MINIREVIEW

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THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 283, NO. 16, pp. 10221–10225, April 18, 2008
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# Getting In and Out from Calnexin/Calreticulin Cycles*

Published, JBC Papers in Press, February 26, 2008, DOI 10.1074/jbc.R700048200

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The *N*-glycan-dependent quality control mechanism of glycoprotein folding was proposed initially by Helenius and coworkers several years ago; with a few minor modifications, it is still valid today (Fig. 1) (1–3).[2] Glycan processing starts immediately after its transfer from a dolichol-P-P derivative to Asn residues in nascent polypeptide chains entering the lumen of the ER.[3] Removal of the outermost and following glucoses by the successive action of GI and GII exposes the $Glc_1Man_9GlcNAc_2$ epitope (Fig. 2). This structure is then recognized by two ER resident lectins (CNX and CRT) that specifically bind monoglucosylated polymannose glycans. This is followed by removal of the innermost glucose by GII, thus liberating the glycoprotein from the lectin anchor. The protein-linked glycan is then reglucosylated by the soluble ER enzyme GT only if the protein moiety displays non-native three-dimensional structures, as this enzyme behaves as a conformational sensor. Cycles of CNX/CRT-glycoprotein binding and liberation, catalyzed by the opposing activities of GT and GII, are terminated once glycoproteins attain their native structures. Glucose-free glycoproteins then continue their transit through the secretory pathway. Alternatively, permanently misfolded glycoproteins may be then transported to the cytosol for proteasomal degradation. Lectin-glycoprotein association not only thwarts Golgi exit of folding intermediates and irreparably misfolded glycoproteins but also enhances folding efficiency by preventing aggregation and promoting proper disulfide bonding. The latter is catalyzed by an oxidoreductase of the protein-disulfide isomerase family (ERp57) that acts exclusively on glycoproteins, as it is loosely associated with CNX/CRT.

GT is the only component of the quality control mechanism that senses protein conformations, as it recognizes hydrophobic amino acid patches exposed in molten globule-like conformers (4, 5). GT may also glucosylate glycoproteins in not fully assembled oligomeric complexes because it also recognizes hydrophobic surfaces exposed as a consequence of the absence of subunit components (6). The aim of this review is to give an overview of recent reports dealing with the entrance and exit of glycoproteins from CNX/CRT cycles.

## Getting In: GII Is Not What It Was Thought to Be

The first step in the pathway leading to the entrance of glycoproteins into CNX/CRT cycles is the removal of the outermost glucose unit from the glycan by the membrane enzyme GI. This reaction occurs almost simultaneously with glycan transfer. The rapid GI-mediated deglucosylation of the protein-linked glycan, as well as the apparent inability of the enzyme to remove *in vivo* (but not *in vitro*) the glucose from the dolichol-P-P-linked glycan, strongly suggests the existence of a supercomplex formed by the oligosaccharyltransferase, GI, and the dolichol derivative, with a very precise orientation of the components.

It was assumed that the sole role of GII was that of removing glucose residues *l* and *n* (Fig. 2). Recent work has suggested, however, a regulatory role for this enzyme. GII is a soluble dimeric protein from yeast to mammals. The $\alpha$ subunit displays the catalytic activity and no ER-retaining/retrieval sequence, and the $\beta$ subunit bears a KDEL-like sequence at its C terminus, from yeast (*Schizosaccharomyces pombe* but not *Saccharomyces cerevisiae*) to mammals (7–9). This subunit has also a sequence stretch with high homology to the mannose-binding domain of the Man-6-P receptor (10). Disruption of the $\beta$ subunit-encoding gene in the fission yeast results in an almost complete loss of activity, probably as a consequence of mislocalization of the $\alpha$ subunit. It has been shown for the rat liver enzyme that the $\beta$ subunit is not required for activity (8, 11). Remarkably, disruption of the $\beta$ subunit gene in *S. cerevisiae* does not affect the enzymatic activity or ER localization of the catalytic component but results in the exclusive *in vivo* production of monoglucosylated protein-linked *N*-glycans (9). Thus, the $\beta$ subunit appears to be required for the complete removal of glucoses in the budding yeast enzyme. In the case of the mammalian enzyme, apparently two *N*-glycans in the same glycoprotein are required for the formation of monoglucosylated *N*-glycans *in vivo* but not for the deglucosylation of these last compounds (12). For both the *S. cerevisiae* and mammalian GIIs, it was hypothesized that an interaction of the Man-6-P receptor-like domain of the $\beta$ subunit with *N*-glycans was responsible for results mentioned above (9, 12). Furthermore, in the case of the mammalian enzyme, it was proposed that, as both bonds to be successively cleaved (Glc$\alpha$1,3Glc,Glc$\alpha$1,3Man) (Fig. 2) are differently oriented in space, a transient separation between the GII single catalytic site and the *N*-glycan occurs after the first cleavage. This separation probably allows recognition of the monoglucosylated epitope by CNX/CRT (12). There is an apparent contradiction between results obtained with *S. cerevisiae* and mammalian cells, as in the former case, the $\beta$ subunit is apparently required for the second cleavage but not for the first one, whereas according to the mechanism proposed for mammalian GII, the same subunit would intervene only in the first cleavage. Furthermore, glycoproteins bearing only one diglucosylated glycan are efficiently completely deglucosylated by purified

* This minireview will be reprinted in the 2008 Minireview Compendium, which will be available in January, 2009. This work was supported by the Howard Hughes Medical Institute and National Institutes of Health Grant GM44500.

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[2] Refs. 2, 3, 19, and 53 are review articles.

[3] The abbreviations used are: ER, endoplasmic reticulum; GI, glucosidase I; GII, glucosidase II; CNX, calnexin; CRT, calreticulin; GT, UDP-Glc:glycoprotein glucosyltransferase; HA, hemagglutinin; EDEM, ER degradation-enhancing $\alpha$-mannosidase-like protein; M8A, $Man_8GlcNAc_2$ isomer A; M8B, $Man_8GlcNAc_2$ isomer B; ERGIC, ER-Golgi intermediate compartment.

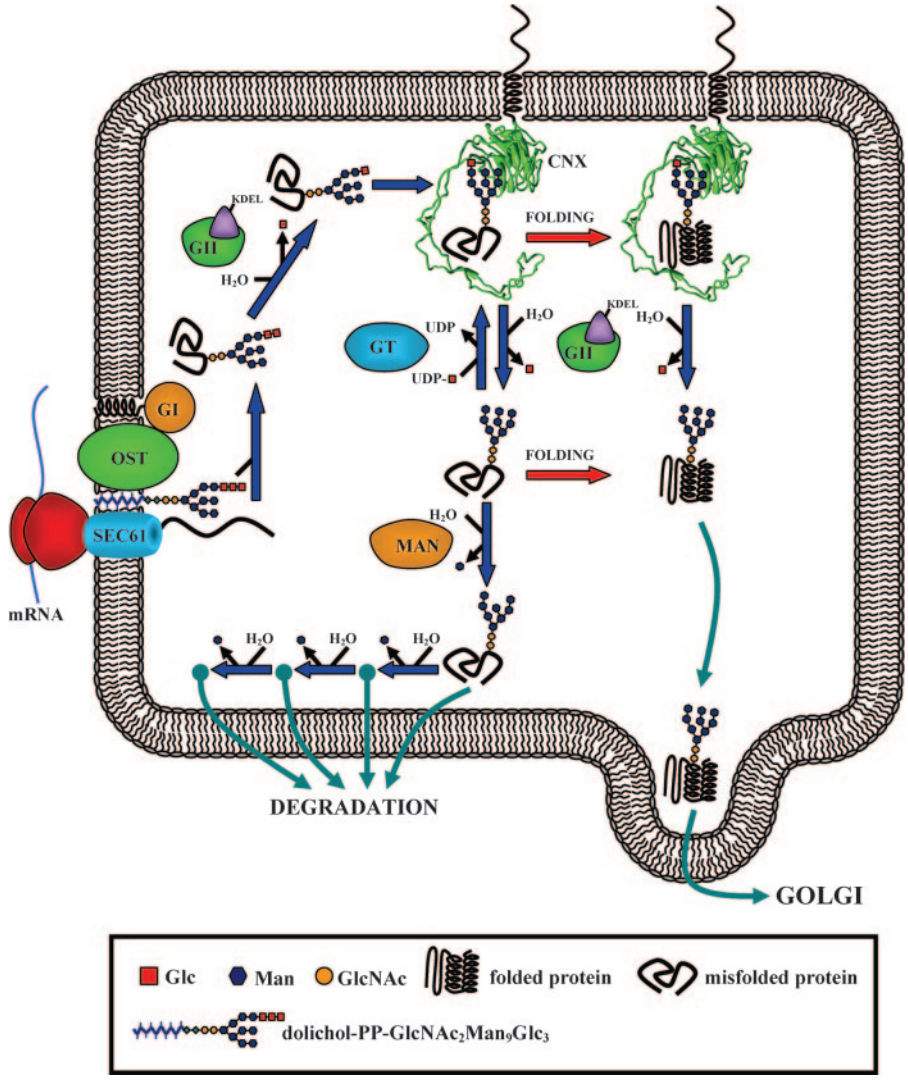


FIGURE 1. **Model proposed for the quality control of glycoprotein folding.** Proteins entering the ER are *N*-glycosylated by the oligosaccharyltransferase (*OST*) as they emerge from the translocon. Two glucoses are removed by the sequential action of GI and GII to generate monoglucosylated species that are recognized by CNX and/or CRT (only CNX is shown), which is associated with ERp57. The complex between the lectins and folding intermediates/misfolded glycoproteins dissociates upon removal of the last glucose by GII and is reformed by GT activity. Once glycoproteins have acquired their native conformations, either free or complexed with the lectins, GII hydrolyzes the remaining glucose residue and releases the glycoproteins from the lectin anchors. These species are not recognized by GT and are transported to the Golgi. Glycoproteins remaining in misfolded conformations are retrotranslocated to the cytosol, where they are deglycosylated and degraded by the proteasome. One or more mannose residues may be removed during the whole folding process.

mammalian GII (13). The putative regulation of the entrance of glycoproteins into CNX/CRT cycles by GII certainly merits further studies.

## What Happens Once Glycoproteins Are in CNX/CRT Cycles?

Although most glycoproteins studied so far interact with the lectins, apparently not all of them are reglucosylated by GT, as some may complete their folding processes taking advantage of the initial binding triggered by the partial deglucosylation of the transferred glycan. GT is not required for the viability of single yeast or mammalian cells grown under normal conditions and even for that of certain multicellular organisms such as plants, but disruption of its encoding gene was found to be embryonically lethal for mice (14–16). These results, together with the strict requirement of the enzyme for the viability of *S. pombe* only when grown under severe ER stress conditions, indicate that a restricted set of glycoproteins absolutely require GT for attaining their proper folding with acceptable efficiency (17). There are alternative quality control and folding efficiency enhancement mechanisms in the ER lumen besides the *N*-glycan-dependent one. Deficiencies in the latter trigger the up-regulation of the former ones.

According to their rates of release from CNX/CRT association, glycoproteins expressed in fibroblasts derived from GT-minus mouse embryos could be classified in three classes; in the first one, the observed rates were similar to those in wild-type cells (18). Glycoproteins in this class represent those with only one cycle of association with the lectins triggered by partial deglucosylation of the transferred glycan. In the second class, glycoproteins heavily dependent on GT-mediated association with CNX/CRT for folding showed an accelerated release from the lectins. GT absence resulted, as expected, in a lower folding efficiency. The most intriguing case was that of glycoproteins in the third class, as they showed a prolonged association with CNX/CRT. It was speculated that the observed results could be due to a protein-protein association between the lectins and glycoproteins or, alternatively, to the fact that a selenocysteine-containing oxidoreductase (Sep15) that forms a 1:1 complex with GT could play a role in assessing and refining the disulfide bond content of glycoproteins in this class (18, 19).

Although $Glc_1Man_9GlcNAc_2$ displays the same affinity for CNX and CRT, it has been known for several years that the set of glycoproteins interacting with one or other of the lectins only partially overlap. The difference observed was at least partially related both to the membrane-bound or soluble status of CNX or CRT, respectively, and to the vicinity of the *N*-glycans to the membranes (20). The use of CNX- or CRT-deficient cells revealed some unexpected results (21): expression of viral and cellular glycoproteins in CRT-null mouse embryonic fibroblasts and in CNX-deficient human T lymphoblastoid cells showed that loss of either CNX- or CRT-glycoprotein interaction or both (by addition of glucosidase inhibitors to wild-type

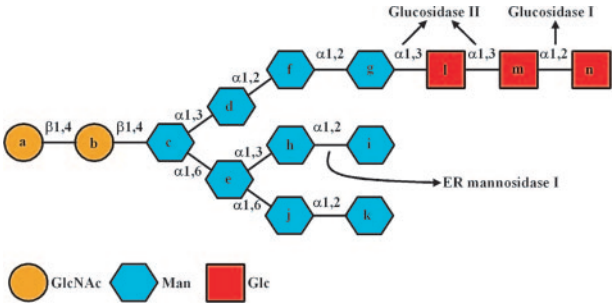


FIGURE 2. **Structure of glycans.** The lettering (*a*–*n*) follows the order of addition of monosaccharides in the synthesis of $Glc_3Man_9GlcNAc_2$-P-P-dolichol. GI removes residue *n*, and GII removes residues *l* and *m*. GT adds residue *l* to residue *g*. M8A lacks residues *g* and *l*–*n*; M8B formed by mammalian or yeast cell ER α-mannosidase I lacks residues *i* and *l*–*n*; and $Man_8GlcNAc_2$ isomer C lacks residues *k* and *l*–*n*. The smallest glycan formed in the *S. pombe* ER ($Man_7GlcNAc_2$) lacks residues *i*, *k*, and *l*–*n*.

cells) affected the process and outcome of glycoprotein production as well as the fidelity of quality control in a variety of ways. Effects were seen on the folding rate (which was accelerated particularly when CRT was absent), in the folding efficiency (which was generally reduced), and in the retention of incompletely folded glycoproteins in the ER (which was affected only when association with both lectins was abolished). CNX seemed to be more important than CRT as folding assistant. Loss of CRT had, in fact, only marginal consequences, whereas loss of CNX resulted in a dramatic impairment of influenza virus HA folding and in a more substantial elevation of other alternative ER resident chaperones, a symptom of ongoing ER stress.

Totally unexpected results were obtained upon studying the interaction of CNX/CRT and other chaperones with cellular and viral glycoproteins expressed in cells lacking functional CNX (22). Three variants of the same cellular glycoprotein differing in folding competence, number of glycans, and solubility status, which were CNX substrates in wild-type cells, failed to interact with CRT when expressed in CNX-null fibroblasts. Instead, they interacted more strongly with BiP. In contrast, four viral glycoproteins (Semliki Forest virus E1 and p62, vesicular stomatitis virus G, and influenza virus HA) gave different results. The first two glycoproteins normally interact with both CNX and CRT, but in CNX-minus cells, they interacted more abundantly with CRT, and their maturation proceeded normally. In the case of HA, a glycoprotein that is deeply dependent on CNX for successful maturation and that normally interacts with both CNX and CRT, the absence of the former lectin resulted in a persistent interaction with CRT. The most surprising result was obtained with G protein that normally interacts only with CNX. Infection of CNX-deficient fibroblasts with vesicular stomatitis virus (viral infection was also used to express E1, p62, and HA) resulted in the interaction of G protein with CRT. As transfection of G protein failed to trigger its interaction with CRT, it was suggested that viral infection somehow subverted the normal glycoprotein recognition by the ER lectins. This result may explain why total inactivation of CNX/CRT cycles affects viral replication and infectivity but not viability of mammalian cells. Additional expression of individual glycoproteins, both of cellular and viral origin and in this last case as a result of both viral infection and transfection, must be studied to substantiate this very interesting finding.

## Getting Out: ER-associated Degradation Components That Recognize Glycoproteins

Exit of properly folded glycoproteins from CNX/CRT cycles poses no conceptual problems, as their conformations do not allow GT-mediated reglucosylation. But, how do cells recognize that glycoproteins are irreparably misfolded or that multimeric complexes are definitively unable to complete their oligomeric structures and pull them out from futile CNX/CRT cycles to allow proteasomal degradation to proceed? Although intensive work has been dedicated to this issue in recent years and substantial progress has been made, the picture that now emerges is rather complex, and no clear answer to the question is yet available.

The observation that addition to mammalian cells of mannose analogs (behaving as ER mannosidase I and/or as polymannose lectin inhibitors) delayed degradation of misfolded glycoproteins prompted the suggestion that a "mannose removal time clock" regulated disposal. As removal of mannose is slower than that of glucose by GI and GII, it was proposed that demannosylation of glycoproteins staying in the ER for relatively long periods, as happens with irreparably misfolded glycoproteins, was a tag identifying molecules to be driven to degradation.

There are at least two proteins in ER-associated degradation that may interact with polymannose chains for pulling misfolded glycoproteins out from CNX/CRT cycles: ER α-mannosidase I and EDEM. Both mammalian and yeast cell ER mannosidases I are membrane proteins that convert $Man_9GlcNAc_2$ to $Man_8GlcNAc_2$ isomer B (M8B) (Fig. 2), but they are not as specific as initially thought because the recombinant species were able to further degrade M8B to smaller glycans. However, high enzyme concentrations not thought to occur *in vivo* were employed in the assays (23). Nevertheless, glycans smaller than M8B have been detected in glycoproteins forced to stay in the ER for rather long time periods as happens with irreparably misfolded and ER resident glycoproteins (24–26). The enzymatic activities responsible for further degradation of $Man_8GlcNAc_2$ glycans in the ER have not been unequivocally identified yet, and they might not even be ER resident proteins. It is known that irreparably misfolded glycoproteins may cycle between the ER and Golgi before being driven to degradation in both yeast and mammalian cells (27–32). Unlike the *S. cerevisiae* Golgi, which is devoid of mannosidase activities, mammalian cell *cis*-Golgi cisternae display three α-mannosidase activities able to degrade $Man_9GlcNAc_2$ to $Man_5GlcNAc_2$ (Fig. 2, residues *a*–*e*, *h*, and *j*) (33, 34). Furthermore, mammalian (but not yeast) cells have an ERGIC/*cis*-Golgi endomannosidase that yields M8A (Fig. 2) and Glc-Man as degradation products of $Glc_1Man_9GlcNAc_2$.

Genome analysis revealed that there are three ER α-mannosidase I homologs in mice and only one in either *S. pombe* or *S. cerevisiae* (35–40). They are called EDEM in mammalian cells and Htm1p or Man1p in yeast. EDEMs were first thought to be membrane-bound, but recent work showed them to be soluble proteins (41, 42). EDEMs display a 450-residue domain that shares 35% sequence identity with the catalytic domain of ER α-mannosidase I. It was first proposed that EDEMs behaved as lectins and not as enzymes, as they lack a particular disulfide bond thought to be required for hydrolytic activity, but further sequencing work detected several active fungal mannosidases

lacking that particular bond. It has been reported that overexpression of EDEMs enhances misfolded protein degradation by pulling those species out from CNX/CRT cycles, whereas a decrease in EDEM amounts, by RNA interference, results in a degradation delay (35, 40).

### Mechanisms Proposed for Misfolded Glycoprotein Escape from CNX/CRT Cycles

Based on a report indicating that the absence of mannose residues *i* or *i* and *k* (Fig. 2) (*i.e.* leaving intact the 3′-branch to which Glc is added by GT) decreased the GT-mediated glucosylation rate (43), a first proposal assumed that impeded reglucosylation of $Man_8GlcNAc_2$ or $Man_7GlcNAc_2$ glycans following GII glucose removal would liberate misfolded glycoproteins from CNX/CRT cycles (44). However, as an earlier report had shown that GII displayed a similar rate trend as GT concerning *N*-glycan composition (45), according to this proposal, misfolded glycoprotein degradation would have to be the outcome of a delicate balance between specificities for glycans and relative amounts of GII and GT. However, a more recent study reported similar GII-mediated deglucosylation rates for $Glc_1Man_9GlcNAc_2$ and the monoglucosylated derivative of M8B (13). Further studies on the GII specificity for glycans are required to elucidate this discrepancy. Nevertheless, it was shown that even glycoproteins bearing the $Man_5GlcNAc_2$ structure (Fig. 2, residues *a*–*g*) were good GT substrates *in vivo* and that the resulting glucosylated glycoproteins interacted with CNX (46).

A second proposal was based on the observation that EDEMs interacted with totally deglucosylated misfolded glycoproteins, whereas CNX associated with monoglucosylated species, and suggested that EDEMs, behaving as lectins, physically interacted with the glycans, thus hindering GT-mediated reglucosylation (41, 47). This proposal implies that EDEMs should be lectins with an extremely broad specificity spectrum, as even glycoproteins synthesized in cells transferring $Glc_3Man_5GlcNAc_2$ (Fig. 2, residues *a*–*g* and *l*–*n*) participate in CNX/CRT cycles (46).

Finally, a third proposal based on the observation that ER α-mannosidase I overexpression accelerated misfolded glycoprotein degradation (48) assumed that extensive demannosylation and specifically removal of residue *g* (Fig. 2), *i.e.* the residue to which GT adds the glucose unit, would prevent GT-mediated reglucosylation and thus CNX/CRT-glycoprotein interaction. The enzymatic activity(ies) responsible for such demannosylation has not been unequivocally identified yet, but several possibilities have been advanced. (*a*) ER mannosidase I concentrates in specific subcellular sites together with misfolded glycoproteins (25). Such concentration was observed recently for a HA-tagged version of ER α-mannosidase I expressed in mammalian cells (49). Confirmation of such concentration for the native enzyme is necessarily required. (*b*) It was proposed recently that EDEMs might display enzymatic activity, as overexpression of EDEM1 and EDEM3 (EDEM2 has not been tested yet) resulted in a more extensive demannosylation of misfolded glycoproteins (35, 50). Moreover, EDEM mutants defective in amino acids known to be essential for ER α-mannosidase I activity failed to increase demannosylation. The main objection to this proposal is that EDEMs have not yet been purified to homogeneity, thus precluding assaying the enzymatic activity of the native species, and additionally, no mannosidase activity could be detected in recombinant EDEM1, -2, or -3, although expression of the homologous lumenal portion of ER α-mannosidase I gave positive results. Furthermore, the inability of EDEM mutants to promote extensive *N*-glycan demannosylation might not be conclusive evidence for the enzymatic activity of the α-mannosidase homologs, as, for instance, mutations might abolish a putative lectin activity responsible for hindering GT-mediated reglucosylation. (*c*) Other possibilities are *cis*-Golgi mannosidases, which as mentioned above are able to convert $Man_9GlcNAc_2$ to $Man_5GlcNAc_2$, and/or ERGIC/*cis*-Golgi endomannosidase that yields M8A, an isomer lacking residue *g* (Fig. 2) (33, 34). A recent study showed that overexpression of not only ER α-mannosidase I and EDEMs but also Golgi α-mannosidase IA, IB, or IC resulted in an enhancement of misfolded glycoprotein demannosylation and degradation (32). It was not shown, however, if the overexpressed proteins localized exclusively to the Golgi or if they were present in the ER as well.

The second proposal for a misfolded glycoprotein escape mechanism from CNX/CRT cycles (see above) is probably the only one applicable to *S. pombe*, which displays a quality control mechanism similar to that occurring in mammalian cells and in which disruption of the EDEM-encoding gene drastically decreased the degradation rate of misfolded glycoproteins (51). No $Man_9GlcNAc_2$ demannosylation was observed in mutants lacking a functional ER α-mannosidase I-encoding gene, thus suggesting that the yeast single EDEM homolog has no α-mannosidase activity. Also, there are no ERGIC/*cis*-Golgi endomannosidase or *cis*-Golgi α-mannosidase activities in *S. pombe*. Finally, even after an extremely long residence in the ER, $Man_9GlcNAc_2$ in misfolded glycoproteins was minimally degraded, $Man_7GlcNAc_2$ being the smallest glycan detected. This last compound still had mannose residue *g* (Fig. 2) (51).

The absence of monoglucosylated glycans is not an absolute condition for glycoprotein degradation: $Glc_1Man_5GlcNAc$ (Fig. 2, residues *b*–*g* and *l*) was found to be a cytosolic by-product of the degradation of misfolded glycoproteins synthesized by mutant Chinese hamster ovary cells known to transfer $Man_9GlcNAc_2$ in protein *N*-glycosylation (52). The glycan came from cytosolic degradation of $Glc_1Man_9GlcNAc_2$ and $Glc_1Man_8GlcNAc_2$, the species that determined CNX/CRT recognition of folding intermediates in those cells. These results show that diversion to degradation of misfolded glycoproteins cannot be ascribed solely to their liberation from CNX/CRT anchors caused by hindering formation of monoglucosylated *N*-glycans.

### Do CNX and CRT Behave Also as Classical Chaperones?

Although binding of most known substrates to CRT and CNX appears to be mediated exclusively by the glycan moiety, in some cases, the lectins may apparently display a behavior more akin to that observed in classical chaperones (53). For instance, under mild cell lysis conditions, some proteins remain associated with CNX/CRT even in the presence of glucosidase inhibitors (54–56). In addition, thermally induced aggregation of non-glycosylated proteins may be suppressed *in vitro* by both CNX and CRT (57, 58). In addition, CNX mutants devoid of lectin activity may associate *in vivo* with class I histocompatibility molecules (59). Interestingly, CRT displays a marked pref-

erence for hydrophobic peptides in *in vitro* binding assays (60). The absence of any obvious binding site for hydrophobic domains in the structure of CNX constitutes a major drawback for the occurrence of polypeptide-based interactions. Nevertheless, the static picture captured in the crystal may hinder alternative conformations that are unfavorable under the crystallization conditions but able to bind proteins displaying non-native conformations. For instance, upon heat shock or calcium depletion, both CRT and CNX undergo conformational changes that induce their oligomerization and increase their ability to bind non-glycosylated substrates (61, 62). ATP provides the energy required for binding and unbinding cycles of classical chaperones, and the role of the nucleotide is played by UDP-Glc in the CNX/CRT lectin-based cycles described in this review. Whether similar binding-unbinding cycles (and their energy purveyor) occur in the putative role of CNX and CRT as classical chaperones is presently unknown.

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