Amgen Inc. v. F. Hoffmann-LaRoche LTD et al
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EXHIBIT Y
PART 2

Lodish Decl. in Support of Opposition to Roche's Motion for Summary Judgment of Invalidity for Double Patenting Over Claim 10 of the '016 Patent

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protein could be detected. Each glycoprotein must therefore be evaluated individually to determine the contribution of the carbohydrates to its properties and functions.

Modulation of physicochemical properties

It has been postulated that, from an evolutionary standpoint, once the glycosylation of proteins was introduced, the rather large, hydrophilic glycans were selected for by virtue of certain favorable physical properties they confer to the protein to which they are attached [196]. The negative charges of sialic acid residues and sulfate groups do, indeed, increase the solubility, and affect the conformation of glycoproteins, as well as the adhesive properties of cells [63]. These effects are of particular importance for the function of the highly glycosylated mucins [197], which may carry oligosaccharides on as many as one third of their amino acids [179, 180]. Mucin regions are often found on cell surface receptors, between the lipid bilayer and the extracellular ligand binding domain. It is thought that the role of these mucin domains, which usually have a rigid, rod-like structure, is to extend the functional domains away from the cell surface [198]. There are other ways by which the carbohydrate in a glycoprotein can modify the physicochemical properties of the molecule. The glycans may rigidify the protein by forming hydrogen bonds with the polypeptide backbone. Since the surface area of the carbohydrate moieties is quite significant when compared to that of the peptide moiety, they may, in addition, influence other properties of proteins, such as heat stability and susceptibility to proteolysis. In the case of a glycoprotein like human α_1-acid glycoprotein (molecular mass 44 kDa), which contains five N-glycans essentially of the tri- and tetra-antennary type, the protein could be almost completely enveloped by the glycans [24].

Perhaps the single general function of N-glycosylation is to aid in folding of the nascent polypeptide chain and in stabilization of the conformation of the mature glycoprotein. As a consequence, it may also affect any, or all, of the functions that depend on conformation. When it is prevented, some (glyco)proteins aggregate and/or are degraded, and are, therefore, not secreted from the cells in which they are synthesized. Other glycoproteins are less affected and are secreted, but have compromised biological activities, while some appear to be totally unaffected. Occasionally, the effect depends on the particular site of glycosylation within the protein chain.

Several recent studies highlight these points. Abolition of one or more of the four N-glycosylation sites of the simian virus hemagglutinin sialidase by site-directed mutagenesis led to an impairment in the folding and assembly of the glycoprotein, which in turn affected its intracellular transport. The severity of the impairment depended on the number of glycosylation sites deleted and their location in the protein [199]. Enzymatic removal of the carbohydrate moiety of β_2 glycoprotein I of human plasma produced large changes in the secondary structure of the native protein (as measured by circular dichroic spectroscopy), pointing to the importance of the carbohydrate in maintaining the conformation of the polypeptide chain [200]. The oligosaccharide at Asn22 of the HA_1 subunit of influenza virus hemagglutinin was shown by mutagenesis experiments to be required for the correct proteolytic cleavage of the latter; such cleavage is indispensable for effective virus spread in the infected host and is a prime determinant in virus pathogenicity [201].

Site-directed mutagenesis of each of the three N-glycosylation sites was also performed on human protein C, an anti-thrombotic serine protease that circulates in serum as a mixture of an inactive single-chain zymogen and an active two-chain enzyme [202]. It demonstrated that glycosylation at different sites affects distinct properties of this complex two-chain protein. Thus, glycosylation at Asn97 in one of the enzyme chains is critical for efficient secretion and influences the degree of glycosylation at Asn329 in the other chain; glycosylation at Asn248, on the other hand, affects the intramolecular cleavage and removal of the dipeptide Lys-Arg required for the activation of the zymogen.

Diminished tyrosinase activity is associated with albinism. In melanocytes from a strain of albino mice with a defect in glycosylation, the enzyme was extremely unstable and temperature-sensitive, thus accounting for the decrease in its activity [203]. Saposin B is a glycoprotein that stimulates the hydrolysis of sphingolipids by specific lysosomal hydrolases. Its deficiency results in tissue accumulation of cerebroside sulfate and a clinical picture resembling metachromatic leukodystrophy. Studies of a patient with this disease revealed that, in the defective saposin B, a single amino acid change (from Asn21-Ser22-Thr23 to Asn21-Ser22-Ile23) led to the elimination of an N-glycosylation site [204]. It was speculated that the absence of the corresponding carbohydrate chain in the mutant glycoprotein exposed a potential proteolytic cleavage site, resulting in its rapid proteolysis.

Although there is evidence that the glycans may stabilize proteins against heat denaturation, the effect on thermostability of the attachment of carbohydrate units to normally unglycosylated enzymes has not been systematically investigated in the past. Recently, the relationship between enzyme stability and glycosylation was examined for two different β-1,3/1,4-glucanases from *Bacillus* species that have been expressed in *E. coli* and in *Saccharomyces cerevisiae* [205]. Both the enzymes secreted from the yeasts were heavily glycosylated, with a carbohydrate content of about 45% (which was N-linked), and were considerably more heat-stable than their unglycosylated counterparts synthesized by *E. coli*. The findings described should be of great interest to industry, for which stabilization of enzymes is a common requirement [206].

A rare variant of fibrinogen with an extra N-glycan, not normally present in this glycoprotein, was shown to result from a unique, congenital mutation in its primary sequence [207]. In the mutated glycoprotein, Ser434 of the Aα chain is replaced with Asn, thus creating a new N-glycosylation site to which a di-branched, complex oligosaccharide, mostly disialylated, is attached. The abnormal fibrinogen (designated fibrinogen Caracas II) could be converted to fibrin, but aggregation of the latter protein, a vital step in the clotting activity of fibrinogen, was greatly impaired.

The presence of *O*-GlcNAc on various proteins known to form multimers, including the nuclear pore proteins, erythrocyte band 4.1 and lens α-crystallin, has prompted speculation that it may be involved in the organization of multiprotein complexes [8].

The carbohydrate may change markedly the quartenary structure of a protein to which it is attached, as demonstrated in the X-ray crystallographic study of the *E. corallodendron* lectin [191]. The heptasaccharide, linked at Asn17 of each of the two subunits of this lectin, prevents the formation of the characteristic dimer observed in other homologous legume lectins (e.g. concanavalin A and pea lectin). As a result, these

subunits adopt a completely different three-dimensional structure.

Not only is glycosylation at a particular site important in directing protein folding and assembly, but the precise structure of the glycan may also be critical. A clear case is that of human chorionic gonadotropin (hCG), one of the family of glycoprotein hormones that also includes lutotropin (LH), follitropin and thyrotropin [115, 116]. These hormones are heterodimers of noncovalently associated α- and β-subunits. Within a given animal species, the α-subunits are identical, while the β-subunits are responsible for biological specificity. The α-subunit normally carries two complex-type N-glycans, whereas the β-subunit has either one (in LH and thyrotropin) or two (in hCG and follitropin) N-units. When the α-subunit is expressed alone it is over-glycosylated, in that it contains predominantly multiantennary, fucosylated structures. This form is virtually unable to associate with the free β-subunit of hCG to form the mature hormone. In contrast, the free α-subunit obtained from cells grown in the presence of swainsonine, an inhibitor of the processing enzyme α-mannosidase II, expressed oligomannose and hybrid-type oligosaccharides and associated readily with the β-subunit [208]. It is therefore most likely that, *in vivo*, the α-subunit combines with the β-subunit before its carbohydrate chains are fully processed. If combination does not occur, processing continues, resulting in forms of the α-subunit that lack the ability to associate to the mature, active hormone.

In bacteria it is likely that interactions between the S-layer glycoproteins and the ambient environment are strongly determined by the physicochemical properties of the carbohydrate residues. Studies of the S-layer glycoproteins of *Halobacteria* suggested that their carbohydrate moieties are involved in the shape determination of these organisms. Thus, upon exposing growing halobacterial cells to bacitracin, an inhibitor of N-glycosylation, they changed shape from rods to spheres [10].

Modulation of biological activity

The ability of carbohydrates to modulate the activities of biologically functional molecules, occasionally even in an all-or-nothing manner, has been established unequivocally during the last decade, even though for most glycoproteins the role of the carbohydrates is still obscure.

Enzymes

In the majority of cases investigated, glycosylation has no effect on the biological activity of enzymes. The first clue that this could be the case was provided by nature itself, when it was found that the enzyme ribonuclease occurs both in unglycosylated and variously glycosylated forms, all of which exhibited the same catalytic activity [1, 3]. Recently, however, there has been an increasing, albeit still small, number of glycoprotein enzymes whose activity and stability was shown to be modulated by their carbohydrate units [73].

Perhaps the best documented case illustrating the effect of carbohydrate on enzymatic activity is that of tPA, a serine protease which converts plasminogen into plasmin and thereby induces clot lysis (fibrinolysis). It has a peptide backbone of 527 amino acids, with four potential glycosylation sites, only three of which may be occupied: Asn117 by an oligomannose unit and Asn184 and Asn448 by complex units [209]. Two major molecular species of tPA occur naturally, type I (glycosylated at all three sites) and type II (glycosylated at Asn117 and Asn448 only); each of these species can further exist in a single-chain and double-chain form. A comparison of the rate of conversion of the single-chain into the double-chain form, the enzymatic activity of the different forms and their susceptibility to plasma protease inhibitors [210, 211] led to the conclusion that these properties are affected by the glycan at Asn184. In a subsequent study, in which recombinant type I and type II tPA with modified glycans was produced in CHO cells grown in the presence of the processing inhibitor deoxymannojirimycin, it was shown that the structure of the carbohydrate at Asn448 also affects the catalytic activity of tPA [212].

Hormones

Earlier work has shown that while the chemically or enzymatically deglycosylated glycoprotein hormones bind to their receptors on target cells with the same affinity as the native ones, their ability to activate the hormone-responsive adenylate cyclase is drastically decreased [213]. Site-directed mutagenesis experiments on hCG cDNA implied that glycosylation at Asn52 of the α-chain alone is sufficient for normal signal transduction [214]. Furthermore, in the absence of this critical oligosaccharide unit, glycosylation at Asn13 of the β-chain resulted in intermediate activity of the hormone, whereas glycosylation at Asn30 of the same chain resulted in an inactive product. Recently it was found that deglycosylated hCG interacted with a different domain of the receptor than the native hormone [215]. This difference may be a factor determining the success or failure of signal transduction from the receptor to the effector system. It could also explain the apparently contradictory finding that, although deglycosylation does not impair binding of the hormone to its receptor, binding of the glycosylated hormone was inhibited by various glycopeptides and oligosaccharides [216].

The role of carbohydrates in the activity of erythropoietin, another glycoprotein hormone, is the subject of intense studies [217], not the least because of the great commercial interest in this compound. Desialylation of erythropoietin enhanced its *in vitro* activity by increasing its affinity for the receptor, but decreased its activity *in vivo*, presumably by decreasing its life-time in circulation. Examination of several preparations of recombinant erythropoietin that differ in the degree of branching of their N-glycans revealed that *in vivo* activity of the hormone increased with the ratio of tetraantennary to biantennary saccharides [218].

However, conflicting results have been obtained on the effect of N-deglycosylation on the *in vitro* activity of erythropoietin [73]. The controversy appears now to be resolved by the clear demonstration, provided by two independent groups, that non-glycosylated hormone has severalfold higher specific activity than the native one [219, 220]. These studies also established a correlation between loss of *in vivo* activity upon N-deglycosylation and the loss of sialic acid. In addition, evidence has been presented that the single O-glycan of erythropoietin does not contribute to the activity of the hormone, either *in vitro* or *in vivo*.

Receptors

As with other classes of biologically active glycoproteins, the role of glycosylation in receptor function is not uniform. Like most membrane glycoproteins, they need, as a rule, to be N-glycosylated to attain a conformation necessary for transport to the cell surface. Loss of one (out of three) glyco-

sylation sites of the human transferrin receptor by site-directed mutagenesis was sufficient to prevent the mutated receptor from leaving the endoplasmic reticulum, where it underwent specific cleavage and subsequent degradation [221]. Similar mutational studies of the β-adrenergic receptor revealed that glycosylation is important for correct its intracellular trafficking [222]. In contrast, no such requirement was observed for cell surface localization of the m2 muscarinic acetylcholine receptor [223].

The effect of receptor glycosylation on ligand binding is also variable. For instance, it plays no role in the binding characteristics of the β-adrenergic receptor [222] and of the acetylcholine receptor mentioned above [223], whereas it is required for binding by the basic fibroblast growth factor receptor [224]. Of the six potential glycosylation sites on the rat LH receptor, site-directed mutagenesis on three of the sites resulted in pronounced decreases in binding, while mutations at the other three sites had no effect [225]. Desialylation was found to increase [226], diminish [227] or have no effect [226, 228] on the affinity of other receptors for their ligand(s). Different glycoforms of the low-molecular-mass mannose-6-phosphate receptor differ in their affinity for the ligand: a more highly glycosylated form with a high content of sialic acid had a lower affinity than the form lacking poly-(*N*-acetyllactosamine) units and most of the sialic acid [229]. Quite surprisingly, the non-glycosylated receptor, produced in the presence of tunicamycin, was reported to have unchanged binding properties [230]. The same change in glycosylation may selectively modify the binding properties of closely related receptors, as shown for the insulin and insulin-like growth factor receptors from CHO cell glycosylation mutants [231].

Carbohydrates may also affect the functional coupling of receptors to effector systems such as adenylate cyclase via guanine nucleotide binding proteins, G-proteins and tyrosine kinase, essential for the transmission of signals from the ligand to the cell. Thus, insulin receptor in which all four potential N-glycosylation sites of the β-subunit have been eliminated by site-directed mutagenesis had similar affinity for its ligand as the wild-type receptor but lost its transmembrane signalling ability, as evidenced by lack of stimulation of glucose transport and glycogen synthesis by the hormone [232]. Reports on the role of glycans in the coupling of the β-adrenergic receptors to adenylate cyclase are conflicting [222, 233, 234], possibly due to differences in experimental approaches.

Lectins

Most lectins from plants or animals are glycoproteins and, whenever tested, their activity was not affected by modification or absence of their glycans. For instance, recombinant *E. corallodendron* lectin expressed in *E. coli* has the same sugar specificity and hemagglutinating activity as the native, glycosylated protein [235]. An exception seems to be ricin, the two-chain toxic lectin from *Ricinus communis*, the B-chain of which has been reported to lose its carbohydrate binding activity when produced in *E. coli* [236]. An unusual case of a different kind is that of concanavalin A. The mature lectin is not glycosylated, but during biosynthesis in the plant an inactive, glycosylated, precursor transiently appears. Transformation of the precursor into mature lectin involves, besides deglycosylation, a complex series of polypeptide cleavages and re-arrangements [237]. However, *in vitro* enzymatic deglycosylation alone is sufficient to render the precursor active [238, 239]. Also, the non-glycosylated precursor expressed in *E. coli* was active without further processing. These and other findings led to the conclusion that glycosylation of the pro-lectin is essential for its intracellular trafficking in the plant, possibly by preventing interactions with glycoproteins on its way from the endoplasmic reticulum to its final destination in the vacuoles [240]. Interestingly, wheat germ agglutinin, another lectin which is nonglycosylated in its mature form, is transiently glycosylated during biosynthesis in the plant; no information on the effect of the carbohydrate on the precursor is available [241].

Other glycoproteins

Two types of T-cell-derived IgE-binding factor have been described, one of which enhances, and the other suppresses, IgE synthesis in mast cells [242]. The factors share a common polypeptide backbone, but only the former is glycosylated. This is perhaps the only known case of such a remarkable change in the activity of a protein caused by the presence of a carbohydrate.

Human granulocyte-macrophage colony stimulating factor (GM-CSF) occupies a central regulatory role in renewing and activating the phagocytic system and is being used clinically to prevent certain types of infection following chemotherapy and radiotherapy. Comparison of three differently glycosylated forms of native GM-CSF, and its aglycosylated, *E. coli* derived form, demonstrated a decrease in activity *in vitro* and in affinity of the factor for its receptor with increasing extent of glycosylation [243, 244]. Removal of the single O-glycan chain present in GM-CSF greatly decreased the activity of the factor, probably because the carbohydrate stabilizes the conformation of the protein, or inhibits its polymerization, and thus protects it against inactivation [245].

Immunological properties

The first documented case illustrating the importance of carbohydrates as immunodeterminants was that of the ABO human blood-type determinants, α-linked *N*-acetylgalactosamine in A type, α-linked galactose in B type and α-linked L-fucose in O type [246]. The A and B blood-type-specific oligosaccharides (antigens) are most abundant in intestinal and gastric mucosa, lungs and salivary glands. Significant amounts are also found in other tissues, for instance kidneys, bladder, and bone marrow. Indeed, much of the early structural work on the human blood group antigens was carried out on the water-soluble ovarian cyst mucins. Molecules carrying the antigens include membrane enzymes, membrane structural proteins and receptors (e.g. EGF receptor in A431 cells). Currently, numerous antigenic determinants on glycoprotein glycans are known [247]. Close to half of the monoclonal antibodies generated against animal cells or cell membranes are directed against the carbohydrates of the surface glycoproteins or glycolipids. Plant glycoproteins are very immunogenic, when they contain the β-1,2 xylose and α-1,3 L-fucose attached to the N-linked pentasaccharide core, which are absent in mammalian glycoproteins [152, 248].

The effect of a glycan on the antigenicity of a glycoprotein can be indirect, resulting from its impact on protein folding. In its absence, altered folding may either eliminate epitopes present on the native glycoprotein or create new ones. Thus, two (out of 11) conformational epitopes of vesicular stomatitis virus glycoprotein were rendered inactive to the corresponding monoclonal antibodies when the glycoprotein was produced in the presence of tunicamycin, an inhibitor of

N-glycosylation [249]. These epitopes are, however, unaffected if the N-glycans of normally formed virus glycoprotein are removed enzymatically. It is thus possible that N-glycans may direct the folding of the glycoprotein into its native conformation and that, once this conformation has been attained, it can be maintained in their absence.

A case of the masking of epitopes by carbohydrates emerged in the course of a clinical trial of GM-CSF [250]. From a total of 16 patients that have been treated with recombinant GM-CSF synthesized in yeasts, four developed serum antibodies directed against native GM-CSF. The antibodies reacted with sites on the native protein backbone that are normally protected by O-glycosylation, but are exposed in the recombinant GM-CSF produced in yeasts and *E. coli*. Masking of antigenic epitopes by carbohydrates may be independent of oligosaccharide size. A single *N*-acetylglucosamine at Asn149 was sufficient to prevent recognition of a peptide epitope of influenza virus hemagglutinin by its antibody [251].

The interaction between an antigen and its antibody can be influenced as well by the presence of carbohydrate on the latter. Moreover, the effect of the carbohydrate on antigen binding, whether enhancement or inhibition, depends on its position. Comparison of a number of anti-(α-1,6 dextran) monoclonal antibodies revealed that those with an occupied N-glycosylation site at Asn58 of the variable region of the heavy chain bound dextran with a 15-fold higher apparent K_a than those that lack this glycosylation site [252]. Introduction, by genetic engineering, of a glycosylation site at Asn54 of the variable region of a non-glycosylated anti-dextran antibody blocked antigen binding, while glycosylation at Asn60 increased the affinity of the antigen–antibody interaction about fivefold [253]. The presence of an occupied glycosylation site in the heavy chain variable region of an antibody against the glycolipid galactosylgloboside abrogated the ability of the antibody to bind antigen [254]. It has been proposed that the presence of carbohydrate affects the conformation of the combining site. However, the possibility that inhibition of antibody binding is caused by the carbohydrate blocking access to the binding site has not been excluded.

The fact that the carbohydrate unit attached to Asn247 of the heavy chain of IgG is highly conserved suggests that it may have an important structural or functional role. It has been found that removal of the carbohydrate by site-directed mutagenesis, by glycosidase digestion of mature IgG or by treating IgG-secreting cells with tunicamycin, does not affect the antigen-binding properties of the antibody. However, the carbohydrate-free IgG lost its ability to bind to Fc receptors on macrophages and exhibited a threefold lower affinity for the complement component $C1_q$ than untreated antibody [255]. In addition, antigen-antibody complexes formed from carbohydrate-deficient antibodies failed to be eliminated from the circulation. Glycosylation is also important for the effector activity of IgM molecules.

Tamm-Horsfall urinary glycoprotein (uromodulin), the major protein of human urine, is a powerful suppressive agent of both the lymphocyte proliferation induced by the lectin L-PHA, and of the one-way mixed lymphocyte reaction [256]. The immunosuppressive power of the glycoprotein resides in its carbohydrate portion [257, 258]. These findings emphasize the potential functional role of complex carbohydrates in regulating the human immune response.

Carbohydrates on cell surfaces often modulate cellular immune functions. One of the earliest demonstrations came from studies with lymphocytes, showing that binding of lectins to their surface carbohydrates had a mitogenic effect on the cells [259]. Treatment of the cells with periodate under conditions that oxidize the side chains at C7, C8 and C9 of sialic acid had a similar effect, as did treatment with galactose oxidase, an enzyme that generates cell-surface aldehydes on C6 of galactose and *N*-acetylgalactosamine residues. Recently, it has been reported that treatment of antigen-presenting cells with galactose oxidase greatly enhanced the immunogenicity of viral, bacterial and protozoal vaccines in mice [260]. The underlying mechanism could be transient Schiff base formation between aldehyde groups on one type of the interacting cells and amino groups on the other type which may be required for activation of T lymphocytes by antigen-presenting cells [261]. It is not clear, however, whether sugar residues on cell-surface glycoproteins or glycolipids (or both) were responsible for this effect.

The interaction between cells bearing the CD2 antigen (a member of the immunoglobulin family, also known as LFA-2) and its counter-receptor LFA-3 (also a member of the immunoglobulin family), is markedly increased by T cell activation [63]; erythrocytes that express LFA-3 adhere *in vitro* to activated T cells expressing CD2, forming typical rosettes, whereas no rosette formation occurs with resting cells. The increase in adhesion between CD2 and LFA-3 on T cell activation has been ascribed to the decrease of the negative charge on the T cell surface, resulting from a decrease in the level of sialic acid. This is based largely on the finding that the effect of activation on the CD2/LFA-3 interaction can be mimicked by by desialylation of the erythrocytes or T cells or by covalent attachment of positively charged groups to the erythrocytes.

CD22, another surface antigen of the immunoglobulin superfamily, found on B lymphocytes, mediates lectin-type adhesion of these cells to several leukocyte subsets [262, 263]. It binds specifically α2,6-linked sialic acid, particularly when present on tri- and tetra-antennary oligosaccharides, and the binding is independent of divalent cations [262, 263]. The properties of CD22 appear distinct from those of either C-type or S-type lectins [264] and, as such, CD22 may represent a novel animal lectin. On T lymphocytes, CD22 recognizes several cell-surface sialoglycoproteins, one of which is the leukocyte common antigen, CD45 [265, 266].

Natural killer cells, a population of lymphocytes capable of lysing target tumor cells, are likely candidates for primary defence of the body against cancer cells. Tumor cells vary greatly in their susceptibilty to natural killer lysis. A number of observations have implicated carbohydrates on the surface of the target cells in this selectivity [267]. For instance, mutant CHO cell lines, synthesizing exclusively oligomannose and hybrid-type N-glycans were more susceptible to lysis by natural killer cells than their parent cells that synthesize complex oligosaccharide chains [268]. Furthermore, oligomannose glycopeptides were efficient inhibitors of such lysis. Similarly, K-562 cells, a standard target for human natural killer cells, exhibited increased sensitivity to the latter cells when grown in the presence of an inhibitor of α-mannosidase I (thus synthesizing exclusively oligomannose glycans) [269].

Recognition determinants

Equally (if not more) intriguing than their effect on the physicochemical properties and biological activities of proteins are the roles of carbohydrate groups in biological recognition, where their structure diversity provides signals for

protein targeting and cell–cell interactions. The messages encoded in these structures are transmitted through interactions with complementary sites on carbohydrate-binding proteins, chiefly lectins [54, 64, 270] and perhaps also glycosyltransferases [271]. Another possibility that is now being explored is that carbohydrate–carbohydrate interactions may provide a basis for cell–cell recognition [272, 273]. In the case of cell-surface determinants, the carbohydrate can be in the form of glycoproteins, glycolipids or both. A clear example of this duality is that of the ABO and I/i blood group determinants which, according to most studies, reside largely in glycoproteins (80–85%), while the rest are on glycolipids [274, 275].

Clearance (traffic) markers

The rapid removal of desialylated glycoproteins from rabbit serum via the hepatic asialoglycoprotein receptor (or lectin), a phenomenon discovered in the late 1960s, is the prototype of the saccharide-based recognition system, although its role in nature has not yet been proven beyond doubt [52]. The lectin involved is an oligomeric protein consisting of two types of subunit, with molecular sizes of 40 and 48 kDa. Each of the subunits is a type II glycoprotein, inserted into the membrane by means of a hydrophobic region, with the carbohydrate-binding domain located extracellularly [276].

Several other systems in which the traffic of glycoproteins is controlled by their carbohydrate constituents are known. A prominent example is the intracellular routing of lysosomal enzymes to their compartment which is mediated by the recognition between Man-6-*P* attached to the oligomannose unit(s) of such enzymes, and the Man-6-*P* receptor(s) [277]. Two such receptors have been described, one cation-independent and of high molecular mass (220 kDa), the other cation-dependent and of low molecular mass (48 kDa). Both Man-6-*P* receptors have the same orientation in the membrane. The extra-cytoplasmic domain of the high-molecular-mass receptor consists of 15 contiguous and similar repeating units, while that of the low-molecular-mass receptor is similar both in size and in sequence to each of the repeating units. A defect in the synthesis of the Man-6-*P* marker recognized by the receptors results in I-cell disease (also called mucolipidosis II or MLII), an inherited lysosomals storage disease [49], characterized by a lack in the lysosomes of those enzymes that normally carry the marker. It is caused by a deficiency of GlcNAc-phosphotransferase, the first enzyme in the pathway of mannose phosphorylation, and is thus a processing disease, the first of its kind to be identified. Therefore, even though the disease is transmitted by a single gene, some 20 enzymes are affected. The enzymes lacking the recognition marker do not reach their destination (the lysosomes) and are, consequently, secreted into the extracellular medium which is one of the biochemical abnormalities of the affected cells. Recent work indicates that the specificity of the GlcNAc-phosphotransferase for certain lysosomal enzymes is based on its ability to recognize a specific lysine residue and a particular tertiary domain of the receptor [45].

Another carbohydrate-specified targeting system is that of the sulfated glycoprotein hormones. Native LH, carrying predominantly mono- and di-sulfated oligosaccharides on its β-subunit, is cleared from the circulation four to five times more rapidly than recombinant LH, produced in CHO cells, that bears only sialylated oligosaccharides. The sulfated oligosaccharides of LH are synthesized by the action of two enzymes, a glycoprotein hormone-specific GalNAc-transferase and a GalNAc-specific sulfotransferase. The former enzyme contains, in addition to the carbohydrate binding site, a peptide binding site which recognizes the sequence Pro-Xaa–Arg/Lys in the acceptor protein, located 6–9 amino acid residues from the N-terminus of an N-glycosylation sequon [278]. The oligosaccharide formed by the two transferases just mentioned, SO_4-4GalNAcβ4GlcNAcβ2Manα, is recognized by a receptor present on hepatic endothelial and Kupffer cells which has only recently been identified [279]. Sulfated oligosaccharides were also found on the common precursor to two other hormones, adrenocorticotropin and melanotropin. It has been hypothesized that the attachment of this structure is a general tag that signals rapid clearance, resulting in a short burst of circulating hormones, thus preventing overloading of receptors [280].

The presence of well-defined carbohydrate binding proteins on cell surfaces is being exploited for drug targeting to specific organs. Gaucher's disease is caused by a deficiency of the enzyme β-glucocerebrosidase, resulting in accumulation of glucocerebroside in Kupffer and endothelial (non-parenchymal) cells of the liver. These cells contain on their surface a receptor (lectin) specific for mannose (and *N*-acetylglucosamine) [281]. After trimming, with the aid of glycosidases, of the complex and hybrid sugar chains of β-glucocerebrosidase down to the pentasaccharide core, mannose residues become exposed and the modified enzyme is recognized by the mannose-specific lectin mentioned above. In this way the administered glucocerebrosidase is effectively delivered to the deficient cells where it is needed [68, 69]. Another example is the uptake of ricin by non-parenchymal cells, which appears to result principally from entry of the glycoprotein toxin using the mannose recognition pathway [282].

It is possible that enveloped viruses, which contain glycoconjugates on their surfaces, may also make use of naturally occurring surface lectins, in addition to the normal receptors, as means of attachment to host cells. Thus, a Sendai virus mutant lacking its attachment protein was shown to enter human hepatoma Hep G2 cells by means of the asialoglycoprotein receptor [283].

Fertilization

The interaction between oligosaccharides on the extracellular coat (zona pellucida) of the egg and a carbohydrate-binding protein on the sperm appears to be involved in the highly precise gamete (eggs and sperm) recognition required for species-specific fertilization in mammals. In mousc it has been shown that the ability of the zona pellucida to bind sperm is conferred by terminal α-linked galactose residues on O-glycans of one of the three zona pellucida glycoproteins, designated ZP3 [283a]. It is possible that the carbohydrate-binding protein on the sperm is a β-1,4 galactosyltransferase, an enzyme recently shown to be an integral sperm plasma membrane component and to selectively recognize oligosaccharides on ZP3 [284].

Infection

The oligosaccharide repertoire on the host cell surface is among the key genetic susceptibility factors in viral and microbial infection and in toxin action. A number of viral, mycoplasmal, bacterial and protozoan pathogens use specific

Table 9. Carbohydrates as attachment sites for infectious agents. GP = glycoprotein; GSL = glycosphingolipid.

Organism	Target tissue	Carbohydrate	
		structure	predominant form
Viruses			
Influenza type A	respiratory tract	NeuAc(α2−6)Gal	GP
B	respiratory tract	NeuAc(α2−3)Gal	GP
C	respiratory tract	9-O-AcNeuAc(α2−3)Gal	GP
Bacteria			
E. coli type 1	urinary tract	Man(α1−3){Man(α1−3)[Man(α1−6)]Man(α1−6)}Man(β1−4)-	GP
P	urinary tract	Gal(α1−4)Gal	GSL
S	neural	NeuAc(α2−3)Gal(β1−3)GalNAc	GSL
CFA/1	intestine	NeuAc(α2−8)-	GSL
K99	intestine	NeuGc(α2−3)Gal(β1−4)Glc	GSL
Actinomyces naeslundii	oral	GalNAc(β1−3)Galβ	GP
Neisseria gonorrhoea	genital	Gal(β1−4)Glcβ	GSL
		NeuAc(α2−3)Gal(β1−4)GlcNAc	GP
Streptococcus pneumonia	respiratory tract	GlcNAc(β1−3)Gal	GP
Fungi			
Candida albicans	skin and mucosa	Gal(β1−4)Glc	GSL
Protozoa			
Entamoeba histolytica	intestine	Gal(β1−4)GlcNAc	GP
Giardia lamblia	intestine	Man-6-*P*	GP

carbohydrate structures (of glycoproteins or glycolipids) on host cells as attachment sites in the initial stages of infection (Table 9). This conclusion is convincingly supported by results of experiments in intact animals that demonstrated the possibility of preventing urinary tract infection by blocking the attachment of the responsible bacteria with an appropriate sugar [60a]. It is possible that *in vivo*, such attachment is counteracted, at least in part, by suitable glycoproteins present in tissues and body fluids. One candidate is Tamm-Horsfall glycoprotein, that binds mannose-specific *E. coli* via its single oligomannose unit [256]. It may thus serve as a vehicle for the clearance of bacteria from the urinary tract. Secretory IgA is capable of preventing the attachment of mannose-specific bacteria to mucosal surfaces by binding of the bacteria via its oligomannose units [285]. Such binding could form the basis of the non-immune antibacterial action of secretory IgA *in vivo*. In a similar manner, colonic mucin could serve as a nonimmune defense against the parasite *Entamoeba histolytica*, preventing adherence to, and invasion through, the intestinal epithelium by binding the parasite via the galactose-specific lectin of the latter [286].

Infectious agents bind via their lectins to sugars not only on epithelial cells, but also on phagocytes. As demonstrated extensively with type-1 fimbriated, mannose-specific *E. coli*, such binding may be followed by activation of the phagocytes and uptake and killing of the bacteria, a phenomenon designated lectinophagocytosis, in analogy to opsonophagocytosis, which requires the participation of immune factors (opsonins) [61]. Recently, it has been found that the oligomannose and hybrid units of the leukocyte surface antigens CD11/CD18 (a family of heterodimeric type-I membrane glycoproteins present in different proportions on all leukocytes) serve as receptors for mannose-specific *E. coli* [287]. It has also been shown that lectinophagocytosis may occur *in vivo* [288]. It may thus provide protection against infection by bacteria to nonimmune hosts or in sites that are poor in opsonins. The latter include lungs, renal medulla and the peritoneal cavity, especially during peritoneal dialysis.

In another mode of lectinophagocytosis, a wide range of microorganisms (bacteria, fungi and protozoa) that express mannose on their surface, bind to the mannose-specific macrophage surface lectin mentioned above. Such binding, too, leads to the uptake of the microorganisms by the phagocytic cell and occasionally also their killing [61]. A particularly interesting example of such a microorganism is the pathogenic fungus, *Pneumocystis carinii*, a major cause of death among AIDS patients [289].

Human immunodeficiency virus (HIV), the causative agent of AIDS, is heavily glycosylated [290]. The major envelope glycoprotein gp120 of the HIV has a key role in infection by the virus through its interaction with the membrane glycoprotein CD4 of T lymphocytes. Glycosylation of gp120 appears to be a prerequisite for CD4 binding: the non-glycosylated protein from cells grown in the presence of tunicamycin does not bind to CD4 and treatment of gp120 with deglycosylating enzymes impairs binding. Of the various inhibitors of glycosylation tested, the most dramatic anti-viral effects observed have been with glucosidase inhibitors; in particular, *N*-butyldeoxynojirimycin was found to have strong anti-viral activity, while lacking cytotoxic activity toward indicator T-cells lines [291]. It is now undergoing clinical testing for control of HIV infections [292].

Leukocyte traffic

Research carried out mainly during the last three years has demonstrated that adhesive interactions mediated by surface carbohydrates and surface lectins play a crucial role in leukocyte trafficking to sites of inflammation and hemostasis and in the migration (homing) of lymphocytes to specific lymphoid organs. In these processes, the carbohydrates serve as 'area codes' which are interpreted by a recently discovered family of cell adhesion molecules that are endogenous lectins [63−65, 293] They are designated as selectins: E-selectin (previously known as ELAM-1), P-selectin (previously PADGEM or GMP-140) and L-selectin (LECAM-1). All

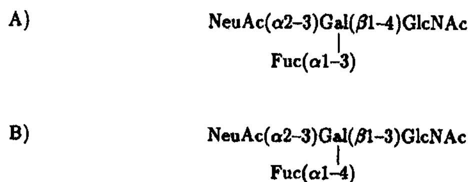

Fig. 6. Structure of (A) sialyl-Lewis^x and (B) sialyl-Lewis^a.

three are highly asymmetric composite proteins with an unusual mosaic architecture, consisting of three types of functional domains. One of these, located at the extracellular tip of the molecule, resembles structurally the animal C-lectins [264]. The others are an epidermal-growth-factor-like domain and one consisting of several short repeating units related to complement-binding protein repeats [64]. Binding of carbohydrate ligands to the lectin-like domain is central to the function of the selectins in cell–cell interactions. Expression of E-selectin on endothelial cells is induced by cytokines; it mediates adhesion of neutrophils and monocytes to endothelium, as well as that of certain tumor cell lines. P-selectin is an integral membrane glycoprotein present in intracellular granules of quiescent platelets and endothelial cells, and is translocated to the cell surface following activation. Like E-selectin, it mediates adhesion of neutrophils, monocytes and subsets of lymphocytes to endothelial cells. L-selectin is the 'homing receptor' for lymphocytes that mediates their tissue-specific adhesion to the high endothelial venules of peripheral lymph nodes. It has been shown that the selectins slow down the movement of leukocytes by inducing rolling of these cells over the endothelial cell surface. During this process, the leukocyte integrins are activated and acquire the ability to bind to their ligands, ICAM-1 and ICAM-2, on endothelial cells.

Both E-selectin and P-selectin bind specifically to sialyl α2,3Le^x (SiaLe^x in brief) and its positional isomer, sialyl-Le^a, on glycoproteins, as well as on glycolipids (Fig. 6); no binding to sialylα2,6 Le^x was observed. Analysis by NMR spectroscopy revealed a remarkable similarity between the conformations of sialyl-Le^x and sialyl-Le^a [294, 295]. Key features required for high-affinity binding are believed to be the carboxyl group of α-2,3-sialic acid, the C4 and C6 hydroxyls of galactose and C2, C3 and C4 hydroxyls of fucose. However, both sialyl-Le^x and sialyl-Le^a are widely distributed and expression of these structures by itself cannot explain the specificity of the individual selectins. It has, indeed, been found that out of several glycoproteins on myeloid cells that carry sialyl-Le^x, P-selectin interacted specifically with a glycoprotein of about 120 kDa, pointing to the role of the carrier molecule in determining the selectin–carbohydrate interaction [296].

The specificity of L-selectin is less well established than that of the two other members of the group. Two ligands for L-selectin, associated with the high endothelial venules, have been identified as glycoproteins, of about 50 and 90 kDa, which are highly sulfated, fucosylated and sialylated. The cDNA for the 50-kDa glycoprotein has very recently been cloned and the derived amino acid sequence was found to be rich in serine and threonine and to contain one potential N-glycosylation site [297]. The peptide backbone can thus be viewed as a scaffold for presentation of mucin-type carbohydrates to the lectin domain of L-selectin. It has additionally been demonstrated that a SiaLe^x-related tetrasaccharide (in which the *N*-acetylglucosamine is replaced by glucose) can interact with L-selectin, as it does with E-selectin and P-selectin [298].

While L-selectin on lymphocytes functions in their homing to peripheral lymph nodes, on the surface of polymorphonuclear cells it participates in the recognition between these cells and stimulated epithelial cells. Its function is believed to be to present carbohydrate ligands to the epithelial E- and P-selectins [299]. Thus, although L-selectin accounts for at most 5% of protein-associated cell-surface SiaLe^x on polymorphonuclear cells (and even less of the total cell surface SiaLe^x if glycolipids are included), its selective removal by mild treatment of the cells with chymotrypsin, or addition of an anti-L-selectin monoclonal antibody to the system, significantly decreased the binding of E-selectin-transfected cells to polymorphonuclear cells.

The clinical importance of selectin–carbohydrate interactions in acute inflammatory responses in humans is illustrated by the finding that the neutrophils of two patients with recurrent bacterial infections (in addition to other abnormalities) had a deficiency in SiaLe^x [300]. The specific biochemical lesion responsible for this defect has not yet been established, but is believed to be a reflection of a general fucosyltransferase deficiency in these patients. In agreement with the ligand activity of SiaLe^x, the neutrophils of the patients were unable to bind to E-selectin. The above findings imply that this inability prevents the neutrophils from migrating to the sites of infection and suggest that inhibitors of the selectins may be potent anti-inflammatory agents [301].

Neural adhesion

Several of the glycoproteins that have been implicated in neural cell adhesion (e.g. N-CAM and L1) express a common carbohydrate epitope, SO_4-3GlcAβ3Galβ4GlcNAcβ3Galβ4GlcNAcβ-R, that is found also on glycolipids [302]. This carbohydrate was shown to be involved in cell–substrate and cell–cell interactions, e.g. between neurons and astrocytes [303], and to promote neurite outgrowth of motor neurons *in vitro* [304]. Carbohydrates also appear to participate in the functional interaction between L1 and N-CAM, resulting in the formation of a complex between the two molecules. Treatment with the glycosylation inhibitor castanospermine, but not with swainsonine, decreased significantly the aggregation between L1 and N-CAM-positive neuroblastoma cells, indicating that the interaction depends on the presence of properly synthesized (although unidentified) complex or hybride-type glycans [305].

A more selective carbohydrate modification of glycoproteins involved in neural cell adhesion is the presence of α-2,8-polysialyl chains on N-CAM [172, 173, 306]. The expression of the highly sialylated form of N-CAM is developmentally regulated. It is abundant in the embryo at the time of neural differentiation and undergoes a post-natal conversion to the adult form with a lower degree of sialylation, resulting mainly from a decrease in the average chain length of the polysialic acid units. The expression of polysialic acid on N-CAMs appears to be critical for regulating a variety of events in the multiple pathways of neuronal development, including neurite fasciculation, neuromuscular interactions and cell migration. Several lines of evidence suggest that these very large, negatively charged carbohydrate chains exert their regulatory effects not only by weakening N-CAM homophilic binding but, more generally, by interfering with cell–cell interactions that depend on the close apposition of cell surface membranes [306, 307].

Other functions

The functions of *O*-GlcNAc remain enigmatic, but there is experimental evidence that it may play a vital role in critical cellular processes, such as nucleocytoplasmic transport and regulation of gene transcription. The levels of *O*-GlcNAc on lymphocyte proteins change rapidly and transiently upon lymphocyte activation; different proteins are affected at different rates, suggesting that the attachment/removal of *O*-GlcNAc is highly regulated [308]. Many transcription factors for genes transcribed by RNA polymerase II are modified by glycosylation, probably by addition of *O*-GlcNAc moieties [309, 310]. The finding that *O*-GlcNAc is present only on the non-phosphorylated form of the transcription factors, and not on the phosphorylated form which is predominant in actively dividing cultured cells, led to the proposal that glycosylation of this type and phosphorylation are mutually exclusive and that the addition of *N*-acetylglucosamine may serve a regulatory function *in vivo* by reversibly blocking potential phosphorylation sites. The recent finding [311], that on the ubiquitous serum response transcription factor the *O*-GlcNAc attachment sites are far removed from the phosphorylation sites, casts some doubt on this proposal. It is conceivable, however, that glycosylation and phosphorylation of the serum transcription factor are interdependent via allosteric mechanisms.

A different form of cyclic addition and removal of carbohydrates to cytoplasmic glycoproteins in response to external stimuli has been described in the ciliate *Paramecium aurelium* [312]; it involves the rapid release and reattachment of α-glucose 1-phosphate to the cytosolic glycoprotein, parafusin, upon stimulation of exocytosis. Since parafusin is an evolutionarily conserved molecule that is present in most eukaryotic cells examined, and the α-Glc-1-*P*-6-Man phosphodiester was shown to be present also in other organisms, e.g. rat liver [313], it is possible that the above reactions represent a novel regulatory mechanism that could be widely applicable [312].

The functional significance of the GlPtdIns anchor, especially that of its glycan, remains obscure. A possible indication of a general function of the GlPtdIns anchor is the finding that treatment of mouse T-lymphocytes with antibodies specific for GlPtdIns-anchored membrane proteins, such as Thy-1, Ly-6 or Qa-2 (a class I histocompatibility antigen), can induce cell proliferation. These and other data indicate that, under some circumstances, transmembrane events can be mediated via GlPtdIns-anchored glycoproteins [18, 314]. It has been suggested that release of glycosyl-phosphatidylinositol from certain GlPtdIns-anchored glycoproteins may play a role in the mechanism of insulin action [315], although this is far from being certain.

In polarized epithelial cells, in which distinct sets of cell-surface components are localized to separate plasma membrane domains, GlPtdIns-anchored proteins are confined to the apical domain and are not present in the basal surface. Introduction of the GlPtdIns anchor into a viral (*Herpes simplex*) glycoprotein changed its localization in transfected cells from basolateral to apical [316]. It has been shown that selective delivery occurs also in hypocampal neurons, where GlPtdIns-anchored Thy-1 is found exclusively on axonal membranes. Thus, GlPtdIns anchorage may be an important factor in establishing cell polarity [317].

Defective GlPtdIns biosynthesis is implicated in a human disease, paroxymal nocturnal hemoglobinuria [314]. Patients with this disease experience periodic hemolysis, due to the absence of two GlPtdIns-anchored proteins, decay-accelerating factor and C8 binding protein, from the cell surface of erythrocytes, platelets and leukocytes. Since the defective cells also lack other GlPtdIns-anchored proteins, it is likely that the molecular basis for nocturnal hemoglobinuria is a defect of GlPtdIns biosynthesis or of anchor attachment; the precise lesion(s) has not yet been determined.

CHANGES DURING DIFFERENTIATION, DEVELOPMENT AND IN PATHOLOGICAL STATES

The expression of certain glycans is strictly controlled, both spatially and temporally, by developmental programs, and is frequently altered in pathological situations, especially in cancer [318–320]. This topic has been thoroughly covered in a recent book [55].

Differentiation and development

A comprehensive picture of differentiation-dependent alterations of cell-surface carbohydrates has been obtained in mouse embryogenesis and in the differentiation of blood cells. The developing embryo expresses carbohydrates that become restricted to specialized tissues in the adult. Re-expression of fetal carbohydrates often occurs in adult tissues that become cancerous [321]. Many of these alterations are found in poly-(*N*-acetyllactosamine) glycans. Early mouse embryos are characterized by an abundance of a large (up to 10 kDa), fucosylated, highly branched poly-(*N*-acetyllactosamine) glycan, called embryoglycan; with development, its amount progressively decreases. Embryoglycan is the carrier of developmentally regulated carbohydrate markers, such as Le^x (also known as SSEA-1) which appears at the 16-cell stage and is believed to be important in the compaction of the embryo. Developmental changes in poly-(*N*-acetyllactosamine) are found in other cells too, e.g. erythrocytes: in the fetal cells, this glycan is linear and tetraantennary and expresses blood type i activity while in adult cells it is branched and biantennary, and acquires blood type I activity.

A highly O-glycosylated sialoglycoprotein, leukosialin, appears to be an early marker in erythroid differentiation. It is abundantly present on an erythroleukemic cell line (probably representing pro-erythroblasts), is a minor component on the surface of erythroblasts in culture and is absent on mature erythrocytes, but is ubiquitous on various leukocytes (except for resting B cells) [168]. The glycans of leukosialin vary in structure not only with cell lineage (erythroid, myeloid and T-lymphoid), but also with different maturation stages along the same lineage, and change dramatically upon T lymphocyte activation. Thus, while leukosialin from resting T-cells expresses almost exclusively the O-linked tetrasaccharide NeuAcα2,3Galβ3(NeuAcα2,6)GalNAc, in activated cells the predominant structure is the hexasaccharide NeuAcα2,3Gal-β3(NeuAcα2,3Galβ4GlcNAcβ6)GalNAc [322]. This change is due to the appearance in the activated cells of (GlcNAc to GalNAc) β-1,6 *N*-acetylglucosaminyltransferase, the 'branching enzyme' of O-glycans, which is not detectable in resting T lymphocytes. The hexasaccharide is also expressed in T lymphocytes from patients with leukemia and immunodeficiency syndromes, for example the rare Wiskott-Aldrich syndrome.

Pathological states, including malignancy

Many pathological states are characterized by changes in the carbohydrate structure of cellular glycoproteins and in

some cases the alterations have been traced to alterations in the activity of specific glycosidases and/or transferases. Congenital dyserythropoietic anaemia type II (or HEMPAS), a rare genetic anaemia in humans, is characterized by the absence of the normal, poly-(*N*-acetyllactosamine)-containing glycans in the erythrocyte membrane glycoprotein band 3 (and band 4.5) and their replacement by unusual, truncated hybrid structures. Red cells expressing such structures aggregate and are removed from circulation. In one patient the defect has been localized to the gene encoding Golgi α-mannosidase II, a critical enzyme in the processing of N-glycans. Cells from two other patients with HEMPAS were characterized by a low level of another processing enzyme, *N*-acetylglucosaminetransferase II [323]. The possibility has therefore been considered that the disease is a genetically heterogenous collection of deficiencies in various steps of N-glycosylation.

The serum IgG, of patients with rheumatoid arthritis contains the same set of biantennary oligosaccharides found in healthy individuals, but in different proportions. There is a significant increase in structures devoid of galactose and terminating in *N*-acetylglucosamine [209, 255, 324]. Women during pregnancy show a negligible concentration of this form at partum and an increase to normal level post-partum. This correlates well with the course of the disease, which enters remission in most women during pregnancy and recurs post-partum. The lower levels of galactose appear to be confined to IgG, since other serum glycoproteins examined, e.g. transferrin, are normally glycosylated. It has been reported that human B cells contain a galactosyltransferase specific for the N-oligosaccharides of IgG and that this enzyme is much less effective in patients with rheumatoid arthritis due to its lower affinity for UDP-Gal [325].

The absence of galactose on the α-1,6 branch of the IgG oligosaccharide could affect the interaction of this branch with the protein mentioned previously; this, in turn, could lead to changes in the conformation of the Fc moiety of IgG and to exposure of new antigenic determinants that may elicit an immune response in the patient, with possible relevance to rheumatoid arthritis. In addition, the sites on the protein originally occupied by the galactose of the α-1,6 branch may interact with galactose still present on other IgG molecules, resulting in the appearance in the patient's serum of complexes typical of the disease, without an actual auto-immune response [255, 324].

Galactose attached in an α-1,3 linkage to Galβ4GlcNAc, displays a unique pattern of distribution in mammals [326, 327]. It is abundant in nonprimate mammals and New World monkeys, but is absent in humans and Old World apes and monkeys, with the possible exception of tPA from human vascular trees and placenta [328]. It was, however, found on several malignant human cell lines and on 50% of malignant breast specimens examined. Since humans normally produce antibodies that recognize Galα3Galβ4GlcNAcβ-R, such antibodies may function in antitumor defense [329].

In several model systems, malignant transformation, tumor cell invasiveness and metastatic potential were shown to be associated with increased levels of GlcNAcβ6Manα6-Manβ4-R branches of complex N-glycans and of GlcNAcβ6 branches attached to the *N*-acetylgalactosamine of Galβ3-GalNAc-Ser/Thr [330, 331]. Detailed studies were carried out on two models: (a) rat fibroblasts and their tumorigenic and metastatic T24H-ras-transfected counterpart, and (b) non-metastatic SP1 mammary carcinoma cells and two of their metastatic sublines. In both models, the metastatic sublines had a nearly double level of the transferase causing branching of O-glycans and a 3–10-fold increase of the β-1,6-branching transferase (GlcNAcT-V) of N-glycans over the non-metastatic lines [332]. Since these two enzymes control the subsequent formation of poly(*N*-acetyllactosamine) chains on O- and N-glycans, they are responsible for the higher levels of these glycans in metastatic cells. The poly-(*N*-acetyllactosamine) chains probably contribute to the metastatic potential by diminishing cell-substratum adhesion, and thereby facilitating tumor cell invasion.

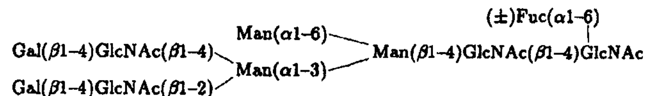

Fig. 7. Structure of N-linked oligosaccharides of human chorionic gonadotropin in patients with choriocarcinoma.

A marked change in the glycosylation of hCG has been observed in patients with choriocarcinoma [333]. The change in the O-chains is quantitative, in that there is a marked increase in those containing the Galβ4GlcNAcβ6 branch attached to Galβ3GalNAc, from about 10% to more than 60%. This is most probably due to an increase in the activity of the branching *N*-acetyglucosaminyltransferase already mentioned. The change in N-units is even more dramatic, in that new structures, not found in normal human glycoproteins, appear (Fig. 7) Similar structures have also been found in γ-glutamyltranspeptidase produced in hepatocellular carcinoma [334]. Their appearance is probably due to a change in the specificity of GlcNAc-transferase-IV, which adds the hexosamine in a β-1,4 linkage to the Manα3 branch and which, in normal cells, does not act on monoantennary oligosaccharides. It has been proposed that these structures could serve as reliable tumor-specific markers and might be used for the diagnosis of certain malignancies, e.g. with the aid of the lectin from *Datura stramonium* or of suitable monoclonal antibodies [333].

In about 50% of patients suffering from various cancers, such as of the pancreas, colon or urinary bladder, there is a loss of expression of the Lewis antigens (Le^a and Le^b) [335], indicating a possible increased risk of cancer among Lewis-negative patients. Tumors which lost expression of the Le^a antigen are associated with more aggressive behaviour of cancers of the bladder, uterus, cervix, colon, head and neck. Loss of expression of blood group A antigen on non-small cell lung cancer is also closely related to the risk of metastasis in patients with A or AB blood types [336]. In that sense, the body's surveillance system for monitoring tumors may be based on detecting changes in glycosylation status [337].

SiaLe^x is expressed in human colon cancer, and the higher the expression rate, the lower the survival rate of the patients after surgery [338]. Also, colon cancer lines with high SiaLe^x epitope expression had a high metastatic activity when transplanted into nude mice; the epitope in these cells was bound to a mucin of 99 kDa. The results suggest a possible role of SiaLe^x in the process of metastasis of human cancer, perhaps by mediating the adhesion of the tumor cells to E-selectin on activated blood vessel epithelium [339].

CONCLUDING REMARKS

The availability of highly refined analytical and preparative techniques for the study of glycoproteins has resulted

during the last decade in a vast increase in the number and types of well characterised compounds of this class, and in the structural elucidation of large numbers of derived carbohydrates. It has thus become clear that living organisms produce a much wider spectrum of protein-linked carbohydrates than previously considered, and that the simple 'rules' that have served in the past as guidelines for research in this area, e.g. that *N*-acetylgalactosamine is confined to O-glycans, sulfate to proteoglycans, and that all N-glycans are linked to proteins via *N*-acetylglucosamine, are no longer valid. The structures of the glycans encountered are exceedingly diverse and we are, as yet, unable to discern the principles that guide their formation. Progress has been made in our understanding of the central role of the polypeptide backbone in specifying glycosylation, although the mechanism(s) by which the information encoded in the amino acid sequence is translated into a particular glycan structure is still not clear. A related problem concerns the molecular basis for the variations in glycosylation of the same protein between different species or cell types, and in the course of development, differentiation and oncogenesis. Answers to these questions are not only of theoretical interest and biomedical relevance, but will have a major impact on the fast-growing biotechnological industry that produces glycoproteins for therapeutic applications.

The intriguing question of the functions of the carbohydrate of glycoproteins, whether soluble or membrane-bound, is still wide open. For a small number of glycoproteins, modulation of the physicochemical properties or biological activities by their glycans has been demonstrated, but for the great majority of these compounds, the role of the carbohydrate remains an enigma. One thing is clear: glycosylation can have markedly different effects on different proteins. This means that each glycoprotein must be examined individually and meticulously for the possible functions of the glycans it carries. Progress in this area will therefore be unavoidably slow. Still unresolved is the question of the biological relevance of microheterogeneity. Very appealing is the idea that the existence of glycoforms may be a means for controlling the biological activity of glycoproteins and nature's way of coping with varied physiological conditions. This concept assumes that unique biological functions can be ascribed to different components of the ensemble of glycoforms, an assumption that has to be supported by more hard data.

The accumulation of evidence on the role of carbohydrates as recognition molecules is most rewarding. It serves as a strong impetus for improving techniques of structural analysis of carbohydrates, for the development of new methods of oligosaccharide synthesis and for lectin research. It also focuses attention on the importance of oligosaccharide conformation in carbohydrate−protein interactions and thus stimulates studies of their three-dimensional structure. We may expect that the knowledge thus accrued will lead to novel approaches for the treatment of various diseases, such as microbial infections, inflammation and cancer. Particularly exciting are the opportunities opened for targeting drugs and genes to desired locations in the body.

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