. This discovery, coupled with the discovery of the role of galectin-8 in autophagic recognition of microbes[103,104], suggests a deep evolutionary

role of galectins as arms of the innate immune system, as galectins evolved long before adaptive immunity in vertebrates.

Galectins are soluble proteins, thus, the ‘sending cell’, i.e. the cell producing the galectin, cannot directly receive signals through the galectin. This is not the case for membrane-bound lectins, e.g. selectins, many other C-type lectins, and Siglecs. Such GBPs have cytoplasmic domains capable of cell signaling upon ligation by ligands and upon cellular activation. For example, the cytoplasmic domain of platelet P-selectin is phosphorylated upon activation[105,106]. In addition, the antibodies to either E- or P-selectin alter endothelial cell morphology[107], and L-selectin ligands induce signaling via L-selectin[108,109]. Overall, the signaling activities of selectins and Siglecs are extensive, as are other C-type lectins involved in innate immune responses[6,7].

Glycans Expressed by Pathogens: Their Antigenicity and Biological Recognition

Our interest in blood cells and the vascular system led us to also explore the types of glycans expressed by parasites that live in the bloodstream. Given the complex interactions and functions of leukocytes, erythrocytes, platelets, and glycoproteins in the blood, it seems almost impossible that parasites can not only live in the bloodstream but thrive. Such blood parasites include the blood flukes, which are parasitic worms (helminths), represented by *Schistosoma mansoni* and other species. Other helminths that reside in other sites in infected individuals and animals include *Trichuris suis*, *Dirofilaria immitis*, and *Haemonchus contortus*. Such parasites were of interest because the worms in infected animals can survive many months and years, suggesting that the immune system is compromised and inefficient in eliminating such pathogens.

Our studies indicated that parasite glycans were unusual and immunogenic in infected animals and humans. This was highly controversial at the time, as it was felt that parasites practiced molecular mimicry[110,111] and shared antigenic determinants between them and their host, thus effectively preventing host immunity against the parasite. However, many studies in our group by Nyame et al and others[112–123] showed that parasitic worm glycans in glycoproteins and glycolipids were unusual and were highly antigenic in infected animals, inducing IgG, IgM, and IgE responses to specific antigens, including the LacdiNAc (GalNAcβ1–4GlcNAc-R), fucosylated LacdiNAc LDNF (GalNAcβ1–4(Fucα1–3)GlcNAc-R), and difucosylated LacdiNAc FLDNF (Fucα1–3GalNAcβ1–4(Fucα1–3)GlcNAc-R), Lewis x antigen or Le^x (Galβ1–4(Fucα1–3)GlcNAc-R), core α3Fucose, and core Xylose (Figure 4). All glycans in helminths lack sialic acid, thus demonstrating a complete difference from the types of glycans typically expressed by human cells. Interestingly, in our early studies we also identified O-linked GlcNAc in parasite glycoproteins[124], which was a major type of modification in many of their glycoproteins. These findings were consistent with the earlier discovery by Hart and colleagues of O-GlcNAc in vertebrate cells[125,126], and further led to a universal appreciation of this common and critical protein modification.

The paradox of worm survival in the face of immunity is not well understood, but it can be hypothesized that it arises from the fact that the titer of anti-carbohydrate antibodies in

infected individuals is not high. Only by repeated exposure to antigens, probably upon parasite death, as can occur in repeated drug-induced killing over the life of an individual living in an endemic area, can an individual acquire protective immunity. But the glycans can also directly affect the immune system in a process we termed glycan gimmickry[127], in which parasite glycans subvert protective aspects of the innate system and induce an unbalanced and weak humoral response.

Another unusual aspect of helminth glycosylation is that their basic motif in glycan synthesis is not the common LacNAc motif (Galβ1–4GlcNAc-R) that most vertebrates use, but instead is the LacdiNAc or LDN (GalNAcβ1–4GlcNAc-R) motif. To understand the synthesis of LDN we identified the major β1–4GalNActransferase responsible in *C. elegans* and orthologues in all worms for LDN synthesis[128]. Interestingly, transfection of CHO Lec8 cells, which lack galactose due to a defect in UDP-Gal transport[129,130], resulted in the formation of unusual poly-LDN chains on their N-glycans, where the β1–4GalNActransferase using UDP-GalNAc replaced the functioning of the endogenous β1–4Galtransferase, further confirming the novel types of structures that can be generated by parasite enzymes[131].

To better understand the immune responses in people and animals upon infections with helminths, we developed natural glycan microarrays of total *S. mansoni* N-glycans, using a technology that we termed *shotgun glycan microarrays* as discussed in more detail below[132] (Figure 5). With this approach we identified a multitude of immune responses to many different glycan epitopes[133]. These studies were also supplemented by using defined glycan microarrays of parasite glycan antigens[134]. This work was also complementary to the outstanding studies by Hokke et al[135–137], in which they generated glycan microarrays of parasite glycans and identified many immunogenic responses. In more recent studies we also generated a shotgun glycan microarray of the nematode *C. elegans*, and identified many glycan structures and their recognition[138].

In addition to inducing antibodies, we and others found that the helminth parasite N-glycans interact with many different glycan-binding proteins (lectins) expressed by our cells[139–142]. In studies with van Die, we showed that several major lectins in dendritic cells and macrophages, including the mannose receptor and DC-SIGN, could interact with glycans expressed by parasitic worms, including *Schistosoma mansoni*, *Trichuris suis*, and *Haemonchus contortus*. The process is probably one we termed glycan gimmickry[127]. Just as importantly, the glycans expressed by parasitic worms in glycoproteins and glycolipids on the surface and secreted by the worms, can regulate and suppress host immune responses[127]. One of the interactors in this regard is the mannose receptor (MR)[143], which recognizes multiple types of oligomannose glycans synthesized by parasitic worms[144]. Ligation of parasite glycoproteins by MR and perhaps other lectins is associated with downregulating the Th1 immune responses and enhancing Th2 immune responses, as others have observed in mice[145–147].

Discovery of Cosmc, a Molecular Chaperone for O-glycan Biosynthesis

Our studies on glycosylation of proteins in parasites and animal cells were consistent with those of others in the field in suggesting that a majority of all secreted proteins are glycoproteins with one or more glycans attached to a wide variety of amino acids[148]. While N-glycosylation has been studied in more detail historically, and shown to be a common modification of secreted and membrane glycoproteins, recent studies now demonstrate that many if not most glycoproteins have at least one site where GalNAc is α-linked to Ser/Thr residues[149,150], and in some cases also to Tyr residues. The addition of GalNAc to Tyr residues was first discovered in the human amyloid precursor protein APP[151], and recent studies suggest it may be a more common modification than earlier realized[152], and can also be recognized by innate immune receptors, such as MGL[153].

While the addition of α-GalNAc requires one of twenty different polypeptide N-acetylgalactosaminyltransferases (ppGalNAcTs)[154], the elongation of the α-GalNAc in all cells is typically determined by a single enzyme, whose gene we identified and termed the core 1 β3-galactosyltransferae of T-synthase[28,29]. This enzyme synthesizes the common core 1 disaccharide, also called the T or TF antigen Galβ1–3GalNAcα1-Ser/Thr. Many tumor cells express the non-elongated GalNAcα1-Ser/Thr, also termed the Tn antigen, and/or its sialylated derivative, the sialylTn antigen (STn - Neu5Acα2–6GalNAc1α-Ser/Thr). In normal cells, the T antigen is elongated through modification by addition of other monosaccharides, including Sia, GlcNAc, Fuc, GalNAc, and others to generate thousands of different O-glycan species on cellular glycoproteins[155–157].

Our laboratory in work by Ju et al discovered that in some tumor cells, e.g. human Jurkat cells, a T leukemic cell line, many glycoproteins have the Tn antigen, not due to a loss of T-synthase, but due to a mutation in a novel gene encoding a molecular chaperone which we named the Core 1 β3-Gal-T-Specific Molecular Chaperone or Cosmc[158]. We subsequently demonstrated that Cosmc is a resident ER protein[159,160] that binds to the unfolded T-synthase during its biosynthesis in the ER[161], and is required for proper folding of the T-synthase (Figure 6). Cosmc does not bind to the T-synthase once it has completed folding and becomes an active enzyme. [The *T-synthase* and *Cosmc* genes are designated *C1Galt1* and *C1Galt1C1*, respectively.] Cosmc is an oligomeric protein that recognizes discrete linear peptide elements within the Cosmc binding region of the T-synthase, or CBRT[162–164]. This reversible binding to the unfolded T-synthase prevents it from incorrectly oligomerizing and becoming partly degraded in the ER, and subsequently exported to the cytoplasm where it is degraded by the 26S proteasome. Once the T-synthase is folded, the CBRT elements are buried and inaccessible to Cosmc.

Cosmc is encoded on the X-chromosome in humans at Xq24. We discovered that in patients with Tn syndrome, where a portion of blood cells of all lineages express the Tn antigen[165–167], there are acquired mutations in *Cosmc* in hematopoietic stem cells[168]. This mutation results in lineages of all blood cells that lack *Cosmc* and hence T-synthase, and thus express the Tn antigen. Others have also reported spontaneous acquired mutations in *Cosmc* in Tn syndrome[169] and other disorders[170,171].

With the knowledge of Cosmc function, we could then explore general O-glycan function by deleting *Cosmc* either generally or in a targeted fashion in mice. Such studies have demonstrated that in the absence of *Cosmc,* mouse embryos uniformly express the Tn antigen in all cells, and the embryos die in utero by E10.5–E12.5. The male *Cosmc*$^{-/y}$ embryos at early states appear normal, but soon develop progressive hemorrhaging in the brain and spinal cord from E10.5 to E12.5, and exhibit growth retardation and death[172]. A targeted loss of Cosmc in endothelial and hematopoietic cells (EHC Cosmc($^{-/y}$) is associated with perinatal death in most animals and with defective lymphangiogenesis, severely prolonged tail-bleeding times and macrothrombocytopenia[173]. The platelets of such animals lack a variety of glycoprotein-related functions, including those associated with GPIbα, integrin αIIb, and GPVI. In addition, there is dysfunctional expression of von Willebrand factor, which contains the Tn antigen on its ~ten O-glycans and is readily degraded systemically. Other studies on targeting loss of T-synthase in mice reveal phenocopying of the Cosmc phenotype, indicating that Cosmc and the T-synthase are in the same pathway and loss of either one causes a loss of extended O-glycans. Many studies using targeted deletions of Cosmc (or T-synthase) in murine blood cells and other tissues, indicate that loss of extended O-glycans causes unique pathological changes, and reveal key functions of O-glycans in normal development and homeostasis[174–178]. Some changes in Cosmc and T-synthase have also been associated with altered O-glycosylation of the hinge region of IgA1 in Henoch-Schönlein purpura nephritis[179] and in IgA nephropathy, a major cause of glomerular nephropathy[180,181]. The Cosmc gene *C1Galt1C1* was implicated by GWAS studies as a risk factor for IgA nephropathy[181] and for ulcerative colitis and Crohn's disease[182].

The unique role of Cosmc in mucin-type O-GalNAc glycosylation pathways afforded development of unique cell lines by Clausen's group in which Cosmc is deleted and cells lack extended O-glycans[149,183]. Interestingly, it appears to be uniformly observed that loss of Cosmc in animal cells leads to a collapse of the typical O-GalNAc glycans to only the Tn and sialyl Tn antigen, confirming that no galactosyltransferase other than the T-synthase appears capable of using the Tn antigen as an acceptor.

Roles of O-glycans in Cancer

In regard to tumorigenesis and metastasis the function of the Tn antigen is somewhat enigmatic, but recent studies are demonstrating extreme pathology associated with Tn expression. In many human solid tumors, the Tn antigen is highly expressed, and is in fact one of the most recognized tumor-associated carbohydrate antigens[184,185,155,186]. In pancreatic cancer the Tn antigen is expressed in a majority of tumors in humans, and in many cases the promoter for the *Cosmc* gene is hypermethylated and silenced. We also observed epigenetic silencing of the *Cosmc* promoter in Tn4 cells, which is an immortalized B cell line expressing the Tn antigen from a male patient with a Tn-syndrome-like phenotype[187]. Recent studies on models of pancreatic cancer and induced Tn expression by knockdown of *Cosmc* or hypermethylation of the *Cosmc* promoter indicate that aberrant Tn expression is associated with oncogenic features, including proliferation, migration, and invasion of pancreatic cancer cells[188–190]. Some studies have also shown that loss of extended O-glycans by deletion of T-synthase promotes spontaneous duodenal tumors[191].

We have also observed extreme pathology in targeted deletion of *Cosmc* in intestinal epithelial cells of mice, in particular it is associated with spontaneous inflammation, induced colitis, and altered gut microbiome in the mucosal layer of the distal colon/rectum, which is often associated with colorectal cancers[192].

Thus, all of the studies to date demonstrate that expression of the Tn antigen on animal tissues, which normally lack expression, is pathological and associated with loss-of-cellular functions and tumorigenesis. Most likely, the loss of extended O-glycans on glycoproteins leads to one or both results – loss of glycan recognition or altered glycoprotein structure/function indirectly through its loss of extended O-glycans. Such conclusions are consistent with earlier studies on the critical roles of O-glycans in specific glycoproteins for expression and stability, in particular the LDL-receptor, which contains multiple O-glycans that are required for its normal expression and function[193–195].

Development of Glycan Microarrays and Shotgun Glycomics to Explore Glycan Recognition in Infection and Immunity

The complexity of human and rodent glycomes is not understood, but our estimates suggest that the number of determinants, i.e. glycan sequences recognizable with high affinity by an antibody, GBP, microbial adhesin, or toxin, numbers in the thousands[196]. The total number of glycans that may carry one or more of these determinants is likely to be in the hundreds of thousands, and that does not include estimates of glycosaminoglycans, which are even more complex. We have studied the recognition of glycans by human immunoglobulins, and the results indicate that there are vast numbers of anti-carbohydrate antibodies of all classes in humans and also begin to suggest that a large portion of all human immunoglobulins recognize glycan antigens[197–199]. But defined arrays are limited in glycan numbers and diversity, and even additions of microbial glycans[101], which are also highly recognized by human immunoglobulins, provide only a small window of insight into the vast repertoire of anticarbohydrate antibodies.

The modern methods of glycan microarrays to explore the recognition of glycan determinants were developed by several groups[200–204]. The Consortium for Functional Glycomics printed covalent glycan microarrays of mammalian-like glycans with alkyl linkers[202]. These superseded earlier efforts using ELISA-type assays and other formats[205]. These resources funded by the NIGMS/NIH led to many hundreds of published studies by investigators exploiting the sensitivity, reproducibility, and reliability of such microarrays for glycan binding studies (http://www.functionalglycomics.org and https://ncfg.hms.harvard.edu). Furthermore, the arrays use extremely small amounts of glycans, extending the use of such precious resources.

The original versions of glycan microarrays mainly focused on defined glycans that were produced chemically, enzymatically, or by chemo-enzymatic approaches. In some cases, these arrays also incorporated naturally-occurring glycans, especially such glycans as those on glycosphingolipids, and milk oligosaccharides. We realized that such arrays are inevitably limited due to obvious limitations of enzymes and chemical synthesis hurdles. Thus, with Song and Smith we developed shotgun glycomics[132,206,207], whereby

naturally-occurring glycans of all types could be isolated from glycoproteins and glycolipids and then be fluorescently-tagged, separated by HPLC methods, and finally printed covalently on slides (Figure 5). Such natural glycan arrays in that case are termed shotgun glycan microarrays and are representative of the glycome of the natural starting materials.

The development of these types of shotgun glycan microarrays required the development of fluorescent linkers with bifunctionality, i.e. they have a reactive moiety to derivatize glycans, and they have a reactive moiety to covalently attach them to supports. The first of these bifunctional linkers we used was 2,6-diaminopyridine (DAP), which allowed us to exploit the reactivity of the aryl amine for reaction with free, reducing glycan tags, and exploit the available residual aryl amine for reaction to N-hydroxysuccinimide-derivatized surfaces[208]. However, DAP was not ideal in some ways due to weak fluorescence and relatively weak reactivity of the residual aryl amine. Thus, we developed a much more robust and versatile compound, the bifunctional fluorescent linker, 2-amino-N-(2-aminoethyl)-benzamide (AEAB)[209]. With this approach we were successful in generating a wide variety of shotgun glycan microarrays, including those developed for *C. elegans* N-glycans[138], human milk oligosaccharides[210,211], pig lung N-glycans[212], and more recently human lung derived N-glycans[213]. The shotgun glycomics approach, while laborious over the short term, provides archival material for longitudinal studies, along with unprecedented insights into both glycomics and functional glycan recognition of endogenous material that is difficult to identify by any other approach.

Over the past few years we have explored the endogenous glycans recognized by human pathogens using shotgun glycomics approaches. Using shotgun glycan microarrays of human samples, we have discovered numerous unique relationships. As discussed above, we observed many novel anti-carbohydrate antibodies to glycan antigens in parasitic worms. We have also used this approach to explore the binding of endogenous human and animal glycans by neonate-specific bovine-human reassortant rotavirus, which were found to recognize blood group containing glycans[214], as well as to discover that influenza viruses bind unique types of sialylated and non-sialylated and phosphorylated glycans[215–220]. Our recent discovery of interactions of influenza viruses with phosphorylated glycans of the human lung suggests that viral interactions with the human glycome are perhaps more complex than thought earlier.

Conclusion and Acknowledgments

The lessons that are being learned in the field of glycosciences about the functional glycome through human and animal genetics and new chemical, biochemical, and biological technologies are astonishing, and the pace at which discoveries are being made in the area of glycoscience is increasing. Worldwide efforts are underway, also, to better define the human glycome and its functions. As co-Directors of the Human Glycome Project, Gordon Lauc and I welcome all to join and help in this important but daunting effort https://human-glycome.org. Here we have highlighted specific aspects of the growth in understanding of glycobiology where our lab has had an impact. Overall, there is increasing appreciation of the unique and important functions of glycans in biology and medicine. Much of this arises from understanding the complexity of the glycome, discovering the amazing biosynthetic

pathways for glycan synthesis, discerning the specific nature of glycan recognition by GBPs, including antibodies and lectins, and recognizing the impact of glycoscience in human health.

In closing, I am grateful that our work has contributed to these developments and to an appreciation of the field, through both research and education. I want to thank all my students, fellows, and colleagues with whom I have had the pleasure to work, for their incredible dedication and terrific efforts over the years. I also want to thank the editors of the textbook *Essentials of Glycobiology*, with whom it was a pleasure to create the 1st, 2nd, and 3rd Editions, now also freely available online at https://www.ncbi.nlm.nih.gov/books/NBK310274/. I also want to thank the International Glycoconjugate Organization for the honor of being the recipient of the IGO Award in 2019.

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Biography

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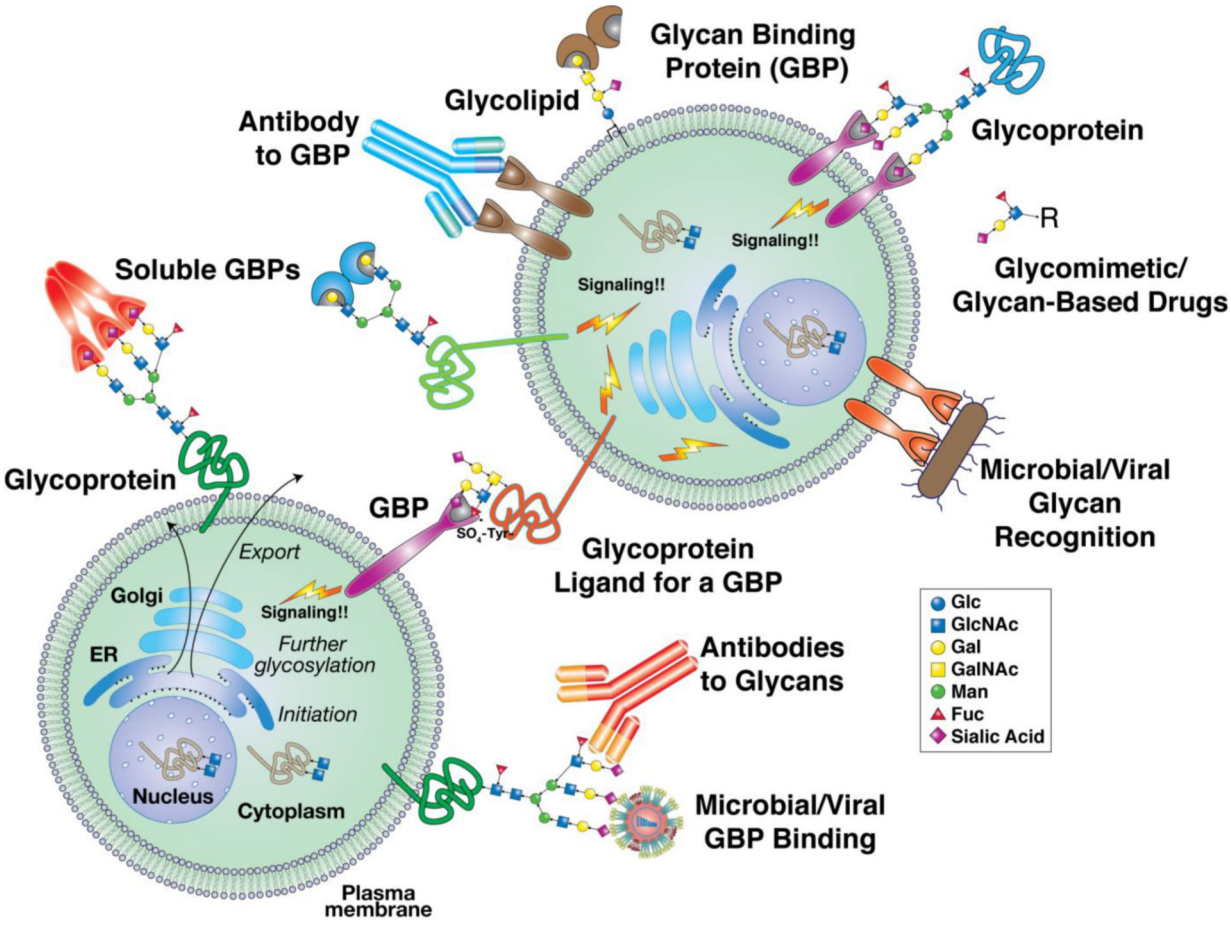

Figure 1.

Complex carbohydrates on glycoproteins and glycolipids within and on cells and in cellular secretions can be bound by glycan-binding proteins (GBPs) and antibodies, as well as cross-recognized by microbes and viruses and their glycans and GBPs, e.g. adhesins and hemagglutinins. Through these direct (and indirect) interactions, glycans can signal cells, regulate cell adhesion, and participate in a wide range of developmental, immunological, hematological, and cellular/tissue pathways. Alteration or disruption of glycosylation pathways or GBP expression, through acquired and heritable disorders, or drug treatments, and also in tumor cells, typically leads to pathological outcomes.

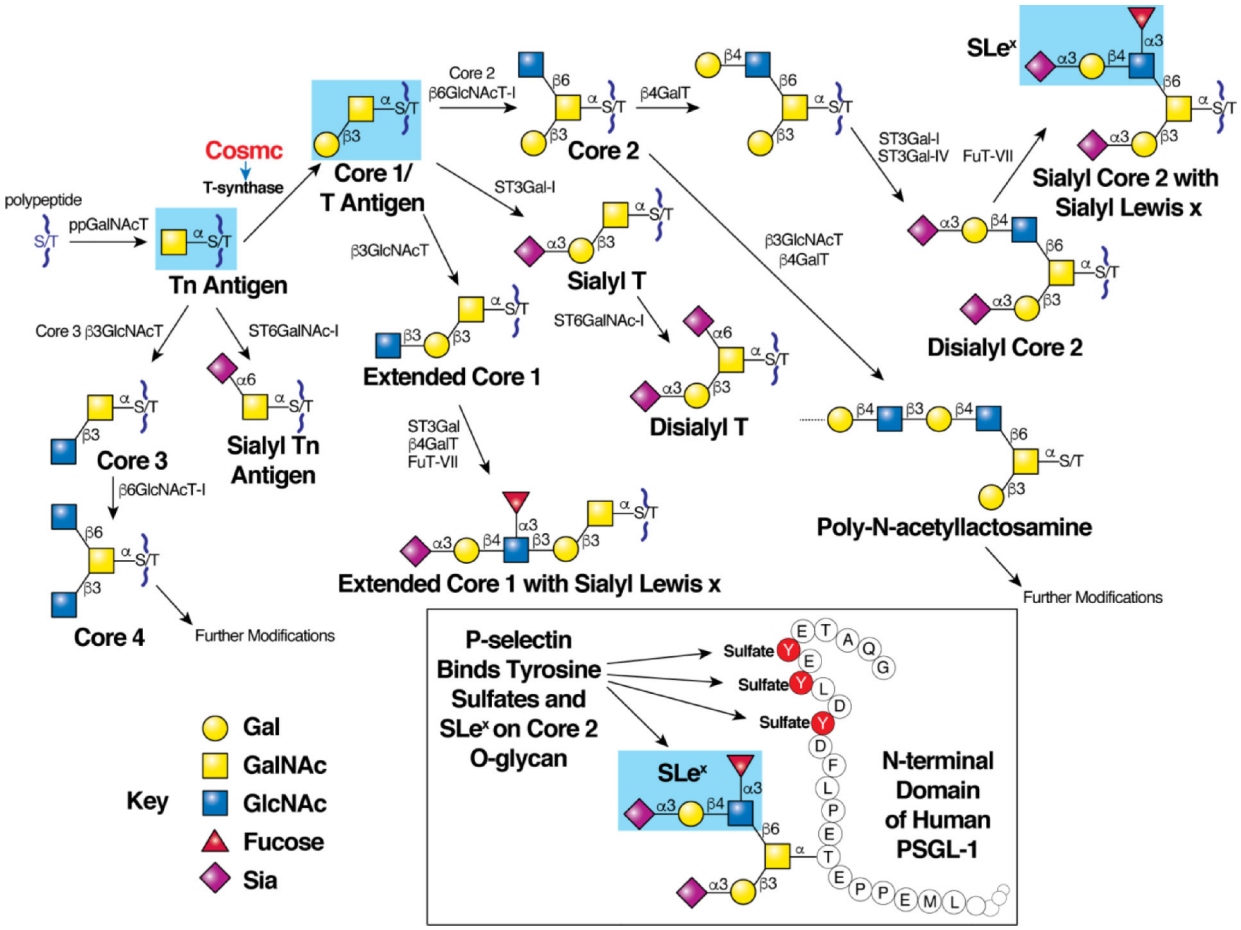

Figure 2.

The biosynthesis of mucin-type O-glycans is initiated post-endoplasmic reticulum and in the Golgi apparatus by the addition of GalNAc by a family of ppGalNAcT enzymes, then subsequently galactose is added by the T-synthase, which requires the molecular chaperone Cosmc in the ER for the correct folding and activity of the enzyme. Subsequent modifications of the Thomsen or T antigen (also called the Thomsen-Friedenreich or TF antigen) occurs by additional enzymatic reactions to generate an incredible diversity of O-glycans, including those with the core 2 O-glycan and SLe^x determinant recognized by P-selectin and other selectins. At the bottom of the figure is a depiction of how P-selectin binds residues within the SLe^x determinant along with sulfated tyrosine residues and peptide determinants at the extreme N-terminus of PSGL-1.

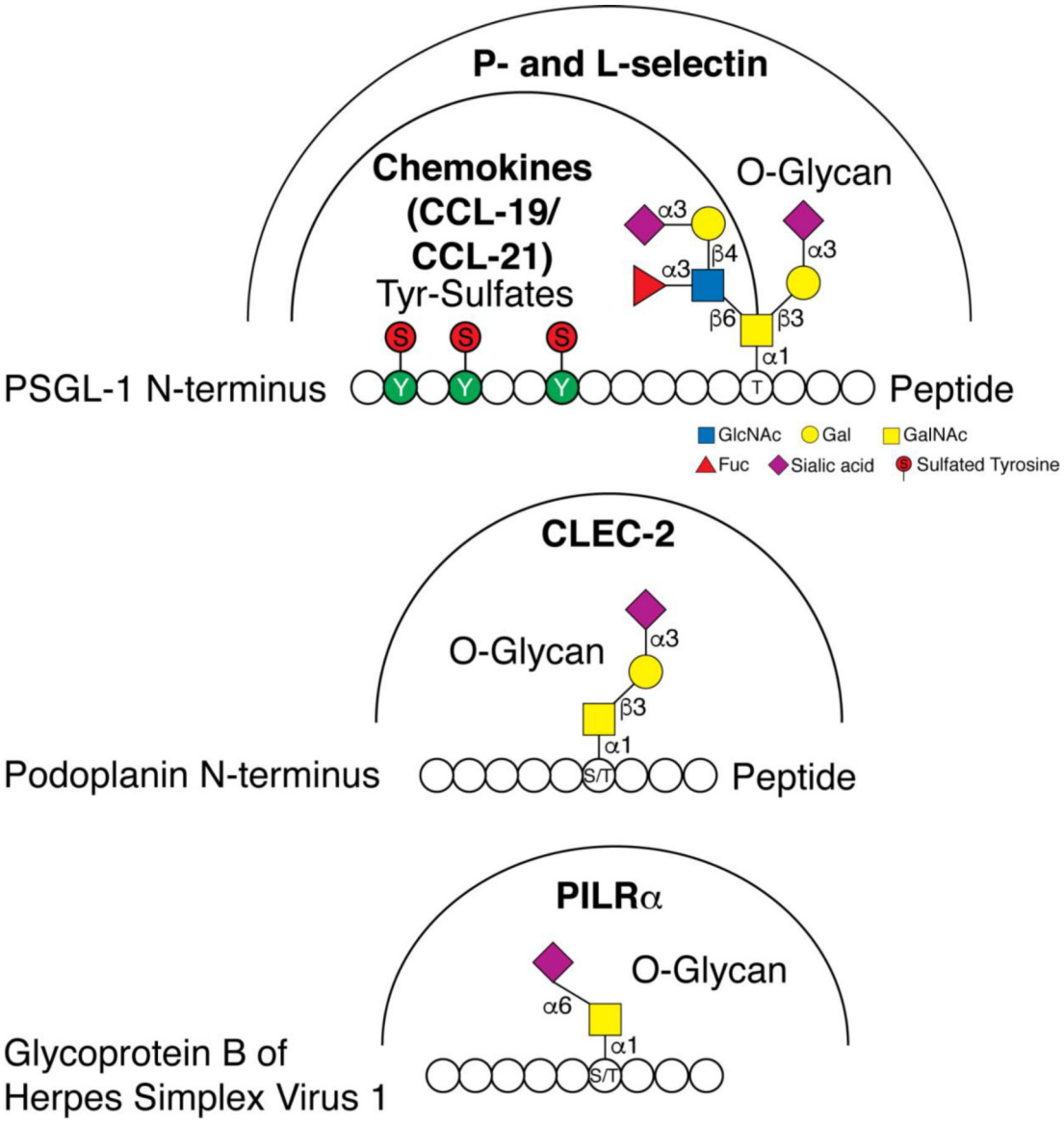

Figure 3.

Several examples are shown in which multiple determinants, including glycan, peptide, and tyrosine sulfate, within glycoprotein ligands contribute to high affinity binding of recognition molecules, such as glycan-binding proteins and chemokines.

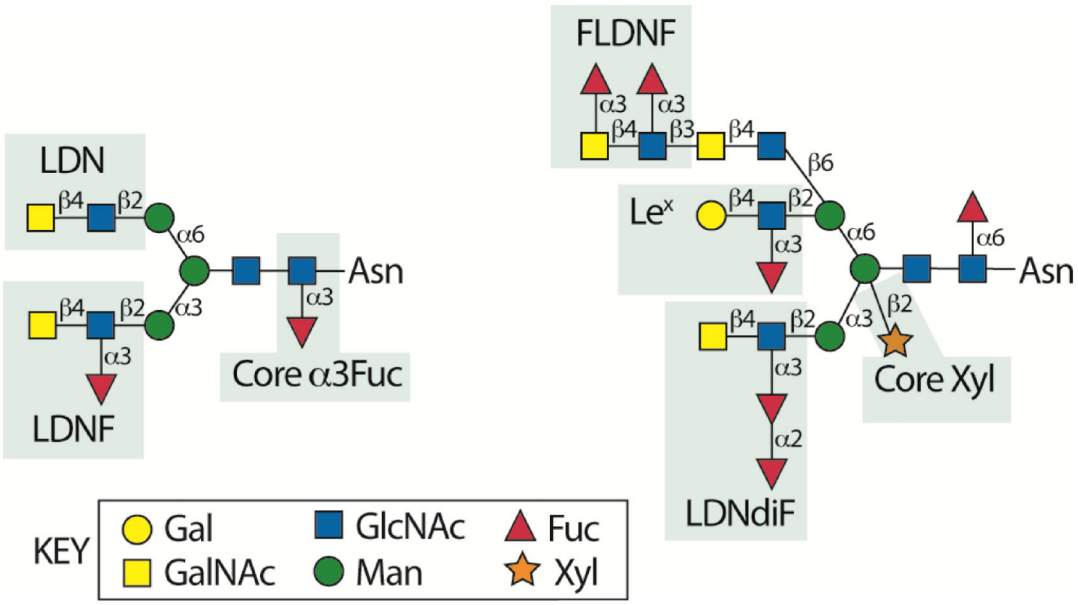

Figure 4.

Examples of antigenic glycan determinants expressed in glycoproteins, Asn- or N-glycans of parasitic helminths, including *Schistosoma mansoni*, and many trematodes and nematodes. The determinants are in colored boxes and their common names are shown, e.g. LDN, FLDNF, etc.

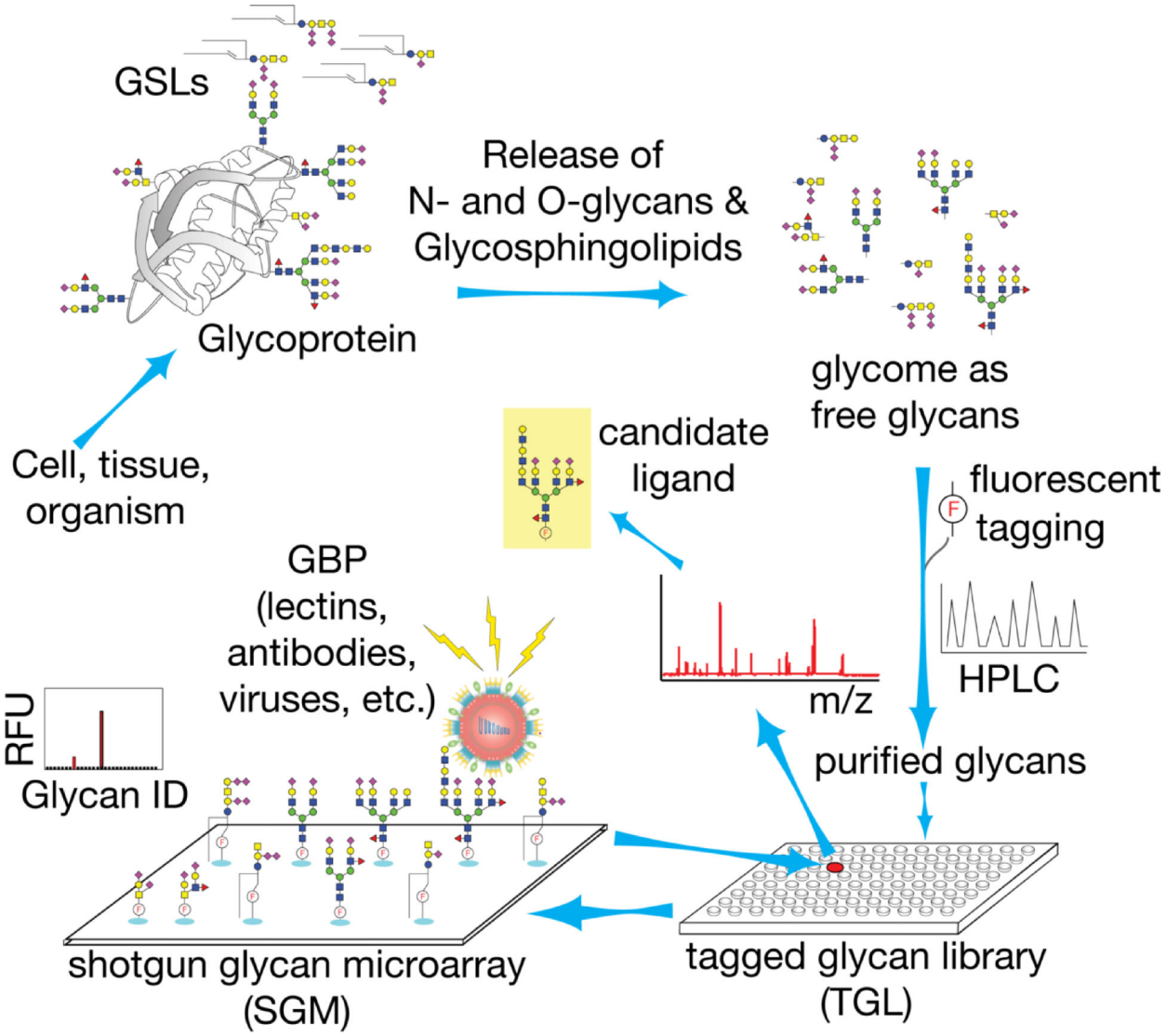

Figure 5.

The technology of shotgun glycomics requires the release of glycans from biological samples of glycoproteins and glycolipids by enzymatic or chemical strategies. The released glycans, which represent the glycome of the source material, can be fluorescently tagged with a bifunctional linker, and then separated by multi-dimensional chromatography and quantified. The separated glycans are preserved in a tagged-glycan library or TGL, from which they can be covalently or non-covalently printed to generate shotgun glycan microarrays or other presentations. The interrogation of the shotgun glycan microarrays with a GBP, lectin, toxin, or virus, for example, can lead to the identification of a novel set of glycans carrying the determinants needed for recognition. The structural characterization of the glycans retrieved from the TGL can be performed by MS and other technologies. Depicted is the concept of shotgun glycomics applied to influenza virus, where the starting material may be the human lung[213].

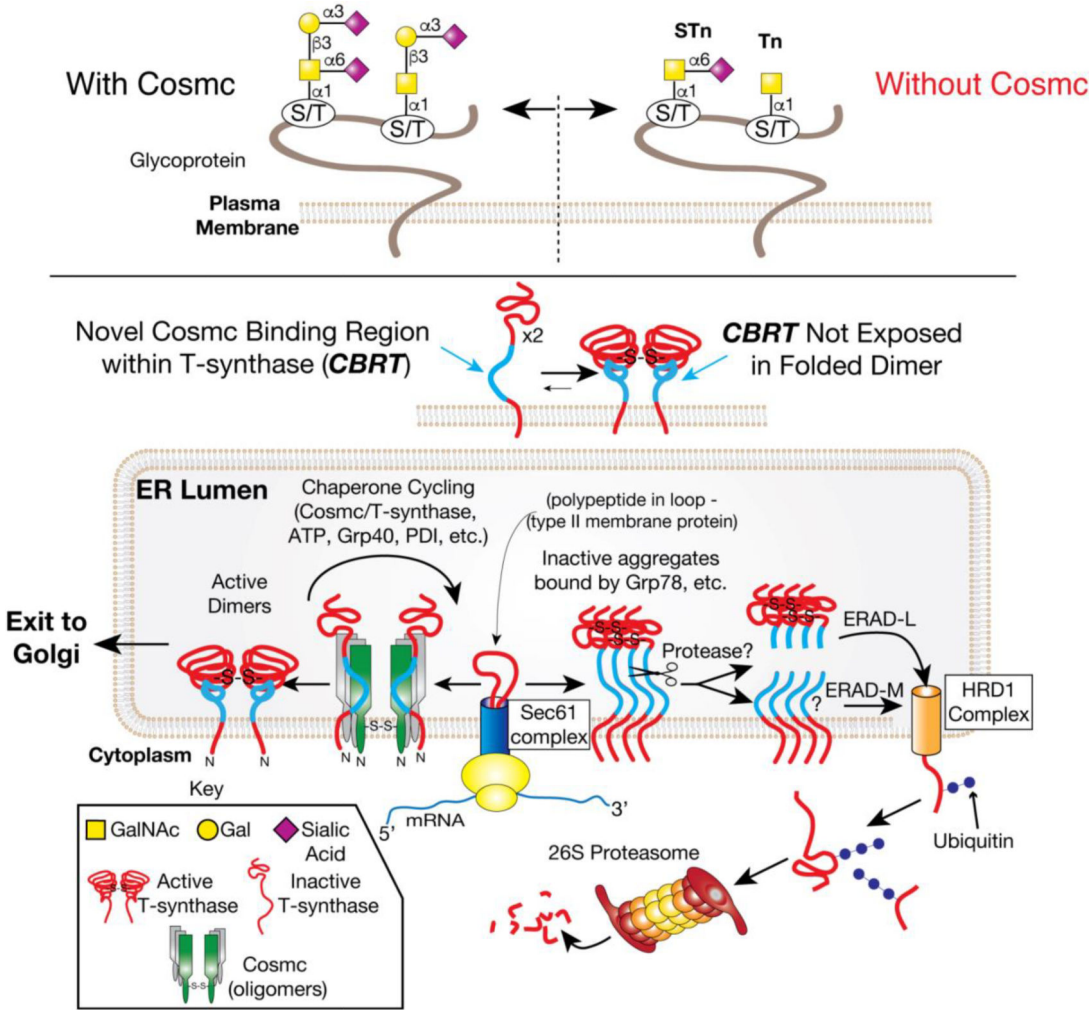

Figure 6.

A depiction of our working model as to how Cosmc functions in the endoplasmic reticulum as a molecular chaperone to assist in folding of newly synthesized T-synthase. Human Cosmc (shown in green), is present in the ER as a disulfide-bonded oligomer, where it can interact with the nascent polypeptide of human T-synthase (shown in red) as it is synthesized. This interaction is reversible and prevents the undesirable association and oligomerization of the T-synthase and inactive forms. The interaction occurs through the Cosmc Binding Region within T-synthase (CBRT), which is accessible in the immature protein but inaccessible once the T-synthase has completed folding and is active. The absence of Cosmc leads to these inactive forms that are proteolyzed eventually in the ER and in the 26S proteasome in the cytoplasm. When Cosmc functions normally, the T-synthase becomes an active dimer that moves to the Golgi apparatus, where it functions to generate normal O-glycans that have core 1 as a precursor with galactose linked to N-acetylgalactosamine. In the absence of Cosmc cells generate the Tn and Sialyl Tn antigen lacking galactose.