

Calnexin, calreticulin and the folding of glycoproteins

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Protein folding in living cells is a complex, error-prone process. Numerous mechanisms are in place to ensure that newly synthesized proteins reach their folded functional form. One of the strategies is the covalent attachment of oligosaccharides to asparagine residues of nascent polypeptide chains. Such N-linked glycosylation occurs for the majority of soluble and membrane-bound proteins that are synthesized in the endoplasmic reticulum (ER). The addition of oligosaccharides can be beneficial during maturation by making folding intermediates more soluble. In addition, it allows the newly synthesized polypeptides to bind to calnexin and calreticulin, unique lectins present in the ER, which serve as molecular chaperones.

Several reviews have been published on calnexin and calreticulin in recent years that provided descriptions of the discovery and molecular characterization of these resident ER chaperones¹⁻⁴. Here, we outline what is currently known about calnexin, calreticulin and accessory enzymes in the process of glycoprotein folding.

Calnexin and calreticulin are lectins

Calnexin is a nonglycosylated type I membrane protein (65 kDa, 573 amino acids) with its substrate-binding domain in the lumen of the ER. It has an 89-residue cytoplasmic tail that is phosphorylated and carries a C-terminal RKPRRE sequence that serves as an ER-localization signal². Calreticulin, the soluble homologue (46 kDa, 400 amino acids), has both a KDEL retrieval sequence and a highly negatively charged region responsible for Ca²⁺ binding and ER retention at its C-terminus^{4,5}. The primary amino acid sequences of calreticulin and the luminal domain of calnexin show extensive stretches of homology.

Although reported to be localized in several cell compartments, calreticulin is clearly more abundant in the lumen of the ER, where it is one of the major proteins. A variety of functions have been ascribed to calreticulin, among them gene regulation, Ca²⁺ sequestration and RNA binding⁴. It is now clear that, like calnexin, it is a molecular chaperone that binds transiently to many glycoproteins in the ER⁶⁻⁹. Both seem to function as monomers, although they may be part of a larger dynamic network of proteins that includes other ER chaperones and folding enzymes*.

Initial experiments in tissue culture cells treated with glycosylation inhibitors showed that calnexin selectively associated with folding intermediates of glycoproteins¹⁰. Further studies using glucosidase inhibitors demonstrated that calnexin bound to partially glucose-trimmed glycoproteins¹¹. Subsequently, it was shown that calnexin¹²⁻¹⁴ and calreticulin^{6,7} specifically associated with monoglucosylated glycoproteins. Biochemical analysis using isolated oligosaccharides has confirmed that they are a new type of lectin and that they bind monoglucosylated core glycans (Glc₁Man₅₋₉GlcNAc₂), whereas they do not bind glycans with two or three or no glucose residues^{15,16}. Both the affinity for the oligosaccharides and the number of binding sites are unknown.

Generating the oligosaccharide ligands

N-linked glycans are added to growing polypeptide chains as 14-residue oligosaccharides (Glc₃Man₉GlcNAc₂)

Calnexin and calreticulin are molecular chaperones in the endoplasmic reticulum (ER). They are lectins that interact with newly synthesized glycoproteins that have undergone partial trimming of their core N-linked oligosaccharides. Together with the enzymes responsible for glucose removal and a glucosyltransferase that re-glucosylates already-trimmed glycoproteins, they provide a novel mechanism for promoting folding, oligomeric assembly and quality control in the ER.

by the oligosaccharyltransferase associated with the translocation machinery¹⁷ (Fig. 1). They are attached to the side chain of asparagine residues in the consensus sequence Asn-X-Ser/Thr. Transfer of the oligosaccharide generally occurs co-translationally as soon as the acceptor site enters the lumen of the ER.

The original core glycans contain a string of three glucose residues. Their removal begins on the growing nascent chain. Glucosidase I removes the terminal α 1,2-linked glucose, followed by elimination of the two remaining α 1,3-linked glucoses by glucosidase II (Fig. 1). Compared with other oligosaccharide-modification reactions in the secretory pathway, glucose trimming is exceptionally efficient; nearly all N-linked chains are completely de-glucosylated. The Golgi complex of some cell types contains an endomannosidase that eliminates any residual glucose residues that may have been missed in the ER¹⁸.

The monoglucosylated glycans that bind to calnexin and calreticulin arise in the ER as an intermediate during the stepwise removal of glucoses. However, the same structure is also formed by re-glucosylation of fully glucose-trimmed oligosaccharides. This occurs through the action of a luminal enzyme, the UDP-Glc:glycoprotein glucosyltransferase^{19,20} (Fig. 1). This enzyme counteracts the function of glucosidase II by adding glucose residues back to the oligosaccharides, thus regenerating monoglucosylated glycans²¹. The existence of de- and re-glucosylation reactions introduces the possibility of a cycle that involves association with, and dissociation from, calnexin and calreticulin (see below).

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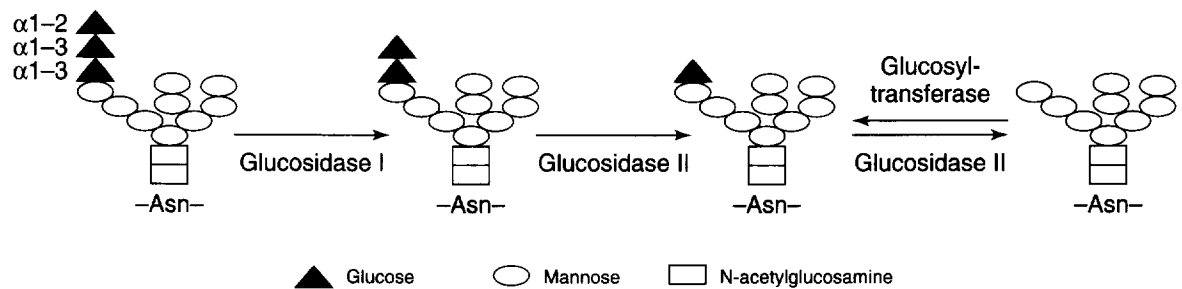


FIGURE 1

N-glycosylation and glucose trimming in the endoplasmic reticulum (ER). The core oligosaccharide ($\text{Glc}_3\text{Man}_9\text{GlcNAc}_2$) is synthesized in the ER membrane and transferred to the consensus N-glycosylation sites (Asn-X-Ser/Thr) on nascent polypeptide chains. Immediately after transfer, the glucose residues are removed by the sequential action of glucosidases I and II. Glucosidase I removes the terminal α 1-2-linked glucose, whereas glucosidase II removes the two remaining α 1-3-linked residues¹⁷. Fully glucose-trimmed high-mannose glycans can be re-glucosylated by UDP-glucose:glycoprotein glucosyltransferase if present on incompletely folded or assembled proteins¹⁹.

The enzymes that generate the monoglucosylated glycoproteins have been known for many years, but their genes have been cloned only recently (Table 1). Glucosidase I is a type-II membrane glycoprotein (92 kDa). Glucosidase II, by contrast, is soluble and does not share any primary sequence homology with glucosidase I. In mammalian cells, it is a heterodimer in which the α subunit (104 kDa) contains the active site, and the β subunit (58 kDa) carries retrieval and retention sequences for ER localization.

UDP-Glc:glycoprotein glucosyltransferase is a soluble enzyme of 170 kDa. It and glucosidase II are the only soluble enzymes involved in the processing of N-linked carbohydrates in the secretory pathway. Its C-terminal 500 amino acids share homology between different species and with other known glucosyltransferases, suggesting that it probably contains the catalytic domain.

The glucosyltransferase only recognizes non-native glycoproteins as substrates^{22,23}. Among the proteins capable of distinguishing native from non-native polypeptides, it is the only one known to modify its substrates covalently. As long as glycoproteins are incompletely folded or misfolded, they are re-glucosylated by the transferase and continue to be bound to calnexin and calreticulin. This is illustrated by the tsO45 vesicular stomatitis virus (VSV) G protein, which has a thermoreversible folding defect. At the nonpermissive temperature, the G protein undergoes continuous de- and re-glucosylation in the ER of infected cells²¹ and it remains calnexin associated²⁴. The majority of glycoproteins undergo re-glucosylation at least once²⁵. While it is not clear how the transferase distinguishes between folded and unfolded substrate glycoproteins, the enzyme can detect very subtle differences between protein conformers (E. S. Trombetta and A. Helenius, unpublished).

Glucosidases I and II are sensitive to 1-deoxynojirimycin, castanospermine and related α -glucosidase inhibitors. By preventing glucose trimming, these agents inhibit binding of newly synthesized glycoproteins to the chaperones^{7,8,11,12,26-28}. Therefore, they have been used to study the maturation of glycoproteins in the absence of calnexin and calreticulin binding^{9,24,27,29}.

A model for transient glycoprotein binding to calnexin and calreticulin

Binding of glycoproteins to calnexin and calreticulin is a transient step during early maturation. Depending on the distribution of glycans in the polypeptide chain, association can begin with the growing nascent chain³⁰. It continues posttranslationally for variable periods of time – ranging from a few minutes to hours. With permanently misfolded proteins, or unassembled subunits of oligomers, the association usually continues until the protein is degraded^{9,11,12,13,31-33}. Final release from calnexin and calreticulin occurs when the polypeptides have reached a fully folded conformation or when they have undergone oligomeric assembly (for reviews, see Refs 2, 34 and 35).

According to one model (Fig. 2a; Ref. 36), glycoprotein folding intermediates are retained in the ER by a cycle of glucose removal and addition coupled to chaperone binding and release. Glucosidase II performs a dual role: it is needed to uncover the monoglucosylated glycans for initial binding and afterwards to de-glucosylate them completely, thus releasing the glycoproteins from the chaperones. The glucosyltransferase allows reassociation by re-glucosylating unbound non-native proteins. The glycoprotein exits the cycle when it has reached its native form and becomes 'invisible' to the glucosyltransferase.

Consistent with the need for glucose removal for substrate release, inhibition of glucosidase II activity inhibits release of substrates from calnexin and calreticulin^{9,13,28,37,38}. It also prevents complete folding and oxidation of bound influenza HA molecules, suggesting that cycling of the substrate on and off the chaperones is important for proper folding⁹.

Do calnexin and calreticulin interact with the polypeptide moiety of substrates?

Most studies with glycosylation and glucosidase inhibitors, mutant cell lines and site-specific mutagenesis of consensus glycosylation sites have confirmed that calnexin and calreticulin binding requires the presence of one or more monoglucosylated N-linked glycans^{6,7,10-14,37,39,40}. In the model outlined above (Fig. 2a), calnexin and calreticulin function as lectins, whereas the glucosyltransferase monitors the folding

TABLE I - PROTEINS INVOLVED IN THE CALNEXIN/CALRETICULIN CYCLE

Protein	Cells defective in the respective gene		
	Mammals	<i>S. cerevisiae</i>	Other
Glucosidase I ^(a) N- [800 a.a.] -C	<i>LEC23</i> ^(b)	<i>gls1-1</i> ^(c) No growth defect	
Glucosidase II ^(d) [α 900 a.a.] α subunit [β 500 a.a.] β subunit	<i>Phar 2.7</i> ^(e)	<i>Δgls2</i> ^(d) No growth defect	
Glucosyltransferase ^(f,g) [1500 a.a.]	ND#	<i>Δkre5</i> * ^(h) Severe growth defect	<i>S. pombe Δgpt1</i> ^(g) No growth defect
Calnexin ⁽ⁱ⁾ N- [570 a.a.] -C	<i>CEM-NKR</i> ⁽ⁱ⁾	<i>Δcne1</i> ** ^(k,l) No growth defect	<i>S. pombe cnx1</i> ^(m,n) Essential
Calreticulin ^(o) [400 a.a.]	ND	***	

#None described.

**KRES* is the only gene found in the *Saccharomyces cerevisiae* genome showing moderate homology with other UDP-Glc: glycoprotein glucosyltransferases. No evidence has been obtained indicating that Kre5p is a functional glycoprotein re-glucosylating enzyme [Ref. (p)].

***CNE1* is the only gene found in the *S. cerevisiae* genome showing some similarity to those encoding calnexin or calreticulin; Cne1p is a membrane protein only marginally related to the calnexin family.

***No protein similar to calreticulin is found in the *S. cerevisiae* genome.

Abbreviations: a.a., amino acids; *S. pombe*, *Schizosaccharomyces pombe*.

References

- ^(a) Kalz-Fuller, B. *et al.* (1995) *Eur. J. Biochem.* 231, 344–351; ^(b) Ray, M. K. *et al.* (1991) *J. Biol. Chem.* 266, 22818–22825; ^(c) Esmon, B. *et al.* (1984) *J. Biol. Chem.* 259, 10322–10327; ^(d) Trombetta, E. S. *et al.* (1996) *J. Biol. Chem.* 271, 27509–27516; ^(e) Reitman, M. L. *et al.* (1982) *J. Biol. Chem.* 257, 10357–10363; ^(f) Parker, K. *et al.* (1995) *EMBO J.* 14, 1294–1303; ^(g) Fernandez, F. *et al.* (1996) *EMBO J.* 15, 705–713; ^(h) Meaden, P. *et al.* (1990) *Mol. Cell. Biol.* 10, 3013–3019; ⁽ⁱ⁾ Bergeron, J. J. *et al.* (1994) *Trends Biochem. Sci.* 19, 124–128; ^(j) Scott, J. and Dawson, J. (1995) *J. Immunol.* 155, 143–148; ^(k) de Virgilio, C. *et al.* (1993) *Yeast* 9, 185–188; ^(l) Parlati, F. *et al.* (1995) *J. Biol. Chem.* 270, 244–253; ^(m) Parlati, F. *et al.* (1995) *EMBO J.* 14, 3064–3072; ⁽ⁿ⁾ Jannatipour, M. and Rokeach, L. A. (1995) *J. Biol. Chem.* 270, 4845–4853; ^(o) Michalak, M., ed. (1996) *Calreticulin*, R. G. Landes; ^(p) Fernandez, F. S. *et al.* (1994) *J. Biol. Chem.* 269, 30701–30706.

status. However, there is an ongoing debate whether binding to the chaperones also involves protein-protein interactions.

One of the reasons for the uncertainty is that some glycoproteins artificially deprived of glycan addition by using tunicamycin, or by mutation of consensus glycosylation sequences, have been shown to co-immunoprecipitate with calnexin^{41–43}. The ε subunit of the T-cell receptor is also invoked as a naturally occurring nonglycosylated protein that binds to calnexin because, when overexpressed together with mutant calnexin, its distribution in cells is altered⁴⁴. Calreticulin has not been reported to bind to nonglycosylated proteins.

For VSV G-protein mutants, the coprecipitation phenomenon was recently reported to depend on aggregation of the misfolded, nonglycosylated protein in the ER⁴⁰. It is commonly observed that, when expressed without N-linked oligosaccharides, many glycoproteins misfold, aggregate and coprecipitate

with ER chaperones^{45,46}. The ε subunit of the T-cell receptor also aggregates when expressed without other subunits of the receptor (J. Hoppa and H. Ploegh, pers. commun.). If aggregation is the reason why nonglycosylated proteins can coprecipitate with calnexin, it remains unclear whether there is a direct interaction between the nonglycosylated protein and calnexin.

Another series of observations has suggested that a glycoprotein-calnexin interaction may be mediated both by carbohydrate-protein and protein-protein interactions. When isolated anti-calnexin immune complexes were treated with endoglycosidase H, the oligosaccharides were removed but the substrate polypeptides did not elute from the immunobeads^{15,39,42}. Clearly, the substrates remained bound by interactions other than those occurring between the oligosaccharides and the chaperone. Most of these experiments were performed with membrane proteins that may remain artificially trapped in a

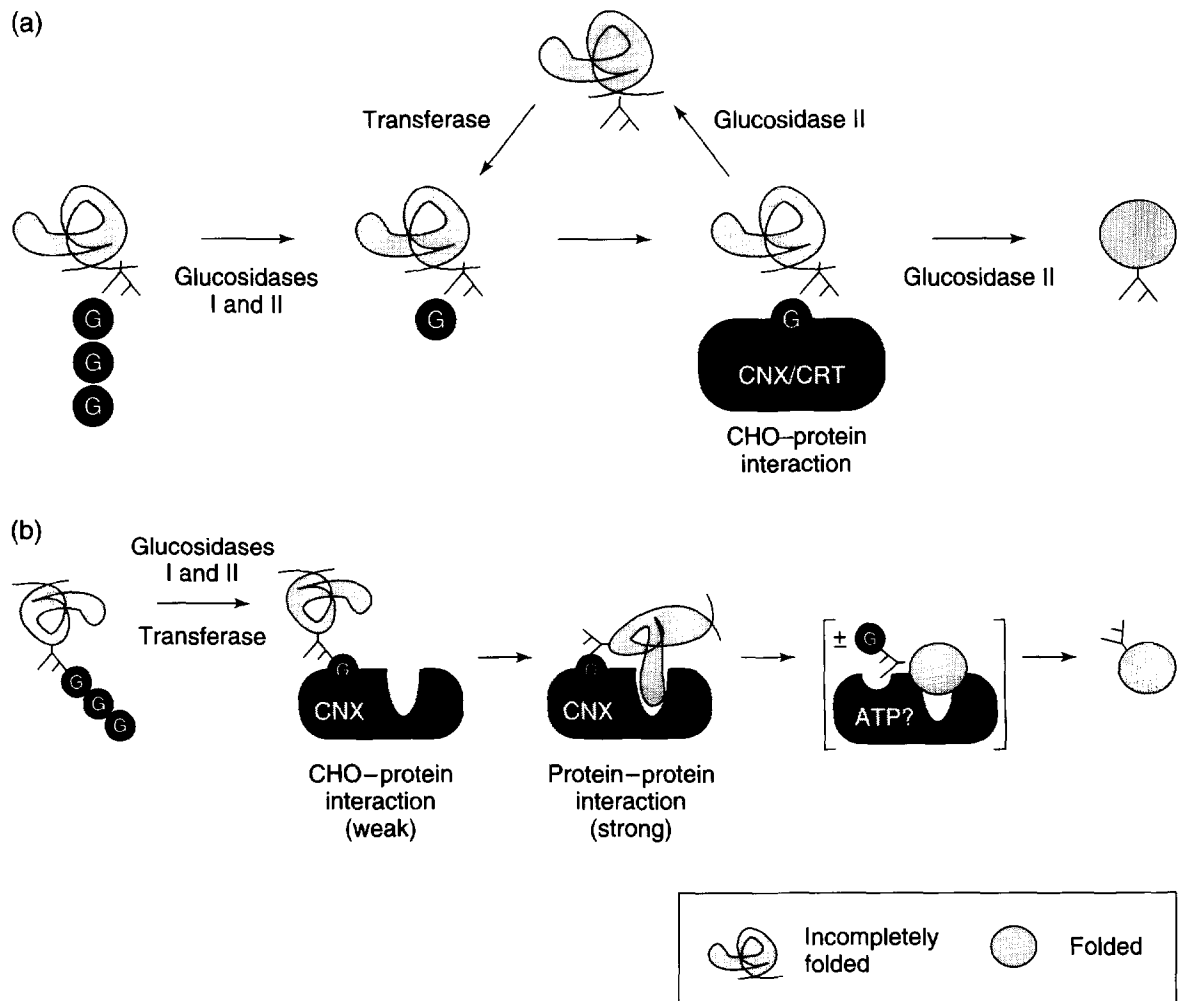


FIGURE 2

Models for calnexin and calreticulin binding to their substrate glycoproteins. (a) Lectin-only model³⁶. Glucose residues (G) in the core oligosaccharides are trimmed by glucosidases I and II. When trimmed to the monoglucosylated form, the oligosaccharides mediate the binding of the glycoprotein to calnexin (CNX) and calreticulin (CRT). These function as lectins. Release of the substrate glycoprotein depends on hydrolysis of the remaining glucose residues by glucosidase II. If the glycoproteins are not completely folded, the high-mannose glycans are selectively re-glucosylated by the glucosyltransferase, allowing the oligosaccharides to rebind to the chaperones. Once glycoproteins reach their mature conformation, they are no longer recognized by the glucosyltransferase. (b) Dual-mode model. According to this model, the monoglucosylated oligosaccharides are merely needed to bring glycoproteins into contact with calnexin (CHO-protein interaction). After the initial carbohydrate-mediated association, stronger protein-protein interactions occur between the chaperone and peptide elements exposed on the surface of the incompletely folded substrate protein. These stabilize the complex until the peptides are hidden in the folded protein. Thus, release of the substrate depends on conformational changes in the substrate glycoprotein that eliminate the protein-protein interaction. It may also involve ATP-mediated conformational changes in calnexin. The model is based in part on reports by Ware *et al.*¹⁵ and Williams³.

common detergent micelle with calnexin after the glycan is removed. Nonetheless, these results may suggest that protein-protein interactions are present. Together with the observation that class-I heavy chains can be chemically crosslinked to calnexin at a site close to the transmembrane region⁴⁷, these findings have inspired a model in which the oligosaccharides merely act to bring substrate and calnexin into close proximity, whereafter a protein-protein interaction, similar to that seen for classical chaperones, takes over^{3,15} (Fig. 2b). Once the complex has been established, the oligosaccharide component is thought to be dispensable for binding.

According to this model, release of the substrate does not occur through the action of glucosidase II, but rather as a result of a conformational change in

the substrate polypeptide. As the peptide segments responsible for binding are buried through folding of the protein, the complex becomes destabilized and dissociates. Release has also been proposed to be modulated by ATP binding to calnexin^{3,15,48}. ATP has been reported to bind to calnexin, but there is no evidence for ATPase activity⁴⁸.

In recent studies using bovine pancreatic ribonuclease (RNase) as a ligand, evidence for the lectin-only model has been found^{37,49}. RNase-calnexin and RNase-calreticulin complexes were treated with N-glycanase F, which removed the N-linked oligosaccharides, or with glucosidase II, which just removed the glucose residues³⁷. In either case, full dissociation of the complexes occurred. It was concluded that it was the interaction between the oligosaccharides and

the chaperones that held these complexes together. This was further supported by observations that the fully folded RNase binds to calnexin and calreticulin³⁷, and the ectodomain of calnexin⁴⁹, as efficiently as does unfolded RNase. Glucosidase-II-dependent release from calnexin and calreticulin also occurred irrespective of the folding status of the RNase³⁷.

The evidence for a lectin-like association both *in vivo* and *in vitro* is strong. It is based not only on the direct biochemical binding studies with isolated oligosaccharides but also on extensive studies in cells and microsomes. As the need for glucosidase II to dissociate the complexes also has been demonstrated experimentally, this suggests that an oligosaccharide-mediated interaction is needed for establishing the complexes and for maintaining them^{9,13,38}. Furthermore, experiments with microsomes have shown that re-glycosylation not only occurs but also results in substrate binding to calnexin¹³. More experiments will be needed to evaluate the possible role of protein-protein interactions – for which the evidence so far is indirect. Only a few proteins have been analysed in detail. To obtain a more complete picture, detailed biochemical and structural information is needed for several soluble and membrane-bound substrate proteins. Both calnexin and calreticulin need to be included in these studies. It will also be important to design binding assays better than those based on co-immunoprecipitation.

Are calnexin and calreticulin functionally distinct?

Since the lectin specificities of calnexin and calreticulin are identical^{15,16}, it is not surprising that there is extensive overlap between their substrate glycoproteins. Not only do they bind to similar sets of proteins but they can also associate simultaneously with the same glycoprotein²⁸ (D. N. Hebert and A. Helenius, unpublished).

Closer analysis of the substrate specificity reveals interesting differences. VSV G protein, which has two N-linked glycans, binds to calnexin but not calreticulin²⁴. The protein associates first with BiP, an Hsp70 analogue, whereafter it is transferred to calnexin. In the case of human class-I MHC molecules, calnexin binds to the free heavy chain but is soon replaced by calreticulin when the heavy chain oligomerizes with the β_2 -microglobulin subunit⁵⁰. Influenza HA, by contrast, associates initially with both calnexin and calreticulin, but calreticulin dissociates from the complex earlier⁹. Mutation of glycosylation consensus sites in different combinations has shown that calreticulin binding to HA depends on three glycans in the top and hinge domain of the protein, whereas calnexin binding depends on the four sites in the stem domain (D. N. Hebert and A. Helenius, unpublished; Fig. 3). The top domain reaches its oxidized form earlier than the stem domain, which explains why calreticulin binding ceases earlier than calnexin binding. Differences are also observed for the T-cell receptor (TCR) subunits: TCR α , TCR β , CD3 δ and CD3 ϵ bind to calnexin^{12,44,51,52}, whereas only the α and β subunits bind to calreticulin, and this for shorter times than they bind to calnexin³⁸.

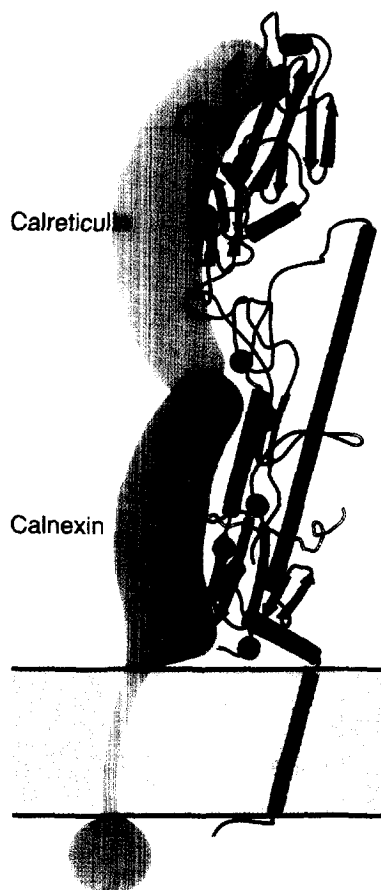


FIGURE 3

Mapping of calnexin- and calreticulin-binding domains on influenza haemagglutinin (HA). The location of N-linked glycans plays a role in differential calnexin and calreticulin binding to HA, suggesting spatial constraints in determining chaperone specificity (D. N. Hebert and A. Helenius, unpublished). The N-linked glycosylation sites are indicated by the red circles. Glycosylation sites in the stem domain were found to be involved in calnexin binding, whereas the glycosylation sites in the globular/hinge region were required for calreticulin binding. The binding of the chaperones to distinct regions of the molecule permitted the formation of ternary HA-calnexin-calreticulin complexes as depicted. The structure of HA was adapted from Ref. 59.

Such differences between the chaperones may reflect differential accessibility of the glycans from the luminal versus membrane-proximal side of a nascent or newly synthesized protein. The shift of the MHC class-I heavy chain from calnexin to calreticulin when it binds to β_2 -microglobulin may, for example, reflect a conformational change that increases the luminal exposure of the glycans. Alternatively, the specificity differences could be explained by exposure of polypeptide determinants that interact differently with calnexin and calreticulin (see Fig. 2b).

How important is the calnexin/calreticulin cycle?

Calnexin and calreticulin associate with a wide variety of proteins, including soluble secretory proteins, complex membrane receptors and ion channels, lysosomal hydrolases and extracellular matrix components (Table 2). They also play a role in the biosynthesis of viral glycoproteins, including those of HIV-1, hepatitis C virus and influenza virus. Their substrate-binding function has been linked to a number of diseases with an ER-storage phenotype, such as cystic fibrosis, familial hypercholesterolaemia and α_1 -antitrypsin deficiency^{34,53}. In these disease states, they serve as part of the machinery that retains the misfolded glycoproteins in the ER.

Calnexin/calreticulin binding increases the efficiency of glycoprotein folding both in live cells and in microsomes. It is not difficult to find evidence of deleterious effects on the maturation of specific glycoproteins when binding to the chaperones is inhibited. Addition of glucosidase inhibitors results in a lowered rate and efficiency of secretion for many glycoproteins⁵⁴. Also, the folding and subsequent

TABLE 2 – GLYCOPROTEINS THAT BIND TO CALNEXIN AND CALRETICULIN

Ligands	Calnexin	Calreticulin	Refs
Endogenous glycoproteins			
α1-antitrypsin	+	ND	a,b
α1-chymotrypsin	+	ND	a
Apo B-100	+	ND	a
CFTR	+	ND	c
Complement 3	+	ND	a
Fibrinogen	+	ND	d
GABA _A receptor	+	ND	e
GLUT-1 glucose transporter	+	+	f
gp80 (MDCK glycoprotein)	+	ND	g
Integrins	+	+	h-j
MHC class I	+	+	k-u
MHC class II	+	ND	v-x
Myeloperoxidase	ND	+	y
Nicotinic acetylcholine receptor	+	ND	z
P-glycoprotein	+	ND	aa
T-cell receptor	+	+	bb-dd
Thyroglobulin	+	ND	ee
Transferrin	+	+	a,ff
Viral glycoproteins			
Cytomegalovirus glycoprotein B	+	ND	gg
HIV gp160	+	+	hh
HA	+	+	ii-ll
VSV-G	+	-	jj, mm,oo
Hepatitis C virus E1/E2	+	ND	pp

^aOu, W. J. *et al.* (1993) *Nature* 364, 771–776; ^bLe, A. *et al.* (1994) *J. Biol. Chem.* 269, 7514–7519; ^cPind, S. *et al.* (1994) *J. Biol. Chem.* 269, 12784–12788; ^dRoy, S. *et al.* (1996) *J. Biol. Chem.* 271, 24544–24550; ^eConnolly, C. N. *et al.* (1996) *J. Biol. Chem.* 271, 89–96; ^fOliver, J. D. *et al.* (1996) *J. Biol. Chem.* 271, 13691–13696; ^gWada, I. *et al.* (1994) *J. Biol. Chem.* 269, 7464–7472; ^hLenter, M. and Vestweber, D. (1994) *J. Biol. Chem.* 269, 12263–12268; ⁱHotchin, N. A. *et al.* (1995) *J. Cell Biol.* 128, 1209–1219; ^jCoppolino, M. *et al.* (1995) *J. Biol. Chem.* 270, 23132–23138; ^kDegen, E. and Williams, D. B. (1991) *J. Cell Biol.* 112, 1099–1115; ^lCapps, G. G. and Zuniga, M. C. (1994) *J. Biol. Chem.* 269, 11634–11639; ^mJackson, M. R. *et al.* (1994) *Science* 263, 384–387; ⁿOrtmann, B. *et al.* (1994) *Nature* 368, 864–867; ^oRajagopalan, S. and Brenner, M. B. (1994) *J. Exp. Med.* 180, 407–412; ^pBalow, J. P. *et al.* (1995) *J. Biol. Chem.* 270, 29025–29029; ^qCarreno, B. M. *et al.* (1995) *J. Immunol.* 155, 4726–4733; ^rNossner, E. and Parham, P. (1995) *J. Exp. Med.* 181, 327–337; ^sTector, M. and Salter, R. D. (1995) *J. Biol. Chem.* 270, 19638–19642; ^tVassilakos, A. *et al.* (1996) *EMBO J.* 15, 1495–1506; ^uSadasivan, B. *et al.* (1996) *Immunity* 5, 1–20; ^vAnderson, K. S. and Cresswell, P. (1994) *EMBO J.* 13, 675–682; ^wSchreiber, K. L. *et al.* (1994) *Int. Immunol.* 6, 101–111; ^xMarks, M. S. *et al.* (1995) *J. Biol. Chem.* 270, 10475–10481; ^yNauseef, W. M. *et al.* (1995) *J. Biol. Chem.* 270, 4741–4747; ^zGelman, M. S. *et al.* (1995) *J. Biol. Chem.* 270, 15085–15092; ^{aa}Loo, T. W. and Clarke, D. M. (1994) *J. Biol. Chem.* 269, 28683–28689; ^{bb}Kearse, K. P. *et al.* (1994) *EMBO J.* 13, 3678–3686; ^{cc}Rajagopalan, S. *et al.* (1994) *Science* 263, 387–390; ^{dd}Van Leeuwen, J. and Kearse, K. (1996) *J. Biol. Chem.* 271, 25345–25349; ^{ee}Kim, P. S. and Arvan, P. (1995) *J. Cell Biol.* 128, 29–38; ^{ff}Wada, I. *et al.* (1995) *J. Biol. Chem.* 270, 20298–20304; ^{gg}Yamashita, Y. *et al.* (1996) *J. Virol.* 70, 2237–2246; ^{hh}Otteken, A. and Moss, B. (1996) *J. Biol. Chem.* 271, 97–103; ⁱⁱHammond, C. *et al.* (1994) *Proc. Natl. Acad. Sci. U. S. A.* 91, 913–917; ^{jj}Peterson, J. R. *et al.* (1995) *Mol. Biol. Cell* 6, 1173–1184; ^{kk}Chen, W. *et al.* (1995) *Proc. Natl. Acad. Sci. U. S. A.* 92, 6229–6233; ^{ll}Hebert, D. N. *et al.* (1996) *EMBO J.* 15, 2961–2968; ^{mm}Hammond, C. and Helenius, A. (1994) *Science* 266, 456–458; ⁿⁿHammond, C. and Helenius, A. (1994) *J. Cell Biol.* 126, 41–52; ^{oo}Cannon, K. S. *et al.* (1996) *J. Biol. Chem.* 271, 14280–14284; ^{pp}Dubuisson, J. and Rice, C. M. (1996) *J. Virol.* 70, 778–786. (+), association demonstrated; (-), does not associate; ND, not determined.

expression at the cell surface of HIV-1 glycoprotein, VSV G protein and both human and murine MHC class I molecules are severely inhibited^{24,26,27,29,55}. However, mammalian cells lacking glucosidases I or II, or calnexin, are viable (Table 1). Apparently, most of the substrates can fold sufficiently well to satisfy the needs of these cells.

It also appears that most of the components of the cycle are dispensable in *Saccharomyces cerevisiae* as the genes for glucosidases I and II, as well as for the calnexin homologue Cne1p, can be disrupted without any effect on growth (Table 1). By contrast, elimination

of Kre5p, an ER protein with partial homology to the glucosyltransferase, severely affects viability. However, there is no evidence that Kre5p or Cne1p are functional homologues of the glucosyltransferase and calnexin. As Kre5p and glucosidases I and II affect the synthesis of cell wall glucan structures, it is possible that their primary function in *S. cerevisiae* is not related to folding (Ref. 56, and J. F. Simons and A. Helenius, unpublished).

In the fission yeast *Schizosaccharomyces pombe*, calnexin and glucosyltransferase are closely related to their homologues in higher species. The calnexin

homologue, *cnx1*, is an essential protein, whereas no phenotype has been associated with disruption of the gene encoding the glucosyltransferase. The fact that mammalian cells devoid of calnexin are viable, whereas *S. pombe* are not, might be explained by the presence of calreticulin in the former. Both *S. pombe* and *S. cerevisiae* seem to lack a calreticulin homologue.

That proteins manage to fold, albeit less efficiently, in certain cells that are devoid of a functional calnexin/calreticulin cycle is probably due to redundancy between folding factors in the ER. The ER is unique among the compartments of the cell in that it contains a large number of different chaperones and folding enzymes at high concentrations. The notion of redundant functions is supported by the observation that BiP is upregulated in castanospermine-treated or glucosidase-II-deficient tissue culture cells^{26,57}.

Chaperones of a new kind

Calnexin and calreticulin fulfil the most stringent definitions of molecular chaperones⁵⁸. They associate transiently with folding intermediates of a large number of different proteins and promote proper folding and assembly through a cycle of binding and release^{9,29}. They help prevent aggregation of proteins, suppress formation of non-native disulphide bonds, inhibit premature degradation and provide retention for the quality-control function in the ER. Thus, their impact on protein biogenesis is similar to that described for the classical chaperones in the Hsp70 and chaperonin families⁵⁸. However, their functional principle appears to be different. Classical chaperones bind to hydrophobic sequences exposed on the surface of incompletely folded proteins. Substrate binding and release is accompanied by a cycle of conformational changes regulated by ATP binding and hydrolysis. For calnexin and calreticulin, an ATP-driven cycle has not been shown. In addition, they do not seem to distinguish between folded and unfolded proteins directly^{37,49}. Their functionality seems to depend on independently acting enzymes that regulate association and dissociation by inducing covalent modifications in the substrate proteins. Recognition of incompletely folded proteins and the release of substrates from calnexin and calreticulin may, as discussed above, be entirely determined by these enzymes.

More than likely, the binding of calnexin and calreticulin to glycoproteins induces specific effects on the folding of the polypeptide chain. It may, for example, slow down the folding rate^{9,29}. It may immobilize specific cysteines, thus preventing incorrect disulphide pairing. It may also prevent premature oligomerization by steric obstruction and assist association of certain folding or assembly intermediates with other ER proteins.

Examples of such effects are starting to appear in the literature. The co-translational binding of calnexin and calreticulin to nascent chains of influenza HA prevents the formation of a specific non-native disulphide linkage between N-terminal cysteines³⁰ and prevents premature oligomeric assembly of HA monomers⁹. In the case of MHC class I, calreticulin may help in mediating the binding of the assembling antigen with TAP and other ER proteins⁵⁰.

Glycoproteins have no doubt evolved to make use of the chaperones in the ER in many ways. They have adjusted their glycosylation patterns to best exploit these lectins. Being so different in structure and properties, each glycoprotein is likely to interact differently with the folding machinery in the ER. As more is learned about calnexin and calreticulin and their accessory enzymes, and as different substrate proteins are analysed, great versatility in the system is likely to emerge. Both of the two current models – the lectin-only and the dual-mode model – may apply, depending on the properties and complexity of the specific substrate glycoproteins.

References

- HELENIUS, A. (1994) *Mol. Biol. Cell* 5, 253–265
- BERGERON, J. M., BRENNER, M. B., THOMAS, D. Y. and WILLIAMS, D. B. (1994) *Trends Biochem. Sci.* 19, 124–128
- WILLIAMS, D. B. (1995) *Biochem. Cell Biol.* 73, 123–132
- MICHALAK, M., ed. (1996) *Calreticulin*, R. G. Landes
- SÖNNICHSEN, B., FÜLLEKRUG, J., VAN NGUYEN, P., DIEKMANN, W., ROBINSON, D. G. and MIESKES, G. (1994) *J. Cell Sci.* 107, 2705–2717
- NAUSEEF, W. M., McCORMICK, S. J. and CLARK, R. A. (1995) *J. Biol. Chem.* 270, 4741–4747
- PETERSON, J. R., ORA, A., VAN NGUYEN, P. and HELENIUS, A. (1995) *Mol. Biol. Cell* 6, 1173–1184
- WADA, I., IMAI, S.-I., KAI, M., SAKANE, F. and KANO, H. (1995) *J. Biol. Chem.* 270, 20298–20304
- HEBERT, D. N., FOELLMER, B. and HELENIUS, A. (1996) *EMBO J.* 15, 2961–2968
- OU, W.-J., CAMERON, P. H., THOMAS, D. Y. and BERGERON, J. J. M. (1993) *Nature* 364, 771–776
- HAMMOND, C., BRAAKMAN, I. and HELENIUS, A. (1994) *Proc. Natl. Acad. Sci. U. S. A.* 91, 913–917
- KEARSE, K. P., WILLIAMS, D. B. and SINGER, A. (1994) *EMBO J.* 13, 3678–3686
- HEBERT, D. N., FOELLMER, B. and HELENIUS, A. (1995) *Cell* 81, 425–433
- ORA, A. and HELENIUS, A. (1995) *J. Biol. Chem.* 270, 26060–26062
- WARE, F. E., VASSILAKOS, A., PETERSON, P. A., JACKSON, M. R., LEHRMAN, M. A. and WILLIAMS, D. B. (1995) *J. Biol. Chem.* 270, 4697–4704
- SPIRO, R. G., ZHU, Q., BHOYROO, V. and SÖLING, H.-D. (1996) *J. Biol. Chem.* 271, 11588–11594
- KORNFELD, R. and KORNFELD, S. (1985) *Annu. Rev. Biochem.* 54, 631–664
- LUBAS, W. A. and SPIRO, R. G. (1988) *J. Biol. Chem.* 263, 3990–3996
- PARODI, A. J., MENDELZON, D. H. and LEDERKREMER, G. H. (1983) *J. Biol. Chem.* 258, 8260–8265
- TROMBETTA, E. S., BOSCH, M. and PARODI, A. J. (1989) *Biochemistry* 28, 8108–8116
- SUH, P., BERGMANN, J. E. and GABEL, C. A. (1989) *J. Cell Biol.* 108, 811–819
- SOUSA, M. C., FERRERO-GARCIA, M. A. and PARODI, A. J. (1992) *Biochemistry* 31, 97–105
- TROMBETTA, E. S. and PARODI, A. J. (1992) *J. Biol. Chem.* 267, 9236–9240
- HAMMOND, C. and HELENIUS, A. (1994) *Science* 266, 456–458
- GAÑAN, S., CAZZULO, J. J. and PARODI, A. J. (1991) *Biochemistry* 30, 3098–3104
- BALOW, J. P., WEISSMAN, J. D. and KEARSE, K. (1995) *J. Biol. Chem.* 270, 29025–29029

- 27 TECTOR, M. and SALTER, R. D. (1995) *J. Biol. Chem.* 270, 19638–19642
- 28 OTTEKEN, A. and MOSS, B. (1996) *J. Biol. Chem.* 271, 97–103
- 29 VASSILAKOS, A., COHEN-DOYLE, M. F., PETERSON, P. A., JACKSON, M. R. and WILLIAMS, D. B. (1996) *EMBO J.* 15, 1495–1506
- 30 CHEN, W., HELENIUS, J., BRAAKMAN, I. and HELENIUS, A. (1995) *Proc. Natl. Acad. Sci. U. S. A.* 92, 6229–6233
- 31 PIND, S., RIORDAN, J. R. and WILLIAMS, D. B. (1994) *J. Biol. Chem.* 269, 12784–12788
- 32 JACKSON, M. R., COHEN-DOYLE, M. F., PETERSON, P. A. and WILLIAMS, D. B. (1994) *Science* 263, 384–387
- 33 QU, D. F., TECKMAN, J. H., OMURA, S. and PERLMUTTER, D. H. (1996) *J. Biol. Chem.* 271, 22791–22795
- 34 HAMMOND, C. and HELENIUS, A. (1995) *Curr. Opin. Cell Biol.* 7, 523–529
- 35 WILLIAMS, D. B. and WATTS, T. A. (1995) *Curr. Opin. Immunol.* 7, 77–84
- 36 HAMMOND, C. and HELENIUS, A. (1993) *Curr. Biol.* 3, 884–885
- 37 RODAN, A. R., SIMONS, J. F., TROMBETTA, E. S. and HELENIUS, A. (1996) *EMBO J.* 15, 6921–6930
- 38 VAN LEEUWEN, J. E. M. and KEARSE, K. P. (1996) *J. Biol. Chem.* 271, 25345–25349
- 39 ZHANG, Q., TECTOR, M. and SALTER, R. D. (1995) *J. Biol. Chem.* 270, 3944–3948
- 40 CANNON, K. S., HEBERT, D. N. and HELENIUS, A. (1996) *J. Biol. Chem.* 271, 14280–14284
- 41 LOO, T. W. and CLARKE, D. M. (1994) *J. Biol. Chem.* 269, 28683–28689
- 42 ARUNACHALAM, B. and CRESSWELL, P. (1995) *J. Biol. Chem.* 270, 2784–2790
- 43 CARRENO, B. M., SCREIBER, K. L., MCKEAN, D. J., STROYNOWSKI, I. and HANSEN, T. H. (1995) *J. Immunol.* 154, 5173–5180
- 44 RAJAGOPALAN, S., XU, Y. and BRENNER, M. B. (1994) *Science* 263, 387–390
- 45 GIBSON, R., SCHLESINGER, S. and KORNFELD, S. (1979) *J. Biol. Chem.* 254, 3600–3605
- 46 HURTLEY, S. M., BOLE, D. G., HOOVER-LITTY, H., HELENIUS, A. and COPELAND, C. S. (1989) *J. Cell Biol.* 108, 2117–2126
- 47 MARGOLESE, L., WANECK, G. L., SUZUKI, C. K., DEGEN, E., FLAVELL, R. A. and WILLIAMS, D. B. (1993) *J. Biol. Chem.* 268, 17959–17966
- 48 OU, W. J., BERGERON, J. J., LI, Y., KANG, C. Y. and THOMAS, D. Y. (1995) *J. Biol. Chem.* 270, 18051–18059
- 49 ZAPUN, A., PETRESCU, S. M., RUDD, P. M., DWEK, R. A., THOMAS, D. Y. and BERGERON, J. J. M. (1997) *Cell* 88, 29–38
- 50 SADASIVAN, B., LEHNER, P. J., ORTMANN, B., SPIES, T. and CRESSWELL, P. (1996) *Immunity* 5, 103–114
- 51 HOCHSTENBACH, F., DAVID, V., WATKINS, S. and BRENNER, M. (1992) *Proc. Natl. Acad. Sci. U. S. A.* 89, 4734–4738
- 52 DAVID, V., HOCHSTENBACH, F., RAJAGOPALAN, S. and BRENNER, M. B. (1993) *J. Biol. Chem.* 268, 9585–9592
- 53 THOMAS, P. J., QU, B. and PEDERSON, P. L. (1995) *Trends Biochem. Sci.* 20, 456–459
- 54 GROSS, V., ANDUS, T., TRAN-HI, T.-A., SCHWARTZ, R. T., DECKER, K. and HEINRICH, P. C. (1983) *J. Biol. Chem.* 258, 12203–12209
- 55 GRUTERS, R. A. *et al.* (1987) *Nature* 330, 74–77
- 56 MEADEN, P., HILL, K., WAGNER, J., SLIPETZ, D., SOMMER, S. S. and BUSSEY, H. (1990) *Mol. Cell. Biol.* 10, 3013–3019
- 57 PAHL, H. L. and BAEUERLE, P. A. (1995) *EMBO J.* 14, 2580–2588
- 58 HARTL, F. U. (1996) *Nature* 381, 571–580
- 59 WILSON, I. A., SKEHEL, J. J. and WILEY, D. C. (1981) *Nature* 289, 366–373

*** Reference added in proof**

TATU, U. and HELENIUS, A. (1997) *J. Cell Biol.* 136, 555–565

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