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Glucose Trimming and Reglucosylation Determine Glycoprotein Association with Calnexin in the Endoplasmic Reticulum

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Summary

To determine the role of N-linked oligosaccharides in the folding of glycoproteins, we analyzed the processing of in vitro translated influenza hemagglutinin (HA) in dog pancreas microsomes. We found that binding to calnexin, a membrane-bound molecular chaperone, was specific to molecules that possessed monoglucosylated core glycans. In the microsomes, these were generated either by glucose removal from the original triglucosylated core oligosaccharide by glucosidases I and II or by reglucosylation of already unglucosylated high mannose glycans. Release of fully folded HA from calnexin required the removal of the remaining glucose by glucosidase II. The results provided an explanation for trimming and reglucosylation activities in the endoplasmic reticulum and established a direct correlation between glycosylation and folding.

Introduction

Recent studies have shown that the endoplasmic reticulum (ER) contains, in addition to members of the classical stress protein families, a unique machinery for the folding and retention of glycoproteins (Ou et al., 1993; Bergeron et al., 1994; Hammond et al., 1994). Central to this pathway is calnexin, a membrane protein that binds transiently to a large variety of newly synthesized glycopolypeptides (Degen and Williams, 1991; Degen et al., 1992; Hochstenbach et al., 1992; Ou et al., 1993; Hammond et al., 1994). Calnexin assists glycoproteins during initial folding, it retains transport-incompetent misfolded glycoproteins, and it may be functionally involved in assembly of some oligomeric proteins in the ER (Anderson and Cresswell, 1994; Bergeron et al., 1994; Hammond and Helenius, 1994a, 1994b; Jackson et al., 1994; Rajogapalan et al., 1994).

The processes of glycosylation and oligosaccharide trimming in the ER have been extensively studied (for reviews see Hubbard and Ivatt, 1981; Kornfeld and Kornfeld, 1985; Elbein, 1991). The 14 oligosaccharide N-linked core glycans begin to undergo trimming immediately after transfer from the dolichol-pyrophosphate precursor to an asparagine side chain in the growing polypeptide chain. The outermost of the three glucoses is rapidly removed by glucosidase I, followed by the hydrolysis of the middle glucose by glucosidase II (Hubbard and Robbins, 1979; Michael and Kornfeld, 1980; Atkinson and Lee, 1984; Hettkamp et al., 1984). The third glucose, which is also removed by glucosidase II, is usually lost a few minutes after chain termination. The glucose-free, high mannose glycans can, however, be reglucosylated by the lumenal enzyme UDP-glucose:glycoprotein glucosyltransferase, which has been shown to reglucosylate unfolded glycoproteins selectively in vitro (Trombetta et al., 1989; Sousa et al., 1992; Trombetta and Parodi, 1992).

Having observed that the binding of glycoproteins to calnexin is blocked by inhibitors of the two ER glucosidases, we recently proposed that calnexin is a lectin that binds to N-linked oligosaccharides (Hammond et al., 1994), that the binding requires the removal of the two outermost glucoses from the core oligosaccharides by glucosidases I and II (Hammond et al., 1994; Helenius, 1994), that release from calnexin requires removal of the third glucose by glucosidase II, and that folding and retention are coupled to a de- and reglucosylation cycle. This model provides an explanation for both the trimming of glycoproteins and the reglucosylation activity in the ER. In addition, it explains a mechanism by which incompletely folded and misfolded glycoproteins are retained in the ER. Oligosaccharide processing would thus have a central role in the quality control and folding system of the cell.

To determine the validity of the hypothesis, we have in this study employed in vitro translation of a glycoprotein, influenza hemagglutinin (HA), in the presence of dog pancreas microsomes. While originally developed to study signal sequence–mediated translocation of polypeptides into the ER (Blobel and Dobberstein, 1975), this cell-free system also supports glycoprotein folding (Scheele and Jacoby, 1982; Bulleid and Freedman, 1988; Marquardt et al., 1993). In its mature form, HA is a homotrimeric type I membrane protein whose structure is well characterized and whose folding and oligomerization in living cells have been extensively analyzed (Wilson et al., 1981; Braakman et al., 1991; Tatu et al., 1995). To fold properly, HA must be glycosylated and acquire intramolecular disulfide bonds (Hurtley et al., 1989; Braakman et al., 1991; Roberts et al., 1993). There are seven N-linked glycans and six intrachain disulfides in each subunit.

The interaction between HA and calnexin was analyzed under a variety of folding conditions. The HA subunits were found to fold in association with calnexin. Calnexin binding required the presence of monoglucosylated core glycans on HA. Glucosidases I and II were thus needed to prepare the newly synthesized HA for binding. Glucosidase II had an additional role in releasing HA from the calnexin complex after completed folding by removing the last of the three glucoses. Evidence was also obtained for the role of the glucosyltransferase in promoting the binding of misfolded HA to calnexin. Therefore, the results of this study confirmed the central predictions from the model and expanded our understanding of calnexin as an ER chaperone.

Results

HA Associates with Microsomal Calnexin

To determine whether HA associates with calnexin in mi-

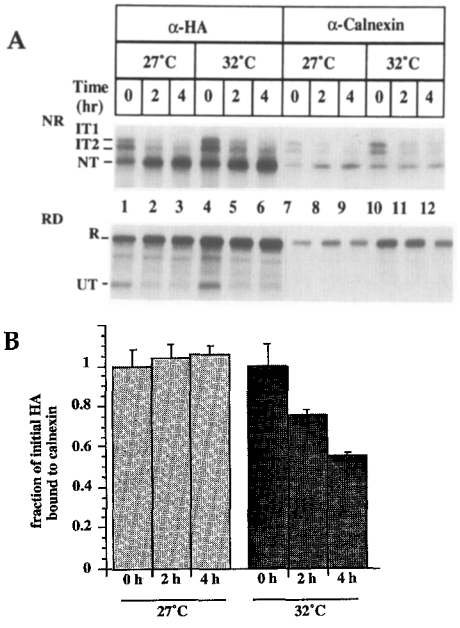

Figure 1. In Vitro Translated HA Associates with Calnexin

^{35}S-labeled HA was translated in a reticulocyte lysate/microsome system in the presence of 4 mM GSSG at 27°C or 32°C. Cycloheximide was added after 1 hr of translation. After 0, 2, or 4 hr of further oxidation, samples were alkylated with NEM and immunoprecipitated with anti-HA (α-HA) or anti-calnexin (α-Calnexin) antibodies. The immunoprecipitated ^{35}S-labeled HA was resolved upon nonreducing (NR) and reducing (RD) SDS–polyacrylamide gels and visualized by fluorography. Bands were quantified by a digital densitometer and plotted as the fraction of initial calnexin-bound HA.

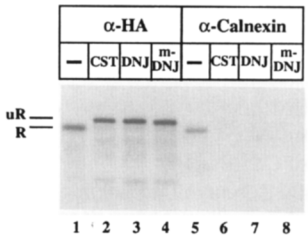

Figure 2. Glucosidase Inhibitors Block Association of HA with Calnexin

Reticulocyte lysate/microsomes were incubated in the absence (minus) (lanes 1 and 5) or presence of glucosidase inhibitors (1 mM CST, lanes 2 and 6; 1 mM deoxynojirimycin [DNJ], lanes 3 and 7; 1 mM N-methyl deoxynojirimycin [m-DNJ], lanes 4 and 8) for 10 min at 27°C. HA mRNA was then added under oxidizing conditions, and the samples were incubated at 27°C for 1 hr. Samples were alkylated with NEM and immunoprecipitated with either anti-HA (α-HA) (lanes 1–4) or anti-calnexin (α-Calnexin) antibodies (lanes 5–8). Immunoprecipitated untrimmed (uR) and trimmed (R) ^{35}S-labeled HAs were resolved by reducing SDS–PAGE and fluorography.

crosomes, we translated synthetic HA mRNA using the reticulocyte lysate system in the presence of dog pancreas microsomes and [^{35}S]methionine (Marquardt et al., 1993). Oxidized glutathione (GSSG) was included in the mixture to raise the redox potential sufficiently to allow HA to form disulfide bonds without inhibiting translation (Scheele and Jacoby, 1982; Marquardt et al., 1993). After 1 hr of translation at either 27°C or 32°C, cycloheximide was added to arrest protein synthesis. The incubation was continued for 0, 2, and 4 hr before N-ethylmaleimide (NEM), a membrane-permeable alkylating agent, was added to prevent oxidation of remaining free sulfhydryl groups (Creighton, 1978; Braakman et al., 1991). The samples were solubilized with nonionic detergent and subjected to immunoprecipitation using anti-HA or anti-calnexin antibodies, and the immunoprecipitates were analyzed by both nonreducing and reducing SDS–polyacrylamide gel electrophoresis (SDS–PAGE) and autoradiography.

In the reduced samples (Figure 1A, RD, lanes 1–6), two principal HA bands were observed. The more rapidly migrating band, UT, represented untranslocated HA (Marquardt et al., 1993), and the slower migrating band, marked R, corresponded to the translocated, glycosylated HA. While the translocated HA remained stable throughout the 4 hr incubation period, a large portion of UT was degraded. The nonreduced samples revealed three HA bands (Figure 1A, NR, lanes 1–6), familiar from previous studies on HA maturation in cells and microsomes (Braakman et al., 1991; Marquardt et al., 1993). IT1 and IT2 corresponded to incompletely oxidized folding intermediates (Braakman et al., 1991). The most rapidly migrating band, called NT for native, corresponded to molecules with all major intrachain disulfides in place.

That the formation of NT was virtually complete by 2 hr showed that a large fraction of HA was able to undergo normal folding and disulfide formation in microsomes. The epitopes expressed in the in vitro translated HA have previously been shown to be identical to those observed in HA generated in living cells (Marquardt et al., 1993). The efficiency of folding, defined as the percentage of total translocated HA converted to NT, reached 80% by 4 hr. UT was not visible in the nonreduced gel because it was aggregated and cross-linked by interchain disulfides that prevented its entry into the resolving gel.

More than one third of the translocated HA was coprecipitable with anti-calnexin antibodies (Figure 1A, RD, lanes 7–9). Depending on the antibody preparation, the amount could reach as high as 80%. To determine whether glucose trimming was needed for HA binding to calnexin, we included inhibitors of glucosidases I and II (Elbein, 1991) in the in vitro translation mixture. Castanospermine (CST), deoxynojirimycin, and N-methyl deoxynojirimycin treatment resulted in the generation of a higher molecular mass HA (Figure 2, lanes 2–4), confirming that trimming was inhibited. Regardless of the inhibitor employed, no HA association with calnexin could be observed (Figure 2, lanes 6–8). The removal of one or more glucoses from the N-linked oligosaccharides of HA was therefore required for HA binding to calnexin.

After completed disulfide formation, the newly synthesized HA dissociated from calnexin, but this reaction was highly temperature dependent and incomplete. At 27°C, the temperature normally used for in vitro translation, no release could be observed (see Figure 1A, lanes 7–9; Figure 1B). At 37°C, most of the HA was released, but it misfolded and entered large disulfide-linked aggregates (data not shown). However, at the intermediate tempera-

ture of 32°C, dissociation of NT was observed (see Figure 1A, lanes 10–12; Figure 1B). Approximately 45% of the HA coprecipitating with calnexin at the beginning of the chase dissociated within 4 hr. Microsomes thus provided a cell-free system in which the folding and glycan-dependent association with calnexin of HA could be analyzed. At 32°C, association with the chaperone was transient, as it is in live cells.

Analyzing Differentially Glucose-Trimmed Forms of HA

Direct biochemical analysis could not be employed to establish which of the trimmed glycoforms supported calnexin association because the amount of HA was too small for oligosaccharide analysis. Nor was it possible to use metabolic labeling, as glycosylation in the microsome system relies on presynthesized endogenous dolichol-pyrophosphate precursors. Instead, we took advantage of the small electrophoretic mobility differences observed among HA molecules at different stages of trimming. The result in Figure 2 indicated that glucose trimming resulted in a detectable shift in gel mobility. The difference between untrimmed HA (Figure 2, lanes 2–4) and calnexin-bound HA (lane 5) suggested a molecular mass difference of 7.5 kDa. Since the combined molecular mass of all 21 glucoses is only 3.8 kDa, their contribution to the apparent molecular mass was exaggerated. Whether this was caused by their exposed position in the longest antenna of the oligosaccharides or by anomalous SDS binding is not clear, but the effect helped to provide the resolution needed to separate different trimming intermediates of HA.

To analyze the glycans further, we subjected the immunoprecipitated HA molecules to glycosidase treatment prior to SDS–PAGE. Endoglycosidase H (endo H), which removes all but a single N-acetyl glucosamine (Figure 3A), was routinely used to confirm that electrophoretic mobility differences were caused by oligosaccharides and not by other covalent modifications. An example is shown in Figure 3B (lanes 14–17), in which all the HA bands from different preparations migrated at an equal rate slightly slower than UT. The mobility differences in the corresponding untreated samples (Figure 3B, lanes 1–4) therefore reflected differences in the N-linked glycans.

Jack bean α-mannosidase, an exoglycosidase, was used to distinguish between incompletely and fully glucose-trimmed oligosaccharides (Hammond et al., 1994). As schematically shown in Figure 3A, it maximally removes five mannoses from the tri-, di-, and monoglucosylated oligosaccharides (which we will call G3, G2, and G1, respectively). In unglucosylated glycans (G0), there are eight accessible α-linked mannoses. Their removal causes an accentuated mobility difference between glucose-containing and glucose-free chains (Hammond et al., 1994). An example of this can be seen by comparing the magnitude of the shift between lanes 3 and 4 with that between lanes 11 and 12 in Figure 3B. By removing the side antennae, α-mannosidase also abolishes any differences that might be caused by mannose trimming in the microsomes. It is of interest to note that inclusion of 1-deoxymannojirimycin, an ER mannosidase inhibitor, had no effect on any of the results. The loss of terminal mannoses, known to occur at a slow rate in the ER, therefore did not play an essential role either in our experiments or in the interpretation of the mobility data.

Homogeneous preparations of G3, G2, and G0 HA were used as reagents and as standards. They had distinct electrophoretic mobilities and displayed characteristic glycosidase-induced shifts (Figure 3B). G3 HA was obtained by translation in the presence of CST (Figure 3B, lanes 1, 9, and 14). G2 HA (Figure 3B, lanes 2, 10, and 15) was obtained in microsomes from which glucosidase II had been

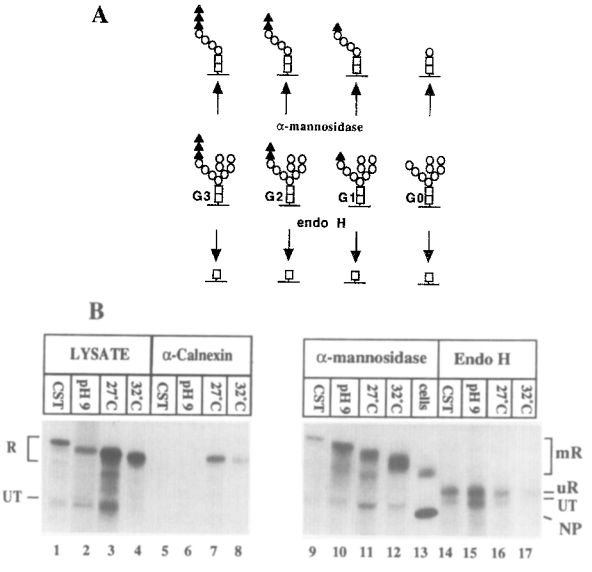

Figure 3. Monoglucosylated HA Associates with Calnexin

(A) The products of glycosidase digestion of the various glucosylated glycans are outlined. Triangles, circles, and squares represent glucoses, mannoses, and N-acetyl glucosamines, respectively. Jack bean α-mannosidase removes terminal α-mannoses. Endo H cleaves ER forms of glycans between the two N-acetyl glucosamine residues.

(B) Lysates containing four differential glucosylated HAs were generated and analyzed separately by reducing SDS–PAGE and fluorography (lanes 1–4). Triglucosylated ^{35}S-labeled HA was generated by translating HA at 27°C under oxidizing conditions into microsomes, which were pretreated for 10 min with 1 mM CST (lanes 1, 5, 9, and 14), as in Figure 2. Diglucosylated ^{35}S-labeled HA was generated by translocating HA into microsomes depleted of glucosidase II. Rough ER microsomes were depleted of soluble lumenal components by alkaline extraction of membranes with a pH 9.0 buffer (Bulleid and Freedman, 1988; Nicchitta and Blobel, 1993). Membranes were resealed and isolated by centrifugation through a neutral pH sucrose gradient before use in the in vitro translation (lanes 2, 6, 10, and 15). More completely trimmed ^{35}S-labeled HA was accumulated by translating HA under oxidizing conditions at 27°C (lanes 3, 7, 11, and 16), as in Figure 1. HA possessing largely unglucosylated glycans was synthesized in the presence of GSSG for 5 hr at 32°C, as in lane 4 of Figure 1. ^{35}S-labeled HA was immunoprecipitated with anti-calnexin (lanes 5–8) or anti-HA (lanes 1–4 and 9–17). Immune complexes of ^{35}S-labeled HA and anti-HA antibodies were digested with α-mannosidase (lanes 9–13) for 2 hr or with endo H (lanes 14–17) for 16 hr at 37°C prior to resolution upon reducing SDS–polyacrylamide gels. An unglucosylated HA standard (lane 13) was obtained from influenza-infected CHO15B cells in which a 2 min ^{35}S-labeled pulse was followed by a chase for 30 min to accumulate ^{35}S-labeled HA in the Golgi complex (Hammond et al., 1994). Reduced (R), untranslocated (UT), mannosidase (mR), and endo H–digested (uR) HAs are designated. The viral nuclear protein (NP) appeared only in the cellular-infected sample.

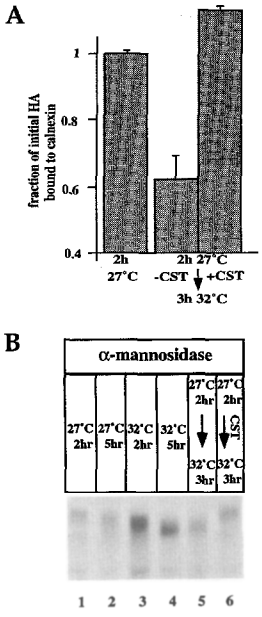

Figure 4. CST Inhibits HA Dissociation from Calnexin

(A) ^{35}S-labeled HA was synthesized for 2 hr at 27°C or 32°C, at which time cycloheximide was added. Samples were either immediately alkylated with NEM or incubated for an additional 3 hr at the temperature of synthesis. Additional samples were first incubated for 2 hr at 27°C and then treated with and without 1 mM CST for 10 min prior to a temperature shift to 32°C for 3 hr. All samples were precipitated with anti-HA and anti-calnexin antibodies. ^{35}S-labeled HA was resolved upon reducing SDS–polyacrylamide gels. HA bands were quantified by digital densitometry. The fraction of initial bound HA was plotted. (B) All samples were digested with α-mannosidase for 2 hr at 37°C prior to resolution by reducing SDS–PAGE. HA translated at 27°C persisted in the G1 form (lanes 1 and 2). Translation or chase at 32°C in the absence of CST initiated further trimming of the glycans, as seen by a rise in the mobility (lanes 4 and 5). The addition of CST prior to the increase in temperature resulted in a decrease in mobility of HA due to the trapping of reglucosylated G0 glycans in the G1 form.

washed out. Unlike glucosidase I, which is an integral membrane protein, glucosidase II could be quantitatively washed out of microsomes because it is soluble or weakly associated with the lumenal membrane surface (Brada and Dubach, 1984). Its quantitative removal was confirmed by Western blotting (data not shown). G0 HA (Figure 3B, lane 13) was obtained from influenza-infected CHO15B cells chased for 30 min (Hammond et al., 1994). Since this mutant cell line contains neither a functional N-acetyl glucosamine transferase (Balch et al., 1986) nor an endomannosidase (Hiraizum et al., 1993), the N-linked glycans are not trimmed beyond a partially trimmed high mannose form free of glucoses. When treated with α-mannosidase, the glycans are trimmed to $Man_1GlcNAc_2$, i.e., the product obtained from all deglucosylated high mannose forms (see schematic in Figure 3A).

Monoglucosylated Glycans Support Calnexin Binding

Since G3 HA failed to associate with calnexin (see Figure 2) (Hammond et al., 1994) and since we have previously shown the same for G0 HA (Hammond et al., 1994), only G2 and G1 HA remained potential ligands for the lectin affinity of calnexin. The G2 HA, translated in the glucosidase II–free microsomes, did not associate with calnexin (Figure 3B, lane 6). The electrophoretic mobility of G2 HA (Figure 3B, lane 2) was clearly less than that of calnexin-bound HA (lane 7). From these observations, we concluded that the diglucosylated (G2) oligosaccharides did not promote calnexin association.

That calnexin-associated HA (Figure 3B, lane 11) had a mobility somewhat faster than the G2 HA (lane 10), but slower than G0 HA (lane 13), was consistent with the glycans being predominantly in the G1 form. The intermediate mobility and the observations that G3, G2, and G0 do not support binding indicated that calnexin must have specificity for G1 glycans. This conclusion is consistent with reports that misfolded vesicular stomatitis virus (VSV) G proteins bind to calnexin and contain predominantly G1 chains (Suh et al., 1989; Hammond and Helenius, 1994b) and that T cell receptor α and β subunits, HA, VSV G protein, and numerous other proteins fail to associate with calnexin in cell lines devoid of functional glucosidase II (Kearse et al., 1994; A. Ora and A. H., unpublished data).

Glucosidase II Is Involved in the Release of HA from Calnexin

Our model for calnexin function not only predicts that binding is mediated by the G1 glycans (a prediction confirmed by the results presented above), but also that removal of glucoses is needed to dissociate the HA from calnexin. To test whether release was glucosidase dependent, HA was allowed to accumulate in stable calnexin complexes at 27°C. The temperature was then shifted to 32°C for 3 hr in the presence or absence of CST, and the extent of calnexin association was determined by anti-calnexin precipitation. In contrast with controls in which about 40% of the initially bound HA dissociated, no release of HA from calnexin occurred in the presence of posttranslationally added CST (Figure 4A). The fraction of calnexin-bound HA actually continued to rise in the presence of CST, suggesting that more G1 glycans were formed.

The electrophoretic mobilities of HA after α-mannosidase treatment showed that the shift from 27°C to 32°C caused accelerated deglucosylation of HA (Figure 4B, lane 5 versus lane 1). Since the HA shown in lane 6 migrated slower than the HA in lane 1, the presence of CST not only blocked the deglucosylation process but reversed it. The bands were broad, however, suggesting heterogeneity among the individual HA molecules, which was expected since each HA has multiple N-linked glycans. This experiment showed that enzymatic glucose removal is required to disengage the HA–calnexin complex.

HA Undergoes Reglucosylation

G1 glycans can arise via two different pathways: by trimming of the original core oligosaccharide and by reglucosylation of G0 glycans. To determine whether HA is a substrate for reglucosylation in microsomes, we generated unfolded HA molecules by translating HA in the presence of 4 mM dithiothreitol (DTT) to inhibit disulfide bond formation. The UDP-glucose:glycoprotein glucosyltransferase only reglucosylates high mannose glycans in glycopro-

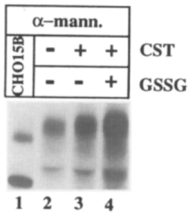

Figure 5. HA Translated under Reducing Conditions Is Largely Unglucosylated, and the Restoration of the Oxidizing Environment Results in the Reglucosylation of Glycans

^{35}S-labeled HA was generated by both in vitro translation and influenza infection of CHO15B cells. CHO15B cells were pulse labeled for 2 min and chased for an additional 30 min to accumulate G0 HA in the Golgi (lane 1). HA was translated and translocated into microsomes under reducing conditions for 2 hr at 27°C. At this point, the samples were either alkylated (lane 2) or treated with 1 mM CST in the absence (minus) (lane 3) or presence (plus) of GSSG (lane 4) for an additional hour before alkylation. NEM-treated lysates were precipitated with anti-HA antibodies. Immune complexes were digested with α-mannosidase (α-mann.) for 2 hr at 37°C prior to resolution by reducing SDS–PAGE.

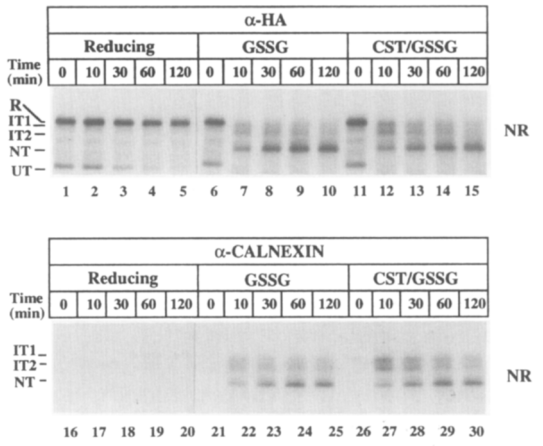

Figure 6. Posttranslationally Oxidized HA Associates with Calnexin

^{35}S-labeled HA was translated under reducing condition (4 mM DTT) for 1 hr at 27°C. At time 0, 4.5 mM GSSG was added in the absence (lanes 6–10 and 21–25) or presence of 1 mM CST (lanes 11–15 and 26–30), or the reducing environment was maintained (lanes 1–5 and 16–20). Samples were alkylated at the designated times. ^{35}S-labeled HA was immunoprecipitated with anti-HA (α-HA) (lanes 1–15) or anti-calnexin (α-Calnexin) (lanes 16–30) antibodies prior to resolution by nonreducing (NR) SDS–PAGE. Reduced HA (R), untranslocated HA (UT), oxidative intermediates (IT1 and IT2), and native oxidized HA (NT) are designated.

teins that are incompletely folded (Sousa et al., 1992), and DTT effectively prevents HA folding in cells and in microsomes (Braakman et al., 1992; Marquardt et al., 1993). The samples were NEM-treated, solubilized, precipitated with anti-HA antibodies, treated with α-mannosidase, and analyzed by SDS–PAGE.

The HA produced was fully reduced and extensively glucose trimmed (Figure 5, lane 2). After α-mannosidase treatment, it migrated only slightly slower than the G0 HA standard (Figure 5, lane 1) but much faster than G2 HA (data not shown), suggesting that most of the molecules still contained some glucose residues. To test whether HA served as a substrate for reglucosylation, we added CST to inhibit glucosidase II. This resulted in a small but reproducible shift to a higher apparent molecular weight in the α-mannosidase-treated HA (Figure 5, lane 3). The increase indicated that reglucosylation was taking place. Evidently, all the enzymes involved, glucosidases I and II and glucosyltransferase, were active in the presence of DTT. The reduced HA contained some G0 glycans, and these served as a substrate for reglucosylation. An equilibrium was likely established in which reglucosylation was balanced by glucosidase II–dependent deglucosylation so that some G0 glycans were present at any given time.

Interestingly, when GSSG was added with CST, the HA was even further glucosylated, judging by the additional increase in apparent molecular weight (Figure 5, lane 4). The addition of GSSG had several profound effects on the system: it resulted in a more efficient immunoprecipitation with anti-HA antibodies because the reduced form of HA is poorly precipitated (Figure 5); it initiated posttranslational folding of HA to the IT1, IT2, and NT forms (Figure 6, lanes 6–10; Marquardt et al., 1993); it induced almost immediate binding of HA to calnexin (Figure 6, lanes 22–25); and it triggered an increase in the reglucosylated form of HA glycans (data not shown). This effect was enhanced by CST, but was not entirely dependent on it (see Figure 5, lanes 3 and 4). Neither the folding of HA nor its binding to calnexin was affected by CST (Figure 6, lanes 12–15 and 27–30). That CST did not interfere with these processes was significant because it demonstrated that CST has no direct effect on substrate binding to calnexin, nor on the folding process per se.

Taken together these experiments strongly supported three further predictions from our model: that unfolded HA can undergo reglucosylation, that reglucosylation is balanced by CST-inhibitable deglucosylation, and that reglucosylated HA can bind to calnexin.

The only unexpected finding in the DTT experiments was that little of the reduced HA was associated with calnexin. Since it did contain some G1 glycans, HA should have been associated with the chaperone. It was evident that DTT not only prevented HA folding, but also somehow interfered with its association with calnexin. The effect was not only seen when DTT was present during translation, but also when it was added posttranslationally. DTT caused the dissociation of already formed HA–calnexin complexes in a CST-insensitive manner (data not shown). This indicated that the complexes did not dissociate via the normal glucosidase-dependent mechanism. Western blots using anti-calnexin antibodies revealed a direct effect of DTT on calnexin. When microsomes were treated with DTT, a slight reduction in SDS–PAGE mobility occurred, indicating that at least one intrachain disulfide bond was sensitive to DTT (data not shown). The calnexin ectodomain contains four cysteines. We concluded that reduction of intrachain disulfide bonds in calnexin greatly lowered its capacity to bind HA. Since the addition of GSSG rapidly restored calnexin function (Figure 6, lanes 22–25), the effect was reversible.

Discussion

It has been known for many years that glycoproteins acquire their N-linked oligosaccharides in the ER and that these undergo extensive processing prior to transport to the Golgi complex (Hubbard and Robbins, 1979; Kornfeld and Kornfeld, 1985; Elbein, 1991). The basic rationale for these co- and posttranslational processes has, however, remained obscure. Why maintain an elaborate glycosylation machinery in the ER when the Golgi complex in the same pathway possesses extensive oligosaccharide-synthesizing capabilities? Why assemble a complex 14-saccharide unit and, after addition to a polypeptide chain, immediately trim it down? Why keep adding glucose residues to glycans that have already undergone full glucose trimming? Our results indicate that the answers to these questions are related to the folding and quality control of the newly synthesized glycoproteins. The N-linked glycans serve at least two purposes in these early processes: to increase the overall solubility of folding intermediates and lower the risk of aggregation (Kern et al., 1993) and to mediate the interaction of glycoproteins with lectin-like chaperones, such as calnexin (Hammond et al., 1994; Helenius, 1994). Evidently, glucosidases I and II and the UDP-glucose:glycoprotein glucosyltransferase modulate their binding to the chaperones and make it reversible once folding is completed.

Rather than using intact cells to study these processes, we took advantage of a classic cell-free translation system. All major ER chaperones and folding enzymes are present in the isolated microsomes, and the milieu can be readily adjusted by inclusion of a redox buffer, ATP, and other required factors (Marquardt et al., 1993). Therefore, the microsomes not only supported translocation of HA and cotranslational modifications such as signal sequence cleavage and N-linked glycosylation, but also chaperone-mediated folding and conformational maturation. The advantages of this system were the wide range of experimental conditions that could be tested, the opportunity to manipulate the composition of the lumen, and the rapid access of inhibitors.

In many respects, folding of HA in microsomes was similar to that described in live infected cells (Braakman et al., 1991). The same intermediates were seen, and the immunochemical properties of the final product (NT) were identical (Marquardt et al., 1993). However, the process was slower and the overall efficiency somewhat reduced. Furthermore, we found that certain events, such as the release of NT from calnexin, were incomplete and highly temperature sensitive. Release without misfolding and aggregation occurred only at an intermediate temperature around 32°C. Our experiments show that the NT that dissociates from calnexin at this temperature proceeds to trimerize (D. N. H. and A. H., unpublished data).

As in live cells, CST and other glucosidase inhibitors prevented the association of HA with calnexin. The two outermost glucoses had to be removed from one or more glycans in the HA before it could bind to calnexin. The monoglucosylated N-linked oligosaccharides were, according to our results, the only form that supported the binding of HA to calnexin. Since CST failed to block binding of already trimmed HA to calnexin, it was clear that CST had no direct effect on calnexin per se. Using microsomes lacking glucosidase II, it could be shown that glucosidase II was critical for preparing the glycoprotein substrate for calnexin binding. Since the substrate for glucosidase II is generated by glucosidase I, one can conclude that both enzymes play a critical role in this process. The same conclusion can be reached from the inhibition of glycoprotein association with calnexin in mutant cell lines devoid of either glucosidase I or glucosidase II (Kearse et al., 1994; A. Ora and A. H., unpublished data).

CST also inhibited the dissociation of HA from calnexin after completed folding. Glucosidase II thus serves a dual function: it removes the middle glucose to allow substrate association with calnexin, and it removes the innermost glucose to allow substrate disengagement from calnexin. This finding demonstrated that the glycan-dependent contact between the substrate and calnexin is not only needed for the formation of a complex, but also for maintaining it. A shift from a lectin-like binding mode to an entirely peptide-based interaction is unlikely.

Our results indicated, moreover, that the removal of the last glucose residues in HA glycans was counteracted by a process of reglucosylation that converted the G0 glycans back to the G1 form. We could observe this reaction only when HA was synthesized in the presence of DTT, which kept the HA unfolded and therefore compatible as a substrate for UDP-glucose:glycoprotein glucosyltransferase. However, we believe that it is an ongoing process for as long as the HA remains incompletely folded. Studies with a temperature-sensitive folding mutant of VSV G protein, tsO45, have indicated that permanently misfolded glycoproteins can enter a futile de- and reglucosylation cycle that keeps them persistently associated with calnexin (Suh et al., 1989; Hammond et al., 1994). Judging by the gel mobility of α-mannosidase-digested HA, the de- and reglucosylation cycle reached an equilibrium in the presence of DTT, with a fraction of the glycans in the G0 state and the rest presumably in the G1 state. When CST was added, a shift toward the G1 state was observed. These findings indicated that a re- and deglucosylation cycle does occur in the microsomes.

The DTT used to keep HA unfolded also had another effect; it reduced the disulfide bond(s) of calnexin and thus inhibited its ligand binding capacity. When the normal redox environment was restored inside the vesicles by the addition of GSSG, calnexin rapidly associated with the HA molecules, causing an apparent shift toward a higher fraction of G1 glycans. These DTT effects are not restricted to HA nor to microsomes. We have observed them for HA, VSV G protein, and a variety of cellular calnexin substrates (U. Tatu and A. H., unpublished data). They contradict a previous report that found that DTT prolonged and enhanced complexes between calnexin and a 70 kDa secretory glycoprotein in MDCK cells (Wada et al., 1994).

Taken together, the findings were all consistent with a model that places glucosidase II and UDP-glucose:glycoprotein glucosyltransferase in a cycle that regulates calnexin binding of newly synthesized glycoproteins (Ham-

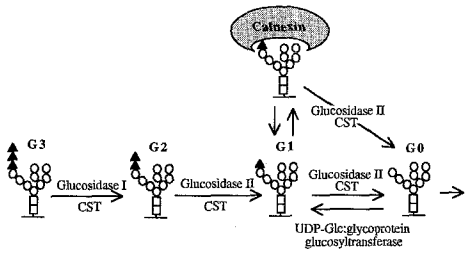

Figure 7. Model of Glucose Trimming and Reglucosylation Relationship to Calnexin Association and Disengagement

Triangles, circles, and squares represent glucoses, mannoses, and N-acetyl glucosamines, respectively. All glucosidase activities can be inhibited with CST.

mond et al., 1994). Figure 7 shows the various steps in the pathway, as we perceive it in light of the present results. The removal of the first α1–2 glucose is required to make a glycan substrate for glucosidase II. This soluble ER enzyme in turn removes the second glucose and allows the glycoprotein to bind to calnexin. Depending on its folding status, the glycoprotein can then enter a de- and reglucosylation cycle. By folding or assembling (or both) into a proper oligomeric protein, the glycoprotein can escape the glucosyltransferase that does not utilize folded and fully assembled proteins as substrates and thus leave the cycle. While it is apparent that the G1 glycans of calnexin-free HA molecules in DTT-containing solutions do serve as substrates for glucosidase II, it is not clear from our results whether the calnexin-bound HA can be deglucosylated without first dissociating from the chaperone. Further studies are needed to clarify this important point. In our model (Figure 7), we have included arrows for both possibilities.

The G1 glycans act as the determinants for chaperone binding. They are located on the surface of folding molecules and oligomerizing assemblies and therefore are easily accessible. Unlike the hydrophobic peptides that are thought to serve as binding sites for hsp70 family members (Rothman, 1989; Blond-Elguindi et al., 1993), the carbohydrates do not get buried during the folding and assembly process. An enzymatic posttranslational modification may therefore be necessary to regulate the on/off cycle of this class of chaperone.

While the G1 glycans are clearly the most important determinants for calnexin binding, additional protein–protein contacts between the chaperone and the substrate glycoprotein cannot be ruled out. Cross-linking studies have demonstrated close proximity between calnexin and the transmembrane domain of a bound substrate molecule, the heavy chain of major histocompatibility complex class I antigens (Margolese et al., 1993). Depending on the properties of individual glycoproteins, folding and assembly may involve additional lumenal components such as BiP/GRP78, protein disulfide isomerase, GRP94, and *cis-trans* peptidyl proline isomerase. Many of these are known to associate transiently with newly synthesized glycoproteins.

Calnexin is not the only lectin in the secretory pathway or even in the ER. The mannose 6-phosphate receptors in the Golgi are clearly responsible for intracellular targeting of lysosomal hydrolases from the Golgi to the endosomes (Kornfeld, 1992). ERGIC-53, a membrane protein that recycles among the ER, the intermediate compartment, and the *cis* Golgi, and a *trans*–Golgi network protein VIP36 have both been shown to have sequence homology with leguminous plant lectins (Fiedler et al., 1994; Fiedler and Simons, 1994). Moreover, we have evidence that calreticulin, an abundant soluble ER homolog of calnexin, interacts selectively with partially trimmed glycoproteins (J. Peterson, A. Ora, P. N. Van, and A. H., unpublished data). Further studies in microsomes, cells, and living organisms are needed to define more fully the significance of the N-linked glycans and their early processing. A more complete understanding of the glycoprotein maturation will be helpful in the generation and design of functional recombinant protein. Also, several human disease states involve folding or quality control defects in glycoproteins at the level of calnexin binding (Le et al., 1994; Pind et al., 1994).

Experimental Procedures

Reagents

The cell-free translation system (rabbit reticulocyte lysate, amino acid mixture lacking methionine, DTT, and RNasin) were purchased from Promega (Madison, WI). The dog pancreas microsomes were a gift from Dr. R. Gilmore (Worcester, MA). Promix ^{35}S metabolic-labeling reagent was purchased from Amersham Corporation (Arlington Heights, IL). GSSG and Zysorbin (fixed and killed Staphylococcus aureus) were obtained from Fluka Chemical Corporation (Ronkonkoma, NY) and Zymed Laboratories (South San Francisco, CA), respectively. CHAPS (3-[(3-cholamidopropyl)-dimethylammonino]-1-propanesulfonate) was acquired from Pierce (Rockford, IL). All other chemicals employed, including jack bean α-mannosidase, were purchased from Sigma Biochemicals (St. Louis, MO).

The polyclonal anti-HA rabbit serum was raised against whole X31 influenza virus (derived from A/Aichi/1968/H3N2) (Doms et al., 1985) and recognized all forms of HA. The polyclonal anti-calnexin rabbit serum was raised against a synthetic peptide corresponding to the cytoplasmic tail of canine calnexin (Wada et al., 1991). A polyclonal rabbit antiserum raised against purified glucosidase II was a gift from Dr. D. Brada (Zurich, Switzerland).

Influenza X31 HA mRNA

The cDNA for influenza X31 HA was cloned into a pBluescript expression system. Uncapped HA mRNA was synthesized utilizing T7 RNA polymerase (Boehringer Mannheim Biochemicals, Indianapolis, IN) as described previously (Marquardt et al., 1993).

Translation, Translocation, and Folding of HA

^{35}S-labeled HA was translated and translocated into dog pancreas microsomes using the following mixture: 52 μl of rabbit reticulocyte lysate, 6 μl of ribonuclease-treated dog pancreas microsomes, 2 μl of amino acid mixture minus methionine, 8 μl of ^{35}S metabolic label, 1 μl of 100 mM DTT, 16 μl of H_2O, 4 μl of RNase inhibitor, and 4 μl of HA mRNA (1μg/μl) (Marquardt et al., 1993). Cotranslational or vectorial folding studies were performed by including 4 mM GSSG in the translation mixture. Posttranslational folding studies were carried out by translating and translocating HA into microsomes at 27°C under reducing condition. After 1 hr of translation, 4.5 mM GSSG was added to initiate synchronous oxidation posttranslationally (Marquardt et al., 1993). Samples were removed at designated times and alkylated with 20 mM NEM to block all free sulfhydryls (Creighton, 1978; Braakman et al., 1991). The samples were then either analyzed directly by SDS–PAGE or immunoprecipitated prior to electrophoresis.

Immunoprecipitation

For anti-HA precipitation, alkylated samples were solubilized in ice-cold 0.5% Triton X-100 in MNT buffer (20 mM MES, 100 mM NaCl, 20 mM Tris–HCl [pH 7.5]), 0.5% Zysorbin containing protease inhibitors (1 mM EDTA and 10 μg/ml each of chymostatin, leupeptin, antipain, and pepstatin). Anti-calnexin precipitations were solubilized in 2% CHAPS in HBS (50 mM HEPES and 200 mM NaCl [pH 7.5]) containing Zysorbin and protease inhibitors. After 1 hr of end-over-end rotation at 4°C, the Zysorbin was pelleted by centrifugation at 2500 × g at 4°C. To the supernatant, 0.1% protein A–Sepharose beads and 0.005% polyclonal anti-HA or calnexin serum were added. The samples were incubated at 4°C with end-over-end rotation for 16 hr. The immune complexes were pelleted at 2500 × g and washed once with 0.05% Triton X-100, 0.1% SDS, 300 mM NaCl, 10 mM Tris–HCl (pH 6.8) for anti-HA precipitations or in 0.5% CHAPS in HBS for anti-calnexin precipitations. The final pellets were resuspended in 50 μl of sample buffer (200 mM Tris [pH 6.8], 3% SDS, 10% glycerol, 0.004% bromophenol blue, 1 mM EDTA), heated at 95°C for 5 min, and centrifuged to remove protein A–Sepharose. Samples were divided into two portions with 100 mM DTT added to the reducing samples, which were then reheated at 95°C for 5 min prior to analysis by SDS–PAGE (7.5%) and fluorography. HA bands were quantified by densitometry with a Visage 200 digital gel scanner when needed.

Soluble Lumenal Protein Extraction

To remove soluble intralumenal proteins from the microsomes, we diluted the microsomes 1:10 in 50 mM CAPS, 50 mM HEPES solution buffered to pH 9.0 and incubated on ice for 30 min (Nicchitta and Blobel, 1993). The microsomes were isolated and resealed by centrifuging at 60,000 × g for 30 min in a Beckman TL-100 ultracentrifuge through a sucrose cushion (0.5 M sucrose, 50 mM triethanolamine [pH 7.4]) with a sample:cushion ratio of 3:1. Pelleted microsomes were resuspended into the reticulocyte lysate mixture described previously (Marquardt et al., 1993).

Glycosidase Digestions

After immunoprecipitation of ^{35}S-labeled HA with anti-HA antibodies, the wash buffer was removed, leaving the pelleted protein A–Sepharose immune complexes. For α-mannosidase, the pellet of each sample was resuspended in 25 μl of 0.05 M sodium citrate buffer (pH 4.4) and 0.28 U of jack bean α-mannosidase. The samples were incubated at 37°C for 2 hr. For endo H digestion, the immune complex pellet was resuspended in 25 μl of 100 mM sodium acetate, 0.2% SDS (pH 5.5) and heated at 95°C for 5 min. The samples were cooled, and 25 μl of 100 mM sodium acetate (pH 5.5) and 0.05 U/μl of endo H were added and incubated at 37°C for 16 hr. Digested samples were analyzed by reducing SDS–PAGE.

Western Blot Analysis

Proteins were transferred to nitrocellulose (Schleicher & Schuell, Keene, NH) with a PolyBlot Transfer System (American Bionetics, Hayward, CA) for 10 min at 400 mA in 192 mM glycine, 25 mM Tris (pH 8.5). The nitrocellulose was blocked with 3% milk in phosphate-buffered saline (PBS) for 16 hr at 4°C. Blots were washed three times between antibody and chemilluminiscence incubations with PBS. The secondary antibody employed was goat anti-rabbit Ig Fc (Pierce) labeled with horseradish peroxidase. ECL (Amersham Corporation, Arlington Heights, IL) was employed as the detection reagent.

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