Calreticulin was identified by immunochemical and sequence analyses to be the higher molecular mass (60 kDa) component of the polypeptide doublet previously observed in a rat liver Golgi endomannosidase preparation obtained by chromatography on a Glcα1→3Man-containing matrix. The affinity for this saccharide ligand, which paralleled that of endomannosidase and was also observed with purified rat liver calreticulin, suggested that this chaperone has lectin-like binding properties. Studies carried out with immobilized calreticulin and a series of radiolabeled oligosaccharides derived from N-linked carbohydrate units revealed that interactions with this protein were limited to monoglucosylated polypeptides. Although optimal binding occurred with Glc3Man3GlcNAc, substantial interaction with calreticulin was retained after sequential trimming of the polypeptide portion down to the Glc2Man3GlcNAc stage. The α1–6-mannose branch point of the oligosaccharide core, however, appeared to be essential for recognition as Glc3Man3GlcNAc did not interact with the calreticulin. The carbohydrate-peptide linkage region had no discernible influence on binding as monoglucosylated oligosaccharides in N-glycosidic linkage interacted with the chaperone to the same extent as in their unconjugated state. The immobilized calreticulin proved to be a highly effective tool for sorting out monoglucosylated polypeptide oligosaccharides or glycopeptides from complex mixtures of processing intermediates. The copurification of calreticulin and endomannosidase from a Golgi fraction in comparable amounts and the strikingly similar saccharide specificities of the chaperone and the processing enzyme have suggested a tentative model for the dissociation through glucose removal of calreticulin-glycoprotein complexes in a post-endoplasmic reticulum locale; in this scheme, deglucosylation would be brought about by the action of endomannosidase rather than glucosidase 1.1

It has become apparent in recent years that N-linked oligosaccharides at an early stage of processing can play an important role in the quality control of the secretory pathway by influencing the folding and assembly of the proteins to which they are attached (1–3). More specifically, attention has been focused on the glucose residues that are initially present on the N-linked carbohydrate unit (Glc3Man3GlcNAc), since it has been noted that inhibition of glucose trimming through a blockade of the ER1-situated glucosidases may lead to accelerated degradation (4, 5) or delayed secretion (6–8) of various glycoproteins. The finding that retention of the triglucosyl sequence can result in such a profound effect on newly synthesized glycoproteins can be rationalized by reports that proteins with N-linked oligosaccharides bind to certain molecular chaperones only subsequent to the processing of the carbohydrate units to the monoglycosylated state (1). Most of the evidence for this lectin-like activity has been obtained through studies on calnexin (9, 10), a membrane-associated chaperone of the ER, although very recent observations, made while our investigations were in progress, have suggested on the basis of electrophoretic examinations that the luminal chaperone calreticulin can also bind glycoproteins after partial deglucosylation (11).

Our attention was drawn to the latter chaperone by the quite unexpected finding, from sequence analyses that the larger (mass of 60 kDa) of the two previously observed polypeptide components present in the ligand affinity chromatography-purified rat liver Golgi endomannosidase preparation (12) was indistinguishable from calreticulin. The copurification of endomannosidase and calreticulin on a Glc-Mann-Affi-Gel suggested that the chaperone has a saccharide affinity and provided the impetus for undertaking a detailed definition of its lectin-like properties toward N-linked oligosaccharides at various processing stages. The results obtained from studies with immobilized calreticulin provided some striking parallels between the saccharide specificity of this chaperone to those previously observed for endomannosidase (13, 14) and suggested the possibility that the two proteins may work in tandem in a post-ER location.
by Van et al., for a protein then termed CaBP3 (18), were chromatographed on a Glc-Man-Affi-Gel column under the conditions previously described (12). Aliquots of the eluted fractions were assayed for endomannosidase activity and were submitted to immunoblotting subsequent to polyacrylamide gel electrophoresis. Prior to carrying out the latter procedure, the protein in the sample to be examined (∼300 μl) was diluted with the addition of 20 to 30 volumes of acetonitrile at −20°C and after 3 h at that temperature collected by centrifugation (2,000 × g for 20 min). For subsequent study, the remainder of the neutralized glycine HCl buffer-eluted fractions that contained the endomannosidase activity were concentrated in an Ultrafree-CL filtration unit (Millipore) as described previously (12).

Preparation of Glycopeptides—The Glc-Man-Affi-Gel purified endomannosidase digestion preparation was submitted to 10% polyacrylamide gel electrophoresis in SDS and then electroblotted onto a polyvinylidene fluoride membrane (19) from Bio-Rad for 6 h (60 V) at 4°C in 10% w/v CAPS, pH 10.6 buffer. After visualizing the two protein bands (60 and 56 kDa) by a brief (1 min) exposure to 0.1% Poron A 5 in 1% acetic acid, they were separately excised, washed with water, and sent frozen to the Harvard University Microchemistry Facility. Under the direction of William S. Lane, solid phase-trypsin digestion was carried out on each protein band (3–μg sample) followed by reverse-phase high performance liquid chromatography of the resulting peptides. Several of the latter were then selected for amino acid sequencing by automated Edman degradation (20).

Polyacrylamide Gel Electrophoresis and Immunoblotting—The electrophoresis was carried out on 12% polyacrylamide gels (1.5 mm thick) overlaid with a 3.5% stacking gel according to the procedure of Laemmli (22). The trocellulose sheet (22), which was incubated with rabbit antiserum raised against rat liver calreticulin at 1:800 dilution, and after washing over the proteins, the proteins were exposed to 125I-labeled protein A as described previously (23). The proteins were resolved from other proteins on nitrocellulose sheet by transferring them in the presence of toluene. The oligosaccharides were isolated from the nitrocellulose gel by extraction with 50% ethanol and removal of the contaminating Man9GlcNAc, iodinated with 125I by the chloramine-T method (24), and after washing exposed to 125I-labeled protein A as described previously (23). The radioactive bands on the nitrocellulose gel were detected by autoradiography, while the standards were stained by India ink.

Preparation of Radiolabeled Oligosaccharides and Glycopeptides—Metabolically radiolabeled [14C]Glc,Man,GlcNAc and [14C]Man,Man,GlcNAc were isolated from thyroid glycoproteins after incubation of slices with [14C]glucose as described previously (14, 24); in this procedure, the protein in the sample to be examined (115,000 dpm) in 300 μl of 0.1 M sodium citrate buffer, pH 5.2, 5.5:3:1 (Solvent A) was equilibrated at room temperature with a 50 mM NaMES, pH 6.8, buffer containing 60 mM NaCl and 40 μM calcium acetate, prior to the application of [3H]labeled oligosaccharides (2,000–20,000 dpm) or glycopeptides (4–10 0 dpm) in 300 μl of this buffer. After addition to the column, the samples were allowed to interact with the immobilized calreticulin for 30 min prior to their elution with the equilibrating buffer at a flow rate of 6 ml/h, while 1-ml fractions were collected; aliquots were taken from each fraction for the determination of radioactivity by scintillation counting.

For chromatography of the series of purified oligosaccharides, H+-labeled Glc,Man,GlcNAc prepared from thyroid slices incubated with [2-3H]mannose, was included with each sample as an internal standard. For further purification, the oligosaccharide fractions resolved by the calreticulin column were desalted, after titration to pH 7.8 with NH4OH, by passage through columns containing Dowex 50-X2, 200–400 mesh (H+ form) overlaid with Dowex 1-X2, 200–400 mesh (acetate). Glycopeptides were freed from salt by adsorption to Dowex 50-X2 resin and subsequent elution with 1.5 n NH4OH, which was then removed by lyophilization. When not in use, the calreticulin-matrix column was kept at 2°C in the presence of 0.02% sodium azide.

Structural and Analytical Procedures—Endomannosidase digestions of oligosaccharides were carried out in a manner similar to that previously reported (13) with rat liver Golgi membranes (25 μg of protein) for 3 h at 37°C in the presence of 2 mM castanospermine, 10 mM EDTA, and 2 mM 1-deoxymannojirimycin, while treatment of glycopeptides with endo H (Genzyme) was performed as described previously (29). The products of these digestions were examined by thin layer chromatography after removal of salt and protein. Reduction of oligosaccharides was achieved with NaBH4 under previously specified conditions (13). Protein was determined by the procedure of Peterson (30) using bovine serum albumin as a standard.

Thin Layer Chromatographic Procedures—Resolution of small oligosaccharides, including fragments from acetylation treatment, was achieved on plastic sheets precoated with cellulose (0.1 mm thickness, Merck) in pyridine/ethyl acetate/water, 5:5:3:1 (Solvent A), whereas larger oligosaccharides were separated on plastic sheets coated with Silica Gel 60 (0.2 mm thickness, Merck) in 1-propanol/acetic acid/water, 3:3:2 (Solvent System E). All chromatography was carried out at a temperature of 20°C, except when specified otherwise. The resultant oligo- and oligosaccharide fractions were obtained by Pronase digestion of the deprotected oligosaccharides. The heptasaccharide fraction obtained by preparative thin layer chromatography was eluted from the plate with water, and the resultant eluates, after extraction with peroxide-free ether to remove scintillants, were passed through small columns of Dowex 50 (H+) and Dowex 1 (acetate).

Radioactivity Measurements—Liquid scintillation counting was carried out with Ultrafluor with a Beckman LS 7500 instrument; double channel measurements were made when 14C- and 3H-labeled oligosaccharides were present together. Detection of radioactive components was accomplished with the use of X-Omatic AR film (Eastman Kodak) at −80°C either by autoradiography of immunoblots on nitrocellulose sheets or by fluorography of thin layer chromatographic plates that were sprayed with a scintillation mixture containing 2-methylnaphthalene (31).

RESULTS

Presence of Calreticulin in the Rat Liver Golgi Endomannosidase Preparation Obtained by Glc-Man-Affi-Gel Chromatography—An examination of amino acid sequences occurring in the electrophoretically resolved protein components (60 and 56 kDa) of the ligand affinity-purified endomannosidase preparation (Fig. 1) indicated quite unexpectedly that the higher molecular mass constituent was closely related to calreticulin, while the faster 56 kDa-band had no similarity to any previ-
In order to determine if calreticulin by itself binds to the Glc-Man-Affi-Gel matrix, we chromatographed a sample of the purified protein on this column under the same conditions as employed for the Triton-solubilized Golgi membranes and observed on the basis of immunoblotting that this protein was indeed retained and eluted under the same conditions as the endomannosidase activity (Fig. 2). Moreover, when a Golgi extract was placed on the Affi-Gel column, the immunologically detected calreticulin and enzymatically monitored endomannosidase activity is present in the unfractinated rat liver Golgi membranes (Fig. 1).

In view of our previously demonstrated understanding that endomannosidase has a high degree of specificity for monoglucosylated polymannose oligosaccharides, which is responsible for its retention on Glc-Man-Affi-Gel (12), we explored the possibility that the binding of calreticulin to this matrix has a similar basis. Indeed, when an incompletely resolved mixture of radiolabeled Man\(_{9}\)GlcNAc and Glc\(_{6}\)Man\(_{9}\)GlcNAc was chromatographed on a calreticulin-Sepharose column, thin layer chromatographic examination of the resulting two peaks indicated that the glucosylated oligosaccharide was bound, while the unglucosylated polymannose component was unretained (Fig. 3).

**Fig. 1.** Immunoochemical identification of calreticulin in the ligand affinity chromatographically purified rat liver endomannosidase preparation and the Golgi membranes from which it was obtained. Subsequent to polyacrylamide gel electrophoresis in SDS, the endomannosidase obtained by Glc-Man-Affi-Gel chromatography (0.3 \(\mu\)g of protein, AG) as well as unfractionated rat liver Golgi membranes (50 \(\mu\)g of protein, GOL) and calreticulin standard (0.7 \(\mu\)g of protein, CRT) were immunoblotted with antisera against rat calreticulin as described under “Experimental Procedures.” The components were detected by autoradiography after reaction of the bound antibody with \(^{125}\)I-labeled protein. For comparison the components of an aliquot of the endomannosidase preparation (1 \(\mu\)g of protein) were visualized by silver staining (AG, Silver) after the electrophoresis. The designated molecular size markers expressed as kDa were Escherichia coli \(\beta\)-galactosidase (116,000), bovine serum albumin (66,000), hen ovalbumin (45,000), and bovine erythrocyte carbonic anhydrase (29,000).

**Fig. 2.** Immunoochemical detection of calreticulin in the eluted fractions from a Glc-Man-Affi-Gel column. Polyacrylamide gel electrophoresis in SDS, followed by immunoblotting with anti-calreticulin serum, was carried out on concentrated aliquots (300 \(\mu\)l) of neutralized fractions eluted with the glycine HCl, pH 3.0, buffer from a Glc-Man-Affi-Gel column (12), which had been loaded with 10 \(\mu\)g of purified rat calreticulin. The numbers on top of the lanes refer to the fraction (4 ml) emerging from the column upon application of the glycine buffer as described previously (12). When Triton-solubilized Golgi membranes were chromatographed on this column, a similar immunoblot of the emerging fractions was obtained; the calreticulin and endomannosidase activity peaks (fraction 2) coincided. The detection of the components by autoradiography and the molecular size markers were the same as in Fig. 1. The lane designated as CRT contained a calreticulin standard.

**Fig. 3.** Separation of monoglucosylated and unglucosylated Man\(_{9}\)GlcNAc on a calreticulin-Sepharose column. A mixture of \(^{14}\)C-labeled Glc\(_{6}\)Man\(_{9}\)GlcNAc and Man\(_{9}\)GlcNAc (14,000 dpm), which was incompletely resolved by thin layer chromatography, was applied to an immobilized calreticulin column under the conditions described under “Experimental Procedures.” Fractions of 1 ml were collected and monitored for radioactivity by scintillation counting (left panel). After desalting equal amounts of the unbound (UN) and bound (BD) peaks as well as a portion of the initial (IN) sample were chromatographed on a silica-coated plate in Solvent System B for 26 h (right panel). The components were detected by fluorography and their migration compared to standard oligosaccharides. The abbreviations employed were as follows: \(G_{m}\), Glc\(_{6}\)Man\(_{9}\)GlcNAc; \(M_{m}\), Man\(_{9}\)GlcNAc; \(M_{6}\), Man\(_{6}\)GlcNAc; \(M_{7}\), Man\(_{7}\)GlcNAc; \(M_{8}\), Man\(_{8}\)GlcNAc. Calreticulin prompted us to determine the specificity of this interaction with a series of purified oligosaccharides, which differed in the number of glucose residues and the size of their polymannose component. In the assay system employed, the
14C-labeled oligosaccharides were individually chromatographed on calreticulin-Sepharose along with 3H-labeled Glc1Man9GlcNAc to serve as reference for their position of emergence from the column (Fig. 4). From these analyses it became evident that binding of Man9GlcNAc to calreticulin occurred only in its monoglucosylated state and, moreover, that effective although somewhat diminished interaction occurred with oligosaccharides in which the polymannose portion had been truncated (Figs. 4 and 5). However, it became apparent from the complete failure of Glc1Man4GlcNAc to be retained by the column that at a minimum a structure like Glc1Man5GlcNAc with its ω1–6 branch point is required for the calreticulin binding (Fig. 5). Peptides containing the Glc1Man9GlcNAc2 carbohydrate unit bound to about the same extent as unconjugated Glc1Man9GlcNAc (Fig. 5), indicating that the di-N-acetylchitobiose segment and the linkage amino acid had little influence on the interaction with calreticulin; indeed, no detectable difference was noted in the binding of Glc1Man9 terminating with an N-acetylglucosamine residue or in a di-N-acetylchitobiose moiety (data not shown). A clear difference in the binding of Glc1Man9GlcNAc in the reduced and unreduced state was, however, noted (Figs. 4 and 5), and this unexpected finding was noted irrespective of whether or not the oligosaccharides were 14C- or 3H-labeled.

Immobilized Calreticulin Can Effectively Separate Free and N-linked Monoglucosylated Polymannose Oligosaccharides from Mixtures of Processing Intermediates—Application of 14C-radiolabeled glycopeptides from partially processed thyroid glycoproteins onto a calreticulin-Sepharose column resulted in a selective binding of Glc1Man9GlcNAc-containing peptides to the immobilized matrix, as revealed by thin layer chromatographic examination of the oligosaccharides released by endoH digestion of the bound and unbound fractions (data not shown). The immobilized calreticulin column also proved to be highly effective in removing Glc1Man9GlcNAc from complicated mixtures of polymannose intermediates even with the additional presence of the tri- and diglucosylated Man5–9GlcNAc components (Fig. 6). Thin layer chromatography indicated that the unbound material was specifically freed from the Glc1Man9GlcNAc oligosaccharide, which was recovered in the bound fraction (Fig. 6). The minor saccharide component migrating ahead of the Glc1Man9GlcNAc in the bound fractions was identified as Glc1Man8GlcNAc, which has been reported to occur as an N-linked processing intermediate (33). The effectiveness of the calreticulin-Sepharose in resolving metabolic intermediates was further made apparent when the cytosolic heptasaccharide fraction from HepG2 cells, which has been reported to consist of Glc1Man9GlcNAc and Man5–9GlcNAc (27), was loaded onto the column (Fig. 7). Thin layer chromato-
truncation of Glc1Man9GlcNAc, about 65% of the initial binding capacity was still observed. The selectivity of the immobilized calreticulin for monoglycosylated polymannose oligosaccharides or glycopeptides made it a highly effective tool for sorting out these components from complex mixtures of processing intermediates. Indeed, the high specificity of the calreticulin stands out in contrast to the mannose/glucose-binding lectins, such as concanavalin A (34), which cannot discriminate between glucosylated and unglucosylated polymannose oligosaccharides (35).

While calreticulin and endomannosidase are believed to function quite differently, namely as molecular chaperone (1, 36, 37) and processing enzyme (13, 14), respectively, our study demonstrates some intriguing similarities that merit comment. The selective retention of these two proteins on a matrix containing Glcα1→3Man an substituents was an expression of a common specific interaction with monoglycosylated polymannose oligosaccharides. The inability of calreticulin to bind tri- and diglycosylated oligosaccharides was mirrored by the previously reported low in vitro reactivity of endomannosidase with such saccharide species. Also relevant was the finding that monoglycosylated oligosaccharides with extensively truncated mannose chains could still effectively interact with both the chaperone and the enzyme (14), particularly since this property stands in pronounced contrast to the specificity of glucosidase II, which is known to require the untrimmed mannose branches for interaction with its substrate (38).

Despite calreticulin is generally believed to be primarily situated in the ER (32), our finding of this protein in the Golgi is consistent with reports indicating its presence at the cell surface (39, 40) and other subcellular compartments (41-43). Indeed, it is apparent that calreticulin takes part in intracellular trafficking which accounts for this wide distribution (43-44) and distinguishes it from calnexin, the other lectin-like chaperone, which is a membrane-bound ER-resident protein (45).
The presence of molecular chaperones with affinity for proteins with monoglucosylated N-linked oligosaccharides has provided the basis for a model (1) that accounts for their preferential association with glycoproteins at an early stage of processing and explains the observed accelerated protein degradation due to impaired folding or oligomerization during a glucosidase blockade (4, 5). However, as this scheme also postulated that dissociation of glycoproteins from the chaperone is brought about by the action of glucosidase II, its relevance would be limited to the ER locale where this enzyme is situated (46). If deglucosylation is required to dissociate the calreticulin-glycoprotein complexes in a more distal location, which would be either the Golgi itself or an ER-Golgi intermediate compartment, another mechanism is required. This has prompted us to propose a tentative modified model (Fig. 8) for such a more distal site in which removal of the glucose is achieved by endomannosidase through the excision of a Glc$_3$Man$_3$ disaccharide. This scheme would take into account the occurrence of calreticulin and endomannosidase in comparable amounts in this location and, more importantly, the fact that endomannosidase in marked contrast to glucosidase II has the capacity to interact with N-linked oligosaccharides in which the mannose chains have been trimmed. The latter characteristic is relevant, as glycoproteins that exit from the ER will have already undergone a substantial degree of processing though the action of ER-resident mannosidases (25, 26). Although the ER may be the primary site for protein folding and oligomerization to take place, a number of instances have already been described in which such quality controlling events take place in more distal compartments (47, 48).

The highly specific lectin-like interaction of molecular chaperones like calreticulin and calnexin with the N-linked oligosaccharides of glycoproteins represents an intriguing example of the biological role of saccharide chains and in particular extends the function of the polymannose-linked glucose residues beyond that of their well-known involvement in the process of cotranslational N-glycosylation (49, 50). Since it is quite likely, however, that the carbohydrate-protein interaction is only one manner in which the binding of chaperones to polypeptide intermediates is mediated (10, 45, 51), definition of the mechanisms utilized by various cell types for their diverse secretory proteins will require extensive further investigation.

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Definition of the Lectin-like Properties of the Molecular Chaperone, Calreticulin, and Demonstration of Its Copurification with Endomannosidase from Rat Liver Golgi

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