Beyond lectins: the calnexin/calreticulin chaperone system of the endoplasmic reticulum

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Summary

Calnexin and calreticulin are related proteins that comprise an ER chaperone system that ensures the proper folding and quality control of newly synthesized glycoproteins. The specificity for glycoproteins is conferred by a lectin site that recognizes an early oligosaccharide processing intermediate on the folding glycoprotein, $Glc_1Man_9GlcNAc_2$. In addition, calnexin and calreticulin possess binding sites for ATP, Ca^{2+}, non-native polypeptides and ERp57, an enzyme that catalyzes disulfide bond formation, reduction and isomerization. Recent studies have revealed the locations of some of these ligand-binding sites and have provided insights into how they contribute to overall chaperone function. In particular, the once controversial non-native-polypeptide-binding site has now been shown to function both in vitro and in cells. Furthermore, there is clear evidence that ERp57 participates in glycoprotein biogenesis either alone or in tandem with calnexin and calreticulin.

Key words: Calnexin, Calreticulin, Endoplasmic reticulum, Quality control, Protein folding, Glycoproteins

Introduction

At first glance, the endoplasmic reticulum (ER) appears an inhospitable environment for protein folding. The concentration of luminal proteins is ~100 mg/ml and the total Ca^{2+} concentration is 5-10 mM, which probably gives rise to a gel-like matrix (Booth and Koch, 1989; Meldolesi and Pozzan, 1998). Nevertheless, proteins that exit the ER are, for the most part, properly folded and assembled, owing to the coordinated activities of a variety of folding enzymes, molecular chaperones and a rigorous quality-control system that retains and disposes of misfolded proteins. Folding enzymes include as many as 17 members of the protein disulfide isomerase (PDI) family that catalyze the formation and interchange of disulfide bonds, peptide prolyl isomerases that catalyze cis-trans isomerization of peptidyl proline bonds, and components of the Asn-linked glycosylation system. The latter, through the co-translational addition and subsequent modification of large $Glc_3Man_9GlcNAc_2$ oligosaccharides (Fig. 1), influences protein solubility, folding pathways, interactions with certain chaperones and degradative processes. ER chaperones include members of the Hsp40, Hsp70, Hsp90, Hsp100 and calnexin/calreticulin families. Chaperones are thought not to alter folding pathways. Rather, they delay folding and prevent off-pathway aggregation, allowing more efficient folding and subunit assembly. They also contribute to quality control by retaining non-native conformers within the ER and targeting them for degradation.

During the past decade, the most intensively studied ER chaperones have been calnexin (Cnx) and calreticulin (Crt) because of their unusual mode of substrate recognition, their intimate relationship with the Asn-linked glycosylation system, and the diversity of functions attributed to them. Cnx is a 90 kDa type I ER membrane protein, the bulk of which lies within the ER lumen (Wada et al., 1991). Crt is its 60 kDa soluble paralog that is localized to the ER lumen by a C-terminal KDEL sequence (Fliegel et al., 1989). Both proteins associate with most, if not all, glycoproteins that pass through the ER. These chaperones have co-evolved with Asn-linked glycosylation in eukaryotes; their binding preference for glycoproteins stems from the fact that Cnx and Crt are lectins specific for a transient oligosaccharide-processing intermediate that possesses a single terminal glucose residue: $Glc_1Man_9GlcNAc_2$ (Fig. 1) (Hammond et al., 1994; Ou et al., 1993; Spiro et al., 1996; Ware et al., 1995). They also bind to ATP, Ca^{2+}, Zn^{2+} and to one of the many thiol oxidoreductases of the ER, ERp57 (Baksh et al., 1995; Corbett et al., 2000; Oliver et al., 1997). Finally, they can associate with polypeptide segments of non-native glycoprotein conformers in a manner similar to other molecular chaperones, although the importance of this mode of association is controversial (Ihara et al., 1999; Saito et al., 1999). Here, I examine how these diverse binding interactions contribute to glycoprotein folding, quality control and degradation.

Chaperone structure and binding sites

The structure of the ER-luminal portion of Cnx, solved by X-ray crystallography (Schrag et al., 2001), reveals two domains: a globular β-sandwich domain that resembles legume lectins; and an extended 140 Å arm domain consisting of two β-strands folded in a hairpin configuration (Fig. 2A). Each β-strand is composed of four tandemly repeated, proline-rich repeats, which is why the extended arm is frequently referred to as the P domain. For Crt, only the arm domain structure has been solved by NMR methods. It is similar to that of Cnx but shorter,

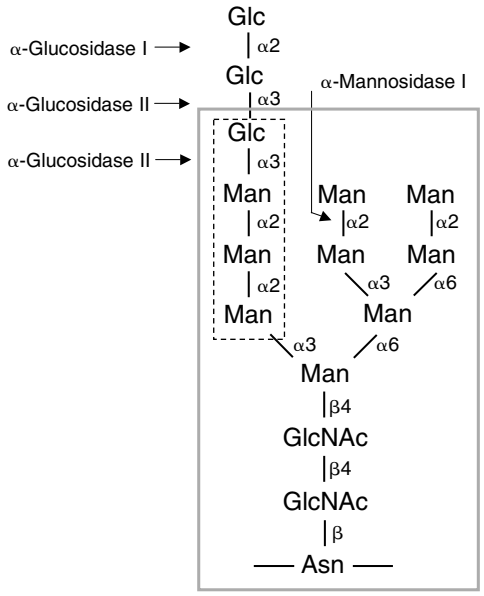

Fig. 1. Composition of the $Glc_3Man_9GlcNAc_2$ oligosaccharide initially transferred to nascent glycoproteins. The outer box surrounds the monoglucosylated $Glc_1Man_9GlcNAc_2$ oligosaccharide recognized by calnexin and calreticulin. It is formed by the initial action of α-glucosidase I followed by cleavage of a second glucose by α-glucosidase II. The lectin sites of Cnx and Crt bind to the terminal glucose residue of the $Glc_1Man_9GlcNAc_2$ oligosaccharide as well as to three underlying mannose residues (dashed box). Also indicated is the terminal mannose residue cleaved by α-mannosidase I to create a signal associated with rapid degradation of misfolded glycoproteins. Glc, glucose; Man, mannose; GlcNAc, N-acetylglucosamine.

consisting of three rather than four tandem repeats (Ellgaard et al., 2001). Crt is thought to possess a globular domain similar to that of Cnx, since the two chaperones share ~39% sequence identity and have identical lectin-binding specificities (Spiro et al., 1996; Vassilakos et al., 1998; Ware et al., 1995). The crystal structure and extensive mapping studies have demonstrated that the oligosaccharide-binding site resides in a cleft on the surface of the globular domain (Leach et al., 2002; Schrag et al., 2001). This site recognizes not only the terminal glucose residue of the $Glc_1Man_9GlcNAc_2$ oligosaccharide but also three underlying mannose residues (Fig. 1) (Spiro et al., 1996; Vassilakos et al., 1998). The terminal glucose residue is coordinated by six residues within the lectin site and mutation of any one of these in either Cnx or Crt results in a dramatic loss of lectin function (Kapoor et al., 2004; Leach and Williams, 2004; Thomson and Williams, 2005).

Cnx and Crt associate with the ERp57 thiol oxidoreductase through the tip of their arm domains (Fig. 2B) (Frickel et al., 2002; Leach et al., 2002; Pollock et al., 2004). Mutant chaperones lacking the most distal set of arm domain repeats exhibit a profound impairment in ERp57 binding (Leach et al., 2002). The site proposed to bind polypeptide sequences in certain non-native proteins has not been well characterized. However, since the isolated globular domains, but not the arm domains, retain the ability to suppress the thermal aggregation of various non-glycosylated proteins, the polypeptide-binding site appears to reside primarily within the globular domain (Leach et al., 2002). Both Cnx and Crt have a high-affinity Ca^{2+}-binding site (K_d = 10 μM for Crt) (Baksh and Michalak, 1991), which has been mapped to the globular domain in Cnx at a location far removed from the lectin site (Fig. 2A). The Ca^{2+} ion plays an important structural role since its absence is associated with reduced melting temperature, acquisition of protease sensitivity and loss of lectin function (Corbett et al., 2000; Li et al., 2001; Vassilakos et al., 1998). In addition, Crt possesses multiple low-affinity (K_d = 2 mM) Ca^{2+}-binding sites that are involved in buffering ER Ca^{2+} stores (Baksh and Michalak, 1991; Nakamura et al., 2001). Both chaperones bind Zn^{2+} at sites within the globular domain (Baksh et al., 1995; Leach et al., 2002) and both bind ATP, although no ATPase activity has been detected. As is the case in other molecular chaperones, ATP may regulate conformational changes in Cnx and Crt. ATP, but not ADP or AMP, renders Crt more resistant to protease digestion and enhances the aggregation-suppression abilities of Cnx and Crt in vitro (Corbett et al., 2000; Ihara et al., 1999; Ou et al., 1995; Saito et al., 1999). The nucleotide-binding site has not been mapped.

Functions of Cnx and Crt

Of the ~100 newly synthesized glycoproteins that associate with Cnx or Crt (David et al., 1993; Van Leeuwen and Kearse, 1996), some interact only with one chaperone whereas others may bind both either simultaneously or sequentially (see Helenius et al., 1997 for a list). Initial association can be co-translational (Chen et al., 1995) and the interaction is maintained during subsequent folding and assembly steps. Release from Cnx or Crt is often closely coupled to export of the glycoprotein out of the ER to the Golgi complex (Anderson and Cresswell, 1994; Degen et al., 1992; Hammond et al., 1994; Ou et al., 1993). The functional consequences of the interaction have been assessed in a variety of ways. Since the $Glc_1Man_9GlcNAc_2$ oligosaccharide recognized by Cnx and Crt is formed by the combined action of glucosidases I and II on the initially transferred $Glc_3Man_9GlcNAc_2$ oligosaccharide, glucosidase-deficient cells or inhibitors of these enzymes such as castanospermine (CST) have been extensively used to prevent Cnx/Crt interactions (Fig. 1). This approach suffers from the fact that all newly synthesized glycoproteins will be affected and this may alter functions independently of blocking Cnx/Crt association. Also, not all interactions with Cnx or Crt are blocked by this treatment (see below). Alternative approaches include the use of Cnx- or Crt-deficient cell lines and expression of glycoproteins of interest in heterologous systems that lack Cnx/Crt interactions.

The studies using glucosidase inhibitors indicate that the folding and assembly of many, but not all, glycoproteins is influenced by Cnx and Crt (see Danilczyk and Williams, 2001; and Parodi, 2000 for listings). In some cases these observations have been confirmed in Cnx- or Crt-deficient cell lines or in expression systems lacking functional chaperone. For example, influenza hemagglutinin (HA) folding, trimerization and export from the ER are all accelerated in Crt-deficient cells but its folding efficiency is reduced owing to the formation of large disulfide-linked complexes. Semliki forest virus glycoprotein maturation is also accelerated in Crt-deficient cells, but again it is less efficient (Molinari et al., 2004). In the case of class I major histocompatibility complex (MHC) molecules, their assembly into mature ternary complexes is impaired in the

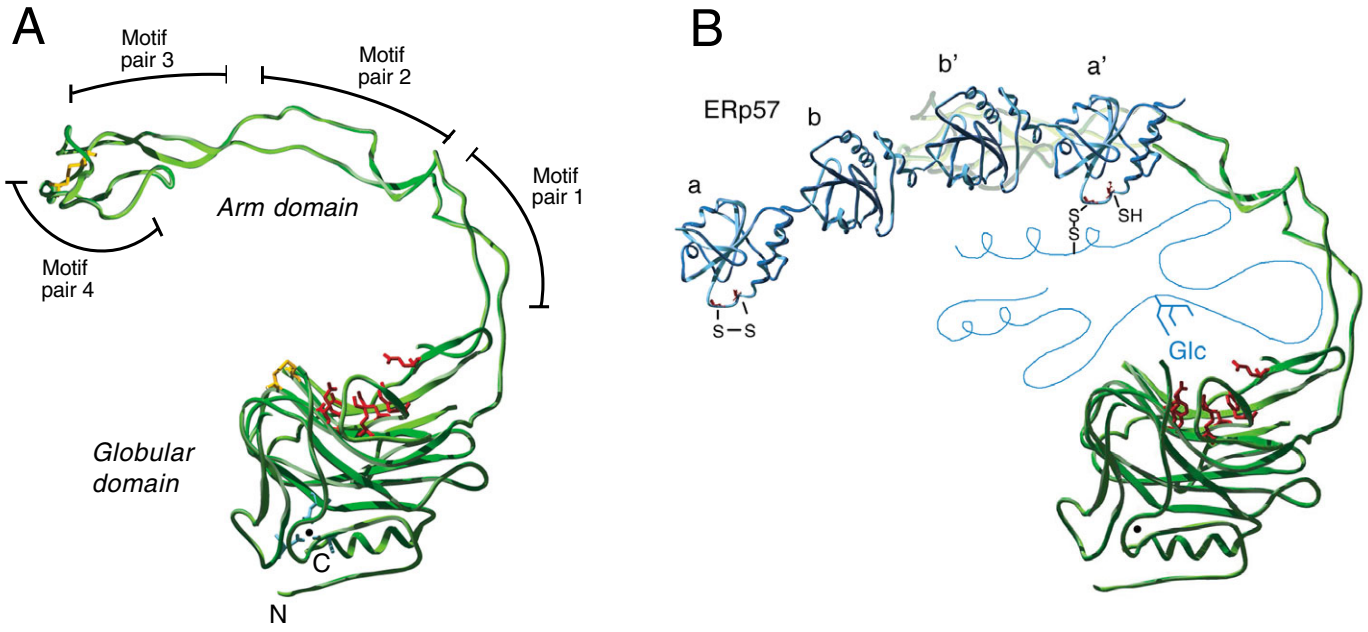

Fig. 2. (A) Crystal structure of the ER luminal domain of calnexin. The globular domain contains the oligosaccharide-binding site with amino acids that contact the terminal glucose residue shown in red. A bound Ca^{2+} is indicated by the black sphere, residues coordinating the ion depicted in cyan. Disulfide bonds are shown in yellow. The extended arm domain consists of two strands, one containing four repeats of motif 1 [I-DP(D/E)A-KPEDWD(D/E)] and the other containing four copies of motif 2 [G-W–P-IN-P-Y]. Each motif-1-repeat is paired with a motif-2-repeat on the opposite strand. The four motif pairs are shown. (B) Model for the interaction of a folding glycoprotein with calnexin or calreticulin. Calnexin (green) is shown associated with a hypothetical model of ERp57 (blue) drawn on the basis of the NMR structure of the PDI 'a' domain (Kemmink et al., 1996). The four domains of ERp57 are indicated: a, b, b′, a′. A folding glycoprotein (thin blue line) may enter the cavity between the arm and globular domains interacting both with the lectin site as well as a polypeptide-binding site. This may sequester it from other folding glycoproteins thereby minimizing aggregation. Aggregation is further suppressed by the ability of the polypeptide-binding site to shield exposed hydrophobic segments. The two CGHC active sites of ERp57 (red) are well-placed to catalyze disulfide-bond formation, reduction or isomerization.

absence of Crt as is their expression at the cell surface (Gao et al., 2002).

Similar experiments using Cnx-deficient cells demonstrated that folding of HA to its fully oxidized state is reduced to only 30% of the level seen in wild-type cells. Furthermore, export to the cell surface is almost undetectable in this instance (Molinari et al., 2004). By contrast, the biogenesis of class I molecules is not affected by Cnx deficiency (Scott and Dawson, 1995). These findings underscore the different dependencies of various glycoproteins on either Cnx or Crt. Indeed, the choice of whether a glycoprotein is recognized by Cnx or Crt has been shown to be related to the number and location of its glycosylation sites (Harris et al., 1998; Hebert et al., 1997), as well as the different topologies of Cnx and Crt. Converting Cnx to a Crt-like soluble molecule or anchoring Crt in the ER membrane switches the substrate specificity of each chaperone to resemble that of the other (Danilczyk et al., 2000; Wada et al., 1995). However, specificity extends beyond chaperone topology and lectin binding, since a soluble form of Cnx cannot substitute for Crt in promoting class I biogenesis (Gao et al., 2002). Not yet elucidated differences in their polypeptide-binding specificities probably also play a significant role in substrate selection.

The more rapid folding observed in several instances of Cnx or Crt deficiency, as well as the appearance of misfolded, aggregated, or disulfide cross-linked species, suggest that these chaperones normally delay folding, help suppress the formation of aggregates and promote correct disulfide-bond formation. Additional phenotypes frequently associated with Cnx/Crt deficiency or CST treatment include more rapid export of non-native glycoproteins from the ER as well as their increased degradation (Gao et al., 2002; Jackson et al., 1994; Moore and Spiro, 1993; Rajagopalan et al., 1994). These findings indicate that Cnx and Crt participate in ER quality control, stabilizing non-native species and retaining them either until a native state can be attained or until degradative processes are engaged.

For the most part, eukaryotic cells in culture can survive in the absence of Cnx and/or Crt, an exception being *S. pombe*, in which deletion of the *Cnx* gene is lethal (Jannatipour and Rokeach, 1995; Parlati et al., 1995). Mammalian cell lines lacking either chaperone are viable and most cells can endure treatment with CST for 24 hours or more. Even in *Dictyostelium discoideum*, the $Cnx^{-}Crt^{-}$ double knockout survives, although growth rates are reduced and the ability to phagocytose particles is profoundly impaired (Muller-Taubenberger et al., 2001). In these cases, survival may be due in part to the unfolded protein response (UPR), in which there is compensatory upregulation of other chaperones and folding factors (Balow et al., 1995; Knee et al., 2003; Molinari et al., 2004).

By contrast, knocking out the Crt or Cnx gene in mice has severe consequences. Crt-deficient mice die by embryonic day 18 and exhibit cardiac defects (Mesaeli et al., 1999). They can be rescued by overexpression of activated calcineurin (Guo et

al., 2002), which is a Ca^{2+}/calmodulin-dependent protein phosphatase involved in the regulation of diverse functions such as T-cell activation, apoptosis, embryonic development, cardiac physiology and metabolism. Given that activation of calcineurin occurs in response to sustained Ca^{2+} release from the ER and that Crt is important for maintaining ER Ca^{2+} levels (Nakamura et al., 2001), this suggests that Crt acts as an upstream regulator of calcineurin in Ca^{2+} signaling (Arnaudeau et al., 2002; John et al., 1998; Lynch and Michalak, 2003). Cnx-deficient mice are viable but have reduced survival. They seem to have no cardiac abnormalities but have neurological problems: abnormal gait, reduced limb coordination and a reduction in the number of large myelinated nerve fibers (Denzel et al., 2002). The contrasting phenotypes of Crt and Cnx deficiency in mice indicate that these chaperones must have some non-overlapping functions. Furthermore, because the phenotypes are more severe than those in cultured cells, the proteins presumably play roles in developmental processes beyond their functions in protein folding – for example, in ER Ca^{2+} homeostasis (Arnaudeau et al., 2002) – as well as influencing cell shape, adhesion and motility (Bedard et al., 2005; Fadel et al., 1999; Opas et al., 1996).

Mechanisms of action: lectin-only or dual-binding?

Since the discovery that interaction of glycoproteins with Cnx or Crt can be impaired by the addition of glycosylation or oligosaccharide-processing inhibitors to cultured cells (Hammond et al., 1994), the primary focus of research into how these chaperones promote glycoprotein folding and participate in ER retention has centered on their lectin function. Ari Helenius and co-workers (Hammond et al., 1994; Hebert et al., 1995) have proposed that interactions between a folding glycoprotein and Cnx/Crt are controlled by the availability of a single terminal glucose residue on Asn-linked oligosaccharide chains (Fig. 3, lectin-only model). As the nascent chain enters the ER through the translocon and is

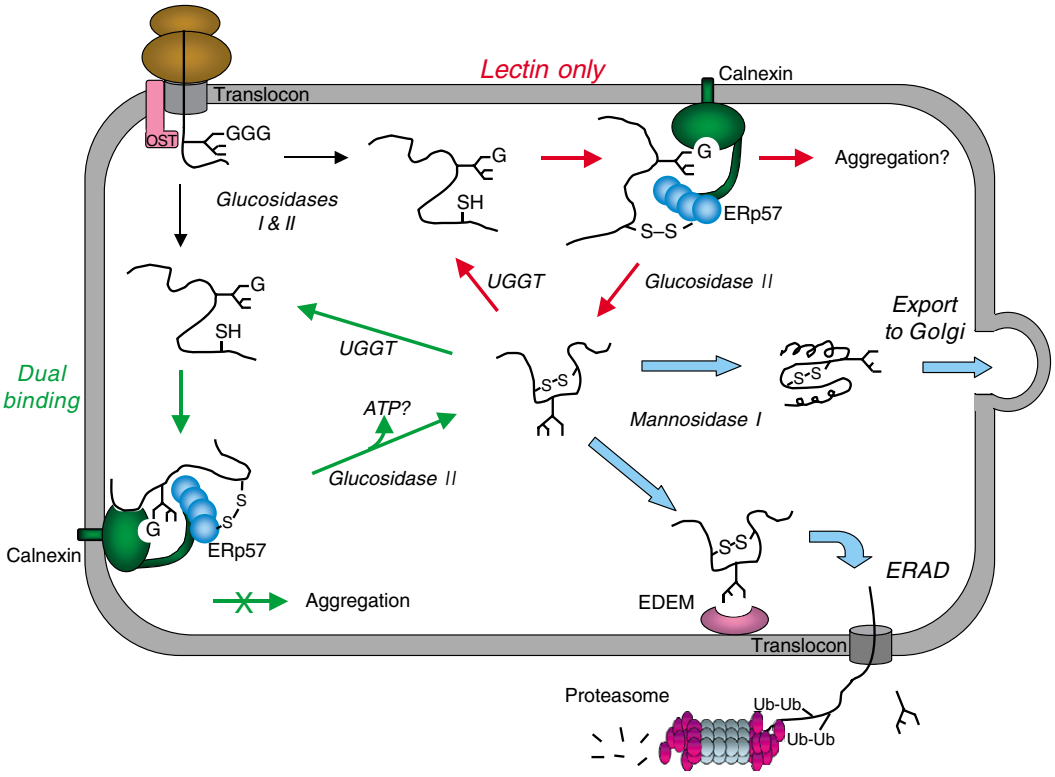

Fig. 3. Proposed mechanisms of action for calnexin and calreticulin. As a nascent polypeptide enters the ER lumen via the translocon pore, Asn residues within Asn-X-Ser(Thr) sequences may be recognized by oligosaccharyl transferase (OST) and glycosylated with the preassembled $Glc_3Man_9GlcNAc_2$ oligosaccharide. The outer two glucoses are then rapidly removed by glucosidases I and II to generate the $Glc_1Man_9GlcNAc_2$ oligosaccharide recognized by Cnx and Crt. In the lectin-only model (red arrows), cycles of glycoprotein release and re-binding are controlled solely by the removal and re-addition of the terminal glucose residue by glucosidase II and UDP-glucose:glycoprotein glucosyltransferase (UGGT), respectively. UGGT is the folding sensor because it only reglucosylates non-native glycoprotein conformers. Chaperone binding serves to retain non-native glycoproteins within the ER and also recruits ERp57 to promote disulfide-bond formation and isomerization. It is unclear whether binding to glycoproteins only through the lectin site is sufficient to suppress aggregation. In the dual-binding model (green arrows), Cnx and Crt recognize non-native glycoproteins through their lectin sites as well as through a polypeptide-binding site specific for non-native conformers. This allows them to prevent off-pathway aggregation reactions similarly to other molecular chaperones. Binding via the polypeptide binding site is influenced by ATP and by the free Ca^{2+} concentration, either of which may regulate the interaction. In both models, folding takes place upon release from the chaperone, followed by further oligosaccharide trimming and export to the Golgi apparatus. For misfolded glycoproteins that remain for prolonged periods in the Cnx/Crt cycle, trimming by α-mannosidase I generates a $Man_8GlcNAc_2$ structure that may be recognized by a putative lectin termed EDEM as part of a signal leading to retrotranslocation and proteasomal degradation [ER-associated degradation (ERAD)]. Ub, ubiquitin.

glycosylated by oligosaccharyl transferase, the monoglucosylated structure is formed by the combined action of glucosidases I and II. In the lectin-only model, this then interacts with Cnx/Crt only through the lectin site, and dissociation is probably governed by the relatively weak affinity of this site (K_d = 1-2 μM) (Patil et al., 2000). Upon release from the chaperone, the glycoprotein is deglucosylated by the further action of glucosidase II. Folding and subunit assembly occur at this stage and, if completed, the glycoprotein may be exported from the ER. However, if incompletely folded, the glycoprotein becomes a substrate for UDP-glucose: glycoprotein glucosyltransferase (UGGT), which re-attaches a single glucose only to non-native glycoprotein conformers (Caramelo et al., 2004; Ritter et al., 2005; Taylor et al., 2004). This signals re-binding to Cnx/Crt and the cycle continues until a native conformation is achieved or until degradative processes engage. Hence, UGGT is the folding sensor in this model.

How does lectin-mediated binding promote glycoprotein folding and quality control? This is an important question since there is no direct masking of hydrophobic sites to suppress aggregation, which is the hallmark of other molecular chaperones. One hypothesis is that a folding glycoprotein might become sequestered between the arm domain and globular lectin domain (Fig. 2B), which would reduce aggregation with other folding glycoproteins (Helenius and Aebi, 2004). Also, by recruiting ERp57 to the proximity of the glycoprotein, disulfide formation and isomerization may be enhanced (High et al., 2000). Quality control is easier to envision, since repeated cycles of interaction with Cnx/Crt would tend to retain a non-native glycoprotein within the ER.

Substantial experimental evidence supports the lectin-only model. Most compelling is the finding that interactions between Cnx/Crt and many glycoproteins can apparently be prevented in glucosidase-deficient cells or in cells treated with glucosidase inhibitors such as CST [summarized in Danilczyk and Williams (Danilczyk and Williams, 2001) and Parodi (Parodi, 2000)]. Furthermore, if glucosidase inhibitors are added after glycoprotein-Cnx and/or -Crt complexes are formed, dissociation is impaired, which lends support to the involvement of glucosidase II in the dissociation stage of the cycle (Hebert et al., 1995). Cycles of deglucosylation and reglucosylation have been demonstrated in cells and microsomes, and shown to be important for glycoprotein folding (Cannon and Helenius, 1999; Wada et al., 1997). Finally, interactions between ERp57 and several glycoproteins are sensitive to treatment with glucosidase inhibitors, which is consistent with it being recruited by Cnx and Crt (Molinari and Helenius, 1999; Oliver et al., 1997). The ability of ERp57 to enhance the oxidative refolding of RNAse in vitro has also been shown to be greatly increased by the simultaneous presence of Cnx or Crt, which suggests synergistic effects resulting from formation of a ternary complex (Zapun et al., 1998).

In an alternative model, the ‘dual-binding’ model, Cnx and Crt interact with folding glycoproteins not only through their lectin sites but also through the polypeptide-binding site (Fig. 3) (Ihara et al., 1999; Ware et al., 1995). The folding-promoting function of Cnx and Crt would thus be similar to that of other molecular chaperones, i.e. they bind to hydrophobic segments of non-native glycoproteins and suppress their aggregation. There is no need to invoke entry between the arm and globular domains although this is certainly a possibility (Fig. 2B). Disulfide bond formation and isomerization could be promoted by recruitment of ERp57, as in the lectin-only model (Fig. 3). Complex dissociation would require both oligosaccharide release and a conformational change in the polypeptide-binding site, possibly regulated by ATP or some other factor. In this model, both the chaperone and UGGT act as folding sensors.

Considerable evidence supports the dual-binding model. For example, immunoisolated complexes of Cnx and several glycoproteins can be enzymatically deglycosylated without any evidence of dissociation of the complex (Arunachalam and Cresswell, 1995; Ware et al., 1995; Zhang et al., 1995). There are also numerous examples of glycoproteins that exhibit unaltered or only modestly diminished interactions with Crt or Cnx when expressed in glucosidase-deficient cells, treated with CST or mutated to remove all glycosylation sites (see Danilczyk and Williams, 2001 for references). This is in marked contrast to the experiments mentioned above in which these treatments completely prevent binding to Cnx or Crt. Such variability has been attributed in part to differences in immunoisolation protocols, since the lysis buffer and wash conditions can profoundly influence recovery of Cnx or Crt with glycoproteins lacking $Glc_1Man_9GlcNAc_2$ structures (Danilczyk and Williams, 2001). Inherent differences in the substrates themselves may also be a factor. Some glycoproteins may bind exclusively through lectin-oligosaccharide interactions, whereas others might use this mode as well as polypeptide-based association. The observed lectin-independent binding of some substrates to Cnx or Crt could simply be due to their inclusion in protein aggregates. This may be the case in some instances (Cannon et al., 1996) but the possibility has been rigorously excluded in others, in which aggregates were tested for by density gradient centrifugation (Danilczyk and Williams, 2001; Wanamaker and Green, 2005).

A further demonstration of polypeptide-based binding interactions comes from the finding that Crt can bind directly to non-glycosylated peptides in cells and in vitro (Jorgensen et al., 2000; Nair et al., 1999). Whether such binding is reflective of a chaperone function or of several other binding functions attributed to Crt is unclear. Experiments more directly relevant to chaperone function have shown that Crt and the soluble ER luminal domain of Cnx can bind to or suppress the thermally induced aggregation not only of glycoproteins bearing monoglucosylated oligosaccharides but also of a variety of non-glycosylated proteins (Culina et al., 2004; Ihara et al., 1999; Rizvi et al., 2004; Saito et al., 1999; Thammavongsa et al., 2005). In these latter studies, Cnx and Crt recognize only non-native conformers of the non-glycosylated proteins, which is consistent with the idea that they function as folding sensors similar to other chaperones. One criticism of this work has been that Cnx and Crt may exhibit a small degree of unfolding at 43-45°C, the temperatures used in the thermal aggregation assays (Li et al., 2001). However, studies using MHC class I molecules and deglycosylated IgY as substrates have been performed at 37°C and 31°C, respectively, temperatures at which there is no evidence of chaperone unfolding (Saito et al., 1999; Thammavongsa et al., 2005). By contrast, other studies have shown that binding of Crt to MHC class I molecules (Wearsch et al., 2004) and binding of Cnx/Crt to RNAse B

(Rodan et al., 1996; Zapun et al., 1997) depend on the presence of monoglucosylated oligosaccharides and that the conformational state of the polypeptide chain has no influence on binding. However, the studies using RNAse B suffer from the drawback that the denatured enzyme exhibits virtually no hydrophobic character and as such is a poor substrate for molecular chaperones that interact with polypeptide chains.

Perhaps the most compelling evidence for lectin-independent interactions comes from recent experiments using Cnx variants that possess point mutations in the lectin site. These lack lectin function but retain the ability to bind ERp57 and suppress the aggregation of thermally denatured non-glycoproteins. When coexpressed with the heavy-chain subunit of MHC class I molecules in *D. melanogaster* SC2 cells, they retain the ability to associate with heavy chains and, furthermore, can stabilize them against rapid degradation (Leach and Williams, 2004).

A final concern regarding the polypeptide binding capacity of Cnx/Crt is the lack of an obvious hydrophobic binding site in the crystal structure of Cnx. However, Cnx was crystallized in the presence of 1 mM Ca^{2+} and in the absence of ATP. ATP binding enhances the ability of Cnx and Crt to suppress the aggregation of denatured, non-glycosylated proteins in vitro, and it induces substantial conformational changes in Cnx and Crt that include an increase in surface hydrophobicity (Ihara et al., 1999; Saito et al., 1999). Furthermore, the free Ca^{2+} concentration in the ER is 400-600 μM. This lower concentration is associated with modest changes in the protease sensitivity of Crt (Corbett et al., 2000), and it strongly enhances the potency of aggregation suppression at physiological temperature (A. Brockmeier, unpublished observations).

Abundant evidence thus indicates that Cnx and Crt can associate with folding glycoproteins through polypeptide-based interactions. Is there evidence that dual lectin- and polypeptide-based interactions with glycoproteins actually occur? Aggregation suppression experiments have been conducted on several glycoprotein substrates either with or without their monoglucosylated glycans. Whereas aggregation of jack bean α-mannosidase, soybean agglutinin or IgY can be suppressed by Cnx or Crt in the absence of glycosylation, the potency of suppression is substantially increased when the glycans are present (Ihara et al., 1999; Saito et al., 1999; Stronge et al., 2001). This suggests that Cnx and Crt are capable of engaging both glycan and polypeptide determinants on the same substrate. Presumably such dual engagement would increase the overall binding affinity. Evidence that this may be advantageous compared with other chaperones comes from an in vitro comparison of the abilities of Cnx and the Hsp70 chaperone BiP to suppress the aggregation of non-glycosylated and monoglucosylated protein substrates. Whereas Cnx and BiP are equally potent at suppressing the aggregation of non-glycosylated substrates, Cnx more potently suppresses that of glycoproteins bearing $Glc_1Man_9GlcNAc_2$ oligosaccharides. This advantage is lost upon substrate deglycosylation (Stronge et al., 2001).

Several pressing issues nevertheless remain: (1) the polypeptide binding specificities of Cnx and Crt need to be defined and compared to further our understanding of the relationship between these chaperones; (2) the polypeptide-binding sites must be precisely localized; and (3) by selectively disabling the polypeptide- and lectin-binding sites through mutagenesis, we must assess the relative impact that these have on the overall folding-promoting and quality control functions of Cnx and Crt.

ERp57 – a thiol oxidoreductase with diverse modes of substrate recognition

ERp57 is a member of the PDI family of proteins. It contains four thioredoxin-like domains: a, b, b′ and a′. The a and a′ domains contain the active site – CXXC – motifs. The N-terminal cysteine residue of the motif forms mixed disulfides with substrate proteins during oxidation and isomerization reactions. Interaction of ERp57 with the arm domains of Cnx and Crt is mediated primarily through its b′ domain and to some extent through its positively charged C-terminus (Fig. 2B) (Russell et al., 2004; Silvennoinen et al., 2004). The frequent finding that association of ERp57 with glycoprotein substrates can be prevented by treatment with CST or expression in Cnx-deficient cells has led to the commonly held view that ERp57 is always recruited to folding glycoproteins through its interactions with Cnx or Crt (Lindquist et al., 2001; Molinari and Helenius, 1999; Oliver et al., 1997). This view is supported by the in vitro finding that the oxidative folding of monoglucosylated RNAse B by ERp57 is dramatically enhanced by the presence of Cnx or Crt (Zapun et al., 1998).

Recent experiments studying the biogenesis of MHC class I molecules have challenged the notion that ERp57 is recruited to substrates by Cnx or Crt. Assembly of MHC class I molecules begins with binding of the Cnx- and ERp57-associated class I heavy chain to a soluble subunit, β_2-microglobulin (β_2m). The heavy-chain-β_2m heterodimer then enters a peptide loading complex consisting of Crt (or Cnx in some cases), ERp57, an ABC peptide transporter termed TAP and tapasin which, among other functions, bridges the class I heavy chain and TAP. Following the loading of a peptide antigen onto the class I molecule, the peptide loading complex disassembles and class I molecules are exported to the cell surface. This class I antigen presentation process occurs in almost all mammalian cell types. Cresswell and co-workers have recently documented a very abundant disulfide-linked complex of ERp57 and tapasin (Peaper et al., 2005). Under normal conditions, this contains ~15% of the cellular ERp57 but, upon upregulation of MHC class I molecule synthesis by interferon γ, as much as 80% of ERp57 may be covalently linked to tapasin. Clearly, tapasin is a highly preferred substrate for ERp57. Importantly, neither CST treatment nor chaperone deficiency in Cnx- or Crt-deficient cell lines has any effect on the level of the ERp57-tapasin complex, which indicates that Cnx and Crt do not recruit ERp57 into the peptide loading complex.

Despite clear in vitro evidence that ERp57 acts as a thiol oxidase, reductase and isomerase (Frickel et al., 2004), demonstrating these functions in cells has been difficult. Along with Crt, ERp57 has been shown to associate with the SERCA2b Ca^{2+} pump, inhibiting pump function in a process dependent on cysteines within an intralumenal loop of SERCA2b (Li and Camacho, 2004). Furthermore, ERp57 forms mixed disulfide complexes with viral glycoproteins and probably also with class I heavy chains (Antoniou et al., 2002; Lindquist et al., 2001; Molinari and Helenius, 1999). Since mixed disulfides are normal intermediates in oxidation and isomerization reactions, this implicates ERp57 in the catalysis

of these processes. We have confirmed such a role during MHC class I biogenesis by depleting ERp57 more than 90% by RNA interference (Y. Zhang and D.W., unpublished). This markedly reduces the rate of formation of heavy-chain disulfide bonds as well as the folding of one of the heavy-chain domains. Remarkably, despite the abundant ERp57-tapasin complex, ERp57 depletion has no apparent effect on the formation of the peptide-loading complex (other than the loss of ERp57) or on the loading of peptide antigens onto MHC class I molecules. These findings need to be extended to peptide-loading complexes containing other class I allotypes and to different species but, at present, the function of the ERp57-tapasin complex remains enigmatic. Further insights into the full range of ERp57 functions will require experiments using additional glycoprotein substrates and RNAi as well as the development of an ERp57-knockout mouse.

The Cnx/Crt chaperone system in ER-associated degradation

Most proteins that misfold or fail to assemble properly are targeted for degradation by ER-associated degradation (ERAD). This involves the identification of the misfolded protein, its unfolding and retrotranslocation into the cytosol, deglycosylation and finally degradation by the proteasome. Several ER chaperones seem to be involved in the initial identification and unfolding steps, including Cnx/Crt, BiP and PDI (reviewed in McCracken and Brodsky, 2003). In the case of Cnx or Crt, misfolded glycoproteins appear to be retained in the ER through repeated rounds of interaction with these chaperones and then are released from such futile folding cycles to undergo ERAD. Mannose trimming occurs during these prolonged folding cycles through the action of ER α-mannosidase I, which gives rise to a specific $Man_8GlcNAc_2$ isomer (Fig. 1). Mannose trimming may enhance ERAD in two ways. First, it promotes exit from the Cnx/Crt cycle because UGGT, the reglucosylation enzyme required for re-entry of many non-native glycoproteins into the cycle, is less effective with mannose-trimmed substrates (Sousa et al., 1992). This presumably increases access of the glycoprotein to other components of the ERAD system. Second, the $Man_8GlcNAc_2$ structure appears to act as a degradation signal (Jakob et al., 1998), possibly recognized by a putative ER lectin termed EDEM (Fig. 3). Overexpression of EDEM enhances ERAD whereas inhibition of α-mannosidase I by the drug kifunensin slows ERAD (Hosokawa et al., 2001; Jakob et al., 2001; Nakatsukasa et al., 2001). Additional studies have indicated a close temporal and physical relationship between release from Cnx and interaction with EDEM (Hosokawa et al., 2001; Jakob et al., 2001; Nakatsukasa et al., 2001). Thus there seems to be a delicate interplay between several oligosaccharide-recognizing proteins in the Cnx/Crt and ERAD pathways.

Although several features of substrate recognition in the ERAD pathway are elucidated, many more questions remain to be addressed. The degradation of some misfolded glycoproteins is not affected at all by blocking mannose trimming, and non-glycosylated mutant proteins can also be recognized by the ERAD system. Furthermore, many native glycoproteins acquire the $Man_8GlcNAc_2$ structure while in the ER but are spared rapid degradation. Clearly, there must be additional sensors of a glycoprotein's conformational state that are involved in making the decision to embark on ERAD. Such a property may reside within EDEM itself or in additional, as yet unidentified, components of the ERAD system.

Future perspectives

Despite tremendous progress in elucidating the functions of Cnx and Crt over the past decade, particularly the role of their lectin capacity in glycoprotein folding and quality control, there are several areas where further work is needed. The ability of these chaperones to recognize the polypeptide backbone of non-native glycoproteins needs to be investigated in terms of their binding specificities, the location of the binding site, modes of regulation and the relationship to the binding of other ligands. In particular, the relative roles of oligosaccharide versus polypeptide binding in promoting proper folding and ER retention need to be clarified. Further experiments must definitively assess the functions of ERp57 in the folding of a variety of glycoproteins, and we should also broaden our view to consider its functions that are independent of Cnx and Crt. The relationship of ERp57 to the many other thiol oxidoreductases of the ER is another area of intense interest. Why are there so many and to what extent do their substrate specificities overlap? Additional attention needs to be focused on the non-chaperone roles that Crt and Cnx may play in the ER. This is a well-developed, albeit somewhat controversial, area for Crt, and readers are directed to comprehensive recent reviews (Bedard et al., 2005; Groenendyk et al., 2004). Less attention has been focused on Cnx in this regard but some recent studies suggest that Cnx is involved in stress-induced apoptosis (Takizawa et al., 2004) and that it can control the function of the SERCA2b Ca^{2+} pump in a phosphorylation-regulated manner (Roderick et al., 2000). Finally, there are many indications that chaperones and folding enzymes are organized as networks within the ER, providing potential assembly lines for protein folding (Meunier et al., 2002; Tatu and Helenius, 1997). It will be of great interest to understand the physical framework of these networks in subdomains of the ER and particularly in terms of potential associations with the nascent chain translocation and Asn-linked glycosylation machineries.

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