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The Molecular Chaperone Calnexin Binds $Glc_1Man_9GlcNAc_2$ Oligosaccharide as an Initial Step in Recognizing Unfolded Glycoproteins*

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Calnexin is a molecular chaperone that resides in the membrane of the endoplasmic reticulum. Most proteins that calnexin binds are *N*-glycosylated, and treatment of cells with tunicamycin or inhibitors of initial glucose trimming steps interferes with calnexin binding. To test if calnexin is a lectin that binds early oligosaccharide processing intermediates, a recombinant soluble calnexin was created. Incubation of soluble calnexin with a mixture of $Glc_{0-3}Man_9GlcNAc_2$ oligosaccharides resulted in specific binding of the $Glc_1Man_9GlcNAc_2$ species. Furthermore, $Glc_1Man_{5-7}GlcNAc_2$ oligosaccharides bound relatively poorly, suggesting that, in addition to a requirement for the single terminal glucose residue, at least one of the terminal mannose residues was important for binding. To assess the involvement of oligosaccharide-protein interactions in complexes of calnexin and newly synthesized glycoproteins, α_1-antitrypsin or the heavy chain of the class I histocompatibility molecule were purified as complexes with calnexin and digested with endoglycosidase H. All oligosaccharides on either glycoprotein were accessible to this probe and could be removed without disrupting the association with calnexin. Furthermore, the addition of 1 M α-methyl glucoside or α-methyl mannoside had no effect on complex stability. These findings suggest that once complexes between calnexin and glycoproteins are formed, oligosaccharide binding does not contribute significantly to the overall interaction. However, it is likely that the binding of $Glc_1Man_9GlcNAc_2$ oligosaccharides is a crucial event during the initial recognition of newly synthesized glycoproteins by calnexin.

Calnexin (previously known as p88 or IP90) is a resident protein of the endoplasmic reticulum (ER)[1] that was originally identified by virtue of its transient association with assembling class I histocompatibility molecules (1). Subsequently, calnexin has been found associated with folding and assembly intermediates of a wide array of soluble and membrane proteins (2–4). These include subunits of the T cell receptor (2, 5), membrane immunoglobulin (2), class II histocompatibility molecules (6, 7), $\alpha\beta_1$ integrins (8), influenza hemagglutinin (HA) (9), vesicular stomatitis virus G protein (9), as well as many monomeric secretory glycoproteins such as α_1-antitrypsin and transferrin (4, 10).

Binding of calnexin to most proteins occurs rapidly following (and possibly during) their synthesis. Its dissociation appears to correlate with folding or assembly events. In the case of transferrin and influenza HA, calnexin binds to incompletely oxidized folding intermediates and dissociates at about the time fully disulfide-bonded molecules are formed (4, 9). For the major secretory glycoprotein of Madin-Darby canine kidney cells, gp80, calnexin dissociation correlates with gp80 precursor folding as judged by the differential susceptibility to proteinase K of calnexin-bound versus released molecules (11). Furthermore, the dissociation of calnexin from the gp80 precursor can be blocked by modulating disulfide bond formation with either dithiothreitol or diamide. In addition to folding, subunit assembly can occur while polypeptides are associated with calnexin. Assembly of the heavy chain, β_2-microglobulin (β_2m), and peptide ligand of mouse class I histocompatibility molecules takes place on calnexin. Formation of the complete ternary complex is required for efficient dissociation of calnexin since incomplete complexes lacking β_2m or peptide exhibit prolonged binding to calnexin (12). By contrast, in human cells, assembly of class I heavy chain-β_2m heterodimers appears to be sufficient to trigger calnexin dissociation (13, 14). Class II histocompatibility molecules assemble into a large complex consisting of three invariant chains and two $\alpha\beta$ dimers while associated with calnexin. Addition of the final $\alpha\beta$ dimer correlates with calnexin dissociation (7). The consistent observation that calnexin interacts with incompletely folded or assembled proteins, but is absent from native (or nearly native) structures, suggests a molecular chaperone function for calnexin.

Although direct evidence demonstrating that calnexin facilitates protein folding or assembly events is lacking, it is clear that calnexin is a component of the quality control system that retains misfolded or incompletely folded/assembled proteins in the ER. For both class I and class II histocompatibility molecules, dissociation of calnexin correlates closely with the transport of these molecules out of the ER (1, 7). Furthermore, incompletely assembled forms of class I molecules (12), the T cell receptor (5), and integrins (8) remain stably associated with calnexin and are not

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[1] The abbreviations used are: ER, endoplasmic reticulum; HA, hemagglutinin; β_2m, β_2-microglobulin; HPLC, high performance liquid chromatography; PBS, phosphate-buffered saline; PAGE, polyacrylamide gel electrophoresis; endo H, endoglycosidase H; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propansulfonic acid.

transported. Misfolded mutant proteins such as those produced by the metabolic incorporation of amino acid analogs (4), the ts045 mutant of vesicular stomatitis virus G protein (9), and a truncated variant of α_1-antitrypsin (10) are also retained as complexes with calnexin. A direct demonstration of calnexin's capacity to retain incompletely assembled proteins was provided by co-expressing calnexin along with free class I heavy chains or heavy chain-β_2m heterodimers in *Drosophila* cells (15). The aberrant transport of these assembly intermediates out of the ER that occurs in *Drosophila* cells was impeded when calnexin was co-expressed. In a separate study, retention by calnexin was demonstrated by expressing either full-length calnexin or a truncated variant that lacks an ER localization signal in cells that also express the T cell receptor ϵ subunit or class I heavy chain subunit. Whereas these subunits were retained in the ER in association with intact calnexin, their association with truncated calnexin resulted in redistribution to the Golgi complex or cell surface (16, 17).

The most extensively studied molecular chaperones are soluble ATPases that are members of the Hsp 60 and 70 families of heat shock proteins. Through ATP-driven cycles of binding and release these chaperones act to stabilize unfolded proteins and prevent their aggregation (18). Calnexin differs substantially from Hsp 60 and 70 chaperones in that it is an integral membrane protein of 574 residues containing a single type 1 transmembrane domain (5, 19). Its unglycosylated ER luminal domain (464 amino acids) contains several regions with homology to calreticulin, and its cytoplasmic domain contains phosphorylation sites for casein kinase II (20, 21) as well as an ER localization signal (-RKPRRE) at the C terminus (16). Like calreticulin, the major calcium binding protein of the ER lumen, calnexin binds calcium (11). This property could potentially be involved in regulating calnexin associations because chelation of calcium *in vitro* has been shown to disrupt complexes of calnexin and a truncated variant of α_1-antitrypsin (10). Similarly, treatment of cells with the Ca^{2+} ionophore A23187 prevents binding of calnexin to the class I H-2L^d molecule (22). By sequence analysis, calnexin has no apparent nucleotide binding sites, and induction by stress has not been demonstrated (23). All of these differences from Hsp chaperones may reflect a unique function and/or mechanism of action of calnexin in protein biogenesis in the ER.

One of the most conspicuous characteristics of calnexin is its apparent specificity for glycoproteins that possess Asn-linked oligosaccharides. Ou *et al.* (4) originally showed that pretreatment of human hepatoma cells with tunicamycin prevents the formation of complexes between calnexin and many newly synthesized secretory glycoproteins. This finding was subsequently reproduced with the integral membrane glycoproteins, influenza HA and vesicular stomatitis virus G. Furthermore, pretreatment with the α-glucosidase I and II inhibitors, castanospermine and 1-deoxynojirimycin, blocks the binding of calnexin to HA or G proteins (9). In contrast, the α-mannosidase inhibitor 1-deoxy-mannojirimycin has no effect. The data obtained with the oligosaccharide processing inhibitors suggests that glucose trimming of newly synthesized glycoproteins is a requirement for calnexin binding. Additional studies showed that the HA glycoprotein isolated from a complex with calnexin likely contains oligosaccharides with one or two terminal glucose residues and that the vesicular stomatitis virus G ts045 mutant, which possesses monoglucosylated oligosaccharides for extended periods in the ER, exhibits prolonged association with calnexin. All of these observations led to the proposal that for calnexin binding to occur, a glycoprotein must possess oligosaccharides that have undergone partial trimming from the initial $Glc_3Man_9GlcNAc_2$ structure to one containing either two or, more likely, a single glucose residue (9, 24).

One interpretation of these results is that calnexin is a lectin with specificity for monoglucosylated oligosaccharides. However, as discussed recently (23), other interpretations are equally plausible. In fact some observations are difficult to reconcile with recognition of monoglucosylated oligosaccharide being a prerequisite for calnexin binding. For example, both the T cell receptor ϵ subunit that lacks Asn-linked oligosaccharides and a recombinant form of the multidrug resistance P glycoprotein in which *N*-glycosylation sites are absent, form stable and long-lived complexes with calnexin (16, 25). Conversely, removal of the transmembrane and cytoplasmic domains from the T cell receptor α subunit almost completely eliminates calnexin binding, but this truncated subunit still possesses its full complement of *N*-linked oligosaccharides (26). Finally, cross-linking experiments have indicated that Asn-linked oligosaccharides are unlikely to be the sole mode of association between calnexin and class I heavy chains (26). This latter study identified a region encompassing the transmembrane domain and three flanking amino acids of the heavy chain as a site of interaction with calnexin.

In an effort to clarify the involvement of Asn-linked oligosaccharides in the binding of calnexin to newly synthesized glycoproteins, we tested the ability of calnexin to function as a lectin by assaying its ability to bind to a series of oligosaccharide-processing intermediates. Additionally, the relative contribution of protein-carbohydrate and protein-protein interactions in maintaining the association between calnexin and newly synthesized soluble or transmembrane glycoproteins was assessed. Our findings indicate that calnexin is indeed a lectin with specificity for the $Glc_1Man_9GlcNAc_2$ oligosaccharide. They also lead us to propose a model in which binding to this oligosaccharide is a critical event that occurs during initial recognition of newly synthesized glycoproteins by calnexin. However, once calnexin-glycoprotein complexes are formed, protein-protein interactions become predominant and oligosaccharide binding contributes minimally, if at all, to the overall association.

EXPERIMENTAL PROCEDURES

Production and Analysis of Dolichol-linked Oligosaccharides—[^{3}H]Dolichol-linked oligosaccharides were produced by high-efficiency labeling of Chinese hamster ovary cells with [2-^{3}H]mannose cleaved from dolichol pyrophosphate and reduced with $NaBH_4$ essentially as described earlier (27). Approximately 5×10^4 cpm of free reduced oligosaccharides were obtained from each 100-mm dish of cells. Oligosaccharides were fractionated with an ISCO dual-pump HPLC system, employing Chemresearch software (ISCO Corp.), with a 150-mm Adsorbosphere HS 3-μm silica column (Alltech) as described (28). Radioactive material eluting from the column was identified with a Radiomatic A-140 detector with either a 1.0- or 2.5-ml detector cell; the former was chosen for experiments requiring optimal peak resolution and the latter for those needing optimal sensitivity.

Preparation of Soluble Hexahistidine-tagged H-2K^b and Calnexin Proteins—Soluble class I H-2K^b heavy chain (with His_6 at the C terminus) in association with β_2m was purified from culture medium of transfected *Drosophila melanogaster* SC2 cells by Ni-NTA-agarose (Qiagen) and Mono Q (Pharmacia Biotech Inc.) anion exchange chromatography as described previously (29). To prepare His_6-tagged soluble calnexin, canine calnexin cDNA under control of the metallothionein promoter in plasmid pRMHa3 (30) was digested with *Dsa*I and *Sal*I to remove the segment corresponding to the transmembrane and cytosolic domains. The deleted segment was replaced with an oligonucleotide cassette such that the final construct encoded the complete ER luminal domain of calnexin terminating at Pro^{462} followed by the sequence SRRSWGSHHHHHH. The plasmid was co-transfected with a neomycin resistance plasmid, phshs-neo, into *Drosophila* SC2 cells, and G418-resistant stable cell lines were obtained (31). Cells grown in Insect-Xpress medium (Whittaker) at 23 °C were treated with 1 mM $CuSO_4$ for 3 days to induce expression of soluble calnexin. Culture supernatants (2 litres) were concentrated 10-fold by ultrafiltration through an Amicon YM30 membrane and were dialyzed against phosphate-buffered saline, pH 7.4 (PBS), prior to loading onto a 5-ml column of Ni-NTA-agarose.

After recirculating the sample overnight, the column was washed extensively with PBS followed by 20 mM imidazole in PBS. Calnexin was eluted with 100 mM imidazole in PBS and then dialyzed against 50 mM Tris-Cl, pH 8.5. The sample was applied to a Mono Q 10/10 anion-exchange column (Pharmacia), which was subjected to a linear 0–600 mM NaCl gradient in 50 mM Tris, pH 8.5. Calnexin eluted at ~400 mM NaCl and was concentrated using a Centricon 30 concentrator (Amicon). Both soluble calnexin and H-2K^b were essentially homogeneous as judged by SDS-PAGE analysis and silver staining. About 0.5–1 mg of each protein was recovered per liter of culture supernatant.

Incubation of Radiolabeled $Glc_{0-3}Man_9GlcNAc_2$ Oligosaccharides with Immobilized Proteins—Each assay employed 6–7.5-μg samples of hexahistidine-tagged calnexin or class I H-2K^b molecule immobilized on 12–15 μl of nickel-agarose in binding buffer (10 mM Hepes-Na, pH 7.5, containing 0.15 M NaCl and 10 mM $CaCl_2$). Unless indicated otherwise, all procedures were performed at 23 °C. The agarose was washed briefly with binding buffer just prior to incubation with oligosaccharides, suspended in 100 μl of binding buffer containing approximately 20,000 cpm of a mixture of $Glc_{0-3}Man_9GlcNAc_2$ oligosaccharides, and incubated for 1 h with agitation on an orbital shaker at 200 rpm. The samples were then centrifuged for 5 min at 2,600 × *g*, and the supernatant was collected. The agarose beads were rinsed briefly with 100 μl of binding buffer and centrifuged as above, and the supernatant was collected. The agarose pellet was saved for later analysis (below). The two supernatants were pooled, 0.1 ml (approximate packed volume) of water-washed Dowex 50W-X8 (H^+ form) was added, and the samples were agitated at 300 rpm for 5 min. The supernatants were recovered; approximately 20 Amberlite MB-3 beads were added; and the samples were mixed at 350 rpm for 15 min. If all of the dye on the MB-3 beads changed color, indicating that salts remained in the sample, the process was repeated with additional beads. The supernatants were evaporated to dryness; dissolved in 200 μl of 80% acetonitrile, 20% water, 0.2% 1,4-diaminobutane; and divided into two equal portions, and each was analyzed separately by HPLC as described above with a 1.0-ml detector cell. In all experiments reported, similar results were obtained with each of the two samples.

To analyze the oligosaccharides that bound to the immobilized proteins, the nickel-agarose beads were subjected to a series of sequential washes. The first wash was performed briefly (1–2 min) at 4 °C with 100 μl of binding buffer followed by centrifugation for 5 min at 2,600 × *g* and recovery of supernatant. Four subsequent washes were then performed, each for 1 h at 23 °C with agitation at 200 rpm, consisting of 100 μl of binding buffer alone followed by binding buffer supplemented with (in order) 0.1 M α-methyl D-galactopyranoside, 0.1 M α-methyl D-mannopyranoside, or 0.1 M α-methyl D-glucopyranoside (Aldrich). The sixth and final wash was performed with agitation overnight at 23 °C with 100 μl of 0.1 M α-methyl D-glucopyranoside in binding buffer. An aliquot of each supernatant (10%) was analyzed by liquid scintillation counting, and the remaining six wash supernatants for each immobilized protein were pooled and treated with Dowex and Amberlite beads as described above. The entire eluate sample was then analyzed by HPLC with a 2.5-ml detector cell, which gives greater detector sensitivity relative to a 1.0-ml detector cell, but also increases peak widths and decreases peak resolution. To detect oligosaccharides, which remained bound to the proteins after the elution procedure, the nickel-agarose beads were boiled for 15 min in 100 μl of binding buffer. No tritium was detected in the resulting supernatants for either H-2K^b-agarose or calnexin-agarose.

Preparation of $[^3H]Glc_1Man_{5,6,7}GlcNAc_2$ Oligosaccharides and Incubation with Calnexin-agarose—Pure $[^3H]Glc_1Man_9GlcNAc_2$ was isolated from a mixture of $[^3H]Glc_{0-3}Man_9GlcNAc_2$ dolichol-linked oligosaccharides by preparative HPLC. $[^3H]Glc_1Man_{5,6,7}GlcNAc_2$ oligosaccharides were then generated by partial digestion with Jack bean α-mannosidase (Boehringer Mannheim), and their monosaccharide compositions were assigned by comparison of their elution times with those of related dolichol-linked oligosaccharides (27, 28). Two isomeric configurations are possible for $Glc_1Man_6GlcNAc_2$, and three are possible for $Glc_1Man_7GlcNAc_2$, but these were not characterized. $[^3H]Glc_1Man_8GlcNAc_2$ was detectable only after brief digestion times, and the quantities generated were insufficient for further analysis. A mixture of $[^3H]Glc_1Man_{5,6,7,9}GlcNAc_2$ oligosaccharides was prepared, and 2,000 cpm were analyzed directly by HPLC (see Fig. 3, starting fraction). Another portion (approximately 4,000 cpm) was dissolved in 100 μl of binding buffer and incubated with calnexin-agarose as described above. After collection of the supernatant (approximately 3,400 cpm) by centrifugation, the pellet was eluted with two aliquots of 100 μl of binding buffer containing 0.1 M α-methyl glucoside at 23 °C for 1 and 16 h, respectively. Approximately 1,000 cpm were recovered in the pooled eluates. Both the supernatant and eluate fractions were desalted and characterized by HPLC as described above, except that individual 0.5-ml fractions were collected, mixed with 4.0 ml of scintillation mixture, and analyzed with a liquid scintillation counter.

Isolation and Analysis of Radiolabeled Calnexin-Class I Heavy Chain Complexes—Transfected *D. melanogaster* Schneider cells expressing canine calnexin and either H-2K^b or D^b class I heavy chains were maintained in Schneiders insect medium (Sigma) supplemented with 10% fetal bovine serum, antibiotics, and 0.5 mg/ml Geneticin (Life Technologies, Inc.). Synthesis of calnexin and class I heavy chains was induced by treatment with 1 mM $CuSO_4$ for 24 h. Following induction, cells (1–3 × 10^7) were incubated for 15 min at 23 °C in methionine-free Graces insect medium (Sigma) and then were resuspended at 5 × 10^7 cells/ml in Met-free Graces medium supplemented with 0.5 mCi/ml [^{35}S]Met (>800 Ci/mmol; Amersham Corp.). Radiolabeling was carried out for 10 min at 23 °C and then the cells were washed twice with PBS, pH 7.4, followed by lysis at 0.2–0.3 × 10^7 cells/ml in PBS containing 0.5% digitonin (Sigma), 10 mM iodoacetamide, 1% aprotinin, 0.25 mM 4-(2-aminoethyl)benzenesulfonyl fluoride, and 10 mg/ml each of chymostatin, leupeptin, antipain, and pepstatin. After 30 min on ice, lysates were divided into 1-ml aliquots and centrifuged at 16,000 × *g* for 10 min. To isolate calnexin-class I heavy chain complexes, lysates were incubated for 2 h at 4 °C with 20 μl of a rabbit antiserum raised against a peptide corresponding to the C-terminal 14 amino acids of canine calnexin (15). Immune complexes were recovered by shaking with 15 μl of protein A-agarose (Life Technologies, Inc.) for 1 h at 4 °C. The agarose beads were washed 3 times with 0.2% digitonin in PBS and resuspended in 50 μl of wash buffer. To this bead-bound complex of calnexin and class I heavy chain was added either 5–20 milliunits (IUB) of endo H (New England Biolabs), 1 M α-methyl D-mannopyranoside, or 1 M α-methyl D-glucopyranoside, and the samples were incubated at room temperature for 1–4 h. Control incubations with no additions were included for each experiment. At the end of the incubation, the beads were centrifuged, the supernatant was removed, and the beads were washed once with 50 μl of wash buffer that was pooled with the supernatant. The supernatants and washed beads were boiled in SDS-PAGE sample buffer containing 100 mM dithiothreitol and proteins were separated by SDS-PAGE using 10% gels and visualized by fluorography (1 M sodium salicylate). In addition to the nondenaturing conditions used for the endo H digestions outlined above, digestions performed under denaturing conditions (0.1% SDS) were included in each experiment to provide size standards for completely deglycosylated class I heavy chains (15).

Isolation and Analysis of Calnexin-α_1-Antitrypsin Complexes—Human HepG2 hepatoma cells were grown in α-minimum essential medium supplemented with 10% fetal bovine serum and antibiotics. Radiolabeling was performed as described for *Drosophila* cells except that the preincubation (30 min) and labeling (10 min) were performed at 37 °C in Met-free α-minimum essential medium supplemented with 9 mM Hepes and 2 mM glutamine. Cell lysis, isolation of anti-calnexin immune complexes, and digestion with endo H were also performed as described above except that 2% sodium cholate (Sigma) replaced digitonin in the lysis buffer and protein A-agarose beads were washed with 0.5% sodium cholate in PBS. Unlike the situation in *Drosophila* cells, calnexin binds to many radiolabeled proteins in HepG2 cells. In order to detect α_1-antitrypsin in the supernatant and bead fractions following endo H digestion of anti-calnexin immune complexes, it was necessary to reisolate with anti-α_1-antitrypsin antibody. Supernatant and bead fractions were boiled in 0.1 ml of PBS containing 0.2% SDS for 10 min and then 1.2 ml of PBS, pH 8, containing 1% Nonidet P-40 was added. The samples were incubated with 10 μl of rabbit anti-α_1-antitrypsin antibody (Calbiochem) for 2 h on ice, and immune complexes were recovered with protein A-agarose beads. The beads were washed 4 times with PBS containing 0.5% Nonidet P-40, and proteins were analyzed by SDS-PAGE as described above. Under these conditions there was no residual endo H activity during the immune isolation of α_1-antitrypsin.

RESULTS

Calnexin Selectively Binds $Glc_1Man_9GlcNAc_2$ from an Oligosaccharide Mixture—To obtain radiolabeled oligosaccharides that could be used in a direct test of binding by calnexin, we took advantage of the fact that the dolichol-linked oligosaccharide pool in Chinese hamster ovary cells can be efficiently labeled with [^{3}H]mannose (27). This pool consists of the full-length dolichol-linked oligosaccharide, $Glc_3Man_9GlcNAc_2$

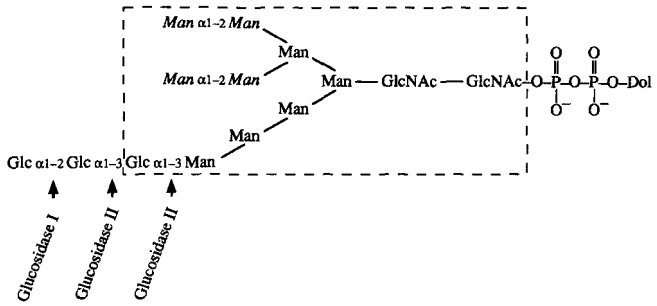

FIG. 1. **Structure of the full-length dolichol-linked oligosaccharide.** This oligosaccharide is transferred to Asn-*X*-Ser(Thr) sequences during translocation of nascent polypeptides into the ER lumen. It is subsequently processed through the action of α-glucosidases I and II within the ER and by α-mannosidases in both the ER and Golgi apparatus. The sites of cleavage by the glucosidases are indicated. The $Glc_1Man_9GlcNAc_2$ oligosaccharide that is bound by calnexin is enclosed by the *box*. Mannose residues removed by digestion with Jack bean α-mannosidase (see Fig. 3) are shown in *italics*.

(Fig. 1), as well as less abundant precursors possessing shorter oligosaccharide chains. After release from dolichol pyrophosphate with mild HCl treatment and reduction with $NaBH_4$, the oligosaccharides can be resolved with an HPLC system that separates neutral oligosaccharides on the basis of increasing size (28). Although the predominant radiolabeled dolichol-linked oligosaccharide is $Glc_3Man_9GlcNAc_2$ (Ref. 27 and references therein), HPLC analysis revealed appreciable quantities of precursors eluting as $Glc_{0-2}Man_9GlcNAc_2$ (Fig. 2*A*). These assignments are based on previous studies in which dolichol-linked oligosaccharides from normal and mutant Chinese hamster ovary lines labeled *in vivo* and *in vitro* were characterized (27, 28, 32). In addition, we found that only species expected to contain glucose residues were labeled after incubation of cells with [^{3}H]galactose, which is converted intracellularly into UDP-[^{3}H]glucose. The relative incorporation of label reflected the anticipated glucose contents of the oligosaccharides (data not shown). Thus, in cells incubated with [^{3}H]galactose, $Glc_3Man_9GlcNAc_2$ was by far the most efficiently labeled dolichol-linked oligosaccharide, with $Glc_{1-2}Man_9GlcNAc_2$ labeled in lesser amounts.

Solutions of [^{3}H]mannose-labeled $Glc_{0-3}Man_9GlcNAc_2$ were incubated with a soluble His_6-tagged form of calnexin, or, as a control, a soluble His_6-tagged form of the class I H-2K^b histocompatibility protein, each immobilized on nickel-agarose. After 1 h, the agarose was removed by centrifugation, and the supernatants were analyzed by HPLC. An incubation without agarose beads was also included. As shown in Fig. 2*A*, four distinct oligosaccharide species ($Glc_{0-3}Man_9GlcNAc_2$) were present in the sample incubated without agarose. After incubation with H-2K^b-agarose, the amount of each oligosaccharide remaining in solution was not significantly altered (data not shown). In contrast, the $Glc_1Man_9GlcNAc_2$ oligosaccharide was selectively depleted after incubation with calnexin-agarose. In several experiments, the amount of $Glc_1Man_9GlcNAc_2$ oligosaccharide recovered was reduced by 20–45%, whereas no depletion of other oligosaccharides from the mixture by calnexin could be detected (data not shown).

To examine material which specifically bound to calnexin, a six-step elution procedure (Table I) was employed with the H-2K^b- and calnexin-agarose samples recovered after the experiment. Each successive step involved a condition expected to be more effective for elution of bound oligosaccharide from calnexin. In total, about 80 cpm was recovered from H-2K^b-agarose, whereas approximately 560 cpm was recovered from calnexin-agarose. Surprisingly, a large fraction of the radioactive material eluted from calnexin-agarose in the initial steps

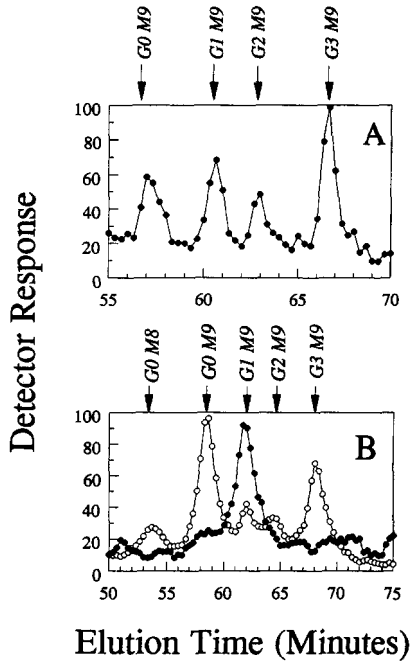

FIG. 2. **Selective binding of $Glc_1Man_9GlcNAc_2$ oligosaccharide by immobilized calnexin.** A mixture of $Glc_{0-3}Man_9GlcNAc_2$ oligosaccharides was dissolved in binding buffer (see "Experimental Procedures"), divided into three equal portions, and incubated for 1 h at 23 °C either alone, with H-2K^b-agarose, or with calnexin-agarose. *Panel A*, HPLC analysis of the oligosaccharide mixture incubated in the absence of immobilized protein. The *arrowheads* indicate the elution times of the various oligosaccharides in the mixture. *Panel B*, HPLC analysis of oligosaccharides eluted from calnexin-agarose (*closed circles*). The elution profile of a mixture of the following oligosaccharide standards is also included (*open circles*): $Man_8GlcNAc_2$, $Man_9GlcNAc_2$, $Glc_1Man_9GlcNAc_2$, $Glc_2Man_9GlcNAc_2$, and $Glc_3Man_9GlcNAc_2$.

TABLE I

Elution of bound radioactive material from immobilized proteins

The agarose samples recovered from the experiment shown in Fig. 2 were sequentially eluted in a six-step procedure as described under "Experimental Procedures." The tritium content of 10% of each eluate was determined by liquid scintillation counting. Values listed in the table reflect the total estimated radioactivity recovered for each step.

Step	Glycoside	Time	Radioactivity eluted			
			Per step		Total	
			H-2K^b	Calnexin	H-2K^b	Calnexin
		h	*cpm*			
1		0.02	50	110	50	110
2		1	20	100	70	210
3	0.1 M αMe-Gal	1	10	120	80	330
4	0.1 M αMe-Man	1	0	80	80	410
5	0.1 M αMe-Glc	1	0	40	80	450
6	0.1 M αMe-Glc	16	0	110	80	560

that employed buffer alone or buffer plus α-methyl galactoside, a compound not expected to inhibit calnexin. These data suggested that the oligosaccharide was not tightly bound to calnexin. After the sequential elution procedure, no additional radioactivity was recovered by boiling the agarose. The fractions eluted from calnexin-agarose were then pooled and analyzed by HPLC (Fig. 2*B*). A single peak was observed that co-eluted with $Glc_1Man_9GlcNAc_2$. In other experiments, the small amount of radioactivity eluting from H-2K^b-agarose was analyzed by HPLC, but no discrete peaks were observed. In contrast, radioactivity recovered from calnexin-agarose always eluted as $Glc_1Man_9GlcNAc_2$.

$Glc_1Man_{5-7}GlcNAc_2$ Oligosaccharides Are Relatively Poor Ligands for Calnexin—The preceding experiments demon-

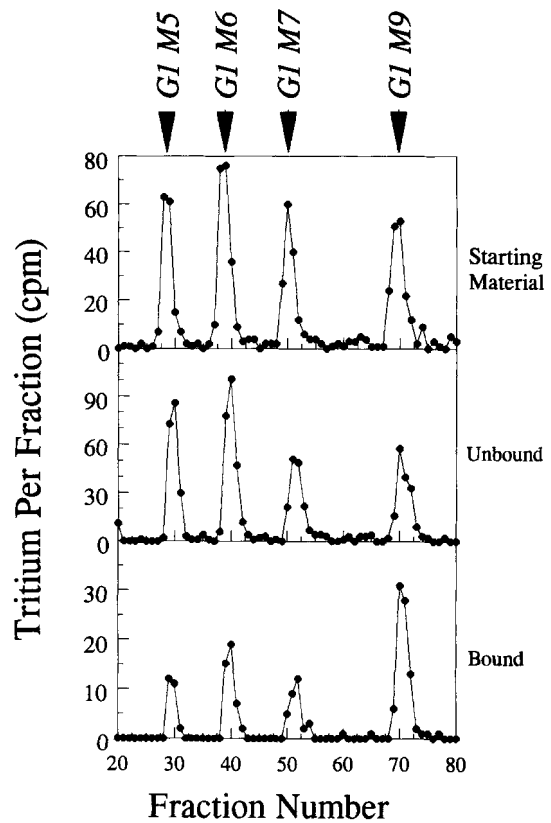

FIG. 3. **Binding of calnexin to mannosidase-treated glucosylated oligosaccharides.** A mixture of [^{3}H]$Glc_1Man_{5,6,7,9}GlcNAc_2$ oligosaccharides was incubated with calnexin-agarose. Unbound material was collected in the supernatant fraction following centrifugation, and bound material was collected by eluting the pellet with 0.1 M α-methylglucoside. The starting, unbound, and bound fractions were analyzed by HPLC. In each case, the flow rate was 1 ml/min, and fractions were collected every 0.5 min. Fraction 20 corresponds to 35 min in the solvent program. The elution positions of the various oligosaccharides are indicated by the *arrowheads*.

strated that calnexin exhibits a strong preference for the $Glc_1Man_9GlcNAc_2$ oligosaccharide over oligosaccharides that possess 9 mannoses and either 0, 2, or 3 glucose residues. The Glcα1,3 determinant is present at the nonreducing terminus of one of three mannose-containing branches present in $Glc_1Man_9GlcNAc_2$ (Fig. 1). To examine whether either of the two remaining branches on $Glc_1Man_9GlcNAc_2$ is important for binding to calnexin, purified [^{3}H]$Glc_1Man_9GlcNAc_2$ was digested with Jack bean α-mannosidase to generate glucosylated molecules with 5, 6, or 7 mannose residues. These were added to a sample of the undigested material, and the resulting mixture of [^{3}H]$Glc_1Man_{5,6,7,9}GlcNAc_2$ oligosaccharides was incubated with calnexin-agarose. The starting mixture, unbound radioactive material in the supernatant, and bound material eluted from the pellet were analyzed by HPLC (Fig. 3). Comparison of the relative amounts of each oligosaccharide in the bound fraction to the relative amounts in the starting material indicated a 3-fold enrichment of the $Glc_1Man_9GlcNAc_2$ species bound to calnexin-agarose. The other oligosaccharides were not significantly enriched. These data indicate that at least one of the mannosidase-sensitive residues on $Glc_1Man_9GlcNAc_2$ is important for binding to calnexin. It is not clear whether the $Glc_1Man_{5-7}GlcNAc_2$ oligosaccharides detected in the bound fraction were specificially bound to calnexin. These species were not significantly depleted from the bound fraction by repeating the experiment with a brief wash with binding buffer prior to the elution steps (data not shown). Thus it is possible that calnexin binds $Glc_1Man_{5,6,7}GlcNAc_2$ although less avidly than $Glc_1Man_9GlcNAc_2$.

Incubation of Calnexin-Glycoprotein Complexes with α-Methyl Glycosides—The finding that calnexin binds the $Glc_1Man_9GlcNAc_2$ oligosaccharide raises the question of whether this is the sole means of interaction between calnexin and newly synthesized glycoproteins or if protein-protein interactions are also involved. Because α-linked glucose and mannose residues participate in the binding of the $Glc_1Man_9GlcNAc_2$ oligosaccharide to calnexin (Figs. 2 and 3), immunoisolated calnexin-glycoprotein complexes were incubated with 1 M α-methyl glucoside or α-methyl mannoside in an effort to inhibit the oligosaccharide component of the interaction. In these experiments, *Drosophila* cells expressing canine calnexin and the heavy chain of either the H-2K^b or D^b mouse class I histocompatibility molecule were used as a convenient source of defined calnexin-glycoprotein complexes. Class I heavy chains are Type 1 transmembrane polypeptides that possess two to three *N*-linked oligosaccharides, and we have shown previously that in the absence of their partner subunits, β_2m and peptide ligand, heavy chains form long-lived complexes with calnexin in mouse cells or in transfected *Drosophila* cells (12, 15). In both mouse and transfected *Drosophila* cells, the calnexin-heavy chain interaction appears to depend on *N*-linked oligosaccharides since it is prevented by treatment of cells with the oligosaccharide processing inhibitor castanospermine.[2] The advantage of using the *Drosophila* transfectants in the present experiments is that the calnexin-heavy chain complex is the predominant species recovered with anti-calnexin antibodies.

Calnexin-heavy chain complexes were immunoisolated from digitonin lysates of metabolically radiolabeled *Drosophila* transfectants using an antibody raised against the carboxyl-terminal 14 amino acids of calnexin followed by collection on protein A-agarose. The agarose-bound calnexin-K^b or -D^b complexes were incubated with either 1 M α-methyl glucoside or α-methyl mannoside and then separated into supernatant (*S*) and agarose bead (*B*) fractions (Fig. 4). Following this treatment, nearly all heavy chains remained in the bead fraction associated with calnexin. Small amounts of heavy chain and calnexin were detected in the supernatant fraction at approximately the same ratio as observed in the agarose bead fraction. The amounts in the supernatant were variable from experiment to experiment and likely reflect the difficulty in sedimenting agarose beads efficiently through viscous solutions of 1 M glycoside. Similar results were obtained when the experiment was repeated using Nonidet P-40 rather than digitonin for cell lysis and immune isolation (data not shown). The inability to dissociate calnexin-heavy chain complexes under conditions known to be effective in dissociating other lectin-glycoprotein complexes (33) suggests that oligosaccharide-protein interactions may not contribute substantially to the calnexin-heavy chain association once complexes are formed. Alternatively, α-methyl glucoside and α-methyl mannoside may be inefficient competitors of the binding of calnexin to the $Glc_1Man_9GlcNAc_2$ oligosaccharide.

N-Linked Oligosaccharides Are Not Involved in Maintaining Stable Complexes between Newly Synthesized Glycoproteins and Calnexin—As an additional approach to assess the relative involvement of protein-oligosaccharide *versus* protein-protein interactions in complexes between calnexin and newly synthesized glycoproteins, endo H was used as a probe to monitor accessibility of *N*-linked oligosaccharides in calnexin-glycoprotein complexes. In the first set of experiments, radiolabeled calnexin-class I heavy chain complexes were isolated from dig-

[2] A. Vassilakos, unpublished observations.

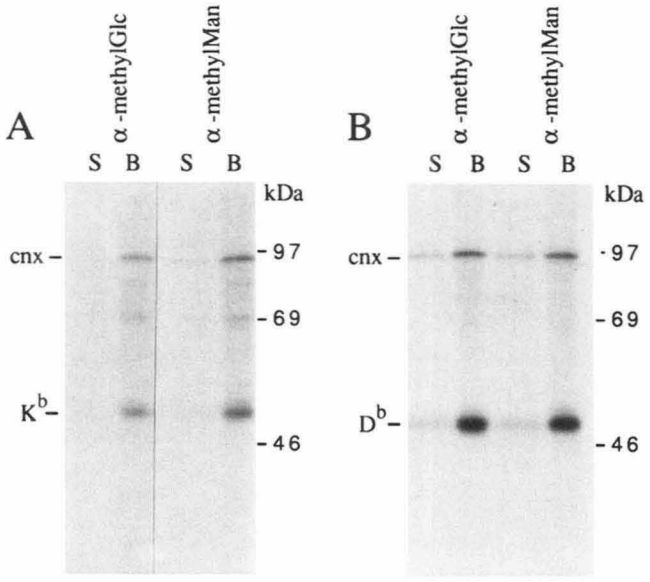

FIG. 4. **Incubation of calnexin-glycoprotein complexes with α-methyl glycosides.** *Drosophila* cells expressing H-2K^b or D^b heavy chains and calnexin were radiolabeled with [^{35}S]Met for 10 min. Cells were lysed in buffer containing 0.5% digitonin and calnexin-heavy chain complexes were isolated with anti-calnexin antibody followed by protein A-agarose. The agarose beads were incubated with either 1 M α-methyl glucoside (*α-methylGlc*) or α-methyl mannoside (*α-methylMan*) and then were centrifuged to form bead-bound (*B*) and supernatant (*S*) fractions. Radiolabeled proteins in these fractions were analyzed by SDS-PAGE. *Panel A*, effect of α-methyl glycosides on calnexin-K^b heavy chain complexes; *panel B*, effect of α-methyl glycosides on calnexin-D^b heavy chain complexes. The mobilities of calnexin (*cnx*), the K^b and D^b heavy chains, as well as molecular mass standards are indicated.

itonin lysates of *Drosophila* transfectants using anti-calnexin antibody followed by collection on protein A-agarose. Fig. 5 depicts the results of digesting calnexin-K^b heavy chain (*panel A*) and calnexin-D^b heavy chain (*panel B*) complexes with endo H and analyzing proteins in supernatant (*S*) and agarose bead (*B*) fractions. Remarkably, the two oligosaccharides on the K^b heavy chain and the three oligosaccharides on the D^b heavy chain were completely accessible to endo H, resulting in total deglycosylation of the heavy chains (compare the endo H-digested agarose bead fraction, *lane 6*, with the deglycosylated heavy chain standard, *lane 2*). Furthermore, removal of heavy chain oligosaccharides by endo H did not result in dissociation of heavy chains from calnexin. All of the deglycosylated heavy chains remained associated with calnexin in the bead-bound complex; none were present in the supernatant fraction (compare the endo H-digested *S* and *B lanes*). The same results were obtained when complexes of calnexin and the H-2L^d heavy chain were digested with endo H and also when the experiments were repeated using Nonidet P-40, CHAPS, or sodium cholate detergents in place of digitonin for cell lysis (data not shown). The experiment was also attempted under detergent-free conditions by delivering endo H via transient permeabilization at high pH into microsomes containing radiolabeled calnexin-heavy chain complexes (34). Subsequent immunoisolation of complexes with anti-calnexin antibodies revealed that, although the efficiency of endo H digestion was quite low, completely deglycosylated heavy chains could be recovered in association with calnexin (data not shown).

Calnexin binds not only to membrane-associated proteins such as class I molecules but also to a number of soluble, secretory glycoproteins (4). Since secretory glycoproteins lack transmembrane segments that have been implicated in the binding of membrane proteins to calnexin (26), they may rely more heavily on oligosaccharide-protein interactions to maintain stable associations with calnexin. Furthermore, experiments involving endo H digestion of complexes containing calnexin and secretory glycoproteins avoid the potential complication of the deglycosylated glycoprotein being unable to dissociate from calnexin due to immobilization within the same detergent micelle. Thus, to evaluate the role of *N*-linked oligosaccharides in the interaction between calnexin and a secretory glycoprotein, anti-calnexin immunoprecipitates from sodium cholate lysates of metabolically radiolabeled HepG2 cells were treated with endo H. Previous studies have shown that such immunoprecipitates contain complexes between calnexin and many secretory glycoproteins including α_1-antitrypsin, α_1-antichymotrypsin, α-fetoprotein, transferrin, complement component C3, and apoB-100 (4). Following separation into supernatant and bead fractions, the endo H-digested samples were boiled in SDS to disrupt calnexin-glycoprotein complexes (and to inactivate endo H) and then were subjected to a second round of immunoprecipitation with antibodies against α_1-antitrypsin (Fig. 5*C*). At least 50% of α_1-antitrypsin molecules present in complexes with calnexin could be completely deglycosylated by endo H (compare the endo H-digested agarose bead fraction, *lane 6*, with the deglycosylated α_1-antitrypsin standard, *lane 2*). Furthermore, the deglycosylated molecules remained associated with calnexin in the bead fraction (compare the endo H-digested *S* and *B lanes*). These results were consistent with those obtained using calnexin-class I heavy chain complexes and suggest that once calnexin-glycoprotein complexes are formed, *N*-linked oligosaccharides are dispensable in maintaining an association with calnexin. The less efficient deglycosylation observed with α_1-antitrypsin relative to class I heavy chains may reflect the fact that a large number of substrates that compete for endo H are present in calnexin immunoprecipitates from HepG2 cells, whereas calnexin-heavy chain complexes constitute the major species immunoprecipitated from *Drosophila* transfectants.

DISCUSSION

Our findings indicate that the ER luminal domain of calnexin has the capacity to bind to the early *N*-linked oligosaccharide processing intermediate, $Glc_1Man_9GlcNAc_2$. The binding is specific because calnexin selected this oligosaccharide from a mixture containing $Glc_{0-3}Man_9GlcNAc_2$ species. Calnexin also preferentially selected this oligosaccharide from a mixture containing $Glc_1Man_{5,6,7,9}GlcNAc_2$. Thus, in addition to recognizing a single terminal glucose, calnexin also appears to recognize at least 1 terminal mannose residue in $Glc_1Man_9GlcNAc_2$ (Fig. 1). More extensive specificity studies and a detailed analysis of the binding kinetics were precluded by the limited quantities of purified, soluble calnexin available.

In none of our experiments was hydrolysis of any oligosaccharide detected, suggesting that calnexin is a lectin rather than an enzyme that modifies oligosaccharide structure, *e.g.* a glycosidase. However, unlike most lectins that bind large oligosaccharides with micromolar dissociation constants (33), calnexin binds the $Glc_1Man_9GlcNAc_2$ oligosaccharide weakly; extensive dissociation of the complex is detectable following short incubations in buffers lacking sugar haptens. Furthermore, primary sequence comparisons revealed that calnexin lacks the carbohydrate-recognition domains characteristic of each of the three major groups of animal lecins. These include the C- (Ca^{2+}-dependent) type, the galectins (S-type), and the P- (mannose 6-phosphate) type lectins (35). Although some lectins, such as concanavalin A, are capable of binding to processing intermediates of Asn-linked oligosaccharides, none exhibit the binding specificity associated with calnexin (35, 36). Consequently, we conclude that calnexin constitutes a new type of

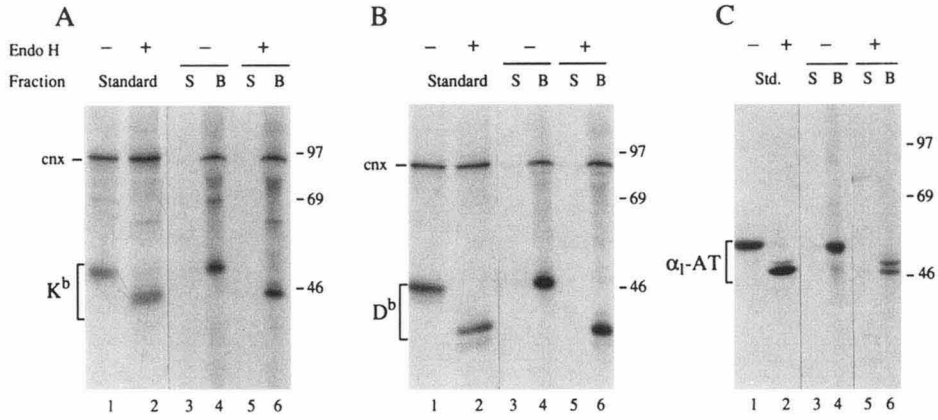

FIG. 5. **Digestion of calnexin-glycoprotein complexes with endo H.** *Panels A* and *B*, radiolabeled calnexin-heavy chain complexes were isolated with anti-calnexin antibody followed by protein A-agarose as described in the legend to Fig. 4. The agarose beads were incubated in the absence or presence of endo H and then were centrifuged to form bead-bound (*B*) and supernatant (*S*) fractions (*lanes 3–6*). In addition, separate samples of calnexin-heavy chain complexes were dissociated in SDS and incubated in the absence or presence of endo H to provide mobility standards of glycosylated and deglycosylated heavy chains, respectively (*lanes 1* and *2*). To confirm that the heavy chain standards were completely deglycosylated, limited endo H digests were performed to visualize all partially deglycosylated species (data not shown). The major heavy chain bands in *lane 2*, *panels A* and *B*, do indeed represent the K^b heavy chain lacking its two oligosaccharides and the D^b heavy chain lacking its three oligosaccharides, respectively. *Panel C*, HepG2 cells were radiolabeled for 10 min with [^{35}S]Met. Cells were lysed in buffer containing 2% sodium cholate, and calnexin-glycoprotein complexes were isolated, digested with endo H, and separated into supernatant and bead-bound fractions as in *A* and *B*. Subsequently, the fractions were boiled in PBS containing 0.2% SDS, adjusted to 1% Nonidet P-40, and subjected to a second round of immunoprecipitation using anti-α_1-antitrypsin antibody (*lanes 3–6*). *Lanes 1* and *2* contain standards of glycosylated and deglycosylated α_1-antitrypsin, respectively. Complete deglycosylation of the α_1-antitrypsin standard was confirmed as described for the class I heavy chains.

lectin with unique specificity for the $Glc_1Man_9GlcNAc_2$ oligosaccharide.

Treatment of cultured cells with either tunicamycin or the glucosidase inhibitors, castanospermine or 1-deoxynojirimycin, results in a dramatic block in the formation of complexes between calnexin and a large array of newly synthesized glycoproteins (4, 9). Consistent with previous speculation (9, 24), the demonstration that calnexin binds selectively to the $Glc_1Man_9GlcNAc_2$-processing intermediate provides a clear molecular explanation for the action of these drugs. The remarkable efficacy of tunicamycin and the glucosidase inhibitors also underscores the crucial role that oligosaccharide binding must play in the formation and/or maintenance of calnexin-glycoprotein complexes.

Two observations lead us to propose that the binding of $Glc_1Man_9GlcNAc_2$ oligosaccharide is important in the initial formation of complexes with calnexin, but it cannot be responsible for maintaining complexes once they are formed. First, the apparent affinity of calnexin for the oligosaccharide is low and it is unlikely that complexes maintained through this interaction alone would survive the prolonged immune isolation procedures used for their purification. It is possible that calnexin could possess more than one carbohydrate binding site or could exist as a homooligomer (features that might increase the avidity of the interaction), but such properties would not be effective for the many glycoproteins having a single oligosaccharide chain. Second, in purified complexes of calnexin with either class I heavy chains or α_1-antitrypsin, all oligosaccharides are accessible to endo H, and their removal is not accompanied by complex dissociation. Consequently, we envision a two-step mechanism for the interaction of newly synthesized glycoproteins with calnexin (Fig. 6).

In this model, the initial interaction between nascent glycoproteins and calnexin occurs through binding of the $Glc_1Man_9GlcNAc_2$ oligosaccharide. This intermediate first appears on nascent chains (37) and, given the rapid association observed between calnexin and some glycoproteins (1, 7, 9, 11), it is possible that the initial binding of calnexin could occur co-translationally. Having been brought in proximity to calnexin through this initial interaction, the unfolded glycoprotein then binds to calnexin through segments of its polypeptide chain. The physical features recognized by calnexin in this second interaction are largely unknown, but they may be hydrophobic segments or patches as is thought to be the case for chaperones of the Hsp 60 and Hsp 70 families (18). Due to the reversibility of carbohydrate binding, dissociation of the oligosaccharide likely occurs as the polypeptide chain and calnexin interact, perhaps aided by new steric constraints placed on the oligosaccharide and calnexin. Subsequently, the polypeptide folds in association with calnexin and in conjunction with folding enzymes until sites for calnexin binding are buried in the folded molecule. Whether folding (and assembly) occurs while the polypeptide is tethered to calnexin or during cycles of calnexin binding and release is unknown. Although calnexin does not possess consensus sequences for nucleotide binding, it has recently been demonstrated that the ER luminal domain of calnexin binds ATP *in vitro* (38). This raises the possibility that calnexin could undergo cyclic interactions with unfolded glycoproteins in a manner analogous to other chaperones. Conceptually, the two step binding model is reminiscent of the mechanism that regulates leukocyte localization in the vasculature. Circulating leukocytes are brought into proximity with endothelial cells via transient selectin-carbohydrate interactions followed by tight adhesion mediated by integrins and Ig superfamily adhesion receptors (39).

The existence of monoglucosylated oligosaccharides on newly synthesized glycoproteins is prolonged by a cycle of deglucosylation by glucosidase II and reglucosylation via an ER enzyme known as UDP-glucose:glycoprotein glucosyltransferase (40, 41). The latter enzyme reglucosylates only nonnative glycoproteins and it has been suggested by Helenius that the purpose of the cycle is to ensure that nonnative glycoproteins continually oscillate between calnexin-bound and unbound states. Once a glycoprotein folds, it is no longer a substrate for reglucosylation and it dissociates from calnexin (24). Although attractive, this model requires that oligosaccharide structure is the main regulator not only of calnexin binding but of release as well. This is inconsistent with our observation that oligosaccharides do not participate in maintaining calnexin-glycoprotein complexes. Rather, we suggest that the function of reglucosylation may be to provide newly synthesized glycoproteins with additional opportunities to bind calnexin on those occasions when

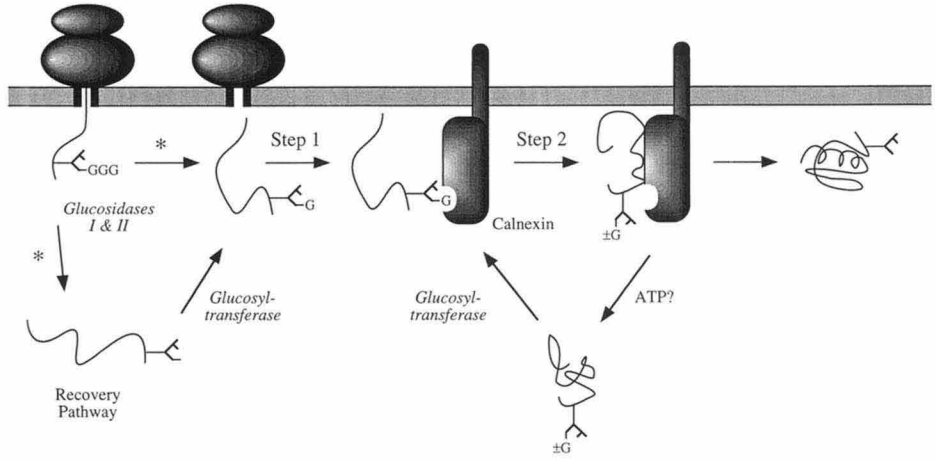

FIG. 6. **Two-step model for binding of calnexin to unfolded glycoproteins.** Following removal of two glucose residues, newly synthesized glycoproteins initially contact calnexin via their monoglucosylated oligosaccharide chains (*Step 1*). Having been placed in proximity to calnexin by this first interaction, the unfolded polypeptide associates directly with additional sites on calnexin (*Step 2*). The oligosaccharide chains are accessible to exogenous probes at this stage. Reglucosylation by UDP-glucose:glycoprotein glucosyltransferase may play an important role in recovering proteins that have lost all three glucoses prior to any contact with calnexin or in facilitating rebinding to calnexin during cycles of folding. *Asterisks* indicate steps blocked by castanospermine and 1-deoxynojirimycin.

all three glucoses are removed before an initial interaction with calnexin can take place (Fig. 6, *Recovery Pathway*). This may explain the increased level of association with calnexin that occurs for some glycoproteins during the 5–10-min period postsynthesis (4, 9). Reglucosylation followed by two-step binding may also be the means whereby a folding glycoprotein can rebind to calnexin if dissociation from calnexin occurs before folding is complete (Fig. 6).

Why has calnexin evolved to utilize oligosaccharide binding for its initial interaction with unfolded glycoproteins? Unlike soluble chaperones of the ER, calnexin is constrained within the ER membrane and, at least in some cell types, it may be associated with components of the translocation apparatus (19). Given this disposition, polypeptide binding sites on calnexin may have limited access to nascent glycoproteins. Oligosaccharide addition, being among the first covalent modifications that occur on nascent chains, ensures that a conserved and well exposed site for calnexin binding is present at an early stage in the folding of nascent chains. Calnexin could even be associated with glucosidase II, poised to capture the monoglucosylated oligosaccharide as soon as it is formed. For some proteins, however, this initial stage of oligosaccharide capture can be bypassed since they bind to calnexin even though they are unglycosylated. This is the case for the T cell receptor ϵ subunit (16) and also for variants of the multidrug resistance P glycoprotein (25) and the class I H-2 L^d heavy chain (42) that lack consensus sequences for *N*-glycosylation. Presumably these proteins have accessible sites on their polypeptide chains for interaction directly with calnexin.

As suggested previously (24), the finding that calnexin binds to the $Glc_1Man_9GlcNAc_2$ processing intermediate may explain why eukaryotes from yeasts to humans initiate *N*-glycosylation with a common, preassembled oligosaccharide (Fig. 1). If the purpose of early attachment of oligosaccharide was solely to ensure that segments of a folding polypeptide remained exposed to solvent, then oligosaccharides of diverse size and composition would likely suffice. Calnexin is an abundant protein in virtually all eukaryotic cell types examined including yeast (in which it has an essential function),[3] plants, worms, and mammals (see Ref. 23 for references). Its intimate association with *N*-linked oligosaccharides as part of its quality control and chaperone functions may be the major factor responsible for preserving the *en bloc* mode of glycosylation that originated in early eukaryotes.

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[3] M. Jannatipour and L. A. Rokeach, submitted for publication.

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