

# Quality Control in the Endoplasmic Reticulum: Folding and Misfolding of Vesicular Stomatitis Virus G Protein in Cells and In Vitro

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**Abstract.** Parallel experiments in living cells and in vitro were undertaken to characterize the mechanism by which misfolded and unassembled glycoproteins are retained in the ER. A thermoreversible folding mutant of vesicular stomatitis virus (VSV) G protein called ts045 was analyzed. At 39°C, newly synthesized G failed to fold correctly according to several criteria: intrachain disulfide bonds were incomplete; the B<sub>2</sub> epitope was absent; and the protein was associated with immunoglobulin heavy chain binding protein (BiP), a heat shock-related, ER protein. When the temperature was lowered to 32°C, these properties were reversed, and the protein was transported to the cell surface.

Upon the shift up from 32°C back to 39°C, G protein in the ER returned to the misfolded form and was retained, while the protein that had reached a pre-Golgi compartment or beyond was thermostable and remained transport competent. The misfolding reaction could be reconstituted in a cell free system using ts045 virus particles and protein extracts from microsomes. Taken together, the results showed that ER is unique among the organelles of the secretory pathway in containing specific factors capable of misfolding G protein at the nonpermissive temperature and thus participating in its retention.

**T**RANSPORT of newly synthesized secretory proteins and membrane glycoproteins from the ER to the Golgi complex occurs, as a rule, only after they have acquired the correct tertiary and quaternary structure (Hurtley and Helenius, 1989; Klausner, 1989; Rose and Doms, 1988). This may explain why different proteins emerge from ER at widely different rates and efficiencies (Lodish et al., 1983), and why proteins that are misfolded or incompletely assembled into oligomers are not secreted or expressed on the cell surface. The capacity to sort proteins by conformational criteria provides the cell with a quality control system that prevents potential damage caused by the transport of defective gene products. It also allows the cell posttranslationally to regulate the expression of proteins and complex protein assemblies.

To study the principles and mechanisms of protein folding and quality control in the ER, we use viral membrane glycoproteins. In its mature form, the vesicular stomatitis virus (VSV) G protein employed in this study is a noncovalently associated homotrimer (Doms et al., 1987; Kreis and Lodish, 1986). Each subunit (67 kD) has a large ectodomain, a single membrane spanning domain and a short carboxy-terminal cytoplasmic tail (Rose and Gallione, 1981; Rose et al., 1980). The protein is co-translationally translocated into

the ER, and two N-linked sugars are added to the nascent chain (Rothman and Lodish, 1977). In the ER lumen, the ectodomain folds and assembles into transport competent trimers (Doms et al., 1987; Machamer et al., 1990). The half-times for the key events are as follows: synthesis, 1–2 min; folding of the ectodomain, 3–4 min; trimerization, 7–9 min; transport to the *cis*-Golgi compartment, 18–20 min (Balch et al., 1986; Doms et al., 1987; Machamer et al., 1990). G protein is subjected to the same posttranslational controls and processing as cellular glycoproteins. If misfolded, the protein remains in the ER (Doms et al., 1988), usually permanently associated with immunoglobulin heavy chain binding protein (BiP) (Machamer et al., 1990), a heat shock-related ER protein thought to have a role in protein folding, assembly and retention (Pelham, 1989; Rothman, 1989). During folding of the wild-type protein, BiP associates transiently with G protein (Machamer et al., 1990).

We focus here on a temperature sensitive folding mutant of G protein called ts045 (Flammond, 1970), which has a single relevant point mutation in the ectodomain (Gallione and Rose, 1985). At the nonpermissive temperature (39°C), G protein misfolds, forms noncovalently associated aggregates, and remains in the ER (Doms et al., 1987). When the temperature is lowered to 32°C (the permissive temperature), the aggregates disassemble into monomers that proceed to trimerize (Doms et al., 1987). The trimers are transported via the Golgi complex to the plasma membrane where they assemble into viral particles (Doms et al., 1987). If the

1. *Abbreviations used in this paper:* BFA, brefeldin A; immunoglobulin heavy chain BiP, binding protein; NEM, *N*-ethyl maleimide; VSV, vesicular stomatitis virus.

temperature is shifted up to 39°C, G protein in the ER reagggregates (Doms et al., 1987) and is retained (Balch et al., 1986). The protein is thus thermoreversible in both directions making it especially useful for studying quality control.

We demonstrate in this study that the ER is distinct among the organelles of the secretory pathway in having a system for dealing with the misfolded protein. The system contains factors that are capable of actively modifying disulfide bonds and antigenic epitopes, and retaining proteins with incorrect conformations. These changes were reproduced in vitro using G protein from tsO45 virus and a mixture of soluble proteins from microsomes. The parallel study of protein folding in the living cell and in vitro was, thus, possible.

## Materials and Methods

### Reagents, Cell Lines, and Viruses

The CHO cell line clone, 15B, and the Indiana serotype VSV mutant tsO45 were obtained as previously described (Balch et al., 1986). Indiana serotype VSV wild-type virus was obtained from Dr. Mike Whitt (Department of Pathology, Yale University). Trans- $^{35}\text{S}$ methionine was obtained from ICN Biomedicals Inc. (Irvine, CA). Brefeldin A (BFA) was a gift from Dr. Jennifer Lippincott-Schwartz (National Institutes of Health, Bethesda, MD). Endoglycosidase D was purchased from Boehringer-Mannheim Biochemicals (Indianapolis, IN), *N*-ethyl maleimide (NEM) from Sigma Chemical Company (St. Louis, MO) and monensin from Calbiochem-Behring Corp. (San Diego, CA). Monoclonal antibody I-14 was obtained from Dr. Doug Lyles (Bowman Gray School of Medicine, Wake Forest, NC); the polyclonal anti-VSV sera from Dr. Mike Whitt and the anti-BiP antibody from Dr. David Bole (Department of Cell Biology, Yale University). The rat liver subcellular fractions were provided by Dr. Elizabeth Sztul (Yale University, Department of Cell Biology). Cyclosporin A was provided by Dr. R. Hanschmacher (Department of Pharmacology, Yale University).

### Methods

**Labeling and Chase Conditions.** Cells were grown and infected with VSV temperature sensitive mutant tsO45 and labeled with  $^{35}\text{S}$ methionine as previously described in detail (Balch et al., 1986). In brief, a 90% confluent 10-cm dish of CHO clone 15B cells were infected with tsO45 at 32°C for 4–5 h before labeling. Before labeling, cells were washed in media without serum and methionine (labeling medium), and then gently scraped with a rubber policeman and resuspended in 1.5 ml labeling media in a glass tube. Cells were incubated at the indicated temperature (32 or 39°C) in a water bath for 10 min after which 100  $\mu\text{Ci}$  of  $^{35}\text{S}$ methionine was added to the media. Labeling was terminated by the addition of cold methionine to a final concentration of 2.5 mM. During the chase, cells were incubated at the indicated temperature in water baths. At the end of the chase period, the cells were lysed by adding an equal volume of prewarmed lysis buffer containing 40 mM MES, 60 mM Tris, 200 mM NaCl, 2% Triton X-100, 2.5 mM EDTA, 2 mM EGTA, pH 7.4, incubating at the chase temperature for 1 min and then transferring the lysate to ice. To prevent proteolysis, the lysis buffer contained 1 mM PMSF and 10  $\mu\text{g}/\text{ml}$  each of chymostatin, leupeptin, antipain, and pepstatin, from a 10 mg/ml stock in DMSO. In some of the experiments, the lysis buffer contains 20 mM NEM. In experiments where BiP precipitation was carried out, the lysis buffer contained hexokinase 75 U/ml and 15 mM glucose.

### Immunoprecipitations

Lysates were spun in a microfuge for 5 min at 1,500 *g* to pellet nuclei and cell debris. Immunoprecipitations were carried out by incubating with the primary antibody for 30 min at 4°C, and then agitating the immune complex with fixed *Staphylococcus aureus* for an additional 30 min at 4°C. The immunoprecipitate was washed with 100 mM Tris, 0.5 M NaCl, pH 8.0, and prepared for SDS-PAGE and autoradiography as previously described (Balch et al., 1986). The amount of G protein precipitated was quantitated by scanning densitometry. Immunoprecipitations from labeled virus preparations followed essentially the same protocol, except in some of the latter experi-

ments protein A Sepharose instead of fixed *Staphylococcus aureus* was used to precipitate the immune complex.

### Preparation of [ $^{35}\text{S}$ ]Methionine-labeled Virus

A 10-cm confluent dish of BHK-21 cells was infected at 32°C with VSV tsO45. Initially, the virus was bound to the cells in alpha MEM, 10 mM Hepes. After binding for 1 h, the inoculum was removed and GMEM, 5% FCS, 10% tryptose phosphate broth was added to the cells. After 2 h, the cells were washed twice with PBS and 5 ml of labeling media (1% Glasgow MEM, 10 mM Hepes, in MEM without methionine) containing 1 mCi  $^{35}\text{S}$ methionine was added to the cells. The infected cells were labeled for 9 h at 32°C after which the media was removed and cells and debris pelleted. The supernatant was collected and layered over a 0.5-ml sucrose cushion (20% sucrose in 10 mM Tris, pH 7.4, with 0.1% BSA) and centrifuged in an SW 50.1 rotor (Beckman Instruments, Inc., Palo Alto, CA) for 1 h at 115,000 *g*. After removing the supernatant and the sucrose cushion, the virus containing pellet was gently resuspended in 100  $\mu\text{l}$  of 10 mM Tris, pH 7.4. Next, the sample containing virus was loaded onto a 13 ml 20–45% wt/vol sucrose gradient in 10 mM Tris, pH 7.4, and centrifuged at 37,000 rpm in an SW 41 rotor (Beckman Instruments, Inc.) for 46 min. The gradient was fractionated, and the fractions containing labeled virus were pooled, BSA was added to a final concentration of 0.01% and the virus was stored in portions at  $-80^\circ\text{C}$ .

### In Vitro Assay

The ability of total cell lysates, microsomes, and cytosol to convert correctly folded G protein to the misfolded conformation in vitro was tested as follows: the subcellular fraction in 1% Triton X-100, 20 mM MES, 30 mM Tris, 10 mM NaCl, 1.25 mM EDTA, 1 mM EDTA, pH 7.4, (45  $\mu\text{l}$  containing 17  $\mu\text{g}$  protein) was prewarmed to the indicated temperature and 5  $\mu\text{l}$  labeled virus was added to the lysate and incubated for 30 min. The reaction was terminated by transferring samples onto ice. The activity in the lysate was initially assessed by monoclonal antibody I-14 immunoprecipitations and SDS-PAGE under reducing and nonreducing conditions. In later experiments, this activity was quantitated by immunoprecipitating with I-14 and determining the amount of I-14 precipitable G protein by counting the immunoprecipitates in a scintillation counter.

### Preparation of CHO Cytosol and Microsome Fractions

CHO cells grown in suspension were harvested by centrifuging at 600 *g* for 5 min. The cell pellet was washed twice with PBS, and washed once with ICT buffer (78 mM KCl, 50 mM Hepes, 4 mM  $\text{MgCl}_2$ , 10 mM EDTA, 8.37 mM  $\text{CaCl}_2$ ). The cells were resuspended in ICT buffer at a ratio of 1 vol of cell pellet to 5 vol of buffer. Cells were homogenized with a ball bearing homogenizer with 0.0013-in clearance (Balch and Rothman, 1985). Nuclei and cell debris were pelleted by centrifuging at 600 *g* for 15 min. The postnuclear supernatant was layered over a 1-M sucrose cushion (in ICT buffer without KCl) and centrifuged at 200,000 *g* for 1 h. The supernatant (cytosol) and the membrane fraction that accumulated in the sucrose cushion were collected separately and stored in portions at  $-80^\circ\text{C}$ .

## Results

### Monitoring the Conformational Change in tsO45 G Protein

To follow the temperature-induced conformational changes in tsO45 G protein, we analyzed changes in antigenic epitopes and in sulfhydryl groups. The immunochemical approach involved precipitation with a conformation-specific, monoclonal anti-G protein antibody called I-14 that reacts with the B<sub>2</sub> epitope in the ectodomain of G protein (amino acids 357–367) (Lefrancois and Lyles, 1982; Vandepol et al., 1986). In wild-type G, the epitope is generated during a late folding step, but before trimerization (Doms et al., 1988). We found that I-14 could be used to distinguish between correctly and incorrectly folded tsO45 G. As shown in Fig. 1 B, when infected cells were labeled at 39°C, the misfolded

form was precipitated only at a 25% background level. When the temperature was shifted from 39 to 32°C, G protein became fully precipitable with a  $t_{1/2}$  of 1–2 min (Fig. 1 *B*, triangles).

The second assay was based on the observation that in misfolded tsO45 G protein aberrant, interchain disulfide bonds formed when cells were lysed in the absence of alkylating agents. After lysis, only trace amounts of the 39°C form of G migrated as a monomer in SDS-PAGE run under non-reducing conditions (Fig. 2, *Nonred* lane 1) because the bulk of the protein failed to enter the stacking gel. In contrast, the nonreduced 32°C form of G was quantitatively recovered at the position of the monomer band (Fig. 2, *Nonred*, lane 2). If the samples were reduced before electrophoresis (Fig. 2, *Red*, lanes 1 and 2) or if the lysis buffer used to solubilize the cells contained 20 mM NEM (data not shown), G protein was quantitatively recovered at the position of the monomer band regardless of the temperature. These results indicated that misfolded, noncovalently aggregated G protein in the lumen of the ER had free sulfhydryl groups on the surface. These exposed groups were readily oxidized by atmospheric oxygen after lysis to form G protein aggregates linked by interchain disulfides. Similar effects have previously been described in other systems (van Driel et al., 1987; Dahms and Kornfeld, 1989). To confirm the presence of free sulfhydryls on the surface of misfolded G, we alkylated unlabeled immunoprecipitates with <sup>14</sup>C-labeled iodoacetamide and found that the G protein from 39°C cells but not from 32°C was labeled (data not shown).

Next, we determined if misfolded G in the ER was associated with BiP. BiP is a heat shock-related protein associated with many misfolded and unassembled proteins in the lumen of the ER (Bole et al., 1986; Dorner et al., 1987; Gething et al., 1986; Hurtley et al., 1989; Pelham 1988; Sharma et al., 1985). We have previously shown that it binds transiently to wild-type G during folding and permanently to a variety of irreversible G protein mutants (Machamer et al., 1990). As shown in Fig. 3, when immunoprecipitations were carried out on ATP depleted lysates with a monoclonal anti-BiP antibody, misfolded G protein coprecipitated with BiP. Quantitation indicated that 30% of misfolded G was precipitable with anti-BiP. After lysis, the association of BiP with misfolded proteins is labile (Hurtley et al., 1989). Therefore, >30% of the misfolded molecules were probably associated with BiP in the ER lumen. When the temperature was shifted from permissive to nonpermissive, G no longer coprecipitated with BiP (Fig. 3, lane 3). We concluded that BiP was complexed with misfolded G protein and that is dissociated after the temperature shift down.

These results provided convenient assays to monitor the conformational changes in tsO45 G, and they provided us with a more detailed picture of the misfolded form and the transition induced by a temperature shift down. At 39°C, G protein was noncovalently aggregated (Doms et al., 1987), did not express the B<sub>2</sub> epitope, had incomplete disulfide bonds, and was associated with BiP. Upon shift-down to 32°C, the aggregates dissociated into monomers in an ATP dependent reaction (Doms et al., 1987), the peptide segment(s) that carry the B<sub>2</sub> epitope acquired their final three-dimensional configuration, the accessible sulfhydryl groups were oxidized or hidden, and BiP dissociated. These events were rapid, and they occurred while the protein was still in the ER.

### Conformational Changes upon Temperature Shift-up

Using the conformation assays, we examined the effects of a shift from permissive to nonpermissive temperature. Infected cells were labeled at 39°C to synchronize transport, and chased at 32°C for different times before raising the temperature again to 39°C. The shorter chase times were long enough to allow G to undergo the full conformational correction, while the longer chase times allowed, in addition, trimerization and exit of G from the ER. The protein was thereafter analyzed for evidence of misfolding. When cells were lysed after short chase times, aberrant disulfides formed (Fig. 2, compare lanes 2 and 3), and the B<sub>2</sub> epitope was lost (Fig. 1 *A*, lanes 2 and 3). From this, we concluded that G protein could misfold after having first folded correctly. However, with increasing chase time at 32°C, the fraction of G that misfolded during a shift-up decreased (Figs. 1 and 2). The  $t_{1/2}$  for the acquisition of thermostability was 20 min (Fig. 1, *A* and *B*, squares).

The kinetics with which G protein became thermostable coincided with the kinetics of ER to Golgi transport (Balch et al., 1986; Doms et al., 1987). To further examine the connection between stabilization and ER to Golgi transport, we took advantage of a 15°C temperature block that results in accumulation of transported proteins in a compartment located between the ER and the *cis*-Golgi compartment (Saraste and Kuismanen, 1984; Balch et al., 1986). We found that G protein accumulated in this compartment was already thermostable (data not shown), suggesting that to become thermostable the protein had to leave the ER but it did not have to reach the *cis*-Golgi complex.

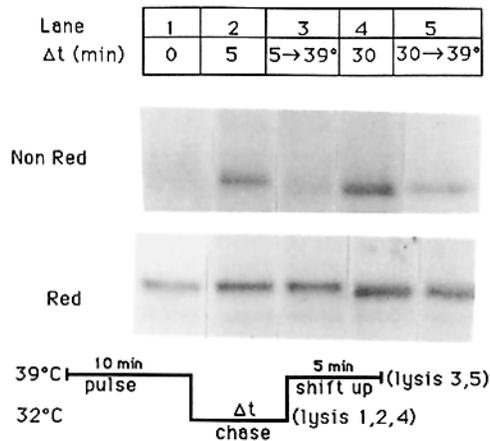
### Experiments with BFA

There were several possible explanations for the thermostability of G upon export from the ER. The protein could undergo further conformational changes and posttranslational modifications, or it could simply be removed from conditions in the ER conducive to misfolding. To test these possibilities, we used BFA, a compound that inhibits the transport of G from the ER to the Golgi complex without interfering with folding and oligomerization (Takatsuki and Tamura, 1985; Doms et al., 1989).

When the temperature was dropped to 32°C for 40 min in the presence of BFA (allowing the ER restricted protein to attain the correct quaternary structure) and shifted up again to 39°C, G protein misfolded. (Fig. 4, *A* and *B*, lane 6). This was in contrast to a prolonged incubation at 32°C in the absence of BFA where the protein became thermostable (Fig. 1 *A*, lane 6, and Fig. 4 *B*, lane 5). Therefore, not only did BFA block ER to Golgi transport, it also blocked the acquisition of thermostability. This indicated that incubation at permissive temperature per se was not the cause for thermostability. Rather, transport of G protein out of the ER was needed to make it thermostable.

To test whether posttranslational modifications after exit from the ER caused the stabilization, we took advantage of the observation that in BFA-treated cells the proteins in the *cis* and medial Golgi are returned to the ER (Doms et al., 1989; Lippincott-Schwartz et al., 1989). BFA thus makes it possible to recall G protein (which has already become thermostable) from the Golgi network to the ER. Cells infected with tsO45 VSV were labeled at 39°C and chased at 32°C in the presence of monensin. This carboxylic ionophore



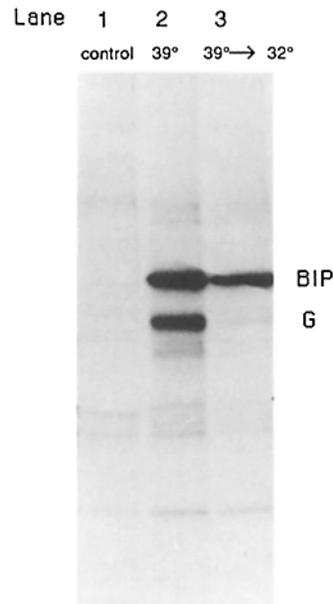


**Figure 2.** Misfolded G protein formed interchain disulfide bonds after lysis. TsO45-infected CHO 15B cells were labeled for 10 min at 39°C and chased at 32°C for 0, 5, and 30 min. At the end of the chase, a fraction of each sample was lysed while the remainder was shifted up again to 39°C for 5 min, and then lysed. The lysis buffer did not contain NEM. The samples were kept at 32°C for 30 min after lysis to facilitate interchain disulfide bond formation and then shifted to ice. Each sample was subjected to SDS-PAGE under reducing and nonreducing conditions. In the lanes from nonreduced gels (*top*) where little or no G protein is present at the expected position, the protein was present as a smear on the stacking gel. Upon reduction (*bottom*), material on the stacking gel disappeared and the protein migrated at the expected position.

blocks the transport of G protein from the medial Golgi compartment to the cell surface (Griffiths et al., 1983) and increases the amount of labeled G protein in the Golgi compartment. Next, BFA was added, and the cells were incubated at 32°C to allow G protein from the medial Golgi network to return to the ER. The temperature was then raised to 39°C, and the protein analyzed for conformation alterations. As shown in Fig. 5 A (lane 3), we found that in cells treated with BFA the B<sub>2</sub> epitope was lost while it was present in cells that had not been treated with the drug (Fig. 5 A, lane 4). Disulfide bonds were also disrupted in the presence of the drug though the difference with and without the drug was not as impressive as in the case of the B<sub>2</sub> epitope (Fig. 5 B, lanes 3 and 4). These results with BFA indicated that posttranslational changes were not responsible for the thermostability. Apparently, G protein became stable simply because it was removed from the ER.

#### Temperature-induced Changes in Mature G Trimers

Although undetected by our conformational assays, at 39°C, subtle conformation change occurs in G protein independent of cellular factors since tsO45 viruses cannot infect cells at 39°C (Keller et al., 1978). Since the key event in virus entry



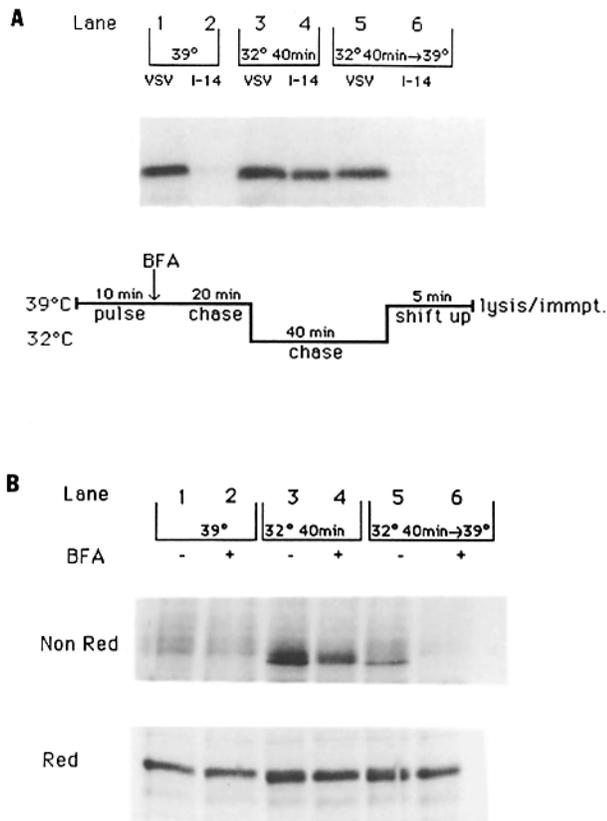
**Figure 3.** BiP associated with misfolded tsO45 G protein. Cells were labeled overnight with [<sup>35</sup>S]methionine, unincorporated radioisotope was removed, and the cells were infected with tsO45 for 4 h. G protein was labeled with [<sup>35</sup>S]methionine for 10 min, and a fraction was shifted to 32°C for 30 min while the remainder was maintained for 30 min at 39°C. Cells were lysed and immunoprecipitations were carried out with a monoclonal antibody against BiP. Lane 1 is a precipitation with a control antibody; lane 2 is a precipitation with anti-BiP antibody from cells maintained at 39°C; and lane 3 is a precipitation with anti-BiP antibody from cells shifted from 39°C to 32°C for 30 min. The positions of BiP and G protein on the gel are indicated.

is a G protein mediated membrane fusion event (White et al., 1983), we examined the fusion activity of tsO45 G protein on the cell surface in a cell-cell fusion assay and found the mutant to be fusion incompetent at 39°C (data not shown). Thus, the conformation of tsO45 G protein was modified at 39°C, but the change was clearly less dramatic than the one observed in the ER since the protein remains a trimer with the B<sub>2</sub> epitope and the correct disulfide bonds (Figs. 1 and 2; Doms et al., 1987). A subtle conformation alteration is probably induced by temperature in all the compartments of the pathway, but only in the ER does it lead to further misfolding. Apparently, the general conditions in the ER or specific resident ER factors drive the protein into the grossly misfolded, aggregated form.

#### Misfolding In Vitro

We next focused our efforts on identifying putative ER factors responsible for the gross misfolding of G. We determined the effects of a temperature shift-up on mature G protein trimers solubilized from [<sup>35</sup>S]methionine labeled tsO45 virus particles in the absence of cellular factors. As expected, we saw no evidence for a gross conformational change using our standard assays (Fig. 6, lane 2). However, when postnuclear cell extracts from CHO 15B cells were added, the full temperature-induced conformational change was observed (Fig. 6, lane 5). G protein was converted to a

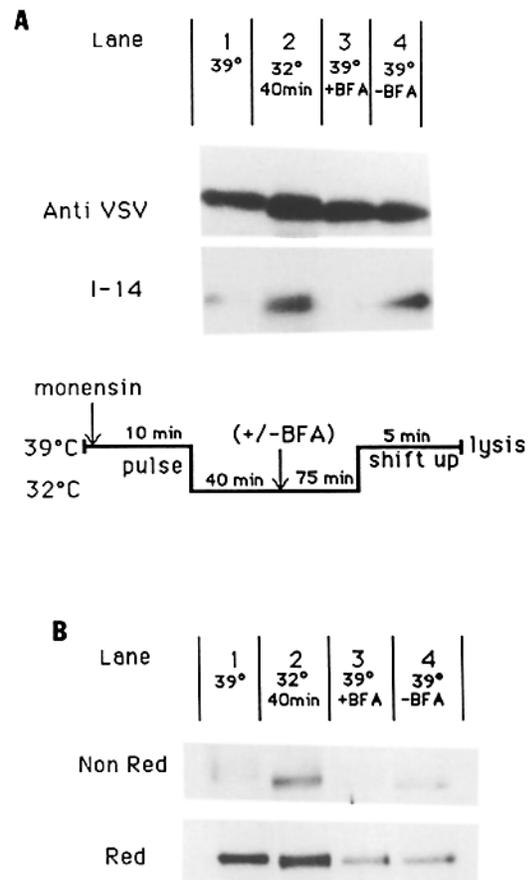
both trimmed and untrimmed forms of the protein (compare lanes 4 and 5). (B) The fraction of G protein precipitated by I-14 after a temperature shift-up (39°C  $\rightarrow$   $\Delta t$  32°C  $\rightarrow$  39°C) was determined by scanning densitometry of gels, and the kinetics with which a thermostable I-14 epitope was acquired is compared to the appearance of the I-14 epitope after a temperature shift-down (39°C  $\rightarrow$   $\Delta t$  32°C). The data for the temperature shift-up curve is from the averaging of three experiments while the curve for the shift-down curve is from the averaging of two experiments. Total G corresponds to the amount precipitated by I-14 after 15 min of chase at 32°C.



**Figure 4.** BFA prevented tsO45 G protein from becoming thermostable. (A) CHO 15B cells infected with tsO45 were labeled for 10 min at 39°C. After the pulse, chase media containing 5  $\mu$ g/ml BFA was added and the temperature was maintained at 39°C for an additional 20 min (lanes 1 and 2). Next, the temperature was dropped to 32°C for 40 min (lanes 3 and 4), and then raised again to 39°C for 5 min (lanes 5 and 6). At each stage of the experiment, samples were collected, lysed, and immunoprecipitated with the anti-VSV polyclonal antibody and I-14 monoclonal. The immunoprecipitates were subjected to SDS-PAGE under reducing conditions. (B) The experiment was carried out as described in (A) except an additional control of cells not treated with BFA was included. Cells were lysed in the absence of NEM and the total lysates were subjected to SDS-PAGE under reducing and nonreducing conditions. In the lanes from nonreduced gels where little or no G protein is present at the expected position, the protein was present as a smear on the stacking gel. Upon reduction, material on the stacking gel disappeared and the protein migrated at the expected position.

form that did not precipitate with monoclonal I-14, and it became cross-linked by aberrant interchain disulfides. Apparently, the cell lysate contained factors that were able to convert the mature tsO45 G from the fusion inactive but relatively intact form to the fully misfolded form normally observed only in the ER. When wild-type virus particles were used in the assay, G protein was stable at 39°C indicating that *in vitro* misfolding was specific to tsO45 (data not shown). While our assays were carried out in the presence of detergent, we found that it was not necessary to have detergent in the assay. Misfolding was also observed when extracts prepared from sonicated microsomes was added to intact tsO45 virus particles (data not shown).

The extent of conversion to the misfolded form in the cell

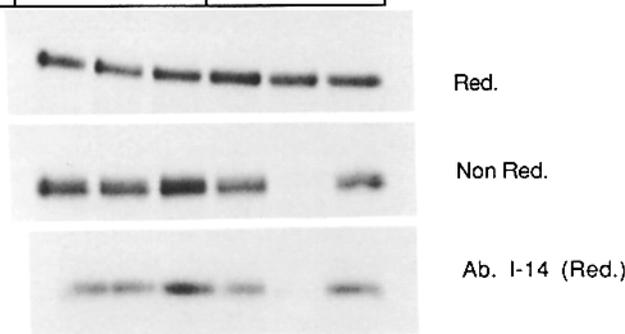


**Figure 5.** TsO45 G protein retrieved from the Golgi body to the ER was misfolded at 39°C. (A) CHO 15B cells were infected with tsO45 VSV and 10 min before the pulse monensin was added to the media at a final concentration of 25  $\mu$ M. Cells were pulsed with [<sup>35</sup>S]methionine for 10 min at 39°C (lane 1) and chased at 32°C for 40 min (lane 2). In the presence of monensin, G protein accumulates in the medial Golgi body at 32°C. Next, the cells were divided into two equal fractions and one was treated with BFA (5  $\mu$ g/ml) while the other was not. After 15 more minutes at 32°C, the monensin was removed by pelleting the cells (600 g for 5 min at 4°C), aspirating the media and adding fresh media with or without BFA. Cells were incubated for an additional 60 min at 32°C (to allow labeled G protein to return to the ER in the BFA-treated sample) and then shifted up to 39°C for 5 min (lanes 3 and 4). At various points in the experiment, cells were lysed and G protein was immunoprecipitated with VSV antisera (*top*) or I-14 monoclonal (*bottom*). (B) The experiment was carried out as described in A, except cells were lysed in the absence of NEM and the total lysates were subjected to SDS-PAGE under reducing and nonreducing conditions. In the lanes from nonreduced gels where little or no G protein is present at the expected position (lanes 1 and 3), the protein was present as a smear on the stacking gel. Upon reduction, material on the stacking gel disappeared and the protein migrated at the expected position.

free mixture could be quantitated by measuring the amount of radioactivity precipitable with I-14 after a 39°C incubation and a 32°C incubation. For optimal sensitivity, the assay was adjusted to a lysate level which gave 60–80% conversion.

Since the active lysate contained a mixture of proteins from all the compartments of the cell, it was important to

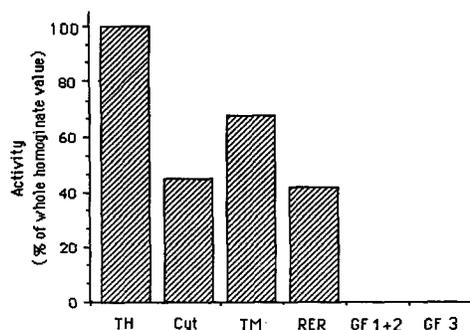
Lane	1	2	3	4	5	6
Lysate		-			+	
Temp. (°C)	32	39	0	32	39	0



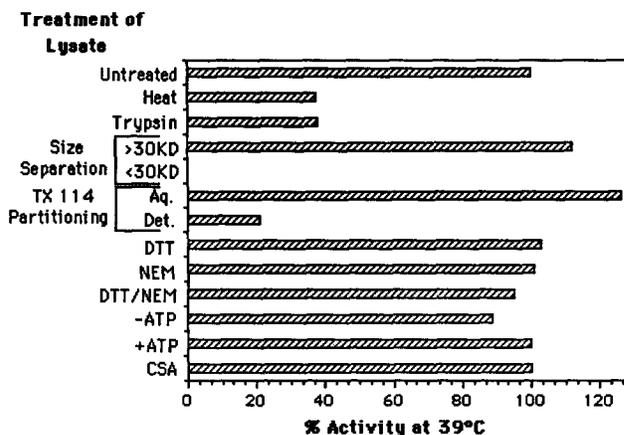
**Figure 6.** Ts045 G protein misfolding could be reconstituted in vitro. [<sup>35</sup>S]Methionine-labeled ts045 virus was incubated in the absence (lanes 1, 2, and 3) or presence (lanes 4, 5, and 6) of a CHO cell detergent lysate (17  $\mu$ g protein) and incubated at 0°C (lanes 3 and 6), 32°C (lanes 1 and 4), and 39°C (lanes 2 and 5) for 30 min (see Materials and Methods). A portion of each sample was immunoprecipitated with monoclonal I-14 and subjected to SDS-PAGE under reducing conditions (*bottom*). The remainder of the lysate was subjected to SDS-PAGE under reducing (*top*) and nonreducing conditions (*middle*) without immunoprecipitating. In the lanes on nonreduced gels where little or no G protein is present at the expected position, the protein was present as a smear on the stacking gel. Upon reduction, material on the stacking gel disappeared and the protein migrated at the expected position.

determine whether the activity was present in the ER. Cell fractions from rat liver were tested. The results in Fig. 7 showed that a total microsomal fraction and a purified ER fraction possessed activity while purified Golgi fractions did not. The cytosol fraction was also found to be active. The results were, nevertheless, consistent with the observation that in the living cell the G protein could only undergo the full conformational change while in the ER.

Some properties of the microsomal activity revealed by our initial characterization are listed in Fig. 8. Heating or trypsin digestion resulted in activity loss, a filter (Amicon Corp., Danvers, MA) with a 30-kD molecular mass cut-off



**Figure 7.** Intracellular distribution of activity mediating the misfolding of ts045 G protein in vitro. Rat liver fractions corresponding to total homogenate (TH), cytosol (Cyt), total microsomes (TM), rough ER enriched (RER), and Golgi-enriched (GF1+2 and GF3) were tested for activity in the in vitro assay. The preparation and characterization of these fractions have been previously described (Sztul et al., 1985). The amount of activity in the different fractions was quantitated by counting the amount of G protein precipitable by I-14. The total activity in the homogenate was determined by using the following formula:  $\{(\text{cpm } 32^\circ\text{C} - \text{cpm } 39^\circ\text{C}) / \text{vol of homogenate tested in assay}\} \times \text{total volume of homogenate}$ . Similarly, the total activity in each of the other fractions is determined, too. In the figure, the total activity in each fraction is expressed as a percentage of the total activity in the homogenate. Typical values for the assay were as follows: when saturating amounts of microsome protein was added to the assay, 3,000 cpm were precipitated by I-14 at 32°C while 300 cpm were precipitated at 39°C.



**Figure 8.** Properties of activity in microsomes mediating the misfolding of ts045 G protein in vitro. The different treatments detailed below were carried out on detergent solubilized CHO total microsomes (17  $\mu$ g protein was used per assay) before using in the in vitro assay as described in Materials and Methods. The amount of activity in each treated lysates was quantitated by counting the amount of G protein precipitable by I-14. The percentage activity was determined using the following formula:  $\{(\text{cpm } 32^\circ\text{C} - \text{cpm } 39^\circ\text{C} \text{ treated}) / (\text{cpm } 32^\circ\text{C} - \text{cpm } 39^\circ\text{C} \text{ untreated})\} \times 100$ . Heat treatment was for 5 min at 100°C. Trypsin treatment was with 100  $\mu$ g/ml for 40 min at 37°C after which 10-fold excess (by weight) of soybean trypsin inhibitor was added to inactivate the trypsin. Size separation was carried out by using a microconcentrator filter (Amicon Corp.) with a 30 kD molecular mass cut-off. Equal volumes of the filtrate and retentate were tested in the assay. Triton X-114 partitioning was carried out as described by Bordier (1981). Volumes of the aqueous and detergent phases were brought up to the same volume and equal volumes were tested in the assay. Solubilized microsomes were pretreated with DTT (0.1 mM for 15 min at room temperature); NEM (1 mM for 5 min at 4°C), or sequentially pretreated first with DTT (0.1 mM for 15 min at room temperature); and next with NEM (1 mM for 5 min at 4°C). Before using in the assay, the free DTT and NEM were separated from microsomal protein by running the treated microsomes over a Sephadex G-25 column and collecting the protein peak in the void volume. ATP depletion was carried out by treating the microsomes with hexokinase (70 U/ml), glucose 20 mM and apyrase (50 U/ml) for 30 min at 37°C. ATP addition consisted of adding back ATP to a final concentration of 5 mM. Cyclosporin A was used at a final concentration of 2  $\mu$ g/ml.

retained the activity. The activity remained in the aqueous phase after a Triton X-114 partitioning. The activity was not inhibited by reduction with DTT, alkylation with NEM or sequential treatment with DTT and NEM. Since protein disulfide isomerase, a soluble ER folding enzyme, is inactivated after sequential reduction and alkylation (Lambert and Freedman, 1984), this enzyme is probably not responsible for the activity. Depletion of the ATP in the extract with hexokinase/glucose and apyrase did not affect the activity, nor did addition of exogenous ATP. The addition of cyclosporin A, an inhibitor of the proline isomerase activity of cyclophilin (Fischer et al., 1989; Takahashi et al., 1989), had no effect making it unlikely that cyclophilin or related molecules may be involved. Taken together, the results indicated that one or more soluble ER proteins larger than 30 kD catalyzed the misfolding. Attempts to purify the proteins are underway.

## Discussion

The folding of newly synthesized polypeptides in the ER occurs during and after vectorial translocation across the membrane. Signal peptide cleavage and glycosylation are frequently needed for proteins to reach their correct and complete three-dimensional structure. Proteins with disulfides, moreover, depend on the action of protein disulfide isomerase, a luminal ER protein, for rapid folding (Anfinsen, 1973; Freedman, 1989). Although direct evidence is still missing, BiP, the ER counterpart of cytosolic hsp70, and other glucose-regulated proteins may also be involved in the folding process (Pelham, 1989; Rothman, 1989). In the case of transmembrane glycoproteins, folding of a single polypeptide chain takes place in three different environments; the lumen, the membrane and the cytosol. Since it is likely to be a complex, facilitated process, the folding of such proteins cannot be studied using standard *in vitro* approaches developed so far (see Jaenicke, 1987).

To investigate cell biological aspects of membrane glycoprotein folding in the ER, we have made use of a thermoreversible folding mutant. Instead of following the entire folding process, we concentrated on the relatively discrete conformational changes induced by temperature shifts. Our results show that this approach can be used in the living cell as well as *in vitro*, and that it provides a system for identifying and characterizing cellular folding factors.

### The Temperature-induced Changes in *tsO45 G*

That *tsO45 G* protein synthesized at nonpermissive temperature did not fold correctly was shown by several criteria: some of its sulfhydryl groups remained reduced and accessible to alkylation, conformation dependent epitopes failed to appear, and BiP did not dissociate as it does after folding of wild-type *G* monomers. The N-linked carbohydrate side chains are, moreover, known to retain an aberrant single glucose as terminal residue (Suh et al., 1989). The protein forms noncovalent aggregates ranging in size from 10 to >50S (Doms et al., 1987; and our unpublished results).

When the temperature was shifted from nonpermissive to permissive, the abnormalities in *G* protein rapidly disappeared. With a half-time of 2 min, the misfolded molecules were converted to correctly folded monomers. These proceeded to trimerize and exit the ER with normal kinetics. The aberrant terminal glucose residues are removed in the

process (Suh et al., 1989), and the bound BiP dissociates. The order in which these changes occurred was not analyzed, but preliminary results suggested that the last event was dissociation of BiP (our unpublished result). Although we have not yet been able to reproduce and study these changes *in vitro*, we have some evidence suggesting that they depend on cellular factors. Not only is the conformation correction dependent on metabolic energy in the form of ATP (Doms et al., 1987), but the intactness of the ER seems to be important (our unpublished results).

The reverse reaction (the misfolding of the *tsO45 G* protein induced by raising the temperature from permissive to nonpermissive) could, however, be studied both in the living cell and *in vitro*. In the ER of the infected cell, a temperature shift-up resulted in the formation of noncovalently associated aggregates indistinguishable from those formed after synthesis at 39°C. Free sulfhydryl groups were exposed, the B<sub>2</sub> epitope was lost, and the protein was retained in the ER. It did not seem to make any difference whether the protein was monomeric or trimeric at the time of shift-up. Thus, the process of aggregate formation and retention is not necessarily linked to synthesis, translocation, and initial folding. It can be activated posttranslationally when a protein misfolds in the ER.

The most interesting feature noted during the shift-up experiments was that the ER was the only compartment within the secretory pathway where the full conformational change that led to misfolding and retention occurred. After reaching the 15°C compartment located between the ER and the *cis*-Golgi network (Saraste and Kuismanen, 1984), the protein no longer underwent the changes. Our experiments with BFA showed that the apparent resistance was not dependent on the time the protein was allowed to fold at 32°C nor on post-translational modifications in post-ER compartment. This was confirmed by the *in vitro* experiments that showed that in the presence of an ER enriched fraction, the conformational change could be reproduced in mature *G* protein derived from virus particles.

### The Folding/Misfolding Factors in the ER

Recent studies on protein folding, assembly, and targeting have resulted in the identification of polypeptides termed chaperonins or polypeptide chain binding proteins that appear to be catalysts of protein folding and quaternary assembly (Pelham, 1989; Rothman, 1989). These proteins belong either to the GroEL family or the hsp70 family (Rothman, 1989). It has been shown that chaperonins in the hsp70 family bind to peptides (Flynn et al., 1989). Rothman (1989) and Pelham (1989) have suggested that the proteins act by successive binding and ATP dependent release from peptide "signals" in the substrate proteins.

While *tsO45 G* protein did not undergo the full conformational change in post-ER compartments at 39°C, we did find evidence for a subtle change in its conformation. The protein was unable to mediate fusion at 39°C. This subtle change may involve the exposure of previously hidden peptide segments, the exposure of hidden disulfide bonds and/or a change in domain positions. It is likely that such a change occurs within the ER as well, and that an ER factor(s) (possibly belonging to the chaperonin family) recognizes the protein as being defective and initiates reactions that lead to the major conformational change and retention. These may be factors

participating in the normal folding and assembly of proteins in the ER, or, alternatively, may be specific factors needed to prevent defective proteins from reaching the cell surface. In other words, it is not clear yet whether the ER proteins involved have as their function to misfold proteins with folding defects, or whether they represent misguided folding factors.

The misfolding activity was associated with soluble proteins and present in the ER and also in the cytosol. Such multi-compartmental distribution is reminiscent of the hsp70 family whose members are distributed in the ER, cytosol, and the nucleus. It was somewhat surprising that heat and trypsin treatment did not completely abolish the activity suggesting that the proteins may be rather resilient. The absence of any effect after ATP depletion or addition argues against BiP and other members of the hsp70 family (Flynn et al., 1989) being responsible for the activity. However, we are not sure if the ATP levels have been lowered below the micromolar range required for the activity of these proteins. Protein disulfide isomerase, another potential candidate for the activity, is probably not involved because the activity was not inactivated by sequential reduction and alkylation (Lambert and Freedman, 1984). The activity was quite stable and easily assayed, and these properties should help in our attempts at further isolation and identification.

### Mechanism of Retention

The reasons for the retention of tsO45 G protein in the ER at nonpermissive temperature are not known, but our results and the observations of Kreis and Lodish (1986) may provide a clue. Kreis and Lodish found that when monoclonal antibodies to the tsO45 G cytoplasmic tail were injected into the cytosol of infected cells, patching of G protein occurred only at the permissive temperature. The interpretation they offered was that the protein was monomeric at nonpermissive temperature and therefore not cross-linkable to complexes larger than dimers by bivalent monoclonal antibodies. Since we now know that the protein, in fact, forms noncovalently associated aggregates (and hence is multivalent) at the nonpermissive temperature, another explanation has to be found. The inability to form immunocomplexes may mean that misfolded G proteins are not mobile laterally in the plane of the ER membrane. The lack of lateral mobility would not only account for the lack of lateral redistribution, but it could explain why G protein cannot exit the ER. Movement of the protein to areas of the ER such as the transitional elements, or other sites where transport vesicles are formed, may be needed for transport to the Golgi complex.

Immobilization of misfolded G protein aggregates could be imposed by a meshwork of interacting resident proteins within the lumen and the membrane of the rough ER (Hortsch et al., 1987). Such an "ER skeleton" could restrict diffusion above a critical aggregate size, or it could fix the misfolded proteins by direct associations with the misfolded proteins or with the bound BiP. Evidence that BiP may have a role in the retention of misfolded proteins has been recently obtained (Dorner et al., 1988). While BiP itself is thought to diffuse freely within the ER (Ceriotti and Colman, 1988), it may not be mobile when associated with misfolded G proteins. The current thinking regarding the mechanisms for BiP's retention in the ER involves transport to a "salvage" compartment located between the ER and the Golgi network

where a receptor recognizes the KDEL tetrapeptide sequence at the COOH terminus of BiP and recycles the protein back to the ER (Pelham, 1989). If misfolded proteins are, indeed, fixed in the ER membrane their retention is unlikely to depend on this system because they would be unable to reach the salvage compartment.

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