Trimming and Readdition of Glucose to N-Linked Oligosaccharides Determines Calnexin Association of a Substrate Glycoprotein in Living Cells*

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To analyze the role of glucose trimming and reglucosylation in the binding of substrate proteins to calnexin in the endoplasmic reticulum (ER) of living cells, we made use of the thermosensitive vesicular stomatitis virus tsO45 glycoprotein (G protein). At nonpermissive temperature the G protein failed to fold completely and remained bound to calnexin. When the cells were shifted to permissive temperature, complete folding occurred accompanied by glucosidase-mediated elimination of calnexin-G protein complexes. If release from calnexin was blocked during the temperature shift by inhibiting the glucosidases, folding occurred, albeit at a reduced rate. In contrast, when unfolded by a shift from permissive to nonpermissive temperature, the G protein was reglucosylated rapidly and became capable of rebinding to calnexin. The rate at which calnexin binding occurred showed a 20-min delay that was explained by accumulation of the G protein in calnexin-free exit sites of the ER. These contained the glucosyltransferase responsible for reglucosylation of misfolded glycoproteins but had little or no calnexin. After unfolding and reglucosylation, the G proteins moved slowly from these structures back to the ER where they reassocciated with the chaperone. Taken together, these results in live cells fully supported the lectin-only model of calnexin function. The ER exit sites emerged as a potentially important location for components of the quality control system.

The observation that calnexin, a resident protein of the endoplasmic reticulum (ER) membrane, associates selectively with incompletely folded glycoproteins that contain monoglucosylated N-linked glycans led a few years ago to the proposal that glycoprotein folding and quality control are linked intimately to oligosaccharide trimming in the ER (1, 2). It was proposed that calnexin is part of a molecular chaperone cycle that includes three accessory factors: glucosidases I, II, and a glucosyltransferase (UDP-glucose-glycoprotein glucosyltransferase) (GT) (for recent reviews, see Refs. 1–3). According to the model, binding and release of newly synthesized glycoproteins from calnexin are regulated by these enzymes. GT, a soluble luminal enzyme, was thought to serve as the folding sensor because it was known to reglucosylate high mannose glycans only on glycoproteins with nonnative conformations (4).

Further studies have shown that virtually all proteins with N-linked oligosaccharides bind transiently to calnexin and/or calreticulin (its soluble luminal counterpart) during folding and oligomeric assembly (for review, see Ref. 3). Studies performed in vitro and in microsomes have supported many features of the original model and have confirmed each of the key steps (5–13).

Although there is accumulating evidence for a lectin-based mechanism for these ER chaperones, it is still unclear what happens in live cells. To examine the connection among folding, reglucosylation, and calnexin binding in situ, we made use of a mutant glycoprotein, the glycoprotein of tsO45 vesicular stomatitis virus (VSV G protein), which has a thermoreversible folding defect (14–17). We induced folding and misfolding of this G protein in the ER by shifting cells between the permissive and the nonpermissive temperatures and followed the removal and readdition of glucose residues to the N-linked glycans as well as binding and release from calnexin. The results confirmed the steps in the proposed calnexin cycle and provided additional information about the timing and cellular location of these events.

EXPERIMENTAL PROCEDURES

Reagents, Cell Lines, and Viruses—The cell line CHO15B and Indiana serotype VSV mutant tsO45 were described previously (18). Promix 35S-labeled cysteine and methionine were purchased from Amersham Pharmacia Biotech. CHAPS was obtained from Pierce. Zysorbin (fixed and killed Staphylococcus aureus) and secondary antibodies were from Zymed Laboratories Inc. (San Francisco). N-Butyldeoxynojirimycin was donated by Searle Inc. (St. Louis). The polyclonal anti-VSV G protein antiserum was raised against purified whole viral protein (19) and the polyclonal anti-calnexin rabbit serum against a synthetic peptide corresponding to a segment of the cytoplasmic tail of canine calnexin (20). Hybridoma producing the monoclonal antibody I-14 were obtained from Dr. Jack Rose (Yale University). All other reagents were purchased from Sigma.

Metabolic Labeling—Cells were infected with tsO45 VSV and labeled metabolically as described (16). Briefly, CHO15B cells grown to 90% confluence in 10-cm dishes were infected with virus at the permissive temperature (30 °C) for 3.5 h and washed twice in phosphate-buffered saline. 1 ml of serum-free medium lacking cysteine and methionine was added. The cells were scraped gently with a rubber policeman into 1.7-ml microcentrifuge tubes and incubated in an Eppendorf Thermomixer. 4 h after infection, cells were radiolabeled with 100 μCi/ml [35S]Promix for 2–10 min as indicated. Labeling was terminated by the addition of cold cysteine and methionine to a final concentration of 5 mM and cycloheximide to 0.5 mM. During the chase, the cells were incubated...
at either 30 or 40 °C with constant shaking. They were then lysed by adding an equal volume of lysis buffer containing 4% CHAPS, 2 × HBS (100 mM HEPES, pH 7.5, 400 mM NaCl), 2 mM phenylmethylsulfonyl fluoride, 40 mM N-ethylmaleimide, 0.5% Zorbin, and protease inhibitors (20 μg/ml each of chymostatin, leupeptin, antipain, and pepstatin).

Immunoprecipitation—Cell lysates were cleared by centrifuging at 12,000 × g for 5 min at 4 °C. The postnuclear supernatant from approximatley 4 × 10^6 cells was precipitated with antibody and protein A-Sepharose beads sufficient to immunoprecipitate >90% of the desired protein. Immunoprecipitates were washed three times for 10 min at room temperature with vigorous shaking in 0.5% CHAPS and HBS (50 mM HEPES, pH 7.5, 200 mM NaCl). The pellets were then suspended in 50 μl of sample buffer (200 mM Tris, pH 6.8, 3% SDS, 10% glycerol, 0.004% bromphenol blue, 1 mM EDTA). Samples were heated to 95 °C for 5 min (in the presence of 100 mM dithiothreitol for reducing gels) and centrifuged at 12,000 × g for 1 min to pellet the Sepharose beads. Immunoprecipitated labeled proteins were then analyzed by SDS-PAGE, visualized with a Molecular Dynamics PhosphorImager, and quantified by densitometry.

Mannosidase Assay—Immune complexes containing 35S-labeled VSV G protein were washed in water and resuspended in 20 μl of 0.05 M sodium citrate buffer, pH 4.4. They were then incubated at 37 °C overnight with 0.09 μl of unit of jack bean α-mannosidase to determine whether the N-linked glycans of G protein contained a terminal glucose residue. α-Mannosidase was supplied as a suspension in 3 M (NH4)2SO4, an equal volume (1 μl) of 3 M (NH4)2SO4 was added to control samples. After digestion, 50 μl of reducing sample buffer was added, and the proteins were processed as described above.

Indirect Immunofluorescence Microscopy—CHO15B cells were plated on Alcian blue-treated glass coverslips (12 mm) 40 h before the experiment. They were fixed for 20 min with 3.7% formaldehyde in serum-free medium + 10 mM HEPES, pH 7.4, and washed twice in serum-free medium. Fixation and all subsequent steps were performed at room temperature. After a 15-min exposure to permeabilization solution (phosphate-buffered saline with 10% calf serum, 0.05% saponin, 15 mM glycine, and 10 mM HEPES, pH 7.4), the cells were incubated with primary antibodies diluted in permeabilization solution for 30 min, washed three times for 5 min in permeabilization solution, and incubated with fluorescein isothiocyanate or Texas red-conjugated secondary antibodies for 30 min. The cells were washed three times in permeabilization solution and once in water and mounted in Mowiol containing 2.5% 1,4-diasabicyclo[2.2.2]octane (Calbiochem) to reduce photobleaching. Cells were viewed with a Zeiss Axiohot 2 fluorescence microscope.

RESULTS

VSV G protein is a type I transmembrane glycoprotein of 67 kDa with a short cytoplasmic tail (21). Its ectodomain is translocated cotranslationally into the ER where the addition of two N-linked glycans, the formation of multiple intrachain disulfide bonds, and homotrimerization occur (22–24). Folding is assisted by two chaperones, BiP at early stages and calnexin at later stages (23, 25). In the tsO45 strain, the G protein is temperature-sensitive for folding because of the presence of a point mutation in the ectodomain (21). Although the protein folds normally at 30 °C, it fails at 40 °C to acquire a full set of disulfide bonds and remains associated with BiP and calnexin (23, 25, 26). At the nonpermissive temperature, 66% of the glycans contain a single terminal glucose residue that is turning over in a process catalyzed by the enzymes glucosidase II and GT (27). At the permissive temperature of 30 °C, the native conformation is formed rapidly, and the protein is transported to the Golgi complex and beyond. As long as the protein is in the ER, the transition between the folded and unfolded states can be brought about simply by raising or lowering the temperature (25).

The effects of temperature on tsO45 G protein folding and calnexin binding are shown in Fig. 1. Infected cells were pulse labeled at 30 °C with [35S]cysteine and methionine, and the G protein immunoprecipitated using anti-VSV antibodies and I-14, a conformation-specific monoclonal antibody to epitope B2 of the G protein (29). The precipitates were analyzed by nonreducing SDS-PAGE. The band marked α. G indicates the position of the fully oxidized G protein; red. G marks the reduced form. Incompletely oxidized folding intermediates are visible as a smear above the fully oxidized protein (23). Monoclonal I-14 against the B2 epitope recognized only the fully oxidized forms of tsO45 G protein. This was consistent with previous observations with wild-type G protein (29). In this case the B2 epitope appears after full oxidation but before trimer formation (23, 24). Because I-14 did not precipitate the incompletely oxidized tsO45 G proteins, nor, as seen in Fig. 1B, the misfolded tsO45 G protein produced at the nonpermissive temperature, it could be used as a convenient indicator for tsO45 G protein folding.

A pulse-chase approach was used to assess the effect of temperature on folding and calnexin binding of tsO45 G protein. Cells were infected with tsO45 VSV at 30 °C for 3.5 h, starved for cysteine and methionine for 30 min, pulse labeled for 2 min at 30 °C with [3S]-labeled cysteine and methionine, and chased at either 30 or 40 °C with an excess of unlabeled cysteine and methionine. Cycloheximide was added during the chase period to prevent further protein synthesis. Samples were removed at various times of chase, alkylated with N-ethylmaleimide, and lysed. Postnuclear supernatants were immunoprecipitated with anti-VSV, anti-calnexin, or I-14. After separation by SDS-PAGE, precipitated proteins were visualized and quantified using a PhosphorImager.

At the permissive temperature of 30 °C, the tsO45 G protein folded with a t1/2 of approximately 7 min judging by precipitation with I-14 (Fig. 1C). By 50 min of chase, nearly all (<95%) of the labeled G protein molecules had assumed a structure recognized by I-14. At 40 °C, tsO45 G protein remained as a heterogeneous, partially aggregated mixture of incompletely folded proteins (20). Except for a faint background that decreased with time (Fig. 1, B and C), the G protein was not immunoprecipitated by I-14. The tsO45 G protein was not degraded during the period examined as precipitation with anti-VSV antibodies showed that the level of the G protein was constant at either temperature (Fig. 1B). A second round of precipitation with the antibodies failed to bring down appreciable amounts of VSV G protein, indicating that the precipitations were essentially quantitative (Fig. 1B, IP Control).

Calnexin Binding to tsO45 VSV G Protein—To characterize the interaction of VSV G protein with calnexin during the chase, samples from the lysates were immunoprecipitated with a polyclonal antibody to calnexin. After separation by SDS-PAGE (Fig. 1B), the amount of coprecipitating G protein was quantified by densitometry (Fig. 1D). Directly after the pulse, some VSV G protein was already bound to calnexin at both temperatures. At 30 °C, the amount of VSV G protein bound to calnexin peaked at 5 min of chase and declined gradually as folding progressed (Fig. 1D). At 40 °C, it increased gradually with time, consistent with the observation by Suh et al. (27) that the fraction of monoglucosylated glycans in the misfolded G protein keeps increasing with time at nonpermissive temperature.

Typically, the fraction coprecipitating with calnexin was 10–15% of total labeled VSV G protein. Only 66% of the glycans are monoglucosylated at any given time (27), which may partially account for this relatively low level of binding. Proteins that have a glucose on only one of the two glycans are so weakly bound to calnexin that the complexes do not withstand these immunoprecipitation and washing conditions (7).

Effects of Glucosidase Inhibitors—Inhibitors of ER glucosidases I and II prevent the trimming of glycans from the core N-linked oligosaccharides and thus inhibit the interaction of newly synthesized glycoproteins with calnexin (5, 30). In the present studies we used N-butyl-1-deoxyxojirimycin (NB-DNJ)
as the inhibitor because when added to live cells, it inactivates the ER glucosidases in 2–5 min.\(^2\)

When NB-DNJ was present 30 min before and throughout a pulse-chase experiment, the efficiency of tsO45 VSV G protein folding at 30 °C to the I-14-precipitable form dropped to 20% of control (Fig. 2, A and B). Similar to wild type (25), efficient folding of the ts045 G protein was dependent on the removal of glucose residues and thus on the involvement of the calnexin cycle for optimal folding.

To determine what would happen if the inhibitor was added when VSV G protein had already been trimmed to the monoglucosylated form and was associated with calnexin, cells infected with tsO45 VSV were pulsed for 5 min and chased at the nonpermissive temperature for 10 min. NB-DNJ was added, and incubation was continued for an additional 10 min. The cells were then shifted to the permissive temperature to initiate folding, and samples were removed for I-14 and anti-calnexin immunoprecipitation at various times.

In the absence of inhibitor, the shift down resulted in a small transient increase in calnexin binding during the first 10 min followed by a decrease in the next 10 min. The I-14 precipitations (Fig. 3C) showed that folding of VSV G protein was completed 10–20 min after shift down \((t_{1/2} \approx 5 \text{ min})\). The I-14 epitope appeared faster than during normal folding at 30 °C, consistent with the suggestion that tsO45 G protein was already partially folded at 40 °C (26).

When NB-DNJ was present during the shift down, tsO45 G protein was not released from calnexin (Fig. 3B). After a small initial increase in binding similar to control cells, the amount of calnexin-bound G protein remained virtually unchanged. Folding of tsO45 G protein was as efficient as in the control samples. However, as shown in Fig. 3C, it was considerably slower, taking place with a \(t_{1/2}\) of about 15 min.

Thus, the results showed that when ER glucosidases were inhibited during the chase, VSV G protein was trapped in calnexin-bound complexes. This indicated that in living cells, glucosidases are needed not only to generate binding-competent trimming intermediates but also to release substrate gly-

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\(^2\) K. S. Cannon and A. Helenius, unpublished observations.
cin was added to prevent trimming of was necessary to prevent transport of folded G protein from the inhibitors, brefeldin A (BFA) and deoxymannojirimycin. BFA used a temperature shift up protocol in the presence of two when a properly folded glycoprotein misfolds in the ER. We nature of tsO45 G protein allowed us to examine what happens that GT does not reglucosylate glycoproteins efficiently with without added deoxymannojirimycin, the glycans on the G protein were reglucosylated poorly. This is consistent with reports that VSV G protein has only two mannose residues that can be removed. Digestion thus accentuates the difference in the molecular weight between glycoproteins that have Glc1 and Glc0 chains so that they can be resolved on SDS-PAGE. (Fig. 4C, sample 1 and 2). VSV G protein has only two N-glycans, so although the molecular weight differences were not large, they were clearly detectable (Fig. 4C).

At the end of the pulse, when G protein was bound to calnexin, mannosidase treatment induced only a small shift in the position of the G protein band (Fig. 4C, sample 1; see marker Glc1). After 60 min of chase, a large portion of the G protein displayed an accentuated shift (see marker Glc0). However, after an additional hour at the nonpermissive temperature, the amount of Glc0 decreased, and Glc1 increased, indicating that reglucosylation had occurred (sample 4). Quantification of the amount of G protein in each lane shown that little or no protein degradation occurred in the course of this experiment. We concluded that VSV G protein was reglucosylated when misfolding was induced by a shift from the permissive to the nonpermissive temperature. This explained why rebinding to calnexin occurred.

We next examined the rates at which unfolding, reglucosylation, and rebinding to calnexin occur upon shift to the nonpermissive temperature (Fig. 5). Unfolding, measured by loss of I-14 recognition, occurred rapidly after the temperature shift (t1/2 = 3 min; Fig. 5). The conversion from the Glc0 to the Glc1 form of G protein had similar kinetics, indicating that GT recognized and modified the G protein as soon as it unfolded. When the amount of G protein associated with calnexin was analyzed, bimodal kinetics were reproducibly observed; a rapid initial phase followed by a slower second phase. This indicated that the cells contained two pools of G protein which differed in the rate with which they bound to calnexin after reglucosylation.

**Cellular Location of G Protein and GT**—To explore the possibility of separate pools, indirect immunofluorescence was used to determine the intracellular distribution of VSV G protein in cells incubated in the presence of BFA. At 4 h postinfection, BFA was added along with cycloheximide to prevent further protein synthesis. If the cells were then held for 2 h at continued for another hour. As shown schematically in Fig. 4A, samples were removed at the end of the pulse (sample 1), after 1 h at 30 °C (sample 2), after 2 h at 30 °C (sample 3), or after 1 h at 30 °C followed by 1 h at 40 °C (sample 4). The folding state of VSV G protein was examined in each sample (Fig. 4B), and the amount of calnexin-bound G protein was determined by coimmunoprecipitation (Fig. 4D).

Virtually all G protein molecules folded to the I-14-positive form within 60 min at the permissive temperature (Fig. 4B, samples 1–3). There was a corresponding decrease in the amount of G protein bound to calnexin (Fig. 4D, samples 1–3). After the shift to nonpermissive temperature, VSV G protein lost its folded structure, was no longer immunoprecipitated by I-14 (Fig. 4B, sample 4), and rebound to calnexin (Fig. 4D, sample 4). Unfolding of a protein that had previously folded and been released resulted in binding to calnexin a second time.

An α-mannosidase assay (5, 30) was used to test whether the re-binding of G protein to calnexin involved reglucosylation, as predicted by the model. The assay took advantage of the differential susceptibility of N-linked glycans to digestion with the exomannosidase depending on whether they contain a terminal glucose residue or not (see Fig. 4C, right). Those glycans that contain a single terminal glucose residue (Glc1) have, at most, five hexoses that can be cleaved by this mannosidase. Glycans without a terminal glucose residue (Glc0) have up to eight mannose residues that can be removed. Digestion thus accentuates the difference in the molecular weight between glycoproteins that have Glc1 and Glc0 chains so that they can be resolved on SDS-PAGE. (Fig. 4C, compare samples 1 and 2). VSV G protein was immunoprecipitated with I-14 or anti-VSV.

**Panel A**

**Panel B**

**Fig. 2. Interaction with calnexin improves the folding of tsO45 G protein. Panel A**, the glucosidase inhibitor NB-DNJ was applied to tsO45 VSV-infected cells for 30 min before and throughout the pulse-chase experiment. After a 2-min pulse with [35S]Promix and chase with cold cysteine and methionine for the indicated times, cells were lysed in 2% CHAPS and HBS and immunoprecipitated with I-14 or anti-VSV. Panel B, VSV G protein bands were measured by densitometry and graphed as a percentage of the maximum G protein recognized by I-14.
30°C, VSV G protein displayed a faint reticular ER pattern and, in addition, a pattern of bright spots (Fig. 6b). The spots did not appear to stain with anti-calnexin (Fig. 6c). As reported previously for G protein in similarly BFA treated cells (33), they most likely corresponded to the ER exit sites (34–36). If, after addition of the drugs at 4 h postinfection, the cells were instead held at 30°C for 1 h and then shifted to the non-permissive for the 2nd h, G protein staining of the ER became more prominent, and the spotty pattern diminished (Fig. 6d), indicating that the misfolded G protein moved back to the reticular ER.

We next examined the steady-state location of the GT in noninfected cells using polyclonal anti-GT antibodies. In addition to weak staining of an ER-like reticulum, a spotty staining pattern was again observed (Fig. 6e). Antibodies to the ER chaperones BiP and calnexin did not appear to stain these spots (Fig. 6f–h). The location of GT staining was unchanged by treatment with BFA and by temperature shifts (not shown), consistent with its localization in ER exit sites.

The results indicated that the G protein was present in two main locations, the reticular ER and a compartment that corresponds to ER exit sites. The GT was present in both of these compartments, whereas calnexin was predominantly in the reticular ER. When the temperature was shifted up, G protein in the exit compartment moved back to the reticular ER. Taken together, this provided an explanation for the bimodal calnexin binding kinetics after temperature shift up and suggested a role for the ER exit sites in quality control of glycoproteins.

**Discussion**

By examining folding and misfolding of tsO45 G protein in living cells, we could follow in detail how calnexin association of substrate glycoproteins is regulated by the turnover of glucose residues. The results provided in vivo support for the notion that the association/dissociation cycle is driven by the opposing actions of glucosidase and the ER glucosyltransferase, which catalyze multiple rounds of reversible covalent modification of the core glycans (2).

The thermoreversible nature of the folding defect allowed analysis of the events associated with folding and misfolding without interference by complexities inherent in the normal co- and post-translational maturation process. When the temperature was shifted from nonpermissive to permissive, the tsO45 G protein, arrested in incompletely folded conformations, underwent folding to the native state. The process involved association with calnexin. When a glucosidase inhibitor was added during the temperature shift, the G protein remained calnexin-bound, confirming that release required glucosidase action. Although trapped in a calnexin complex, the G protein continued to fold but at a considerably lower rate than when allowed to participate in the normal binding and release cycle.

In contrast, after a shift from the permissive to the nonpermissive temperature, the previously folded G proteins underwent misfolding. This resulted in almost instantaneous addition of glucose by the GT followed by binding to calnexin. Apparently, GT recognized the proteins as misfolded and converted the high mannose glycans to the monoglucosylated form.

Taken together, these observations were fully consistent with the lectin-only model of calnexin binding (2) and with evidence that has been obtained previously in cell-free systems (5–13). Although we cannot exclude the possibility of protein-protein interactions between substrate glycoprotein and calnexin, it is not necessary to invoke such interactions to explain the data obtained. Contrary to an alternative model that emphasizes direct protein-protein interactions during substrate binding (1, 11, 37), the dissociation and association of tsO45 G protein with calnexin appeared to be determined solely by the presence and absence of glucose and not by the folding status of the protein.

That post-translational reglucosylation of newly synthesized glycoproteins occurs in live cells was first demonstrated by Parodi and Cazzulo (38) in *Trypanosoma cruzi*. They estimated that an average glycoprotein is reglucosylated at least once before it leaves the ER (39). Using tsO45 G protein synthesized in COS cells at nonpermissive temperature, Suh et al. (27) reported that 66% of the glycans are monoglucosylated and that the glucose residues are turning over. This established that glycoproteins are subject to continuous de- and reglucosylation as long as they remain incompletely folded in the ER and...
suggested that the addition of glucose might somehow prevent defective proteins from leaving the ER. The present in vivo studies extend previous observations by showing that a glycoprotein that has been folded, glucose trimmed, and released from calnexin can, upon reglucosylation, again bind to calnexin. Evidence for reglucosylation has been obtained for influenza hemagglutinin and for transferrin in microsomes (5, 8) and for VSV G protein in live cells (27). Van Leeuwen and Kearse (40) showed that T cell receptor α and β subunits, when expressed alone without other subunits of the receptor, incorporate [3H]glucose post-translationally in live cells and are associated with calnexin. Our findings add to these by showing that reglucosylation is a direct result of misfolding and that it leads to rebinding of substrate glycoproteins to calnexin, confirming that each of the major steps in the proposed calnexin chaperone cycle occurs in live cells.

Using a pulse and chase approach with labeled glucose, Van Leeuwen and Kearse (40) have shown in live cells that glucose residues turn over in less than 15 min. In microsomes, a de-glucosylation rate of less than 7 min has been reported (8). We were able to show that the readdition of glucose occurred almost immediately when tsO45 G protein was rendered misfolded. The $t_{1/2}$ of the process was about 2 min and essentially indistinguishable from the rate of misfolding. This suggests that the re- and deglucosylation cycle in the ER may be even more rapid than thought previously.

Although reglucosylation of the G protein occurred almost instantly after misfolding, association with calnexin showed more complex kinetics. A small fraction bound to calnexin immediately, the majority with a $t_{1/2}$ of about 20 min. A likely explanation for the delay was that some G protein was located in a calnexin-free compartment and had to move back to the reticular ER before it could bind to the chaperone. In agreement with previous reports on BFA-treated cells (33), most of the G protein was located in punctate structures distributed throughout the cytoplasm. Although enriched in p58, a marker for the intermediate compartment (33), these structures did not contain detectable amounts of calnexin. However, after the temperature shift, the G protein migrated slowly back to the reticular ER, thus explaining the delayed calnexin association.

There is reason to believe that the organelles in which the G protein accumulates correspond to so-called exit sites and are
For that can neither maintained at 30 °C (prevent mannose trimming, respectively. The cells were then incubated nojirimycin were added to halt protein synthesis, block transport, and VSV 30 °C. At 4 h postinfection, cycloheximide, BFA, and deoxymane- and Texas red-conjugated fluorescent secondary antibodies. indicated proteins followed by species-specific fluorescein isothiocya-

panels a–d, CHO15B cells maintained at 37 °C were fixed and stained with antibodies against the

lowed by species-specific fluorescein isothiocyanate- and Texas red-

ganelle. We observed that when synthesized at 15 °C, tsO45 G protein folded correctly and entered a compartment that corre-

sults add credence to the idea that exit sites play an active role

connections than on retrograde transport vesicles because for-

that the 15 °C compartment lacked the machinery needed to

form a conformational change aided by an ER folding factor or by ER-like conditions present in the exit ports. The I-14 epitope is lost, and the proteins are converted to a conformation that allows them to be “seen” by the GT. After reglucosylation, they are ready to bind to calnexin, but since there is little calnexin in these locations, binding is delayed until the G proteins have been moved back into the reticular part of the ER which contains the bulk of the calnexin. That this is the course of events is supported by the observation that GT, unlike cal-

newer giving the monoclonal antibody I-14.

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De- and Reglucosylation Determine Calnexin Binding

FIG. 6. Immunofluorescence localization of G protein, BIP, calnexin, and GT. Panels a–d, CHO15B cells were infected with tsO45 VSV 30 °C. At 4 h postinfection, cycloheximide, BFA, and deoxyman-

associated intimately with the ER (34–36). The spotty appear-

ance and the presence of p58 (ERGIC53) (33) indicate that they are related to the intermediate compartment (41). However, our previous studies using tsO45 (28) imply that they are not the classical intermediate compartment as defined by a 15 °C block (41) but rather represent an earlier, more ER-like orga-

nelle. We observed that when synthesized at 15 °C, tsO45 G protein folded correctly and entered a compartment that corre-

responded to the classical intermediate compartment. When the temperature was shifted to the nonpermissive, the G pro-

tein in this compartment failed to misfold (28). We concluded that the 15 °C compartment lacked the machinery needed to

unfold tsO45 G protein upon a temperature shift. The compart-

ment differs thus from organelles in which G protein accumu-

late in BFA-treated cells.

We propose that at the permissive temperature, G proteins undergo normal calnexin-assisted folding and accumulate in

exit ports of the ER. Because of the BFA-induced block in the formation of transport vesicles, they cannot move further.

When the temperature is raised to the nonpermissive, they undergo a conformational change aided by an ER folding factor or by ER-like conditions present in the exit ports. The I-14 epitope is lost, and the proteins are converted to a conformation that allows them to be “seen” by the GT. After reglucosylation, they are ready to bind to calnexin, but since there is little calnexin in these locations, binding is delayed until the G proteins have been moved back into the reticular part of the ER which contains the bulk of the calnexin. That this is the course of events is supported by the observation that GT, unlike cal-

newer, located in spots with a distribution similar to that of

calnexin in these locations, binding is delayed until the G

proteins in the ER and in the putative exit sites raises some interesting questions about quality control in the early secre-

tory pathway. Late conformational maturation steps and con-

formation-dependent sorting may not occur exclusively in the reticular ER but also in exit sites and en route to the cis-Golgi complex. This has been suggested for influenza hemagglutinin because homotramer formation seems in part to take place in post-ER, pre-Golgi organelles (42). Furthermore, both mis-

folded tsO45 G protein and the transport-incompetent mutant ΔF508 CFTR are known to accumulate in exit sites or in the intermediate compartment (20, 43). It is unclear at present what determines the concentration of these and other cargo proteins and exclusion of calnexin in the exit ports (see Ref. 44). The mechanism by which the misfolded G protein returns from the exit ports to the reticular ER is also not clear. It is more likely to depend on diffusion through remaining membrane connections than on retrograde transport vesicles because forma-

tion of retrograde vesicles depends on the COP I coat protein complex, the assembly of which is inhibited by BFA. Our re-

results add credence to the idea that exit sites play an active role not only in cargo capture but also conformation-based quality control.
De- and Reglucosylation Determine Calnexin Binding

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