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N-linked oligosaccharides are necessary and sufficient for association of glycosylated forms of bovine RNase with calnexin and calreticulin

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Calnexin and calreticulin are lectin-like molecular chaperones that promote folding and assembly of newly synthesized glycoproteins in the endoplasmic reticulum. While it is well established that they interact with substrate monoglucosylated *N*-linked oligosaccharides, it has been proposed that they also interact with polypeptide moieties. To test this notion, glycosylated forms of bovine pancreatic ribonuclease (RNase) were translated in the presence of microsomes and their folding and association with calnexin and calreticulin were monitored. When expressed with two *N*-linked glycans in the presence of micromolar concentrations of deoxynojirimycin, this small soluble protein was found to bind firmly to both calnexin and calreticulin. The oligosaccharides were necessary for association, but it made no difference whether the RNase was folded or not. This indicated that unlike other chaperones, calnexin and calreticulin do not select their substrates on the basis of folding status. Moreover, enzymatic removal of the oligosaccharide chains using peptide *N*-glycosidase F or removal of the glucoses by ER glucosidase II resulted in dissociation of the complexes. This indicated that the lectin-like interaction, and not a protein–protein interaction, played the central role in stabilizing RNase–calnexin/calreticulin complexes.

Keywords: endoplasmic reticulum/glucosidase/glycans/protein folding

Introduction

Calnexin, a membrane protein, and calreticulin, its soluble homologue, are molecular chaperones present in the endoplasmic reticulum (ER). They associate transiently with folding intermediates and unassembled subunits of most newly synthesized membrane and secretory glycoproteins (Degen and Williams, 1991; Ou *et al.*, 1993; Nauseef *et al.*, 1995; Peterson *et al.*, 1995; Wada *et al.*, 1995). The association promotes efficient folding of the substrate proteins and prevents their degradation and premature oligomerization (Hammond and Helenius, 1994; Kearse *et al.*, 1994; Romagnoli and Germain, 1995; Hebert *et al.*, 1996; Vassilakos *et al.*, 1996). It also plays a role in the quality control process that prevents export of immature proteins from the ER to the Golgi complex and beyond (Hammond *et al.*, 1994; Jackson *et al.*, 1994; Rajagopalan *et al.*, 1994).

While all other known chaperones associate with the peptide moiety of their substrates, calnexin and calreticulin are lectins. They bind glycoproteins that have partially trimmed, monoglucosylated ($Glc_1Man_{9-5}GlcNAc_2$) core glycans (Hammond *et al.*, 1994; Hebert *et al.*, 1995; Peterson *et al.*, 1995; Ware *et al.*, 1995; Spiro *et al.*, 1996). This trimming intermediate is initially generated in the ER by the sequential removal of two of the three terminal glucoses from the core oligosaccharide by α-glucosidases I and II (Kornfeld and Kornfeld, 1985). Monoglucosylated glycans are also formed in the ER by the action of UDP-glucose:glycoprotein glucosyltransferase, a lumenal ER enzyme that specifically reglucosylates glucose-free, high mannose *N*-linked glycans present on incompletely folded glycoproteins (Parodi *et al.*, 1984; Sousa *et al.*, 1992; Trombetta and Parodi, 1992). That the binding involves a direct oligosaccharide–protein interaction, with a specificity for monoglucosylated oligosaccharides, has been confirmed *in vitro* using calnexin ectodomains or calreticulin (Ware *et al.*, 1995; Spiro *et al.*, 1996).

Although it is now widely accepted that the lectin-like activity is central to calnexin and calreticulin functions as chaperones, it remains unclear whether additional interactions occur between substrate glycoproteins and the chaperones and, if so, whether such interactions are functionally relevant. Different possibilities have been envisaged, including a combination of oligosaccharide–protein and protein–protein interactions. According to one view (Ware *et al.*, 1995; Williams, 1995), after initial contact is established via the glycan, calnexin is believed to associate in a more stable fashion with hydrophobic peptide moieties on the surface of the incompletely folded proteins. The substrate protein remains associated with the chaperone until it has folded and lost the conformational features responsible for attachment.

To address the mode of substrate binding to calnexin and calreticulin, we used bovine pancreas RNase, a soluble, well-characterized, single domain protein. A series of mutants were generated that differed in the degree of glycosylation and folding state. When these model substrates were expressed in microsomes, it was found that glycoforms with two *N*-linked glycans co-immunoprecipitated with both calnexin and calreticulin. Formation of such stable complexes was dependent on glycan trimming by glucosidases. The chaperones did not distinguish between folded and unfolded versions of the protein. By treating the complexes with glycosidases [glucosidase II and peptide:*N*-glycosidase F (PNGase F)], we could show that the monoglucosylated glycans in these RNase variants were both necessary and sufficient for stable interaction with calnexin and calreticulin.

Results

Synthesis of RNase glycoforms in microsomes

Bovine pancreas RNase is a small, soluble protein with a compact, single domain structure stabilized by four intramolecular disulfide bonds (Williams *et al.*, 1987). Although the overall three-dimensional structure of pancreatic RNases from various species is very similar (Lenstra *et al.*, 1977), the number and location of *N*-linked oligosaccharides vary (Beintema *et al.*, 1976). Bovine pancreatic RNase B is identical to RNase A except for a single covalently linked glycan at Asn34. The oligosaccharide is fully exposed on the surface and has no effect on the structure or activity of the RNase (Puett, 1973; Biondi *et al.*, 1980; Berman *et al.*, 1981; Williams *et al.*, 1987; Joao *et al.*, 1992). It does not affect the refolding of the denatured enzyme *in vitro* (Puett, 1973; Grafl *et al.*, 1987). Folding of RNase can be followed in different ways, including monitoring its conversion into a trypsin-resistant form (Lang and Schmid, 1986).

In order to generate different glycoforms and to produce variants that cannot fold properly, several constructs were designed (Figure 1). In a construct named 2Asn:WT, a second consensus glycosylation site was introduced by mutating Tyr76 to Asn. This corresponds to a glycosylation site present in pancreatic RNases of several species, including human, pig and whale (Beintema *et al.*, 1976; Ribo *et al.*, 1994). In a construct called QN:WT, Asn34 was changed to Gln, but the glycosylation site at position 76 was retained. In MUT, Cys58, Cys65, Cys72 and Cys84 were mutated to Ala to impair disulfide bond formation and folding. The Cys→Ala mutations were also introduced into the glycosylation mutants, resulting in the constructs called QN:MUT and 2Asn:MUT, as shown in Figure 1A.

To characterize the translocation and glycosylation of the various forms of RNase designed, their mRNAs were translated *in vitro* in the presence of dog pancreas microsomes and ^{35}S-labeled methionine and cysteine. The translation products were solubilized with CHAPS, immunoprecipitated with polyclonal anti-RNase antiserum and analyzed by SDS–PAGE. Previous studies have shown that microsomes support folding and oligomerization of translocated proteins when conditions are optimized for disulfide bond formation (Scheele and Jacoby, 1982; Marquardt *et al.*, 1993; Hebert *et al.*, 1995, 1996). Glycoproteins undergo productive interactions with calnexin and calreticulin (Hebert *et al.*, 1995, 1996).

When the wild-type RNase was translated, two labeled proteins were seen, corresponding to RNase A and B (Figure 1B, lane 1). That two forms were generated was expected because the consensus sequence for *N*-linked glycosylation at Asn34 is known to be used in only about one out of six chains synthesized *in vivo* (Plummer and Hirs, 1963; Plummer, 1968). The proteins were resistant to proteinase K in the absence of detergent, indicating that they had been translocated into the microsomes. As expected, RNase B, the larger of the proteins, constituting 20% of the total, was sensitive to endoglycosidase H digestion and precipitable with concanavalin A–Sepharose (data not shown). When expressed in the presence of 1 mM castanospermine (CST) or deoxynojirimycin (DNJ), which inhibit the α-glucosidases responsible for glucose trimming in the ER (Elbein, 1991), only the RNase B band displayed an upward molecular weight shift (lane 4). This confirmed that the protein was subject to glucose trimming in the microsomes.

Expression of 2Asn:WT and 2Asn:MUT resulted in the

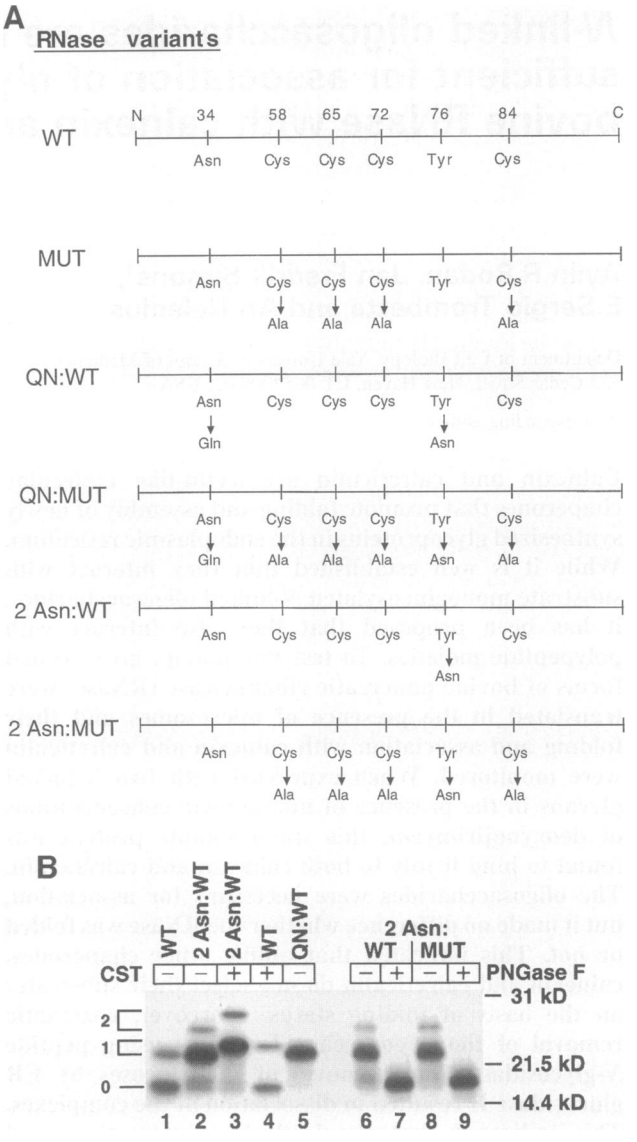

Fig. 1. Expression of RNase variants in microsomes. (**A**) Wild-type (WT) RNase is schematically depicted with Asn34, Cys58, Cys65, Cys72 and Cys84 and Tyr76 indicated. Numbers refer to amino acid positions in the signal-cleaved polypeptide. Arrows indicate mutations. In the folding-deficient mutant RNase (MUT), the indicated cysteines were mutated to alanines. In QN:WT and QN:MUT, the first glycosylation site at Asn34 was abolished by mutation to Gln, while a second site was created by mutating Tyr76 to Asn. Both glycosylation sites were present in 2Asn:WT and 2Asn:MUT. (**B**) ^{35}S-labeled RNase was translated under reducing conditions in the absence (lanes 1, 2 and 5) or presence (lanes 3 and 4) of 1 mM CST. After 1 h, CHX, unlabeled methionine and 4.5 mM GSSG were added for an additional 30 min. RNase was then alkylated with 20 mM NEM, the microsomes were lysed with 2% CHAPS and RNase was precipitated with anti-RNase antibody. In lanes 1–5, RNase was directly analyzed by 15% SDS–PAGE. In lanes 6–9, samples were mock (lanes 6 and 8) or PNGase F (lanes 7 and 9) treated prior to SDS–PAGE. The number of glycans on RNase is indicated on the left in this and subsequent figures. Molecular weight standards are indicated at the right: lysozyme (14.4 kDa), trypsin inhibitor (21.5 kDa) and carbonic anhydrase (31 kDa).

formation of RNase A, RNase B and an additional slower migrating band that constituted 15% of total RNase (Figure 1B, lanes 2, 6 and 8). The slower mobility of the additional band was clearly due to an additional *N*-linked glycan. Treatment with PNGase F, which removes the entire *N*-linked glycan, (Plummer *et al.*, 1984) resulted in conversion of this band to a band that co-migrated with RNase A (lanes 7 and 9). The effect of CST (lane 3) was also consistent with the presence of two glycans, because the mobility shift caused by this glucosidase inhibitor was larger than seen for RNase B, which carries one *N*-linked glycan. In Figure 1B and subsequent figures the glycoforms are marked with the numbers 0, 1 and 2 to indicate the number of glycans.

When QN:WT (Figure 1, lane 5) and QN:MUT (not shown) were translated, both were produced almost entirely in a form co-migrating with wild-type RNase B, indicating that Asn76 was more efficiently glycosylated than Asn34. This was likely the reason why little RNase A was produced whenever constructs contained Asn76.

The results showed that the RNase variants were correctly translated and translocated and that they underwent normal glycosylation and glucose trimming.

Association of RNase with calnexin and calreticulin

It was apparent that the RNase glycoforms were extensively glucose trimmed by the end of the translation period and therefore unlikely to remain chaperone associated. To slow down the trimming process and at the same time uncouple folding from trimming, low concentrations of DNJ were added to the translation mixture. At micromolar concentrations DNJ was found to partially inhibit trimming. Based on the mobility of RNase in SDS–PAGE and its sensitivity to α-mannosidase, maximum amounts of monoglucosylated glycans accumulated at a DNJ concentration of 5 μM (compare Figure 2A, lanes 5 and 6 and 11 and 12, with Figure 1B, lanes 2 and 3). As shown below, this led to accumulation of sufficient monoglucosylated glycans to allow isolation of calnexin and calreticulin complexes.

The various RNase glycoforms precipitated using anti-calnexin, anti-calreticulin and anti-RNase antibodies were analyzed by SDS–PAGE (Figure 2A). In this and subsequent figures the anti-RNase precipitates were loaded at a level 10- or 20-fold lower (indicated in the figure legends) than the anti-calnexin and anti-calreticulin precipitates. The doubly glycosylated RNases, either the wild-type (2Asn:WT) or the Cys→Ala mutant (2Asn:MUT), were found to bind more efficiently than the other forms (Figure 2A, lanes 1–4 and 7–10). Association with the chaperones was inhibited by high concentrations of DNJ or CST, but required small concentrations of DNJ (Figure 2B, lanes 1, 2, 4 and 5), indicating that it was dependent on partial glucose trimming.

While RNase A did not show any detectable association, a small fraction of the singly glycosylated forms were sometimes seen in anti-calnexin and anti-calreticulin immunoprecipitates. This suggested that weak interactions between calnexin/calerticulin and the singly glycosylated RNases were possible. The nature of these associations remained unclear, however, because some of the binding was insensitive to CST treatment and thus apparently

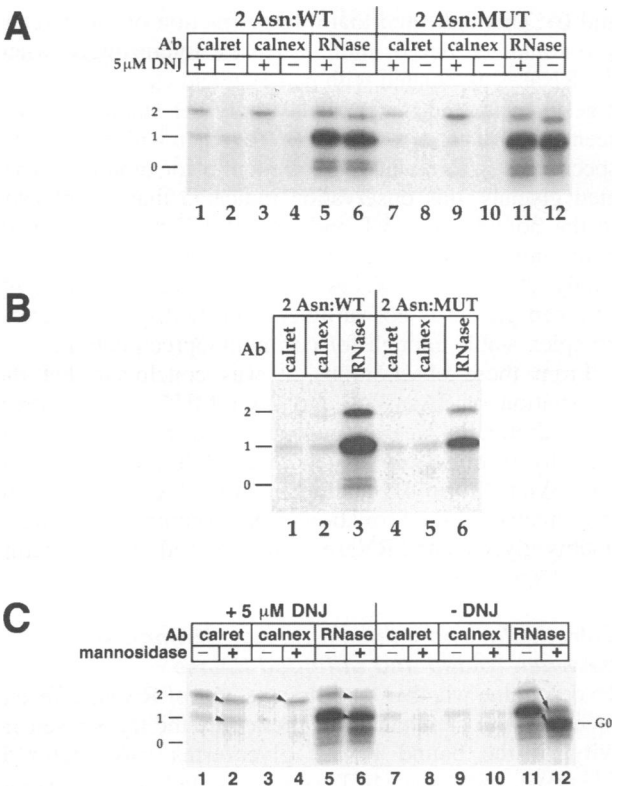

Fig. 2. Monoglucosylated RNase associates with calreticulin and calnexin in a glycan-dependent manner. (**A**) ^{35}S-labeled 2Asn:WT (lanes 1–6) or 2Asn:MUT (lanes 7–12) RNase were translated under reducing conditions in the absence (lanes 2, 4, 6, 8, 10 and 12) or presence (lanes 1, 3, 5, 7, 9 and 11) of 5 μM DNJ. After 1 h, CHX, unlabeled methionine, 1 mM DNJ and 4.5 mM GSSG were added for an additional 30 min, followed by alkylation and microsome lysis in 2% CHAPS. Precleared samples were divided into three equal aliquots and precipitated with anti-calreticulin (lanes 1, 2, 7 and 8), anti-calnexin (lanes 3, 4, 9 and 10) or anti-RNase (lanes 5, 6, 11 and 12) antibody, followed by reducing SDS–PAGE. In all panels, anti-RNase-precipitated samples were diluted 20-fold compared with anti-calreticulin- and anti-calnexin-precipitated samples prior to electrophoresis. (**B**) Synthesis, precipitation and SDS–PAGE analysis were performed as in (A) except that translation was carried out in the presence of 1 mM CST. (**C**) 2Asn:MUT RNase was synthesized in the presence of 5 μM DNJ (lanes 1–6) or in its absence (lanes 7–12) and precipitated as in (A). RNase was then mock treated (lanes 1, 3, 5, 7, 9 and 11) or digested with α-mannosidase (lanes 2, 4, 6, 8, 10 and 12) prior to SDS–PAGE analysis.

independent of glucose trimming (Figure 2B, lanes 1, 2, 4 and 5).

To determine whether the glycans in the co-immunoprecipitated 2Asn:WT and 2Asn:MUT possessed terminal glucose residues, we made use of α-mannosidase digestion, which accentuates mobility differences in SDS–PAGE between glycoproteins with glucose-containing and glucose-free *N*-linked glycans (Hammond *et al.*, 1994; Hebert *et al.*, 1995). When the RNase translated in the absence of DNJ was precipitated by anti-RNase and treated with α-mannosidase, the molecules exhibited large downward molecular weight shifts (Figure 2C, lanes 11 and 12, see arrows), confirming that the glycans had been trimmed to a glucose-free form.

However, when samples were analyzed after translation in the presence of 5 μM DNJ, the majority of the glycosylated RNase glycoforms underwent only minor shifts in mobility, as denoted by the arrow heads (lanes 5

and 6). This indicated that a large fraction of the glycans still contained one or more glucoses. Interestingly, when the RNases associated with calnexin and calreticulin were digested with the mannosidase, only the small shift was seen (lanes 1–4, arrow heads). Together with the known specificity of calnexin and calreticulin for monoglucosylated glycans, this observation indicated that the glycans in the bound 2Asn:WT and 2Asn:MUT molecules were both monoglucosylated. The inefficient binding of the singly glycosylated RNase suggested that one partially trimmed glycan is not sufficient to establish a chaperone complex stable enough for co-immunoprecipitation.

From these observations, it was concluded that the association of 2Asn:WT and 2Asn:MUT with calnexin and calreticulin was dependent on the presence of two partially trimmed glycans, both most likely in a monoglucosylated form. By adding 5 μM DNJ, conditions were thus created for the formation of stable complexes between doubly glycosylated RNase molecules and the two lectin-like chaperones.

Calreticulin and calnexin do not distinguish between folded and unfolded RNase

To determine whether the folding status of RNase affected its binding to calnexin and calreticulin, the trypsin sensitivity of the bound RNase glycoforms was analyzed. 2Asn:WT and 2Asn:MUT were translated under reducing conditions to prevent disulfide formation (Creighton, 1988). Oxidation and folding was then initiated by addition of 4.5 mM GSSG (Scheele and Jacoby, 1982; Marquardt *et al.*, 1993). Samples were removed at different times, treated with trypsin or mock treated and analyzed by SDS–PAGE after immunoprecipitation.

Before oxidation neither 2Asn:WT nor 2Asn:MUT were resistant to trypsin (Figure 3A and B, lanes 7). However, after addition of GSSG, increasing amounts of singly and doubly glycosylated 2Asn:WT became trypsin resistant (Figure 3A, lanes 8–12), indicating that the protein had reached its folded state. As expected, the cysteine mutant 2Asn:MUT failed to become trypsin resistant (Figure 3B, lanes 7–12; Lang and Schmid, 1986).

Since trypsin treatment allowed us to distinguish between folded and unfolded RNase molecules, the folding state of RNase that was bound to calnexin and calreticulin could be determined. The results in Figure 3C show that 2Asn:WT co-precipitated with anti-calnexin and anti-calreticulin was resistant to trypsin (lanes 2 and 6), while the co-precipitating 2Asn:MUT was sensitive (lanes 4 and 8). Calnexin and calreticulin were thus able to form stable complexes with both unfolded and fully folded substrate proteins, demonstrating that binding was not determined by the substrate protein's folding state.

Removal of oligosaccharides dissociates the complexes

To assess whether the carbohydrate–chaperone interaction was sufficient to maintain the association, complexes between RNase and calreticulin or calnexin were treated with glycosidases. The glycosidases were added to detergent-solubilized microsomes prior to immunoprecipitation with anti-calnexin, anti-calreticulin or anti-RNase antibodies. By performing the digestion before the immunoprecipitation, potential interference by the

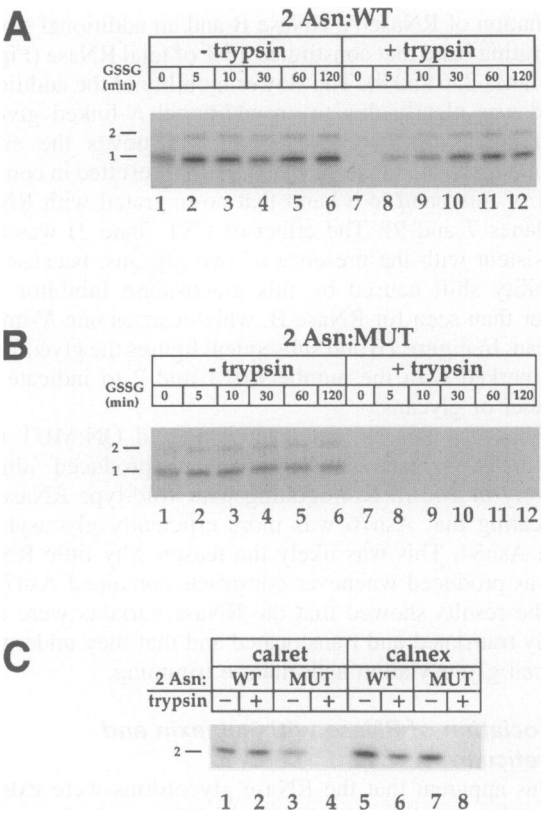

Fig. 3. Calreticulin and calnexin bind folded and unfolded RNase. ^{35}S-labeled 2Asn:WT (**A**) or 2Asn:MUT (**B**) RNase was translated under reducing conditions in the presence of 5 μM DNJ. At the indicated time points following initiation of oxidation with 4.5 mM GSSG, aliquots were removed and lysed with 1% TX-100. The RNase was mock treated (lanes 1–6) or digested with 100 μg/ml trypsin (lanes 7–12) for 10 min at room temperature. Soybean trypsin inhibitor at 200 μg/ml and 10 mM PMSF were added to stop the reaction and the RNase was precipitated with anti-RNase antibody. RNase was analyzed by reducing SDS–PAGE. (**C**) 2Asn:WT and 2Asn:MUT RNase were translated in the presence of 5 μM DNJ, post-translationally oxidized in the presence of 1 mM DNJ for 30 min and precipitated as described in Figure 2A. Following precipitation, the RNase was mock treated (lanes 1, 3, 5 and 7) or digested with 100 μg/ml trypsin (lanes 2, 4, 6 and 8) for 10 min at room temperature. Trypsin was quenched as above and the RNase was analyzed by reducing SDS–PAGE.

antibody–protein A beads was eliminated and the risk of non-specific association to the beads of partially folded, and therefore poorly soluble, proteins was lowered.

In the first set of experiments, PNGase F was used. 2Asn:MUT was translated in the presence of 5 μM DNJ and post-translational oxidation was induced by adding GSSG as described above. The microsomes were lysed with CHAPS and the lysate treated with PNGase F to remove the *N*-linked glycans. The samples were then subjected to immunoprecipitation with anti-calreticulin, anti-calnexin and anti-RNase antibodies followed by SDS–PAGE analysis. 2Asn:WT was not included in this experiment since the glycans of the fully folded protein, in contrast to the folding-incompetent 2Asn:MUT, were not accessible to cleavage by PNGase F.

Precipitation with anti-RNase showed that the glycoforms of 2Asn:MUT were quantitatively deglycosylated as a result of PNGase F action (Figure 4, lanes 5 and 6). Moreover, after removal of the glycans the RNase was no

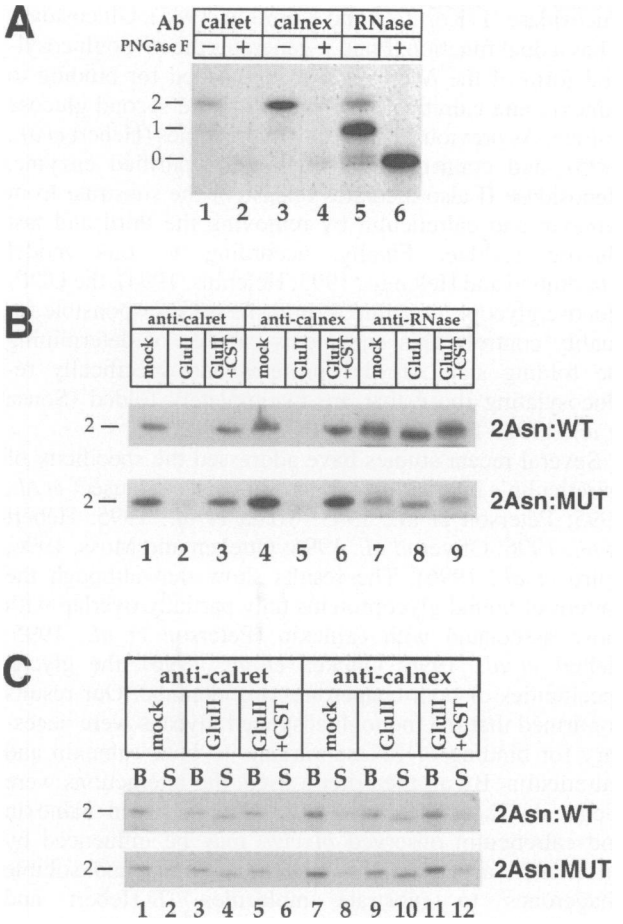

Fig. 4. Removal of *N*-linked glycans or glucoses abolishes association of RNase with calreticulin and calnexin. (**A**) 2Asn:MUT RNase was translated in the presence of 5 μM DNJ and post-translationally oxidized as described in Figure 2A. Following microsome lysis with 2% CHAPS, samples were mock treated (lanes 1, 3 and 5) or digested with 0.2 U PNGase F (lanes 2, 4 and 6) for 5 min at room temperature. Samples were placed on ice, diluted 25-fold with 2% CHAPS and precipitated with anti-calreticulin (lanes 1 and 2), anti-calnexin (lanes 3 and 4) or anti-RNase (lanes 5 and 6) antibody. The RNase was analyzed by reducing SDS–PAGE. Anti-RNase-precipitated samples were diluted 20-fold prior to electrophoresis. (**B**) 2Asn:WT (top) and 2Asn:MUT (bottom) RNase were translated in the presence of 5 μM DNJ and post-translationally oxidized as described in Figure 2A. Following microsome lysis with 2% CHAPS, samples were mock treated (lanes 1, 4 and 7) or digested with purified glucosidase II in the absence (lanes 2, 5 and 8) or presence (lanes 3, 6 and 9) of CST for 5 min at room temperature. Samples were placed on ice, diluted 25-fold with 2% CHAPS and precipitated with anti-calreticulin (lanes 1–3), anti-calnexin (lanes 4–6) or anti-RNase (lanes 7–9) antibody. The RNase was analyzed by reducing SDS–PAGE. Anti-RNase-precipitated samples were diluted 20-fold prior to electrophoresis. (**C**) 2Asn:WT (top) and 2Asn:MUT (bottom) were translated and co-immunoprecipitated with calreticulin (lanes 1–6) or calnexin (lanes 7–12) as described in Figure 2A. Immune complexes were washed twice with 0.5% CHAPS in PBS. Calreticulin-containing complexes were resuspended in PBS, whereas calnexin-containing complexes were resuspended in 0.5% CHAPS in PBS. Complexes were mock treated (lanes 1, 2, 7 and 8) or digested with glucosidase II in the absence (lanes 3, 4, 9 and 10) or presence (lanes 5, 6, 11 and 12) of 0.4 mM CST. The calreticulin-bound complexes were digested for 5 min and the calnexin-bound complexes for 30 min. The supernatant and bead fractions were separated, the supernatant fraction reprecipitated as described in Materials and methods and the samples analyzed by reducing SDS–PAGE.

longer precipitable with calnexin or calreticulin (lanes 2 and 4). Evidently, the loss of the glycans led to dissociation of the complexes that existed between the RNase and either chaperone. This indicated that the oligosaccharide chains were the principal groups involved in holding the complexes together. This was true even though 2Asn:MUT was unfolded and therefore potentially able to form peptide–peptide interactions.

In live cells, the release of substrates from the chaperone complexes coincides with removal of the remaining single glucose residue by the lumenal enzyme α-glucosidase II (Hammond *et al.*, 1994). We were interested in knowing whether this enzyme would have access to the glucose residues on chaperone-bound RNase *in vitro* and whether it could cause dissociation of the complexes.

2Asn:WT and 2Asn:MUT were translated as above. The microsomes were lysed and heterodimeric glucosidase II, purified to homogeneity from rat liver, was added at room temperature. Control samples were treated with glucosidase II in the presence of CST, a specific inhibitor of this α-glucosidase.

That glucosidase II digested the remaining glucoses was shown by the slightly increased mobility of the glycosylated RNase bands upon SDS–PAGE (Figure 4B, compare lanes 7 and 8). In the CST-containing controls and in mock-treated samples, no such mobility shift was observed (lanes 7 and 9). The mobility shift in the glucosidase II-treated samples was not due to contaminating proteolytic activity, since RNase A and glycoforms translated in the presence of CST were unaffected (not shown).

Glucosidase II had a dramatic effect on RNase association with calnexin and calreticulin. Like PNGase F, it abolished RNase interaction with calnexin and calreticulin (Figure 4B, lanes 2 and 5). Neither the wild-type nor the folding-incompetent mutant were co-precipitated with antibodies to the two chaperones after glucosidase digestion. In the mock-treated samples (Figure 4B, lanes 1 and 4) and in samples that were treated with glucosidase II in the presence of CST (lanes 3 and 6), the RNases were still associated with the chaperones. This indicated that the glucosidase digested the glucoses and induced RNase dissociation from calnexin and calreticulin. The chaperones did not protect the substrate proteins from glucosidase action.

Glucosidase II treatment of the complexes was also performed by adding the enzyme after immunoprecipitation. In this case, the RNase–chaperone complexes were coupled to protein A–Sepharose beads via anti-chaperone antibodies. The resulting suspension was treated with glucosidase II, after which the supernatant and the bead fractions were separated by centrifugation. The bead and supernatant fractions were analyzed separately by SDS–PAGE.

In mock-treated samples and in samples containing glucosidase II and CST together, only trace amounts of RNase were observed in the supernatant (Figure 4C, lanes 2, 6, 8 and 12). Judging by the SDS–PAGE mobility, the RNase remained undigested. However, in the glucosidase II-treated samples ~50% of both the 2Asn:WT and 2Asn:MUT was released into the supernatant (lanes 3 and 4, and 9 and 10). The faster gel mobility of the released RNase showed that it had been digested by the enzyme.

The RNase that remained in the bead fractions was, on the other hand, still undigested (lanes 3 and 9).

Thus, it was apparent that glucosidase II was not as efficient at digesting the glucoses of chaperone-associated RNase when the RNase was bound to immunobeads as when the complexes were free in solution. However, the result confirmed that when cleavage occurred it led to loss of association between the RNase and both calreticulin and calnexin. It was concluded from the glycosidase experiments that the glycans were necessary and sufficient for stabilizing complexes formed with calnexin and calreticulin.

Discussion

The mode of substrate binding to calnexin and calreticulin is an important issue because it reflects the functional principle of this unique pair of chaperones. Do they work by mechanisms similar to other known molecular chaperones or are they different? Members of the well-characterized HSP70 and chaperonin families interact with hydrophobic peptide determinants that are exposed on the surface of incompletely folded substrate polypeptides but hidden in the folded conformation (see Rothman, 1989; Gething and Sambrook, 1992; Hartl, 1996; Zhu *et al.*, 1996). In these chaperones a cycle of substrate binding and release is driven by conformational changes induced by ATP binding and hydrolysis.

Models with features similar to these have been proposed for calnexin (Wada *et al.*, 1994; Ou *et al.*, 1995; Ware *et al.*, 1995; Williams, 1995; Vassilakos *et al.*, 1996). It has been suggested that the oligosaccharide-mediated interaction constitutes a prelude to stronger protein–protein contacts that persist until the substrate is folded and its surface is free of hydrophobic peptide binding sites (Ware *et al.*, 1995; Williams, 1995; Vassilakos *et al.*, 1996).

The observations in this study support a different mechanism. No difference in calnexin and calreticulin binding was observed whether the RNase was folded or not, as long as the glycans had the appropriate monoglucosylated composition. This suggested that, unlike the classical chaperones, these do not distinguish between folded and unfolded glycoproteins. Moreover, the bound RNases were found to dissociate from calnexin and calreticulin when the *N*-linked glycans were removed by PNGase F. The same effect was obtained by hydrolyzing the terminal glucose residues with purified glucosidase II. Glucosidase II not only had access to the *N*-linked glycans, but catalyzed efficient dissociation of the RNase from calnexin and calreticulin whether it was folded or not. No additional factors were needed for release to occur. The findings strongly argued against protein–protein interactions being important in stabilizing the association. For the doubly glycosylated RNase forms used in our studies, the lectin-like interactions were both necessary and sufficient for the formation of stable complexes with calnexin and calreticulin.

Taken together, the results support a model in which calnexin and calreticulin are part of a complex pathway which involves other ER factors (Hammond and Helenius, 1993; Helenius, 1994). These include several enzymes that catalyze a cycle of covalent modifications of newly synthesized glycoproteins. Glucosidase I initiates the trimming by removing the first glucose residue in the *N*-linked core glycan, thus making it a substrate for glucosidase II (Kornfeld and Kornfeld, 1985). Glucosidase II has a dual function. First, it generates the monoglucosylated form of the *N*-linked glycans needed for binding to calnexin and calreticulin by removing the second glucose residue. As previously shown in microsomes (Hebert *et al.*, 1995), and confirmed here using the purified enzyme, glucosidase II also mediates release of the substrate from calnexin and calreticulin by removing the third and last glucose residue. Finally, according to this model (Hammond and Helenius, 1993; Helenius, 1994), the UDP-glucose:glycoprotein glucosyltransferase is responsible for quality control. This enzyme is capable of determining the folding state of glycoproteins and specifically re-glucosylating those that are incompletely folded (Sousa *et al.*, 1992; Trombetta and Parodi, 1992).

Several recent studies have addressed the specificity of calreticulin's interaction with its substrates (Nauseef *et al.*, 1995; Peterson *et al.*, 1995; Wada *et al.*, 1995; Hebert *et al.*, 1996; Oliver *et al.*, 1996; Otteken and Moss, 1996; Spiro *et al.*, 1996). The results show that although the pattern of bound glycoproteins only partially overlap with those associated with calnexin (Peterson *et al.*, 1995; Hebert *et al.*, 1996; Otteken *et al.*, 1996), the glycan specificities of both chaperones are identical. Our results confirmed that the monoglucosylated glycans were necessary for binding of RNase variants to both calnexin and calreticulin. By all the criteria used, the interactions were identical. The differences in binding between calnexin and calreticulin observed *in vivo* may be influenced by differential access of the membrane-bound and soluble chaperones to substrate molecules (D.Hebert and A.Helenius, in preparation).

That monoglucosylated glycans are needed for productive binding of substrates to calnexin has been repeatedly described (Hammond *et al.*, 1994; Kearse *et al.*, 1994; Hebert *et al.*, 1995; Ora and Helenius, 1995; Romagnoli and Germain, 1995; Zhang *et al.*, 1995; Otteken and Moss, 1996; Vassilakos *et al.*, 1996). However, some uncertainty has been introduced by results showing that calnexin can be co-immunoprecipitated with certain non-glycosylated proteins (Loo and Clarke, 1994; Arunachalam and Cresswell, 1995; Carreno *et al.*, 1995; Kim and Arvan, 1995). A recent study using vesicular stomatitis virus G protein indicates, however, that glycan-independent interactions may be neither specific nor direct, because they may only occur after aggregation of folding-incompetent proteins in the ER (Cannon *et al.*, 1996). Indeed, the only naturally non-glycosylated protein that has been proposed to associate with calnexin, TCRε (Rajogapalan *et al.*, 1994), has been found to aggregate when expressed alone in the absence of other T cell receptor subunits (J.Hoppa and H.Ploegh, personal communication).

Our observation that glycosidase treatment results in dissociation of the RNase–calnexin and –calreticulin complexes is not consistent with previous reports using endoglycosidase H and various calnexin–substrate complexes (Arunachalam and Cresswell, 1995; Ware *et al.*, 1995; Zhang *et al.*, 1995). The substrate proteins used were transmembrane proteins (murine MHC class I heavy chain K^b and D^b, human MHC class I HLA heavy chain, MHC class II DRα and DRβ and invariant chain) and a soluble

protein, α_1-antitrypsin. It was found that although endoglycosidase H efficiently hydrolyzed the glycans, it failed to release the substrates from immunoprecipitates obtained using anti-calnexin antibodies and protein A beads. The results were taken as evidence for the presence of protein–protein interactions and led to the suggestion that the carbohydrate–protein interaction is only needed to bring about initial contact between the substrate and calnexin, to be followed by stronger protein–protein contacts (Ware *et al.*, 1995; Williams, 1995).

Interpretation of these results is complicated by the fact that, in the case of the membrane proteins, the substrate molecule and calnexin are likely to be trapped in a common detergent micelle. It is not clear whether two integral membrane proteins can partition away from the micelle and from each other when a direct molecular contact between them is abolished. While the detergent molecules are known to equilibrate rapidly and move from one micelle to the next, there is no evidence that proteins can do so. That the MHC heavy chains and calnexin are, indeed, in the same micelle has been indicated by chemical cross-linking between residues close to or in the transmembrane domain (Margolese *et al.*, 1993).

With a soluble substrate like RNase or α_1-antitrypsin the detergent problem may not arise, nor if the chaperone itself is a soluble protein, like calreticulin. Why then the discrepant results between α_1-antitrypsin and RNase in respect to calnexin binding after glycosidase digestion? Perhaps the α_1-antitrypsin was not released into the supernatant because it was incompletely folded and poorly soluble after release from calnexin by the glycosidase. This does not apply for the RNase, which was found to bind to calnexin and calreticulin and to be released in its folded, soluble form. Alternatively, different proteins may show different binding modalities.

The only direct evidence for a protein–protein interaction has been provided by the aforementioned chemical cross-linking result (Margolese *et al.*, 1993). The cross-link between calnexin and MHC class I heavy chain was formed close to, or within, the transmembrane domains of the two proteins. No cross-links were seen in the ectodomains. Yet, there are several indications that it is the ectodomain that serves as the primary substrate binding site in calnexin. First, it is known that calnexin binds to many soluble glycoproteins which have no membrane anchor (Ou *et al.*, 1993). Second, recombinant calnexin ectodomains are fully capable of forming complexes with glycoprotein substrates (Rajagopalan *et al.*, 1994; Ware *et al.*, 1995) and a soluble recombinant calnexin devoid of the transmembrane domain rescues calnexin-deficient *Schizosaccharomyces pombe* mutant strains (Parlati *et al.*, 1995). Furthermore, calnexin shares sequence homology and function with calreticulin, which does not have a membrane anchor. While a membrane association may contribute to the stability of calnexin–substrate complexes, the specific sites of contact are likely to be in the ectodomain.

Since glucosidase II and PNGase F induced rapid and quantitative modification of the chaperone-bound RNases, they must have access to the relevant parts of the glycans. This poses an apparent problem; how can they access groups that are at the same time responsible for binding the substrate to the chaperones? There are two possible explanations. First, the enzymes may only access the glycans when they are temporarily dissociated from the chaperone. For a protein with multiple glycans, like the chaperone-bound RNase, this does not mean that the substrate glycoprotein must be free in solution to be processed, because one or more glycans may remain bound while others are transiently dissociated. Second, the structure of the complexes may be so open or flexible that the core oligosaccharide can be accessed by the enzymes while bound to the chaperones.

It is interesting in this context that two glycans were needed before stable binding of RNase to the chaperones could be observed. RNase molecules with a single monoglucosylated glycan probably also bind, but the affinity may be too low to withstand the conditions used during solubilization and immunoprecipitation. Many lectins are multivalent and this greatly increases their affinity for ligands (Weis and Drickamer, 1996). It would not be surprising if calnexin and calreticulin also have multiple binding sites.

The functions of classical chaperones and calnexin/calreticulin seem to be similar in that both sequester folding intermediates, slow down the folding rate and prevent aggregation (Hartl, 1996; Hebert *et al.*, 1996; Vassilakos *et al.*, 1996). Both operate through a cycle of binding and release which may be repeated multiple times. The molecular mechanisms appear, however, to be fundamentally different. Calnexin and calreticulin do not seem to distinguish between folded and unfolded substrate proteins. Furthermore, the association and dissociation cycle is mediated by covalent modification of the substrate (the removal and addition of glucose residues). While the results do not exclude the possibility of protein–protein interactions between RNase and the chaperones, they indicate that the oligosaccharide–protein interactions are sufficient for formation of stable complexes. More experiments using a variety of proteins will be needed to determine whether protein–protein interactions, in addition to the well-established glycan–protein interactions, play any substantive, functionally relevant role in glycoprotein attachment to calnexin and calreticulin.

Materials and methods

Reagents

The MEGAscript™ *in vitro* Transcription Kit was purchased from Ambion (Austin, TX). The rabbit reticulocyte cell-free translation system, including amino acid mixture lacking methionine, was from Promega (Madison, WI). Dog pancreas microsomes and recombinant PNGase F were from Boehringer-Mannhein (Indianapolis, IN). Dithiothreitol (DTT) and molecular weight standards were from Bio-Rad (Hercules, CA) and oxidized glutathione (GSSG) from Fluka Chemical Corp. (Ronkonkowa, NY). Promix ^{35}S metabolic labeling reagent and the Sequenase™ Version 2.0 DNA Sequencing Kit were from Amersham Corp. (Arlington Heights, IL). CHAPS (3-[3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate) was from Pierce (Rockford, IL). Endoglycosidase H was from Calbiochem Corp. (La Jolla, CA). Restriction enzymes and DNA modification enzymes were from New England Biolabs (Beverly, MA) and Boehringer-Mannheim and used according to manufacturer's directions. All other chemicals were purchased from Sigma (St Louis, MO).

Polyclonal anti-RNase A rabbit serum was from Biogenesis (Sandown, NH). It precipitated both unglycosylated and glycosylated forms of RNase. Polyclonal anti-calnexin rabbit serum was raised against a synthetic peptide corresponding to the cytoplasmic tail of canine calnexin (Wada *et al.*, 1991). Polyclonal rabbit anti-human calreticulin antiserum was from Affinity Bioreagents (Neshanic Station, NJ).

Plasmid construction

The cDNA for RNase A was kindly provided by Dr David Schultz (Schultz and Baldwin, 1992). This was used as a template to generate a PCR fragment using oligonucleotide primers CAC**GGTACC**CAGGC-CGCCGGU and **GGATCC**GAC (restriction sites in bold). The fragment was cloned into the *Kpn*I and *Bam*HI sites of a T7 vector, pF(BGL)-PRPRBPTI (Weissman and Kim, 1992). The resulting open reading frame consisted of the first 18 amino acids of the bovine pancreatic trypsin inhibitor signal sequence followed by the last four amino acids of the RNase A signal sequence and full-length RNase A. This construct, referred to as WT, has a consensus glycosylation site at Asn34.

RNase A/B variants

All mutations were introduced by PCR mutagenesis and confirmed by dideoxy sequencing. Wild-type RNase sequences in the T7 pF(BGL) vector were replaced by PCR fragments by standard restriction enzyme cleavage and ligation. The constructs created were as follows. A folding-deficient mutant (MUT) was generated by mutating cysteine codons 58, 65, 72 and 84 to alanine. Doubly glycosylated RNase (2Asn:WT and 2Asn:MUT) was made by engineering a second glycosylation site into the WT or MUT background by substituting Asn for Tyr76. To generate RNase molecules containing a glycosylation site at position 76 but not at position 34, Asn34 of 2Asn:WT or 2Asn:MUT was mutated to Gln, resulting in the QN:WT and QN:MUT constructs.

Translation of RNase A/B and post-translational oxidation

Plasmid DNA was linearized with *Eco*RV beyond the 3′-end of the cDNA. *In vitro* transcription of uncapped mRNA was done using the MEGAscript™. *In Vitro* Transcription Kit according to the manufacturer's directions. mRNA was translated in reticulocyte lysate in the presence of microsomes according to the manufacturer's instructions. Typical volumes used were: 3 μl microsomes, 26 μl reticulocyte lysate, 1 μl 0.2 mM amino acid mixture without methionine, 0.5 μl 100 mM DTT, 4 μl ^{35}S Promix methionine and cysteine, 2 μl mRNA and H_2O to a final volume of 50 μl. Translation was carried out for 1 h at 27°C. DNJ at 5 μM or CST at 1 mM was included in the translation mix when indicated. Translation was stopped with 0.5 mM cycloheximide (CHX) and 1 mM methionine. To initiate post-translational oxidation, GSSG was added to a final concentration of 4.5 mM (Marquardt *et al.*, 1993), which proceeded for 15 or 30 min at 27°C. In some experiments, 1 mM DNJ was included to prevent post-translational glucose trimming. After oxidation, samples were chilled and alkylated with 20 mM *N*-ethyl maleimide (NEM) to block free sulfhydryls (Creighton, 1978; Braakman *et al.*, 1991). Microsomes were lysed in ice-cold 2% CHAPS in HBS (50 mM HEPES and 200 mM NaCl, pH 7.5) containing 10 mM PMSF and 10 μg/ml each of chymostatin, leupeptin, antipain and pepstatin (CLAP).

Immunoprecipitation

Lysed microsomes were precleared for 1 h at 4°C with 2% protein A–Sepaharose beads by end-over-end rotation. Proteins were precipitated with 2% protein A–Sepharose beads (v/v) and either anti-RNase, anti-calnexin or anti-calreticulin antibody overnight at 4°C with end-over-end rotation. Beads were pelleted and washed with 1 ml buffer A (10 mM Tris–HCl, pH 8.0, 300 mM NaCl, 0.05% Triton X-100, 0.1% SDS, 0.01% NaN_3) for 10 min at room temperature; in some experiments different washing conditions were used, as indicated. The beads were then resuspended in buffers for further incubation or in SDS–PAGE sample buffer (200 mM Tris–HCl, pH 6.8, 3% SDS, 10% glycerol, 1 mM EDTA, 0.004% bromophenol blue). Some samples were reduced by addition of 25 mM DTT. Samples were heated to 95°C and analyzed by 15% SDS–PAGE and fluorography. RNase bands were in some cases quantified by densitometry with a Visage 200 digital gel scanner.

Trypsin treatment

Translation and post-translational oxidation was carried out as described above. Aliquots were removed before oxidation and at several time points following initation of oxidation. At each point, microsomes were lysed by the addition of Triton X-100 to a final concentration of 1%. The volume was increased by 70-fold dilution in 50 mM Tris–HCl, pH 8.0, and the samples were mock treated or digested with 100 μg/ml trypsin for 10 min at room temperature. The reaction was stopped by addition of 200 μg/ml soybean trypsin inhibitor, 10 mM phenylmethylsulfonyl flouride (PMSF) and 10 μg/ml CLAP. RNase A/B was immunoprecipitated with anti-RNase antibody as described above and the products analyzed by 15% non-reducing SDS–PAGE. Alternatively, RNase A/B was immunoprecipitated with anti-calreticulin, anti-calnexin or anti-RNase antibody prior to trypsin digestion. Immune complexes were resuspended in 20 μl 50 mM Tris–HCl, pH 8.0, and incubated for 10 min at room temperature with buffer or 100 μg/ml trypsin. The trypsin was inhibited as above and the products analyzed by reducing and non-reducing 15% SDS–PAGE.

Glycosidase digestions

α-Mannosidase digestions were performed as described (Hammond *et al.*, 1994; Hebert *et al.*, 1995). For quantitative removal of oligosaccharide with PNGase F, immune complexes were washed with buffer A and with phosphate-buffered saline (PBS). The immune complexes were then resuspended in 20 μl PBS, 0.5% SDS and boiled for 5 min. The SDS was quenched by addition of 180 μl 1% CHAPS in PBS. Samples were either mock treated or digested with 1 U PNGase F overnight at 37°C in the presence of 10 mM PMSF and 10 μg/ml CLAP. Reducing sample buffer was added and the products were analyzed by 15% SDS–PAGE.

For removal of oligosaccharide or glucose prior to immunoprecipitation, RNase A/B was translated and post-translationally oxidized as described above. Following treatment with NEM, microsomes were lysed by the addition of 10% CHAPS in PBS to a final concentration of 2%. The mixture was divided and mock treated or digested with 0.6 U PNGase F to remove the oligosaccharide.

Microsomal glucosidase II was isolated to homogeneity from rat liver as described (Trombetta *et al.*, 1996). It was added at a final concentration of 0.2 U/ml to microsome lysates in the presence or absence of 0.4 mM CST. Incubations were carried out for 5 min at room temperature in the presence of 10 mM PMSF and 10 μg/ml CLAP. Samples were placed on ice and lysed and diluted 25-fold with 2% CHAPS on ice. Preclearing, immunoprecipitation with anti-calreticulin, anti-calnexin or anti-RNase antibody and SDS–PAGE were performed as above.

Glucosidase II digestions were also performed after co-immunoprecipitation of RNase with calreticulin or calnexin. Immune complexes were washed twice with 0.5% CHAPS in PBS and resuspended in 20 μl of the same solution. RNase was mock treated or digested with glucosidase II in the absence or presence of 0.4 mM CST for 5 min. To recapture calnexin or calreticulin that may have dissociated from the antibodies or antibody that had dissociated from the protein A beads, additional antibody and beads were added for 3 h with shaking at 4°C. The beads were pelleted and reducing sample buffer was added to the supernatant. The beads were washed once in 1 ml buffer A to remove residual supernatant and resuspended in reducing sample buffer.

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