Relationship between Calnexin and BiP in Suppressing Aggregation and Promoting Refolding of Protein and Glycoprotein Substrates*

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Calnexin (CNX) is a membrane protein of the endoplasmic reticulum (ER) that has been defined primarily as a lectin, yet it is capable of functioning as a molecular chaperone with non-glycosylated proteins in vitro. Here, we assess the relative contributions of the oligosaccharide- and polypeptide-binding sites of CNX to its in vitro chaperone functions by comparing it with the Hsp70 chaperone of the endoplasmic reticulum, BiP. Both proteins were equally effective in preventing the aggregation of non-glycosylated citrate synthase, indicating that the polypeptide-binding site of CNX is capable of functioning at a level similar to that of Hsp70. However, when confronted with glycoprotein substrates, the lectin site of CNX provided a significant advantage over BiP in suppressing aggregation. CNX also cooperated with BiP and the J domain of Sec63p in the ATP-dependent refolding of glycoprotein and non-glycosylated substrates. The lectin site of CNX was essential for refolding of the glycoprotein. These findings reinforce the function of CNX as a bona fide chaperone and illustrate how its lectin site confers advantages relative to other chaperones when confronted with glycoprotein substrates.

Calnexin (CNX) is a membrane protein of the endoplasmic reticulum (ER) that interacts transiently with newly synthesized Asn-linked glycoproteins. This preference for glycoproteins is due to the fact that CNX is a lectin that binds to monoglucosylated oligosaccharides of the form Glc₃Man₉GlcNAc₂ (1, 2). These oligosaccharides are formed transiently in the ER following attachment of the precursor oligosaccharide, Glc₃Man₃GlcNAc₂, to nascent polypeptide chains. In vivo studies have shown that CNX binds primarily to folding intermediates rather than to native conformers (3–5), and it can enhance the efficiency of glycoprotein folding and subunit assembly (6–9). Furthermore, CNX plays a role in the ER quality control system, preventing the export of incompletely folded or misfolded glycoproteins (10, 11). Based on these observations, it is generally thought that CNX functions as a molecular chaperone for Asn-linked glycoproteins.

How CNX effects its molecular chaperone and quality control functions is the subject of considerable debate. In one view, CNX functions solely as a lectin, binding only to glycoproteins bearing Glc₃Man₉GlcNAc₂ oligosaccharides. Dissociation of CNX from the glycan is caused by the action of glucosidase II, which removes the terminal glucose residue of the oligosaccharide. Rebinding of CNX occurs upon re-addition of the glucose, an action carried out by UDP-Glc:glycoprotein glucosyltransferase (1, 12). In this model, CNX does not function as a classical molecular chaperone that prevents the aggregation of folding intermediates by binding to exposed hydrophobic polypeptide segments. Instead, CNX is thought to retain glycoprotein folding intermediates and act as a scaffold for the binding of other ER chaperones and folding catalysts. For example, the thiol oxidoreductase Erp57 binds to CNX, and as shown in vitro, this interaction promotes disulfide formation/isomerization in glycoproteins associated with CNX (13, 14).

An alternative view is that CNX does indeed function as a molecular chaperone by virtue of a polypeptide-binding site that it possesses in addition to its lectin site (2, 15). This dual binding model is supported by a variety of in vivo studies in which CNX has been shown to associate with proteins that lack the appropriate monoglucosylated oligosaccharides (11, 16–23). Furthermore, we have demonstrated that a soluble form of CNX, consisting of its entire ER luminal domain (S-CNXX), is capable of binding to unfolded but not native forms of non-glycosylated proteins in vitro and that this interaction potently suppresses the thermally induced aggregation of the unfolded conformers (15). The aggregation-suppressing function of S-CNXX is enhanced in the presence of ATP and is attenuated when its lectin site is occupied by a synthetic monoglucosylated oligosaccharide. Consequently, we proposed that S-CNXX possesses a polypeptide-binding site that recognizes some feature of non-native proteins and that it is differentially regulated by ATP and monoglucosylated oligosaccharides. In addition, S-CNXX was found to collaborate with ATP-dependent factors in rabbit reticulocyte lysate (RRL) to promote the refolding of thermally inactivated citrate synthase (CS), a non-glycosylated protein. However, the active components of RRL that collaborate with S-CNXX were not identified, and no corresponding ER factors were identified that might substitute for the active components of RRL. Collectively, these results strongly support the view that CNX functions as a molecular chaperone in vitro in addition to its lectin functions. That CNX probably engages in similar functions in vivo has recently been suggested by the finding that when the formation of monoglucosylated oligosaccharides is completely blocked, CNX is still capable of specific associations via polypeptide-based interactions with a large
variety of newly synthesized proteins within the ER (24).

The in vitro studies on the molecular chaperone functions of CNX have raised a number of questions. How do these functions compare with those of other “classical” molecular chaperones such as those of the Hsp70 family? Does the existence of both lectin and polypeptide-binding sites confer an advantage to CNX over other molecular chaperones that bind solely through polypeptide-based interactions, e.g. either by increasing the avidity of CNX for glycoprotein substrates or by increasing the number of potential substrates that are able to bind to CNX? Finally, is it possible to identify other molecular chaperones of the ER that are capable of replacing RRL and collaborating with CNX in the refolding of denatured proteins?

To address these issues, we examined the in vitro chaperone properties of the ER Hsp70 protein, BiP, and compared them with those of S-CNX. Remarkably, despite the availability of purified preparations of BiP for the past decade, there has been a paucity of studies focused on its chaperone functions in vitro. Only very recently has it been shown that BiP can act synergistically with protein disulfide isomerase in the oxidative folding of an antibody Fab fragment (25). However, its ability to cooperatively with protein disulfide isomerase in the oxidative folding of nascent polypeptides within the lumen of the ER.

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The Lectin Site of S-CNX Confers Enhanced Potency Relative to BiP in Suppressing the Aggregation of Glycoproteins Containing Glc3-Man9GlcNAc2 Oligosaccharides—The dual binding model for the interaction of CNX with glycoproteins predicts that engagement of both the lectin and polypeptide-binding sites of CNX should increase its binding avidity for monoglycosylated glycoproteins relative to binding through either site alone (15). This may confer an advantage over other molecular chaperones that bind to glycoproteins solely through polypeptide-based interactions. To test this hypothesis, the relative abilities of S-CNX and BiP to prevent the aggregation of denatured glycoproteins were compared. In these experiments, jack bean α-mannosidase and chicken IgY were used as substrates since they contain the monoglycosylated oligosaccharides recognized by the lectin site of S-CNX. α-Mannosidase is a heterodimer of 44- and 64-kDa subunits and contains at least one oligosaccharide of the form Glc3-Man9GlcNAc2 on its 64-kDa subunit (32). The oligosaccharides of the IgY heavy chain are more heterogeneous, with 27.1% being monoglycosylated (Glc3-Man9GlcNAc2) (33).

As shown in Fig. 2A, when α-mannosidase was denatured in 6 M GdnHCl and then diluted out of the denaturant, it aggregated rapidly at room temperature. S-CNX was capable of preventing the aggregation of α-mannosidase, with essentially complete suppression occurring at an ~2-fold molar excess of S-CNX over α-mannosidase dimer (Fig. 2A, upper panel). By comparison, a 32-fold molar excess of BiP was needed to suppress the aggregation of α-mannosidase to the same extent (Fig. 2A, lower panel). Thus, S-CNX is ~16 times more effective than BiP in suppressing the aggregation of this glycoprotein substrate.

A similar but less dramatic difference between S-CNX and BiP was observed with the IgY substrate. When reduced and chemically denatured IgY was diluted rapidly into solutions containing various concentrations of S-CNX or BiP and heated to 45 °C, S-CNX was consistently 2–4-fold more potent than BiP in suppressing IgY aggregation (Fig. 2B). For example, whereas a stoichiometric concentration of S-CNX was capable of suppressing IgY aggregation completely, a 4-fold excess of BiP was required to achieve the same effect. Therefore, when presented with monoglycosylated glycoprotein substrates, S-CNX appears to have a significant advantage over a molecular chaperone that is restricted solely to polypeptide-based interactions.

To confirm that the lectin-carbohydrate interaction is responsible for the enhanced potency of S-CNX over BiP in preventing aggregation, we repeated the experiments under identical conditions, except that deglycosylated α-mannosidase and IgY were used instead of the glycosylated proteins. Glycans were removed by treatment with Endo H, and complete digestion was verified by the increased mobility of the treated proteins following SDS-polyacrylamide gel electrophoresis analysis and by their loss of reactivity when blotted with concanavalin A (data not shown). Deglycosylation of α-mannosidase profoundly impaired the ability of S-CNX to suppress aggregation (Fig. 3A, upper panel). A 32-fold molar excess of S-CNX suppressed the aggregation of deglycosylated α-mannosidase by ~50%, whereas a 1:2 molar ratio (S-CNX/α-mannosidase) was able to sustain this level of aggregation suppression for the glycosylated form (compare Figs. 2A and 3A, upper panels). The reduced efficacy of S-CNX was unlikely to be a consequence of deglycosylation creating a much more aggregation-prone substrate since BiP was still capable of completely suppressing the aggregation of deglycosylated α-mannosidase at a 32-fold excess, the same molar ratio as with glycosylated α-mannosidase (Fig. 3A, lower panel).

When a similar experiment was performed with deglycosylated IgY (Fig. 3B), S-CNX again became less effective than BiP. A 4-fold molar excess of...
S-CNX suppressed aggregation to the same extent as equimolar concentrations of BiP. This contrasts with the 2–4-fold greater potency that S-CNX possessed over BiP with glycosylated IgY (compare Figs. 2B and 3B). Collectively, these experiments suggest that the greater ability of S-CNX over BiP to suppress the aggregation of denatured monoglucosylated glycoproteins.

**Fig. 2.** S-CNX is more effective than BiP in suppressing the aggregation of monoglucosylated glycoproteins. A, after denaturation in 6 M GdnHCl, α-mannosidase (Man) was diluted to a final concentration of 0.3 μM at room temperature in the presence of the indicated concentrations of S-CNX, BiP, or IgG. Aggregation was measured over a period of 5 min. B, IgY was denatured in 6 M GdnHCl and 40 mM dithiothreitol, and aggregation was initiated by dilution to a final concentration of 0.67 μM in the presence of the indicated concentrations of S-CNX, BiP, or IgG at 45 °C. Aggregation was measured over a period of 60 min.

**Fig. 3.** S-CNX is less effective than BiP in suppressing the aggregation of deglycosylated glycoproteins. α-Mannosidase (A) and IgY (B) were treated with Endo H to remove monoglucosylated N-glycans. The deglycosylated proteins were chemically denatured, and their aggregation was measured in the presence of S-CNX, BiP, or IgG as described in the legend to Fig. 2. dg-Man, deglycosylated α-mannosidase; dg-IgY, deglycosylated IgY.
Functional Relationship between Calnexin and BiP

The Aggregation-suppressing Functions of S-CNX and BiP Are Regulated Differently by ATP and Monoglucosylated Oligosaccharides—Previously, we demonstrated that the ability of S-CNX to suppress the aggregation of non-glycosylated proteins is enhanced by the addition of ATP and attenuated when its lectin site is engaged with the tetrasaccharide Galα1–3Manα1–2Manα1–2Man (G1M3) (15). The addition of other nucleotides or non-glycosylated oligosaccharides has no effect. However, no ATPase activity could be detected in highly purified preparations of S-CNX (15). In contrast, Hsp70 family members, stimulated by their Hsp40 co-chaperones, hydrolyze ATP in a cycle that regulates their binding to and release from unfolded proteins (34). Interestingly, different Hsp70 proteins seem to vary with respect to the effect that ATP has on their abilities to suppress protein aggregation (35–38). Consequently, to characterize further the functional differences between S-CNX and BiP, we compared the effects of ATP and G1M3 on their abilities to suppress the thermal aggregation of citrate synthase.

Consistent with our previous findings, Fig. 4 demonstrates that the presence of 3.0 mM ATP significantly enhanced the ability of S-CNX to suppress the aggregation of CS, whereas the addition of 40 μM G1M3 attenuated this function. In contrast, the presence of ATP had no significant effect on the ability of BiP to suppress CS aggregation. Even in the presence of the J domain of Sec63p, no effect on the aggregation of CS by BiP was observed. This result was not due to the inability of purified BiP to hydrolyze ATP since the BiP preparation possessed ATPase activity that was stimulated 2–4-fold by the addition of a 2–4-fold molar excess of purified J domain (data not shown). Not surprisingly, the G1M3 oligosaccharide did not affect aggregation suppression by BiP since this chaperone lacks a binding site for monoglucosylated oligosaccharides. Thus, BiP and S-CNX differ substantially in the manner by which ATP and monoglucosylated oligosaccharides affect their abilities to suppress the aggregation of unfolded proteins.

S-CNX, BiP, and the J Domain of Sec63p Cooperate in the Refolding of Citrate Synthase—Previous experiments in our laboratory demonstrated that S-CNX is capable of maintaining heat-inactivated CS in a folding-competent state and acts in conjunction with additional ATP-dependent chaperones in RRL to refold the denatured substrate (15). However, the individual factors in RRL participating in the refolding of CS were not identified, and it was unclear whether ER chaperones could replace RRL in cooperating with S-CNX. To address these issues, CS was incubated at 43 °C for 60 min in the presence of equimolar concentrations of either S-CNX or mouse IgG. Under these conditions ~50% of the initial CS activity was lost. The temperature was then reduced to 23 °C to allow renaturation to occur either alone or in the presence of various additional chaperone systems. When CS was thermally inactivated in the presence of IgG, only a minimal recovery of activity was observed, even when RRL was present in the refolding mixture (Fig. 5, upper panel). This indicates that once denatured, chaperones present in RRL are unable to refold CS. In contrast, when S-CNX was present during inactivation, ~40% of the lost CS activity could be recovered in the presence of RRL and ATP, similar to our previous results (15). S-CNX present on its own during inactivation and reactivation failed to promote refolding, indicating that a cooperation with other chaperone systems is required. Furthermore, as described previously, the critical role of S-CNX is during the thermal inactivation stage since its inclusion along with RRL only during reactivation failed to enhance productive refolding (data not shown).

We then investigated whether various ER chaperone systems could replace RRL during the refolding stage. The addition of canine Grp94, the Hsp90 family member within the ER, along with ATP was unable to substitute for RRL (Fig. 5, upper panel). However, when RRL was replaced with purified yeast BiP and the Sec63p J domain, 30% of the lost CS activity could be recovered (Fig. 5, middle panel). This degree of reactivation was dependent on both ATP and the J domain since no reactivation was observed in the absence of ATP, and reactivation reached only 18% when the J domain was omitted. The J domain alone or in combination with ATP had no effect (data not shown). The dependence of BiP function on both the Sec63p J domain and ATP is consistent with many other studies on refolding of substrates by Hsp70 and Hsp40 proteins, wherein the J domain of Hsp40 promotes binding and release cycles of Hsp70 through stimulation of its ATPase activity (34). Since the addition of the G1M3 oligosaccharide diminished the ability of S-CNX to prevent the aggregation of thermally denatured CS, we tested the effect of the presence of G1M3 during thermal inactivation of CS or during BiP/Sec63p J domain-mediated reactivation. The addition of G1M3 to S-CNX during thermal inactivation reduced the level of CS reactivation by ~50% (Fig. 5, lower panel), whereas there was no inhibitory effect observed when it was included in the refolding mixture only (middle panel).

Collectively, these results indicate that S-CNX and the BiP/Sec63p chaperone systems can cooperate in the refolding of a non-glycosylated protein. Furthermore, the role of S-CNX in
this process is to maintain CS in a folding-competent state during thermal inactivation, whereas BiP and the J domain participate directly in refolding.

S-CNX Cooperates with Other Chaperones in Refolding Glycosylated α-Mannosidase, an Effect Dependent on Its Lectin Site—Since the lectin site of S-CNX participated significantly in its ability to suppress the aggregation of monoglucosylated glycoproteins (Fig. 2, A and B), it was of considerable interest to determine if lectin-carbohydrate interactions are also involved when S-CNX is included during refolding of a glycoprotein substrate. Initially, we examined whether S-CNX was capable of participating in the reactivation of chemically denatured α-mannosidase in conjunction with RRL. α-Mannosidase was denatured with 6 M GdnHCl and then diluted out of the denaturant into a reactivation mixture containing S-CNX or mouse IgG, RRL, and ATP. Fig. 6 (upper panel) shows that >30% of the α-mannosidase activity was recovered upon incubation with a mixture containing S-CNX, RRL, and ATP. Removal of any one of these three components resulted in minimal recovery of activity. Therefore, neither S-CNX nor RRL was capable of reactivating α-mannosidase on its own; rather cooperation between the two chaperone systems was required. To determine whether the participation of the lectin site of S-CNX was involved in its interaction with the glycoprotein, we repeated the experiment with α-mannosidase that had been deglycosylated with Endo H. The complete system of S-CNX, RRL, and ATP failed to recover any activity, consistent with a role for the lectin site of S-CNX in this process. As an alternative approach, glycosylated α-mannosidase was chemically denatured, and the G1M3 oligosaccharide was added to the reactivation mixture. Although G1M3 partially attenuates the polypeptide-based binding of S-CNX as observed with CS (see Fig. 4, upper panel; and Fig. 5, lower panel), it will also compete for lectin-carbohydrate interactions when a monoglucosylated substrate such as α-mannosidase is used. Indeed, the presence of G1M3 completely abolished the reactivation of α-mannosidase (Fig. 6, upper panel), in contrast to the partial inhibition of CS refolding that was observed when G1M3 attenuated polypeptide binding alone (Fig. 5, lower panel). This result is consistent with the view that the lectin site of S-CNX is crucial for its ability to participate in the refolding of a glycoprotein substrate.

In the preceding experiment, we were unable to replace RRL with BiP and the Sec63p J domain (data not shown). Consequently, to investigate the involvement of the lectin site of S-CNX in glycoprotein folding using entirely defined components, we subjected α-mannosidase to a thermal inactivation-reactivation protocol similar to that employed for CS reactivation (Fig. 5). α-Mannosidase was heated at 45 °C for 90 min in the presence of S-CNX or mouse IgG, and ATP. Removal of any one of these three components resulted in minimal recovery of activity. Therefore, neither S-CNX nor RRL was capable of reactivating α-mannosidase on its own; rather cooperation between the two chaperone systems was required. To determine whether the participation of the lectin site of S-CNX was involved in its interaction with the glycoprotein, we repeated the experiment with α-mannosidase that had been deglycosylated with Endo H. The complete system of S-CNX, RRL, and ATP failed to recover any activity, consistent with a role for the lectin site of S-CNX in this process. As an alternative approach, glycosylated α-mannosidase was chemically denatured, and the G1M3 oligosaccharide was added to the reactivation mixture. Although G1M3 partially attenuates the polypeptide-based binding of S-CNX as observed with CS (see Fig. 4, upper panel; and Fig. 5, lower panel), it will also compete for lectin-carbohydrate interactions when a monoglucosylated substrate such as α-mannosidase is used. Indeed, the presence of G1M3 completely abolished the reactivation of α-mannosidase (Fig. 6, upper panel), in contrast to the partial inhibition of CS refolding that was observed when G1M3 attenuated polypeptide binding alone (Fig. 5, lower panel). This result is consistent with the view that the lectin site of S-CNX is crucial for its ability to participate in the refolding of a glycoprotein substrate.
one systems. The addition of the G1M3 tetrasaccharide during thermal inactivation in the presence of S-CNX resulted in a complete block of α-mannosidase reactivation. This result confirms the participation of the lectin site of S-CNX in reactivation of a denatured glycoprotein substrate by defined ER chaperone systems.

**DISCUSSION**

Although the functions of CNX as a lectin and as a factor involved in protein folding and quality control in the ER are widely accepted, its proposed role as a classical molecular chaperone with non-native substrates through polypeptide-based interactions is controversial (15, 39–41). In this study, we compared the ability of S-CNX to suppress the aggregation and to promote the folding of non-native substrates in vitro with that of a bona fide molecular chaperone of the ER, the Hsp70 family member BiP. BiP has been shown to recognize non-native folding intermediates through a binding site that can accommodate 9-mer peptides with large hydrophobic residues at alternate positions (42). However, despite intensive analysis of the functions of other Hsp70 family members, the ability of BiP to suppress protein aggregation in vitro has not been examined previously. Our experiments show that, like other Hsp70 proteins, BiP potently suppresses the thermally induced aggregation of a non-glycosylated substrate (citrate synthase) when present in stoichiometric amounts. Most importantly, S-CNX was found to be just as effective as BiP in preventing the aggregation of citrate synthase. This result supports the notion that S-CNX functions as a true molecular chaperone since its ability to bind to polypeptide segments of a non-native protein can be just as potent as that of a member of the well-characterized Hsp70 chaperone family.

We also compared the regulatory effects of ATP on the aggregation-suppressing functions of BiP and S-CNX. "Hsp70 proteins cycle between an ATP-bound state that has low affinity for unfolded polypeptide and a high affinity ADP-bound state. Conversion between these states is regulated by the intrinsic ATPase of Hsp70 and by co-chaperones that accelerate the ATPase activity (Hsp40/DnaJ) or facilitate ADP-to-ATP exchange (GrpE/Bag1) (43). However, conflicting data have been reported on the effects of ATP on Hsp70 proteins during in vitro aggregation experiments. Whereas the addition of ATP reduced the ability of E. coli DnaK or mammalian Hsc70 to suppress the aggregation of certain denatured substrates (36, 38, 44), either no effect or the opposite effect was observed in other studies using either Hsc70 or S. cerevisiae Ssa2p (35, 37). More consistent results were observed upon addition of both ATP and the Hsp40/DnaJ co-chaperone, wherein an increase in aggregation suppression was observed (35–37). This was most likely a result of enhanced ATPase activity shifting the equilibrium of the various Hsp70 proteins to the higher affinity ADP state. In the present case, ATP did not significantly alter the aggregation-suppressing ability of BiP for either thermally denatured citrate synthase or chemically denatured α-mannosidase (data not shown). Furthermore, no change in aggregation suppression was observed upon addition of both ATP and the J domain of Sec63p, despite the fact that the J domain was capable of stimulating the ATPase activity of BiP. This atypical behavior of BiP was also reflected in recent experiments by others. It was demonstrated that the Sec63p J domain did not stimulate the binding of BiP to a peptide substrate in solution in the presence or absence of ATP (26). It was postulated that the J domain-activated state of BiP might be too short-lived to be detectable in solution. If this is the case, it would provide an explanation for the apparent lack of effect of the J domain on the aggregation-suppressing functions of BiP and S-CNX."

**Fig. 6. Reactivation of denatured α-mannosidase.** A, α-mannosidase or deglycosylated α-mannosidase was denatured in 6 M GdnHCl. Reactivation was initiated by diluting ~220-fold (0.1 μM final concentration) into reactivation buffer (at 4°C) containing the indicated combinations of 0.8 μM S-CNX or IgG, 1 mM ATP, 10% RRL, and 40 μM G1M3. α-Mannosidase activity was assayed at various times over a 90-min period. Values shown are the means ± S.D. of three independent experiments. B, α-mannosidase (0.25 μM) was thermally denatured at 45°C for 90 min to ~50% activity in the presence of equimolar concentrations of S-CNX, IgG, or S-CNX and 40 μM G1M3. Reactivation was initiated by shifting the temperature to 23°C in the presence of various combinations of 1 mM ATP, 1.5 μM BiP, and 3.0 μM Sec63p J domain (J) as indicated. α-Mannosidase activity was assayed at various times over a 60-min period. Values shown are the means ± S.D. of three independent experiments.
BiP in our aggregation suppression assays. It should be noted, however, that in our experiments focused on refolding of thermally denatured substrates, both ATP and the Sec63p J domain were essential for optimal BiP function, as expected for an Hsp70 protein.

In contrast to BiP, ATP enhanced the aggregation-suppressing ability of S-CNX for both CS and α-mannosidase. ATP (but not ADP) is known to cause a conformational change in CNX, potentially increasing the affinity of its peptide-binding site for substrates (15). Therefore, S-CNX and BiP differ fundamentally in the regulation of their polypeptide binding by adenine nucleotides.

Given that CNX has a demonstrated capacity to function as a molecular chaperone in vitro, the question arises as to its role within the ER relative to other molecular chaperones. Are its functions simply redundant compared with those of other polypeptide-binding chaperones such as BiP or Grp94, or does the dual oligosaccharide- and polypeptide-binding properties of CNX provide it with unique capabilities? Support for a unique role was provided by experiments in which S-CNX and BiP were equally potent in suppressing the aggregation of a non-glycosylated substrate, but S-CNX performed much more efficiently than BiP when presented with glycoprotein substrates possessing monoglucosylated oligosaccharides. This advantage could be attributed to lectin-oligosaccharide interactions because removal of the monoglucosylated glycans from either the α-mannosidase or IgY substrate resulted in a substantial attenuation of the aggregation-suppressing ability of CNX, while leaving the ability of BiP to suppress aggregation largely unaffected. A similar contribution from the lectin site of CNX was observed in the refolding of chemically denatured α-mannosidase. Molecular chaperones present in RRL were unable to refold this monoglucosylated substrate on their own. However, the addition of S-CNX permitted a substantial degree of refolding. This effect of BiP could not be replaced by Grp94, the Hsp90 homolog within the ER. As was observed in the aggregation suppression assays, the lectin site of S-CNX was crucial for refolding of the α-mannosidase substrate, which again underscores the flexibility of its dual mode of binding compared with exclusively polypeptide-binding chaperones. However, it is clear from the aggregation suppression assays that α-mannosidase is a particularly challenging substrate for the polypeptide-binding sites of either BiP or S-CNX. It will be of interest to examine the folding of other monoglucosylated proteins that interact more avidly with the polypeptide-binding site of S-CNX such as IgY and soybean agglutinin (15). In such cases, one might expect to observe a less strict dependence on the S-CNX lectin site for refolding.

The demonstration that the CNX and BiP chaperone systems have the capacity to cooperate in the folding of denatured substrates in vitro suggests the potential for such collaboration in vivo. Indeed, several studies examining chaperone interactions with nascent glycoproteins in cultured cells have documented sequential or simultaneous associations of BiP and CNX with the human major histocompatibility complex class I molecule (3); thyroglobulin (19); acid phosphatase (17); and the viral glycoproteins vesicular stomatitis virus G (7), influenza hemagglutinin (5), and Semliki Forest virus E1 (46). Furthermore, chemical cross-linking of intact cells revealed the presence of a large network of associated ER chaperones, including CNX, BiP, calreticulin, and Grp94 (5). The in vitro experiments described in this study provide a starting point for understanding the roles these various chaperones play in the complex process of nascent glycoprotein folding and quality control within the ER. Our findings suggest that CNX and undoubtedly calreticulin as well occupy a unique niche within the ER folding and quality control machinery as a consequence of their dual mode of substrate recognition.

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