Calnexin and calreticulin promote folding, delay oligomerization and suppress degradation of influenza hemagglutinin in microsomes

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Calnexin (CNX) and calreticulin (CRT) are molecular chaperones that bind preferentially to monoglucosylated trimming intermediates of glycoproteins in the endoplasmic reticulum. To determine their role in the maturation of newly synthesized glycoproteins, we analyzed the folding and trimerization of *in vitro* translated influenza hemagglutinin (HA) in canine pancreas microsomes under conditions in which HA's interactions with CNX and CRT could be manipulated. While CNX bound to all folding intermediates (IT1, IT2 and NT), CRT was found to associate preferentially with the earliest oxidative form (IT1). If HA's binding to CNX and CRT was inhibited using a glucosidase inhibitor, castanospermine (CST), the rate of disulfide formation and oligomerization was doubled but the overall efficiency of maturation of HA decreased due to aggregation and degradation. If, on the other hand, HA was arrested in CNX–CRT complexes, folding and trimerization were inhibited. This suggested that the action of CNX and CRT, like that of other chaperones, depended on an 'on-and-off' cycle. Taken together, these results indicated that CNX and CRT promote correct folding by inhibiting aggregation, preventing premature oxidation and oligomerization, and by suppressing degradation of incompletely folded glycopolypeptides.

Keywords: calnexin/calreticulin/endoplasmic reticulum/protein folding

Introduction

Molecular chaperones are proteins that associate specifically with incompletely folded or unassembled proteins, and increase the efficiency by which they acquire their correct three-dimensional structure. The best characterized molecular chaperones are members of two stress protein families, Hsp60 and Hsp70. They associate with a large number of different proteins. *In vivo* and *in vitro* evidence indicate that they directly improve the efficiency of protein folding (reviewed in Rothman, 1989; Gething and Sambrook, 1992; Hartl *et al.*, 1992; Hendrick and Hartl, 1993).

We are studying two less characterized chaperones; calnexin (CNX), a membrane protein, and calreticulin (CRT), its soluble homolog (see Michalak *et al.*, 1992; Bergeron *et al.*, 1994). They are localized in the endoplasmic reticulum (ER) where they bind selectively and transiently to newly synthesized glycoproteins (Ou *et al.*, 1993; Hammond *et al.*, 1994; Peterson *et al.*, 1995; Wada *et al.*, 1995). Their preference for glycoproteins is based on a

lectin-like affinity for monoglucosylated *N*-linked oligosaccharides (Glc₁Man_{9–7}GlcNAc₂) (Hammond *et al.*, 1994; Hebert *et al.*, 1995; Ora and Helenius, 1995; Peterson *et al.*, 1995; Ware *et al.*, 1995).

We have found that binding of substrate glycoproteins to and release from CNX and CRT depends on the trimming and reglucosylation of the *N*-linked glycans (Hammond *et al.*, 1994; Helenius, 1994; Hebert *et al.*, 1995). The monoglucosylated oligosaccharides are generated by trimming the two outermost glucose residues from the core oligosaccharides (Kornfeld and Kornfeld, 1985). Glucosidase I removes the first of the three glucoses and glucosidase II the second, and eventually also the third. Monoglucosylated glycans can also be generated by UDP-glucose:glycoprotein glucosyltransferase, a luminal enzyme that adds a glucose residue to glucose-free, high mannose chains in glycoproteins that are incompletely folded (Sousa *et al.*, 1992). We have proposed that these three enzymes, together with CNX and CRT, provide an ER-specific folding and retention machinery (Hammond *et al.*, 1994; Helenius, 1994).

Although CNX and CRT are referred to routinely as molecular chaperones, there is little direct data to show that they actually influence the folding of substrate proteins. Their key role is often thought to be the retention of folding intermediates and misfolded proteins in the ER (Bergeron *et al.*, 1994; Rajogapalan *et al.*, 1994). Inhibitors of the α -glucosidases which prevent glycoprotein binding to CNX and CRT are, however, known to cause delays in the folding and intracellular transport of glycoproteins (reviewed in Helenius, 1994), suggesting a more direct chaperone-like function. For example, if CNX binding is blocked, formation of intrachain disulfide bonds is partially inhibited in vesicular stomatitis virus G protein (Hammond and Helenius, 1994), and the oxidation of MHC class I heavy chain is delayed (Tector and Salter, 1995).

To investigate whether CNX and CRT have a direct role in the conformational maturation of glycoproteins, we have monitored the folding of *in vitro* translated influenza hemagglutinin (HA) in dog pancreas microsomes. The system was first optimized so that it could be used to follow both folding and oligomeric assembly of HA. The binding of CNX and CRT to various HA intermediates was determined, and the effects of their binding on folding and trimerization assayed. The results indicated that CNX and CRT interact with different but overlapping populations of folding intermediates, and that they have a variety of effects on the rates and efficiency of folding, oligomeric assembly and degradation.

Results

HA folds and trimerizes in microsomes

To evaluate the role of CNX and CRT in HA maturation *in vitro*, we first had to find conditions in which the microsomal system would not only support the folding of

Fig. 1. HA trimerizes at 32°C in microsomes. ^{35}S -Labeled HA was translated under oxidizing conditions at 27, 32 or 37°C. After 2 h, cycloheximide was added and the samples were chased for up to an additional 6 h. At the designated times, the samples were treated with NEM, lysed and precipitated with anti-HA and anti-HA trimeric antibodies. HA was analyzed by non-reducing (NR) and reducing (RD) SDS-PAGE. Oxidative intermediates (IT1 and IT2), fully oxidized HA (NT) and reduced HA (R) were resolved.

HA but also its assembly into homotrimers. The most important change from previous protocols (Marquardt *et al.*, 1993; Hebert *et al.*, 1995) proved to be an increase in temperature. Experiments were performed at three different temperatures: 27°C (the temperature previously used to study *in vitro* translation and folding of HA); 37°C (the physiological temperature); and 32°C, an intermediate temperature. After translation and incubation, samples were removed from the translation mixture at different times. Remaining free thiols were alkylated with *N*-ethylmaleimide (NEM) to block further disulfide bond formation (Braakman *et al.*, 1991; Marquardt *et al.*, 1993). The samples were then lysed, precipitated with either polyclonal anti-HA antibodies or trimer-specific HA monoclonal antibodies, and analyzed by SDS-PAGE (non-reducing and reducing) and fluorography.

As shown in Figure 1, only the intermediate temperature of 32°C provided conditions in which the HA folded efficiently to the fully oxidized form (called NT for native) and, in addition, proceeded to trimerize. About 15% of the labeled HA became immunoprecipitable with trimer-specific monoclonal antibodies (lanes 17–20). That these were authentic trimers was confirmed by trypsin digestion and velocity gradient centrifugation (our unpublished

Fig. 2. CRT association with HA is glucosidase dependent. HA was translated under oxidizing conditions in the absence (–) or presence (+) of the glucosidase inhibitor, CST, for 1 h at 27°C. Alkylated lysates were analyzed either directly (Lysate) or after precipitation with anti-HA (α-HA), anti-CNX (α-CNX) or anti-CRT (α-CRT) antibodies upon reducing SDS-PAGE. Untrimmed triglycosylated (uR), reduced (R) and untranslocated (UT) HA are designated (Marquardt *et al.*, 1993).

data). As anticipated for mature trimers (Copeland *et al.*, 1986), the HA precipitated by trimer-specific antibodies was not degraded by trypsin but merely clipped into the two native fragments HA₁ and HA₂, and in sucrose velocity gradients roughly one-tenth of total HA sedimented at velocity of 9S_{20,w}.

Together with previous data (Marquardt *et al.*, 1993; Hebert *et al.*, 1995), these results indicated that the entire pathway of maturation and glycan trimming that HA normally undergoes in the ER—from the earliest full-length oxidative intermediates (IT1 and IT2) to the fully assembled homotrimer—can be reproduced in microsomes provided that the conditions and temperature are adjusted appropriately. The reason why only a fraction of the labeled HA trimerized was likely to be due to the low amount of HA synthesized during *in vitro* translation. Many of the microsomal vesicles may not have contained sufficient HA molecules to support efficient trimer formation. HA trimerization is known to be a concentration-dependent process (Braakman *et al.*, 1991).

CNX and CRT associate with different HA intermediates

To determine whether both CNX and CRT bound to HA during folding and trimerization in microsomes, [^{35}S]-methionine-labeled HA was translated in the presence or absence of castanospermine (CST) for 1 h at 27°C. CST is an α -glucosidase inhibitor that prevents glucose trimming and HA binding to CNX and CRT (Elbein, 1991; Hammond *et al.*, 1994; Peterson *et al.*, 1995). Samples were alkylated and either lysed with the zwitterionic detergent CHAPS and immunoprecipitated with anti-HA, anti-CNX or anti-CRT antibodies (Figure 2, lanes 3–8), or solubilized directly with SDS-containing sample buffer (lanes 1–2). When the intensity of the HA bands was compared after SDS-PAGE under reducing conditions, the fraction of HA that precipitated with each antibody could be estimated.

The amount of HA brought down with anti-HA antibodies (Figure 2, lanes 3 and 4) was 90% of total HA in

Fig. 3. HA dissociates from CNX and CRT at 32°C. ^{35}S -Labeled HA was generated under oxidizing conditions as described in Figure 1 with cycloheximide added after 2 h of translation. The samples were alkylated and lysed, and HA was precipitated with anti-CN α (α-calnexin) or anti-CRT (α-calreticulin) antibodies prior to analysis by non-reducing (NR) and reducing (RD) SDS-PAGE. Folding intermediates (IT1 and IT2), fully oxidized HA (NT) and reduced HA (R) are designated.

the lysate (lanes 1 and 2). The slower electrophoretic mobility of HA in lanes 2 and 4 compared with HA in lanes 1 and 3 confirmed that CST inhibited trimming of the glycans. A comparison of HA bands in lanes 1 and 2 demonstrated that CST had no effect on the efficiency of HA translation.

The amount of HA precipitated with anti-CN α antibodies was 60% of total HA (lane 5). When CST was present, none of the HA was precipitated (lane 6). When immunoprecipitations were performed with anti-CRT antibodies (lane 7), 36% of the HA in the CST-free samples was co-precipitated. Again, the untrimmed HA in the CST-containing sample was not precipitated (lane 8). Thus, both CN α and CRT associated with large fractions of the labeled HA, and the association was inhibited by CST.

The extent of glucose trimming of the CRT-associated HA and the trimeric HA was determined using jack bean α-mannosidase which accentuates the electrophoretic mobility difference between HA molecules with unglucosylated and glucosylated glycans (Hammond *et al.*, 1994; Hebert *et al.*, 1995). The CRT-associated HA migrated with a mobility intermediate between the untrimmed (G3) HA and the fully glucose-trimmed (G0) HA standard (data not shown). It had the same mobility as the CN α -associated HA, consistent with an HA population rich in monoglucosylated oligosaccharides (Hammond *et al.*, 1994; Hebert *et al.*, 1995; Peterson *et al.*, 1995). Trimeric HA precipitated using the trimer-specific monoclonal antibody migrated with the glucose-free HA standard (data not shown). This was expected because trimerization in live cells occurs after release of HA from CN α (Tatu *et al.*, 1995), and release from the lectin chaperones depends on removal of the final glucose residue.

To compare further HA's association with CN α and CRT, [^{35}S]methionine-labeled HA was synthesized for 2 h at 27, 32 and 37°C and chased for up to an additional 6 h. SDS-PAGE analysis of alkylated, non-reduced samples after immunoprecipitation with anti-CN α anti-

bodies confirmed that IT1, IT2 (the incompletely oxidized folding intermediates) and NT, all associated with CN α at 27 and 32°C (Figure 3 'NR', lanes 1–8). The disulfide cross-linked HA aggregates generated at 37°C, which migrate at the top of the gel, were co-precipitated with CN α (lanes 9–12). During the chase at 32°C, a large fraction of HA initially bound to CN α dissociated from the lectin. This is best appreciated by comparing lanes 5 and 8 in Figure 3 'RD'. Only 25% of the HA initially associated with CN α remained bound after 6 h of chase. Release of HA from CN α also occurred at 27°C (compare lanes 1 and 4). It is noteworthy that at this temperature the dissociated HA was neither degraded nor trimerized (see Figure 1), but remained monomeric unlike the HA dissociating at 32°C.

HA's association with CRT was only observed in the 27 and 32°C samples. Its association was limited to the first 4 h at 32°C, and was thus more transient than CN α association. Accordingly, it involved almost exclusively IT1, the earliest full-length folding intermediate of HA (Figure 3 'NR', lanes 13 and 17). We concluded that although both lectins associated with monoglucosylated forms of HA, CRT bound preferentially to the early folding intermediate IT1 whereas CN α bound to both early and late intermediates. Moreover, the two chaperones differed in that CN α associated with misfolded, disulfide cross-linked forms of HA, while CRT did not.

HA's folding is arrested when permanently bound to CN α and CRT

Having established that the major events in the *in vitro* folding pathway reflected those occurring in live cells and that CRT and CN α displayed somewhat different binding specificities, experiments were performed to determine what role CN α and CRT play in the folding of HA. We again used CST as an inhibitor of glucosidases, and analyzed whether it affected the folding of HA.

First, we tested how HA folded when CST was used to block the dissociation of the HA–CST and HA–CRT complexes (Hebert *et al.*, 1995). CST was added to

Fig. 4. Post-translationally added CST inhibits dissociation of CNX and CRT complexes, formation of disulfide bonds and subsequent HA trimerization. ^{35}S -Labeled HA was translated in the presence of microsomes at 32°C . After 2 h, cycloheximide was added and the samples were divided into two aliquots, of which one was incubated in the absence of CST (-CST) and the other in its presence (+CST) for the additional designated times. The samples were then alkylated, lysed with detergent, and aliquots were precipitated with anti-HA, anti-CN, anti-CRT or anti-trimeric antibodies prior to electrophoresis using non-reducing (NR) and reducing (RD) SDS-PAGE. This experiment was repeated six times with indistinguishable results.

microsomes after 2 h of translation under oxidizing conditions. At this time, most of the HA was bound to CNX and CRT. The disassembly of the complexes was largely prevented by CST addition. When the effect of CST on HA folding in these samples was evaluated in the non-reduced gels, a dramatic effect was observed. While conversion of HA from the incompletely oxidized intermediate forms IT1 and IT2 was normal in control samples (lanes 15–18 and 22–25), in CST-containing samples the HA remained incompletely oxidized (Figure 4, lanes 19–21 and 26–28). Most of the IT1 and IT2 persisted over 6 h. Trimerization was also blocked judging by the lack of HA precipitable with the trimer-specific monoclonal antibody (Figure 4, lanes 33–35).

These observations indicated that HA molecules trapped in CNX and/or CRT complexes failed to undergo normal disulfide bond formation. The conformational freedom of the bound glycopolypeptides was apparently constrained by the interaction with CNX and CRT. This implied that a process of release and rebinding akin to that observed for

Hsp60 and Hsp70 chaperone families is occurring with CNX and CRT. Unlike the classical chaperones, which rely on ATP hydrolysis, the 'on-and-off' cycle is, however, mediated by a covalent modification in the *N*-linked glycan moieties of the substrate protein and induced by two enzymes with opposing activities; glucosidase II and UDP-glucose: glycoprotein glucosyltransferase.

CST accelerates HA's folding and trimerization

Instead of preventing the dissociation of HA–lectin complexes, CST could also be used to prevent complex formation (Hammond *et al.*, 1994; Peterson *et al.*, 1995). In this case, CST was included in the translation mixture to prevent the removal of any of the three glucoses in the core glycans (see Figure 2) (Hebert *et al.*, 1995). The consequences on the folding of HA are seen in Figure 5A. The generation of the oxidized NT form was considerably faster than in control samples. While HA folded with a half-time of 80 min in the absence of CST at 32°C , in its presence folding was nearly twice as fast ($t_{1/2}$ 43 min). CNX/CRT binding appeared to inhibit the oxidation of HA.

When trimerization was analyzed, a similar acceleration was observed (Figure 5B). At 32°C , trimers formed with a half-time of 1.2 h in the presence of CST and 3.1 h in its absence. The difference was even larger at 27°C (Figure 5C) where trimerization was non-existent in the absence of CST (Figure 1 and Figure 5C, lanes 1–6), but rapid trimerization was observed in the presence of CST (Figure 5C, lanes 7–12).

These results indicated that when HA folded without association with CNX and CRT, oxidation of cysteines and oligomerization occurred more rapidly. In other words, binding to CNX and CRT suppressed rapid folding and disulfide bond formation, and prevented rapid trimerization.

CST decreases HA's folding efficiency

While the addition of CST during translation did not seem to have a noticeable effect on the overall efficiency of HA folding at 32°C , we found that folding at 37°C clearly was compromised (our unpublished data). This suggested that, while slowing down the rate of folding, CNX–CRT could have a favorable effect on the efficiency of folding, at least when conditions for folding were suboptimal.

To pursue this possibility, we changed the folding conditions so that HA would be folded entirely post-translationally. As previously described (Marquardt *et al.*, 1993; Hebert *et al.*, 1995), fully reduced HA can be generated in microsomes by allowing translation to occur in the presence of dithiothreitol (DTT). Folding can then be induced by the addition of oxidized glutathione (GSSG). As GSSG enters the microsomes, the reduced HA immediately begins to acquire disulfide bonds, it binds to CNX, and about one-third proceeds to fold to the fully oxidized NT form. While folding under these conditions is clearly suboptimal, it was easily quantified because the amount of HA did not increase during the folding period due to continued translation.

To test whether binding to CNX–CRT was critical for HA folding under these conditions, CST was included in the translation and oxidation mixtures. After addition of GSSG, the fraction of HA that converted to NT was quantified. It was found that at 27 and 32°C folding was

Fig. 5. Co-translational CST causes rapid folding and trimerization of HA. ^{35}S -Labeled HA was translated in the presence (+CST) or absence (-CST) of CST at 32°C. After 2 h, cycloheximide was added. Alkylated and lysed HA was precipitated with anti-HA (A) or anti-trimeric (B) antibodies prior to resolution upon non-reducing and reducing SDS-PAGE. Autoradiograms were analyzed by densitometry. (A) The total amount of NT was obtained in arbitrary units, and (B) the fraction of trimeric HA was determined as the fraction of anti-trimeric precipitable HA over total HA. (C) ^{35}S -Labeled HA was translated under oxidizing conditions in the absence (-CST) or presence (+CST) of CST at 27°C. Alkylated and lysed HA was removed at the designated times, precipitated with trimer-specific antibodies and resolved on reducing SDS-PAGE.

<60% of CST-free controls (Figure 6A). At 37°C, none of the HA folded properly when CST was present, whereas 7% folded in its absence. We concluded that the lack of glucose trimming affected the efficiency of folding particularly under suboptimal folding conditions.

Fig. 6. CST lowers folding efficiency and increases degradation of HA. ^{35}S -Labeled HA was translated under reducing conditions either in the absence (-CST) or presence (+CST) of CST for 1 h at 27°C. Cycloheximide and 4.5 mM GSSG were added for an additional 1 h at 27, 32 or 37°C. At this time, HA should be folded sufficiently since the half-time of post-translational folding for HA at 27°C is 7.5 min (Marquardt *et al.*, 1993). HA was then alkylated, lysed and precipitated with anti-HA antibodies and resolved by non-reducing and reducing SDS-PAGE. Autoradiograms were analyzed by densitometry with the folding efficiency (A) determined as NT (after 1 h of oxidation at the respective temperature) over the total amount of HA after 1 h of translation under reducing conditions. (B) The total amounts of HA remaining after 1 h of oxidation at the respective temperatures from the reducing gels are displayed.

CST increases degradation of HA

Finally, we also determined whether CST caused a change in the level of HA degradation. Studies in living cells have indicated that ER degradation of glycoproteins is often increased in the presence of glucosidase inhibitors (Moore and Spiro, 1993). Quantification of HA degradation in microsomes was performed by densitometric analysis of labeled HA bands. By subtracting the labeled HA remaining after a 1 h incubation from the initial amount, we could estimate the extent of proteolysis (Figure 6B).

In the absence of CST, the amount of degradation was insignificant at 32°C. In contrast, at 37°C, about one-third of the HA was lost in an hour. At all temperatures, degradation was increased by >30% when CST was present during translation. We concluded that binding to CNX and CRT protected HA from ER degradation in microsomes. Whether the protection was caused by more efficient folding or by direct steric protection of HA against proteases is unclear.

Discussion

The most important result from these studies was that the interaction with CNX and CRT clearly influenced HA's folding and trimerization. When HA was prevented from binding to these lectins, the rates of folding and oligomeric assembly were accelerated and maturation was less efficient. When, on the other hand, HA was arrested in CNX and CRT complexes, folding and oligomeric assembly were inhibited. These results indicated that CNX and CRT are not only responsible for retention of incompletely folded glycoproteins in the ER but, like other chaperones, they directly affect the outcome of the maturation process.

When trapped in CNX and CRT complexes by the inhibition of glucosidase II, HA failed to proceed in its folding pathway. This suggested that the degree of folding that a substrate glycopolyptide has while bound may be limited. Apparently, a substrate needs to be able to dissociate from these chaperones to proceed through the normal folding pathway. Like chaperones in the Hsp60 and 70 families, CNX and CRT are thus likely to function through an 'on-and-off' cycle whereby substrates are repeatedly bound and released. During the 'on' phase of the cycle, contacts with the chaperone probably limit the steric freedom of the substrate glycopolyptide. In the case of HA, these interactions prevent global folding.

The degree of restraint imposed by CNX and CRT is likely to depend on a glycoprotein's structure, on the number of glycans as well as additional factors. In the case of HA, folding involves the formation of four small (64–76, 97–139, 281–305 and 473–477) and two large disulfide loops (52–277 and 14–466) (Wilson *et al.*, 1981). Closing of the large loops may require a high degree of steric freedom. The presence of numerous potential CNX and CRT attachment sites, in the form of seven *N*-linked glycans uniformly distributed over the length of the protein, may serve further to enhance the constraints imposed on the HA glycopolyptide. Local folding and the formation of some of the small disulfide loops may, however, occur while HA is bound.

Previously, we have observed that post-translational addition of CST under reducing conditions was not effective in inhibiting subsequent HA oxidation (Hebert *et al.*, 1995). This difference was probably due to the starting point at which CNX association of HA occurred; when allowed to bind post-translationally by addition of an oxidizing agent to reduced microsomes, the HA was clearly more extensively glucose trimmed and probably more folded at the time of attachment than it was under the conditions used here in Figure 4. In other words, the ability of the chaperones to restrain a protein's further folding may depend on its folding status at the time of binding.

Whereas substrate binding to GroEL and Hsp70 is determined by direct interaction with a substrate's polypeptide chain (Landry *et al.*, 1992), CNX and CRT associations are determined primarily by the *N*-linked glycans. Unlike the on-and-off cycles of GroEL and Hsp70s, which depend on ATP hydrolysis, the CNX–CRT cycle relies on enzymatic removal and re-addition of glucose residues to the substrate protein. Although additional contacts between the polypeptide chain and the CNX molecule may contribute to the binding (Arunachalam and Cresswell, 1995; Ware

et al., 1995; Zhang *et al.*, 1995), it is apparent that the specificity for complex formation is provided by the carbohydrate moieties. Consequently, as we have shown here and elsewhere (Hammond *et al.*, 1994; Hebert *et al.*, 1995), both binding and dissociation are determined by the action of α -glucosidases.

We have proposed that the responsibility for quality control in the CNX and CRT pathway rests with the UDP-glucose: glycoprotein glucosyltransferase (Hammond *et al.*, 1994; Helenius, 1994). This enzyme is capable of distinguishing between folded and unfolded glycoproteins, and it uses the latter as a substrate for reglucosylation (Sousa *et al.*, 1992; Sousa and Parodi, 1995). The CNX–CRT pathway thus differs from classical chaperones in that, instead of involving an on-and-off cycle which depends on a set of co-chaperones (such as DnaJ, GrpE or GroES) and on ATP hydrolysis (Martin *et al.*, 1991; Todd *et al.*, 1994; Weissman *et al.*, 1994), it relies on a set of independently functioning enzymes and substrate binding proteins.

In many important respects, maturation of HA in microsomes mirrors that of newly synthesized HA in living cells. The folding intermediates, the glucose trimming, the transient chaperone interactions and the orderly progression from subunit folding to trimerization can all be reproduced. The overall rate of maturation is, however, slower. All four key events; translation, folding to NT, release from CNX–CRT and trimerization occur more slowly than in live cells. Translation at 27°C was about four times slower in our system than in cells (our unpublished data). Based on the rate of NT formation after post-translational addition of GSSG to microsomes at this temperature, we can derive an approximate half-time of 7 min compared with 5 min in live cells (Braakman *et al.*, 1991; Marquardt *et al.*, 1993). With rates in the range of 2–3 h, the release of HA from CNX and its trimerization were the steps most affected in microsomes. The slow rate of these late events may have numerous causes. It could be the result of inefficient glucosidase action resulting in a slow 'off' rate of substrate glycoproteins. Trimerization and the release could also be coupled so that slow trimerization, due to the low concentration of HA, would force the fully oxidized monomer to stay in the CNX–CRT cycle longer.

The subphysiological optimal temperature found in microsomal maturation of HA may have its explanation in reduced chaperone action in the isolated microsomes compared with the ER of live cells. Preliminary observations have indicated that chaperone dependence of HA folding increases rapidly above 32°C (our unpublished data). It should be noted, in this context, that *in vitro* refolding experiments with denatured polypeptides are, as a rule, only successful at even lower temperatures (Creighton, 1990; Jaenicke, 1991).

Chaperone-bound HA did not undergo trimerization even when folded to the fully oxidized NT form. In contrast, when CST was used to prevent the lectin binding, trimerization of HA occurred rapidly. The observation that CNX and CRT may actually have a role in preventing premature trimer formation is supported by previous observations in living cells that trimerization occurs after dissociation of NT from CNX (Tatu *et al.*, 1995). Recent reports on MHC class I indicated that complexes between

MHC class I heavy chains and CNX dissociated prior to the association with β_2 -microglobulin (Nossner and Parham, 1995; Tector and Salter, 1995). Others have, however, reported that CNX is associated with oligomeric intermediates of MHC class I and II in the ER (Degen *et al.*, 1992; Anderson and Cresswell, 1994).

Although CNX and CRT both bind to glycoproteins with monoglucosylated *N*-linked oligosaccharides (Hammond *et al.*, 1994; Nauseef *et al.*, 1995; Peterson *et al.*, 1995; Wada *et al.*, 1995; Ware *et al.*, 1995), a difference in their interaction with HA emerged from our co-precipitation studies: CRT bound preferentially to IT1 while CNX associated with IT1, IT2 and some NT. Accordingly, CRT was found to dissociate more rapidly from HA as folding proceeded. Given the apparent identity in glycan specificity, this suggested that other factors affect binding of the two chaperones. One could be differential accessibility of the glycan moieties to the membrane-associated CNX and the lumenally located CRT. Preliminary results indicate that the two lectins may actually bind to different sets of glycan moieties on HA (our unpublished data).

Materials and methods

Reagents

The components of the cell-free translation system (rabbit reticulocyte lysate, amino acids lacking methionine, DTT and RNasin) were purchased from Promega Inc. (Madison, WI). The canine pancreas microsomes were a generous gift of Dr R.Gilmore (Worcester, MA). Multiple reticulocyte preparations along with two microsome preparations were employed during the course of these experiments. Promix ³⁵S-metabolic labeling reagent, GSSG (oxidized glutathione) and Zysorbin (fixed and killed *Staphylococcus aureus*) were purchased from Amersham Corp. (Arlington Heights, IL), Fluka Chemical Corp. (Ronkonkoma, NY) and Zymed Laboratories (S. San Francisco, CA), respectively. CHAPS, {3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate}, was obtained from Pierce (Rockford, IL). All other chemicals and enzymes were purchased from Sigma Biochemicals (St Louis, MO).

Two polyclonal HA antibodies were used together in α -HA precipitations to obtain quantitative precipitations of HA: a polyclonal raised against whole X31 influenza virus (a strain of virus derived from A/Aichi/1968/H3N2) (Doms *et al.*, 1985) and an anti-peptide polyclonal antibody raised against a peptide corresponding to the N-terminal sequence of HA. The polyclonal anti-CN_X rabbit serum was raised against a synthetic peptide corresponding to the cytoplasmic tail of CN_X (Wada *et al.*, 1991). A mouse monoclonal antibody specific for trimeric HA (Copeland *et al.*, 1986) that recognizes the N2 epitope of HA was used. The α -CRT antibody was obtained from Affinity Bioreagents Inc. (Golden, CO).

Influenza HA mRNA

The cDNA of influenza X31 HA was cloned into a pBluescript expression system (Stratagene Inc., La Jolla, CA). Uncapped HA mRNA was synthesized using the T7 promoter with T7 RNA polymerase (Boehringer Mannheim, Indianapolis, IN) as described previously (Marquardt *et al.*, 1993).

Translation, translocation and folding of HA

³⁵S-Labeled HA was translated and translocated into dog pancreas microsomes as described in Hebert *et al.* (1995). For co-translational folding, HA was translated in the presence of 4.0 mM GSSG; for post-translational folding it was translated in the absence of GSSG for 1 h, at which time 50 mM cycloheximide and 4.5 mM GSSG were added. Samples were treated with 20 mM NEM to block free thiols by alkylation prior to immunoprecipitation and resolution upon SDS-PAGE of [³⁵S]HA. CST (1 mM) was employed when indicated.

Immunoprecipitations

For anti-HA precipitations, 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 μ g/ml each of chymostatin, leupeptin, antipain and pepstatin). For anti-CN_X and anti-CRT precipitations, the samples were solubilized in 2% CHAPS in HBS [50 mM HEPES and 200 mM NaCl (pH 7.5)] containing Zysorbin and protease inhibitors (Ou *et al.*, 1993; Peterson *et al.*, 1995). After 1 h of end-over-end rotation at 4°C, the Zysorbin was pelleted by centrifugation at 2500 g at 4°C. To the supernatant, 1.6% protein A-Sepharose beads and 1.6% polyclonal HA (whole virus and N-terminal each for anti-HA), 1.6% polyclonal CN_X, 0.7% polyclonal CRT or 2% monoclonal trimeric HA antibodies were added and the samples were incubated with end-over-end rotation for 16 h at 4°C. The immune complexes were pelleted and washed with 0.05% Triton X-100, 0.1% SDS, 300 mM NaCl, 10 mM Tris-HCl (pH 6.8) for anti-HA and anti-trimeric precipitations or in 0.5% CHAPS in HBS for anti-CN_X and anti-CRT precipitations. The final samples were treated as described (Hebert *et al.*, 1995) prior to analysis by 7.5% non-reducing and reducing SDS-PAGE. HA bands were quantified by densitometry with a Visage 200 digital scanner when required.

Glycosidase digestion

Immune complexes of ³⁵S-labeled HA were digested directly with 2.8 mU of jack bean α -mannosidase in 0.05 M sodium citrate buffer (pH 4.4) per 25 μ l sample as described previously (Hammond *et al.*, 1994; Hebert *et al.*, 1995).

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