Calnexin and calreticulin are membrane-bound and soluble chaperones, respectively, of the endoplasmic reticulum (ER) which interact transiently with a broad spectrum of newly synthesized glycoproteins. In addition to sharing substantial sequence identity, both calnexin and calreticulin bind to monoglucosylated oligosaccharides of the form Glc$_1$Man$_5$–9GlcNAc$_2$, interact with the thiol oxidoreductase, ERp57, and are capable of acting as chaperones in vitro to suppress the aggregation of non-native proteins. To understand how these diverse functions are coordinated, we have localized the lectin, ERp57 binding, and polypeptide binding sites of calnexin and calreticulin. Recent structural studies suggest that both proteins consist of a globular domain and an extended arm domain comprised of two sequence motifs repeated in tandem. Our results indicate that the primary lectin site of calnexin and calreticulin resides within the globular domain, but the results also point to a much weaker secondary site within the arm domain which lacks specificity for monoglucosylated oligosaccharides. For both proteins, a site of interaction with ERp57 is centered on the arm domain, which retains ~50% of binding compared with full-length controls. This site is in addition to a Zn$^{2+}$-dependent site located within the globular domain of both proteins. Finally, calnexin and calreticulin suppress the aggregation of unfolded proteins via a polypeptide binding site located within their globular domains but require the arm domain for full chaperone function. These findings are integrated into a model that describes the interaction of glycoprotein folding intermediates with calnexin and calreticulin.

As the site of synthesis of proteins destined for secretion, cell surface expression, and residency in the secretory pathway, the endoplasmic reticulum (ER) contains an array of folding enzymes and molecular chaperones that facilitate the folding of newly synthesized proteins. Peptidylprolyl cis-trans-isomerase and members of the protein disulfide isomerase family enzymatically catalyze rate-limiting steps in the folding pathway of polypeptides, whereas molecular chaperones such as Grp94 and BiP function by preventing aggregation through cycles of binding and release of unfolded polypeptides. Another set of chaperones present in the ER, calnexin (CNX) and calreticulin (CRT), interact preferentially with glycoproteins that bear Asn-linked oligosaccharides, enhancing their folding and subunit assembly (1–4). This preferential binding is caused by the presence within CNX and CRT of a lectin site with specificity for the oligosaccharide-processing intermediate, Glc$_1$Man$_9$GlcNAc$_2$ (5–8). However, oligosaccharide binding is not an absolute requirement for their association with diverse glycoproteins that transit the ER. Both molecules have been shown to bind in vitro and in vivo to nonglycosylated proteins and peptides as well as to glycoproteins lacking the Glc$_1$Man$_9$GlcNAc$_2$ oligosaccharide (9–22).

CNX, a type I transmembrane protein, and its soluble paralog CRT are Ca$^{2+}$-binding proteins that share sequence similarity that is most pronounced in a central segment containing two proline-rich sequence motifs repeated in tandem (23–26). Motifs 1 and 2, which are repeated three times each in CRT and four times each in CNX, have consensus sequences of I-D/P/D/E-A-KPEDWD(D/E) and G-W-P-IN-P-Y, respectively. In addition, there are three segments of high sequence similarity, A, B, and C, with the last two flanking the repeat motifs (Fig. 1).

In cultured cell systems, the association of CNX and CRT with newly synthesized glycoproteins is transient, with dissociation occurring at or near acquisition of a native structure. CNX and CRT also associate in prolonged fashion to misfolded or incompletely assembled glycoproteins (for review, see Refs. 1–4). These interactions have been shown to promote proper glycoprotein folding and subunit assembly, either to enhance or inhibit the degradation of non-native glycoprotein conformers, and to retain misfolded or incompletely folded glycoproteins in the ER (1–4). How these diverse functions are accomplished is controversial. In one model, the interactions of glycoproteins with CNX and CRT are controlled entirely by the presence of the single terminal glucose residue on the Glc$_1$Man$_9$GlcNAc$_2$ oligosaccharide (5, 27). Cycles of release and rebinding are regulated by glucose removal and readdition catalyzed by the action of the ER enzymes glucosidase II and UDP-glucose: glycoprotein glucosyltransferase, respectively. In this model, CNX and CRT do not function as classical molecular chaperones but are proposed to retain unfolded glycoproteins and coordinate the activities of other ER chaperones and folding enzymes. Such a view is supported by the finding that CNX and CRT interact with the ER resident thiol oxidoreductase, ERp57 (28). Alternatively, a second “dual binding” model has been proposed in which unfolded glycoproteins interact with both the lectin site and a polypeptide binding site in CNX and CRT (8, 29). In this model, the polypeptide component of the interaction serves a molecular chaperone function to suppress aggregation of the unfolded substrate. Support for this model...
Mapping Functional Sites of Calnexin and Calreticulin

Our laboratory as were the ER luminal domain of CNX (CNX 1–411) and the CNX 1–391 and CNX 204–391 constructs (7). All other constructs were generated for this study by PCR using CDNs encoding dog CNX luminal domain and full-length rabbit CRT as templates in the pGEX-3X vector (Amersham Biosciences). All constructs were sequenced and transformed into BL21Gold(DE3) E. coli cells for protein expression.

The following primers were used to prepare CRT deletion constructs for this study. Underlining signifies regions of CRT sequence. Primer A, 5'-TCGATGCAAGGCTGTCGAGG-3', amplifies from the GST sequence just 5' of the multiple cloning site. Primer B, 5'-GGAACTGTATGTGGCACAGG-3', amplifies from the GST sequence just 3' of the multiple cloning site. Other primers were: primer C, 5'-GGCTGTCAGGACACCTCACTCCTATATTCTGCTTCTCGGCCC-3'; primer D, 5'-TGGAGGCTCTTCAATGACTCTGGGGTGTTGTAATCG-3'; primer E, 5'-TGGAGGCCCTCTTCATATCTGCTCGTCCGAGG-3'; primer F, 5'-GGATCCGGCGGAAATTAAGGCAAGTATGCGTGC-3'; primer G, 5'-GGATCCGGCGGAAATTAAGGCAAGTATGCGTGC-3'; primer I, 5'-GGGCGCCGCTTCCCGGCTCCCTTGGGGGTGTAAGGCAGCCC-3'; and primer J, 5'-GGGCGCCGCTTCCCGGCTCCCTTGGGGGTGTAAGGCAGCCC-3'.

The C-terminal deletions, CRT 1–341, CRT 1–282, and CRT 1–238, were prepared using primer A as the 5'-primer and primers C, D, and E, respectively, as the 3'-primers. The N-terminal deletions, CRT 86–401, CRT 191–401, and CRT 205–401, were prepared using primers F, G, and H, respectively, as the 5'-primers and primer J. The double deletion construct, CRT 86–341, was prepared using primer F as the 5'-primer and primer C as the 3'-primer. CRT 270–341 was prepared using CRT 270–401 as template, primer A as the 5'-primer, and primer C as the 3'-primer. CRT 191–341 and CRT 191–282 were prepared using primer G as the 5'-primer and primers C and D, respectively, as the 3'-primers. All of the above PCR products were digested with XhoI and SalI and subcloned into pGEX-3X. CRT (repeats), CRT 1–189/284–401, was prepared with CRT 1–401 as template by a two-step sequence overhang extension method (39). First, CRT 1–189 and CRT 284–401 were prepared using 5'-primers A and J and 3'-primers I and B, respectively. These two PCR products were then mixed and subjected to PCR in the presence of flanking primers A and B. The resulting product was subcloned into the parent CRT 1–401 pGEX-3X vector using AccI and Stul. Primers J and 1 code for a GSOSG linker (parentheses within primer sequence).

The following primers were used to prepare CNX deletion constructs. Underlining signifies regions of CNX sequence. Primer A (above); primer B (above); primer 1, 5'-CATGAACTCCCTCATAGTTAGGA-TTGGTCAATATCGGACG-3'; primer 2, 5'-CATGAACTCCCTCATAGTTAGGA-TTGGTCAATATCGGACG-3'; primer 4, 5'-CATGATCCCCCGGTTATTTGAGG-CCAGAGAAGGACG-3'; primer 5, 5'-CATGATCCCCCGGTTATTTGAGG-CCAGAGAAGGACG-3'; primer 6, 5'-GATGAACTCCCTCATAGTTAGGA-TTGGTCAATATCGGACG-3'; primer 7, 5'-GATGAACTCCCTCATAGTTAGGA-TTGGTCAATATCGGACG-3'; primer 8, 5'-GATGAACTCCCTCATAGTTAGGA-TTGGTCAATATCGGACG-3'; primer 9, 5'-GATGAACTCCCTCATAGTTAGGA-TTGGTCAATATCGGACG-3'; primer 10, 5'-GATGAACTCCCTCATAGTTAGGA-TTGGTCAATATCGGACG-3'.

CNX N-terminal deletion constructs CNX 1–374 and CNX 1–256 were prepared using primer A as the 5'-primer and primers 1 and 2, respectively, as the 3'-primers. These two PCR products were digested with BamHI and EcoRI and subcloned into pGEX-3X. The C-terminal deletions CNX 137–461, CNX 257–461, CNX 271–461, and CNX 328–461 were prepared using primers 3, 4, 5, 8, respectively, as the 5'-primers and primer B as the 3'-primer. Double deletions CNX 257–387 and CNX 257–325 were prepared using primer 5 as the 5'-primer and primers 6 and 7, respectively, as the 3'-primers. CNX 328–387 was prepared using primer 5 as the 5'-primer and primer 6 as the 3'-primer. The latter seven PCR products were digested with SmaI and EcoRI and subcloned into pGEX-3X. The resulting product was subcloned into the parent CNX 1–461 pGEX-3X vector using BamHI and EcoRI. Primers 9 and 10 code for a GSOSG linker (parentheses within primer sequence).

**Purification of GST Fusion Proteins**—Depending on the specific GST fusion protein to be purified, protein expression was induced with 0.1–0.5 mM isopropyl-1-thio-β-D-galactopyranoside for 0.5–3 h at temperatures ranging from 25 to 37 °C.Cell pellets were resuspended in buffer A (10 mM Hepes, pH 7.5, 150 mM NaCl, 5 mM CaCl2), sonicated, and...
and centrifuged at 15,000 \times g. Supernatant fractions were applied to glutathione-agarose beads for 1 h followed by elution with buffer A containing 10 mM reduced glutathione. Eluted samples were subjected to NAP-25 gel filtration (Amersham Biosciences) to remove glutathione and concentrated using a Centricon-10 membrane concentrator (Millipore). Protein concentrations were determined using the Bio-Rad protein assay. All proteins exhibited purity greater than 90% by SDS-PAGE.

Formation of disulfide bonds in the various deletion mutants was confirmed by first removing GST by factor Xa cleavage and then monitoring mobility differences in the CNX or CRT segments by SDS-PAGE in the absence or presence of diethylthreitol treatment (data not shown). ERp57 Radiolabeling and Binding Assay—30 \mu M ERp57 was radio-labeled in 100 \mu l of 0.1 M NaHCO_3, pH 8.0, using a 100-fold molar excess of NHS-\[^{[\text{35}S]}\text{C}l\]acetate (40). Radiolabeled protein (specific activity \sim 15,000 cpm/nmol) was washed free of NHS-\[^{[\text{35}S]}\text{C}l\]acetate using repeated cycles of dilution in buffer A and centrifugation in a Centricon-10 membrane concentrator. GST fusion constructs, immobilized on glutathione-agarose beads and suspended at a concentration of 1 \mu M, were incubated for 30 min at room temperature in 100 \mu l of buffer A containing 0.1% Nonidet P-40, 100 \mu g/ml bovine serum albumin, and either 0.1 \mu M ERp57 or 1.5 \mu M \[^{[\text{35}S]}\text{C}l\]Ac-ERp57. Agarose beads were centrifuged for 5 s at 10,000 \times g, washed twice with buffer A, and resuspended in buffer A containing 10 mM reduced glutathione. The radioactive eluates were analyzed by liquid scintillation counting and compared with either GST-CNX 1–401 or GST-CRT 1–401 full-length positive controls as well as with a GST negative control. Results are expressed as the average percent binding above GST background relative to the full-length controls on an equimolar basis for replicate experiments. Radioactivity recovered in eluted samples ranged from 500 to 1,000 cpm. Nonradioactive samples were analyzed by Western blotting with an anti-ERp57 antibody and the ECL detection system (Amersham Biosciences). Binding specificity for the radioactive assay was confirmed by competition with an excess of nonradioactive ERp57. The addition of a 10-fold or 20-fold molar excess of nonradioactive ERp57 resulted in 81 or 92% inhibition of radioactive ERp57 binding, respectively.

Aggregation Assays—Citrate synthase (CS) and malate dehydrogenase (MDH) were desalted using NAP-25 gel filtration with buffer A as the mobile phase. 1 \mu M CS or MDH was mixed with 1 \mu M GST fusion construct in buffer A in a total assay volume of 500 \mu l. Samples were maintained at 45°C in quartz cuvettes, and aggregation was monitored by measuring light scattering at 360 nm over a period of 1 h.

Oligosaccharide Binding Assay—Radiolabeled \[^{[\text{35}S]}\text{Glc}_\text{Man}_\text{GlcNAc}_2\] oligosaccharide was prepared from the dolichol-PP-oligosaccharide fraction of \text{Saccharomyces cerevisiae} strain \text{alg8} (41) essentially as described previously (9). Binding experiments were performed for 30 min at 4°C in 100 \mu l of buffer A containing 2,000 cpm of \[^{[\text{3H}]\text{Glc}}\text{Man}_\text{GlcNAc}_2\] and 1 \mu M immobilized GST fusion protein. The agarose beads were centrifuged for 5 s at 10,000 \times g, washed once with buffer A, and then incubated at 50°C for 5 min in 10 mM Hepes, pH 7.5, 150 mM NaCl, and 1 mM EGTA to elute bound oligosaccharide. After heating, the beads were centrifuged for 5 s at 10,000 \times g, and the supernatant fraction was analyzed by liquid scintillation counting. All experiments included CNX 1–461 or CRT 1–401 full-length positive controls plus a GST negative control. Results are reported as the average percent binding above GST background compared with full-length control on an equimolar basis for replicate experiments. Typically, with 2,000 cpm input oligosaccharide, 300 cpm (15%) was bound by full-length GST-CN X or GST-CRT.

**RESULTS**

**Mapping the Oligosaccharide Binding Sites of CNX and CRT**—Previous studies have provided conflicting information concerning the location of the lectin sites in CNX and CRT. Deletion mutagenesis suggested that the lectin sites may reside within the repeat segments (arm domain) of both proteins (7, 32), whereas the x-ray structure of CNX crystals soaked in glucose suggested that the monosaccharide was bound to the globular domain with most contacts occurring within box A (35). Both studies suffered from drawbacks in that \[^{[\text{3H}]\text{Glc}}\text{Man}_\text{GlcNAc}_2\] binding by the repeat segments represented only a small fraction of that bound by the full-length proteins, and glucose used in the structural studies represented a non-physiological ligand that binds with more than 1,000-fold lower affinity than \[^{[\text{3H}]\text{Glc}}\text{Man}_\text{GlcNAc}_2\] (7). To resolve this issue, we utilized an expanded mutagenesis approach that combined progressive N- and C-terminal deletion mutants along with mutants representing the individual arm and globular domains defined by the CNX crystal structure. All mutants were prepared as GST fusions through their N termini and, after immobilization on glutathione-agarose beads, were used in binding assays with the physiological \[^{[\text{3H}]\text{Glc}}\text{Man}_\text{GlcNAc}_2\] ligand. The constructs are depicted in Fig. 2 along with a graphical representation of the results for the \[^{[\text{3H}]\text{Glc}}\text{Man}_\text{GlcNAc}_2\] binding assay. Note that the integrity of the various fusion proteins was assessed by assaying for the presence of disulfide bonds (see “Experimental Procedures”) and for their ability to bind at least one ligand, \textit{i.e.} oligosaccharide, ERp57, or unfolded polypeptide (see Figs. 2–6). Three of the 29 constructs (CNX 328–461, CNX 257–325, and CNX 328–387) did not pass these tests and may either lack all ligand binding sites or may be misfolded. Indeed misfolding is likely for the last two constructs because these CNX segments interact with one another to form the extended arm domain (35).

For CRT, the C-terminal deletion construct CRT 1–341 exhibited wild type oligosaccharide binding, indicating that the highly acidic C-terminal tail of CRT is not part of the lectin site (Fig. 2). Further deletion to a point flush with the repeat motifs, CRT 1–282, dramatically reduced binding to 6% of control, illustrating the importance of box C to lectin function. Further deletion to remove motif 2 repeats (CRT 1–238) or the entire repeat segment (CRT 1–182) completely eliminated oligosaccharide binding. Of the N-terminal deletions, CRT 86–401 bound oligosaccharide very poorly. Although this may suggest an important role for residues 1–85, this is probably not the case because further deletion of box A to form CRT 139–401 resulted in an increase to 42% of control binding. This was a surprising finding because the majority of residues contacting glucose in the CNX structure are present in box A, and all of these residues are conserved in box A of CRT. Further deletion to remove box B (CRT 191–401) reduced binding to 11%, indicating the importance of box B to lectin function. Again, deletion into the repeats reduced binding to low levels (CRT 205–401 and CRT 270–401). The various N- and C-terminal double deletion constructs including CRT 191–341, CRT 139–320, CRT 139–273, and the repeat or arm domain itself (CRT 191–282) all retained low but reproducible oligosaccharide binding ranging from 4 to 11%. Collectively, these findings indicate that regions flanking either side of the repeats contribute predominantly to lectin function. However, consistent with previous results, the repeat motifs alone represent a site of \[^{[\text{3H}]\text{Glc}}\text{Man}_\text{GlcNAc}_2\] binding, although it is weak compared with the full-length molecule (7, 32).

Based on the assumption that the nonrepeat regions of CRT would form a globular lectin-like domain analogous to that observed in the CNX crystal structure, we created a construct that lacks the repeat motifs, designated CRT 1–189/284–401. This construct exhibited binding at 75% of control, clearly indicating that the nonrepeat segments comprise the primary lectin domain of CRT.

Similar results were obtained for CNX (Fig. 2). A C-terminal deletion removing 70 amino acids including box C, CNX 1–391, reduced oligosaccharide binding to 57% of control, suggesting that CNX is somewhat less sensitive to the loss of box C than CRT. Additional deletions into the repeat motifs, CNX 1–374 and CNX 1–256, further decreased binding to 15 and 0%, respectively. Two N-terminal deletions that retain the repeat motifs, CNX 137–461 and CNX 257–461, exhibited 7 and 5% binding, respectively, demonstrating the importance of flanking segments N-terminal to the repeats. Again, further deletion
Mapping the lectin sites of CRT and CNX. GST fusion constructs of CRT and CNX were immobilized on glutathione-agarose beads and incubated for 30 min at 4°C with [3H]Glc1Man9GlcNAc2 oligosaccharide. Beads were washed once, and bound oligosaccharide was eluted and quantified by liquid scintillation counting. Oligosaccharide binding is expressed as a percentage of the binding observed for full-length CRT, CRT 1–401, or for the complete ER luminal domain of CNX, CNX 1–461 (~300 cpm). In all cases background binding to immobilized GST alone has been subtracted (~50 cpm). Error bars represent the S.E. in a minimum of four replicate experiments.
into the repeats, CNX 271–461 and CNX 328–461, reduced binding to low levels. Double deletions that retain box B and the repeats (CNX 204–391) or just the repeats (CNX 257–387, corresponding to the arm domain) possessed 17 and 8% residual binding, respectively. Neither motif 1, CNX 257–325, nor motif 2, CNX 328–387, alone exhibited any detectable binding. A construct representing the globular lectin-like domain of CNX lacking the arm domain, CNX 1–255/390–461, retained 75% of the oligosaccharide binding observed with the full-length CNX 1–461 control. Therefore, as observed with the CRT deletion mutants, these data support the view that the repeat motifs represent a weak oligosaccharide binding site, but the globular domain comprised of nonrepeat segments constitutes the primary carbohydrate recognition domain.

To determine whether the low percentage of oligosaccharide binding by the CRT and CNX repeat motifs represents a true binding site for monoglucosylated oligosaccharide, we tested competitor oligosaccharides for their abilities to inhibit $^{[3]H}$Glc$_1$Man$_9$GlcNAc$_2$ binding by full-length CRT 1–401 or the CRT repeat motifs CRT 191–282. For this experiment the amounts of immobilized CRT 1–401 and CRT 191–282 were raised to 5 $\mu$m to increase the amount of bound oligosaccharide and thus the sensitivity of the binding assay. CRT 1–401 bound 1,540 cpm of $^{[3]H}$Glc$_1$Man$_9$GlcNAc$_2$, whereas CRT 191–282 bound 15% of that amount (238 cpm), consistent with their relative binding shown in Fig. 2. Addition of the monoglucosylated oligosaccharide inhibitor Glc$_1$-Man$_3$Man at 10 mM completely blocked the binding of radioactive ligand to either protein. In contrast, the addition of 10 mM Man$_1$–3Man, which lacks a terminal $\alpha$-linked glucose residue, completely blocked binding of the radioactive ligand to CRT 191–282 but only reduced binding to full-length CRT 1–401 by 25%. This indicates that oligosaccharide binding by the repeat motifs is not specific for the monoglucosylated species.

**ERp57 Binding Sites of CNX and CRT**—Initially, we tested whether CRT and the soluble ER luminal domain of CNX (CNX 1–461) could bind to ERp57 when fused through their N termini to GST and immobilized on glutathione-agarose. As shown in Fig. 3A, purified ERp57 bound to both GST-CNX 1–461 and GST-CRT but not to GST alone. Binding occurred in the absence of Zn$^{2+}$; hence this interaction is distinct from the Zn$^{2+}$-dependent binding observed by Corbett et al. (33). Furthermore, the addition of the Glc$_3$Man$_3$ tetrasaccharide, a potent competitor of Glc$_1$Man$_3$GlcNAc$_2$ binding to the lectin sites of CNX and CRT (7), did not interfere with ERp57 binding, suggesting that the lectin and ERp57 binding sites are distinct.

To localize the ERp57 binding sites of CNX and CRT, the GST-fused deletion mutants employed in the lectin mapping studies were used in a quantitative ERp57 binding assay. In this assay, ERp57 was radiolabeled with NHS-$[^{14}$C]acetate, and its binding to immobilized GST fusion proteins was quantified. The deletion mutants are depicted in Fig. 3B along with a bar graph representing their relative binding to $[^{14}$C]Ac-ERp57. The construct CRT 1–341 bound ERp57 similarly to full-length CRT, indicating that the acidic C-terminal tail of CRT is not required for ERp57 binding. A further C-terminal deletion that retained the repeat motifs 1 and 2, CRT 1–282, exhibited only 11% binding, suggesting that residues 283–341 (box C) are important for interaction with ERp57. However, this particular construct overemphasized the importance of the 283–341 segment because the repeat motifs alone (CRT 191–282) exhibited greater binding (see below). The additional C-terminal deletions, CRT 1–238 and CRT 1–182, which possess only the first three repeats or no repeats, respectively, exhibited little if any ERp57 binding. This suggests that the tandem repeat segments are important elements for association with ERp57. N-terminal truncations revealed that residues 1–138 participated little in the interaction because CRT 86–401 and CRT 139–401 retained 92 and 97% binding, respectively. Further deletion suggested some contribution by residues 139–190 (box B) and the first copy of repeat 1 because CRT 191–401 and CRT 205–401 exhibited somewhat impaired binding of 66 and 55%, respectively. However, the greatest contribution to binding was provided by the second and third copies of repeat 2. Their removal in the CRT 270–401 construct resulted in a complete loss of ERp57 binding. Dual N- and C-terminal deletions confirmed the importance of the repeats as well as some involvement of flanking residues. Combined removal of residues 1–85 and 342–401 in the CRT 86–341 construct had little impact on binding. However, further removal of flanking boxes A and B (CRT 191–341) or flanking box C and the last motif 2 repeat (CRT 139–273) reduced binding to 45 and 50%, respectively.

The critical role of the repeats was demonstrated further by comparing ERp57 binding to the repeats alone (CRT 191–282, corresponding to the arm domain) with binding to a construct completely lacking the repeat motifs, CRT 1–189/284–401. The former retained 41% binding, whereas the latter showed a low level of binding representing 15% of control. Within the repeats, the first copy of motif 1 and the last copy of motif 2 appear not to be critical because their absence in the CRT 205–401 and CRT 139–273 constructs did not have a substantial impact on binding.

The repeat motifs were also crucial for ERp57 binding to CNX. As shown in Fig. 3B, deletion of box C and additional residues C-terminal to the repeats had no effect on ERp57 binding (CNX 1–391) indicating that CNX is more tolerant to the removal of C-terminal flanking residues than CRT. However, the complete removal of the repeat motifs, CNX 1–256, resulted in a total loss of binding. In the case of N-terminal deletions, the construct in which the first 136 residues were removed, CNX 137–461, retained 100% ERp57 binding and further deletion until flush with the repeat motifs, CNX 257–461, reduced binding to 67%. Additional truncation to remove all motif 1 repeats, CNX 328–461 completely eliminated binding. Therefore, the repeats are essential for binding, and N-terminal flanking residues participate as well but to a lesser degree. The same trend was observed with double deletion constructs in which CNX 204–391 retained 100% of ERp57 binding. This construct contains both the box B N-terminal flanking segment and the repeats, comprising about 40% of the luminal S-CN X sequence. Truncation to the repeats alone, CNX 257–387 (corresponding to the arm domain), retained 54% binding. Neither motif 1 alone, CNX 257–325, nor motif 2 alone, CNX 328–387, supported binding. However, as observed for CRT, the removal of the first copy of motif 1 or the last copy of motif 2 had relatively little impact on binding as illustrated by the CNX 271–461 and CNX 1–374 constructs. The construct lacking the repeat motifs, CNX 1–255/390–461, which corresponds to the globular lectin domain, showed less than 5% binding, even lower than the case for the corresponding CRT construct. Thus, for both CRT and CNX, the predominant site of ERp57 binding is located within the distinctive arm domain comprised of the repeat motifs.

**ERp57 Binding in the Presence of Zn$^{2+}$**—It was reported previously that CRT binds to ERp57 through an N-terminal segment (residues 1–182) in a Zn$^{2+}$-dependent manner (33). To investigate the relationship between this observation and the Zn$^{2+}$-independent binding we mapped to the repeat motifs, comparative binding experiments were performed in the presence or absence of 100 $\mu$M Zn$^{2+}$. As shown in Fig. 4, the addition of Zn$^{2+}$ caused a ~50% increase in ERp57 binding to full-
FIG. 3. Location of the ERp57 binding sites of CRT and CNX. A, CRT and the ER luminal domain of CNX fused to GST were immobilized on 1 μM glutathione-agarose beads and incubated with 0.1 μM ERp57 in the presence or absence of 100 μM Glc1Man3 (G1M3) tetrasaccharide. Beads were washed twice, and bound ERp57 was eluted and analyzed by SDS-PAGE and Western blotting with anti-ERp57 antibody. B, GST fusion constructs of CRT and CNX were immobilized on glutathione-agarose beads and incubated with 14C-Ac-ERp57. Beads were washed twice, and bound ERp57 was eluted and quantified by liquid scintillation counting. ERp57 binding is expressed as a percentage of the binding observed for either CRT 1–401 or CNX 1–461 (~500–1,000 cpm). In all cases, background binding to immobilized GST has been subtracted (typically 100 cpm). Error bars represent the S.E. in a minimum of four replicate experiments.
length CRT (CRT 1–401) and to the ER luminal domain of CNX (CNX 1–461). Consistent with previous findings for CRT (33), the N-terminal construct, CRT 1–182, which exhibited no ERp57 binding in the absence of Zn\(^{2+}\), bound ERp57 in the presence of Zn\(^{2+}\). In contrast, neither a central CRT segment (CRT 139–273) nor a C-terminal segment (CRT 270–401) exhibited increased ERp57 binding in the presence of Zn\(^{2+}\). Similarly, Zn\(^{2+}\)-dependent ERp57 binding could be localized to the N-terminal 256 residues of CNX (CNX 1–256). For both proteins, constructs lacking the repeat motifs, CRT 1–189/284–401 and CNX 1–255/390–461, also exhibited increased ERp57 binding in the presence of 100 \(\mu M\) Zn\(^{2+}\) (data not shown). These findings suggest that the addition of Zn\(^{2+}\) organizes an additional binding site for ERp57 localized to those segments of CNX and CRT N-terminal to the repeat motifs.

**Mapping the Polypeptide Binding Sites in CNX and CRT**

Incubation of the nonglycosylated proteins, CS or MDH, at 45 °C results in aggregation that can be monitored by an increase in light scattering at 360 nm. We showed previously that CRT and the ER luminal domain of CNX (CNX 1–461) bind specifically to unfolded conformers of CS and MDH and that equimolar concentrations of these chaperones suppress the thermally induced aggregation of both substrates efficiently (29, 30). Furthermore, the addition of 1–3 mM ATP enhances but is not required for this aggregation suppression function. By incubating purified GST-fused deletion mutants of CRT and CNX with CS or MDH and monitoring aggregation suppression it should be possible to localize a region within CRT and CNX which is responsible for their interaction with unfolded protein substrates.

We first determined whether fusion to GST adversely affects the aggregation suppressing abilities of GST-CRT and GST-CN X 1–461 with the unfused chaperones. At a molar ratio of 1:1, GST-CRT and GST-CN X 1–461 were just as effective as CRT and CNX 1–461 at suppressing the thermal aggregation of CS or MDH. These experiments were performed in the absence of ATP and, because efficient aggregation suppression was observed, nucleotide was not included in subsequent experiments. Additionally, in all experiments using GST-fused deletion mutants, the thermal aggregation of the constructs themselves was tested to ensure that any observed light scattering was not caused by unstable constructs (data not shown).

As shown in Fig. 6, progressive C-terminal deletions of CRT suggested that a major contribution to aggregation suppression was provided by the conserved flanking residues 283–341 (box C) because CRT 1–341 fully suppressed aggregation, but CRT 1–282 permitted 50–75% aggregation. An additional contribution from the repeat motifs was suggested by the CRT 1–182 construct, which was even more deficient in aggregation suppression than CRT 1–282. N-terminal deletions also localized an antiaggregation site to the C-terminal third of CRT. Construct CRT 270–401, containing the last motif 2 repeat and box C, fully retained the ability to suppress aggregation. Of the N- and C-terminal double deletions, CRT 139–320, which includes box B, the full set of repeat motifs, and most of box C, exhibited only slightly impaired suppression of CS and MDH aggregation. Comparison of this construct with CRT 139–273 showed that the loss of the last motif 2 repeat and box C greatly reduced the antiaggregation activity pointing to these elements as a site of interaction with unfolded substrates. However, a construct almost completely comprised of the last motif 2 repeat and box C, CRT 270–341, was incapable of preventing aggregation.
aggregation, suggesting that it can function to prevent aggregation only when in the context of extended N- or C-terminal flanking regions. These flanking regions are highly charged and may enhance the solubility of the CRT 270–341 segment.

A disadvantage of this progressive deletion approach to localize a site of interaction with unfolded substrates is that truncation within the presumed globular domain of CRT (non-repeat regions) may expose hydrophobic segments that can interact with unfolded substrates. To minimize this concern, we tested the individual arm and globular lectin domains of CRT for their abilities to suppress CS and MDH aggregation. Fig. 6 shows that the arm domain (CRT 191–282) completely lacked the ability to suppress aggregation, whereas the lectin domain (CRT 1–189/284–401) suppressed the aggregation of CS and MDH to 29 and 15%, respectively. This indicates that the polypeptide binding site resides primarily within the globular lectin domain but requires the repeat motifs for full activity.

In the case of CNX, the removal of box C was less deleterious to its ability to suppress aggregation compared with CRT. CNX 1–391 only permitted 25–40% aggregation compared with 50–75% for the comparable CRT 1–282 construct (Fig. 6). Furthermore, removal of the last motif 2 repeat (CNX 1–374) had little additional impact on aggregation suppression. In contrast, complete removal of the repeats in the CNX 1–256 construct essentially abolished aggregation suppression, suggesting a greater involvement of the repeats than was observed for CRT. It was difficult to prepare sufficient quantities of some of the N-terminal deletion constructs for aggregation suppression assays. However, CNX 137–461, which contains the repeat motifs and boxes A, B, and C, could be produced in large amounts, and it retained almost full aggregation suppression function. Interestingly, CNX 328–461, which contains the repeat 2 motifs and box C, lacked the ability to suppress aggregation in contrast with the CRT 270–401 construct, which retained full function. The involvement of box B was suggested by the CNX 204–391 and CNX 257–387 constructs. The former, which contained box B and the repeats, retained substantial function, whereas the latter, containing only the repeats (i.e. the intact arm domain), lost all function. Comparison of the arm domain and globular lectin domain of CNX revealed that at a construct: substrate molar ratio of 1:1 the globular lectin domain, CNX 1–255/390–461, reduced CS and MDH aggregation to 48 and 54%, respectively. In contrast, the arm domain, CNX 257–387, completely failed to suppress aggregation. Although the globular domain exhibited impaired antiaggregation activity relative to the entire ER luminal domain, it was more effective at suppressing aggregation at a 2-fold molar excess (e.g. aggregation of CS and MDH was reduced to 34 and 37%, respectively), whereas the arm domain remained ineffective at this concentration (data not shown). Therefore, as observed for CRT, the polypeptide binding site of CNX resides predominantly within the globular lectin domain but is enhanced by the presence of the arm domain.

**DISCUSSION**

A recently reported x-ray crystal structure shows that the ER luminal portion of canine CNX consists of two domains, a \( \beta \)-sandwich globular domain that contains boxes A, B, and C and a 140 Å hairpin extension (arm domain) formed by the repeat motifs (35). A structurally similar arm domain is seen in the NMR structure (36) of the CRT repeat motifs (Fig. 7). In both proteins, the motif 1 repeats form one strand of the hairpin, and the motif 2 repeats form the other strand. Each motif 1 repeat interacts with a corresponding motif 2 repeat in a head-to-tail fashion. Upon soaking the CNX crystal in glucose, the monosaccharide was detected within a concave depression in the globular domain (35). Six glucose contact residues were identified, four in box A, one between boxes A and B, and one in box C, all highly conserved among CNXs and CRTs (Fig. 7). The presence of a lectin site within the concave surface of the globular domain is consistent with its structural similarity to the galectin and legume lectin families. A \( \text{Ca}^{2+} \) binding site was also identified within the globular domain (Fig. 7A), which is different from a site mapped previously to the repeat motifs using \( \text{EGCa}^{2+} \) overlay and ruthenium red binding (42). Also, there are two disulfide bonds in the CNX structure, one in the globular domain, shown to be labile, and the second near the tip of the arm domain (35). Given this structural information, it remained to be established how CNX and CRT utilize their two domains to function as molecular chaperones for glycoproteins. Consequently, we set out to map the lectin, ERp57 binding, and polypeptide binding sites of CNX and CRT.

When CNX deletion mutants were assayed for their binding to the natural oligosaccharide ligand \( \text{[H]Glc}_{2} \text{Man}_{n} \text{GlcNAc}_{2} \), we found that 75% of the CNX oligosaccharide binding capa-

**Fig. 5.** Aggregation suppression by native and GST-fused forms of CRT and the ER luminal domain of CNX. 1 μM CS or MDH was incubated at 45 °C alone or in the presence of 1 μM GST, CRT, GST-CRT, CNX 1–461, or GST-CN 1–461 as indicated. Aggregation was monitored by measuring light scattering at 360 nm.
bility could be localized to its globular domain (CNX 1–255/390–461). This is consistent with the location of bound glucose in the x-ray crystal structure of CNX (35). Although a corresponding globular lectin domain has not yet been demonstrated for CRT, we showed for the first time that a CRT deletion construct lacking the repeat motifs and corresponding to the

Fig. 6. Polypeptide binding sites of CRT and CNX. The ability of GST-fused CRT and CNX deletion constructs to suppress CS or MDH aggregation was determined at a chaperone:substrate ratio of 1:1. Results are expressed as the percent residual aggregation of CS (filled bars) and MDH (open bars) compared with GST control after a 60-min incubation at 45°C as monitored by light scattering at 360 nm. Error bars represent the S.E. in a minimum of four replicate experiments.
globular domain of CNX (CRT 1–189/284–401) also retained 75% of the oligosaccharide binding observed with full-length CRT. This finding, coupled with the sequence similarity between CNX and CRT, suggests that CRT possesses a globular lectin domain akin to that of CNX (Fig. 7A). In agreement with previous results (7, 32), we detected a weak lectin site within the repeat motifs (arm domains) of both CNX and CRT. Deletion constructs that correspond to the repeat motifs alone (CNX 257–387 and CRT 191–282) were able to bind oligosaccharide, albeit much more weakly than the full-length controls. However, through the use of competitor oligosaccharides that either possess or lack a terminal α1-3-linked glucose we could demonstrate that oligosaccharide binding by the repeats differed from that observed by the globular domain in that it lacked specificity for monoglucosylated oligosaccharides. Collectively, these findings establish that the globular domain contains the lectin site for the physiologically relevant oligosaccharide and help to clarify previous studies that reported oligosaccharide binding by the repeat motifs.

Using a similar approach, we localized the Zn2+-independent ERp57 binding site to the arm domains of both CNX and CRT. We found that although the globular domains exhibited minimal binding, the arm domains retained about 50% of the ERp57 binding exhibited by the full-length controls. Additional deletion mutants revealed that the first repeat 1 motif and the last motif 2 repeat, which exhibit paired interactions at the

![Functional sites on CNX and CRT.](image-url)
base of the arm domains of both CNX and CRT, are not required for ERp57 binding (Fig. 7A). This suggests that ERp57 binds to segments of the arm domain which are located distal to the globular domain. During the preparation of this manuscript, Ellgaard and co-workers (34) described the use of NMR chemical shift mapping to localize ERp57 interaction with CRT residues 225–251. This segment corresponds to the last copy of motif 1 and the first copy of motif 2, representing the tip of the arm domain. In conjunction with the present findings, it is likely that ERp57 binds primarily to the tips of the arm domains of both CRT and CNX. However, because the arm domains retain only 50% of the binding observed with full-length controls, there appears to be a role for the globular domains as well, presumably in constraining the orientation or conformation of the arm domains. Taken together, the oligosaccharide and ERp57 mapping studies show that the globular domain is the main site of interaction with monoglucosylated Asn-linked oligosaccharide, whereas the arm domain acts to tether the ERp57 oxidoreductase.

In addition to the Zn\(^{2+}\)-independent ERp57 binding site described above, we observed that in the presence of 100 \(\mu\)M Zn\(^{2+}\), ERp57 bound to the N-terminal portion of the CRT globular domain (CRT 1–182). This corresponds to a site of interaction previously described by Corbett et al. (33). In their study, Zn\(^{2+}\)-independent ERp57 was not observed, which may reflect differences in the binding assays employed. Using 1 mM Zn\(^{2+}\), they measured changes in fluorescence of Cascade Blue-labeled ERp57 in response to CRT addition, which requires significant changes in the environment of the fluorophore. We also show that CNX has a Zn\(^{2+}\)-dependent site of interaction with ERp57 localized to the N-terminal portion of its globular domain (CNX 1–256). It should be noted that the addition of 10–100 \(\mu\)M Zn\(^{2+}\) causes a conformational change in CRT resulting in increased hydrophobicity (43), increased chaperone activity (30), and, above 2 mM, quantitative precipitation (44). In the presence of 100 \(\mu\)M Zn\(^{2+}\), CNX aggregates rapidly at elevated temperatures and exhibits reduced solubility at room temperature, suggesting that like CRT, it exposes hydrophobic stretches upon Zn\(^{2+}\) binding. Given these solubility changes, we considered the possibility that Zn\(^{2+}\)-dependent ERp57 binding to CRT and CNX is nonspecific. However, Zn\(^{2+}\)-dependent ERp57 binding was preserved despite the inclusion in our assays of 0.1 mg/ml bovine serum albumin and 0.1% Nonidet P-40 to minimize nonspecific absorption. The physiological significance of this second mode of ERp57 interaction is unclear. It is known that Zn\(^{2+}\) is present within the ER (45), but whether its concentration is sufficient to induce conformational changes in CRT and CNX to influence ERp57 binding remains an open question.

Efforts to localize a polypeptide binding site within CRT and CNX identified their globular lectin domains as the region primarily responsible for suppressing the aggregation of nonglycosylated proteins. This was supported by the finding that N- and C-terminal deletion mutants lacking either of the conserved boxes B or C exhibited impaired aggregation suppression and by the fact that the globular domain constructs (CRT 1–189/284–401 and CNX 1–255/390–461) retained the ability to suppress aggregation, whereas the arm domains (CRT 191–282 and CNX 257–387) did not. However, although the arm domains themselves were incapable of suppressing aggregation, they clearly enhanced aggregation suppression by the globular domains. This suggests that polypeptide segments of non-native folding intermediates may interact with both domains of CRT and CNX (Fig. 7A). Because molecular chaperones interact with folding intermediates through binding sites that possess some hydrophobic character (46), we examined the surface of CNX for hydrophobic patches. Candidate hydrophobic interactions do exist on the same face of the globular domain as the lectin site, and additional sites are present along the length of the arm domain. Further site-directed mutagenesis within these regions will be required to determine more precisely how CRT and CNX bind to nonglycosylated folding intermediates.

Taken together, our findings suggest a model whereby CRT and CNX interact with non-native conformers of glycoproteins during folding within the ER. As shown in Fig. 7B, we envision that a monoglucosylated glycoprotein substrate could occupy the large cavity formed by the globular domain and the arm domain. This would permit contacts through the lectin site as well as through polypeptide binding sites residing within both domains. The arm domain, which is presumably quite flexible, would perform the additional function of bringing ERp57 in proximity to free thiol groups to form mixed disulfide intermediates in both oxidation and isomerization reactions. The arm may also physically constrain the glycoprotein, impeding rapid diffusion away from the chaperone. In this context, the previously described enhancement of aggregation suppression by CRT and CNX in the presence of ATP (29, 30) might be caused by the closing of the arm domain over substrate in a manner analogous to the \(\alpha\)-helical “lid” segment of HSP70 (47). Given progress in delineating the lectin, ERp57 binding, and polypeptide binding sites of CRT and CNX, we are in a position to test how these functions are integrated into a functional chaperone and in turn regulated by cofactors such as Ca\(^{2+}\), Zn\(^{2+}\), and ATP. Selective ablation of each of these functions by mutagenesis and assessing the consequences using \(in\) \(vitro\) and \(in\) \(vivo\) assays of glycoprotein folding and quality control should be most informative.

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\(^2\) M. Leach and D. Williams, unpublished observations.
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