Interactions between Newly Synthesized Glycoproteins, Calnexin and a Network of Resident Chaperones in the Endoplasmic Reticulum

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Abstract. Calnexin is a membrane-bound lectin and a molecular chaperone that binds newly synthesized glycoproteins in the endoplasmic reticulum (ER). To analyze the oligomeric properties of calnexin and calnexin-substrate complexes, sucrose velocity gradient centrifugation and chemical cross-linking were used. After CHAPS solubilization of Chinese Hamster Ovary cells, the unoccupied calnexin behaved as a monomer sedimenting at 3.5 $S_{20,W}$. For calnexin-substrate complexes the $S$-values ranged between 3.5–8 $S_{20,W}$, the size increasing with the molecular weight of the substrate. Influenza hemagglutinin, a well-characterized substrate associated with calnexin in complexes that sedimented at 5–5.5 $S_{20,W}$. The majority of stable complexes extracted from cells, appeared to contain a single calnexin and a single substrate molecule, with about one third of the calnexin in the cell being unoccupied or present in weak associations. However, when chemical cross-linking was performed in intact cells, the calnexin-substrate complexes and calnexin itself was found to be part of a much larger heterogeneous protein network that included other ER proteins. Pulse-chase analysis of influenza-infected cells combined with chemical cross-linking showed that HA was part of large, heterogeneous, cross-linked entities during the early phases of folding, but no longer after homotrimer assembly. The network of weakly associated resident ER chaperones which included BiP, GRP94, calreticulin, calnexin, and other proteins, may serve as a matrix that binds early folding and assembly intermediates and restricts their exit from the ER.

The ER maintains an efficient machinery for protein translation, folding, oligomeric assembly, and quality control (Gething and Sambrook, 1992; Helenius et al., 1992b). The lumen provides an exclusive, highly specialized environment for the controlled folding and maturation of membrane proteins and soluble proteins most of which are destined for export to other organelles or for secretion. The redox environment and the ionic milieu are carefully maintained and concentration of molecular chaperones and folding enzymes such as protein disulfide isomerase (PDI), GRP 94, calnexin, calreticulin, and ERp72 is very high. At nearly millimolar concentrations, the most abundant of them greatly outnumber the substrate proteins.

The chaperone-assisted folding and the formation of disulfide bonds begin already while growing nascent chains enter the lumenal compartment (Bergman and Kuehl, 1979; Chen et al., 1995). It continues posttranslationally often with the formation of additional disulfide bonds and in many cases with the assembly of oligomers (Hurtley and Helenius, 1989; Braakman et al., 1991; Gething and Sambrook, 1992). Proteins that fail to fold or oligomerize properly are, as a rule, prevented from export, and get degraded (Hurtley and Helenius, 1989; Klausner, 1989; Hammond and Helenius, 1995).

During folding, polypeptides with N-linked oligosaccharides interact transiently and specifically with two homologous lectin-like chaperones that are unique to the ER, the membrane-bound calnexin and the soluble calreticulin. A transmembrane protein of 64 kD, calnexin interacts transiently with a large number of different glycoproteins during their folding and maturation (Ou et al., 1993). Calreticulin (46 kD), a soluble protein interacts with an overlapping but not identical group of proteins (Nauseef et al., 1995; Peterson et al., 1995; Wada et al., 1995). Both bind specifically to partially trimmed, monoglucosylated forms of the N-linked core glycans present on folding intermediates (Hammond et al., 1994; Hebert et al., 1995; Peterson et al., 1995; Wada et al., 1995; Spiro et al., 1996). They promote proper folding, prevent premature oligomerization, inhibit degradation, and mediate quality control for a variety of glycoproteins (David et al., 1993; Ou et al., 1993; Hammond and Helenius, 1994; Jackson et al., 1994; Kearse...
Materials and Methods

Cells, Viruses, and Chemicals

CHO15B cells and the Lec 23 cells used in this study are mutant cells that lack the medial-Golgi enzyme N-acetyl glucosamine transferase and glucosidase I, respectively (Gottlieb et al., 1975; Ray et al., 1991). They were grown as described (Balch et al., 1986; Ora and Helenius, 1995). The X31 influenza virus was used to infect CHO15B cells (Braakman et al., 1991). Infected cells were used 4–6 h after infection for the pulse-chase experiments. Media and reagents for cell culture were obtained from Gibco BRL (Grand Island, NY). Brefeldin A (BFA) was purchased from Epicenter Technologies (Madison, WI). It was used at a final concentration of 5 μg/ml, from a stock of 1 mg/ml in ethanol. CHAPS and the chemical cross-linking reagents [Dithiobis(succinimidylpropionate) (DSP), Disuccinimidyl Glutarate (DSG), and Disuccinimidyl Suberate (DSS)] were purchased form Pierce (Rockford, IL). Castanospermine (CST), Cycloheximide (CHX), N-ethylmaleimide (NEM), and Triton X-100 were purchased from Sigma Chem. Co. (St. Louis, MO). [35S]-Promix containing cysteine and methionine was purchased from Amersham Corp. (Arlington Heights, IL).

Antibodies and Immunoprecipitations

The rabbit anti-influenza virus serum, the antiserum against the NH2-terminal 1-12 amino acid sequence of X31 influenza hemagglutinin (HA) referred to as NHA1, and the rabbit anti-calnexin COOH-terminal peptide antibodies have been described (Doms et al., 1985; Hammond et al., 1994; Peterson et al., 1995). The polyclonal rabbit anti-rat-calreticulin antiserum was a gift from Dr. Hans Dieter Söling (Göttingen, Germany). The latter was affinity purified against rat calreticulin for use in immunoprecipitations (Peterson et al., 1995). The rabbit anti-calreticulin polyclonal antiserum and the rat anti-GP49 monoclonal antisem in the cross-linking experiments in intact cells was purchased from Affinity Bioreagents (Neshanic Station, NJ). The mouse anti-Bip monoclonals were purchased from StressGen (Victoria, Canada).

Pulse-chase Analysis of HA Folding and Metabolic Labeling

The folding of HA was followed by the method described by Braakman et al., 1991). For studying calnexin binding the cells were lysed with 2% CHAPS in 0.2 M NaCl, 50 mM Hapes, pH 7.5 (HBS buffer, Hammond et al., 1994). For overnight labeling, the cells were starved for 15 min for methionine and cysteine and then 4 ml of complete α-MEM containing 0.3 mM of radiolabeled methionine and cysteine (Amersham Promix) was added to each 60-mm dish.

Velocity Sedimentation on Sucrose Gradients

Monomeric and trimeric forms of HA were resolved on 5–25% continuous sucrose gradients in 20 mM MES, 100 mM NaCl, 30 mM Tris HCl, pH 8.6 (MNT buffer) with 0.1% Triton X-100 (Copoland et al., 1986). In experiments where the gradient fractions were to be used for cross-linking, the sucrose gradients were made in HBS buffer containing 0.1% Triton X-100. The cell lysates were layered on top of 5-ml gradients and centrifuged in an SW 55 rotor at 42,000 rpm for 15 h at 4°C (Beckman Instrs., Fullerton, CA). After centrifugation the gradients were fractionated manually from top to bottom. To characterize the size of the large cross-linked complex (Fig. 6), the cross-linked cell lysates were sedimented on a 5–25% sucrose gradient in TLS 55 rotor tubes. The tubes were sedimented at 160,000 g (50,000 rpm) for 3 h at 4°C in a Beckman table top ultracentrifuge. The gradients were fractionated into 12 fractions and precipitated with anti-HA antibodies.
Calnexin-Substrate Complexes

When the experiments in CHO cells described above were repeated without cycloheximide or castanospermine treatment, the endogenous calnexin-substrate complexes could be analyzed. The distribution of calnexin in the gradients was now clearly bimodal (Fig. 1C). In addition to the peak at 3–4 S\textsubscript{20,W} which cosedimented with the unoccupied calnexin (A and B), a more prominent rapidly sedimenting population of calnexin molecules was apparent. Calnexin was distributed as heterogeneously sized complexes over the 3–8 S\textsubscript{20,W} range.

The proteins complexed to calnexin could be visualized when the cells were pulse-labeled for 5 min before solubilization (Fig. 2). As expected (Ou et al., 1993; Peterson et al., 1995), a large number of different cellular proteins were found coprecipitating with calnexin. These were present as a relatively discrete peak in the 4–8 S\textsubscript{20,W} range centered at ~5.5 S\textsubscript{20,W}. The substrate proteins that had a higher apparent molecular weight were present in the faster sedimenting complexes indicating that their size determined the increment in sedimentation rate over that of calnexin alone. Given the relatively narrow size distribution and the low overall sedimentation rates, it seemed likely that most of the CHAPS-solubilized complexes contained a single calnexin and a single substrate molecule.

Analyzing Calnexin-Hemagglutinin Complexes by Cross-linking

To further analyze the properties of calnexin substrate complexes, we studied a single well-defined substrate molecule, influenza HA. This 84-kD glycoprotein associates with calnexin cotranslationally and remains associated for ~4 min after chain completion until all the intrachain disulfides are formed (Hammond et al., 1994; Chen et al., 1995; Tatu et al., 1995). After dissociating from calnexin, HA monomers proceed to assemble into noncovalent homotrimers that are transported to the Golgi complex and eventually to the plasma membrane.

Using sucrose gradients, we have previously shown that calnexin-HA complexes sediment at 5–5.5 S\textsubscript{20,W}, calnexin-free HA subunits at 4.5–5 S\textsubscript{20,W}, and mature HA trimers at 8.9 S\textsubscript{20,W} (Tatu et al., 1995). Fig. 3A shows the sedimentation of the folding intermediates (IT1, IT2), the fully oxidized NT, and the trimers of HA on sucrose gradients. As shown in Fig. 3B, anti-calnexin antiserum brought down IT1, IT2, and some of the fully oxidized HA (NT) from the 5–5.5 S\textsubscript{20,W} region of the gradient. Calnexin itself was seen as a weakly labeled band just above IT1 (Fig. 3B).

To characterize the oligomeric structure of the detergent solubilized complexes, influenza-infected cells were pulsed for 5 min, lysed, and fractionated on similar sucrose gradients. Fractions 5–7, which contained the calnexin and calreticulin complexes with HA, were pooled and aliquots were subjected to chemical cross-linking using the cleavable cross-linker DSP. Together with uncross-linked controls, the samples were then immunoprecipitated with anti-HA, anti-calnexin, and anti-calreticulin, respectively, and analyzed in nonreduced form by SDS-PAGE and fluorography.

As shown in Fig. 3C, anti-HA precipitates of uncross-linked samples contained IT1, IT2, and NT (lane 1). The
Transient Association of HA with Larger Complexes

Next, we used cross-linking in situ to determine whether additional associations existed between the newly synthesized HA and resident ER proteins. We pulse-labeled influenza-infected cells for 3 min and chased for various times. The cells were then incubated with the cross-linker DSP (which is membrane permeable), lysed with CHAPS, immunoprecipitated with different antibodies, and analyzed by SDS-PAGE and fluorography. In the 5% gels used in this experiment, the separation of IT1, IT2, NT, and G (G is the Golgi form of HA) was improved, and large cross-linked complexes were more completely resolved than above.

The results shown in Fig. 4A, demonstrated that the newly synthesized HA could be cross-linked inside the ER of the cell. The products turned out to be quite different than those seen after solubilization. When DSP was added within the first 2 min of chase, cross-linking was very efficient as shown by the virtually complete disappearance of the monomeric HA species (lanes 6 and 7, Nonreduced). That the HA was present in the material on top of the stacking gel was shown by boiling the samples in the presence of DTT, thus breaking the DSP links (lanes 6 and 7, Reduced).

The result indicated that early folding forms of HA were efficiently cross-linked to covalent complexes most of which were too large to get into the gel. The same large HA-containing complexes could also be precipitated by anti-calnexin (Fig. 4B, lanes 6 and 7), suggesting that the 1:1 calnexin-HA complexes observed by velocity centrifugation in CHAPS lysates (Fig. 3C) were part of much larger cross-linkable complexes in situ.

As HA maturation proceeded during the chase, the pattern of cross-linked products began to change. After 5–20 min of chase, the HA appeared in the form of three discrete bands (Fig. 4A, lanes 8–10). Unlike the previous large complexes, these were not coprecipitated with anti-calnexin. One of them corresponded to the noncross-linked monomeric species, the two others to HA homodimers and homotrimers familiar from previous studies (Doms and Helenius, 1986). All three bands were precipitable with trimer-specific monoclonal antibodies to HA (Copeland et al., 1986) (Fig. 4B, anti-trimer). Moreover, within each band, a slight shift occurred with time from a slower to a somewhat faster migrating band. This shift is caused by mannose trimming of N-linked glycans in the Golgi complex (Balch et al., 1986). The shift revealed that the trimeric HA had moved from pre-Golgi compartments to the Golgi complex.

Similar large cross-linked complexes were obtained using another cross-linker DSG, indicating that the results were not cross-linker dependent (not shown). Moreover, when DSP was included in the lysis buffer, similar large cross-linked complexes were observed (not shown). Thus, the interactions between the HA and its cross-linking partners were apparently stable for some time after solubilization and dilution. Together with experiments described below (Fig. 7B), this result indicated that the extensive cross-linking of early folding forms of HA was not just due to a high local protein concentration in the ER.
To arrive at a size estimate for the cross-linked complexes containing HA, velocity sedimentation on sucrose gradients was carried out following cross-linking. After precipitating with anti-HA antibodies, the fractions were analyzed on SDS-PAGE in Nonreduced form (Fig. 5). Sedimentation standards (urease, 18.9 S\textsubscript{20,w}; and BSA, 4.5 S\textsubscript{20,w}) were analyzed on identical gradients. While the uncross-linked HA was found in fractions 2, 3, 4, and 5, the cross-linked complexes containing HA were present throughout the gradient indicating heterogeneous size. The cross-linked complexes ranged in size from \(~8\) to more than 40 S\textsubscript{20,w}.

Taken together, the results showed that early folding forms of HA (IT1, IT2, and NT) are cross-linked to large, heterogeneous calnexin-containing complexes. Evidently, the HA-calnexin and HA-calreticulin complexes present at this stage of maturation were part of extensive structures that could be stabilized by cross-linking in situ and in lysates immediately after solubilization. As folding proceeded, HA reached its trimeric form and was no longer directly associated with this structure. Instead, cross-linking now occurred between the subunits of the mature HA homotrimer.

**The Effects of Brefeldin A**

That the HA trimers escaped cross-linking after trimer formation could mean that they were still in the ER but dissociated from the chaperone complexes, or it could be simply explained by their export out of the ER. To test whether HA trimers retained in the ER would cross-link...
to the large complexes, we employed BFA, a drug that prevents transport of HA and other proteins from the ER to the Golgi complex (Klausner et al., 1992). As shown in Fig. 6 (lanes 6–10, + DSP), BFA had no effect on the cross-linking of HA either early or late in the chase. That the inhibitor was working, was demonstrated by the lack of conversion of HA to the mannose-trimmed form marked G in the figure.

The result indicated that HA trimers, even though present in the ER, did not get cross-linked to large complexes of ER proteins. The interaction of early folding forms with constituent ER proteins was thus qualitatively different than that of assembled trimers. It was concluded that dissociation of HA from the chaperone machinery proceeded or coincided with trimer assembly, and that this led to a situation where cross-linking no longer occurred. We have previously shown that HA dissociates from calnexin just before trimer formation takes place (Tatu et al., 1995).

Composition of In Situ Cross-linked Complexes

To determine which luminal ER proteins were part of the HA-containing, cross-linked complexes formed in situ, long term (36 h) labeling of CHO cells with [35S]methionine and cysteine was performed so that the resident proteins would be labeled. The cells were then infected with influenza virus, and 5 h postinfection the HA synthesized was
labeled using a brief 5-min pulse. Half of the cells were cross-linked with DSP, and the other half used as an uncross-linked control. The lysates were immunoprecipitated with antibodies against five different antigens; HA, calnexin, calreticulin, BiP, and GRP94. A background control received protein A beads without antibody ("PA control"). The immunoprecipitates were analyzed in nonreduced form to determine whether cross-linking had occurred (NR), and in reduced form (R) to determine the identity of the cross-linked components.

Judging by the amount of cross-linked HA precipitated with anti-calnexin and anti-calreticulin compared to anti-HA, almost all the HA was covalently cross-linked to either one or both of these chaperones (Fig. 7, Reduced, lanes 7–9). A small amount of BiP was also present in the complexes precipitated with anti-HA (bottom right panel, lane 7). We have previously shown that ~5–10% of HA misfolds and binds to BiP (Hurtley et al., 1989). No detectable HA-precipitation occurred with anti-GRP94, but precipitations with this antibody were not very efficient (bottom right panel, lane 11).

The converse experiment using anti-HA did not bring down detectable amounts of labeled cellular proteins except a small amount of BiP (bottom right panel, lane 7). Considering the excess of chaperones present in the ER over HA, this was not unexpected since only a small fraction of each would bind at any given time to the newly synthesized HA molecules. From these results, we tentatively concluded that the large cross-linkable complexes containing HA also contained calnexin and calreticulin and possibly some BiP. Whether other ER proteins such as PDI were present, remains to be determined.

To rule out the possibility that the cross-linking of newly synthesized HA into large complexes containing ER chaperones in situ was merely a result of the high protein concentration, we carried out a cross-linking experiment in castanospermine-treated cells. Castanospermine inhibits HA association with calnexin and calreticulin by preventing trimming of glucose residues from the N-linked core oligosaccharides (Elbein, 1991; Hammond et al., 1994; Peter-
son et al., 1995). If cross-linking would simply occur as a result of high protein concentration, the lack of specific binding of HA to these chaperones should not prevent formation of large calnexin and calreticulin complexes containing HA. In castanospermine-treated cells, the newly synthesized HA failed, however, to precipitate with anti-calnexin, anti-calreticulin, or anti-BiP antibodies regardless of whether the cells had been treated with DSP or not (Fig. 7B, lanes 2 and 3 without DSP, lanes 6 and 7 after DSP cross-linking and reduction). The result indicated that to be cross-linked with a complex containing calnexin and calreticulin, HA had to be specifically associated with these chaperones. It was not enough that they were present in the same compartment.

Figure 7. Composition of the folding complex. (A) CHO15B cells were prelabeled for 36 h and then infected with the Influenza virus. 6 h after infection the cells were pulse labeled for 5 min and the pulse was stopped with ice cold PBS containing NEM. The cells were scraped from the dish and divided into two aliquots. One aliquot was cross-linked with DSP as in Fig. 4. The cells were lysed with 2% CHAPS in HBS after cross-linking, and the lysates from the cross-linked and the control samples were divided into aliquots. These were precipitated with antibodies against HA (lanes 1 and 7), calnexin (CNX, lanes 2 and 8), calreticulin (CRT, lanes 3 and 9), BiP (lanes 4 and 10), GRP94 (lanes 5 and 11) and with Protein A beads without any antibody (lanes 6 and 12). The precipitates were analyzed in nonreduced (top panels) and the reduced form (bottom panels) by 5% SDS-PAGE and fluorography. (B) Two confluent dishes of CHO cells were infected with Influenza virus. 4 h after infection one dish was preincubated with 1 mM castanospermine for 1 h and then pulse labeled for 5 min in the presence of 1 mM castanospermine. The other untreated dish was pulse labeled without castanospermine for 5 min. Cross-linking was performed as above and cells were lysed with 2% CHAPS in PBS. Lysates were divided into four aliquots and immunoprecipitated as above. The immunoprecipitates were analyzed in the reduced form by 7.5% SDS-PAGE and fluorography.
Constitutive Association of Chaperone Proteins in the ER

Interestingly, the precipitations shown in Fig. 7 revealed extensive cross-linking of calreticulin with BiP and GRP94, and of GRP94 with BiP (see lanes 3 and 4). To examine whether these associations were dependent on the presence of newly synthesized substrate proteins, the cells were prelabeled as described. Then cycloheximide was added for 5 h before cross-linking in order to clear the ER of substrate proteins.

It was evident from the results (Fig. 8, A and B) that, under conditions where no newly synthesized proteins were present, the chaperones were associated with each other in a variety of cross-linkable combinations. The anti-calnexin immunoprecipitates after reduction of the large cross-linked complexes formed (Fig. 8, lane 2) showed, for example, bands corresponding to calnexin, calreticulin, BiP, and GRP94. In addition to BiP, anti-BiP precipitates showed GRP94 and some calreticulin. Also, in Lec 23 cells where calnexin and calreticulin do not bind to substrates (Ora and Helenius, 1995) calreticulin could be cross-linked to calnexin, BiP, and GRP94 (Fig. 8A, lane 3).

Discussion

The results indicated that calnexin and possibly calreticulin formed stable, discrete 1:1 complexes with substrate glycoproteins in the ER. These complexes were resistant to detergent solubilization, gradient centrifugation, and immunoprecipitation. In the ER of intact cells, calnexin and calreticulin were, however, part of a larger network of interacting proteins which included other ER chaperones. While this network was stabilized by weaker contacts that dissociated upon detergent solubilization it could be analyzed using chemical cross-linkers in situ.

In CHAPS solubilized samples, substrate-free calnexin molecules were found to sediment at 3–4 S_{20,W}. No evidence for the presence of additional, tightly bound subunits was observed although a shoulder of faster sedimenting forms were occasionally seen. This indicated that calnexin is a monomer when it is not associated with a substrate molecule. Judging by the sedimentation, we estimated that no more than about a third of the calnexin in CHO cells was normally in an unoccupied state. The rest was associated with a variety of different substrate proteins (see also Ou et al., 1993; Peterson et al., 1995).

The sedimentation rate (4–8 S_{20,W}) in sucrose gradients after solubilization with CHAPS, indicated that complexes between calnexin and its substrate glycoproteins were only somewhat larger than calnexin alone. The sedimentation profiles suggested that most of them contained no more than a single substrate molecule. The molecular weight of the substrate proteins determined how much faster the complex sedimented than calnexin itself. Very few of the complexes were in the 12 S_{20,W} range previously reported by Ou et al. (1993) to be the average size of calnexin-substrate complexes after cholate solubilization (Ou et al., 1993). The majority of complexes were in the size range observed for calnexin/α1-antitrypsin complexes (Le et al., 1994) i.e., smaller than originally assumed.

The 1:1 stoichiometry was found to apply to the majority of stable calnexin-HA and calreticulin-HA complexes extracted from virus-infected cells. While monomeric HA has an S-value of ~4.5 S_{20,W} (Doms and Helenius, 1986), the calnexin-HA complexes sedimented at ~5–5.5 S_{20,W}. When the gradient fractions were treated with the chemical cross-linker, the main cross-linked, HA-containing spe-
cies had a MW of 140–160 kD, consistent with a 1:1 complex. Calreticulin-HA dimers somewhat smaller than the calnexin-HA dimers could also be demonstrated. Only a small amount of larger cross-linked species were observed, possibly representing ternary complexes containing both cross-linked calnexin, calreticulin, and HA. Previously we have shown that on sucrose gradients calnexin associated folding intermediates IT1 and IT2 sediment somewhat faster than calnexin associated NT (Tatu et al., 1995). Together with the observation that calreticulin preferentially binds to IT1 and some IT2 (Hebert et al., 1996), it seems probable that the folding intermediates form ternary complexes of HA, calnexin, and calreticulin. That ternary complexes can occur, has been recently shown for HIV gp160, a protein which has 24 N-linked glycans and therefore a large number of potential attachment sites for calnexin and calreticulin (Otteken and Moss, 1996). Others have shown that calnexin can bind to oligomeric assembly intermediates such as assemblies of α, β, and invariant chains of MHC class II antigens (Anderson and Cresswell, 1994). If present in lysates from CHO cells, such large oligomeric, calnexin-containing complexes were too few to be detectable in our gradients.

The observation that calnexin and calreticulin interact with their substrates as monomers renders them somewhat unusual because most lectins are homoooligomeric (Weis and Drickamer, 1996). Even if lectins have extended binding sites that recognize more than just a single terminal residue (as calnexin and calreticulin do [Ware et al., 1995; Spiro et al., 1996]), the affinity for individual glycans generally reaches only the millimolar range which is not sufficient for stable association (Weis and Drickamer, 1996). Most lectins have, therefore, either multiple lectin domains in the same polypeptide chain or they are multimeric. For example, other membrane-bound lectins in the secretory pathway, the two mannose-6-P receptors and ERGIC 53, are oligomeric (Schweizer et al., 1988; Zhongmin et al., 1992). The members of the calnexin family of lectins have, however, conserved sequence repeats (Wada et al., 1995) which could provide as many as 3–4 glycan-binding domains per polypeptide.

While calnexin and calreticulin seem to bind to their substrates as monomers, the in situ cross-linking experiments indicated that they share weaker interactions with larger chaperone entities. Particularly obvious were cross-links between calreticulin and BiP, calreticulin and GRP94, calreticulin and calnexin, as well as BiP and GRP94. Similar contacts have been previously reported in pancreatic acinar microsomes (Baksh et al., 1995) and in chondrocytes (Nakai et al., 1992). Since inhibition of protein synthesis had no effect on the cross-linking patterns, associations are present whether substrate proteins are synthesized or not. They probably represent authentic interactions in a dynamic network throughout the rough ER. Inhibition of substrate binding to calnexin and calreticulin by castanospermine inhibited cross-linking of HA to them further supporting that the cross-links represent highly specific interactions and ruling out the possibility of nonspecific cross-links simply due to high protein concentrations in the ER.

The existence of a network of resident ER proteins has been postulated for some time (Kreibich et al., 1978; Hortsch et al., 1987; Booth and Koch, 1990; Sambrook, 1990; Helenius et al., 1992a; Hammond and Helenius, 1995). At a concentration exceeding 100 mg/ml, the luminal resident proteins, most of which are chaperones and folding enzymes, easily outnumber the newly synthesized substrate proteins present in the ER (Koch, 1987; Marquardt et al., 1993). Electronmicrographs of the ER reveal a lace-like material spanning the luminal space. The majority of resident proteins have COOH-terminal KDEL sequences that provide a signal for retrieval from the Golgi complex (Pelham, 1991), and most of them have long negatively charged sequences that constitute low affinity, high capacity calcium-binding sites. Both of these sequence elements contribute to efficient localization of calreticulin in the ER lumen (Sonnichsen et al., 1994). While providing structure to the lumen and the membrane of the rough ER, the matrix is probably very dynamic and highly responsive to the fluctuations in free calcium and ATP concentrations (Sambrook, 1990; Nigam et al., 1994).

Pulse-chase experiments combined with in situ cross-linking showed that early full-length folding intermediates of HA were connected to the network. Once the HA reached its mature trimeric conformation, it was no longer cross-linked. While BiP and GRP94 were part of larger cross-linked structures, there was no evidence that they interacted directly with HA (Hurtley et al., 1989).

The ER matrix is likely to provide a structural framework for the resident chaperones and associated substrates, and functional structure necessary for efficient quality control. Thus, it may serve as a complex, mixed-bed, “affinity chromatography matrix” that transiently absorbs incompletely folded and assembled proteins, and prevents their nonspecific aggregation with each other. The substrate proteins undergo controlled on- and off-cycles with selected chaperone components of the matrix. Glycoproteins will, for example, associate with calnexin and calreticulin, with glucosidase II and UDP-glucose:glycoprotein glucosyl transferase driving the on- and off-cycle (Hammond and Helenius, 1993). These interactions may promote folding, suppress irreversible aggregation, and at the same time limit the mobility of the newly synthesized folding and assembly intermediates, preventing their premature exit from the ER.

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