Polypeptide Substrate Recognition by Calnexin Requires Specific Conformations of the Calnexin Protein*

Vilasack Thammavongsa, Laura Mancino, and Malini Raghavan

From the Department of Microbiology and Immunology and the Graduate Program in Immunology, University of Michigan Medical School, Ann Arbor, Michigan 48109-0620

Calnexin is an endoplasmic reticulum chaperone that binds to substrates containing monoglucosylated oligosaccharides. Whether calnexin can also directly recognize polypeptide components of substrates is controversial. We found that calnexin displayed significant conformational liability for a chaperone and that heat treatment and calcium depletion induced the formation of calnexin dimers and higher order oligomers. These conditions enhanced the chaperone activity of calnexin toward glycosylated and non-glycosylated major histocompatibility complex (MHC) class I heavy chains, and enhanced calnexin binding to MHC class I heavy chains. In contrast to these observations, calnexin binding to oligosaccharide substrates has been reported to be impaired under calcium-depleting conditions. Calnexin dimers were induced in HeLa cells upon heat shock and under calcium-depleting conditions, and heat shock enhanced calnexin binding to MHC class I heavy chains in HeLa cells. Virus-induced endoplasmic reticulum stress also resulted in the appearance of calnexin dimers. Tunicamycin treatment of HeLa cells induced a slow accumulation of calnexin dimers, the appearance of which correlated with enhanced calnexin binding to oligosaccharide substrates containing monoglucosylated oligosaccharides. Calreticulin, and calnexin have been shown to function as molecular chaperones by enhancing the assembly of a variety of cellular and viral substrates. This function is attributed to the ability of calnexin/calreticulin in the ER to bind to glycoproteins carrying monoglucosylated oligosaccharides and to retain such proteins in the ER. Newly synthesized glycoproteins carrying three glucose residues on the oligosaccharide chain (GlcNAc2Man7–9Glc3) are trimmed by the sequential action of ER glucosidases I and II. Glucosidase II can also catalyze the removal of the terminal glucose residue, and the resulting non-glycosylated glycoproteins either traffic out of the ER if properly folded, or become associated with the ER luminal enzyme UDP-glucose:glycoprotein glucosyltransferase, if misfolded. UDP-glucose:glycoprotein glucosyltransferase binds to misfolded but not native structures, causing the regeneration of monoglucosylated oligosaccharide-containing glycoproteins. Thus, UDP-glucose:glycoprotein glucosyltransferase is an important protein folding sensor in the ER, and calnexin and calreticulin have been suggested to cooperate with UDP-glucose:glycoprotein glucosyltransferase by binding to and ensuring ER retention of glycoproteins that are tagged by UDP-glucose:glycoprotein glucosyltransferase as being misfolded.

In the studies described here, we investigated whether calnexin could also function as a direct polypeptide folding sensor in the ER. In vitro analyses have indicated that both calnexin and calreticulin are capable of direct functional interactions with non-glycosylated polypeptide substrates, inhibiting their aggregation (3, 4). However, more recent studies in our laboratory have indicated that specific conditions are required for efficient observation of the binding of calreticulin to polypeptide substrates lacking monoglucosylated oligosaccharides (5). Calreticulin had surprising conformational liability for a chaperone, with heat treatment or calcium depletion inducing the conversion of calreticulin monomers into specific oligomeric forms. We observed that conditions that enhanced oligomerization of calreticulin also enhanced the ability of calreticulin to bind glycosylated and non-glycosylated polypeptide substrates and enhanced the chaperone activity of calreticulin toward such substrates. However, oligomerization per se was not required for enhanced polypeptide substrate recognition by calreticulin. In this study, we examined whether calnexin shares some of the properties we have described for calreticulin. We investigated the conformations of calnexin under different conditions and their effects on polypeptide substrate recognition by calnexin. Many parallels were observed between calreticulin and calnexin, emphasizing the likely existence of multiple conformational states of these chaperones with distinct substrate recognition properties.

EXPERIMENTAL PROCEDURES

Peptides and Proteins—Major histocompatibility complex (MHC) class I-specific peptides were obtained synthetically and labeled with iodoacetamidofluorescein as described previously (6). Human soluble
HLA-A2/β2-microglobulin (β2m) heterodimers were purified from insect cells as described previously (7). An Escherichia coli construct expressing soluble HLA-A2 heavy chains was obtained from Dr. David Garboczi (8), and constructs encoding chimp β2m (identical to the human β2m sequence at the amino acid level) and the HLA-E heavy chain were obtained from Dr. Pamela Bjorkman. Inclusion bodies were purified as described (8) and solubilized in 6 M guanidine hydrochloride. For purification of HLA-A2 and HLA-E heterodimers from the inclusion bodies, 3 μM heavy chains and 6 μM β2m were refolded in 50 ml of buffer containing 100 mM Tris-HCl, 400 mM L-arginine, 2 mM EDTA, 0.5 mM oxidized glutathione, and 5 mM reduced glutathione, pH 8.0, overnight at 4 °C. After high speed centrifugation, refolded class I heterodimers were further purified by gel filtration chromatography using a Superdex 200 column (Amersham Biosciences) in 50 mM Tris-HCl and 150 mM NaCl, pH 7.5. The presence of both heavy chains and β2m at the expected elution position was verified by SDS-PAGE of the eluted fractions.

Soluble Calnexin—An E. coli construct encoding a glutathione S-transferase-soluble calnexin fusion was obtained from Dr. David Williams and purified as described (9). This protein retains the ability to bind oligosaccharide substrate, to bind ERp57, and to suppress the aggregation of polypeptide substrates as described (9, 10).

Cells—HeLa cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum and antibiotics. FCA4 and Huh7 (11) cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, 1-glutamine, nonessential amino acids, and penicillin/streptomycin. FCA4 cells were maintained in G418 (500 μg/ml; Invitrogen).

Co-immunoprecipitation Analyses to Detect Calnexin-Class I Complex Formation—Calnexin, HLA-A2, or HLA-A2 + calnexin (10 μM each in a total volume of 60 μl) were heated in aggregation assay buffer (50 mM Tris-HCl, 150 mM NaCl, and 1 mM CaCl2, pH 7.5) at 50 °C or in the indicated conditions at 37 °C for 1 h. For analyses under calcium-depleting conditions, calnexin (in 50 mM Tris-HCl and 150 mM NaCl, pH 7.5) was preincubated in the presence of 2 mM EGTA for 1 h at 37 °C, followed by the addition of HLA-A2 and incubation for an additional 1 h. The samples were diluted with 50 mM Tris-HCl and 150 mM NaCl to 360 μl and incubated with 15 μl of HC-10 ascites (12) per immunoprecipitation for 1 h at 4 °C, followed by centrifugation. The supernatants were incubated overnight at 4 °C with washed protein G beads. The beads were washed three times with aggregation assay buffer containing 0.7% Triton X-100, resuspended in SDS-PAGE buffer, and separated by SDS-PAGE (10% gels). Immunoprecipitated proteins were visualized by Coomassie Blue staining. To assess calnexin-non-glycosylated class I complex formation at 37 °C, refolded HLA-A2 and calnexin (2 μM each) were incubated in 50 mM Tris, 150 mM NaCl, and 1 mM CaCl2, pH 7.5, at a final volume of 100 μl at 37 °C for 1 h. Samples were then diluted into 1 ml of buffer and incubated with 5 μl of HC-10 ascites at 4 °C for 4 h, followed by an additional 2-h incubation with washed protein G beads. The beads were washed three times with buffer containing 0.5% Triton X-100, resuspended in SDS-PAGE buffer, aliquoted into two equal fractions, and separated by SDS-PAGE (10% gels). Gels were silver-stained to verify recovery of class I heavy chains and immunoblotted with anti-calnexin antisera (Stressgen Biotechnologies Corp., Victoria, British Columbia, Canada) to detect associated calnexin.

Enzyme-linked Immunosorbent Assay (ELISA) to Detect Calnexin-Class I Complexes—The indicated amounts of refolded HLA-A2 or HLA-E were coated onto 96-well ELISA plates overnight at 4 °C. Supernatants were removed, and blocking buffer (phosphate-buffered saline and 1% fetal calf serum) was added at 37 °C for 1 h. 10 μg of purified calnexin was added in 50 mM Tris, 150 mM NaCl, and 1 mM CaCl2, pH 7.5, and incubated at 37 °C for 4 h. Plates were washed three times with phosphate-buffered saline and 0.5% Tween, followed by incubation with anti-calnexin antisera and by incubations with horseradish peroxidase-conjugated goat anti-rabbit antibody. Detection of complexes was conducted by measuring the enzymatic degradation of H2O2 using o-phenylenediamine (Rainbow Scientific, Inc.).

Temperature or Calcium Dependence of Calnexin Oligomerization in HeLa, Huh7, and FCA4 Cells—Calnexin oligomerization in cells was analyzed following procedures described previously for calreticulin (5). Briefly, the indicated cells in 150-cm2 culture flasks under the indicated conditions were lysed in 400 μl of lysis buffer (1% digitonin (Waco Chemical & Supply Co.; for analysis in HeLa cells) or 1% Triton X-100 (for analyses in Huh7 and FCA4 cells), 10 mM Tris, 1 mM phosphate, 130 mM NaCl, and 10 mM iodoacetamide, pH 7.5), and calnexin was visualized by immunoblotting with anti-calnexin antisera using a colorimetric alkaline phosphatase kit (Bio-Rad).

MHC Class I Interactions with Calnexin in HeLa Cells—HeLa cell lysates were prepared as described above, and immunoprecipitation analyses were conducted as described previously (5) using the rabbit anti-calnexin polyclonal antiserum.

RESULTS

Calnexin Undergoes Structural Changes upon Heat Treatment and under Calcium-depleting Conditions—Studies by Ou et al. (13) suggested that purified soluble calnexin undergoes conformational changes following calcium binding. Using a canine soluble calnexin comprising residues 1–461 of the mature protein (9), we analyzed calnexin structure by native PAGE at different temperatures and in the absence or presence of calcium (Fig. 1). In the absence of calcium, at least five calnexin species were resolved even at 37 °C, forming a ladder of bands (Fig. 1A, upper panel). The size of the fastest migrating band is consistent with that of a calnexin monomer (~65 kDa), and the size of the second band is consistent with that of a calnexin dimer (~125 kDa). Heat treatment enhanced the formation of high molecular mass calnexin bands and a corresponding reduction in the intensities of calnexin monomer/dimer bands (Fig. 1A, compare lanes 4 and 6). Compared with the calcium-depleting condition, the presence of 1 mM Ca2+...
revealed higher levels of calnexin monomers and lower levels of dimers/oligomers at 37 °C (Fig. 1, A, compare upper and middle panels; and B, corresponding quantifications) and slower kinetics of temperature-induced calnexin oligomerization (Fig. 1B, lower panel). In the presence of 5 mM Ca²⁺, calnexin monomers were the predominant species observed even at 47 °C (Fig. 1, A, lower panel; and B). Thus, both heat treatment and calcium depletion enhanced calnexin dimer/higher order oligomer formation at the expense of monomers. Both low temperatures and high concentrations of calcium stabilized calnexin monomers. The extent of dimers and oligomers observed was also a function of calnexin concentration. Higher concentrations of calnexin promoted dimer/oligomer formation (data not shown).

**Heat Treatment and Calcium Depletion Enhance the Ability of Calnexin to Bind to Polypeptide Substrates**—Calcium depletion has been previously shown to abrogate calnexin binding to oligosaccharide substrates (9). Because calcium binding and heat treatment influence calnexin structure (Fig. 1), we wanted to examine the effects of these conditions upon calnexin interactions with polypeptide substrates. We used a soluble MHC class I heterodimer (HLA-A2, the heavy chain and the β₂m light chain) that was expressed in and purified from insect cell supernatants (7). Although the HLA-A2 heavy chain is glycosylated, it is not expected to contain monoglycosylated oligosaccharide structures that calnexin can recognize. These structures are transient intermediates during glycoprotein trafficking through the ER. Because insect cells lack the class I-specific peptide transporter, class I molecules purified from insect cells are peptide-deficient, and the heavy chains are ther-
Calnexin is able to bind to non-glycosylated HLA-A2 at physiological temperature. A, left and right panels, direct protein loading of 2 μg of calnexin (CNX; lanes 1) or refolded non-glycosylated HLA-A2 (A2; lanes 2) and immunoprecipitations with antibody HC-10 of calnexin alone in buffer (lanes 3), buffer alone (lanes 4), or calnexin and non-glycosylated HLA-A2 (lanes 5). Each protein was at 2 μM in aggregation assay volume (final volume of 100 μl). Mixtures were then incubated at 37 °C for 1 h, and proteins were immunoprecipitated with HC-10 ascites. Left panel, silver-stained gel; right panel, immunoblot with anti-calnexin antisera. Fig. 3, upper right panel shows a silver-stained SDS-polyacrylamide gel of the co-immunoprecipitation analysis. An additional band consistent with the size of the HC-10 immunoprecipitated HLA-A2 heavy chains upon heat treatment and under calcium-depletion conditions was observed with HLA-A2 samples that were refolded in the presence of heat-treated calnexin.

To further confirm these results, we assessed whether calnexin-class I complexes could be observed using ELISA-based detection. Refolded non-glycosylated HLA-A2 heterodimers were analyzed by SDS-PAGE to verify the presence of both heavy chains and β2m (Fig. 3B, upper left panel, second lane) and then coated overnight onto 96-well ELISA plates in 50 mM Tris and 150 mM NaCl, pH 7.5. 10 μg of purified soluble calnexin in 100 μl of buffer containing 50 mM Tris, 150 mM NaCl, and 1 mM CaCl2, pH 7.5, was then added to the wells, and complexes were allowed to form during incubation at 37 °C for 4 h. Detection of calnexin-class I complexes was performed by the addition of anti-calnexin antiserum. Fig. 3B (upper right panel) shows that the amount of calnexin that was detectable increased with the amount of HLA-A2 heterodimers coated onto the plate. The binding signal was specific, as equal amounts of β2m or bovine serum albumin coated onto the plates yielded significantly lower absorbance signals. A parallel set of analyses was undertaken with non-glycosylated HLA-E (Fig. 3B, lower panels). These analyses verify that calnexin is indeed able to recognize and bind to non-glycosylated MHC class I heavy chains under physiological conditions.

Heat Treatment and Calcium Depletion Enhance Calnexin Chaperone Activity toward a Protein Substrate—It was a possibility that the enhanced binding observed between calnexin and peptide-deficient HLA-A2 heavy chains upon heat treatment and under calcium-depleting conditions reflected nonspecific associations induced by conditions that generated unfolded/aggregated forms of calnexin. To rule out this possibility, we assessed calnexin function in a MHC class I refolding assay (Fig. 4). HLA-A2/β2m heterodimers were purified after baculovirus-mediated expression in insect cells, diaлизed into buffer containing 6 M guanidine hydrochloride, and concentrated to 100 μM. 2 μl of protein was diluted with rapid stirring into 100 μl of aggregation assay buffer (Fig. 4A, left panel, lanes 9 and 10) or buffer containing the indicated proteins at 2 μM (lanes 1–8). Samples were then centrifuged to separate soluble and insoluble HLA-A2 heavy chains. Pellets were resuspended in a volume equal to the supernatant, and both fractions were analyzed by SDS-PAGE and Coomassie Blue staining (Fig. 4A, left panel). When refolding was conducted in the presence of buffer alone, the majority of HLA-A2 heavy chains precipitated (Fig. 4A, left panel, lanes 9 and 10). The presence of the control protein (IgG) did not significantly alter the
extent of rescue of the HLA-A2 heavy chain (Fig. 4A, left panel, lanes 7 and 8). At a 1:1 ratio relative to HLA-A2, a protective effect of calnexin was observed. Higher levels of HLA-A2 heavy chain were recovered in the soluble fraction compared with the IgG and buffer controls (Fig. 4A, left panel, compare lane 5 with lanes 7 and 9). The amount of HLA-A2 heavy chains recovered in the supernatant was highest in the presence of calnexin that had been preincubated at 47 °C for 1 h (Fig. 4A, left panel, compare lane 3 with lanes 5, 7, and 9). Fig. 4A (right panel) shows a quantification of HLA-A2 heavy chain recovery in the supernatant and pellet fractions (averaged over two independent experiments) following refolding under the indicated conditions. These analyses indicate that the chaperone activity of calnexin was not destroyed by the heat treatment, but was in fact enhanced.

To investigate whether HLA-A2 heavy chains that were rescued by calnexin were maintained in a folding-competent state, the assembly of heavy chains with peptides and β2m was assessed. For these analyses, we used a fluorescent HLA-A2-specific peptide (LLDC2PTAAV) and native gel-based fluoroimaging analyses for quantification of assembled HLA-A2β2m-LLDC2PTAAV complexes (5). The remaining supernatants recovered from the analyses shown in Fig. 4A were incubated overnight at 4 °C with excess LLDC2PTAAV and β2m, followed by native PAGE and fluoroimaging analyses to visualize HLA-A2β2m-LLDC2PTAAV complexes. Under these conditions, significantly higher HLA-A2-LLDC2PTAAV complex assembly was apparent under conditions in which calnexin or heat-treated calnexin was present during refolding, with heat-treated calnexin being more efficient (Fig. 4B, left panel). Fig. 4B (right panel) shows a quantification of the relative intensities of the fluorescent bands (averaged over two independent refolding analyses), with the signal obtained for HLA-A2 refolding with heat-treated calnexin set to 100%. Inhibition of HLA-A2 aggregation by calnexin (Fig. 4A, right panel) correlated with a subsequent increase in HLA-A2-peptide complex assembly (Fig. 4B, right panel), indicating that heavy chains rescued from precipitation by calnexin were being maintained in a folding-competent state. Untreated calnexin was ~2-fold less efficient than heat-treated calnexin, and heat-treated calnexin was at least 4-fold more efficient than the control protein IgG in maintaining a folding-competent form of the HLA-A2 heavy chain.

To examine whether calnexin could also enhance the refolding efficiency of a non-glycosylated form of HLA-A2, we used HLA-A2 heavy chains that were separately expressed in E. coli and purified as inclusion bodies using established procedures (8). HLA-A2 heavy chains in guanidine hydrochloride (in the absence of β2m) were incubated in refolding buffer alone (upper and lower panels, lanes 6 and 7) or in buffer containing β2m-LLDC2PTAAV, and assembly of HLA-A2β2m-LLDC2PTAAV complexes was analyzed by fluoroimaging of native gels. D, the fluorescence signals from lanes 1–5 in C were quantified and averaged over two independent experiments. The fluorescence signal observed with HLA-A2 samples that were refolded in the presence of heat-treated calnexin was arbitrarily set to 100% to allow a comparison of the relative refolding efficiencies of the other conditions.

However, as shown above in Fig. 4, heat-treated calnexin was also more efficient in inhibiting precipitation of non-glycosylated HLA-A2 heavy chains compared with untreated calnexin (Fig. 5, A, upper panel, compare lanes 2 and lane 4; and B, quantifications averaged over two independent experiments). Inhibition of HLA-A2 aggregation was also enhanced when refolding was undertaken in the presence of calnexin and EGTA (Fig. 5, A, lower panel, compare lanes 2 and 4; and B, quantifications averaged over two independent experiments).

To determine whether non-glycosylated HLA-A2 heavy chains rescued by calnexin, heat-treated calnexin, and calcium-depleted calnexin were competent in their ability to form heavy chainβ2m-peptide complexes, assembly assays were undertaken in the presence of β2m and peptide as described for Fig. 4. Significantly higher HLA-A2β2m-LLDC2PTAAV complex assembly was apparent under conditions in which heat-shocked or EGTA-treated calnexin was present during refolding (Fig. 5, C and D). Altogether, these observations suggest that calnexin is able to recognize substrates independent of oligosaccharide-mediated recognition and that this polypeptide-specific chaperone activity is enhanced by heat treatment and calcium depletion. Furthermore, the non-glycosylated substrates rescued by calnexin are maintained in a conformation that is competent for subsequent folding and assembly.

Calnexin Oligomerization and Polypeptide Binding in Cells—Calreticulin has been previously shown to oligomerize via the formation of disulfide-linked dimers, which served as a useful tool for observation of the induction of calreticulin dimers in cells (5, 14). We investigated
whether oligomerization of calnexin also proceeds through the formation of covalent calnexin dimers. There are four conserved cysteine residues in calnexin (positions 161, 195, 361, and 367; numbering based on the canine calnexin sequence), which form two internal disulfide bonds, Cys$^{161}$-Cys$^{195}$ and Cys$^{361}$-Cys$^{367}$ (15). Canine and human calnexins contain an additional cysteine at position 442. To further assess the formation of disulfide-linked dimeric calnexin structures, we analyzed heat-shocked calnexin (canine) by native PAGE or by reducing and nonreducing SDS-PAGE (Fig. 6A). Upon heat treatment, native PAGE revealed a ladder of calnexin species (Fig. 6A, lane 2), whereas ~65- and ~125-kDa species were the major species that were resolved by nonreducing SDS-PAGE (lane 4). The ~125-kDa species was not visualized by SDS-PAGE under reducing conditions (Fig. 6A, lane 6), indicating that it was a disulfide-linked dimer of calnexin. The high molecular mass calnexin species induced by heat shock accumulated as the dimer upon incubation with SDS (Fig. 6A, lanes 7–10). Although the species corresponding to the calnexin dimer was SDS-resistant, it converted to the monomer under reducing conditions (Fig. 6A, compare lanes 4 and 6). N-Ethylmaleimide-modified calnexin was significantly abrogated in its ability to form a dimer upon heat treatment (Fig. 6A, lane 12). However, the modified protein was still able to form higher order oligomers, although with slightly reduced efficiency compared with untreated calnexin. Thus, the presence of a free sulphydryl (Cys$^{442}$) group is important for stable calnexin dimer formation. However, higher molecular mass oligomers could assemble even in the absence of a disulfide-linked dimer. Consistent with this possibility, Cys$^{442}$ is not conserved and occurs as a cysteine only in human and canine calnexins.

Because oligomerization of canine calnexin in vitro involved a disulfide-linked dimeric intermediate, we could use dimerization as a probe for the induction of higher order calnexin structures in HeLa (human) cells under conditions that induced calnexin oligomerization and that enhanced polypeptide substrate binding in vitro. We also analyzed the extent of calnexin-class I complex formation in cells under these conditions.

HeLa cells were heat-shocked for 2 h at 42 or 45 °C (Fig. 6B) or treated with 5 μM thapsigargin for 20 h (Fig. 6C). Thapsigargin is a drug that depletes intracellular calcium stores by inhibiting ER calcium ion pumps. Lysates were prepared and separated by SDS-PAGE, and subsequent immunoblot analyses were performed with anti-calnexin antisera. In lysates from cells that were cultured at 37 °C, a single major band was visualized by immunoblot analyses with anti-calnexin antibody, corresponding to a 90-kDa species, consistent with a calnexin monomer. (The calnexin in HeLa cells contains transmembrane and cytosolic domains and migrates more slowly than soluble calnexin.) Upon heat shock of cells and analyses of lysates by nonreducing SDS-PAGE, a second band was induced, corresponding to a 180-kDa species (Fig. 6B, upper panel, lanes 5 and 6, bands indicated by the asterisk), which was not visualized under reducing conditions following any of the treatments (Fig. 6B, lanes 1–3), consistent with it being a disulfide-linked dimer. This species was also induced upon thapsigargin treatment of HeLa cells at 37 °C (Fig. 6C, upper panel, lane 4), but was not visualized under reducing conditions (Fig. 6C, upper panel, lane 2). The dimer was also not induced in murine fibroblast cells (K42 cells) (data not shown) (16). Because murine calnexin lacks the counterpart of Cys$^{442}$, the absence of induction of disulfide-linked calnexin dimers in murine cells is consistent with an involvement of Cys$^{442}$ in the formation of a disulfide-linked calnexin dimer. Additional higher molecular mass species were also visualized upon heat shock of HeLa cells (Fig. 6B, upper panel, lane 6, arrows), the identity of which is unknown, but which could correspond to disulfide-linked complexes of calnexin with cellular proteins.

In vitro, heat treatment of calnexin or calcium depletion enhanced the ability of calnexin to bind MHC class I heavy chains, coincident with the observed enhancement in calnexin dimers and higher order structures. We wanted to see whether the appearance of dimeric calnexin species in cells also coincided with an increase in substrate association, as was observed in vitro. Equal amounts of protein in the lysates were immunoprecipitated with anti-calnexin antisera, and calnexin was visualized by immunoblot analyses with antibody HC-10. The data are representative of at least two independent analyses. IgG HC, IgG heavy chain; IgG LC, IgG light chain.

**FIGURE 6.** Oligomerization of canine calnexin proceeds via the formation of a disulfide-linked dimeric intermediate, and disulfide-linked calnexin dimers are induced in HeLa cells upon heat shock and under calcium-depleting conditions. A, heat-treated (lanes 2, 4, and 6) or untreated (lanes 1, 3, and 5) calnexin (12 μM) was separated by native PAGE or nonreducing or reducing SDS-PAGE as indicated and visualized by Coomassie Blue staining. The calnexin dimer (*), calnexin monomer (○), and high molecular mass calnexin species (arrows) are indicated. Heat-treated calnexin was subjected to native PAGE analyses following addition of the indicated amounts of SDS (lanes 7–10). Untreated or N-ethylmaleimide (NEM)-modified calnexin (12 μM) was heat-treated prior to native PAGE (lanes 1 and 12). B and C, upper panels, lysates (50 μg) from HeLa cells that were maintained at 37 °C (B, lanes 1 and 4; and C, lanes 1 and 3), heat-shocked for 2 h at 42 °C (B, lanes 2 and 5) or at 45 °C (B, lanes 3 and 6), or incubated at 37 °C in the presence of 5 μM thapsigargin (thap) for 21 h (C, lanes 2 and 4) were analyzed by reducing and nonreducing SDS-PAGE as indicated, and calnexin was visualized by immunoblot analyses with anti-calnexin antisera. The calnexin dimer (*), calnexin monomer (○), and high molecular mass calnexin species (arrows) are indicated. Lower panels, proteins in lysates from HeLa cells that were maintained at 37 °C (B, lanes 1 and 4; and C, lanes 1 and 3), heat-shocked for 2 h at 42 °C (B, lanes 2 and 5) or at 45 °C (B, lanes 3 and 6), or treated with 5 μM thapsigargin for 24 h (C, lanes 2 and 4) were immunoprecipitated with anti-calnexin antisera. Immunoprecipitated samples (IP) or lysates as indicated were separated by 10% SDS-PAGE, and proteins were transferred to polyvinylidene difluoride membrane. Complexes of MHC class I heavy chains (Class I HC) and calnexin (cnex) were visualized by immunoblot analyses with antibody HC-10. The data are representative of at least two independent analyses. IgG HC, IgG heavy chain; IgG LC, IgG light chain.**
**Polypeptide Substrate Binding by Calnexin**

**DISCUSSION**

The precise way in which calnexin and calreticulin contribute to protein folding and quality control is a matter of considerable debate. On one hand, these chaperones are known lectins with a marked preference for binding to proteins containing monoglycosylated N-linked glycans that are components of a glycan-based quality control system that specifically monitors the folding of glycoproteins (27). On the other hand, it has been shown that calnexin and calreticulin bind non-glycosylated proteins in vitro via protein-protein interactions and can inhibit their

---

**FIGURE 7. Oligosaccharide depletion and viral protein expression enhance calnexin oligomerization.** A, upper panel, immunoblot analyses with anti-calnexin antibody were performed as described in the legend to Fig. 6 (B and C) following SDS-PAGE under reducing or nonreducing conditions. Lysates were obtained from HeLa cells that were cultured at 37 °C without (lanes 1 and 4) or with 10 μg/ml tunicamycin (TUN) for 24 h (lanes 2 and 5) or 48 h (lanes 3 and 6). Lower panel, shown are the results from immunoblot analyses with antibody HC-10 of lysates or from anti-calnexin immunoprecipitations (cnx IP) with HeLa cells that were cultured at 37 °C without (lanes 1 and 4) or with tunicamycin for 24 h (lanes 2 and 5) or 48 h (lanes 3 and 6). The deglycosylated class I species that appeared upon treatment with tunicamycin is indicated (*). IgG LC, IgG heavy chain; class IHC, class I heavy chain; IgG LC, IgG light chain; B, lysates from FCA4 cells (lanes 1 and 4) and Huh7 cells (lanes 2 and 5) cultured at 37 °C and Huh7 cells heat-shocked (HS) at 45 °C for 2 h (lanes 3 and 6) were separated by SDS-PAGE under reducing (lanes 1–3) and nonreducing (lanes 4–6) conditions and analyzed by immunoblotting with anti-calnexin antibody (10C5). B, upper panel, lanes 1–3, compare untreated cells (lanes 1–3) or with 10 μM Glc1–3Man (M1), Glc1–3Manα1–2Manα1–2Man (M3), or buffer alone (∗) for 1 h prior to heat treatment at the indicated temperatures. Proteins were then visualized by native PAGE. The data are representative of at least two independent analyses.

Tunicamycin Treatment Results in Accumulation of Calnexin Oligomerization Intermediates and Enhanced Calnexin Binding to Deglycosylated MHC Class I Heavy Chains—Tunicamycin is a compound that inhibits glycosylation of newly synthesized proteins in the ER. Tunicamycin treatment of cells is generally observed to reduce calnexin binding to various cellular and viral substrates (for example, see Refs. 1, 17, and 18). Tunicamycin treatment also generally impairs glycoprotein folding, enhances accumulation of misfolded glycoproteins in the ER, and is routinely used for induction of ER stress. (Some recent examples of the use of tunicamycin for induction of ER stress include Refs. 19–21.) We examined calnexin conformation in cells that were treated with tunicamycin. Tunicamycin treatment enhanced calnexin dimer formation, with greater levels of dimers accumulating at 48 h compared with 24 h (Fig. 7A, lower panel, lanes 2 and 3, asterisks). In cells that were treated with tunicamycin for 48 h, enhanced deglycosylated class I heavy chain binding to calnexin could be visualized, coincident with the increase in calnexin dimer formation (Fig. 7A, lower panel, lane 6 with lane 4). The glycosylated class I species that persisted at 48 h likely corresponds to a class I species that has trafficked outside of the ER prior to the start of the tunicamycin treatment and is therefore not accessible for calnexin binding. Previous findings that calnexin-substrate interactions in cells are largely oligosaccharide-dependent (1, 17, and 18) were based upon experiments undertaken in cells that were subjected to a short pulse of tunicamycin. Our data indicate that tunicamycin treatment induced a slow conversion of calnexin from a monomeric to a dimeric form (and possibly higher order structures). The appearance of calnexin dimers coincided with enhanced visualization of complex formation between calnexin and a deglycosylated MHC class I substrate (Fig. 7A).

Calnexin Oligomers Are Present under Conditions of ER Stress Induced by Viral Protein Expression—Cellular ER stress can also be induced by viral infection (22–24). RNA replication of hepatitis C virus (HCV) subgenomic replicons has been shown to be permissive in the human hepatoma cell line Huh7 (25). FCA4 cells (derived from the parental Huh7 cell line) stably express all the nonstructural genes and support both the replication and translation of HCV subgenomic replicons (11). The replication activities of the HCV subgenomic replicon were shown to induce ER stress (22). We examined the oligomerization state of calnexin in FCA4 cells. Under nonreducing conditions, calnexin dimers were apparent in FCA4 cells, but were almost completely absent in Huh7 cells that were cultured at 37 °C (Fig. 7B, compare lanes 4 and 5). Huh7 cells were heat-shocked to mark the positions of calnexin dimers following separation by nonreducing SDS-PAGE (Fig. 7B, lane 6). It is noteworthy that oligomerization of calreticulin was also enhanced in FCA4 cells (data not shown). These data indicate that physiologically relevant events such as virus-induced ER stress influence calnexin conformation in cells.

Oligosaccharide Substrates Themselves Influence the Structure of Calnexin—Heat treatment and calcium depletion could trigger calnexin dimer formation via destabilization and partial unfolding of monomeric calnexin structures. HCV replicons express all of the HCV nonstructural proteins, and a few of these proteins are capable of inducing ER stress and activating NF-κB by an ER overload stress response, which involves alteration of Ca2⁺ homeostasis and elevation of reactive oxygen species in the mitochondria (26). It is possible that fluxes in ER calcium stores in these virally infected cells directly affect the structural and functional state of calnexin. But what mechanisms trigger tunicamycin-induced dimers? The extent of calnexin oligomerization in vitro was strongly influenced by the protein concentrations (data not shown). Chaperone up-regulation by tunicamycin could influence cellular concentrations of dimers. However, only small differences in calnexin levels, if any, were detectable in tunicamycin-treated cells compared with untreated cells (Fig. 7A, upper panel, lanes 1–3). We examined the possibility that, in cells, the presence of oligosaccharide substrates themselves could stabilize calnexin into an “oligosaccharide-bound” compact conformation. Removal of these substrates could trigger destabilization of calnexin monomers, causing partial unfolding and a slow accumulation of dimers and higher order oligomers. Native PAGE was used to monitor formation of calnexin oligomers in the presence of 200 μM Glc1–3Man, an oligosaccharide that binds poorly to calnexin, or 200 μM Glc1–3Manα1–2Manα1–2Man, an oligosaccharide that binds with higher affinity (9). Preincubation of calnexin with Glc1–3Manα1–2Manα1–2Man prior to heat shock at 45 °C reduced the appearance of oligomeric calnexin, whereas preincubation with Glc1–3Man only slightly reduced calnexin oligomerization compared with calnexin that was heat-shocked in the absence of oligosaccharide (Fig. 7C, compare lanes 6 and 7). When calnexin was incubated with Glc1–3Manα1–2Manα1–2Man at 40 °C, the dimeric form of calnexin re-equilibrated to monomers (Fig. 7C, lane 4). This effect was not observed in the presence of Glc1–3Man (Fig. 7C, lane 3). This result suggests that stabilization of calnexin into a monomeric structure is enhanced in the presence of ligands specific to the calnexin lectin domain.
aggregation (3, 4). There is also evidence for direct binding of calnexin to polypeptide substrates in cells (28).

Here, we have shown that the functional properties of calnexin are not fully defined by the assumption of a single stable "oligosaccharide-binding" conformation. Rather, calnexin appears to exist in multiple conformational states (Fig. 1), the extent of each being dependent upon the environmental conditions. The conformational states of calnexin determine its functional properties. Untreated calnexin (calnexin that was neither heat-treated nor calcium-depleted) had lower chaperone activity toward polypeptide substrates compared with heat- and EGTA-treated calnexins (Figs. 4 and 5), and polypeptide-binding activity was reduced with untreated calnexin (Fig. 2). In contrast to these observations, calcium depletion has been previously shown to significantly reduce the ability of calnexin to bind a substrate containing a monoglycosylated oligosaccharide (9). Thus, polypeptide and oligosaccharide recognition by calnexin appears to involve distinct conformations of the calnexin protein. Because calcium depletion enhances calnexin susceptibility to proteolytic digestion (13), calcium depletion or heat treatment could enhance polypeptide binding by calnexin by inducing partial unfolding of the lectin domain. On the other hand, oligosaccharide binding appears to be favored by conditions that enhance structural stability (folding) of the monomeric lectin domain (15). Additionally, oligosaccharide substrates themselves appear to be important for maintaining the structural stability of calnexin (Fig. 7).

The experiments described here for calnexin parallel those we have recently described for calreticulin (5). Both chaperones require a partial unfolding of their lectin domains to expose hydrophobic surfaces that are capable of interactions with substrate proteins. The partial unfolding events that expose the polypeptide-binding site also expose surfaces capable of self-association into dimers and higher order oligomeric structures. By analogy to calreticulin (5), we believe that dimer/oligomer formation per se is not essential for calnexin polypeptide-binding activity. Calnexin can effectively suppress heat-induced aggregation of nonglycosylated substrates at low concentrations (2 μM), conditions under which no significant dimerization/oligomerization was observable by gel filtration chromatography (4). The partial unfolding events that expose the calnexin polypeptide-binding site also expose surfaces capable of self-association into dimers and higher order oligomeric structures. By analogy to calreticulin (5), we believe that dimer/oligomer formation per se is not essential for calnexin polypeptide-binding activity. Calnexin can effectively suppress heat-induced aggregation of nonglycosylated substrates at low concentrations (2 μM), conditions under which no significant dimerization/oligomerization was observable by gel filtration chromatography (4). The partial unfolding events that expose the calnexin polypeptide-binding site also expose surfaces capable of self-association into dimers and higher order oligomeric structures. By analogy to calreticulin (5), we believe that dimer/oligomer formation per se is not essential for calnexin polypeptide-binding activity. Calnexin can effectively suppress heat-induced aggregation of nonglycosylated substrates at low concentrations (2 μM), conditions under which no significant dimerization/oligomerization was observable by gel filtration chromatography (4). The partial unfolding events that expose the calnexin polypeptide-binding site also expose surfaces capable of self-association into dimers and higher order oligomeric structures. By analogy to calreticulin (5), we believe that dimer/oligomer formation per se is not essential for calnexin polypeptide-binding activity. Calnexin can effectively suppress heat-induced aggregation of nonglycosylated substrates at low concentrations (2 μM), conditions under which no significant dimerization/oligomerization was observable by gel filtration chromatography (4).

FIGURE 8. Calnexin conformations and their substrate recognition characteristics. Oligosaccharide binding by calnexin is favored by conditions that enhance the structural stability (folding) of calnexin monomers. Oligosaccharide substrates themselves stabilize calnexin in a compact monomeric conformation (A). Polypeptide binding is favored by conditions that induce partial unfolding of calnexin monomers (B). Oligosaccharide dissociation could trigger transient partial unfolding of calnexin monomers (B), exposing a polypeptide-binding site that can directly bind to peptide components of the substrate via hydrophobic protein-protein interactions (B). Oligosaccharide rebinding can result in re-equilibration to A. Partially unfolded calnexin monomers can assemble into dimers (C) and higher order calnexin oligomers (D). These conformations are favored by high concentrations of calnexin and conditions that are destabilizing for monomers. These conformations can also function in the direct recognition of polypeptide components of substrates, inhibiting protein aggregation and promoting folding, in particular under ER stress conditions.

37 °C under normal physiological conditions, it is also possible that the calnexin polypeptide-binding conformation is transiently induced during a calnexin cycle. Based upon the results shown in Fig. 7, the binding of substrates containing monoglucosylated oligosaccharides would be expected to stabilize calnexin in its monomeric conformation (indicated by the structure shown in Fig. 8A). However, dissociation of a bound oligosaccharide substrate could cause local unfolding events in calnexin, transiently inducing a conformation in which the polypeptide-binding site of calnexin is exposed, allowing calnexin to function as a folding sensor for the same substrate (indicated by the structure shown in Fig. 8B). Dissociation of the polypeptide component of the substrate would allow calnexin to bind the oligosaccharide component of the same or a different substrate, which in turn would drive the conformation back to that indicated in Fig. 8A. Thus, the calnexin cycle may involve serial binding mechanisms through oligosaccharide- and polypeptide-based interactions that require distinct conformations of the calnexin protein (Fig. 8, A and B).

Prolonged ER stress such as heat shock or calcium or oligosaccharide depletion causes the accumulation of dimers and higher order calnexin structures (Fig. 8, C and D), which appear to be functional for polypeptide substrate binding and inhibition of polypeptide substrate aggregation. By undergoing a specific mode of self-association (oligomerization), calnexin must inhibit its own nonproductive aggregation. What consequences could result from an enhancement in polypeptide substrate binding by calnexin under conditions of ER stress? When the ER is overwhelmed, the stress-induced forms of calnexin may assist by preventing aggregation of substrates independent of the enzyme network required to maintain substrate monoglucosylation in cells. Enhanced binding could translate to enhanced suppression of aggregation and maintenance of substrates in a folding-competent state, as observed in vitro (Figs. 2, 4, and 5). Terminally misfolded proteins that become calnexin-associated could also be targeted for degradation via an ER-associated degradation pathway.

Acknowledgments—We thank Dr. David Williams for the calnexin construct, Dr. Peter Snow for the HLA-A2 baculovirus, Dr. David Garboczi for the E. coli construct expressing HLA-A2, Dr. Pamela Bjorkman for the E. coli constructs expressing HLA-E and B2m, and Dr. C. Seeger for the Huh7 and FCA4 cell lines. We are grateful to Drs. Colin Duckett, Tom Kerppola, Alice Telesnitsky, Mike Imperiale, and Xiaohui Xu for generously allowing equipment usage. We thank the University of Michigan Biomedical Research Core Facilities for peptide syntheses and purifications and the University of Michigan Hybridoma Core for HC10 ascites production.
REFERENCES

1. Hammond, C., Braakman, I., and Helenius, A. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 913–917
2. Ellgaard, L., Molinari, M., and Helenius, A. (1999) Science 286, 1882–1888
3. Saito, Y., Ihara, Y., Leach, M. R., Cohen-Doyle, M. F., and Williams, D. B. (1999) EMBO J. 18, 6718–6729
4. Ellgaard, L., Molinari, M., and Helenius, A. (1999) Mol. Cell 4, 331–341
5. Rizvi, S. M., Mancino, L., Thammavongsa, V., Cantley, R. L., and Raghavan, M. (2004) Mol. Cell 15, 913–923
6. Lapinski, P. E., Neubig, R. R., and Raghavan, M. (2001) J. Biol. Chem. 276, 7526–7533
7. Mancino, L., Rizvi, S. M., Lapinski, P. E., and Raghavan, M. (2002) Proc. Natl. Acad. Sci. U. S. A. 99, 5931–5936
8. Garboczi, D. N., Utz, U., Ghosh, P., Seth, A., Kim, J., VanTienhoven, E. A., Biddison, W. E., and Wiley, D. C. (1996) J. Immunol. 157, 5403–5410
9. Vassilakos, A., Michalak, M., Lehrman, M. A., and Williams, D. B. (1998) Biochemistry 37, 3480–3490
10. Leach, M. R., Cohen-Doyle, M. F., Thomas, D. Y., and Williams, D. B. (2002) J. Biol. Chem. 277, 29686–29697
11. Ou, W. J., Bergeron, J. J., Li, Y., Kang, C. Y., and Thomas, D. Y. (1995) J. Biol. Chem. 270, 18051–18059
12. Jorgensen, C. S., Ryder, L. R., Steino, A., Hoijrup, P., Hansen, J., Beyer, N. H., Heegaard, N. H., and Houen, G. (2003) Eur. J. Biochem. 270, 4140–4148
13. Schrag, J. D., Bergeron, J. J., Li, Y., Borisova, S., Hahn, M., Thomas, D. Y., and Cygler, M. (2001) Mol. Cell 8, 633–644
14. Miyawaki, A., Llopis, J., Heim, R., McCaffery, J. M., Adams, J. A., Ikura, M., and Tsien, R. Y. (1997) Nature 388, 882–887
