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REVIEW ARTICLE

# Calnexin, Calreticulin, and ERp57

*Teammates in Glycoprotein Folding*

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## Abstract

In eukaryotic cells, the endoplasmic reticulum (ER) plays an essential role in the synthesis and maturation of a variety of important secretory and membrane proteins. For glycoproteins, the ER possesses a dedicated maturation system, which assists folding and ensures the quality of final products before ER release. Essential components of this system include the lectin chaperones calnexin (CNX) and calreticulin (CRT) and their associated co-chaperone ERp57, a glycoprotein specific thiol-disulfide oxidoreductase. The significance of this system is underscored by the fact that CNX and CRT interact with practically all glycoproteins investigated to date, and by the debilitating phenotypes revealed in knockout mice deficient in either gene. Compared to other important chaperone systems, such as the Hsp70s, Hsp90s and GroEL/GroES, the principles whereby this system works at the molecular level are relatively poorly understood. However, recent structural and biochemical data have provided important new insights into this chaperone system and present a solid basis for further mechanistic studies.

**Index Entries:** Calnexin; calreticulin; endoplasmic reticulum; ERp57; lectin; molecular chaperone; oxidoreductase.

## INTRODUCTION

The addition of N-linked glycans to newly synthesized polypeptide chains occurs in the endoplasmic reticulum (ER). Here the 14-saccharide core glycan (Fig. 1), $Glc_3Man_9GlcNAc_2$, is added to the growing nascent polypeptide chain from a dolicholpyrophosphate-linked precursor in a reaction catalyzed by the oligosaccharyl transferase complex *(1,2)*. The modified asparagine side chain occurs within the Asn-Xxx-Ser/Thr consensus sequence, where Xxx denotes any amino acid except proline *(3)*. Although only relatively few of the soluble ER-resident proteins seem to be glycosylated, the majority of extracellular proteins produced in mammalian cells carry N-linked glycans *(4)*. The large number of different glycoproteins that traffic through the ER are involved in many fundamental intra- and intercellular processes. Consequently, mutations in

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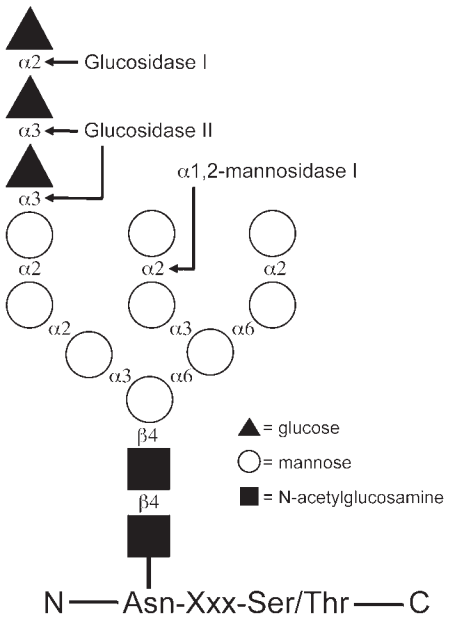



Fig. 1. Schematic representation of the N-linked core oligosaccharide attached to an asparagine side chain in an Asn-Xxx-Ser/Thr amino acid consensus sequence. The linkage for each individual glycosyl residue is indicated along with cleavage sites for various ER enzymes that modify the sugar structure.

proteins of the N-glycosylation pathway are known to cause severe disease phenotypes, and a number of congenital disorders of glycosylation are known *(5,6)*.

The sugar itself can directly modulate protein structure and function, e.g., by increasing properties such as stability and solubility *(7–11)*. In addition, N-linked glycans act as tags in intracellular trafficking *(12)*, and accordingly different lectins act as sorting receptors in the secretory pathway *(13–15)*. Despite the many different and widely recognized functions of *N*-linked glycans, the role of the N-linked glycan in protein folding in the ER has only become fully appreciated within the past 10 yr. Specifically, it has been found that modifications of the core glycan to generate a monoglucosylated ($Glc_1Man_9GlcNAc_2$) form determine the interaction with two homologous lectin chaperones, calnexin (CNX) and calreticulin (CRT).

Studies in tissue culture cells and ER-derived microsomes have demonstrated that the interaction with CNX and CRT protects glycoproteins from aggregation and premature degradation *(16–20)*. Repeated cycles of binding to and release from CNX and CRT, controlled by enzymes that modify the core oligosaccharide, ensure the retention of substrate glycoproteins in the favorable folding environment of the ER until correctly folded. If permanently misfolded, these substrate proteins are degraded. At least for certain glycoproteins, the removal of a mannose residue in the middle branch of the glycan is a signal for degradation *(21)*. A recently discovered mannose-binding lectin, EDEM, specifically accelerates the degradation of glycoproteins and could well be directly involved in targeting $Man_8GlcNAc_2$-containing proteins for degradation *(22–28)*. Overall, the oligosaccharide and the different enzymes that modify it play important roles in glycoprotein folding and degradation. These aspects have been covered in great detail in other reviews *(29–33)*.

Cell biological studies have provided most of our current knowledge about the CNX/CRT chaperone system. However, more recent work has revealed structural information at atomic resolution for both CNX and CRT, along with details about their interaction with the co-chaperone ERp57. Moreover, other studies have explored the possibility that protein–protein interactions, in addition to the glycan-mediated interaction, contribute to the function of CNX and CRT as molecular chaperones. Taken together, these investigations have considerably increased our understanding of the molecular basis for glycoprotein folding and have also raised interesting new questions. This review describes the function of the CNX/CRT chaperone system. In particular, we emphasize the role of molecular properties of CNX, CRT, and ERp57 in determining the mechanism of chaperone function in the living cell.

## ER QUALITY CONTROL

The endoplasmic reticulum (ER) plays a fundamental role in the synthesis, folding, and assembly of numerous important proteins, such as cell-surface receptors, membrane channels, extracellular matrix components, serum proteins, and antibodies. The environment in the ER is optimal for the correct folding and maturation of such proteins. For many proteins the maturation process involves co- and posttranslational modifications, such as signal-peptide cleavage, glycophosphatidylinositol (GPI)-anchor addition and N-linked glycosylation. Furthermore, the oxidizing milieu of the ER supports the formation of disulfide bonds. This stabilization of protein conformation is likely to help proteins maintain their structure in the extracellular environment. Finally, the ER is rich in chaperones and enzymes, which are crucial in assisting the process of correct protein folding *(34,35)*.

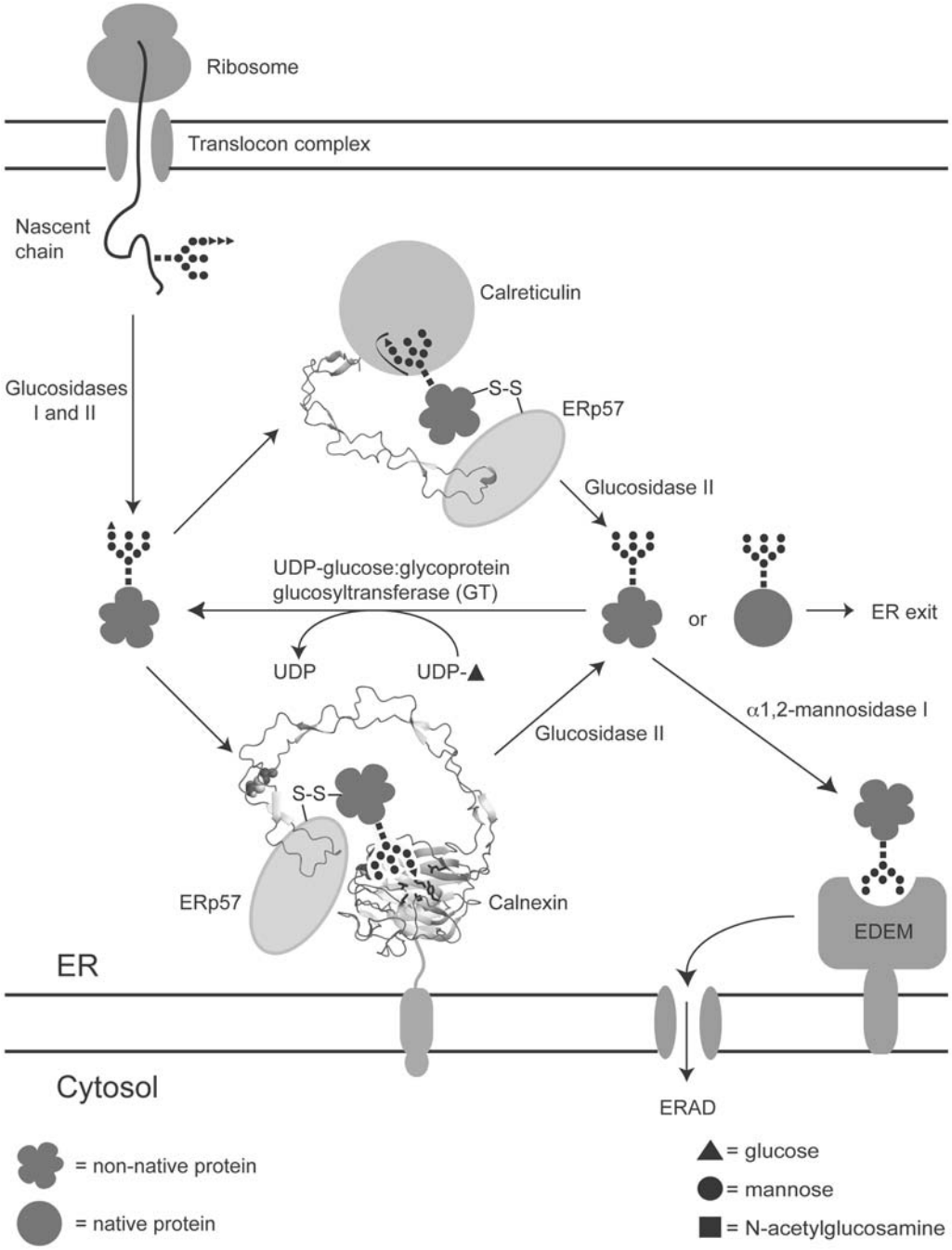
Still, incorrectly folded or incompletely assembled proteins are common side products during protein synthesis in the ER. Such products, which could be harmful to the cell if allowed to proceed along the secretory pathway to the cell surface or another cellular location, are subject to a stringent quality control (QC) system *(36,37)*. A number of general chaperones, including BiP, a member of the Hsp70 family of chaperones, and protein disulfide isomerase (PDI), a thiol-disulfide oxidoreductase, recognize and retain proteins that expose non-native features. This system ensures that misfolded and incorrectly assembled proteins are retained in the ER and eventually degraded. Many so-called ER storage diseases are known, which arise from the ER retention of mutant alleles of certain proteins. Such diseases include cystic fibrosis and emphysema (for reviews, *see* refs. *38–41*).

Defective ER-retained proteins are typically degraded by the proteasome after selective retrotranslocation to the cytosol and ubiquitination *(42)*. This process is referred to as ER-associated degradation (ERAD). Moreover, the cell responds to increased misfolding of proteins in the ER by the unfolded protein response (UPR) (reviewed in ref. *43*). Signaling from the ER to the nucleus leads to increased transcription of genes encoding ER chaperones to help alleviate the folding problem. In addition, another signaling pathway involving phosphorylation of the translation initiation factor eIF2α leads to attenuation of protein translation to further reduce the load on the ER folding machinery. Lastly, yet other genes involved in degradation are upregulated, as recently shown in the case of EDEM (*28)*. Several investigations show that the processes of ERAD and UPR are closely coordinated (*44–46)*.

## THE CALNEXIN/CALRETICULIN CYCLE

The QC system described above applies to all proteins that encounter the lumen of the ER or are inserted into the ER membrane. CNX, CRT, and ERp57 are important factors of this general ER QC system *(47)*.

CNX and CRT cooperate with a number of enzymes in the process of assisting glycoprotein folding. The first step of this so-called CNX/CRT cycle (*see* Fig. 2) involves binding to either chaperone through the monoglucosylated glycan, $Glc_1Man_9GlcNAc_2$, present on nascent chains and on newly synthesized glycoproteins. This form of the sugar appears either as a trimmed intermediate of the triglucosylated core oligosaccharide or by readdition of a glucose residue to the fully deglucosylated glycan (*see* below). Sequential trimming of the two outermost glucose residues on the core oligosaccharide is executed by glucosidases I and II. The importance of monoglucosylation for CNX and CRT binding has been shown in living cells, in microsomes and in vitro for a number of different proteins (*see* for instance refs. *48–56*). Many such studies have used inhibitors of glucosidase II, such as castanospermine and deoxynojirimycin, to prevent the formation of the monoglucosylated, trimmed intermediate of the original core oligosaccharide. Under these experimental conditions, access to CNX and CRT is prevented and proteins are retained in the ER where they often form high molecular weight complexes




and eventually are targeted for degradation. In addition to the initial binding through the monoglucosylated glycan, protein–protein interactions of CNX and CRT with their substrates could well contribute to the chaperone function of both proteins (*see* below) *(57,58)*.

The process of disulfide bond formation in glycoprotein substrates of CNX and CRT is assisted by the thiol-disulfide oxidoreductase ERp57, which is found noncovalently associated with both proteins in vivo *(47,59,60)*. The importance of ERp57 in the CNX/CRT cycle has

Fig. 2. The calnexin/calreticulin cycle. The folding of newly synthesized glycoproteins in the ER is assisted by calnexin (CNX) and calreticulin (CRT). Both proteins bind to the monoglucosylated form of the glycoprotein generated after the initial removal of two glucoses by glucosidases I and II. Here, we have used the available three-dimensional structures of CNX (the lumenal domain) and CRT (the P-domain) to depict parts of the two molecules (*see also* Fig. 4). The glycan binds to the lectin domain of both chaperones—in CRT the glycan binding site is represented by the curved black line. Other interactions can occur through protein-protein contacts (not depicted). Disulfide bond formation in glycoprotein substrates of CNX and CRT is catalyzed by the associated thiol-disulfide oxidoreductase ERp57, with which substrates form transient mixed disulfide intermediates. In both CNX and CRT, the tip region of the P-domain mediates the interaction with ERp57. Removal of the remaining glucose, in a reaction catalyzed by glucosidase II, prevents interaction with CNX and CRT. Upon release, one of three possible fates awaits the glycoprotein. First, if the protein has reached its native conformation it is not longer retained in the ER and is free to travel along the secretory pathway. Second, if the protein is still not correctly folded, it can be recognized by the UDP-glucose:glycoprotein glucosyltransferase (GT). This enzyme uses UDP-glucose as a sugar donor to reglucosylate non-native glycoproteins carrying high-mannose glycans. Consequently, it acts as a folding sensor in CNX/CRT cycle. The readdition of a glucose residue to the N-linked glycan promotes reassociation with CNX and CRT. An important role of CNX and CRT is to retain glycoproteins in the ER, where the conditions for folding are favorable. Finally, prolonged ER-retention increases the chances of encountering the ER α1,2-mannosidase I. This enzyme removes a mannose residue in the middle branch of the glycan to generate a form with eight mannoses (*see also* Fig. 1). A novel ER lectin, EDEM, is likely to recognize this form of the glycan and thereby extract the glycoprotein from the CNX/CRT cycle. Moreover, EDEM directs the glycoprotein for degradation by the ERAD pathway.

become apparent from studies showing that the protein interacts with soluble secretory proteins, as well as integral membrane proteins, carrying N-linked glycans (*59,61)*. As observed for substrates of CNX and CRT, both the association and release of substrates from ERp57 are modulated by glucose trimming (*61–63)*. That the addition of glucosidase inhibitors can result in impaired disulfide bond formation was recently shown in the case of CD1d heavy chain, which interacts with CNX and CRT during folding *(64)*. Furthermore, the direct involvement of ERp57 in the oxidative folding of glycoproteins is evident from the finding that the protein forms transient mixed disulfides with CNX- and CRT-associated glycoproteins during folding in living cells *(63)*. Thus, by interacting with CNX and CRT, ERp57 functions as a specialized thiol-disulfide oxidoreductase for glycoproteins.

Glycoproteins are released from CNX and CRT by the action of glucosidase II, which removes the terminal glucose of the glycan *(49,54)*. This step most likely occurs irrespective of the folding state of the glycoprotein, and prevents its renewed association with CNX and CRT. If not correctly folded at this stage, the glycoprotein is recognized by UDP-glucose:glycoprotein glucosyltransferase (GT). This enzyme works as a folding sensor and only re-adds a glucose residue to the oligosaccharide on non-native glycoproteins *(29)*. In this way, the action of GT ensures that proteins in a misfolded conformation can reassociate with CNX and CRT *(52,54,65)*. GT has been shown to detect misfolding on a domain level *(66)*, and even local folding defects in one domain can be distinguished so that only glycans in structurally destabilized regions are reglucosylated (C. Ritter, K. Quirin and A. Helenius, personal communication). Therefore, surveillance can take place on a domain-by-domain basis and it can be hypothesized that glycosylation sites in

domains with a tendency for misfolding have been maintained throughout evolution in order to optimize the folding efficiency of glycoproteins carrying such domains.

Overall, cycles of binding and release slow down the rate of folding but increase its efficiency for many glycoproteins by keeping them exposed to the ER QC system *(20)*. Like other protein QC systems in the cell, the detection of non-native glycoproteins in the ER by GT relies on features of the polypeptide chain that distinguish it from proteins in a native conformation. The benefit of using sugars as "reporter molecules" for the protein folding status likely relates to the fact that the modifications of the core oligosaccharide that control the fate of glycoproteins in the ER are independent of the specific protein. Thereby the glycan, which is present in a large number of molecules, works as a highly versatile tag that can be modified and recognized by a relatively small number of ER-resident enzymes and lectins.

## SUBSTRATE INTERACTIONS OF CALNEXIN AND CALRETICULIN

A wide variety of important cellular and viral glycoproteins are known substrates of CNX and CRT. These include HIV gp120 and gp160 *(67–70)*, class I major histocompatibility complex (MHC) heavy chain *(17,71–77)*, T-cell receptor subunits *(71,78)*, $\alpha_1$-antitrypsin *(50,79,80)*, tyrosinase *(81–83)*, the prion protein *(84,85)*, and the cystic fibrosis transmembrane conductance regulator (CFTR) *(86,87)*. Although many glycoproteins associate with both CNX and CRT, and despite having the same glycan specificity, these two lectins can have distinct roles in glycoprotein maturation. For instance, they can bind to the same protein at different stages of the folding process as seen for both the MHC class I heavy chain *(88,89)* and influenza hemagglutinin (HA) *(18)*. For the latter, association has been shown to depend on the position of the sugar within the molecule. Whereas CRT associates preferentially with the glycans of the HA top domain, CNX associates more efficiently with those glycans present in the membrane-proximal stem domain *(90)*.

The set of substrates bound by the two proteins is largely, but not completely, overlapping (*51,91)*. In line with the results obtained for HA, the difference in substrate recognition pattern has been shown to depend on the presence of the membrane anchor in CNX (*92,93)*. However, the finding that the lumenal domain of CNX cannot complement the function of CRT in MHC class I assembly when expressed in CRT-deficient cells indicates that the two proteins possess protein specific functions despite their many similarities *(76)*.

As mentioned previously, the exact mode of interaction by CNX and CRT with their glycoprotein substrates is not entirely clear. Studies performed in vitro using RNaseB as a model glycoprotein and studies of glycoprotein folding in the protozoan parasite *Trypanosoma cruzi* suggest that the function of CNX and CRT as molecular chaperones can be attributed solely to their lectin activity *(53,55,94)*. However, assays of aggregation and refolding using purified proteins as substrates show that CNX and CRT could have an additional function as classical chaperones, characterized by protein–protein contacts with hydrophobic regions exposed by non-native polypeptide chains *(57,58)*. As observed for classical chaperones, CNX and CRT can suppress aggregation and preserve proteins in a folding competent state, independent of their glycosylation status (monoglucosylated or nonglycosylated) *(57,58)*. However, the efficiency of suppressing aggregation was enhanced for monoglucosylated proteins, potentially indicating an avidity effect of two binding sites, one for the glycan and one for the polypeptide chain of the substrate *(95)*. Whereas native conformers were shown not to interact with CNX and CRT, complexes were formed with misfolded conformers.

The effects of cofactors, such as $Zn^{2+}$, adenosine triphosphate (ATP) and the monoglucosylated glycan on this bonafide chaperone function of CNX and CRT, have also been investigated in vitro. These cofactors are known to modulate structural properties of

CNX and CRT (*see* below). Although ATP was shown to enhance the ability to suppress protein aggregation, addition of the monoglucosylated glycan inhibited this function *(57,58)*. The latter result indicated that occupation of the oligosaccharide binding site is capable of influencing the protein–protein interactions proposed to mediate the chaperone function.

Overall, these experiments support the notion that protein–protein based contacts of CNX and CRT with their substrates, in addition to an initial glycan based interaction, play an important role during folding *(57,58)*. Moreover, ATP binding by CNX and CRT, and potentially $Zn^{2+}$ binding in the case of CRT, could promote substrate interaction by leading to the exposure of hydrophobic surface. Substrate release would then occur by ATP hydrolysis or dissociation, both processes possibly mediated by a co-chaperone.

## MOLECULAR PROPERTIES OF CALNEXIN AND CALRETICULIN

Besides its well-established role as a molecular chaperone, CRT is a major $Ca^{2+}$ storage protein and plays an important role in $Ca^{2+}$ homeostasis in the ER *(96,97)*. Thus, the embryonic lethal phenotype observed in CRT-deficient mice is connected to the function of the protein in $Ca^{2+}$ signaling during cardiac development *(99,100)*. Although CNX also binds $Ca^{2+}$ *(101,102)*, the protein does not seem to play a significant role in $Ca^{2+}$ homeostasis and its major function is that of a molecular chaperone. CNX gene-deficient mice do not show as serious defects as CRT knockout mice. However, 50% die within 2 d of birth, whereas the rest display severe motor disorders, a selective loss of large myelinated fibers and die within 3 mo *(103)*.

Biochemical studies of CNX and CRT are abundant, and below the most important biochemical properties of the two proteins are summarized. Together with structural data, these molecular characteristics constitute the basis for understanding the mechanism of chaperone function of CNX and CRT in detail.

### *Primary Structure*

The molecular cloning of CRT (46.5 kDa, 400 residues) *(104,105)* and CNX (65.4 kDa, 572 residues) *(101,102)* has revealed that the two proteins are highly similar (Fig. 3). The main differences are found in their carboxyl terminal regions. Whereas CRT is a soluble luminal protein with a carboxyl terminal KDEL retrieval sequence, CNX is a type I transmembrane protein with a predicted transmembrane region spanning residues 463–485, followed by a 87-residue carboxyl terminal cytosolic tail. However, the luminal domain of CNX is highly similar to CRT. Based on sequence analysis, both proteins have been suggested to consist of three regions, the N-, P-, and C-domains (Fig. 4) *(104–107)*. The N-domain (CRT residues 1–188, CNX residues 1–253) was originally predicted to comprise a β-sheet rich globular structure *(104,105)*. The unique P-domain (CRT residues 189–283, CNX residues 254–388) constitutes the signature sequence for this family of proteins, and has obtained its name due to the many prolines present in this region. It consists in its entire length of two short sequence repeats, the type 1 and type 2 repeats. Each type of repeat is present in three and four copies in CRT and CNX, respectively. In both proteins, the arrangement of the repeat sequences is such that all type 1 repeats are clustered together, followed by a cluster of type 2 repeats (Figs. 3 and 4). The distinguishing feature of the CRT C-domain (residues 284–400) is the enrichment of acidic amino-acid residues in the approx 60 carboxyl terminal residues. This region is known to play a role in low-affinity $Ca^{2+}$ binding *(106,108,109)*. In contrast, the C-domain of CNX, residues 389–462, does not display noticeable traits. Here, the membrane-proximal region of approx 20 residues is likely to represent a linker sequence between the membrane anchor and a globular lectin domain (*see* below).

The sequences of both CNX and CRT encode cysteine residues that form intrachain disulfide bonds. In CNX the four cysteines pair to form

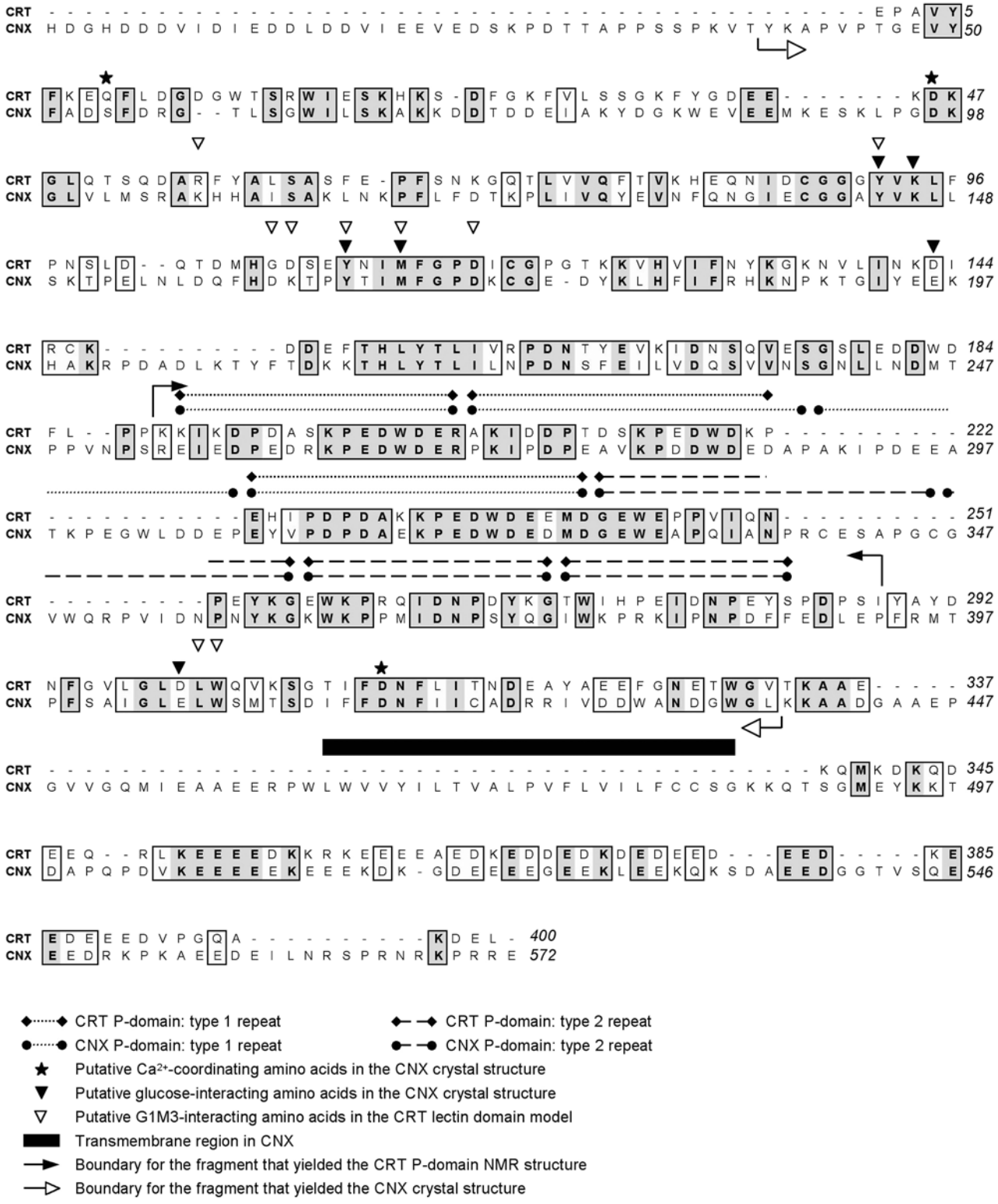

CRT P-domain: type 1 repeat
CNX P-domain: type 1 repeat
CRT P-domain: type 2 repeat
CNX P-domain: type 2 repeat
Putative Ca2+-coordinating amino acids in the CNX crystal structure
Putative glucose-interacting amino acids in the CNX crystal structure
Putative G1M3-interacting amino acids in the CRT lectin domain model
Transmembrane region in CNX
Boundary for the fragment that yielded the CRT P-domain NMR structure
Boundary for the fragment that yielded the CNX crystal structure

Fig. 3. Amino-acid sequence alignment of human CNX and human CRT. Both sequences are shown without amino terminal signal sequences and numbered accordingly. Repeat sequences of the P-domain are indicated above the alignment. Amino-acid residues proposed to coordinate $Ca^{2+}$ in the crystal structure of the lumenal domain of CNX are labeled with an asterisk, and amino-acid residues proposed to interact with the glycan are marked with a triangle. The black bar denotes the transmembrane region in CNX. Boundaries for the NMR structure of the CRT P-domain (residues 189–288) and for the crystal structure of the lumenal domain of CNX (residues 40–437) are indicated above and below the respective sequences with arrows.

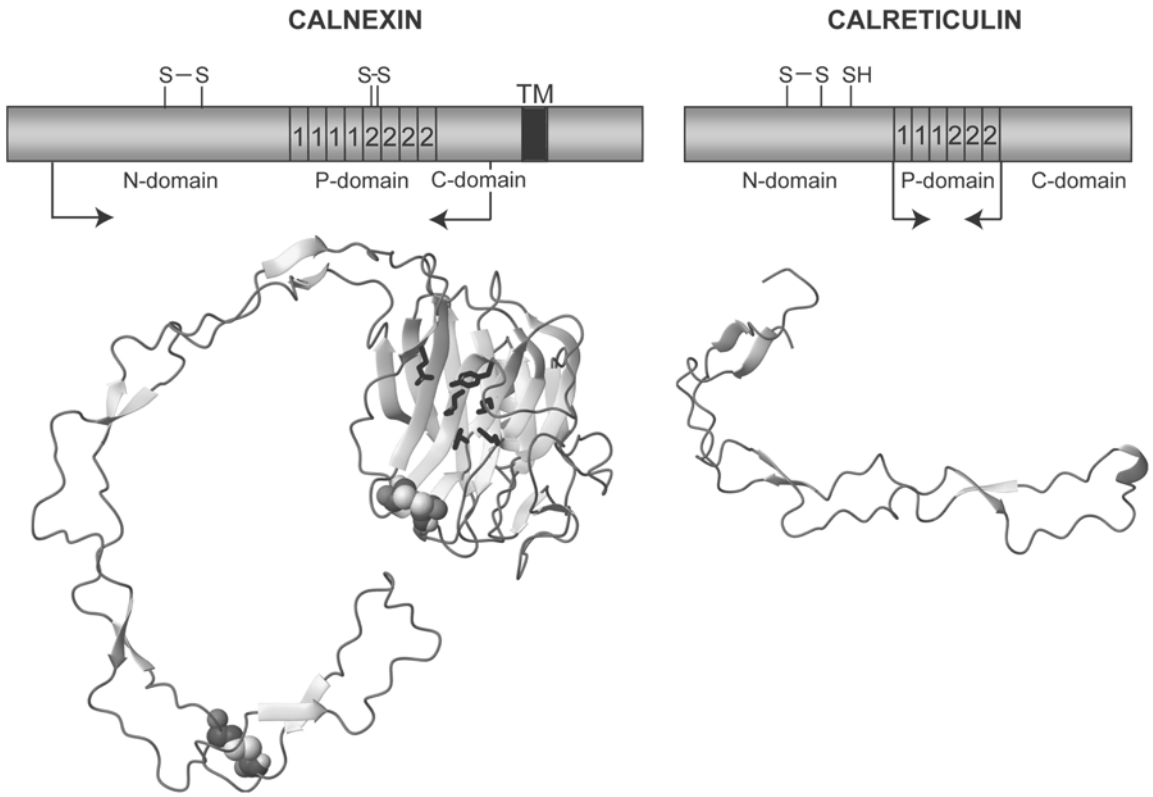



Fig. 4. Overview of the primary amino-acid sequence of CNX and CRT, and the three-dimensional structures solved for the two proteins. In the schematic representation, the position of type 1 and type 2 repeats of the P-domain is indicated along with the position of free and disulfide-bonded cysteine residues. 'TM' denotes the transmembrane region in CNX. The arrows indicate the boundaries of the fragments for which the three-dimensional structures are shown below. In the representation of the three-dimensional structure of the lumenal domain of CNX, residues involved in the binding of the glucose are shown as black stick models and the cysteines forming the two disulfide bonds are shown as CPK models. This figure was prepared using the program MOLMOL.

the Cys140–Cys174 and Cys340–Cys346 disulfides *(110)*. In CRT a free cysteine is present at position 146, whereas the Cys89–Cys120 disulfide is equivalent to the Cys140–Cys174 disulfide in CNX *(111)*. Although certain animal CRT sequences contain a conserved N-glycosylation site at residue 326, human placental CRT has been shown not to be glycosylated *(111)*. Animal CNXs do not contain potential sites for N-linked glycosylation. A unique feature of the carboxyl terminal cytosolic tail of CNX is the presence of sites for phosphorylation by casein kinase II at Ser534 and Ser544 and by extracellular-signal regulated kinase-1 at Ser563 *(112,113)*. Phosphorylation at these positions has been shown to regulate the association of CNX with ribosomes *(114)*.

Both CNX and CRT have a tissue-specific isoform in testis. The CNX isoform, calmegin, interacts with nascent chains of glycoproteins and is required for male fertility in mice *(115,116)*, whereas the function of the recently discovered CRT isoform, CRT2, has not yet been investigated *(117)*. The sequence similarity of both testis isoforms with the respective ubiquitously expressed forms is high (>60%), and it is very likely that the isoforms also function as lectin chaperones *(117–119)*.

## $Ca^{2+}$ Binding

Different cofactors have been found to interact with CNX and CRT in vitro and thereby modulate the structural properties of both proteins. The effects of metal binding by CRT have been thoroughly characterized by a variety of methods, such as circular dichroism, intrinsic fluorescence, 8-anilino-1-naphtalenesulfonate (ANS) binding and equilibrium dialysis. Of twelve different divalent cations tested for binding to CRT, only $Ca^{2+}$ and $Zn^{2+}$ were shown to bind specifically *(109)*. CNX binds $Ca^{2+}$ *(101,102,120,121)*, whereas there is little evidence that the protein binds other metals.

Owing to its role in $Ca^{2+}$ homeostasis, the $Ca^{2+}$-binding properties of CRT have been particularly well investigated (*see* for instance, refs. *106,108,109,122–125*). The protein has two distinct classes of binding sites: one high-affinity binding site that complexes 1 mol $Ca^{2+}$/mol protein with a $K_d$ of 0.05–11 μ*M*, and several low-affinity sites, located in the acidic C-terminal domain, that bind approx 20 mol $Ca^{2+}$/mol protein with a $K_d$ of 2 m*M* *(106,108,109)*. As pointed out earlier *(107)*, the rather broad range of $K_d$ values determined for high-affinity $Ca^{2+}$ binding by CRT is likely a result of the different experimental conditions employed in these studies.

$Ca^{2+}$ binding by CRT seems to have only little effect on the content of regular secondary structure elements and on the global structure of the protein *(109,123,125,126)*. However, upon occupancy of the high-affinity $Ca^{2+}$ binding site, local structural effects are observed that indicate a more compact conformation with increased thermal stability *(109,125)*. These results can be rationalized based on the crystal structure of the lumenal domain of CNX, which coordinates one $Ca^{2+}$ ion with a proposed role of structural stabilization *(110)*. In addition, CRT shows increased resistance toward proteolytic digestion by a variety of proteases at high $Ca^{2+}$ concentrations *(124,125)*. A similar result has been reported for CNX *(121)*.

## $Zn^{2+}$ Binding

CRT contains one high-affinity binding site for $Zn^{2+}$ (apparent $K_d$ of 0.05 μ*M*) and 14 low-affinity binding sites (apparent $K_d$ of 310 μ*M*) *(109)*. $Zn^{2+}$ binding by CRT has been mapped to the N-domain *(127)* and has been shown to induce a considerable conformational change in the protein resulting in the exposure of hydrophobic surface *(57,109)*. In accordance with these results, CRT is found to aggregate at concentrations of $Zn^{2+}$ above 600 μ*M* *(109,127)*, whereas CNX aggregates already in the presence of 100 μ*M* $Zn^{2+}$ *(128)*. In CRT the observed conformational change is accompanied by an increased susceptibility toward proteolytic digestion by trypsin and chymotrypsin and a decreased thermal stability *(125)*.

As proposed by several authors, the exposure of hydrophobic surface by CRT observed

in vitro in the presence of concentrations as low as 50 μ*M* $ZnCl_2$ could potentially be important for the ability of the protein to suppress aggregation through protein–protein interactions with its substrates *(57,124,125)*. Unfortunately, although $Zn^{2+}$ is present in the ER lumen, little is known about its concentration and all the mammalian zinc transporters described to date are localized to cellular compartments other than the ER. However, recent studies show that the ER of *Schizosaccharomyces pombe* harbors a zinc transporter thought to regulate ER zinc homeostasis in this organism *(129,130)*.

## *ATP Binding*

Another potentially important cofactor of CNX and CRT is ATP. Both proteins bind ATP in vitro *(57,58,121,131)*. Although a weak ATPase activity has been reported for both proteins *(57,58)*, others have been unable to detect any such activity *(121,124)*. For both proteins, the binding of ATP is accompanied by a decrease in intrinsic fluorescence emission and enhanced binding of ANS, indicating exposure of hydrophobic surface *(57,58)*. Whereas the presence of ATP protects the carboxyl terminal region of CRT from proteolysis *(124)*, both destabilizing and stabilizing effects of ATP binding by CNX have been reported *(121,131)*.

Currently, the physiological significance of ATP binding by CNX and CRT remains unclear. Classical chaperones such as those belonging to the Hsp70 family, including the ER-resident protein BiP, are ATPases. Cycles of ATP binding, hydrolysis and nucleotide exchange control substrate binding and release, and are, in turn, regulated by cofactors that either influence ATPase activity or act as nucleotide exchange factors. The reported ATPase activity of <0.1 pmol/min/μg for CNX and CRT is very low *(57,58)*. In comparison, BiP, which is considered a weak ATPase, has an activity of 5.2 pmol/min/μg *(132)*. Thus, if ATP hydrolysis plays a role for the chaperone function of CNX and CRT, positive regulators of ATPase activity and nucleotide exchange are likely to exist.

## *Glycan Binding*

A number of studies performed in vitro have characterized the direct interaction of CNX and CRT with isolated glycans using biochemical methods. All support the notion that both proteins are lectins that specifically interact with the monoglucosylated form of the core glycan (Fig. 1) *(50,131,133–135)*. These studies also show that CNX and CRT interact with glycans with increasing affinity when comparing oligosaccharides of different lengths as follows: Glc(G1) << Glcα1-3Man(G1M1) << Glcα1-3Manα1-2Man(G1M2) < Glcα1-3Manα1-2Manα1-2Man(G1M3). Therefore, the glycan interaction is likely to involve the entire α1-3 branch of the oligosaccharide, which forms a continuous molecular surface in the NMR structure of $Glc_1Man_9GlcNAc_2$ *(136)*. Direct affinity measurements have shown that the G1M3 tetrasaccharide binds CRT with comparable affinity to an IgG molecule carrying the entire monoglucosylated glycan *(135)*. However, glycan binding by CNX and CRT could potentially also involve contacts to the mannose residues on the α1-6 branch of the glycan *(50,133)*.

Detailed biophysical analysis of the binding of IgG carrying a single monoglucosylated glycan to CRT demonstrated a $K_d$ of approx 2 μ*M (137)*. Furthermore, this study showed that—at least under these experimental conditions using a native monoglucosylated protein—no contribution to the binding reaction was observed from protein–protein interactions. As proposed by Surolia and coworkers *(137)*, the relatively low affinity of CRT for the glycan could be advantageous to allow for rounds of glycoprotein association and dissociation. This feature would permit trimming of the remaining glucose of the glycan by glucosidase II to occur on the nonbound substrate. Such a model is supported by the finding that RNaseB bound by the lumenal domain of CNX is protected effectively from glucosidase II and PNGaseF digestion *(53)*.

In vitro, the binding of the oligosaccharide by either CNX or CRT depends critically on the

presence of $Ca^{2+}$ *(131,137)*. Thus, it is possible that the deleterious effect of $Ca^{2+}$ depletion from the ER, observed for the folding of glycoproteins known to interact with CNX and CRT, directly reflects structural changes in the lectin domain, which render both proteins incapable of ligand interaction *(138)*.

## STRUCTURAL STUDIES OF CALNEXIN AND CALRETICULIN

The biochemically determined properties of CNX and CRT have been put into new perspective by recent structural studies on both molecules. For CNX, the crystal structure of a fragment comprising residues 40–437, encompassing most of the lumenal domain, has been reported to a resolution of 2.9 Å (Fig. 4) *(110)*. This highly unusual structure contains two separate entities: a globular β-sandwich domain homologous to legume lectins and an extended hairpin fold of approx 140 Å in length, corresponding to the P-domain. The globular domain comprises a concave and a convex β-sheet with six and seven β-strands, respectively. In this part of the molecule, the CNX model also shows a putative $Ca^{2+}$ binding site, with the $Ca^{2+}$ ion proposed to play a role in structural stabilization rather than ligand interaction. The only regular secondary structure elements in the P-domain are four short, anti-parallel β-sheets where each β-strand contains three residues. The disulfide bond connecting the cysteine residues 340 and 346 is found close to the tip of the P-domain.

By soaking the crystal in glucose and determining its location in the electron density, it was found that the concave β-sheet of the globular domain harbors a monovalent glycan binding site. Modeling the binding of the G1M3 tetrasaccharide indicated that steric hindrance is likely to prevent the access of glucosidase II to its sugar substrate. This finding supports the idea that the glycoprotein must dissociate from the lectin for cleavage by glucosidase II to occur. The disulfide bond connecting the cysteine residues 140 and 174 is present in the globular domain where it connects two β-strands in the vicinity of the proposed glycan-binding site. This feature can account for the observed sensitivity of oligosaccharide binding by CNX toward reduction *(121,131)*.

Interestingly, legume lectins, galectins and neurexin 1β possess a fold closely related to the globular domain of CNX *(110)*. The laminin G-like domain of neurexin 1β contains alternative splice sites in loops connecting the β-strands and the absence or presence of inserted sequences at these sites determines ligand interactions *(139)*. Similarly, the P-domain of CNX is introduced at the same topological position as one of the splice sites in neurexin 1β and also establishes a protein–protein interaction (*see* below).

For CRT, the NMR structure of the P-domain, residues 189–288, has been solved (Fig. 4) *(140,141)*. Like the CNX P-domain it shows an extended hairpin fold, in the case of CRT with a length of approx 110 Å. Three short antiparallel β-sheets and three small hydrophobic clusters stabilize the structure. This threefold repetition of structural elements and the four-fold repetition of similar structural features in the CNX P-domain closely reflects the repetitive nature of the P-domain sequence in the two molecules (Figs. 3 and 4). Roughly, each type 1 repeat sequence pairs up with a type 2 repeat sequence in the structure of both P-domains by forming interactions across the hairpin. In addition, it has recently been shown that a short fragment corresponding to one type 1 repeat and one type 2 repeat, and comprising the β-sheet and the hydrophobic cluster at the tip of CRT P-domain, constitutes an independently folding structure *(142)*. It is tempting to speculate that the unique sequence of the P-domain has evolved by the sequential insertion of such "12" units into a loop region of the globular lectin domain.

Currently, the structure corresponding to the CNX lectin domain is not known for CRT. However, the crystal structure of the lumenal domain of CNX and the sequence similarity between the two proteins (Figs. 3 and 4) strongly indicate that the CRT N-domain and

residues 284–337 of the C-domain will form a globular domain with structural similarity to the CNX lectin domain. Indeed, this is exactly what the recently modeled structure of the CRT lectin domain also suggests *(135)*. Furthermore, biochemical and biophysical analysis of full-length CRT has shown that the molecule is asymmetric and elongated *(126)*. Based on this information, the known structural data for both proteins and the overall high conservation of sequence and function between the two proteins, it can be assumed that both CNX and CRT show a two domain structure comprising a globular lectin domain and a long protruding P-domain. In addition, the residues of CNX proposed to be involved in the binding of glucose and $Ca^{2+}$ are largely conserved in CRT. Likewise, residues in CRT proposed to bind the G1M3 tetrasaccharide are well conserved in CNX (Fig. 3).

Whereas the crystal structure of the lumenal domain of CNX revealed the lectin function of the globular domain, the function of the P-domain remained unclear despite detailed structural analysis obtained by both X-ray crystallography and NMR spectroscopy. However, it has now been shown that the P-domain of both CNX and CRT binds to ERp57 *(128,142,143)*. Using NMR spectroscopy and deletion mutants of both CNX and CRT, these studies have mapped the site of interaction with ERp57 to the distal end of the P-domain. Furthermore, the $K_d$ of the interaction between ERp57 and the CRT P-domain was determined to approx 9 μ*M (142,143)*. Although this affinity is quite weak, it is likely that a more stable tertiary complex results in the presence of a cysteine-containing glycoprotein substrate through the formation of transient mixed disulfide bonds between the glycoprotein and ERp57.

## THE ROLE OF ERP57

The growing family of ER thiol-disulfide oxidoreductases, most of which contain one or more thioredoxin-like domains, promotes the proper oxidation, isomerization and reduction of disulfide bonds *(144)*. The reactions involving these proteins proceed through intermolecular disulfide-bonded intermediates and both ERp57 and PDI form mixed disulfides with viral glycoproteins during folding in living cells (*63)*. Therefore, rather than regulating the redox state of other factors in the ER, these proteins directly catalyze the oxidative folding of proteins.

### *Molecular Studies of ERp57*

The best characterized of the redox-active proteins in the ER is PDI. It comprises four thioredoxin-like domains—termed a, b, b′, and a′—followed by an acidic carboxyl terminal c-domain, which also harbors the KDEL ER-retention motif. Thioredoxin is a small 12 kDa protein, which functions as a disulfide reductase in the cytosol. The three-dimensional structures of thioredoxin and related domains show a typical α/β fold with a five-stranded β-sheet surrounded by four α-helices (for a review, *see* ref. *145*). In PDI, the a and a′ domains are catalytically active and both contain two cysteine residues in a characteristic 'CXXC' sequence motif. Within the thioredoxin superfamily these cysteines are redox-active and switch between the dithiol and disulfide forms.

ERp57 is the closest known homolog of PDI, with which it shares the same domain composition except for the absence of the acidic c-domain. Consequently, the QDEL ER-retention motif in ERp57 is located directly at the carboxyl terminal end of the a′ domain *(146)*. PDI and ERp57 show an overall amino acid identity of 29%, with the a and a′ domains being most closely related, whereas the b′ domains are the most divergent. In PDI, the b′ domain has been shown to bind peptides, a feature that is likely to be related to its function as a bonafide chaperone. Since the ERp57 b′ domain does not possess a similar function, it is tempting to speculate that this domain in ERp57 could have evolved to constitute a binding site for CNX and CRT, as proposed recently by Freedman and colleagues *(145)*. Currently, no structural data are available for ERp57. However, NMR structures of the PDI a and b domains show

that they both comprise typical thioredoxin-like folds *(149,150)*. Preliminary NMR data and assignments have been reported for the a′ domain of ERp57 *(151)*.

Using in vitro assays for redox activity, ERp57 has been shown to have disulfide reductase and isomerase activity *(152–155)*. Only one in vitro study has investigated the catalytic activity of ERp57 using an endogenous substrate of the protein, namely partially folded MHC class I heavy chain molecules *(156)*. Toward this substrate, ERp57 was shown to exhibit reductase activity, and it was proposed that this activity might be involved in reducing heavy chain molecules prior to retrotranslocation and degradation *(156)*. However, it remains to been seen exactly which type(s) of disulfide exchange reaction(s) ERp57 performs in vivo.

## PDI and Glycoprotein Folding

As mentioned previously, ERp57 acts as a glycoprotein specific redox-active enzyme through its cooperative interaction with CNX and CRT. The finding that ERp57-enhanced disulfide bond formation in vitro is dependent on CNX and CRT stresses the functional cooperation of both lectin chaperones with ERp57 *(157)*. No positive effects on glycoprotein refolding by PDI were observed upon addition of CNX and CRT. Although PDI has been shown to be involved in glycoprotein folding *(63)*, substrate interaction of PDI in vivo is not regulated by glucose trimming *(59–61,63,158)*. These studies all indicate that PDI functions independently of CNX and CRT, which is in agreement with findings that were unable to detect complexes of either lectin with PDI *(60,143)*. However, other data have shown that PDI interacts with CRT *(123,159)*. It is possible that this apparent discrepancy is a result of the different experimental conditions used because the interaction is strongly influenced by changes in $Ca^{2+}$ concentrations *(123)*. Thus, fluctuations in the free concentration of $Ca^{2+}$ in the ER could affect protein folding, either directly by inducing conformational changes in CNX and CRT resulting in altered substrate interactions or indirectly by modulating the binding of co-chaperones *(97,123)*.

## ERp57 in MHC Class I Assembly

Recently, several investigations have focused on the interaction of ERp57 with glycoproteins during CNX- and CRT-dependent folding (*see*, for instance, refs. *64,160–162*). To date, the best-studied glycoprotein substrate of ERp57 in vivo is the MHC class I complex. This heterodimer is comprised of the glycosylated heavy chain and the associated protein, $\beta_2$-microglobulin. The MHC class I complex plays a critical role in the immune response by delivering for instance virus- and tumor-derived antigenic peptides to the cell surface for presentation to receptors on cytotoxic T-cells. The process of MHC class I folding, assembly and peptide loading takes place in the ER, and has been shown to involve CNX, CRT and ERp57 together with the more specialized proteins transporter associated with antigen processing (TAP) and tapasin. During the early stages of folding the newly synthesized heavy chain interacts with CNX. Upon binding of $\beta_2$-microglobulin, CRT replaces CNX and together with TAP, tapasin and ERp57 form the so-called peptide loading complex (PLC). In a tapasin-dependent process, the class I molecules are then loaded with peptide, the PLC dissociates and the mature MHC class I complex can traffic to the cell surface (for recent reviews, *see* refs. *77,163,164*).

ERp57 is involved at various stages of MHC class I assembly. The heavy chain contains two disulfide bonds and folds and oxidizes while associated with CNX, but prior to incorporation into the PLC *(165–167)*. Because a ternary complex of heavy chain, CNX and ERp57 has been observed already at this early stage of maturation, it is possible that ERp57 is involved in catalyzing disulfide bond formation in the heavy chain *(167)*. Moreover, ERp57 is an important component of the PLC *(167)*. The association of ERp57 with the PLC has been shown to be tapasin dependent and correlate with CRT association *(169,171)*. The latter finding suggests that a preformed complex of CRT and

ERp57 could enter into the PLC *(171)*. The functional cooperation between ERp57 and tapasin is stressed by the finding that the two proteins form an intermolecular disulfide bond in the PLC (*172)*. Analysis of cysteine-lacking mutants of ERp57 and tapasin has led to the conclusion that the two proteins in concert mediate the isomerization and/or re-oxidation of the disulfide bond in the peptide-binding groove of MHC class I heavy chain *(172)*. Although it is presently not known exactly how this process occurs, it has been shown to be important for the correct loading of the MHC class I $\beta_2$-microglobulin heterodimer with high affinity peptides *(172)*.

## FROM MOLECULAR PROPERTIES TO IN VIVO FUNCTION

The insight gained from the many biochemical and cell biological studies of CNX, CRT and ERp57 is providing a more and more detailed picture of the processes involving these three chaperones. Our understanding is now so advanced that many functional features observed in vivo can be rationalized based on known biochemical and structural properties. As described previously, the present data strongly indicate that CNX and CRT show a similar overall structural organization. Functionally, the lectin domain is responsible for binding the glycan attached to substrate glycoproteins, whereas the P-domain binds ERp57 at its tip. These features, in combination with the properties of both chaperones determined in vivo, have led us to present a basic model for the cooperative interaction of either chaperone and ERp57 with the nascent chain or non-native conformers of newly synthesized glycoproteins in the ER *(143,173)*.

We propose that substrate binding occurs through the interaction of the monoglucosylated glycan with the lectin domain of either CNX or CRT. This feature allows the polypeptide chain a certain degree of conformational freedom in the process of folding. The structural arrangement of the lectin domain and the elongated P-domain with ERp57 bound at the tip sequesters the polypeptide chain of the substrate in a partially protected space during folding. This partial shielding of the bound glycoprotein substrate should help to suppress intermolecular aggregation that could result from interactions with other ER folding intermediates. The role of the P-domain is likely to involve positioning of ERp57 for the formation of intermolecular disulfide bonds with a variety of bound substrate proteins. The conformational plasticity observed in the NMR structure of the CRT P-domain could allow both chaperones to adapt to substrates of varying size and shape *(140)*. This complements the idea that the overall molecular flexibility of CRT characterized biophysically by Bouvier and colleagues is advantageous for the protein in its function as a molecular chaperone by allowing transient and dynamic interactions with a number of substrates *(109,123,125,126)*. An additional function of the P-domain could be to constrain diffusion of the substrate after dissociation from CNX and CRT. This would allow for a more rapid reassociation, provided that the remaining glucose on the glycoprotein substrate has meanwhile not been trimmed by glucosidase II. Taken together, the function of CNX and CRT is likely to be dictated by their distinctive structural features and the close cooperation with ERp57, which leads to the productive oxidative folding of glycoprotein substrates.

This basic model supports the view that CNX and CRT function as molecular chaperones by keeping their substrates "out of trouble" rather than by actively promoting folding. However, it leaves room for the incorporation of additional features of both proteins relating to the binding of cofactors and protein–protein contacts with substrate glycoproteins. The prediction of protein–protein interactions during CNX- and CRT-assisted glycoprotein folding is appealing because it provides explanations for results otherwise not easy to rationalize. For instance, when performing immunoprecipitation experiments to identify glycoprotein substrates of CNX in vivo, it has been observed

that certain complexes persist even after removal of the glycan by glycosidase treatment with Endo H or PNGase F *(50,78,174,175)*. Therefore, it could be that the initial binding of CNX and CRT to the glycoprotein substrate occurs as a result of the lectin activity, but that subsequent protein-protein contacts stabilize the complexes. Polypeptide-based contacts could also help explain the rather high efficiency observed for coimmunoprecipitation of substrate proteins with CNX and CRT in spite of the relatively low affinity measured for the interaction between CRT and monoglucosylated IgG *(137)*.

Currently, however, the data relating to the function of CNX and CRT as bona fide molecular chaperones are not easy to assess definitively. For instance, the physiologic role of potentially important cofactors implicated in this function, such as $Zn^{2+}$ and ATP, is presently not evident. It has also been argued that immunoprecipitation is not the method of choice to study interactions that involve nonnative conformers or nascent chains owing to their hydrophobic nature *(29)*. For example, overexpression of protein or addition of tunicamycin, which inhibits glycosylation and turns on the UPR, often results in protein misfolding and aggregation. Potentially, complexes of CNX and nonglycosylated proteins immunoisolated under such conditions that perturb the folding environment in the ER lumen are nonphysiologic in nature. Besides, interactions of membrane proteins with CNX could possibly be preserved if caught in the same detergent micelle *(20)*.

The fact that the experimental conditions employed in immunoprecipitation experiments can influence results considerably contributes an additional difficulty. When performing immunoprecipitations under mild detergent conditions *(176)*, interaction of nonglycosylated proteins or proteins carrying di- or triglucosylated glycans with CNX and CRT can be observed. In contrast, when performing similar experiments under more stringent detergent conditions, no binding to CNX was observed *(177)*. No matter which experimental conditions are employed, any substrate interaction observed with CNX and CRT should be critically assessed to ensure that it is transient and that it results in the formation of a fully mature and correctly folded substrate protein. Only such interactions are expected to occur as a result of the chaperone function of CNX and CRT. In contrast, long-lasting interactions with substrate proteins are likely to be nonproductive and of nonspecific nature.

## PERSPECTIVES

Since 1989 when Suh and colleagues observed that a misfolded variant of the vesicular stomatitis virus G-protein was retained in the ER in a monoglucosylated form *(178)*, our understanding of the processes involved in glycoprotein folding in the ER has increased tremendously. In particular, the roles of the different proteins in the CNX/CRT cycle have become much more evident. The publication of three-dimensional structures of CNX and CRT has provided substantial new insight into the molecular mechanism of the chaperone function of these proteins and has significantly changed the way we think about these two lectin chaperones.

The next goals for the field should involve the determination of a high-resolution structure of the CRT lectin domain, if possible in complex with the G1M3 glycan. More detailed information about the interaction of CNX and CRT with ERp57, as well as structure determination of ERp57, or individual domains thereof, will be important. Additional experiments will be needed to clarify the exact role of the P-domain in glycoprotein folding. Despite being structurally similar, the P-domains in CNX and CRT differ with a "12" repeat unit in size. Whether this difference also conveys functional distinctions is presently unclear. Addressing such questions relating to the P-domain should also promote a better understanding of how the cooperation of CNX and CRT with ERp57 assists disulfide bond formation in substrate glycoproteins.

The direct mapping of specific residues in CNX and CRT that decide the interaction with ATP, $Zn^{2+}$ and polypeptide regions of substrate proteins will also be essential. Such information could for instance be obtained by structure determination in the presence of these co-factors, which might also provide direct information on the local and global conformational changes that occur upon cofactor binding. These results should in turn allow the design of mutations that specifically remove the co-factor interactions individually. Provided that such mutants can be shown to retain structural and functional properties of the wild-type protein, they will become valuable tools for the characterization of functions related to cofactor binding both in vivo and in vitro. Application of this experimental strategy will be particularly important to investigate functions such as ATP binding, which can otherwise not easily be targeted in vivo without perturbing the cellular environment. To fully appreciate the role of ATP binding by CNX and CRT the identification of potential co-chaperones for ATP hydrolysis, nucleotide exchange or dissociation will be critical. Taken together, the results of such experiments should provide us with a more detailed understanding of how the CNX/CRT chaperone system functions at the molecular level.

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