The Lectin Chaperone Calnexin Utilizes Polypeptide-based Interactions to Associate with Many of Its Substrates in Vivo*

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Calnexin and calreticulin are molecular chaperones of the endoplasmic reticulum that bind to newly synthesized glycoproteins in part through a lectin site specific for monoglycosylated (Glc₃Man₉GlcNAc₂) oligosaccharides. In addition to this lectin-oligosaccharide interaction, in vitro studies have demonstrated that calnexin and calreticulin can bind to polypeptide segments of both glycosylated and nonglycosylated proteins. However, the in vivo relevance of this latter interaction has been questioned. We examined whether polypeptide-based interactions occur between calnexin and its substrates in vivo using the glucosidase inhibitor castanospermine or glucosidase-deficient cells to prevent the formation of monoglycosylated oligosaccharides. We show that if care is taken to preserve weak interactions, the block in lectin-oligosaccharide binding leads to the loss of some calnexin-substrate complexes, but many others remain readily detectable. Furthermore, we demonstrate that calnexin is capable of associating in vivo with a substrate that completely lacks Asn-linked oligosaccharides. The binding of calnexin to proteins that lack monoglycosylated oligosaccharides could not be attributed to nonspecific adsorption nor to its inclusion in protein aggregates. We conclude that both lectin-oligosaccharide and polypeptide-based interactions occur between calnexin and diverse proteins in vivo and that the strength of the latter interaction varies substantially between protein substrates.

Glycoprotein folding within the endoplasmic reticulum (ER)¹ is facilitated in part by the membrane-bound chaperone calnexin (CNX) and its soluble homolog calreticulin (CRT) (1). These proteins are unique among molecular chaperones in that they utilize a lectin site as a means to associate with unfolded glycoproteins (2–4). The lectin site is specific for Glc₃Man₉GlcNAc₂ oligosaccharides, which exist transiently in the dissociation process (5, 6).

In contrast with the preceding results, many lines of evidence have suggested that CNX and CRT can also associate with non-native proteins via protein-protein interactions. First, several studies show that complexes between CNX and either membrane-bound or soluble glycoproteins cannot be dissociated by enzymatic removal of oligosaccharides (3, 18, 19). Second, there are several examples of CNX interacting with proteins that either lack Asn-linked oligosaccharides naturally (20) or have lost them through mutagenesis or under-glycosylation (21–23). Third, after treatment of cells with castanospermine or glucosidase inhibitors added after complexes with CNX or CRT were formed prevented or slowed glycoprotein dissociation, thus implicating glucosidase activity in the dissociation process (5, 6, 17).

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¹ The abbreviations used are: ER, endoplasmic reticulum; CAS, castanospermine; CNX, calnexin; CRT, calreticulin; HA, influenza hemagglutinin; H chain, heavy chain of class I histocompatibility molecule; PAGE, polyacrylamide gel electrophoresis; CHO, Chinese hamster ovary; mAb, monoclonal antibody; CHAPS, 3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonic acid.
teins via polypeptide-based interactions in addition to lectin-oligosaccharide binding has been largely discounted. It has been speculated that the lack of dissociation of CNX-substrate complexes after complete deglycosylation may be due to the trapping of the two species within the same detergent micelle (1, 35) or that the substrate, being nonnative, might become insoluble upon dissociation (1, 35, 36). Similarly, it has been suggested that the association of CNX with nonglycosylated proteins in vivo may arise through nonspecific inclusion of CNX within misfolded protein aggregates (1, 36, 37). However, apart from a single instance in which CNX was detected in association with aggregates of nonglycosylated vesicular stomatitis virus G protein (37), there has been no direct evidence to support such speculations. Finally, the in vitro studies demonstrating direct binding of nonglycosylated peptides to CRT or the molecular chaperone functions of CNX and CRT with nonglycoproteins have been questioned in terms of their relevance to the in vivo situation (1).

In an effort to address the question of the existence of polypeptide-based interactions between CNX and its diverse substrates in vivo, we chose to utilize the same methodology used most commonly in previous studies to demonstrate the apparent exclusivity of lectin-oligosaccharide interactions, i.e. block the formation of monoglucosylated oligosaccharides and assess by co-immuno-isolation if complexes between diverse substrates and CNX can be detected. We reasoned that upon loss of the lectin-oligosaccharide interaction, any remaining polypeptide-based association might be too weak to survive rigorous immuno-isolation conditions. Consequently, care was taken to employ mild, yet highly specific isolation procedures. Using either pharmacologic or genetic methods to block the formation of the Glc₃Man₉GlcNAc₂ oligosaccharide in diverse cell types, we show that although many complexes were lost, a large number of CNX-substrate complexes remained readily detectable. Complementary results were also obtained using a substrate that lacked oligosaccharides through mutation of its Asn-X-(Ser/Thr) sequence. Interactions with CNX (and CRT) were maintained in the absence of any detectable aggregation. We conclude that in addition to the well established lectin-oligosaccharide interaction, polypeptide-based association does indeed exist in vivo between CNX or CRT and a diverse array of protein substrates.

EXPERIMENTAL PROCEDURES

Cell Lines and Antibodies—Murine BW5147 thymoma cells, their glucosidase II-deficient variant Pha⁺².7 (38) (both provided by Dr. R. Kornfeld, Washington University), and L cells were grown in Dulbecco's modified Eagle's minimum essential medium. Murine EL-4 thymoma cells and the human C1R cell line, that stably expresses the HLA-B27 molecule (39) (provided by Dr. P. Cresswell, Yale University), were cultured in RPMI 1640, CHO-K1 and its glucosidase I-deficient variant CHO-Lec23 (40) were obtained from Dr. A. Helenius, Swiss Federal Institute of Technology, and were grown in a minimum essential medium. Stably transfected Drosophila melanogaster Schneider cells that express CNX along with H-2Kb heavy (H) chains in the presence of mouse β₂-microglobulin (41) were maintained in Schneider's insect medium (Sigma). All media were supplemented with 10% fetal bovine serum and antibiotics.

A rabbit antiserum (anti-8) directed against the C terminus of the H-2Kb H chain, which reacts with all conformational states of H₂Kb, was provided by Dr. Brian Barber, University of Toronto. Antiserum UCSF#2 reacts with the cytoplasmic tail of class I HLA H chains and was provided by Dr. Frances Brodsky, Stanford University. mAb P1N1.1, which reacts with invariant chain, was obtained from Dr. Tania Watts, University of Toronto. Two rabbit antisera were used to isolate CNX. One was directed against the C-terminal 14 amino acids (anti-C-CNX), and the second was raised against the entire 462-residue ER luminal domain (anti-N-CNX). mAb 12CA5, which reacts with the influenza hemagglutinin (HA) epitope tag on CNX(HA) and the CNX 1–387(HA), mutant was provided by Dr. Paul Hamel, University of Toronto.

Construction of N-Glycosylation Mutants and Expression in L Cells—The N-glycosylation mutants of the H-2Kb H chain were generated by mutating the consensus glycosylation sequence, Asn-X-(Ser/Thr), using the QuikChange™ site-directed mutagenesis kit (Stratagene) and full-length H-2Kb cDNA in pcDNA3 (Invitrogen) as template. To remove the glycosylation site at residue 86 with Lys using the mutagenic oligonucleotide (mutated base in lowercase) 5′-GC AGA TAT CTT AAG AGG AGG AGC CGT CTG CGC-3′. The glycosylation site at residue 86 was removed by substituting Asn-86 with Lys using the mutagenic oligonucleotide 5′-GGG AAG GAG CAG AGC AAG GGC GCC-3′ as the mutagenic oligonucleotide. To add a glycosylation site at position 256, Tyr-256 was changed to Lys with the mutagenic oligonucleotide 5′-GGG AAG GAG CAG CAT ACA TGC CAT GTG TAC C-3′. Recombinant plasmids were introduced into L cells using the SuperFect™ transfection reagent (Qiagen), and stably transfected cells were established by G418 selection. Transient expression of HA-tagged calnexin (CNX(HA)) and a truncated ER luminal segment of calnexin (CNX(1–387(HA))) in L cells was conducted as described previously (41).

Metabolic Radiolabeling and Immuno-isolation—BW5147, Pha⁺².7, CHO-K1, CHO-Lec23, or L cells at a density of 1 × 10⁶ cells/100-mm dish were incubated in Met-free medium for 60 min at 23 °C to deplete intracellular Met pools. They were then radiolabeled at 23 °C for the times indicated in the various figure legends by the addition of 400 μCi/ml [³⁵S]Met. Castanospermine (CAS), when added, was present throughout the prelabeling and labeling periods. Cells were lysed for 30 min at 4 °C in 1 ml of lysis buffer containing either 1% digitonin or 1% CHAPS in PBS, pH 7.4, 10 mM iodoacetamide, 60 μg/ml Pefabloc® (Roche Molecular Biochemicals), and 10 μg/ml each of leupeptin, antipain, and pepstatin. For isolation of CNX and associated molecules, lysates were incubated with preimmune or anti-CNX antibodies for 2 h. Immunoprecipitates were collected for 1 h using protein A-agarose beads and analyzed by SDS-PAGE as described previously (41). For detection of calnexin-associated Kb, D, HLA-B27, and invariant chain, digitonin lysates were subjected to sequential immunoprecipitation (42). Briefly, CNX-substrate complexes were recovered with anti-CNX antiserum, dissociated by heating at 42 °C for 1 h in 0.2% SDS, adjusted to 2% Nonidet P-40, 5% skim milk, and incubated with anti-class I H chain or anti-β₂-microglobulin antibodies. Immune complexes were collected and analyzed as above.

Radiolabeling of transfected Drosophila cells with [³⁵S]Met, lysis, and immuno-isolation was carried out as described previously (14). Briefly, after induction of the metallothionein promoter with 1 μM CuSO₄ for 16 h, Drosophila cells were incubated for 1 h in Met-free Schneider's medium in the presence or absence of 1 μM CAS. Cells were then incubated with 0.5 μCi/ml [³⁵S]Met for 5 min in the presence or absence of CAS and lysed in digitonin lysis buffer. Lysates were incubated with anti-class I H chain or anti-C-CNX antibodies, and immune complexes were collected on protein A-agarose followed by SDS-PAGE analysis using 10% gels (43). Radioactive proteins were visualized by fluorography.

Glycerol Density Gradient Centrifugation—L cells (1 × 10⁶) expressing wild type or nonglycosylated H-2Kb were radiolabeled with [³⁵S]Met for 30 min, lysed in 1 ml of 1% digitonin buffer, and centrifuged briefly at top speed in an Eppendorf microcentrifuge to remove insoluble material. A 0.5-ml aliquot of lysate was loaded onto a 12-ml, 10–40% (w/v) linear glycerol gradient prepared in digitonin buffer. The gradients were centrifuged at 4 °C for 15 h at 35,000 rpm using a Beckman SW 41 rotor. Fractions (0.75 ml) were collected from the top of the gradients, and K⁺ H chains were immune-isolated from each fraction using anti-8 antisera. As a control for the total amount of K⁺ molecules loaded onto the gradient, an additional 0.5-ml sample of lysate was also immune-isolated with anti-8 antisera.

Immunoblotting—For detection of H-2Kb-CNX complexes by immunoblotting, 1 × 10⁶ L cells expressing wild type K⁺ or various K⁺ glycosylation mutants were lysed in digitonin lysis buffer and immune-isolated with anti-8 antisera and protein A-agarose. After SDS-PAGE analysis, proteins were transferred to nitrocellulose membrane (44), and the membrane was incubated with rabbit anti-N-CNX antisera at 1:5,000 dilution followed by donkey anti-rabbit IgG horseradish peroxidase conjugate at 1:10,000 dilution (Jackson Laboratories). Immune complexes were visualized using enhanced chemiluminescence (Amer sham Pharmacia Biotech).

RESULTS

Calnexin Associates with Many Substrate Proteins when the Formation of Monoglucosylated Oligosaccharides Is Blocked—
To establish whether CNX associates with its substrates only via its lectin site or whether protein-protein interactions also contribute to this association, we examined the formation of CNX-substrate complexes in glucosidase I- or glucosidase II-deficient cell lines and in the presence of the glucosidase inhibitor, CAS. In an effort to minimize protein aggregation and to preserve potentially weak protein-protein interactions, metabolic radiolabeling of cells was conducted at 25 °C, and lysis was performed using the mild detergents digitonin and CHAPS. Furthermore, immune complexes were washed for the minimum number of times (typically three) required to preserve CNX-substrate interactions while minimizing recovery of nonspecifically associated proteins.

Initially, the BW5147 mouse lymphoma cell line and its glucosidase II-deficient mutant, PhaR2.7, were radiolabeled with \[^{35}S\]Met, and digitonin lysates were subjected to immunoprecipitation with two separate anti-CNX antibodies. The anti-CNX antibody recognizes the last 14 residues of the cytoplasmic tail of CNX, and the anti-N-CNX antibody is directed against the entire ER luminal domain (residues 1–462). As reported previously (15, 16), in addition to CNX, which appeared as a major band of 90 kDa, a large number of newly synthesized proteins co-isolated as complexes with CNX from the parental BW5147 cells (Fig. 1A). A similar pattern of proteins was observed with the two independent anti-CNX antibodies (Fig. 1A, lanes 2 and 3). A substantial number of these proteins were lost or reduced in intensity in the glucosidase II-deficient PhaR2.7 cells, reflecting their apparent requirement for monoglucosylated oligosaccharides for stable association with CNX. However, it is noteworthy that many other proteins remained firmly associated with CNX (Fig. 1A, lanes 5 and 6). A similar result was obtained when parental BW5147 cells were treated with CAS to block glucosidase activity (Fig. 1A, compare lanes 2 and 3 with lane 7). Indeed, the patterns of CNX-associated proteins were remarkably similar in the PhaR2.7 and CAS-treated BW5147 cells. It is conceivable that these proteins arise as a result of nonspecific interactions either with the precipitating immunoglobulins or with protein A-agarose beads. However, we consider this possibility unlikely since a similar spectrum of proteins was obtained with the two independently generated anti-CNX antibodies (containing different arrays of immune globulins), and they were absent from control isolations performed with preimmune serum and protein A-agarose beads (Fig. 1A, lanes 1 and 4). To confirm these findings, we also compared glucosidase I-deficient Lec23 cells to their parental CHO cell line (Fig. 1B). Lec23 cells have been shown to possess little or no glucosidase I activity, and no monoglucosylated oligosaccharides could be detected on glycoproteins (40). Remarkably, despite the block in formation of monoglucosylated oligosaccharides, there was no obvious reduction in the number of CNX-associated proteins recovered with each antiserum, although some differences in the patterns of recovered proteins were apparent (Fig. 1B, compare lane 2 with lanes 5 and lane 3 with lane 6). Again, these proteins were absent in control isolations performed with preimmune antiserum.

To further exclude the possibility that the proteins remaining associated with CNX in glucosidase-deficient cells or after CAS treatment were due to nonspecific associations with immune complexes, HA-epitope tagged CNX (CNX(HA)) and a soluble variant (CNX(1–387(HA))) were prepared and transfected into mouse L cells. We showed previously that the CNX(1–387(HA)) variant fails to form complexes with newly synthesized proteins (41). The transfectants were radiolabeled with \[^{35}S\]Met, lysed in 1% CHAPS, and subjected to immunoprecipitation with anti-HA mAb. As shown in Fig. 1C, lane 1, a
large number of newly synthesized proteins were recovered with the anti-HA mAb from cells expressing full-length CNX(HA). Consistent with the experiments presented in Fig. 1, panels A and B, many proteins were also recovered in association with CNX(HA) after treatment of cells with CAS (Fig. 1C, lane 3). In contrast, only trace levels of proteins were recovered in association with the binding-impaired CNX-(1–387(HA)) variant (Fig. 1C, lane 2). This was also the case when the CNX-(1–387(HA)) variant was isolated from CAS-treated cells (data not shown). That the CNX-(1–387(HA)) variant was recovered under identical conditions of immune isolation as the CNX(HA) construct establishes that the CNX-associated proteins remaining after CAS treatment are indeed bona fide complexes and not merely proteins nonspecifically adsorbed to anti-HA immune precipitates.

Our finding that CNX remains capable of specific association with many proteins under conditions where the formation of monoglucosylated oligosaccharides is blocked contrasts with a number of previous studies. For example, Ora and Helenius (16) compared CNX-associated proteins in CHO versus glucosidase I-deficient Lec23 cells and showed that many fewer proteins associated with CNX in parental CHO cells than we observed and that none of those could be detected in the Lec23 mutant cells. To determine if the disparity in results arises from differences in the radiolabeling and immune isolation methods, we directly compared CNX-associated proteins in CHO and Lec23 cells using our mild conditions and those of Ora and Helenius (16). The major differences in methodology included our radiolabeling of cells at 23 °C versus 37 °C, our use of digitonin versus CHAPS during cell lysis and washing of immune complexes, our elimination of a pre-clearance step with fixed Staphylococcus aureus cells, and our recovery of immune complexes over a 3-h period versus the overnight isolation employed by Ora and Helenius (16). The results of this comparison are depicted in Fig. 1D. Consistent with the results presented in Fig. 1B, we observed few differences in the patterns of CNX-associated proteins recovered from CHO cells relative to glucosidase I-deficient Lec23 cells (compare lanes 2 and 4). In contrast, the procedure of Ora and Helenius (16) recovered far fewer CNX-associated proteins from parental CHO cells, and most of these were not detectable in isolates from Lec23 cells (Fig. 1D, compare lanes 6 and 8). These results demonstrate that the ability to detect CNX-associated proteins either from wild type cells or from cells deficient in the ability to produce monoglucosylated oligosaccharides is highly dependent on the particular conditions chosen for radiolabeling, cell lysis, and immune isolation.

**Differential Effects of Castanospermine on Defined CNX-Glycoprotein Complexes**—To obtain further insight into the nature of proteins that remain associated with CNX under conditions that block the formation of monoglucosylated oligosaccharides, we examined the interactions of CNX with several defined glycoproteins. Initially, complexes between CNX and the mouse class I histocompatibility molecules, H-2Kb and H-2Db, were studied using the EL4 thymoma cell line. Murine class I molecules consist of three subunits that assemble within the ER, a ~46-kDa transmembrane heavy (H) chain that possesses 2 or 3 Asn-linked glycans, the soluble 12-kDa β2-microglobulin subunit, and an 8–10-residue peptide ligand. EL4 cells were incubated with or without CAS and screened for proteins associated with CNX by immuno-isolation with anti-CNX antibodies. As expected, many proteins co-isolated as complexes with CNX (Fig. 2A, lane 3). After CAS treatment, many of these complexes were lost, but others were preserved (Fig. 2A, lane 4). To confirm that CAS was effective in inhibiting glucosidase activity in this and preceding experiments, the H-2Kb and H-2Db proteins were isolated directly, and their H chain subunits were shown to exhibit the characteristic decrease in mobility after CAS treatment that reflects unprocessed N-linked oligosaccharides (Fig. 2A, compare lanes 5 and 6 and lanes 7 and 8). The interactions of H-2Kb and H-2Db H chains with CNX were completely blocked after CAS treatment. This was evidenced by the loss of the intense 46-kDa species in the anti-CNX immunoprecipitate from CAS-treated cells (Fig. 2A, compare lanes 3 and 4) and also by the absence of H chains after their immune isolation from solubilized anti-CNX immunoprecipitates (Fig. 2A, compare lanes 9 and 10 and lanes 11 and 12). Thus, murine class I molecules in EL4 cells appear to depend extensively on lectin-oligosaccharide interactions for their stable association with CNX.
A different situation was observed when we expressed the K<sup>b</sup> H chain, β<sub>2</sub>-microglobulin, and CNX in <i>D. melanogaster</i> Schneider cells. These cells possess the glucosidases and glycosyltransferase required for a functional deglucosylation-re-glucosylation cycle (45–47), but they lack the specialized transporter associated with antigen processing that transports peptide ligands for class I molecules from the cytosol to the lumen of the ER. We showed previously that under these conditions the H chain assembles with β<sub>2</sub>-microglobulin but remains peptide-deficient, and the heterodimer is retained in the ER in association with CNX (14, 48). In transfected <i>Drosophila</i> cells, the K<sup>b</sup> molecule is the major substrate associated with CNX, and it can be identified directly in anti-CNX immunoprecipitates. When these cells were incubated in the absence or presence of CAS, the K<sup>b</sup> H chain mobility was reduced in response to CAS treatment, reflecting a block in glucose trimming (Fig. 2B, lanes 5 and 6). However, when CNX-K<sup>b</sup> complexes were recovered with anti-CNX Ab, a significant fraction of K<sup>b</sup> molecules remained associated with CNX after CAS treatment (Fig. 2B, lanes 1 and 2), and these molecules possessed the slower electrophoretic mobility indicative of unprocessed oligosaccharides. To confirm that the change in mobility of the CNX-associated K<sup>b</sup> H chain after CAS treatment was due to carbohydrate modification, the immune complexes were digested with endoglycosidase H to remove Asn-linked oligosaccharides, after which they possessed the same mobility with or without CAS treatment (Fig. 2B, lanes 3 and 4). It is noteworthy that CNX-K<sup>b</sup> complexes could also be detected in the absence or presence of CAS by immuno-isolating K<sup>b</sup> molecules and visualizing the associated CNX band (Fig. 2B, lanes 5 and 6). Similar results were obtained with the D<sup>b</sup> H chain (Fig. 2C). It is conceivable that the altered glycans resulting from CAS treatment promoted aggregation of K<sup>b</sup> and D<sup>b</sup> molecules and the nonspecific inclusion of CNX in such aggregates. We showed previously that a mix of disulfide-linked and non-disulfide-linked aggregates of K<sup>b</sup> or D<sup>b</sup> H chains can readily be detected under conditions of nonreducing SDS-PAGE when samples are heated only to 55 °C (14). Under these conditions, no aggregated D<sup>b</sup> molecules could be detected at the top of the separating gel (Fig. 2C). Thus, in <i>Drosophila</i> cells, incompletely assembled class I H chains are capable of associating with CNX in the apparent absence of monoglucosylated oligosaccharides on the H chain. The difference relative to EL4 cells is probably related to detection sensitivity since H chains are the major substrates associated with CNX in <i>Drosophila</i> cells.

Since the murine K<sup>b</sup> and D<sup>b</sup> H chains possess two or three glycans, respectively, there was the formal possibility that CAS treatment may not fully block the formation of monoglucosylated oligosaccharides at all sites, thereby permitting some degree of interaction with CNX. Consequently, we examined CNX association with the human class I molecule, HLA-B27, which has only a single glycan at position 86. C1R cells stably expressing HLA-B27 were treated for 1 h with 1 mM CAS and lysed, and CNX-associated proteins were isolated with anti-CNX Ab. As shown in Fig. 3A, CAS treatment resulted in the loss of many but not all complexes of newly synthesized proteins with CNX. To assess the fate of CNX-HLA-B27 complexes, immune complexes containing CNX and associated proteins were dissociated, and the released proteins were subjected to a second round of immune isolation with anti-HLA antibody. As shown in Fig. 3B, left panel, equivalent amounts of HLA-B27 H chains were recovered from CNX complexes in the absence or presence of 0.25 or 1 mM CAS. Furthermore, the CNX-associated H chains from CAS-treated cells exhibited the reduced mobility indicative of blocked glucose trimming. This was most apparent at 1 mM CAS; at the lower concentration of 0.25 mM, the mobility shift was less pronounced, suggesting an incomplete block in glucose trimming (Fig. 3B, left and center panels). We also assessed the tendency for HLA-B27 to form insoluble aggregates after CAS treatment. However, no H chains could be sedimented after centrifugation at 100,000 × g (Fig. 3B, right panel). Therefore, for a singly glycosylated glycoprotein that clearly lacked monoglucosylated oligosaccharide interactions with CNX were fully maintained.

We extended these experiments to include an endogenous glycoprotein of C1R cells, the invariant chain. Invariant chain is the major substrate of CNX in these cells, and it can be readily observed as an intense ~35-kDa band in anti-CNX immune isolates (Fig. 3A, lanes 2 and 3). Invariant chain also
appeared to be present at reduced intensity in anti-CNXY immunocomplexes after 1 mM CAS treatment (Fig. 3A, lanes 5 and 6). To confirm this, immune complexes containing CNX and associated proteins were isolated from cells treated with 0–1 mM CAS, dissociated, and subjected to re-immuno-isolation with anti-invariant chain mAb. In this experiment, the bulk of invariant chain molecules remained associated with CNX after CAS treatment (Fig. 3C, left panel), and they exhibited the reduced electrophoretic mobility that accompanies a block in glucose trimming (Fig. 3C, left and center panels). Invariant chain also did not form insoluble aggregates after CAS treatment (Fig. 3C, right panel).

We conclude that for three different glycoproteins, the presence of monoglycosylated oligosaccharides is not required for association with CNX. Furthermore, the associations detected in CAS-treated cells are unlikely to be a consequence of the nonspecific inclusion of CNX in large glycoprotein aggregates.

**Calnexin Associates with Nonglycosylated Class I Molecules**

Although CNX fails to bind to purified Glc_3Man_9GlcNAc or Glc_3Man_9GlcNAc oligosaccharides *in vitro* (3), the possibility remained that the CNX-substrate complexes observed in CAS-treated or glucosidase-deficient cells could be mediated through lectin interactions with di- or trisaccharides. To address this issue, we tested the association of CNX with Kb H chains mutated to lack Asn-linked oligosaccharides. In addition, we examined the consequence of varying the number of N-linked glycans on CNX binding. The various Kb glycosylation mutants containing 0, 1, 2, or 3 glycans are depicted in Fig. 4A. The wild type Kb H chain and glycosylation mutants were stably expressed in murine L cells (which contain β_2-microglobulin and all other components required for normal assembly of class I molecules). Fig. 4B shows that the various forms of the Kb H chain were synthesized in L cells and possessed the electrophoretic mobilities expected based on their different numbers of oligosaccharide chains. We also established that the differentially glycosylated H chains associated normally with β_2-microglobulin and that the singly, doubly, and triply glycosylated proteins were all transported from the ER to the Golgi apparatus at comparable rates (data not shown). In the case of the nonglycosylated molecule, its stability was similar to wild type Kb, and it could be detected at the cell surface by flow cytometry, albeit at lower levels than observed for the wild type protein (data not shown).

Collectively, these findings suggest that changes in the number of N-glycans do not cause major misfolding of the Kb molecule.

The various L cells transfectants were radiolabeled and lysed in digitonin buffer, and CNX-associated proteins were isolated with anti-CNXY Ab. Kb H chains were then recovered from the anti-CNXY immunoprecipitate in a second round of immune complex association with anti-H chain antiserum. As demonstrated in Fig. 4C, left panel, all of the Kb glycosylation mutants associated with CNX, including the nonglycosylated mutant. The same result was obtained when Kb H chains were isolated from nonradiolabeled cell lysates, and the presence of associated CNX was detected by immunoblotting (Fig. 4C, right panel).

However, a comparison of the relative amounts of Kb mutants synthesized with the relative amounts recovered in association with CNX indicated that the nonglycosylated mutant formed complexes with CNX somewhat less efficiently than the glycosylated forms (compare Fig. 4, B with C, left panel). This suggests that the presence of one or more N-linked glycans increases the stability of the CNX-Kb interaction.

Because aggregation is a common fate for glycoproteins that have been treated with tunicamycin to block N-glycosylation or that have been mutated to lack Asn-linked glycans, it was essential to determine whether the co-isolation of the nonglycosylated Kb protein with CNX occurred because of a specific protein-protein interaction or was due merely to the nonspecific inclusion of CNX in large glycoprotein aggregates. To test this possibility, transfected cells expressing the wild type Kb H chain or its nonglycosylated mutant were radiolabeled and lysed in digitonin lysis buffer, and the lysates were subjected to sedimentation through glycerol density gradients. Fractions were collected, and Kb molecules were isolated from each fraction. As shown in Fig. 4D, the wild type Kb H chain was detected mainly in fractions 5 and 6. A similar distribution was observed for the nonglycosylated Kb H chain; there was no evidence of large aggregates that sediment near the bottom of the gradient. When CNX-H chain complexes were isolated from fractions with anti-CNXY Ab, the nonglycosylated H chain was again detected.
found to sediment primarily in fractions 5 and 6, indicating that CNX-unglycosylated H chain complexes are not found in large aggregates (data not shown). Therefore, we conclude that the association between CNX and nonglycosylated Kβ H chains is a specific polypeptide-based interaction that can occur independently of lectin-oligosaccharide binding.

**DISCUSSION**

Our findings indicate that when care is taken to preserve weak interactions during metabolic radiolabeling and immune isolation, complexes between CNX and a diverse array of proteins lacking monoglycosylated oligosaccharides can be detected. This was observed when the formation of Glc3Man5GlcNAc2 oligosaccharides was blocked with the glucosidase inhibitor CAS or through the use of cells lacking either glucosidase I or glucosidase II. Complexes were not likely due to weak interactions between di- and triglycosylated oligosaccharides on the glycoprotein substrate and the CNX lectin site because interactions were maintained even when a nonglycosylated substrate was tested. Also, CNX does not bind detectably to these oligosaccharides *in vitro* (3). Furthermore, the association of CNX with proteins lacking monoglycosylated oligosaccharides was not due to the nonspecific inclusion of CNX into protein aggregates nor was it due to nonspecific adsorption of proteins onto anti-CNX immune precipitates. Consequently, we conclude that CNX is capable of associating with diverse proteins *in vivo* through polypeptide-based interactions in addition to its well-characterized lectin-oligosaccharide interaction.

It is conceivable that the association between CNX and various proteins that lack monoglycosylated oligosaccharides is indirect, being mediated by the thiol oxidoreductase Erp57, which is known to bind to CNX and CRT. Previous studies have shown that CNX- or CRT-bound Erp57 forms mixed disulfides with various glycoproteins during their oxidative folding (49). However, we consider such an indirect association to be unlikely for three reasons. First, it has been demonstrated that Erp57-glycoprotein-mixed disulfides fail to form when cells are treated with CAS (49), the same conditions we use to detect lectin-independent CNX-substrate complexes. Second, under nonreducing SDS-PAGE conditions we have consistently been unable to detect covalent complexes between various defined glycoproteins and Erp57 at a level that could account for the amount of CNX-associated glycoprotein observed in CAS-treated cells. For example, note that in Fig. 2C there is a strong CNX band co-isolating with the D1b H chain in CAS-treated cells, but there is no significant band corresponding to a covalent Erp57-H chain complex (∼105 kDa) that could have mediated this association with CNX. Finally, we and others (30–34) have readily detected associations between CNX or CRT and nonglycosylated proteins or peptides *in vitro* in the complete absence of any added Erp57. Consequently, we favor the view that the interactions we observe *in vivo* between CNX and proteins lacking monoglycosylated oligosaccharides are directly mediated through a polypeptide binding site on CNX.

It is noteworthy that the interactions between various proteins and CNX were affected to markedly different extents when the formation of monoglycosylated oligosaccharides was blocked. Examination of the patterns of proteins recovered in anti-CNX immune complexes (Figs. 1, 2A, and 3A) revealed that some proteins were completely lost, others appeared to be present at reduced levels, and others seemed unaffected. This situation was also observed when complexes between CNX and specific glycoproteins were examined. The murine class I molecules, H-2Kb and H-2Db, could not be recovered in association with CNX after CAS treatment of EL4 cells, although some interaction could be detected when overexpressed in *Drosophila* cells. However, unglycosylated H-2Kb remained firmly associated with CNX. This likely reflects different conformational states between the glycosylated and unglycosylated proteins with the accompanying presentation of different polypeptide determinants to CNX. In contrast to the glycosylated murine class I molecules, the human class I molecule, HLA-B27, and the human MHC class II invariant chain were largely unaffected in their interactions with CNX after CAS treatment. Collectively, these findings suggest that different glycoproteins exhibit different dependencies on lectin-oligosaccharide interactions for their stable association with CNX. In other words, in the absence of lectin-oligosaccharide binding, different proteins exhibit differences in the strength of their polypeptide-based interactions with CNX.

Consistent with the above *in vivo* findings, substrate-specific differences have also been observed when the interactions of CNX or CRT with unglycosylated protein or peptide substrates were studied *in vitro*. For example, when the ability of CNX to suppress the aggregation of various unfolded proteins was assessed, equimolar amounts of CNX effectively suppressed the aggregation of the naturally unglycosylated proteins citrate synthase and malate dehydrogenase (33). However, more than a 3-fold molar excess was required to fully suppress the aggregation of enzymatically deglycosylated soybean agglutinin (33), and a 30-fold molar excess was only partially effective in preventing the aggregation of enzymatically deglycosylated α-mannosidase. CNX did not bind to any of these proteins without their prior denaturation. An analysis of the binding of 39 different peptides to CRT also revealed its marked preference for certain peptides. In general, hydrophobic peptides lacking acidic residues were favored, and there also appeared to be a minimum length requirement (32). A preference for binding to hydrophobic peptides is a common characteristic among molecular chaperone families, and for some chaperones such as the cytosolic Hsp90 and Tric proteins, there are clear preferences for certain protein substrates over others (50).

Differences in the strength of polypeptide-based interactions between CNX or CRT and various glycoprotein substrates coupled with variations in the stringency of isolation conditions can account for many of the conflicting results reported in the literature concerning the association of these chaperones with specific glycoproteins after treatment with glucosidase or glycosylation inhibitors. For example, if glycosylation is prevented or if glucosidases are inhibited, complexes are not detected between CNX or CRT and the α and β subunits of the T cell receptor (51), influenza hemagglutinin (2), vesicular stomatitis virus G glycoprotein (52), ribonuclease B (35), myeloperoxidase (9), cruzipain (53), and tyrosinase (13). However, complexes can readily be detected at normal or reduced levels under conditions of deglycosylation or glucosidase inhibition with the ε and δ subunits of the T cell receptor (20, 25), P glycoprotein (23), erythrocYTE AE1 (54), acid phosphatase (27), major histocompatibility complex class I-α and -β chains (19), major histocompatibility complex class II invariant chain (24), major histocompatibility complex class I H chain (this study), and human immunodeficiency virus gp160 (10). Other interesting examples of variability include the finding that CAS treatment almost completely prevented the formation of complexes between CNX and coagulation factors V and VIII but only partially inhibited the formation of complexes with CRT (26). Furthermore, CAS prevented the formation of complexes between CNX and the α subunit of the acetylcholine receptor in one study (55) but had little effect on complex formation in another (28). The main difference appeared to be the use of

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Tritions X-100 for cell lysis in the former study as opposed to the milder CHAPS detergent in the latter. In addition to these studies that focused on complexes of specific glycoproteins with either CNX or CRT, variable results have also been reported when the entire spectrum of CNX- or CRT-associated proteins were examined. In agreement with our current study, Kearse et al. (51) observe strong association of many proteins with CNX in the glucosidase II-deficient Phan27 cell line and in CAS-treated wild type cells even though associations with T cell receptor-α and -β were virtually eliminated. In contrast, Hele- nius and co-workers (12, 16) observe an almost complete elimination of CNX- or CRT-associated proteins in Phan227 cells and in CAS-treated wild type cells. Likewise, in glucosidase I-deficient Lec23 cells, we observed very few changes in the number of CNX-associated proteins relative to wild type CHO, whereas Ora and Helenius documented a substantial loss of these complexes (16). The opposite outcomes of the latter two studies can clearly be attributed to differences in the radiolabeling, lysis, and immune-isolation conditions employed (Fig. 1D). The milder conditions used in our study preserved interactions that were lost after the more stringent procedures used by Ora and Helenius (16).

The preceding survey illustrates how conflicting conclusions can arise when generalizations are made on the basis of studying the association of a single glycoprotein with either CNX or CRT or when only a single type of isolation condition is employed. It is exceedingly unlikely that all of the examples of complexes between CNX or CRT with unglycosylated or non-glucose-trimmed proteins can be dismissed on the basis of their nonspecific inclusion in aggregates, trapping within detergent micelles, and insolubility of folding intermediates, as has frequently been claimed (1, 35–37). Indeed, in the present study, we have eliminated the possibilities of insolubility and aggregation through the use of high speed sedimentation and glycerol density gradient ultracentrifugation analyses. Furthermore, it is not possible to explain our results by the mere “trapping” of membrane proteins within the same detergent micelle, as CNX. Such a phenomenon should be applicable to all of the specific membrane proteins we studied, and yet we observed no such trapping of CNX with mouse class I molecules isolated from CAS-treated EL4 cells (Fig. 2A). Rather, a view more consistent with the data is that CNX and CRT possess both a lectin site and a polypeptide binding site and that the latter binds with varying affinities to polypeptide segments of different glycoproteins. Such a dual binding model (3) has the advantages that it accommodates the in vitro demonstrations of polypeptide interactions between diverse substrates and either CNX or CRT (32–34) and it evokes the possibility of an enhanced avidity of chaperone-glycoprotein interactions via contacts through two binding sites.

In fact, a lectin-only type of interaction is rather difficult to rationalize in light of the stable complexes that occur between CNX or CRT and various monoglycosylated glycoproteins. Recent studies have demonstrated that the affinity of CRT for IgG carrying a single Glc1Man9GlcNAc2 oligosaccharide is 1–2 μM (56). Glycans with dissociation constants greater than about 1 μM are typically retarded on immobilized lectin columns rather than binding tightly (57). Such chromatographic behavior has been documented for the interaction of monoglucosylated oligosaccharides with immobilized CRT (4) and is consistent with our observation that Glc1Man9GlcNAc2 bound to immobilized CNX is readily released upon washing (3). An increased affinity could result if CNX and CRT are oligomeric and capable of binding to multiple oligosaccharides on a glycoprotein substrate. Indeed there are several reports of enhanced CNX interactions when a glycoprotein is converted from a singly to a doubly glycosylated form (35, 37, 54). This could be due either to oligomeric CNX or to a bivalent CNX interaction that is induced by the precipitating anti-CNX antibody. Although we and others provide gel filtration evidence suggestive of CNX or CRT oligomers (33, 34, 58), this appears not to be the case. First, no increase in apparent affinity was observed when CRT was incubated in vitro with IgG possessing two monoglucosylated oligosaccharides (56). Second, recent biophysical studies demonstrate that CRT is monomeric and possesses a highly asymmetric structure that accounts for its anomalous gel filtration behavior (59). Third, CNX behaves as a monomer, as evidenced by sedimentation through a sucrose density gradient (60), and it is highly asymmetric, with a single lectin site as revealed by its recently solved x-ray crystallographic structure. Finally, using epitope-tagged variants of CNX or CRT in transfected cells, we have consistently been unable to co-isolate these proteins as mixed oligomers with the endogenous chaperones.

Despite the weak lectin-oligosaccharide binding affinity and the apparent lack of CNX or CRT oligomers, complexes of these chaperones even with singly glycosylated glycoproteins (such as the human class I molecule, α-fetoprotein, and the nicotinic acetylcholine receptor α subunit) are stable to detergent solubilization and immune isolation. Such stable associations can most readily be explained by a dual binding mechanism that encompasses both lectin-oligosaccharide and polypeptide-based interactions. Further proof of this model must await the identification of the polypeptide binding sites on CNX and CRT and the elucidation of their fine binding specificities.

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