N-glycan Biosynthesis:
Basic Principles and Factors Affecting Its Outcome

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1. Abstract

Carbohydrate chains are the most abundant and diverse of nature's biopolymers and represent one of the four fundamental macromolecular building blocks of life together with proteins, nucleic acids, and lipids. Indicative of their essential roles in cells and in multicellular organisms, genes encoding proteins associated with glycosylation account for approximately 2% of the human genome (1). It has been estimated that 50%-80% of all human proteins carry carbohydrate chains -glycans - as part of their structure. Despite cells utilize only 9 different monosaccharides for making their glycans, their order and conformational variation in glycan chains together with chain branching differences and frequent post-synthetic modifications can give rise to an enormous repertoire of different glycan structures of which few thousand is estimated to carry important structural or functional information for a cell (2). Thus, glycans are immensely versatile encoders of multicellular life. Yet, glycans do not represent a random collection of unpredictable structures but rather, a collection of predetermined but still dynamic entities that are present at defined quantities in each glycosylation site of a given protein in a cell, tissue or organism.

In this chapter, we will give an overview what is currently known about N-glycan synthesis in higher eukaryotes, focusing not only on the processes themselves but also on factors that will affect or can affect the final outcome - the dynamicity and heterogeneity of the N-glycome. We hope that this review will help understand the molecular details underneath this diversity, and in addition, be helpful for those who plan to produce optimally glycosylated antibody-based therapeutics.

Key words: Endoplasmic reticulum, Golgi apparatus, N-glycosylation, organelle homeostasis

2. Introduction

Asparagine-linked (N-linked) glycosylation is an essential protein modification, affecting a number of basic cellular processes such as protein folding, its half-life, trafficking and

immunogenicity as well as its interactions between cells, cells and extracellular matrix components or pathogens (3). In all eukaryotic cells, N-glycans are synthesized in two specialized organelles, the endoplasmic reticulum (ER) and the Golgi apparatus. Together, these organelles harbor dozens of functionally distinct glycosyltransferases and glycosidases that sequentially modify the growing oligosaccharide chain (4-7). Yet, it is much less clear how this sequence of enzymatic reactions is orchestrated to guarantee faithful synthesis of N-glycans, considering that enzymes do not use any template, can compete with each other for the same substrate and/or the acceptor, and even localize in the same Golgi compartment. Another puzzling issue is an intrinsic micro-heterogeneity of glycans made by the cell. For example, an N-glycan attached to a specific asparagine of a given protein can be different from an N-glycan attached to the same site in another protein molecule. Distinguishing this "background noise" from dynamic changes that are functionally important e.g. during embryonic development, cell differentiation and aging can sometimes be problematic. Nevertheless, unlike other polymerization events in the cell, glycosylation apparently need not be a high-fidelity system, since cells normally tolerate such micro-heterogeneity without facing problems in cell survival or proliferation.

The other side of the coin is that this variation can sometimes lead to a devastating disease. Congenital disorders of glycosylation (CDGs) are a rare, yet diverse group of serious, often multiorgan diseases characterized by defects in glycosylation (8,9). More than 140 different CDG syndromes are known as of today, the severity of which varies from prenatal death to survival into adulthood with relatively normal life span. Disturbed N-glycosylation forms the largest group of the CDGs. It is divided into two groups (Type I and Type II) based primarily on the genetic defect and the site it is affecting. Type I CDGs are characterized by defects in the synthesis of N-glycans in the endoplasmic reticulum (ER), while the Type II CDGs have problems in their processing in the Golgi apparatus. In addition to CDGs, glycosylation changes play an important role in many other human diseases including autoimmune diseases, inflammation, tumorigenesis and its progression (10). Yet, the underlying mechanistic details that cause these changes are incompletely understood, as are also the reasons why some changes lead to disease and some not. Partly, this is due to the dynamic and variable nature of the glycan themselves, their cell- and tissue-specific expression (11) as well as the lack of tools that would allow glycan editing at will in a specified glycosylation site or protein itself.

3. Biosynthesis of N-glycans in the endoplasmic reticulum

Building blocks for N-glycan synthesis

The early steps in N-glycan biosynthesis in the endoplasmic reticulum (ER) are conserved in all three domains of life (12), whereas their processing and maturation differs markedly. All N-glycans share a common core structure (asn-GlcNAc2Man3-) which is further elongated in a species- and tissue-specific manner (11) by adding few other subterminal or terminal sugar residues to the core structure. Depending on the sugar residue and the linkage type used, these

additions can significantly influence the structure of the N-glycan (11). The main sugar residues utilized as building blocks are N-acetylglucosamine (GlcNAc), mannose (Man), galactose (Gal), fucose (Fuc) and sialic acid (N-acetylneuraminic acid (Neu5Ac) being the predominant form), of which the latter two act as chain-capping residues. In some instances, N-acetylgalactosamine (GalNAc) residues can be used to construct an N-glycan. Glucose (Glc) residues are also temporarily incorporated to the growing N-glycan during its synthesis in the ER, yet they are invariably removed as glucose residues have not been detected in a mature N-glycan isolated from cultured cells or tissues (13). Occasionally, mature N-glycans can also be modified by the addition of sulphate or phosphate, generating determinants that modulate cell adhesion or glycoprotein localization in the cells. Interestingly, despite the deletion of the CMAH gene (needed for N-glycolylneuraminic acid (Neu5Gc) synthesis) 3 million years ago, this neuraminic acid variant is still regularly detected in trace amounts in human glycans (14). This is due to dietary consumption of Neu5Gc-containing animal products (e.g., red meat and dairy products) and its incorporation into newly synthesized glycans (15). Perhaps unsurprisingly, the highest Neu5Gc levels are detected in epithelial and endothelial cells that line the intestine and blood (and lymph) vessels, respectively.

Precursor synthesis and its attachment to nascent polypeptide chains

The N-glycosylation of nascent polypeptides in the ER lumen relies on prior assembly of a lipid-linked oligosaccharide (LLO) precursor (Glc_3Man_9-$GlcNAc_2$) onto a membrane-embedded dolichyl phosphate (Dol-P) carrier (Fig. 1). This set of events is orchestrated by the Alg-family of ER-localized, membrane-associated glycosyltransferases (16). They stepwise assemble the LLO using nucleotide sugars (UDP-GlcNAc, GDP-Man, Dol-P-Man and Dol-P-Glc) as donor substrates. The LLO assembly begins on the cytoplasmic face of the ER membrane by the formation of a $GlcNAc_2$ -PP-Dol intermediate from GlcNAc-1-phosphate and GlcNAc. These additions are catalyzed by Dpagt1 (Alg7) and Alg13p/Alg14p UDP-GlcNAc-transferases, respectively. The three enzymes exist as hexamers with a stoichiometry of 2:2:2 (17). Alg14 appears to be the central unit, capable of recruiting other enzymes to the complex (18). Next, ER mannosyltransferases (Alg1, Alg2 and Alg11) that also form complexes with each other (19) add five mannose residues from GDP-Man donors to form a $Man_5GlcNAc_2$-PP-Dol intermediate. Thus, LLO precursor synthesis on the cytoplasmic face of the ER membrane involves three main enzyme complexes, one formed by Dpagt1/Alg13/Alg14 and the other two either by Alg1/Alg2 and Alg1/Alg11. This arrangement likely ensures that each mannose residue will be linked correctly to the precursor despite the co-existence of several competing enzymes on the same membrane.

The next step involves translocation of the $Man_5GlcNAc_2$-PP-Dol intermediate into the ER lumen, a process that is thought to mediated by a protein termed as the Rft1, but it is still uncertain whether it acts as a bona-fide flippase protein (20). In the ER lumen, mannosyltransferases (Alg3/Alg9/Alg12) and glucosyltransferases (Alg6/Alg8/Alg10) further elongate the LLO precursor by attaching four additional mannose residues and three glucose residues, respectively. This completes the precursor synthesis and yields the

$Glc_3Man_9GlcNAc_2$-PP-Dol structure, which will be used later as the donor substrate for *en bloc* transfer of an N-glycan to a suitable polypeptide chain. It is noteworthy that unlike the initial catalytic steps on the cytosolic face of ER, the completion of the LLO precursor synthesis in the ER lumen does not use nucleotide sugars as donors. Rather, membrane-embedded Dol-P-Man and Dol-P-Glc are used as sugar donors in this case. Their synthesis takes place also on the cytoplasmic side of the ER membrane (from GDP-Man and UDP-Glc, respectively) before they are translocated (flipped) to the luminal side (20).

The most preferred acceptor asparagine residues for N-glycosylation are the ones within the Asn-X-Ser/Thr motif (where X ≠ proline) (21). Of these two, the Asn-X-Thr sequon is preferred over Asn-X-Ser, mainly because the interaction between the side chain methyl group of threonine and the asparagine-lysine (NK) motif in the binding pocket of the oligosaccharyltransferase (OST) increases the stability of the complex (22-24). The identity of the amino acid X and flanking amino acids also contribute to the glycosylation of a given sequon. In addition, the position of the sequon within the polypeptide, the secondary and tertiary structure of the protein, and its final destination in a cell can impair or enhance the likelihood of whether that site becomes glycosylated or not (25). Thus, the presence of sequons alone cannot be used as an adequate predictor of N-glycosylation. Indeed, roughly one third of the identified sequons in secreted glycoproteins remain non-glycosylated (26).

The transfer of the completed precursor oligosaccharide is catalyzed by ER membrane-localized OST complex. It is an octamer consisting of a single catalytic subunit and seven accessory subunits, each important for optimal glycosylation efficiency. Most multicellular animals (sponges are an exception) possess two such complexes due to an ancient duplication of the gene encoding the catalytic subunit. The STT3A and STT3B (for OST-A and OST-B complexes, respectively) have different kinetic properties, acceptor substrate preferences and partially nonoverlapping roles in glycosylation (27). The accessory subunit compositions between the two complexes also differ. OST-A complex associates with Sec61 core components of the ER translocon complex and co-translationally glycosylates the nascent polypeptide in accessible sequons during polypeptide chain translocation into the ER lumen. Sequons within the last ~ 50-55 residues of the C-terminus are, however, inside the translocon and hence inaccessible for STT3A. Instead, STT3B transferase in OST-B complexes can post-translationally add N-glycans to such sequons. It also can use internal sequons that are skipped by the STT3A as acceptors (28,29). Often, these include closely spaced sequons adjacent to signal cleavage site or sequons with cysteine residues nearby or inside the motif (i.e. the N-C-T/S motif (27).

N-glycan processing in the ER and quality control

The newly attached $Glc_3Man_9GlcNAc_2$ N-glycan structure is further modified once the polypeptide is translocated to the ER lumen and begins to fold. The first step involves removal of the terminal glucose residue by a transmembrane enzyme α-glucosidase I. The second glucose is then rapidly removed by the soluble α-glucosidase II (30,31) The resulting mono-

glucosylated glycan is a preferred ligand for the carbohydrate-recognizing molecular chaperones calnexin and calreticulin. These chaperones readily associate also with the protein ERp57 (32), a disulfide isomerase that catalyzes the formation of inter- and intramolecular disulphide bonds, thereby helping proper folding of the nascent glycoprotein. Calnexin binding appears to happen irrespective of the folding state of the glycoprotein (33), suggesting that it most likely interacts with the nascent polypeptide as soon as it arrives in the ER lumen. During the folding process (Fig. 2), the last glucose residue is removed by the α-glucosidase II. Unfolded or misfolded proteins display exposed hydrophobic patches that are recognized by UDP-glucose-glycoprotein glucosyl-transferase (UGGT) (34), an enzyme that can glucosylate the same mannose residue again in that N-glycan. By doing so, it re-creates the $Glc_1Man_9GlcNAc_2$ structure that is again acted upon by the chaperone-disulfide isomerase complex. This removal and re-addition of glucose residues can continue for several cycles until the protein is properly folded. Once this is achieved, the glycan is finally trimmed by ER mannosidase I (ERMan1) that removes the terminal mannose residue from the middle branch of the N-linked oligosaccharide. The resulting $Man_8GlcNAc_2$ structure then can be recognized by the ERGIC-53 (LMAN1), a mannose-specific lectin of the LMAN1/MCFD2 cargo receptor complex (35), thereby facilitating packaging and transport of the native N-glycosylated glycoprotein into COPII (Coat Protein complex II)-coated vesicular carriers that ferry cargo from the ER to the Golgi via the ER-Golgi intermediate compartment (ERGIC) (36,37).

An ER stress caused by various factors (e.g. altered calcium homeostasis, redox state and glucose deprivation or mutations) is characterized by accumulation of misfolded or unassembled proteins in the ER and can be detrimental to cell viability. Metazoan cells can, however, cope with this stress by launching an ER stress response that suppresses the rate of translation and increases expression of molecular chaperones to ease protein folding in the ER. In a case these maneuvers still fail, terminally misfolded glycoproteins will be directed to degradation via an ER-associated degradation (ERAD) pathway (38). It starts when ERMan1 mannosidase and EDEM (ER degradation enhancing mannosidase-like) proteins (EDEM1/2/3 in mammals) are recruited to cleave off two mannose residues (instead of one) from an N-glycan. As a result, the glycan becomes unrecognizable by ERGIC-53, thereby preventing glycoprotein transport to later secretory compartments. The exposed α(1,6)-linked mannose in the $Man_7GlcNAc_2$ structure is now recognized by the OS-9/XTP3-B lectin complex that directs the bound glycoprotein to the transient ERAD (ER-associated degradation) protein complex at the ER membrane with ubiquitin ligase activity. ERAD complex then ensures that the glycoprotein is returned back to the cytoplasmic side of the ER membrane by tagging it for proteasomal degradation through ubiquitination (38).

The other route for degrading of misfolded glycoproteins relies on malectin, a membrane-associated, ER stress-induced lectin first identified in 2008 (39). It is highly conserved in metazoans (40) and it shows high specificity towards di-glucosylated N-glycans ($Glc_2Man_9GlcNAc_2$) (41,42). Malectin forms a stable complex with ribophorin I, a subunit of the OST complex with proposed chaperone activity based on its ability to recognize misfolded

protein backbones (43,44) This complex seems to act as an early intervention mechanism for detecting and capturing nascent non-native N-glycoproteins before it delivers them to proteasomal degradation if initial attempts to fold will fail (45). Whether this malectin-ribophorin I -mediated removal mechanism involves a unique retro-translocation machinery different from the ERAD machinery is currently unclear.

4. N-glycan processing in the Golgi apparatus

Correctly folded glycoproteins entering cis-Golgi compartment carry typically an N-glycan with 8 mannose residues left ($Man_8GlcNAc_2$). While some N-glycans may exit the Golgi without being modified, their proportion is normally low in humans (46). Partly, this is due to a presence of a quality control mechanism that is present in the Golgi. Golgi membranes harbor a mannose-binding lectin VIP36, that can recycle high mannose type N-glycans back to the ER (46). In support of this, VIP36 also interacts with the ER-localized BiP chaperone (47). By doing so, VIP36 can halt secretion of improperly glycosylated glycoproteins to post-Golgi compartments. Another mechanism to prevent high mannose type N-glycoproteins from passing through the Golgi takes over when a mono-glycosylated N-glycan ($Glc_1Man_9GlcNAc_2$) carrying glycoprotein arrives in the Golgi. The glycan part is cleaved internally by the Golgi endo-α-mannosidase between the two Man residues of the Glcα1-3Manα1-2Manα1-2 moiety, thereby yielding a $Man_8GlcNAc_2$ isomer that is different from that produced by ERMan1 in the ER (48). Interestingly, experimental evidence also suggests that the calreticulin-based glycoprotein quality control may be functional also in the Golgi compartment, as calreticulin was found to co-localize with endo-α-mannosidase in the ERGIC and cis/medial-Golgi compartment at least in cultured rat liver cells (49).

Normally, the vast majority of N-glycans are processed in the Golgi to complex and/or hybrid type N-glycans by a distinct set of glycosidases and N-acetylglucosaminyltransferases, also termed as MGAT1-5 (50, 51). The processing involves complex mutual interplay between the MGAT homomers and heteromers, mannosidase II (ManII) acting as a central hub (51). Thus, upon arriving in the Golgi, ER-derived MGAT homomers form heteromeric complexes not only with other MGATs but also with relevant UDP-N-acetylglucosamine transporters. Thereby, they organize into multi-enzyme/multi-transporter assemblies in the Golgi membranes. Their interplay likely involves either distinct or dynamic complexes (51) to facilitate efficient processing and branching of N-glycans in the cis- and medial-Golgi.

The processing begins in the cis-Golgi by the removal of three mannose residues to yield the $Man_5GlcNAc_2$ structure. Golgi mannosidases IA-C are responsible for the cleavages. Then, the first GlcNAc is added by MGAT1, using nucleotide-activated N-acetylglucosamine as a donor substrate. Once this GlcNAc is added, two additional mannose residues are removed by the Golgi α-mannosidase II. This creates a scaffold for MGAT2 to add a second GlcNAc to the exposed mannose residue, yielding a precursor for all complex-type N-glycans. MGAT4 and MGAT5 can then initiate the synthesis of third and fourth GlcNAc branches, respectively. Alternatively, MGAT3 can add a bisecting GlcNAc at the tri-mannosyl core structure (Fig. 3, middle). If this bisecting GlcNAc is added before MGAT4 and MGAT 5 have added theirs,

the synthesis of the third and fourth GlcNAc branches by MGAT4 and MGAT 5 is halted (52). Bisecting GlcNAc also cannot be further elongated with any other sugar residue. Its addition also significantly alters the conformation of an N-glycan and suppresses the addition of terminal sugar residues such as sialic acid and fucose. The human natural killer-1 epitope (HSO3-3GlcAβ1-3Galβ1-4GlcNAc), a sulfated trisaccharide structure that is extensively expressed in the nervous system, is another terminal epitope suppressed by the bisecting GlcNAc (53). Bisecting GlcNAc is also known to inhibit α-mannosidase II, suggesting that this addition may be one reason for the synthesis of hybrid type N-glycans. Yet, not all hybrid-type N-glycans contain a bisecting GlcNAc. It is therefore possible that rapid elongation of the first GlcNAc branch by galactose can also inhibit the necessary removal of two terminal mannoses by α-mannosidase II and thus, the build-up of the other GlcNAc branches.

Normally, the GlcNAc branches are further elongated by adding galactose, N-acetylglucosamine and sialic acid. Galactose is added to the GlcNAC nearly always with the β(1,4)-linkage. This structure, termed as N-acetyllactosamine (LacNAc), can be repeated several times in one branch, forming poly-LacNAc structures. Poly-LacNAc motifs in turn can act as substrates for making additional branches to the antennae. This is done by a set of special enzymes called GCNT2s A-C (54) by adding extra GlcNAc residues with the β(1,6)-linkage to internal galactose residues. These GlcNAc residues can also be subsequently elongated by β(1,4)-galactosyltransferases to form additional LacNAc structures. This kind of branched N-glycan is termed as an I-branched glycan. They are most frequently found in adult erythrocytes, mucosal epithelia, and cells of the eye and olfactory bulb (54). GalNAc residues are also occasionally found in N-glycans of mammals forming LacdiNAc (GalNAcβ(1,4)GlcNAc) type structures.

The antennae are often capped with a sialic acid by various sialyltransferases. This blocks further elongation of the branches except in the case of polysialylation. Polysialylated N-glycans are commonly detected in neural cell adhesion molecules (NCAMs) of the nervous system (55). Fucose is another residue that cannot be elongated further. It can be added by specific Golgi fucosyltransferases either to the asparagine-linked GlcNAc to produce the ‘core fucosylated’ N-glycan, or to GlcNAc residues of the antennae.

In specific cases, sugar residues of the antennae can undergo further modifications such as sulphation, phosphorylation and O-acetylation (56,57). For example, lysosomal acid hydrolases carry N-glycans with a phosphate that directs the enzymes to lysosomes. Lysosomal enzymes share common conformational lysine-containing motifs that are recognized by the cis-Golgi-localized GlcNAc-1-phosphotransferase enzyme. In the first catalytic step, GlcNAc-1-phosphotransferase transfers GlcNAc-1-P from UDP-GlcNAc to the C6 hydroxyl group of selected mannose residue present in the high mannose-type N-glycan (58,59). In the second step, N-acetylglucosamine-1-phosphodiester α-N-acetyl-glucosaminidase (NAGPA) cleaves the GlcNAc, leaving only the phosphate group linked to the mannose. Man-6-phosphate (M6P) tag is the ligand for transmembrane Man-6-P receptors (MPRs) residing in the trans-Golgi network (TGN). Once recognized by the MPR, the receptor

escorts the lysosomal hydrolase with its ligand to endosomes and eventually to lysosomes in clathrin-coated vesicles. In lysosomes, the enzyme is released at low pH and the receptor is recycled back to the trans-Golgi. Mutations that impair tagging of mannose with phosphate lead to lysosomal storage diseases, a group of over 70 rare diseases characterized by accumulation of macromolecules in lysosomes (60).

N-glycosylation of immunoglobulins

N-glycosylation is also an important modification of all immunoglobulin isotypes and contributes affecting their binding characteristics and effector functions. Although their synthesis might not be different in any way from other N-glycans, there are some special issues that are worth of discussing. N-glycans attached to immunoglobulin G (IgG) are best characterized owing to IgG abundance in the serum and successful production of many IgG-based therapeutic antibodies by the biopharma industry. IgG N-glycans are typically found in the Fc region but a minor proportion (15–25%) of serum IgG can contain N-glycans within their variable domains. These so-called "Fab glycans" differ from the Fc region N-glycans by having higher proportion of terminally galactosylated and sialylated N-glycans with a bisecting GlcNAc, while having a lower abundance of core-fucosylated N-glycans (61). Yet, it is not clear why the number of antennae in IgG N-glycans seems to be limited to only two antennae (or three if the bisecting GlcNAc is considered also as an own branch). One possibility that may explain this is that antibody producing plasma cells do not express the MGAT4 or MGAT5 enzymes needed for further branching. Another explanation could be that addition of bisecting GlcNAc (or some other regulatory system) will prevent further branching of IgG N-glycans. The existence of such a system would be logical, given that an increase in N-glycan "bulkiness" brought about by additional branching might interfere with the folding and pairing of the Fc regions in the ER, and thereby alter its conformation known to be important for its binding to Fc receptors and antibody effector functions. Similarly, it is unclear why the Fab N-glycans display higher proportion of more mature (more completely processed) N-glycans than those of Fc N-glycans. Whether this difference stems from better accessibility of the Fab glycans over Fc glycans, or something else such as increased extracellular glycosylation or decreased degradation of glycosidases, remains to be explored.

5. Golgi micro-environment is important for normal processing and maturation of N-glycans

Despite the rather homogenous nature of high mannose type N-glycans arriving in the Golgi, N-glycans leaving the Golgi are much less so. For example, a single glycoprotein can carry complex, hybrid and high-mannose N-glycans on the same polypeptide. Hybrid and complex type N-glycans can also display variable number of antennae in their structure that may, or may not, carry sialic acid and/or fucose. While we do not have a clear picture at the molecular level what determines the outcome in each case, this heterogeneity reflects both protein- and cell-specific processing of N-glycans brought about for example by epigenetic changes that

determine what enzymes are expressed by the cell. Other factors that also modulate N-glycan biosynthesis are discussed below.

Golgi pH homeostasis

The environmental cues outside or inside the cells can also contribute to N-glycan diversity. Unlike the ER, the other secretory pathway compartments, including the ER-Golgi intermediate compartment (ERGIC), the Golgi apparatus itself and secretory vesicles, have uniquely acidic lumens, their pH decreasing along the pathway towards the plasma membrane (62). This pH gradient is crucial for their efficient functioning in membrane trafficking, glycosylation, proteolysis, protein sorting, or cargo transport (63). Altered glycosylation due to abnormal Golgi pH is also responsible for several human disorders identified recently (64). Proper pH in the Golgi lumen appears to be important especially for the activity and assembly of the glycosyltransferase complexes in the Golgi. Previously, we have shown that the trans-Golgi β(1,4)GalT1 galactosyltransferase not only forms homomers in the ER, but also heteromers with either ST3Gal3 or ST6Gal1 sialyltransferases upon its arrival in the Golgi (65,66). Interestingly, these two heteromeric complexes assemble only in the acidic pH of the Golgi lumen (pH< 6.5). In the former case, complex formation could be prevented by increasing Golgi pH only by 0.2 pH units (65,66). This increase was also sufficient to redirect the ST3Gal3 enzyme from the Golgi to post-Golgi compartments, consistent with their oligomerization-driven retention in the organelle (67). The loss of the enzyme heteromers and enzyme mislocalization also coincided with reduced α(2,3)-sialylation and increased α(2,6)-sialylation of carcinoembryonic antigen (CEA) (67). A similar decrease and increase in α(2,3)- and α(2,6)-sialylation, respectively, in CEA N-glycans has also been observed in cancer tissues *in vivo* (68). Since Golgi resting pH is often elevated in cancer cells (69), these findings suggest that Golgi resting pH may be used to regulate what linkage type will be used to link sialic acids to an N-glycan. This kind of switch from one linkage type to another one can have dramatic effects on cell behavior. For example, increased expression of α(2,6)-linked sialic acid in N-glycans can inhibit tumor cell apoptosis and activate growth factor pathways (9 and references therein).

Interestingly, the formation of the β(1,4)GalT1/ST6Gal heteromer was shown to increase markedly the catalytic activity of the β(1,4)GalT1 perhaps via substrate channeling (65). Alternatively, heteromer formation may also increase accessibility of the donor or acceptor substrates to the active site of the β(1,4)GalT1, even though it is not directly involved in homodimer formation (70). Yet, the active site is more exposed in the β(1,4)GalT1/ST6Gal heterodimers than it is in homodimers (71). In addition to 3D structures, this view is supported by the observation that a single mutation in the active site (H243) was able to abolish homodimer formation but not heterodimer formation.

Acidic Golgi resting pH is also needed the keep certain glycosyltransferases active. Accordingly, Golgi acidity (pH < 6.5) is essential for the full catalytic activity of ST6Gal1

sialyltransferase but not for β(1,4)GalT1, nor the MGATs (65). Partly, this can be explained by the pH-sensitive interactions between the β(1,4)GalT1 and the two sialyltransferases acting on N-glycans (66). Yet, it is likely that pH-dependent conformational changes in the tertiary structure of ST6Gal1 also contribute to the activity loss if the Golgi resting pH is close to neutral. Collectively, these data suggest that the main role of the decreasing pH gradient from the cis-to-trans side of the Golgi compartments (pH 6.7-pH 6.3) is to orchestrate mutual interactions between glycosyltransferases, to promote their active conformation, and to get them correctly localized, in accord with their suggested oligomerization-mediated retention in the Golgi (72).

Golgi ion homeostasis

Golgi lumen contains high amounts of calcium, magnesium and manganese ions (73-75). The presence of these divalent cations is important for cargo concentration and sorting (76) as well as for glycosylation (77). The cations are transported into the Golgi lumen by the SERCA2 and SPCA1/2 type Ca^{2+}/Mn^{2+} pumps. Of these two, SERCA2 is enriched in the cis-Golgi, while SPCA1 is mainly present in the trans-Golgi (75). Unlike SERCAs, SPCAs are also engaged in Mn^{2+} transport (75,78). In addition to SPCAs, recent evidence suggests that TMEM165 mutations in patients cause a type II congenital disorder of glycosylation in humans by interfering with Mn^{2+} and Ca^{2+}/H^{+} transport (79,80). Manganese is an essential trace metal and important co-factor needed for the catalytic activity of many inverting Golgi glycosyltransferases such as β(1,4)GalT1. The DXD motif typically present in these enzymes plays a key role in Mn^{2+} -mediated donor substrate (UDP-Gal) binding (81). Based upon the recent structure of the β(1,4)GalT1 homodimer (70), Mn^{2+} appears to regulate transitions of the lid and the "Trp loop" that define the open (inactive) and closed (active) states of the enzyme. Accordingly, the Met340H mutant form of the enzyme that binds Mn^{2+} 25 times more avidly, blocks the β(1,4)GalT1 in the closed state, inactivates the enzyme and prevents its ability to form homodimers.

Golgi redox state

Reactive oxygen species and low oxygen tension (hypoxia) also contribute to Golgi glycosylation potential. Most often, their effects are mediated by hypoxia-inducible factors (HIF1-3) that regulate the expression of a number of N-glycosylation-associated genes, including MGATs (MGAT2, MGAT-3 and MGAT5a and 5b), fucosyltransferases (FuT1, 2 and 7), sialyltransferases (ST3Gal1 and ST6Gal1) as well as nucleotide sugar transporters for UDP-galactose, CMP-sialic acid and UDP-N-acetylglycosamine (82-84). Based upon these observations, Taniguchi et al. (85) introduced the term "Glyco-redox" to link altered glycosylation with oxidative stress generated by hypoxia or reactive oxygen species (ROS). Their close association may also contribute to neurodegenerative disorders such as Parkinson disease, Alzheimer's disease, and amyotrophic lateral sclerosis (ALS). Hypoxia (or HIFs) may also induce cleavage of cell surface N-linked glycans and thereby affect cell-extracellular matrix interactions (85-87). Oxidative stress and altered glycosylation have also been linked

to high-fat diet, obesity and the onset of type II diabetes mellitus (88-90). Marth and co-workers showed in their studies (89,90) that high levels of free fatty acids inhibit the expression of MGAT4a, a glycosyltransferase needed for β(1,4)-GlcNAc branching of N-glycans as well as GLUT-2 glucose transporter in in pancreatic β-cells. The β(1,4)-GlcNAc branch is normally required for cell surface localization of the glucose transporter, and thus for glucose transport into cells. Without the β(1,4)-GlcNAc branch, the GLUT-2 remains intracellular, leading to decreased glucose import, insulin export and accumulation of glucose in the blood.

Recent evidence indicates that hypoxia can modulate N-glycosylation also in a HIF-independent manner via affecting oxidative potential of the Golgi lumen (91). Surprisingly, in normoxic conditions it is higher than that of the ER (the main site of disulfide bond formation in the cells). In hypoxic cells, however, Golgi oxidative potential equals that of the ER in normoxic cells. The cells also displayed less sialic acid in their cell surface N-glycans. Interestingly, this was shown to be associated with reduced formation of surface exposed disulphide bonds in ST6Gal1 (and likely also in some other sialyltransferases including ST3Gal3), loss of its catalytic activity and inability to interact with β(1,4)GalT1 (91). Therefore, the high oxidative potential in the Golgi lumen appears to be necessary for the catalytic activity of certain sialyltransferases. This “redox switch” guarantees that the ST6Gal1 remains inactive until it reaches the Golgi compartment where it is expected to function. Likewise, the β(1,4)GalT1 enzyme acquires full activity also in the acidic Golgi compartment after interacting with theST6Gal1 sialyltransferase.

6. Concluding remarks

N-glycosylation is a frequent and complex modification of proteins, and essential for both uni- and multicellular life. It regulates a plethora of cellular functions that range from protein folding, trafficking, sorting, localization, half-life, and signaling to proliferation, migration and adhesion with its surroundings. Therefore, it is also not surprising that we currently know a vast number of human disorders that are caused by, or are associated with, altered N-glycosylation. While previous work has provided us a clear overall picture of the basic principles in N-glycan biosynthesis, there is a big gap in our understanding of the factors that underlie cell-, tissue- or organism-specific glycosylation patterns and their dynamic variability that starts during embryonic development continuing thereafter throughout our lives. We currently know that factors such as pH, redox potential, and changes in ion fluxes mainly in the Golgi compartment fundamentally affect and regulate the functioning and activity of glycosyltransferases expressed in a cell. Yet, there are many questions that remain unanswered. For example, how and why cells have evolved such a complex way to make their N-glycans, needing removal of some sugar residues and replacing them with others instead of adding the right sugar in the beginning? Perhaps there is an evolutionary reason, as yeasts (an early eukaryote) produce mainly high mannose type N-glycans which needed to be modified to different ones in order to provoke immune responses only against them and other pathogens, and thereby survive. And what is the reason (or cause) of producing N-glycans which differ

between two identical protein molecules? Does it result from a “sloppy” machinery that is prone to mistakes, or is there some purpose or benefit behind? Increasing biodiversity perhaps? Or is it a mark of ongoing evolution and trials to find the best fit for changing conditions? Is it regulated, or random? List in endless.

Nevertheless, these examples emphasize the need to understand in much more detail how glycans are made, how their synthesis is regulated and to what extent. An important issue also to keep in mind when one aims to produce optimally glycosylated antibodies for therapeutic use is to realize that yeasts, other lower eukaryotes or bacteria might not be the best choices to be used as hosts, as glycosylation is not just a simple outcome of enzymes present. It requires also conditions that support their full activity and complex mutual interplay necessary for their efficient functioning. Finally, we infer that there is an urgent need for developing more effective glycoengineering tools to edit glycans at will and thereby improve physicochemical and pharmacological properties of glycoprotein-based therapeutic compounds.

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8. Compliance with Ethical Standards

Conflict of Interest: The authors declare that they have no conflict of interest.

Ethical approval: This article does not contain any studies with human participants or animals performed by any of the authors.

9. List of abbreviations

Alg	Yeast Asparagine-linked glycosyltransferase
ALS	Amyotrophic lateral sclerosis
Asn	Asparagine
BiP	Binding protein
CDG	Congenital disorders of glycosylation
CEA	Carcinoembryonic antigen
CMAH	Cytidine monophospho-N-acetylneuraminic acid hydroxylase
CMP-Sia	Cytidine-5′-monophospho-N-acetylneuraminic acid
COPII	Coat protein complex II
Dol	Dolichol

Dol-P	Dolichol phosphate
Dol-P-Glc	Dolichol monophosphate glucose
Dol-P-Man	Dolichol monophosphate mannose
Dpagt1	Dolichyl-phosphate N-acetylglucosamine-phosphotransferase 1
EDEM	ER degradation enhancing mannosidase-like protein
ER	Endoplasmic reticulum
ERAD	ER-associated degradation
ERGIC	ER-Golgi intermediate compartment
ERGIC-53	ER-Golgi intermediate compartment 53 kDa protein
ERManI	ER mannosidase I
ERp57	Endoplasmic reticulum resident disulfide isomerase
Fuc	Fucose
FUT	Fucosyltransferase
Gal	Galactose
GalNAc	*N*-acetyl-D-galactosamine
GCNT2	N-acetyl-lactosamine-β-1,6-N-acetylglucosaminyltransferase
GDP-Man	Guanosine-5′-diphosphate-α-D-mannose
Glc	Glucose
GlcNAc	*N*-acetyl-D-glucosamine
GLUT-2	Glucose transporter type 2
HIF	Hypoxia-inducible factor
LacNAc	*N*-Acetyl-D-lactosamine
LLO	Lipid-linked oligosaccharide
LMAN1	Lectin mannose-binding 1
M6P	Mannose-6-phosphate
Man	Mannose
MCFD2	Multiple coagulation factor deficiency protein 2
MGAT	Mannosyl-glycoprotein-N-acetylglucosaminyltransferase
MPR	Mannose-6-phosphate receptor
NAGPA	N-acetylglucosamine-1-phosphodiester α-N-acetylglucosaminidase
Neu5Ac	5-*N*-Acetylneuraminic acid (sialic acid)
Neu5Gc	5-*N*-Glycolylneuraminic acid (sialic acid)
OS-9	Protein OS-9; amplified in osteosarcoma 9
OST	Oligosaccharyltransferase complex
ROS	Reactive oxygen species
Rtf1	A transmembrane protein encoded by the yeast Rft1 gene
Sec61	A three-subunit (Sec61α, Sec61β, and Sec61γ) protein translocation complex
Ser	Serine
SERCA2	Sarcoplasmic/endoplasmic reticulum calcium ATPase 2
Sia	Sialic acid
SPCA1/2	Secretory pathway Ca(2+)-ATPase type 1and 2
ST3Gal1	Beta-galactoside α-2,3-sialyltransferase 1

ST3Gal3	Beta-galactoside α-2,3-sialyltransferase 3
ST6Gal1	Beta-galactoside alpha-2,6-sialyltransferase 1
STT3A/B	Catalytic A and -B subunits of the oligosaccharyltransferase complex
TGN	Trans-Golgi network
Thr	Threonine
TMEM165	Transmembrane protein 165
UDP- GlcNAc	Uridine-5′-diphosphate-*N*-acetyl-α-D-glucosamine
UDP-Gal	Uridine-5′-diphosphate-α-D-galactose
UDP-Glc	Uridine-5′-diphosphate-α-D-glucose
UGGT	UDP-glucose-glycoprotein glucosyltransferase
VIP 36	Vesicular integral-membrane protein 36
XTP3-B	XTP3-transactivated gene B lectin

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Figure legends and figures

Fig. 1. A schematic representation of the N-glycan biosynthetic pathway in the ER and the Golgi apparatus. The figure shows gradual maturation of an N-glycan and the various steps involved. For more details, please see the text.

Fig. 2. A glycan-based quality control system in the ER that distinguishes correctly folded glycoproteins from unfolded or misfolded ones. CNX, calnexin; CRT, calreticulin,G-I-II, α-glucosidases I and II.-

Fig. 3. Three examples depicting the main N-glycan types present of the cell surface in higher eukaryotes. High-mannose type N-glycan is characterized by having not underwent any processing in the Golgi compartment. Hybrid-type N-glycan typically has only one branch that has been processed in the Golgi. Bulky complex-type N-glycans in turn have two to five branches that are made and terminated in the medial and trans-Golgi cisternae.

Fig. 1

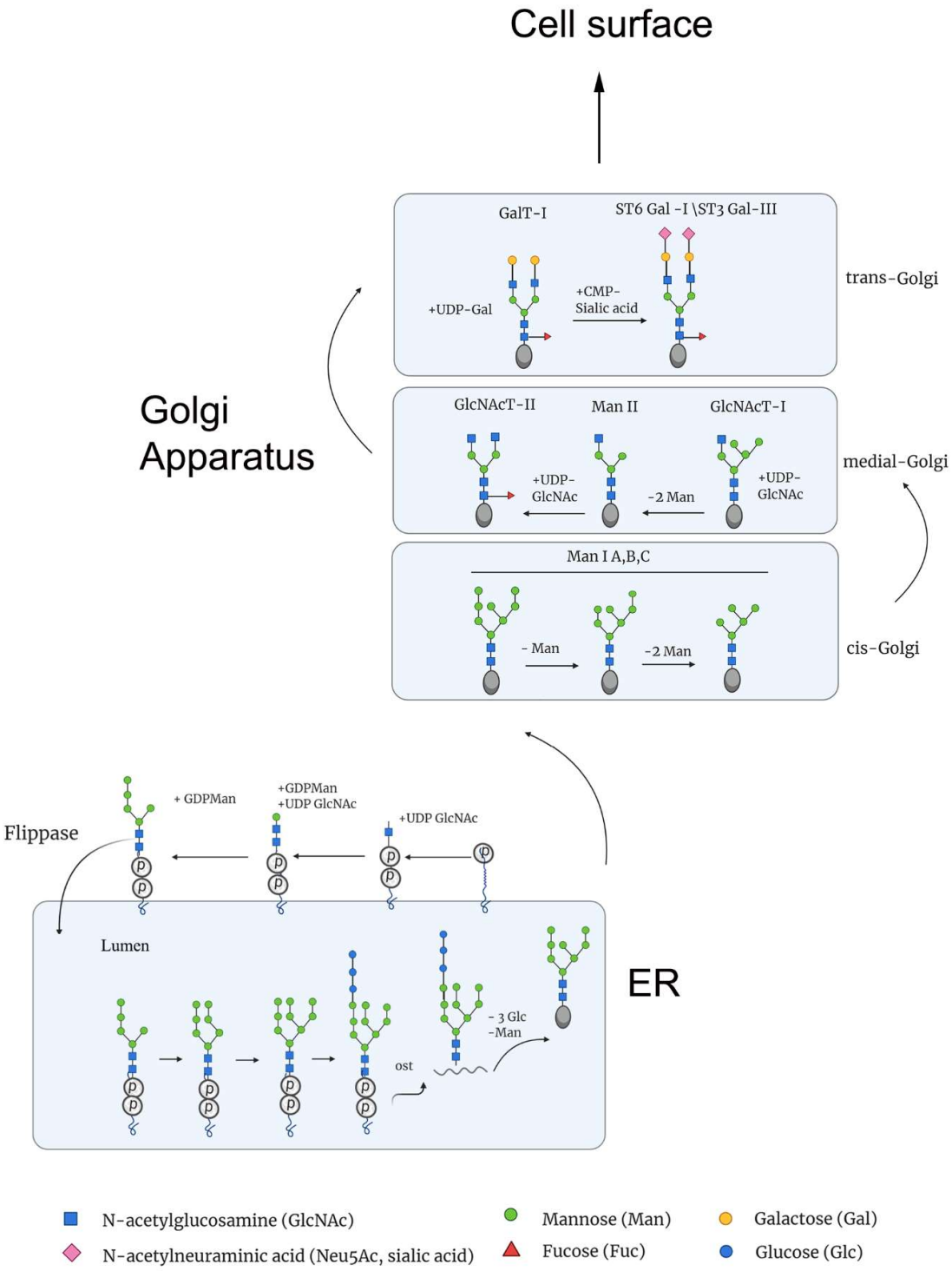

Cell surface
Golgi Apparatus
GalT-I
ST6 Gal -I \ST3 Gal-III
+UDP-Gal
+CMP-Sialic acid
trans-Golgi
GlcNAcT-II
Man II
GlcNAcT-I
+UDP-GlcNAc
-2 Man
+UDP-GlcNAc
medial-Golgi
Man I A,B,C
- Man
-2 Man
cis-Golgi
Flippase
+ GDPMan
+GDPMan
+UDP GlcNAc
+UDP GlcNAc
Lumen
- 3 Glc
-Man
ost
ER
N-acetylglucosamine (GlcNAc)
N-acetylneuraminic acid (Neu5Ac, sialic acid)
Mannose (Man)
Fucose (Fuc)
Galactose (Gal)
Glucose (Glc)

Fig. 2

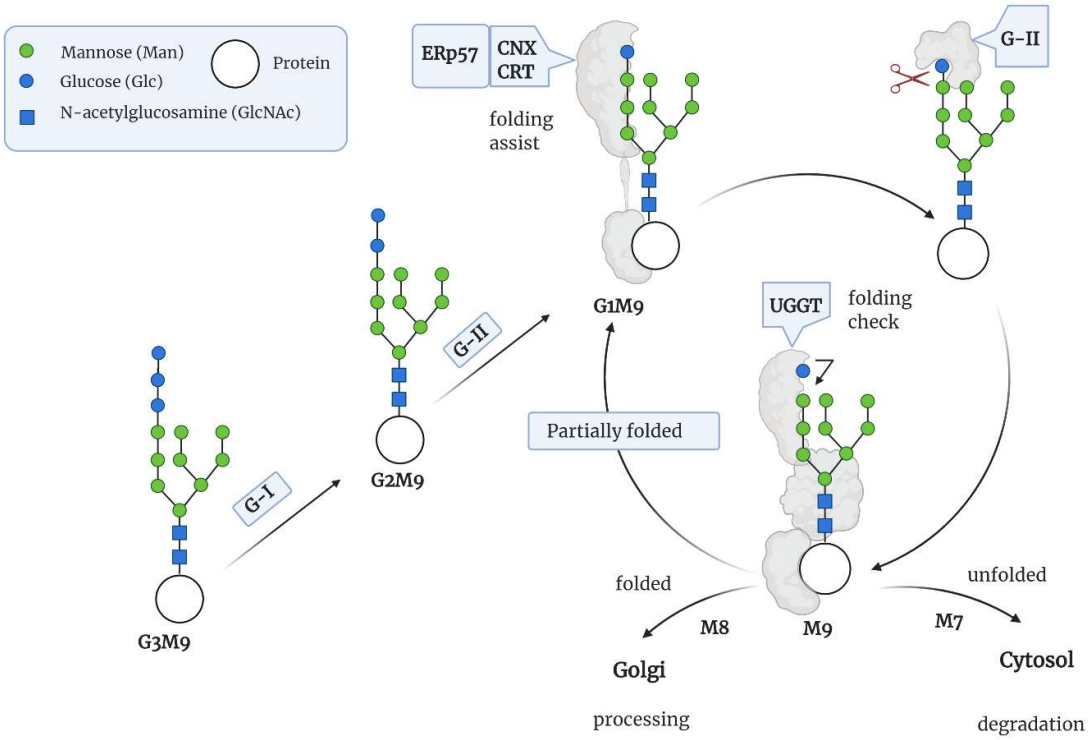
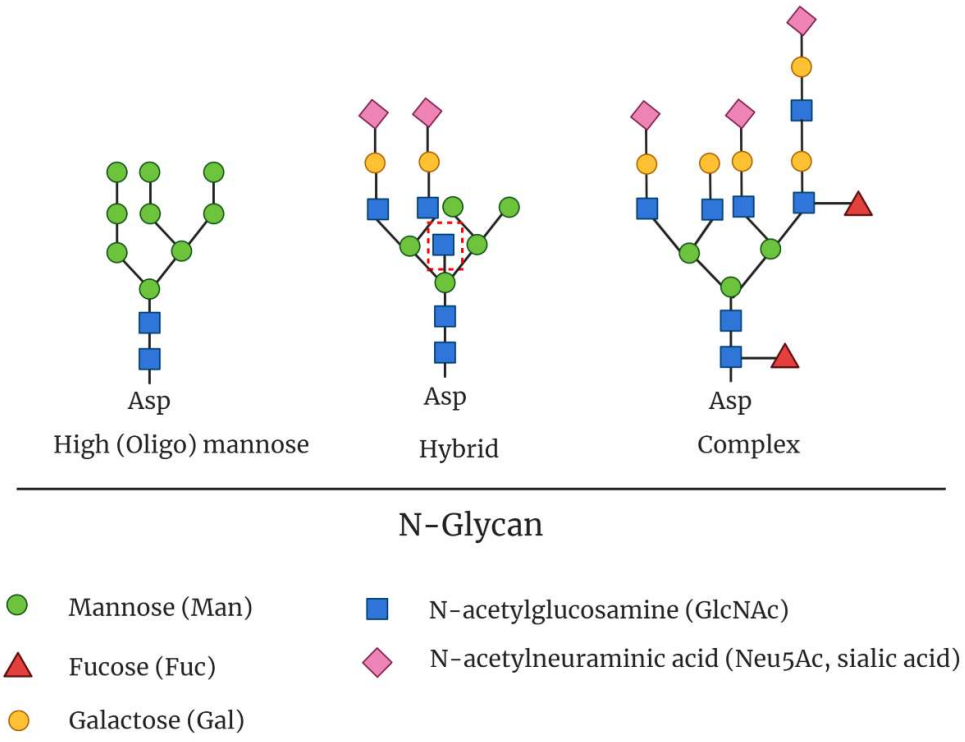

Mannose (Man)
Glucose (Glc)
N-acetylglucosamine (GlcNAc)
Protein
ERp57
CNX
CRT
folding
assist
G-II
G1M9
UGGT
folding
check
G-II
G2M9
G-I
G3M9
Partially folded
folded
unfolded
M8
M9
M7
Golgi
Cytosol
processing
degradation

Fig. 3


Asp
High (Oligo) mannose
Asp
Hybrid
Asp
Complex
N-Glycan
Mannose (Man)
Fucose (Fuc)
Galactose (Gal)
N-acetylglucosamine (GlcNAc)
N-acetylneuraminic acid (Neu5Ac, sialic acid)