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# Concepts and Principles of O-Linked Glycosylation

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**ABSTRACT:** The biosynthesis, structures, and functions of O-glycosylation, as a complex posttranslational event, is reviewed and compared for the various types of O-glycans. Mucin-type O-glycosylation is initiated by tissue-specific addition of a GalNAc-residue to a serine or a threonine of the fully folded protein. This event is dependent on the primary, secondary, and tertiary structure of the glycoprotein. Further elongation and termination by specific transferases is highly regulated. We also describe some of the physical and biological properties that O-glycosylation confers on the protein to which the sugars are attached. These include providing the basis for rigid conformations and for protein stability. Clustering of O-glycans in Ser/Thr(/Pro)-rich domains allows glycan determinants such as sialyl Lewis X to be presented as multivalent ligands, essential for functional recognition. An additional level of regulation, imposed by exon shuffling and alternative splicing of mRNA, results in the expression of proteins that differ only by the presence or absence of Ser/Thr(/Pro)-rich domains. These domains may serve as protease-resistant spacers in cell surface glycoproteins. Further biological roles for O-glycosylation discussed include the role of isolated mucin-type O-glycans in recognition events (e.g., during fertilization and in the immune response) and in the modulation of the activity of enzymes and signaling molecules. In some cases, the O-linked oligosaccharides are necessary for glycoprotein expression and processing. In contrast to the more common mucin-type O-glycosylation, some specific types of O-glycosylation, such as the O-linked attachment of fucose and glucose, are sequon dependent. The reversible attachment of O-linked GlcNAc to cytoplasmic and nuclear proteins is thought to play a regulatory role in protein function. The recent development of novel technologies for glycan analysis promises to yield new insights in the factors that determine site occupancy, structure-function relationship, and the contribution of O-linked sugars to physiological and pathological processes. These include diseases where one or more of the O-glycan processing enzymes are aberrantly regulated or deficient, such as HEMPAS and cancer.

**KEY WORDS:** glycosylation, O-glycan, selectin, ZP3, mucin, oligosaccharide, glycosyltransferase, sialyl Lewis X, blood group, immunological recognition, MHC.

**Abbreviations: BOGP,** bovine oviduct-specific sialoglycoprotein; **CII,** collagen type II; **CDG,** carbohydrate-deficient glycoprotein; **CHO,** Chinese hamster ovary; **CTL,** cytotoxic T-lymphocyte; **DAF,** decay accelerating factor; **Dol-P,** dolichol phosphate; **EGF,** epidermal growth factor; **eIF,** elongation initiation factor; **ER,** endoplasmic reticulum; **ERGIC,** intermediate ER-Golgi compartment; **Fuc,** fucose; **Fuc-TVII,** α1-3fucosyltransferase VII; **GA,** glucoamylase; **Gal,** galactose; **GalNAc,** *N*-acetylgalactosamine; **GalNAc-T,** *N*-acetylgalactosaminyltransferase; **G-CSF,** granulocyte colony-stimulating factor; **Glc,** glucose; **GlcNAc,** *N*-acetylglu-

cosamine; **GlyCAM-1,** glycosylated cell adhesion molecule-1; **GPA,** glycophorin A; **GPI,** glycosyl phosphatidyl inositol; **hCG,** human chorionic gonadotrophin; **HEMPAS,** hereditary erythroblastic multinuclearity with a positive acid serum lysis test; **HEV,** high endothelial venules; **HRI,** heme-regulated protein synthesis inhibitor; **HSP-1,** horse seminal plasma protein-1; **Hyl,** hydroxylysine; **Ig,** immunoglobulin; **IgAN,** IgA nephropathy; **IGF,** insulin growth factor; **IL,** interleukin; **LDL,** low density lipoprotein; **LH,** luteinizing hormone; **LPH,** lactase phlorizin hydrolase; **LR,** LDL-receptor; **Man,** mannose; **mFA1,** murine fetal antigen 1; **MHC,** Major Histocompatibility Complex; **MALT,** mucosa-associated lymphoid tissue; **MCP,** membrane cofactor protein; **MCP-1,** monocyte chemotactic protein-1; **NGFR,** nerve growth factor receptor; **NMR,** nuclear magnetic resonance; **OSM,** ovine submaxillary mucin; **PGM,** phosphoglucomutase; **PNGase F,** protein *N*-glycosidase F; **POMC,** pro-opiomelanocortin; **PSGL-1,** P-selectin glycoprotein ligand-1; **sLe$^x$,** sialyl Lewis X; **t-PA,** tissue-type plasminogen activator; **TSH,** thyroid stimulating hormone; **WAS,** Wiskott-Aldrich syndrome; **Sia,** sialic acid; **TfR,** transferrin receptor; **Xyl,** xylose; **ZP3,** zona pellucida protein 3.



## I. INTRODUCTION

More than half of all proteins and many lipids in biological systems are glycosylated. In the case of glycoproteins, the molecular volume occupied by an oligosaccharide is frequently as large as that of the protein domain to which it is attached. Therefore, it is remarkable that it is only relatively recently that the importance of carbohydrates in biology has been appreciated. Several factors have contributed to the rapid expansion of the field of glycobiology in recent years. These include the development of a novel semiautomated technology to sequence carbohydrates, the increasing number of novel structures that have been identified, and the recognition that glycosylation has multiple functions.

The three main posttranslational modifications of proteins that involve carbohydrates are N- and O-linked glycosylation and glycosyl phosphatidyl inositol (GPI) anchors. Examples of each type of glycosylation, with an emphasis on the O-linked oligosaccharides, are shown in Figure 1. For instance, a complex triantennary N-linked sugar is attached to a protein domain (indicated as a grey ribbon) of corresponding size. Similarly, a GPI-anchor at the carboxyterminus is shown. Different types of O-glycans are also compared in Figure 1. These include a sialylated core 1 mucin-type O-linked sugar, an O-linked GlcNAc and an O-linked Fuc, and two O-glycans linked to OH-lysine and OH-proline in collagen and in plant proteins, respectively. N-linked glycosylation is initiated in the endoplasmic reticulum and further processing takes place in the Golgi system. Three main classes of N-linked structures occur: oligomannose, complex- and hybrid-type sugars. For an in-depth review of the structures and functions of N-linked sugars the reader is referred to a number of recent reviews (Kornfeld and Kornfeld, 1985; Rademacher et al., 1988; Varki, 1993; Opdenakker et al., 1993; Rudd and Dwek, 1997). O-linked glycosylation is normally initiated in the Golgi apparatus, most commonly by a *N*-acetyl galactosaminyltransferase that transfers a *N*-acetylgalactosamine (GalNAc) residue to the side chain of a serine or a threonine residue. Subsequently a stepwise enzymatic elongation by specific transferases yields several core structures, which are further elongated or modified by sialylation, sulfatation, acetylation, fucosylation, and polylactosamine-extension. Eight core structures have been identified (Yamashita et al., 1995; Hounsell et al., 1996) and are shown in Figure 2. In addition, a number of other structures, such as O-linked fucose (Harris and Spellmann, 1993), have been described and O-GlcNAc residues have been identified on nuclear gly-

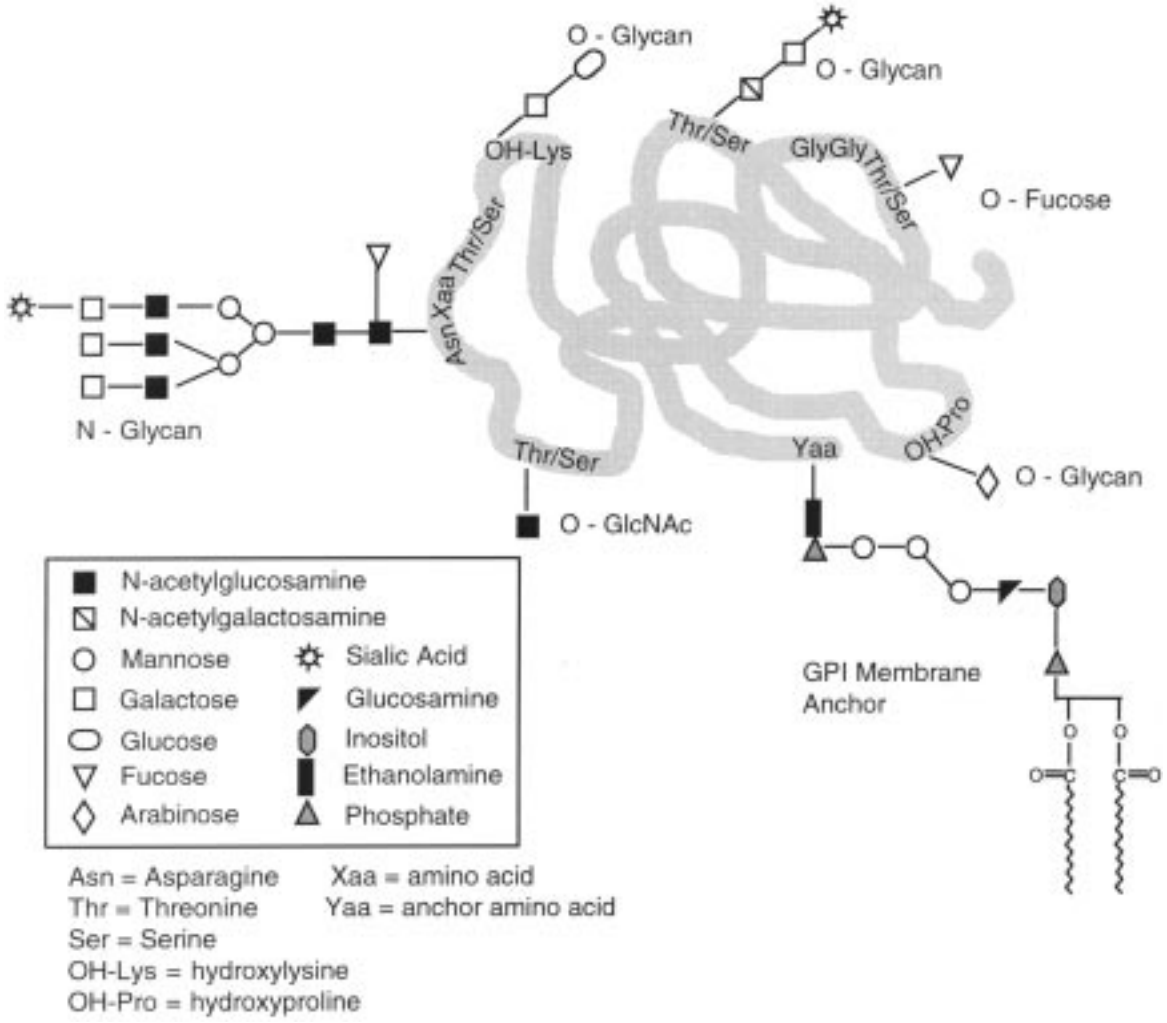


**FIGURE 1.** Comparison of N- and O-linked glycosylation. A hypothetical protein domain of ~20 kDa is shown with the attachment of possible oligosaccharides. N-linked glycans usually possess a considerable volume compared with the protein module and are attached at specific Asn-Xaa-Ser/Thr sequon-sites. Similarly, glycosylphosphatidylinositol (GPI)-binding sites are determined by the primary structure of the protein and mediate the anchoring of glycoproteins to cell membranes through one of the following six amino acids: cysteine, asparagine, aspartic acid, glycine, alanine, or serine. A larger variety of possible attachment sites (threonine and serine residues) and of possible prototypic structures for O-linked sugars exist. The most common type in mammals is the mucin-type oligosaccharide attached to a serine or a threonine through a GalNAc-residue. O-GlcNAc glycosylation occurs often on cytoplasmic and nuclear proteins as a reversible regulatory modification, whereas arabinosylation of hydroxyproline occurs in plants. Hydroxylysines in collagens carry a galactose-linked glycan. O-linked fucose occurs together with O-linked glucose on specific sequons in the EGF domains of several proteins. (Modified from Rudd et al. [1995A].)

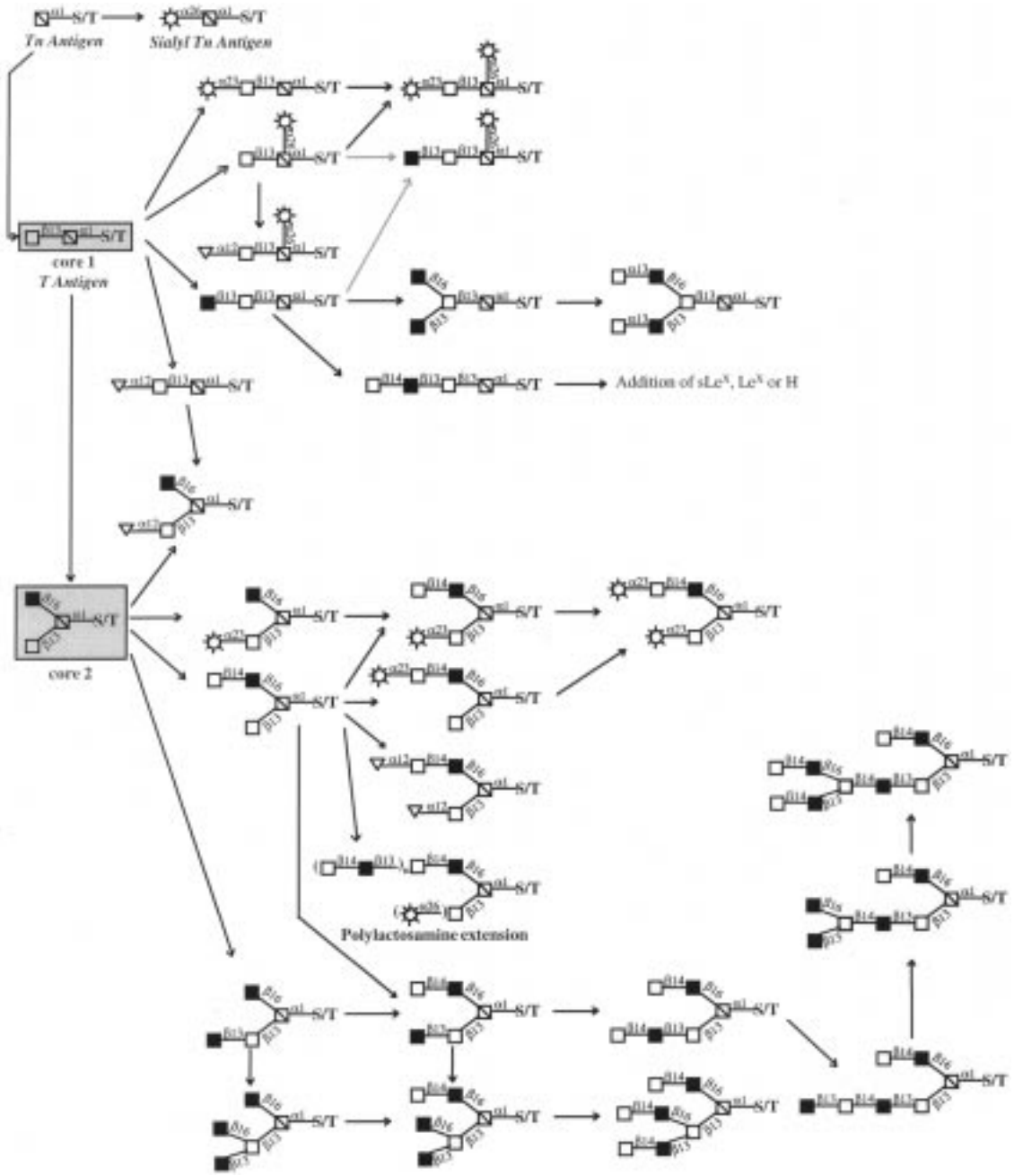


**FIGURE 2.** Biosynthesis of O-linked sugars. Mucin-type O-linked oligosaccharides are attached to serine or threonine by a GalNAc residue. In particular cancer cells, this GalNAc-residue is not elongated and is then called Tn antigen. It may be modified by sialylation to yield the sialyl-Tn antigen. In the synthesis of O-linked sugars, the monosaccharides and the linkage attached to this first GalNAc-residue define the core structures, of which eight have been identified to date. Further elongation and termination by various glycosyltransferases yield a large number of O-linked glycans, of which a number are shown here. These sugars may define various antigenic determinants, for example, Lewis-type antigens and blood group determinants (see also Figures 3, 5, and 7) and F1$\alpha$, an antigen detected in gastric carcinoma. O-linked mannosylation occurs in yeasts. Based on the reviews by Schachter and Brockhausen (1989), Yamashita et al. (1995) and Hounsell et al. (1996), and on Häusler et al. (1992) for the O-mannosylation.

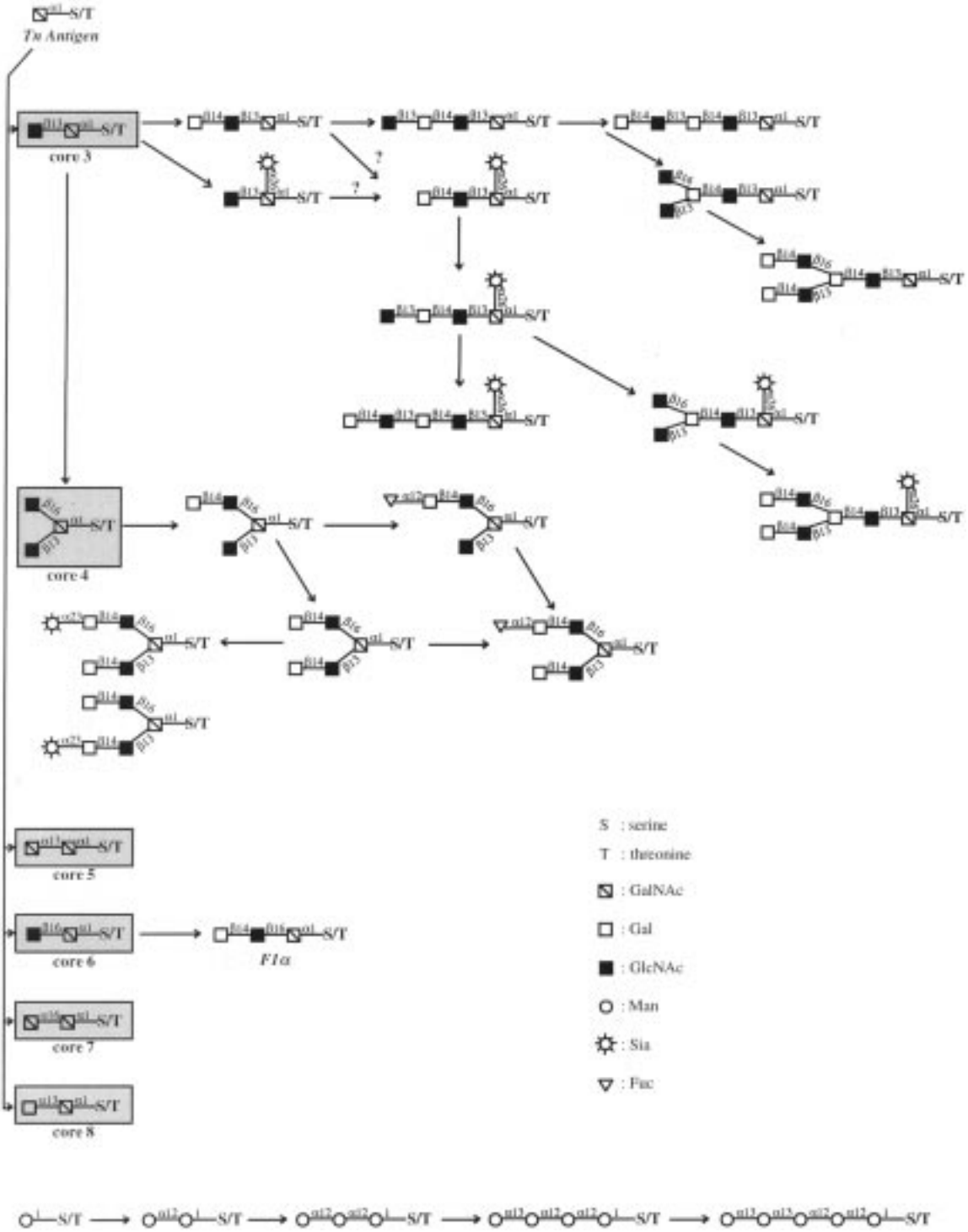


**FIGURE 2** (continued).

coproteins (Hart, 1997). In yeast species, commonly O-linked oligomannose structures are found (Herscovics and Orlean, 1993).

In this review the structures and functions of O-linked sugars are discussed. In particular, protein modules or motifs containing O-glycans have been examined to define general trends in the functions of O-linked sugars. These include conformational alterations of the protein structures and the influence of glycosylation in particular pathologies.

## II. STRUCTURES AND BIOSYNTHESIS OF O-LINKED GLYCANS

### A. Introduction

Various types of O-linked sugars can be distinguished. By definition, mucin-type glycans (commonly found on mucins but also on other glycoproteins) have GalNAc at the reducing terminus and are found on many secreted and membrane-bound glycoproteins in 'higher' eukaryotes. In contrast to N-glycosylation, O-glycosylation does not begin with the transfer of an oligosaccharide from a dolichol precursor, but with the addition of a single monosaccharide, in this case GalNAc. The latter is transferred from UDP-GalNAc into the polypeptide chain to the hydroxyl on some Ser or Thr residues of the fully folded and assembled protein within a ternary complex formed between the protein, UDP-GalNAc and GalNAc transferase (GalNAc-T, EC 2.4.1.41). The location of the subcellular compartment where O-linked glycosylation is initiated is still controversial and may depend on the type of GalNAc-T. It may range from subregions of the ER, a proximal Golgi compartment, an intermediate ER-Golgi compartment (ERGIC), and beyond the ERGIC in the Golgi apparatus (Perez-Vilar et al., 1991; Roth et al., 1994; Roth, 1995).

Elongation of this GalNAc generates eight different core structures (to date) (Hounsell et al., 1996), and these may be further elongated and modified (Figure 2). If this elongation does not occur (e.g., in particular transformed cells), the Tn-antigen (GalNAcα1-Ser/Thr) is generated, which might be modified to sialyl-Tn (sTn) by α2-6 sialylation (Yamashita et al., 1995). The addition of the monosaccharide residues to GalNAc is commonly believed to take place in the Golgi complex. The location of the glycosyl transferases may be cell-type specific (Roth et al., 1986) and vary depending on the stage of cell differentiation (Perez-Vilar et al., 1991). In contrast to mammalian O-glycosylation, mostly oligomannoses are found in yeast and the first step in the biosynthesis occurs in the endoplasmic reticulum.

Some specific types of O-glycosylation have also been identified that deviate from the common pathway. For instance, on a specific consensus-sequence in the epidermal growth factor (EGF) protein domains, O-linked fucoses and glucoses are found (Harris and Spellmann, 1993). This constitutes one of the few examples of O-linked glycosylation for which there is a clear consensus sequence. Proteins of the cytoplasm and nucleus can be reversibly glycosylated with an O-linked GlcNAc, which might function in a similar manner as phosphorylation (Hart, 1997). Some special types of O-linked glycosylation are Gal on hydroxylysines of collagen in the sequence -Gly-Xaa-Hyl-Gly- (Michaëlsson et al., 1994), and the arabinosylation of hydroxyprolines in some vegetal proteins (Kieliszewski et al., 1995). O-linked glycans are found even in bacteria, for example, digalactosyl 2,4-diacetamido-2,4,6 trideoxyhexose on pilines of *Meningococcus species* (Stimson et al., 1995). The xylose-linked polysaccharides of proteoglycans are not discussed here.

Compared with N-linked structures, the analysis of O-glycosylation has proven to be more difficult. In the first place, sites of N-glycosylation can be more readily predicted from the amino acid sequence. For N-linked glycosylation to Asn to occur the primary sequence of the protein must contain a specific sequon (-Asn-Xaa-Ser/Thr- or rarely -Asn-Xaa-Cys-; Xaa is any amino acid except Pro). In contrast, any Ser or Thr residue is a potential site for O-glycosylation, and the rules by which occupied O-glycosylation sites can be predicted are only beginning to be understood (see Section II.B.1). Secondly, while N-linked sugars in a glycoprotein are usually well separated from each other on characteristic sections of peptide, O-linked sugars are frequently located in clusters in short regions of peptide chain that contain repeating units of Ser, Thr, and Pro. Therefore, in contrast to *N*-glycans, the site-specific attachment of O-glycans cannot normally be identified by analyzing isolated glycopeptides that contain a single glycosylation site within a unique section of polypeptide chain. One possible means of solving this problem is the use of automated Edman degradation (Gooley et al., 1997).

The release of O-glycans from proteins using anhydrous hydrazine is also more difficult than hydrazinolysis from N-linked sites because the Galβ1-4GalNAc linkage is particularly labile and degradation of O-glycans takes place readily at high pH. Therefore, for maximum recovery of O-glycans, it is essential to exclude traces of water from the hydrazine reaction and use a lower temperature (65°C) than is optimal for the release of *N*-glycans (95°C) (Patel et al., 1993). In contrast to *N*-glycans, which can be released intact from the protein with peptide *N*-glycanase-F, no equivalent enzyme is available to release O-linked sugars. Endo-α-*N*-acetyl galactosaminidase can cleave the O-glycosidic linkage to Ser or Thr. However, this enzyme is very specific and releases only unsubstituted Galβ1-3GalNAc.

## B. Biosynthesis of Mucin-Type O-Glycans

### *1. Addition of the First GalNAc*

The first step in the biosynthesis of mucin-type O-linked glycans is the addition of a GalNAc-residue to a Ser/Thr by a GalNAc-transferase (UDP-GalNAc::polypeptide *N*-Acetyl-galactosaminyl transferase, GalNAc-T). Despite extended searches and the application of statistical methods on a large number of known glycoproteins, no general consensus-sequence for mucin-type O-glycosylation has been found (Elhammer et al., 1993; Hansen et al., 1995). These statistical studies, however, yielded some general rules (see further), a database of O-glycosylated proteins (O-GLYCBASE 2.0) and an algorithm to predict O-glycosylation sites is available on internet (Hansen et al., 1997).

The difficulties in finding a consensus-sequence for the O-linked glycosylation are due to the complex regulation of the addition of the first GalNAc. Various GalNAc-T initiate O-glycosylation. In 1993 the cDNA of bovine GalNAc-T1 was cloned independently by Hagen et al. (1993) and Homa et al. (1993). Then it was shown that different GalNAc-T activities exist (Sørensen et al., 1995). GalNAc-T2 was purified from human placenta and its cDNA cloned (White et al., 1995), and also the cDNA of GalNAc-T3 was cloned (Bennett et al., 1996). This list is growing steadily: the cDNA of GalNAc-T4 has been found recently by PCR in a murine spleen cDNA library by Hagen et al. (1997), and Hagen and Nehrke (1997) and

Bennett et al. (1997) reported other isoforms of GalNAc-T.

GalNAc-T1, GalNAc-T2, and GalNAc-T3 have broad but different substrate-specificities. For instance, GalNAc-T3 can glycosylate the HIV-V3 peptide, but GalNAc-T1 and 2 cannot recognize this peptide as a substrate. The specificities of these three enzymes have also been analyzed *in vitro* by Wandall et al. (1997). They showed that these three GalNAc-T have overlapping but different specificities with different $K_m$-values for the same site on the same substrate. In contrast, the recombinant form of GalNAc-T4 was shown to have a more restricted specificity, as it glycosylates only one peptide out of an array of 11 (six of which were glycosylated by GalNAc-T1) (Hagen et al., 1997). Some of these GalNAc-T encoding DNAs were also cloned from other species (reviewed by Clausen and Bennett, 1996).

In addition, the different glycosyltransferases are expressed in a tissue-specific manner. The GalNAc-T3 is expressed in the pancreas and testis and less in the placenta, kidneys, colon, and intestine (Bennett et al., 1996). In the liver and the kidney, mainly the GalNAc-T2 is expressed. GalNAc-T4 from the mouse is expressed mainly in the sublingual gland, stomach, colon, small intestine, and cervix, and intermediate levels were seen in the kidney, ovary, lung, and uterus (Hagen et al., 1997). It is also shown that different organs contain different transferase activities (Sørensen et al., 1995). Therefore, one protein can be differently glycosylated, according to the tissue-specific expression of the different glycosyltransferases.

Based on statistical analysis of occupied O-glycosylation sites and on the data of the GalNAc-T, we can deduce the following general rules:

1. The site-specificity of mucin O-glycosylation is **tissue specific**, as there are different GalNAc-T with overlapping but different specificities, and these GalNAc-T have a different tissue-specific expression pattern. This means that no simple consensus sequence can be found.
2. O-glycosylation is mainly a posttranslational and postfolding event. Therefore, only **exposed** serines and threonines will be glycosylated. In accordance with this, the following observations were made: (1) mucin-type O-glycosylation occurs mostly on β-turns of the secondary structure and on other regions with an extended conformation (role of Pro), and (2) in regions with low hydrophobicity (residues like Trp, Leu, Ile, and Phe are rarely seen in the vicinity of O-glycosylation sites). (3) No large amino acids are found in the neighborhood of O-glycans, probably due to steric hindrance.
3. There is a **primary sequence preference** for the mucin-type O-glycosylation. This preference is different for Ser or Thr, as Thr seems to be glycosylated more efficiently than Ser. Many Ser, Thr, Pro, and Val residues occur in the 8 positions before and after an O-glycosylated Thr, and in particular Pro is often found at positions –1 and +3. For Ser, a specificity window of 15 or 39 amino acids was shown; in many cases Pro, Thr, or Ser was present at positions –9, –8, and +1. In addition, Cys was never found in positions –2 to +2, and Trp never in positions –1 and +1. Met, Asp, and Asn were rarely seen in the neighborhood of mucin-type O-glycosylation sites. Positive charges are not seen in the immediate vicinity of the attachment site (maybe the GalNAc-T carries a positive charge); and a negative charge at position –1 was never documented. These rules were established by comparison of different sites on known

glycoproteins, without reference to the tissue specificity.

## 2. Biosynthesis of the Core Structures and Further Elongation and Termination

After the addition of the first GalNAc, further elongation and termination (e.g., by sialylation) leads to a large number of structures, synthesized by various glycosyltransferases, which were surveyed by Field and Wainwright (1995). Already eight core structures have been defined (Figure 2), and these can be further elongated with a 'backbone structure' (Schachter and Brockhausen, 1989; Yamashita et al., 1995; Hounsell et al., 1996). Additionally, the oligosaccharides can be modified, for instance by sialylation, fucosylation, sulphatation, methylation or acetylation.

A well-studied enzyme of this pathway is the UDP-GlcNAc::Galβ1-3GalNAcα1-R β1-6GlcNAc transferase (core 2 GlcNAc-T). This enzyme catalyzes the addition of a GlcNAc to core 1 to form core 2. It was purified in 1991 (Sangadala et al., 1991), and the differential expression in mouse embryos was studied (Granovsky et al., 1995). It is highly expressed in the embryonal and extra-embryonal tissues in the E7-stade (7th day of the gestation). Later, its expression is reduced to certain mesodermal tissues, and afterward to tissues that produce mucins and cartilage. In Chinese hamster ovary (CHO) cells, no core 2 GlcNAc-T activity is detected. However, when butyrate is added, a cAMP-dependent core 2 GlcNAc-T activity is shown (Datti and Dennis, 1993). Another way to increase the core 2 GlcNAc-T activity is to express a cloned core 2 transferase in these cells (Bierhuizen et al., 1994). This provides a method by which the glycosylation of recombinant proteins can be controlled.

The core 2 GlcNAc-T is thought to be rate limiting for the biosynthesis of polylactosamine extensions on O-linked glycans in SP-1 tumor cells, because core 1 sugars are poor acceptors for polylactosamine. In the same way, GlcNAc-TV is essential for the biosynthesis of polylactosamine on N-linked sugars. A small increase in these β1-6 branching GlcNAc-T seems to result in a dramatic increase of the expression of polylactosamine extensions (Yousefi et al., 1991). It was also shown that the developmental expression of polylactosamine (and poly-$Le^x$ and poly-$Le^y$) in the endoderm differentation is regulated by these two β1-6 branching GlcNAc-T (Heffernan et al., 1993). After the synthesis of the core 2, a β1-4Gal-T acts to produce a β1-6 linked lactosamine that can be extended into polylactosamine by β1-3GlcNAc-T(i) and β1-4Gal-T (Figure 2). This structure can also be sialylated and/or fucosylated and can carry the sialyl Lewis x ($sLe^x$) determinant (Moore et al., 1994). It is, for instance, demonstrated that the expression of $sLe^x$ on the P-selectin glycoprotein ligand-1 from neutrophils and its binding to P-selectin is dependent of core 2 GlcNAc-T (Li et al., 1996B; Kumar et al., 1996) and occurs on the polylactosamine-extensions (Moore et al., 1994; see also Section IV.A). The importance of the core 2 GlcNAc-T is also highlighted by studies that show that its expression is enhanced during the activation of T-lymphocytes, and also in leukemia cells vs. normal leukocytes. In AIDS and the Wiskott-Aldrich syndrome, both immunodeficiency diseases, the expression of the core 2 GlcNAc-T is aberrantly regulated (Brockhausen et al., 1991).

The elongation and termination of the O-linked core glycans is complex and was reviewed in an excellent review by Schachter and Brockausen (1989). On the basis of the latter review and more recent studies (Yamashita et al., 1995; Hounsell et al., 1996), a flowchart was made with the biosynthesis of a num-

ber of the O-linked glycans (Figure 2). Briefly, we discuss here the biosynthesis of some important antigenic determinants (Figure 3).

The Lewis x ($Le^x$), Lewis y ($Le^y$), and sialyl Lewis x ($sLe^x$) determinants and the blood group antigens A, B, and H of type 2 are all derived from the structure Galβ1-4GlcNAc-R. This structure occurs on N- and O-linked sugars and also on glycosphingolipids. Fucosylation of the GlcNAc yields the $Le^x$ structure. This structure cannot be further sialylated or fucosylated. Fucosylation of the Gal yields the H-antigen, which can be modified to $Le^y$, A or B antigens. The blood group ABO antigens of type 1 and also $Le^a$ (Galβ1-3(Fucα1-4)GlcNAc-R) and $Le^b$ (Fucα1-2Galβ1-3(Fucα1-4)GlcNAc) can be synthesized from the structure Galβ1-3GlcNAc (Schachter and Brockhausen, 1989). The biologic importance of these determinants is discussed in Sections IV.A and IV.C.

Finally, 9-O-acetylation is an important modification of sialic acids of different types of glycans (N- and O-linked, and also on gangliosides). This highly regulated modification was shown to occur on O-linked sugars. For instance, the expression of 9-O-acetylation of sialic acids of sialomucins in murine erythroleukemia (MEL) cells is primarily regulated by the rate of synthesis of the mucins (Shi et al., 1996A), and an increase in 9-O-acety-

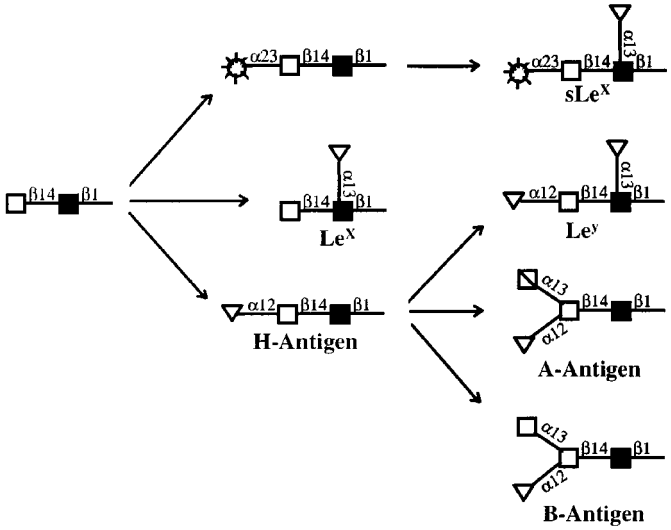


**FIGURE 3.** Synthesis of O-linked antigenic determinants. O-glycans may carry antigenic determinants at the nonreducing terminus. The $Le^X$, $Le^Y$, $sLe^X$, and ABO blood group type 2 determinants are all synthesized on the minimal structure Galβ1-4GlcNAcβ1-. The synthesis of the latter determinants is shown here. The type 1 blood group determinants and $Le^a$ (Galβ1-3(Fucα1-4)GlcNAcβ1-) and $Le^b$ (Fucα1-2Galβ1-3(Fucα1-4)GlcNAcβ1-) are synthesized in analogous way from Galβ1-3GlcNAcβ1-. These determinants are important for the biological functions, for example, the recognition of $sLe^X$ by selectins, or the determination of blood groups. (Based on Schachter and Brockhausen [1989].)

lation is correlated with a higher sensitivity for the alternative complement pathway lysis, as it inhibits the binding of factor H, an inhibitor of this pathway (Shi et al., 1996B). It can also be used as a marker for mouse $CD4^+$ T cells, as it is expressed at the surface of naive $CD4^+$ T cells and not of $CD8^+$ T cells, and the activation of the $CD4^+$ T cells is associated with a substantial decrease of 9-O-acetylation (Krishna and Varki, 1997).

## C. Non-Mucin O-Linked Sugars in Mammals

Mucin-type O-glycans are not the only O-linked oligosaccharides in mammalian cells. Other types include mainly the O-linked fucose and O-linked glucose sugars found in the epidermal growth factor (EGF) domains of different proteins, and O-linked GlcNAc on cytosolic and nuclear proteins.

The glycosylation and other posttranslational modifications of EGF modules were reviewed by Harris and Spellman (1993). Xylα1-3Xylα1-3Glcβ1-Ser and shorter sugar chains (Xylα1-3Glcβ1-Ser and Glcβ1-Ser) have been found on the Ser of the conserved consensus sequence -Cys-Xaa-Ser-Xaa-Pro-Cys- in the EGF domains (Figure 4) of several proteins like the bovine blood coagulation factors VII and IX (Hase et al., 1988), human coagulation factors VII and IX, and human and bovine protein Z (Nishimura et al., 1989), tissue plasminogen activator (t-PA, Harris et al., 1991), thrombospondin (Nishimura et al., 1992A), and murine fetal antigen 1 (mFA1, Krogh et al., 1997). The enzyme activity that catalyzes the addition of the second xylose to the first xylose was detected by Minamida et al. (1996). The functions of these sugars are unknown, except that it was found after mutagenesis of the glycosylated Ser from human factor VII to an Ala that the coagulation activity decreases by an unknown mechanism (the catalytic activity was not affected) (Bjoern et al., 1991). The role of these sugars remains controversial, as it is unknown whether these effects are due to the lack of glycosylation or to the mutation of the amino acid.

On the same EGF domains another type of O-linked sugars was found, also on a well-defined consensus sequence. O-linked fucose was found attached to the serine or threonine of the sequence -Cys-Xaa-Xaa-Gly-Gly-Thr/Ser-Cys- of urokinase (Kentzer et al., 1990), t-PA (Harris et al., 1991), factor VII (Bjoern et al., 1991) and factor XII (Harris et al., 1992). The tetrasaccharide Siaα2-6Galβ1-4GlcNAcβ1-3Fucα1-Ser was found on human (but not bovine) factor IX (Nishimura et al., 1992B; Harris et al., 1993). The fucosyltransferase cDNA, responsible for the initiation of this modification, has been cloned recently (Wang et al., 1996). In Figure 4, the 3D structure of an EGF domain is shown with the modifications by Glc- and Fuc-linked oligosaccharides.

Hajjar and Reynolds (1994) showed that the O-linked fucose is essential for the binding and degradation of t-PA by HepG2 cells (a hepatoma cell line). This has not been confirmed yet, but O-linked fucosylation might mediate the circulatory clearance of glycoproteins by the liver. The consensus sequence may be a protein module that is recognized by a specific fucosyltransferase and that, by its glycosylation, confers a particular function to the recognized glycoproteins (in this case hepatic clearance). The search for and discovery of such sites for O-linked glycosylation must remain a challenge because it will enable better understanding of the biological functions of particular O-linked structures.

Another type of O-linked glycosylation is the addition of O-linked GlcNAc (O-GlcNAc) to serines and threonines of cytoplasmic and nuclear proteins (discussed in more detail in Section IX). This addition is catalyzed by the highly conserved O-GlcNAc transferase, cloned independently by two groups (Kreppel et al., 1997; Lubas et al., 1997),

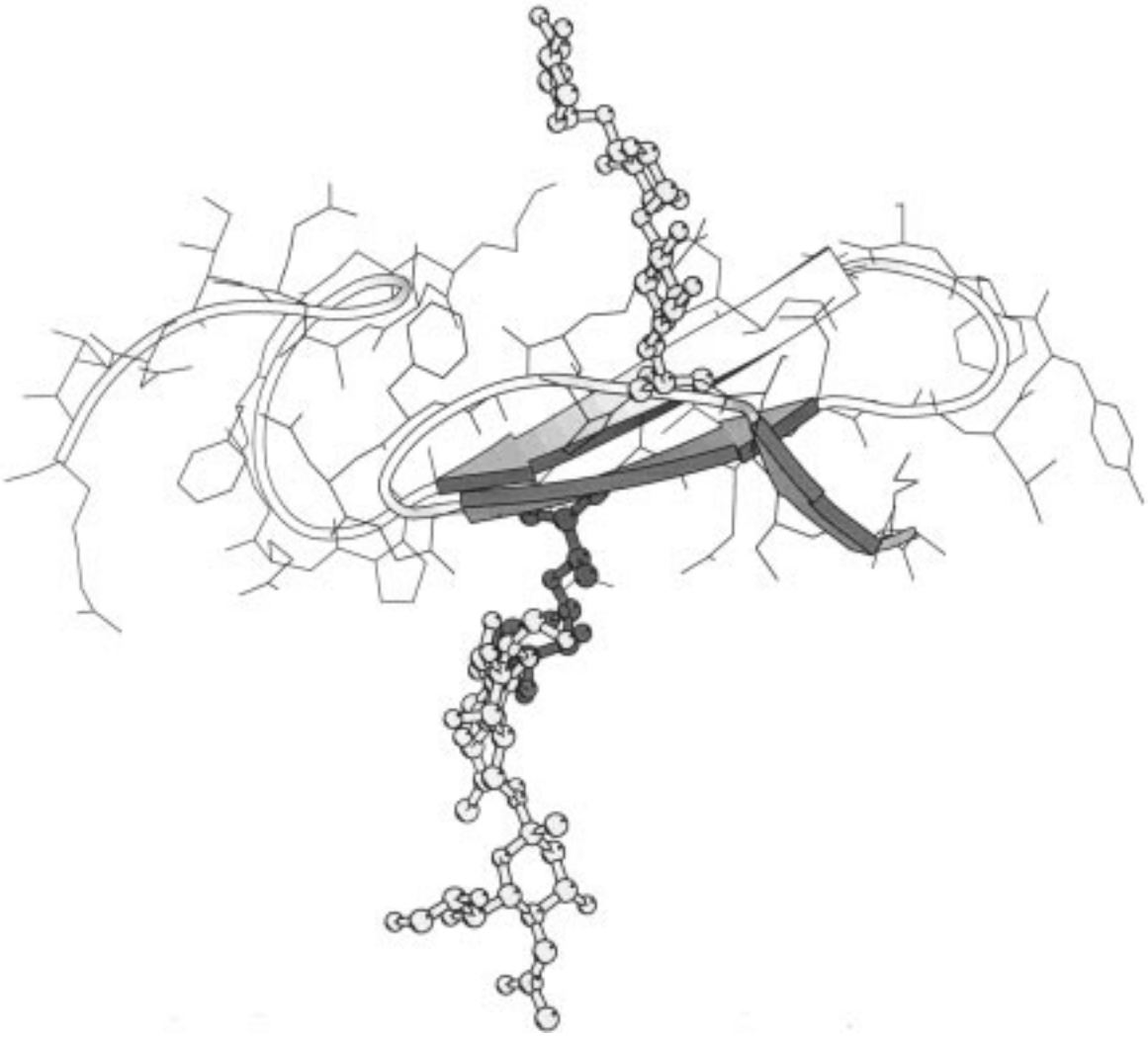

**FIGURE 4.** 3D structure of an EGF-domain. Multiple glycoproteins (e.g., tissue-type plasminogen activator, urokinase, blood coagulation factors VII, IX, XII, protein Z, murine fetal antigen 1, thrombospondin) contain an EGF domain that is posttranslationally modified by Asn- and Asp-hydroxylation, O-fucosylation, and O-glucosylation. These modifications are conserved and sequon determined, in contrast to most other types of O-glycosylation. Therefore, EGF-domains are rather unique molecular modules in terms of O-linked glycosylation. The O-linked fucose on this protein module might function in the binding of the glycoprotein to hepathic receptors that determine the circulatory clearance. The figure shows (Xylα1-3)Xylα1-3Glcβ1- linked to a serine and Siaα2-6Galβ1-4GlcNAcβ1-3Fucα1- (the fucose is in dark grey) linked to a serine of an EGF domain. The latter tetrasaccharide was only found in human factor IX; in other EGF domains only Fucα1-Ser/Thr was detected.

which is especially enriched in the pancreas. The specificity for O-GlcNAc glycosylation is similar to that of proline-directed kinases, and O-GlcNAc sites are often found nearby proline or valine residues, typical in Ser/Thr-rich domains. Often a negatively charged residue is present in the vicinity of the O-GlcNAc (Haltiwanger et al., 1990). This type of glycosylation is abundant but also quite unusual, as (1) it occurs on cyto-

plasmic and nuclear proteins and (2) it is reversible. Consistent with this, a soluble *N*-acetyl-β-D-glucosaminidase has been characterized (Dong and Hart, 1994). It probably has a regulatory role like phosphorylation (see Section IX; and reviewed by Hart, 1997).

Another unusual type of glycosylation in mammalian cells is the O-linked mannosyl type. Clusters of Siaα2-3Galβ1-4GlcNAcβ1-2Man1-Ser/Thr have been found in Ser/Thr-rich domains of the bovine peripheral nerve α-dystroglycan (Chiba et al., 1997). It seems probable that the Sia of this sugar is important for the binding of α-dystroglycan to laminin, as desialylation (but not de-N-glycosylation) results in a diminished binding to laminin. Similar sugars (Siaα2-3Galβ1-4GlcNAcβ1-3Man1-Ser/Thr) have been found in rat brain proteoglycans (Krusius et al., 1986; Margolis and Margolis, 1993).

## D. Biosynthesis of O-Linked Sugars in the Yeast *Saccharomyces cerevisiae*

As mentioned already, the glycosylation in yeasts (oligomannose-structures) is different from that in mammalian cells. For the N-linked glycans, the core structure ($Man_3GlcNAc_2$) can be elongated with 8 to about 14 mannoses, or up to 200 mannoses (Herscovics and Orlean, 1993). A possible pathway for the O-linked oligomannose glycans in yeasts is described by Häusler et al. (1992) and is shown in Figure 2. The first step is the addition of a Man to a Ser/Thr. This reaction is different from the addition of the first GalNAc in mammalians, as (1) it happens in the endoplasmic reticulum and (2) the sugar donor is dolichol-phosphate-Man (Dol-P-Man), and not a sugar nucleotide. The synthesis of Dol-P-Man from GDP-Man and Dol-P is controlled by the *dpm*1-gene. The addition of the Man to Ser/Thr is controlled by *pmt* genes. The first gene of this family to be described, *pmt*1, encodes a Dol-P-D-Man::protein mannosyltransferase (Strahl-Bolsinger and Tanner, 1991). Other *pmt* genes have been identified, up to *pmt*7. Their role in O-glycosylation has only been demonstrated for *pmt*1, *pmt*2, and *pmt*4. Disruption of *pmt*1 results in a decrease of the *in vivo* O-mannosylation to 40 to 50%, whitout other phenotypic changes (Strahl-Bolsinger et al., 1993). The disruption of some combinations of *pmt* genes can be lethal (Gentzsch and Tanner, 1996), and therefore it is concluded that O-glycosylation in yeast is vital. The substrate specificity of PMT1 has been studied, but in common with the observations for mammalian O-glycosylation, no consensus sequences are known yet. A Gly just before the Thr seems to inhibit the O-mannosylation of this Thr, and also an acid residue in the neighborhood inhibits the O-mannosylation. Also, a Pro after the Ser/Thr has a negative influence on the glycosylation efficiency (Strahl-Bolsinger and Tanner, 1991), which is contrary to the effect of Pro for the O-glycosylation in mammalian cells.

The addition of a third mannose is controlled by *mnt*1 and occurs in the Golgi apparatus. This gene is identical to *Kre*2, and the disruption of this gene yields an enhanced resistance against the killer toxin K1 of *Saccharomyces cerevisiae* (Häusler et al., 1992). This shows the importance of O-mannosylation for the killer toxin resistance. Further elongation is probably controlled by *mnn* genes, which are also important for the biosynthesis of N-linked oligosaccharides.

We can conclude that the O-glycosylation in yeasts is different from that of mammalian cells. First, the sugars have a different structure, second, the sequence specificity is different, and third, the first step of the biosynthesis is localized in the endoplasmic reticulum (vs. the Golgi apparatus in mammalians). This might be important, as it implies that the first step can occur cotransla-

tionally, in contrast to the posttranslational (and postfolding) O-glycosylation in mammalian cells.

## III. EFFECTS OF O-LINKED GLYCOSYLATION ON PROTEIN STRUCTURE AND STABILITY

O-linked glycosylation may play a major role in determining the secondary, tertiary, and quaternary structure of the fully folded protein. Otvos et al. (1995) showed that glycosylation distorts the α-helicity of a synthetic peptide homologous to a part of a rabies virus glycoprotein, and that the O-linked glycosylation of Thr is more efficient in perturbing the secondary peptide structure than N-linked glycosylation of Asn. This could be expected as an O-linked sugar is closer to the peptide backbone compared to an N-linked glycan.

### A. O-Glycans Maintain Protein Tertiary Structure

Examples of molecules in which changes in tertiary protein structures are brought about by O-linked glycosylation of the protein chain are the mucins and related molecules that contain domains high in Ser/Thr content (up to 1 in 3 residues) and, in some cases, limited amounts of N-linked sugars (Gowda and Davidson, 1994; Kramerov et al., 1996). Often, molecules with Ser/Thr-rich domains also possess a high proline content (these domains are hereafter referred to as Ser/Thr/Pro-rich domains). Sections of peptide with a high Pro-content and many sugars attached to serines or threonines may assume a 'bottle brush'-like structure.

Mucins are usually secreted by mucosal or glandular cells and possess specific rheological and hydrodynamic properties that can be ascribed to extensive O-linked glycosylation (reviewed by Carraway and Hull, 1991; Strous and Dekker, 1992). As a result, mucin-rich fluids are highly viscous and function, for instance, in the lubrification of mucosal membranes and of the surface of the eye. The major secreted mucin in the colorectum is MUC2 and its production has been confirmed at both mRNA and mature product levels to be localized in the goblet cells in the colorectal mucosa (Corfield and Warren, 1996; Tytgat et al., 1994). In addition, the examination of mucin gene mRNA has shown major expression of MUC4 and smaller amounts of MUC3. However, the nature of the mature mucin products for these genes has not yet been characterized (Corfield et al., 1996). The oligosaccharide chains in mature colorectal mucins consist of 65 to 75% for 4 to 12 monosaccharides per chain and 30 to 40% neutral chains. Core 3, GlcNAcβ1-3GalNAcα1-Ser/Thr, is the predominant core linked to the mucin polypeptide. Poly-*N*-acetyllactosamine "backbones" are found and branching at galactose residues as in I chains is also common. Sialylation is largely through α2-6 linkages to galactose (Corfield et al., 1995). Sulfate is a major substitution in these chains. Both Gal-3-O-$SO_4$ and GlcNAc-6-O-$SO_4$ have been identified, but other linkages may also exist. Shorter oligosaccharides, in particular Siaα2-6GalNAc- (sialyl-Tn), have also been demonstrated as normal components. A unique glycosylation feature of the colonic mucins is the presence of O-acetyl esters on positions 7, 8, and 9 of sialic acids. More than 50% are O-acetylated and 30% contain oligo-O-acetyl forms with either 2 or 3 acetyl esters per sialic acid residue (Corfield et al., 1995). The complex pattern of glycosylation varies with the location throughout the normal

colon. This has been based on immunohistochemical analysis with well-defined antibodies and lectins, but detailed structural analyses remain to be carried out.

Ocular mucins have been detected and partly characterized in man and dog. They share properties with respect to mucin gene peptide and glycosylation patterns (Corfield et al., 1997; Gipson and Inatomi, 1997). The secreted mucins on the ocular surface are principally derived from the conjunctival goblet cells and make a significant contribution to the mucus gel layer in the preocular tear film (Corfield et al., 1997). They are products of the MUC5AC gene in man and dog, together with at least one other unidentified gene product. The mature mucins show polydispersity typical for most secreted mucins and exist as a family of glycoforms that can be separated by ion-exchange chromatography (Berry et al., 1996; Hicks et al., 1997). The glycosylation of the conjunctival goblet cell mucins appears to be characteristic for the eye, as similar patterns of short oligosaccharides, with 1 to 3 monosaccharides form a major portion of the chains found in both man and dog. The structure of all oligosaccharides has not yet been characterized, but GalNAcα1-Ser/Thr (Tn antigen) and Siaα2-6GalNAcα1-Ser/Thr (sialyl-Tn antigen) have been identified. These two structures are of interest as they are uncommon in normal glycoproteins (e.g., human IgA) and have been identified as markers for malignancy in some mucins and glycoproteins. The importance of these short oligosaccharides with respect to the interaction of ocular mucins with bacteria and leukocytes as part of the normal defense processes has been proposed (Corfield et al., 1997).

Mucin-like glycoproteins have also been found in cobra venom secretions. These poisonous mucins carry partially fucosylated polylactosamine extensions (up to 20 repeats of the structures -3Galβ1-4GlcNAcβ1- and -3Galβ1-4(Fucα1-3)GlcNAcβ1-). Such mucins do not enhance the viscosity, but instead increase the solubility of other proteins in the poison (Gowda and Davidson, 1994). The extensive O-linked glycosylation of the repetitive Ser and Thr residues elongates the peptide structure and results in a filamentous protein. The prolines might play a double role: they may confer a signal function for the interaction of the protein with the glycosyltransferases of the Golgi apparatus (see Section 2.A) and, because the nitrogen of the proline does not carry a hydrogen atom, there is no possibility of forming hydrogen bounds that are necessary for the formation of the classic α-helix. This, together with repulsion of sialic acid groups, contributes to the extension of the peptide structure.

Using electron microscopy, Rose et al. (1984) have shown that the ovine submaxillary mucin (OSM) possesses a filamentous morphology and that desialylation with *Clostridium perfringens* sialidase does not alter this structure. In contrast, elimination of all the O-linked oligosaccharides induces a major change in the tertiary structure of the protein that is converted from a linear to a globular shape. It appears that the presence of O-linked oligosaccharides determines the filamentous structure of mucins, but that the precise length and structure of the terminal residues of the glycans are less important. Essentially the same conclusions were drawn from a morphological study of the porcine submaxillary mucin (PSM) and other mucins (Shogren et al., 1986; Shogren et al., 1989). It was suggested that the multiple interactions between the Ser/Thr-linked GalNAc and the peptide backbone form the structural basis of the elongation of the peptide chain (Li et al., 1996A).

O-glycosylated Ser/Thr-rich protein domains also lead to an elongated morphology of other protein chains. Typical examples include the P-selectin glycoprotein ligand-1 (PSGL-1) (Li et al., 1996A), leukosialin (=CD43), and CD45 (Cyster et al., 1991; Shimizu and Shaw, 1993) (see also Figure 8). In these cases, the extended selectin ligand structures enhance the reversible "velcro-ef-

fect" in the early phase "rolling" of leukocyte adhesion to endothelial cells (see Section IV.A.).

Another protein in which O-linked glycosylation plays an essential role in the tertiary structure is the neurotrophin receptor (nerve growth factor receptor, NGFR). Signal transduction through this receptor is crucial for neuronal survival and apoptosis. At the extracellular side, the receptor possesses a ligand binding domain and a "stalk region" that couples the four identical parts of the binding region to the transmembrane domain. This stalk region contains one O-linked core 1 (Galβ1-3GalNAcα1-Ser/Thr) structure that is sialylated extensively. Desialylation results in a lower ligand affinity, and elimination of the carbohydrate further lowers the affinity. In addition to a possible role in signaling to the intracellular milieu, it is likely that the sugar helps to orient the ligand binding domain away from the cell surface (Chapman et al., 1996). A similar function for N-glycans has been suggested by a study of CD2 and LFA-3 that are present on T-cells and antigen-presenting cells and interact through homologous binding surfaces (van der Merwe et al., 1995). Molecular modeling (Rudd et al., in preparation) indicates that the N-linked sugars at Asn126 on CD2 and Asn167 on CD48 are located close to the membrane surface where they may orient the binding site by limiting the conformational space available to the protein. This may provide a physical explanation for the confinement cone of 52° described for CD2 (Dustin et al., 1996) that promotes trans-interactions with CD48 on other cells and discourages cis-interactions with other CD2 molecules on the same cell.

## B. O-Glycans Modulate Aggregation

In common with N-glycans, O-linked oligosaccharides might also influence the aggregation of proteins and their quaternary structure. For example, it has been shown with the OSM that the filamentous form with O-linked sugars aggregates at low ionic strength. This aggregation does not occur in the deglycosylated globular form (Rose et al., 1984). Two other examples are horse seminal plasma protein-1 (HSP-1) and lactase phlorizin hydrolase. The latter is a human intestinal lactose-hydrolase with N- and O-linked sugars. When the N-linked carbohydrates are removed, then the enzyme with O-linked sugars aggregates with the glycotype without O-links (Naim and Lentze, 1992). HSP-1 is a heparin- and gelatin-binding protein from horse seminal plasma. It occurs in two forms that differ in their O-linked glycosylation. One form associates with HSP-2 and the quaternary complex acquires heparin binding activity. The other form does not associate with HSP-2, and thus does not bind to heparin sepharose. However, these two forms do not differ in their gelatin binding capacity (Calvete et al., 1995). Another physiological relevant example is IgA1 (see also Sections VII.B, X.C, and Figure 9). If IgA1 contains more asialo-core 1 (or agalactosyl core 1, Tn) O-linked sugars than usual, it is more sensitive to aggregation, which can be the cause of IgA nephropathy (IgAN; Iwase et al., 1996). Last but not least, the O-GlcNAc-glycosylation of cytoplasmic and nuclear proteins might also influence aggregation (see Section IX.).

## C. O-Glycans Maintain Protein Stability and Confer Protease and Heat Resistance

O-linked glycosylation is important for the stability of glycoproteins. Using NMR, Pieper et al. (1996) assessed this aspect on the T1 fragment of glycophorin $A^N$. As indicated by its name, this 18-kDa protein is

heavily glycosylated. The core 1 O-linked sugars interact with the peptide and are constrained into a particular conformation. Reciprocally, this results in the stabilization of the conformation of the peptide backbone. Another example is granulocyte colony stimulating factor (G-CSF). This protein contains one O-linked sugar that protects the molecule against polymerization at 37°C and against heat denaturation (Oh'eda et al., 1990). It is possible that the sugar chain protects a cysteine (in the ionized state) against oxidizing radical attack (Hasegawa, 1993). It has been shown too that the sugar influences the G-CSF bioactivity (see Section V). A third example that illustrates the role of O-linked sugars on protein stability is the *Aspergillus awamori* gluco-amylase. This enzyme carries 2 N-links and about 40 O-linked oligosaccharides. Neustroev et al. (1993) showed that it is mainly the O-linked glycans that are essential for the stability of the enzyme. Also in this case, the sugars have been shown to be essential for the function of the enzyme in the hydrolysis of raw starch (Goto et al., 1995, see also Section VI).

O-linked glycosylation confers protease and heat resistance to glycoproteins, for example, to the mucins (Gowda and Davidson, 1994; Kramerov et al., 1996). The decay accelerating factor (DAF; CD55) is another typical example. When the O-linked sugars are removed from DAF, the molecule is proteolyzed (Coyne et al., 1992). The cellular heat resistance is partially due to O-linked sugars. Of course, heat shock proteins play a central role in thermoresistance, but Ménoret et al. (1995) documented that heat resistance is associated with the expression of blood group A and H antigens at the erythrocyte cell surface. In this case, the resistance is independent of heat shock proteins.

In conclusion, O-linked glycosylation has a pronounced effect on protein structure in terms of secondary, tertiary (elongation of the protein backbone), and quaternary structure (aggregation). In some cases this structural influence has already been associated with functional differences. These aspects are reviewed below.

## IV. O-LINKED GLYCOSYLATION FUNCTIONS IN RECOGNITION

O-linked oligosaccharides are crucial elements in some glycoprotein-protein interactions, as has been shown by the loss of recognition function after deglycosylation. Typical examples are the selectin-ligands and the zona pellucida glycoproteins.

### A. O-Linked Glycosylation of Selectin Ligands

Selectins form a family of adhesion molecules that assist in governing leukocyte circulation in the organism. P- and E-selectins are detected on blood platelets and activated endothelial cells and direct, for example, migrating leukocytes to sites of infection and inflammation. L-selectin is expressed on leukocytes and is important in the transendothelial migration of leukocytes in the high endothelial venules (HEV). Leukocyte migration through the blood vessels starts by "rolling" on the endothelia. This phenomenon is a reversible slowing down and/or halting of leukocytes that otherwise would be drawn by the hydrodynamic forces of the circulation. Further (irreversible) steps in transendothelial leukocyte migration are mediated by, for example, integrins (Springer, 1994). At the extracellular side the selectins possess a number of domains that are homologous with complement regulating proteins, for example, the epidermal growth factor (EGF) domains and the C-type lectin domain. The latter recognizes

the carbohydrate part of the ligands (Roitt et al., 1996).

### 1. PSGL-1 (P-Selectin Glycoprotein Ligand-1)

PSGL-1 is the most important ligand of P-selectin, but can also bind to E-selectin (Asa et al., 1995). It is a thin, extended membrane protein (Li et al., 1996A) with a mucin-like serine-, threonine-, and proline-rich domain (Ser/Thr/Pro-domain). Many of the serines and threonines carry O-linked oligosaccharides, some of which have polylactosamine extensions with a terminal sialyl-Lewis-x structure (Moore et al., 1994; Li et al., 1996B). Most probably these O-linked sugars are responsible for the molecular morphology (Li et al., 1996A; see Section III.A): the extended structure arises from the interactions of the GalNAc residues with the peptide skeleton.

PSGL-1 also contains three N-linked oligosaccharides. These are not necessary for the binding to P-selectin (Li et al., 1996B). The minimal structure for low-affinity binding of PSGL-1 to its P-selectin receptor is the sialyl-Lewis-x (Siaα2-3Galβ1-4(Fucα1-3)GlcNAc = $sLe^x$) (Hooper et al., 1996), the 3D-structure of which is presented in Figure 5. In neutrophils this structure occurs almost exclusively on the polylactosamine extension (Moore et al., 1994). Maly et al. (1996) showed that the α1-3Fucosyl transferase (Fuc-TVII) is essential for the expression of $sLe^x$ on selectin ligands. For instance, Fuc-TVII knock-out mice have a phenotype of deficiency in leukocyte adhesion and of leukocytosis. It has also been demonstrated that recombinant CHO-derived PSGL-1 only binds to P- and E-selectins if the CHO cells are cotransfected with core 2 β1-6 GlcNAcTransferase and an α1-3 Fucosyltransferase (Fuc-TIII, Fuc-TIV or Fuc-TVII) (Li et al., 1996B; Kumar et al., 1996). The GlcNAc transferase is essential for the generation of the core two oligosaccharides; the fucosylation follows later in the biosynthesis. It is concluded that in the mouse Fuc-TVII is essential but that it can be replaced by other α1-3 fucosyl transferases. The importance of the $sLe^x$ structure and function was illustrated further by injection in rats that were poisoned with cobra venom. Exogenous $sLe^x$ diminished the neutrophil-dependent lung damage. Similar results in this animal model were obtained by pretreatment with monoclonal antibodies against P-selectin (Mulligan et al., 1993).

The sialyl-Lewis-x structure has also been found on cells that do not express PSGL-1. This structure is certainly not sufficient for high-affinity binding to P-selectin. For this to happen tyrosine-sulfation is also needed (Hooper et al., 1996; Li et al., 1996B). O-linked oligosaccharide sulfation is also required for the binding of GlyCAM-1 to its receptor, E-selectin, and is discussed below. In contrast, tyrosine-sulphation is not necessary for binding of PSGL-1 to E-selectin.

### 2. L-Selectin Ligands

The most prominent ligands of L-selectin are CD34 and GlyCAM-1 (glycosylated cell adhesion molecule-1) (Hooper et al., 1996). GlyCAM-1 possesses two Ser/Thr-rich domains with a cluster of O-linked oligosaccharides that induce protein extension. These oligosaccharides have terminal $sLe^x$ structures. For recognition by L-selectin three structures are needed: sialic acid, fucose, and 6-*O*-sulfatation of $sLe^x$ Gal or GlcNAc (Imai et al., 1993; Hooper et al., 1996). These criteria must also be met for CD34 that occurs in the sulfated form only in the high endothelial venules (HEV).

Colostrum contains GlyCAM-1 in nonsulfated form (Dowbenko et al., 1993). These

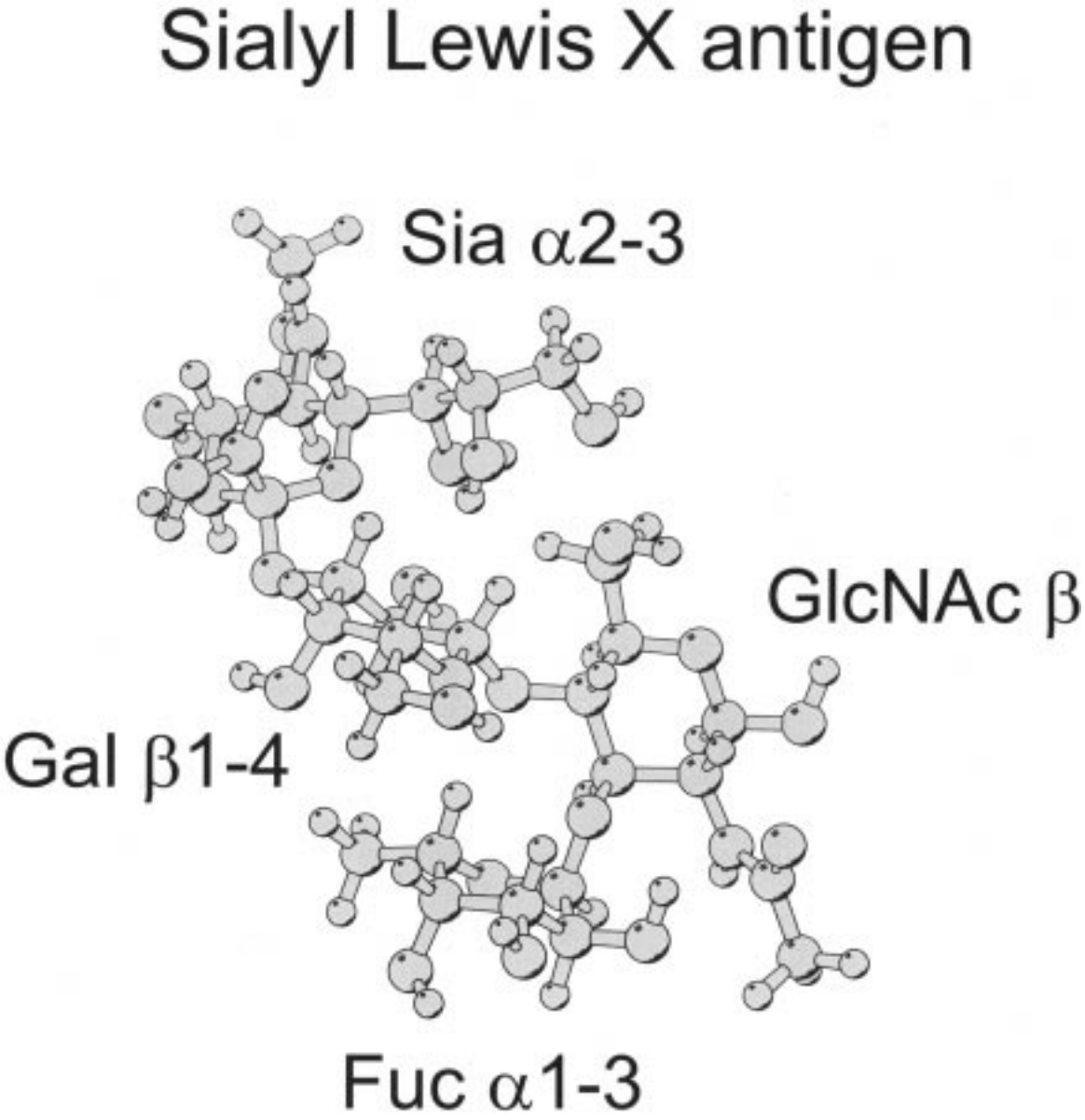


**FIGURE 5.** 3D structure of Sialyl Lewis X antigen. The sialyl Lewis X antigen ($sLe^X$, see Figure 3 for the biosynthesis) functions in cell-cell recognition. By the binding to selectins $sLe^X$ is responsible for the low-affinity cellular interaction. Additional modifications (e.g., tyrosine sulfatation or sulfatation of $sLe^X$ itself) are necessary for higher affinity binding, depending on the type of selectin. (Based on Wormald and Edge [1993].)

molecules are therefore not recognized by L-selectin. Presumably, the function of GlyCAM-1 in milk is completely different from its role in immune cell recognition. Maybe it protects the intestinal mucosa of the newborn against rotavirus entry. Indeed, these viruses have receptors for O-linked sugars (see Section IV.F). Based on these examples of various functions of GlyCAM-1, it is tempting to define the peptide part as a "scaffold" for the functional sugars.

Another ligand of L-selectin is MAdCAM-1 (Yang et al., 1995). This glycoprotein is pre-

sent on the HEV of mucosa-associated lymphoid tissue (MALT) and contributes to local leukocyte traffic. MAdCAM-1 possesses three immunoglobulin domains and one Ser/Thr-rich domain. The latter contains the carbohydrate structures that are recognized by L-selectin. In addition, MAdCAM-1 can bind the α4β7-integrin LPAM-1. This integrin is related to VLA-4 (α4β1 integrin) and binds to VCAM-1 (VLA-4 ligand) and fibronectin. The binding of MAdCAM-1 to LPAM-1, however, is sialylation independent (Yang et al., 1995). Finally, the last known L-selectin ligand, an *O*-sialoglycoprotein, has not yet been identified. The latter molecule plays a role in the aggregation of neutrophils on endothelial cells during inflammation (Bennett et al., 1995B). The interaction of L-selectin with this *O*-sialoglycoprotein induces a low affinity binding that results in a β2-integrin-mediated high-affinity interaction.

### 3. E-Selectin Ligand

The E-selectin ligand is not yet known (Hooper et al., 1996), although sulfated Lewis bloodgroup antigens (e.g., 3-*O*-sulfate-Le$^a$: $SO_4$-3-Galβ1-3(Fucα1-4)GlcNAcβ1-) bind well to E-selectin. It is not certain that the ligand of E-selectin is a mucin-like glycoprotein (Izumi et al., 1995).

Mucin-like glycoproteins on cancer cells have been implicated in metastasis. Izumi et al. (1995) demonstrated that the interaction between E-selectin and O-linked sugars (sLe$^x$) is crucial in the hepatic colonization by certain human colon carcinoma cells: higher expression levels of sLe$^x$ at the cell surface of the tumor cells increases the metastatic potential. Other cell lines show a differential interaction with the various selectins (Mannori et al., 1995). Some cells bind to P-selectin and adhere to platelets, whereas others adhere to neutrophils through L-selectin. The latter can result in dual effects, depending on the secondary activation signals for the granulocytes. The neutrophils might become more tumoricidal or, by the activation and release of, for example, proteolytic activity, they might assist in the metastasis of the cancer cells. It has been demonstrated that the treatment of HL60 leukemia cells with an inhibitor of elongation of O-linked glycosylation (benzyl-α-GalNAC) decreases the selectin-dependent adhesion and increases the integrin-dependent binding to fibronectin (Kojima et al., 1994).

In summary, selectin ligands contain a Ser/Thr/Pro-rich domain that carries a number of O-glycans. This confers an elongated conformation to the ligand, important to 'capture' the leukocyte in the bloodstream. More, the sugars of the Ser/Thr/Pro-rich domain carry the minimal structural determinant for binding to the selectins, and these determinants can be located at the end of polylactosamine extensions. Depending on the selectin type, further modifications (sialylation and sulfatation) result in high-affinity binding. The expression of the binding determinants can also be regulated, which is clearly shown for GlyCAM-1. These types of interactions play key roles in the normal trafficking of leukocytes of our immune system, in leukocytosis and in pathological processes such as metastasis and tissue-specific colonization by cancer cells (Springer, 1994; Kraal and Mebius, 1996; Opdenakker et al., 1998).

## B. Function of O-Linked Oligosaccharides in Fertilization

O-linked glycosylation is essential in the recognition events between the oocyte and the fertilizing spermatozoon. The oocyte is surrounded by a glycoprotein layer, the zona pellucida. On a specific place in this zona pellucida, the crater region, the sperm cell

binds for fertilization. This binding event results in the acrosomal reaction, which consists of exocytosis of permeabilizing molecules. In many species the binding is mediated by O-linked oligosaccharides of a glycoprotein of the crater region, that is, zona pellucida protein 3 (ZP3) (Yurewicz et al., 1991; Youakim et al., 1994; Gong et al., 1995; Kinloch et al., 1995; Yonezawa et al., 1995). After the penetration of the sperm cell, the activated oocyte immediately releases hexosaminidase that starts to cleave the O-links of the other sperm cells and thus prevents polyspermia. The species specificity of the binding is mainly determined by the structure of the oligosaccharide chains on ZP3. These sugars depend on the ZP3 polypeptide backbone and it is interesting to note that there is a high species-specific divergence in the amino acid sequences of the ZP3 region involved in the binding (Kinloch et al., 1995).

In the mouse the carboxyterminal part of mZP3 is essential for sperm cell binding. This region is encoded by gene exon 7 and contains five serines (Ser-329, –331, –332, –333, –334) as potential O-linked attachment sites. The O-linked glycosylation motif of five serines with an ultimate Ser that is separated by one residue from the other four serines is a recurring pattern. This sequence Ser-Xaa-Ser-Ser-Ser-Ser might signal the species-specific attachment of the O-linked sugar. In these cases of O-linked sites, there are no local prolines involved, but instead usually a conserved region of nine amino acids (of which four are cysteines) confer the right conformation for O-linked sugar attachment. So far, it is unclear whether O-links with a terminal α-Gal or a terminal β-GlcNAc mediate the binding with the sperm, although there is more evidence for terminal α-Gal. Oocytes, and not sperm cells, express the α1-3 Gal transferase (Johnston et al., 1995) and Litscher et al. (1995) have shown that synthetic oligosaccharides with a terminal Gal inhibit the binding of sperm cells to oocytes, whereas oligosaccharides with terminal GlcNac are ineffective as inhibitors of binding.

In the mouse the β1-4 galactosyltransferase (GalTase) has been identified as one of the sperm receptors of the mZP3 (Gong et al., 1995). There are two forms of the GalTase: one has a short cytoplasmic domain, is localized in the Golgi apparatus, and has a biosynthetic function; the other possesses a long cytoplasmic domain and is situated at the surface of the sperm cells. The latter one initiates the binding to ZP3. When several (multivalent) interactions between GalTase and the ZP3 structures occur, the GalTase aggregates on the cell membrane. This leads to the activation of the sperm cell by a G-protein complex through the cytoplasmic part of the GalTase (Gong et al., 1995). This is a clear example of the increasing number of phenomena that imply a requirement for multivalency and aggregation for signal transduction (see Section IV.F).

Transgenic overexpression of GalTase in the mouse (Youakim et al., 1994) leads to an increased binding of spermatozoa to ZP3. However, this binding is less intimate than expected. This is due to an increased binding of epididymal glycosides in the transgenic animals. These glycosides bind to the sperm GalTase. Before the fertilization can occur, these glycosides need first to be removed and this is more difficult when GalTase is overexpressed. A second reason for a lower fertility of these animals is the increased sensitivity of the spermatozoa for ZP3. Due to the higher number of surface GalTase molecules, the aggregation and ensuing signal transduction are facilitated, which induces a premature acrosome reaction in the spermatozoa.

There is still discussion as to whether in the pig N-linked oligosaccharides mediate oocyte-sperm binding. Yurewicz et al. (1991) reported that purified O-linked ZP3 sugars (but not N-linked sugars) inhibit sperm bind-

ing. At least some N-linked sugars contain anionic sulphated/sialylated polylactosamine extensions and the O-linked structures have sialylated polylactosamine. These polylactosamines are not involved in the sperm cell binding. Yonezawa et al. (1995), however, separated (pig) PZP3α from PZP3β after removal of the polylactosamines. In that case only the PZP3α mediates binding through N-linked sugars. The discrepancies between these results are not yet clear. In addition, a special receptor has been discovered on pig sperm cells, the spermadhesin AWN-1. AWN-1 binds sialylated core 1 O-linked oligosaccharides (Siaα2-3/6Galβ1-3GalNAc) but also (with a lower affinity) certain N-linked structures such as Siaα2-3/6Galβ1-4GlcNAc. Desialylation results in a fivefold lower affinity (Dostalova et al., 1995). We conclude that O-linked and possibly also N-linked oligosaccharides of PZP3 play a role in sperm cell adhesion to oocytes in the pig and that AWN-1 is a receptor for these sugars.

AWN-1 and also other spermadhesins (AQN-1, -2, -3, PSP-1, AWN-2) may become glycosylated themselves (Calvete et al., 1994). AWN-1 carries mutually exclusively N- or O-linked sugars. By this glycosylation the molecule loses the capacity to bind to the zona pellucida and to certain other molecules (e.g., soybean trypsin inhibitor). After capacitation of the sperm cell for binding to the oocyte, only nonglycosylated AWN-1, which can bind ZP3, remains on the sperm cell surface. This suggests a decapacitation function for the glycosylation of AWN-1. This is further suggested by the presence of acid β-D-galactosidases in epididymal secretions. These enzymes catalyze alterations of sperm surface molecules (Tulsiani et al., 1995). It is hypothesized that deglycosidases play a role in the capacitation. Focarelli and Rosati (1995) documented the role of O-linked glycosylation of sperm cells in another organism, *Unio elongatulus*.

In the bovine species an oviduct-specific sialoglycoprotein (BOGP) with sperm protecting-activity has been described. BOGP contains one N-linked and 15 O-linked sugars. Satoh et al. (1995) showed that desialylation and further deglycosylation destroys the activity of the glycoprotein.

In the human an acrosome-reaction inhibiting glycoprotein (ARIG) has been described (Drisdel et al., 1995). It is present in semen and inhibits premature acrosome reactions. The inhibitory capacity of this molecule is completely dependent on the N- and O-linked sugars such that removal of either N-links or O-links completely abolishes the bioactivity.

We conclude that glycosylation in general and O-linked structures in particular play a crucial recognition role in reproduction. The importance is in the generation of a species barrier and the avoidance of polyspermia by activation of signal transduction. Glycosylation fulfills a function in the fertilization of an oocyte by a sperm cell but is also essential for the capacitation and viability of the spermatozoon.

## C. O-Linked Glycosylation in Specific Immunological Recognition

A basic concept of our defense system is the observation that foreign (non-self) proteins induce an immune response. For this to occur, the recognition of non-self epitopes of the foreign molecule or its derivatives is essential. Antibodies can recognize three-dimensional epitopes, whereas T-cell-dependent antigens are processed into short linear peptides. Glycosylation of these epitopes has a profound influence on this immunological recognition. Here we discuss first the influence of O-linked glycosylation on the binding of an epitope to the major histocompatibility complex (MHC) and the recognition by the T-cell receptor, as well as the effect

on the immune response, and then we summarize some data on glycosylation in antigen-antibody recognition.

The MHC-II complex, which is expressed in antigen-presenting cells, is intracellularly complexed with the invariant chain $I_i$. Only when a peptide is bound in the MHC-II groove will the MHC-II be freed from the $I_i$ chain and presented at the cell surface where the recognition by the T cell receptor of $CD4^+$ T-lymphocytes can take place. Studies with monoclonal antibodies have shown that the oligosaccharide chain on $Thr_{187}$ of $I_i$ is completely covered in the $I_i$-MHC-II complex and this is not the case with free $I_i$. This suggests that the O-linked sugar might function in the stabilization of the $I_i$-MHC-II complex (Nguyen et al., 1993). As a consequence, O-linked glycosylation may play a role even before the recognition of the antigen.

It has also been demonstrated that the N-linked glycosylation of MHC-I influences the recognition by the T cell receptor. In this case, the presence of N-linked sugars on MHC-I seems to hinder the recognition of the MHC-I-bound peptide by the T cell receptor (Shen and Kane, 1995). This is in accordance with findings that relatively voluminous N-linked oligosaccharides downmodulate the activity (e.g., enzymatic function or signaling activities) of many molecules (Opdenakker et al., 1993).

The glycosylation of peptides profoundly influences the binding to MHC molecules. A number of remarkable examples of molecular mimicry are described below.

A peptide of wild-type (WT) Sendai virus nucleoprotein binds to MHC-I complex $H\text{-}2D^b$ with the Asn at position 5 (P5-Asn) as anchor (Haurum et al., 1995). P5-Asn lies in the MHC complex groove and forms there three hydrogen bridges with the MHC molecule. When P5-Asn is glycosylated it can no longer bind to $H\text{-}2D^b$. When P5-Asn is replaced by Ser (yielding peptide K2), there is also no binding, but when this Ser is glycosylated with GlcNAc (K2-O-GlcNAc) the binding is restored. This GlcNAc thus mimics the Asn residue in that the GlcNAc amide replaces the Asn amide group. Another example is the VF13N synthetic peptide derived from the rabies virus glycoprotein. When glycosylated, this peptide binds less well to the MHC-II complex. N-linked glycosylation seems to be more efficient in this respect than O-linked glycosylation, although O-linked glycosylation is more efficient in breaking the α-helicity of the peptide (Otvos et al., 1995, see Section III).

Sometimes the glycosylation of a particular peptide residue has no effect on the binding with MHC, but instead on the recognition of the peptide-MHC by the T cell receptor. Cytotoxic T lymphocytes (CTL) are an important arm in our immune defense against viruses, particular bacteria, and tumors. They are equipped with specific receptors for short peptides derived from pathogen (or tumor) proteins presented on the surface of the infected (or transformed) cell by the MHC-I. CTL preferentially recognize peptides derived from cytosolic and nuclear proteins, a fact that reflects the cytoplasmic site for degradation by the proteasome. Because O-glycosylation is a common modification of cytosolic and nuclear proteins (see Section IX), it is possible that CTL might recognize O-GlcNAc substituted glycopeptides as part of their wide-ranging repertoire of specificities.

An example of this is the synthetic peptide K3 (Haurum et al., 1994). K3 binds efficiently to MHC-I complexes and possesses reactivity with a particular T cell clone. The K3-O-GlcNAc (O-α-GlcNAc bound to position 4 of the peptide) binds both $H\text{-}2K^b$ and $H\text{-}2D^b$ and is also reactive with a specific T cell clone, which does not or only barely cross-react with the K3 specific CTL-clone. Furthermore, K3-O-GalNAc binds to $H\text{-}2D^b$ too, but it is not recognized by the K3-O-GlcNAc-specific CTL clone. This illustrates

that the glycosylation at a position that is not essential for MHC recognition might still have an influence on T cell recognition. Haurum et al. (1994, 1995) have shown that CTL elicited in response to synthetic O-GlcNAc-substituted glycopeptides are truly glycan specific, and Abdel-Motal et al. (1996) reported that direct immunization with glycopeptides can generate an MHC-unrestricted hapten-specific CTL response. This is consistent with the X-ray crystal structure of glycopeptide:MHC complexes (Figure 6) that show that the glycan is oriented toward the T cell receptor when it is bound to the MHC molecule (Glithero et al., in preparation). This is an important observation because it shows that in principle, a "self" peptide can be made "foreign" by the addition of a single O-linked saccharide. Thus, situations in which there is aberrant glycosylation pose the potential threat of initiating an autoimmune reaction.

Molecular mimicry has been proposed as one of the basic mechanisms of autoimmunity. O-linked glycosylation plays an important role in this mechanism. Typical examples of this are the T cell responses against OH-Lys glycosylated collagen type II and the cross-reaction of anti-GlcNAc antibodies with nonglycosylated cytokeratin peptides. Collagen type II (CII) is posttranslationally modified by hydroxylation of prolines and lysines. OH-Lys is further modified by glycosylation (Galβ1-*O*-Lys or Glcα1-2Galβ1-*O*-Lys). This modification occurs in the immunodominant part of CII and is essential for the T cell response and is a crucial event in arthritis (Michaëlsson et al., 1994). Immunization of mice with the immunodominant fragment of CII induces arthritis and the severity correlates with the glycosylation of the CII fragment.

An example of molecular mimicry in the humoral immune system are the anti-GlcNAc antibodies. These antibodies are generated as a result of microbial infections by *Streptococcus* species. Indeed, GlcNAc forms an immunodominant epitope on the *Streptococcus* group-specific A carbohydrate (Shikhman and Cunningham, 1994). However, these antibodies have been shown to cross-react with a cytokeratin peptide (SFGSGFGGGY), even if this peptide is not glycosylated or if it is chemically synthesized. In addition, this peptide binds to GlcNAc-specific lectins such as wheat germ agglutinin (WGA), *Datura stramonium* agglutinin (DSA), and others (Shikhman et al., 1994). This is a remarkable example of molecular mimicry in which a carbohydrate epitope, GlcNAc-glycopeptide, resembles a cytokeratin peptide.

Recognition of glycosylated antigenic determinants might be relevant for cancer therapy. In the malignant state, the cancer cells, in general, underglycosylate the glycoproteins; this results in immunoreactive carbohydrate epitopes (see Section X.B). Another example in which the glycosylation influences recognition by antibodies is leukosialin (also known as CD43 and sialophorin). Epitope specificity of CD43 depends mainly on the linear protein sequence because recombinant CD43, produced in *Escherichia coli* and hence nonglycosylated, is recognized by almost all anti-CD43 antibodies. However, desialylation of glycosylated CD43 results in a drastic decrease of the binding by these same antibodies (Cyster et al., 1991). This suggests that the sialylation maintains the conformation of the peptide epitopes without necessarily being involved in the recognition event.

Of extreme importance in medicine are the blood groups. More that 20 blood group systems have been described in humans (Roitt et al, 1996), the most important of which is the ABO system. The ABO blood groups are completely determined by sugar epitopes that are carried by different glycoproteins such as the anion transport protein and the Glc transporter. Blood group O contains the minimal H antigen (Figures 3 and 7), which in blood group A is extended with GalNAc, and in blood group B with Gal. Each individual, although tolerant for self-epitopes, produces antibodies against the epitopes that he or she

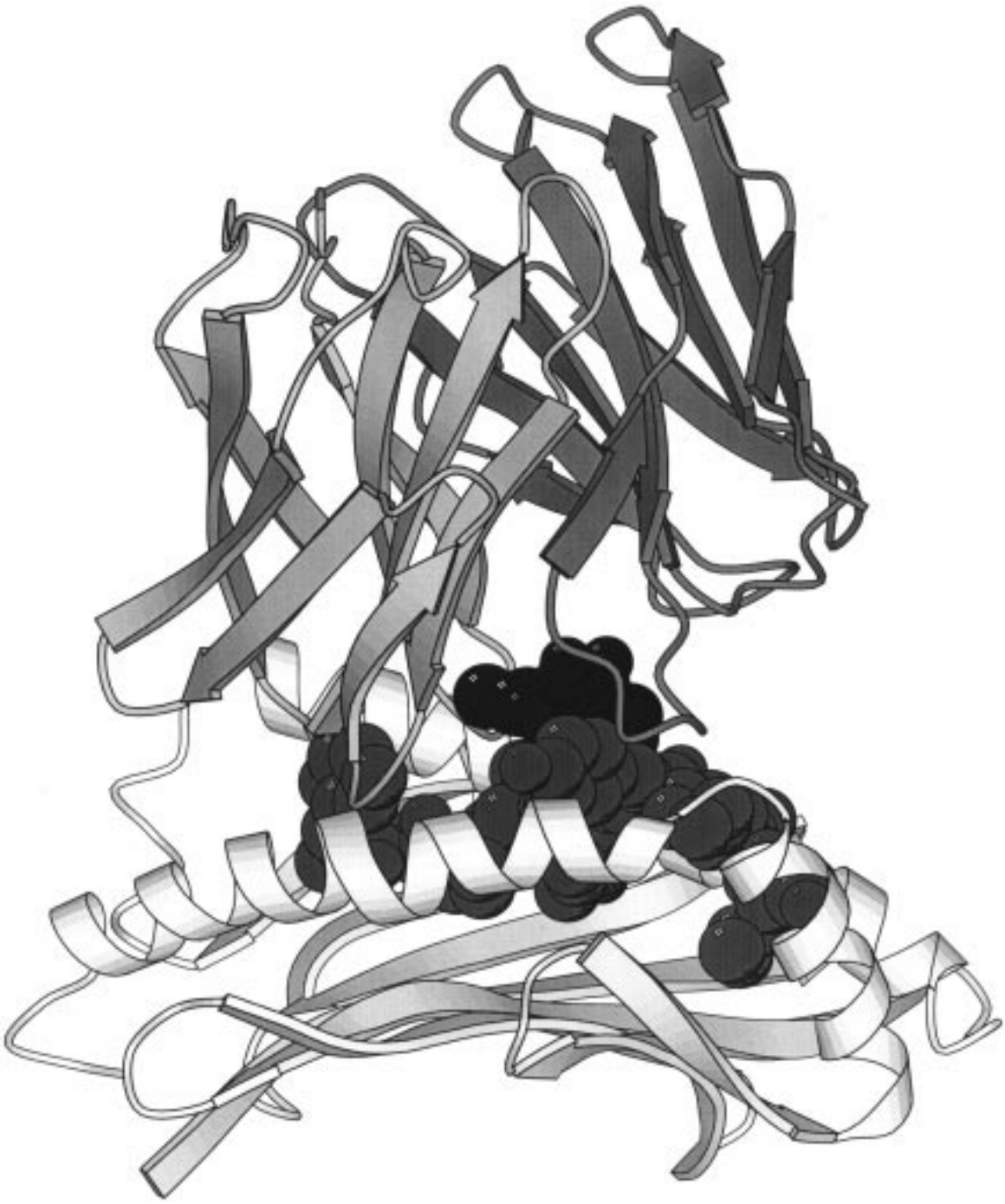

**FIGURE 6.** Orientation and recognition of a glycopeptide in the interaction between class I MHC and the T-cell recepor. The K3-O-GlcNAc peptide (FAPS-O-GlcNAcNYPAL, indicated as balls) binds to mouse class I MHC H-2D$^b$ (at the bottom). This complex is recognized by a glycopeptide-specific H-2D$^b$ restricted T-cell receptor (at the top) on a sugar-specific CTL clone. As can be seen on this X-ray cristallographic structure (based on Glithero et al., in preparation), the GlcNAc-residue (in black) is oriented toward the T-cell receptor, which explains the sugar specificity of the T-cell clone. The same glycopeptide with a GalNAc instead of the GlcNAc is recognized by another specific T-cell clone.

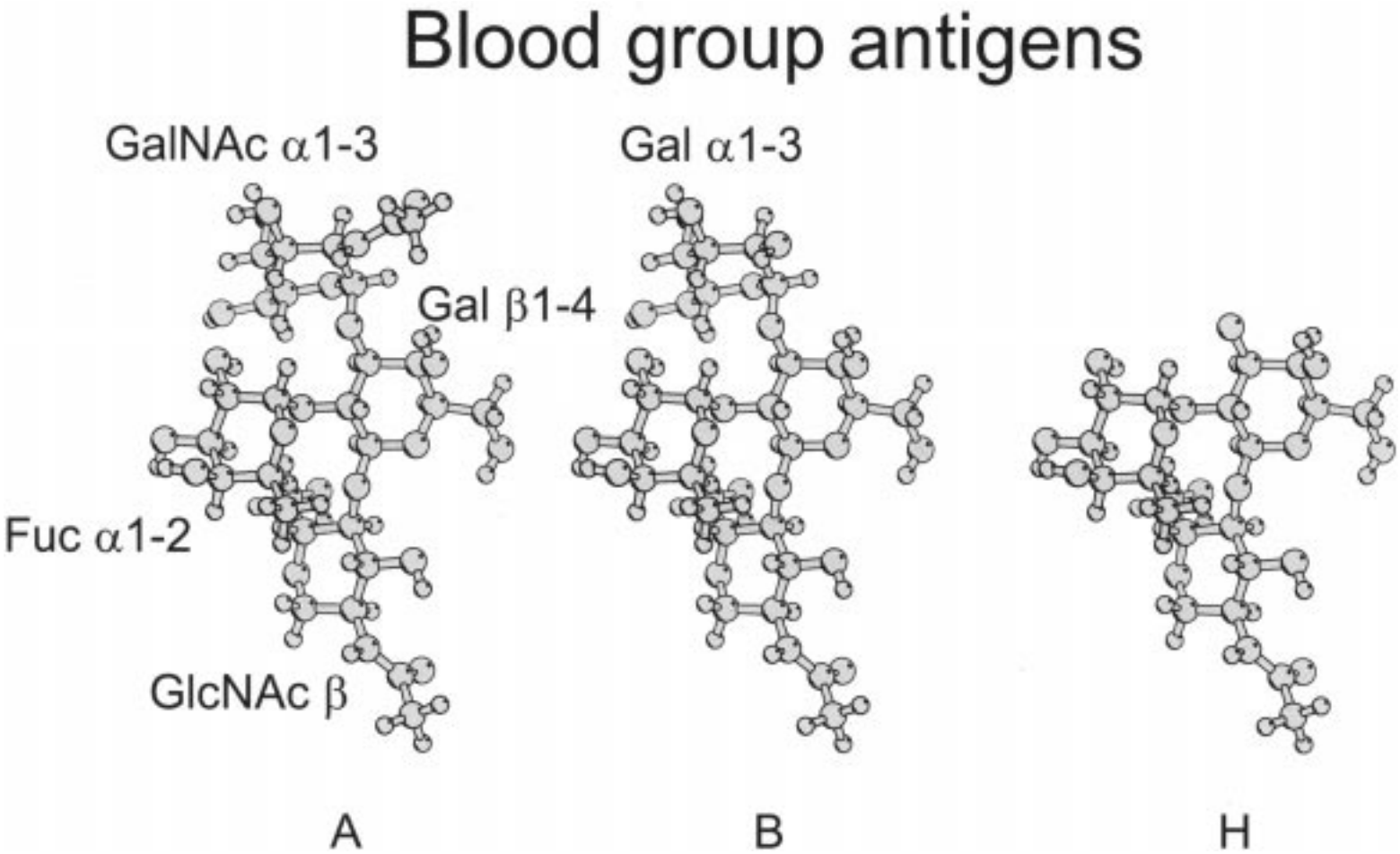


**FIGURE 7.** 3D structures of blood group antigens A, B, and H. The major blood groups A, B, and O are determined, respectively, by the A, B, and H oligosaccharide antigens. These are present on different glycoproteins such as the anion transport protein and the the glucose transporter of erythrocytes. An individual is immunologically tolerant for self-epitopes and produces antibodies against the other blood group epitopes that occur abundantly in the environment on microbial oligosaccharides. The H-antigen is the minimal structure, the A-antigen possesses an additional GalNAc, and the B-antigen a Gal. (Based on Bush et al. [1986].)

does not have, probably by regular contact with microbial sugar structures. Individuals with blood group A thus make antibodies against the blood group B antigen and vice versa. Another important blood group system is the Rhesus system; the antigenic determinants of this system, however, are protein epitopes and are not discussed here. There are also other minor blood group systems such as the MN system and the Miltenberger blood groups Mi.I and Mi.II. The MN blood groups, as well as the Miltenberger groups (Ugorski et al., 1993), are determined by polymorphic protein epitopes that are localized at the aminoterminus of glycophorin A (GPA, literally "the sugar-carrier A") (Roitt et al., 1996). In 1994, Jaskiewicz et al. showed that glycosylation influences these epitopes. The following epitopes were identified on GPA: for M: $Ser_1$-$Ser_2$-$Thr_3$-$Thr_4$-$Gly_5$-$Val_6$-$Ala_7$-$Met_8$-$His_9$ and for N: $Leu_1$-$Ser_2$-$Thr_3$-$Thr_4$-$Glu_5$-$Val_6$-$Ala_7$-$Met_8$-$His_9$ and the rarely occurring $M^g$: $Leu_1$-$Ser_2$-$Thr_3$-$Asn_4$-$Glu_5$-$Val_6$-$Ala_7$-$Met_8$-$His_9$. In GPA-M and GPA-N the O-linked sugars Siaα2-3Galβ1-3(Siaα2-6)GalNAcα1-Ser/Thr are carried by $Ser_2$, $Thr_3$, and $Thr_4$. In GPA-$M^g$, the replacement of $Thr_4$ by an Asn results in lack of glycosylation of $Ser_2$ and $Thr_3$. The anti-M antibodies recognize $Val_6$ and $Met_8$ on the normally glycosylated GPA under the condition that there is a Gly and no Glu at position 5. Desialylation of GPA destroys this recognition, but further deglycosylation restores the reactivity that becomes independent of the residue at position 5. In addition, these antibodies do not recognize N but instead $M^g$, although the amino acid sequence of $M^g$ more resembles the one of N than that of M. These data show that the O-linked glycosylation at the aminoterminus of GPA takes part, although not exclusively, in the recognition of these blood group antigens by anti-M antibodies.

In summary, O-linked glycosylation has a profound effect on the antigenic properties of peptides. O-linked glycosylation can generate a neo-epitope (e.g., CII), or can have as an effect the hiding of an epitope (e.g., VF13N). O-linked glycosylation can mimic other epitopes (molecular mimicry of cytokeratins). It can change the properties of an epitope even without really being part of the epitope (CD43 and GPA). For a better understanding of the specific immune response we need to recognize the possibility that (O-linked) glycopeptides can act as antigens.

## D. Influence of O-Linked Glycosylation on Glycoprotein Clearance

Glycosylation is crucial for the circulatory clearing of many glycoproteins. Several organs (liver, kidney) or systems (endothelia, lymph system) contribute to this. This phenomenon has been best documented for glycoprotein hormones (Drickamer, 1991). On the hepatocytes there are different receptors that are specific for particular forms of glycosylation. A major advantage of such receptors is that one receptor type can clear a whole range of glycoproteins, and the clearing of each individual glycoprotein can be controlled by the glycosylation.

A typical receptor is the hepatic asialoglycoprotein receptor, which not only recognizes desialylated N-linked oligosaccharides but also GalNAc structures as they occur on some O-linked structures. As a consequence, desialylation and the presence of nonsialylated oligosaccharides (which rarely occurs on plasma proteins) determine the clearance time (Drickamer, 1991). Another important receptor is the $SO_4$-GalNAc-receptor; it rapidly clears glycoproteins with this (N-linked) sugar structure (e.g., luteinizing hormone [LH].) Rapid clearing of LH is important to generate a circadian rhythm (Hooper et al., 1996). Other similar receptor types exist, but it is not yet clear whether these bind O-linked or

N-linked structures. A possible role in clearance was also described but not confirmed for the O-linked fucose in EGF-modules (Hajjar and Reynolds, 1994).

## E. Other Examples of O-Linked Structures and Recognition

A number of interesting examples of O-linked glycosylation in molecular recognition are from glycoproteins that were mentioned earlier in another context. The O-linked oligosaccharide of the neurotrophin receptor plays a role in the ligand recognition and possibly also in signal transduction (Chapman et al., 1996; see Section III.A). The O-linked glycosylation of human chorionic gonadotrophin (hCG) is essential for the thyrotrophic activity and the N-linked carbohydrate for signal transduction (see Section V). CD22 belongs to the immunoglobulin superfamily (see also Figure 8) and is present on particular $IgM^+$ B lymphocytes. It is a lectin-like molecule that recognizes the minimal structure Siaα2-6Galβ1-4Glc(NAc). The α2-6 linkage of the sialic acid is essential, as an α2-3 sialic acid is not recognized. CD22 recognizes the structure on O-linked and N-linked glycoproteins and glycolipids. Multimerization of CD22 results in a better binding of disialylated biantennary structures or glycoproteins with several of the above structures (Powell and Varki, 1994; Powell et al., 1995). CD22 binds to various cellular glycoproteins and can trigger the activation of B and T lymphocytes. An example of a CD22 ligand is CD45. Because CD45 is subject to differential splicing and differential glycosylation, depending on the activation and the tissue distribution of the T cell (Lai et al., 1991), these two regulation mechanisms can govern the binding to CD22 (Powell et al., 1995). A similar macrophage lectin is sialoadhesin, which recognizes glycoprotein and glycolipid Siaα2-3Gal, for instance, on glycophorin (Nath et al., 1995). The structures of these lectins have been deciphered: for sialoadhesin the first immunoglobulin domain is essential and sufficient for the interaction with the sugars. In CD22, the second immunoglobulin domain is necessary too, presumably to ensure the correct folding of the first domain. The oligosaccharide recognition site in these molecules contains the special inter-β-sheet disulfide bridge structure (Nath et al., 1995). In addition to sialoadhesins, macrophages also carry $Ca^{2+}$-dependent (C-type) lectins that recognize Gal/GalNAc (Sakamaki et al., 1995). These lectins might be important in the recognition of tumor cells by macrophages. Tumor cells often have an altered glycosylation pattern and carry incompletely glycosylated mucins with many exposed Gal/GalNAc structures (see also Section X.B).

## F. Multivalent and Multimeric Binding and Signal Transduction: the Role of O-Linked Glycosylation

In the interactions of a receptor with a glycoprotein ligand multimeric and multivalent binding are important. For instance, with a multivalent ligand, if the same sugar structure is repeated severalfold on the same molecule, the interaction is stronger, and even more if the receptor is multimeric. The glycosylation of the ligand may also be important for signal transduction.

Typical examples of this are the selectin ligands and CD22 (see Sections IV.A and IV.E, respectively). PSGL-1 has on its Ser/Thr-rich domain several O-linked oligosaccharides. Some of these contain the structure that is recognized by P-selectin ($sLe^x$). Multiple interactions of P-selectin and PSGL-1 are important to resist the relative strong circu-

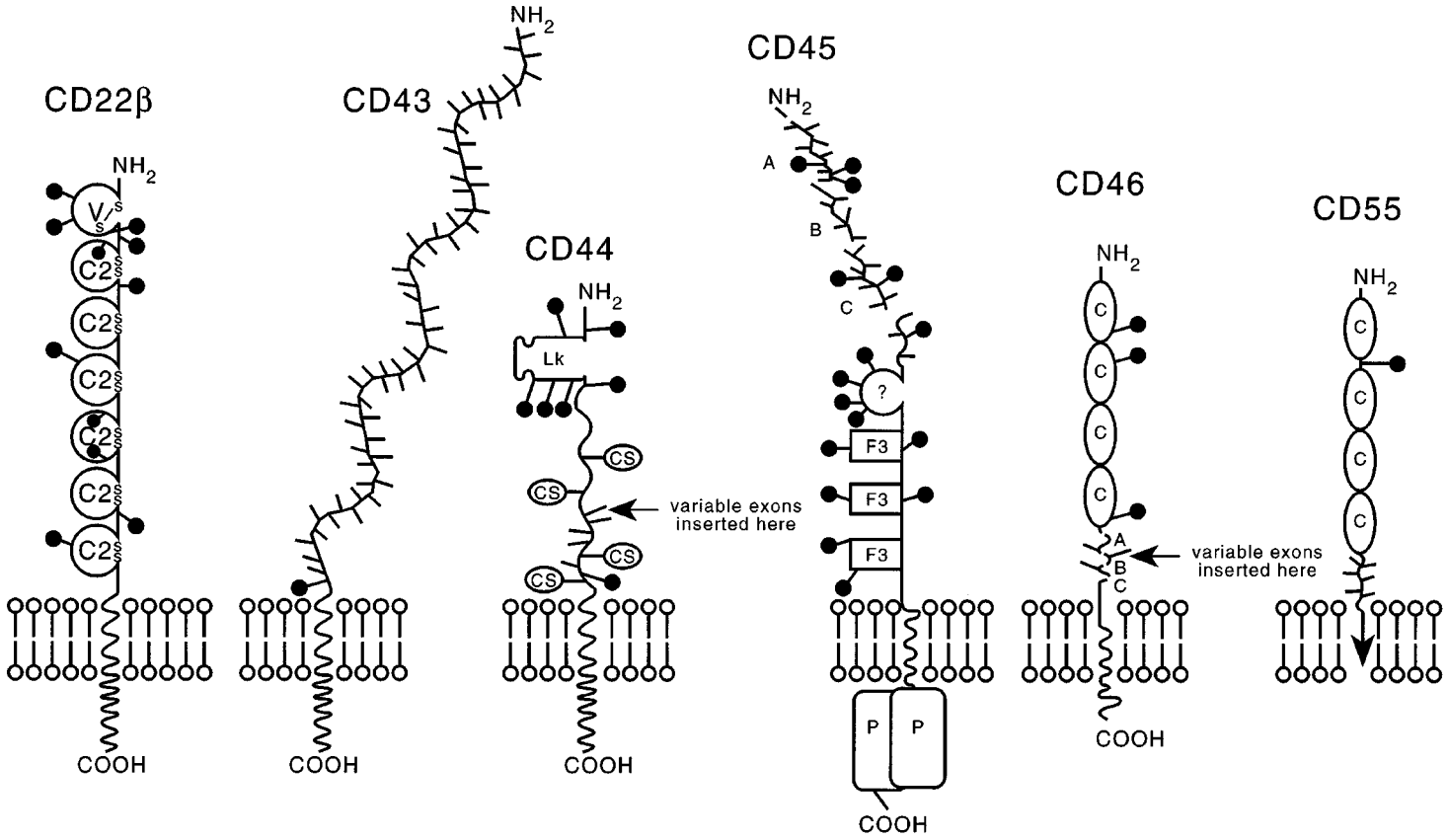


**FIGURE 8.** O-linked glycosylation in prototypic membrane-bound CD molecules. Sialoadhesin (CD22), leukosialin or sialophorin (CD43), the leukocyte homing receptor (CD44), the leukocyte common antigen (CD45), the membrane cofactor protein (CD46), and the decay accelerating factor (CD55) were compared in terms of modular domain structure, N-linked glycosylation sites (indicated as ball and stick), and serine/threonine rich attachement sites for O-linked glycosylation (sticks only). For the proteins CD44, CD45, and CD46, a number of alternative splicings give rise to protein variants. In these particular examples the alternative splicing is in serine/threonine-rich domains, and the functional implications are discussed in the text. Not all O-linked attachment sites are indicated, as information on the site specificity is not yet available. (Courtesy of Dr. N. Barclay [Barclay et al., 1997].) Abbreviations for the domains are as follows: V, variable immunoglobulin domain; C2, constant immunoglobulin domain 2; F3, fibronectin type III; CS, chondroitin sulphate; C, complement control protein (also called SCR, short consensus repeat); P, phosphotyrosinase phosphatase (PTPase) domain; Lk = link domain.

latory shear stress forces on the leukocytes. Many tiny binding forces together generate a relatively strong interaction and enable the rolling and halting of cells.

Rotaviruses, which cause gastroenteritis in the newborn, bind to sialylated O-linked sugars with a preference for Siaα2-3Galβ1-4GlcNAc (Willoughby, 1993). This is a low-affinity binding event, although the avidity increases considerably with multivalent sugar ligands such as mucins. The high affinity seems to depend less on the exact sugar structure than on the multivalency and clustered sialic acid residues. The virus may present many molecules of the still unknown receptor, acting as a multimeric sugar receptor. Reciprocally, colostral mucins (see Section IV.A) might function in trapping the virus and in newborn host defense.

As outlined in the discussion of the interaction between ZP3 and GalTase in the mouse, multivalency is crucial in fertilization. Furthermore, Gong et al. (1995) showed that aggregation of GalTase, or multivalent clustering of this receptor, is necessary for signal transduction. There are also examples of the importance of N-linked sugars in signal transduction. Sialylation of N-linked sugars of hCG is necessary for signaling (but not for binding to the hCG receptor). As a result, desialylated hCG is an inhibitor of hCG (Nemansky et al., 1995).

From these examples, it is clear that multivalency of the ligand and multimerization of receptors are important in biological systems. This has been shown both for binding interactions and for signal transduction by hormones and cytokines.

## V. O-LINKED GLYCOSYLATION INFLUENCES THE ACTIVITY OF SIGNALING MOLECULES

For a number of signaling molecules, explicitly hormones and cytokines, it has been demonstrated that glycosylation contributes to the specific activity of these molecules. In most cases, this influence is not spectacular: usually a difference of 2- or 3-fold between the glycosylated and aglycosyl forms. These obvious small differences become much more significant when they are amplified in a cascade such as the cytokine network (see also the next section on enzymes). It is also relevant to notice here that many cytokines possess lectin-like domains that might function in multimerization and in the generation of high local concentrations by binding to glycosylated extracellular matrix molecules (George, 1994; Opdenakker et al., 1995). Typical examples of cytokines with lectin activity are tumor necrosis factor-α (Lucas et al., 1994) and interleukin-2 (Sherblom et al., 1989).

The influence of N-linked glycosylation on signal molecules and enzymes has been reviewed recently (Opdenakker et al., 1993; 1995): usually the relatively large N-linked sugars downmodulate the specific activities. Here we discuss the effects of O-linked glycosylation. A general explanation for the effects of this type of glycosylation on activity does not exist yet. A possible explanation is the lack of information about the existence of different recognition patterns or "modules" for O-linked glycosylation. Indeed, with some known exceptions (O-fucosylation, O-glucosylation), the protein motifs or sequons that are the recognition signals for the attachment of mucin-type O-linked sugars are not yet completely understood, as the regulation of the attachment of the first GalNAc-residue is extremely complex (see Section II.A).

Some reasons for the slow progress in understanding the functions of O-linked sugars have been reviewed (Opdenakker et al., 1993; 1995): the need for manifold and sensitive assay systems, the basic understanding that protein glycosylation cannot be effectively probed by *in vitro* mutagenesis (e.g., knocking-out of glycosylation sites by mutation of the glycosylated amino acid) and

the lack of interest in glycosylation studies in the early recombinant DNA era. Unfortunately, too many early studies appeared showing "no effect" of carbohydrates. As the development of novel reagents and technology increased and sensitive assay systems have been established, more examples have unfolded that show the importance of glycosylation on the bioactivity of glycoproteins.

O-linked glycosylation is important for the biological activity of interleukin-5 (IL-5). Although it was originally thought that glycosylation was not important for its bioactivity (Kunimoto et al., 1991; Tominaga et al., 1990), a more recent report indicated that both O- and N-linked sugars influence the activity of IL-5 (Kodama et al., 1993). Consistent with earlier findings on other cytokines, removal of the N-linked sugar resulted in a 2.8-fold increase of the biological activity (and a decrease in thermostability). Desialylation increases the activity fivefold and removal of the O-linked sugars yields a 10-fold increase in its activity. IL-5 is thus an example in which the O-linked glycosylation decreases the biological activity.

Granulocyte-colony stimulating factor (G-CSF) has one O-linked sugar that increases the molecular stability (see Section III.C; Hasegawa, 1993). This sugar also influences the colony stimulating capacity of G-CSF (Nissen et al., 1994). Lenogastrim is a recombinant form of human G-CSF produced in Chinese hamster ovary (CHO) cells and hence is glycosylated (one O-link on $Thr_{133}$). This form possesses colony stimulating activity at a 16-fold lower dose when compared with filgastrim, the aglycosyl form of human G-CSF produced in *Escherichia coli*. At concentrations which are active *in vivo*, lenogastrim retains a more than twofold higher specific activity than filgastrim. It should, however, been noticed that these differences can also be due to, for example, different folding. Moreover, in terms of the clinical use in the treatment of leukopenia, it is also relevant to notice that lenogastrim yields larger colonies.

For two other cytokines, IL-2 and monocyte chemotactic protein-1 (MCP-1), it is less clear that the O-linked glycosylation has an influence on the bioactivity. Riske et al. (1991) mention that the activity of IL-2 with or without O-linked glycosylation is similar, but their results indicate a 2.3-fold difference in activity. IL-2 produced in eukaryotic C127 cells (with O-linked sugars) is more active than IL-2 produced in *Escherichia coli*. In their study of human MCP-1, Ishii et al. (1995) report that the O-linked sugars do not influence the activity, but from the data it is clear that bacteria-derived MCP-1 (without O-links) needs a higher concentration for half-maximal activity than the MCP-1 produced in a baculovirus expression system. Without addressing the important issue of glycoprotein quantification (Opdenakker et al., 1993; 1995) in such analyses, a minimal conclusion of these studies is that structure-function analysis of the role of carbohydrates in glycoproteins needs extensive and sensitive testing.

Glycosylation influences the activity of hCG. Although Huth et al. (1994) report that the N- and O-linked sugars of hCG do not influence the folding and the biological activity, the sialylation of the N-linked carbohydrates helps to transduce the hormone signal but is not essential for the binding with the lutotropin/choriogonadotropin receptor (Nemansky et al., 1995). The O-linked glycosylation is important for the thyrotropic effects of hCG (Yoshimura and Hershman, 1995). With the exception of a carboxyterminal peptide extension (β-CTP) containing four O-linked oligosaccharides in hCG, the hCG and hLH posses a very similar β-subunit. hCG has a lower thyrotropic activity than hLH, but after removal of the β-CTP the thyrotropic activity is the same for both molecules. Similarly, desialylation or deglycosylation of hCG increases its thyrotropic activity. Similar observations were made with

thyroid stimulating hormone (TSH): its activity increases with desialylation (Joshi et al., 1995). These examples illustrate that O-linked glycosylation suppresses the bioactivity of particular hormones. In addition, desialylation of hCG results in a shorter half-life in the circulation.

In conclusion, O-linked glycosylation has a clear influence on the activity and the stability of particular signal molecules: for some cytokines or hormones the O-linked sugars increase the activity; in other cases they decrease the specific activity. The effect of O-linked glycosylation is not evident in all investigated molecules. This illustrates the difficulties encountered with such studies but also suggests that many functions of sugars have yet to be discovered.

## VI. EFFECTS OF O-LINKED GLYCOSYLATION ON THE ACTIVITY OF ENZYMES

The effects of N-linked glycosylation on enzyme activities have been well established with a number of examples. The first indications that removal of N-linked sugars from t-PA enhances its activity were confirmed in a number of other studies (Opdenakker et al., 1986; Parekh et al., 1989; Mori et al., 1995) and led to the conclusion that, in general, N-linked glycans usually downmodulate the activity of enzymes. This finding has been corroborated by several other studies some of which have already been mentioned. One particular study of bovine pancreas ribonuclease is important in this aspect, because it pioneered the use of individual glycoforms to address structure-function relationships (Rudd et al., 1994A). For a relatively small number of enzymes the influences of O-linked glycosylation on the catalytic activities have been described: the examples of lactase-phlorizin hydrolase (LPH) and gluco-amylase are discussed below.

Human LPH is an intestinal brush border membrane enzyme that hydrolyzes lactose. The two glycotypes $LPH_N$ (with N-links) and $LPH_{N/O}$ (with N- and O-links) have been defined above (Naim and Lentze, 1992). Both forms possess the same affinity for the substrate (same $K_m$), but $LPH_{N/O}$ has a higher catalytic velocity ($V_{max}$ four times higher than in $LPH_N$). The O-linked sugar induces a higher enzyme activity by fastening the catalysis. The difference in O-glycosylation may be related to the differentiation stage of the intestinal epithelial cells because the differentiation concurs with alterations in the expression levels of the Golgi-transferases; a possible function of the N-linked sugars consists in preventing aggregation because both forms aggregate after removal of the N-glycosylation (see Section III.B).

The example of gluco-amylase (GA) from *Aspergillus awamori* var. *kawachi* has already been discussed in relation to the increased stability imposed by O-linked sugars (Goto et al., 1995; see Section III.C). GA consists of two domains: an aminoterminal catalytic domain (GAI) with two N-linked oligosaccharides and a Ser/Thr-rich domain (Gp-I) that carries several O-linked oligomannose structures. Two different mutants of the *Aspergillus* species have been described: one which produces a hyperglycosylated form of GA rich in Man (GA MU-H) and another that produces a hyperglycosylated GA in which the mannoses have been replaced by Glc (GA MU-L). Both forms have the same catalytic activity as the native GA against dissolved starch. The GA MU-H is, however, more active against raw nondissolved starch than GA MU-L. When the GA MU-H is treated with protein N-glycanase F (PNGase F) its activity does not change. After treatment with both PNGase F plus α-mannosidase, the activity toward raw starch is reduced considerably. The replacement of Man by Glc, or the

removal of the O-linked mannosides results in a reduced activity against raw (but not against dissolved) starch. The comparison of the activities of the enzyme in water vs. $D_2O$ and glycerol indicate that the O-linked mannoses play a role in breaking clusters of water molecules. The latter is important in the hydration of raw starch. Indeed it is known that Man breaks water clusters, whereas Glc stabilizes these. This might well explain the difference in activities between GA MU-H and GA MU-L. The O-linked sugars of the GA enzyme thus fulfill an important role in the hydrolysis of raw starch.

In general, we can conclude that the O-linked glycosylation of enzymes can influence the enzymatic activity. This may be mediated by an influence on the affinity of the substrate or by increasing the catalytic velocity. From the two mentioned examples it appears that O-linked glycosylation can upregulate the activity of enzymes. This is in sharp contrast to the observation that the voluminous N-linked sugars usually downmodulate the activities of enzymes and signal molecules.

## VII. THE ROLE OF SER/THR-RICH DOMAINS

### A. Ser/Thr-Rich Domains on Cell Surface Glycoproteins

Three aspects of Ser/Thr-rich domains have already been discussed. First, the multiple O-linked glycans confer an elongated structure to the peptide backbone, and this is reflected in a number of physicochemical properties (e.g., mucins). Second, the O-linked sugars can also carry functional determinants (e.g., selectin ligands), and third, the organization of these determinants in clusters confers functional multivalency. Here, we discuss an additional regulation level: the differential occurrence of these Ser/Thr/Pro-rich domains in proteins (Figure 8). Indeed, in some cases the presence or absence of this domain has been shown to confer a prominent difference in glycoprotein function.

CD44 is a membrane molecule that binds hyaluronic acid (HA) and assists in leukocyte circulation, homing, and cell interaction. In line with the general rule (for N-linked sugars), CD44 possesses N-linked oligosaccharides which may be expected to downregulate the HA-binding capacity (Katoh et al., 1995). Another regulatory mechanism of CD44 is the differential splicing of a Ser/Thr-rich domain (Bennett et al., 1995A). Therefore, CD44 may occur as two variants: CD44H and CD44E. In contrast to CD44H, CD44E contains the extension regions encoded by the variable exons V8-V9-V10. These regions are Ser/Thr-rich and contain many O-linked sugars. It now appears that CD44H binds stronger to HA than CD44E. Removal of the O-linked glycans from CD44E results in an increased binding that is similar to that of CD44H. In addition, inclusion of the fourth immunoglobulin domain of ICAM-1 (intercellular adhesion molecule-1, a domain that is not Ser/Thr-rich) into CD44H, at the site of the native Ser/Thr-rich domain of CD44E, does not downregulate the binding capacity to HA. In contrast, the inclusion of a mucin-like domain (of CD34) does decrease the binding. This indicates that (in addition to the N-linked sugars) the O-linked sugars of the differently spliced domain play an important role in the HA-binding capacity of CD44.

Membrane cofactor protein (MCP or CD46) is another example of differential splicing of Ser/Thr-rich domains. CD46 is a complement-regulating protein and acts as a cofactor of factor I, which inactivates the complement factors $C_{3b}$ and $C_{4b}$. Although originally no function was ascribed to the Ser/Thr/Pro-rich domain (Post et al., 1991), later it has been documented that this do-

main has a regulatory function (Iwata et al., 1994). CD46/MCP usually occurs as three splicing variants ($ST^{ABC}$, $ST^{BC}$, and $ST^{C}$), depending on the presence of three differentially spliced exons A, B, and C, which together form a Ser/Thr-rich domain. The $ST^{C}$ variant is most efficient in inhibiting the classic pathway of complement activation (by binding to $C_{4b}$), whereas $ST^{ABC}$ is less effective in inhibiting the classic pathway but more effective in inhibiting the alternative pathway of complement activation (by binding to $C_{3b}$). $ST^{BC}$ possesses an activity intermediate between $ST^{ABC}$ and $ST^{C}$. This regulation — by differential splicing and O-linked glycosylation of CD46 — determines the complement inhibiting activity and might be relevant in pathological conditions. Cancer cells most often have $ST^{ABC}$, which implies a diminished sensitivity for complement activation through the alternative pathway. A related complement-regulating molecule, CD55 (or decay accelerating factor, DAF, see Section III.C), also has a Ser/Thr-rich domain. The domain in CD55 is essential for activity and serves as a nonspecific spacer to raise the functional domain above the cell membrane (Coyne et al., 1992).

There are a number of other molecules that have a differentially spliced Ser/Thr-rich domain, but the function of this domain remains unclear, although cell differentiation might govern which splicing variant is produced. For example, the chicken receptor (LR8) for low-density lipoproteins (LDL) contains a Ser/Thr-rich domain with O-linked sugars when it is expressed in somatic cells. This domain is not present when LR8 is expressed in oocytes (Bujo et al., 1995) or in male germ cells (Lindstedt et al., 1997). In humans the absence of the Ser/Thr-rich domain of the LDL receptor can result in familial hypercholesterolemia (Lelli et al., 1995). Sometimes there seem to be differences between species in the splicing of these Ser/Thr-rich domains. For instance, the mouse monocyte chemotactic protein-1 (MCP-1, encoded by the JE gene) has a carboxyterminal Ser/Thr-rich domain (Ernst et al., 1994) that has not been found yet in the human counterpart protein (Van Damme et al., 1992). Two zinc-metalloproteases have a differentially spliced Ser/Thr-rich domain. The testicular angiotensin-converting enzyme (testis ACE) has such a domain, which is not present in somatic ACE (Ehlers et al., 1992). The human and mouse inducible matrix metalloproteinase gelatinase B also possess a Ser/Thr-rich domain, whereas the closely related and constitutive gelatinase A lacks this domain (Masure et al., 1993). In the latter case, gene duplication instead of alternative splicing generates two enzymes with the presence of a Ser/Thr-rich domain as the main difference.

Differential splicing of Ser/Thr-rich domains with O-linked sugars thus may be a regulatory mechanism for the activity of various classes of molecules: membrane receptors, cytokines and enzymes. In addition, one can conceptually define this Ser/Thr-rich domains as a separate protein module for O-linked glycosylation. It is clear that further study of these domains is imperative.

## B. Ser/Thr-Rich Domains of Immunoglobulins

The hinge regions of the immunoglobulins IgD and IgA1 contain O-glycosylated Ser/Thr/Pro-rich domains located between the Fab and Fc regions. In IgA1 (Figure 9) this section of the peptide has five O-glycosylation sites that contain mainly sialylated GalGalNAc sugars (Field et al., 1994). The O-glycans may have both general and specific functions. For example, repulsion between the charged sugars in regions containing O-linked sugars may serve to extend the peptide chain (Gerken et al., 1989). The

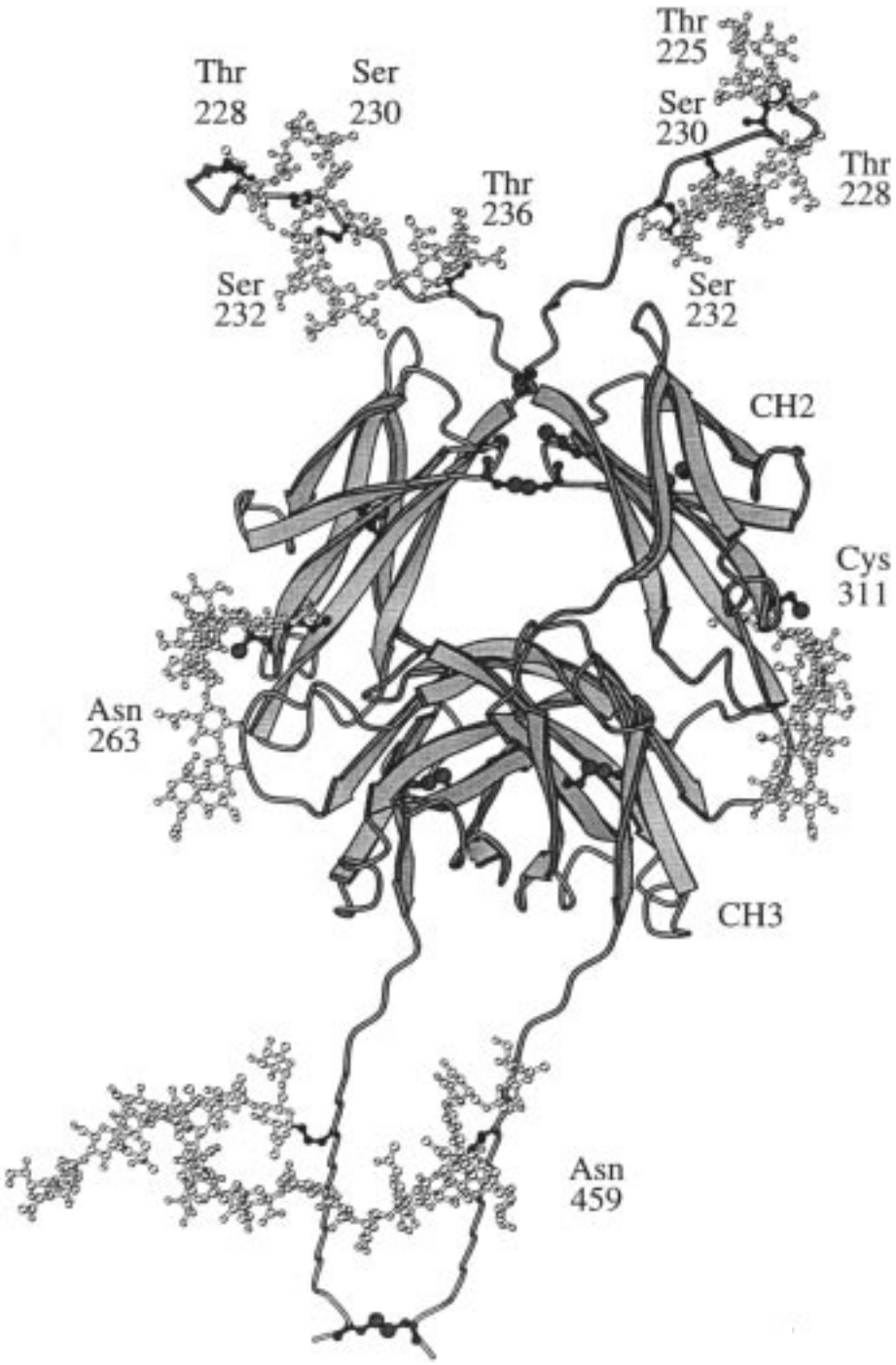


**FIGURE 9.** 3D structure of the Fc-region of an IgA1 antibody. The Fc region of IgA1 is composed of the constant domains CH2 and CH3 of two heavy chains linked together by disulfide bridges. CH2 and CH3 are N-glycosylated at $Asn_{263}$ and $Asn_{459}$, and the hinge-region, between CH1 and CH2, contains three O-linked sugars at $Thr_{228}$, $Ser_{230}$, and $Ser_{232}$, while $Thr_{225}$ and $Thr_{236}$ are partially glycosylated. These O-linked glycans are involved in IgA nephropathy (see Section X.C). (Modified from Mattu et al. [1998].)

energy-minimized structure of the hinge region of IgA1 suggests that the conformation of the sugars allows them to protect the hinge region from cleavage and indeed the hinge region of IgA1 is resistant to many common proteases (Mestecky and Kilian, 1985). Only a limited number of highly specific proteases have been identified that are able to cleave IgA1 at its extended hinge region. These are secreted by a number of pathogenic bacteria such as those that cause meningitis (*Neisseria meningitidis*), respiratory tract infections (*Haemophilus influenzae* and *Streptococcus pneumonia*), gonorrhoeae (*Neisseria gonorrhoeae*), conjunctivitis (*Haemophilus aegyptius*), and dental plaque formation (*Streptococcus sanguis*) (Plaut, 1983; Killian and Reinholdt, 1986; Mulks and Shoberg, 1994). *In vitro*, both IgA1 and IgD bind to a lectin site on T-lymphocytes through the O-glycans in the hinge region (Rudd et al., 1994B). The involvement of the IgA1-glycosylation in pathology is discussed in Section X.C.

## VIII. ROLE OF O-LINKED GLYCOSYLATION IN PROTEIN EXPRESSION AND PROCESSING

Glycosylation is important for the expression and processing (in terms of posttranslational modifications) of particular proteins. As already indicated above, glycophorin A (GPA) carries both N- and O-linked sugars. These are important for the expression of GPA in CHO cells. Moreover, O-linked sugars play a role in the "processing" of particular blood-clotting factors, of the insulin-like growth factor-II (IGF-II) and of pro-opiomelanocortin (POMC).

With the use of glycosylation-deficient CHO cells, Remaley et al. (1991) showed that GPA can only be expressed when O-linked sugars are attached. Expression remains possible whitout N-glycosylation or if the extension of O-linked glycans is hindered. Obviously, N-linked glycosylation is not necessary for GPA expression, but O-glycans are. However, expression of GPA without O-linked oligosaccharides is possible on the condition that specific types of N-links are present. As a consequence, both O-linked and specific types of N-linked glycosylation permit the recombinant expression of GPA, and this independently of each other (Ugorski et al., 1993).

O-linked glycosylation seems to influence the proteolytic processing of proteins. An example of this phenomenon is IGF-II. Certain mesenchymal tumors secrete "big IGF-II", which is a form of pro-IGF-II. This form also occurs in the serum of normal persons albeit in much lower concentrations. The normal molecule has an O-linked sugar on $Thr_{75}$ and is cleaved into IGF-II. The big-IGF-II of mesenchymal tumors does not carry this sugar and is not clipped further (Daughaday et al., 1993). This indicates that O-linked sugars might be important in modulating the proteolytic cleavage of precursor proteins.

A more controversial example is POMC. The 77 residue aminoterminal part of POMC (N-$POMC_{1-77}$) is cleaved between $Arg_{49}$ and $Lys_{50}$ into N-$POMC_{1-49}$ and Lys-γ3-MSH (Lys-γ3-melanocyte-stimulating hormone) by the POMC converting enzyme. This processing only takes place if there is no O-linked sugar on $Thr_{45}$ or if this sugar is extended incompletely. When there is a normally extended sugar at this position, the N-POMC is insensitive for the POMC convertase (Birch et al., 1991). However, one has to relate these results to the observations that replacement of $Thr_{45}$ by Ala has no influence on the cleavage between $Arg_{49}$ and $Lys_{50}$ in AtT-20 cells (Noel et al., 1991). We wish to stress here once more that *in vitro* mutagenesis studies

to alter glycosylation sites are in no way ideal examples to study the role of sugars, as the primary protein structure is then changed too. Only comparisons of the same protein structure with or without carbohydrate can be conclusive about the role of sugars. Currently, there exist several means to generate such glycoprotein variants for comparative glycosylation studies. Enzymatic deglycosylation under native conditions (e.g., with PNGase F or with sialidase plus endo-β-*N*-acetylgalactosaminidase), expression of recombinant glycoproteins in cell lines with specific defects in the glycosylation machinery and expression in the presence and absence of glycosylation inhibitors (e.g., tunicamycins, benzyl-α-GalNAc or monensins) are all examples of relevant methods for this purpose. Chemical deglycosylation is much less ideal because the protein structure is most often damaged or destroyed.

Another, equally controversial, example relates to the activation of clotting factor X and Xa. This occurs through proteolysis and is catalyzed by factor IXa and its cofactor VIIIa in the intrinsic cascade, and by factor VIIa and tissue factor in the extrinsic cascade. Generation of the factor X activation peptide leads to the formation of the prothrombinase complex and finally to thrombin generation. The activation of factor X is dependent on the attached sialic acid residues: desialylation considerably reduces the activation (Sinha and Wolf, 1993). This has, however, not been corroborated by Bharadwaj et al. (1995): their results show that removal of Sia has no effect on the activation of factors IX and X. Even when disregarding these inconclusive results, it has been shown that the removal of the O-linked sugars from factor X decreases the $k_{cat}$ of the activation reaction sevenfold (Inoue and Morita, 1993).

O-linked glycosylation plays a role in the generation of soluble forms of receptors. The function of such soluble receptors is not always clear. The receptor of transferrin (TfR) is a typical example: it is synthesized and transported to the cell membrane where it is subject to cycles of internalization in the endosomes and recycling. In the endosomes a protease can cleave the receptor into a soluble form, sTfR (Rutledge et al., 1994B). The cleavage occurs at residue 100 and results in the removal of the aminoterminal part that contains the transmembrane domain. The protease is sensitive to the sugar chain on $Thr_{104}$: replacement of this residue by Asp, Gly, Glu, or Met (so that position 104 can no longer be glycosylated) results in an increased sensitivity of TfR for the protease (Rutledge et al., 1994A). They confirmed this data by showing that replacement of $Thr_{104}$ by large and charged amino acids also has a negative influence on the cleavage. Moreover, expression of the wild-type TfR in the glycosylation-deficient CHO ldlD cells showed an enhanced cleavage of the receptor, and it was also shown that desialylation abolishes the protective effects of the sugar (Rutledge and Enns, 1996).

In conclusion, glycosylation influences the expression and processing of glycoproteins, although this might not be sufficiently documented in a number of cases. As protein synthesis and processing are essential processes in the life cycle of all eukaryotic cells, their regulation must attract the interest of all cell biologists. The examples further illustrate that studies employing *in vitro* mutagenesis to knock out glycosylation sites must be interpreted with caution.

## IX. O-GLYCOSYLATION OF CYTOPLASMIC AND NUCLEAR PROTEINS

Independently of the activity of the glycosylating machinery in the endoplasmic reticulum and the Golgi apparatus, particular cytoplasmic and nuclear proteins may become O-glycosylated. With only one exception (so far), this glycosylation is GlcNAcα1-Ser/Thr. Hart and colleagues (1995, 1997) have reviewed this type of O-linked glycosy-

lation, and some important elements are summarized here.

The O-GlcNAc-glycan of an O-GlcNAc-glycosylated protein has a turnover that is faster than that of the peptide to which the sugar is attached. This implies that the process may be reversible. Additional evidence for this is the existence of an *N*-acetyl-β-D-glucosaminidase (Dong and Hart, 1994). Many of the O-GlcNAc-carrying glycoproteins are phosphorylated. In addition, O-GlcNAc glycosylation commonly occurs in sequences that are recognized by particular kinases, for example, the growth factor and proline-directed kinases. Therefore, it has been suggested that O-GlcNAc-attachment might be an alternative regulatory modification that is analogous to regulatory protein phosphorylation. O-GlcNAc glycosylation might also play a role in the multimerization of specific proteins.

Nucleoporins carry O-GlcNAc, and antibodies or lectins (e.g., wheat germ agglutinin) that are directed against this structure block the energy-necessitating step of macromolecular transport through the nuclear membrane pores. This suggests that O-GlcNAc functions in the transport process.

O-GlcNAc glycosylation is also a major modification of chromatin proteins. In the polythene chromosomes of *Drosophila* salivary glands the O-GlcNAc glycosylation seems to decrease at sites where active transcription is taking place. Moreover, various transcription factors seem to carry O-GlcNAc. For instance, c-Myc and the oestrogen receptor have this structure in their transactivation domains. This strongly suggests a role for O-GlcNAc glycosylation in the regulation of transcription. Furthermore, Chou et al. (1995) have documented that O-GlcNAc glycosylation occurs on $Thr_{58}$ of c-Myc, exactly the residue that is often mutated in lymphoma.

The carboxyterminal domain (CTD) of eukaryotic RNA-polymerase II is phosphorylated extensively (form IIo) or glycosylated extensively with O-GlcNAc (form IIa). Form IIa initiates transcription and thereafter is deglycosylated and phosphorylated into form IIo. This example of reciprocity between O-glycosylation and phosphorylation illustrates that posttranslational modifications by glycosylation have an important regulatory function and that further research in this area may be expected to yield important insights in biological processes (reviewed by Hart, 1997).

Many cytoplasmic proteins carry an O-GlcNAc-sugar. Human erythrocyte band 14 protein, talin, cytokeratin 13, and rat and mouse spinal cord neurofilaments are examples of this. In these molecules O-GlcNAc glycosylation might function in the macromolecular assembly. The microtubuli-associated protein, Tau, also contains O-GlcNAc and this modification appears to play a role in Alzheimers disease. Griffith and Schmitz (1995) demonstrated that the O-GlcNAc glycosylation in the detergent-insoluble fraction of cytoskeletal proteins increases in Alzheimer patients. In addition to Tau, the β-amyloid precursor membrane protein also has O-GlcNAc (Griffith et al., 1995). Both these proteins precipitate in the "senile plaques" of Alzheimer patients. The role of the O-GlcNAc glycosylation might be in the assembly of multimeric complexes, as has been shown for a number of viral proteins (Hart et al., 1995).

Human keratin 8 and 18 are cytoplasmic proteins that carry O-GlcNAc glycosylation. The O-GlcNAc in keratin 18 is localized on three serine residues of the head domain and is obviously not related to the filament assembly (Ku and Omary, 1995). During colchicine or nocodazol-induced growth arrest from G2 into M, the cellular O-GlcNAc glycosylation and the phosphorylation strongly increase. The network of keratin filaments are then disassembled and form aggregates (Chou and Omary, 1993). Also during cell activation the O-GlcNAc glycosylation seems to change. Kearse and Hart (1991) showed that

when T-lymphocytes are activated with concanavalin A, the nuclear O-GlcNAc content increases and the cytoplasmic content decreases. This fluctuation has a wave-like pattern, restores after a while, and seems not to be dependent on the transport of glycoproteins from the cytoplasm to the nucleus. For particular glycoproteins this wave is dependent on *de novo* protein synthesis. Although the exact meaning of this fluctuation at present escapes our understanding, O-GlcNAc glycosylation seems to play a role during cell mitosis and activation.

Another interesting and clear example is the cytoplasmic $p^{67}$ protein. The O-GlcNAc glycosylation of this protein is regulated by a specific deglycosidase (Figure 10, Chakraborty et al., 1994). In reticulocytes the activity of this $p^{67}$ deglycosidase is dependent on the presence of hemin. As long as hemin is present in the lysate, the $p^{67}$ deglycosidase remains inactive. Once activated, however, it remains irreversibly active, irrespective of hemin. As long as the $p^{67}$ deglycosidase remains inactive, the $p^{67}$ retains its O-GlcNAc, remains active, and inhibits the eIF-2 kinase HRI ("heme-regulated protein synthesis inhibitor"). The function of the latter HRI consists in phosphorylating the translation elongation factor eIF-2α. Translation can proceed as long as the eIF-2α is not phosphorylated. Thus, when the $p^{67}$ deglycosidase is activated, the O-GlcNAc is removed and the $p^{67}$ is degraded. After this, the HRI is not inhibited any longer and starts to phosphorylate (=inactivate) the eIF-2α. This results in translation inhibition. Alternatively, as long as $p^{67}$ remains glycosylated, it remains active and mRNA translation is possible. This is an example of stabilization of cellular metabolism by glycosylation.

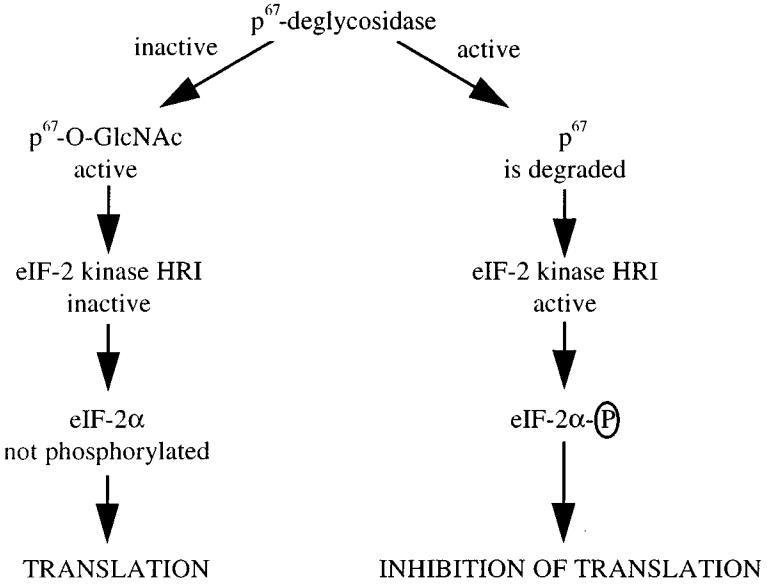


**FIGURE 10.** Regulation of translation by $p^{67}$-deglycosidase. The $p^{67}$-deglycosidase plays an essential role in the regulation of translation. When the deglycosidase is active, the O-GlcNAc of $p^{67}$ is removed and the degradation of $p^{67}$ follows. As a consequence, the heme-regulated protein synthesis inhibitor (HRI) of the eukaryotic peptide chain initiation factor 2 (eIF-2) kinase is activated, which results in the phosphorylation of eIF-2. This finally results in the inhibition of translation. The example illustrates that O-linked glycosylation of p67 is pivotal in the regulation of translation.

*Saccharomyces cerevisiae* phosphoglucomutase (PGM) is, as far as we know, at present the only cytoplasmic protein that carries a glycan structure different from O-GlcNAc. It contains Glc that is linked via a phosphodiester to O-linked mannose (Dey et al., 1994). So far, no functional role for this structure on the enzymatic activity has been detected, but the structure itself is influenced by environmental factors (carbon source and temperature). The Glcα1-phosphate is coupled to the mannose by the specific UDP-Glc:glycoprotein Glcα1-phosphotransferase. When the yeast is grown in Glc-containing medium and the Glc is consumed, then the cellular PGM increases, but the PGM-glucosylation decreases. Glucosylation also decreases during growth in medium with Gal as carbon source or during heat shock (in the latter case even in the presence of abundant Glc). The decrease in glucosylation is therefore presumably not due to the leveling off of the donor UDP-Glc, but to a regulation of the phosphoglucose transferase (the phosphodiesterase does not seem to play a major role). The enzyme associates with the sarcolemmal membranes of skeletal muscle through glucosylation, and this association correlates with the increase in cytoplasmic $Ca^{2+}$. Therefore, the hypothesis was formulated that the enzyme plays a role in the supply of Glc-6-phosphate as an energy source for transmembrane $Ca^{2+}$ transport. The fact that many yeasts with mitochondrial defects also possess a mutated PGM supports this hypothesis.

Thus, it is thus clear that O-GlcNAc glycosylation, like phosphorylation, plays an important regulatory role in intracellular processes. This type of glycosylation is essential in the regulation of transcription (RNA polymerase II) and translation ($p^{67}$). It is crucial in the control of cell division and differentiation. Therefore, further molecular studies of this type of O-linked glycosylation are crucial to understand the "life and death" of cells.

## X. O-LINKED GLYCOSYLATION AND PATHOLOGY

### A. Enzyme Defects

In the previous sections numerous examples have been given showing how O-linked glycosylation influences the activities of glycoproteins and might play a role in pathology. The possible relationship between O-GlcNAc-glycosylation and Alzheimer disease and the role of glycosylation in autoimmunity has already been mentioned. The emphasis in this section is on diseases that are associated with defects in carbohydrate-modifying enzymes. Nature often provides us with examples of enzyme defects under the form of specific genetic disorders and these can be used to test our man-made hypotheses. In the terminology of molecular medicine, we discuss the "inborn errors of O-linked glycosylation". In cell biology nomenclature some of the diseases that are discussed below might be denominated as diseases of the lysosomes, the Golgi apparatus and the endoplasmic reticulum; they may be grouped separately and have a place aside the mitochondrial and the peroxisomal diseases.

#### *1. Lysosomal α-N-Acetylgalactosaminidase Deficiency*

The lysosomal α-*N*-acetylgalactosaminidase (GalNAc'ase) functions to degrade glycopeptides in the lysosomes. Deficiency leads to an accumulation of glycopeptides that can be visualized as lysosomal inclusions filled with diffusely amorphous and filamentous material (Kanzaki et al., 1991). This can further lead to angiokeratoma diffusum with glycoaminoaciduria. The defect in the degradation of glycopeptides has its effects on

the central nervous system and can lead to infantile neuroaxonal dystrophy.

### 2. GlcNAc Transferase II Deficiency and HEMPAS

Hereditary erythroblastic multinuclearity with a positive acid serum lysis test (HEMPAS) is a disorder that leads to various gradations of anaemia. The erythropoiesis is inefficient or disturbed: in the bone marrow abnormal and multinucleated erythroblasts are found. Another feature of this disease is that peripheral erythrocytes are sensitive to lysis in acidified serum. There are two reasons for this. First, particular IgM antibodies bind to abnormally glycosylated constituents of the erythrocyte membranes. Second, the complement system is aberrantly regulated (Tomita and Parker, 1994). The abnormal O-glycosylation is the result of a deficiency of the GlcNAc transferase II (an enzyme that is necessary for the biosynthesis of polylactosamine) (Fukuda et al., 1987). By defect, particular membrane constituents are underglycosylated and are recognized by specific IgM molecules. This results in the classic activation of the complement cascade. The alternative complement activation is induced by acidification. HEMPAS might be caused by a defect in the O-glycosylation of GPA and constitutes an example in which enzyme-dependent underglycosylation causes pathology.

### 3. Alteration in O-Linked Sugar Biosynthesis and Tn-Polyagglutinylation Syndrome

Tn-polyagglutinylation syndrome is a disease in which the red blood cells agglutinate because of an enzymatic defect in the O-glycosylation machinery (Blumenfeld et al., 1992). The GalNAcβ1-3Gal transferase is insufficient. This enzyme normally elongates GalNAcα1-Ser/Thr (=Tn) to Galβ1-3GalNAcα1-Ser/Thr (core 1). Concomitantly, the α2-6sialyltransferase is induced. This is exceptional, because the latter enzyme predominantly occurs in tissues that synthesize mucins and not in hematopoietic cells. The latter enzyme generates sTn from Tn. Together this results in the expression of Tn and sTn on glycophorins A and B aside the normal sialylated core 2 O-linked sugars. Tn is recognized by anti-Tn antibodies that are omnipresent in all sera. This results in the agglutination of erythrocytes. Thus, this syndrome is the result of alterations of enzymes in the biosynthetic pathway of O-linked oligosaccharides.

### 4. Induction of Core 2 GlcNAc Transferase and Heart Disease

Nishio et al. (1995) showed that diabetes and hyperglycaemia induces the glycosylating enzyme core 2 GlcNAc transferase in rat heart muscle. This enzyme adds GlcNAc to core 1 (Galβ1-3GalNAcα1-Ser/Thr and forms core 2 (Galβ1-3(GlcNAcβ1-6)GalNAcα1-Ser/Thr). The induction of this enzyme is supposed to result in the deposition of glycoconjugates in the heart. The induction of the enzyme can be prevented by insulinotherapy. This is another example how an excess of glycoconjugates may lead to pathology: in this case, however, it is not due to a deficient degradation of glycopeptides, but instead it is the result of an increased synthesis.

### 5. Carbohydrate-Deficient Glycoprotein (CDG) Syndromes

CDG syndromes are a group of diseases caused by defects of the enzymes that cata-

lyze the glycosylation of glycoproteins in the endoplasmic reticulum and the Golgi apparatus. So far, more attention has been paid in this group of diseases to N-linked glycosylation, but it is not excluded that O-linked sugars are affected too. They were first described in 1980 and they became known as "Jaeken diseases" (Jaeken et al., 1980). These pathologies are syndromes with multiple defects in the connective tissues, in the clotting and fibrinolytic systems and with a multiendocrinopathy (Jaeken et al., 1993; de Zegher and Jaeken, 1995). Two enzyme defects have been identified: type I CDG is caused by a deficiency in the phosphomannomutase (Van Schaftingen and Jaeken, 1995) and type II CDG is the result of a defect in GlcNAc transferase II (Jaeken et al., 1994).

### *6. Glycosylation Defects in the Wiskott-Aldrich Syndrome*

Wiskott-Aldrich syndrome (WAS) is an X-linked recessive disease characterized by immunodeficiency, incomplete lymphocyte maturation, and platelet function defects. Two glycosyltransferases are abnormally expressed: the core 2 GlcNAc transferase and the α2-6sialyl transferase. Both these transferases are selectively regulated, and the expression levels in the differentiation stages of lymphocytes and platelets are quite different in WAS patients when compared with normal individuals (Higgings et al., 1991). This results in a major alteration of the O-linked sugars of, for example, leukosialin (CD43). The incomplete lymphocyte maturation in WAS patients is apparent as a dysregulation of O-glycosylation of these cells. Moreover, it was shown by Tsuboi and Fukuda (1997) that transgenic mice, overexpressing the core 2 GlcNAc transferase, exhibit a reduced T-cell immune response. This may be one of the causes for immunodeficiency in the WAS.

## B. Mucins and Cancer

The glycosylation of cancer cells is often disturbed. This is quite understandable if one takes in account that the glycosylation machinery (Golgi apparatus and endoplasmic reticulum) constitutes about 25% of most cells. It is unlikely that such a major cell compartment would escape from changes during the transformation process. The best characterized glycosylation changes in cancer cells are in the O-linked sugars of mucins. These alterations correlate with invasion and metastasis. In gastric mucins prominent differences have been documented during oncogenesis, which depend on the depth of the invasion (Yamashita et al., 1995). Major variations in the various O-linked mucin sugars depend on the origin, differentiation, and selection of the cancer cells or on the differences in glycosylation in adjacent tissues, which are induced by the cancer cells (Yamashita et al., 1995; Reed et al., 1994). For example, sialylation might contribute to the adhesion of pancreatic cancer cells (Sawada et al., 1993). Mucin glycosylation is also altered in breast carcinoma (Hanisch et al., 1996), and the glycans of the mucin MUC-1 show a more complex pattern with the presence of core 2 (with a varying extent of sialylation) in normal breast epithelial cell lines, while MUC-1 in corresponding carcinoma cell lines contains the more simple core 1 and O-linked GalNAc (Lloyd et al., 1996). It is at present perhaps premature to extract general trends but in most cases malignancy goes along with a decrease of the elongation of O-linked sugars and a decrease of the ratio GlcNAc/GalNAc. The latter might be relevant for the accessibility of the cancer cells for macrophages that carry a Gal/GalNAc-specific $Ca^{2+}$-dependent lectin

on their surface. Indeed, Sakamaki et al. (1995) have shown that cells with surface GalNAc are more readily recognized by macrophages.

The differences in glycosylation of cancer cells may be important for therapeutic and diagnostic applications. With the alteration of glycosylation the antigenic properties of cancer cells are changed. Monoclonal antibodies against the sTn structure may be useful in diagnostics and immunotherapy (Moyana and Xiang, 1995). Other authors caution that the sTn epitopes also occur on normal cells (Reed et al., 1994). The specificity of some tumor-specific antibodies can also be explained by the availability of peptide antigens in tumor cells that are masked by longer carbohydrate chains in normal cells (Lloyd et al., 1996). As a promising lead, the application of such antibodies in animal models and also in humans has led to a reduction of metastases (Longenecker et al., 1994). Glycosylation analysis of O-linked sugars may suggest new ways to fight cancer.

## C. O-Linked Glycosylation of Antibodies

The glycosylation of antibodies is altered in some pathologies. In rheumatoid arthritis the content of agalactosyl IgG (IgG(O), an N-linked sugar) is increased and is a marker for the disease (Rudd et al., 1995B). In IgA nephropathy (IgAN) the serum content of IgA1 increases. In addition to N-linked sugars, this immunoglobulin contains O-linked structures in the hinge region. In normal conditions there are core 1 structures (Galβ1-3GalNAcα1-Ser/Thr = T) that are eventually sialylated (see Section VII.B and Figure 9). In IgAN these core 1 sugars are less sialylated (Iwase et al., 1996) or the terminal Gal is absent; in other words, the IgA1 then carries the Tn structure. This results in the kidney deposition of IgA1 in IgAN (Allen et al., 1995). Allen et al. (1997) showed also a reduced β1-3 galactosyltransferase activity in B-cells (not in T-cells or monocytes) from IgAN patients compared with controls.

The O-linked sugars of IgA1 and IgD play a role in other autoimmune diseases too. Rudd et al. (1995B) showed that, in systemic lupus erythematosus, rheumatoid arthritis, Behçet's disease, and in IgAN, the T lymphocytes express a lectin that binds the O-linked sugars.

## XI. GENERAL CONCLUSIONS

O-linked glycosylation is a complex and highly regulated posttranslational modification. Although no general consensus protein sequence can be found (in contrast to the N-linked glycosylation sites), some general rules for the site-specificity of mucin-type O-glycans have been established, based on statistical data and data from the cloning of GalNAc-transferases. Further analysis of the site-specificity of O-glycosylation is needed, and the regulation of the processing of the O-linked glycans (e.g., the attachement of polylactosamine glycans) is not always well understood. Developmentally regulated modifications of the sugars, for example, acetylation of sialic acid, can considerably affect the functions of the sugars.

The physicochemical and biological properties of serine-, threonine- and proline-rich domains containing clustered O-linked glycans are profoundly influenced. Besides a striking influence on the three-dimensional conformation, the sugars can carry biologically relevant determinants, of which the synthesis can be regulated. The clustering of these determinants confers polyvalency, which is important for signal transduction, while the differential splicing of some of these O-glycan-rich domains provides an additional regulation level of glycoprotein targeting and activity.

Several other protein recognition elements such as the -Cys-Xaa-Ser-Xaa-Pro-Cys- for O-glucosylation and -Cys-Xaa-Xaa-Gly-Gly-Thr/Ser-Cys- for O-fucosylation of EGF-domains and the -Ser-Xaa-Ser-Ser-Ser-Ser- motif of ZP3 in fertilization are recognized by O-link specific glycosyltransferases and deserve the status of sequon. This might signify that these sequons can be seen as protein modules that carry a sugar and that have a particular function. This further underlines the relevance of examining the site-specificity of O-glycosylation.

Functions of O-linked sugars include recognition in different processes like selectin binding in leukocyte circulation, fertilization, and glycoprotein clearance. They influence immunological recognition of antigens and signal transduction and play a role in the processing and expression of glycoproteins. In contrast to N-linked glycans, which invariably downmodulate the activity, the O-linked glycans can up- or downmodulate the activity of enzymes and signaling molecules like hormones and cytokines. The O-linked GlcNAc residues, which are reversibly attached to nuclear and cytoplasmic proteins, play a regulatory role and can be compared with phophorylation. In addition to alterations of O-linked glycosylation associated with cancer and autoimmune diseases, a number of "inborn errors of O-linked glycosylation" have been defined to cause specific pathologies.

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