Lectin-deficient Calnexin Is Capable of Binding Class I Histocompatibility Molecules in Vivo and Preventing Their Degradation*

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Calnexin (CNX) is a membrane-bound lectin of the endoplasmic reticulum (ER) that binds transiently to newly synthesized glycoproteins. By interacting with oligosaccharides of the form Glc1Man9GlcNAc2, calnexin enhances the folding of glycoprotein substrates, retains misfolded variants in the ER, and in some cases participates in their degradation. Calnexin has also been shown to bind polypeptides in vivo that do not possess a glycan of this form and to function in vitro as a molecular chaperone for nonglycosylated proteins. To test the relative importance of the lectin site compared with the polypeptide-binding site, we have generated six calnexin mutants defective in oligosaccharide binding using site-directed mutagenesis. Expressed as glutathione transferase fusion proteins, these mutants were still capable of binding ERp57, a thiol oxidoreductase, and preventing the aggregation of a nonglycosylated substrate, citrate synthase. They were, however, unable to bind Glc1Man9GlcNAc2 oligosaccharide and were compromised in preventing the aggregation of the monoglucosylated substrate jack bean α-mannosidase. Two of these mutants were then engineered into full-length calnexin for heterologous expression in Drosophila cells along with the murine class I histocompatibility molecules Kβ and Dβ as model glycoproteins. In this system, lectin site-defective calnexin was able to replace wild type calnexin in forming a complex with Kβ and Dβ heavy chains and preventing their degradation. Thus, at least for class I molecules, the lectin site of calnexin is dispensable for some of its chaperone functions.

Calnexin (CNX) is a type I transmembrane protein of the ER that binds transiently to newly synthesized glycoproteins. Its ER luminal portion is comprised of a globular lectin domain that binds Ca2+ and recognizes the monoglucosylated Glc1Man9GlcNAc2 oligosaccharide (1, 2) and an arm-like extension that binds the thiol oxidoreductase ERp57 (3, 4). Calnexin has high sequence similarity to calreticulin (CRT), a soluble ER lectin of the same specificity (5), and considerable functional overlap (6–8). It is also similar to a testis-specific membrane protein termed calmegin (9). CNX and CRT function as chaperones by enhancing the in vivo folding of their substrates as has been demonstrated in a number of model systems including the assembly of major histocompatibility complex class I molecules (8, 10), the nicotinic acetylcholine receptor (11), and influenza hemagglutinin (12). They also act as components of the quality control machinery, retaining misfolded or incompletely folded substrates within the ER (13, 14). Recently it has been shown that CNX has a role in ER-associated degradation by binding and delivering substrates to the ER degradation-enhancing mannosidase-like protein, a mannose-specific lectin that targets glycoproteins for retrotranslocation to the cytosol where they are degraded by the proteosome (15, 16). The signal for degradation is the conversion of the oligosaccharide from Man9GlcNAc2 to a Man8GlcNAc2 form by an ER mannosidase, as shown by the block of degradation by the mannosidase inhibitor kifunensine (17).

There are two conflicting models for the function of CNX and CRT as ER chaperones, the lectin-only (18, 19) and the dual-binding models (1, 20). In the lectin-only model, binding of CNX and CRT to folding glycoproteins is regulated by the availability of the terminal glucose residue on the Glc1Man9GlcNAc2 oligosaccharide. After the initial formation of the monoglucosylated oligosaccharide by the action of glucosidase II, cycles of dissociation and rebinding are catalyzed by the further action of glucosidase II, which removes the glucose residue, and by UDP-glucose:glycoprotein glucosyltransferase, which reattaches it selectively to incompletely folded glycoproteins (21, 22). According to the lectin-only model, CNX and CRT bind to glycoproteins that remain in the cycle and promote their folding by recruitment of various folding factors. This view is supported by the finding that both CNX and CRT can recruit the thiol oxidoreductase ERp57 to oxidize disulfide bonds in the folding glycoprotein (23). In addition, the lectin-only mode of binding is supported by the finding that treatment of cells with castanospermine, a glucosidase inhibitor that prevents the formation of monoglucosylated oligosaccharides, results in the elimination of CNX/CRT interactions with many glycoprotein substrates (reviewed in Ref. 6).

In the dual-binding model, CNX and CRT are proposed to possess a polypeptide-binding site in addition to the lectin site that allows them to function as more typical chaperones, promoting folding by preventing aggregation. Several lines of evidence support the dual-binding model. First, it has been observed that in the presence of castanospermine, select substrates remain associated with CNX or CRT under mild isolation conditions (reviewed in Ref. 24). Also, in Lec23 and Pha2p7 cells, which do not express glucosidases required for...
the formation of monoglucosylated glycans, an interaction can be observed between CNX and many substrates (24, 25). In vitro studies have further shown that CNX and CRT can function as molecular chaperones by preventing the aggregation not only of glycoproteins but of nonglycosylated substrates as well (20, 26). Interestingly, when challenged with a denatured monoglucosylated substrate, CNX has a marked advantage in preventing aggregation over a conventional peptide-binding chaperone such as BiP, an ER Hsp70, suggesting that the lectin site can act in concert with an additional polypeptide-binding site (27). These two binding modes allow for the promotion of folding and assembly of a diverse spectrum of glycoproteins, while retaining improperly folded substrates and in some cases targeting them for ER-associated degradation.

To further address the relative importance of the lectin versus polypeptide-based interactions in the chaperone functions of CNX, we have engineered point mutants within the lectin site and assessed their ability to bind radiolabeled Glc,Man,GlcNAc. These mutants were then tested for their abilities to bind ERp57 and to prevent the aggregation of glycoylated or nonglycosylated substrates. Select mutants were then co-expressed with class I histocompatibility molecules in Drosophila cells to assess their ability to replace wild type CNX. The results indicate that lectin-deficient CNX retains many aspects of its in vitro and in vivo chaperone functions.

EXPERIMENTAL PROCEDURES

Plasmids and Mutagenesis—The generation of CNX point mutants for in vitro experiments utilized a plasmid in which glutathione S-transferase (GST) was fused to the N terminus of the soluble ER luminal domain of CNX/CNX(1–461). This plasmid (pGEX-cSNX) has been described previously (28). For the production of CNX point mutants for expression in Drosophila melanogaster cells, cDNA encoding full-length CNX in the Drosophila expression vector pRMHa3 was used (designated pRMHa3-CNXX) (13). Additional pRMHa3 plasmids encoding the murine class I heavy chains H-2Kb and H-2Db as well as β2-microglobulin (ß2m) have been described previously (13). QuikChange site-directed mutagenesis (Stratagene) was used to mutate six CNX residues involved in oligosaccharide binding utilizing both the pGEX-cSNX and pRMHa3-CNXX plasmids as templates. The following mutagenic primers were used to either introduce or remove a restriction site as indicated; mutagenic bases are shown in lowercase letter: Y165A, 5′-GAATGTGGTGGTGCTGcagGTGAAACTGCT-3′ (addition of PstI site); K167A, 5′-GGTGCTATATGGGGACTG-CC-3′ (addition of BstBI site); Y186A, 5′-CCACGC-AAGGcAgcTGGATATTGG-3′ (addition of NheI site); M189A, 5′-CCCTTATTACGAGTTGCGGcCCTAAATAATGGG-3′ (addition of AatII site); E217A, 5′-GGGGTATATGGGACAaAaaCTGcATCATC-AAGcG-3′ (loss of SphI site); and E246A, 5′-GGGCTATATGGGcGaGcTGTGTGCT-3′ (addition of Nhel site). The construction of the GST-fused domain of CNX, pGEX-cSNX (257–367), was previously described (3).

Purification of GST Fusion Proteins—Plasmids were transfected into BL-21 Gold (Stratagene) Escherichia coli cells, and protein expression was induced with 0.5 mM isopropyl-β-D-thiogalactopyranoside for 3 h at 30 °C. The cell pellets were resuspended in 10 mM Hepes, pH 7.5, 150 mM NaCl, 5 mM CaCl2 (Buffer A), sonicated, and centrifuged at 10,000 × g. The supernatant fractions were applied to glutathione agarose beads (Sigma) for 1 h followed by washing with buffer A and elution with buffer A containing 20 mM reduced glutathione. The samples were then subjected to NAP-25 gel filtration (Amersham Biosciences) to remove glutathione and concentrated using a Centricon 10 membrane concentrator (Amicon). All of the proteins were judged free of contaminant aggregates (as determined using the Bio-Rad protein assay).

Oligosaccharide Binding Assay—The dolichol-PP-oligosaccharide fraction of Saccharomyces cerevisiae strain slg8 (29) was used to isolate radiolabeled [3H]Glc,Man,GlcNAc, as described previously (1). Binding to GST-fused CNX proteins was performed for 60 min at 4 °C in 100 μl of buffer A containing 30% (vol/vol) of [3H]Glc,Man,GlcNAc (1 μl, 50,000 cpm) and GST fusion protein immobilized on glutathione-agarose beads. The beads were centrifuged for 5 s at 10,000 × g, oligosaccharide in the supernatant fraction was quantified by liquid scintillation counting. All of the experiments included the entire GST-fused ER luminal domain of CNX (CNX(1–461)) as a positive control plus a GST-negative control. The results are reported as the average percentages of binding above GST background compared with CNX(1–461) control on an equimolar basis for replicate experiments. Typically, with 2000 cpm input oligosaccharide, 300 cpm (15%) was bound by full-length GST-CNX(1–461).

ERp57 Binding Assay—ERp57 was expressed from a pET vector transformed into BL-21 Gold E. coli cells by inducing with 0.5 mM isopropyl-β-D-thiogalactopyranoside for 3 h at 30 °C (23). The cell pellets were resuspended in buffer A, sonicated, and centrifuged at 15,000 × g. The supernatant was applied to a MonoQ anion exchange column (Amersham Biosciences), and ERp57 was eluted with a NaCl gradient. ERp57-containing fractions were pooled and then concentrated using a Centricon 10 membrane concentrator (Amicon) changing the buffer to 0.1 M NaHCO3, pH 8.0, by repeated dilutions of the concentrated protein. ERp57 was radiolabeled with N-hydroxysulfosuccinimidyl-[111] ioacetate as described previously (3). GST fusion constructs, immobilized on glutathione-agarose beads and suspended at a concentration of 1 μg, were incubated for 30 min at room temperature in 100 μl of buffer A containing 0.1% Nonidet P-40, 100 μg/ml bovine serum albumin, and 0.1 μM ERp57 or 1 μM [35S]AE-seRgp57. Agarose beads were centrifuged for 5 s at 10,000 × g, washed twice with buffer A, and eluted with buffer A containing 10 mM reduced glutathione. For nonradiolabeled samples, the eluted fractions were lyophilized, acetic acid-saturated with ethanol and analyzed by Western blotting with an anti-ERp57 antibody (gift from Dr. D. Thomas, McGill University) and the ECL detection system (Amersham Biosciences). Radioactive eluates were quantified by liquid scintillation counting and compared with the GST-fused ER luminal domain of CNX (CNX(1–461)) as a positive control plus a GST-negative control. The results are expressed as the average percentage of binding above GST background relative to the CNX(1–461) control. Typically radioactivity recovered in eluted samples ranged from 500 to 1000 cpm.

Aggregation Assays—Citrate synthase (CS; Sigma) and jack bean α-mannosidase (α-M; Sigma) were desalted using NAP-25 gel filtration (Amersham Biosciences). Desalted α-Man was diluted to 0.36 μM in a total assay volume of 500 μl, and aggregation was assessed by monitoring light scattering at 360 nm for 1 h at 45 °C. α-M was rapidly diluted to 0.36 μM with 500 μl of GST fusion construct (0.60–0.72 μM) containing buffer A. Aggregation was monitored as for CS but at 15 °C for 5 min.

Cell Lines and Antibodies—In the pRMHa3 vector, all of the cDNAs are under the control of the metallothionein promoter (30). Stably transfected Drosophila cell lines were established by co-transfecting the calcium phosphate method a phshsneo plasmid containing the neomycin-resistance gene along with one or more pRMHa3 plasmids encoding the H-2Kb heavy chain, the H-2Dβ heavy chain, β2m, CNX, or CNX point mutants. To ensure that all of the proteins were expressed within one cell, the cell lines were cloned using the soft agar technique (31). After screening for expression by metabolic radiolabeling and immunosialylation, clones with comparable protein expression levels were chosen for all subsequent experiments. D. melanogaster Schneider cells were maintained in Schneider’s insect medium with 10% fetal bovine serum and antibiotics. Stably transfected cell lines were cultured in the presence of 500 μg/ml Geneticin (Invitrogen). The following mAbs were used for the isolation of class I molecules: mAb 28-14-8S, which recognizes a conformational epitope in the α2 domain of free or fixed D. melanogaster Dβ heavy chain (32), and mAb 20-8-4S, which recognizes mAb 28-14-8S but at 15°C. Both mAbs were used to immunoprecipitate α-chain containing heavy chains associated with β2m (33). A rabbit antisera (anti-HC) directed against inclusion bodies of soluble Dβ recognizes unfolded Dβ heavy chains and was provided by Dr. Hidde Ploegh (Harvard Medical School) (34). A rabbit antisera (anti-ß2m) directed against the C terminus of the H-2Kb H chain, which reacts with all conformational forms of the class I heavy chain, was provided by Brian Barber (Vanderbilt University) (35). For isolation of CNX, a rabbit antisera directed against the native 462 residue ER luminal domain of dog calnexin was used (24).

Metabolic Radiolabeling, Immunosialylation, and Gel Electrophoresis—Expression of transfected cDNAs in Drosophila cells was induced by incubation with 1 mM CuSO4 for 16 h, and then cells were incubated for 5 min in 100 mM NaCl, 5 mM CaCl2, containing 1% digitonin, 10 mM iodoacetamide, 1% aprotinin, and 10 μg/ml each of chymostatin, leupeptin, antipain, and pepstatin. For experiments with Kβ, the ly-
sates were precleared for 1 h with a preimmune serum followed by protein A-agarose beads. The lysates were incubated for 2 h at 4°C with anti-class I or anti-CNX antibodies followed by recovery of immune complexes by incubation for 1 h with protein-A agarose beads. Immune complexes were analyzed by SDS-PAGE using 10% or 12.5% gels, and the radioactive proteins were visualized by fluorography. For quantification of bands, the fluorograms were scanned and then analyzed using National Institutes of Health Image software.

RESULTS

Lectin Site Mutants of Calnexin Are Impaired in Oligosaccharide Binding—From the x-ray structure of CNX co-crystallized with α-L-glucose (2), six residues that presumably contact the terminal glucose of the natural Glc$_3$Man$_9$GlcNAc$_2$ oligosaccharide were identified (Fig. 1). Tyr$_{165}$, Lys$_{167}$, Tyr$_{186}$, Glu$_{217}$, and Glu$_{426}$ all form hydrogen bonds to glucose hydroxyl groups, whereas the hydrophobic Met$_{189}$ lies underneath the glucose ring within van der Waals’ contact distance (2).

To assess the contributions of these individual interactions to Glc$_3$Man$_9$GlcNAc$_2$ binding, each of the six residues was mutated to Ala in the context of the soluble, luminal domain of CNX fused to GST. The purified proteins were immobilized on glutathione beads and incubated with radiolabeled [3H]Glc$_3$Man$_9$GlcNAc$_2$. From the binding results shown in Fig. 2A, five of the six point mutants were sufficient to drop oligosaccharide binding to a background level seen with the arm domain alone. We have previously shown that the arm domain construct exhibits a low level of oligosaccharide binding that is not specific for monoglucosylated species and thus represents the background binding of oligosaccharide in a completely lectin-deficient mutant (3). Only the M189A mutant exhibited slight binding above the background of the arm domain. Therefore, the loss of even a single hydrogen bonding residue is sufficient to ablate lectin function.

Lectin Site Mutants of Calnexin Are Capable of Binding ERp57 and Preventing Aggregation—To ensure that the point mutations had not severely affected the folding of CNX, we tested each for binding of the oxidoreductase ERp57. Because the binding site for ERp57 is known to be within the arm domain, we tested each lectin site mutant construct for binding of ERp57 using immobilized GST fusion constructs of the ER luminal segment of CNX (wt CNX), the indicated lectin site point mutants, and the arm domain were incubated with [3H]Glc$_3$Man$_9$GlcNAc$_2$ for 30 min at 4°C. After washing beads once, bound oligosaccharide was eluted by chelation of calcium necessary for binding and quantified by liquid scintillation counting. Oligosaccharide binding is expressed as a percentage of the specific binding observed for wild type CNX (350–500 cpm) after subtraction of background binding for GST alone (50–100 cpm). The values are the averages of four replicate experiments with error bars representing the standard error.

FIG. 1. X-ray crystal structure of the ER luminal segment of calnexin. The ER luminal segment of CNX is composed of a β-sandwich lectin domain and an arm-like extension consisting of two tandemly repeated sequence motifs. The six amino acid residues that contact glucose in the co-crystal are shown in stick representation and are labeled. The structural calcium ion is shown as a small sphere on the convex side of the lectin domain.

FIG. 2. Oligosaccharide and ERp57 binding by lectin-site mutants of calnexin. A, immobilized GST fusion constructs of the ER luminal segment of CNX (wt CNX), the indicated lectin site point mutants, and the arm domain were incubated with [3H]Glc$_3$Man$_9$GlcNAc$_2$ for 30 min at 4°C. After washing beads once, bound oligosaccharide was eluted by chelation of calcium necessary for binding and quantified by liquid scintillation counting. Oligosaccharide binding is expressed as a percentage of the specific binding observed for wild type CNX (350–500 cpm) after subtraction of background binding for GST alone (50–100 cpm). The values are the averages of four replicate experiments with error bars representing the standard error. B and C, the ER luminal segment of CNX or the lectin site mutants fused to GST were immobilized on glutathione-agarose beads (1 M) and incubated with 0.1 μM ERp57 or 1 μM [14C]Ac-ERp57. The beads were washed twice, and bound ERp57 was eluted with 10 mM reduced glutathione and either separated by SDS-PAGE and detected by Western blotting with an anti-ERp57 antibody (B) or detected directly by scintillation counting (C). The error bars represent the standard errors in a minimum of three replicate experiments. The asterisk denotes a degradation product of ERp57.
domain of CNX (3, 4), it should not be affected by point mutants within the globular lectin domain unless the mutants are severely misfolded. In all six mutants, binding of ERp57 was not substantially impaired as assessed either qualitatively by pull-down and Western blotting (Fig. 2B) or quantitatively by assessing the amount of radiolabeled ERp57 bound to GST-immobilized CNX mutants (Fig. 2C).

It is possible that point mutations within the lectin site could affect binding of unfolded polypeptides, because we have previously shown that the globular domain is the site of non-native polypeptide binding, although assisted by the presence of the arm domain (3). To address this possibility, the ability of the six GST-fused CNX mutants to prevent the thermal aggregation of CS was tested. As shown in Fig. 3A, GST had no effect on CS aggregation, whereas GST fused to the ER luminal domain of CNX almost completely suppressed aggregation at a CS:CNX ratio of 1:0.5 and fully suppressed aggregation at a ratio of 1:2. When tested at a CS:CNX ratio of 1:0.5, all six mutants were able to prevent the aggregation of CS at levels not significantly different from that of wild type CNX, indicating that their polypeptide-binding site was not impaired by the point mutation (data not shown).

**Lectin Site Mutants of Calnexin Are Impaired in Preventing the Aggregation of a Monoglucosylated Substrate**—Because CNX normally interacts with monoglucosylated glycoproteins, we tested to see whether the lectin site mutants were able to prevent the aggregation of chemically denatured jack bean α-Man, which possesses an Asn-linked oligosaccharide of the form Glc1Man9GlcNAc2. We have shown previously that suppression of α-Man aggregation is strongly influenced by the presence of its monoglucosylated oligosaccharide (27). As shown in Fig. 3C, GST had no effect on α-Man aggregation, whereas GST fused to the ER luminal domain of CNX effectively suppressed aggregation at α-Man:CNX ratios of 1:1 or 1:2. In contrast to GST-CN, all six mutants exhibited a profound impairment in the ability to suppress aggregation of α-Man at a molar ratio of 1:1 (Fig. 3D). This demonstrates that the disruption of the lectin site is sufficient to compromise the ability to suppress aggregation of a monoglucosylated substrate. The small degree of residual aggregation suppression observed with most of the mutants is likely due to interaction via the polypeptide-binding site because CNX can suppress the aggregation of deglycosylated α-Man albeit at higher molar ratios (27).

**Lectin Site Mutants of Calnexin Are Able to Bind Class I Histocompatibility Molecules in Vivo**—To further examine the role of the lectin site of CNX, we assessed the in vivo function of two of the point mutants. Using a heterologous expression system in *Drosophila* cells, we were able to assess the effect CNX and its lectin site mutants have on the biogenesis of mouse class I histocompatibility molecules. Mouse class I histocompatibility molecules.
Characterization of Lectin-deficient Calnexin

FIG. 4. Lectin-deficient E217A CNX associates with free class I heavy chain D^b. Drosophila cells expressing D^b H chain in the absence or presence of CNX or E217A CNX were incubated for 5 min with [35S]Met. D^b H chains and CNX were immunoisolated with the mAb 28-14-8S and a polyclonal antibody against the luminal segment of canine CNX, respectively, and analyzed by reducing SDS-PAGE followed by autoradiography. The mobilities of the heavy chain, CNX, and molecular mass markers are indicated. act, wild type; IP, immunoprecipitation.

FIG. 5. Effects of Y165A and E217A CNX on the stability of free class I heavy chain D^b. Drosophila cells expressing D^b heavy chain in the absence or presence of CNX, Y165A CNX, or E217A CNX were incubated for 5 min with [35S]Met and then chased with excess unlabeled Met for the periods indicated. A, D^b heavy chains were immunoisolated from cell lysates with the conformation-sensitive mAb 28-14-8S and analyzed by reducing SDS-PAGE followed by fluorography. The mobilities of the D^b heavy chain and CNX are indicated. B, fluorograms from three independent experiments were quantified by densitometry, and the averaged amounts of D^b heavy chain signal at each time point are expressed as percentages of the amount present in the pulse sample. The error bars are omitted for clarity; standard errors were 7% or less.

tcomotability molecules consist of three subunits: a type I membrane heavy chain, which possesses two or three Asn-linked glycans; a soluble subunit termed β_m; and a peptide ligand of 8–9 amino acids. In Drosophila cells, class I molecules do not acquire peptide ligands, and hence their study is limited to heavy chain folding and assembly with β_m. Endogenous CNX and CRT are expressed in Drosophila cells, but despite the presence of all enzymes required for a functional deglucosylation-reglucosylation cycle (36–38), we have shown that no interaction with mouse class I heavy chains can be observed (13). Thus, Drosophila cells represent an operationally CNX- and CRT-deficient environment for mouse histocompatibility molecules. Co-transfected mammalian CNX and CRT are able to bind transiently to class I heavy chains in Drosophila cells and assist in their maturation (8, 10, 13).

Initially, we tested whether a lectin site mutant was capable of forming complexes with the class I heavy chain H-2D^b. In this experiment, Drosophila cells expressing D^b heavy chains alone or in the presence of wild type CNX or the lectin-deficient E217A mutant were radiolabeled, and the cell lysates were immunoisolated with anti-D^b or anti-CNX antibodies. As shown in Fig. 4, treatment of lysates expressing wild type CNX with either antibody succeeded in recovering both the D^b heavy chain and CNX. The same result was observed for lysates of cells expressing the E217A mutant. Confirmation of this finding is shown in Fig. 5. In this pulse-chase experiment, lysates of cells were isolated with anti-D^b antibodies, and complexes with CNX were recovered not only with wild type CNX but with the E217A mutant and the Y165A mutant. Therefore, an intact lectin site is not required for CNX to associate with D^b heavy chains in vivo.

Lectin Site Mutants of Calnexin Retain the Ability to Protect Class I Histocompatibility Molecules against Degradation—Calnexin has been implicated in influencing the degradation fate of some of its substrates. Major histocompatibility complex class I heavy chains expressed in CNX-deficient semi-permeabilized mammalian cells are degraded more rapidly than in the parent cell line (39). Furthermore, an increase in the degradation of some model substrates is observed when their interaction with CNX is inhibited by using glucosidase inhibitors (12, 40–42) or when CNX levels are lowered by siRNA (43). Using the Drosophila system we showed previously that co-transfected CNX is capable of preventing the loss of D^b heavy chains recovered by the conformationally sensitive mAb 28-14-8S (8, 13). As seen in Fig. 5, which depicts a pulse-chase immunoisolation experiment, D^b heavy chains lose the folded epitope recognized by mAb 28-14-8S in the absence but not in the presence of CNX. For D^b alone, heavy chains recovered with mAb 28-14-8S decreased progressively during the chase period, with only 35% remaining by 180 min, whereas wild type CNX stabilized folded D^b heavy chains such that 81% remained following 180 min of chase (Fig. 5B). The loss of the folded epitope recognized by 28-14-8S is followed by degradation because the total D^b heavy chains recovered by a combination of mAb 28-14-8S and a conformation-insensitive anti-HC antibody decreases over the chase period (data not shown). To assess whether an intact lectin site of CNX is required to prevent this unfolding and degradation, we examined the ability of lectin-deficient CNX mutants Y165A and E217A to stabilize folded D^b heavy chains. Remarkably, the two lectin-deficient mutants of CNX were equally as potent as the wild type molecule in stabilizing D^b heavy chains with 72 and 95% of heavy chains remaining after 180 min for Y165A and E217A, respectively (Fig. 5B). Thus, CNX mutants with defective lectin sites are still competent to bind free class I heavy chains in vivo and to stabilize them against unfolding and degradation.

To extend our findings to a second major histocompatibility
complex class I molecule, we prepared *Drosophila* cell lines that express K\(^b\) heavy chains plus its dimeric partner β\(_2\)m either alone or along with wild type CNX or the CNX mutants Y165A or E217A. In this experiment degradation was monitored by determining the loss of total heavy chains recovered by immunoprecipitation. In addition, the appearance of a faster mobility mature form was monitored. In *Drosophila* cells, Asn-linked oligosaccharides are processed in the Golgi apparatus to smaller species relative to their immature ER forms, and thus heavy chains that have reached the Golgi apparatus migrate more rapidly by SDS-PAGE when compared with ER forms. The various *Drosophila* transfecteds were subjected to pulse-chase radiolabeling, and K\(^b\) molecules were isolated with anti-8 antiserum, which recognizes the total pool of K\(^b\) H chains and then separated by reducing SDS-PAGE. The mobilities of mature and immature heavy chain are indicated. B, the total signal for both the immature and mature bands at each time point for three replicate experiments was quantified by densitometry and expressed as the percentage of the amount present in the pulse sample for each cell line. C, the signal of the lower, mature band at each time point for three replicate experiments was quantified and expressed as a percentage of the amount present in the pulse sample for each cell line. The cell lines are indicated as in B. For both plots the error bars are omitted for clarity; the data points represent the mean values with a standard error of 8% or less.

**Fig. 6.** Effects of Y165A and E217A CNX on the maturation and degradation kinetics of K\(^b\)heavy chains. *Drosophila* cells expressing K\(^b\) heavy chain and β\(_2\)m in the absence or presence of CNX, Y165A CNX, or E217A CNX were radiolabeled with [\(^{35}\)S]Met for 5 min and then chased in the presence of excess unlabeled Met for the indicated times. A, K\(^b\) heavy chains were immunoprecipitated from cell lysates with anti-8 antisera, which recognizes the total pool of K\(^b\) H chains and then separated by reducing SDS-PAGE. The mobilities of mature and immature heavy chain are indicated. B, the total signal for both the immature and mature bands at each time point for three replicate experiments was quantified by densitometry and expressed as the percentage of the amount present in the pulse sample for each cell line. C, the signal of the lower, mature band at each time point for three replicate experiments was quantified and expressed as a percentage of the amount present in the pulse sample for each cell line. The cell lines are indicated as in B. For both plots the error bars are omitted for clarity; the data points represent the mean values with a standard error of 8% or less.

We have shown that the lectin site of CNX can be disrupted by mutating single residues known from x-ray crystallographic studies to interact with glucose and presumably with the terminal glucose of the natural Glc\(_3\)Man\(_9\)GlcNAc\(_2\) ligand (2). These residues are involved either in hydrogen bond interactions in the case of Tyr\(_{165}\), Lys\(_{167}\), Tyr\(_{186}\), Glu\(_{217}\), and Glu\(_{426}\) or in hydrophobic stacking interactions with the sugar ring in the case of Met\(_{189}\). The mutations do not affect the ability of CNX to bind to its oxireductase partner Ehp57 or to prevent the aggregation of nonglycosylated substrates. However, direct binding of oligosaccharide and the prevention of aggregation of a monoglucosylated substrate is impaired, suggesting that the disruption of one or two hydrogen bonds or the loss of stacking interactions provided by the mutated residue is sufficient to lower dramatically the affinity for the entire glycan.

These findings are consistent with previous studies on the legume class of lectins to which calnexin is structurally related (2). Mutation of a single hydrogen-bonding or sugar-stacking residue within the lectin site of various legume lectins results in loss of glycan binding. For the lectin from *Erythrina coral-lodendron* (EcorL), mutation of the hydrogen-bonding residues Asp\(_{89}\) or Asn\(_{133}\) to Ala (44) and mutations in equivalent residues in the *Phaselus vulgaris* phytohemagglutinin lectin (45), *Pisum sativum* lectin (46), and *Griffonia simplicifolia* lectin II (47) result in a loss of sugar binding. A mammal-specific animal lectin with a role in ER-Golgi trafficking, ERGIC-53, is homologous to the legume lectins, and mutation of the conserved Asn residue also results in the loss of lectin activity (48). In addition, the mutation of Phe\(_{331}\) of EcorL, which stacks against galactose in a co-crystal complex with lactose (49), to a smaller, nonaromatic residue results in loss of saccharide binding (44).

To determine whether an intact lectin site is required in vivo for the interaction with a natural substrate we transfected two of the lectin site mutants of CNX, Y165A and E217A, into a *Drosophila* heterologous expression system along with the mouse class I histocompatibility molecules, H-2K\(^b\) and H-2D\(^b\). The CNX lectin site mutants, like wild type CNX, were capable of binding to the glycosylated class I heavy chain, thereby demonstrating that the lectin site is not required for the association. These findings are complementary to previous work demonstrating that CNX is capable of interacting with class I molecules in a glycan-independent manner. For example, complexes between CNX and either K\(^b\) or D\(^b\) could not be dissociated by enzymatic removal of heavy chain oligosaccharides (1). Furthermore, in the presence of castanospermine to block the formation of monoglucosylated oligosaccharides, CNX remained associated with human HLA-B27 molecules in the human cell line C1R-B27 as well as with K\(^b\) and D\(^b\) molecules expressed in *Drosophila* cells (24). Finally, using K\(^b\) glycosylation mutants expressed in mouse L cells that possess 0–3 Asn-linked sites, it was shown that CNX interacts with all four heavy chains, including the unglycosylated one, and that these interactions are not due to nonspecific inclusion into protein aggregates (24). Collectively, these results from diverse systems are consistent with the model that CNX functions via a lectin-glycan interaction and a polypeptide-binding interaction, with the latter not merely being an artifact of the *Drosophila* expression system.
A dual mode of interaction has advantages over a lectin-only association in that it permits CNX or CRT to function as classical chaperones, suppressing aggregation by binding selectively to polypeptide segments of non-native folding conformers. Such aggregation suppression by CNX or CRT has been demonstrated both in vitro (20, 26, 27, 50) and in vivo (10, 12). Dual binding would also serve to enhance the avidity of CNX or CRT association with glycoprotein substrates relative to binding via either interaction alone. Indeed we showed previously that CNX has a marked advantage over the classical Hsp70 chaperone BiP in preventing the in vitro aggregation of a monoglucosylated substrate, suggesting that the lectin and polypeptide binding interactions cooperate in their anti-aggregation function (27). Lectin-only interactions are also difficult to rationalize in light of the numerous observations that CNX or CRT complexes with various glycoproteins are stable to immune isolation and repeated washing steps. The dissociation constant of CNX for IgG carrying a single GlcMManGlcNAc 2 oligosaccharide is only 1–2 μM (51). Glycans with dissociation constants in this range are typically retarded on lectin columns rather than binding tightly (52), and we have shown that the binding of GlcMManGlcNAc 2 oligosaccharide to immobilized CNX is readily lost upon washing (1).

The most remarkable aspect of the present study is the finding that lectin-deficient mutants of CNX retain the ability to bind class I heavy chains and protect them against degradation. These findings support the view that polypeptide interactions between CNX or CRT and folding glycoproteins vary substantially in the affinity with which their polypeptide segments associate with CNX or CRT. In this context, the class I histocompatibility molecules used in the present study may represent examples of glycoproteins that do not depend strongly on lectin-glycan interactions for association with CNX. It will be of particular interest to extend these studies to glycoproteins that are much more dependent on lectin-glycan interactions. One might anticipate that more dramatic effects of ablating the lectin function of CNX would be observed with such substrates.

Acknowledgments—We thank Dr. Hidde Ploegh for the gift of the anti-HC antiserum and Dr. David Thomas for the gifts of anti-ERP57 antiserum and the plasmid encoding ERp57.

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