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Enzyme action in glycoprotein synthesis

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Abstract. Just a few decades ago, the saccharides bound to glycoproteins were considered little more than an irritation. They increased the difficulty of purifying and characterizing proteins, making proteins run as several bands on gels and smearing them on columns. They were considered a nuisance and were typically cleaved away to reveal the 'important part', the protein moiety, for structural (e.g. via X-ray crystallography or nuclear magnetic resonance) and functional studies. We now realize that that the saccharide is often as important as the protein itself, and that glycosylation can have many effects on the function, structure, physical properties and targeting of a protein. There are a myriad of reviews and books on this subject, reflecting the nearly overwhelming number of articles in print discussing saccharide structures, glycoprotein processing enzymes and the biological implications of glycosylation. This review discusses, in turn, the extent and biological relevance of glycosylation; the structures observed; how glycosylated proteins are formed in vivo; the clinical relevance of glycosylation, in terms of the correlations between disease states and unusual glycosylation patterns; and, finally, the molecules, both natural and synthetic, that can be used to study the roles of carbohydrates in glycoprotein structure and function or to disrupt various carbohydrate recognition processes and enzymatic reactions in the glycoprotein synthetic pathway.

Key words. Glycosyltransferase; biosynthesis; processing; inhibition; oligosaccharide; carbohydrate; cloning.

Extent of glycosylation in nature

Although originally thought to be solely the domain of eukaryotes, both N-linked and O-linked glycosylation have been found in eubacteria [1–3] and the archaea [4–6]. This is of particular clinical interest in that many pathogens hide their presence from the immune system or interact with their host cells by displaying normal eukaryotic saccharides on surface glycoproteins or glycolipids. Polysialic acids, normally displayed in developing neural tissues, are displayed by certain *Escherichia coli* strains and by *Neisseria* species, including *N. meningitidis*, an organism responsible for bacterial meningitis. The Lewis x antigen (Le^x; NeuAcα2,3Galβ1,4-GlcNAcβ-R) is displayed by *Helicobacter pylori*, the etiological agent of gastric ulcers. Although the function of Le^x on the *H. pylori* membrane is not known, in the eggs of schistosomes, which also display Le^x, the antigen induces the host's production of interleukin (IL)-10 [7], which inhibits inflammatory T cells (Th_1).

The biosynthetic pathways for glycosylation are often quite different between prokaryotes and eukaryotes, however, and the difference stems from the need to prevent activated sugars from diffusing away. Eubacteria and the archaea have no membrane-bound organelles and must use nondiffusable intermediates, typically lipid-linked sugars, when building polysaccharides externally. Alternatively, they may build the polysaccharides in the cytoplasm on a membrane-bound acceptor – typically a lipid – from nucleotide-activated sugars, and then transfer them to the outside en bloc. In many cases, however, bacteria must simply be creative and use an external energy source to fuel reactions. For example, a *Streptococcus* involved in dental caries uses the relatively high free energy ($\Delta G^\circ_{hydrolysis} = -6.6$ kcal/mol) of the sucrose glycosidic bond to fuel the transglycosidation reaction

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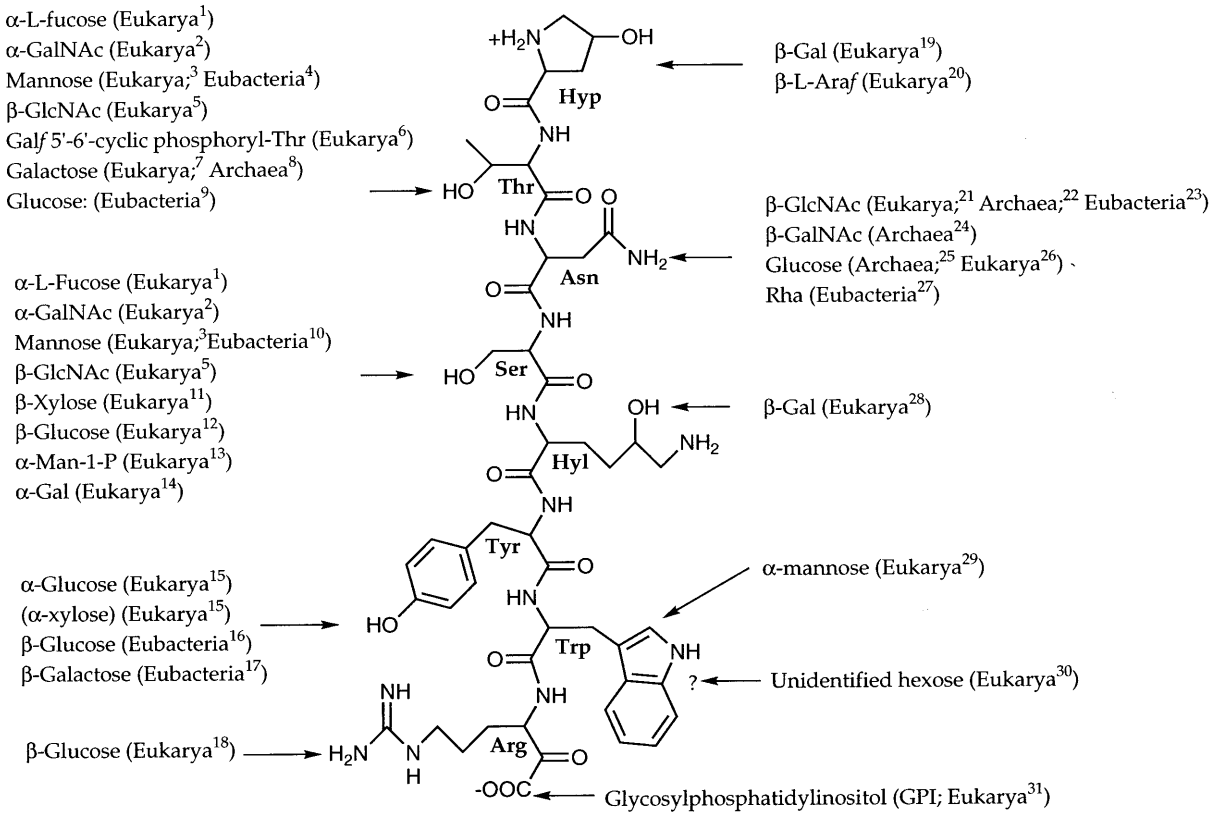

Figure 1. Carbohydrate-protein linkages known, to date, in prokaryotes and eukaryotes. [1]EGF-like domains; [2]mucins; [3]α-linked, in yeast mannoproteins; unidentified linkage in coral mucins; [4]α-linked in *Mycobacterium* MPT32p; [5]Eukarya: many cytoplasmic, nuclear proteins; [6]trypanosomal cell-surface proteins; [7]α-linked: vent worm collagen; [8]unknown linkage: halobacterium; [9]*Clostridium difficile* toxin B: uses to inactivate host Rho protein; [10]unknown linkage: *Flavobacterium proteins*; [11]proteoglycans (exc. keratan sulfate); [12]various clotting factors; [13]*Leishmania* secreted proteoglycan; [14]plant cell wall protein; [15]α-glucose, xylose: bound to tyrosine in glycogenin; unclear if xylosylation is physiologically relevant [155]; [16]*Thermoanaerobacter* (formerly *Clostridium*) S-layer proteins; [17]*Thermoanaerobacter* S-layer proteins; [18]plant starch initiator protein; [19]algae; [20]arabinogalactan protein; [21]most N-linked proteins; [22]*Halobacterium* cell-surface glycoprotein; [23]*Streptococcus* cell wall protein; [24]*Halobacterium* cell-surface glycoprotein; [25]unidentified linkage in *Halobacterium* cell-surface glycoprotein; [26]α-linked in laminin; [27]unidentified linkage in *Bacillus stearothermophilus* S-layer; [28]collagen; [29]human RNase U_s; [30]stick insect neuropeptide; [31]widespread in eukaryotes.

required to create a glucan matrix on teeth. This review will concern itself primarily with glycosylation in eukaryotes. Review of glycosylation in prokaryotes, however, is instructive when trying to determine the rationale behind mechanisms that eukaryotes use.

Types of N-glycans and O-glycans

A major stumbling block to the study of glycoproteins has been microheterogeneity: a single protein species can have hundreds of isoforms that differ only in saccharide composition. Although the number of glycoforms is fairly overwhelming, they do fall into a few classes. These are categorized first by the residues that the sugars are linked to. The linkages observed in eukaryotes, eubacteria and the archaea are shown in figure 1. N-linked glycans in eukaryotes fall into three categories shown in figure 2. (Some consider hybrid and complex types which contain xylose linked α1,2 to the innermost mannose, found in plants, to be a fourth.) Apart from the chitobiose (GlcNAcβ1,4GlcNAc) core, the high-mannose type saccharides contain almost entirely mannose, in a variety of linkages. (They may contain small amounts of other sugars. In the fission yeast, for example, they are capped with Galα1,2 [8].) Yeasts are remarkable in their use of high-mannose oligosaccharides. *Saccharomyces cerevisiae* can add 50–150 mannose residues to a single glycosylation site [9], linked primarily α1,6 and α1,2 and capped with Manα1,3. A general illustration for yeast high-mannose type saccharides is shown in figure 3. Complex-type saccharides have all but the innermost three mannose

residues trimmed away, and are extended typically with galactose and *N*-acetylglucosamine (GlcNAc) and then capped with galactose, *N*-acetylgalactosamine (GalNAc), fucose and/or sialic acids. Hybrid-type chains have the characteristics of high-mannose glycans on the mannose α1,6 branch, but look like complex-type chains on the mannose α1,3 branch. In mammals, both hybrid- and complex-type saccharides may have fucose linked α1,6 to the asparagine-linked GlcNAc and/or GlcNAc attached β1,4 to the innermost mannose.

O-linked glycans are less easy to categorize, being much more varied. Many of the O-linked saccharides found in mammals have GalNAc at the reducing terminus, α-linked to serine or threonine. Called 'mucin-type', as the linkage was first discovered in mucins, this group contains a variety of core structures, shown in figure 4 [10]. In addition, many other sugars are known to be O-linked, as shown in figure 1.

Extension of the core structures of both N- and mucin-type O-linked glycans is most commonly (in mammals) via addition of LacNAc (Galβ1,4GlcNAc) or polyLacNAc chains [(Galβ1,4GlcNAcβ1,3)N], which may be fucosylated (α1,3) at the GlcNAc residues. LacdiNAc (GalNAcβ1,4GlcNAc) has been found less frequently in mammals (and when observed, is found at the nonreducing terminus), although recent studies suggest that LacdiNAc might be more prevalent than originally thought [11]. Noncore chitobiose has also been found, but is quite rare. It has been observed in batroxobin [12], a serine protease in pit viper venom, and an enzyme catalysing its formation has been cloned from the pond snail [13]. In some erythrocyte proteins, the chains may be branched at the galactose of LacNAc with β1,6-linked *N*-acetylglucosamine to form a structure called the I-antigen. Branch formation is developmentally regulated, being virtually absent in proteins from embryonic tissues [14]. The length of the polyLacNAc chain is also regulated; typically a single unit in most tissues, the chains are longer in erythrocyte membrane glycoproteins and in transformed cells [15]. The chains, especially of N-linked glycans, usually end in type 1 (Galβ1,-3GlcNAc) or type 2 (Galβ1,4GlcNAc) saccharides that are capped with fucose, *N*-acetylgalactosamine, galactose and sialic acids in any of a variety of linkages (see fig. 5). On top of this, there may be a myriad of other modifications: sulfation, methylation, phosphorylation, O-acetylation and addition of GlcNAc-, mannose- or GalNAc-1-phosphate.

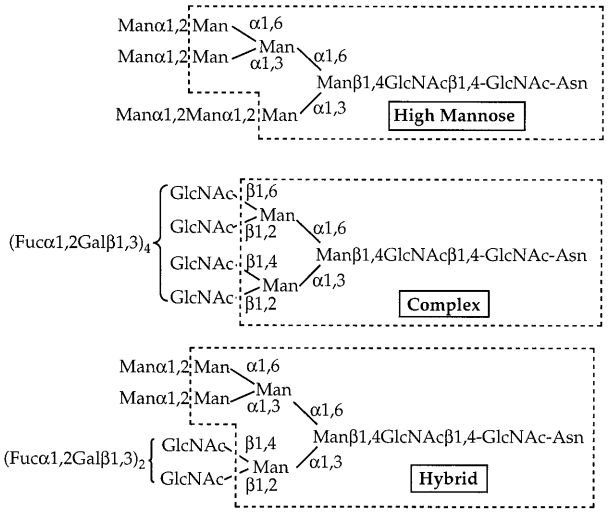

Figure 2. N-Glycan core structures. (Representative glycans are shown; core structures are boxed).

Biological relevance of glycosylation

Glycosylation is an extremely important modification at the protein, cellular and organismal levels. An excellent and thorough review of the biological consequences of glycosylation was written by Varki [16]. At the protein level, one can point to cases where it affects nearly every property of a protein (see table 1). There are many cases in which it has been shown to increase the stability of proteins toward denaturation and/or proteolytic degradation, including a recent nuclear magnetic resonance (NMR) study indicating that O-fucosylation of threonine in the *Pars intercerebralis* major peptide C stabilizes the folded structure of the polypeptide [17]. Glycosylation can greatly alter the physical characteristics of the protein in solution. This is most obvious in the case of mucins, which are responsible for the sliminess of

Table 1. Effects of glycosylation on proteins.

Changes in physical properties
Stability
resistance to proteolysis
resistance to denaturation
Solution properties
increased viscosity
lowered freezing point
Increased solubility
Changes in folding
prevents aggregation
interacts with chaperones
nucleates β turns?
Changes in activity
Altered recognition
protein–protein
protein–saccharide
Increased/decreased multimerization
Changes in targeting
intracellular
extracellular
altered Clearance

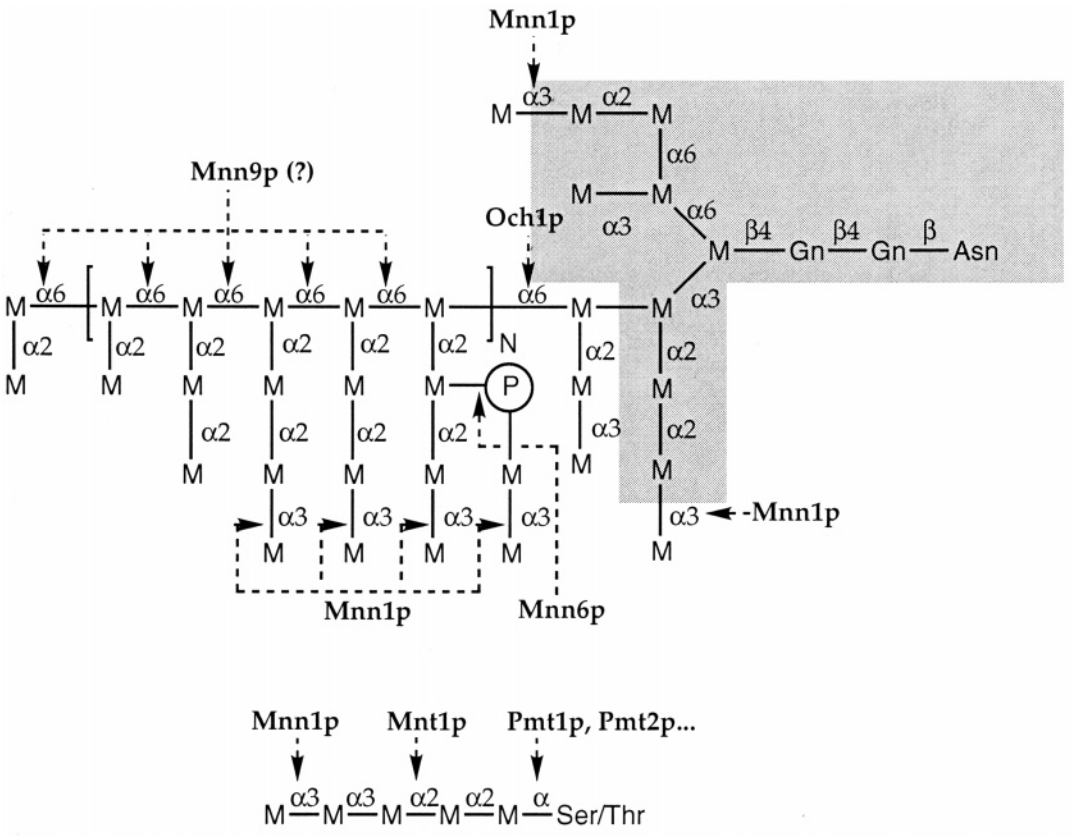

Figure 3. N- and O-linked glycans in yeast (adapted from Tanner and Lehle [9]). The (cloned) enzymes that are thought to catalyse the linkages are shown in bold. The N-glycan core structure is emphasized with a grey box. In addition, a family of α1,2 mannosyltransferases have been cloned and assayed [156] (Ktr1p, Ktr2p, Ktr3p and Yur1p), but their roles in the elaboration of these structures are unknown.

mucous secretions. The activity of many proteins is affected by their glycosylation states. For example, two haemorrhagic toxins from *Crotalus viridis* (western rattlesnake) are metalloproteases that lose activity upon deglycosylation [18]. Likewise, deglycosylated haptoglobin loses its binding affinity for hemoglobin [19]. Recognition of glycoproteins is often based on the saccharides they bear. Many immunoglobulins recognize foreign saccharide epitopes. It is the recognition of Galα1,3Gal-R that is responsible, at least in part, for many cases of xenograft rejection [20, 21], and N-glycans fucosylated α1–3 at the core GlcNAc (Fucα1,3GlcNAc-Asn) may contribute to the anaphylactic response to bee stings [22]. The selectins, adhesion molecules responsible for recruitment of leukocytes to sites of inflammation and also implicated in tumour metastasis, bind to sialylated Lewis antigens [23, 24]. Many proteins do not fold properly when unglycosylated, and instead aggregate. Thus, glycosylation may help both to improve solubility and aid folding. Since glycosylation is cell-type specific, every protein is in essence carrying a postmark indicating where it came from, so it is not surprising that there are many cases in which glycosylation affects targeting of a protein. Proteins labelled with mannose-6-phosphate are shuttled to lysosomes, for example, and the decoration of some pituitary hormones with GalNAc-4-SO_4 is thought to play a part in their retrieval from the blood by hepatic endothelial cells [25]. The importance of saccharides with regard to targeting is underscored by the number of carbohydrate receptors borne by hepatic and other cells [26, 27]. Clearance of 'old' proteins (desialylated) is brought about by asialoglycoprotein receptors in the liver, for example, while mannosylated proteins may be taken up by a mannose-specific receptor in macrophages. The necessity of glycosylation for cell–cell communication and tissue organization was demonstrated by early studies of the effect of tunicamycin, an inhibitor of the initial step in N-linked glycosidation, on embryogenesis. Embryos treated with tunicamycin are halted at the blastula stage [28]. Later studies with *Mgat1* knockout mice (a knockout of the enzyme *N*-acetylglucosaminyltransferase I that catalyses the first step toward complex and hybrid N-glycan synthesis) supported this: again, the mice died in utero, and many of them showed

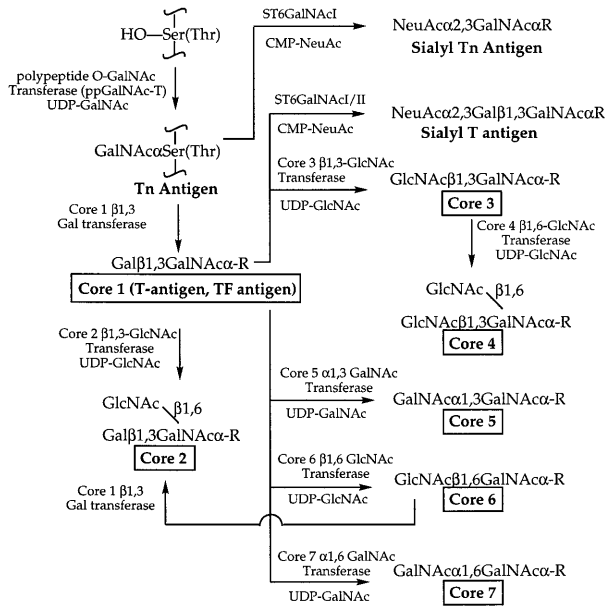

Figure 4. O-glycan cores and enzymatic routes between them. (Adapted from I. Brockhausen [157]).

a curious inverted left–right assymetry prior to death [29, 30].

There are many cases in the literature where removal of the sugar groups seems to have little effect. Because of the large range of saccharide functions, it is difficult to say that in these cases the sugars are of little importance. Some of the functions can be very difficult to assess, especially when they are only important on an organismal level (such as the effect of saccharides on serum clearance) or at a particular developmental stage.

Glycoprotein synthesis in vivo

N-glycosylation

Asparagines are glycosylated cotranslationally in the lumen of the rough endoplasmic reticulum by a multimeric enzyme, oligosaccharyltransferase (OST) [31], which moves a preconstructed oligosaccharide block from dolichyl pyrophosphate to the side-chain amide nitrogen of asparagine. This is reminiscent of archaeal N-glycosylation: Paul and coworkers demonstrated that in the Halobacteria, N-glycosylation of at least some cell-surface proteins occurs via transfer of a prebuilt tetrasaccharide from short-chain (C_{60}) dolichyl phosphate (not pyrophosphate) to asparagine [32]. Synthesis of the dolichol block in eukaryotes is shown schematically in figure 6, and the structure of dolichol is illustrated in figure 7. Many of the enzymes which catalyse the synthesis of the lipid-linked core have been cloned, and these are shown in table 2. The core five mannose and two GlcNAc residues of the dolichol-linked oligosaccharide block appear to be added on the cytoplasmic face of the endoplasmic reticulum (ER), using nucleotide-activated sugars. Hanover and Lennarz found that the first two intermediates were inaccessible to β1,4-galactosyltransferase added to the outside of sealed ER microsomes [33], indicating lumenal synthesis; however, no mannose transporter has been found in the ER. In addition, Snider and Rogers [34] provided evidence that the mannose units were added on the outer face when they showed that intermediates from the monomannose to the pentamannose form are accessible to concanavalin A (ConA) and that addition of sugars to the growing block is sensitive to protease K in sealed microsomal vesicles. Convincing evidence for cytoplasmic generation of the first two *N*-acetylglucosaminylated intermediates came from Abeijon et al. [35], who showed that even in the presence of an inhibitor (5-bromouracil) of UDP-GlcNAc (uridine disphosphate, UDP) transport to the microsomal lumen, GlcNAc pyrophosphoryldolichol (GlcNAc-P-P-Dol) and $GlcNAc_2$-P-P-Dol were synthesized from exogenously added UDP-GlcNAc. These and other studies together suggest that the dolichol-linked intermediate is synthesized on the outer face up until the addition of the fifth mannose, at which point the block is translocated to the lumenal face, and this transport most likely requires a protein carrier. Although the rate of unassisted flip-flop is very slow for dolichyl phosphate [36], it has recently been shown that there is a protein enriched in the ER capable of transposing mannosylphosphorylcitronellol, an analogue of mannosylphosphoryldolichol [37], and it seems likely that a similar (or perhaps the same?) 'flippase' is responsible for inverting the $Man_5GlcNAc_2$-P-P-Dol block. The final four mannose and three glucose residues are added by lumenal enzymes using dolichyl phosphate-activated sugars, which also appear to be made cytoplasmically and then translocated [38]. These last, lipid-linked steps are reminiscent of prokaryotic synthetic machinery. Prokaryotes use lipid-linked sugar intermediates extensively, although eukaryotes have moved toward longer lipids, using mainly C_{90-100} dolichol rather than the C_{55} polyisoprenol preferred by eubacteria [39] (see fig. 7) or the C_{60} dolichol used by archaea [40]. Interestingly, many eukaryotic glycosyltransferases will still accept C_{55} dolichols almost equally well [41], although they require saturation of the α-isoprenoid unit [42].

Oligosaccharyltransferase appears to act cotranslationally (or shortly thereafter), requiring merely 12–14 residues to pass into the ER lumen for glycosylation to occur [43]. OST will accept Asn-Xaa-Thr/Ser, and occasionally also accepts Asn-Xaa-Cys, such as in human protein C [44] and the von Willebrand factor [45]. Several

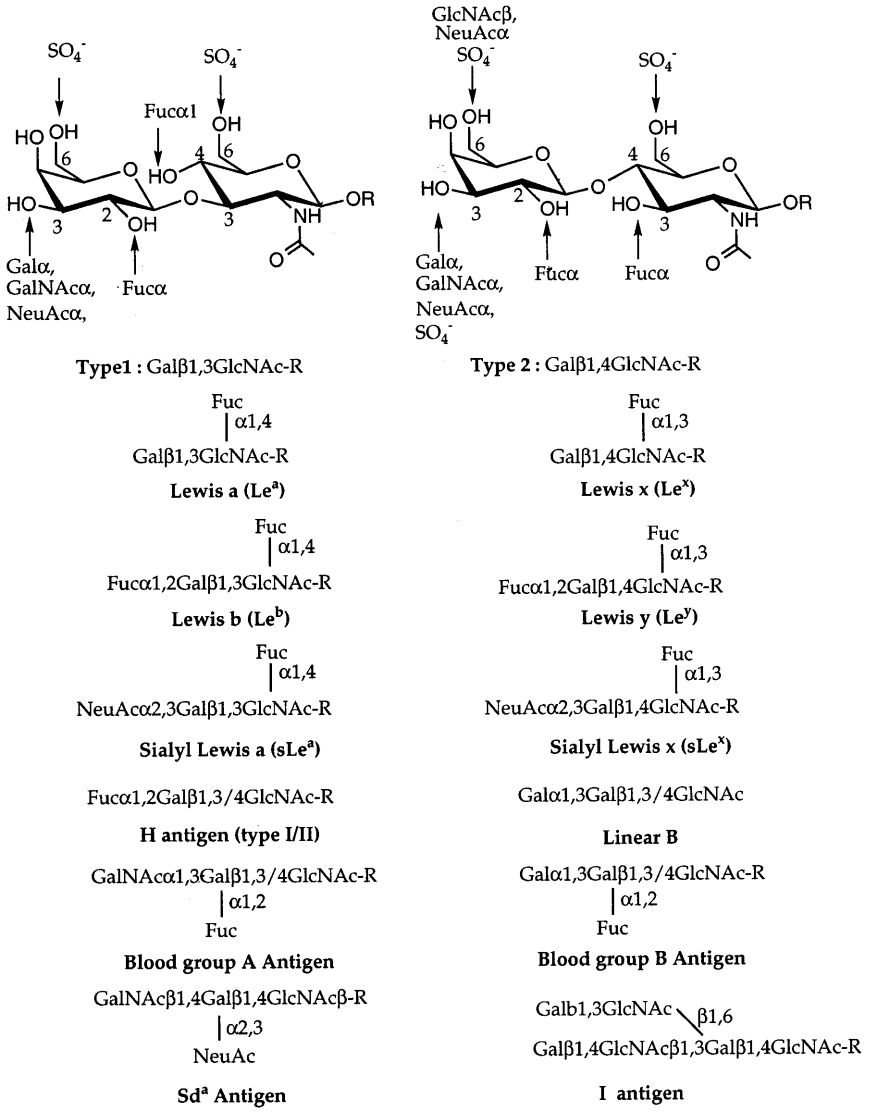

Figure 5. Common modifications to type I and type II terminal structures, and some of the resulting antigens.

studies have indicated that the specificity seems to arise from the ability of this structure to form an asx-turn (reviewed by Imperiali [46]), as peptide substrates that can adopt such a conformation tend to be good substrates, whereas those that do not are poorly glycosylated. Interestingly, when N-glycosylation occurs in archaea, it is also generally at the same consensus sequence [32, 47, 48], and this sequence may even be conserved in eubacteria [49]. Since secondary structure predictions indicated that β turns might be present in 70% of glycosylation sites in folded proteins [50], it has been postulated that glycosylation acts as a conformational switch, forcing the nascent peptide to twist from an Asx turn to a β turn. There is some NMR evidence to suggest that this may be the case [51], though studies of 44 crystal structures of glycoproteins have indicated that β turns are found at a much lower percentage (32%) of glycosylation sites than originally believed [52].

Figure 8 shows the initial processing of the N-linked glycan core. Soon after transfer to the polypeptide, the outer three glucose residues are cleaved away. The outermost α1,2-glucose is removed by α-glucosidase I, while the inner two α1,3-glucose residues are removed by α-glucosidase II. Both glucosidases are localized in the ER. One cannot help but wonder why these sugars are added in the first place if they are simply to be removed again, particularly in view of the fact that some trypanosomes can do without them [53]. Thus eukaryotes

have had the possibility of eliminating the terminal glycosylation steps, but they have not done so. From an organic chemistry perspective, this is reminiscent of protecting group chemistry, and Hoflack et al. [54] showed that the glucose-protected intermediate is less susceptible to phosphodiester cleavage. In addition, since it appears that there is a protein transporter [37] for dolichol-linked intermediates, it is possible that the larger oligosaccharyl-P-P-dolichol molecules are transported inefficiently, and so lumenal addition of four mannose residues and three glucose residues might enable concentration on that side. Accordingly, the Man_{6-9} and glucosylated intermediates were not observed on the cytoplasmic face of the ER in Snider's ConA localization studies [34]. Finally, the chaperone calnexin binds to the glucose of unfolded/misfolded proteins in the ER, and there is in fact a glucosyltransferase in the ER capable of adding glucose back to misfolded proteins [55]. Unless the glucose is removed, the protein will accumulate in the ER, which allows a sort of 'quality control' process to occur (reviewed by Helenius [56]).

Between the ER and the Golgi, all of the α1,2-linked mannose residues may be removed by a few mannosidases resident in the ER and the Golgi resident α-mannosidase I [57]. At least one endomannosidase is present in the Golgi as well, which allows cleavage between the two mannose residues of Glcα1,3Manα1,2Man-R [58].

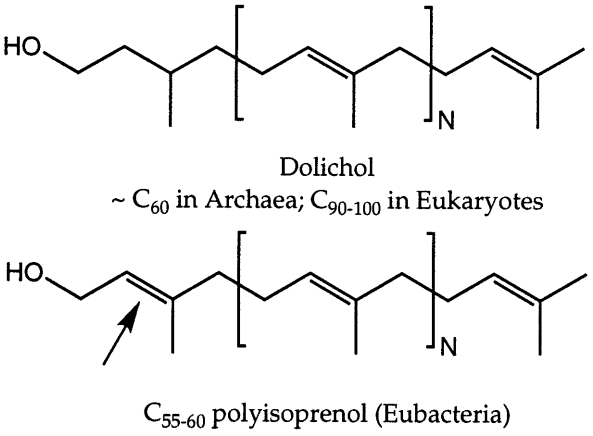

Figure 7. Structure of dolichol (used by eukaryotes and the archaea) and polyisoprenol used by eubacteria (and accepted by at least some archaeal enzymes [5]).

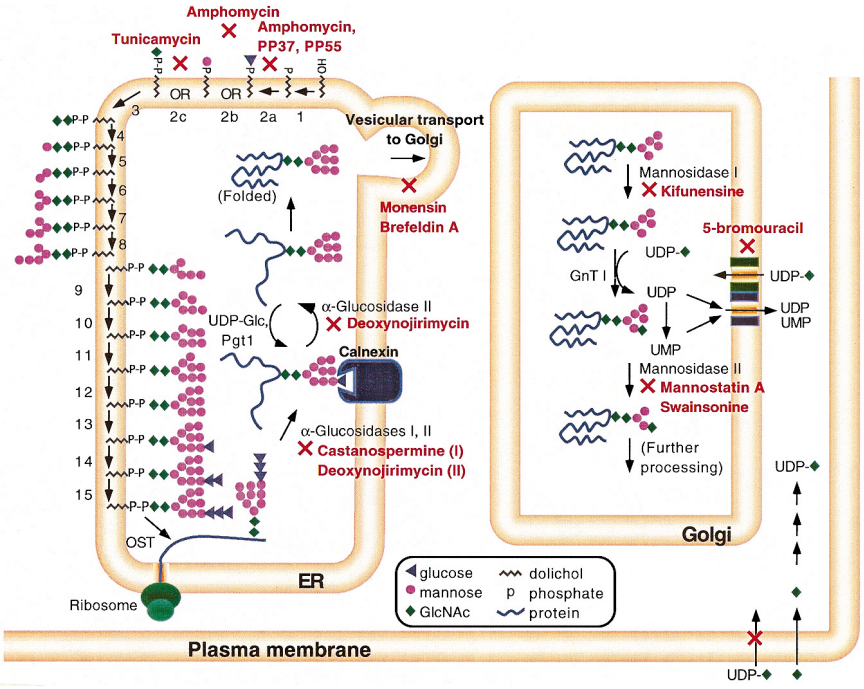

Figure 6. Synthesis of the N-linked glycoproteins. Formation of the dolichol block, transfer to the protein and initial processing reactions. [Enzymes: 1: dolichol kinase (yeast Sec59p); 2a: mannosylphosphoryldolichol synthase (yeast Dpm1p); 2b: glucosylphosphoryldolichol synthase (yeast Alg5p); 2c: dolichyl phosphate GlcNAc-phosphotransferase (yeast Alg7p); 3: GlcNAc-P-P-Dol GlcNAc transferase; 4–8: GDP-Man-dependent mannosyltransferases; 6–12: Dol-P-Man-dependent mannosyltransferases; 13–15: Dol-P-Glc-dependent glucosyltransferases].

Table 2. Cloned enzymes involved in dolichol block synthesis.

Enzyme	GenBank accession #	Preferred acceptor	Source
Dolichol kinase (yeast Sec59p)	M25779	Dolichol	*Saccharomyces cerevisiae* [189]
GlcNAc-1-phosphotransferase (yeast Alg7p)	1. Y00126 2. M36899 3. M96635 4. X65603, S41875 5. U09454 6. Z82022	Dolichyl phosphate	1. *Saccharomyces cerevisiae* [190, 191] 2. Hamster [192, 193] 3. *Leishmania amazonensis*[194] 4. Mouse [195] 5. *Schizosaccharomyces pombe* [196] 6. Human (Eckert V. unpub.)
Mannosyltransferase I (yeast Alg1p)	J05416	GlcNAc-GlcNAc-P-P-Dol	*Saccharomyces cerevisiae* [197, 198]
Putative α1,3-ManT (yeast Alg2p)	X87947	$Man_2GlcNAc_2$-P-P-Dol	*Saccharomyces cerevisiae* [199]
Putative α1,3-ManT (yeast Alg3p)	-	$Man_3GlcNAc_2$-P-P-Dol	*Saccharomyces cerevisiae* [200]
Putative α1,3-GlcT (yeast Alg6p)	Z74910, Y13140	$Man_9GlcNAc_2$-P-P- Dol	*Saccharomyces cerevisiae* [201]
Putative α1,3-GlcT (yeast Alg8p)	X75929	$GlcMan_9GlcNAc_2$-P-P-Dol	*Saccharomyces cerevisiae* [202]
Putative ManT (yeast Alg9p)	X96417	$Man_6GlcNAc_2$-P-P-Dol	*Saccharomyces cerevisiae* [203]
Dol-P-Man synthase (yeast Dpm1p)	1. AF007874 2. AF007875 3. L19169, J04184 4. AF007873 5. Z54162 6. U54797	Dolichyl phosphate	1. *Caenorhabditis briggsae* [204] 2. Human [204] 3. *Saccharomyces cerevisiae* [205] 4. *Schizosaccharomyces pombe* [204] 5. Trypanosome [206] 6. *Ustilago maydis* [207]
Dol-P-Glc synthetase (yeast Alg5p)	X77573	Dolichyl phosphate	*Saccharomyces cerevisiae* [208]

Further processing of the glycan is shown in figure 8. The successive action of a series of *N*-acetylglucosaminyltransferases (GnTs) and mannosidases elaborate the structure further into hybrid- or complex-type glycans. Many of these modifications are interdependent: GnTs III, IV and V require the prior action of GnT II, while the action of GnT III prevents the action of GnT IV and V and mannosidase II [59]. The glycans are further elongated and capped in the *trans* Golgi and the Golgi network by the competing reactions of a variety of other enzymes.

O-glycosylation

Mucin-type O-glycosylation is initiated by the direct transfer of GalNAc or another sugar from a nucleotide sugar to serine or threonine. The search for an O-GalNAcylation consensus sequence was relatively unsuccessful, and it was eventually concluded that *O*-GalNAc transferase preferred serine/threonine/proline-rich regions but simply had a very broad specificity [60, 61] . Only recently has it been realized that there is more than one UDP-GalNAc:protein *N*-acetylgalactosaminyltransferase (ppGalNAc-T). Five (disregarding species differences) have been cloned so far (table 3). Once the chain has been initiated, the saccharide can be elongated with a variety of glycosyltransferases, many of which can accept both N- and O-linked saccharide substrates.

A special case of O-glycosylation is the cytoplasmic/nuclear *O*-GlcNAcylation of proteins (on Ser or Thr), a modification predicted to have a regulatory role complementary to (or perhaps reciprocal to) protein phosphorylation. Pulse-chase studies have shown that the sugar moiety turns over faster than the protein itself [62]. In addition, GlcNAcylation has been shown to modify the activities of several proteins, and the sites of GlcNAcylation are often the same sites modified by protein kinases [63]. *O*-GlcNAcylation was shown to be critical to cell viability in a study from Snow and coworkers in which transfection of CHO cells with a gene for cytoplasmically localized β1,4-galactosyltransferase, which caps the GlcNAc residue with galactose, was found to be lethal within one cell cycle [64].

GPI-anchors

GPI (glycophosphoinositide) anchoring, or 'glypiation', is a recently discovered posttranslational modification in which a preformed glycolipid structure (shown in fig. 9a) is attached to the C-terminal region of a protein by transamidation. It appears to be a strictly eukaryotic modification, and is used more commonly in the lower eukaryotes than in mammals. The general core structure (ethanolamine-P-6-Manα1,2Manα1,6Manα1,4GlcNα1,6-inosPtd) of GPI is constant between species, though there are species-

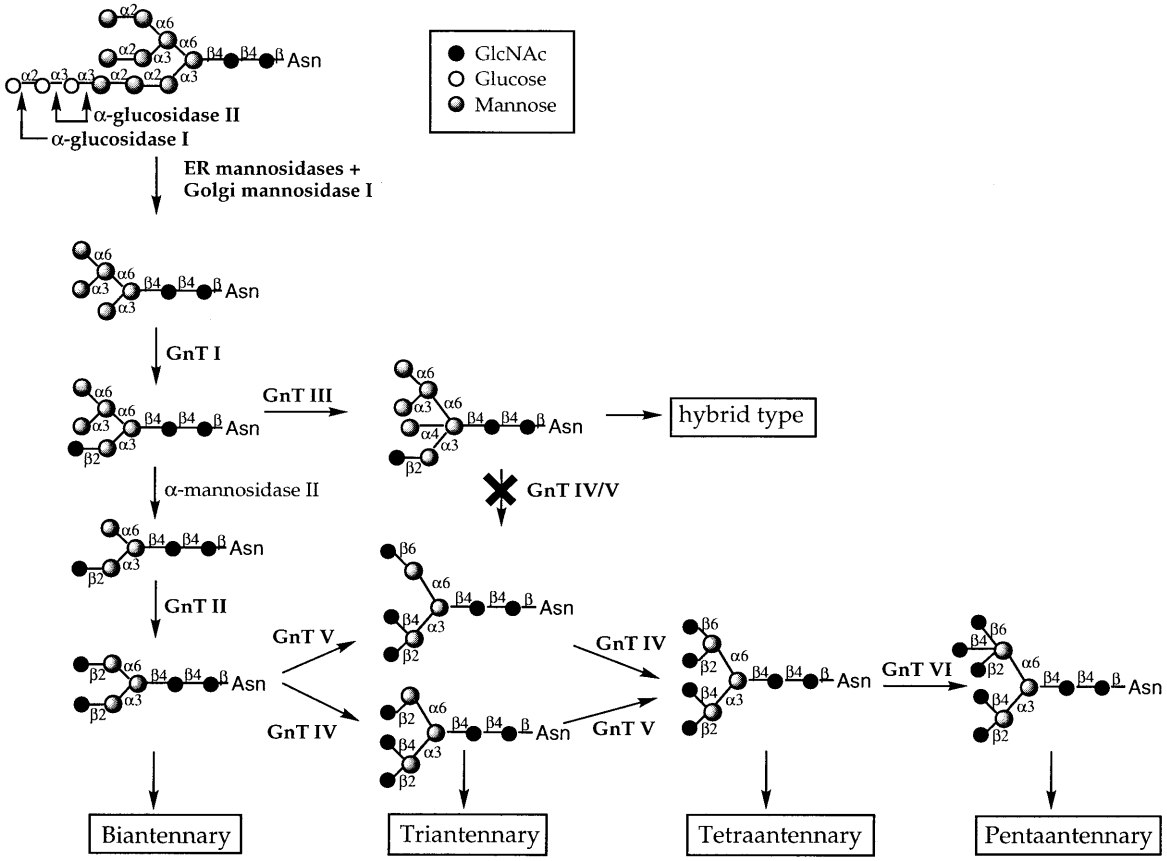

Figure 8. Initial processing of the N-linked saccharide core.

specific differences in the lipids attached and the modifications of the constituent sugars. Although there is not a clear-cut consensus for glypiation, some general features of glypiated proteins have been identified (see fig. 9b) [65]. The amino acid to which GPI is linked (the ω residue) should be small and preferably polar (Asp, Cys, Ser, Gly, Asn; occasionally Ala); the $\omega+2$ should also be small (Ala, Gly, or Ser). The $+1$ site can be quite variable, based on mutagenesis studies (all amino acids tested were accepted to a certain degree except proline), but works best with small amino acids, which are usually found in the natural sites. After the $\omega+2$ site, there are typically a few charged amino acids, and then a stretch of 15 to 20 hydrophobic ones. The GPI anchor appears to be synthesized on the cytoplasmic face of the endoplasmic reticulum, based on the phosphoinositol phospholipase C (PI-PLC) sensitivity and ConA accessibility of the intermediates in sealed microsomal vesicles (though there may simply be a rapid flippase) [66]. GPI biosynthesis has been reviewed recently by Takeda and Kinoshita [67].

Glycosyltransferases

There are estimated to be hundreds of glycosyltransferases responsible for elaboration of the many saccharide structures observed. Glycosyltransferases have been found in nature that accept nucleotide sugars (typically cytidine 5′-phospho-β-NeuAc, guanosine-5′-diphospho-β-L-Fuc and -α-D-Man, and uridine-5′-diphospho-α-D-Glc, -α-D-GlcNAc, -α-D-Gal, -α-D-GalNAc, -α-D-Xyl, -α-D-GlcA, though plants, eubacteria and archaea are different in their nucleotide usage), dolichyl phosphosugars (dolichyl phospho-1-Glc, -GlcNAc or -Man), sugar phosphates and disaccharides as sugar donors. Most of the transferases involved in glycoprotein elaboration, however, use either dolichyl phosphosugars or nucleotide sugars. Some of them do not show absolute specificity in their choice of sugar donor. The sialyltransferases, for example, are somewhat promiscuous. Those tested can accept *N*-glycolylneuraminic acid (NeuGc) [68, 69] and recently cells were labelled with ketones by feeding them *N*-levulinoyl mannosamine, which is converted to *N*-levulinoyl neuraminic acid (NeuAc) in the cell and added to glycoproteins in place of NeuAc [70].

Table 3. Cloned eukaryotic glycoprotein glycosyltransferases. (A few of the glycosyltransferases shown are primarily involved in the synthesis of glycolipids. They have been included either because they are homologous to glycoprotein glycosyltransferases or because they show significant reactivity with glycoprotein substrates).

Enzyme	GenBank accession #	Preferred acceptor	Source
Galactosyltransferases			
β1,4 GalT	1. X14558, M13214, M25398 2. L12565 3. M22921, X55415, X13223, X14085, D29805 4. D00314, J03880, M26925	**GlcNAc**βR	1. Bovine [209–213] 2. Chicken [214] 3. Human [215–218] 4. Mouse [219–221]
α1,3 GalT (blood group B GalT)	AF006673	Fucα1,2**Gal**βR	Human [222]
α1,3 GalT	1. J04989 2. M26925, M85153 3. L36152, L36535 4. J05421, M60263	**Gal**β1,4GlcNAcR	1. Bovine [223] 2. Mouse [224, 225] 3. Porcine [226] 4. Human (pseudogene) [227, 228]
α1,2 GalT	Z30917	**Mannose** (presumed)	*Schizosaccharomyces pombe* [8]
Fucosyltransferases			
α2FucT I (*H*)	1. M35531 2. U90553, Y09883 3. L50534 4. X80226 5. L26009, AB006137	**Gal**β-R	1. Human [229, 230] 2. Mouse [Domino S. E., unpublished, and Hitoshi S., unpublished] 3. Pig [231] 4. Rabbit (RFTI) [232] 5. Rat (FTA) [233] and Soejima, M., unpublished
α2FucT II (*secretor*)	1. U17894, D87942 2. AB006612 3. AB006609 4. AB006611 5. Y09882 6. AB006610 7. X80225 8. X91269 9. AB006138	**Gal**β-R	1. Human [234–236] 2. Chimpanzee pseudogene [Koda Y., unpublished] 3. Gibbon [Koda Y., unpublished] 4. Gorilla pseudogene [Koda Y., unpublished] 5. Mouse [Hitoshi S., unpublished] 6. Orangutan [Koda Y. unpublished] 7. Rabbit (*RFTII*) [232] 8. Rabbit (*RFTIII*) [237] 9. Rat (FTB) [233] and Soejima M., unpublished
α3FucT III	1. X87810 2. U78737 3. X53578	Galβ1,4(3)**GlcNAc**-R	1. Bovine [238] 2. Hamster [Zhang A., unpublished] 3. Human [239]
α3FucT IV	1. U73678 2. M65030, M58597 3. U33457, D63380 4. U58860	Galβ1,4**GlcNAc**-R	1. Chicken [240] 2. Human [241–243] 3. Mouse [244, 245] 4. Rat [Sajdel-Sulkowska E. M., unpublihsed]
α3FucT V	M81485	Galβ1,4**GlcNAc**-R	Human [246] (Bovine, Hamster: see FucT III)
α3FucT VI	L01698, M98825	Galβ1,4**GlcNAc**-R	Human [75, 247] (Bovine, Hamster: see FucT III)
α3Fuc T VII	1. U08112, X78031 2. U45980	Galβ1,4**GlcNAc**-R	1. Human [248, 249] 2. Mouse [250]
GlcNAc-Asn α6FucTI	1. D89289 2. D86723	*N*-glycan core	1. Human [251] 2. Porcine [252]
GlcNAc transferases			
GnT I (Mgat1p)	1. U65791, U65792 2. M55621 3. M73491, L07037 4. M57301 5. D16302	see figure 6	1. Hamster [253] 2. Human [254, 255] 3. Mouse [256, 257] 4. Rabbit [258] 5. Rat [259]
GnT II (Mgat2p)	1. U21662 2. L36537, U15128	see figure 6	1. Rat [260] 2. Human [261]
GnT III (Mgat3p)	1. D10852 2. D13789, L48489 3. L39373	see figure 6	1. Rat [262] 2. Human [263, 264] 3. Mouse [265]
GnT V (Mgat5p)	1. D17716, S80050 2. L14284	see figure 6	1. Human [266, 267] 2. Rat [268]
Core 2 β1,6GnT (C2GnT)	1. U41320 2. M97347 3. U19265	Galβ1,3**GalNAc**α-R	1. Bovine [Li, C.-M., unpublished] 2. Human [269, 270] 3. Mouse [Warren, C. E., unpublished]

Table 3. (continued).

Enzyme	GenBank accession #	Preferred acceptor	Source
I antigen β1,6GnT	Z19550, L19659	Galβ1,4GlcNAcβ1,3**Gal**-R	Human [271]
OGT (ppOGnT)	1. U77412 2. U77413 3. U76577	**HO**-Ser/Thr	1. *Caenorhabditis elegans* [272] 2. Human [272] 3. Rat [273]
GlcNAc-R β1,4GlcNAc T	X80228	Glc**NAc**-R	Snail (*Lymnaea stagnalis*) [13]
GalNAc transferases			
ppGalNAc-T1	1. L07780 2. U41514, X85018 3. U73820 4. U35890	**HO**-Ser/Thr	1. Bovine [274, 275] 2. Human [276, 277] 3. Mouse [278] 4. Rat [279]
ppGalNAc-T2	X85019	**HO**-Ser/Thr	Human [277]
ppGalNAc-T3	1. X92689 2. U70538	**HO**-Ser/Thr	1. Human [280] 2. Mouse [281]
ppGalNAc-T4	1. Y08564 2. U73819	**HO**-Ser/Thr	1. Human [282] 2. Mouse [283]
α1,3 GalNAc T (blood group A)	J05175	Fucα1,2**Gal**β-R	Human [222, 284]
UDP-GalNAc:GM_3/GD_3, β1,4-GalNAc T	1. M83651 2. L25885, U18975 3. D17809	NeuAcα2,3**Gal**β1,4Glcβ-ceramide	1. Human [285] 2. Mouse [286, 287] 3. Rat [288]
CT/Sd^a antigen GalNAc T	L30104	Siaα2,3**Gal**β1,4GlcNAc-R	Mouse [289]
Sialyltransferases			
ST3Gal I	1. X80503 2. L13972, L29555 3. X73523 4. M97753	**Gal**β1,3GalNAc-R (on O-linked glycoproteins; lipids to a lesser extent)	1. Chick [290] 2. Human [291, 292] 3. Mouse [293] 4. Pig [294]
ST3Gal II	1. U63090, X96667 2. X76989 3. X76988	**Gal**β1,3GalNAc-R (on O-linked glycoproteins or lipids to equal extent)	1. Human [295, 296] 2. Mouse [297] 3. Rat [297]
ST3Gal III	1. L23768 2. X84234 3. M97754	**Gal**β1,3(4)GlcNAc-R (N-linked glycoprotein)	1. Human [298] 2. Mouse [299] 3. Rat [300]
ST3Gal IV	1. X74570, L23767 2. D28941, X95809	**Gal**β1,4(3)GlcNAc-R (N-linked glycoprotein)	1. Human [301, 302] 2. Mouse [Kono M., unpublished; Sasaki K., unpublished]
ST6Gal I	1. X75558 2. X17247, G29388, X54363,A17362, X62822 3. D16106 4. M18769	**Gal**β1,4GlcNAc-R	1. Chicken [303] 2. Human [304–307] 3. Mouse [308] 4. Rat [309]
ST6GalNAc I	X74946	**GalNAc**αR Galβ1,3**GalNAc**αR NeuAcα2,3Galβ1,3 **GalNAc**αR	Chicken [310]
ST6GalNAc II	1. X77775 2. U14550 3. X93999	Galβ1,3**GalNAc**αR NeuAcα2,3Galβ1,3	1. Chicken [311] 3. Mouse [312]
ST6GalNAc III	L29554	**GalNAc**αR NeuAcα2,3Galβ1,3 **GalNAc**αR	2. Human [Soutiropoulou, unpublished] Rat [313]
ST8Sia I	1. U73176 2. X77922, L43494, D26360, L32867, 3. X84235, L38677 4. D45255, U53883	**NeuAc**α2,3(GalNAcβ1,4) Galβ1,4G lcβ-ceramide (Ganglioside)	1. Chicken [Daniotti, J. L., unpublished] 2. Human [314–316] 3. Mouse [Tsuji, S., unpublished; Furukawa K., unpublished] 4. Rat [317, 318]
ST8Sia II	1. U33551, U82762 2. X83562 3. L13445	**NeuAc**α2,8(3)-R	1. Human [319] 2. Mouse [320, 321] 3. Rat [322]
ST8Sia III	1. X80502 2. U55938	**NeuAc**α2,3Galβ1,4GlcNAcR	1. Mouse [323] 2. Rat [324]
ST8 Sia IV	1. AF008194, 2. Z46801 3. L41680 4. X86000 5. U90215	**NeuAc**α2,8(3)-R	1. Chicken [Bruses, J. L., unpublished] 2. Hamster [325] 3. Human [326] 4. Mouse [327] 5. Rat [Phillips G. R., unpublished]
ST8 Sia V	1. U91641 2. X98014	**NeuAc**α2,3(GalNAcβ1,4) Galβ1,4Glcβ-ceramide (ganglioside)	1. Human [328] 2. Mouse [329]
Mannosyltransferases			

Table 3. (continued).

Enzyme	GenBank accession #	Preferred acceptor	Source
α1,2ManT (Mnt1p or Kre2p)	1. X69919 2. M81110, X62647	Manα-R	1. *Candida albicans* [Buurman, E. T. unpublished] 2. *Saccharomyces cerevisiae* [330–332]
α1,2ManT (Ktr1p)	X62941	Manα-R	*Saccharomyces cerevisiae* [156]
α1,2ManT (Ktr2p)	L17083	Manα-R	*Saccharomyces cerevisiae* [156]
a1,2ManT (Ktr3p)		Manα-R	*Saccharomyces cerevisiae*
α1,2ManT (Yur1p)		Manα-R	*Saccharomyces cerevisiae* [156]
Mannose phosphate transferase (Mnn6p)	U43922	see figure 3	*Saccharomyces cerevisiae* [333]
α1,3ManT (Mnn1p)	L23753	Manα-R (capping enzyme)	*Saccharomyces cerevisiae* [334]
α1,6ManT (Och1p)	D11095	$Man_5GlcNAc_2$-R	*Saccharomyces cerevisiae* [335]
Putative α1,6ManT (Mnn9p) 2. L23752	1. U63642	?	1. *Candida albicans* [Southard S. B. unpublished] 2. *Saccharomyces cerevisiae* [334]
Mnn10p: Putative ManT?	L42540	?	*Saccharomyces cerevisiae* [336]
Pmt1p (protein mannosyl transferase)	L19169	**HO**-Ser/Thr	*Saccharomyces cerevisiae* [337]
Pmt2p	L05146	**HO**-Ser/Thr	*Saccharomyces cerevisiae* [338]
Pmt3p	X83797	**HO**-Ser/Thr	*Saccharomyces cerevisiae* [73]
Pmt4p	X83798	**HO**-Ser/Thr	*Saccharomyces cerevisiae* [73]
Pmt5p	X92759	**HO**-Ser/Thr	*Saccharomyces cerevisiae* [Dommaschk, U.; unpublished]
Pmt6p	Z49133	**HO**-Ser/Thr	*Saccharomyces cerevisiae* [339]
Glucosyltransferases			
Glycoprotein glucosyltransferase (Gpt1p)	1. U20554 2. U38417	$Man9GlcNAc_2$-Asn of unfolded protein	1. *Drosophila* [340] 2. *Schizosaccharomyces pombe* [341]
Glucuronic acid transferase			
HNK-1 antigen β1,3-GlcA transferase	D88035	**Gal**β1,4GlcNAc-R	Human [342]

Dolichol-dependent glycosyltransferases are used minimally in higher eukaryotes, and are responsible primarily for adding sugars to other lipids such as the dolichol block for N-linked glycan synthesis and GPI. Many of the O-linked chains in yeast and other fungi are initiated directly via dolichyl phosphomannose [71], although elongation of the chain then proceeds through guanosine diphosphate (GDP)-mannose. Dolichyl phosphomannose is synthesized on the cytoplasmic face of the ER from GDP-mannose and dolichyl phosphate. The dolichol-linked sugar is then transposed to the lumenal face, where glycosyltransferases move the sugar to a protein acceptor. Many of the enzymes catalysing these reactions have been cloned, including the dolichyl phosphomannose synthase (DPM1) in yeast and other organisms (see table 3) and a host of dolichyl-phosphomannose:protein mannosyltransferases (PMT1–6), shown in table 3 and reviewed by Gentzsch and Tanner [72]. These mannosyltransferases are somewhat homologous and have individual peptide substrate specificities [73]. Dolichol-dependent enzymes have complex topologies, typically spanning the membrane multiple times.

The glycosyltransferases which utilize nucleotide sugars are typically type II membrane proteins, with a short N-terminal cytoplasmic domain that can vary in length due to alternate transcription initiation sites. The purpose of such polymorphism is unknown, although both the site of transcriptional initiation and messenger RNA (mRNA) processing are regulated [74]. The cytoplasmic tail is followed by a single transmembrane segment, a 'stem' region which is susceptible to proteolytic cleavage (which commonly results in secretion of the solubilized enzyme), and finally, the large C-terminal catalytic domain. Although they have similar overall structures, they often show little relation to one another at the genetic or amino acid level. Within classes, there can be a high degree of homology. The α1,3-fucosyltransferases III, V and VI, for example, show 85–91% homology at the amino acid level [75], though α1,3-fucosyltransferase IV shows little homology to any of them. Glycosyltransferases are specific sometimes for the protein, as in the cases of polysialic acid synthase (ST8SiaII or IV) modification of the neural cell adhesion molecule (NCAM) [76], and GalNAc addition to pituitary hormones [77]. Many can in fact be inhibited by the deglycosylated forms of their normal protein substrates. *N*-acetylglucosamine-1-phosphotransferase, the enzyme that adds GlcNAc-1-phosphate to mannose on proteins destined for lysosomes, is inhibited by deglycosylated lysosomal enzymes [78]. The optimal pH values for the glycosyltransferases are similar, falling between 6 and 7. Most glycosyltransferases require Mn^{2+}, but many, such as human fucosyltransferase V [79], will also work with Mg^{2+}, Ca^{2+}, Co^{2+} or other divalent cations. As a rule, they also suffer from extreme product inhibition by the released nucleotides. For this reason, cells either destroy or sequester the products. The nucleotides may

be hydrolysed, and in fact yeast are dependent on the activity of a Golgi resident guanosine diphosphatase for glycosylation [80]. Likewise, acid phosphatase, a *trans*-Golgi resident enzyme, hydrolyses cytidine monophosphate (CMP), a product of sialyltransferase action, and uridine diphosphatase is colocalized with galactosyltransferase in the *trans* Golgi [81]. However, hydrolysis of the product is not always necessary. The sugar nucleotide substrates are generally made in the cytoplasm, and thus must be transported into the ER/Golgi lumen [82]. Many of the transporters that pass nucleotide sugars into the ER and Golgi apparatus are actually part of an antiporting system, which exchange sugar nucleotides for the released nucleotides [83]. In this manner, the level of free nucleotides is kept low. Several putative transporters have recently been cloned, based on their ability to restore sugar nucleotide transport to transport-defective mutants. These are the UDP-GlcNAc [84], GDP-Man [85], CMP-NeuAc [86] and UDP-Gal transporters [87, 88].

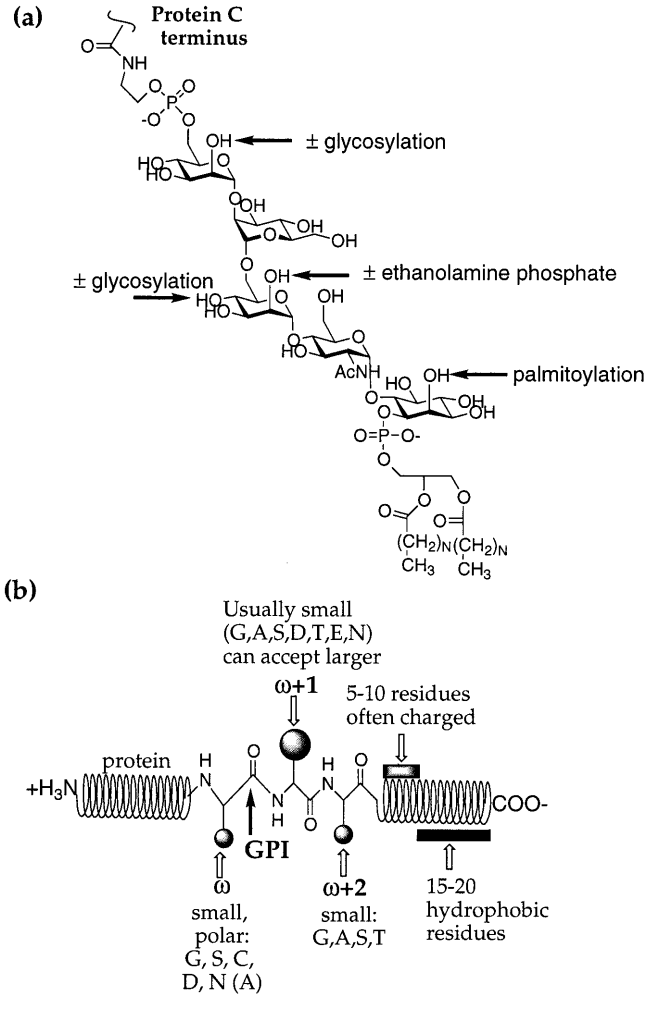

Figure 9. (*a*) Structure of the GPI anchor. (*b*) Consenus sequence for protein glypiation. The large arrow marks the amide attacked by the ethanolamine of GPI.

A large variety of glycosyltransferases have also been cloned, mostly as a result of expression-cloning methods and homology-based polymerase chain reaction (PCR) approaches. Table 3 lists those cloned to date, along with their preferred acceptors. Others have been isolated, and a review discussing the preparation of these was written by Sadler and coworkers [89].

Further processing of glycoproteins

Saccharides can be further embellished with a variety of groups. One of the most common modifications is sulfation. Sulfotransferases may sulfate the glycoprotein at a variety of positions, some of which are shown in figure 5. The protein may also be sulfated at mannose (6- and 4-) [90, 91] or at the chitobiose core [92], or on the terminal GalNAc of pituitary hormones [93]. Sulfation is not limited to sugars; tyrosine residues may also be sulfated. Both P- and L-selectins, molecules involved in leukocyte homing, bind to ligands that contain both sulfate groups and the sialyl Le^x structure [24]. The location of the sulfate, however, helps determine the specificity of the interaction (fig. 10). L-selectin binds to sulfated sialyl Le^x on GlyCAM (cell adhesion molecule, CAM) [94], while P-selectin binds to unsulfated sialyl Le^x. However, its glycoprotein ligand, PSGL-1, is modified by a number of sulfated tyrosine residues necessary for high-affinity P-selectin binding [95–97]. Although a number of the enzymes that sulfate glycosaminoglycans and hyaluronic acid have been cloned, none of those responsible for sulfating N-linked glycans or the mucin-type O-glycans have been cloned. Many have, however, been isolated and

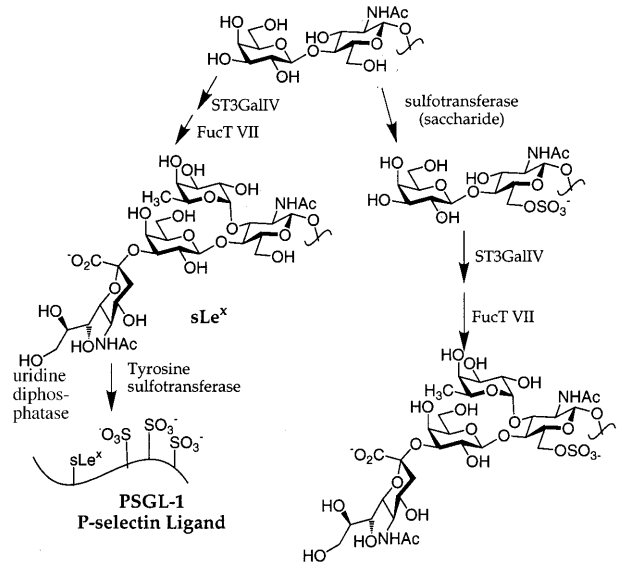

Figure 10. Sulfation of the L-selectin and P-selectin ligands.

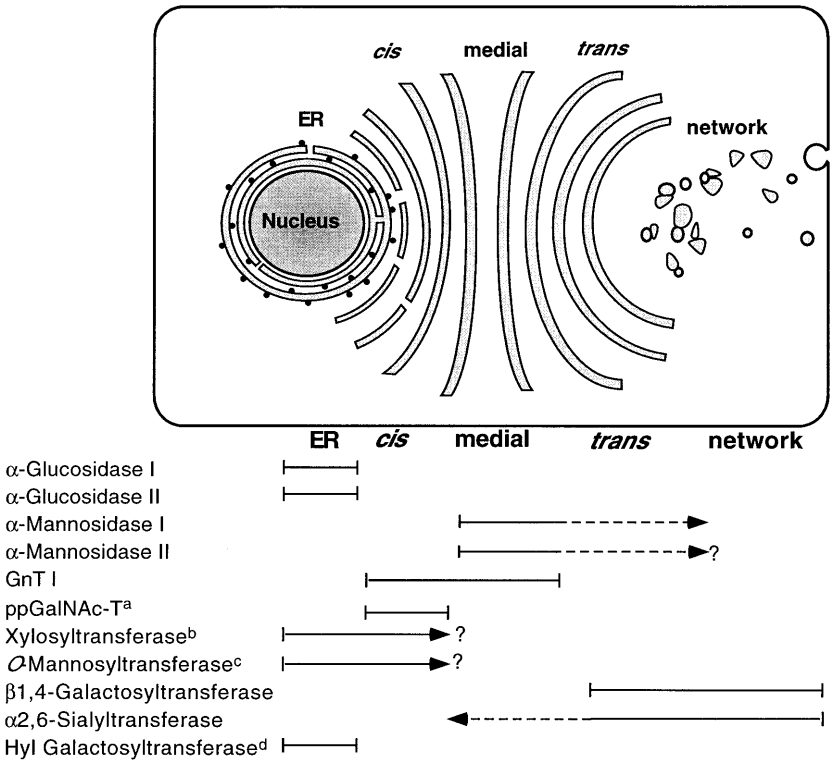

Figure 11. Localization of various glycosylation reactions. Data is taken mostly from Roth's recent review [101] or from primary publications where noted. a: Piller et al. [158]; b: Vertel et al. [159]; c: Haselbeck and Tanner [160]; d: Blumenkrantz et al. [161]. In cases where the exact boundaries are unknown, the limits are left as question marks. In cases where the limits are known to vary with cell type, the location found most often is shown in solid lines, while the locations occasionally observed are shown in dotted lines.

characterized, such as the enzyme that sulfates pituitary glycoprotein hormones [93]. All use 3′-phosphoadenosine 5′-phosphosulfate (PAPS) as a sulfate donor, and are found in the Golgi apparatus.

When the glycoprotein eventually leaves the Golgi and exits the cell, it may still undergo further enzymatic modification. Many glycosidases are found in the tissues and serum, including α- and β-galactosidases, α and β-glucosidases, α-fucosidase, α-mannosidase, β-glucuronidase, *N*-acetylglucosaminidase and *N*-acetylgalactosaminidase [98]. In fact, it was the observed reuptake of lysosomal glycosidases by fibroblasts that stimulated the search for a lysosomal protein (Man-6-phosphate) receptor. The concentration of glycosidases in the plasma varies greatly with the state of health of the individual [99].

Enzyme localization. It was noted many years ago that glycosyltransferases showed not only distinct tissue distributions but were also found in specific locations within the cell, based on immunolocalization of the enzymes and their products and subcellular fractionation studies (reviewed by Roth [100, 101] and shown schematically in fig. 11). Curiously, the subcellular distribution of at least some of these enzymes is also cell-type dependent. This was shown rather well by Roth et al. [102], who immunolocalized sialyltransferase in slices of intestinal tissue. They showed that, within the same slice, an absorptive cell had sialyltransferase and sialic acid distributed throughout the Golgi (excepting the first cisterna), while an adjacent goblet cell had both enzyme and sialic acid localized in just the *trans* Golgi. After the discoveries of simple targeting signals such as K(H)DEL for ER localization and mannose-6-phosphate for lysosomal targeting, it was assumed that something similar – simple and obvious – would be found that targeted the glycosyltransferases to one location or another. It quickly became clear that this was not the case. The often rapid secretion of glycosyltransferases that have been proteolytically cleaved from the membrane indicates that the signal for targeting lies somewhere in the cytoplasmic, transmembrane or stem regions [103]. Various groups have performed deletion studies with glycosyltransferases, and found that the region(s) important for trafficking differ from enzyme to enzyme and even from cell line to cell line. This subject is reviewed extensively by Colley [104]. Two

major hypotheses have been formed regarding the mechanism of Golgi retention. The first is the 'bilayer thickness' model, proposed by Bretscher and Munro [105] and Masibay et al. [106] in 1993. The idea is that the membranes get thicker from the ER to the plasma membrane due to the presence of higher concentrations of cholesterol. This has been supported by the work of Orci et al., who showed via electron microscopy that filipin, which binds to cholesterol, forms a gradient from the ER to the Golgi [107], and by Nezil and Bloom, who showed that membranes increase in width upon addition of cholesterol [108]. Enzymes with longer transmembrane segments should therefore localize in regions with thicker membranes. Studies have shown that increasing the thickness of the transmembrane domain indeed causes migration of the enzyme to the cell surface [106], and hydrophobicity plots of the transmembrane regions of plasma membrane and Golgi proteins have shown that Golgi proteins generally have shorter transmembrane domains [105]. An alternative hypothesis was presented by Nilsson et al. [109], called 'kin recognition'. This model suggests that it is protein–protein interactions that keep transferases out of transport vesicles and in the compartments where they belong. Strongest support for this model comes from a study by the same group, in which the cytoplasmic tail of the normally medial Golgi resident GnT I was replaced by the that of the ER resident invariant chain Ii33p. The GnT I was localized to the ER, since the ER localization signal of Ii33p is in the cytoplasmic domain. Interestingly, the *medial* Golgi enzyme mannosidase II colocalized with it. The reciprocal experiment, attaching the cytoplasmic tail of Ii33p to Golgi mannosidase II, had a similar effect on GnT I. Placing the cytoplasmic tail on the normally *trans*-Golgi resident β1,4-galactosyltransferase gene, however, caused no localization of either of the *medial* Golgi enzymes to the ER [110].

It has been known for many years that there is not only a gradient of cholesterol in the Golgi, but also a gradient of pH [111], with the *trans* Golgi reaching a pH of about 6 to 6.5 [112]. Some results suggest that in cystic fibrosis patients, the pH gradient is compromised, leading to an increase in sulfation of respiratory mucins and a decrease in mucin sialylation [113], although this is not incontrovertible. Other studies have suggested that the chloride channel that is defective in cystic fibrosis patients has little to do with the acidification of vesicles [112]. The differences may be due to differences between the cell lines and protein probes used in the various studies. Barasch et al. note that drop in mucin sialylation may be due, in part, to a reduction in the activity of sialyltransferases at higher pH, but also seems to be due to improper targeting of the sialyltransferase [113]. It is quite possible, therefore, that the pH gradient itself contributes to proper localization of glycosyltransferases, perhaps by causing aggregation of the integral membrane proteins.

Who gets what: regulation of glycosylation. The saccharide added to a protein depends on a variety of factors. Glycosylation is dependent on the species, cell type, developmental stage and site within the protein itself. For example, γ-glutaminyltranspeptidase from mouse kidney bears mainly biantennary saccharides, while that from human kidney bears mainly triantennary sugars [114]. The same protein produced in human liver is heavily sialylated and has no bisecting GlcNAc (β1,4) at the innermost mannose, while the kidney version is fucosylated and bisected.

The glycoforms found at a given site on a given protein depend on a myriad of factors, such as the concentration of glycosyltransferases available and their substrate specificities. Glycosyltransferases may compete for a substrate, and the action of one may preclude the other. For example, Sharma et al. recently made transgenic mice and pigs that overexpressed α1,2-fucosyltransferase and displayed decreased levels of the immunogenic linear B antigen (Galα1,3Galβ) [115]. The concentration of glycosyltransferases appears to be regulated at the transcriptional level: in general, the levels of mRNA correlate with transferase activity. There are exceptions; Perng et al. found that GnT V mRNA levels were very high in the brain, but enzyme activity was very low [116], and Brockhausen et al. found that although core 2 *N*-acetylglucosaminyltransferase (C2GnT) mRNA levels were increased far above normal in the breast cancer cell line MCF-7, the enzyme activity was somewhat less than normal [117]. However, the glycosyltransferase that wins may not always be the one that is present at the highest concentration. It might simply have more available substrate, indicating control at the level of the sugar nucleotide transporter, or perhaps the sugar nucleotide synthase; or

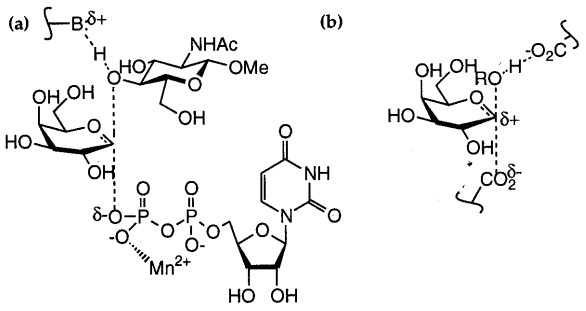

Figure 12. Putative transition state structures of (*a*) β1,4-galactosyltransferase; (*b*) β-galactosidase.

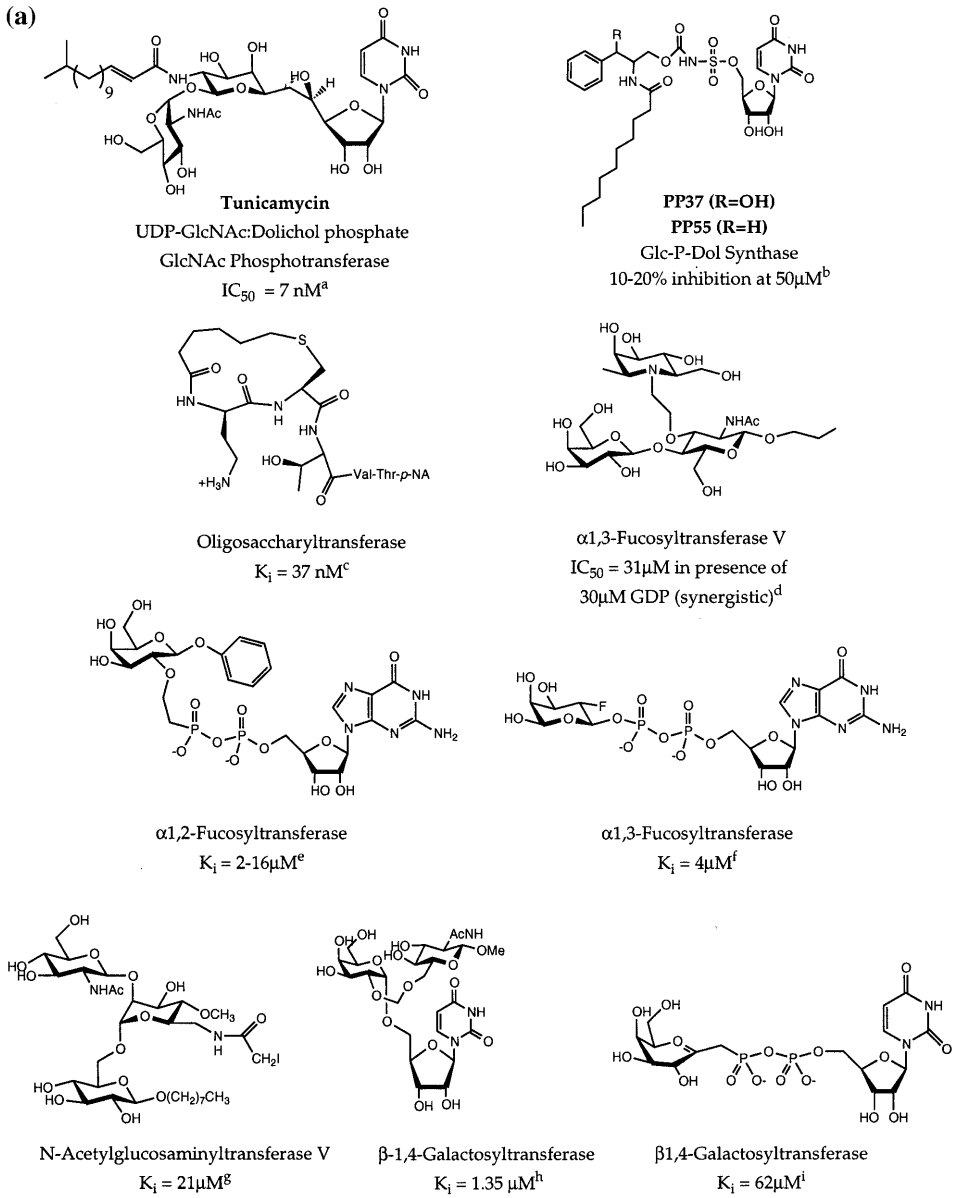

Figure 13. Glycosyltransferase (*a*) and glycosidase (*b*) inhibitors (the enzymes inhibited and the respective K_i or IC_{50} values are given). a: Heifetz [162]; b: Paul et al. [163]; c: Hendrickson et al. [164]; d: Qiao et al. [165]; e: Palcic et al. [166]; f: Murray et al. [143]; g: Lu et al. [167]; h: Hashimoto et al. [168]; i: Schmidt and Frische [169]; j: Tropea et al. [170]; k: Wong et al. [171]; l: Elbein et al. [172]; m: Pan et al. [173]; n: Dorling et al. [174]; o: Cottaz et al. [175]; p: Tsuji et al. [176]; q: Asano, et al. [177]; r: Dong et al. [178]; s: Bernotas et al. [179]; t: Wong et al. [151]; u: Asano et al. [180]; v: Moris-Varas, et al. [181]; w: Jeong, et al.[182]; x: Ichikawa and Igarashi [183]; y: Schedler et al. [184]; z: Takayama et al. [185]; aa: Knapp, et al. [186]; bb: McCarter et al.[176]; cc: Sollis et al. [187]; dd: von Itzstein et al. [148]; ee: Kim et al. [188].

alternatively the glycosyltransferase might be localized earlier in the Golgi apparatus and thus have a temporal advantage. The residence time of a protein in a given compartment will also affect the saccharides it ends up with. Glycosyltransferases show a certain degree of protein specificity. In some cases, the glycosyltransferases recognize specific peptide motifs: the pituitary hormone GalNAc transferase recognizes Pro-Leu-Arg, for example [118], and the transferase that adds fucose to serine or threonine recognizes the motif Cys-Xaa-Xaa-Gly-Gly-Ser/Thr-Cys [119]. In many cases, though, the glycosyltransferases do not appear to recognize a specific motif, but are simply encouraged or discouraged from adding sugars to a certain site due to factors such as steric hindrance. The processing of high-mannose-type oligosaccharides to form complex or hybrid structures is strongly affected by steric hindrance from the folded protein. This was clearly illustrated in a study by Hsieh et al. [120], in which the E1 and E2 glycoproteins of the Sindbis virus were produced in a line of Chinese hamster ovary (CHO) cells unable to make complex N-glycans due to defective GnT I and therefore produce Endo

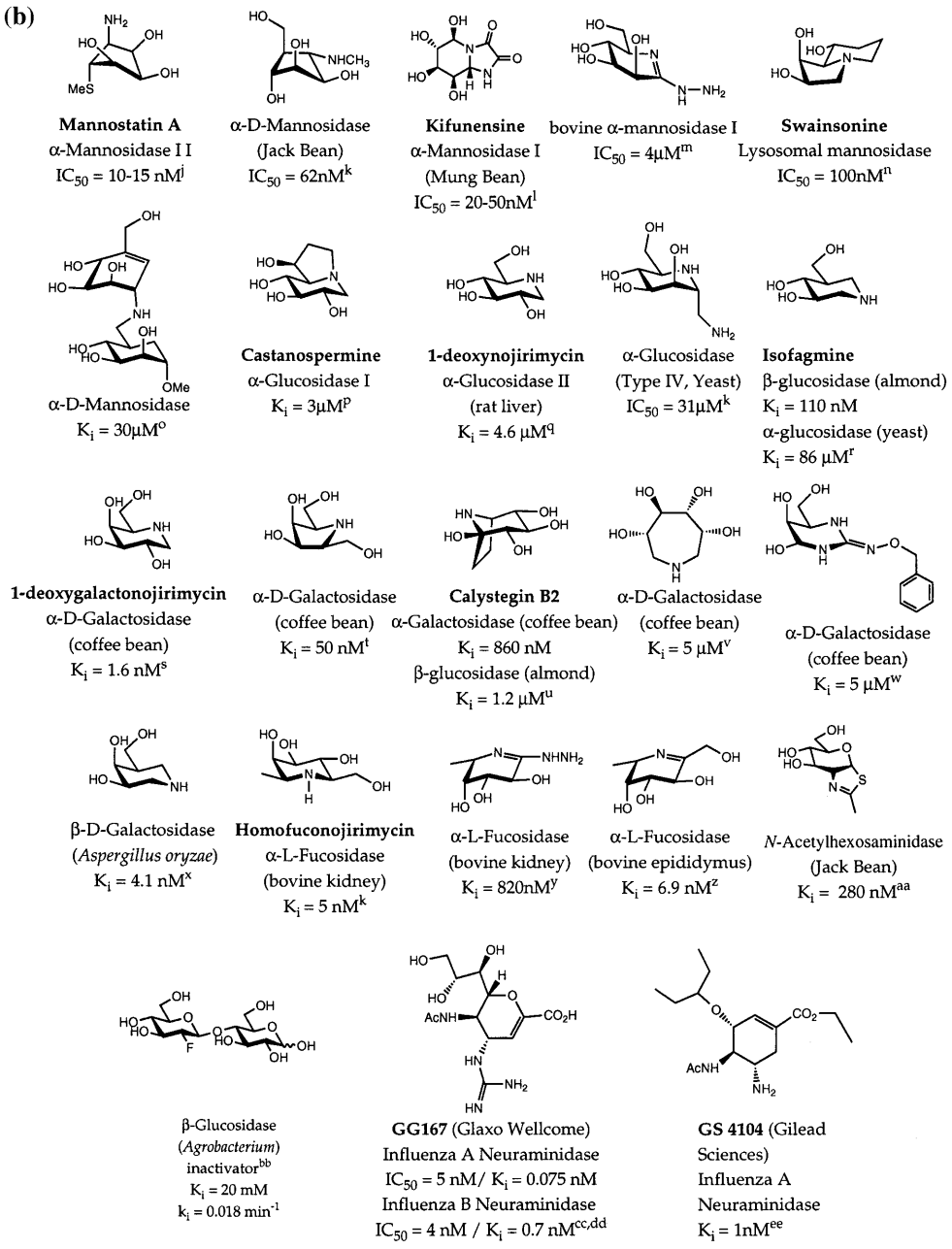

Figure 13b. (see legend, previous page).

H-sensitive glycans. (The endoglycosidase Endo H selectively cleaves the core chitobiose.) Endo H was able to cleave most of the glycans on these proteins which, when produced in wild-type CHO cells, are normally converted to complex-type glycans. The glycosylation sites which normally bear high-mannose-type glycans in wild-type CHO cells tended to be refractory to Endo H cleavage. The preference of Endo H for the complex sites was abolished in the presence of detergent or following pronase digestion of the glycoproteins. This indicates that the sites that usually contain high-mannose oligosaccharides are too hindered by the surrounding protein for Endo H to reach the chitobiose, and suggests that the processing enzymes are likewise inhibited.

Glycosylation and disease states

A number of diseases are associated with defects in glycoprotein processing or catabolism, such as I-cell disease (a defect in GlcNAc-1-phosphotransferase), paroxysmal nocturnal haemoglobinurea (a defect in GPI synthesis) and the carbohydrate-deficient glyco-

protein syndromes (CDGS). There are many other disease states that, while not shown to be directly associated with errors in glycoprotein synthesis, are correlated with unusual glycosylation patterns. Bizarre changes in glycosylation are often typical for cancer cells [121], from display of truncated mucin-type saccharides [122, 123], to increases in the degree of branching of N-linked chains [124, 125], to display of molecules not normally seen on that cell type, such as polysialic acid-decorated proteins [126]. Abnormally high expression of Lewis and sialylated Lewis blood group structures have also been found on various tumour cell lines; this expression is correlated with high metastatic potential [121, 127]. The changes vary with the cell line. For example, many T-cell leukaemias (both established cell lines and cells isolated from the blood of leukaemic individuals) show an increase in the display of core 2 structures [128], while some breast cancer lines show minimal core 2 antigen, have no C2GnT (the enzyme responsible for core 2 formation) activity and contain undetectable levels of C2GnT mRNA [117]. Instead, they express mRNA for a competing enzyme, ST3GalI, 2- to 3-fold higher than normal cells and have 8–10-fold higher enzymatic activity. This explains the prevalence of sialylated T-antigen structures in these cells. Changes in glycosylation have been associated with increases in levels of the cognate glycosyltransferases. The increase in concentration of sialylated T-antigen observed in certain colonic cancer cell lines [129] correlates with an increase in ST3GalI activity and mRNA transcription. Concentrations of GnT V have also been shown to be elevated in tumour cells, explaining the increase in multiantennary N-glycans [130]. In addition, the tissue and serum levels of glycosidases are also elevated in cancer, and this is thought to play a part in metastasis, both by degrading the extracellular matrix and allowing the tumour cells to migrate and by reducing cell–cell adhesions by deglycosylation of membrane glycoproteins [99].

A number of pathogens bind to sugars of the host cell and/or use sugars to mask themselves from the immune system. At least some strains of *H. pylori*, the causative agent of stomach ulcers, bind to the lining of the stomach via the Lewis b antigen on the epithelial surface [131]. In addition, *H. pylori* displays the Le^x antigen itself [132], as do schistosomes, which appear to use it for modulating the host's immune response [7]. Influenza viruses A and B bind to NeuAc on the surface of cells, while the influenza C virus binds to 9-*O*-acetyl-NeuAc. Some strains of *E. coli* and *Neisseria* (*meningitidis* and *gonorrhoeae*) display polysialic acid on their surfaces, which presumably masks them from the immune system.

Some pathogens are also able to modify the glycosylation of their host cells. The influenza virus, for example, not only binds to sialic acid on the cell surface but can also cleave it off with neuraminidase. This is probably necessary for the release of young virions from the cell, prevention of viral aggregation, and viral migration [133].

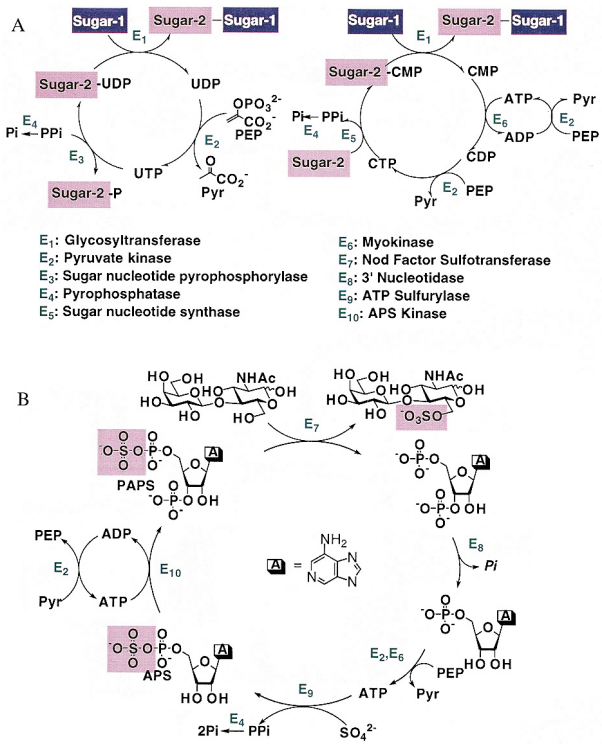

Figure 14. (*A*) Enzymatic synthesis of saccharides using sugar-nucleotide recycling. (*B*) Enzymatic synthesis of sulfated saccharides with regeneration of PAPS.

Inhibition of glycoprotein processing

In many of these disease states, specific inhibition of key glycosyltransferases or glycosidases (or other saccharide-processing enzymes such as the sulfotransferases, acetylases and so forth) within the pathogen or its target may be of therapeutic interest. For example, since the increased branching in N-glycans of tumour cells correlates with metastatic potential, selective inhibition of GnT V might help prevent metastasis. Similarly, inhibition of α1,3-fucosyltransferases might be of great help in the treatment of cancers by inhibiting the high levels of production of Lewis and sialylated Lewis blood group structures observed in tumours. The potential of this approach has been demonstrated by studies in which tumour metastasis has been reduced by treatment with inhibitors of glycosylation: tunicamycin, a UDP-GlcNAc:dolichyl phosphate GlcNAc-1-phosphotransferase inhibitor, reduced melanoma cell binding to ep-

ithelial cells in culture and in vivo [134]. Since complex saccharides cannot be formed without the prior action of the Golgi α-mannosidase II, and since elevated tissue and serum levels of glycosidases are associated with cancer and correlate with increased metastatic potential, there have been efforts to find glycosidase inhibitors for chemotherapeutic agents as well. This approach has been shown to be somewhat effective in studies of swainsonine (α-mannosidase II inhibitor) inhibition of tumour growth in vivo [135]. In this study, swainsonine was effective at inhibiting tumour growth, but only when partnered with the interferon-inducing reagent polyinosinic:polycytidylic acid. Similarly, castanospermine, an α-glucosidase I inhibitor, also prevented metastasis in vivo [136]. α-Glucosidase inhibitors such as castanospermine and deoxynojirimycin are also effective at inhibiting the proliferation of the human immunodeficiency virus (HIV) in vitro, though they show much less efficacy in vivo (reviewed by Jacob [137]). Although originally thought to inhibit viral coat protein gp120 processing, recent evidence indicates that they may inhibit viral entry into the cell after CD4 binding [138]. The biological functions of glycosidases and the use of glycosidase inhibitors as antiviral and anticancer agents have been reviewed by Winchester and Fleet [139]. Unfortunately, nonspecific inhibition of glycoprotein processing can lead to high toxicity. Tunicamycin, for example, shows potent neurological toxicity and can cause apoptosis in cultured sympathetic neurons [140], and swainsonine also causes neurological malfunction ('locoism') [141]. It is necessary, therefore, to design inhibitors that can specifically target enzymes later in the processing pathway.

Inhibition of bacterial glycosyltransferases and glycosidases could also be of potentially great value, particularly since the mechanisms of glycosylation are quite different in eukaryotes and prokaryotes, and there is thus a good chance that the prokaryotic glycosyltransferases could be inhibited selectively.

Design and synthesis of selective inhibitors to target specific enzymes requires, at the very least, some knowledge of the catalytic mechanism of the enzyme to be inhibited. Glycosyltransferases collect sugars activated with good leaving groups at their anomeric positions, and catalyse the replacement of the leaving group with the nucleophilic group of the acceptor. They may be either retaining (i.e. accept nucleotide sugars in one anomeric configuration and release saccharides of the same configuration) or inverting. In the cases where the reaction has been studied (β1,4-galactosyltransferase [142], for example, and human fucosyltransferase V [79, 143]), glycosylation appears to proceed through a transition state with substantial sp^2 character, based on secondary deuterium isotope effects and on evaluation of the structures that inhibit them. It has been proposed that the transition states of the enzymatic reactions proceed through a half-chair (or twist boat) conformation, shown for the β1,4-galactosyltransferase in figure 12a [144]. Glycosidases may also be retaining or inverting, and several studies have suggested that the reactions catalysed by these enzymes, too, have transition states with substantial sp^2 character [144, 145]. Recently, for example, Tanaka et al. studied hydrolysis of glucosyl fluorides by several inverting and retaining glucosidases, and secondary isotope effects suggest that the reaction proceeds, in all cases, via an S_N1-like mechanism [146]. Thus, the transition states for glycosi-

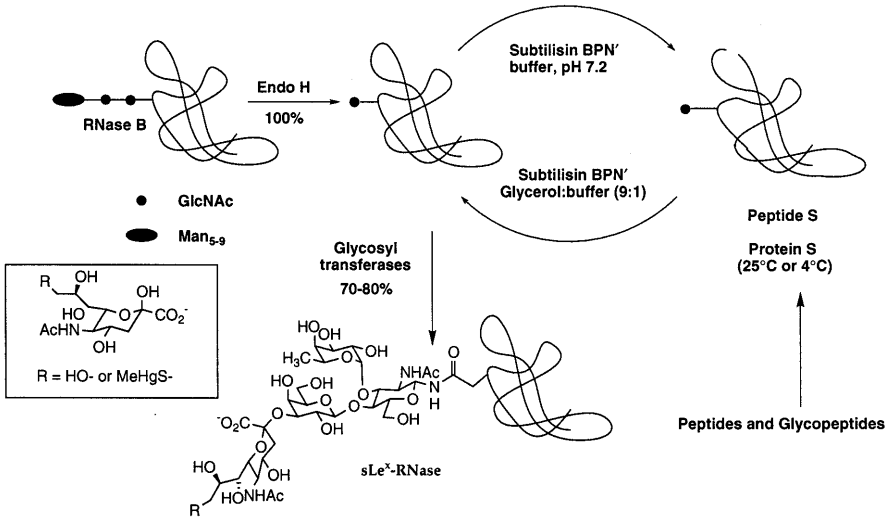

Figure 15. Enzymatic approaches to the synthesis of homogeneous glycoproteins.

dase reactions, like glycosyltransferase reactions, are expected to have a half-chair conformation (fig. 12b), and many of the same types of compounds will inhibit both classes of enzyme. A variety of scaffolds have been used to simulate the proposed transition states of glycosidases and glycosyltransferases [147], including 5-, 6- and 7-membered ring azasugars, which (when protonated) mimic the positive charge of the putative oxocarbenium; guanidinosugars, which should mimic both the sp^2 character and the positive charge of the transition state; and unsaturated carbocycles, which mimic the shape but not the charge. For glycosyltransferase inhibitors, these are typically bound to a nucleoside through pyrophosphate or a pyrophosphate mimic such as malonate, tartrate, a monosaccharide or a phosphonate. A number of glycosidase and glycosyltransferase in-hibitors, both natural and synthetic, are shown in figure 13. Many of these, while effectively inhibiting enzymes in vitro, are not expected to inhibit the enzymes in vivo, due to poor stability and bioavailability: the pyrophosphate-containing glycosyltransferase inhibitors are particularly problematic in this regard. Of particular note are the influenza sialidase inhibitors. The best of these, GG167, was designed rationally, based on the crystal structure of neuraminidase [148]. It has been tested in vivo, in both animal models and human clinical trials [133]. The compound works very well prophylactically, and reduces fever (though not viral titres) in patients treated within a day of viral inoculation. GG167 administered 2 days after inoculation had little effect, though, perhaps due to the overwhelming viral load already present. GG167 must be delivered nasally, but a similar compound developed by Gilead Sciences (GS4104) can be delivered orally and is currently in clinical trials.

Enzymatic synthesis of oligosaccharides, glycopeptides and glycoproteins

The enzymes involved in the processing and synthesis of carbohydrates in vivo have also been used in the copeptides and glycoproteins [149–152]. The use of sugar nucleotide-dependent glycosyltransferases coupled with regeneration of sugar nucleotides has been shown to be a general method for the synthesis of oligosaccharides on large scale (fig. 14a). The regioselective sulfation of sugars using PAPS coupled with a regeneration system has also been developed for the synthesis of oligosaccharide sulfates [153] (fig. 14b). The use of glycosidases alone or coupled with glycosyltransferases provides an alternate route to oligosaccharides. Glycosyltransferases have also been used in the solid-phase synthesis of glycopeptides and oligosaccharides and, combined with the use of engineered subtilisin for peptide bond formation, in the synthesis of glycoproteins (fig. 15) [150, 154]. It appears that enzymatic synthesis of oligosaccharides, glycopeptides and glycoproteins will become an important approach to prepare glycoconjugates for functional studies and for therapeutic evaluation.

Conclusions

Much has been learned about glycoprotein synthesis and processing. The complexity of the enzyme network required is perhaps a bit surprising. Although this makes delineation of the biochemical pathways difficult, one should find the complexity encouraging, for a couple of reasons. First, the complexity of the system allows cells to mark themselves specifically. In disease states such as cancer, these markers give us the potential to target certain cells specifically via immunotherapy and related techniques. In addition, although the number of enzymes required to create all of the linkages may seem daunting, it is also heartening: if specific inhibitors can be designed, it may become possible to inhibit the production of a limited set of structures without having the severe toxicity effects seen with broad glycosylation inhibitors such as tunicamycin and swainsonine. The cloning of many of these enzymes has also brought the chemoenzymatic synthesis of saccharides, saccharide analogues and glycoproteins within reach. These techniques are rapidly becoming viable alternatives to the chemical preparation of these synthetically difficult compounds.

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