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The glycan code of the endoplasmic reticulum: asparagine-linked carbohydrates as protein maturation and quality-control tags

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The majority of proteins that traverse the secretory pathway receive asparagine (Asn)-linked glycosylations. Glycans are bulky hydrophilic modifications that serve a variety of structural and functional roles within the cell. Here, we review the recent growing knowledge on the role of Asn-linked glycans as maturation and quality-control protein tags in the early secretory pathway. The carbohydrate composition encodes crucial information about the structure, localization and age of glycoproteins. The 'glycan code' is encoded by a series of glycosidases and carbohydrate transferases that line the secretory pathway. This code is deciphered by carbohydrate-binding proteins that possess distinct carbohydrate binding properties and act as molecular chaperones or sorting receptors. These glycosidases and transferases work in concert with resident secretory pathway carbohydrate-binding proteins to form a network that assists in the maturation and trafficking of both native and aberrant glycoproteins within the cell.

Protein glycosylation promotes the maturation and quality control of proteins in the lumen of the secretory pathway. More than half of all eukaryotic proteins are believed to be glycosylated, and an estimated 1% of all mammalian genes are involved in the glycosylation process [1–3]. The average glycoprotein contains three consensus sites of glycosylation, with greater than two-thirds of these sites being utilized. Asn-linked glycans are large, flexible and hydrophilic modifications that can extend ~3 nm away from the glycoprotein (Figures 1 and 2). They can play important structural and functional roles for a protein, as well as acting to sterically protect a protein from proteolysis or antigenic recognition. Here, we focus on the ability of Asn-linked glycans to act as modular tags to recruit carbohydrate-binding factors, which serve a variety of functions in the maturation and the quality-control processes of the cell. Special attention will be given to the composition of the Asn-linked carbohydrate signal and the role that glycans play in the recruitment of molecular chaperones and quality-control sorting receptors (Table 1). We follow the maturation of a glycoprotein from its initial translation and glycosylation until it leaves the endoplasmic reticulum (ER) either as a properly folded and assembled unit or as a substrate for degradation owing to improper maturation (Figure 3).

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Glycan addition and processing in the endoplasmic reticulum

Asn-linked glycosylation begins with the assembly of the complete dolichol-linked oligosaccharide donor (OS-PP-Dol), a 14-residue oligosaccharide (Figure 2) [4]. This process begins on the cytoplasmic face of the ER and is finished within the lumen, where the Asn-linked glycan is then transferred *en bloc* by the oligosaccharyltransferase (OST) to an Asn residue in the consensus sequence Asn-X-Ser/Thr (Figure 3, step 1). The transfer reaction requires that the glycosylation consensus sequence form a turn so that the hydroxyl-containing side-chains of Ser or Thr can increase the nucleophilic properties of the relatively chemically unreactive Asn amide side chain (Figure 2b) [4]. This explains why the Pro residue cannot be located at the X position, and how the requirements for the enzymatic reaction select for sequences that can form turns, which are likely to be localized at the surface of the protein.

The OST is an integral membrane protein complex, which has been studied most extensively in yeast. It comprises eight different subunits: Ost1p, Ost2p, Ost3p/Ost6p, Ost4p, Ost5p, Wbp1p, Swp1p and Stt3p. The organization of the OST of multicellular eukaryotes is less clear. Currently, six subunits have been identified, with homologs of the nonessential Ost4p and Ost5p being absent from vertebrate genomes [5]. Crosslinking studies have identified Stt3p as the central subunit involved in the transfer of the glycan to the polypeptide [6]. The mammalian genome possesses two homologs of both Stt3p and Ost3p/Ost6p. Cell-type-specific complexes containing these various homologs differ in their enzymatic properties, providing a possible mechanism to correlate OST

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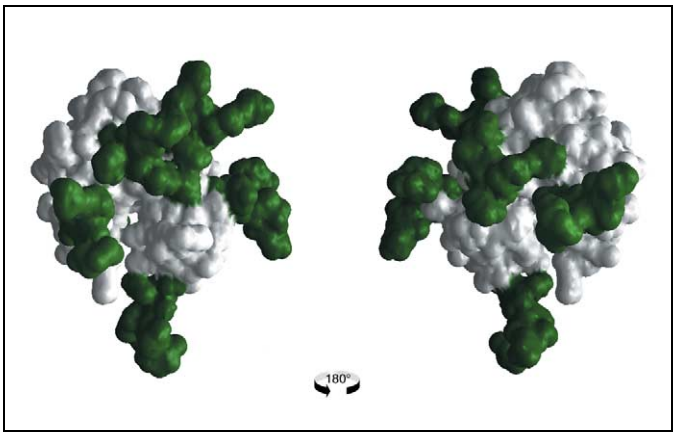

Figure 1. A model of the extracellular domain of the human high-affinity IgE receptor α chain, with its asparagine (Asn)-linked glycans, depicting the structure (FcεRIα; PDB code 1F2Q) as it would appear on the surface of the cell. The 176-amino-acid protein is displayed in shaded white and the seven Asn-linked carbohydrates are depicted in green. At each carbohydrate site, nine sugar residues are displayed. The carbohydrates generally extend away from the protein and comprise nearly 50% of the molecular mass of the glycoprotein. Two views are shown 180° apart. The 1–7 saccharides seen at each site in the crystal structure have been extended to nine saccharide residues at each site using molecular modeling to provide a picture of how the glycoprotein would appear at the cell surface as well as within the lumen of the endoplasmic reticulum.

function with the secretory protein load found in different cell types [7].

While Asn-linked glycosylation is commonly included within the general category of posttranslational modifications, the majority of the Asn-linked glycans are initially transferred co-translationally. This process is aided by the proximal location of the OST to the Sec61 protein channel, which mediates the translocation of nascent chains into the ER, and the lack of complex protein structure on the nascent chain as it emerges from the translocon [8,9]. Rapid folding can sequester the consensus sites, resulting in inefficient glycan transfer or hypo-glycosylation [10]. In addition, incomplete OS-PP-Dol chains are also transferred inefficiently, leading to the expression of hypo-glycosylated products in cells defective in the assembly of the complete OS-PP-Dol donor [11].

The OST contains at least two binding sites, including one for the glycosylation consensus site and another for OS-PP-Dol [12]. It also likely binds to the Sec61 translocon. Immediately after the glycan is transferred, the membrane protein glucosidase I removes the terminal α1–2-linked glucose, generating the $Glc_2Man_9GlcNAc_2$ species. This frees the transferred carbohydrate from rebinding to the OST carbohydrate-binding site, and allows the OST to properly reload with another OS-PP-Dol in readiness for the arrival of the next consensus site.

Carbohydrate trimming to enter a lectin-based chaperone system

Following translocation into the ER lumen and glycosylation, proteins encounter chaperones, which facilitate the maturation process and detection of defective cargo. The first lectin chaperones that a nascent glycoprotein meets are calnexin and calreticulin. Calnexin is a type I membrane protein and calreticulin is its soluble paralog. Both proteins possess a single binding site for glycans that have been trimmed sequentially by glucosidase I and then glucosidase II to a mono-glucosylated state (Figure 3, steps 2 and 3) [13,14]. Binding to the lectin chaperones is inhibited after the final glucose is removed by glucosidase II (Figure 3, step 4). Calreticulin binds to a single mono-glucosylated glycan with micromolar affinity [15]. The topology of the chaperones and the location of the glycans on the maturing protein dictate which chaperone binds to a given glycan on a glycoprotein [16,17]. Calnexin binds to membrane-proximal glycans, whereas calreticulin binds to glycans that project deeper into the lumen.

The X-ray structure of the ectodomain of calnexin

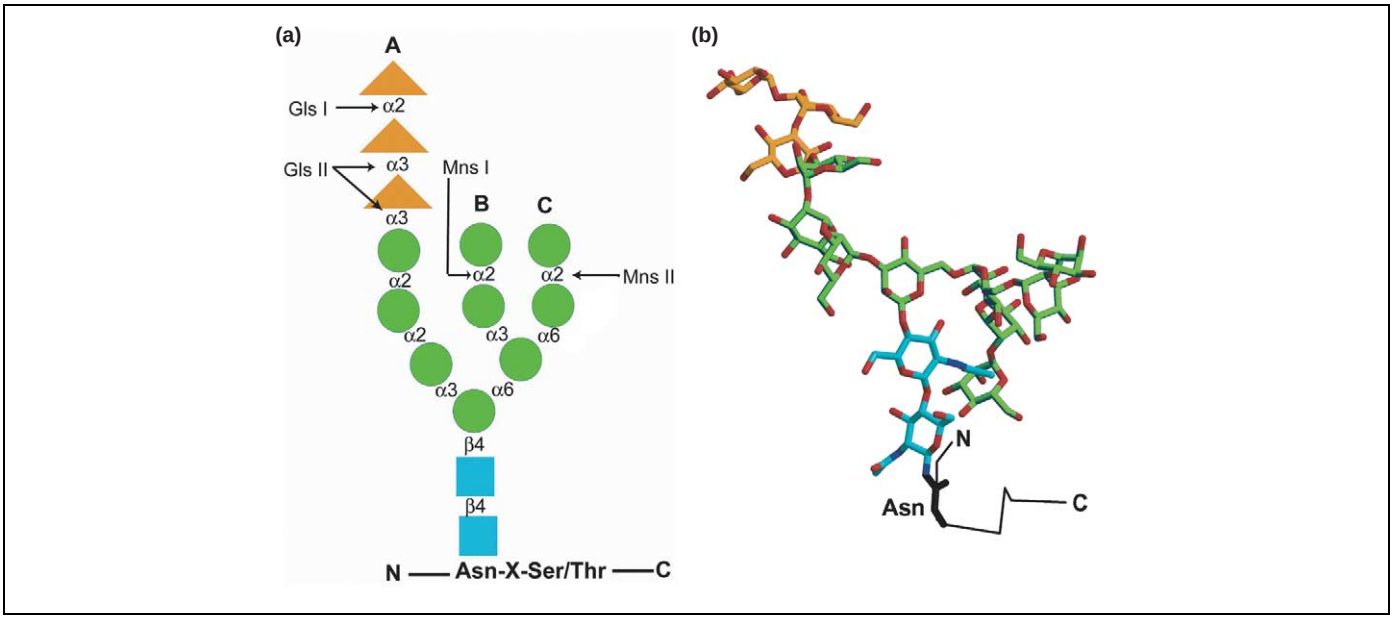

Figure 2. Composition and structure of the transferred asparagine (Asn)-linked glycan. **(a)** Depiction of the initial composition ($Glc_3Man_9GlcNAc_2$) of the carbohydrate transferred by the oligosaccharyl transferase (OST), with glycosidic linkages identified. The sites of action of various glycosidases are shown. Glucose (orange), mannose (green) and *N*-acetylglucosamine (cyan) monosaccharides attached to asparagine (black) on the protein are indicated. Gls and Mns denote glucosidase and mannosidase, respectively. **(b)** Atomic model based upon the coordinates of glucoamylase (PDB code 1GAI), with terminal sugars added by steric modeling. Note the turn observed in the protein backbone.

Table 1. Role of the individual glycan structures in the endoplasmic reticulum (ER)

Glycan structure	Mode of generation	Function
$Glc_3Man_9GlcNAc_2$	Transferred by the OST	Complete glycan that is efficiently transferred by the OST from the OS-PP-Dol.
$Glc_2Man_9GlcNAc_2$	Glucosidase I	Inhibits transferred glycan from rebinding to the OST; no known binding partner; allows the freed glycoprotein to begin folding.
$Glc_1Man_9GlcNAc_2$	Glucosidase II or GT	Binds chaperones calnexin and calreticulin; aids in the recruitment of the oxidoreductase ERp57; protection from aggregation, inhibits/slows global folding and retains glycoprotein in the ER.
$Man_9GlcNAc_2$	Glucosidase II	Releases from calnexin and calreticulin for folding and maturation.
$Man_{8B}GlcNAc_2$	ER mannosidase I	Targets substrates to the ERAD pathway when coupled to a malfolded protein.
$Man_xGlcNAc_2$	ER mannosidases I and II	Binds to ERGIC-53 in the ER for targeting to the ERGIC when coupled to a properly folded protein.

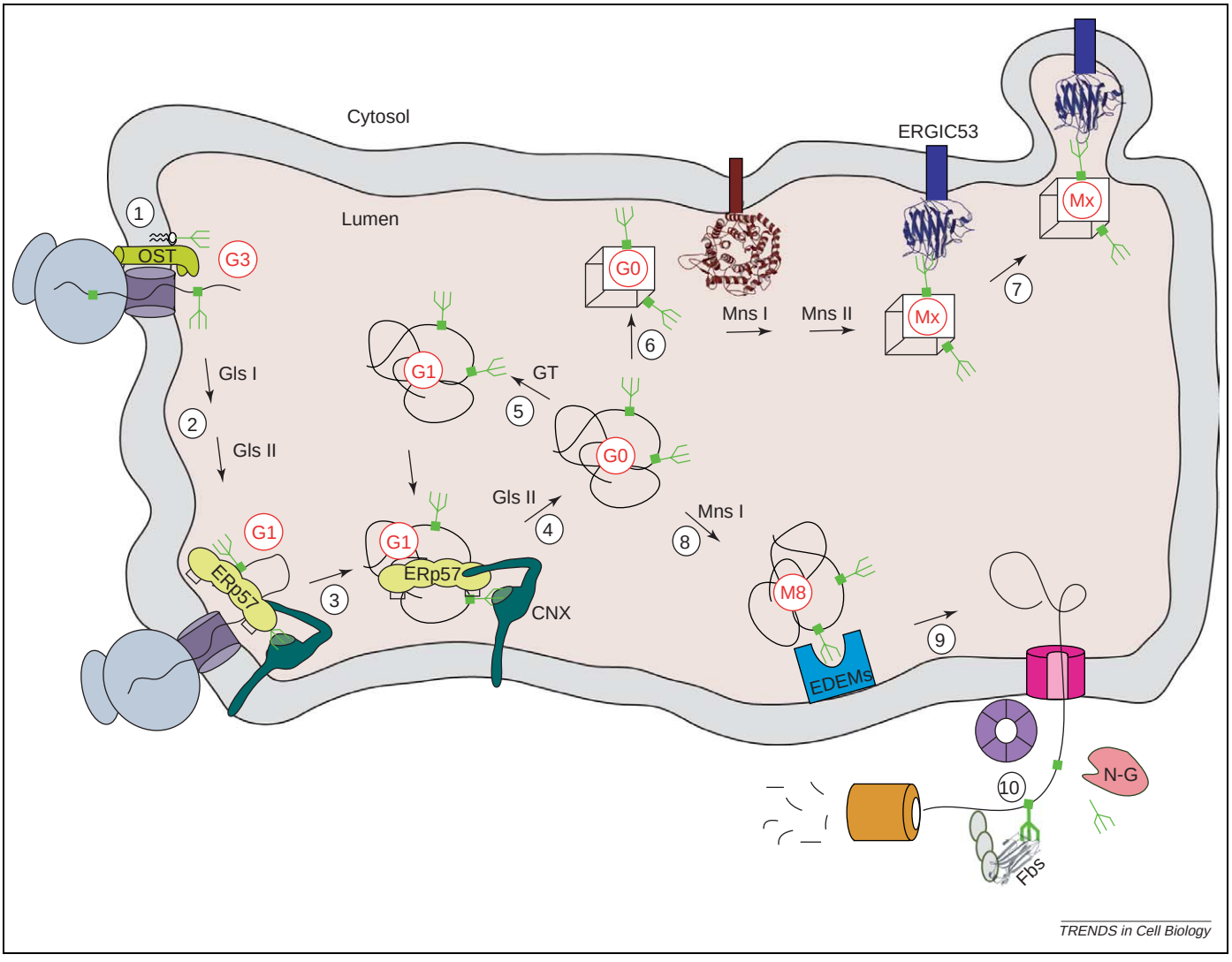

Figure 3. Lectin chaperones and sorting receptors of the endoplasmic reticulum (ER). Asn-linked glycans (green branched structure, with composition designated by red circles such as G3 for triglucosylated or $Glc_3Man_9GlcNAc_2$) are co-translationally transferred by the oligosaccharyl transferase (OST) to the growing nascent chain (step 1). Glycans are immediately trimmed by glucosidase I and II (Gls I and II) to their mono-glucosylated state (G1) to initiate co-translational calnexin (CNX) binding (step 2). While calreticulin binding can also commence co-translationally, it is not depicted for clarity. Calnexin binding to the mono-glucosylated substrate continues post-translationally (step 3). Removal of the final glucose by glucosidase II supports substrate release from calnexin (step 4). The freed protein can fold and assemble to its native conformation (step 6). Proper maturation includes mannose trimming by ER mannosidases I and II (Mns I and Mns II) to generate an undefined high-mannose glycan signal (Mx), which is recognized by ERGIC-53 for packaging into coatomer COPII-containing vesicles for anterograde transit (step 7). Alternatively, if the calnexin-released substrate contains non-native structure, it can be re-glucosylated to the mono-glucosylated state by UDP-glucose: glycoprotein glucosyltransferase (GT) (step 5) which supports rebinding to calnexin. Non-native glycoproteins, which are retained in the calnexin binding cycle, will eventually be trimmed by ER mannosidase I (Mns I) to a $Man_{8B}GlcNAc_2$ state (M8) for binding to EDEM or EDEM2 (EDEMs) (step 8). EDEM-bound substrates extracted from the calnexin binding cycle are targeted for retrotranslocation to the cytosol (step 9). The AAA-ATPase P97 (purple) drives the dislocation of the aberrant chain through an undefined translocon. In the cytosol (step 10), glycans can be recognized by an Fbs-containing E3 enzyme and polyubiquitinated (gray ovals), or deglycosylated by N-glycanase (N-G) before proteasomal (orange) degradation. Glycosidases, transferases, lectin chaperones and their associated oxidoreductase ERp57 are designated. Ribbon diagrams of ERGIC53, mannosidase I and Fbs1 are shown (PDB codes 1R1Z, 1FMI, and 1UMI, respectively).

demonstrates that calnexin comprises two distinct domains [14]. The first domain forms a globular β-sandwich structure, which is a signature of the carbohydrate-binding domains of leguminous lectins. The second domain, termed the P-domain, forms a long hairpin and extends away from the carbohydrate-binding domain. The P-domain comprises two series of four repeats in calnexin and three repeats in calreticulin, producing a slightly longer protrusion in the case of calnexin compared with calreticulin (14 versus 11 nm) [14,18]. This provides a longer reach for the membrane-tethered chaperone. In all other ways, the lumenal structures of calnexin and calreticulin are thought to be largely similar.

Calnexin and calreticulin interact initially with their substrates co-translationally as the protein is being translocated into the ER through the Sec61 translocon (Figure 3, step 2) [19–22]. The timing of the interactions with chaperones is dictated by the location of glycans on the nascent chains. Prior interaction with the hsp70 family member BiP can be bypassed if a glycan is located within approximately the first 50 amino acids of the glycoprotein [20]. Generally, calnexin and calreticulin binding increases the efficiency of glycoprotein folding by protecting the protein from aggregation [23,24]. In addition, their binding retains immature or aberrant proteins in the ER [25]. Trapping glycans in their mono-glucosylated state by the posttranslational addition of glucosidase inhibitors prevents substrate release [26]. The folding, oxidation and oligomerization of the trapped substrate are inhibited, indicating that, similar to the traditional ATP-driven chaperones, global folding occurs after release from the lectin chaperones (Figure 3, step 4) [23]. Therefore, calnexin and calreticulin binding act to slow the folding process, allowing for a more efficient overall process of maturation.

The localization of glycans can also help to direct the folding reaction by recruiting chaperones to distinct locations, thereby delaying the folding of the chaperone-bound domain [21,23]. This could be of particular importance in proteins that possess nonsequential folding domains. For instance, *influenza* hemagglutinin (HA) possesses three highly conserved glycans at its N-terminus, which ensures early binding by calnexin. This helps to protect and delay the folding of the N-terminus, allowing the medial domain of HA to fold unhindered. Calnexin finally releases the N-terminus posttranslationally so that the C- and N-termini can join to complete the folding process. This study suggests that some glycans might have evolved to be localized to certain locations in the sequence to aid the overall folding reaction [21].

A glycoprotein-specific oxidoreductase

Proteins that traverse the oxidizing environment of the ER can also be covalently modified by the formation of disulfide bonds. This oxidation reaction is catalyzed by proteins that act as oxidizing agents or electron acceptors. Alternatively, these oxidoreductases can reduce disulfide bonds by providing a source of electrons. This activity can support the isomerization of disulfides such that the native links can be reached or disulfide bonds can be reduced before the retrotranslocation of aberrant proteins to the cytosol for degradation. The oxidoreductase ERp57 acts on glycoproteins specifically since it is positioned near to the mature glycoprotein through its binding to the acidic tip of the P-domain arm of calnexin and calreticulin (Box 1) [27–29]. Therefore, an additional role of calnexin and calreticulin is to deliver the oxidoreductase ERp57 to distinct locations on maturing glycoproteins.

A single glucose residue as a quality-control signal

Glycan signals are also used to target aberrant proteins for destruction. The hallmark for an unfolded protein and binding by traditional chaperones is exposed hydrophobic regions, yet binding with the lectin chaperones is dictated by hydrophilic interactions. Therefore, how are calnexin and calreticulin binding connected to the signatures of immature, malfolded or unassembled proteins? This link is, at least in part, provided by UDP-glucose: glycoprotein glucosyltransferase (GT), which transfers a glucose onto non-glucosylated proteins possessing non-native conformations (for review, see [30]). This re-glucosylation step supports rebinding to calnexin and calreticulin and is the central decision in the retention of aberrant glycoproteins in the ER (Figure 3, step 5). Interestingly, GT activity is absent in the budding yeast *Saccharomyces cerevisiae* yet essential in the fission yeast *Schizosaccharomyces pombe* under stress conditions where it is regulated by the unfolded protein response (UPR) [30]. The presence of GT might also explain the species dependence on calnexin, where calnexin is essential for *Schizosaccharomyces pombe* viability, but not for *Saccharomyces cerevisiae* [30].

GT is a 174-kDa protein comprising two functional domains [31]. The transferase activity is localized to the C-terminal catalytic domain, whereas the N-terminal 75% of the protein acts as the folding sensor. *In vitro* studies using purified GT and model substrates have demonstrated that GT recognizes exposed hydrophobic residues on near-native folding intermediates [32–34]. Re-glucosylation occurs on the terminal mannose of the 'A' branch (Figure 2); this mannose remains intact in the ER and is only removed in the Golgi. Conflicting data exist as to the requirements for the proximity of the non-native domain and the re-glucosylated glycan [35,36]. The GT enzyme currently appears to be the only glycan encoder (transferase or glycosidase) that can translate a malfolded protein signal into a glycan signal by modifying its composition, demonstrating its importance in the carbohydrate-based quality-control network.

Anterograde transport lectin sorting receptors

Lectins of the early secretory pathway are also involved in the selective anterograde trafficking of glycoprotein cargo. Studies by the Hauri laboratory have shown that the type I membrane mannose-binding lectin ERGIC-53 assists in the transport of glycoproteins from the ER to the ER-Golgi intermediate compartment (ERGIC) (Figure 3, step 7) [37]. ERGIC-53 binds to high-mannose glycans and aids in the efficient secretion of glycoproteins, including cathepsin C and the blood-coagulation factors V and VIII [38–40]. This lectin recycles between the ER and

Box 1. ERp57, a glycan specific oxidoreductase

Oxidoreductases are characterized by the presence of multiple thioredoxin domains possessing CxxC motifs. Protein disulfide isomerase (PDI) is the most abundant oxidoreducatse in the endoplasmic reticulum (ER), with its thioredoxin domains organized in an a, b, b′ and a′ order. The CxxC sequences are localized to the catalytically active a and a′ domains. The b and b′ domains appear to form the signature thioredoxin fold but lack the CxxC motif, indicating that they are not involved directly in the oxidation or reduction reactions. ERp57 possesses the same modular a, b, b′ and a′ organization. However, PDI has an acidic C-terminus, with a KDEL ER-retention signal. By contrast, the lysine-rich C-terminal tail of ERp57 is basic and has a QDEL retention signal. Recent studies indicate that the basic tail along with the C-terminal b′ and a′ domains of ERp57 appear to play a role in interacting with the acidic tip of the P-domain of calnexin and calreticulin [29]. However, conflicting results exist as to the importance of the b domain and the basic tail in supporting these interactions.

ERGIC, where it binds to substrates in the neutral and calcium-rich ER, and release of its cargo is triggered by the more acidic and lower-calcium environment of the ERGIC [41]. Two less-characterized ERGIC-53-related proteins have also been discovered, termed VIP36 (vesicular integral membrane protein) and ERGL (ERGIC-53-like), suggesting that the use of glycans as trafficking signals is not limited to the function of ERGIC-53.

ER-associated glycoprotein degradation

Protein folding is error-prone, and amino acid mutations or deletions can further decrease folding efficiency. Non-native polypeptides generated as physiologic and pathologic byproducts of protein biogenesis need to be rapidly degraded to avoid accumulation and/or deposition in the ER lumen. The ER-associated degradation (ERAD) process is operated by the cytosolic proteasome and therefore requires recognition of the folding-incompetent polypeptides by molecular chaperones and enzymes in the ER lumen (Figure 3, steps 8–9) and dislocation across the ER membrane for further modifications in the cytosol such as ubiquitylation and deglycosylation before disposal (step 10). Accordingly, gene disruption or pharmacological inhibition of several factors acting on both sides of the ER membrane affect ERAD [42]. The compartmentalization of the degradation process protects nascent polypeptides and protease-sensitive folding intermediates expressed in the ER from unwanted disposal.

Mannose trimming to generate a degradation tag

Folding-competent and folding-incompetent polypeptides enter co-translationally in the calnexin/calreticulin cycle [19]. While the folding-competent proteins are eventually released as native polypeptides in the secretory pathway, the folding-incompetent ones are retained, temporarily protected from the degradation machinery, and subjected to additional folding attempts. Their Asn-linked glycans are eventually trimmed of terminal mannoses by the ER enzyme α-mannosidase I. Although removal of the α-1,2-linked mannose from the B branch of the core oligosaccharide might represent *per se* a strong degradation signal [4], the ER mannosidase I can cleave several terminal mannoses from the protein-bound B and C branches of the glycan [43–45]. As a generally accepted concept, mannose trimming is a symptom of persistent ER retention and facilitates disposal of the coupled non-native polypeptide, which is utilized both in yeast and mammalian cells [46,47].

Although mannoses of the B and C branches seemingly contribute little to calreticulin binding [15], it has been proposed that their removal makes the associated misfolded polypeptide a weaker ligand for calnexin and calreticulin [48]. Certainly, mannose trimming generates a sub-optimal substrate for glucosidase II and GT (reviewed in [30,42,49]), so that, when a polypeptide with low mannose content is released from calnexin and calreticulin, the re-glucosylation necessary to re-associate is less efficient. Either way, mannose trimming facilitates exclusion of non-native polypeptides from the calnexin cycle and degradation, whereas selective inhibition of the ER α-mannosidase I with the alkaloid kifunensine retards release of folding-defective polypeptides from the calnexin cycle and delays ERAD [50–52].

EDEM decodes the N-glycan degradation tag to interrupt futile folding attempts

The slower progression of misfolded polypeptides through the calnexin cycle and their reduced affinity for calnexin/calreticulin upon mannose removal might offer an advantage to mannose-binding lectins present in the ER lumen (termed EDEM [53] and EDEM2 [54,55] for: ER degradation enhancing α-mannosidase like proteins in mammals, and Htm1p/Mnl1p for: mannosidase like protein in yeast [47,56]). EDEM and EDEM2 (and EDEM3, whose function in the ER has not yet been established [54,55]) are members of the glycosylhydrolase family 47, also comprising the ER α-mannosidase I and the ER Golgi α-mannosidases IA, IB and IC. Although structural modeling shows no difference in the presence and position of catalytic residues between EDEMs and the active hydrolase ER α-mannosidase I [57], *in vitro* and *in vivo* data show that mammalian EDEM [44,54] and its yeast homolog Htm1/Mnl1 [58] are enzymatically inactive. They are proposed to act as a lectin quality-control receptor in the ER lumen by a mechanism analogous to glycan-recognition during catalysis by the hydrolase, but no experimental data have yet been published that show glycan specificity.

EDEM and EDEM2 are inducible proteins, whose intralumenal concentration is increased upon stress-induced activation of the transcription factor Xbp1 [55,59,60]. Elevation of EDEM and EDEM2 expression might give EDEM variants further advantage in this competition for binding misfolded polypeptides and eventually lead to accelerated extraction of folding-defective polypeptides from the calnexin cycle [55,61,62]. Consistently, deletion of the yeast EDEM homolog [63] and downregulation of EDEM transcripts [61] or lack of transcriptional EDEM induction [59,60] in mammalian cells all inhibit ERAD of glycoproteins.

EDEM and EDEM2 lack canonical sequences for ER retention and possibly rely on association with ER-resident chaperones for proper intracellular localization. While EDEM2 is a soluble ER protein [54,55], the

uncleaved signal peptide was found to anchor EDEM at the ER membrane in Cos cells and was suggested to mediate formation of a functional complex with calnexin [62]. In several other cell lines, however, the signal peptide of EDEM is efficiently cleaved, resulting in EDEM release from the ER membrane, thus challenging the model predicting that a physical association with calnexin is required for EDEM activity [55].

A glycan tag to direct misfolded glycoproteins to the cytosolic proteasome

Most misfolded proteins in the cytoplasm, including those arriving from the ER, are decorated with polyubiquitin chains to promote degradation by the 26S proteasome. Polyubiquitylation occurs by the concerted action of activating E1, conjugating E2 and ligating E3 enzymes located in the cytosol [64]. There are several E3 variants, and one of the best-characterized E3 complexes, the SCF (for: Skp1, Cul1, Roc1) contains a Fbs1 (F-box sugar recognition) protein in the adult brain and testis or a more ubiquitously expressed Fbs2 protein that confers specificity for high-mannose Asn-linked glycoproteins (Figure 3, step 10) [65,66]. Asn-linked glycans can therefore serve as degradation tags even after release of ERAD candidates into the cytosol. Alternatively, the glycan might be removed by peptide:N-glycanase before delivery to the proteasome [67,68], but removal is not an absolute requirement for degradation by proteasomes [69].

Concluding remarks

Glycans play diverse roles inside and outside of the cell. Here, we have focused on the function of Asn-linked glycans in the early secretory pathway, where the glycan processing steps are more homogenous than in downstream locations. The past decade has seen a large increase in our understanding of the role glycans play in directing the maturation and quality-control traffic in the ER. The glycan composition is used as a signal to recruit chaperones and sorting receptors to assist in the maturation, quality-control and degradation processes (Table 1). The glycan A branch is involved in chaperone binding, whereas the B and C branches control trafficking out of the ER for anterograde trafficking to the Golgi or retrotranslocation to the cytosol. The necessity and role of each individual glycan are highly variable. While some glycans are absolutely required for proper maturation, others are totally dispensable. Further studies will be needed to provide guidelines to predict the need and role of a given glycan on a protein. Interestingly, studies have also demonstrated that the carbohydrate signal can also be exploited to modulate the function of mature glycoproteins in the ER, including the calcium regulation of the SERCA pump and peptide loading of major histocompatibility complex (MHC) class I [70,71]. The large number of human diseases associated with defects in glycosylation underscores the importance of understanding glycosylation and its associated functions at the molecular level [72].

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