

## Current ideas on the significance of protein glycosylation

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### Abstract

Carbohydrate has been removed from a number of glycoproteins without major effect on the structure or enzyme activity of the protein. Thus carbohydrate has been suggested to underly a non-primary function for proteins, such as in relatively non-specific interactions with other carbohydrates or macromolecules, stabilization of protein conformation, or protection from proteolysis. This non-specific concept is consistent with both the general similarity in carbohydrate structure on very diverse glycoproteins and the frequent structural microheterogeneity of carbohydrate chains at given sites. The concept is supported in a general sense by the viability of cells whose glycosylation processes have been globally disrupted by mutation or pharmacological inhibitors.

In contrast to the above observations, other studies have revealed the existence of specific, selective receptors for discrete oligosaccharide structures on glycoproteins which seem to be important for compartmentalization of the glycoprotein, or the positioning of cells on which the glycoprotein is concentrated. Sometimes multivalency in the carbohydrate-receptor interaction is crucial. There are additional possible roles for carbohydrate in the transduction of information upon binding to a receptor. The possibility of specific roles for carbohydrate is supported by the existence of numerous unique carbohydrate structures, many of which have been detected as glycoantigens by monoclonal antibodies, with unique distributions in developing and differentiated cells.

This article attempts to summarize and rationalize the contradictory results. It appears that in general carbohydrate does in fact underlie only roles secondary to a protein's primary function. These secondary roles are simple non-specific ones of protection and stabilization, but often also satisfy the more sophisticated needs of spatial position control and compartmentalization in multicellular eukaryotic organisms. It is suggested that there are advantages, evolutionarily speaking, for the shared use of carbohydrate for non-specific roles and for specific roles primarily as luxury functions to be executed during the processes of cell differentiation and morphogenesis.

### Introduction

New information about the significance of protein glycosylation is rapidly accumulating. This can be attributed to advancements in methods to detect and isolate new structures, to determine structure, and to modify carbohydrate structures experimentally. Nevertheless, the field continues to experience profound unrest. Many studies have failed to un-

cover significant cellular or molecular consequences for dramatic changes in carbohydrate structures. Other studies have pointed to isolated specific roles but no sweeping generalizations of function have been possible. Interpreters of the former studies would attribute apparent specific effects to secondary consequences of changes in carbohydrate on protein structure. Interpreters of the latter studies would argue that the former studies

simply failed to ask the right questions of function. It is clear that there is much to be done to extend our knowledge for this particular type of posttranslational modification.

The purpose of this article is to outline the scope of investigations presently being carried out in this subject area and to generalize the possible significance of these new findings. In particular, this article will attempt to compare and contrast the two prevailing views in the field. As intimated above, one view is that glycosylation plays a relatively non-specific role in maintaining and preserving polypeptide function. The alternate view is that carbohydrate structures participate in numerous specific interactions with discrete protein receptors, and that these interactions lead to predictable modifications in the localization or activity of the glycoprotein. These opposing views will be simply referred to as the *non-specific* and *specific* models. The importance of this issue is dramatized by the fact that nearly all secretory, extracellular matrix, cell surface and lysosomal proteins are glycosylated.

There are many published comprehensive reviews on aspects of this subject (1-3, 14, 20, 43, 105, 110, 115, 130, 175) and the reader is referred to these for more detailed summaries and documentation. Since this article will in part serve as an introduction and a background to the others in this volume, an overview of general features of the glycosylation process has been provided to precede the summary of existing and new ideas in the field which follows.

### General structures of protein-linked carbohydrate

Oligosaccharides linked to asparagine occur in cellular slime molds, yeast, higher plants, insects, and humans, and several essential features of these structures are highly conserved throughout this phylogenetic range (14). Typically though not invariably, a  $\text{Glc}_3\text{Man}_9\text{GlcNAc}_2$  structure is synthesized on a polyisoprenoid derivative (dolichol-P-P) and transferred *en bloc* to asparagine residues on polypeptide acceptors forming an N-glycosidic linkage (hence N-linked). The three Glcs are usually trimmed by a tandem pair of  $\alpha$ -glucosidases to yield a high mannose N-linked structure. The high-mannose structure is variably modified, beginning with removal of mannoses in a typical sequence by

a tandem pair of  $\alpha$ -mannosidases. One pathway retains most mannoses but permits additional substitutions of e.g., sulfate and/or phosphate. A second pathway trims down to  $\text{Man}_5\text{GlcNAc}_2$  and makes limited additions such as GlcNAc, and sometimes Gal and core-linked Fuc. These are referred to as hybrid structures. The third pathway continues the second pathway to build complex structures usually terminated by sialic acid. There can be two, three or four chains emanating from the branching mannose (and secondary branching Mans) and these structures are accordingly referred to as biantennary, triantennary or tetraantennary complex N-linked oligosaccharides. The sialic acids, which appear not to precede echinoderms in their evolutionary appearance, comprise a diverse family of sugars (100). Numerous additional substituents including sulfate, phosphate, methyl groups and others have been documented (8, 9, 161).

Each step in the glycosylation pathway is catalyzed enzymatically; there is usually a unique enzyme for each linkage position formed as monosaccharides are accreted or deleted (47). N-linked glycosylation is not template driven. Only the first step might be considered so, inasmuch there is a necessary but not sufficient requirement of an x-asn-x-ser/thr-x sequence for the oligosaccharide acceptor. Protein structure or conformation has also been shown to influence whether a site becomes glycosylated as well as whether it becomes high mannose, hybrid, or complex (4, 7) or whether it receives a phosphodiester-linked  $\text{GlcNAc-PO}_4$  (7) or  $\text{GalNAc-SO}_4$  (175). A relationship between position in the polypeptide chain (distance from N- to C-terminus) and whether an oligosaccharide becomes high mannose or complex has been observed (6). Finally these relations are not absolutely determinate, for spontaneous heterogeneity of carbohydrate structures at particular positions has been documented (12, 5, 37). Changes in structure also result from extracellular influences (see below).

The other major structural class of oligosaccharides is O-linked on ser or thr (25, 26). A largely distinct set of enzymes catalyzes the synthesis of these structures. These oligosaccharides are built by monomer addition from sugar-nucleotide donors directly on the polypeptide rather than on a lipid precursor. Trimming does not appear to play a major role. Gal and GalNAc are typical proximal sugars and Man is rarely found. O-linked structures

are found both in branched and straight chain forms. O-linked structures often resemble the oligosaccharides of glycolipids (45), and in fact these may be built from shared enzymes. An experimental consideration of the role of these oligosaccharides on protein must inevitably involve an examination of homologous glycolipids.

Although the N-linked and O-linked structures discussed above account for 95–100% of incorporation of labelled sugar precursors into cellular glycoproteins, numerous additional small classes of oligosaccharides are also found. For example, GalGlc is found on hydroxylysine and hydroxyproline of collagens. GlcNAc-PO<sub>4</sub>-ser is found on a lysosomal enzyme in *D. discoideum* (18) and GlcNAc-asn is found on certain glycoproteins of what is probably the nuclear envelope (19).

Archeobacterial proteins are also glycosylated. A major class of these glycosylations shares some homology with N-glycosylation and may be evolutionarily related (10, 38, 11). A polyisoprenoid lipid carrier is utilized prior to *en bloc* transfer. The ancient character of the mammalian N-linked glycan suggests important functional attributes for his posttranslational modification.

Several physical methods have been applied to ascertain the conformation of oligosaccharides on proteins (30, 31). Studies in model systems have implied that there is a preferred conformation at least for the core sugars of N-linked oligosaccharide chains. This conformation is stabilized by hydrogen bonding with the polypeptide chain, which orients the structure, and limited intrachain hydrogen-bonding. The existence of preferred conformations implies that shape may be important for specific interactions of protein-linked carbohydrate with receptors.

### Cellular sites of glycosylation

N-linked glycosylation appears to occur only in the rough endoplasmic reticulum (RER) and hence only to proteins which possess a signal sequence (150). The process is co- or post-translational (14). Mannose trimming begins in the RER but can be concluded in Golgi. Modification of high-mannose forms or conversion to hybrid or complex forms occurs in the Golgi. Nearly all glycosylation is believed to be restricted to these two compartments.

Certain steps in glycosylation have, however, been postulated to occur in the SER, secretory vesicles, nuclear envelope and cell surface (e.g., 43, 139, 173). Of course, deglycosylation occurs in lysosomes as a part of protein turnover (102).

O-linked glycosylation appears to be initiated and concluded in the Golgi (25, 26, but see 27). As for N-linked glycosylation, there has been some resolution of processes between the cis, medial and trans elements of the Golgi apparatus.

Nearly all proteins which pass through the RER and Golgi become glycosylated in some form. One exception is the secretory protein serum albumin (32), which is synthesized in hepatocytes. There is no evidence for a glycosylated precursor.

It should be recognized that at least some glycoproteins, e.g., cell surface transferrin receptor, can probably be reexposed to the Golgi compartment subsequent to primary passage through this organelle (112). This provides new opportunities for glycosylation subsequent to initial synthesis.

Occasional reports have appeared in the literature regarding the presence of glycoproteins in cytoplasmic compartments topologically discontinuous with the lumen of the RER and Golgi. Histones and other nucleoplasmic proteins (68, 77, 78), a ribosomal protein (79) and certain mitochondrial proteins (132, 174) have been suggested to be glycoproteins. These findings contradict the dogma that glycosylation is strictly an RER and Golgi dependent event unless it is postulated that modified proteins subsequently translocate across the membrane back to the cytoplasmic space. Further work remains to be performed to confirm the existence of oligosaccharides on these proteins and to characterize their structure as a means for understanding their enzymatic basis.

It must be appreciated that the description of the glycosylation pathway as we now understand it suggests many interesting kinds of regulatory mechanisms. For example, each step of the pathway can be influenced by enzyme synthesis and turnover, acceptor accessibility, as well as the supply of donor sugar, cofactors such as lipids, divalent cations, etc. The question of accessibility is particularly interesting because sequentially acting enzymes tend not to be mixed but are distributed vectorially through the RER and the multiple compartments of the Golgi. Movement of polypeptides through these compartments appears to be mediated by

ATP-dependent dissociative movement of vesicles (142). Thus glycosylation may conceivably be regulated by the pathway of movement of the acceptor protein. There is also an indication that some glycosyltransferases form supramolecular complexes. Thus, it is possible that certain carbohydrate structures are dictated not only by vesicle movement, but also by which enzyme cluster initially captures the acceptor protein (122).

### Existing ideas about carbohydrate function

Ideas about the role of protein glycosylation may be divided into two general groups: 1) those which imply specificity associated with particular carbohydrate structures, and 2) those which stress the general similarities of oligosaccharide structures, and the presence of microheterogeneity in the structure of particular oligosaccharides, in suggesting that combined carbohydrate bulk creates a common microenvironment for resident polypeptides much as the lipid bilayer provides an environment for integral membrane proteins. Most effort has been devoted to the former category of ideas because of the greater ease of formulating hypotheses of this type.

### Non-specific functions

A non-specific role for oligosaccharides that is dependent on their generalized chemical properties has been suggested in several contexts. It was appreciated early on that carbohydrate afforded a stabilizing effect on protein solubility and against heat denaturation (see, e.g. 34, 38 and 35). It was also realized that oligosaccharides afford resistance to proteolytic degradation (166), presumably by a mechanism of steric hindrance, and these ideas have been reinforced in numerous studies up to the present (e.g., 12, 101, 102). There are in addition several instances where carbohydrate is known to interfere sterically with protein-protein interaction not involving proteolysis (74, 75).

Predating this understanding, it was known that carbohydrate is a major constituent of the extracellular matrix in the form of polysaccharides known as glycosaminoglycans (GAGs) (3). In the past 15 years, it has been discovered that most GAGs are

covalently attached to polypeptides, resulting in the formation of glycoproteins which, due to the specialized nature of the attached carbohydrate, are known as proteoglycans. GAGs are anionic polymers which hydrate to form enlarged domains which can interact with one another, as well as with polycationic polymers such as collagen and with other glycoproteins (3). A three dimensional space is thus defined by polysaccharide, protein, and the bound water which provides an environment for cells. Convection and diffusion are modified, and fluctuations in ionic strength and pH are minimized. These structures depend on the polyanionic nature of the carbohydrate, and the general chemistry rather than the specific monosaccharide identities and sequences may be dominant in governing the properties of the system.

Carbohydrate oligomers are also known to self-associate in the formation of mucous gels and in yeast and plant cell walls (152–154, 22). In the former, the anionic nature of the carbohydrate appears to be important although in the algal and yeast gels the interactions are non-coulombic.

The collective oligosaccharide density on cell surface proteins is sufficiently great so as to invite comparison with the carbohydrate density of the matrix space. This observation has led to the suggestion that cell surface carbohydrate might also establish a convection and diffusion-limited region around the cell in an area thick enough to be named, in certain cell types, the glycocalyx or fuzz. The polyanionic nature of the carbohydrate found on some cell surfaces probably results in marked cation and pH differences in this region relative to the surrounding medium (177). It has recently been proposed that the heterogeneity of carbohydrate structure found on proteins of this region might result in minor differences in protein function, leading to a broader range in the ionic strength and pH values optimal for protein function (36, 37). As such, protein oligosaccharides might subservise a homeostatic process used by cells to maintain their viability in potentially changing milieus. It has also been suggested that a layer of non-immunogenic protein-associated carbohydrate may play an immuno-protective role for underlying cell surface antigens, especially on neoplastic cells (27).

Plasma membrane carbohydrate, owing to inter-carbohydrate associations, might also influence the lateral diffusion of certain glycoproteins. Inter-

molecular association at the cell surface has been suggested to be so extensive in an archeobacterial system that it can influence the shape of the included cell (11). An extension of intermolecular association to contact between two cells in close mutual proximity leads to a possible mechanism for cell adhesion. Interaction of oligosaccharides might be due to an apposition of the hydrophobic faces of two monosaccharides such as mannose (126). Implicit in this model is the idea of a large number of weak interactions equalling in strength a smaller number of strong interactions. This mechanism is feasible in polymeric systems with repeating similar subunits. Cell surface oligosaccharides could simulate a polymer inasmuch as they are co-anchored in the same membrane. The interactions involved in these phenomena may be relatively non-specific.

The notion of a 'non-specific' role for carbohydrate has received perhaps its strongest support from studies on cells whose glycosylation processes have been globally altered by mutation or drugs. The most dramatic studies have involved mutant cells selected on the basis of resistance to the toxic effects of certain lectins. Many mutations leading to loss of lectin recognition have been identified in CHO and other cells (20). Some have been characterized and in fact most steps in the pathway to complex, N-linked glycan formation are mutable. The striking finding is that mutant cells are largely viable in culture. Most mutant CHO cells remain adherent under appropriate conditions, proliferate, and are tumorigenic. Mutants defective in early steps leading to the formation of high-mannose glycans are also viable (24). This result was not, however, found in a yeast mutant (40).

Selected activities of cells have also been investigated in some mutant strains. For example, the half-life of cell surface proteins was found not to be dramatically affected (23). Similar results were found following treatment of cells with sialidase (107).

A considerable literature details the consequences of carbohydrate modification on the secretory process. Though several examples of apparently specific effects will be enumerated in a later section, the number of cases where little or no effect is found is noteworthy (82, 83, 98, 161). In some cases detailed kinetic analysis has found only small effects on secretory rates (159).

Similar results have been found regarding accumulation of receptors in the plasma membrane and in their function at that site (88, 99, 90, 164). Genetic modification of the gene for the LDL receptor has deleted a sequence required for a cluster of O-linked glycans and even for this poorly-studied glycan type no consequence on function could be detected (163). Mutants in the enzymatic pathway of O-glycosylation still place the LDL receptor, albeit in a less stable form, on the cell surface (165).

These negative results have suggested that carbohydrate cannot underlie essential housekeeping functions in cells. This implies a non-specificity of function in the sense that obvious functions cannot specifically be attributed to the carbohydrate when it is present. This represents a shift in meaning from the above definition of nonspecificity inasmuch as specific receptors may be involved in functions non-essential in the tissue culture environment. Thus it has alternatively been suggested that carbohydrate may be more important for particular differentiated functions, which are not expressed in cell culture systems such as the CHO cell line. There are but few investigations into this possibility. For example, in the cellular slime mold *D. discoideum*, mutations have eliminated two developmentally regulated glycoantigens (28, 29). Nevertheless, the cells develop reasonably normally to produce fruiting bodies and viable spores. The result may imply that carbohydrate is either non-specific or subserves subtle modulatory roles or that their roles are redundant with alternate (or back-up) mechanisms. Alternatively, the value of these carbohydrate structures may only become evident under the competitive conditions of natural survival outside of the laboratory.

Although the above ideas have been grouped together under 'non-specific mechanisms', this is not meant to imply that carbohydrate structure per se is completely irrelevant. 'Non-specific' should be interpreted relative to the types to be discussed below, were specific, saturable recognition mechanisms related in principle to enzyme-substrate or hormone-receptor interactions are apparently involved. In any case, the non-specific mechanisms tend to rely on the bulk chemical properties of the participating oligo(poly)saccharides.

### Specific mechanisms

This phrase implies interactions between receptors and ligands (usually the oligosaccharide) of saturability and high selectivity and avidity. Selectivity can be achieved by high affinity monovalent interactions or multiple weaker interactions. Selective interaction is often equated with molecular or, on a more complex level, cellular recognition. The phrase also implies that a specific function can be attributed to the carbohydrate structure. Perhaps the strongest indication for specific roles are the variety of structures which have been detected on protein carbohydrate.

The following sections outline how several characterized structures are thought to function in specialized processes. In some cases, a receptor protein capable of recognizing a given structure is known.

#### *Intracellular routing to lysosomes*

In 1965 Eylar (80) presented the idea that protein glycans constitute a 'chemical passport' signalling export of a protein to the cell surface or for secretion. Though this idea has since been supplanted by the 'signal hypothesis' of Blobel and Sabatini (150), it has served as a precursor to more refined ideas of chemical signals leading to the accumulation of glycoproteins in specific compartments (81). For example, a mannose-6-phosphate moiety has been suggested to be responsible for associating proteins with receptors which in turn lead to protein accumulation in lysosomes (134). The key to this understanding was the finding of mutant cells which appeared to secrete constitutively certain lysosomal enzymes that were distinct in their oligosaccharide structures: namely that normal enzymes possessed multiple phosphate residues 6-linked to non-peripheral Mans in high-mannose structures, and that mutant enzymes lacked these phosphate moieties. This suggested that there was a Man-6-PO<sub>4</sub> receptor in membranes of the Golgi, and plasma membrane (since lysosomal enzymes can also be absorbed from the medium and translocated to lysosomes), which was responsible for binding the Man-6-PO<sub>4</sub> recognition marker on certain glycoproteins. Two such receptors, one of 215kD and the other of 46kD, have been discovered (157, 158). These are located in the Golgi and/or on

the cell surface in concordance with their proposed functions. From studies on receptor movement and the effects of inhibitors there is fairly good evidence that the Man-6-PO<sub>4</sub> moiety, together with one or both of these receptors, plays a recognition role in the intracellular routing. However, not all lysosomal enzymes seem to be involved because some copies of the altered enzymes still accumulate in mutant cells (157), and some lysosomal enzymes are not affected. It has been suggested that there is lysosomal heterogeneity with only one class of lysosome employing this recognition marker. It is nevertheless apparent that for some lysosomal enzymes in some cell types the Man-6-PO<sub>4</sub> receptor does not function alone, even as an intermediate signal, for lysosomal routing. There must be other recognition signals on the enzyme polypeptide which lead to its potential lysosomal accumulation.

It is interesting to note that the distinctive carbohydrate structure plays a compartmentalization role separate from the enzyme's function in catalytic degradation. Furthermore, carbohydrate is employed to perform a common role shared by multiple proteins. A posttranslational modification such as glycosylation is well-suited to the demands of a mechanism for compartmentalization.

#### *Other intracellular compartments*

The RER, Golgi, smooth endoplasmic reticulum, and nuclear envelope are also intracellular destinations for proteins which traverse the RER cotranslationally. These compartments contain numerous characteristic and uniquely distributed glycoproteins, but there is not enough information to evaluate the possibility that carbohydrate structure is involved in routing or maintaining the positions of the glycoproteins.

Several cases of RER-specific glycoproteins have been considered. Cells infected with Rotavirus synthesize, under control of the virus, several proteins including VP7, an integral membrane glycoprotein whose distribution is restricted to the RER (95). This protein together with the membrane of the RER form a temporary coat for the virus. In a genetically modified form of VP7, a hydrophobic region, which presumably serves as a transmembrane anchor defining the proteins as an integral membrane glycoprotein, was deleted. The truncated gene encoding VP7 was introduced to cells by

transfection. Truncated VP7 was synthesized, but was then secreted despite this fact that glycosylation at its single site toward the C-terminus retained its high-mannose character. These results show that the key factor which retained the glycoprotein in the RER was the transmembrane sequence and was not related to the glycosylation site. The results would also imply that glycosylation is not essential to the process of secretion.

Several nuclear envelope glycoproteins have been found to express an unusual carbohydrate structure consisting of GlcNAc-asn (19), which is an expected product of endo-glycosidic degradation. It is at present unknown where this structure is synthesized and thus whether glycosylation precedes or follows arrival into the nuclear envelope.

### *Secretion*

The secretion of protein appears to involve at least two pathways: a constitutive pathway and a pathway which includes a secretory vesicle which is stored cytoplasmically (144). A range of high mannose and complex bi-, tri- and tetra-antennary N-linked structures has been observed on both secretory and plasma membrane associated glycoproteins. Most studies have been directed, however, toward N-linked glycans since these structures are easier to modify and detect than are O-linked and other, unknown structures.

One approach to evaluate the potential role of N-linked glycans has involved the use of pharmacological inhibitors of several steps, namely the initial steps of synthesis, and deglycosylation and demannosylation. Tunicamycin blocks N-linked glycosylation completely (as well, possibly, as other types of glycosylation (15)), whereas deoxynojirimycin and swainsonine block certain steps leading from high mannose structures to the complex type. Though the effects are not simple it tentatively appears that lack of N-linked glycosylation has a measureable affect on the rate of secretion of some proteins from hepatocytes or hepatoma cells (82, 85, 84). Secretion from these cells appears not to involve a stored secretory vesicle. Modification of glycosylation by deoxynojirimycin or swainsonine appears in some cases to affect the rate of movement from the RER to the Golgi and, in other cases, from the Golgi to the extracellular space (162, 159, 84). Some glycoproteins and albumin are not affected at all. This result has led to speculation that there is a

receptor which can recognize and retain glycoproteins bearing the high mannose marker. This mechanism would not be universal for all proteins. Alternatively, these differences may reflect spurious differences in rates of dissociation from glycosylation enzymes. It seems reasonable to consider that glycans may be designed to play a modulatory role in the kinetics of secretion for only selected proteins. Since secretion of these proteins is not regulated by storage in a secretory vesicle, this mechanism may be used by the cell as a fine-tuning mechanism for controlling release of protein. It is plausible that the passive constitutive pathway for secretion (95, 144) does not involve carbohydrate per se, but that modifications of this pathway or its kinetics do. Further work remains to be done to compare carbohydrate structure on different glycoproteins and to consider the possible involvement of neighboring polypeptide regions in the interaction with a hypothetical receptor.

### *Secretory vesicles*

Entry of protein into stored secretory vesicles requires a specific signal as shown by the observation that some hormone polypeptides produced by pituitary cells are stored whereas others are constitutively secreted. It has been observed that peptide hormones which are stored in pituitary gonadotropes and thyrotropes are sulfated whereas related peptide hormones released from placental cells, but not stored there, are not sulfated. This sulfate has subsequently been shown to reside at one or more non-reducing termini of N-linked complex oligosaccharides in the position usually occupied by sialic acid. This substitution may be directed by a substitution of the GalNAc for underlying Gal (156). The functional significance of the relationship between GalNAc-SO<sub>4</sub> and delivery to a vesicle which is stored cytoplasmically prior to release by a secretagogue remains to be explored. An exception to this structure-function correlation is posed by human FSH, which is stored in human pituitary gonadotropes but, unlike the LH stored in these cells, is not sulfated. Possibilities consistent with a role for GalNAc-SO<sub>4</sub> are that FSH may segregate to a discrete vesicle population relative to LH, or that the two hormones colocalize and that some intermolecular association substitutes for the lack of this carbohydrate modification.

### *Plasma membrane*

There is no consensus oligosaccharide structure discovered thus far which distinguishes plasma membrane from secreted glycoproteins. Although this may suggest the possibility that plasma membrane and secretory glycoproteins may in fact share a common signal, on account of their topologically equivalent destinations, inhibitor experiments have shown that an absence of N-linked glycans does not consistently block the appearance of glycoproteins on the cell surface (88, 90). There are, however, some instances of inhibition (86, 87, 89, 91–93), which allow for the possibility that N-linked glycans may act as a signal or modulator for the export of specific proteins to the plasma membrane. Alternatively, the failure of a few altered glycoproteins to be exported may be explained by enhanced proteolysis, by a secondary effect on conformation, or by the display of another signal elsewhere on the polypeptide (165).

A special class of extrinsic plasma membrane glycoproteins is known, however, for which a glycan structure appears to be significant for localization. This includes glycoproteins which are recognized by the cell surface protein ligatin, which recognizes a Man-6-PO<sub>4</sub>-1-Glc, linked to a high-mannose backbone (160). This structure appears to be important for the cell surface association of these glycoproteins since Glc-1-PO<sub>4</sub> can elute them from cells and a high concentration of Ca<sup>++</sup>, which can elute ligatin, also elutes the associated glycoproteins. Glycoproteins associated by this mechanism were first recognized ultrastructurally on the surface of the intestinal mucosa of the suckling rat, but have since been discovered on the surfaces of a variety of cell types in sea urchin, chicken and mouse. It is unclear whether the Glc-1-PO<sub>4</sub> marker serves as a routing signal for export to the cell surface, using ligatin as an intermediate carrier, or whether it serves a recognition role for secondary association with the cell surface once secretion in soluble form has already occurred. Though the identity of many of the ligatin associated glycoproteins is unknown, the first to be discovered in the intestinal mucosa was  $\beta$ -N-acetylhexosaminidase. In this case, the primary function of the glycoprotein can be viewed as an enzymatic one. The appended carbohydrate appears to serve a role in localization, contributing to

the spatial organization of the system.

Consideration of the possible role of carbohydrate in other molecular associations with the plasma membrane will be considered below in sections on hormone-receptor interactions and cell-substratum (e.g., extracellular matrix) and cell-cell adhesion.

### *Extracellular localization*

It has been proposed that specific carbohydrate/protein interactions might designate the localization of secreted glycoproteins to specific sites in the extracellular matrix (3). This idea has received support recently from the detection of animal lectins in matrices, basement membranes, and mucous secretions (140). Lectins, which are multivalent carbohydrate binding proteins with oligosaccharide specificity, are believed to crosslink other molecules to contribute to, e.g., the viscosity of certain mucous structures (44). Binding of matrix proteins to carbohydrate or GAGs has been reported (21, 32). A recent report suggests that the neural adhesion protein N-CAM has a binding site for heparan sulfate-like GAGs (171).

### *Cell surface receptor recognition of soluble ligands*

In the early 1970s, Ashwell and Morrell demonstrated that desialylation of serum glycoproteins, resulting in the exposure of a penultimate galactose in mammalian species, caused rapid removal of proteins by hepatocytes once these altered proteins were restored to the blood (reviewed in 105, 106). This suggested that normal turnover of serum proteins is regulated by the structure of their glycans, which would be regulated by interaction with sialidases. Though a carbohydrate binding receptor for (non-reducing) terminal Gal/GalNAc has been characterized in hepatocytes, it has not been possible to prove this hypothesis directly. This mechanism is not universal because not all serum proteins, albumin being a notable example, are enzymatically glycosylated. Further work must be done to determine whether this system actually functions in serum protein turnover. It is interesting to note that a family of serum proteins (IgA<sub>1</sub>) binds this receptor by way of O-linked oligosaccharides, but only upon denaturation (175). It has also been recently shown that liver endothelial cells can



desialate a serum protein, thus rendering it susceptible to uptake by hepatocytes via the Gal/GalNAc receptor (178).

Additional studies have revealed the presence of a distinct carbohydrate binding protein in Kupffer and other macrophage-like cells (105, 108, 109, 175). Following recognition, these and other carbohydrate binding proteins become internalized together with their ligands, which carry a Man/GlcNAc/Fuc specificity, but later return to the cell surface free of ligand. In addition, cell surface localized Man-6-PO<sub>4</sub> receptors have been suggested to be responsible for hepatic uptake of enzymes supplied to the fetal blood from placental fluids (111).

It has been suggested that receptors on the Kupffer cell and other types of macrophages may also recognize cells which present the appropriate configuration of carbohydrate residues (110). Binding in these circumstances may lead to phagocytosis. This kind of binding has been detected in model systems (130).

Several examples of a role for protein glycan in other forms of intermolecular recognition have been offered. These include glycans on the Fc portion of IgG being involved in the recognition by complement proteins and cell surface receptors (70, 129), glycans of human chorionic gonadotropin being involved in activation of its receptor subsequent to the actual binding event (71–73), and interaction between anti-thrombin III and heparin leading to activation of inhibition of thrombin protease activity (176).

#### *Cell-cell and cell-matrix recognition*

As early as 1945, Burnett (119) and his colleagues observed the importance of cell surface protein-bound sialic acid in virus binding and infection. This phenomenon is sufficiently complex, however, that it is necessary to discuss the kinds of evidence which are involved in concluding that carbohydrate plays a direct, rather than a regulatory, role in this and other examples of adhesion. Any demonstration that carbohydrate comprises part of a physical bridge in cell adhesion (or any molecular binding) requires that four general criteria be satisfied: 1) removal or alteration of the carbohydrate diminishes cell adhesion; 2) reconstitution of the carbohydrate either on the cell or on an artificial surface re-

stores cell adhesion; 3) a specific receptor for the carbohydrate is identified on the cell (complementary) surface; 4) inactivation of the receptor diminishes cell adhesion. Satisfaction of these criteria can be complicated because cells appear to have multiple adhesion systems, rendering it difficult to assay one independently of the others. Removal of carbohydrate can have other effects on cells. For example, cell viability can be reduced by drugs or mutations affecting glycosylation, and transport of polypeptides to the cell surface and other mechanisms of turnover and placement of glycoproteins can be altered. Specific receptors may be difficult to detect because individual receptor-ligand interactions may have low affinity (e.g., 126). A rationale for this generalization is that, because of the large area of cell surfaces, cells can theoretically interact with other surfaces using a large number of low affinity associations. This may in fact be desirable for cell interactions which would need to be reversible. The results of several approaches where cell reassociation has been competed with model sugars have shown that the competing sugars must be crosslinked to form multivalent structures in order to be competitive (172). A multivalency requirement has also been implicated in the Man-6-PO<sub>4</sub> receptor system (155).

Examination of cell-cell and cell-substratum adhesion in several systems has identified candidate molecules which participate in the formation of the physical bridge (43, 135). In some cases independent approaches have converged on the same candidate molecules. The candidate molecules are usually glycoproteins and in some cases the relative roles of carbohydrate and protein have yet to be resolved. In other studies, candidates have not yet been identified.

The simplest adhesion systems involve parasite/host interaction. Considerable evidence has been adduced that various parasites such as viruses, bacteria, protozoa, etc., possess carbohydrate binding proteins which can interact with sugar structures on the surfaces of cells with which the parasites associate (167, 168, 116–119). In some cases it is suspected that this sugar is associated with glycolipid but in other cases Man is believed to be involved on the basis of competition studies (e.g., 114). This would imply the involvement of glycoprotein because this sugar is rare in glycolipid. The use of carbohydrate as attachment points on cells presumably reflects

the stability of these structures on cell surfaces and their specific associations with target cells suitable for the parasite. These would of course be useful features for any mechanism of specific cell-cell interaction.

Species-specific cell-cell adhesion (the so-called secondary type) in the phylogenetically-primitive marine sponge is suspected to involve carbohydrate ligands (133). Ca-free sea water elutes a fraction from cells of *Microciona prolifera* which is required for their reaggregation. This fraction is the source of a purified proteoglycan which is referred to as aggregation factor. Hypotonic shock elutes another molecular species (the so-called baseplate or aggregation factor receptor) which is also required for reaggregation and may recognize the proteoglycan by a mechanism involving glucuronic acid on the proteoglycan. These preliminary findings were ascertained by competition studies with model sugars and enzymatic treatments, and it will be interesting to confirm these findings using purified molecules (179). Results of comparable studies in another marine sponge, *Geodia cydonium*, are even more intriguing (133). Bound  $\beta$ -glucuronic acid is also believed to be important in aggregation factor/baseplate recognition but its position is reversed so that the key sugar is on the baseplate glycoprotein. Beta-glucuronidase and  $\beta$ -glucuronyltransferase can be eluted from extracellular material suggesting a mechanism for the dynamic regulation of cell recognition. An investigation into the tissue specificity of cell recognition showed that  $\beta$ -galactosidase treatment of non-aggregating cells permitted their aggregation in the presence of aggregation factor. A protein was then isolated which could inhibit aggregation of aggregation-competent cells in a  $\beta$ -galactosidase sensitive fashion. Finally, a  $\beta$ -galactoside binding protein was isolated from extracellular material which rendered an effect similar to that of the glycosidase. Though the significance of these observations is as yet unclear, it has been suggested that carbohydrate structures may underlie specific mechanisms of cell recognition in multiple ways in these species.

Cell-substratum adhesion in another primitive multicellular system, aggregating cellular slime mold cells, appears to involve a multivalent carbohydrate binding protein termed discoidin I. The involvement of discoidin I was initially recognized

based on sequence homology between this protein and an oligopeptide sequence of vertebrate fibronectin which has been shown to contain its cell binding site. This oligopeptide, which blocks fibronectin binding to cells, also affects association of *Dictyostelium* cells with their substrata (148). The cells become incapable of migrating properly on a surface. The effect can be mimicked by anti-discoidin I. The effect is also replicated if the cells are transformed with a gene which encodes an mRNA which hybridizes with authentic discoidin I mRNA and blocks its translation (149). The evidence is very strong that this carbohydrate binding protein is involved in cell-substratum association. What is unclear, however, is the role of the carbohydrate binding activity. There is evidence that it does not associate with the cell via this site (103). It is unclear whether the carbohydrate-binding site might associate the protein with elements of certain substrata, but it is apparent that this is not necessary. There is recent evidence that food bacteria are recognized by this lectin and hence discoidin I might assist in the adhesion of cells to their food source (104).

Perhaps the best documented instance of a direct role for carbohydrate in cell-cell adhesion occurs in higher organisms and involves the protein galactosyltransferase and bound GlcNAc, sometimes associated with oligolactosamine (120, 121, 74, 124). This pair of molecules has been implicated in adhesion between mouse sperm and egg, neural crest cells and substratum, and embryonal carcinoma cells. Each of the four criteria cited above has been addressed by experimental approaches using antibodies, enzymes and drugs to inactivate the receptor and the ligand. A genetic approach to modify receptor or ligand has yet to be applied. Though the latter approach with its inherent specificity is very important, the evidence using the approaches already employed is strong. The system is interesting because the receptor-ligand interaction can be readily reversed by supplying substrate for the galactosyltransferase enzyme activity.

An independent approach to the problem of mouse sperm-egg recognition has resulted in the isolation, from the jelly coat of the egg, of a glycoprotein, ZP3, which can, upon presentation to the sperm, block its ability to recognize eggs (125). It was found that careful alkaline borohydride treatment to strip O-linked glycans without altering

the polypeptide chain neutralized the inhibitory activity of ZP3. Finally, it was shown that the released glycans possessed inhibitory activity and in addition bound to sperm, suggesting that the carbohydrate itself served as the recognition site in the jelly coat for sperm. Further work remains to be done to identify the sperm receptor (169) as well as to relate the findings to the above results implicating the galactosyltransferase.

Carbohydrate of a different type has also been implicated in cell-cell adhesion between mature mammalian cells. For example, it has been suggested that cell surface carbohydrate may be important for recognition between certain lymphocytes and certain high endothelial venule surfaces (HEV) in lymphatic tissues (136). Pretreatment of lymphocytes with specific sugars renders them incapable of binding to HEVs in an *in vitro* assay, and glycosidase treatment of HEVs renders them incapable of being recognized by lymphocytes. There is conflicting evidence from these studies regarding the nature of the carbohydrate structure involved. Additional results have indicated that one lymphocyte subclass recognition site involves a previously studied homing receptor, the ubiquitin-conjugated glycoprotein defined by the MEL-14 antigen. Recent work has found carbohydrate-derivatized polyacrylamide surfaces to which lymphocytes will specifically bind (137). An effort is underway to compare the specificity of this interaction with that to HEVs. The exciting prospect is that the manipulability of the artificial surface (130) will allow dissection of multiple adhesion mechanisms and permit more rapid investigation of the nature of the carbohydrate recognition system believed to be responsible for lymphocyte recognition preparatory to extravasation.

Carbohydrate has been suggested to perform an opposite kind of role in the function of the protein N-CAM in associating neural cells (170). Fab fragments from anti-N-CAM inhibit reassociation of dissociated neural cells and isolated N-CAM can self associate. There are several forms of N-CAM which differ in their degree of sialylation. The more heavily sialylated forms show less affinity for one another in the self association assays. Thus it has been postulated that sialylation is a posttranslational modification which modulates the strength of adhesion mediated by this glycoprotein and *in vivo* evidence has now been provided (127). Recent-

ly it has also been suggested that N-CAM can bind carbohydrate in the heparan sulfate proteoglycan (128) and that this recognition is involved in intercellular (171) and cell-substratum adhesion.

It is too soon to speculate whether binding to a carbohydrate ligand may have secondary effects on cells as has been suggested for, e.g., binding of human chorionic gonadotropin (71–73). This hormone does not depend upon its carbohydrate for binding to its receptor but the carbohydrate is important for activating the cellular response.

The selected examples discussed here indicate that protein-linked (or possibly lipid-linked) carbohydrate may play a fundamental role in multicellular organization in a wide phylogenetic range of organisms. Though in all of the examples cited there is a specificity in the adhesion, in some cases this is due to multiple possibly low-affinity binding interactions. In the extreme, each single binding event may approach non-specificity, with specificity accruing through multivalency. Perhaps this implies that the formalization of non-specific carbohydrate-mediated events obscures an actual continuum of specificity in these events between these two extremes. It is unclear whether the involvement of carbohydrate in adhesion is universal. Several other adhesion molecules, including glycoproteins such as gp80 in *D. discoideum* (164), CAM 80/120 in mammalian cells, etc. and glycolipids (146), are glycosylated but the role of carbohydrate is not known. If the use of carbohydrate is universal, then the specificity inherent in the examples discussed would suggest an underlying structural code which may be important for the affiliated processes of cell sorting and morphogenesis, to which adhesion makes a very important contribution. This may explain some of the interesting morphogenetic effects circumstantially suggested by many studies involving carbohydrate structure perturbations on developing cells.

A role for carbohydrate in cell adhesion choice or sorting correlates with its postulated role in intracellular or extracellular compartmentalization of proteins. Cell sorting and molecular compartmentalization can be regarded as sorting at different levels. It will be interesting to learn whether the proteins on which these carbohydrates are located exist only to place the carbohydrate or whether the carbohydrate piggybacks on another essential function of the protein. Were the latter

true, this would strengthen the analogy with the role of carbohydrate in routing or molecular compartmentalization. It may then become feasible to generalize carbohydrate's role as important for the three-dimensional compartmentalization of proteins, and the cells, with which they are associated. As such, carbohydrate may be the chemical structure cells used to address and organize space as they evolved from prokaryotes to unicellular eukaryotes, and continued to exploit as they evolved to form multicellular tissues and organs.

### *Developmental functions*

Numerous studies have implicated protein-linked carbohydrate in more complicated cellular phenomena (43, 122). Glycosylation is modulated coincidentally with numerous developmental processes (122, 53–67, 69, 96, 161). Carbohydrate interactions have been implicated in mammalian morula compaction (131, 122, 123). Proliferation has been reported to require glycosylation (41) or to be inhibited by glycopeptides and a defined fragment of heparan sulfate (143, 151). Oligosaccharides released from plant cell walls can stimulate cell proliferation and alter cell differentiation in plant cells (145). Myogenesis and chondrogenesis have been speculated to be affected by GAGs linked to proteoglycans (e.g., 113), but conclusions in this field are very controversial. Attachment of cells to substratum-associated carbohydrate can result in its covalent modification (213, 130). Neuritogenesis has been shown to be inhibited *in vitro* by tunicamycin (16), though the specificity of the effect is difficult to assess, as tunicamycin-sensitivity does not convincingly demonstrate the involvement of *protein-linked* carbohydrate (15, 17). A recent report suggests that alteration of processing of N-linked oligosaccharides with swainsonine, without affecting cell viability, inhibits the ability of a line of melanoma cells to metastasize to the lung (52). This finding is consistent with another report documenting carbohydrate differences between metastatic and non-metastatic cell lines (49).

### **Future developments**

There is much new territory to explore in the field. The use of monoclonal antibodies and lectins which recognize carbohydrate-dependent epitopes

continues to reveal an unexpected diversity of carbohydrate structures with surprisingly intricate distributions in embryonic and adult tissues. This has recently been appreciated in amphibian (53) and mouse (54, 55) embryos and in the simpler model system *Dictyostelium discoideum* (96, 161). Tumor cells also differ in many cases in specific carbohydrate structures on both lipid and protein (44–46, 49–51, 180). Several of the antibodies employed recognize carbohydrate epitopes whose corresponding structures have been determined. The antibodies provide unrivaled sensitivity and specificity relative to standard methods for detection of unique carbohydrate structures. Caution must be applied, however, because 1) it is theoretically possible that unique structures at the monosaccharide level may present structures similar enough at the atomic level to be immunologically cross-reactive and 2) identical or similar structures may be immunologically distinguished by conformational restrictions imposed by the surrounding polypeptide environment. Thus inference of structure based on antibody data must be confirmed directly by structural studies. In any case, antibodies and lectins may *model* potential receptors in cells which might specifically recognize carbohydrate-associated structures.

The significance of the association of distinct carbohydrate structures with distinct embryonic or tumor cell types is absolutely unclear at present. Ultrastructural localization has placed some epitopes intracellularly in specific compartments and others on the cell surface or extracellularly in the surrounding matrix. A cell surface localization is a necessary condition for a direct role in cell recognition and, on the strength of suggestions from other systems, this has been promiscuously interpreted to imply a role in cell recognition. This reflects the primitive status of our general understanding of the significance of protein glycosylation. Thus the potential developmental roles of protein-linked carbohydrate in morphogenesis, differentiation, adherence and proliferation will likely be a primary target of investigation in this field in the future.

Interest in developmental involvement of carbohydrate is based not only on a large bank of indirect data but also on an intrigue in the special kinds of epigenetic regulatory mechanisms with which carbohydrate might be associated. For example, glycosylation offers the cell a mechanism to

regulate the structure of multiple proteins without altering gene expression. In addition, monosaccharide residues may be processed independently of the associated polypeptide (141, 142) either by recycling through the Golgi (112) or at locations near the site of function (130, 138), far from the point of origin in the RER and Golgi. It can be envisaged that the cell responded to the evolutionary requirements for cell differentiation by using epigenetic pathways such as glycosylation and other post-translational modifications (13). Consistent with these speculations, there are, as cited above, numerous examples of glycosylation steps modified by differentiation (56–67, 69), as well as by the anchorage state of the cell and the pH and composition of the surrounding medium (39, 36).

An example of what the future may bear is exemplified by a group of developmental arrest mutants recently characterized in *D. discoideum* (147). Isolated on the basis of failure to accumulate an early regulated enzyme, many of these mutants appear to be altered in posttranslational modifications, including glycosylation. Thus many potentially necessary roles played by glycosylation in development remain unexplored.

## Summary

The nearly ubiquitous glycosylation of extracellular and cell surface proteins and proteins from certain organelles may reflect a primitive, *non-specific* function supporting protein structure and protection. This carbohydrate carries out a shared function, however, when it becomes modified in subtle ways without affecting non-specific functions, to allow discrimination by receptors. Recognition by discriminate receptors allows the carbohydrate to subserve *specific* functions.

Carbohydrate would appear to be used by cells to carry out secondary functions (both specific and non-specific) for proteins. These include specialized instances of compartmentalization, rate of transport through the cell, intermolecular association, conformation control and general protection. Interaction of the cell with the environment, which is in a sense secondary to the life of the cell, would also appear to involve protein-linked carbohydrate. This might include compartmentalization or sorting of cells into tissues. In several instances it has

been shown that carbohydrate interaction is multivalent, composed of multiple weak associations which only in aggregate are strong (155, 123, 172).

It would appear that most protein-linked carbohydrate is not solely responsible for the essential housekeeping functions of cells required for life in the tissue culture environment. Perhaps this is because specification of its structure, which is non-template-directed, is not precise enough. Glycosylation presumably has instead afforded the cell, during the evolution of developmental mechanisms, an economy in allowing the structural modification of many preexisting proteins using the same enzymatic mechanism. It provides the cell with another control point over structure which can be exerted on many proteins at the site of function (away from the site of synthesis) without necessitating a change in gene expression. Change in structure by glycosylation may be very suitable for the regulation of the 'luxury' functions of cell differentiation and morphogenesis. An economy of shared non-specific and specific functions of carbohydrate would thus offer an unifying explanation for the often contradictory conclusions yielded from the varied experimental approaches summarized in this article.

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