

# Differential Effects of Mutations in Three Domains on Folding, Quaternary Structure, and Intracellular Transport of Vesicular Stomatitis Virus G Protein

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**Abstract.** The vesicular stomatitis virus glycoprotein (G protein) is an integral membrane protein which assembles into noncovalently associated trimers before transport from the endoplasmic reticulum. In this study we have examined the folding and oligomeric assembly of twelve mutant G proteins with alterations in the cytoplasmic, transmembrane, or ectodomains. Through the use of conformation-specific antibodies, we found that newly synthesized G protein folded into a conformation similar to the mature form within 1–3 min of synthesis and before trimer formation. Mutant proteins not capable of undergoing correct initial folding did not trimerize, were not transported, and were found in large aggregates. They had, as a rule, muta-

tions in the ectodomain, including several with altered glycosylation patterns. In contrast, mutations in the cytoplasmic domain generally had little effect on folding and trimerization. These mutant proteins, whose ectodomains were identical to the wild-type by several assays, were either transported to the cell surface slowly or not at all. We concluded that while correct ectodomain folding and trimer formation are prerequisites for transport, they alone are not sufficient. The results suggest that the cytoplasmic domain of the wild-type protein may facilitate rapid, efficient transport from the ER, which can be easily affected or eliminated by tail mutations that do not detectably affect the ectodomain.

**V**IRAL spike glycoproteins have often been used as model proteins to study transport in the secretory pathway. The most commonly used are the vesicular stomatitis virus (VSV) G protein and the influenza hemagglutinin (HA)<sup>1</sup>. Both are initially synthesized on membrane-bound polysomes and are cotranslationally inserted into the endoplasmic reticulum (ER) membrane (Rothman and Lodish, 1977). The newly synthesized monomers assemble into noncovalently associated trimers in the ER with a half-time of ~7–10 min after synthesis (Copeland et al., 1986; Gething et al., 1986; Kreis and Lodish, 1986; Doms et al., 1987). Only trimers are detected in the Golgi apparatus, suggesting that trimerization might be a prerequisite for transport from the ER (Doms et al., 1987; Copeland et al., 1988). Misfolded or unassembled forms of VSV G protein or influenza HA are retained in the ER, as are a number of other cellular membrane and secretory proteins (Peters et al., 1985; Machamer et al., 1985; Gething et al., 1986; Copeland et al., 1986; Bole et al., 1986; Doms et al., 1987). These findings have raised the possibility that folding and the correct quaternary structure play a role in directing protein transport. In fact, they could help explain why different proteins are transported out of the ER with different rates and efficiencies.

1. *Abbreviations used in this paper:* ER, endoplasmic reticulum; HA, hemagglutinin; TX100, Triton X-100; wt, wild-type.

With the availability of a reliable assay to quantitatively determine the oligomeric state of VSV G protein (Doms et al., 1987), it is now possible to address the relationship between folding, oligomerization, and transport in more detail. In this study we have examined 12 VSV G proteins with mutations in the ectodomain, transmembrane domain, or cytoplasmic domain. The mutant proteins display a variety of transport phenotypes, ranging from complete block in the ER to near normal transport to the cell surface. Three general observations emerged. (a) Mutations in the cytoplasmic domain did not generally affect folding of the ectodomain or subsequent trimerization, but they did slow down or prevent exit from the ER. (b) Mutations in the ectodomain blocked initial folding and therefore trimerization and transport. These mutant proteins accumulated in the ER in the form of aggregates. (c) Although rather tolerant to changes in the transmembrane domain, the G protein's folding and trimerization was affected by a drastic shortening of the transmembrane sequence.

## Materials and Methods

### Cell Lines and Viruses

COS-1 cells were maintained in DME supplemented with 5% FCS as described (Rose and Bergmann, 1982). The Chinese hamster ovary (CHO) cell line clone 15B, which lacks the Golgi-associated carbohydrate-pro-

cessing enzyme *N*-acetylglucosamine transferase I, was grown as described, as were infectious stocks of VSV Indiana and ts O45 (Balch et al., 1986).

### Transfection of COS Cells

COS cells were transfected with the appropriate plasmid DNA essentially as described previously (Adams and Rose, 1985a). Briefly, 10 µg of plasmid DNA with 0.5 mg DEAE-dextran in 1 ml of PBS was added to subconfluent monolayers of COS cells on 5-cm dishes. After 30 min, the DNA was removed and the cells were placed in 1 ml of DME containing 5% FCS and 100 µM chloroquine for 3 h at 37°C. The cells were then placed in normal growth medium for 40 h. The mutant genes have all been described previously (see Table I) except for TMS. In this gene, the codon for the first arginine in the cytoplasmic domain of G protein was replaced by a stop codon. As a result, the resulting G protein lacks the cytoplasmic domain altogether (Pitta, A., and J. K. Rose, unpublished observations).

### Trimerization Assay

The quaternary structure of the wild-type (wt) and mutant G proteins was determined by sucrose density gradient centrifugation as described (Doms et al., 1987). Cells were labeled with 50–100 µCi [<sup>35</sup>S]methionine in methionine-free medium for 5 min to 2 h as indicated in the results. The cells were then washed with PBS and placed in normal growth medium containing 2.5 mM cold methionine for the indicated time. Cells were lysed with 1% Triton X-100 (TX100) in 20 mM MES, 30 mM Tris, 100 mM NaCl (MNT buffer), pH 5.8, unless otherwise indicated. The lysates were placed on ice and spun in an Eppendorf microfuge for 1 min at high speed (10,000 g). 200-µl aliquots were loaded onto 5-ml continuous 5–20% (wt/vol) sucrose gradients in MNT buffer with 0.1% TX100. The gradients were spun in SW55 or 50.1 rotors (Beckman Instruments, Inc., Palo Alto, CA) for 16 h, 47,000 rpm at 4°C. Fractions were collected from the bottom and the G protein in each was immunoprecipitated as described below. The precursor form of HA (HAO) was purified and used as a 9-S sedimentation marker (Doms and Helenius, 1986).

### Antibodies

Well-characterized mouse mAbs to VSV G protein were generously provided by Leo Lefrancois and Douglas Lyles. Their production and characterization are described in Lefrancois and Lyles (1982a, b, 1983) and in Vandepol et al. (1986). The rabbit polyclonal serum against VSV was raised by three sequential intradermal injections of UV-inactivated VSV in complete Freund's adjuvant.

### Immunoprecipitations

After fractionation of sucrose gradients, 0.5 ml of detergent solution (1% NP-40, 0.4% sodium deoxycholate, 0.3% SDS, 12.5 mM EDTA, 50 mM Tris, pH 8.0) was added to each 350-µl gradient fraction. A rabbit polyclonal serum against G protein was then added (3 µl) and the solution was incubated for 30 min at 37°C. Fixed *Staphylococcus aureus* (50 µl of a 10% slurry) was then added for 30 min at room temperature. The resulting immune complexes were washed twice with RIPA buffer containing 1% NP-40, 1% DOC, 0.1% SDS, 150 mM NaCl, and 10 mM Tris, pH 7.4. The pellet was resuspended in 25 µl of 10 mM Tris, pH 6.8, and solubilized in 2× sample buffer for SDS-PAGE. Immunoprecipitations from cell lysates followed essentially the same protocol.

### pH Assay

We have previously shown that VSV G protein trimers acquire stability to ultracentrifugation with a characteristic pH dependence (Doms et al., 1987). To determine the pH dependence with which mutant G protein trimers underwent this stabilizing conformational change, transfected COS cells were metabolically labeled with [<sup>35</sup>S]methionine, as above, and then incubated in excess cold methionine for 30–120 min as indicated. The cells were lysed at the indicated pH (5.8–7.4) and the lysate centrifuged on continuous sucrose gradients at the same pH. Centrifugation conditions and immunoprecipitations were the same as described above.

### Results

To determine the rate and efficiency of trimerization, we expressed wt and mutant G proteins in COS cells by transfection

with the appropriate plasmid DNA (Rose and Bergmann, 1982). The cells were pulsed with [<sup>35</sup>S]methionine and chased in the presence of excess cold methionine for various times. TX100 lysates were subjected to sucrose density gradient centrifugation to separate trimers from monomers, and the fractions from the gradients were immunoprecipitated with polyclonal anti-G antibodies. The precipitates were analyzed by SDS-PAGE followed by fluorography, and when necessary the amount of radiolabeled G protein was quantitated by scanning densitometry of the autoradiograms.

We have previously shown that mature VSV G protein consists of a noncovalently associated homotrimer that sediments at 8 S<sub>20,w</sub> (Doms et al., 1987). When extracted from the membrane with detergent, it is stable to sucrose density gradient centrifugation in the presence of TX100 provided that the pH is <6.3. At higher pH, it completely dissociates to 4 S<sub>20,w</sub> monomers on the gradient. The acid stabilization of G protein trimers corresponds to a conformational change related to its acid-induced membrane fusion activity (White et al., 1983; Doms et al., 1987).

### Trimerization of wt VSV G Protein in COS Cells

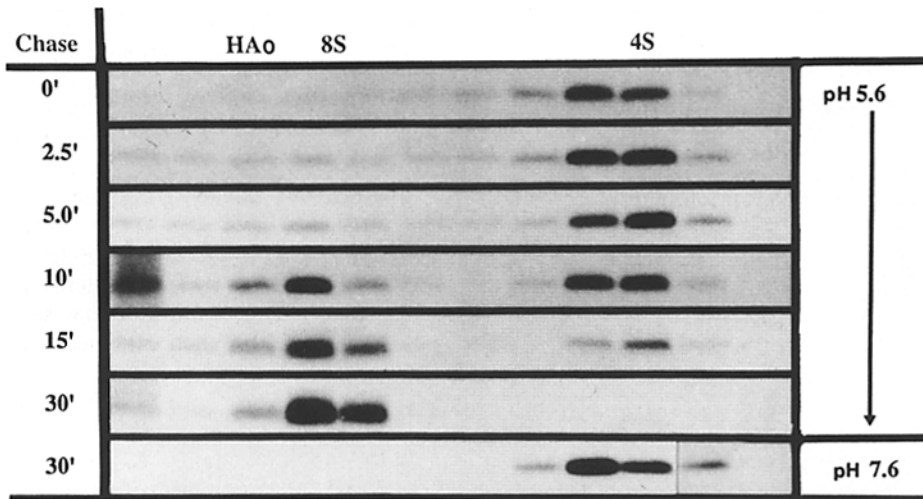
As shown in Fig. 1, newly synthesized wt G protein shifted quantitatively from the monomer peak to the trimer peak with a half-time of ~15 min (this estimate includes the 10-min chase plus half of the pulse time). The process of trimerization in the transfected COS cells was thus very similar to that previously characterized for VSV-infected CHO cells (Doms et al., 1987). In agreement with the CHO results, it was also found that the trimers were formed before the acquisition of endoglycosidase H resistance (not shown), and that they dissociated to monomers when centrifuged at pH 7.4 (Fig. 1). The only significant difference observed was the slightly slower rate of trimerization in the COS cells, which could be due to a difference in cell type or by a lower level of G expression. In fact, the rate at which wt trimers formed in HeLa cells more closely approximated the trimerization kinetics previously described in CHO cells (not shown).

### The Panel of Mutants

G protein has three topological domains: (a) the NH<sub>2</sub>-terminal ectodomain (462 amino acids), which projects into the ER lumen and contains both the N-linked carbohydrates; (b) the hydrophobic transmembrane domain (20 amino acids); and (c) the COOH-terminal cytoplasmic domain (29 amino acids). Each of the mutant proteins used in this study had point mutations, deletions, or insertions in one of these domains. Their transport phenotypes have been previously described and found to range from near normal transport to the cell surface to partial or complete inhibition (Rose and Bergmann, 1983; Adams and Rose, 1985b; Gabel and Bergmann, 1985; Gallione and Rose, 1985; Puddington et al., 1986; Scullion et al., 1987; Machamer and Rose, 1988a, b). Table I contains a summary of the mutants, the amino acid sequence changes, and their transport phenotypes.

### Mutant Proteins with Alterations in the Cytoplasmic Domain

The G proteins with modified cytoplasmic COOH-terminal domains were first screened for their ability to form trimers. Transfected COS cells were labeled for 20 min, followed by



**Figure 1.** Kinetics of wt VSV G trimerization. Transfected COS cells were labeled with [<sup>35</sup>S]methionine for 10 min and then chased for various times before lysis in TX100. Aliquots of the cell lysate were applied to pH 5.8 continuous sucrose density gradients and centrifuged for 16 h at 47,000 rpm in an SW 50.1 rotor. Fractions were collected and the G protein in each was immunoprecipitated and analyzed by SDS-PAGE and fluorography. The region of each fluorograph showing the G protein precipitated across each gradient is shown. The bottom of the gradient is to the left, and the 8-S and 4-S positions (determined from sedimentation standards as described in Doms and Helenius, 1986) are indicated.

a 0-, 30-, or 120-min chase. Cells were lysed in a pH 5.8 buffer and the quaternary structure of the labeled G protein determined. The 120-min samples were also sedimented on pH 7.4 gradients to determine whether the trimers would display normal dissociation properties.

All of the mutant G proteins (G $\mu$ , 1473, TMR, TMS, and GHA) were found to form trimers with high efficiency (Figs. 2 and 3). In addition, four of the mutants trimerized more

quickly than the wt (Fig. 3). In HeLa cells, however, only a slight difference in trimerization kinetics was seen between the mutants and wt (not shown). Only the TMS mutant appeared to form trimers more slowly than wt G protein. At early times of chase, significant fractions of this protein pelleted to the bottom of the centrifuge. Because of variable recovery of the aggregate, we assessed the fraction of G protein sedimenting to the bottom of the tube by routinely im-

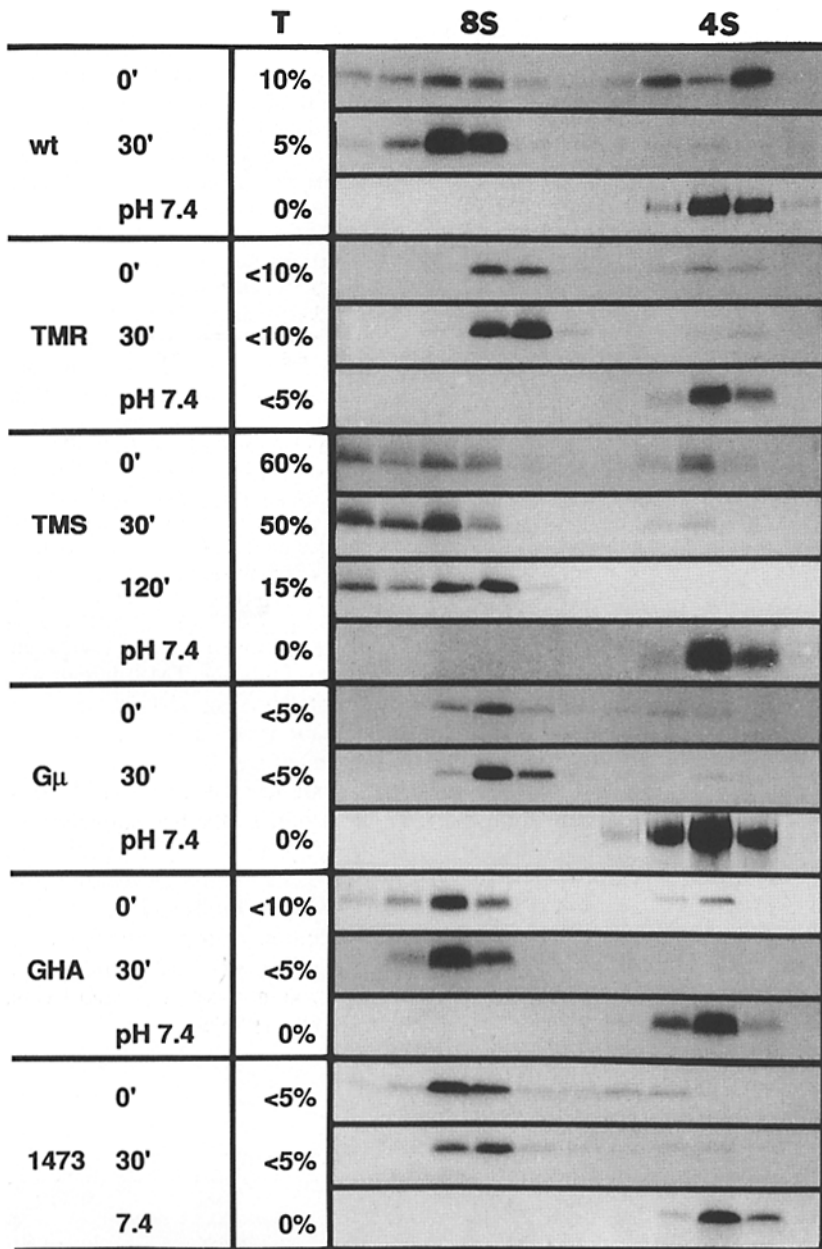
**Table I.** G Protein Mutants Used: Sequence Changes and Transport Phenotypes

	Sequence	% Resistant to endoglycosidase H			References				
		30 min	60 min	120 min					
<b>Cytoplasmic</b>									
wt	RVGIHLCEIKLKHTTKRQIYTDIEMNRLGK	80	100	100	1-5				
TMR	R	0	44	65	6				
TMS	-	0	≤5	≤10	7				
G $\mu$	KVK	15	35	60	3				
GHA	RGNIRCNICI	15	50	75	3				
1473	PSRDRSRHDKIH	0	0	0	1				
<b>Transmembrane</b>									
wt	...KSSIASFFFIIIGLIIGLFLVLR...	80	100	100	1-5				
TM14	...KSSIASFFFII-----FLVLR...	ND	100	100	4				
TM12	...KSSIASFFFI-----LVLR...	ND	0	30*	4				
<b>Ectodomain</b>									
		Glycosylation Sites							
wt	-	179	-	336	-	80	100	100	1-5
tsO45 32°C	-	179	-	336	-	80	100	100	2
tsO45 40°C	-	179	-	336	-	0	0	0	2
QN1	117	179	-	336	-	30	70	90	5
QN1/TA1,2	117	-	-	-	-	30	30	40	5
EN2/TA1,2	-	-	217	-	-	10	10	10	5
VT	-	179	-	336	387	0	0	0	5

Reference numbers cited in table correspond to the following references:

- (1) Rose and Bergmann, 1983.
- (2) Gallione and Rose, 1985.
- (3) Puddington et al., 1986.
- (4) Adams and Rose, 1985b.
- (5) Machamer and Rose, 1988a, b.
- (6) Scullion et al., 1987.
- (7) Pitta and Rose, unpublished data.

\*, at 3 h.



**Figure 2.** Oligomeric structure of G protein cytoplasmic tail mutants. COS cells expressing wt, TMR, TMS, G $\mu$ , GHA, or 1473 were pulse labeled with methionine for 20 min and then chased in cold medium for 0, 30, or 120 min before lysis in TX100 at pH 5.8. The lysates were applied to pH 5.8 and pH 7.4 sucrose density gradients. The gradients were fractionated and the G protein in each was immunoprecipitated and analyzed by SDS-PAGE and fluorography. The 0- and 30-min chase points for each protein are shown, as is the 30-min chase point when centrifuged on a pH 7.4 sucrose gradient. The average amount of G protein that pelleted to the bottom of the tube is also shown. This was determined both by quantitation of the G protein actually immunoprecipitated from the pellet, as well as by monitoring the recovery of the G protein from the gradient by comparing the amount immunoprecipitated from the fractions with the amount precipitated from an aliquot of the cell lysate before centrifugation. The bottom fraction is to the left, and the 8S and 4S positions are shown.

munoprecipitating an aliquot of the cell lysate before centrifugation, allowing us to monitor recovery of G protein from the gradient. Recoveries ranged from 80–100% except for TMS, the missing fraction of which was frequently recovered from the bottom of the tube. The average amount of G protein in the pellet from several experiments is shown in Fig. 2.

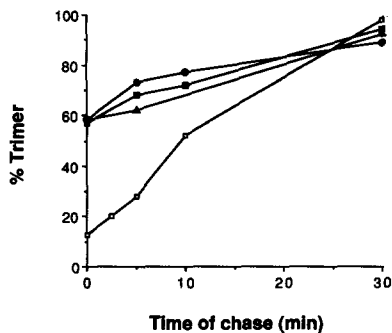
While the mutants were thus trimerization competent, none of them have a normal transport phenotype (Fig. 2). Mutant 1473 is completely inhibited in transport, only 10% of TMS is transported after 2 h, and G $\mu$ , TMR, and GHA are transported efficiently but four- to eightfold more slowly than wt G protein (Puddington et al., 1986). The results showed that trimerization alone is not sufficient for transport. Nor was trimer formation kinetically linked with transport. In fact, there was no apparent correlation between the

rate at which a mutant protein trimerized and the rate at which it is transported (Fig. 3).

#### **Conformation of the Cytoplasmic Domain Mutants**

The transport defect in the cytoplasmic domain mutants could, generally speaking, have two different causes: either the alterations prevented some interaction involving the cytoplasmic domain itself, or the defects could somehow affect the rest of the protein, rendering it incapable of transport. To evaluate these possibilities we examined the conformation of the mutant trimers.

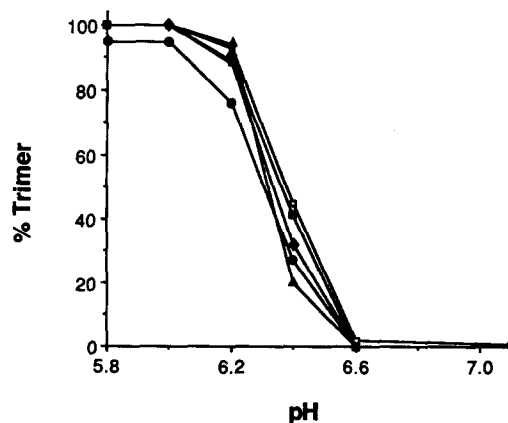
The mature, trimeric wt G protein catalyzes membrane fusion at pH values <6.2 (Florkiewicz and Rose, 1984; Riedel et al., 1984). Fusion activity is accompanied by a characteristic increase in the stability of the trimer (Doms et al., 1987). Studies with fusion proteins from Influenza A and



**Figure 3.** Kinetics of wt and mutant VSV G trimerization. COS cells transfected with the plasmids encoding wt, TMR, GHA, G $\mu$ , or 1473 were pulse labeled for 10 min and then chased for various times. The cells were lysed with TX100 at pH 5.8 and subjected to density gradient centrifugation to separate trimeric from monomeric G protein. The fraction of the G protein sedimenting at the 8-S position was determined by scanning densitometry of fluorograms. The results are the average of 2–3 experiments.  $\square$ , wt;  $\blacktriangle$ , GHA;  $\blacksquare$ , G $\mu$ ;  $\bullet$ , 1473.

other viruses have shown that the pH threshold of the corresponding conformational change is characteristic to each strain of virus and, more importantly, quite sensitive to subtle changes in the ectodomain structure (Daniels et al., 1985; Doms et al., 1986). We determined the pH dependence of G trimer stabilization during gradient centrifugation in the presence of TX100. It was found that TMR, GHA, G $\mu$ , and 1473 demonstrated a pH dependence of trimer stabilization nearly identical to wt G trimers (Fig. 4). This suggested that those regions of the molecule involved in the pH-activated conformational change were correctly folded.

We next compared wt and mutant G trimers using a panel of conformation-specific mAbs. The rationale for this approach was the observation that mAbs selected for specificity



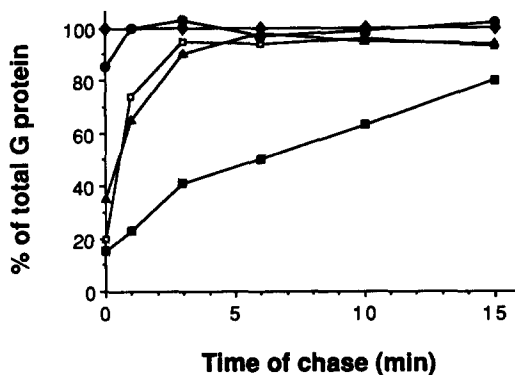
**Figure 4.** pH dependence of the conformational change. Individual dishes of COS cells were transfected with the appropriate plasmid. 48 h after transfection, the cells were labeled for 1 h, chased for 1 h, and then lysed with 1% TX100 in MNT buffer at the indicated pH. The lysate was then centrifuged on a 5–20% (wt/vol) continuous density sucrose gradient that was at the same pH as the lysate. The gradients were fractionated, the G protein in each fraction was immunoprecipitated with a polyclonal rabbit serum, and the amount of G protein in each was determined by SDS-PAGE and scanning densitometry of fluorographs. The results are the average of 2–4 experiments.  $\square$ , wt;  $\blacktriangle$ , GHA;  $\blacklozenge$ , TMR;  $\blacksquare$ , G $\mu$ ;  $\bullet$ , 1473.

to mature proteins frequently fail to react with misfolded or misassembled forms of the antigen (see Copeland et al., 1986; Gething et al., 1986). A panel of six mAbs directed against major antigenic epitopes on the mature VSV G protein was kindly provided by Douglas Lyles and Leo Lefrancois (Table II). With the exception of monoclonal II7, all were neutralizing and thus reactive with native epitopes in the ectodomain of mature G protein (Lefrancois and Lyles, 1982a, b, 1983; Vandepol et al., 1986).

**Table II. Immunoprecipitation of wt and Mutant G Proteins with Polyclonal and Monoclonal Antibodies**

Antibody epitope	RAB	I1	I14	I17	IN2	IN5	IN6
	all	A <sub>1</sub>	B <sub>2</sub>	NA	Ind/NJ	Ind/NJ	Ind/NJ
Protein							
wt trimer	+++	+++	+++	++	++	++	++
wt monomer	+++	+++	+++	++	++	++	++
tsO45 32°C	+++	+++	+++	++	++	++	++
tsO45 40°C	++	+	+	++	+	+	+
TMR	+++	+++	+++	++	++	++	++
TMS	+++	++	++	++	++	++	+
G $\mu$	+++	+++	+++	++	++	++	++
GHA	+++	+++	+++	++	++	++	++
1473	+++	+++	+++	++	++	++	++
TM14	+++	+++	+++	++	++	++	++
TM12	+++	–	–	+++	–	–	–
QN1	+++	+++	+++	++	++	++	++
QN1/TA1,2	+++	++	++	++	++	++	++
EN2/TA1,2	+++	+	+	+++	–	+	+
VT	+++	–	–	+++	–	–	–

Cells were labeled for 60 min and then chased in complete medium for 60 min before lysis at pH 7.4. Aliquots of the lysate were immunoprecipitated with the indicated polyclonal and monoclonal antibodies. The amount precipitated was quantitated by scanning densitometry and expressed relative to the amount precipitated by the rabbit serum. +++,  $\geq 75\%$  of that seen by the polyclonal; ++,  $\geq 25\%$ ; +,  $\geq 10\%$ ; and –,  $< 10\%$ . Cells infected with tsO45 were labeled at 40°C for 10 min and then chased at 40°C for 5 min. The cells were then lysed at 40°C or transferred to 32°C for 20 min before lysis. The rabbit serum (RAB) immunoprecipitated all of the mutants to the same extent except for tsO45 at the nonpermissive temperature.



**Figure 5.** Acquisition of antigenic epitopes. CHO clone 15B cells were infected with VSV. 4 h after infection, the cells were placed in methionine-free medium for 5 min, then labeled with [<sup>35</sup>S]methionine for 3 min. The cells were chased in complete medium containing excess cold methionine for the indicated times before lysis with TX100 at pH 7.4. The total amount of G protein at each time point was determined by TCA precipitation, and the amount precipitated by the mAbs II, I13, and I17 was expressed relative to the total. The fraction of G protein in trimeric form was determined by centrifugation as described in Fig. 1. □, II; ▲, I14; ●, I17; ◆, total G; ■, % trimer.

To determine whether the monoclonals were conformation specific, we analyzed their ability to immunoprecipitate different forms of G protein. The monoclonals were all found to precipitate trimers as well as monomers obtained by dissociating trimers in neutral pH velocity gradients (Table II). They were thus unable to distinguish between trimers and artificially generated monomers, suggesting that gradient-induced dissociation did not lead to significant changes in the major antigenic epitopes.

Several of the monoclonals were, however, found to react selectively with different ER forms of G protein. In pulse-chase experiments, monoclonals II and I14 failed to precipitate G protein immediately after a 3-min labeling period, but acquired the ability to do so within a 1–3-min chase (Fig. 5). The newly synthesized monomer presumably folded during the chase period, and stable epitopes were expressed. In contrast, monoclonal I17 precipitated G protein efficiently already at 0 min of chase, suggesting that it reacted with an epitope that was either conformation independent or very rapidly generated during G protein folding (Fig. 5). It is noteworthy that the polyclonal anti-G antibodies also displayed slightly lower activity against newly synthesized G protein; this is not an uncommon observation with antisera prepared against native antigens.

The antibodies were also tested for their ability to precipitate a misfolded form of G protein encoded by the temperature-sensitive, thermo-reversible point mutant tsO45. At the nonpermissive temperature (40°C), tsO45 G aggregates in the ER immediately after synthesis (Doms et al., 1987). At permissive temperature (32°C, or after a shift from nonpermissive to permissive temperature) all the G protein monomerizes, forms normal trimers with wt kinetics, and is transported to the cell surface (Balch and Keller, 1986; Doms et al., 1987). As shown in Table II and Fig. 6, it was found that five of the monoclonals recognized trimeric tsO45 G protein in cells grown at permissive temperature (32°C), but showed low affinity for the misfolded G protein present at nonper-

missive temperature. The difference in specificity was best illustrated by monoclonal II, which immunoprecipitated 85% of the total G protein synthesized at permissive temperature but only 10% of G protein synthesized at nonpermissive temperature. This is consistent with the observation that these antibodies stain the ER of cells expressing tsO45 at 40°C very weakly. However, after the cells had been shifted to 32°C, the antibodies give a strong Golgi-like staining pattern consistent with their ability to precipitate correctly folded trimers more efficiently than misfolded forms (not shown). Monoclonal I17 differed, once again, from the other monoclonals in precipitating both forms equally well.

When tested against the proteins with mutated cytoplasmic domains, we found that none of the antibodies detected qualitative or quantitative differences between mutant trimers G $\mu$ , I473, GHA, and TMR and the wt trimers (Table II). Together with the correct functional response of the mutant proteins to acidic pH, the antigenic integrity of their ectodomains argued that the mutations in the cytoplasmic moiety had little effect on the general folding or oligomeric structure of the G protein. The mutant protein TMS, in which the entire cytoplasmic domain is lacking, was somewhat different. It trimerized more slowly than wt protein and gave an intermediate reactivity with the conformation-specific antibodies (Table II).

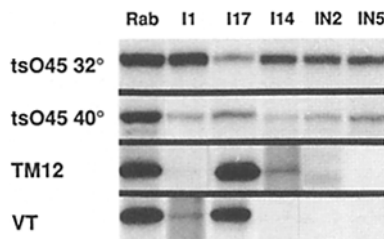
### G Proteins with Altered Transmembrane Domains

The VSV G transmembrane domain is 20 residues long with charged amino acids on both sides. In an earlier study, Adams and Rose (1985b) showed that when the hydrophobic region is shortened by site-directed mutagenesis to 14 residues, the G protein (TM14) assumes a normal transmembrane configuration and is transported to the cell surface normally (Table I). However, when the membrane-spanning domain is shortened to 12 residues,  $\leq 30\%$  of the protein (TM12) acquires endoglycosidase H resistance after a 3-h chase.

We analyzed the quaternary structure and antigenic properties of TM14 and TM12 at various times after synthesis. TM14 was normal in terms of trimer formation and the trimers showed normal pH sensitivity (Fig. 7). It was also antigenically indistinguishable from the wt protein (Table II). By contrast, TM12 sedimented as a heterogeneous aggregate after 0, 30, and 60 min of chase (Fig. 7). When compared to the wt molecule by immunoprecipitation with the panel of conformation-specific mAbs, we found that only the polyclonal rabbit serum and I17 were able to precipitate TM12 G protein efficiently (Table II and Fig. 6). Weak reactivity was observed with II and I14. We concluded that TM14, which was efficiently transported, was normal with respect to folding and trimerization, whereas TM12 was incorrectly folded, aggregated, and hence failed to trimerize and exit the ER after 60 min of chase.

### G Proteins with Mutations in the Ectodomain

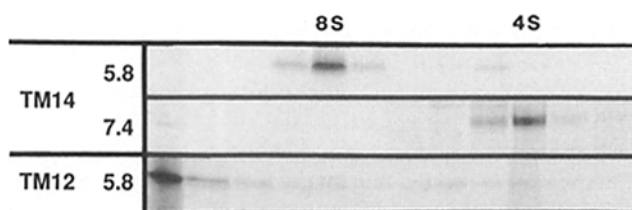
A number of G protein mutants with changes in the ectodomain have been described (Gallione and Rose, 1985; Machamer et al., 1985; Machamer and Rose, 1988a, b). The best studied is the temperature-sensitive mutant tsO45, which differs at 5 positions from its parent wt. One of the amino acid changes, Ser for Phe at position 204, has been identified as the ts lesion (Gallione and Rose, 1985). As mentioned



**Figure 6.** Immunoprecipitation of tsO45, TM12, and VT. Cells expressing TM12 or VT were labeled for 60 min and chased for 60 min. The cells were lysed with TX100 at pH 7.4 and aliquots were immunoprecipitated with the indicated antibodies. CHO cells infected with the temperature-sensitive mutant tsO45 were labeled at 40°C for 10 min, then chased at 40°C for 5 min. At this point, some of the cells were lysed at 40°C and aliquots immunoprecipitated as above. The remaining cells were shifted to 32°C for 20 min before lysis. Under these conditions, >90% of the tsO45 G protein trimerizes.

above, tsO45 G protein is found in aggregated form in the ER of infected cells at the nonpermissive temperature. It forms trimers and is transported normally after shift to permissive temperature (32°C) in the presence of ATP (Doms et al., 1987). To determine if the aggregation was caused solely by the single amino acid change identified in tsO45 and not by other viral proteins, we expressed the recombinant gene R-ts-R (Gallione and Rose, 1985), which contains the amino acid change at position 204, and a wild type recombinant, ts-R-ts. As shown in Fig. 8 A, the mutant protein R-ts-R when expressed in COS cells behaved in the same way as the tsO45 protein in virus-infected CHO cells. It was aggregated and aberrantly folded at the nonpermissive temperature judging by its antigenic and sedimentation properties (Table II). After a temperature down shift the phenotype reversed and antigenically normal trimers were observed (Fig. 8 A and Table II). The G protein containing the wt sequence (ts-R-ts) was, as expected, trimeric at both 40 and 32°C.

We next analyzed the oligomeric and antigenic structures of four glycosylation mutants that had changes in the number and location of N-linked sugars (Table I). Wt G protein possesses two N-linked oligosaccharides at positions 179 and 336 (Table I). Glycosylation is known to play an important role in the transport of G protein to the cell surface. In tunicamycin-treated cells the G protein is not glycosylated and is found in aggregated form, presumably due to misfolding (Gibson et al., 1979; Machamer et al., 1985). We have



**Figure 7.** Oligomeric structure of the transmembrane mutants TM12 and TM14. COS cells expressing TM12 or TM14 were labeled for 1 h and chased for 1 h before lysis at pH 5.8 or 7.4. The distribution between monomeric, trimeric, and aggregated forms was determined by centrifugation as in Fig. 1.

shown that glycosylation at either of the two sites alone is, however, sufficient to make G protein transport competent (Machamer et al., 1985). By removing existing consensus sequences for core glycosylation and/or introducing new ones as outlined in Table I, we have generated a set of mutants which range in phenotype from partial transport to complete inhibition (Machamer et al., 1985, 1988a, b). Some of these mutants were analyzed here with respect to trimerization and folding.

As shown in Fig. 8 B and Table 1, mutant VT, which is blocked completely in transport from the ER, failed to form trimers after 1 h of chase. The G protein was recovered in a heterogeneous aggregate in a manner similar to that observed above for TM12 and tsO45 (see Figs. 7 and 8 A). Because of difficulty in recovering the aggregated material from the bottom of the centrifuge tube, we show the average amount of G protein recovered in the bottom fraction from several experiments in Fig. 8 B. VT was also incorrectly folded judging by its reactivity with the panel of monoclonals (Fig. 6 and Table II). Furthermore, the aggregate was present irrespective of gradient pH (Fig. 8 B). EN2/TA1,2 also aggregated but to a somewhat lesser extent. As seen in Fig. 8 B, ~30% of the protein was found in the trimer peak, with 65% sedimenting to the bottom of the tube. However, it is likely that a greater proportion of the protein actually existed in an aggregated state. Because recovery of the EN2/TA1,2 G protein from the gradient was only 60% (compared to 80–90% for the other mutant G proteins) and since recovery of aggregates from the bottom of the centrifuge tube was generally found to be difficult and variable, we presumed that the missing 40% was in the aggregate.

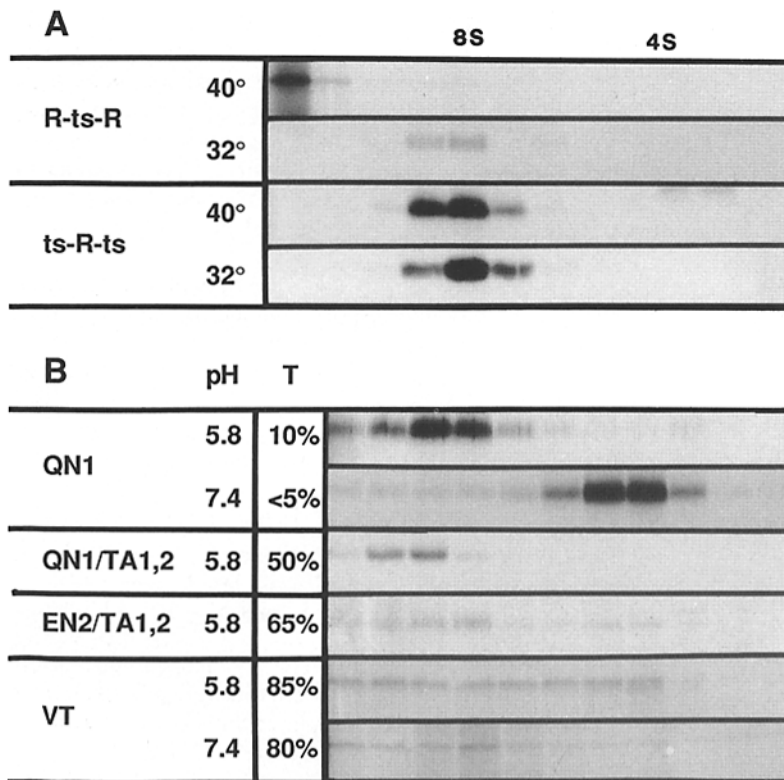
The third glycosylation mutant, QN1/TA1,2, is transported to the surface slowly and with only 50% efficiency (Machamer and Rose, 1988a). We found that 50% of the G protein sedimented as trimers and the rest was aggregated. Only the trimerized fraction of G acquired resistance to endoglycosidase H (not shown). These results are in agreement with previous observations that only 50% of QN1/TA1,2 acquires endoglycosidase H resistance and is transported to the cell surface (Machamer and Rose, 1988a), and show that only the correctly folded trimeric form is transported.

QN1, which is transported efficiently but more slowly than wt G, has an additional N-linked chain. It was found to form trimers relatively efficiently (Fig. 8 B). The trimers dissociated at pH 7.4 to monomers as expected for correctly folded G protein trimers, and the antigenic properties were identical to wt (Table II).

In summary, the ectodomain mutants that displayed transport-inhibited phenotypes were generally aggregated and misfolded. Trimerization, when it occurred, correlated with transport in as much as only the fraction of mutant proteins that were trimerized were able to exit the ER.

## Discussion

Oligomerization of membrane proteins plays a role in their transport through both the endocytic and biosynthetic pathways (Carlin and Merlie, 1986; Mellman, 1987). The IgG Fc receptor, for example, is rapidly internalized and delivered to lysosomes only when cross-linked into a higher-order structure (Ukkonen et al., 1986). In the biosynthetic pathway, an increasing number of oligomeric membrane proteins



**Figure 8.** Oligomeric structure of G proteins with mutations in the ectodomain. (A) COS cells were transfected and labeled with [<sup>35</sup>S]methionine 48 h later. Cells expressing tsO45 G protein or the revertant, tsO45R, were labeled for 30 min and chased for 30 min at the indicated temperature before lysis at pH 5.8. Lysis was performed at 32 or 40°C as indicated. The oligomeric structure of the G protein was determined by sucrose density gradient centrifugation as described in Fig. 1. (B) Cells expressing the glycosylation mutants were labeled for 60 min and then chased for 2 h. The oligomeric structure of the metabolically labeled G protein was determined by sucrose gradient centrifugation at pH 5.8 or 7.4, as in Fig. 1. The average amount of proteins pelleting to the bottom of the tube is shown (T), and was determined as described in Fig. 2.

have been identified, which apparently acquire their final quaternary structure shortly after biosynthesis (Bole et al., 1986; Carlin and Merlie, 1986; Kreis and Lodish, 1986). We have found that two well-characterized viral spike glycoproteins, the influenza HA and the VSV G protein, form trimers in the ER (Doms et al., 1987; Copeland et al., 1988). The panel of VSV G protein transport mutants described here has allowed us to examine the relationship between folding, trimerization, and subsequent transport from the ER into the secretory pathway.

### Folding before Trimer Formation

As nascent polypeptides are translocated into the ER, they undergo a series of modifications catalyzed by ER enzymes including signal peptidase, oligosaccharide transferase (Kornfeld and Kornfeld, 1985), protein disulfide isomerase (Freedman, 1984), and probably proline isomerase (Bächinger, 1987; Evans et al., 1987; Lang et al., 1987). These enzymes induce covalent modifications and promote faster and more accurate polypeptide folding. At some point during or after the initial folding, the newly synthesized chains acquire enough structure to specifically recognize other subunits and form mature multimers (Bergman and Kuehl, 1979). In membrane proteins such as influenza HA and neuraminidase, where the x-ray structure is known, the individual subunits have discrete, highly folded domains which may fold before oligomeric assembly (Wilson et al., 1981; Varghese et al., 1983). Regions intimately involved in inter-subunit contacts may, on the other hand, acquire their final secondary and tertiary structure only during assembly.

Our results showed that a significant degree of folding of

G protein occurs before trimer formation. Newly synthesized G protein was found to express conformation-specific epitopes A and B within 1–3 min after synthesis. This presumably reflects the rate of initial folding of the monomer before trimerization. Similar results have been reported for subunits of influenza HA (Copeland et al., 1988; Yewdell et al., 1988), the acetylcholine receptor (Merlie and Lindstrom, 1983; Carlin and Merlie, 1986), and for the mumps virus HN glycoprotein (Waxham et al., 1986). In the case of influenza HA and some other proteins, additional epitopes are expressed only after or coincident with oligomerization, suggesting either additional conformational changes or the creation of new epitopes at subunit interfaces (Bächi et al., 1985; Copeland et al., 1986; Gething et al., 1986). Initial folding of subunits is probably important for oligomerization because one would expect that structurally defined domains are needed to provide sites of recognition and attachment during oligomerization. Whether oligomerization depends on cellular factors or whether it is a self-assembly event governed by random subunit-subunit collisions remains to be seen, but it is a highly specific and frequently very efficient process.

### Mutants Defective in Initial Folding

Correct initial folding constitutes a requirement for trimer formation in G protein; this was illustrated by our transport mutants. Several of the G mutants analyzed in this study were translocated and core glycosylated normally, but failed to acquire the conformation-specific antigenic epitopes characteristic of the wt monomers and trimers. These included the transmembrane mutant TM12, the temperature-sensitive mu-



tant tsO45, and the glycosylation mutants VT, QNI/TA1,2, and to a more limited degree EN2/TA1,2. Being misfolded, these proteins failed to form trimers and were not transported from the ER (tsO45, VT) or transported very inefficiently (TM12, QNI/TA1,2, and EN2/TA1,2). Instead, they were found in aggregates that were not dissociated by nonionic detergent. For proteins transported inefficiently, only the fraction of protein forming trimers was transported.

While many secretory and membrane glycoproteins are transported with no core sugars, others, including the San Juan strain of VSV G protein, depend on the addition of N-linked oligosaccharides (Leavitt et al., 1977). We have shown here and in previous studies (Machamer and Rose, 1988a, b) that the requirement for oligosaccharide is manifested at the level of initial folding. Relocation of glycosylation sites can sometimes restore proper folding and trimerization (e.g., QNI/TA1,2). In the absence of detailed structural information on VSV G protein, we can only speculate as to the reasons why changes in glycosylation can affect folding. N-linked oligosaccharides may, through their polarity or large bulk, help segments of the polypeptide acquire a correct orientation during folding (Gibson et al., 1979; Sliker et al., 1986), while oligosaccharides inserted in other locations may prevent protein folding or assembly (Schuy et al., 1986).

One of the two transmembrane mutations, TM12, also affected initial folding. Since this mutant has a severely shortened transmembrane sequence, the phenotype might be rationalized on the basis of abnormal interactions with the membrane at the base of the spike protein. Part of the normal ectodomain could, for instance, be pulled into the lipid bilayer, thus preventing its normal folding.

Finally, the total lack of a cytoplasmic domain in the TMS protein retarded proper folding of the ectodomain. In addition, trimeric TMS displayed reduced reactivity with the conformation-specific antibodies. In contrast, a mutant influenza HA which also lacks its cytoplasmic domain is transported with normal kinetics (Doyle et al., 1986). Until more is known about the structural relationships between the domains of these proteins, we must conclude that some membrane proteins differ in their sensitivity to perturbations in their transmembrane and cytoplasmic regions.

### *The Misfolded State*

Aggregated, misfolded ER forms have been observed for other glycoproteins. They occur, for instance, as a side product during synthesis of influenza HA (Copeland et al., 1986). In the case of G protein, the aggregates are heterogeneous and sometimes disulfide linked (Doms et al., 1987; Machamer and Rose, 1988b). Though it is not yet known whether the aggregates contain cellular proteins, it is intriguing to speculate that they may interact with cellular factors whose role it is to bind and retain them in the ER. Factors involved in "refolding" errant subunits may also be involved (Bole et al., 1986) although we have no such indication. Indeed, misfolding and aggregation are not always irreversible processes. Misfolded tsO45 G protein can be rescued from the aggregate by reducing the temperature and supplying ATP (Doms et al., 1987). Some G protein glycosylation mutants can also be rescued at reduced temperature (Gibson et al., 1979; Machamer and Rose, 1988b).

### *Trimer Formation*

The G protein mutants examined in this study allowed us to make several predictions regarding which domains of the molecule are important in trimer assembly and transport. First, we found that transport mutants that displayed incorrect folding had changes in either the transmembrane or ectodomain. Second, transport mutants with defects in the cytoplasmic tail displayed, as a rule, normal folding and trimerization. This difference suggested that the ectodomain folds independently of the COOH-terminal domain. Indeed, both G and HA mutants are rather tolerant to changes in both their cytoplasmic and transmembrane domains (Adams and Rose, 1985a, b; Doyle et al., 1985, 1986; Roth et al., 1986; Puddington et al., 1986). While these regions may be important for the final stability and transport of the G and HA oligomers (Doms and Helenius, 1986), they do not seem to play important roles in initiating trimer formation. In fact, the wt cytoplasmic domain of G protein may slow the rate of trimerization, perhaps due to charge repulsion arising from its large number of basic residues. When eliminated (TMR) or replaced with less highly charged sequences (G $\mu$ , GHA, and 1473), the resulting G proteins all formed trimers more quickly than the wt protein. However, wt G protein trimerized nearly as quickly as the mutant proteins when expressed in HeLa cells. Thus, trimerization kinetics might be affected in part by differences in cell type or expression levels.

### *Transport-defective Trimers*

The final class of mutant proteins identified in this study formed trimers but were transported slowly or inefficiently out of the ER. This group of mutants had alterations in the cytoplasmic domain. Mutant 1473 was completely blocked in transport, only 10% of TMS was transported after 2 h, while GHA, G $\mu$ , and TMR were transported efficiently but at one-fourth to one-eighth the wt rate (Puddington et al., 1986). The mutants acquired all the antigenic epitopes characteristic of the mature G protein and, with the exception of TMS, they trimerized with wt kinetics. Furthermore, the mutant trimers underwent the stabilizing acid-induced conformational change with the same pH dependence as wt G trimers. Thus, their ectodomains were normal by the available criteria. However, differences not detected by our assays cannot be ruled out.

Several conclusions could be drawn from these data. First, trimer formation was not directly linked to transport. This point was illustrated by GHA, G $\mu$ , and TMR, all of which formed trimers with wt kinetics but were transported slowly nevertheless. Second, correct folding and trimerization of the ectodomain is apparently required but not alone sufficient for transport of G protein from the ER. This was best shown by 1473, which formed correctly folded trimers but was completely blocked in ER to Golgi transport (Gabel and Bergmann, 1985). Third, for transport to occur rapidly and efficiently, the cytoplasmic domain must fulfill certain structural requirements independently of the ectodomain. However, the cytoplasmic domain does not contain a signal that is absolutely essential for transport.

It has recently been proposed that proteins destined to reside in the ER possess retention signals, while proteins lacking such signals are exported by default via the bulk flow of

membrane (see Pfeffer and Rothman, 1987). Indeed, BiP and other peripheral ER proteins may be retained due to a specific sequence (KDEL) located at the COOH terminus of these proteins (Munro and Pelham, 1987). In this case, the receptors for this sequence must be in the lumen. Pääbo et al. (1987) have shown, on the other hand, that deletion of sequences from the cytoplasmic COOH terminus of E19, a resident ER membrane protein of adenovirus, resulted in transport of the protein to the surface. This suggests that retention may also involve interactions at the cytoplasmic surface of the membrane. The cytoplasmic sequence of the mutant 1473 also appears to serve as a retention signal, perhaps by interacting with cytoplasmic proteins or cytoplasmic domains of ER proteins. Indeed, when this sequence is placed on the cytoplasmic side of other membrane proteins, they too are retained in the ER (Guan et al., 1988).

Our results with the other cytoplasmic domain mutants are not entirely consistent with the bulk flow hypothesis. When the cytoplasmic domain is shortened, for instance, to a single arginine residue (TMR), the G protein trimerizes and is transported efficiently, albeit at one-eighth the rate of wt G. According to the bulk-flow hypothesis, the wt protein should be exported at the bulk-flow rate, while the mutants would somehow be slowed by interactions in the ER. However, it is difficult to imagine how the single remaining arginine could serve as a retention signal. While a full explanation for the results described here and in other studies is not yet apparent, we favor the idea that the wt cytoplasmic domain of G protein facilitates accelerated transport of correctly folded and trimerized G protein from the ER, perhaps by allowing it to concentrate at transport sites. Mutants with modifications in the tail would not be accelerated, but would conceivably follow along at the bulk rate. The function played by the G tail would thus be analogous to that of the cytoplasmic domain of the LDL receptor. The COOH-terminal domain of this plasma membrane protein is crucial for clustering of the receptor into coated pits as a prelude to rapid, accelerated internalization (Lehrman et al., 1985). In some tail mutants, the LDL receptor is internalized but with a significantly lower rate that approximates the known bulk rate of plasma membrane internalization (Steinman et al., 1983; Davis et al., 1986). Likewise, the rate at which mutant TMR is transported from the ER might more accurately reflect the rate at which large membrane proteins exit the ER by bulk flow.

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