Glycomics Hits the Big Time

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Cells run on carbohydrates. Glycans, sequences of carbohydrates conjugated to proteins and lipids, are arguably the most abundant and structurally diverse class of molecules in nature. Recent advances in glycomics reveal the scope and scale of their functional roles and their impact on human disease.

By analogy to the genome, transcriptome, or proteome, the "glycome" is the complete set of glycans and glycoconjugates that are made by a cell or organism under specific conditions. Therefore, "glycomics" refers to studies that attempt to define or quantify the glycome of a cell, tissue, or organism (Bertozzi and Sasisekharan, 2009). In eukaryotes, protein glycosylation generally involves the covalent attachment of glycans to serine, threonine, or asparagine residues. Glycoproteins occur in all cellular compartments. Glycans are also attached to lipids, often ceramide, which is comprised of sphingosine, a hydrocarbon amino alcohol and a fatty acid. Complex glycans are mainly attached to secreted or cell surface proteins, and they do not cycle on and off of the polypeptide. In contrast, the monosaccharide O-linked N-acetylglucosamine (O-GlcNAc) cycles rapidly on serine or threonine residues of many nuclear and cytoplasmic proteins. Identifying the number, structure, and function of glycans in cellular biology is a daunting task but one that has been made easier in recent years by advances in technology and by our growing appreciation of how integral glycans are to biology (Varki et al., 2009).

The scope of the glycomics challenge is immense. The covalent addition of glycans to proteins and lipids represents not only the most abundant posttranslational modification (PTM), but also by far the most structurally diverse. Although it is commonly stated that more than 50% of all polypeptides are covalently modified by glycans (Apweiler et al., 1999), even this estimate is far too low because it fails to include that myriad nuclear and cytoplasmic proteins are modified by O-GlcNAc (Hart et al., 2007). Even though the generic term "glycosylation" is often used to categorize and lump all glycan modifications of proteins into one bin, side by side with other posttranslational modifications such as phosphorylation, acetylation, ubiquitination, or methylation, such a view is not only inaccurate, but also is completely misleading. If one only considers the linkage of the first glycan to the polypeptide in both prokaryotic and eukaryotic organisms, there are at least 13 different monosaccharides and 8 different amino acids involved in glycoprotein linkages, with a total of at least 41 different chemical bonds known to be linking the glycan to the protein (Spiro, 2002). Importantly, each one of these unique glycan:protein linkages is surely as different in both structure and function as protein methylation is from acetylation.

Of course, this modification is not only about a single linkage. When structural diversity of the additional oligosaccharide branches of glycans and the added diversity of complex terminal saccharides on glycans, such as fucose or sialic acids (about 50 different sialic acids are known [Schauer, 2009]), are taken into account, the molecular diversity and varied functions of protein-bound glycans rapidly increase exponentially. Just the "sialome" (Cohen and Varki, 2010) rivals or exceeds many other posttranslational modifications in abundance and structural/functional diversity. In addition, chemical modifications, such as phosphorylation, sulfation, and acetylation, increase the glycan structural/functional diversity even more. Thus, categorizing glycosylation as a single type of posttranslational modification is neither useful nor at all reflective of reality.

Dynamic Structural Complexity Underlies Glycan Functions

Glycoconjugates provide dynamic structural diversity to proteins and lipids that is responsive to cellular phenotype, to metabolic state, and to the developmental stage of cells. Complex glycans play critical roles in intercellular and intracellular processes, which are fundamentally important to the development of multicellularity (Figure 1). Unlike nucleic acids and proteins, glycan structures are not hardwired into the genome, depending upon a template for their synthesis. Rather, the glycan structures that end up on a polypeptide or lipid result from the concerted actions of highly specific glycosyltransferases (Lairson et al., 2008), which in turn are dependent upon the concentrations and localization of high-energy nucleotide sugar donors, such as UDP-N-acetylglucosamine, the endpoint of the hexosamine biosynthetic pathway. Therefore, the glycoforms of a glycoprotein depend upon many factors directly tied to both gene expression and cellular metabolism.

There are at-least 250 glycosyltransferases in the human genome, and it has been estimated that about 2% of the human genome encodes proteins involved in glycan biosynthesis, degradation, or transport (Schachter and Freeze, 2009). Biosynthesis of the nucleotide sugar donors is directly regulated by nucleic acid, glucose, and energy metabolism, and the compartmentalization of these nucleotide sugar donors is highly regulated by specific transporters. Protein glycosylation is therefore controlled by rates of polypeptide translation and protein folding, localization of and competition between glycosyltransferases,

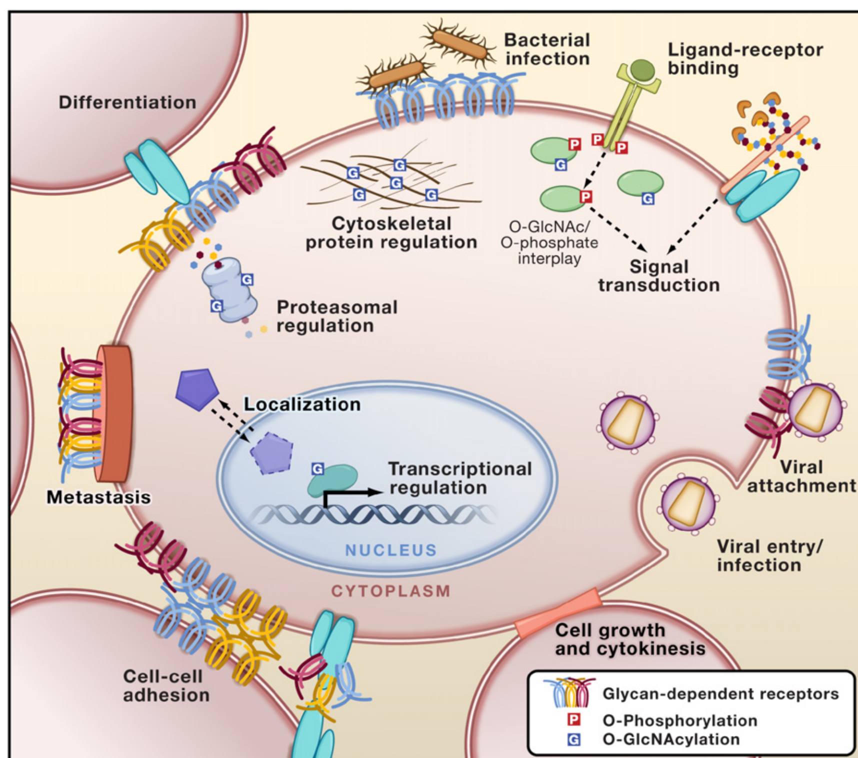

Figure 1. Glycans Permeate Cellular Biology
Complex glycans at the cell surface are targets of microbes and viruses, regulate cell adhesion and development, influence metastasis of cancer cells, and regulate myriad receptor:ligand interactions. Glycans within the secretory pathway regulate protein quality control, turnover, and trafficking of molecules to organelles. Nucleocytoplasmic O-linked N-acetylglucosamine (O-GlcNAc) has extensive crosstalk with phosphorylation to regulate signaling, cytoskeletal functions, and gene expression in response to nutrients and stress.

cellular concentration and localization of nucleotide sugars, the localization of glycosidases, and membrane trafficking. Thus, individual glycosylation sites on the same polypeptide can contain different glycan structures that reflect both the type and status of the cell in which they are synthesized. For example, the glycoforms of the membrane protein Thy-1 are very different in lymphocytes than they are in brain, despite having the same polypeptide sequence (Rudd and Dwek, 1997). Conversely, even small changes in polypeptide sequence or structure will alter the types of glycan structures attached to a polypeptide. For example, histocompatibility antigen polypeptides with more than 90% sequence homology contain different N-linked glycan profiles at individual sites, reflective of their allelic type, even when they are synthesized within the same cells (Swiedler et al., 1985). Thus, site-specific protein glycosylation is highly regulated by gene expression of glycan-processing enzymes, by polypeptide structure at all levels, and by cellular metabolism.

Technology of Glycomics

A detailed understanding of cellular processes will require a detailed appreciation of the glycans modulating proteins and pathways. Although this ultimate goal of glycomics is laudable, we are a very long way from having the technology to completely characterize the glycome of even a simple cell or tissue. Not only is the glycome much more complex than the genome, transcriptome, or proteome, as noted above, it is also much more dynamic, varying considerably not only with cell type, but also with the developmental stage and metabolic state of a cell. Even very conservative estimates indicate that there are well over a million different glycan structures in a mammalian cell's glycome. However, upon considering "functional glycomics," it is estimated that the binding sites of glycan-binding proteins (GBPs), such as antibodies, lectins, receptors, toxins, microbial adhesions, or enzymes (Figure 1), can accommodate only up to two to six monosaccharides within a glycan structure (Cummings, 2009). Therefore, the number of specific glycan substructures that bind to biologically important GBPs in a cell may be fewer than 10,000, a number that is within the realm of current analytical and, if targeted, chemical or enzymatic synthetic capabilities.

Until recently, the lack of tools and the inherent complexity of glycans have been major barriers preventing most biologists from embracing the importance of glycans in biology. Recent technological advances have significantly lowered these barriers. Indeed, the tools of glycomics and the subfields of glycoproteomics, glycolipidomics, and proteoglycomics have all progressed substantially in recent years (Krishnamoorthy and Mahal, 2009; Laremore et al., 2010). Major technological advances, many of which are shared with proteomics, have recently allowed semiquantitative profiling of glycans and glycoproteins (Krishnamoorthy and Mahal, 2009; Vanderschaeghe et al., 2010). Some of these advances are the result of the National Institute of General Medical Science's (NIGMS) support of the Consortium for Functional Glycomics (CFG), which has served to focus and assist more than 500 researchers on issues related to glycomics (Paulson et al., 2006; Raman et al., 2006).

Kobata and colleagues were among the first to profile N-glycans, well before the current concepts of glycomics were conceived. Despite the lack of many modern methods, their pioneering work was characterized by a high level of rigor in defining the arrays of N-glycan structures present in cells and tissues and on specific proteins (Endo, 2010). Currently, a wide variety of high-resolution and highly sensitive methods are available, including capillary electrophoresis (CE), high-performance liquid chromatography (HPLC), and lectin microarrays.

Glycans are often profiled after their release from polypeptides, which results in the loss of any information about proteins and sites to which they were attached. Even though it is much more difficult, it is also much preferable to perform

glycopeptide profiling (glycoproteomics) to first identify attachment sites prior to detailed profiling or structural analysis of the glycans present on a polypeptide. The ultimate goal of glycoproteomics, which is to define all of the molecular species (glycoforms) of glycoproteins in a cell or tissue, has not yet been realized for any glycoprotein with more than one glycan attachment site. N-glycans are generally released from proteins by peptide-N-glycosidase F (PNGase F), which cleaves most, but not all, N-glycans. Unfortunately, no such broadly specific enzyme exists for O-glycans, which are generally released by chemical methods, such as alkali-induced β elimination, or by hydrazinolysis. However, for relatively pure glycoproteins, so called "top-down" mass spectrometric methods, which do not involve prior release of the glycans, may eventually prove useful, as instrumentation and methods improve (Reid et al., 2002).

Due to the small sample sizes involved, most CE or HPLC separation methods require chemical modification of released glycans with fluorescent compounds. CE and HPLC methods provide high-resolution separation of glycans, and when combined with laser-induced fluorescent detection (LIF), tagged glycans can be detected in the low femtomole range. High pH anion-exchange chromatography (HPAEC) with pulsed-amperometric detection separates glycans with high resolution and detects them with high sensitivity without chemical modification, but the high alkalinity employed can be problematic for some labile structures.

Lectins, which are defined as carbohydrate-binding proteins that are neither antibodies nor enzymes, have a wide range of glycan binding specificities, suitable for partial characterization of a glycome. Lectin microarrays use methods and equipment similar to that employed for nucleic acid arrays. Given the large number of different lectins available, lectin microarrays can provide information about the glycome in a high-throughput fashion, which is particularly useful in profiling glycans produced by infectious organisms (Hsu et al., 2006). In the future, it is highly likely that glycomics will play a central role in combating infectious disease. However, many technical issues remain to be resolved, such as standardization required for clinical use, the development of purified recombinant lectins, and better definition of the specificities of many lectins (Gupta et al., 2010).

Both matrix-assisted laser desorption ionization (MALDI) and electrospray mass spectrometry have played a key role in glycan profiling and in glycoproteomics (An et al., 2009; North et al., 2010; Zaia, 2010). For biomarker discovery, affinity enrichment approaches, based upon chemical modification and solid-phase extraction of N-linked glycoproteins, have proven useful in profiling N-linked glycoprotein sites from serum-or even from paraffin-embedded tissues (Tian et al., 2009). Recently, using lectin binding combined with advanced mass spectrometric methods, thousands of N-glycan attachment sites have been mapped, a prerequisite for understanding their functions (Zielinska et al., 2010).

Given the structural diversity of glycans, all of these glycomic approaches generate vast amounts of data. Glycan bioinformatics has made great strides within recent years with major efforts from several laboratories (Aoki-Kinoshita, 2008). At least four major publicly available carbohydrate databases (Glycosciences.de, KEGG GLYCAN, EurocarbDB, and CFG) are now maintained, and efforts to structure them in a uniform format have been in progress for quite some time. In addition, the Carbohydrate-Active EnZyme database (CAZy) has played a key role in providing a global understanding of carbohydrate active enzymes, documenting their evolutionary relationships, providing a framework for elucidating common mechanisms, and establishing the relationship between glycogenomics and glycomes expressed by cells (Cantarel et al., 2009). Moreover, recent advances in bioinformatic analysis tools for complex glycomic mass spectrometry data sets have allowed complex data to be presented in formats useful to nonexperts in all fields of biology (Ceroni et al., 2008; Goldberg et al., 2005).

Perhaps one of the most important contributions to the field of functional glycomics has been the development of well-defined glycan microarrays, which currently display more than 500 different glycan structures (Smith et al., 2010). The NIGMS-supported Consortium for Functional Glycomics (CFG) has generated and made publicly available custom-made DNA microarrays that represent glycosyltransferases and glycan-binding proteins. The CFG also has developed databases that present phenotypic and biochemical data on glycosyltransferase knockout mice. Even though knocking out a single glycosyltransferase gene often affects hundreds of glycoconjugates and myriad biological processes, these mutant mice have proven valuable in revealing the fundamental biological importance of glycans. The microarrays and the databases produced by the CFG member community at large are publically available on the CFG website (http://www.functionalglycomics.org) and have resulted in a profound increase in our understanding of the binding specificities of GBPs, including lectins key to inflammation and immunity, and on infectious microbes or viruses. However, a major barrier preventing glycan biology from being incorporated more into the mainstream is the continued failure by the community to adopt a universally standard glycan structural format and database that are easily accessed worldwide. Most importantly, glycan databases must eventually be incorporated into standard interactive databases that are supported by public agencies (such as NCBI or EMBL) before glycan biology can be fully integrated into the wider research community.

From Glycomics to Biology

Glycans are directly involved in almost every biological process and certainly play a major role in nearly every human disease (Figure 1). Genetic studies in tissue culture cells indicate that specific complex glycan structures are generally not essential to a cell growing in culture, indicating that most of the functions of complex glycans are at the multicellular level. In contrast, the cycling monosaccharide, O-GlcNAc, on nuclear and cytoplasmic proteins, is essential even at the single cell level in mammals (Hart et al., 2007).

The critical roles of glycans in mammals are now well established not only by the dearth of mutations in glycan biosynthetic enzymes that survive development, but also by the severe phenotypes generated when such mutations are not lethal. These severe phenotypes are clearly illustrated by the congenital disorders of

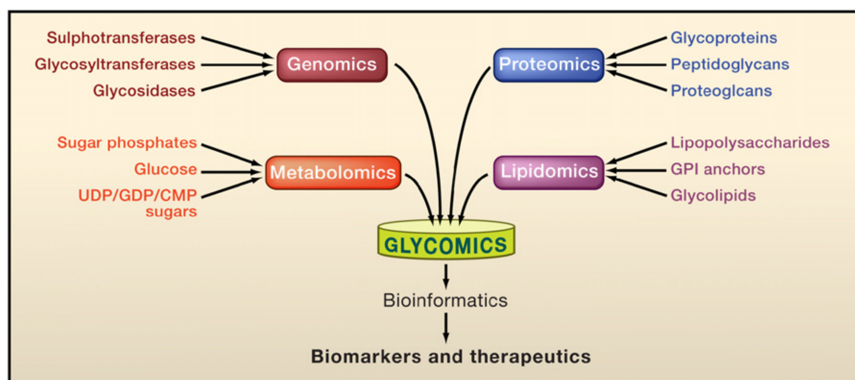

Figure 2. Glycomic Complexity Reflects Cellular Complexity
Given that glycan structures are regulated by metabolism and glyco-enzyme expression and glycans modify both proteins and lipids, functional glycomics also requires the tools of genomics, proteomics, lipidomics, and metabolomics (modified after Packer et al., 2008).

glycosylation (CDGs) (Schachter and Freeze, 2009), which are associated with severe mental and developmental abnormalities. Also, the severe muscular dystrophy that results from defective O-glycosylation of α-dystroglycan (Yoshida-Moriguchi et al., 2010) further illustrates how a mutation in a glycan biosynthetic enzyme results in a devastating disease. The interplay between O-GlcNAcylation and phosphorylation on nuclear and cytoplasmic proteins plays a key role in the etiology of diabetes, neurodegenerative disease, and cancer (Hart et al., 2007; Zeidan and Hart, 2010).

It has long been appreciated that alterations in cell surface glycans contribute to the metastatic and neoplastic properties of tumor cells (Taniguchi, 2008). The functions of many receptors are modulated by their glycans, such as modulation of Notch receptors by the action of specific glycosyltransferases (Moloney et al., 2000), which regulate Notch's activation by its ligands, affecting many developmental events. Selectins, which specifically bind to a subset of fucosylated and sialylated glycans, play a critical role in leukocyte homing to sites of inflammation. Indeed, a selectin inhibitor is currently in phase two clinical trials for vaso-occlusive sickle cell disease (Chang et al., 2010). Siglecs, which are a family of cell surface sialic acid-binding lectins, play a fundamental role in regulating lymphocyte functions and activation. Recent studies on galectins, a family of β-galactoside-binding lectins, have shown that they play a critical role in the organization of receptors on the cell surface and play important roles in immunity, infections, development, and inflammation (Lajoie et al., 2009). Proteoglycans and glycosaminoglycans play a key role in the regulation of growth factors, in microbial binding, in tissue morphogenesis, and in the etiology of cardiovascular disease. Proteoglycans are perhaps the most complicated and information-rich molecules in biology, and progress in proteoglycomics has begun to accelerate (Ly et al., 2010). Nearly all microbes and viruses that infect humans bind to cells by attaching to specific cell surface glycans. Glycomics and glycan arrays will have a substantial impact upon future research toward both diagnosing and preventing infectious disease.

Some of the most important drugs on the market are already the result of glycomics. The anti-flu virus drugs Relenza and Tamiflu are structural analogs of sialic acids that inhibit the flu virus neuraminidase and the transmission of the virus. Natural heparin, a sulfated glycosaminoglycan, and chemically defined synthetic heparin oligosaccharides have long been widely used in the clinic as anticoagulants and for many other clinical uses. Hyaluronic acid, a nonsulfated glycosaminoglycan, is used in the treatment of arthritis. Many recombinant pharmaceuticals, including therapeutic monoclonal antibodies, are glycoproteins, and their specific glycoforms are key to their bioactivity and half lives in circulation and to their possible induction of deleterious immune responses when they do not contain the correct glycans. Given this landscape, the pharmaceutical industry and the US Food and Drug Administration are rapidly realizing the critical importance, in terms of both bioactivity and safety, of carefully defining the glycoforms of any therapeutics derived from glycoconjugates.

Glycoproteomics, Glycolipidomics, and Biomarkers

Clinical cancer diagnostic markers are often glycoproteins, but most current diagnostic tests only measure the expression of the polypeptide. Clearly, given the long known alterations in glycans associated with cancer, it is highly likely that cancer markers that detect specific glycoforms of a protein will have much higher sensitivity and specificity for early detection of cancer (Packer et al., 2008; Taniguchi, 2008). Thus, the convergence of glycomics and glycoproteomics is key to the discovery of biomarkers for the early detection of cancer (Taylor et al., 2009). Recently, the Food and Drug Administration has approved fucosylated α-fetoprotein as a diagnostic marker of primary hepatocarcinoma. In addition, fucosylated haptoglobin may be a much better marker of pancreatic cancer than simply monitoring the expression of the haptoglobin polypeptide. Indeed, The National Cancer Institute has begun an initiative to discover, develop, and clinically validate glycan biomarkers for cancer (http://glycomics.cancer.gov/). System biology analyses of the glycome to identify biomarkers of human disease will, by necessity, also employ many of the same methods used by genomics, proteomics, metabolomics, and lipidomics (Figure 2) (Packer et al., 2008). Due to the critical roles of glycans in cardiovascular disease and lung disease and in the functions of blood cells, the National Heart Lung and Blood Institute (NHLBI) has recognized an acute need to train more researchers in the area of glycosciences by creating a "Program of Excellence in Glycosciences," which will not only support collaborative research, but will also provide hands-on laboratory training in the methods of glycosciences to fellows.

Thus, though our knowledge about the biology of glycans and glycomics continues to lag behind more mainstream fields of genomics and proteomics, technological advances in glycomics in the

last 5 years have begun to accelerate the integration of glycobiology into the other major fields of biomedical research. A complete mechanistic understanding of the etiology of almost any disease will depend upon the elucidation of the functions of all posttranslational modifications but will especially depend upon our understanding the many roles of glycans, the most abundant and structurally diverse type of posttranslational modification.

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