

EXHIBIT X

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

REVIEW/

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THE OLIGOSACCHARIDES OF GLYCOPROTEINS: BIOPROCESS FACTORS AFFECTING OLIGOSACCHARIDE STRUCTURE AND THEIR EFFECT ON GLYCOPROTEIN PROPERTIES

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In this review, we organize the recent data concerning the effects of bioprocess factors on the oligosaccharide structure of human therapeutic glycoproteins, with particular emphasis on the influence of the host cell. We also discuss the effect of oligosaccharide structure on glycoprotein properties, including antigenicity, immunogenicity and plasma clearance rate.

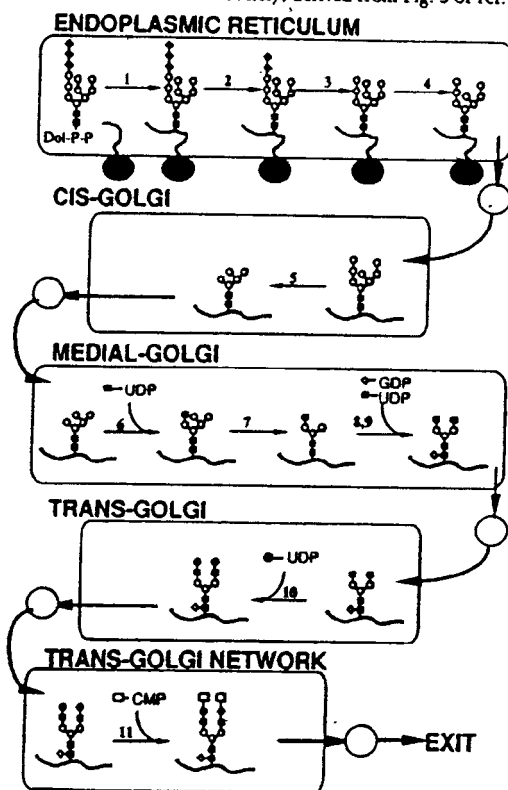
The majority of proteins secreted by mammalian cells are glycoproteins. These proteins possess oligosaccharides covalently attached through an asparagine side chain ("asparagine-linked" or "N-linked") or through a threonine or serine side chain ("O-linked"). A given glycoprotein may contain only N-linked oligosaccharide chains, only O-linked oligosaccharide chains, or both.

The oligosaccharide structures of glycoproteins can have a profound effect on properties critical to the development of glycoprotein products for human therapeutic use, including plasma clearance rate, antigenicity, immunogenicity, specific activity, solubility, resistance to thermal inactivation, and resistance to protease attack (reviewed in refs. 1-3).

BIOPROCESS FACTORS AFFECTING GLYCOPROTEIN OLIGOSACCHARIDE STRUCTURE

N-linked oligosaccharide processing by yeast, plant, insect and mammalian cells. The review article by Kornfeld and Kornfeld⁴ serves as an excellent, detailed introduction to the assembly of N-linked oligosaccharides. In summary, N-linked glycosylation begins with the synthesis of a lipid-linked oligosaccharide moiety (Glc₃Man₉GlcNAc₆-P-P-Dol; abbreviations are given in the legend to Fig. 1), and its transfer *en bloc* to a nascent polypeptide chain in the endoplasmic reticulum (ER) (Fig. 1, reaction 1). Attachment occurs through asparagine, generally at the tripeptide recognition sequence Asn - X - Ser/Thr. In a few

FIGURE 1 A potential pathway of mammalian N-linked oligosaccharide processing. The enzymes are: (1) oligosaccharyltransferase, (2) α -glucosidase I, (3) α -glucosidase II, (4) ER α (1,2)mannosidase, (5) Golgi α -mannosidase I, (6) N-acetylglucosaminyltransferase I, (7) Golgi α -mannosidase II, (8) N-acetylglucosaminyltransferase II, (9) α (1,6) fucosyltransferase, (10) β (1,4)galactosyltransferase, (11) α (2,3) sialyltransferase. The symbols are: ■, N-acetylglucosamine (GlcNAc); ○, mannose (Man); ●, glucose (Glu); ◐, fucose (Fuc); ●, galactose (Gal); □, sialic acid (NeuAc). Dol-P-P is dolichylidiphosphate. The co-substrates for reactions 6,8,9,10 and 11 are the energized forms of the monosaccharides where: UDP is uridine diphosphate, GDP is guanosine diphosphate and CMP is cytidine monophosphate. (Reprinted from ref. 3 with permission of the American Chemical Society; derived from Fig. 5 of ref. 4).



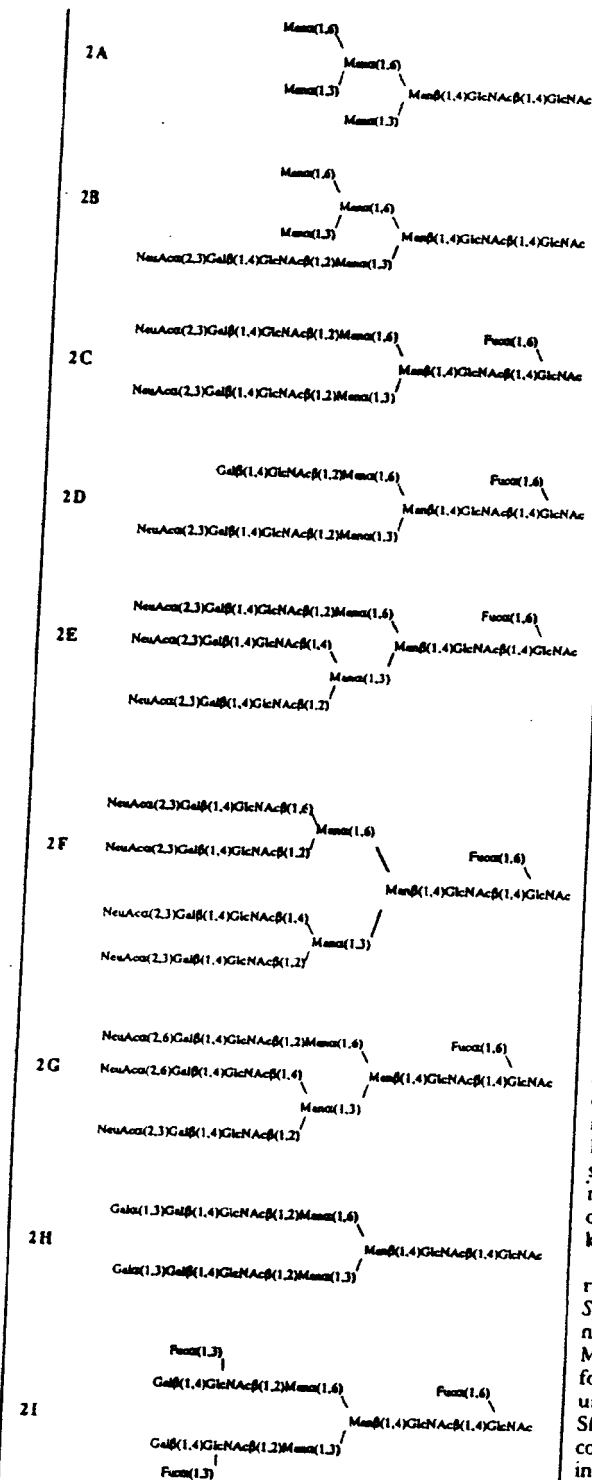


FIGURE 2 Mammalian N-linked oligosaccharides. (A-F) Some high mannose-type (A), hybrid-type (B), and complex-type structures (C-F) from t-PA expressed by recombinant CHO cells⁴⁸. (G) From human transferrin isolated from serum¹¹⁴. (H) From interferon- β 1 expressed by recombinant C127 cells⁴⁴. (I) From human α -amylase isolated from saliva⁵¹.

exceptional cases, N-linked glycosylation has been identified at sites other than Asn - X - Ser/Thr (reviewed in ref. 5). For example, protein C is N-glycosylated at the sequence Asn - X - Cys⁶.

A series of trimming reactions is catalyzed by exoglycosidases in the ER (Fig. 1, reactions 2, 3 and 4). Further processing of N-linked oligosaccharides by mammalian cells continues in the compartments of the Golgi, where a sequence of exoglycosidase- and glycosyltransferase-catalyzed reactions generate high-mannose (Fig. 2A), hybrid-type (Fig. 2B) and complex-type (Fig. 2C-2I) oligosaccharide structures. One example of mammalian Golgi processing is represented in Figure 1, reactions 5 through 11; the outcome of this pathway is the complex-type structure of Figure 2C.

Yeast, insect, plant and mammalian cells share the features of N-linked oligosaccharide processing in the ER, including attachment of $\text{Glc}_2\text{Man}_9\text{GlcNAc}_2\text{-P-P-Dol}$ (Fig. 1, reaction 1) and subsequent truncation to a $\text{Man}_9\text{GlcNAc}_2$ structure (product of reaction 4 in Fig. 1). However, oligosaccharide processing by these different cell types diverges in the Golgi apparatus. For example, plant cells produce complex-type oligosaccharides containing GlcNAc and Gal in linkages similar to those found in mammalian N-linked oligosaccharides, but plant-derived oligosaccharides do not possess sialic acid, a common constituent of mammalian complex-type oligosaccharides. Furthermore, plant-derived N-linked oligosaccharides frequently contain xylose, a monosaccharide not normally found in mammalian N-linked oligosaccharides (Fig. 3A and 3B) (reviewed in ref. 7).

N-linked oligosaccharide processing in the Golgi by yeast is very different from mammalian Golgi processing (reviewed in refs. 4, 8, and 9). In most strains of yeast, including *Saccharomyces cerevisiae*, the oligosaccharide chains are elongated in the Golgi through stepwise addition of mannose, leading to elaborate high mannose (mannan) structures sometimes containing more than 50 mannose monomers (Fig. 3C). The elaborate nature of these yeast N-linked oligosaccharide structures is often reflected in the molecular weights of mammalian proteins produced in recombinant yeast. For example, the molecular weight of aglycosyl HIV gp120 is 60 kD, while the molecular weight of gp120 is 120 kD when produced in mammalian cells¹⁰ and is up to 600 kD when produced in *S. cerevisiae*¹¹. Mutant *S. cerevisiae* strains permit synthesis of recombinant proteins with truncated N-linked, high mannose oligosaccharides (reviewed in refs. 8, 9 and 11). For example, the *mnn9* mutant synthesizes a truncated structure only slightly larger than a mammalian high-mannose structure (Fig. 3C). Thus, the molecular weight of gp120 produced in the *mnn9* mutant is reduced to 120 kD¹¹.

The capability of insect cells for N-linked oligosaccharide processing in the Golgi is poorly understood. *Spodoptera frugiperda* Sf9 cells possess the glycosidases necessary to trim the $\text{Glc}_2\text{Man}_9\text{GlcNAc}_2$ precursor to $\text{Man}_3\text{GlcNAc}_2$ (Fig. 3D)¹⁵. However, the capability of Sf9 for further processing to complex-type oligosaccharides is uncertain. Recombinant glycoproteins produced using Sf9 cells frequently have lower molecular weights than the corresponding native mammalian glycoproteins, suggesting limitations in Sf9 glycosyltransferase activities relative to mammalian cells. For example, the protein component of erythropoietin (EPO) has a molecular weight of 18.4 kD^{14,15}; EPO isolated from human urine or produced by recombinant CHO cells has an apparent molecular weight near 35 kD¹⁶, while EPO produced using the Sf9/baculovirus expression system has a molecular weight of 26.2 kD¹⁷. Other examples illustrating lower molecular

weights of Sf9-produced proteins include tissue plasminogen activator (t-PA)¹⁸, influenza virus hemagglutinin¹³, human acid β -galactosidase¹⁹, and human interferon- β ²⁰. Recent publications suggesting synthesis of N-linked complex-type oligosaccharide structures by Sf9 cells await further confirmation^{13,21}.

The tripeptide sequence Asn - X - Ser/Thr is not a sufficient condition for covalent attachment of an N-linked oligosaccharide. Gavel and Heijne⁵ surveyed the locations of N-linked oligosaccharides in 147 glycoproteins and found that 10% of 465 of the Asn - X - Ser/Thr sites were not glycosylated.

Differences in occupancy of Asn - X - Ser/Thr sites have been observed between mammalian cells and yeast in the glycosylation of human interleukin-1 β (hIL-1 β). The single N-glycosylation site of hIL-1 β at Asn-123 is unoccupied when this molecule is synthesized by human macrophages²², but is N-glycosylated when synthesized by recombinant *S. cerevisiae*^{23,24}.

O-linked oligosaccharide processing by yeast, insect and mammalian cells. The initial step in O-glycosylation by mammalian cells is the covalent attachment of N-acetyl-galactosamine to serine or threonine via an α 1 linkage. This reaction is catalyzed by the enzyme UDP-GalNAc:polypeptide N-acetyl-galactosaminyltransferase (reaction 1 in Fig. 4) (reviewed in ref. 25). No O-glycosylation sequence has been identified analogous to the Asn - X - Ser/Thr template required for N-glycosylation. In further contrast to N-glycosylation, no preformed, lipid-coupled oligosaccharide precursor is involved in the initiation of mammalian O-glycosylation. Nucleotide sugars (e.g. UDP-GalNAc and UDP-Gal) serve as the substrates for the first and all subsequent steps in O-linked processing.

Following the covalent attachment of GalNAc to serine or threonine, several different processing pathways are possible for mammalian O-linked oligosaccharides in the Golgi (reviewed in refs. 25 and 26). The most common pathway of O-glycosylation for cell-surface glycoproteins and plasma glycoproteins is outlined in Figure 4. O-linked oligosaccharide structures from this pathway are evident in interleukin-2 (IL-2) from human lymphocytes and recombinant CHO, BHK, and Ltk⁻ cells^{27,28}, EPO from recombinant CHO cells²⁹, immunoglobulin A from pooled human serum³⁰, granulocyte colony-stimulating factor (G-CSF) from recombinant CHO cells³¹ and plasminogen from human, bovine and porcine serum³².

The biosynthesis of O-linked oligosaccharide structures in yeast is significantly different from mammalian O-glycosylation (reviewed in refs. 8 and 9). Yeast O-glycosylation begins in the ER with the covalent attachment of mannose to the recipient serine or threonine residue via a lipid intermediate (Dol-P-Man), an initiation step completely distinct from mammalian O-glycosylation. Up to four additional mannose residues are attached in the Golgi to yield a structure which has no analogue in mammalian systems [Man α (1,3)Man α (1,3)Man α (1,2)Man α (1,2)Man-Ser/Thr]³³.

Yeast and mammalian cells do not have identical protein recognition sequences/structures for initiation of O-linked glycosylation. Recombinant hIGF-I expressed in *S. cerevisiae* is O-glycosylated at Thr-29^{34,35}. In contrast, normal human serum hIGF-I is not O-glycosylated^{33,34}.

The capability of insect cells for O-glycosylation is poorly understood. A recent report suggests that Sf9 insect cells are capable of performing the first two O-glycosylation steps outlined in Figure 4 to produce pseudorabies virus gp50 containing GalNAc and Gal β (1,3)GalNAc, but unlike mammalian cells, they are unable to sialylate this structure³⁶. A difference between mammalian and insect cells in the initiation of O-linked glycosylation has been observed in one case; recombinant Sf9 cells secrete

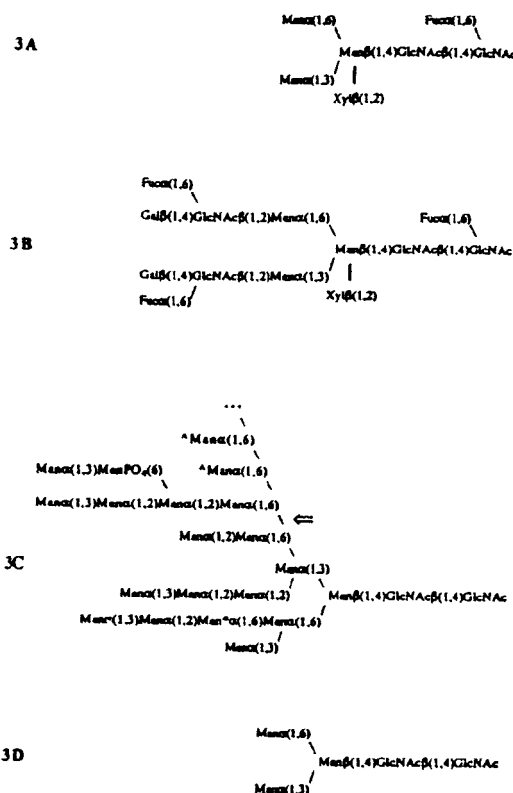


FIGURE 3 N-linked oligosaccharides from plant, yeast and insect cells. (A-B) From the plant enzyme laccase. (C) General structure of the N-linked oligosaccharides of *S. cerevisiae* glycoproteins¹². The structure below the arrow (\Leftarrow) represents a core structure including 12 mannose units and a potential phosphorylation site (*). Above the arrow (\Leftarrow) is the outer chain which can be extended to over 10 repeats of Man α (1,6), as indicated (...). The first Man α (1,6) of the outer chain is shown with an attached Man α PO $_4$ group. Each Man α (1,6) of the outer chain may have such a group or a truncated derivative, as indicated (*), potentially raising the total number of mannose to more than 50. A glycoprotein produced by wild-type *S. cerevisiae* may have glycosylation sites containing both the core and outer chain, as shown above, or just the core structure. Microheterogeneity of oligosaccharide structure at a particular glycosylation site is observed both in the core and in the outer chain due to variability in both the number of mannose linkages and the degree of phosphorylation. The *S. cerevisiae* *mnn9* mutant is unable to synthesize the outer chain. Therefore, glycoproteins produced by that mutant possess only the core structure below the arrow (\Leftarrow) (reprinted from ref. 3 with permission of the American Chemical Society). (D) From influenza virus hemagglutinin expressed by baculovirus-infected *Spodoptera frugiperda* (Sf-9) cells¹³.

only nonglycosylated IL-2³⁷, while cultured mammalian cells secrete a mixture of nonglycosylated and O-glycosylated IL-2²⁸.

N-linked and O-linked oligosaccharide syntheses are cell-type dependent among mammalian cells. Among mammalian cells, oligosaccharide processing is species dependent and cell-type dependent within a given species (reviewed in refs. 1, 38 and 39). The influence of cell type on glycosylation appears to be related primarily to the presence, concentration, kinetic characteristics, and compartmentalization of the individual glycosyltransferases and glycosidases (reviewed in refs. 1 and 40). This section

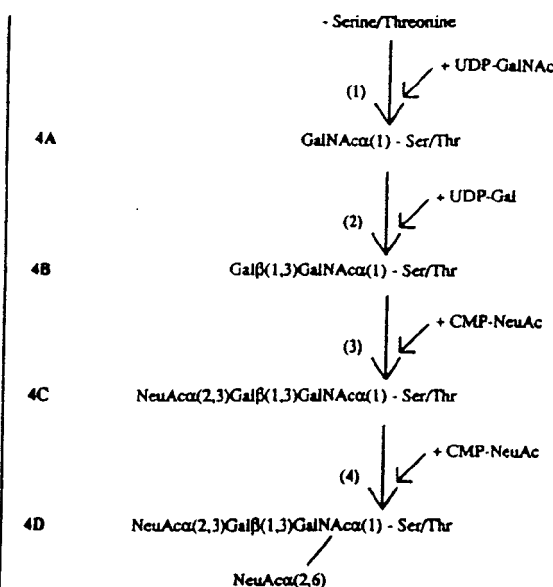


FIGURE 4 A common mammalian O-linked glycosylation pathway. This pathway is evident in the O-linked oligosaccharides of IL-2^{27,28}, EPO²⁹, immunoglobulin A³⁰, G-CSF³¹ and plasminogen³². Enzymes are: (1) UDP-GalNAc:polypeptide N-acetylglucosaminyltransferase, (2) UDP-Gal:GalNAc(1,0)-Ser/Thr β(1,3)galactosyltransferase, (3) CMP-N-acetylneuraminate:β-D-galactoside α(2,3)sialyltransferase, (4) CMP-N-acetylneuraminate:α-D-N-acetylgalactosaminide α(2,6)sialyltransferase. (Reprinted from ref. 3 with permission of the American Chemical Society.)

will focus on the mechanisms underlying cell-type dependent glycosylation by cultured mammalian cells.

Differences in oligosaccharide structure among mammalian cells are frequently attributable to differences in the presence of specific N-acetylglucosaminyltransferase (GnT) activities. For example, differences in branching structure are observed in the complex-type, N-linked oligosaccharides of t-PA produced by a human colon fibroblast (hcf) cell strain compared to those of t-PA produced by a Bowes melanoma cell line. These differences suggest that Bowes melanoma cells express GnT III and VII, but not GnT IV, while hcf cells express GnT IV, but not GnT III and VII⁴¹.

The presence or absence of α(1,3)galactosyltransferase provides another basis for differences in oligosaccharide processing among mammalian cells. This enzyme constructs the linkage galactose bonded α(1,3) to galactose [i.e., Galα(1,3)Gal] (Fig. 2H). The Galα(1,3)Gal structure is found on the cell surfaces and some secreted proteins of New World monkeys and non-primate mammals (e.g. rodents, pigs, sheep, cows), but is not found on most cells and proteins of humans, Old World primates, or anthropoid apes^{42,43}. For example, Galili and coworkers have detected the presence of the Galα(1,3)Gal epitope on the surface of SP/2 myeloma cells and on secreted mouse monoclonal antibodies^{42,43}. This structure is also present on the N-linked oligosaccharides of recombinant interferon-β⁴⁴ and recombinant t-PA⁴⁵ synthesized by the mouse C127 cell line. In contrast, the Galα(1,3)Gal epitope is not found on the cell surface of many cultured human cells, including normal human fibroblasts and the HL60 and HeLa cell lines⁴². This epitope is also not apparent in glycoproteins from recombinant CHO cells,

including human interferon-β⁴⁴, EPO^{46,47} and t-PA⁴⁸ nor is it found in EPO produced by recombinant BHK cells⁴⁹. These data suggest that α(1,3)galactosyltransferase activity in these two rodent cell types is diminished or absent. The CHO cell line does harbor DNA that hybridizes with a probe for a murine α(1,3)galactosyltransferase, but northern blot analysis reveals no evidence of α(1,3)galactosyltransferase message in CHO cells⁵⁰.

Differences in sialylation and fucosylation have also been observed among mammalian cells. Two distinct sialic acid linkages, NeuAcα(2,3)Gal and NeuAcα(2,6)Gal, are found in N-linked glycoproteins isolated from human urine or plasma and from cultured human cell lines (Fig. 2C). For example, EPO isolated from human urine contains about 60% of its sialylated structures with the NeuAcα(2,3)Gal linkage and 40% with the NeuAcα(2,6)Gal linkage⁴⁷. In contrast, recombinant CHO cells generate N-linked oligosaccharides containing only the NeuAcα(2,3)Gal linkage, while recombinant C127 cells generate N-linked oligosaccharides containing only the NeuAcα(2,6)Gal linkage, suggesting that only one of the two sialyltransferases is active in each cell type⁴⁷. Human cells can possess an α(1,3)fucosyltransferase activity leading to generation of a Fuca(1,3)GlcNAc linkage (Fig. 2I)⁵¹. CHO cells apparently possess the α(1,3)fucosyltransferase gene, but it is not normally expressed⁵². The feasibility of altering oligosaccharide processing capabilities through expression of exogenous glycosyltransferase genes has recently been demonstrated independently by several groups, who have introduced α(2,6)sialyltransferase⁵³, α(1,3)fucosyltransferase⁵⁴ and α(1,3)galactosyltransferase⁵⁰ into CHO cells.

While the examples noted above have focused on oligosaccharide processing differences due to the presence or absence of specific glycosyltransferases, differences in oligosaccharide processing can also result from cell-specific differences in the relative activities of competing glycosyltransferases. For example, mammalian cells isolated from carcinomas, or transformed by oncogenic viruses or cellular oncogenes, frequently express increased N-linked oligosaccharide branching due to increased GnT V activity^{55,56}.

Differences in O-oligosaccharide processing have been noted among mammalian cells. For example, significant differences are observed in the O-linked oligosaccharide structures of leukosialin synthesized by the K562 erythroid, HL-60 promyelocytic or HSB-2T lymphoid cell lines⁵⁷.

Differences in O-glycosylation site occupancy are also displayed among mammalian cells. For example, recombinant CHO cells secrete a ratio of glycosylated to nonglycosylated IL-2 on the order of 9:1, while recombinant BHK and Ltk⁻ cells and cultured human lymphocytes produce approximately equal proportions of glycosylated and nonglycosylated IL-2^{27,28}.

Alterations of cell-type dependent glycosylation can result from spontaneous mutations during serial cultivation of mammalian cells. A series of CHO clones has been isolated possessing a variety of mutations affecting N- and O-glycosylation⁵⁸⁻⁶⁰. Most CHO glycosylation mutants maintain a growth rate comparable to their parent population^{58,60}. Mutation usually diminishes a glycosylation capability—for example, the LEC 2 CHO mutant has reduced capacity for sialylation of both N- and O-linked sugars due to reduced ability to transport sialic acid into the Golgi^{58,60}. However, a mutation may lead to a new glycosylation capability. For example, the LEC 10 and 11 CHO mutants express an α(1,3)fucosyltransferase activity not expressed in the parent CHO population⁵⁹. These results provide a note of caution in the selection of clonal populations in conjunction with gene amplification.

N-linked and O-linked glycosylation are affected by the cell culture environment. The outcome of N-linked glycosylation is potentially influenced by cell culture variables such as glucose concentration, ammonium ion concentration and the hormonal content of the medium (reviewed in ref 2). Among the potential mechanisms to explain such effects are: (1) depletion of the cellular energy state, (2) disruption of the local ER and Golgi environment, (3) interference with vesicle trafficking, and (4) modulation of glycosidase and glycosyltransferase activities.

For example, control of mRNA transcription rate and/or stability is presumed to be responsible for many of the effects of hormones on oligosaccharide processing. Recently, this hypothesis has been confirmed in experiments demonstrating increases in specific glycosyltransferase mRNA and corresponding increases in the intracellular concentrations of their respective enzymes in response to cellular stimulation by dexamethasone⁶¹ or retinoic acid⁶².

Downstream processing protocol may influence the set of isolated glycoforms. Glycoprotein oligosaccharides reside on the protein surface, where they may interact with both the protein and the solvent. Therefore, the effects of oligosaccharides on protein surface chemistry are often significant. For example, proteins possessing oligosaccharides are almost always much more soluble than their aglycosyl counterparts^{63,64}. Significant differences in solubility between glycoforms are also possible, as demonstrated by the reduced solubility of EPO in the absence of terminal sialylation⁶⁵.

Since sialic acid is negatively charged at neutral pH, differences in sialic acid content among glycoforms will influence their behavior in ion exchange chromatography. Oligosaccharides also have a significant effect on glycoprotein retention time in chromatographies based on hydrophobic interactions between column support and protein (e.g., see ref. 41).

Thus, the protocol for protein purification may have a profound effect on the distribution of purified glycoforms, and the reproducibility of glycoform distribution will be dependent upon the reproducibility of the purification procedure. These considerations also point to the possibility of adjusting the purification protocol to isolate a subset of glycoforms with enhanced therapeutic potential—for example, if the most highly sialylated subset of glycoforms is valued because of increased *in vivo* circulatory half-life, then one could develop a purification protocol to purify that subset.

EFFECTS OF OLIGOSACCHARIDES ON GLYCOPROTEIN PROPERTIES

The efficacy of a human therapeutic glycoprotein is dependent upon many properties that are potentially affected by oligosaccharide structure. For example, the oligosaccharides of glycoproteins usually enhance protein solubility, and often promote resistance to protease attack and resistance to irreversible thermal inactivation. These latter properties are affected by oligosaccharides on a protein-specific basis due to the influence of oligosaccharides on protein surface chemistry and protein tertiary structure (reviewed in ref. 3).

Specific activity is also frequently affected upon partial or complete removal of oligosaccharides, but is rarely abolished. In fact, the absence of oligosaccharides often enhances specific activity as measured *in vitro*, as exemplified by EPO^{29,66} and t-PA^{67,68}. Oligosaccharides may influence specific activity by affecting glycoprotein tertiary structure. Alternatively, oligosaccharides may promote or inhibit ligand-receptor interactions through a steric effect

or through a charge effect related to the presence of sialic acid on the oligosaccharides of the ligand and/or the receptor (reviewed in refs. 1 and 3).

Below, we present a more detailed discussion of the potential effects of oligosaccharide structure on three properties critical to the efficacy of human therapeutic glycoproteins, antigenicity, immunogenicity and circulatory half life.

Effect of oligosaccharides on glycoprotein antigenicity. Oligosaccharide structures can serve as a basis for antibody recognition (reviewed in ref. 69). Many mammalian circulating antibodies are targeted against specific oligosaccharide determinants. For example, approximately 1% of circulating human IgG is specific for the terminal Gal α (1,3)Gal β (1,4)GlcNAc epitope—that is, approximately 100 μ g/ml of human IgG recognize the "Gal α (1,3)Gal" structure generated by mammalian cell lines possessing α (1,3)galactosyltransferase (Fig. 2H). As a second example, most humans have circulating antibodies against N-linked yeast mannan chains with the general form shown in Figure 3C^{70,71}.

Oligosaccharides may also contribute indirectly to glycoprotein antigenicity⁷². For example, an oligosaccharide may inhibit access to an antigenic site by steric hindrance or by charge interactions contributed by sialic acid⁷³. Alternatively, the antigenicity of a glycoprotein may be altered due to an effect of the oligosaccharides on protein conformation. Such conformational effects have been proposed to explain the differences in antigenicity between glycosylated and aglycosyl forms of human chorionic gonadotropin^{74–76} (hCG), ovine luteinizing hormone⁷⁶ and Semliki forest virus glycoprotein⁷⁷.

Effect of oligosaccharides on glycoprotein immunogenicity. While oligosaccharides clearly affect glycoprotein antigenicity, the effect of oligosaccharides on glycoprotein immunogenicity (ability to elicit an immune response) is less clear. Antibodies to oligosaccharide are elicited in animals when the oligosaccharide is conjugated to an immunogenic protein. For example, antibodies to plant-specific oligosaccharide structures similar to that shown in Figure 3A were raised in rabbits immunized with plant glycoproteins⁷⁸. As an additional example, antibodies against a common O-linked yeast structure, Man α (1,3)-Man α (1,2)Man α (1,2)Man, were raised in rabbits injected with that oligosaccharide conjugated to the plant protein edestin⁷⁹.

Human EPO produced by recombinant CHO cells is the only recombinant therapeutic glycoprotein for which extensive clinical trial data involving antibody development has been published. No antibodies against recombinant EPO have been detected in patients receiving this product, even after chronic administration up to 18 months^{80–82}. Takeuchi and coworkers⁴⁷ have reported the oligosaccharide structures from human urinary EPO and recombinant CHO-derived EPO to be essentially identical, with the exception that the CHO-produced material contains sialic acid only in an α 2,3 linkage, while the human urinary material contains both the α 2,3 and α 2,6 linkages. The absence of antibodies to EPO from recombinant CHO is an important and encouraging result. The detailed analyses of N- and O-linked oligosaccharides from CHO-produced t-PA⁴⁸, interferon- β ⁴⁴ and IL-2²⁸ have also revealed oligosaccharide structures similar to those found on their native human glycoprotein counterparts, although no corresponding data concerning immunogenicity has been published.

Proteins which are normally glycosylated can potentially have altered immunogenicity when administered in aglycosyl form due to the tendency of aglycosyl proteins to aggregate. Protein aggregation enhances immunogenicity

through a mechanism that is poorly understood. For example, increased protein aggregation has been correlated with increased immunogenicity of clinically administered human growth hormone preparations⁸³. In addition, deliberate aggregation of muscle creatine kinase⁸⁴ and cytochrome c⁸⁵ has been demonstrated to significantly increase the immunogenicity of these proteins, eliciting antibodies that recognize the native, unaggregated proteins. In some cases, it may be possible to avoid the insolubility problems associated with aglycosyl proteins through a carefully chosen formulation buffer or through covalent attachment to the protein of an oligosaccharide substitute, such as polyethylene glycol. For example, site-specific attachment of polyethylene glycol to human recombinant *E. coli*-produced IL-2 at its lone O-linked glycosylation site increases the solubility while significantly reducing the immunogenicity of human IL-2 administered in mice⁸⁶.

Antibody development has been reported in patients receiving the *E. coli*-produced human glycoproteins GM-CSF⁸⁷ and interferon beta-ser⁸⁸⁻⁹⁰. However, these studies are inconclusive concerning the effect of missing oligosaccharides on immunogenicity, since the results may be complicated by other factors—for example, amino acid point mutations, differences in N-terminal protein processing and potential impurities.

Effect of oligosaccharides on glycoprotein *in vivo* circulatory half-life. Oligosaccharides play a significant role in defining the *in vivo* glycoprotein clearance rate, a critical property in determining the efficacy of an injected therapeutic protein. High *in vitro* specific activity will be of little consequence if an injected protein is too rapidly eliminated from the circulatory system. For example, the specific activity of EPO is increased upon desialylation, but the *in vivo* activity is abolished due to rapid *in vivo* clearance^{91,92}.

Several circulatory clearance mechanisms are associated with high affinity receptors recognizing terminal monosaccharides of glycoprotein oligosaccharides. The asialoglycoprotein receptor found on hepatocytes binds glycoproteins exhibiting terminal galactose or GalNAc, including desialylated, complex-type N-linked oligosaccharides (e.g., Fig. 2D and 2H) and desialylated O-linked oligosaccharides (e.g., Fig. 4A and 4B) (reviewed in ref. 93). It is presumed that this receptor serves a major role in the turnover of serum glycoproteins⁹³. The functional receptor is an oligomer possessing multiple sites for Gal and GalNAc attachment. Binding of a glycoprotein to this receptor is enhanced by the presence of multiple terminal Gal and GalNAc moieties; for a given asialoglycoprotein, binding will increase with increasing numbers of oligosaccharide groups and with increased oligosaccharide branched structure. The receptor guides bound glycoprotein to lysosomes via endocytosis, and the free receptor is then recycled to the cell surface⁹³. The asialoglycoprotein receptor is thought to be primarily responsible for the rapid *in vivo* clearance noted for desialylated EPO⁹². The loss of *in vivo* biological activity of desialylated GM-CSF also apparently results from rapid clearance by the asialoglycoprotein receptor⁹⁴.

A mannose receptor has been identified on the surface of several cell types, including liver endothelial cells and resident macrophage cells, especially those of the spleen, lung and liver (Kupffer cells) (reviewed in refs. 95 and 96). Oligosaccharides terminating in mannose are infrequently found on the surface of mammalian cells or on mammalian glycoproteins, but are abundant on the surfaces and proteins of lower organisms, such as yeast (Fig. 3C). The mannose receptors of liver endothelial cells and resident macrophages apparently represent a means for

recognizing and eliminating cells and glycoproteins bearing high-mannose-type oligosaccharides⁹⁶. The mannose receptor exhibits affinity for terminal monosaccharides according to the order Man \approx Fuc > GlcNAc > Glu >> Gal. Because of the affinity of the receptor for fucose, the receptor is frequently referenced as the mannose/fucose receptor⁹⁶. The receptor directs bound glycoprotein to lysosomes via endocytosis and is recycled to the cell surface. The mannose receptor is apparently responsible for circulatory clearance of human glycosylated α -amylase on the basis of its N-linked oligosaccharides possessing terminal fucose bonded $\alpha(1,3)$ to GlcNAc (Fig. 2H)⁹¹. The mannose receptor also represents one means of human t-PA clearance due to the high-mannose oligosaccharide at Asn-117⁹⁷. Enzymatic removal of the high-mannose oligosaccharide at Asn-117 using Endo-H⁹⁸ or site-directed mutagenesis to eliminate the glycosylation site at Asn-117⁹⁹ results in enhanced t-PA circulatory half-life. The mannose receptor will presumably represent a major clearance mechanism for glycoproteins produced in recombinant *S. cerevisiae* and insect cells which possess terminal mannose or GlcNAc moieties (Fig. 3C-3D).

Glycoprotein oligosaccharides can also affect clearance rate by mechanisms which do not involve high-affinity receptors. Human proteins with molecular weights less than about 70 kD are continuously removed from the circulation by the kidney. The filtration rate through the kidney glomerular tubules is sensitive to protein tertiary structure as well as molecular weight, and is inhibited by the presence of surface charge (reviewed in ref. 100). Oligosaccharides can prolong glycoprotein circulatory half life by increasing both size and surface charge^{101,102}. For example, α 1-acid glycoprotein with its normal complement of five sialylated, complex-type oligosaccharides is cleared slowly from the rat circulatory system—97% remains in the circulatory system 10 minutes after injection. In contrast, aglycosyl α 1-acid glycoprotein (molecular weight = 23 kD) is cleared rapidly through the kidney—36% remains in the circulatory system 10 minutes after injection.¹⁰¹

Antigenicity generated by the presence or absence of oligosaccharide provides another basis for an effect on glycoprotein clearance. The formation of immune complexes of antigen and antibody leads to clearance of antigen via mechanisms involving the complement system and cellular receptors (reviewed in refs. 103–105). These clearance mechanisms have been postulated to explain the rapid clearance of therapeutic proteins during some clinical trials where an immunogenic response was elicited^{106–109}. However, more rapid protein clearance has not been reported in all cases where an immunogenic response was observed to an injected therapeutic protein⁹⁰. Some investigators have proposed that specific non-precipitating circulating antibodies may in some cases lead to enhanced circulatory half-life of the target protein, by serving as physiological carriers which inhibit normal clearance mechanisms such as kidney filtration¹¹⁰.

CONCLUSIONS

Differences in the N-linked and O-linked oligosaccharide structures produced by mammalian, yeast, insect and plant cells, as documented in the first part of this review, make it difficult to be enthusiastic about the use of yeast, insect and plant cells as hosts for the production of human therapeutic glycoproteins that require substantial circulatory residence time. These cells synthesize oligosaccharide structures with terminating mannose, GlcNAc and/or galactose moieties that should be recognized by the high affinity receptors of the circulatory clearance mechanisms associated with hepatocytes and resident macrophages.

Additional immunogenic effects of glycoproteins possessing yeast-, insect- or plant-specific oligosaccharide structures are possible, although as yet unproven.

Differences in oligosaccharide processing among prospective mammalian host cells could be significant, although the clinical data necessary to draw firm conclusions is not available at this time. For example, it seems likely that the Gal α (1,3)Gal moiety will have an effect on glycoprotein circulatory half-life, given the prevalence in human blood of antibodies directed against that epitope. However, it would be premature to conclude at this time that Gal α (1,3)Gal will necessarily lead to more rapid clearance. In fact, it is conceivable that circulatory half-life could be improved if the antibodies to Gal α (1,3)Gal are non-precipitating. A recent study examining the effect of Gal α (1,3)Gal on t-PA clearance rate in chimpanzees¹¹¹ is inconclusive on this issue due to the complexity of t-PA clearance.^{97-99,112,113} Questions concerning the effect of Gal α (1,3)Gal on clearance extend beyond the use of certain rodent cell lines to produce human therapeutic proteins, as the Gal α (1,3)Gal determinant is also likely to be found on proteins produced by transgenic cows, sheep and pigs.

Detailed N-linked and O-linked oligosaccharide structures have been determined for several glycoproteins produced using recombinant CHO cells, including EPO, t-PA, interferon- β 1 and IL-2. A pleasant surprise from these recent analyses has been the remarkable degree to which the oligosaccharide structures from the CHO-produced glycoproteins correspond to the structures of those same proteins isolated from human urine or produced using normal human diploid cells. As a result, Chinese hamster ovary cells have emerged as the cell line of first choice for the synthesis of recombinant human therapeutic glycoproteins, although CHO cells do possess deficiencies that may limit their applicability in specific cases, such as limited capability for γ -carboxylation and inability for oligosaccharide sulfation.

Acknowledgments

We gratefully acknowledge the thoughtful comments and editorial assistance of Thomas Monica and Steven Williams. This project was supported through a National Science Foundation Presidential Young Investigator Award to CG (EET-8857712), and through Merck and 3M Faculty Development Awards to CG. The present text is a significantly abbreviated version of reference 3, printed with the permission of the American Chemical Society.

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