Quality Control in the Secretory Pathway: Retention of a Misfolded Viral Membrane Glycoprotein Involves Cycling between the ER, Intermediate Compartment, and Golgi Apparatus

Craig Hammond and Ari Helenius

Department of Cell Biology, Yale University School of Medicine, New Haven, Connecticut 05610

Abstract. Proteins synthesized in the ER are generally transported to the Golgi complex and beyond only when they have reached a fully folded and assembled conformation. To analyze how the selective retention of misfolded proteins works, we monitored the long-term fate of a membrane glycoprotein with a temperature-dependent folding defect, the G protein of tsO45 vesicular stomatitis virus. We used indirect immunofluorescence, immunoelectron microscopy, and a novel Nycodenz gradient centrifugation procedure for separating the ER, the intermediate compartment, and the Golgi complex. We also employed the folding and recycling inhibitors dithiothreitol and AlF_4^-, and coimmunoprecipitation with calnexin antibodies. The results showed that the misfolded G protein is not retained in the ER alone; it can move to the intermediate compartment and to the *cis*-Golgi network but is then recycled back to the ER. In the ER it is associated with calnexin and BiP/GRP78. Of these two chaperones, only BiP/GRP78 seems to accompany it through the recycling circuit. Thus, the retention of this misfolded glycoprotein is the result of multiple mechanisms including calnexin binding in the ER and selective retrieval from the intermediate compartment and the *cis*-Golgi network.

FIDELITY in the expression of proteins in the cell is ensured at many levels from transcription of the gene to intracellular transport of the final product. Here we focus on one of the posttranslational levels of "quality control," the sorting that permits properly folded and assembled proteins to leave the ER and proceed to the Golgi complex and beyond, while simultaneously retaining incompletely folded, misfolded, and unassembled proteins (for review see Hurtley and Helenius, 1989). The reason for selectivity at this level in the secretory pathway is the need to keep newly synthesized proteins in contact with the extensive and efficient folding machinery of the ER until they are conformationally mature, and to prevent deployment of nonfunctional and incomplete proteins that often arise as side products of protein biogenesis.

How the cell is able to distinguish between properly folded and assembled proteins and incomplete proteins is only partially understood. Since a huge variety of different soluble and membrane-bound proteins, glycoproteins, and oligomers with widely different properties are subjected to quality control, the main sorting criteria must involve common structural features shared by many newly synthesized polypeptides. Previous studies have suggested that such features include exposed free sulfhydryl groups (Alberini et al., 1990; Sitia et al., 1990), exposed binding sites for certain chaperones (Dorner et al., 1988; Hurtley et al., 1989), and gross aggregation (Tooze et al., 1989; Marquardt and Helenius, 1992). BiP/GRP78, the HSP70 analogue in the ER lumen, binds to hydrophobic sequences exposed on the surface of partially folded or unassembled proteins (Haas and Wabl, 1983; Bole et al., 1986; Flynn et al., 1991; Blond-Elguindi et al., 1993), and calnexin (a membrane-bound chaperone also called p88 and IP90) (Degen and Williams, 1991; Wada et al., 1991; Hochstenbach et al., 1992) binds to glycoproteins that have undergone partial trimming of glucose residues in their N-linked oligosaccharide side chains (Ou et al., 1993; Hammond et al., 1994). Both of these chaperones associate transiently with folding and assembly intermediates, and more permanently with misfolded proteins.

In this study, we have used a well-characterized, thermoreversible folding mutant, the G protein of vesicular stomatitis virus ts045, to study the fate of a misfolded membrane glycoprotein. At nonpermissive temperature, this protein is known to have free sulfhydryl groups, to be conformationally immature, to be present in a complex, and to bind both BiP/GRP78 and calnexin (Doms et al., 1987; De Silva et al., 1990; Machamer et al., 1990; Hammond et al., 1994). Our results revealed that retention of this protein occurs at several levels of the early secretory pathway. The glycoprotein is first retained in the rough ER in association with

Address all correspondence to Dr. Ari Helenius, Dept. of Cell Biology, Yale University School of Medicine, 333 Cedar St., New Haven, CT 06510.

calnexin and BiP/GRP78. Leaving calnexin behind, it can, however, move to the intermediate compartment, located in the secretory pathway between the ER and the Golgi complex. Some of it is transported as far as a *cis*-Golgi-associated compartment. Our results indicate that a system is in place to retrieve G protein to the ER by retrograde transport, thus preventing its movement to the cell surface.

Materials and Methods

Materials

The tsO45 strain of vesicular stomatitis virus (VSV)[1] was used and propagated as described in De Silva et al., 1990. [^{35}S]Translabel (>1,000 Ci/mmole) was purchased from ICN Biomedicals (Irvine, CA). Endoglycosidase H was purchased from Boehringer Mannheim (Indianapolis, IN). Brefeldin A was purchased from Epicentre Technologies (Madison, WI). Texas red and fluorescein-conjugated goat anti-mouse, -rat, and -rabbit antibodies were purchased from Jackson Immunoresearch Laboratories (West Grove, PA) and Zymed Laboratories (San Francisco, CA). Colloidal gold-conjugated goat anti- mouse antibodies were purchased from Janssen Chimica (Beerse, Belgium). All other chemicals were purchased from Sigma Chem. Co. (St. Louis, MO). The following people generously provided antibodies: Dr. David Bole, University of Michigan (Ann Arbor, MI) (rat monoclonal anti-BiP); Dr. Thomas Kreis, University of Geneva (Geneva, Switzerland) (P5D4 monoclonal anti-VSV G, monoclonal anti-β COP); Dr. Kai Simons, EMBL (polyclonal anti-VSV G); Dr. Jaakko Saraste, University of Bergen (Bergen, Norway) (anti-p58); Dr. David Vaux, EMBL (Heidelberg, Germany) (anti-protein disulfide isomerase [PDI]); Dr. Daniel Louvard, Pasteur Institute (Paris, France) (polyclonal anti-ER); Dr. Kelly Moremen, University of Georgia (Athens, GA) (polyclonal anti-mannosidase II); Dr. Bruno Goud, Pasteur Institute (purified anti-rab 2). Additional polyclonal antibodies to VSV G were raised in rabbits using viral protein purified as described (Metsikko et al., 1986). Polyclonal antibodies to calnexin were raised in rabbits against a peptide consisting of the COOH-terminal 19 amino acids deduced from canine calnexin sequence (Wada et al., 1991).

Indirect Immunofluorescence Microscopy

CHO cells were plated on alcian blue-treated glass cover slips 40 h before experiments. Cells were fixed briefly at experimental temperature then for 20 min at room temperature in PBS containing 3% paraformaldehyde. All subsequent processing was done at room temperature. Cells were washed in PBS and residual paraformaldehyde was quenched with 50 mM NH_4Cl. Cells were then permeabilized with 0.1% Triton X-100 in PBS for 10 min. For staining with antibodies to β-COP, cells were permeabilized for 4 min in PBS containing 0.1% Triton X-100 and 0.05% SDS. Cells were next blocked in PBS containing 10% goat serum for 20 min. The cells were incubated with primary antibodies diluted in PBS+10% goat serum for 30 min, washed in PBS for 15 min, then incubated with conjugated secondary antibodies for 30 min. The cells were rinsed first in PBS then water and mounted in Mowiol (Calbiochem Corp., La Jolla, CA) containing 2.5% 1,4-diasabicyclo[2.2.2.]octane to prevent photobleaching.

Electron Microscopy

Immunogold labeling and sample preparation were done as previously described (Martin and Helenius. 1991). The mouse monoclonal antibody to the cytoplasmic tail of VSV G was used as an undiluted hybridoma supernatant.

Pulse Labeling and Endoglycosidase H Digestion

Subconfluent dishes of CHO cells were infected at 32°C with tsO45 at a multiplicity of infection of 30, then incubated for 2 h on a water bath at 39.5°C in bicarbonate-free MEM media buffered to pH 7 with 20 mM Hepes and containing 5% fetal calf serum (complete media). Cells were next washed twice in 39.5°C PBS, incubated for 15 min in methionine-free media, then pulse labeled for 10 min in methionine-free media containing 200 μCi/ml [^{35}S] Trans label at 39.5°C. Chases were initiated by removing the pulse media and adding complete media containing 4.5 mM methionine at either 39.5°C or 32.°C. Endoglycosidase H digestion, immunoprecipitation, and alkylation of G protein were performed as described (Braakman et al., 1991).

Subcellular Fractionation

CHO cells were plated in 100-mm diam dishes 20 h before experiments, then infected, pulse labeled, and chased as described above. At the end of the chase period the cells were rapidly cooled by transferring the dishes to ice and adding ice-cold PBS. Cells were washed twice in homogenization buffer (10 mM triethanolamine, 10 mM acetic acid, 250 mM sucrose, 1 mM EDTA, and 1 mM dithiothreitol [DTT]), then scraped with a rubber policeman in 800 μl of homogenization buffer containing 10 μg/ml each of chymostatin, leupeptin, antipain, and pepstatin. The cells were homogenized by passing them 12 times through a 25-gauge needle on a 1-ml syringe. A post nuclear supernatant (PNS) was prepared by centrifuging for 5 min at 1,500 *g* at 4°C. The PNS was loaded on preformed nycodenz (Nycomed Pharma; Oslo, Norway) gradients. Nycodenz gradients were prepared for the Beckman SW 41Ti rotor by initially making a step gradient containing 2.65 ml each of 24, 19.33, 14.66, and 10% isotonic nycodenz solutions. Nycodenz solutions were made by diluting a 27.6% stock of nycodenz in 10 mM Tris, pH 7.4, 3 mM KCl, and 1 mM EDTA with a saline buffer containing 0.75% NaCl, 10 mM Tris, pH 7.4, 3 mM KCl, and 1 mM EDTA as described (Rickwood et al., 1982). The step gradients were diffused to form linear gradients by sealing the top of the tubes and turning them horizontally at room temperature for 45 min. The linear gradients were then centrifuged for 4 h at 37,000 rpm in a Beckman L8-M ultracentrifuge to generate a nonlinear density profile. The PNS was loaded on top of the gradients and centrifuged for 1.5 h at 37,000 rpm. 15 fractions were collected from each gradient. The density of the fractions was determined from their refractive index (Rickwood et al., 1982). One third of each fraction was mixed with an equal volume of 1% Triton X-100 and immunoprecipitated with polyclonal anti-VSV G antiserum. For coprecipitation of VSV G with calnexin, fractions were mixed with an equal volume of 2% CHAPS and immunoprecipitated with anti-calnexin antiserum as described (Ou et al., 1993). The distribution of p58, PDI, and calnexin in the gradients was determined by western blotting using ECL detection (Amersham Corp., Arlington Heights, IL) followed by scanning densitometry. Mannosidase II activity was measured as described (Storrie and Madden, 1990).

Results

The tsO45 G Protein Is Transport Defective at the Nonpermissive Temperature

The G protein of tsO45 VSV has a point mutation (phe 204 →ser) in its ectodomain which makes it temperature sensitive for folding (Gallione and Rose, 1985). At the permissive temperature of 32°C, it associates transiently with BiP/GRP78 and calnexin, and matures in the ER into normal noncovalently associated homotrimers (3 × 67 kD) (Rose and Gallione, 1981; Doms et al., 1987; Machamer et al., 1990; Hammond et al., 1994). The trimers move rapidly and efficiently through the secretory pathway to the plasma membrane (see Balch, 1986 and references therein). In contrast, at the nonpermissive temperature of 39.5°C the G protein remains incompletely and heterogenously oxidized (De Silva et al., 1990), it does not acquire mature ectodomain epitopes defined by conformation-specific monoclonal antibodies (Doms et al., 1988), and it forms heterogeneous complexes with BiP/GRP78 and calnexin (Doms et al., 1987; De Silva et al., 1990; Hammond et al., 1994). Upon a shift from 39 to 32°C, a large fraction of the glycoprotein folds, trimerizes, and proceeds normally to the cell surface where virus budding takes place (Knipe et al., 1977; Balch et al., 1986; Doms et al., 1987).

1. *Abbreviations used in this paper*: CGN, *cis*-Golgi network; DTT, dithiothreitol; PDI, protein disulfide isomerase; PNS, post-nuclear supernatant; VSV, vesicular stomatitis virus.

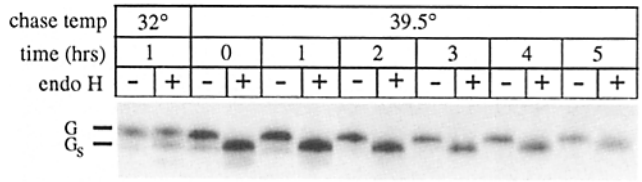

Figure 1. Sensitivity of tsO45 G to digestion by endoglycosidase H. Infected CHO cells were pulse labeled with [^{35}S]methionine for 10 min at 39.5°C then chased at either 32°C or 39.5°C for the indicated times. Cells were lysed and immunoprecipitated with antibodies to VSV G. Half of the immunoprecipitated sample was digested with endoglycosidase H. Samples were analyzed using 7.5% reducing SDS-PAGE. G_s is a soluble form of G protein resulting from intracellular proteolytic cleavage.

To determine the long-term fate of the misfolded protein at nonpermissive temperature, we infected CHO cells with tsO45 VSV and pulsed them with [^{35}S]methionine for 10 min at 39.5°C. The pulse was followed by chase up to 5 h at 39.5°C. Control samples were chased for one hour at the permissive temperature (32°C). The cells were solubilized, postnuclear lysates immunoprecipitated using polyclonal anti-G protein antibodies, and aliquots digested with endoglycosidase H prior to SDS-PAGE. While the G protein in the control dish was found to become resistant to endoglycosidase H digestion during the chase (Fig. 1), the G protein held at 39.5°C remained sensitive, in agreement with previous studies (Chen and Huang, 1986). This confirmed that the misfolded G protein did not reach the medial Golgi complex even after long chase times. The experiment also revealed that the misfolded G protein was relatively stable at 39.5°C; quantitation by densitometry showed that it was degraded with a half-time of 4 h.

Misfolded G Protein Is Not Confined to the ER

When visualized by indirect immunofluorescence microscopy in infected CHO cells that had been held at 39.5°C for 2 h, the distribution of misfolded G was mainly ER-like (Fig. 2 *a*). However, about 20–30% of the most brightly stained cells showed, in addition, punctate accumulations of G protein distributed throughout the cytoplasm. After 4.5 h at nonpermissive temperature, 60–80% of the cells showed such spots (Fig. 2 *b*). Similar results were obtained in BHK cells. The spots were more abundant and more highly stained in cells with higher expression of G protein suggesting that the G protein in them represented over-flow from the ER. This was consistent with the observation that cycloheximide added for 2.5 h after the first 2 h to block synthesis of additional G protein inhibited the increase both in number and intensity of the spots (not shown).

At the 4.5-h chase time point, G protein staining could also be seen in the region of the Golgi complex, which in these cells is often located on top of the nucleus. When the cells were shifted to the permissive temperature for 20 min in the presence of cycloheximide, the G protein from the ER and the spots moved into a pattern that was perinuclear and Golgi-like (Fig. 2 *c*). Later G protein moved to the cell surface with a concomitant loss of staining in the Golgi region (Fig. 2 *d*).

We concluded that the misfolded G protein was not confined to the reticular ER, but could move to distinct organelles distributed in various parts of the cytoplasm including the Golgi region. Since it moved to the cell surface upon

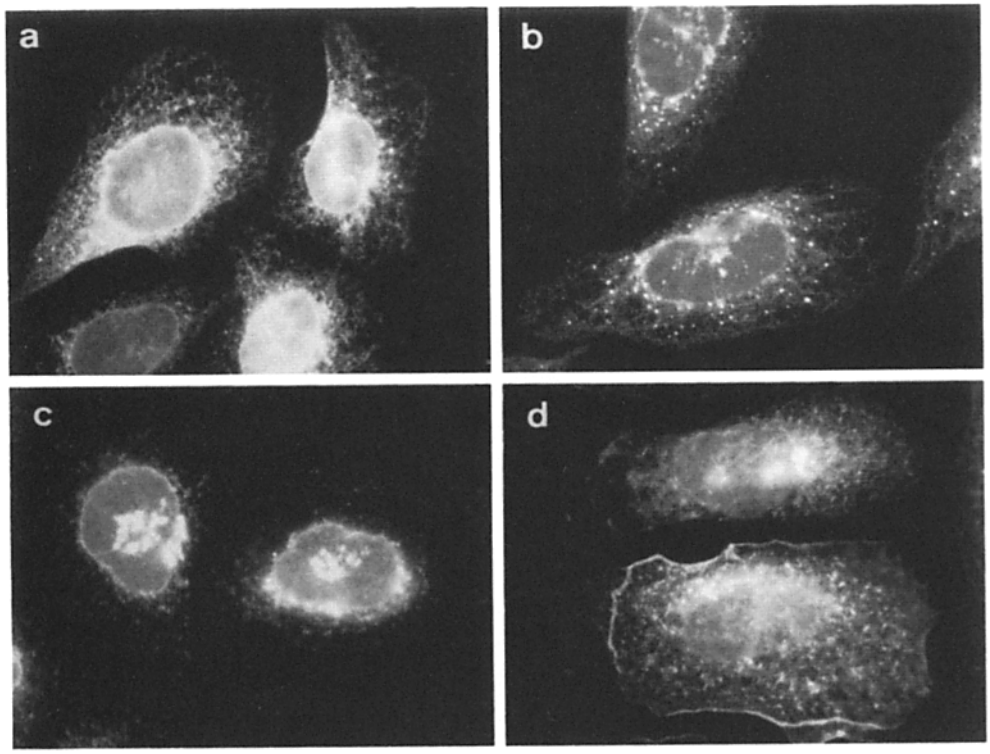

Figure 2. Indirect immunofluorescence microscopy of tsO45-infected CHO cells using polyclonal antibodies to VSV G. Cells were held at 39.5°C for either 2 (*a*) or 4.5 h (*b*). When cells were first held at 39.5°C for 4.5 h then shifted to 32°C in the presence of 500 μM cycloheximide for 20 (*c*) or 120 min (*d*), G was seen to move to the Golgi complex and cell surface. All cells were fixed in 3% paraformaldehyde and permeabilized with 0.1% Triton X-100 prior to staining.

temperature shift, the G protein in these pre-Golgi structures remained in functional contact with the secretory pathway.

The Intermediate Compartment and the CGN Contain Misfolded G Protein

To identify the G protein-containing organelles, we employed double-label immunofluorescence microscopy using antibodies to known ER, intermediate compartment, Golgi, and lysosome antigens. The peripheral G protein-containing spots were clearly positive for markers of the intermediate compartment (Chavrier et al., 1990; Duden et al., 1991; Saraste and Svensson, 1991; Krijnse-Locker et al., 1994). Extensive overlap was seen in the staining pattern with p58 (Fig. 3, *a* and *b*), β-COP (Fig. 3, *c* and *d*), and rab 2 (Fig. 3, *e* and *f*). The spots did not stain with antibodies to either the Golgi enzyme mannosidase II (Fig. 3, *i* and *j*) or lgp 120, a lysosomal membrane protein (not shown). They also failed to stain with antibodies to several ER marker proteins; anti-calnexin (Fig. 3, *g* and *h*), anti-ER antibodies (Louvard et al., 1982) (not shown), and anti-PDI (not shown). On the basis of this analysis, we concluded that the spots represented the intermediate compartment. This identification was consistent with observations that showed that the spots were unaffected by brefeldin A and expanded in size after nocodazole treatment. These responses differentiate the intermediate compartment from elements of the Golgi complex (Lippincott-Schwartz et al., 1990).

The immunofluorescence analysis also showed that G protein was clearly present in the ER. There was a fine reticular staining throughout the cytoplasm and the nuclear membrane was also stained. In addition, some G protein was observed in perinuclear compartments that colocalized with the Golgi marker mannosidase II (Fig. 3, *i* and *j*). As the G protein did not convert to its endoglycosidase H-resistant form (Fig. 1), the Golgi compartments in question comprised, most likely, the *cis*-most elements (the *cis*-Golgi network or CGN [Mellman and Simons, 1992]).

Electronmicroscopy after plasma membrane permeabilization and pre-embedding immunolabeling using monoclonal antibodies to the cytoplasmic tail of G protein, showed that the most heavily labeled organelles after 4 h at 39.5°C included tight clusters of smooth vesicles and short tubules (Fig. 4 *b*). These had a morphology similar to that described for the intermediate compartment (Saraste and Kuismanen, 1984; Schweizer et al., 1988; Lotti et al., 1992). Gold labeling was also seen in the ER cisternae and the nuclear envelope. Occasionally, gold labeling was seen in membranes close to but peripheral to the Golgi stacks. No gold labeling of mitochondria, the plasma membrane, or endosomes was seen. After 2.5 h at 39.5°C most cells showed G protein confined to the ER (Fig. 4 *a*) with very little labeling of smooth membrane organelles.

BiP/GRP78 was the only marker antigen whose distribution was inconsistent with the known composition of the intermediate compartment and CGN. Anti-BiP/GRP78 antibodies stained not only the ER, but also the G protein-containing spots and the Golgi-associated elements (Fig. 3, *k* and *l*). When double staining was done with anti-BiP/GRP78 and anti-mannosidase II, discrete areas of costaining were clearly seen (not shown). This distribution was only observed in tsO45-infected cells held at 39.5°C. When nonin-

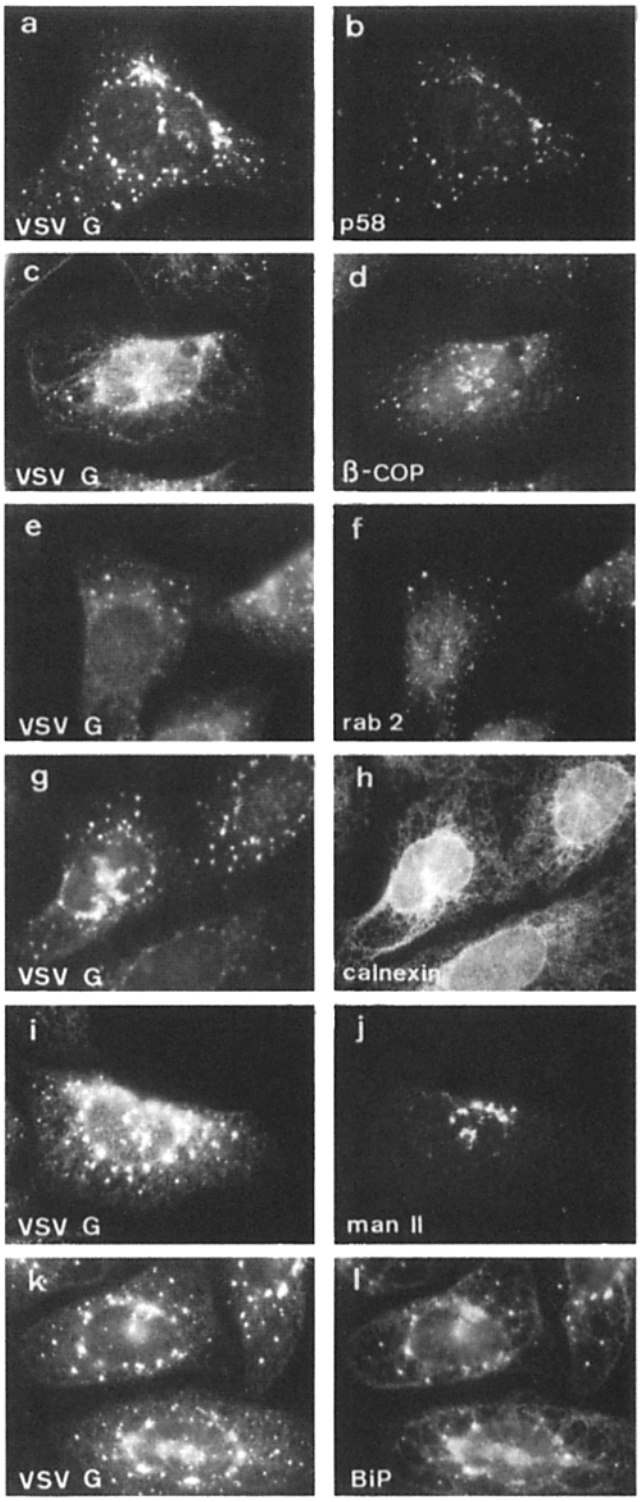

Figure 3. Indirect double-label immunofluorescence characterization of organelles containing misfolded G protein. Cells were held at 39.5°C for 4.5 h then fixed and labeled with monoclonal (*a*, *e*, *g*, and *i*) or polyclonal (*c* and *k*) antibodies to VSV G and antibodies to the following: p58 (*b*), β-COP (*d*), rab 2 (*f*), calnexin (*h*), mannosidase II (*j*), and BiP/GRP78 (*l*).

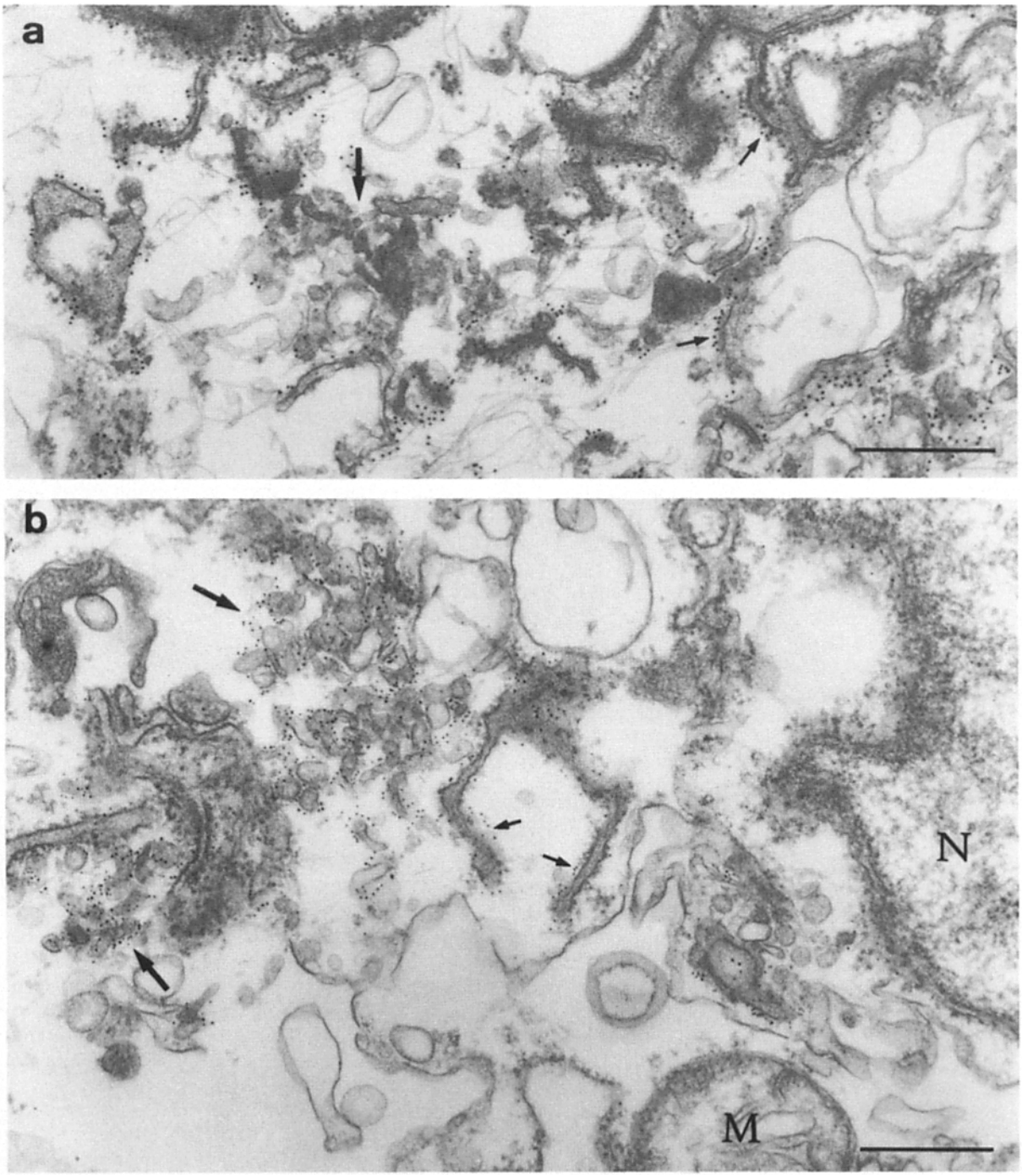

Figure 4. Immunogold labeling of infected CHO cells. Cells were held at 39.5°C for 2.5 (*a*) or 4 h (*b*), rapidly cooled to 4°C, fixed with 3% paraformaldehyde then permeabilized by nitrocellulose ripping of the plasma membrane. The cells were labeled with a monoclonal antibody (P5D4) to the cytoplasmic tail of VSV G and detected by gold-coupled goat anti-mouse antibodies (*b*) or goat anti-mouse antibodies followed by gold-coupled protein A. After labeling, cells were EPON embedded and prepared for thin section EM. Labeling of the ER (*small arrows*) and clusters of tubulo-vesicular elements (*large arrows*) are indicated. Nuclei and mitochondria are marked N and M respectively. Bars, 0.4 μm.

fected control cells at 39.5°C or cells infected with tsO45 at permissive temperature were stained, the BiP/GRP78 was confined to an ER-pattern. This suggested that the presence of BiP/GRP78 in the intermediate compartment and CGN was caused by the presence of misfolded G protein. We have previously shown that misfolded G protein forms a stable complex with BiP/GRP78 at nonpermissive temperature (De Silva et al., 1990). It, therefore, seemed likely that BiP/GRP78's unusual distribution was caused by its association with the misfolded G protein.

We concluded that the tsO45 G protein was not confined exclusively to the ER. A substantial fraction moved from the

ER into the intermediate compartment and the CGN. This is supported by the observations of Schweizer et al. (1990) who found in infected Vero cells vesicles containing both the intermediate compartment marker p53 and misfolded G. Most likely, the G protein was part of larger complexes which contained BiP/GRP78. Judging by the endoglycosidase H sensitivity, little if any of the G protein passed beyond the CGN. That the G protein remained misfolded during long incubations at nonpermissive temperature was confirmed by its lack of reactivity with a monoclonal antibody I14, which recognizes a late folding epitope (B2) present in the properly folded tsO45 G at 32°C (Doms et al., 1988; De Silva et al., 1990).

Separation of ER, Golgi and Intermediate Compartment

To follow the movement of misfolded G protein more quantitatively, we developed a cell fractionation procedure based on nonlinear Nycodenz gradients. This method was used to separate ER, the intermediate compartment and the Golgi complex from each other in cell homogenates. The distribution of markers for the Golgi complex (mannosidase II), ER (PDI and calnexin), and intermediate compartment (p58) is shown in figure 5 *A*. The mannosidase II activity was found in a sharp peak centered in fraction 2 (1.039 g/ml), near the top of the gradient (Fig. 5 *A* 1). PDI (Fig. 5 *A* 1) and calnexin (Fig. 5 *A* 2) were detected in a broad peak between fractions 9 and 12 (1.085–1.104 g/ml) in the heavy portion of the gradient. Some soluble PDI, released during homogenization, was present at the top of the gradient (fractions 1 and 2). The majority of the intermediate compartment marker p58 was found in a broad peak between fractions 2 and 6 (1.039–1.062 g/ml), close to the Golgi fractions (Fig. 5 *A* 2). A small amount was also detected in the heavy, ER-containing fractions, consistent with reports showing the presence of some p58 in the ER (Saraste and Svensson, 1991).

Although some overlap between the Golgi and intermediate compartment occurred it these gradients, the resolution was sufficient to follow the movement of misfolded G protein in the early secretory pathway using a pulse–chase approach. Cells were infected with tsO45 VSV, and incubated for 2 h at 39.5°C to allow G protein synthesis to reach a high enough level for metabolic labeling. They were then pulse labeled for 10 min and chased for either 0 or 2.5 h, making the total time at the nonpermissive temperature comparable to that used in the immunofluorescence experiments. Following the chase, postnuclear supernatants were prepared, fractionated, and immunoprecipitated with anti-G protein antibodies.

Immediately after the pulse, the majority of G protein was found in a single peak which coincided with the ER (Fig. 5 *B* 1). After the 2.5-h chase, its distribution was markedly changed; more than 25% of the G protein had moved to the lighter fractions containing the intermediate compartment and the Golgi complex (Fig. 5 *B* 2). These results were fully consistent with the morphological data showing that some of the misfolded G protein was transported to the intermediate compartment.

When samples that had been chased for 2.5 h at 39.5°C were separated on gradients and the fractions lysed in CHAPS detergent, some of the misfolded G protein could be coprecipitated with the ER chaperone, calnexin (Hammond et al., 1994). Although G protein was present both in the Golgi/intermediate compartment fractions and in the ER fractions (Fig. 6 *a*), G protein coprecipitating with calnexin was confined to the heavy, ER-containing part of the gradient (Fig. 6 *b*). This was consistent with the immunofluorescence experiments which showed the presence of calnexin exclusively in the ER (Fig. 3, *g* and *h*). Although calnexin associated with the misfolded tsO45 G protein in the ER, it did not accompany the protein to the intermediate compartment. This implied that the misfolded G protein existed in both calnexin-free and calnexin-bound forms.

Heterogeneity of tsO45 G Protein Complexes

The presence of separate calnexin-bound and BiP/GRP78-bound forms of tsO45 G protein was supported by coprecipitation analysis using antibodies to G protein and the two chaperones. When either pulse labeled for 30 min or pulsed and chased for 2.5 h at 39.5°C misfolded G protein itself migrated heterogeneously on nonreducing SDS-PAGE with relative mobilities ranging between those of the fully folded and oxidized G protein (G_{ox}) and the fully reduced G protein (G_{red}) (De Silva et al., 1990) (Figs. 7 and 8, lane *1*). Large disulfide-bonded aggregates of labeled G protein on top of the stacking and separating gels were also present (Figs. 7 and 8, lane 1). When cells were lysed in CHAPS, immunoprecipitated with antibodies to G protein, and the immunoprecipitates washed with low stringency, additional labeled bands were seen (Fig. 7, lane *1*). These included bands with the mobilities of calnexin and BiP/GRP78, indicating that the chaperones were associated with the misfolded G protein. When the same lysates were precipitated with anti-calnexin antibodies a strong calnexin band was observed (Fig. 7, lanes *2* and *5*). In addition, the antibody coprecipitated a large amount of G protein which migrated in nonreducing gels as fully oxidized or partially reduced forms (Fig. 7, lanes *2* and *5*). Some BiP/GRP78 was also present in the anti-calnexin immunoprecipitates, but the amount was less than that coprecipitated with anti-G protein antibodies. This suggested that most of the BiP/GRP78-containing G protein complexes were devoid of calnexin. The anti-calnexin antibodies failed to coprecipitate G protein in disulfide linked aggregates (Fig. 7, lane *2*). Similar results were obtained from coprecipitation of samples that had been chased for 2.5 h at 39.5°C (not shown).

In agreement with our previous results (Machamer et al., 1990), antibodies to BiP/GRP78 coprecipitated partially reduced forms of G protein (Fig. 7, lane *3*). It is important to note that the anti-BiP/GRP78 antibody used did not give quantitative immunoprecipitation. Therefore it is likely that the BiP/GRP78 complexes were much more abundant than suggested by the immunoprecipitation in Fig. 7. The limited amount of anti-BiP/GRP78 antibody available to us did not allow the use of large amounts for each precipitation, or immunoprecipitation of multiple samples. Because of the small amount of G protein coprecipitated with BiP/GRP78, we cannot rule out the possibility that BiP/GRP78 may be associated with some of the disulfide-linked aggregates seen in Fig. 7, lane *1*.

The results of the coimmunoprecipitation experiments suggested that tsO45 G protein is present in complexes of at least three different types: complexes containing calnexin, G protein and some BiP/GRP78; complexes containing BiP/

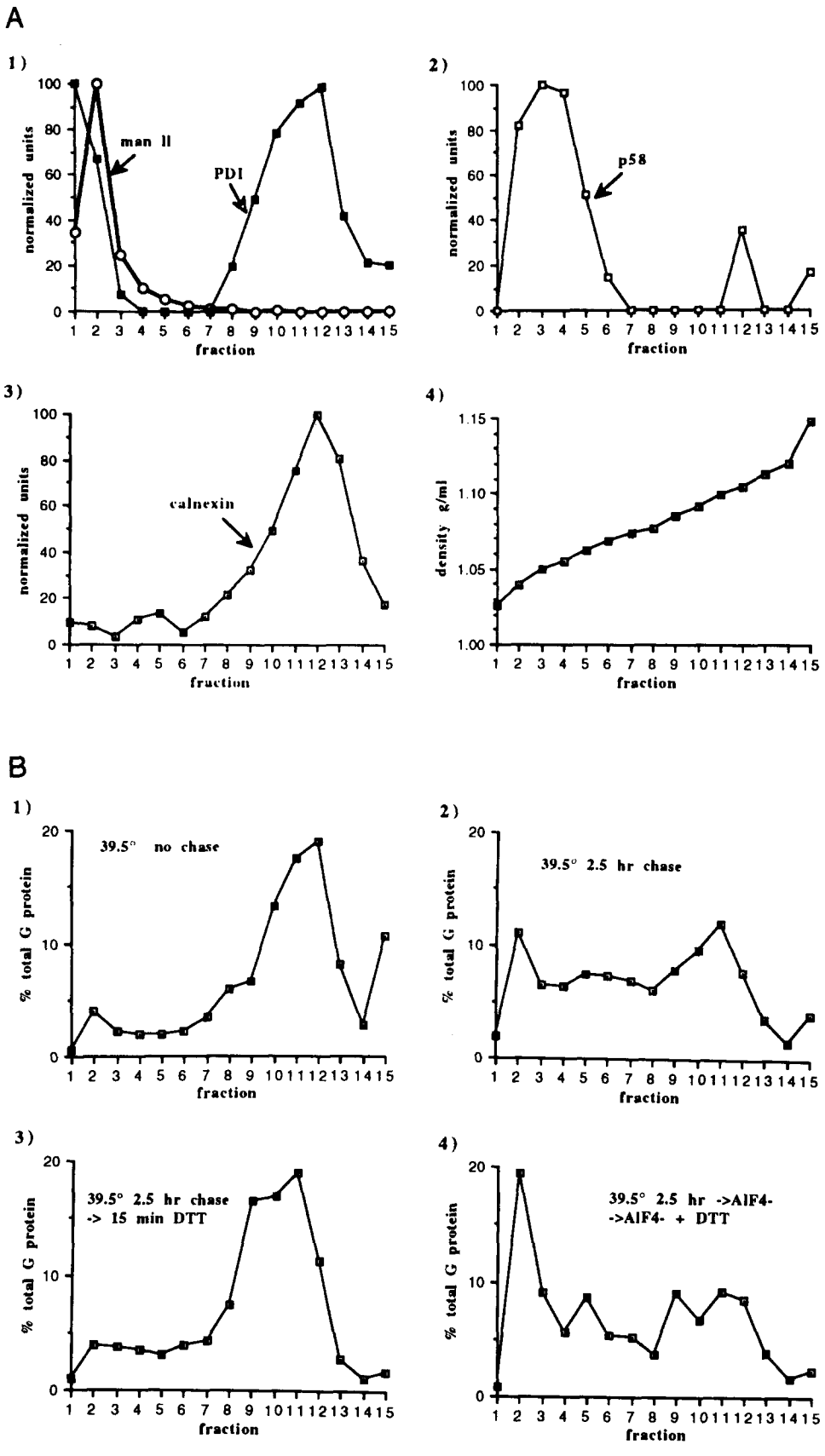

Figure 5. Separation of pulse-labeled CHO cells using nycodenz density gradients. (*A*) Gradient characterization. Markers for the ER are PDI (*1, filled squares*) and calnexin (*3*). The marker for medial Golgi is mannosidase II activity (*1, open circles*). The distribution of the intermediate compartment is shown by the marker protein p58 (*2*). Soluble PDI, released during homogenization, was present at the top of the gradient 1 (fraction *1*). The density profile of the gradient is shown in *4*. (*B*) Pulse–chase analysis of misfolded G. Cells were pulse labeled with [^{35}S]methionine for 10 min at 39.5°C and chased at 39.5°C as follows: no chase (*1*), 2.5-h chase (*2*), 2.5-h chase followed by 15 min chase in 5 mM DTT (*3*), 2.5-h chase followed by first chasing for 5 min in AlF_4- (50 μM $AlCl_3$ + 30 mM NaF) then chasing an additional 15 min in both AlF_4- and 5 mM DTT (*4*). Following the chase, samples were rapidly cooled on ice and separated on nycodenz gradients. Fractions were immunoprecipitated with polyclonal antibodies to VSV G and analyzed by SDS-PAGE and fluorography. The amount of G protein in each fraction was quantified by scanning densitometry and expressed as a percent of total G protein.

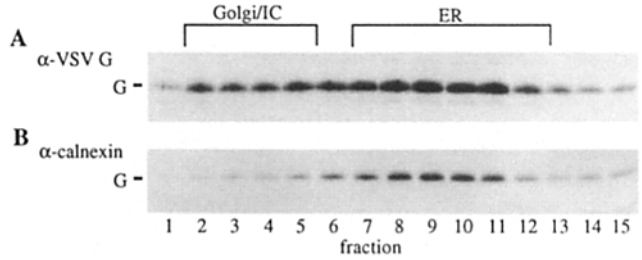

Figure 6. Coprecipitation of misfolded G protein with calnexin. Cells were pulsed labeled for 10 min at 39.5°C, chased at the same temperature for 2.5 h, then separated on nycodenz gradients. Fractions were lysed in 2% CHAPS detergent and immunoprecipitated with antibodies to either G protein (*A*) or calnexin (*B*). Samples were analyzed using reducing 7.5% SDS-PAGE and fluorography. The distribution of ER, intermediate compartment (*IC*) and Golgi in the gradients is indicated (also see Fig. 5).

GRP78 and G protein; and disulfide-linked aggregates devoid of calnexin but possibly containing BiP/GRP78. Since calnexin is localized exclusively in the ER (Fig. 3 *h*; Fig. 5 *A* 3), and since BiP/GRP78 is visible in the intermediate compartment and CGN only after prolonged synthesis misfolded G protein, we think that the G protein that exits the ER corresponds to one or both of the latter type of complexes.

Return of Misfolded G Protein to the ER

What happens to the misfolded G proteins that exit the ER? Do they simply accumulate in the different pre-Golgi compartments or do they get recycled back to the ER? To answer these questions, we used a membrane permeable reducing agent, DTT, which we have shown to reduce newly synthesized proteins in the ER of living cells (Braakman et al., 1992; Tatu et al., 1993). As long as DTT is present, proteins that depend on disulfides for folding are retained in the ER although transport of other proteins to the cell surface continues (Tatu et al., 1993).

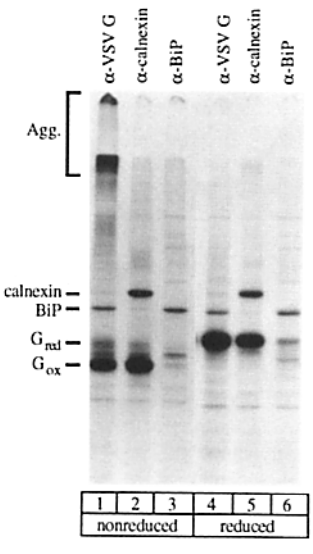

Figure 7. Immunoprecipitation of complexes containing misfolded G protein, calnexin and BiP/GRP78. Cells were metabolically labeled for 1 h, infected, then pulse labeled for 30 min at 39.5°C. The cells were then rapidly cooled, alkylated with 20 mM NEM and lysed in 2% CHAPS containing 30 U/ml apyrase to preserve BiP/GRP78 interactions. Lysates were immunoprecipitated with monoclonal antibodies to VSV G (lanes *1* and *4*), BiP/GRP78 (lanes *3* and *6*) and a polyclonal antibody to calnexin (lanes *2* and *5*). Immunoprecipitates were washed at 4°C under low stringency conditions (0.5% CHAPS, 200 mM NaCl, 50 mM HEPES, pH 7.3) and analyzed on 7.5% nonreducing (lanes *1–3*) and reducing (lanes *4–6*) SDS-PAGE.

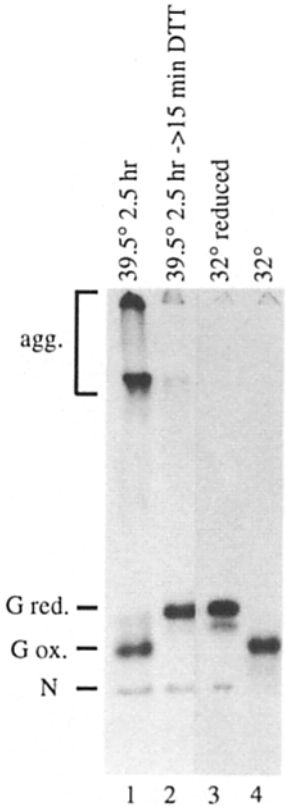

Figure 8. Intracellular reduction of misfolded G protein by DTT. Cells were pulse labeled for 10 min at 39.5°C and chased for either 2.5 (lane *1*) or 2.5 h followed by 15 min in 5 mM DTT (lane *2*). Cells were then rapidly cooled on ice, washed with PBS containing 20 mM NEM and lysed in the presence of 20 mM NEM. Lysates were immunoprecipitated with polyclonal anti-VSV G antibodies and analyzed on nonreducing 7.5% SDS-PAGE. Control samples were pulsed at 32°C and chased for 20 min before alkylation, lysis and immunoprecipitation. Samples were either reduced in vitro by boiling in 10 mM DTT and realkylating (lane *3*) or left fully oxidized (lane *4*). VSV-N (*N*) protein is a nonspecific background band.

When DTT was added to cells containing misfolded tsO45 G protein, the protein was rapidly reduced judging by its mobility on nonreducing SDS-PAGE (Fig. 8, lane *2*). Before DDT addition, the misfolded G protein showed a typical pattern of heterogeneous migration in the gel (Fig. 8, lane *1*). The mobility of G protein synthesized at the permissive temperature and reduced in vitro (Fig. 8, lane *3*) or left fully oxidized (lane *4*) is shown for reference. Apparently, the misfolded G protein was sensitive to DTT not only in the ER but also in the intermediate compartment and the CGN (see below).

When the distribution of G proteins in the DTT-treated cells was analyzed by immunofluorescence, a dramatic change in the intracellular distribution was observed. The staining in the intermediate compartment had disappeared with a corresponding increase in reticular ER staining (compare Fig. 9, *a* and *b*). The staining in the Golgi region was also reduced judging by the intensity of costaining with mannosidase II (not shown). Thus, addition of DTT seemed to cause redistribution of the misfolded G protein and its accumulation in the ER.

That the G protein, upon DTT reduction, moved from the intermediate compartment and CGN to the ER was confirmed using the cell fractionation procedure. Fifteen min of DTT treatment caused a major redistribution of labeled G protein from the intermediate compartment and Golgi fractions to the denser ER fractions (compare Fig. 5 *B*, 2 and 3).

The observed accumulation of the misfolded G protein in the ER could be interpreted in two ways: either DTT activated an otherwise nonexistent pathway of retrograde transport of G protein from the intermediate compartment and the Golgi complex to the ER, or it simply interrupted an ongoing

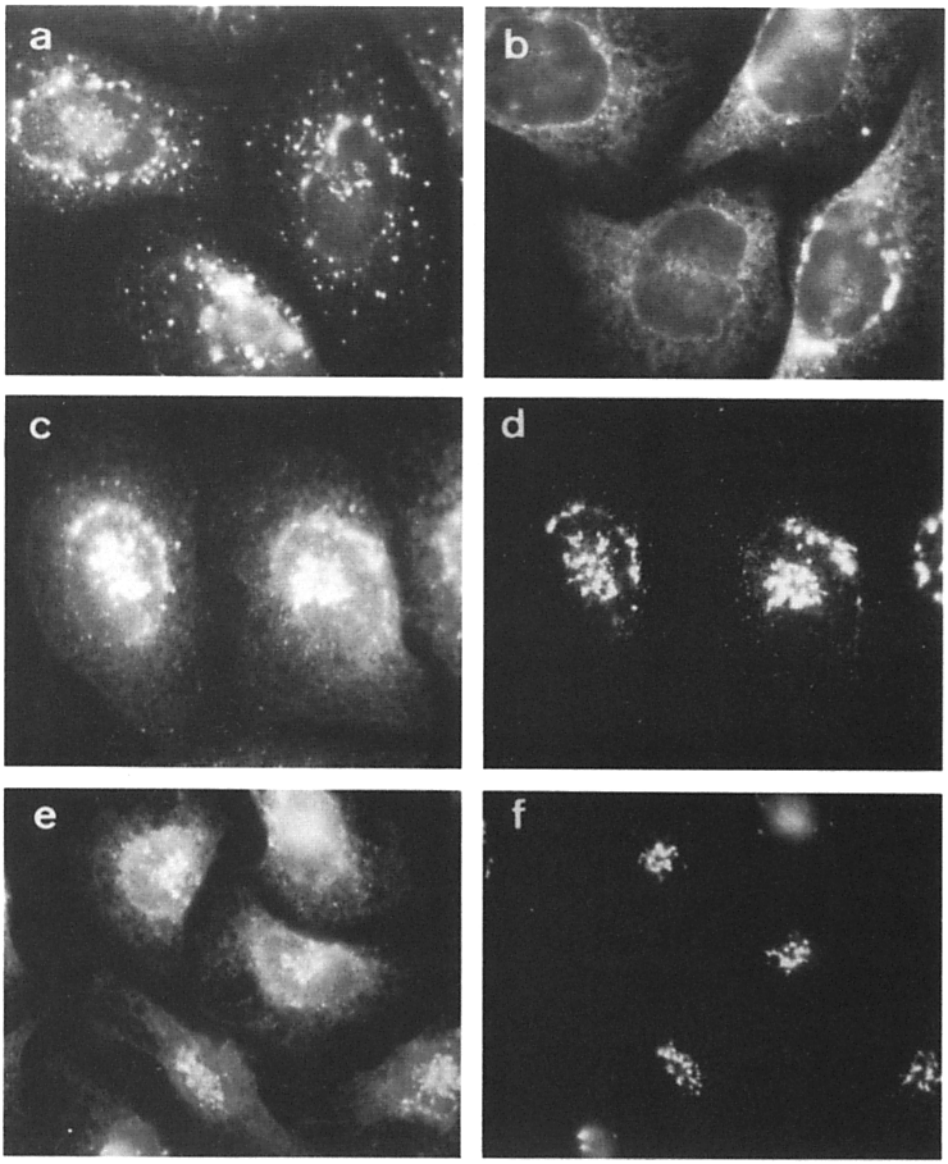

Figure 9. Effect of DTT and AlF_4^- on the distribution of misfolded G protein. Cells were held at 39.5°C for 4.5 h (*a*) then incubated at the same temperature for 15 in in 5 mM DTT (*b*), AlF_4^- (*c* and *d*), or AlF_4^- followed by an additional 15 min in both AlF_4^- and DTT (*e* and *f*). Cells were then fixed and labeled using a monoclonal antibody to VSV G (*a–c*, and *e*). *d* and *f* show double labeling with antibodies to mannosidase II of the cells in *c* and *e*, respectively.

cyclic movement of G proteins between these compartments by preventing the G protein's exit from the ER.

Retrograde Transport of G Protein is Blocked by AlF_4^-

The ongoing retrograde transport from the Golgi to the ER is known to be blocked by AlF_4^-, an activator of heterotrimeric G proteins (Donaldson et al., 1991). To analyze the effects of this inhibitor on G protein distribution, infected cells were incubated at 39.5°C for 4.5 h, whereafter they were treated with AlF_4^- at the nonpermissive temperature for 15 min. Indirect immunofluorescence showed the loss of G protein from the intermediate compartment (compare Fig. 9, *a* and *c*), and a corresponding increase of G protein staining in the perinuclear, mannosidase II-positive structures. The majority of misfolded G protein in the intermediate compartment apparently moved to the Golgi apparatus.

When such AlF_4^--treated cells were treated with DTT, the pattern of G protein did not change (compare Fig. 9, *c* and *e*). Thus, although DTT did cause reduction of the G protein (not shown), the protein failed to recycle back to the ER. The lack of retrograde movement was confirmed using nycodenz gradient fractionation (Fig. 5 *B* 4). Instead of moving to the ER fractions as observed with DTT alone, the G protein in AlF_4^--treated cells remained in the lighter portions of the gradient.

These results are consistent with the presence of an ongoing transport cycle of excess misfolded G protein from the ER via the intermediate compartment to the CGN, and from there back to the ER. Exit from the ER is relatively slow and inefficient and it is inhibited by DTT reduction of the G protein. AlF_4^-, on the other hand, blocks the cycle at the level of CGN as already shown for other retrograde transport events (Donaldson et al., 1991). The putative cycling pathway and the effects of the two inhibitors are shown schematically in Fig. 10.

Discussion

Together with previous data, these results outline a step-by-step itinerary for the newly synthesized, misfolded tsO45 G

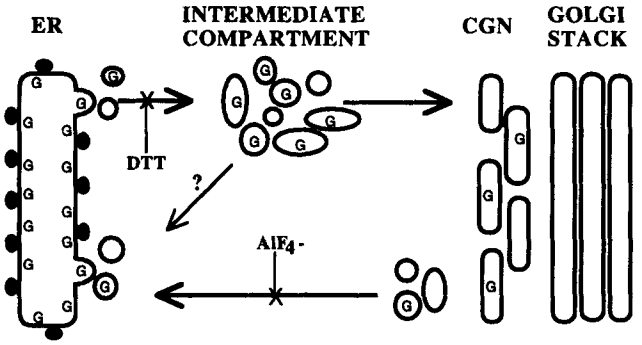

Figure 10. Retention of misfolded G protein in the early secretory pathway. The proposed model depicts most of the G protein retained in the ER. This population of G is largely bound to the ER chaperone calnexin. Some G protein, probably in a complex with BiP/GRP78, escapes from the ER to enter the intermediate compartment and the CGN. This G protein is returned to the ER from the CGN and perhaps directly from the intermediate compartment. DTT further misfolds G protein and prevents its exit from the ER while AlF_4- prevents recycling from the CGN.

protein. At nonpermissive temperature, this mutant glycoprotein is synthesized and glycosylated normally. Like its wild-type counterpart, it begins to acquire intra-chain disulfide bonds already on the nascent chain, and it rapidly associates with two ER chaperones, BiP/GRP78 and calnexin (De Silva et al., 1990, 1993; Hammond et al., 1994). BiP/GRP78 probably binds first since it associates preferentially with less oxidized folding intermediates of the G protein (Machamer et al., 1990; Fig. 7). It is followed by calnexin, which binds transiently to a more fully oxidized population of intermediates (Fig. 6) (Hammond and Helenius, unpublished observations).

Previous studies have suggested that the folding defect in tsO45 G protein manifests itself during the latter part of the posttranslational folding period (De Silva et al., 1993). It prevents full intra-chain disulfide oxidation (De Silva et al., 1990) and dissociation from multi-molecular complexes containing calnexin and/or BiP/GRP78 (De Silva et al., 1990; Hammond et al., 1994). The folding defect results in the lack of specific conformational epitopes A1, B2, IN2, IN5, IN6 (Doms et al., 1988), and a tendency to form aberrant, interchain-disulfided bonded complexes (Fig. 7). The protein also fails to trimerize (Doms et al., 1987), reach a DTT-resistant conformation (Fig. 7) (Tatu et al., 1993), and leave the ER in a timely fashion (for references see Balch et al., 1986).

Our analysis showed that the misfolded tsO45 G protein is present in three forms: complexes associated mainly with calnexin, complexes associated with BiP/GRP78, and interchain cross-linked aggregates devoid of calnexin but possibly containing BiP/GRP78. The association with calnexin, which is only transient with the wild-type G protein, is prolonged for tsO45 because 60–78% of the core oligosaccharides remain monoglucosylated instead of rapidly losing all three of the terminal glucose residues (Suh et al., 1989; Hammond et al., 1994). The reason for this is the continuous reglucosylation of the trimmed high mannose N-linked sugars by UDP-glucose:glycoprotein glucosyltransferase, a lumenal ER enzyme (Trombetta and Parodi, 1992). This enzyme selectively reglucosylates misfolded glycoproteins in the ER (Sousa et al., 1992). According to our recent data, reglucosylation confers continuous rebinding specificity to folding-defective glycoproteins, such as tsO45 G protein, and prevents their dissociation from calnexin (Hammond et al., 1994). Since calnexin is localized exclusively in the ER, it may be responsible for retaining a large portion of the misfolded G protein. It has recently been shown to retain unassembled oligomeric subunits of MHC class I molecules and the CD3 complex (Jackson et al., 1994; Rajagopalan et al., 1994).

The persistent binding of G protein to BiP/GRP78 has a different explanation. It is probably caused by continuous exposure of hydrophobic peptide elements which may be only transiently exposed during normal folding of G protein. BiP/GRP78 and other members of the HSP70 family are thought to bind to their substrates via such segments (Flynn et al., 1991; Blond-Elguindi et al., 1993). Association with BiP/GRP78 may also help to retain G proteins in the ER; it is known that even without its KDEL-retention sequence, BiP/GRP78 is only slowly transported from the ER (Munro and Pelham, 1987).

Our results showed that when the amount of misfolded G protein increased in the ER, some started to move to the intermediate compartment and to the CGN. Since the transported G protein was devoid of calnexin, and since it apparently carried BiP/GRP78 along with it, we assume that the G-protein transported corresponds to the BiP/GRP78-associated variety; however, we have not directly demonstrated the existence of BiP/GRP78-G protein complexes in the intermediate compartment. The extent of escape from the ER seemed to correlate more closely with the expression level of G protein than with the time after infection. Therefore, we think that the appearance of G protein in the intermediate compartment and CGN is caused by some type of a saturation phenomenon in the ER. The available calnexin could, for example, become saturated and fail to retain excess protein.

Calnexin is not the only factor, however, responsible for retaining misfolded glycoproteins in the ER. This is illustrated by the observation that tsO45 G protein synthesized in the presence of castanospermine did not leave the ER. Since this inhibitor blocks tsO45 G protein binding to calnexin (Hammond et al., 1994), the retention must involve other components. Furthermore, the efficient accumulation of all the recycled G protein in the ER when DTT was added indicated that there was extra retention capacity, at least for proteins that are fully reduced. Thus, though calnexin is probably one of the key factors that retain tsO45 G in the ER, other mechanisms are able to retain similar amounts of misfolded protein.

It is worthwhile to mention in this context that VSV infection causes a block in host cell protein synthesis (Dunigan and Lucas-Lenard, 1983). One of the consequences is that a cellular stress response cannot take place. Normally, this response, triggered by the accumulation of misfolded proteins, would result in massive synthesis of additional ER chaperones. In an uninfected cell, the ER can adapt by growing in size and accomodating the increase in misfolded proteins (Pacifici and Iozzo, 1988). Whether the escape of G protein from the ER is promoted by such a handicap unique to VSV-infected cells, or whether it reflects a normal constitutive process, is presently unclear. In any case, our results

show that cells possess a back-up quality control system which prevents transport of the misfolded proteins beyond the CGN.

A recycling pathway between the ER and elements of the Golgi complex has been previously observed in several systems. The proteins that cycle between the ER and the Golgi include the intermediate compartment markers p53 and p58 (for review see Hauri and Schweizer, 1992), soluble ER proteins with KDEL retention signals (Pelham, 1991), recombinant membrane proteins with COOH-terminal retention sequences (Jackson et al., 1993), and the KDEL receptor encoded by the ERD2 and ELP-1 genes (Hsu et al., 1992; Lewis and Pelham, 1992).

The case of tsO45 G protein is somewhat different; it is recycled because it is incompletely folded or misfolded, not because it contains known ER "salvage" signals like the others. The general importance of this retreival mechanism may be that misfolded proteins that have escaped from the ER, continue to be reexposed to the folding machinery in the ER and thus have further chances to fold. Also, being dysfunctional, they are prevented from further transport to the cell surface or other destinations in the cell. That the quality control functions of the secretory pathway extend beyond the ER has previously been suggested by studies on unassembled MHC class I heavy chains (Hsu et al., 1991), and partially assembled T cell receptors (Minami et al., 1987). The latter do not return to the ER but continue to lysosomes where they are degraded.

The mechanism by which the G protein is selectively returned from the CGN to the ER is not known, but we think its apparent association with BiP/GRP78 may be important. Like most lumenal ER proteins, BiP/GRP78 has a COOH-terminal KDEL sequence and is thought to be retreived from the Golgi by the KDEL receptor (Hsu et al., 1992; Lewis and Pelham, 1992). It is not far-fetched to think that the same mechanism might retrieve BiP/GRP78–G protein complexes. One of BiP/GRP78's functions may, in fact, be the selective retreival of misfolded proteins that have escaped from the ER. BiP/GRP78 binds to many such proteins (Haas and Wabl, 1983; Bole et al., 1986; Hurtley et al., 1989; Ng et al., 1989; Machamer et al., 1990), and conditions that compromise the association are known to lead to increased secretion of proteins that would otherwise be retained (Hendershot et al., 1987; Dorner et al., 1988; Suzuki et al., 1991).

Taken together, our results show that retention systems are in place not only in the ER but also in other compartments of the early secretory pathway. Retreival from the CGN serves as a back-up quality control mechanism for misfolded proteins that somehow escape the ER retention systems. Understanding how this sorting works is important because it constitutes part of the process that ensures fidelity of protein expression. In a more practical sense, quality control is a crucial issue in developing more efficient means for the production of recombinant glycoproteins in mammalian cells. It also plays a role in the etiology of diseases with an ER-storage phenotype (Amara et al., 1992).

The authors thank Drs. Jan Simons and Beate Sodiek for critically reading the manuscript, Drs. Aravinda de Silva and Stefano Bonatti for many helpful discussions, and Henry Tan for help with photography.

This study was supported by grants from the National Institutes of Health (5 RO1 GM38346-13 and PO1 CA46128).

Received for publication 10 February 1994 and in revised form 14 April 1994.

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