

# Role of N-linked oligosaccharide recognition, glucose trimming, and calnexin in glycoprotein folding and quality control

(influenza hemagglutinin/vesicular stomatitis virus glycoprotein/glucosidase inhibitors/chaperones/endoplasmic reticulum)

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**ABSTRACT** Using a pulse–chase approach combined with immunoprecipitation, we showed that newly synthesized influenza virus hemagglutinin (HA) and vesicular stomatitis virus G protein associate transiently during their folding with calnexin, a membrane-bound endoplasmic reticulum (ER) chaperone. Inhibitors of N-linked glycosylation (tunicamycin) and glucosidases I and II (castanospermine and 1-deoxynojirimycin) prevented the association, whereas inhibitors of ER  $\alpha$ -mannosidases did not. Our results indicated that binding of these viral glycoproteins to calnexin correlated closely with the composition of their N-linked oligosaccharide side chains. Proteins with monoglucosylated oligosaccharides were the most likely binding species. On the basis of our data and existing information concerning the role of monoglucosylated oligosaccharides on glycoproteins, we propose that the ER contains a unique folding and quality control machinery in which calnexin acts as a chaperone that binds proteins with partially glucose-trimmed carbohydrate side chains. In this model glucosidases I and II serve as signal modifiers and UDP-glucose:glycoprotein glucosyltransferase, as a folding sensor.

The lumen of the endoplasmic reticulum (ER) provides a highly specialized compartment for the folding and oligomeric assembly of secretory proteins, plasma membrane proteins, and proteins destined for the various organelles of the vacuolar system. Their conformational maturation is a complex process determined not only by the amino acid sequence but also by post- and cotranslational modifications, by the intralumenal milieu, and by a variety of chaperones and folding enzymes present (for recent reviews, see refs. 1 and 2). The ER possesses an efficient but still poorly understood “quality control” system to ensure that transport is limited to properly folded and assembled proteins (3).

Of the covalent modifications that occur in the ER, the cotranslational addition of N-linked oligosaccharides in the form of a 14-saccharide core unit (Glc<sub>3</sub>Man<sub>9</sub>GlcNAc<sub>2</sub>) is one of the most common (4). Glycosylation inhibitors, mutant cell lines, and site-specific mutagenesis of consensus glycosylation sequences have shown that it is crucial for the folding of many, but not all, glycoproteins (5, 6). Without added sugars, proteins misfold, aggregate, and get degraded without transport to the Golgi complex. For optimal folding and secretion, some glycoproteins must undergo a series of early trimming steps involving the removal of the three terminal glucose residues (7–9). Trimming, catalyzed by glucosidases I and II, begins on the nascent chain and is followed by a series of ER  $\alpha$ -mannosidase cleavages (4).

As part of our studies on the folding of influenza hemagglutinin (HA) and vesicular stomatitis virus (VSV) G protein in living cells, we have analyzed the interaction between these well-characterized viral glycoproteins and a mem-

brane-bound ER chaperone called calnexin (or p88 or IP90). Calnexin, a 64-kDa type I membrane protein, has been shown to interact transiently with a variety of membrane and soluble glycoproteins in the ER, and it is thought to have a chaperone-like function in the retention and oligomeric assembly of several important membrane glycoproteins (10–15). The results reported here lead us to propose a hypothesis which includes calnexin as one of several key players in a folding and quality control system. The model helps explain why proteins need carbohydrates for maturation and why N-linked oligosaccharides undergo trimming in the ER.

## MATERIALS AND METHODS

**Cell Lines, Viruses, and Reagents.** The X31 strain of influenza virus, wild-type VSV, and the temperature-sensitive tsO45 mutant of VSV were all propagated and used as previously described (16, 17). Chinese hamster ovary (CHO) 15B cells are defective in *N*-acetylglucosaminyltransferase I and thus allow trimming of N-linked carbohydrates to Man<sub>5</sub>GlcNAc<sub>2</sub> without further modification (17, 18). All reagents were purchased from Sigma with the exceptions of 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS) from Pierce and 1-deoxynojirimycin and 1-deoxymannojirimycin from Boehringer Mannheim. A mixture of [<sup>35</sup>S]methionine and [<sup>35</sup>S]cysteine used for metabolic labeling was purchased from ICN. The rabbit anti-calnexin antibody, raised against a C-terminal peptide, was kindly provided by John J. M. Bergeron (McGill University).

**Metabolic Labeling and Immunoprecipitation.** Infection of CHO and CHO 15B cells with X31 influenza virus or VSV and subsequent metabolic labeling have previously been described in detail (16, 17). With the exception of the experiment shown in Fig. 5, cells were metabolically labeled for 1 hr prior to infection to allow detection of calnexin, which is not efficiently labeled with the short pulse times used for viral proteins. Cells were lysed and precipitated with anti-calnexin antibodies as described (15). Viral proteins were precipitated as described (16). SDS/PAGE, fluorography, and quantitation was performed as described (16).

**Glycosidase Digestion.** After immunoprecipitation of samples, the wash buffer was removed by washing the staphylococcal protein A-Sepharose beads once with deionized water. Then 20  $\mu$ l of 0.05 M sodium citrate buffer, pH 4.4, and 0.28 unit of jack bean  $\alpha$ -mannosidase were added to each sample. Samples were incubated at 37°C for 24 hr before analysis by SDS/PAGE.

Abbreviations: ER, endoplasmic reticulum; dNM, 1-deoxynojirimycin; HA, influenza virus hemagglutinin; VSV, vesicular stomatitis virus; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate.

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## RESULTS

To analyze the interaction between the viral glycoproteins and ER chaperones, we used a pulse-chase approach already extensively utilized in our laboratory to study the folding and oligomerization of HA and G protein (16, 17). Both are type I membrane proteins with the majority of their mass in the lumen of the ER. HA has seven N-linked carbohydrates and six intrachain disulfides (19), whereas G protein has two N-linked carbohydrates and, most likely, seven intrachain disulfides (20). CHO 15B cells were first labeled with [<sup>35</sup>S]methionine for 60 min to incorporate radioactivity into resident luminal proteins. The cells were then infected with virus and, 5 to 6 hr after infection, pulsed with [<sup>35</sup>S]methionine for 2–3 min and chased in medium containing 5 mM unlabeled methionine and 0.5 mM cycloheximide. At different chase times, the cells were treated with a membrane-penetrating alkylating agent, *N*-ethylmaleimide, to block any free sulfhydryl groups, and lysed with a zwitterionic detergent, CHAPS. The postnuclear supernatant was immunoprecipitated by using antibodies to the viral glycoproteins or calnexin.

When analyzed under nonreducing conditions, the anti-HA precipitates gave a series of heterogeneous bands on SDS/PAGE faster than the fully reduced proteins (Fig. 1*a*). According to the previously established nomenclature (16), the incompletely oxidized folding intermediates were labeled IT1 and IT2, while the band corresponding to the fully oxidized HA was labeled NT for native. The glycoproteins that had reached the medial Golgi apparatus or beyond were distinguished as a separate faster-migrating band labeled G. As previously described, the shift from IT1 and IT2 to NT, which occurs within the first 10 min of chase, is caused by disulfide bond formation and folding of the glycopolyptide chains (16). The move from NT to G is caused by trimming of N-linked oligosaccharides in the Golgi apparatus.

When the lysates were precipitated with anti-calnexin, a labeled calnexin band at 90 kDa could be observed (Fig. 1*b*). Below it were HA bands corresponding to IT1, IT2, and NT. More than 80% of IT1 and IT2 could be coprecipitated with calnexin, while the Golgi form of HA did not coprecipitate

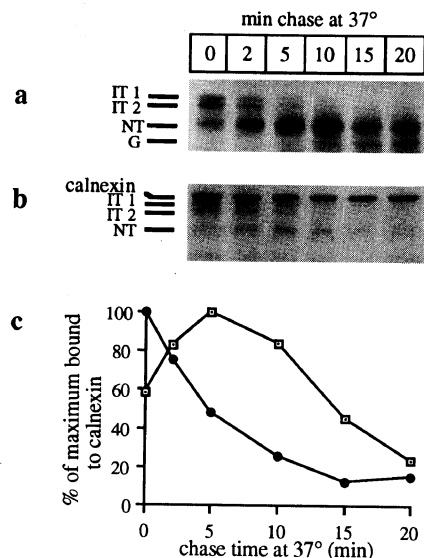


FIG. 1. Kinetics of HA and G association with calnexin. CHO 15B cells were metabolically labeled for 1 hr, then infected with X31 influenza virus or VSV. At 5.5 hr after infection, cells were pulse-labeled with [<sup>35</sup>S]methionine for 2 min and chased at 37°C for 0 to 20 min. Lysates were precipitated with antibodies to HA (*a*) or calnexin (*b*) and analyzed by nonreducing SDS/7.5% PAGE. (*c*) Quantitation by scanning densitometry of fluorographs from anti-calnexin precipitations of HA (●) and VSV G (□). Amount of viral protein precipitated is expressed as a percentage of the maximal amount bound.

with anti-calnexin. Precipitation of NT was only partial (25% at 0-min chase). As already shown for other glycoproteins (10, 15, 21, 22), it was apparent that HA associated transiently with calnexin. Quantitation by densitometry (Fig. 1*c*) indicated that the  $t_{1/2}$  for calnexin binding of HA was 5 min, with minimal binding observed after 15 min. For G protein, the binding peaked about 5 min after the pulse and dropped rapidly thereafter. These results showed that the viral proteins do not undergo initial folding as free monomers in solution, but they do so in association with calnexin.

Recent studies by Bergeron and co-workers (15) have shown that in HepG2 cells only glycoproteins associate with calnexin. This conclusion was supported by our observation that tunicamycin, a glycosylation inhibitor, blocked the association of HA and G protein with calnexin (Fig. 2). Since both proteins are unable to fold properly when synthesized in the presence of tunicamycin (23, 24), it was apparent that incomplete folding alone is not sufficient for their attachment to calnexin; the proteins must also have N-linked carbohydrates.

This raised the possibility that calnexin binds to the N-linked oligosaccharides of newly synthesized proteins. To explore it further, we tested the effects of castanospermine and 1-deoxynojirimycin (dNM), which inhibit glucosidases I and II and thus prevent the trimming of the three glucoses from the core oligosaccharide (7). The mobility differences displayed by the HA bands in inhibitor-free controls (Fig. 3*a*, lanes 1 and 2) and castanospermine- and dNM-treated cells (lanes 3 and 4, 5 and 6, respectively), showed that both compounds inhibited the trimming of N-linked sugars. In both reduced and nonreduced samples, the mobility of HA was slower than in the controls. Castanospermine had a greater effect than dNM, suggesting that dNM, being less efficient (7), allowed one or two glucoses to be removed, as previously described (8).

The inhibitors did not seem to have a noticeable effect on HA folding; the ratios between IT1, IT2, and NT were comparable to controls. Cleavage of the labeled HA into its two subunits HA1 and HA2 when the cells were exposed to trypsin (25), indicated, in agreement with previous studies (26, 27), that the HA molecules were transported normally to the cell surface in the presence of castanospermine (not shown).

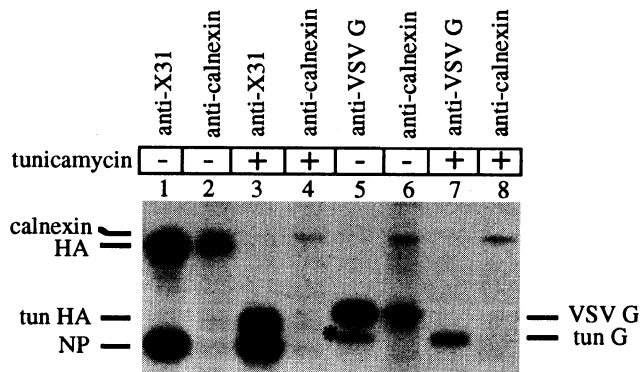


FIG. 2. Tunicamycin inhibits binding of calnexin to HA and G. CHO 15B cells infected with X31 influenza virus (lanes 1–4) or VSV (lanes 5–8) were pretreated with tunicamycin at 5  $\mu$ g/ml for 45 min and then pulse labeled in the presence of the inhibitor for 5 min at 37°C. Lysates were immunoprecipitated with antibodies against X31 HA, VSV G, or calnexin and analyzed by reducing SDS/7.5% PAGE. The positions of tunicamycin-treated HA and G are marked tun HA and tun G, respectively. The band below G protein in lane 5 (\*) is a soluble form of G, Gs. Influenza virus nucleoprotein is marked NP. Because of the limited availability of anti-calnexin antibodies, only a fraction of the total calnexin was precipitated.

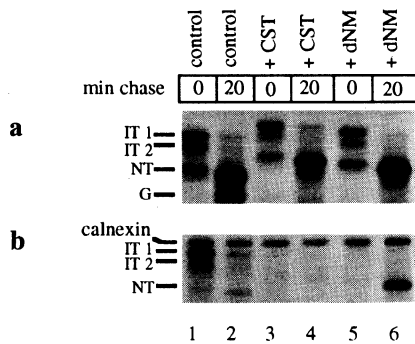


FIG. 3. Glucosidase inhibitors block the interaction between HA and calnexin. CHO 15B cells were pretreated for 45 min with either 1 mM castanospermine (CST) or 1 mM dNM, then pulse labeled for 3 min and chased for either 0 or 20 min in the presence of the inhibitors. Lysates were immunoprecipitated with antibodies to HA (a) or calnexin (b) and analyzed by nonreducing SDS/7.5% PAGE.

However, the most interesting observation was that the glucosidase inhibitors blocked the binding of HA to calnexin (Fig. 3b, lanes 3–5). In contrast, 1-deoxymannojirimycin, an inhibitor of ER  $\alpha$ -mannosidases, had no effect on the binding of HA to calnexin (data not shown).

This result indicated that calnexin binds proteins that have partially or fully deglycosylated oligosaccharide chains. If the dNM effect on trimming was only partial, as suggested by the intermediate mobility shift between control and castanospermine-treated samples, we reasoned that trimming by both glucosidase I and II was needed to make a glycopolyptide capable of binding to calnexin. In other words, calnexin was most likely to bind HA or G molecules that had either monoglucosylated or fully deglycosylated oligosaccharides.

To distinguish between these possibilities we used two approaches. One took advantage of the fact that misfolded proteins that remain in the ER tend to have predominantly  $\text{Glc}_1\text{Man}_9\text{GlcNAc}_2$  oligosaccharide side chains (28, 29). Suh *et al.* (28) have shown that the sugar side chains of G protein of tsO45 VSV are predominantly in the monoglucosylated form when synthesized at the nonpermissive temperature. This mutant G protein has a defect in the ectodomain which makes it temperature conditional for folding. When precipitating with calnexin antibodies, we found this misfolded G protein associated with calnexin (Fig. 4). Unlike the wild-type G protein, whose binding to calnexin was transient, the tsO45 G protein remained calnexin associated for at least 60 min (Fig. 4, lane 7). When glucosidase inhibitors were present during the pulse, binding of tsO45 G protein to calnexin did

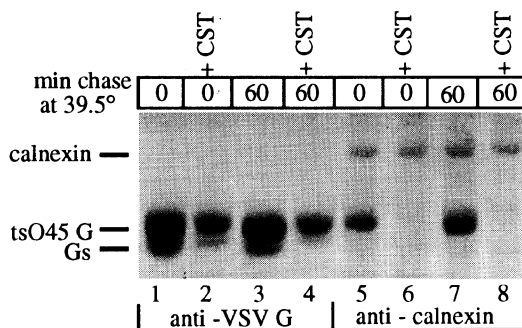


FIG. 4. Misfolded G protein binds to calnexin. Binding is inhibited by castanospermine. CHO cells were infected with the tsO45 mutant of VSV, pulse labeled at the nonpermissive temperature (39.5°C) for 10 min, and chased either 0 or 60 min at 39.5°C. Where indicated the cells were pretreated with 1 mM castanospermine (CST) and maintained in the inhibitor during the pulse and chase. Lysates were precipitated with antibodies to VSV G (lanes 1–4) or calnexin (lanes 5–8) and analyzed by reducing SDS/7.5% PAGE.

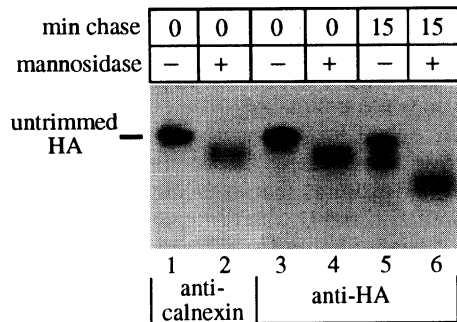


FIG. 5. N-linked oligosaccharides on calnexin-bound HA contain terminal glucose residues. CHO 15B cells were pulse labeled for 2 min and chased for 0 or 15 min. Lysates were immunoprecipitated with antibodies to either calnexin (lanes 1 and 2) or HA (lanes 3–6). The immunoprecipitated HA was digested with jack bean  $\alpha$ -mannosidase and analyzed by reducing SDS/7.5% PAGE. Calnexin is not detected in lanes 1 and 2 because cells were not prelabeled in this experiment.

not take place (Fig. 4, lanes 6 and 8), confirming that incompletely folded proteins do not bind to calnexin unless they have partially trimmed N-linked sugars.

In the second approach, we treated HA that had been coprecipitated with anti-calnexin antibodies with jack bean  $\alpha$ -mannosidase. It is an exoglycosidase that removes all terminal  $\alpha$ -mannoses from N-linked carbohydrates but does not digest the portions of the triantennary core oligosaccharides that retain terminal glucose residues (30, 31). The results in Fig. 5 show that the calnexin-associated HA molecules had a small mobility shift (lane 2) after mannosidase treatment. This shift was, most likely, caused by digestion of terminal mannose residues from the two glucose-free antennae of the core oligosaccharides. Following a 15-min chase, by which time nearly all labeled HA is dissociated from calnexin (Fig. 1), treatment with  $\alpha$ -mannosidase resulted in a larger mobility shift (lane 6), indicating that terminal glucose residues had been removed from the majority of the oligosaccharides, leaving the mannose residues in all three antennae of the side chain susceptible to digestion. The intermediate mobility shift seen after mannosidase treatment indicated that calnexin-bound HA had at least one glucose left on many of its oligosaccharides. We concluded that calnexin possesses binding activity specific for proteins with partially deglycosylated N-linked side chains, with the monoglucosylated form being the most likely ligand. A direct chemical analysis of the oligosaccharides present in calnexin-bound proteins was precluded by the small amounts of anti-calnexin antibodies available to us.

A final piece of information about the binding specificity was obtained from cells chased for 20 min in the presence of dNM. In the presence of this relatively inefficient inhibitor, some of the labeled HA that was not initially associated with calnexin (Fig. 3a, lane 5) was seen to associate (Fig. 3a, lane 6). The bound HA had the mobility of oxidized and glucose-trimmed NT. Apparently, trimming was merely delayed by dNM, and molecules that were already extensively folded but still present in the ER started to bind to calnexin belatedly. This observation showed that proteins do not necessarily need to bind to calnexin directly after their translocation; they can engage the chaperone long after they have been synthesized. Moreover, it was evident that a protein that was already folded could bind to calnexin if it had the appropriately trimmed N-linked sugars.

## DISCUSSION

Our results showed that HA and G protein bind to calnexin during the first 5–10 min after synthesis. This is a period

during which most of their post-translational folding takes place (16, 32). Intrachain disulfide bonds are formed and conformational epitopes appear. When they dissociate from calnexin, they are extensively folded and oxidized and are ready to leave the ER. Our results are similar to those already published for class I major histocompatibility complex antigens and other proteins that associate with calnexin (12, 14, 21). For both HA and G, the timing of release is close to the half-time of trimerization [ $t_{1/2} = 7-15$  min for HA,  $t_{1/2} = 6-8$  min for G (16, 33)], leaving open the possibility that oligomerization occurs while the glycoproteins are calnexin-bound.

That binding to calnexin includes both soluble and membrane-bound proteins, and that it is restricted to glycoproteins, was recently demonstrated by Bergeron and co-workers (15) for secretory proteins in Hep G2 cells. Our results with viral glycoproteins confirmed the latter observation and revealed an additional level of specificity; the N-linked side chains must be partially trimmed by glucosidases. We concluded that preferential binding most likely involves proteins with monoglucosylated oligosaccharides, irrespective of their folding status. Incomplete folding alone does not result in binding.

Suh *et al.* (28), who studied the oligosaccharides of misfolded tsO45 G protein at nonpermissive temperature, observed that not only are the majority of the oligosaccharides of this protein monoglucosylated but the single glucose residues in these oligosaccharides are rapidly turning over. Apparently, they are subject to a cycle where they are continuously removed by glucosidase II and replaced by a glucosyltransferase. An abundant and ubiquitously expressed glucosyltransferase in the ER lumen, called UDP-glucose:glycoprotein glucosyltransferase, has, in fact, been isolated and characterized by Parodi and co-workers (34, 35) as a soluble homodimer with 150-kDa subunits. This enzyme has a remarkable property: it uses as its substrate  $\text{Man}_9\text{-}\gamma\text{GlcNAc}_2$  oligosaccharide side chains, but only when these are linked to misfolded or incompletely folded glycoproteins (36). We think this enzyme may be intimately associated with calnexin binding of newly synthesized and misfolded proteins.

One model for glycoprotein maturation and quality control consistent with the available data is outlined below and depicted schematically in Fig. 6. Here, calnexin plays a central role as a chaperone, and the N-linked oligosaccharides serve as signals of a glycopolyptide's folding status. When a nascent polypeptide enters the lumen of the ER, the oligosaccharide transferase attaches the core saccharide units to asparagines in the consensus sequences. The sugar moieties are immediately trimmed by glucosidase I, which removes the  $\alpha 1,2$  terminal glucose residue, thus making the chain a substrate for glucosidase II, which proceeds to remove one or both of the remaining  $\alpha 1,3$  glucose residues.

These trimming events occur almost immediately after chain addition, in many cases on the nascent chain (see ref. 4).

In the likely case that the protein is still unfolded when all three glucoses are removed, the oligosaccharide serves as a substrate for the UDP-glucose:glycoprotein glucosyltransferase, which adds a glucose residue to the side chain. The added glucose is subsequently removed by glucosidase II and the protein enters the re- and deglucosylation cycle observed by Suh *et al.* (28). Several misfolded transport-incompetent proteins in the ER have been shown to retain mainly  $\text{Glc}_1\text{Man}_9\text{GlcNAc}_2$  oligosaccharides (28, 29, 37), and it is known that even for normal proteins the innermost glucose is removed considerably more slowly than the two terminal glucoses (38). The glycoprotein is released from the re- and deglucosylation cycle when it has folded and is no longer a substrate for the glucosyltransferase. Alternatively, the oligosaccharide may be modified by mannosidases, as mannose removal is known to make oligosaccharides less effective or entirely incompatible as glucose acceptors for the transferase (35).

The re- and deglucosylation cycle would be futile were it not linked to the binding of proteins with monoglucosylated oligosaccharides to calnexin. Calnexin's role, according to our model, and as already suggested by others (10, 15, 22), is to serve as a chaperone for the newly synthesized glycoproteins during their folding and/or oligomeric assembly so that they do not aggregate, or leave the ER prematurely. Left to their own devices, nonglycosylated proteins are known to either aggregate irreversibly or leave the ER without assembling into oligomers (see refs. 3, 23, and 24).

The model implies that glucose trimming provides a way for resident ER factors to keep track of the folding and maturation status of newly synthesized glycoproteins. The detailed configuration of the N-linked oligosaccharides may reflect the degree of folding, the state of oligomerization, the time spent in the ER, etc. It is interesting to note in this context that glucosidase inhibitors not only prevent glycoproteins from binding to calnexin, they also increase the degradation rate of some proteins (39).  $\alpha$ -Mannosidase inhibitors have the opposite effect: they enhance the rate of ER degradation of some transport-incompetent glycoproteins (40). This suggests that ER degradation may also be connected to trimming and/or calnexin association.

It is important to point out that, since calnexin has been shown to exist in a complex with at least three other resident ER membrane proteins (11), it is possible that it is not itself the component of the complex responsible for binding to partially folded proteins. However, cross-linking studies show only calnexin coupled to newly synthesized major histocompatibility complex class I heavy chain (10), suggesting close contact. Whether calnexin binds directly to monoglucosylated oligosaccharides remains an interesting and, as yet, open question. Its sequence has no homology with known lectins. Our data are most consistent with a lectin-like

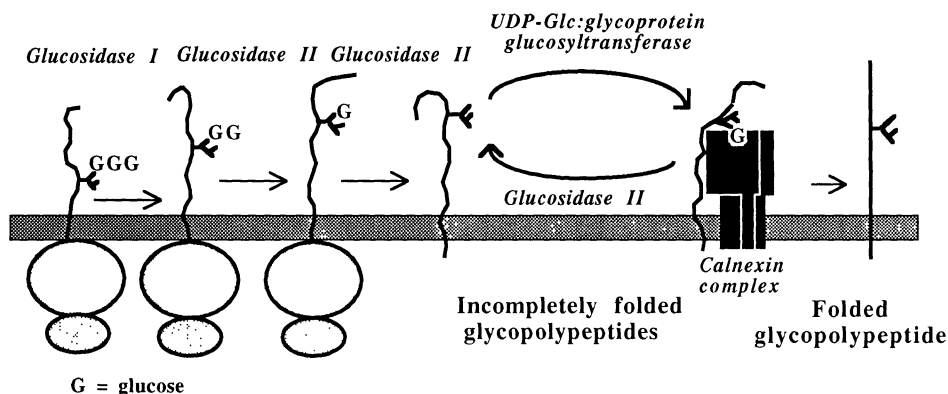


FIG. 6. Proposed pathway for calnexin-dependent folding and quality control as depicted for a transmembrane protein with one N-linked oligosaccharide. There are four main stages to the model: (i) stepwise trimming of glucoses, (ii) calnexin binding of proteins with monoglucosylated side chains, (iii) reglucosylation of glucose-free side chains on incompletely folded glycopeptide chains, and (iv) release of folded chains from the re- and deglucosylation cycle.

activity for calnexin, but we cannot exclude that calnexin recognizes structural characteristics in polypeptides that appear and disappear coincidentally with the processing of oligosaccharides. A direct demonstration of lectin activity for calnexin will require further investigation.

In mature folded glycoproteins, the carbohydrate side chains are generally located on the surface. They are often structurally dispensable. If calnexin does attach to proteins via these appendices, it may give the newly synthesized chains extensive conformational and steric freedom to fold and interact with other folding enzymes and chaperones such as protein disulfide isomerase and immunoglobulin heavy chain binding protein/glucose-regulated protein (BiP/Grp78). In other cases it is known that calnexin-associated proteins can assemble with other subunits to form quaternary complexes (12). We have shown previously that VSV G protein associates transiently during folding with BiP/Grp78 (32), which raises the possibility that both chaperones cooperate in assisting the folding of this protein. Present in the ER membrane as monomers or small oligomers (15), calnexins may provide dynamic, multivalent, and functionally flexible scaffolds designed to assist the folding and oligomeric assembly of glycoproteins.

Finally, it is important to emphasize that, although all newly synthesized glycoproteins so far tested bind transiently to calnexin, only a subgroup show folding and secretion defects in the presence of glucosidase inhibitors which inhibit this interaction (6). Glucosidase inhibitors have only mild effects on cell viability and secretion in many cases (6). Cell lines with mutations in glucosidases and N-linked glycosylation are usually relatively normal (41). Evidently, when prevented from binding to calnexin many glycoproteins find alternative ways of folding and assembling in the ER lumen. The high concentrations of classical chaperones and redox enzymes present in the ER may allow them to fold without the calnexin pathway (1, 2). The apparent redundancy in the ER folding machinery does, however, raise a question as to the real function of the calnexin system. While more work is needed to provide a full answer, it seems most likely to us that, while intimately involved in folding and oligomeric assembly of glycoproteins, the calnexin system may have evolved mainly to ensure quality control, an important but yet elusive function of the ER.

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