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Review

N-linked sugar-regulated protein folding and quality control in the ER

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ABSTRACT

Asparagine-linked glycans (N-glycans) are displayed on the majority of proteins synthesized in the endoplasmic reticulum (ER). Removal of the outermost glucose residue recruits the lectin chaperone malectin possibly involved in a first triage of defective polypeptides. Removal of a second glucose promotes engagement of folding and quality control machineries built around the ER lectin chaperones calnexin (CNX) and calreticulin (CRT) and including oxidoreductases and peptidyl–prolyl isomerases. Deprivation of the last glucose residue dictates the release of N-glycosylated polypeptides from the lectin chaperones. Correctly folded proteins are authorized to leave the ER. Non-native polypeptides are recognized by the ER quality control key player UDP-glucose glycoprotein glucosyltransferase 1 (UGT1), re-glucosylated and re-addressed to the CNX/CRT chaperone binding cycle to provide additional opportunity for the protein to fold in the ER. Failure to attain the native structure determines the selection of the misfolded polypeptides for proteasome-mediated degradation.

Contents

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1. Introduction

The ER is the site for maturation of secretory and membrane proteins in eukaryotic cells, which represent about one third of the total proteome of the cell [1–3]. Although several proteins have been reported to acquire spontaneously their folded structure in vitro, a large number of newly synthesized polypeptides in the cell requires assistance to reach the final and biologically active conformations [4–6]. The lumen of the ER contains resident molecular chaperones and folding factors to optimize the folding efficiency [7]. The folding state of a protein is evaluated by quality control mechanisms: properly folded proteins are secreted or targeted to their final intra- or extra-cellular destination, whereas misfolded proteins are recognized as aberrant products and targeted for the ER-associated degradation ((ERAD) [8,9] and reviewed by Benyair et al., in this issue). Most of the proteins designated for the secretory pathway are subjected to co- or post-translational glycosylation of asparagine residues side chains ((N-glycosylation) [10,11] and reviewed by Shrimal et al., in this issue). The rapid removal of terminal glucose and mannose residues from protein-bound oligosaccharides and the regulated addition of a specific glucose residue dictate the sequential schedule of events occurring during maturation and selection for degradation. Here we present the body of knowledge concerning the sugar-regulated mechanisms that govern protein folding and quality control in the ER lumen.

2. Protein folding in the ER

2.1. N-glycosylation plays a key role in protein folding

The majority of the client proteins entering the ER lumen exhibit -N-X-S/T- (asparagine-any amino acid but proline–serine/threonine) sequences within the polypeptide chain [11,12]. The asparagine residue of this consensus motif is rapidly modified through the covalent attachment of a pre-formed oligosaccharide composed of three residues of glucose, nine mannoses and two N-acetylglucosamines (Glc3Man9GlcNAc2) (Fig. 1). The transfer of the 14-subunits oligosaccharide is catalyzed by the oligosaccharyltransferase complex (OST) (Fig. 2, **step 1**) [13] and thoroughly described in the article by Shrimal and Gilmore in this issue. N-glycosylation increases the solubility of the newly synthesized polypeptides and processing of the protein-bound oligosaccharides creates the signal required for the recruitment of ER-resident lectin chaperones that regulate glycopolypeptide folding [14–17].

2.2. N-glycan processing dictates the binding to the ER-resident lectin chaperones

2.2.1. α-Glucosidase I generates di-glucosylated glycans

2.2.1.1. Organization of α-glucosidase I. The modification of protein-bound oligosaccharides by ER-resident glycanases dictates the fate of newly synthesized polypeptides. As soon as the glycan is added to nascent chains, the first glucose is trimmed by α-glucosidase I [18–20] (Fig. 2, **step 2**), a single pass transmembrane ER protein with the catalytic domain facing the ER lumen [21]. The crystal structure of an eukaryotic α-glucosidase I (the Cwh41p *S. cerevisiae* ortholog) reveals a globular protein of two domains connected by a linker: the N-domain is largely comprised of a 13 strand super β-sandwich and additional helices, and the C-domain contains 12 helices in an (α/α6) toroid bundle with an additional subunit termed C′-domain. The catalytic residues are proposed to lie in the center of the (α/α6) barrel [22].

2.2.1.2. α-Glucosidase I generates the ligand for malectin. For a long time, the di-glucosylated form of protein-bound oligosaccharides generated by the action of α-glucosidase I was considered an extremely short-lived trimming intermediate lacking biological significance. The discovery of malectin, a membrane-bound ER-resident lectin specifically binding di-glucosylated glycans, changed this view [23]. Malectin is induced under conditions of ER stress [24] and is proposed to preferentially associate with off-pathway non-native conformers of well-studied glycoproteins like *influenza* hemagglutinin (HA) and *null* Hong Kong (NHK), a folding-defective variant of the secretory protein α-1-antitrypsin (α1AT). The putative capacity of malectin to detect terminally misfolded proteins so early after their expression in the ER lumen and to distinguish them from non-native intermediates of folding programs is a peculiar property that merits further investigation and may require the formation of a functional complex with the oligosaccharyl transferase complex subunit ribophorin I [24–26]. This conclusion is reinforced by the observation that the overexpression of ribophorin I enhances malectin association with misfolded NHK [26].

2.2.2. α-Glucosidase II induces the entry of glycoproteins into the CNX/CRT cycle

2.2.2.1. Organization of α-glucosidase II. α-Glucosidase II is a heterodimeric protein, with two non-covalently bound subunits. GIIα is the catalytic subunit and GIIβ contains an ER retention sequence in mammals and *S. pombe* but not in *S. cerevisiae* [27]. GIIα activity requires the presence of GIIβ as shown in *S. pombe* and mammalian cells, as well as in cell free assays [28,29]. GIIβ contains a mannose-6-phosphate receptor homology (MRH) domain, which was proposed to regulate GIIα activity by interacting with the mannoses on the B and C branches of the nascent protein glycan [29].

2.2.2.2. Regulation of α-glucosidase II trimming for entry in the CNX/CRT cycle. α-Glucosidase II trims the second and the third glucose of the N-linked glycan (Fig. 2, **steps 3 and 5**). The two trimming events play opposing roles. After the first trimming step, maturing substrates associate with the lectin chaperones CNX and CRT, which recognize mono-glucosylated glycans. The second trimming step releases the protein from the lectin chaperones due to the reduced affinity of these chaperones to glycans lacking terminal glucose residues (Fig. 2, **step 5**) [30–32]. A series of de-glucosylations by α-glucosidase II (**step 5**) and re-glucosylations by a glucosyltransferase, the UGT1 (**step 6**) can occur [32,33]. Re-glucosylation by UGT1, which will be detailed in Section 3, directs the rebinding of CNX or CRT to the mono-glucosylated substrate, hence the name CNX/CRT binding cycle.

Despite having one catalytic site, the rates of the first and the second trimming events by the α-glucosidase II are different as the first cleavage occurs faster than the second cleavage [34,35]. Unlike the rapid trimming by α-glucosidase I, trimming by α-glucosidase II appears to be more regulated. Work in ER-derived microsomes and mammalian cells showed that more than one glycan on client proteins is required for recognition by α-glucosidase II [20].

The activity of α-glucosidase II appears to be influenced by the number of mannoses on a glycan because reduced activity of α-glucosidase II in rat liver microsomes was observed towards substrates possessing a reduced number of mannose residues [36]. This was more recently supported by the observation that trimming of α1,2-bonded mannose residues in *S. pombe* resulted in a prolonged accumulation of mono-glucosylated proteins. The glycans with highest de-glucosylation rates harbored nine mannoses and the de-glucosylation rate decreased with glycans containing seven, six and five mannoses [37]. On the contrary, another study using well defined synthetic glycopeptides as substrates in a cell free assay concluded that the lower number of mannose residues

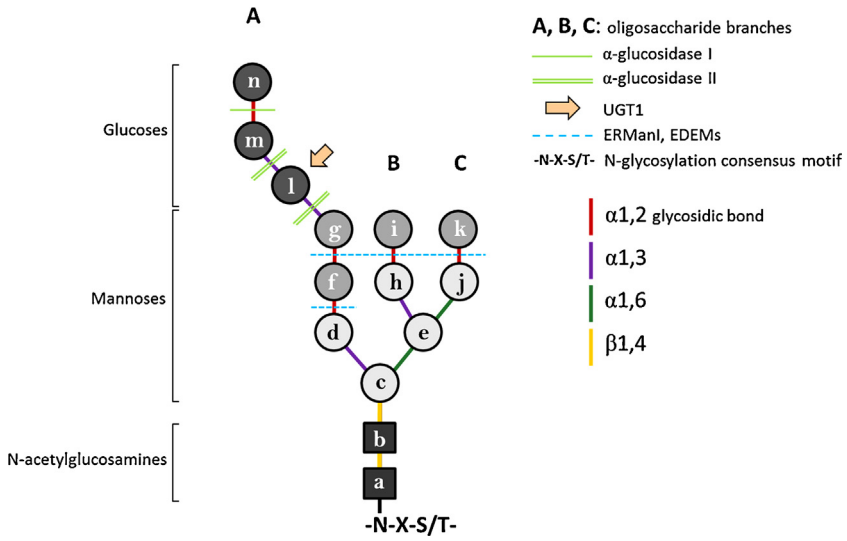

Fig. 1. N-glycan composition and processing. The pre-formed oligosaccharide is composed of three glucoses (dark gray circles), nine mannoses (light gray circles) and two N-acetylglucosamines (black squares). The oligosaccharide branches are shown with A, B and C. The oligosaccharide processing enzymes are listed in the figure and their action is shown. The type of glycosidic bond is shown in color.

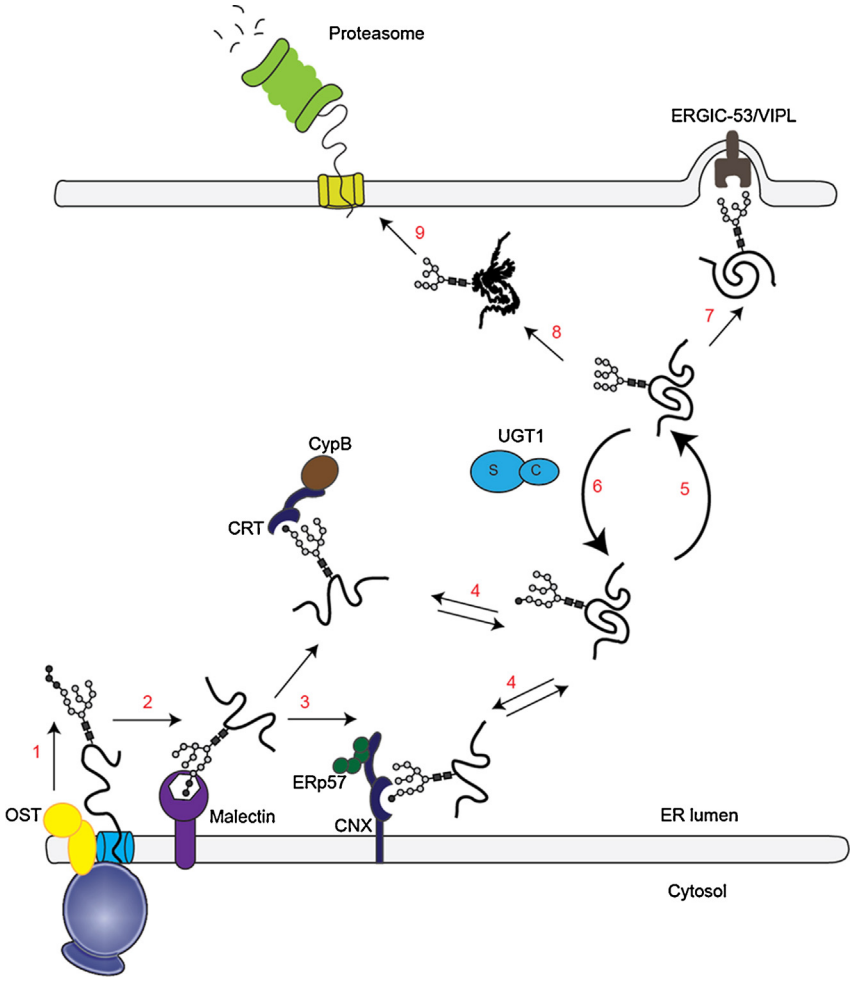

Fig. 2. Scheme of the CNX/CRT cycle. Upon addition of the 14-subunits oligosaccharide catalyzed by the OST complex (**step 1**), the first glucose is removed by α-glucosidase I (**step 2**). Di-glucosylated polypeptides associate with malectin. α-Glucosidase II cleaves the second glucose from the glycan (**step 3**), generating mono-glucosylated polypeptides, which interact with CNX and CRT. Binding and release from lectin chaperones could occur (**step 4**). The release from the ER lectins is determined by the second cleavage by α-glucosidase II, which removes the last glucose residue (**step 5**). Re-glucosylation by UGT1 dictates the re-association of the polypeptides with CNX or CRT (**step 6**). Correctly folded polypeptides are exported from the ER (**step 7**). Terminally misfolded proteins are further processed by mannosidases (**step 8**) and eventually retrotranslocated for proteasomal degradation (**step 9**). The substrate-binding and catalytic domains of UGT1 are shown with s and c, respectively.

did not change α-glucosidase II trimming kinetics, as the trimming by α-glucosidase II of Glc1Man8 was identical or greater than that of Glc1Man9 [35]. Mannose trimming has been proposed to act as a timer that allows proteins either to exit to the Golgi or to be targeted for degradation [11,38]. Reducing the α-glucosidase II activity with increasing mannose trimming could prevent premature release of the protein from the CNX/CRT cycle and thus provide additional time for the proteins to fold by prolonging the binding to these chaperones before they are targeted for trafficking to the Golgi or deemed misfolded and targeted for degradation.

2.2.3. The roles of CNX and CRT

2.2.3.1. Structure, mechanism of binding and similarities between CNX and CRT. CNX is a type I membrane protein. CRT is its soluble paralogue possessing 39% sequence homology [39]. CNX and CRT share similar structural properties: they both have an N-terminal globular domain that contains the lectin binding site and a long arm domain. In CNX, the globular domain is a β-sandwich of concave and convex β-sheets and the arm domain, shaped in an overall hairpin like structure, is termed the P-domain. The latter protrudes 140 Å away from the globular domain and has two different proline rich motifs named 1 and 2, of four copies each [40]. The four copies of motif 1 on one strand interact in a head to tail fashion with the four copies of motif 2 on the other strand of the hairpin [40]. In CRT, the P-domain is similar but shorter than calnexin and has three instead of four tandem repeats [41]. The tip of the P-domain interacts with the oxidoreductase ERp57 as will be detailed later in Section 2.3.1.2 [42]. CNX co-crystallized with glucose has an extensive hydrogen-bonding pattern of the glucose with residues in the globular domain.

In addition to structural similarities, CNX and CRT have similar lectin binding specificities; as shown by in vitro binding studies as they both bind to the glucose as well as the three mannoses on branch A of the glycan with similar affinities in a calcium dependent manner [43]. The oligosaccharide-binding sites of CNX and CRT are similar but not identical [44]. Four out of the six residues that mediate binding in CNX are conserved in CRT.

The crystal structure of CRT with a glycan further confirmed previous observations that CRT binds to the glucose and the three-mannose residues of the glycan, which is mediated by extensive hydrogen bonding between the glucose ring and residues in the sugar-binding site of CRT. The rest of the tetra-saccharide was also involved in hydrogen bonding but to a lesser extent than the glucose [45]. More in depth analysis of the mechanism of binding to CRT proposed that mono-glucosylated glycans are responsible for recruiting substrates to bind to CRT, while the proline-rich domain stabilizes the interaction by locking the substrate in a closed conformation [46]. This might explain why the oxidation of substrates is arrested or delayed when chaperone binding is chemically trapped [32,47,48]. These results are suggestive of global folding events occurring after the substrate is released from the lectin chaperones [49].

CNX and CRT are involved in several processes such as calcium homeostasis, immunity, cancer and apoptosis [50,51]. Here, we will only describe their roles as chaperones in the ER. Early studies proposed a "lectin only" model to describe their chaperone function [52]. The studies relied on cell lines deficient in either α-glucosidase I or α-glucosidase II, or employed glucosidase inhibitors such as castanospermine and deoxynojirimycin-derivatives [31,32,53]. These studies showed that inhibition of glucose trimming prevented substrate binding to these lectin chaperones supporting the requirement of mono-glucosylated glycans for association. It was also proposed that these lectins bind to glycoproteins independently of their conformation [54] and do not bind non-glycosylated proteins [55]. However, later work proposed that they could act in a similar fashion to classical chaperones by binding to peptide regions of the substrates or to non-glycosylated proteins [56–58].

2.2.3.2. Salient differences in the roles of CNX and CRT. Despite the similarities between CNX and CRT, striking differences have been attributed to their roles or binding specificities. The location and the number of glycans recognized by each of the lectins could be quite distinct [59–61]. Furthermore, even though CNX and CRT share some of the same substrates, some proteins are exclusively clients. One explanation for these differences is assigned to the different topologies. It was observed that CRT, when fused to the membrane segment of CNX, acquired the substrate specificity of CNX [62]. Different peptide-binding specificities could also contribute to the different substrate selection by the two ER lectins [43].

Consistent with different substrate specificity and/or with specific functions in the ER lumen is the fact that CNX clients seem to associate with BiP rather than with CRT upon CNX deletion [63] and that CNX and CRT knockouts (KO), that in cultured cells are well tolerated, have different phenotypes in mice. CRT KO is embryonic lethal due to defective heart development [64], whereas CNX KO mice are viable and show motor disorders, associated with a dramatic loss of large myelinated nerve fibers [65]. Finally, the depletion of either of the lectins may have opposing effects on folding. Depletion of CRT enhances the maturation of a subset of viral and cellular proteins with a minor decrease in folding efficiency. To the contrary, CNX depletion prevents the maturation of a substrate like HA, while its absence does not affect the maturation of other proteins [61]. These differences highlight the unique roles of each of these lectins despite their similarities.

2.3. CNX and CRT engage enzymes required for proper protein folding

Specialized ER-resident enzymes catalyze the formation of native intra- and inter-molecular disulfide bonds (Fig. 3A) and the isomerization of peptidyl–prolyl bonds (Fig. 3B) of folding polypeptides. These are rate-limiting steps that are mediated by members of the protein disulfide isomerase (PDI) family and of the peptidyl–prolyl isomerase (PPI) family, respectively. Recent reports showed that at least one member from each family (i.e., the oxidoreductase ERp57 [42,66] and the PPI CypB [67]) might form functional complexes with CNX and CRT. In this scenario, CNX/CRT-ERp57 complexes would promote the oxidative folding of nascent polypeptides, whereas CNX/CRT-CypB complexes would promote isomerization of peptidyl–prolyl bonds to attain the native polypeptide structure [67–70].

2.3.1. The PDI family: formation of native disulfide bonds

The mammalian PDI family comprises about 20 members that can be either catalytically active or inactive. They all have in common one or more thioredoxin-like structural domains and are localized primarily in the ER (Table 1) [71–73].

The thiol-reactive members of the PDI family contain at least one catalytic thioredoxin-like domain termed **a** domain. Each **a** domain conventionally consists of a Cys-Xxx-Xxx-Cys (CXXC) motif [74]. In some cases, the CXXC motif shows cysteine to serine replacement (Table 1). Additionally, PDIs might possess one or more non-catalytic **b** domains. These are structurally similar to the **a** domains and may engage substrates, but do not contain any active cysteine residue [72,75]. ERp27 and ERp29 contain only a **b** domain and are catalytically inactive (Table 1). Furthermore, a linker domain, termed **x** domain, stabilizes the protein structure and has been identified in several PDI family members (such as in PDI and ERp57, Table 1) [76,77]. The most common organization of the functional domains is **abb′xa′**, where the prime symbol stands for the relative position within the protein sequence (Table 1).

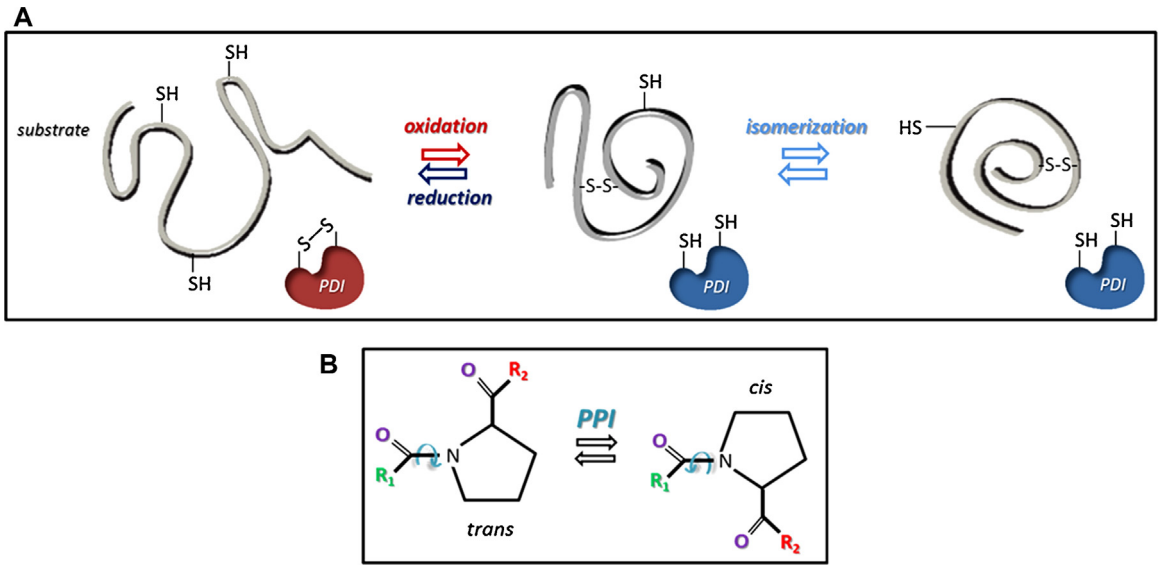

Fig. 3. Disulfide and peptidyl–prolyl bonds. Mechanisms of disulfide bond oxidation, reduction and isomerization catalyzed by PDI proteins (*upper panel*). Mechanism of peptidyl–prolyl *cis–trans* isomerization catalyzed by PPI proteins (*lower panel*).

Depending on the luminal redox environment, thiol-reactive PDIs can hypothetically catalyze the oxidation, the reduction or the isomerization of disulfide bonds (Fig. 3A) [78]. The sequence of the catalytic site, but also the surrounding amino acids in the 3D structure may help in predicting if the given enzyme acts as an oxidase (i.e., it favors the formation of covalent bonds between substrate cysteine residues), as a reductase or as an isomerase. For example, while nearly all oxidases possess a histidine residue within the active site sequence, the presence of a proline residue (as in ERdj5, in TMX1 and in TMX4 (Table 1)) strongly hints at a reductive activity of the enzyme [78,79]. In this review, we will focus on ERp57 role in protein folding because of the specific engagement of this

Table 1
PDI and PPI family members.

Protein	Domains	Active sites	Activities	References
PDI	*abb′xa′*	CGHC, CGHC	*O, R, I, C*	[70–78,81,83,86–89]
ERp57	*abb′xa′*	CGHC, CGHC	*O, R, I*	[41,42,64–66,70–73,76,77,81–85,90–98]
ERp72	$a^0abb'xa'$	CGHC, CGHC, CGHC	*O, R, I*	[68–73]
P5	*aa′b*	CGHC, CGHC	*O, R, I*	[70–73]
ERp44	*abb′*	CRFS	*Ero1 binding*	[71–73]
ERdj5	$Ja''ba^0aa'$	CSHC, CPPC, CHPC, CGPC	*R*	[70–73,78,79]
PDIp	*abb′xa′*	CGHC, CTHC	*O, R, I, C*	[70–73]
PDILT	*abb′xa′*	SKQS, SKKC	*Ero1 binding*	[70–73]
PDIr	ba^0aa'	CSMC, CGHC, CPHC	*O, R, I*	[70–73,78,79]
ERp46	a^0aa'	CGHC, CGHC, CGHC	*?*	[70–73]
ERp18	*a*	CGAC	*O, VKOR binding*	[70–73]
hAGR2	*a*	CPHS	*?*	[72,73]
hAGR3	*a*	CQYS	*?*	[72,73]
TMX1	*at*	CPAC	*R, VKOR binding*	[71–73,78,79]
TMX2	*ta*	SNDC	*?*	[71–73]
TMX3	*abt*	CGHC	*O*	[71–73]
TMX4	*at*	CPSC	*O, R, VKOR binding*	[71–73,78,79]
TXNDC15	*at*	CRFS	*?*	[72,73]
ERp29	*b*	–	*?*	[70–73,89]
ERp27	*bb′*	–	*ERp57 binding*	[71–73]
N33/Tusc3		CSVC	*OST complex subunit*	[13,80]
IAP/MagT1		CVVC	*OST complex subunit*	[13,80]
ERp90		–	*ERFAD interactor*	[151]

Protein	Inhibitors	References
FKBP13	FK506	[70,101–104]
FKBP19	FK506	[70,101–104]
FKBP22	FK506	[70,101–104]
FKBP23	FK506	[70,101–104]
FKBP60	FK506	[70,101–104]
FKBP65	FK506	[70,101–104]
CypB	Cyclosporine A	[67–70,101–106]
CypC	Cyclosporine A	[102]

List of the PDI family members (*upper table*).
a, active thioredoxin-like domain; ***b***, inactive thioredoxin-like domain; ***x***, linker domain; ***J***, J-domain; ***t***, transmembrane domain. The aminoacidic composition of the PDI active sites is shown in the third column. ***O***, oxidation; ***R***, reduction; ***I***, isomerization; ***C***, chaperone activity.
List of the ER-resident PPI members (*lower table*).

PDI family member in functional complexes with the ER lectins CNX and CRT. However, as a short note, the relevant function of the OST-associated oxidoreductases N33/Tusc3 and IAP/MagT1 in insuring efficient nascent protein glycosylation by delaying formation of substrate disulfide bonds should also be mentioned. This activity seems to be particularly important during brain development as dysfunction of these PDI family members is linked to mental retardation [13,80].

2.3.1.1. ERp57. ERp57 exhibits great similarity in domain composition (**abb′xa′**) and length (481 amino acids) with PDI [81]. The two oxidoreductases have an amino acid identity of about 29%, showing a large degree of variation at the level of the **b′** domain. In ERp57, the **b′** domain lacks the hydrophobic substrate-binding pocket insuring the PDI chaperone-like activity [82–84] and contains, instead, the binding region for the CNX and CRT P-domains [85].

2.3.1.2. Interaction between ERp57 and CNX or CRT. Whereas an association of PDI with the P-domain of CRT has originally been reported [86,87], subsequent studies showed that this interaction requires unphysiologic calcium concentration and should, therefore, not have a physiologic relevance [83,88,89]. In contrast, a large set of data proved a direct involvement of ERp57 in the CNX/CRT cycle [81,82,90–92]. The carbohydrate-dependent association between either CNX or CRT, newly synthesized glycoproteins and ERp57 [81] led to the discovery that the two lectins and the oxidoreductase work in combination to assist glycoproteins folding [91,93,94] and Section 2.3.1.3).

The peculiarities of ERp57-CRT and ERp57-CNX interactions have been revealed by TROSY-NMR spectroscopy. These studies established that ERp57 associates with the P-domain of CRT, more precisely with a region of the ER lectin comprising the residues 189–288. The interaction is short-lived, has low affinity (K_d of 9 μM) [82] and is stabilized by the presence of folding substrates [42]. Isothermal titration calorimetry (ITC) allowed the mapping of the minimal domain required for ERp57–CRT interaction to the distal part of the CRT P-domain (residues 221–256) [41]. The crystal structure of the ERp57 **bb′** domain revealed a set of positively charged amino acids in the external part of the ERp57 **b′** domain, which represents the moiety that binds to the negatively charged region of the CRT P-domain [84]. The CNX P-domain (residues 337–353) is the motif required for ERp57 binding [88], with an interaction affinity calculated in a K_d of 26 μM using ITC technique [78,84].

Altogether, these studies demonstrated that ERp57-CNX/CRT help with the productive maturation of glycoproteins by creating a strategic environment for protein folding.

2.3.1.3. ERp57 role in the CNX/CRT cycle. CNX and CRT bind the cargo protein oligosaccharides and ERp57 engages the folding proteins in mixed disulfides that represent short lived intermediates of the oxidative folding process [93]. The use of the trapping mutant version of ERp57 revealed that proteins containing small, disulfide-rich glycoproteins with a surprisingly low level of secondary structures are endogenous substrates of ERp57 [95].

At least in cultured cells, deletion of ERp57 is well-tolerated showing that most cellular glycoproteins can engage other folding assistants to eventually attain their native structure [96]. For example, ERp72 can efficiently replace ERp57 to ensure efficient maturation of Semliki forest virus glycoproteins [97]. In this context, the impaired maturation of *influenza* hemagglutinin or of proteins that require an extensive rearrangement of disulfides in cells lacking ERp57 [95,97] is a paradigm for those cellular proteins that are strictly dependent on CNX, CRT and ERp57 and whose incapacity to attain the functional conformation causes the embryonic or the early mortality in the corresponding knockout mice [64,65,98].

2.3.2. The PPI family: peptidyl–prolyl bonds isomerization

Peptide bonds connecting any amino acid with a proline residue can adopt both a *cis* or a *trans* conformation, with a weak preference for the *trans* conformation [99]. The isomerization of misarranged peptidyl–prolyl bonds can be a rate-limiting step during protein folding (Fig. 3B) [96,100] and is catalyzed by members of the PPI family, which comprises three sub-groups, parvulins, cyclophilins (Cyps) and FK506 binding proteins (FKBPs) (Table 1) [101,102]. The mammalian ER contains six members of the FKBPs and two members of the Cyps sub-groups (Table 1) [102–104].

2.3.2.1. Cyclophilin B. Cyclophilin B (CypB) is a protein of 216 residues that is inhibited by cyclosporine A [103]. Its function as a molecular chaperone has been established in studies on rhodopsin maturation and export in *D. melanogaster* [105]. Recently, it was shown that CypB and BiP enhance IgG maturation and secretion in B cells and that CypB inactivation by cyclosporine A delayed IgG synthesis [106]. Moreover, CypB was found to assist the oxidoreductase ERp72 in the folding of IgG C_H1, which was reported to have a high content in *cis* prolines [70].

2.3.2.2. Interaction between CypB and CNX or CRT. Several groups identified CypB as a component of multiprotein complexes containing CNX and CRT within the ER lumen (Fig. 2) [67–70]. Crystallography studies mapped the interaction site of CypB to the tip of the CNX and CRT P-domains. As such the association of ERp57 and of CypB to CNX and/or CRT is mutually exclusive [67]. The association of CypB with CNX/CRT requires a positively charged lysine-rich motif of CypB. The CypB-CNX and the CypB-CRT binding affinities have a K_d of about 10 μM. This data supports an active role of CypB in the CNX/CRT cycle by promoting the correct orientation of peptidyl–prolyl bonds in nascent polypeptides emerging in the ER lumen.

2.4. Regulation of the chaperone function and sub-cellular localization of CNX and CRT

Post-translational modifications such as phosphorylation and palmitoylation have been described to play a major role in defining the functions of CNX, the only ER lectin displaying a long cytosolic tail. These modifications have been implicated in modulating its location depending on the functional demands for CNX [17,107,108].

Several phosphorylation sites have been identified for CNX on its cytosolic C-terminal tail. These sites are modified by kinases such as casein kinase II (CKII) and the mitogen activated protein kinase ERK1. Phosphorylation of CNX has been proposed to increase its association with ribosomes possibly facilitating/promoting CNX association with nascent chains [107]. CNX phosphorylation is enhanced under conditions of ER stress that cause protein misfolding which prolongs interaction of CNX with maturing substrates such as α1AT thus delaying protein secretion [109].

Palmitoylation of CNX also plays a role in the regulation of its chaperone function and location, but data are somewhat controversial. In one study, palmitoylation was reported to target CNX to the ribosome–translocon complex to position it to better bind nascent chains entering into the ER lumen and ensure their proper maturation and secretion. Consistently, non-palmitoylated CNX shows reduced binding to substrates compared to palmitoylated CNX [110]. In contrast, another study proposed that non-palmitoylated CNX plays a more prominent role in folding while palmitoylated CNX interacts with the calcium channel SERCA2b and is implicated in calcium regulation [108]. This is demonstrated by a change in

the localization of CNX. Palmitoylation caused enrichment of CNX at the mitochondrial-associated membrane (MAMs), thus enhancing its role in calcium signaling while de-palmitoylation relocates CNX to the rough ER or the ER quality control compartment, thus enhancing its role in folding and quality control [108].

As some inconsistencies appear to exist concerning the role of palmitoylation in determining the function of CNX, further investigations are required to clarify the role of this modification. Post-translational modifications of CNX influence its localization and functions. Whether such mechanisms of regulation also occur for other ER resident proteins including CRT is an interesting issue that requires further investigation.

3. Role of UGT1 as a folding sensor

Release from the lectin chaperones CNX and CRT is followed by trimming of the innermost glucose residue by α-glucosidase II that prevents immediate re-association of the newly synthesized polypeptide to CNX and CRT. Here, a decision has to be made whether a protein is properly folded and should be targeted for anterograde trafficking or whether its forward transport should be prevented in order to maintain it longer in the folding environment or to select it for clearance from the ER lumen. Making such decisions requires the capacity to distinguish between folding intermediates to be retained in the folding environment (Fig. 2, **step 6**), native proteins to be released from the ER (**step 7**), and terminally misfolded proteins to be eventually destroyed (**steps 8 and 9**). Such virtues have been proposed to be possessed by UGT1. UGT1 has been described as a folding sensor because it can recognize structural imperfections such as exposed hydrophobic domains on maturing proteins, a function that is performed by its N-terminal domain stretching to about 80% of the protein [111,112]. Upon recognition of structural defects on cargo proteins, the UGT1 transfers a glucose residue onto the A branch of the glycans via its C-terminal catalytic domain (Fig. 2, **step 6**). This forces immature proteins to rebind to CNX and CRT for another round of folding attempts under the assistance of the associated enzymes ERp57 and CypB. An element of recognition by UGT1 on the glycan is the innermost GlcNAc residue [111]. That other yet undetermined elements are also recognized by UGT1 cannot be excluded.

Cycles of de-glucosylation and re-glucosylation occur [30], indicative of a role of UGT1 in retaining intermediates of folding programs and folding-defective polypeptides in the ER to enhance folding efficiency and to hamper secretion of aberrant proteins [32,33,113,114]. UGT1 also contributes to the luminal retention of unassembled subunits of multimeric complexes, thus promoting efficient and complete assembly [115] and, at least for some polypeptides, is required for structural maturation necessary for dissociation from CNX and CRT [116].

Significantly, UGT1 deletion poorly affects the stringency of ER quality control, which is ensured from sequentially operating retention machineries relying on the intervention of luminal chaperones such as BiP [117]. However, the sequential domain lysosomal protein prosaposin is an obligate substrate of UGT1 as it misfolds and is found in aggresome-like structures in its absence [118]. Prosaposin is efficiently reglucosylated by UGT1 as demonstrated using a cell-based reglucosylation assay. A recent investigation also showed that UGT1 promotes solubility of substrates prone to aggregation in the ER, which further hints at a potential role of UGT1 in alleviating cellular damage caused by aggregation [119].

There is discrepancy in the literature on substrate features that elicit UGT1 activity. A study showed that UGT1 is highly affected by the amino acid sequence proximal to the glycan to be reglucosylated [120], and that structural defects recognized by UGT1 are localized near the region containing the glycan [121]. Another study suggests that UGT1 can recognize glycans that are more distant to the region containing the folding defect [122]. To shed more light on this issue, structural analysis of the UGT1 substrate recognition domain may provide better understanding of the ability of UGT1 to differentiate between native and non-native substrates.

Earlier studies agree that UGT1 prefers substrates that are incompletely folded over native proteins; however, the data is conflicting about the severity of the structural imperfection that UGT1 prefers. Seminal UGT1 studies using purified UGT1 or microsomal membranes suggested that UGT1 recognizes severely misfolded substrates such as those that are urea denatured when compared to its native counterpart [123–126]. Yet, later studies showed that UGT1 has better affinity for nearly native proteins, late folding intermediates or proteins that contain only minor structural perturbations [127–130]. The latter conclusion would suggest that UGT1 would have better affinity for proteins on the pathway to fold, and might ignore proteins deemed severely misfolded. However, a recent cellular study found that UGT1 modifies terminally misfolded ERAD substrates such as NHK, T-cell receptor α-subunit, and an alpha-N-acetylgalactosaminidase (α-NAGAL) mutant more efficiently than on-pathway wild type substrates [114]. Additional studies are required to scrutinize the specificity for UGT1 in its native environment. A more detailed description of the UGT1 specificity is presented by Ito et al., in this issue.

UGT2 is a human homologue of UGT1 that shares 55% identity with UGT1, especially in the C-terminus domain that contains the catalytic site. However, its function remains unclear. It was originally proposed that UGT2 lacks re-glucosylation activity [131] yet, a recent study showed that UGT2 has re-glucosylation activity and similar specificity to UGT1. This study used a cell free re-glucosylation assay [132]. Further investigations are required to confirm the function of UGT2.

3.1. Proposed mechanisms of how substrates exit the CNX and CRT cycle

It is still not fully understood how the re-glucosylation/de-glucosylation cycles are terminated. Extensive mannose trimming results in exit of folding-defective proteins from the CNX/CRT cycle (Fig. 2, **step 8**). Trimming of the mannose to which the glucose is added in the A-branch would stop re-glucosylation [133,134], and trimming of the mannose residues of the B and C branches may decrease the affinity of UGT1 for glycans [124]. Moreover, removal of terminal α1,2-bonded mannose residues exposes an α1,6 mannose residue on the C-branch of the protein-bound oligosaccharide that recruits the MRH domain-containing lectins XTP3-B and OS-9 that target aberrant polypeptides for ERAD [11]. The selection of glycosylated ERAD substrates is discussed more thoroughly in the article by Benyair et al., in this issue.

4. Export from the ER: proposed models and roles of ERGIC-53, VIP36 and other lectins in glycoprotein sorting

After leaving the CNX/CRT binding cycle, proteins that have folded properly will be targeted for trafficking to the Golgi via the ER/Golgi intermediate compartment (ERGIC) (Fig. 2, **step 7**). Exit from the ER to the Golgi could occur either by bulk flow, which allows the cargo to be incorporated into COPII vesicles without any sort of selection process or by receptor mediated transport, which involves interaction of the proteins of the coat of the vesicles with membrane receptors that bind to the cargo [135].

In the bulk flow model, it is proposed that proteins leave the ER via the fluid or the membrane of forming vesicles without the intervention of receptors. In this transport model, cargo is not

concentrated [136]. The flow of cargo out of the ER is completely controlled by factors that retain immature or non-native proteins such as the lectin chaperones CNX and CRT. Native and properly assembled proteins that no longer bind chaperones or ER factors are free to exit in COPII vesicles by bulk flow.

In the receptor mediated transport model, receptor interaction with cargo involves glycans. ERGIC-53, VIPL and VIP36 are classified as lectin receptors due to their carbohydrate binding capability. These lectins share similar structural characteristics in their carbohydrate binding domain [137]. Yet, the specificity of these lectin receptors is not identical because the composition of the glycan to which these lectin receptors bind can vary. While VIP36 and VIPL bind to high mannose type oligosaccharides having a de-glucosylated branch A [137,138], ERGIC-53 has a lower binding affinity and broader specificity and can bind to glycans with both glucosylated and de-glucosylated branch A [137]. Structural analysis suggested that the broad specificity of ERGIC-53 is due to a shallower sugar binding pocket in ERGIC-53 and the outward orientation of the 3-OH of the terminal mannose residue, which allows a glucose residue to fit in the binding pocket of ERGIC-53 without steric hindrance [139]. Although they have been shown to bind different substrates, ERGIC-53 and VIP36 appear to share some substrates such as α1AT [138,140]. Substrates specific to ERGIC-53 include the hemophilia associated blood coagulation factors V and VIII, as well as cathepsin Z and C [141–143]. VIP36 binds to α-amylase and clusterin [144,145] and the more recently identified substrate guanylyl cyclase C [146]. While some of the carbohydrate and protein signals that are utilized by selective-transport route have been identified [147,148], what determines that a substrate follows the bulk flow route or the selective receptor mediated transport route is not fully understood.

5. Concluding remarks

While a great wealth of information has been acquired on the role of glycans in maturation and quality control of proteins expressed in the ER of Eukarya, the recent discovery of additional lectins and quality control factors highlights both the complex principles governing protein production and the needs for further studies. Many questions remain to be answered and perhaps models to be re-visited regarding the mechanism of action of the known folding and quality control machineries. To understand how these respond to variation in the quantity and the quality of the cargo load is crucial for designing therapeutic strategies for diseases caused by production, accumulation or destruction of aberrant polypeptides or to intervene to contrast infection by pathogens that exploit the host cell protein factory during their infection cycle. For instance, one strategy has been the use of α-glucosidase I and II inhibitors to inhibit viral infectivity [149] based on the findings that viral glycoproteins are more dependent on CNX/CRT assistance for folding than cellular proteins [63]. Inhibiting glucosidase trimming prevents the entry of viral proteins into the CNX/CRT cycle [150]. This leads to misfolding of viral proteins and their eventual degradation and subsequent activation of the immune response. Derivatives of the iminosugars castanospermine and deoxynojirmycin have been widely used to inhibit the infectivity of a vast range of both DNA and RNA viruses. Some of these inhibitors have entered clinical trials [149]. Optimization of the lectin chaperone pathway may also help to correct the folding of defective proteins associated with loss of function diseases [96] or increase the expression of recombinant proteins used as therapeutics. Future studies will shed more light on how exploiting the components of the quality control network can ameliorate disease phenotypes or provide more effective therapies.

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