Calnexin is a membrane protein of the endoplasmic reticulum (ER) that functions as a molecular chaperone and as a component of the ER quality control machinery. Calreticulin, a soluble analog of calnexin, is thought to possess similar functions, but these have not been directly demonstrated in vivo. Both proteins contain a lectin site that directs their association with newly synthesized glycoproteins. Although many glycoproteins bind to both calnexin and calreticulin, there are differences in the spectrum of glycoproteins that each binds. Using a Drosophila expression system and the mouse class I histocompatibility molecule as a model glycoprotein, we found that calreticulin does possess apparent chaperone and quality control functions, enhancing class I folding and subunit assembly, stabilizing subunits, and impeding export of assembly intermediates from the ER. Indeed, the functions of calnexin and calreticulin were largely interchangeable. We also determined that a soluble form of calnexin (residues 1–387) can functionally replace its membrane-bound counterpart. However, when calnexin was expressed as a soluble protein in L cells, the pattern of associated glycoproteins changed to resemble that of calreticulin. Conversely, membrane-anchored calreticulin bound to a similar set of glycoproteins as calnexin. Therefore, the different topological environments of calnexin and calreticulin are important in determining their distinct substrate specificities.

Calnexin (CNX) and calreticulin (CRT) are resident ER proteins that bind transiently to many newly synthesized glycoproteins as they pass through the ER (1, 2). CNX is a type I membrane protein, whereas CRT resides as a soluble molecule within the ER lumen. CRT and the luminal domain of CNX share extensive amino acid sequence similarity with the highest degree of identity located within a central segment consisting of two tandemly repeated sequence motifs (1, 3). The repeat sequences contain a high affinity Ca\(^{2+}\) binding site and also form the bulk of a lectin site that specifically recognizes a monoglucosylated Asn-linked processing intermediate, Glc\(_{1}\)Man\(_{9}\)GlcNAc\(_{2}\) (4–6). As a consequence of their lectin functions, both CNX and CRT exhibit a marked preference for binding to Asn-linked glycoproteins (7, 8). Indeed, treatment of cells with tunicamycin or with castanospermine, an inhibitor that prevents the formation of the Glc\(_{1}\)Man\(_{9}\)GlcNAc\(_{2}\) oligosaccharide, abrogates the association of CNX and CRT with most glycoproteins (7–11). CNX is thought to function as a molecular chaperone, since its expression enhances the in vivo folding and assembly of class I histocompatibility molecules (12), the nictin acid ethylcholine receptor (13), and the vesicular stomatitis G glycoprotein (9). It also prevents the aggregation of various unfolded proteins in vitro (14). In addition to its chaperone function, CNX participates in quality control, retarding the export of incompletely assembled protein subunits from the ER (15–17). CRT is believed to possess similar chaperone and quality control functions, since the simultaneous inhibition of CNX and CRT binding by castanospermine treatment is accompanied by impaired folding and subunit assembly, more rapid degradation, and premature release of glycoproteins from the ER in a variety of model systems (12, 13, 18–23). However, CRT's individual role in these processes has never been examined.

A prevalent view of how CNX and CRT associate with folding glycoproteins is that the interaction is regulated by the availability of monoglucosylated oligosaccharides. Following the initial attachment of the Glc\(_{1}\)Man\(_{9}\)GlcNAc\(_{2}\) oligosaccharide to a nascent polypeptide chain, ER glucosidases I and II remove the two outer glucose residues to create the Glc\(_{1}\)Man\(_{9}\)GlcNAc\(_{2}\) species that is recognized by the lectin site of CNX and CRT. Once formed, complexes of a glycoprotein with CNX or CRT are dissociated through the further action of glucosidase II, which removes the single remaining glucose residue (24). Another resident ER enzyme, UDP-glucose:glycoprotein glucosyltransferase, regulates the rebinding of the glycoprotein to CNX and CRT by adding back a single glucose residue to recreate the Glc\(_{1}\)Man\(_{9}\)GlcNAc\(_{2}\) structure (24–26). The glucosyltransferase is selective in that it only reglucosylates incompletely folded glycoproteins (27). Hence, cycles of glucose removal and readtion regulate CNX and CRT binding to nontnative glycoproteins with the glucosyltransferase acting as the folding sensor. In this model, CNX and CRT do not function as classical chaperones, but rather the lectin-oligosaccharide interaction itself is thought to enhance folding or subunit assembly, stabilize intermediates, and exert quality control (2, 8, 24). CNX and CRT may also promote folding by recruiting folding catalysts such as the thiol oxidoreductase, ERp57, to the vicinity of the folding glycoprotein (28, 29).

The question of whether CNX and CRT recognize the polypeptide portion of glycoproteins is controversial. On the one hand, in vivo studies have shown that CNX and CRT do not discriminate in their binding between reduced and native forms of RNase B and that complexes with RNase B can be dissociated solely by oligosaccharide modification (30, 31). Al-
ternatively, there is abundant evidence indicating that CNX and CRT are capable of binding to the polypeptide portion of other glycoprotein substrates (4, 32–36) and that they can discriminate between native and nonnative conformations of both glycosylated and nonglycosylated proteins (14, 37). This raises the possibility that in conjunction with the regulated lectin binding and release cycle, CNX and CRT also bind to unfolded polypeptide segments and promote folding in a manner analogous to classical chaperones.

Given the identical lectin specificities of CNX and CRT, it is not surprising that there is overlap in the glycoproteins that they bind and that they can, in some instances, associate simultaneously with the same glycoprotein (2). However, it is clear from an examination of the overall spectrum of glycoproteins co-isolated with CNX or CRT that there are distinct differences in binding specificity (11, 38, 39). Furthermore, it has been demonstrated that the vesicular stomatitis virus G glycoprotein binds to CNX but not to CRT (11) and that CRT dissociates more rapidly than CNX as the folding/assembly of the T cell receptor (39) and the influenza virus hemagglutinin (40) proceeds. Also, during the assembly of class I histocompatibility molecules, only CNX binds to the newly synthesized heavy chain, but it is partially or completely replaced by CRT upon subsequent heavy chain assembly with β2-microglobulin (41, 42). These observations suggest that the two proteins may collaborate during the biogenesis of various glycoproteins and raise the question of what the functional relationship is between CNX and CRT. Do they possess distinct functions that are utilized at different stages in glycoprotein biogenesis? Alternatively, are they functionally interchangeable but bind differentially to certain glycoproteins by virtue of their distinct membrane versus soluble dispositions or through differences in polypeptide binding specificity?

To address these questions, we first asked whether CRT alone is capable of enhancing protein folding and participating in quality control processes in vivo using the well characterized mouse class I histocompatibility molecule as a model glycoprotein. We then compared the results with those previously obtained for CNX to determine the extent to which the functions of these two proteins are interchangeable. Furthermore, we examined the influence of the different topological environments of CNX and CRT by removing the cytoplasmic and transmembrane segments of CNX and assessing the impact on its chaperone/quality control functions and its substrate binding specificity. We found that CRT does indeed function as an apparent chaperone and component of the ER quality control machinery and that these functions are largely interchangeable with those of CNX. Furthermore, CNX retains its functions when expressed as a soluble molecule, but its substrate specificity is altered to resemble that of CRT.

**EXPERIMENTAL PROCEDURES**

**Cell Lines and Antibodies—** *D. melanogaster* Schneider cells were maintained in Schneider’s insect medium (Sigma) with 10% fetal bovine serum and antibiotics. Stably transfected derivatives were cultured in Schneider’s insect medium (Sigma) with 10% fetal bovine serum and antibiotics. Mouse L cells were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum and antibiotics.

The following mAbs were used for the isolation of class I molecules: mAb 20-8-4S, which reacts with H-2Kb heavy (H) chains associated with β2m-microglobulin (β2m) (43) and mAb 28-14-8S, which recognizes a conformational epitope in the αα domain of free or β2m-associated Dα1 H chains (44). A rabbit antisera (anti-β2) directed against the C terminus of the H-2Kb H chain, which reacts with all conformational states of Kβ, was provided by Dr. Brian Barber (University of Toronto) (45). Unassembled mouse class I H chains were isolated using a rabbit antisera (anti-HC) provided by Dr. Hidde Ploegh, Harvard University (46). A rabbit antisera raised against the C-terminal 14 amino acids of CNX was used to isolate full-length CNX (15), whereas CNX mutants lacking the C terminus were detected with a rabbit antisera (app90) directed against the N-terminal signal sequence, the tandemly repeated sequence motifs, and the Tm segment, respectively, of CNX. The region containing the repeat motifs binds oligosaccharide and is also the site of high affinity calcium binding. Restriction sites used in construction of the mutants are indicated. CNX 1–387 is a truncated form of the ER luminal domain that retains the repeated sequence motifs and hence the ability to bind oligosaccharide and calcium (6). It is fused to the -KDEL ER localization motif (underlined). B, HA-tagged calnexin mutants expressed in L cells. CNX with the influenza hemagglutinin epitope, -YPYDVPDYA-, inserted in its cytoplasmic tail has been described previously (38). The CNX:Δcyt(HA) mutant has 87 residues of the 89-residue cytoplasmic tail deleted and replaced with the HA epitope fused to the ER localization signal -DEKMKP (underlined). C, HA-tagged calreticulin mutants expressed in L cells. CRT containing the HA epitope has been described previously (38). CRT: CNX:Δcyt(HA) has also been described previously (38) and is a membrane-anchored form of CRT fused at residue 340 to the Tm- and HA-tagged cytoplasmic segments of CNX (residues 450–573).

![Fig. 1. Calnexin and calreticulin mutants. A, calnexin constructs expressed in Drosophila cells. Lightly shaded, hatched, and stippled boxes represent the N-terminal signal sequence, the tandemly repeated sequence motifs, and the Tm segment, respectively, of CNX.](http://www.jbc.org/)

**Construction of Calnexin and Calreticulin Mutants and Expression in Drosophila and L Cells—** Fig. 1A depicts the full-length and soluble forms of canine CNX that were expressed in *D. melanogaster* cells. The insertion of full-length CNX cDNA into the *Drosophila* expression vector pRHa3 (CNX:pRHa3) has been described previously (15). A truncated form of the ER luminal domain corresponding to CNX residues 1–387 (designated CNX 1–387) was generated by inserting an
oligocassettes, 5′-CGCGGATAGGACGACGGTAAATTGACCG-3′/5′-GATCCGGTTACCTTACGCTGCTCTATT-3′, containing the KDEL ER localization signal and a stop codon flanked by BspMII and KpnI sites cloned into CNX-pRMHa3 cleaved at the unique BspMII and KpnI sites (KpnI being at the 3′ end of the cDNA in the pRMHa3 multiple cloning site). Rabbits were immunized with the purified ER-KDEL-HA complex and boosted twice with the pBluescript vector harboring the KDEL tag. Horse anti-rabbit serum and rabbit anti-CNX and CRT antibodies were obtained from Dr. M. Michalak (University of Alberta). For expression in Drosophila cells, the KpnI/Eco136I restriction fragment of pBl-2C, containing full-length CRT cDNA, was subcloned into the KpnI and HincII sites of the pRMHa3 expression vector.

In the pRMHa3 vector, cDNAs are under the control of the metallothionein promoter (47). Stably transfected D. melanogaster Schneider cell lines were established by co-transfecting a phexcetra plasmid containing the neomycin resistance gene plus multiple pRMHa3 plasmids encoding CRT, CNX, or CNX and CRT and also a deglucosylation-reglucosylation system (53–55), it is unclear why Drosophila CNX and CRT could not be detected in association with murine class I molecules as assessed either by chemical cross-linking (15) or by co-immunoprecipitation with anti-H chain mAb Abs (12). Consistent with this observation, these cells were unable to support the efficient folding or assembly of class I molecules, and they lack the necessary quality control capacity to retard the export of incompletely assembled class I molecules from the ER. Since Drosophila cells possess genes encoding CNX and CRT and also a deglucosylation-reglucosylation system (53–55), they cannot be studied as a useful model to assess the functions of Calnexin and Calreticulin in murine class I biogenesis. Indeed, co-expression of mammalian CNX along with class I H chain and β2m in Drosophila cells revealed that CNX promotes efficient H chain folding and assembly with β2m, stabilizes H chain conformation, and functions as a component of the quality control machinery to retain assembly intermediates within the ER (12, 15). It is important to note, however, that these cells do not acquire peptides in Drosophila cells and hence their assembly cannot be studied beyond the formation of H chain-β2m heterodimers (56).

Unlike CNX, which binds rapidly to newly synthesized free H chains and is present throughout the whole process of murine class I assembly, CRT has only been detected with the products of some class I alleles and only after assembly of H chains with β2m (54, 52). Hence, its role in facilitating H chain folding or assembly with β2m, if any, remains unclear. In fact, CRT’s ability to function as a molecular chaperone has never been clearly demonstrated; nor has its functional relationship with CNX, apart from its identical lectin specificity and ERp57 binding, been assessed.

To examine the functions of CRT in mouse class I biogenesis and to compare it to those of CNX, Drosophila cells were co-transfected with cDNAs encoding rabbit CRT or dog CNX complexes, transfected with Kb or Dd H chains, and anti-μ or anti-β2m mAbs. Initially, the abilities of CRT and CNX to augment the assembly of Kb H chains with β2m were examined. Cells expressing Kb H chains and β2m in the absence or presence of co-expressed CRT or CNX were subjected to pulse-chase radiolabeling, and the levels of newly synthesized Kb H chains were measured using three different antibodies: a rabbit antiserum (anti-8) that recognizes 8-mer saline, pH 7.4, 10 mM sodium acetate, 1% nonfat dry milk, and 10 μl/ml each of chymostatin, leupeptin, antipain, and pepstatin. Lysates were incubated for 2 h at 4 °C with amounts of anti-class I or anti-CNX antibodies previously determined to recover in excess of 97% of their respective antigens in a single round of immunosorption. Immune complexes were incubated for 1 h with protein A-agarose beads and were analyzed by SDS-PAGE using 10% gels (49). Radioactive proteins were visualized by fluorography. For quantitation of bands, fluorograms were scanned using an EPSON 1000C scanner and analyzed using NIH Image software.

L cells at a density of 5 × 106 cells/60-mm dish were radiolabeled for 30 min with 50 μCi/ml [35S] Met, lysed for 30 min at 4 °C in 1 mL of lysis buffer, and incubated with anti-HA antibodies for 2 h. Immune complexes were collected on protein-A-agarose and analyzed by SDS-PAGE.
Functions of Calnexin and Calreticulin

CNX has also been shown to increase the yield of folded class I H chains (12). However, since CRT has only been detected in association with H chain-β₂m heterodimers, its capacity to interact with free H chains and influence folding are unknown. To address this issue, we examined H chain folding in control and CRT- or CNX-transfected cell lines by monitoring the formation of a conformational epitope in the α₃ domain of the Db H chain defined by mAb 28-14-8S. Note that in these experiments Dβ₂m folding was measured in the absence of β₂m using Drosophila transfectants expressing only free H chains. Fig. 3A depicts a pulse-chase radiolabeling experiment followed by immunosioanalysis of total or 28-14-8S-reactive H chains. Similar to results obtained previously (12), CNX enhanced H chain folding by >2-fold; all Dβ₂m H chains folded into a 28-14-8S-reactive conformation in the presence of CNX, whereas only 50–60% of H chains acquired the epitope in its absence (Fig. 3B). CRT also enhanced folding of H chains, although the effect was slower and somewhat less efficient than observed in the presence of CNX. Densitometric analysis revealed that 80% of H chains acquired the 28-14-8S epitope after a 5-min pulse in the presence of CNX, but this level was reached only after a 20-min chase in the presence of CRT (Fig. 3B).

In addition to promoting H chain folding, CNX has been shown to stabilize free H chains against unfolding and/or degradation (15, 18). Consequently, as a final assessment of the functional relationship between CNX and CRT, we compared the abilities of CNX and CRT to stabilize free Dβ₂m H chains. Cells expressing Dβ₂m H chains in the presence and absence of CNX or CRT were subjected to pulse-chase radiolabeling followed by immunosioanalysis of 28-14-8S reactive H chains (Fig. 4). In control cells expressing only Dβ₂m, the half-life of 28-14-8S reactive H chains was 80 min. In contrast, co-expression of CNX stabilized Dβ₂m H chains such that 85% of the mAb-reactive H chains remained after 160 min of chase. CRT was just as effective as CNX in stabilizing free Dβ₂m H chains. These effects of CNX and CRT occurred primarily through stabilization of the 28-14-8S-reactive conformation rather than through pre-
vention of H chain degradation, since immunoisolation of H chains with a conformation-insensitive Ab revealed minimal differences in degradation rates during a 160-min chase period (data not shown).

Overall, these findings indicate that CRT can largely replace CNX in enhancing H chain folding and assembly with β2m, in retaining H-chain-β2m heterodimers in the ER, and in stabilizing free H chain conformation.

Quality Control and Chaperone Functions of Soluble Calnexin—Since CRT, a soluble analog of CNX, appears to function in a manner that is largely interchangeable with CNX, the question arises as to whether CNX can also function as a soluble molecule or if its cytoplasmic and transmembrane segments are essential to its overall functions as a molecular chaperone and component of the ER quality control machinery.

To address this issue, the soluble form of CNX depicted in Fig. 1A was constructed (designated CNX 1–387). This mutant lacks not only cytoplasmic and transmembrane segments but also residues 388–462 of its ER luminal domain. It contains the -KDEL ER localization signal at its C terminus, and it retains the ability to bind Glc3Man9GlcNAc2 oligosaccharide (6). Immunoblots of lysates from stably transfected D. melanogaster cells revealed that CNX 1–387 was expressed at a level comparable with full-length CNX and that it was detected in cell lysates but not in the culture medium, indicative of its intracellular retention (data not shown).

To test whether CNX 1–387 retains the quality control function of CNX, we examined the ER to Golgi transport rates of peptide-deficient Kb-β2m heterodimers in the presence of full-length CNX or CNX 1–387. In this experiment, acquisition of resistance to digestion with endoglycosidase H (Endo H) was used to measure the rate of heterodimer transport from the ER to the medial Golgi cisterna. Endo H cleaves immature oligosaccharides that are present on class I molecules within the ER but is unable to remove mature oligosaccharides that have been processed as class I molecules pass through the medial Golgi cisterna. It is important to note that Endo H treatment reverses the electrophoretic mobilities of immature (Endo Hs) and mature (Endo Hr) class I molecules when compared with the experiment depicted in Fig. 2A. As shown in Fig. 5A (β2m-associated panel), the transport rate of Kb heterodimers was substantially slowed from a half-time of 18 min in CNX-deficient cells to 80 min in cells expressing full-length CNX. Kb heterodimers were also transported out of the ER with a t1⁄2 of 80 min in cells expressing CNX 1–387. Comparable trends were observed when CNX and CNX 1–387 were co-expressed with peptide-deficient Db heterodimers (data not shown). Therefore, CNX's quality control function was not impaired by removal of its cytoplasmic and transmembrane segments and residues 388–462 of its ER luminal domain.

We also tested whether CNX 1–387 retains the molecular chaperone functions of full-length CNX as assessed by its ability to enhance H chain assembly with β2m, to promote H chain folding, and to stabilize the conformation of free H chains. H chain-β2m assembly was analyzed using specific antibodies to
were incubated with [35S]Met for 5 min and then with excess unlabeled transport of Kb- 
expressing free Db H chains in the absence or presence of CNX

CNX 1–387 to stabilize the conformation of free H chains, cells

This was a very rapid process happening largely within the

cells. The cells were radiolabeled for 45 min with [35S]Met and then lysed with buffer containing 1% digitonin. The HA-tagged molecules and associated proteins were immunoisolated with anti-HA mAb 12CA5. Dots indicate the mobilities of CNX and CRT constructs. Lane 1 represents an anti-HA immunoprecipitate of lysate from untransfected L cells.

detect either total or β2m-associated Kβ H chains. The results obtained for control cells lacking CNX and for cells expressing either full-length CNX or CNX 1–387 are depicted in Fig. 5, A and B. CNX 1–387 was just as effective as full-length CNX in enhancing the efficiency of Kβ-β2m assembly. The folding of free Dβ H chains in the presence of CNX or CNX 1–387 was assessed by monitoring the formation of the conformational epitope defined by mAb 28-14-8S and comparing it to the total amount of Dβ H chains. Similar to the results described for H chain assembly with β2m, CNX 1–387 resembled full-length CNX in its ability to enhance H chain folding (Fig. 3, compare CNX:19KTm,lanes 2 and 5). This was a very rapid process happening largely within the 5-min pulse labeling period. Finally, to assess the ability of CNX 1–387 to stabilize the conformation of free H chains, cells expressing free Dβ H chains in the absence or presence of CNX or CNX 1–387 were subjected to pulse-chase radiolabeling followed by immunoisolation of 28-14-8S reactive H chains (Fig. 4, A and B). Again, there was no significant difference in the abilities of full-length CNX and CNX 1–387 to prevent the loss of the folded epitope. Collectively, these results indicate that CNX does not require its cytoplasmic tail, its transmembrane segment, and residues 388–462 of its ER luminal domain to function as a molecular chaperone that stabilizes free H chains and promotes H chain folding and assembly with β2m.

Substrate Specificities of Calnexin, Calnexin Truncation Mutants, and Calreticulin—Since CNX’s cytoplasmic tail, its transmembrane region, and residues 388–462 of its ER luminal domain are not required for its quality control or chaperone functions, we questioned whether these segments might influence the spectrum of proteins with which CNX interacts. To address this issue, various CNX truncation mutants were expressed transiently in mouse L cells and compared with similarly expressed CNX and CRT. To distinguish the transfected protein products from endogenous CNX and CRT, a HA epitope tag was inserted near the carboxyl terminus of each construct (Fig. 1, B and C). The L cell transfectants were radiolabeled with [35S]Met, and then cell lysates were incubated with anti-HA mAb 12CA5. Dots indicate the mobilities of CNX and CRT constructs. Lane 1 represents an anti-HA immunoprecipitate of lysate from untransfected L cells.

FIG. 6. Effect of transmembrane and cytoplasmic segments on the substrate specificities of calnexin and calreticulin. HA-tagged CNX (CNX(HA), lane 2), the soluble ER luminal portion of CNX (CNX:Δcyt,Tm(HA), lane 3), a truncated ER luminal segment of CNX (CNX:Δcyt,Tm,388–462(HA), lane 4), CRT (CRT(HA), lane 5), CNX with a truncated cytoplasmic tail (CNX:Δcyt(HA), lane 6), CNX with a truncated cytoplasmic tail but containing the Tm segment of the adenovirus E3/19K glycoprotein (CNX:19KTm,Δcyt(HA), lane 7), or membrane-bound CRT possessing CNX’s transmembrane and cytoplasmic segments (CRT:CNX,Tm,cyt(HA), lane 8) was transiently expressed in L cells. The cells were radiolabeled for 45 min with [35S]Met and then lysed with buffer containing 1% digitonin. The HA-tagged molecules and associated proteins were immunoisolated with anti-HA mAb 12CA5. Dots indicate the mobilities of CNX and CRT constructs. Lane 1 represents an anti-HA immunoprecipitate of lysate from untransfected L cells.
CNX 1–387 in Figs. 3–5). This construct also served to demonstrate that the various proteins co-isolated with CNX and CRT were specifically associated, since these co-isolated proteins could not be detected when the CNX:Δcyt,Tm,388–462(HA) mutant was immunosolated under identical conditions.

The finding that the soluble ER luminal segment of CNX (CNX:Δcyt,Tm(HA)) associated with a similar spectrum of proteins as CRT suggested that CNX’s Tm segment somehow influences substrate specificity. To determine if this is due to some unique property of CNX’s Tm segment, such as a site of protein interaction, or if it is simply a consequence of anchoring CNX within the ER membrane, we replaced CNX’s Tm segment with the Tm segment of the adenovirus E3/19K glycoprotein (CNX:19KTm,Δcyt(HA); see Fig. 1B). As shown in Fig. 6, the spectrum of proteins associating with CNX anchored by the E3/19K Tm segment (lane 7) closely resembled that observed for CNX anchored by its own Tm segment (CNX(HA)) and CNX:Δcyt(HA), lanes 2 and 6). This finding suggests that the primary basis for the difference in proteins associating with CNX and CRT is their different topological environments rather than some specific property of CNX’s Tm segment.

To confirm this finding, we examined a membrane-anchored form of CRT to determine if its pattern of associated proteins would be similar to that of CNX. This construct consisted of CRT residues 1–340 fused to the Tm and cytoplasmic segments of CNX (designated CRT-CNXTm/Δcyt(HA)). As shown in Fig. 6 (lane 8), this chimera lacked the distinctive pattern of associated proteins observed with soluble CRT (Fig. 6, lane 5); rather, it closely resembled the pattern observed with CNX or the CNX:Δcyt(HA) mutant (Fig. 6, lanes 2 and 6).

It is conceivable that the altered patterns of proteins associated with soluble CNX or the membrane-anchored form of CRT could be due to the oligomerization or aggregation of these mutants with either the endogenous CRT or CNX of mouse L cells; i.e. the CRT-like pattern observed with the ER luminal segment of CNX could be due to its association with endogenous CRT, and the CNX-like pattern observed with membrane-anchored CRT could be a consequence of its association with endogenous CNX. However, this possibility was excluded by immunoblotting anti-HA precipitates of CNX:Δcyt,Tm(HA) and CRT-CNXTm/Δcyt(HA) with antibodies directed against endogenous CNX or CRT. No interactions with either of the endogenous chaperones could be detected (data not shown).

**DISCUSSION**

To date, the concept that CRT functions in vivo to facilitate the folding of newly synthesized glycoproteins and to retain incompletely folded or misfolded glycoproteins within the ER has been based on indirect and correlative evidence. For example, CRT has been shown to bind transiently to folding intermediates but not to native forms of glycoproteins in a variety of in vivo studies (11, 26, 34, 42, 58). CRT also binds to ERp57 and enhances its thiol oxidase activity toward RNase B in vitro (29). Furthermore, its primary sequence similarity and identical lectin specificity with CNX, which clearly does participate in glycoprotein folding and quality control, has suggested similar functions for CRT. In the present study, we show for the first time that CRT is indeed capable of enhancing glycoprotein folding in vivo and that it can retain incompletely folded/assembled glycoproteins within the ER. By heterologous expression of mouse class I subunits in D. melanogaster cells in the absence or presence of co-expressed CRT, we demonstrate that CRT enhances the folding of class I H chains as well as their subsequent assembly with β2m-microglobulin. CRT also stabilizes free H chains and impedes the export of incompletely assembled H chain-β2m heterodimers from the ER.

The finding that CRT stabilizes free H chains and promotes free H chain folding is surprising given that CRT has not been detected in association with either mouse or human H chains prior to assembly with β2m (42, 51). Remarkably, we have also been unable to detect a CRT-free H chain complex in Drosophila cells by co-immunoprecipitation. This raises the question of whether CRT plays a more significant role in the earliest stages of class I biogenesis in mouse and human cells than was previously thought, its involvement being unappreciated given its weak association with free H chains. Alternatively, the participation of CRT in free H chain folding and stabilization that we observe in Drosophila cells may be a consequence of providing H chains with only a single chaperone. In mouse or human cells, where both CNX and CRT are present, CNX may be utilized preferentially due to its membrane disposition or perhaps its proximity to the sec 61 translocon that translocates nascent H chains into the ER (59). The ability of CRT to substitute for CNX under conditions of CNX depletion provides a likely explanation for why a human CNX-deficient cell exhibits minimal alterations in the assembly or intracellular transport of class I molecules (60, 61).

Overall, our experiments indicate that CRT’s chaperone and quality control functions are largely interchangeable with those of CNX. The two molecules are virtually indistinguishable in their abilities to promote the assembly of H chains with β2m, to retard the export of peptide-deficient H chain-β2m heterodimers from the ER, and to stabilize the conformation of free H chains. Only in promoting free H chain folding does CNX function more efficiently than CRT. Given this interchangeability of soluble CRT with membrane-bound CNX, we questioned whether CNX’s cytoplasmic and transmembrane segments contribute to either its chaperone or quality control functions. Our results indicate that neither segment is required, since a truncated form of CNX’s ER luminal domain consisting of residues 1–387 functions essentially as well as full-length CNX. This finding is consistent with the observation that expression of CNX’s ER luminal domain complements the lethal phenotype accompanying the disruption of the CNX gene in Schizosaccharomyces pombe (62). Interestingly, the truncated ER luminal construct failed to form stable complexes with a variety of glycoproteins expressed in L cells, although the complete luminal domain (residues 1–463) was capable of stable substrate association. This may be due to the loss of a short stretch of hydrophobic amino acids (Phe400–Val421) that has previously been suggested to play a role in the binding of CNX to polypeptide segments of nonnative glycoproteins (38). However, we cannot exclude the possibility that the reduced binding of this truncated form of CNX to diverse glycoproteins is due to weaker lectin-oligosaccharide interactions, since this mutant retains approximately half of the lectin activity observed with the intact ER luminal domain (6).

Although CRT and CNX possess identical lectin binding specificities (6) and our current findings suggest that they are essentially redundant in their chaperone and quality control functions, it is clear that they divide the labor of chaperoning the synthesis of newly synthesized glycoproteins. Numerous studies have documented overlapping but distinct substrate binding specificities for the two chaperones (2). This raises the important question of what determines their selectivities for different glycoproteins. Most attempts to address this issue have focused on the structural characteristics of the glycoprotein substrates, particularly the number and location of N-linked oligosaccharide chains. Extensive mutagenesis studies of the seven glycosylation sites in influenza hemagglutinin revealed that CRT binds preferentially to N-glycans located at the top (membrane-distal) domain of the molecule, whereas CNX binds equally well to the top and membrane-proximal
stem domains (40). Consistent with this observation, Harris et al. (34) showed that of the three glycosylation sites in the mouse Ld H chain, removal of the site in the membrane-distal α1 domain (residue 86) ablates CRT binding, whereas CNX binding is unaffected. These findings suggest that the ER luminal versus membrane-bound locations of CRT and CNX may influence their glycoprotein binding preferences. Alterations in polypeptide determinants also appear to influence CNX and CRT binding. Point mutations at residue 134 in the human class I HLA-A21 molecule (63) and at residue 227 in the mouse Ld molecule (34) were accompanied by a loss of CRT binding, but there was no effect on CNX interactions. The glycosylation state of these molecules was not altered by the mutations; nor were they misfolded as evidenced by their capacity to bind both β2-m and several conformation-sensitive mAbs. These findings are most readily explained by a polypeptide component to CNX and CRT binding in addition to the lectin-oligosaccharide interaction. There is considerable evidence to support such a model, whether via its own or a foreign transmembrane segment, is important for interaction with lymphocyte I H chains.

In the present study, we approached the issue of glycoprotein binding specificity from the chaperone side of the interaction. We found that although CNX's transmembrane segment is not required for its chaperone or quality control functions, it clearly constrains interactions. Since it has been shown to bind to an ER luminal site is indeed quite close to the ER membrane, and at least a portion of CNX molecules appears to be further constrained to the environment of the translocon (59). CNX's lectin site is indeed quite close to the ER membrane, and at least a portion of CNX molecules appears to be further constrained to the environment of the translocon (59). CNX's lectin site is indeed quite close to the ER membrane, and at least a portion of CNX molecules appears to be further constrained to the environment of the translocon (59). CNX's lectin site is indeed quite close to the ER membrane, and at least a portion of CNX molecules appears to be further constrained to the environment of the translocon (59). CNX's lectin site is indeed quite close to the ER membrane, and at least a portion of CNX molecules appears to be further constrained to the environment of the translocon (59).
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