Oligosaccharide Binding Characteristics of the Molecular Chaperones Calnexin and Calreticulin†

Aikaterini Vassilakos,‡ Marek Michalak,§ Mark A. Lehrman,|| and David B. Williams*,‡

Department of Biochemistry, University of Toronto, Toronto, Ontario M5S 1A8, Canada, Department of Biochemistry, University of Alberta, Edmonton, Alberta T6G 2S2, Canada, and Department of Pharmacology, University of Texas Southwestern Medical Center, Dallas, Texas 75235-9041

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ABSTRACT: Calnexin and calreticulin are homologous molecular chaperones of the endoplasmic reticulum. Their binding to newly synthesized glycoproteins is mediated, at least in part, by a lectin site that recognizes the early N-linked oligosaccharide processing intermediate, $Glc_1Man_9GlcNAc_2$. We compared the oligosaccharide binding specificities of calnexin and calreticulin in an effort to determine the basis for reported differences in their association with various glycoproteins. Using mono-, di-, and oligosaccharides to inhibit the binding of $Glc_1Man_9GlcNAc_2$ to calreticulin and to a truncated, soluble form of calnexin, we show that the entire Glcα1−3Manα1−2Manα1−2Man structure, extending from the α1−3 branch point of the oligosaccharide core, is recognized by both proteins. Furthermore, analysis of the binding of monoglucosylated oligosaccharides containing progressively fewer mannose residues suggests that for both proteins the α1−6 mannose branch point of the oligosaccharide core is also essential for recognition. Consistent with their essentially identical substrate specificities, calnexin and calreticulin exhibited the same relative affinities when competing for binding to the $Glc_1Man_9GlcNAc_2$ oligosaccharide. Thus, differential glycoprotein binding cannot be attributed to differences in the lectin specificities or binding affinities of calnexin and calreticulin. We also examined the effects of ATP, calcium, and disulfide reduction on the lectin properties of calnexin and calreticulin. Whereas oligosaccharide binding was only slightly enhanced for both proteins in the presence of high concentrations of a number of adenosine nucleotides, removal of bound calcium abrogated oligosaccharide binding, an effect that was largely reversible upon readdition of calcium. Disulfide reduction had no effect on oligosaccharide binding by calnexin, but binding by calreticulin was inhibited by 70%. Finally, deletion mutagenesis of calnexin and calreticulin identified a central proline-rich region characterized by two tandem repeat motifs as a segment capable of binding oligosaccharide. This segment bears no sequence homology to the carbohydrate recognition domains of other lectins.

Calreticulin and calnexin are homologous calcium binding proteins that reside within the endoplasmic reticulum (ER).[1] Whereas calreticulin is a soluble, lumenal protein, calnexin is a type I membrane protein with most of its mass lumenally disposed (*1*, *2*). Segments of these proteins share amino acid sequence identity ranging from 42% to 78% (*3*). The most striking and highly conserved segment contains two tandem repeat motifs, repeated four times each in calnexin and three times each in calreticulin (*2*−*4*). This region, which in calreticulins is known as the proline-rich P domain, also contains a site for high-affinity calcium binding (*5*, *6*).

Both calreticulin and calnexin bind transiently to diverse membrane or secretory glycoproteins that are translocated into the ER. They also exhibit prolonged interaction with mutant glycoproteins that fail to fold or assemble correctly (*1*, *7*−*10*). During these interactions, calnexin functions as part of the ER quality control system that prevents the export of misfolded or incompletely assembled glycoproteins along the secretory pathway (*11*−*13*). A number of recent studies have provided evidence that calnexin also acts as a molecular chaperone that facilitates the folding and assembly of glycoproteins. Specifically, in the absence of calnexin binding, the formation of fully oxidized forms of the vesicular stomatitis virus G glycoprotein or the heavy chain of the human class I histocompatibility molecule is impaired or

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* To whom correspondence should be addressed: Department of Biochemistry, Medical Sciences Building, University of Toronto, Toronto, Ontario M5S 1A8, Canada. Tel (416) 978-2546; Fax (416) 978-8548; e-mail david.williams@utoronto.ca.

‡ University of Toronto.

§ University of Alberta.

|| University of Texas Southwestern Medical Center.

[1] Abbreviations: CNX, calnexin; CRT, calreticulin; DTT, dithiothreitol; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; ER, endoplasmic reticulum; GST, glutathione S-transferase; NP-40, Nonidet P40; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; HPLC, high-performance liquid chromatography; PCR, polymerase chain reaction.

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delayed, respectively (*14, 15*). Likewise, murine class I histocompatibility molecules synthesized in the absence of calnexin assemble inefficiently due to heavy-chain misfolding and aggregation (*16*). Calnexin also protects many nascent glycoproteins against rapid intracellular degradation (*11, 17, 18*). Although calreticulin-deficient mouse embryos have been produced (giving rise to an embryonic lethal phenotype), the effect of calreticulin depletion on the folding of nascent glycoproteins has not yet been studied (*19*). However, it is likely that calreticulin possesses chaperone properties. When the interactions of calreticulin and calnexin with influenza hemagglutinin are simultaneously prevented, the overall efficiency of hemagglutin maturation decreases (*20*).

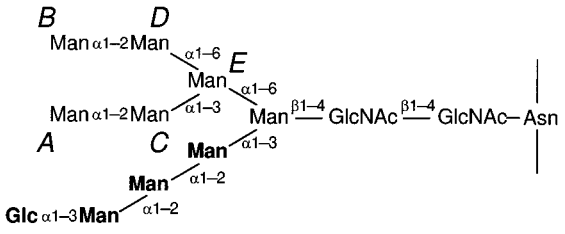
One of the most remarkable aspects of calnexin and calreticulin function is their striking preference for binding to Asn-linked glycoproteins. The basis for this selectivity arises from the fact that both proteins are lectins that recognize the oligosaccharide processing intermediate $Glc_1Man_9GlcNAc_2$ (*21*−*24*). Substrate specificity studies have identified the single terminal glucose residue as a critical determinant recognized by both chaperones since oligosaccharides containing 0, 2, or 3 glucose residues fail to bind (*21, 22*). Oligosaccharide binding is clearly crucial for the formation of complexes between glycoproteins and calnexin or calreticulin. If formation of the $Glc_3Man_9GlcNAc_2$ oligosaccharide is blocked with tunicamycin or if production of the $Glc_1Man_9GlcNAc_2$ species is prevented by treatment with the glucosidase inhibitors castanospermine or deoxynojirimycin, the binding of calnexin/calreticulin to the vast majority of proteins is inhibited (*8, 9, 23, 25*). It has been suggested that these chaperones interact with nascent glycoproteins in cycles of binding and release regulated by glucosidase II, which removes the single glucose residue from the $Glc_1Man_9GlcNAc_2$ oligosaccharide, and by UDP-glucose:glycoprotein glucosyltransferase, which reattaches it (*24*). However, it is not apparent how such a lectin-only mode of interaction can give rise to the observed molecular chaperone functions of these molecules, e.g., the suppression of aggregates. There is abundant evidence suggesting that calnexin and calreticulin recognize the polypeptide segments of newly synthesized glycoproteins as well (*21, 26*−*33*). Consequently, we have proposed an alternative model that incorporates both oligosaccharide and polypeptide interactions into the above cycle (*21*). Since there are in vitro binding experiments that support either a lectin-only (*34, 35*) or a lectin/polypeptide dual mode of binding (*21, 26*−*33*), it is possible that different modes of interaction may be used for individual glycoproteins. Clearly, further experiments are required to resolve this issue.

A number of studies have examined cofactors that may influence the structure and function of calnexin and calreticulin. Mg-ATP has been shown to bind to calnexin, resulting in oligomerization and increased sensitivity to protease digestion (*36*). In vivo studies have suggested a role for ATP in maintaining the association of calnexin with a soluble glycoprotein in Madin-Darby canine kidney cells, but it is unclear if this effect is due to a direct interaction of nucleotide with calnexin (*37*). Indirect evidence suggests that ATP may bind to calreticulin as well, but in contrast to its effect on calnexin in vivo, ATP triggered the dissociation of calreticulin from a variety of denatured proteins in vitro (*33*). Both calnexin and calreticulin bind Ca^{2+} (*5, 6*). In the case of calnexin, Ca^{2+} appears to have the opposite effect of Mg-ATP since a monomeric, protease-resistant conformation accompanies Ca^{2+} binding (*36*). Ca^{2+} is also required for the binding of calnexin to glycoproteins as assessed either in vivo (*38*) or in vitro (*39*). The effects of Ca^{2+} on the structure of calreticulin have not been established but it does not appear to be required for the binding of calreticulin to denatured proteins in vitro. Indeed, the addition of Ca^{2+} enhanced the ATP-induced dissociation of calreticulin from denatured proteins (*33*). By contrast, Ca^{2+} has been shown to be required for the binding of calreticulin to laminin (*40*). Although preliminary and sometimes conflicting, these studies nevertheless implicate ATP and Ca^{2+} as cofactors that affect the structure and function of calnexin and calreticulin. Whether or not these cofactors exert their effects by modulating the lectin functions of these chaperones remains to be addressed.

It is unclear why the ER of most eukaryotic species contains two homologous chaperones with lectin properties. This may reflect redundancy in the ER quality control and protein folding systems or the two chaperones could conceivably function in a coordinated fashion. Although redundant action is suggested by several studies in which calreticulin and calnexin were shown to bind to the same glycoprotein substrates (*9, 10, 41*−*43*), other work has demonstrated differences either in the spectrum of glycoproteins bound (*9, 42*−*44*) or in the stage of glycoprotein maturation recognized by each chaperone (*20, 45*).

We have undertaken a detailed examination of the oligosaccharide binding sites in calnexin and calreticulin in an effort to better understand the nature of their interaction with glycoproteins and to discern any differences that might account for their differential binding to various glycoproteins. Our data indicate that binding interactions between calnexin or calreticulin and the $Glc_1Man_9GlcNAc_2$ oligosaccharide are much more extensive than previously thought (*21, 22*) and include the entire glucosylated branch of the oligosaccharide. Furthermore, the two proteins bind the $Glc_1Man_9GlcNAc_2$ oligosaccharide with similar affinity and are indistinguishable in terms of their oligosaccharide binding specificities. We also examined the effect of Ca^{2+} and Mg-ATP on oligosaccharide binding and found that bound Ca^{2+} is essential to the lectin functions of both proteins. We were unable to reproduce previously reported conformational changes associated with Mg-ATP binding to calnexin and, consistent with this finding, observed only minimal enhancements of oligosaccharide binding to calnexin or to calreticulin in the presence of nucleotide. Finally, we attempted to localize the lectin sites in calnexin and calreticulin and found that, although the extent of oligosaccharide binding was sensitive to N- or C-terminal truncation, a central homologous segment distinguished by two tandem repeat motifs could be identified that retained the ability to bind oligosaccharide. Given that the oligosaccharide binding sites of these proteins are similar in terms of primary sequence, specificity, binding affinity, and the effect of cofactors, it is unlikely that they are responsible for differences observed in the binding of calnexin and calreticulin to nascent glycoproteins. Rather, such differences may be due to differential recognition of the polypeptide chains of glycoproteins or to the distinct topological environments wherein the two chaperones reside.

A



Fragment	Method	Mannose Residues Removed
1. $Glc_1Man_9GlcNAc_2$	------	none
2. $Glc_1Man_8GlcNAc_2$	ER α1,2 mannosidase	A
3. $Glc_1Man_7GlcNAc_2$	Golgi α1,2 mannosidase I	A, B
4. $Glc_1Man_7GlcNAc_2$	Acetolysis	B, D
5. $Glc_1Man_4GlcNAc_2$	Acetolysis	A, B, C, D, E

B

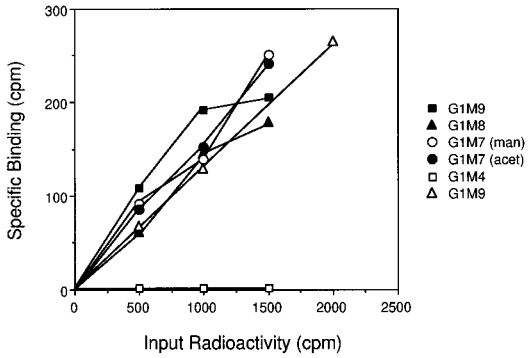


FIGURE 1: Binding of $Glc_1Man_{4,7,8,9}GlcNAc_2$ oligosaccharides to calnexin. (A) Structure of the $Glc_1Man_9GlcNAc_2$ oligosaccharide recognized by calnexin. The indicated treatments were used to remove specific mannose residues (A−E) for the production of $Glc_1Man_{4,7,8,9}GlcNAc_2$ oligosaccharides. (B) Identical samples of immobilized CNX-His were incubated with increasing amounts of each of the $Glc_1Man_{4,7,8,9}GlcNAc_2$ oligosaccharides described in panel A. Bound radioactivity was determined and the results are presented as specific binding vs input radioactivity. G1M9, $Glc_1Man_9GlcNAc_2$; G1M8, $Glc_1Man_8GlcNAc_2$; G1M7 (man), $Glc_1Man_7GlcNAc_2$ prepared by Golgi α-mannosidase treatment; G1M7 (acet), $Glc_1Man_7GlcNAc_2$ prepared by acetolysis; G1M4, $Glc_1Man_4GlcNAc_2$.

EXPERIMENTAL PROCEDURES

Preparation of Homogeneous [^{3}H]$Glc_1Man_{4,7,8,9}GlcNAc_2$ Oligosaccharides. Dolichol-linked [^{3}H]oligosaccharides from the *Saccharomyces cerevisiae alg8* strain (*46*), prepared essentially as described by metabolic labeling with [^{3}H]-mannose (*21*), were used as starting material. Oligosaccharides were cleaved from dolichol pyrophosphate with mild acid, reduced with sodium borohydride, and desalted (*21*). The material from *alg8* cells was mostly $Glc_1Man_9GlcNAc_2$ (which yielded acetolysis fragments migrating as Man_2, Man_3, and $Glc_1Man_4GlcNAc_2$ by HPLC) but also included smaller amounts of oligosaccharides tentatively identified as being $Man_{<9}GlcNAc_2$, which were readily separated from $Glc_1Man_9GlcNAc_2$ by preparative HPLC (*21*). For other specific oligosaccharide fragments the $Glc_1Man_9GlcNAc_2$ was treated as indicated, and the fragments were isolated by preparative HPLC. The structures of these fragments are indicated in Figure 1A. $Glc_1Man_8GlcNAc_2$ lacking the terminal mannose of the center branch was prepared by digestion with purified recombinant *S. cerevisiae* ER α1,2-mannosidase (the kind gift of A. Herscovics, McGill University). $Glc_1Man_4GlcNAc_2$ was obtained by acetolysis, a treatment that selectively cleaves α1,6-linked mannose residues. Incomplete cleavage of α1,6-mannosyl linkages also yielded appreciable quantities of $Glc_1Man_7GlcNAc_2$. A distinct isomer of $Glc_1Man_7GlcNAc_2$ was obtained by digestion with purified, recombinant Golgi mannosidase I (kindly provided by K. Moreman, University of Georgia), which removes all exposed α1,2-linked mannose residues. In all cases the purified fragments had the expected elution times based on the known properties of the HPLC column, which separates oligosaccharides on the basis of increasing sugar content. To verify that $Glc_1Man_4GlcNAc_2$ was, in fact, glucosylated, it was shown to be fully resistant to jack bean α-mannosidase treatment. By comparison, $Man_4GlcNAc_2$ was highly susceptible, whereas $Man_3GlcNAc_2$ was resistant as expected.

[^{3}H]$Glc_{0-3}Man_9GlcNAc_2$ mixtures were isolated from [^{3}H]-mannose-labeled CHO cells as described (*21*).

Preparation of Soluble Proteins: His-Tagged Proteins. The soluble class I histocompatibility H-2K^b heavy chain (with His_6 at the C-terminus) in association with β_2-microglobulin was purified from the culture medium of transfected *Drosophila melanogaster* SC2 cells by Ni-NTA−agarose (Qiagen) and Mono Q (Pharmacia) anion-exchange chromatography as described previously (*47*).

Expression of soluble calnexin in baculovirus-infected Sf9 cells was accomplished using the MAXBAC baculovirus expression system from Invitrogen. The soluble calnexin construct (CNX-His) encoded the N-terminal signal sequence and ER lumenal domain of calnexin followed by the sequence SRRSWGSHHHHHH (*21*). This was cloned into the pVL1393 vector and was kindly provided by T. Jensen and J. R. Riordan, Mayo Clinic, Scottsdale, AZ. Sf9 cells were maintained at 27 °C in Grace's insect cell medium (Life Technologies) supplemented with 3.33 g/L lactalbumin hydrolysate, 3.33 g/L yeastolate, 2 mM glutamine, and 10% fetal bovine serum. For infection with recombinant baculovirus for protein production, the cells were grown at 24 °C in roller bottles containing 150 mL of a 50:50 mixture of supplemented Grace's medium and Insect Xpress medium (Biowhittaker). The recombinant virus was added to a confluent culture of Sf9 cells (15 mL was added to each roller bottle) and incubated with slow rotation for 72 h. At 60 and 72 h, the protease inhibitor AEBSF ([4-(2-aminoethyl)benzenesulfonyl fluoride], Calbiochem) was added to the medium at a concentration of 0.2 mM. Culture supernatant (typically 1 L from 7−8 roller bottles) was collected and cellular debris was removed by centrifugation at 2500*g* for 20 min at 4 °C. CNX-His was subsequently purified from the culture supernatant essentially as described previously (*21*).

PCR was used to synthesize cDNA encoding full-length mature calreticulin. PCR products were purified by polyacrylamide gel electrophoresis and ligated into pRSETB (Invitrogen) to generate a carboxy-terminal fusion of calreticulin with His_6. Transformed *Escherichia coli* K38 cells were grown overnight at 30 °C and then were collected by centrifugation for 15 min at 3000*g* in a GSA rotor. The pellet was suspended in phosphate-buffered saline (PBS)

containing 0.1% Triton X-100 followed by sonication for 3 × 45 s. The suspension was then centrifuged for 12 min at 8000*g*. The supernatant fraction was filtered and loaded onto a Zn^{2+}-chelating Sepharose column (Pharmacia) equilibrated with a buffer containing 50 mM Na_2HPO_4, pH 7.0, and 100 mM NaCl. Protein fractions were eluted with a 0−1 M imidazole gradient, analyzed by SDS−PAGE, pooled, and then concentrated using an Amicon YM30 membrane. CRT-His was further purified using an FPLC Resource Q column. The sample was loaded at a flow rate of 1 mL/min onto a 6 mL column equilibrated with buffer containing 20 mM Na_2HPO_4, pH 7.0, and 100 mM NaCl. CRT-His was eluted with a 100−750 mM NaCl gradient containing 50 mM Na_2HPO_4, pH 7.0. Protein fractions were analyzed by SDS−PAGE, pooled, and concentrated.

Glutathione S-Transferase Fusion Proteins. GST−rabbit calreticulin fusions (full-length or encompassing calreticulin residues 1−182, 1−273, 139−273, 139−401, and 270−401) were expressed and purified from *E. coli* as previously described (*5*). GST−CRT (139−320) was generated by digestion of the pGEX vector (Pharmacia) containing CRT fragment 139−401 with *Bsi*WI and *Eco*RI to remove nucleotides corresponding to CRT residues 321−401. The digest was treated with mung bean nuclease to generate blunt ends and the plasmid was recircularized using T4 DNA ligase. The ligation reaction was transformed into DH5α cells.

GST−calnexin constructs (GST−CNX) were prepared as follows. Canine calnexin cDNA in the Bluescript vector was modified by first removing a *Dsa*I site in the vector followed by insertion of an *Eco*RI 8mer linker into the *Dsa*I restriction site positioned within the calnexin coding sequence at the lumenal side of the transmembrane domain. A DNA fragment containing the sequence for the entire lumenal domain of calnexin was generated by *Bsp*HI digestion and incubation with Klenow to generate a blunt 5′ end, followed by *Eco*RI digestion. The calnexin fragment (encoding amino acids 1−461) was subcloned into the *Sma*I and *Eco*RI sites of pGEX 3X (Pharmacia). This construct, GST−CNX, was used to generate a C-terminal deletion (aa 1−391) by digesting with *Bgl*II (partial digest) and *Eco*RI. The digests were incubated with Klenow fragment to generate blunt ends and then the vector was gel-purified, recircularized using T4 ligase, and used to transform DH5α cells. The deletion mutant 204−391 was generated by *Bgl*II/*Bam*HI digestion of mutant 1−391, incubation with Klenow, and recircularization of the plasmid as above.

Fusion proteins were expressed in *E. coli* and isolated by single-step purification using glutathione−agarose (Sigma). A 1 mL aliquot of overnight cultures was diluted to 10 mL and grown for 90 min. Isopropyl thio-β-D-galactoside (0.1 mM) was added to induce synthesis of fusion protein and cultures were incubated for an additional 3 h. Bacteria were isolated by centrifugation for 10 min at 4000*g* and resuspended in 0.5 mL of solubilization buffer [10 mM Hepes, pH 7.5, containing 0.15 M NaCl, 10 mM $CaCl_2$, and 1% Nonidet P40 (NP-40)]. Cells were disrupted by sonication and cell debris was removed by centrifugation for 5 min at 12000*g*. Fusion proteins were isolated by incubation for 30 min at 4 °C with 50−100 μL of glutathione−agarose. The beads were washed twice with 1 mL of solubilization buffer and three times with binding buffer (10 mM Hepes, pH 7.5, containing 0.15 M NaCl and 10 mM $CaCl_2$). Purified proteins were left bound to the agarose beads for use in oligosaccharide binding assays. They were typically >80% pure as assessed by Coomassie blue staining of SDS−polyacrylamide gels.

Incubation of Radiolabeled Oligosaccharides with Immobilized Proteins. Each assay employed 5 μg samples of CNX-His, CRT-His, GST−CRT, or GST−CNX molecules immobilized on 5−10 μL of Ni−agarose or glutathione−agarose in binding buffer (10 mM Hepes, pH 7.5, containing 0.15 M NaCl, and 10 mM $CaCl_2$). Unless indicated otherwise, all procedures were performed at 21−23 °C. Agarose beads were washed briefly with binding buffer just prior to each experiment and then were suspended in 100 μL of binding buffer that contained either no additions, competitive inhibitors (protein or mono-, di-, and oligosaccharides), nucleotides, dithiothreitol (DTT), or ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA). Incubations involving DTT or EGTA included a 30 min pretreatment at 30 °C prior to addition of oligosaccharide. Unless otherwise indicated, approximately 4000 cpm of oligosaccharides isolated from the dolichol-oligosaccharide fraction of Chinese hamster ovary cells (mixture of $Glc_{0-3}Man_9GlcNAc_2$; *21*) or 2000 cpm of oligosaccharides isolated from *alg8* yeast cells (predominantly $Glc_1Man_9GlcNAc_2$) was added. The samples were incubated for 1 h with agitation on an orbital shaker at 200 rpm, centrifuged for 5 s at 10000*g*, and the supernatant fraction was removed. The agarose beads were rinsed briefly with 200 μL of binding buffer and centrifuged as above, and the supernatant fraction was removed using a Hamilton syringe. The agarose beads were boiled for 5 min in 100 μL of binding buffer and bead-associated radioactivity was analyzed by liquid scintillation counting. Results are reported as specific binding (radioactivity in each sample minus background radioactivity associated with control incubations using either GST or K^b-His).

EGTA and ATP Treatment of Calnexin—Assessment of Protease Sensitivity and Oligomerization State. To test the effects of EGTA and ATP on the protease sensitivity of calnexin, 5 μg samples of CNX-His or GST were immobilized on 5−10 μL of nickel−agarose or glutathione−agarose, respectively, in binding buffer. For ATP-treated samples, beads were suspended in 20 μL of binding buffer containing 30 μg/mL proteinase K, either 1 or 10 mM ATP, and 5 mM $MgCl_2$. For samples treated with EGTA, the agarose beads were washed three times with 1 mL of calcium-free binding buffer and then suspended in 20 μL of calcium-free binding buffer containing 30 μg/mL proteinase K and 10 mM EGTA. pH 7.5 was maintained in all of the incubations. After incubation at 30 °C for 30 min, the samples were boiled in sample buffer and analyzed by SDS−PAGE. Resolved proteins were visualized by Coomassie Blue staining.

For determination of oligomerization state, 5 μg samples of CNX-His or GST (not immobilized) were incubated for 30 min at 30 °C in 100 μL of binding buffer containing 10 mM ATP and 5 mM $MgCl_2$ or in calcium-free binding buffer containing 10 mM EGTA as indicated. After incubation, samples were analyzed on 7% nondenaturing polyacrylamide gels (Laemmli gels omitting the SDS) and resolved proteins were visualized by Coomassie Blue staining.

Table 1: Inhibition of $Glc_1Man_9GlcNAc_2$ Binding to Calnexin[a]

inhibitor[b]	concentration providing 50% inhibition (mM)
GlcαpNO_2Ph, -OCH_3	≫2.5[c]
ManαpNO_2Ph	≫2.5
Glcα1−3Man	0.6
Glc α1−3Glc	0.7
Glcα1−4Glc	1.7
Glcα1−6Glc	≫2.5
Manα1−3Man	≫2.5
Glcα1−3Manα1−2Man	0.08
Manα1−3Manα1−6Man	≫2.5
Glcα1−3Manα1−2Manα1−2Man	0.0045

[a] Equal samples of CNX-His immobilized on Ni−agarose (1 μM final concentration) were incubated with [^{3}H]$Glc_1Man_9GlcNAc_2$ oligosaccharide and various nonradioactive mono-, di-, and oligosaccharides as indicated. Radioactivity specifically bound to calnexin was determined and the results are presented as the concentration of inhibitor that produced 50% inhibition of binding. [b] The disaccharides lactose and sucrose provided no detectable inhibition at 2.5 mM. Polymeric yeast mannan inhibited binding by 25% at a concentration of 0.75 mg/mL. [c] No detectable inhibition at a concentration of 2.5 mM.

RESULTS

To study the oligosaccharide binding properties of calnexin and calreticulin, the soluble ER lumenal domain of calnexin was expressed with a His_6 sequence at its C-terminus (CNX-His) and calreticulin was expressed either with a His_6 sequence at its C-terminus (CRT-His) or as a fusion protein with glutathione S-transferase (GST−CRT). The recombinant proteins were immobilized either on Ni−agarose or glutathione−agarose. In preliminary experiments it was determined that CNX-His, CRT-His, and GST−CRT bound [^{3}H]$Glc_1Man_9GlcNAc_2$ oligosaccharide 20−40-fold above background binding to the corresponding control proteins, H-2K^b-His and GST, and that binding was saturable (data not shown). All subsequent experiments were performed with subsaturating levels of oligosaccharide and the results were expressed in terms of specific binding [radioactivity bound to CNX-His, CRT-His, or GST−CRT minus radioactivity bound to the corresponding control protein (typically 10−20 cpm)].

Oligosaccharide Binding Specificity of Calnexin. We showed previously that the ER lumenal domain of calnexin, when incubated with a mixture of $Glc_{0-3}Man_9GlcNAc_2$ oligosaccharides, bound selectively to the $Glc_1Man_9GlcNAc_2$ species (*21*). Thus, the single glucose residue is an important determinant for recognition by calnexin (see Figure 1A for oligosaccharide structure). To identify additional components of the oligosaccharide that are recognized by calnexin, a variety of mono-, di-, and oligosaccharides were tested for their abilities to inhibit the binding of [^{3}H]$Glc_1Man_9GlcNAc_2$ to immobilized CNX-His (Table 1). The efficacy of each compound was expressed as the concentration that inhibits oligosaccharide binding to 1 μM calnexin by 50%. No inhibition was observed with the monosaccharides, α-methyl glucoside or α-pNO_2Ph glucoside. By contrast, disaccharides containing glucose α1−3-linked to either glucose or mannose inhibited oligosaccharide binding. These findings indicate that a penultimate sugar residue is essential for inhibition and that calnexin does not distinguish between C_2 epimers of the penultimate residue. Preference for the 1−3 linkage was evidenced by the lack of inhibition with Glcα1−6Glc and a 3-fold poorer inhibition with Glcα1−4Glc. Consistent with our previous findings, the terminal glucose residue is an important determinant for recognition by calnexin since Manα1−3Man was not an inhibitor.

Glcα1−3Glc and Glcα1−3Man are present at the respective nonreducing termini of the $Glc_2Man_9GlcNAc_2$and $Glc_1Man_9GlcNAc_2$ oligosaccharide processing intermediates occurring early in glycoprotein biosynthesis. However, only the $Glc_1Man_9GlcNAc_2$ intermediate is specifically recognized by calnexin (*21*). Presumably, additional interactions occur that provide for the observed specificity of calnexin. To test this possibility, tri- and tetrasaccharides that reflect the structure of the glucosylated arm of the $Glc_1Man_9GlcNAc_2$ oligosaccharide (see Figure 1A) were tested as inhibitors. Glcα1−3Manα1−2Man and Glcα1−3Manα1−2Manα1−2Man were ∼8-fold and ∼130-fold more potent inhibitors than the disaccharides, respectively (Table 1). These findings indicate that calnexin recognizes all four sugar residues on the glucosylated α3 branch of the $Glc_1Man_9GlcNAc_2$ oligosaccharide (Figure 1A, boldface type).

Interestingly, yeast mannan (a heterogeneous polymannose structure containing an α1−6 backbone and branched α1−2 and α1−3 linkages) also inhibited oligosaccharide binding, suggesting that additional mannose residues may be involved in interactions with calnexin (Table 1). This possibility was tested by generating a series of radiolabeled oligosaccharides containing 4−9 mannose residues and assaying for their binding to immobilized CNX-His. The method of generation of these oligosaccharides and their structures are shown in Figure 1A. In Figure 1B, specific binding of each oligosaccharide is plotted as a function of input radioactivity to normalize for any differences in specific activities of the oligosaccharides. With the exception of $Glc_1Man_4GlcNAc_2$, all of the oligosaccharides bound to calnexin. The slopes of the curves for each of the oligosaccharides that bound are within the range of variability observed for $Glc_1Man_9GlcNAc_2$ (compare the two $Glc_1Man_9GlcNAc_2$ curves in Figure 1B), suggesting that calnexin binds to each with similar affinity. Clearly, calnexin does not require the two outermost mannose residues (A and B in Figure 1A) nor the penultimate mannose residue D for binding. Given that there was no binding to $Glc_1Man_4GlcNAc_2$, it appears that an internal mannose residue on the nonglucosylated arm of the $Glc_1Man_9GlcNAc_2$ oligosaccharide is also important for recognition by calnexin.

The finding that radiolabeled $Glc_1Man_4GlcNAc_2$ does not bind to calnexin in a direct binding assay stands in apparent contrast to the observation that the Glcα1−3Manα1−2Manα1−2Man tetrasaccharide (contained within $Glc_1Man_4GlcNAc_2$) is the most potent inhibitor of calnexin binding to $Glc_1Man_9GlcNAc_2$ (Table 1). This likely reflects the fact that radiolabeled $Glc_1Man_4GlcNAc_2$ in the direct binding assay is present at much lower concentrations (up to 10^4 -fold lower depending on intracellular mannose pool size) than the concentration of tetrasaccharide employed in the binding inhibition experiments. Presumably, calnexin can bind to the $Glc_1Man_4GlcNAc_2$ species but it is a much poorer ligand than the intact $Glc_1Man_9GlcNAc_2$ oligosaccharide.

Comparison of Calnexin and Calreticulin Binding to the $Glc_1Man_9GlcNAc_2$ Oligosaccharide. Spiro and co-workers recently reported that calreticulin, like calnexin, binds the

Table 2: Inhibition of $Glc_1Man_9GlcNAc_2$ Binding to Calreticulin[a]

inhibitor	concentration providing 50% inhibition (mM)
$Glc\alpha OCH_3$	$\gg 2.5$[b]
Glcα1−3Man	0.5
Glcα1−3Glc	0.4
Glcα1−4Glc	1.8
Glcα1−6Glc	$\gg 2.5$
Glcα1−3 Manα1−2Man	0.005
Glcα1−3Manα1−2Manα1−2Man	0.001

[a] Equal samples of GST−CRT immobilized on glutathione−agarose (0.7 μM final concentration) were incubated with $[^3H]Glc_1Man_9GlcNAc_2$ oligosaccharide and potential inhibitors as indicated. Specifically bound radioactivity was determined and the results are presented as the concentration of inhibitor that produced 50% inhibition of binding.[b] No detectable inhibition at a concentration of 2.5 mM.

$Glc_1Man_9GlcNAc_2$ oligosaccharide (*22*). We confirmed this finding by incubating CRT-His, immobilized on Ni−agarose, with a mixture of radiolabeled $Glc_{0-3}Man_9GlcNAc_2$ oligosaccharides, eluting bound oligosaccharide, and analyzing by HPLC. Calreticulin bound specifically to the $Glc_1Man_9GlcNAc_2$ species (data not shown).

To further compare the oligosaccharide binding specificities of calnexin and calreticulin, we tested a variety of mono-, di-, and oligosaccharides for their abilities to inhibit the binding of $[^3H]Glc_1Man_9GlcNAc_2$ to GST−CRT immobilized on glutathione−agarose (Table 2). The results were remarkably similar to those obtained above with CNX-His (compare Table 2 with Table 1). The smallest inhibitory compounds were the disaccharides, Glcα1−3Man and Glcα1−3Glc, with calreticulin exhibiting a distinct preference for the 1−3 linkage over the 1−4 or 1−6 linkages. Furthermore, the trisaccharide, Glcα1−3Manα1−2Man, and the tetrasaccharide, Glcα1−3Manα1−2Manα1−2Man, were ∼100-fold and ∼500-fold more potent inhibitors than the disaccharides, respectively. These findings indicate that calreticulin, like calnexin, recognizes all four sugar residues on the glucosylated α3 branch of the $Glc_1Man_9GlcNAc_2$ oligosaccharide.

Spiro and co-workers have also reported that the innermost α6-linked Man residue of the $Glc_1Man_9GlcNAc_2$ oligosaccharide is essential for recognition by calreticulin (residue E in Figure 1A) (*22*). This is consistent with our finding that calnexin is unable to bind the $Glc_1Man_4GlcNAc_2$ species that lacks this residue (Figure 1B). Collectively, the results suggest that calreticulin and calnexin share identical oligosaccharide binding specificities. If so, it would be expected that they would exhibit the same relative binding affinities for the $Glc_1Man_9GlcNAc_2$ oligosaccharide. To test this possibility, GST−CRT was immobilized on glutathione agarose and incubated with radiolabeled oligosaccharide and increasing amounts of soluble CNX-His (Figure 2). CNX-His inhibited oligosaccharide binding to GST−CRT with 50% inhibition occurring at 0.7 μM CNX-His, the same concentration as the immobilized GST−CRT in the assay. Since no association of calnexin and calretriculin could be detected under the conditions of the binding assay (data not shown), the observed inhibition occurred through competition for oligosaccharide ligand rather than through some allosteric interaction between the two proteins. Therefore, these findings suggest that the two lectins possess similar binding affinities for $Glc_1Man_9GlcNAc_2$.

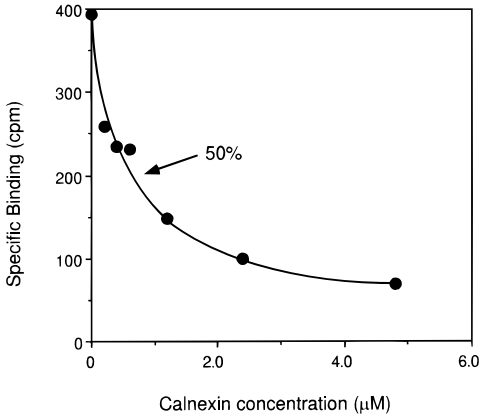


FIGURE 2: Calnexin and calreticulin bind oligosaccharide with similar affinities. Identical samples of GST−CRT immobilized on glutathione−agarose (0.7 μM) were incubated with a subsaturating amount of $[^3H]Glc_1Man_9GlcNAc_2$ and increasing concentrations of soluble CNX-His (as indicated). Specifically bound radioactivity was determined and plotted against the concentration of soluble calnexin in the incubation.

Effects of EGTA, ATP, and DTT on the Lectin Functions of Calnexin and Calreticulin. A previous report indicated that the ER lumenal domain of calnexin binds both Ca^{2+} and Mg-ATP and that removal of Ca^{2+} or addition of Mg-ATP leads to oligomerization of calnexin and an increase in its sensitivity to exogenous protease (*36*). Consequently, it was of interest to test whether the conformational alterations induced by these treatments affects oligosaccharide binding. However, we first wished to confirm that the removal of Ca^{2+} and the addition of Mg-ATP are accompanied by conformational changes in calnexin. As shown in Figure 3A, CNX-His was incubated with or without EGTA or Mg-ATP as indicated and its sensitivity to digestion with proteinase K was determined. Consistent with previous work (*36*), treatment of CNX-His with EGTA resulted in increased sensitivity to proteinase K digestion. This increased sensitivity was only marginally reversed upon readdition of calcium (data not shown). Surprisingly, Mg-ATP did not alter the susceptibility of CNX-His to digestion. Neither treatment affected the protease sensitivity of GST, which was included as a control. Identical samples were also examined by nondenaturing PAGE to determine whether EGTA or Mg-ATP treatment influenced the oligomerization state of these molecules (Figure 3B). Treatment of CNX-His with EGTA, but not Mg-ATP, resulted in increased oligomerization of calnexin. These results confirm that removal of bound Ca^{2+} from calnexin by EGTA treatment alters the conformation of calnexin such that the molecule is more sensitive to proteolysis and results in the formation of higher order oligomers. However, the lack of effect of Mg-ATP is in direct contrast to what has been previously reported (*36*).

Calreticulin also binds Ca^{2+} (*5*) and has been suggested to bind Mg-ATP as well (*33*). We attempted to assess conformational changes associated with removal of bound Ca^{2+} or the addition of Mg-ATP using a variety of proteases as conformational probes. Unfortunately, we were unable to obtain information on conformational changes since calreticulin was extremely sensitive to proteolysis regardless of whether these cofactors were present (data not shown). Nevertheless, we proceeded to test whether the removal of bound Ca^{2+} or the addition of Mg-ATP influences the ability

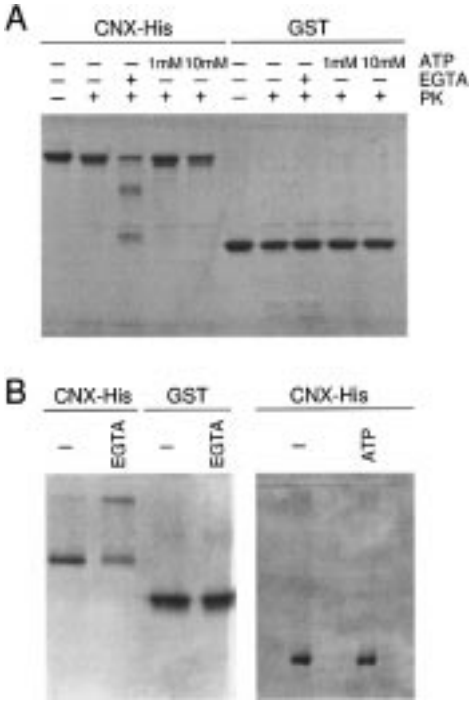


FIGURE 3: Effects of EGTA and Mg-ATP on the conformation of calnexin. (A) Identical samples of CNX-His immobilized on Ni−agarose were incubated with or without 30 μg of proteinase K, 10 mM EGTA, or 1 or 10 mM ATP as indicated for 30 min at 30 °C. Total proteins were analyzed by SDS−PAGE and visualized with Coomassie blue staining. As a control protein, GST bound to glutathione−agarose was subjected to identical treatments. (B) Identical samples of CNX-His (not immobilized) were incubated with or without 10 mM EGTA or 10 mM ATP as indicated for 30 min at 30 °C. Incubation mixtures were analyzed by nondenaturing PAGE and proteins were visualized with Coomassie blue. GST was included as a control.

of either calnexin or calreticulin to bind the $Glc_1Man_9GlcNAc_2$ oligosaccharide.

As shown in Figure 4, incubation with EGTA abolished oligosaccharide binding by CNX-His. This effect was completely reversible when 10 mM Ca^{2+} was reintroduced after EGTA treatment. Thus, the conformational changes associated with removal of bound Ca^{2+} appear to interfere with the function of calnexin's lectin site. Similar to the results obtained with calnexin, EGTA treatment profoundly inhibited oligosaccharide binding by CRT-His. Again, this effect could be reversed upon readdition of Ca^{2+}, although not as completely as observed for calnexin.

CNX-His was also incubated with radiolabeled oligosaccharide and a variety of nucleotides. To exclude the possibility that the recombinant CNX-His was purified with bound ATP, CNX-His was treated with apyrase and found to have no effect on oligosaccharide binding (data not shown). There was also no effect of adding 1 mM ATP on oligosaccharide binding in either the presence or absence of 5 mM Mg^{2+}. At 10 mM ATP there was a 60% enhancement of oligosaccharide binding, but this effect was independent of Mg^{2+}. A similar enhancement was observed with 10 mM ATPγS, ADP, and AMP (data not shown). The high concentrations of nucleotide required to produce this enhancement, the lack of Mg^{2+} requirement, and the fact that ATP, ADP, AMP, and ATPγS produced the same effect all suggest that the enhancement is nonspecific. Interestingly, the phenomenon was not observed with 10 mM GTP.

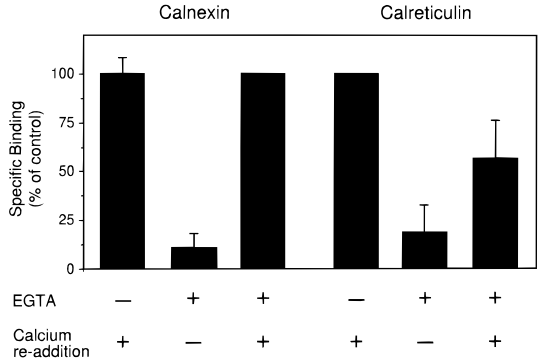


FIGURE 4: Treatment of calnexin and calreticulin with EGTA abolishes oligosaccharide binding. CNX-His and CRT-His immobilized on Ni−agarose were incubated with 10 mM EGTA for 30 min at 30 °C. The samples were cooled to room temperature and radiolabeled $Glc_1Man_9GlcNAc_2$ oligosaccharide either was added directly or the beads were first washed to remove EGTA and then buffer containing 10 mM $CaCl_2$ was added prior to addition of oligosaccharide. These treatments did not result in any loss of CNX-His or CRT-His from the Ni−agarose beads (data not shown). As a control, the immobilized proteins were treated in the same manner as in the EGTA washout−$CaCl_2$ addition experiment but with the omission of EGTA. Specific oligosaccharide binding was determined and expressed as a percentage of the control value. Control values were 380 cpm for CNX-His and 150−360 cpm (depending on the experiment) for CRT-His.

Oligosaccharide binding by CRT-His was also assayed in the presence of 10 mM ATP. Similar to the situation with CNX-His, an enhancement of oligosaccharide binding was reproducibly observed, although it was somewhat less than that detected with CNX-His, i.e., 15−30% (data not shown).

Calnexin contains at least one disulfide bond that has been implicated in its chaperone function (*24*, *36*, *37*). Calreticulin also contains a disulfide bond but its location differs from that in calnexin (*48*). To address whether disulfide bonds are required for the lectin properties of calnexin or calreticulin, samples of CNX-His and CRT-His immobilized on Ni−agarose were incubated with 10 mM DTT for 30 min at 30 °C. The DTT was removed with several rapid washes in binding buffer prior to incubation with radiolabeled oligosaccharide. These conditions were sufficient to reduce disulfide bonds in CNX-His and CRT-His as evidenced by their slower mobility on SDS−PAGE relative to unreduced samples (data not shown). Although disulfide reduction has been previously shown to perturb calnexin function in vivo or in microsomes (*24*, *37*) and to prevent ATP binding by calnexin's ER lumenal domain in vitro (*36*), it did not alter the oligosaccharide binding properties of CNX-His (Figure 5). By contrast, treatment of CRT-His with DTT inhibited oligosaccharide binding by 70%. Thus, the single disulfide bond in calreticulin appears to play an important role in the maintenance of a functional oligosaccharide binding site.

Mapping Oligosaccharide Binding Segments in Calnexin and Calreticulin. To map the oligosaccharide binding segments in calnexin and calreticulin, deletion mutants were constructed as fusion proteins with glutathione S-transferase. Linear representations of calnexin and calreticulin are shown in Figure 6A with homologous regions depicted by large rectangles and the positions of two characteristic tandem repeat motifs indicated by the numbers 1 and 2. The deletion constructs are depicted in Figure 6B along with the results of binding assays with radiolabeled oligosaccharide. Oli-

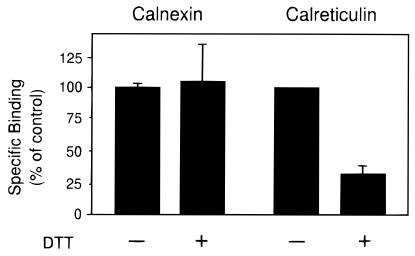


FIGURE 5: Effect of disulfide reduction on oligosaccharide binding by calnexin and calreticulin. CNX-His and CRT-His immobilized on Ni−agarose were incubated in the absence or presence of 10 mM DTT for 30 min at 30 °C. This treatment did not result in any loss of protein from the agarose beads (data not shown). The beads were subsequently washed several times in binding buffer to remove the DTT and then were incubated with radiolabeled oligosaccharide. Specific binding was determined and expressed as a percentage of the control sample that was not exposed to reducing agent. Control values were 120 cpm for CNX-His and 180−380 cpm (depending on the experiment) for CRT-His.

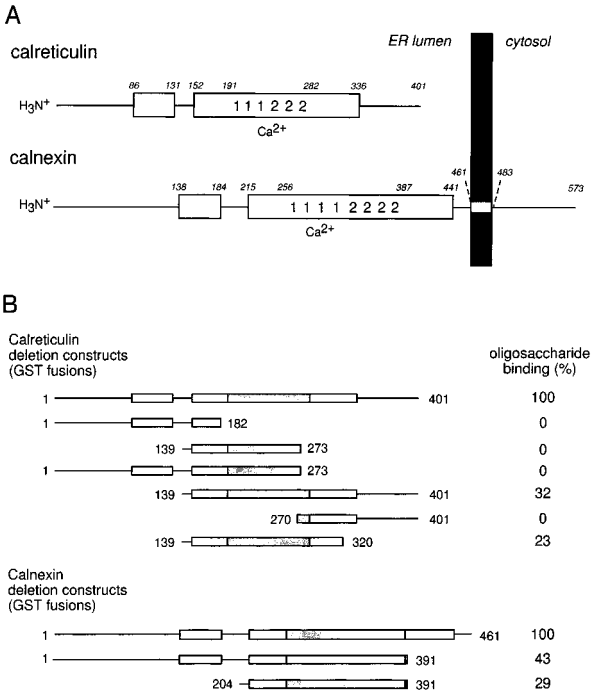


FIGURE 6: Location of the oligosaccharide binding site in calnexin and calreticulin. (A) Schematic representations of calnexin and calreticulin showing their topology as well as regions of homology between the two molecules (large rectangles). Repeat motif 1 (I-DPD/EA-KPEDWDD/E) and motif 2 (G-W- -P-I-NP-Y) are indicated, as are the high-affinity calcium binding sites (*1*, *2*, *5*, *6*). In calreticulin, the segment containing the repeat motifs is also know as the proline-rich P domain (*2*). (B) Mapping oligosaccharide binding segments of calnexin and calreticulin. Deletion mutants of calnexin and calreticulin were expressed and purified as GST fusion proteins, immobilized on glutathione−agarose, and assayed for specific oligosaccharide binding. For these experiments, 4000 cpm of oligosaccharides purified either from Chinese hamster ovary cells or from *alg8* yeast cells was used. Binding was compared to GST−CRT and GST−CNX in each experiment and is expressed as a percentage of the binding observed with these full-length controls. Values are the mean of 2−3 independent experiments. Shaded boxes denote the segment containing the two repeat motifs.

gosaccharide binding is expressed as a percentage of the binding observed with the full-length ER lumenal constructs. Given the small amounts of oligosaccharide available, it was not possible to assess the binding affinities for each deletion construct.

Both GST−CRT (residues 1−401) and GST fused to the ER lumenal domain of calnexin (residues 1−461) bound oligosaccharide, indicating that for both chaperones fusion to GST supports proper folding of their lectin sites (Figure 6B). For calreticulin, constructs encompassing segments of the protein other than the tandem repeat sequences failed to bind oligosaccharide. These constructs correspond to the N-terminal 45% (residues 1−182) and the C-terminal 33% (residues 270−401) of the calreticulin polypeptide chain. By contrast, a central segment (residues 139−320) that encompassed all of the repeat sequences retained the ability to bind oligosaccharide, although binding was reduced to about one-quarter that of the full-length molecule. Further truncation of this functional segment at its C-terminus (139−273), which removed part of the last motif 2 sequence, abolished oligosaccharide binding. Similar results were obtained for calnexin. Calnexin mutant 204−391, which also spanned the repeat motifs, was capable of binding oligosaccharide at about 30% of the level observed for the full-length construct. Thus, although N- and/or C-terminal truncation of calnexin and calreticulin reduced oligosaccharide binding, a homologous region centered on the tandem repeat motifs could be identified as a segment that retains the ability to bind oligosaccharide.

DISCUSSION

Previously we demonstrated that the ER lumenal domain of calnexin has the capacity to bind to the early N-linked oligosaccharide processing intermediate, $Glc_1Man_9GlcNAc_2$ (*21*). Recently, the same observation was made for its soluble ER homologue, calreticulin (*22*). In both studies, the single terminal glucose residue was shown to be crucial for recognition by these chaperones. To explore further the relationship between calnexin and calreticulin, we set out to compare their oligosaccharide binding specificities in detail. The results demonstrate that the lectin sites in both chaperones recognize the entire glucosylated α3-linked branch of the oligosaccharide, consisting of the sequence Glcα1−3Manα1−2Manα1−2Man. Furthermore, not only the terminal glucose residue but also the 1−3 linkage to the penultimate mannose residue were shown to be important recognition elements. We cannot exclude the possibility that residues located more internally than the tetrasaccharide are recognized as well, such as the *β*-linked mannose residue of the core (see Figure 1A), because potential inhibitors larger than the tetrasaccharide were not available for testing. However, the Asn-linked GlcNAc residue can be eliminated as a possible recognition element since this residue was reduced to the corresponding alcohol in these studies, and oligosaccharides lacking this residue have previously been shown to bind well to calreticulin (*22*).

The similarities in binding specificity between calnexin and calreticulin also extend to the α6-linked branch of the oligosaccharide (residues A−E in Figure 1A). That there might be interactions beyond those observed with the glucosylated arm of the oligosaccharide was initially suggested by the finding that polymeric yeast mannan inhibits calnexin binding to the $Glc_1Man_9GlcNAc_2$ oligosaccharide (Table 1) and also binds directly to calreticulin (*49*). Subsequent binding studies using glucosylated oligosaccharides possessing progressively fewer mannose residues revealed that calnexin is unable to bind detectably to the

$Glc_1Man_4GlcNAc_2$ oligosaccharide but binds the $Glc_1Man_{7,8,9}GlcNAc_2$ species (Figure 1B). Thus terminal mannoses are not essential for binding but an internal Manα1Man linkage appears to be important for recognition by calnexin. The same results were recently obtained by Spiro and co-workers for calreticulin. In addition, they were able to identify the determinant essential for recognition by calreticulin as the most internal Manα1−6Man branch point (ref 22; residue E in Figure 1A). Therefore, calnexin and calreticulin appear to possess identical oligosaccharide binding specificities. This conclusion is also supported by our demonstration that the two chaperones compete for the $Glc_1Man_9GlcNAc_2$ oligosaccharide with the same relative binding affinities.

It is interesting to examine the binding specificities of calnexin and calreticulin in light of the recently determined NMR structure of the $Glc_1Man_9GlcNAc_2$ oligosaccharide (*50*). The structure is highly extended from the single terminal glucose to the GlcNAc residue involved in linkage to asparagine. Consequently, the glucosylated α3-linked branch that is recognized by both chaperones is completely accessible. By contrast, the Manα1−6Man branch point that is also important for recognition is largely inaccessible. Molecular dynamics simulations suggest that the α6-linked Man could be involved in an extensive hydrogen-bond network involving water molecules and the α3-linked Man of the glucosylated branch, resulting in the glucosylated branch adopting a single conformation. Loss of the α6-linked Man could result in a more flexible glucosylated branch and hence a structure poorly recognized by either chaperone (R. Woods, M. Wormald, and R. Dwek, personal communication). The structure of the $Glc_3Man_4GlcNAc_2$ oligosaccharide is currently being determined to test the validity of this suggestion.

Using deletion analysis, we attempted to identify segments of calnexin and calreticulin that bind oligosaccharide. Constructs containing N- or C-terminal segments that lacked all or a portion of the central tandem repeat motifs failed to bind oligosaccharide. By contrast, constructs containing the complete motifs but lacking N- and/or C-terminal segments retained oligosaccharide binding capability, albeit at ∼20−45% the level of the full-length proteins. These findings identify a central homologous region of the two proteins containing their distinctive tandem repeat motifs as a segment capable of binding oligosaccharide. However, the reduced binding observed with deletion constructs containing this segment suggests either that the optimal lectin site may be larger or that the constructs may misfold to varying extents. We favor the latter possibility since the deletion constructs were significantly more susceptible to proteolysis than the full-length fusion proteins (data not shown).

The repeat motifs are also the site of high-affinity calcium binding for both calnexin and calreticulin (*5*, *6*). Colocalization of oligosaccharide and calcium binding sites is reminiscent of the carbohydrate-recognition domains of C-type lectins. This class of lectin requires calcium for oligosaccharide binding in a process involving direct interactions between sugar hydroxyl groups and the metal ion (*51*). However, calnexin and calreticulin lack the consensus sequence characteristic of the carbohydrate-recognition domains in C-type lectins or, for that matter, in other major lectin classes as well. Consequently, these proteins appear to possess a novel type of lectin binding site that involves the motif 1 and motif 2 tandem repeats and that may bind oligosaccharide in a manner distinct from other classes of lectins. It is noteworthy that the same tandem repeat motifs have been found in calmegin, a Ca^{2+} binding protein specifically expressed during male meiotic germ cell development that is required for sperm fertility (*52*−*54*), suggesting that calmegin may also function as a lectin.

Several properties of calnexin and calreticulin have been described that have been proposed to influence their chaperone functions. They are calcium-binding proteins (*5*, *6*), contain at least one disulfide bond (*24*, *36*, *48*), and appear to bind adenosine-containing nucleotides (*33*, *36*). We examined these properties to determine their effect on lectin function. Our results indicate that calcium is essential for the lectin properties of calnexin since EGTA treatment abrogated oligosaccharide binding. Consistent with previous findings (*36*), EGTA treatment also increased calnexin's sensitivity to protease digestion and enhanced its assembly into oligomeric forms. Since calnexin is not a C-type lectin, the results are most easily interpreted in terms of calcium being required to maintain the overall conformation of calnexin, including its lectin site. It is interesting that calcium readdition following EGTA treament could fully restore oligosaccharide binding but not calnexin's resistance to protease digestion. Presumably the readdition of calcium permits the folding of the lectin site to full functionality but flanking portions of the protein do not fully acquire a compact, protease-resistant conformation. Previous reports indicating the importance of calcium in the binding of calnexin to glycoproteins both in vitro (*39*) and in vivo (*38*) are consistent with our findings, because it is well established that calnexin binding to most glycoproteins depends on a functional lectin site (*23*, *25*). We also determined that bound calcium is essential for oligosaccharide binding by calreticulin. It is difficult to relate this finding to glycoprotein binding since there are conflicting reports concerning the role of calcium in the binding of calreticulin to various proteins. Calcium did not appear to be required for calreticulin binding to various denatured proteins or glycoproteins (*33*), yet it was essential for the binding of calreticulin to glycosylated laminin (*40*).

We also established that DTT treatment had no effect on oligosaccharide binding by calnexin, indicating that its disulfide bond(s) is not essential for its lectin properties. Interestingly, addition of DTT to intact microsomes under conditions where calnexin is reduced resulted in a loss of binding to in vitro translated/translocated influenza hemagglutinin (*24*). The opposite effect was observed when MDCK cells were treated with DTT. Interactions between calnexin and the secretory glycoprotein, gp80, were dramatically prolonged (*37*). Thus, although not required for calnexin's lectin function, the disulfide bond(s) in calnexin may be required for some other aspect of its function as a molecular chaperone. Possibilities include the structural maintenance of a polypeptide binding site (*21*, *26*, *27*) or a binding site for putative cochaperones or regulatory factors. In contrast to calnexin, DTT treatment substantially inhibited calreticulin's ability to bind oligosaccharide. Calreticulin possesses a single disulfide bond between Cys^{120} and Cys^{146} (*48*). Only the former residue is conserved in calnexin, indicating that the two chaperones differ in the location of their disulfide bonds. Apparently, the disulfide bond in

calreticulin plays a significant role in maintaining a functional lectin site. The loss of this bond may be the reason for the reduced oligosaccharide binding observed with deletion constructs lacking the N-terminal 138 residues of calreticulin (Figure 6B).

The ER lumenal domain of calnexin has been reported to bind Mg-ATP with an accompanying increase in its protease sensitivity and the formation of oligomers (*36*). We were unable to reproduce such conformational effects in the present study. Furthermore, our findings indicate that the presence of Mg-ATP does not significantly affect calnexin's lectin function. While the addition of Mg-ATP slightly enhanced oligosaccharide binding, the effect appeared to be nonspecific. This was suggested by the high concentration of nucleotide required to enhance oligosaccharide binding, the lack of a requirement for Mg^{2+}, and the ability of a range of adenosine-containing nucleotides to produce the same effect. Calreticulin has also been suggested to bind Mg-ATP based on its ATP-dependent dissociation from various denatured proteins in vitro (*33*). However, as was the case for calnexin, only a slight enhancement in oligosaccharide binding was observed in the presence of Mg-ATP. These results do not rule out the possibility that ATP binding or hydrolysis may be involved in other aspects of calnexin or calreticulin function, such as polypeptide-mediated binding or release of bound glycoproteins, much like other well-characterized chaperones (*55*).

Given that the lectin sites of calnexin and calreticulin exhibit the same oligosaccharide binding specificities and affinities, are similarly affected by cofactors, and are related in primary sequence, it is interesting to speculate on the basis for their observed differences in binding to glycoprotein substrates. While there are some common substrates for calnexin and calreticulin (*9, 10, 41*−*43*), there are a growing number of examples where binding to some substrates is exclusive to one or the other chaperone (*9, 16, 42*−*45*). For example, the binding of human class I histocompatibility molecules to either calnexin or calreticulin appears to be dictated by the assembly state of these molecules. Calnexin binds exclusively to the free heavy-chain subunit, whereas calreticulin appears to bind only following assembly of the heavy chain with the β_2-microglobulin subunit (*45*). Murine class I molecules differ in that calnexin appears to be the main chaperone associated with both free and β_2-microglobulin-associated heavy chains (*16, 56*), although low levels of calreticulin binding to the β_2-microglobulin-associated form have recently been reported (*57*). The results presented here would suggest that the lectin specificities of calnexin and calreticulin are not sufficient to provide the observed differences in overall substrate specificity. One possible explanation could be that different spatial orientations of oligosaccharide chains in different glycoproteins might favor interaction either with the membrane-constrained calnexin or with the lumenally oriented calreticulin. The fact that human class I heavy chains possess a single N-linked oligosaccharide versus two or three oligosaccharides in the murine homologues could potentially account for their differences in chaperone interaction. Furthermore, the transfer of human class I molecules from calnexin to calreticulin at the time of assembly with β_2-microglobulin could be a consequence of a different orientation of its oligosaccharide in the two-folding states (*43*). A much simpler explanation is that calnexin and calreticulin differ in their recognition of polypeptide segments of unfolded glycoproteins. That calreticulin is capable of binding to polypeptide in addition to the $Glc_1Man_9GlcNAc_2$ oligosaccharide is well established. For example, calreticulin has been purified from cell extracts by affinity chromatography using the peptide KLGFFKR as ligand (*30*). This peptide corresponds to a highly conserved region in the cytoplasmic domain of the α subunit of integrins. In addition, we have reported that calreticulin binds to protein disulfide isomerase via its P domain, a region that also contains the lectin site (*32*). However, in contrast to its lectin properties, this interaction was inhibited by Ca^{2+}. Calnexin has also been implicated in binding to polypeptide segments because its interactions with glycoproteins are maintained following complete removal of oligosaccharide chains through endoglycosidase H digestion. Of the wide array of membrane and soluble glycoproteins tested in this manner (*21, 27, 28, 31*), only ribonuclease B has been shown to dissociate from calnexin upon deglycosylation (*34, 35*). Assessment of the possibility that calnexin and calreticulin differ in their recognition of peptide motifs within unfolded or unassembled glycoproteins will be greatly facilitated by the reconstitution of their binding interactions with diverse glycoproteins in vitro.

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