Leading Edge
Review

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Glycosylation in Cellular Mechanisms of Health and Disease

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Glycosylation produces an abundant, diverse, and highly regulated repertoire of cellular glycans that are frequently attached to proteins and lipids. The past decade of research on glycan function has revealed that the enzymes responsible for glycosylation—the glycosyltransferases and glycosidases—are essential in the development and physiology of living organisms. Glycans participate in many key biological processes including cell adhesion, molecular trafficking and clearance, receptor activation, signal transduction, and endocytosis. This review discusses the increasingly sophisticated molecular mechanisms being discovered by which mammalian glycosylation governs physiology and contributes to disease.

Glycans are one of the four basic components of cells and may also be the most abundant and diverse of nature's biopolymers. Existing as covalent linkages of saccharides often attached to proteins and lipids, glycans constitute a significant amount of the mass and structural variation in biological systems. The field of glycobiology is focused upon understanding the structure, chemistry, biosynthesis, and biological function of glycans and their derivatives. Glycobiology has a long history that began with investigations of the basic constituents of cells and the nature of the polysaccharide carbohydrate component. Clinical applicability arose early with the discovery of the human blood groups, although evidence that these were glycan antigens came later (Landsteiner, 1931). In addition, the antithrombotic glycan heparin is one of the most commonly used drugs (Linhardt, 1991; Shriver et al., 2004), with current estimates of a billion doses prescribed annually. As glycobiology is increasingly interrelated with other disciplines, nomenclature and terminology within the field continue to evolve. The word glycan is now often used to encompass oligosaccharide, polysaccharide, and carbohydrate, as not all glycans are oligomers and the term carbohydrate can be confused with components of intermediary energy metabolism. More recently, in parallel with approaches to define genomics, proteomics, and lipidomics, the term "glycomics" has emerged, which refers to the study of the glycan structures that compose an organism's "glycome."

The mammalian glycome repertoire is estimated to be between hundreds and thousands of glycan structures, and could be larger than the proteome. Although the diversity of glycan structures theoretically is vast, constraints are provided by the mechanisms of glycan synthesis and regulation. Mammalian glycans are formed by an endogenous portfolio of cellular enzymes and substrates that have been retained in an evolutionary investment encompassing millions of years and spanning 1%–2% of the genome. Vertebrates, and especially mammals, have evolved a highly complex glycan repertoire that is structurally distinct from that of invertebrates, lower eukaryotes, and prokaryotes. It is increasingly evident that the variation in glycomes among organisms is a molecular basis for interspecies recognition systems. Glycans of nonvertebrate organisms, for example, can modulate the development and activation of the mammalian immune system (Cobb and Kasper, 2005). Mammalian glycans are remarkably well conserved, but species-specific variations also exist, and these differences may be involved in the emergence of distinct traits including susceptibilities to infectious pathogens (Gagneux and Varki, 1999; also see the Essay by A. Varki, page 841 of this issue). Engineering new chemical modifications into glycans of living cells may improve the ability to detect glycan function and contribute to future diagnosis and treatment of disease (Prescher et al., 2004; also see the Minireview by J. Prescher and C. Bertozzi, page 851 of this issue). Indeed, glycosylation defects in mice as well as humans and their links to disease have shown that the mammalian glycome contains a significant amount of biological information (Lowe and Marth, 2003; Freeze, 2006). This review focuses upon the involvement of mammalian glycans in the molecular and cellular mechanisms that control health and disease.

Structure and Topology of Mammalian Glycosylation

Nine monosaccharides are used in the enzymatic process of glycosylation in mammals. Conserved biosynthetic pathways provide all nine monosaccharides from sugars and precursors ubiquitously present in the diet. Except in cases of rare genetic defects, dietary intake of monosaccharides or mammalian glycans has not been

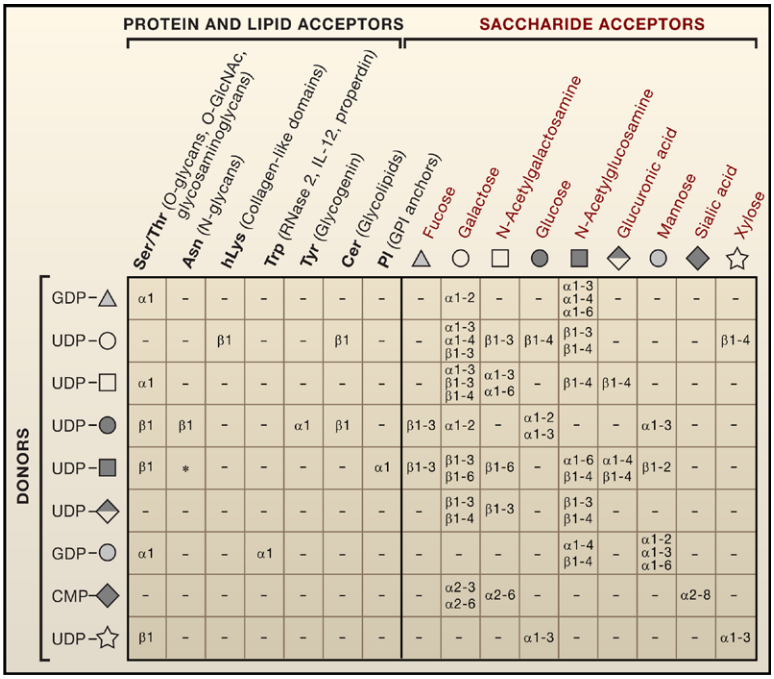

Figure 1. Mammalian Glycan Linkages Produced by Glycosylation

There are nine nucleotide sugar donors and multiple protein and lipid acceptor motifs for glycosyltransferases, which produce 14 different glycans in stereoisomeric configurations (α or β) linked at the number 1 position of the donor sugar ring. The attached monosaccharide frequently then becomes a saccharide acceptor in 1 of 49 other glycosyltransferase reactions. This results in glycosidic bonds with α or β configurations of the donor saccharide linked through position 1 or 2 to position 2, 3, 4, or 6 of an acceptor saccharide. Glycan diversification is dictated by the combinatorial and regulated application of this enzymatic potential. This includes hyaluronan synthesis, which occurs by copolymerization of two nucleotide sugar donors. The formation of iduronic acid by the epimerization of glucuronic acid subsequent to glycosylation is not depicted. Although some disaccharide sequences are found on multiple types of glycans, others are specific to one or few glycan types. Those potential mammalian disaccharide sequences that have not been observed in nature are indicated (–). N-glycosylation (*) is initiated by transfer en bloc of a presynthesized dolichol lipid-linked oligosaccharide precursor. Ser/Thr, serine/threonine; Asn, asparagine; hLys, hydroxylysine; Trp, tryptophan; Tyr, tyrosine; Cer, ceramide; PI, phosphatidylinositol.

rigorously established to have a beneficial effect on human health or in the treatment of disease. Biosynthetic pathways control the production and endogenous functions of different glycan structures. The structural diversity of the mammalian glycome is produced predominantly in the secretory pathway of the cell. Moreover, it is within the Golgi apparatus that glycans become increasingly oligomeric and branched as they transit through this latter portion of the secretory system bound mostly for the cell surface and extracellular compartments.

Glycosylation produces different types of glycans (or glycoconjugates) that are typically attached to cellular proteins and lipids (Figure 1). Protein glycosylation encompasses N-glycans, O-glycans, and glycosaminoglycans (frequently termed proteoglycans). N-glycans are linked to asparagine residues of proteins, specifically a subset residing in the Asn-X-Ser/Thr motif, whereas O-glycans are attached to a subset of serines and threonines (Schachter, 2000; Yan and Lennarz, 2005). Although glycosaminoglycans are also linked to serine and threonine, they are linear, are produced by different biosynthetic pathways, and are often highly sulfated (Esko and Selleck, 2002). Lipid glycosylation in the secretory pathway is also a prevalent modification and creates glycolipids (glycosphingolipids) that include the sialic acid-bearing gangliosides (Maccioni et al., 2002). Glycosylphosphatidylinositol (GPI)-linked proteins share a common membrane-bound glycolipid linkage structure that is attached to various proteins (Kinoshita et al., 1997). Hyaluronan is a unique glycan type unattached to either proteins or lipids that is secreted into extracellular compartments (Weigel et al., 1997). Less common types of protein glycosylation also occur, for example, on lysine, tryptophan, and tyrosine residues of specific proteins, such as glycogen, which was the first identified glycoprotein. In addition, although technically not glycosylation, acetyltransferase and sulfotransferase enzymes residing in the secretory pathway frequently attach acetyl and sulfate groups to selected saccharides residing on some oligosaccharide chains and can thereby modulate glycan structure and function (Klein and Roussel, 1998; Fukuda et al., 2001).

Some forms of glycosylation occur outside of the secretory pathway. Among most eukaryotic organisms, N-acetylglucosamine has been found linked to serine and threonine residues (O-GlcNAc) on many cytoplasmic and nuclear proteins (Hart, 1997). Similar to protein phosphorylation, GlcNAcylation is an enzymatic modification that typically has a shorter half-life than that of the attached proteins. This reflects the presence of a regulated cytoplasmic N-acetylglucosaminidase, which removes O-GlcNAc, leaving the serine or threonine residue subsequently available for another round of GlcNAcylation or sometimes phosphorylation. O-GlcNAc is a highly regulated posttranslational modification required for the viability of many mammalian cell types perhaps by acting as a nutrient sensor, preventing protein phosphorylation, or regulating protein turnover (Zhang et al., 2003; O'Donnell et al., 2004; Zachara and Hart, 2004).

It is useful to distinguish secretory and cytoplasmic glycosylation from glucuronidation, the latter being an enzymatic process linking a single glucuronic acid to bile salts and xenobiotics (molecules that are foreign to cells) (Tukey and Strassburg, 2000). In contrast to glycosylation, glycation refers to the covalent linkage of saccharides such as glucose to proteins by a nonenzymatic

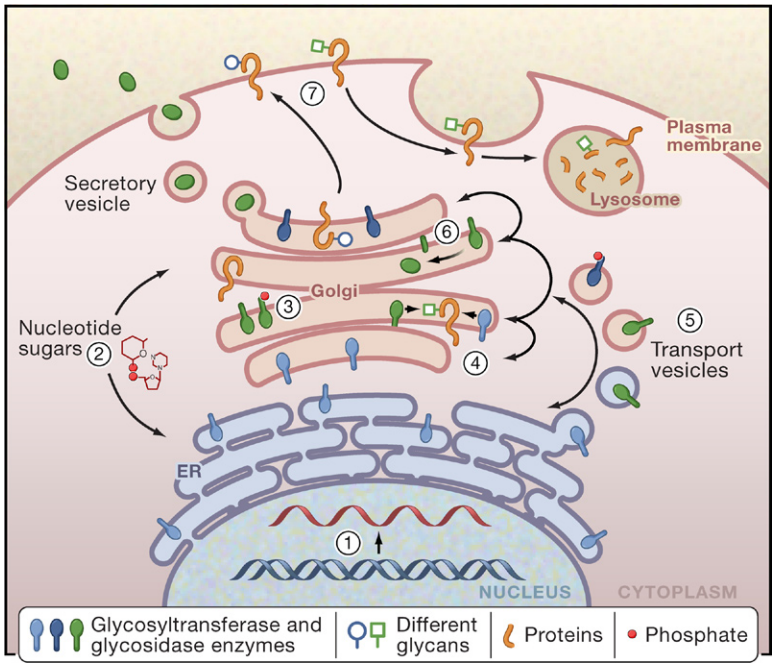

Figure 2. Cellular Regulation of Glycan Expression

Representation of multiple mechanisms that alter cellular glycosyltransferase or glycosidase expression, strucure, and activity, which can thereby regulate the formation of glycans. These include (1) control of glycosyltransferase and glycosidase gene transcription, (2) synthesis and transport of nucleotide sugar donors to the ER and Golgi (sugar transporters not depicted), (3) modulation of enzymatic structure through phosphorylation, (4) relative amounts of enzymes that compete for identical substrates, (5) intracellular enzyme trafficking and altered access to substrates, (6) proteolysis within the lumen of the Golgi resulting in secretion of catalytic domains, and (7) glycan turnover at the cell surface by endocytosis coincident with expression of different glycans from altered glycan synthesis. Effects of glycosyltransferase and glycosidase cytoplasmic tail phosphorylation (3) and intraluminal proteolysis (6) on cellular glycosylation remain to be established. Structural information on the composition of glycans can be obtained from Figure 1.

and irreversible process that is elevated in various diseases and may be a factor in the pathology of aging (Suji and Sivakami, 2004).

Regulation of Mammalian Glycosylation

Glycans are constructed in an ordered sequential manner involving the distinct substrate specificities of glycosyltransferase and glycosidase enzymes. Glycosyltransferases synthesize glycan chains, whereas glycosidases hydrolyze specific glycan linkages. Although glycosyltransferases are the anabolic component of glycosylation, both types of enzymes collaborate to determine the structural outcome in pathways of glycan biosynthesis. Such properties are exemplified by mammalian N-glycan biosynthesis (Kornfeld and Kornfeld, 1985). Similarly, the biosynthetic pathways for the production of O-glycans, glycosaminoglycans, and glycolipids are comprised of single enzymatic steps that typically rely upon glycan structures produced by the previous enzyme to produce the substrate for the next (Schachter, 2000; Maccioni et al., 2002; Esko and Selleck, 2002). Although a one enzyme/one saccharide linkage paradigm applies to almost all biosynthetic steps, numerous glycosyltransferase isozymes exist, and these underlie the breadth of glycan participation among different cell types and physiological processes.

Glycosylation in the secretory pathway is a dynamic process with multiple mechanisms that alter glycosyltransferase and glycosidase expression and structure, as well as their accessibility to substrates. Thus, in concert with protein and lipid turnover, glycosylation can regulate glycan variation (Figure 2). Structural variations in the glycan repertoire at the cell surface produce numerous biomarkers, some of which correlate with differentiation, cell activation, and disease. For example, elevated levels of truncated O-glycans (known as T antigens) can be prognostic for reduced survival of patients with certain types of cancer (Hakomori, 2002; Kobata and Amano, 2005).

Gene transcription has a major impact on glycan formation, which is reflected by the cell-type-specific and developmentally modulated RNA expression profiles observed among many glycosyltransferases and glycosidases. Microarray approaches that detect the transcript levels of enzymes involved in constructing the glycome will be increasingly useful in categorizing these changes and perhaps provide predictive information on cellular glycan expression patterns (Comelli et al., 2006). Transcriptional regulation of RNA abundance occurs among glycosyltransferase genes encoding, for example, GlcNAcT-V and Core 2 GlcNAcT-I. The GlcNAcT-V gene promoter bears Ets transcription factor binding elements that induce transcription in response to signals emanating from several key regulators of cell proliferation, including the *Her-2/Neu* oncogene. Core 2 GlcNAcT-I is induced by the T-bet transcription factor in T helper type 1 lymphocytes (Chen et al., 1998; Underhill et al., 2005). Although multiple transcriptional networks regulate glycosyltransferase and glycosidase gene expression, the effect of this regulation on cellular processes remains largely unknown.

The expression of mammalian glycans is regulated at both a posttranscriptional and posttranslational level. Currently, this does not appear to involve modulation of enzymatic activity, as glycosyltransferases and glycosidases are constitutively active. Nevertheless, some must be properly glycosylated themselves to be active, which suggests a possible mechanism of catalytic regulation

in vivo. Mechanisms altering the intracellular location of glycosyltransferases and glycosidases can be an effective means of regulating glycan formation by controlling access to acceptor substrates. Major changes in the glycome are induced by the loss of some chaperones and multiprotein complexes that alter glycosyltransferase trafficking between the endoplasmic reticulum and Golgi (Wu et al., 2004; Ju and Cummings, 2005; Foulquier et al., 2006). A potential means by which glycosyltransferases and glycosidases may be regulated is through phosphorylation of their cytoplasmic tails, which might modulate intermolecular interactions leading to differential substrate access and intracellular trafficking. There is also evidence of competition among glycosyltransferases in vivo for substrates in the secretory pathway, which can modify glycan formation. Some glycosyltransferases that generate different saccharide linkages have distinct specificities for nucleotide sugar donors but the same acceptor substrate specificity; whereas others bear identical donor specificity but act on different acceptor substrates. Glycosyltransferases of the former type can be mutually exclusive in the assembly line of glycan formation; whichever enzyme modifies the substrate first can thereby redirect the synthetic pathway and alter the structural outcome. This was observed by the in vivo blockade of Core 2 GlcNAcT function due to endogenous expression of ST3Gal-I in T cells. Both glycosyltransferases can act on the same acceptor substrate, and loss of ST3Gal-I elevated Core 2 O-glycan synthesis without a change in Core 2 GlcNAcT enzyme activity (Priatel et al., 2000).

Disengagement of glycosyltransferases and glycosidases from their membrane-anchored locations can occur by proteolysis and would be expected to abolish their activities in glycan formation, although evidence of this potential form of glycan regulation currently awaits further experimentation. The enzymes of mammalian glycosylation are predominantly type 2 transmembrane glycoproteins that contain a luminal catalytic domain linked to a luminal membrane-proximal "stem" domain. Cleavage by secretory proteases within the stem domain results in secretion of a catalytic domain fragment. This fragment can be found in most body fluids and can be induced, for example, in response to inflammation (McCaffrey and Jamieson, 1993). The purpose of this proteolysis is unknown, and the range of enzymes affected is unclear. Although such glycosyltransferase fragments retain enzymatic activity, and hence their ability to bind to available acceptor substrates, they are not likely to be catalyzing glycan formation among extracellular compartments, as the concentration of nucleotide sugar donors outside of the cell's secretory pathway is far below enzyme substrate binding affinities.

The hydrolysis of glycans on mammalian glycoproteins and glycolipids is associated with their degradation in lysosomes. However, endogenous mechanisms that cleave glycans at the cell surface may exist. Hydrolysis of mammalian cell-surface glycans is in fact a feature of some pathogen infection strategies such as sialic acid binding and cleavage by influenza virus (Gagneux and Varki, 1999; also see the Minireview by L. Comstock and D. Kasper, page 847 of this issue). Proteolysis and trafficking to the cell surface would place mammalian glycosidase enzymes in the region of the cell-surface glycocalyx, where some glycans might be hydrolyzed. At least one mammalian glycosidase that cleaves sialic acids from glycans is a transmembrane protein that reaches the plasma membrane (Wang et al., 2004). Although examples of cell-surface glycoprotein alterations consistent with removal of specific glycan linkages have been described—such as the highly reproducible reduction in some sialic acid linkages following immune activation of mammalian lymphocytes—this may be explained by endocytosis and turnover in which newly synthesized glycoproteins bear different glycans due to modulation of glycosyltransferase or glycosidase function.

The biosynthesis and availability of nucleotide sugar donor substrates can exert broad control over mammalian glycan formation. Blockade of donor biosynthesis or functional loss of donor-specific transporters normally residing the endoplasmic reticulum and Golgi membranes can abolish cellular glycans that contain, for example, fucose or sialic acid linkages (Lubke et al., 2001; Smith et al., 2002; Schwarzkopf et al., 2002). In contrast, glucosamine supplementation to the hexosamine biosynthetic pathway can elevate synthesis of some donor substrates and increase production of various glycans in mammalian cells (Zachara and Hart, 2004; Lau et al., 2005). Precisely how this occurs may reflect multiple factors including increased catalysis and changes in gene expression. The impact of such augmented glycosylation upon mammalian physiology is not yet known, although this matter is worthy of careful investigation. With a number of regulatory mechanisms available, several and perhaps all of those discussed above are involved in modulating mammalian glycan expression.

Determinants of Mammalian Glycan Function

Few biological roles for mammalian glycosylation had been established even a decade ago. The rapid pace of discovery since then reflects the application of genetic tools and approaches to expand upon the existing foundation of enzymatic, biochemical, and structural knowledge. Glycosylation, like phosphorylation, produces numerous structural modifications, each of which may be capable of signaling. Likewise, absence of a single kinase or glycosyltransferase affects the modification of multiple proteins and lipids. In studies of phosphorylation, this is commonly interpreted as disruption of a signal transduction cascade. In glycosylation, the specificity of most glycosyltransferases and glycosidases for substrates is defined by glycan structure instead of protein and lipid determinants. Therefore, single enzymes can glycosylate multiple, seemingly unrelated, proteins and lipids. How then does glycan formation achieve a

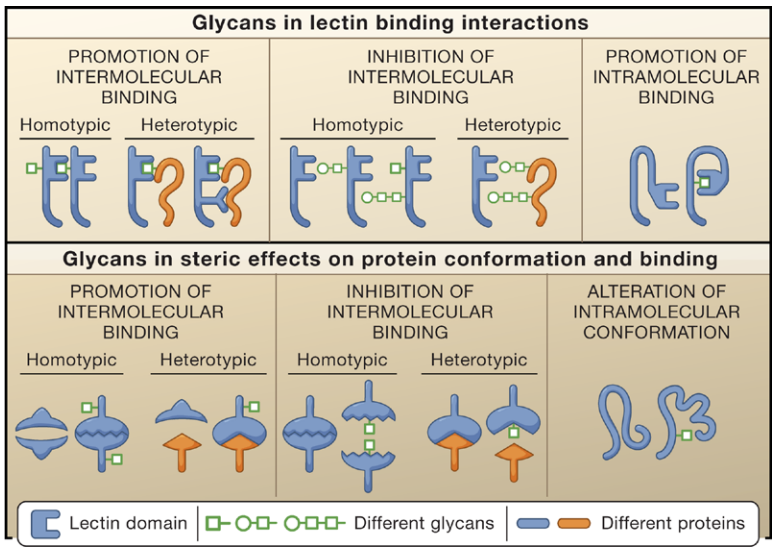

Figure 3. Glycans Modulate Molecular Interactions

Lectin binding and steric mechanisms involving glycan structures in the control of protein-protein interactions are depicted. Glycans can modulate intramolecular and intermolecular binding comprising both homotypic and heterotypic interactions. The participation of protein conformation and protein-protein binding in concert with lectin binding is also denoted. Most of these interactions depicted reflect various degrees of experimental support among the current literature.

high level of specificity in cellular function? The answer may come from combining knowledge of glycan synthesis and regulation with the phenotypes observed in intact organisms bearing defects in glycan formation.

Cultured cells bearing various enzymatic defects in the pathways of glycosylation typically lack significant phenotypes, yet a high degree of evolutionary conservation is typical among mammalian glycosyltransferase and glycosidase orthologs (Amado et al., 1999; Kikuchi and Narimatsu, 2006). Glycosylation in the secretory pathway positions most glycans at the cell surface and extracellular compartments where cell-cell communication is occurring among various cell types in intact organisms. Such intercellular physiology is not typically replicated in immortalized cell-line monoculture systems. GlcNAcT-1 glycosyltransferase deficiency, for example, which was well tolerated among cell lines in vitro, resulted in severe embryonic defects with situs inversus of heart loop formation, aberrant vascularization, and other morphogenic abnormalities in mouse ontogeny, indicating the need to use intact organisms to study mammalian glycosylation (Ioffe and Stanley, 1994; Metzler et al., 1994). Since these findings, dozens of mouse lines have been created bearing germline defects in specific steps of the various glycosylation pathways. Remarkably, most of these inherited glycan deficiencies result in discrete phenotypes that reflect the dysfunction of specific cell types and diverse biological systems (Lowe and Marth, 2003).

Glycans possess distinct structural elements that govern interactions with other molecules. Glycans can promote or inhibit intra- and intermolecular binding that includes both homotypic and heterotypic interactions (Figure 3). Furthermore, mammalian glycans can be so substantial in size and frequency of attachment that they contribute the majority of mass and charge comprising some glycoproteins and glycolipids. For example, the neural cell adhesion molecule NCAM has a uniquely large negatively charged and developmentally regulated glycan structure known as polysialic acid that inhibits its homotypic NCAM protein-protein binding (Hoffman and Edelman, 1983). It has been shown that polysialic acid on NCAM must be regulated in mouse development for selective axonal trafficking, emotional and cognitive memory, and brain morphogenesis. Comparative studies of phenotypes have indicated that the polysialic acid glycan component is required for the proper execution of almost all of the physiological functions attributed to NCAM (Angata et al., 2004; Weinhold et al., 2006). Furthermore, mucins represent an example of a class of glycoproteins bearing a large number of O-glycan linkages that can induce steric effects that extend the conformation of a peptide backbone and may thereby serve to prominently display a large number of glycan decoys for pathogen lectin receptors in human resistance to oral and mucosal infection (Tabak, 1995).

Glycosylation determines ligand abundance for endogenous mammalian lectins. Lectins are glycan binding proteins that are typically highly selective for specific glycan structures and have therefore been extremely useful in studying glycan variation (Goldstein, 2002; Sharon and Lis, 2004). An expanding number of mammalian lectins have been identified and are classified by sequence motifs such as those that define the C-type lectins, S-type lectins, P-type lectins, and the Siglecs (Crocker and Varki, 2001; Drickamer and Taylor, 2003). It is likely that other lectin domains exist, as some proteins with apparent lectin activity do not contain canonical glycan binding motifs. Both lectin binding by chaperones and steric effects of glycans contribute substantially to protein folding prior to trafficking to the Golgi and beyond by reducing protein aggregation and retaining nascent unfolded glycoproteins in the endoplasmic reticulum (Parodi, 2000; Helenius and Aebi, 2004).

The interaction between glycans and lectins typically occurs with lower affinity than protein-protein interactions but with significant avidity given that most lectins can bind multiple glycan moieties and do so with high specificity. Glycans in the region of the glycocalyx can reach millimolar concentrations, and, when bound by endogenous lectin receptors, such interactions can be

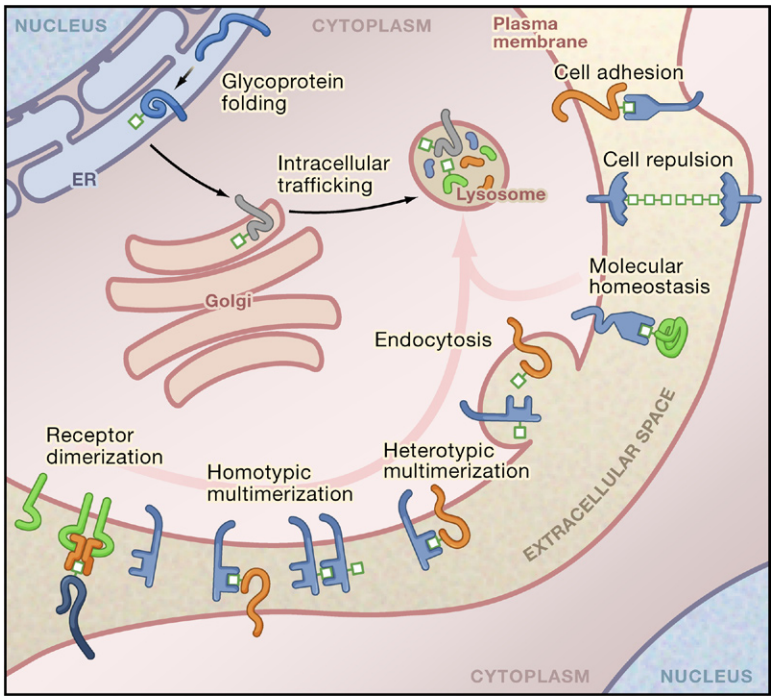

Figure 4. Cellular Mechanisms of Glycan Function

Glycans produced in the secretory pathway participate in multiple mechanisms of cellular regulation. The influence of glycans on protein-protein interactions encompasses a number of cellular functions that span from nascent protein folding and intracellular trafficking to roles in extracellular compartments where cell-cell communication is modulated by adhesion, molecular and cellular homeostasis, receptor activation and signal transduction, and endocytosis.

difficult to disrupt by lower concentrations of glycan ligand mimetics. Moreover, some mammalian lectins at the cell surface can appear fully occupied, or "masked," by binding to endogenous glycan ligands (Crocker and Varki, 2001). Yet even in the presence of glycan ligands residing on numerous glycoproteins, recent studies have observed that endogenous lectin binding in situ on primary cell surfaces can be surprisingly selective for ligands presented by distinct glycoproteins, implying that protein sequence and conformation contribute to mammalian lectin binding selectivity (Collins et al., 2004; Han et al., 2005; Ohtsubo et al., 2005). This may be further understood when glycoprotein ligands are visualized at the atomic level. Technical limitations at present, however, restrict acquisition of three-dimensional structures of glycoproteins to those produced by expression systems that lack most mammalian glycans.

Regulation of Cellular Mechanisms by Glycans

By binding to lectins and sterically modulating molecular interactions, mammalian glycans participate in multiple cellular mechanisms that contribute to health and disease. These basic structural paradigms have been found to regulate protein folding, cell adhesion, molecular trafficking and clearance, receptor activation, signal transduction, and endocytosis (Figure 4). Numerous functions of mammalian glycans are now evident, and some may have arisen early in the evolution of multicellular and vertebrate organisms.

Cell Adhesion

Lectin binding can evoke cell-cell adhesion and aggregation among primitive eukaryotic multicellular organisms (such as sponges) and may have contributed to cell-based kin recognition in the evolution of the earliest metazoans. Mammalian lectin involvement in cell-cell adhesion is best characterized for the selectins and their glycan ligands that include a key fucose linkage on the sialyl-Lewis X oligosaccharide (Lowe, 2003). This cell adhesion system is highly regulated on specific cell surfaces including the endothelium of the vasculature and on most leukocytes, thereby contributing to leukocyte trafficking responses that are essential in immune-system homeostasis, hematopoiesis, and inflammation (Rosen, 2004). Glycosylation can also modulate cell-cell adhesion in early mammalian embryos, and loss of some glycans disrupts fertilization, by mechanisms that are less well resolved. However, these results indicate that other lectin-ligand binding interactions governing cell-cell adhesion likely exist (Surani, 1979; Akama et al., 2002; Shur et al., 2004). When cellular portfolios of glycosyltransferases and glycosidases are altered in embryogenesis and disease and act upon a different assemblage of protein and lipid substrates, the production of rare glycoprotein and glycolipid epitopes can occur. Such unusual glycan-dependent epitopes often define today's known oncofetal and stem cell biomarkers that reflect the various binding specificities of different monoclonal antibodies. Glycan biomarkers of biologic and pathogenic processes further include selectin-dependent cell adhesion that is associated in some contexts with tumorigenic activity (Varki and Varki 2001; Chen et al., 2005).

Self/Nonself Recognition

The ability of mammalian lectins to recognize glycans from divergent organisms such as bacteria, yeast, and invertebrates underlies a mechanism of self/nonself recognition. This is exemplified by Toll-like receptor activation of the innate immune system from binding to bacterial glycan ligands (Barton and Medzhitov, 2003). Many lectins are expressed on cells of the mammalian innate immune system, and several bind to glycans specifically expressed among phylogenetically older organisms. Perhaps defects in mammalian glycosylation can influence self/nonself recognition and in some contexts lead to autoimmune disease. In this regard, the absence in

mice of α-mannosidase II, a glycosidase necessary in nonimmune cells for the normal synthesis of mammalian N-glycan structures bearing multimeric branching patterns, was found to induce a syndrome diagnostic of systemic lupus erythematosus (Chui et al., 2001). Therefore, in this model of autoimmune disease, pathogenesis may reflect endogenous epitope (N-glycan) modification (Wakeland et al., 2001). Innate immune-system recognition of endogenous glycans has been observed following the enzymatic breakdown of the glycan hyaluronan into smaller fragments upon dermal injury, which then activate the Tlr4 Toll-like receptor (Taylor et al., 2004). Normal mammalian glycan structures can also function as self-antigens in facilitating the development of immune cell types and their responses to infection, such as in the establishment of NKT cells by endogenous glycolipid ligands for CD1d (Zhou et al., 2004). Moreover, a significant proportion of endogenous mammalian glycans may participate in binding to major histocompatibility complexes and glycopeptide recognition by T cell receptors (Carbone and Gleeson, 1997). This may reflect a steric mechanism of glycan function in peptide processing and loading onto major histocompatibility complexes, in contrast with the role of glycans in endogenous lectin receptor binding.

Molecular Trafficking and Clearance

Glycan recognition by vertebrate lectins was discovered in studies of glycoprotein clearance from vascular circulation and retains commercial relevance in therapeutic glycoprotein engineering. Glycoprotein turnover by the liver can be dramatically accelerated by lectins known as asialoglycoprotein receptors, which bind to and internalize glycoproteins lacking or bearing insufficient sialic acid linkages (Ashwell and Harford, 1982). Yet the purpose of these receptors remains puzzling, and recent findings suggest that some sialic acid-bearing glycans can also be recognized by these lectins (Park et al., 2005). Given that serum glycoprotein levels appear normal in mice lacking the hepatic asialoglycoprotein receptor, endogenous ligands within normal mammals have not been identified yet (Ishibashi et al., 1994). Moreover, multiple asialoglycoprotein receptors exist among endothelial cells, hepatocytes, dendritic cells, macrophages, and Kupffer cells whose functions are currently unknown. Possible roles of asialoglycoprotein receptors include the modulation of acute inflammation responses and the phagocytosis of some pathogens bearing particular glycan linkages. More recently, both platelets and the von Willebrand factor (vWF) glycoprotein were identified as endogenous ligands for mammalian asialoglycoprotein receptors in the presence of a defect in ST3Gal-IV sialyltransferase expression, which resulted in decreased thrombosis in the mouse (Ellies et al., 2002). Unexpectedly, deficiency of a separate sialyltransferase, ST6Gal-I, failed to alter vWF and platelet homeostasis, which implies that the activities of specific glycosyltransferases and glycoproteins underlie the specificity in this lectin system. The development of glycan array technologies can facilitate both broad and quantitative characterization of glycan binding specificities among isolated lectins and thus may aid in identifying glycoprotein ligands that dictate the biological roles of these and other endogenous mammalian lectins (Bochner et al., 2005).

Genes encoding some lectins include multiple exons encoding different extracellular domains and motifs with distinct glycan binding specificities. The Man/GalNAc-4-SO_4 receptor of macrophages and endothelial cells may bind mannose linkages or sulfated GalNAc residues in this way, the latter being restricted to a small number of glycoprotein hormones such as lutropin that are released in a pulsatile manner from the pituitary gland in the brain. Sulfated glycoprotein hormone clearance by the Man/GalNAc-4-SO_4 receptor thereby modulates hormone bioactivity levels in vivo, which control female fecundity involving ovulation and embryo implantation (Mi et al., 2002). The role of mannose recognition by this lectin is unclear, although its absence from mice also induces serum glycoproteins typical of inflammation (Lee et al., 2002). Not all biological effects involving glycans influence cell adhesion or molecular trafficking and clearance from the extracellular milieu. In contrast, some glycans (particularly glycosaminoglycans) serve to sustain reservoirs of extracellular growth factors and cytokines for use in modifying receptor binding and activation.

Receptor Activation

Glycosylation modulates interactions of receptors and ligands with themselves, coregulatory molecules, and distinct membrane domains of intact cells, thereby altering signal transduction. For example, fibroblast growth-factor receptors bind to specific heparan sulfate glycosaminoglycans on some proteoglycans, and this interaction facilitates the copresentation of ligand monomers to achieve receptor dimerization and activation (Ornitz et al., 1992). Glycosaminoglycans exist as multiple subtypes that include not only heparan sulfate but also chondroitin sulfate, dermatan sulfate, and keratin sulfate and are major constituents of the extracellular matrix. Alteration of the sulfation pattern on glycosaminoglycans by endogenous sulfatases provides an example of a contrasting receptor activation mechanism. Here, selective desulfation unmasks Wnt binding domains, thus facilitating ligand presentation of Wnt to its receptor Frizzled (Ai et al., 2003). Multiple receptor systems appear to employ glycosaminoglycans on proteoglycans to modulate receptor activation in regulating morphogenesis and organogenesis signaling during early ontogeny. Moreover, the ability of glycosaminoglycans to bind to receptor ligands and retard their diffusion has emerged as a mechanism of establishing morphogen gradients that specify the differentiation of cell types during embryogenesis (Belenkaya et al., 2004).

Perhaps all types of mammalian glycans are involved in modulating receptor activation. Glycolipid variation regulates the activation of various cell-surface growth-

factor receptors by mechanisms that also modulate ligand binding and receptor dimerization (Miljan and Bremer, 2002). More unusual and rare glycan linkages are also regulatory. For example, Notch receptor trafficking, ligand binding, and activation is regulated by an O-fucose linkage produced in the endoplasmic reticulum and elaborated further in the Golgi by the Fringe glycosyltransferase (Haines and Irvine, 2003). In contrast, it can be difficult to identify the molecular constituents involved when a genetic defect in glycan formation alters the glycosylation of multiple proteins and/or lipids. This provides the impetus for developing glycoproteomics and glycolipidomics technologies that can extend the identification of the cellular proteome and lipidome to more precisely characterize biologically relevant molecules (Dell and Morris, 2001).

The phenotypes associated with glycan linkage defects have often revealed the roles of glycans in important cellular processes that were not previously thought to be regulated by protein glycosylation. Loss of the GlcNAcT-V glycosyltransferase, for example, was found to induce coclustering of T cell receptors at the cell surface, reducing the threshold for immune activation and causing autoimmune disease (Demetriou et al., 2001). This was attributed to the loss of T cell receptor binding to galectin-3, one of a family of lectins implicated in maintaining a spatially confined arrangement of cell-surface glycoproteins (Morris et al., 2004). In addition, receptors for EGF and TGF-β on epithelial cells, the IgM B cell antigen receptor (BCR), and the glucose transporter 2 glycoprotein on pancreatic β cells are all profoundly modulated by mammalian glycosylation, indicating that different glycans produced in the Golgi maintain these receptors at the cell surface by reducing their rates of endocytosis (Partridge et al., 2004; Ohtsubo et al., 2005; Collins et al., 2006; Grewal et al., 2006).

Endocytosis

Endocytosis plays a critical role in cell biology by providing access to material from extracellular compartments, directing molecular cargo to distinct organelles, terminating or modifying signals emanating from the cell surface, and inducing the turnover of cell-surface molecules. Recent studies have found that mammalian glycans produced in the Golgi modulate the endocytosis of cell-surface glycoproteins and thereby control receptor expression and hence thresholds for cell signaling. The glycan linkage produced by GlcNAcT-V, for example, retards EGF and TGF-β receptor endocytosis, thereby altering receptor activation and signaling among epithelial carcinoma cells (Partridge et al., 2004). Different cell types appear to use distinct glycans to alter rates of endocytosis, and selectivity can further exist among the cell-surface glycoproteins of a given cell type. Pancreatic β cells, but not hepatocytes, for example, appear to use a lectin mechanism to decrease the rate of endocytosis involving glucose transporter 2—but not other similarly glycosylated proteins—in preventing the onset of type 2 diabetes (Ohtsubo et al., 2005).

Lectins and their ligands can modulate cell-surface receptor activation coincident with the regulation of receptor endocytosis. This is perhaps best characterized for CD22 (Siglec-2), a mammalian B cell-specific lectin that modulates BCR activation and thereby alters humoral immune responses. Like most Siglecs, CD22 contains both an extracellular lectin domain that binds to specific sialic acid-bearing glycans and intracellular protein sequence motifs that can bind to intracellular signal transduction proteins and regulate phosphorylation. The cytoplasmic domain of CD22 plays a negative regulatory role by recruiting the Shp-1 phosphatase and thereby downmodulating immune signaling when CD22 is associated with the BCR (Tedder et al., 1997). Genetic deficiency of the ST6Gal-I glycosyltransferase in the mouse results in loss of CD22 Siglec ligands and diminishes BCR activation and signaling. This correlated with increased colocalization of BCRs with CD22, constitutive Shp-1 recruitment to CD22, decreased protein phosphotyrosine levels, elevated BCR trafficking to clathrin microdomains, and enhanced endocytosis of BCRs (Collins, et al., 2006; Grewal et al., 2006). In mice deficient for both CD22 and ST6Gal-I, BCR signaling as well as microdomain association and endocytotic rate were restored to normal. Moreover, the reduced level of humoral immunity in mice with ST6Gal-I deficiency further prevented the development of autoimmune disease, unlike findings in their normal counterparts that bore lower levels of BCR-CD22 interactions. This lectin-ligand system therefore modulates the threshold of B cell immune activation in a mechanism linked to BCR trafficking and endocytosis.

Diseases of Glycosylation

Endocytosis and trafficking to lysosomes are typically involved in degrading proteins and glycans. Defects in these catabolic steps include glycosidase deficiencies that form the bases for cellular storage disorders such as Gaucher's, Niemann-Pick type C, Sandhoff's, and Tay-Sachs diseases. A subset of these maladies can now be clinically treated using a small-molecule analog of a plant-derived organic compound that reduces glucosyltransferase I activity. This compound represents the first drug marketed for human disease therapy that inhibits an endogenous mammalian glycosyltransferase as a mechanism of action (Butters et al., 2005). Remarkably, unlike protein kinase inhibitors that compete with the donor substrate ATP, glycosyltransferase inhibitors generally do not compete with nucleotide sugar donor substrates but are competitive with specific glycan acceptor substrates.

Defects in the anabolic process of glycan formation are more typically considered as human diseases of glycosylation. I cell disease was the first to be identified and was shown to result from failure to produce the mannose 6-phosphate modification on N-glycans in the Golgi. This modification acts as a signal that is necessary for trafficking of hydrolases to the lysosome. Absence of this

signal therefore also causes a storage disorder due to a deficiency in protein catabolism (Kornfeld and Sly, 1985). The genetic basis of I cell disease encompasses mutations in the gene encoding the GlcNAc-phosphotransferase α/β subunit precursor (Kudo et al., 2006).

An increasing number of diseases of glycosylation are being discovered, especially in the pediatric clinic among children during the first few years of life. The congenital disorders of glycosylation (CDGs) refer to a large number of syndromes that include severe morphogenic and metabolic defects associated with general failure to thrive, most of which have been linked to distinct steps in glycan formation (Jaeken and Carchon, 2004). Those among the most prevalent grouping (CDG type 1a) are due to hypomorphic mutations in the *PMM2* gene. Mutations in this gene diminish synthesis of the dolichol-oligosaccharide precursor, which is essential for initiation of N-glycosylation, and hence decrease the frequency of N-glycosylation. A small number of CDG subtypes reflect defects in the formation of nucleotide sugar donor substrates, and some can be treated by dietary intake of precursor saccharides, as exemplified by mannose ingestion, which reverses signs of CDG type 1b disease (Niehues et al., 1998). At present, at least 20 separate genes and more than 100 allelic variants have been identified among the CDGs, many of which involve hypomorphic mutations that impact on glycosyltransferase and glycosidase enzyme activities, as well as genetic defects in synthesis and transport of nucleotide sugar donors (Aebi and Hennet, 2001; Freeze, 2006).

How many diseases of glycosylation exist in the human population, and what are their frequencies of occurrence? The answers are not yet known. Their detection is serendipitous due to infrequent clinical application of serum transferrin isoelectric focusing and other electrophoretic techniques to the analysis of specific glycoproteins. Although these are presently essential diagnostic tools, they are nevertheless unable to detect most glycan linkage defects. Thus, human glycosylation disorders identified so far are primarily severe syndromes, many of which reflect the disruption of early steps in the pathways of glycan biosynthesis. In contrast, mouse models of defective glycosylation have been engineered to ablate both early and late biosynthetic steps. From these studies, it has become evident that the later the defect in glycan synthesis, the less likely it is that a single glycosyltransferase or glycosidase deficiency will cause a severe multisystemic disorder leading to dysmorphic features, failure to thrive, and lethality. The only interspecies and biochemically comparable model documented at present is loss of GlcNAcT-II glycosyltransferase activity, which is the basis for the human CDG-IIa syndrome. Mice lacking GlcNAcT-II activity closely phenocopy human CDG-IIa disease signs, and strain-associated variations in disease severity were also observed (Wang et al., 2001). These findings suggest that animal models may be useful in studying the molecular and pathogenic bases for human diseases of glycosylation.

Connections between glycans and human disease are now being made every year and have expanded to include mild as well as severe syndromes, with timing of onset that can span from early neonatal to adult life. The defects and symptoms further often imply cell- and tissue-specific dysfunction. Human spondylocostal dystosis has been recently linked to inactivation of the Lunatic Fringe glycosyltransferase corresponding to a defect in Notch signaling events essential in ontogenic patterning of the axial skeleton during embryogenesis (Sparrow et al., 2006). Humans lacking a functional ST3Gal-V glycosyltransferase, also known as GM3 synthase, develop an early neurological disorder termed infantile-onset symptomatic epilepsy (Simpson et al., 2004). Mice lacking this enzyme at first appear unaffected, but later, some exhibit seizures as adults. Human hereditary multiple exostoses is an autosomal-dominant bone disease characterized by multiple cartilaginous tumors that occur throughout childhood and is caused by defects in the glycosyltransferases required for synthesis of the heparan sulfate glycosaminoglycan (Duncan et al., 2001). Paroxysmal nocturnal hemoglobinuria usually occurs in adulthood and results from somatic mutation within the bone-marrow stem cell population resulting in a defect in GPI anchor synthesis (Bessler et al., 1994).

The participation of lectins and altered lectin binding would also be expected to contribute to pathogenesis among human diseases and reflect the physiologic functions of glycosylation; however, few examples currently exist. This may imply that the roles of human lectins overlap in vivo, a possibility consistent with the minimal phenotypic consequences of most lectin deficiencies thus far induced and studied in the mouse. Alternatively, some lectin mutations in humans may have a more severe impact and disrupt embryogenesis, resulting in early lethality. However, some human muscular dystrophies are due to mutations in laminin as well as in glycosyltransferases operating in the pathway of O-mannose glycan formation on α-dystroglycan (McGowan and Marinkovich, 2000; Yoshida et al., 2001; Michele et al., 2002). These defects occur coincident with disruption of α-dystroglycan attachment to laminin in what resembles a lectin binding mechanism.

Diseases that alter glycosylation have revealed new mechanisms regarding how glycan formation is regulated. Inherited deficiencies in the conserved oligomeric Golgi (COG) complex members COG1 and COG7 in humans result in a severe childhood disease and perinatal death, respectively (Wu et al., 2004; Foulquier et al., 2006). Cells from these patients lack expression of many different glycans, which may contribute to the pathogenic basis of these syndromes. And the elusive cause of human Tn syndrome, an adult hematologic disorder, was recently identified as a genetic mutation in the *COSMC* gene encoding a protein with chaperone function required for β1-3 galactosyltransferase activity in O-glycan biosynthesis (Ju and Cummings, 2005). Human diseases of glycosylation are not necessarily linked to

changes in glycosyltransferase and glycosidase activity. Endogenous sporadic or germline modifications of the primary structure of single proteins may also contribute. A recent analysis of glycoprotein mutations in the human disease database revealed a larger-than-random occurrence of amino acid changes in proteins that predict a gain-of-N-glycosylation, and those that were tested indeed acquired an N-glycan concurrent with glycoprotein dysfunction (Vogt et al., 2005).

Summary and Future Directions

Research on the function of mammalian glycosylation demonstrates that glycans are involved in multiple disciplines spanning ontogeny, immunology, neurobiology, hematology, and metabolism and are linked to a number of genetic diseases. It is increasingly apparent that biological networks exploit both glycosylation and phosphorylation by using cell-surface glycans to organize plasma membrane receptors and control the recruitment of intracellular signal transduction mediators. Hence, discoveries encompassing glycobiology will contribute to the knowledge needed to decipher the biological systems that comprise living organisms.

In some cases, glycans are the only source of variation between otherwise identical glycoproteins produced within the same cell, resulting in "microheterogeneity." Such heterogeneity and its regulation may play a role in setting thresholds for molecular interactions that govern cellular responses. For example, certain glycoforms representing IgG microheterogeneity are increased in arthritis (Parekh et al., 1985). In addition, the anti-inflammatory property of IgG binding to Fc receptors is elevated among the subset of total circulating IgGs that are sialylated, compared to the fraction of IgGs that have fewer or no sialic acid linkages (Kaneko et al., 2006). When glycosylation does occur, it may not always alter the folding, interactions, function, or fate of all proteins similarly modified. The function of an enzyme in modifying many substrates does not dictate that all such modifications will manifest a biological purpose. This possibility is conspicuous in recent analyses of mouse models of glycan deficiencies in which the phenotype can be attributed to the modulation of single glycoproteins including CD22, glucose transporter 2, TGF-β1 receptor, and von Willebrand factor (Ellies et al., 2002; Ohtsubo et al., 2005; Wang et al., 2005; Collins et al., 2006; Grewal et al., 2006). Glycans attached to some proteins and lipids may be physiologically inert. Such nondeleterious glycosylation might impart subtle characteristics to glycoproteins and glycolipids that become advantageous in response to new selective pressures applied by exogenous and pathogenic stimuli. In contrast, those endogenous glycans with essential physiologic purpose would further maintain the sequence conservation evident among glycosyltransferase and glycosidase gene orthologs.

Although it is not possible to predict a cell's glycan repertoire from analyses of genomic DNA sequences, it is clear that the highly ordered, regulated, and conserved pathways of glycan diversification have evolved to play specific biological roles, many of which are essential and have persisted throughout mammalian speciation. As biomedical connections to glycobiology expand, therapeutic rationales for modulating and detecting glycan production and variation continue to emerge. Glycomics-based initiatives can assist by facilitating the development of techniques to produce complex glycan structures, detect glycan interactions with other molecules, and enable glycan-based methodologies to modulate cell processes (Schweizer and Hindsgaul, 1999; Seeberger and Werz, 2005; Paulson et al., 2006). Research encompassing glycobiology is highly interdisciplinary and increasingly successful in explaining how extracellular signals originate and how cell-cell communication is established among multicellular organisms. The amount of the genome invested in glycosylation compared to what has been understood thus far implies that only a small fraction of glycan function has been decoded. The integration of glycobiology into mainstream education and research will further prepare the next generations of scientists to oversee the merging of now disparate biological disciplines into a cohesive rendering of the molecular mechanisms that govern physiology and disease.

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