

Role for Adenosine Triphosphate in Regulating the Assembly and Transport of Vesicular Stomatitis Virus G Protein Trimers

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Abstract. We have characterized the process by which the vesicular stomatitis virus (VSV) G protein acquires its final oligomeric structure using density-gradient centrifugation in mildly acidic sucrose gradients. The mature wild-type VSV G protein is a noncovalently associated trimer. Trimers are assembled from newly synthesized G monomers with a $t_{1/2}$ of 6–8 min. To localize the site of trimerization and to correlate trimer formation with steps in transport between the endoplasmic reticulum (ER) and Golgi complex, we examined the kinetics of assembly of the temperature-sensitive mutant VSV strain, ts045. At the nonpermissive temperature (39°C), ts045 G protein is not transported from the ER. The phenotypic defect that inhibited export from the ER at the nonpermissive temperature was found to be the accumulation of ts045 G protein

in an aggregate. After being shifted to the permissive temperature (32°C), the ts045 G protein aggregate rapidly dissociated ($t_{1/2} < 1$ min) to monomeric G protein which subsequently trimerized with the same kinetics as the wild-type G protein. Only trimers were transported to the Golgi complex. Kinetic studies, as well as the finding that trimerization occurred under conditions which block ER to Golgi transport (at both 15 and 4°C), showed that trimers were formed in the ER. Depletion of cellular ATP inhibited both the dissociation of the aggregated intermediate of ts045 G protein as well as the formation of stable trimers. The results indicate that oligomerization of G protein occurs in several steps, is sensitive to cellular ATP, and is required for transport from the ER.

IT has become increasingly clear that many integral membrane proteins, including most viral envelope glycoproteins, are multimeric. The process of folding and assembly to form the correct quaternary structure has emerged as an important factor in the selective transport of glycoproteins from the endoplasmic reticulum (ER)¹ to the plasma membrane (Kvist et al., 1982; Mains and Sibley, 1983; Merlie and Lindstrom, 1983; Merlie, 1984; Copeland et al., 1986; Gething et al., 1986; Kreis and Lodish, 1986; Minami et al., 1987). The mechanisms by which newly synthesized proteins are transported from the ER and targeted to the plasma membrane and other cellular destinations are, however, unknown.

Viral envelope glycoproteins are particularly useful models in studying protein transport. The G protein of vesicular stomatitis virus (VSV), the subject of this paper, is a glycoprotein of 67 kD anchored to viral and cellular membranes via a single transmembrane anchor sequence close to the COOH terminus (Rose et al., 1980; Rose and Gallione, 1981). VSV G protein is responsible for attaching the virus to the surface of host cells and triggering virus–membrane fusion in the mildly acidic endosomal compartment after endocytic uptake (White et al., 1983). The biosynthesis, posttranslational

modification, and intracellular transport of G have been analyzed in great detail in many different cell types (Rothman and Lodish, 1977; Li et al., 1978; see Balch et al., 1986, for additional references). Several mutant forms of VSV have been described in which transport of G protein from the endoplasmic reticulum to the plasma membrane is inhibited (Flammond, 1970; LaFay, 1974; Knipe et al., 1977; Schnitzer et al., 1979). A particularly well-studied VSV mutant is the thermoreversible ts045 strain in which a single-point mutation in the ectodomain of G prevents its exit from the ER at nonpermissive temperature (39°C) (Gallione and Rose, 1985).

Although the G protein has been thoroughly characterized in terms of its transport and post-translational processing, the quaternary structure has not been unambiguously determined. Detergent-solubilized VSV G protein sediments as a monomer (Crimmins et al., 1983), whereas chemical cross-linking studies and antibody-binding experiments indicate that at least part of the G is dimeric or trimeric (Dubovi and Wagner, 1977; Kreis, 1986; Kreis and Lodish, 1986). Recent studies by Kreis and Lodish (1986) have suggested that the ts045 G protein is a monomer in the ER at the nonpermissive temperature, and that at least a small fraction assembles to an oligomeric form (to dimers or trimers) after shift to the permissive temperature.

1. *Abbreviations used in this paper:* DMS, dimethylsulferimidate; ER, endoplasmic reticulum; HA, hemagglutinin.

Here we have characterized the oligomeric structure of wild-type and ts045 G protein using quantitative methods based on sucrose density-gradient centrifugation. We demonstrate that G protein is a trimer, that oligomerization occurs in the ER, and that ER-associated ts045 G protein is an aggregate at the nonpermissive temperature. Aggregate disassembly and trimerization of ts045 G (and possibly wild-type G) require metabolic energy.

Materials and Methods

Materials

The Chinese hamster ovary (CHO) cell line clone 15B and the Indiana serotype of the VSV mutant strain ts045 were obtained as previously described (Balch et al., 1986). L-[³⁵S]Methionine (>800 Ci/mol) was purchased from Amersham Corp., Arlington Heights, IL. Dimethylsuberimidate (DMS) was from Pierce Chemical Co., Rockford, IL.

Methods

Labeling of Cells. Cells were maintained, infected with VSV, and labeled with [³⁵S]methionine as previously described in detail (Balch et al., 1986). Briefly, confluent monolayers of 15B cells were infected with either wild-type or ts045 VSV. After 45 min at 32°C, the cells were placed in a 37°C (wild type) or 39°C (ts045) incubator. After 4 h of infection, the cells were washed with methionine-free medium, and the cells were suspended by gentle scraping with a rubber policeman into 1 ml of labeling medium at the appropriate temperature. For experiments which required depletion of cellular ATP, the labeling medium lacked glucose (Balch et al., 1986). Resuspended cells were transferred to a glass test tube preequilibrated in a water bath at the indicated temperature (32°C for wild type and 39°C for ts045). After 5 min of preincubation, [³⁵S]methionine was added at a final concentration of 50 μCi/ml and incubated for 3 min (wild type) or 10 min (ts045) with mixing at the indicated temperature. Chase conditions were obtained where indicated by the addition of unlabeled methionine to a final concentration of 2.5 mM.

Growth and Preparation of [³⁵S]Methionine Virus. A single 10-cm dish of confluent CHO cells were infected with wild-type VSV as above. 4 h after infection, the cells were washed with methionine-free medium and labeled with 0.5 mCi [³⁵S]methionine for 3 h. The medium was collected, spun in a microfuge for 5 min at 1,500 g to remove cells and cell debris, and placed on a 30%/60% sucrose step gradient in 20 mM morpholinethane sulfonic acid (MES), 30 mM Tris, and 100 mM NaCl (MNT buffer) at pH 7.4. The virus-containing supernatant was centrifuged at 4°C for 1.5 h at 24,000 rpm in an SW28 rotor (Beckman Instruments, Inc., Fullerton, CA). [³⁵S]Methionine-labeled virus was collected at the 30%/60% interface, divided into aliquots, and kept at 4°C or frozen in liquid N₂ prior to storage at -80°C.

Trimerization Assay. After labeling, cells infected with wild-type virus were chased for increasing time in the presence of excess unlabeled methionine at 32°C or 37°C as indicated in the Results. Cells infected with ts045 and labeled at 39°C were rapidly shifted to 32°C by first placing the tube containing 1 ml of medium in an ice-H₂O mixture for 5 s to reduce the temperature to 32°C, and then transferred to a 32°C water bath. Temperature equilibration was complete within 10 s. For each time point, aliquots of the cell suspension were added to an equal volume of lysis buffer (40 mM MES, 60 mM Tris, 200 mM NaCl, 1% Triton X-100, 2.5 mM EDTA, 2 mM EGTA, pH 5.5) at the indicated incubation temperature. The final pH of the lysate was ~5.8. 100 μl was loaded on an ice-cold 5–20% (wt/wt) continuous sucrose gradient in MNT buffer containing 1.25 mM EDTA, 1 mM EGTA, and 0.1% Triton X-100 at pH 5.8. In some cases, 50,000 cpm of purified [³⁵S]methionine-labeled influenza hemagglutinin (HA), a trimeric integral membrane protein (224 kD) with a sedimentation value of 9S, was added to serve as an internal sedimentation marker (Doms and Helenius, 1986). Samples were centrifuged in an SW50.1 rotor (Beckman Instruments, Inc.) for 15 h at 45,000 rpm at 4°C. Gradients were fractionated from the bottom of the tube by puncture using a gradient collection device (Beckman Instruments, Inc.) and the protein in each fraction precipitated by addition of an equal volume of ice-cold 20% (wt/vol) trichloroacetic acid. After fractionation, the centrifuge tubes were resealed with silicon sealant, and the material that pelleted to the bottom of the tube was solubilized with the gel sample buffer by incubation at 37°C for 1 h with intermittent shaking. All

samples were prepared for SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and autoradiographed, and the G protein was quantitated by densitometry as described previously (Balch et al., 1986).

Depletion of Cellular ATP. 50-μl aliquots of cell suspension in labeling medium without glucose were transferred to 1-dram glass vials fitted with rubber sleeve stoppers and preflushed with N₂. Under these conditions, intracellular ATP is rapidly reduced (*t*_{1/2} of 2–3 min) to a final steady-state ATP concentration of 15–20% of the O₂ control after 10 min. We have described this technique in detail in an earlier communication (Balch et al., 1986).

pH Dependence of Trimer Stabilization and Chemical Cross-linking. The pH dependence of trimer stabilization was determined for both intact virus and cell-surface G protein. Two approaches were used with identical results. [³⁵S]Methionine-labeled virus (see above) or cells labeled for 30 min and chased for 60 min were lysed in pH 7.4 lysis buffer and brought to the desired pH by addition of pretitrated volumes of 1.0 N acetic acid. Alternatively, labeled virus or cells were added directly to lysis buffer at the indicated pH. Acid-treated samples were returned to pH 7.4 by addition of base, or loaded directly onto 5% to 20% (wt/wt) sucrose gradients in 0.1% Triton X-100 in MNT buffer adjusted to the same pH as the sample. Centrifugation was performed as described above and the G protein in each fraction determined by SDS-PAGE. Aliquots of trimeric and monomeric G were collected and recentrifuged under various conditions as described in the Results.

Chemical Cross-linking with DMS. For chemical cross-linking of G protein with DMS, the mature virus or a cell lysate containing radiolabeled surface G protein (see above) was adjusted to pH 5.8 or 7.4 and loaded on pH 5.8 or 7.4 gradients, respectively. Under these conditions, the G protein quantitatively sedimented as a trimer (pH 5.8) or monomer (pH 7.4) (see Results). Aliquots of the peak fractions were adjusted to pH 7.8 by addition of base, and incubated with 0.5 mM DMS for 45 min. The reaction was quenched by addition of methylamine-hydrochloride, the protein was precipitated with TCA, and the results were analyzed using SDS-gel electrophoresis (Dubovi and Wagner, 1977).

Results

Native G Protein Is Trimeric

To determine the oligomeric state of mature G protein, [³⁵S]methionine-labeled wild-type VSV was solubilized with Triton X-100 and subjected to sucrose-gradient centrifugation in this nonionic detergent. By analogy with other viral fusion proteins, G is likely to undergo acid-induced conformational changes during activation of its fusion activity at acid pH (Doms et al., 1985; White et al., 1983). Therefore, we performed the initial solubilization and gradient centrifugations above and below pH 6.2, the pH threshold for activation of G-induced fusion (White et al., 1981). As shown in Fig. 1, the sedimentation coefficients of G proved to be quite different at pH 7.4 or 5.8. In neutral pH gradients G protein was quantitatively recovered in a 4S peak, and in acidic gradients in an 8S peak. When the pH dependence of the shift in sedimentation was determined, it was found to be only slightly different from the pH dependence of fusion activity (Fig. 2). This suggested that the 8S form appeared as a consequence of a conformational change related to fusion activity. At intermediate pH values both the 4S and 8S forms were present with no evidence of intermediate peaks.

The sedimentation coefficient observed at low pH (8S) was most consistent with a trimeric form of G protein (3 × 67 kD). The G nearly cosedimented with trimeric influenza HA (3 × 76 kD), which was used as an internal standard in the gradients (HA0 in Fig. 1) (Doms and Helenius, 1986). No significant levels of other VSV proteins were observed at the position of the 8S peak, eliminating the possibility of a heteroaggregate. To further characterize the oligomeric structure of G in both gradient peaks, chemical cross-linking with

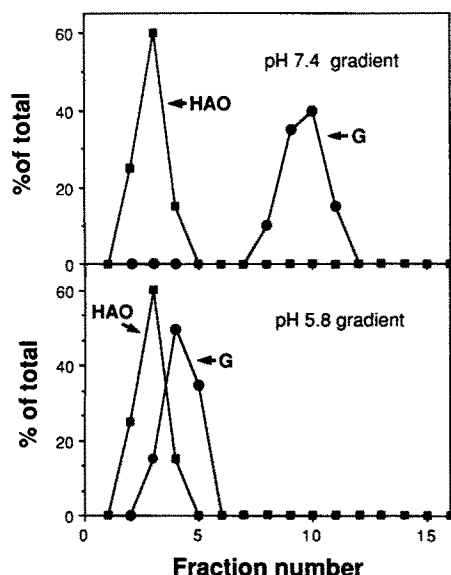


Figure 1. Sedimentation profile of G protein in acid and neutral pH sucrose density gradients. Purified [³⁵S]methionine-labeled VSV was solubilized in Triton X-100 at pH 5.8 or 7.4, and the lysates centrifuged on sucrose density gradients containing Triton X-100 at pH 5.8 or 7.4, respectively (see Materials and Methods). Influenza hemagglutinin (HAO), with a sedimentation coefficient of 9S, was included as an internal standard. Gradients were fractionated from the bottom and the amount of G protein in each determined by SDS-PAGE and scanning densitometry of the autoradiogram as described in Materials and Methods.

DMS was performed. As shown in Fig. 3, SDS-PAGE analysis of cross-linked 8S G protein revealed three bands with apparent molecular masses consistent with monomeric, dimeric, and trimeric forms of G. No higher-order forms were seen. Importantly, the 4S peak from neutral gradients showed only monomers either before or after cross-linking. In non-cross-linked 8S samples (Fig. 3, lane A) a small amount

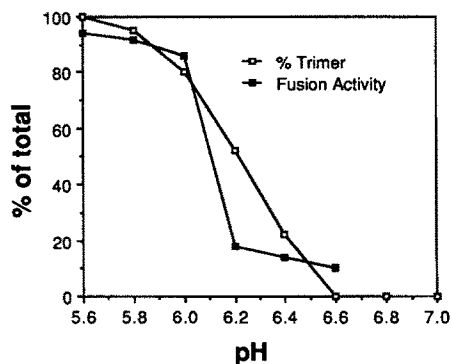


Figure 2. Relationship between pH dependence of VSV-membrane fusion and appearance of the 8S oligomer of G protein. pH-dependent VSV-induced cell-cell fusion was carried out as described by White et al. (1981). The pH dependence with which trimers were observed in sucrose-density gradients was determined by lysis of virus and centrifugation at the indicated pH as described in Materials and Methods. The distribution of G protein across the gradient was determined as in Fig. 1.

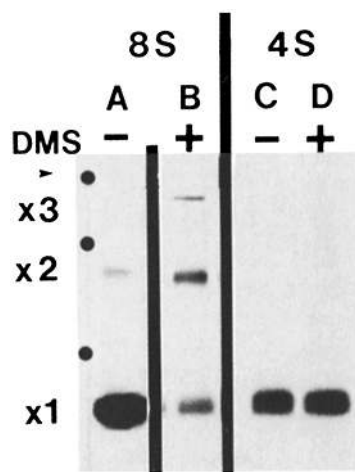


Figure 3. Cross-linking of 4S and 8S G protein from purified virus. Fractions from sucrose gradients containing equimolar amounts of 4S or 8S G protein were cross-linked with DMS and analyzed using SDS-gel electrophoresis as described in Materials and Methods. The portion of the autoradiogram containing oligomers of G protein is shown. The monomer ($\times 1$), dimer ($\times 2$), and trimer ($\times 3$) forms of G protein are indicated. The arrowhead marks the top of the separating gel, and the dots show the positions of monomeric (76 kD), dimeric (152 kD), and trimeric (228 kD) influenza HA.

of non-disulfide-linked, dimeric G protein was observed which probably represents an SDS-resistant form. Such SDS-PAGE-resistant oligomers can often be detected upon analysis of other multimeric integral membrane proteins (Silverberg and Marchesi, 1978; Doms and Helenius, 1986). Taken together, the sedimentation and cross-linking results showed that the 8S form of G protein is a homotrimer and that the 4S form corresponds to a monomer.

Experiments were next performed to determine whether the low pH in the gradients stabilized a native trimer present in the viral membrane against the dissociative effects of sucrose density-gradient centrifugation, or whether it induced the formation of trimers from monomers during solubilization. It was first observed that regardless of whether G protein was initially solubilized at acid or neutral pH, it sedimented as a trimer on acid pH gradients and as a monomer on neutral pH gradients (Table I). Thus, the sedimentation properties were independent of the solubilization pH, but dependent on the gradient pH. The sedimentation properties of G protein were next found to be independent of the concentration of G protein applied to the gradient; G was quantitatively recovered as trimers even after 1,000-fold dilution of the VSV before solubilization and centrifugation on acid gradients (Table I). This concentration independence strongly argues against an acid pH-induced trimerization of monomeric G protein during centrifugation. We also found that monomeric G protein recovered from the 4S peak of pH 7.4 gradients did not yield, even at high concentrations, an 8S peak when recentrifuged on a pH 5.8 gradient (Table I). This suggested that dissociated monomers were, in fact, incapable of reassociating to trimers at acid pH. In contrast, trimeric G from the 8S peak of a pH 5.8 gradient readily dissociated to the 4S form when recentrifuged on a pH 7.4 gradient (Table I).

Table 1. Effects of Lysis and Gradient pH on the Sedimentation of VSV G Protein

pH	pH		Portion of total G		
	Lysis	Gradient 1	Gradient 2	8S	4S
				%	%
7.4	7.4	—	<5	>95	
7.4	5.8	—	>95	<5	
5.8	7.4	—	<5	>95	
5.8	5.8	—	>95	<5	
7.4	5.8	5.8	>95	<5	
7.4	5.8	7.4	<5	>95	
7.4	7.4	7.4	<5	>95	
7.4	7.4	5.8	<5	>95	
7.4*	5.8	—	>95	<5	

VSV was lysed with Triton X-100 at the indicated pH and applied to sucrose gradients at pH 5.8 or 7.4. After centrifugation, the distribution of G protein between the 8S and 4S peaks was determined by SDS-PAGE and scanning densitometry. In some experiments, the G protein was removed from the gradient and recentrifuged on a second sucrose gradient with the indicated pH.

* G protein was diluted 1,000 times by using a larger volume of lysis buffer. Aliquots were then centrifuged as above.

We concluded from these results that (a) the viral G protein was a trimer in its native form, (b) it was not dissociated when solubilized using Triton X-100 at pH 5.8 or 7.4, (c) it dissociated during sucrose-gradient centrifugation at pH 6.6–7.4 but remained intact if the gradient pH was 5.8, and

(d) that dissociation, once it had taken place, was irreversible. These results are consistent with previous sucrose gradient studies at neutral pH showing that G sediments as a monomer (Crimmins et al., 1983), and cross-linking studies which have indicated that G protein can be detected as higher order oligomers (Dubovi and Wagner, 1977; Kreis and Lodish, 1986).

Trimerization of G Protein in the Infected Cell

The stability of native G trimers in acid sucrose gradients offered a novel method for analyzing the oligomerization pathway of newly synthesized G in infected cells. Prior to trimerization G protein monomers were expected to sediment at 4S, after trimerization at 8S. In order to establish clearly the relationship between the quaternary structure of G protein and its transport between the ER and the Golgi compartment, we monitored trimerization and transport in the Chinese hamster ovary (CHO) mutant clone 15B. CHO clone 15B lacks the *medial* Golgi compartment-associated carbohydrate processing enzyme *N*-acetylglucosamine transferase I (Tr I) needed for terminal glycosylation of the two asparagine-linked high-mannose oligosaccharides acquired by G in the ER (Gottlieb et al., 1975; Dunphy et al., 1985). As a consequence of this defect, the high-mannose (man₉GlcNAc₂) oligosaccharides are trimmed to the man₅GlcNAc₂ form by Golgi mannosidase, but are not processed further during subsequent transport to the surface. Because Golgi α -mannosidase is probably associated with the *cis*-Golgi, use of

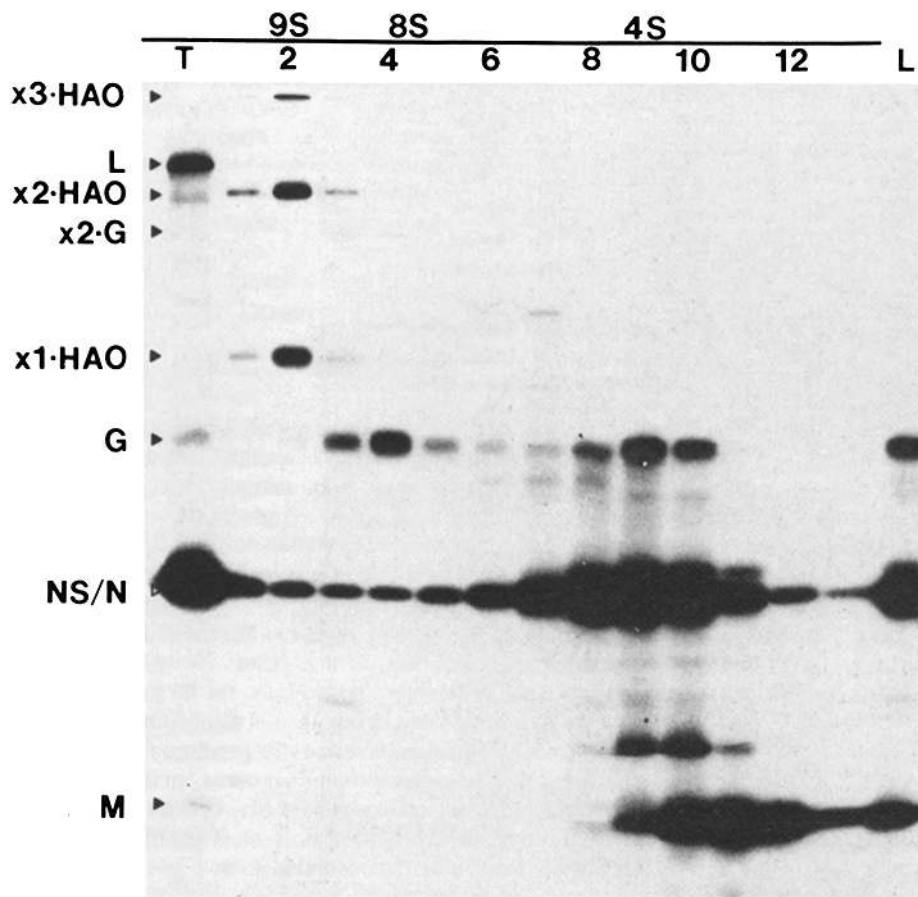


Figure 4. G protein sediments independently of other viral proteins. Cells infected with wild-type VSV were labeled for 3 min and then chased in the presence of excess unlabeled methionine for 5 min. The cells were then lysed and the lysate centrifuged on a pH 5.8 sucrose-density gradient as in Fig. 1. Influenza HAO, a trimeric 9S protein partially resistant to SDS-induced dissociation, was included as an internal standard. The monomer ($\times 1$ -HAO), dimer ($\times 2$ -HAO), and trimer ($\times 3$ -HAO) forms are indicated. G protein from the 8S peak migrated on the gel as a monomer (G) and undissociated dimer ($\times 2$ -G). The locations of the other viral proteins L, N, NS, and M are shown and did not change with different times of chase.

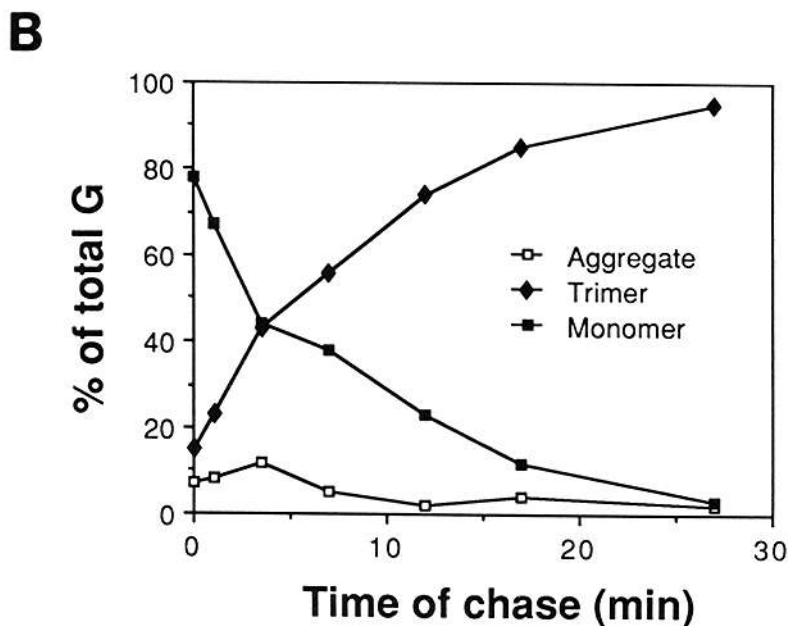
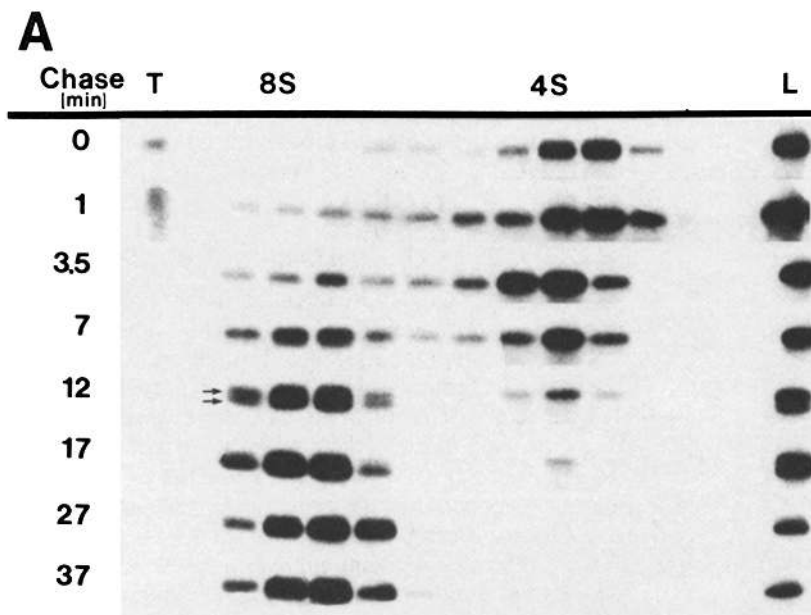


Figure 5. Kinetics of wild-type VSV G trimerization. (A) CHO cells infected with wild-type VSV were labeled with [³⁵S]methionine for 3 min, and chased in an excess of cold methionine. Transport was terminated by mixing with an equal volume of pH 5.5 lysis buffer at the indicated time and temperature, incubated for an additional 1 min before transfer to ice, and the oligomerization profile of G protein determined by density-gradient centrifugation on pH 5.8 sucrose gradients as described in Materials and Methods. The protein in the first 12 fractions was precipitated with TCA and run on the gel in its entirety. 20% of the material which pelleted to the bottom of the tube (in the lane marked T) was also run. Finally, 20% of the material loaded on each gradient is indicated under the lane marked L. All samples were analyzed by SDS-gel electrophoresis, and the portion of the exposed autoradiogram containing G protein for each time period of incubation is shown. Arrowheads at the 12-min chase point show the clear distinction between trimmed and untrimmed VSV G trimers. (B) The fraction of G protein found in the monomeric, trimeric, and aggregate (pellet) fractions was determined from the data presented in A by scanning densitometry as described in Materials and Methods.

clone 15B simplified the analysis of ER to Golgi transport because the more slowly moving untrimmed ER form of G can be readily distinguished from the faster-moving trimmed form by SDS-PAGE (Balch et al., 1986; Balch and Keller, 1986).

Wild-type VSV-infected clone 15B cells were pulsed for 3 min with [³⁵S]methionine and chased in the presence of cold methionine. At different times of chase the cells were solubilized and the lysates were subjected to sucrose gradient centrifugation at pH 5.8. The pellet fraction and each fraction from the gradient were analyzed using SDS-PAGE. A typical gel is shown in Fig. 4; it shows the fractionation of a lysate after a 7-min chase at which time about half of the G protein is seen in the trimer peak and half in the monomer peak. Because the virus efficiently inhibits host protein synthesis, all major labeled bands in the gel corresponded to VSV proteins as indicated. The bands marked as *HA0* show the position

of this trimeric, 9S internal standard added to the gradient sample (Doms and Helenius, 1986). Recoveries of G protein from gradients were generally >80%.

The time course of G protein conversion to the trimeric form at different times of chase is shown in Fig. 5, (A and B). At the earliest time points (Fig. 5, 0 and 1 min of chase), a small amount of G (<5–10%) was consistently recovered from the sucrose gradients in an aggregated form at the bottom of the tube. Approximately 80–90% of the G protein was, however, monomeric at these early chase points and was recovered in the 4S peak (Fig. 5). Trimers migrating at 8S appeared in the gradients with a $t_{1/2}$ of 6–8 min (32°C) after synthesis, though small amounts could be detected even at 1 min of chase. By 17 min of chase, virtually all of the G was trimeric. The SDS-PAGE mobility of G protein trimers at early times of chase corresponded to the mobility of the untrimmed, ER-associated man₉GlcNAc₂ species (see Fig. 5,

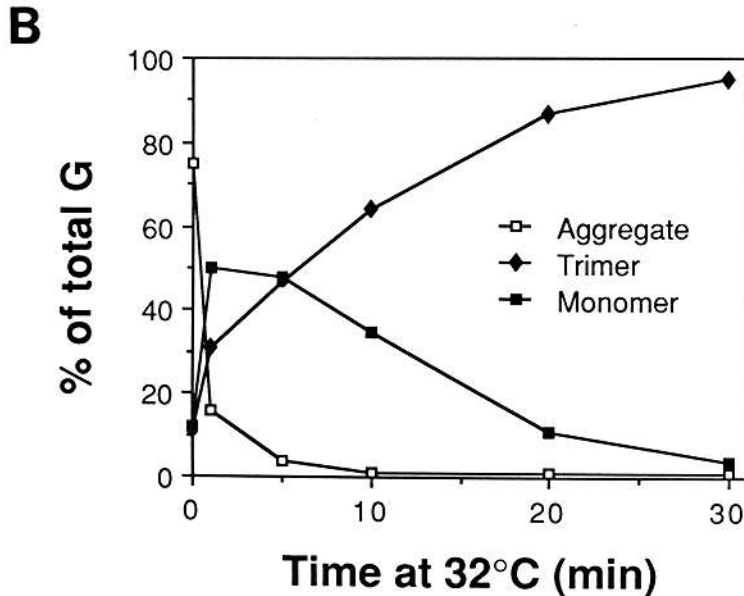
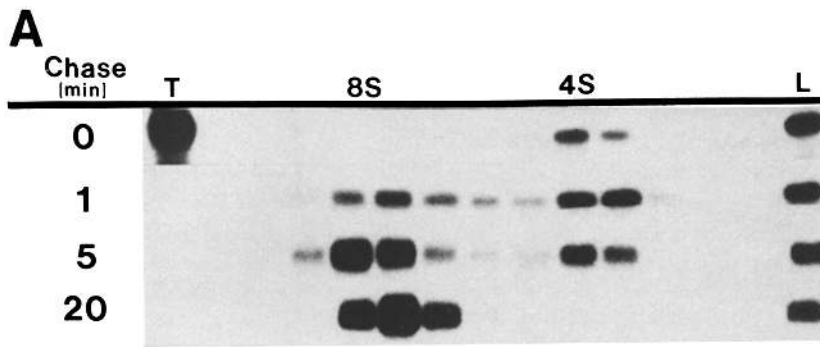


Figure 6. Kinetics of ts045 VSV G trimerization at 32°C. (A) CHO clone 15B cells were infected with ts045 VSV, labeled for 10 min at 39°C as described in Materials and Methods, and then transferred to 32°C. At the indicated time cells were mixed with an equal volume of pH 5.5 lysis buffer, and maintained at the indicated temperature for 1 min prior to transfer of lysates to ice. Samples were analyzed as described in Fig. 5. The protein that sedimented to the bottom of the tube is shown in lane T, and 20% of the starting material is shown in lane L. (B) The amount of G protein that sedimented as the aggregate, trimer, or monomer was determined from the data presented in A and from two additional experiments. The average of the three experiments is presented.

3.5 min). This suggests that the trimers were formed before G protein had reached the *cis*-Golgi compartment. After 12 min of chase at 32°C, the 8S peak contained increasing amounts of the trimmed $\text{man}_5\text{GlcNAc}_2$ oligosaccharide form of G protein, indicating that these trimers had reached the *cis*-Golgi compartment (arrows, 12-min chase in Fig. 5 A). After 30 min of chase, all the trimers were recovered in the trimmed form. Trimmed G protein was never observed in the monomeric peak, suggesting that monomers did not reach the *cis*-Golgi or compartments beyond it in the secretory pathway. The results are consistent with trimerization occurring either in the ER, in transit to the *cis*-Golgi, or immediately upon arrival in the *cis*-Golgi.

***ts045* G Protein Forms an Aggregate at the Nonpermissive Temperature**

Although the oligomerization profile described above supported the hypothesis that G protein underwent maturation to a trimer in the ER, it was difficult to prove this point owing to the rapid, continuous exit of wild-type G protein after synthesis. To obtain further information about the oligomerization process, we studied the fate of G protein in cells infected with the mutant VSV strain ts045. At the nonpermissive temperature (39°C) the mutant G is synthesized and core-glycosylated, but fails to be transported out of the ER (Lafay, 1974; Knipe et al., 1977). Upon shift to the permissive temperature

(32°C), the G protein held in the ER is rapidly and efficiently transported to the Golgi complex and the plasma membrane with kinetics identical to wild-type G protein (Balch et al., 1986; Balch and Keller, 1986). We have previously shown that transport of ts045 G protein from the ER to the *cis*-Golgi compartment occurs in two operationally defined steps, an initial ATP and temperature-sensitive step followed by a subsequent "transfer" step, which is insensitive to incubation at the nonpermissive temperature and the decreased levels of ATP that inhibit the initial step (Balch et al., 1986; Balch and Keller, 1986).

To define the relationship between the oligomerization pathway of ts045 G protein and steps in ER to Golgi transport, 15B cells were infected with ts045 and labeled with [^{35}S]methionine at the nonpermissive temperature, solubilized at the nonpermissive temperature, and analyzed by sucrose-gradient centrifugation and SDS-PAGE as described above. Under these conditions, virtually all (85–95%) of the G protein was recovered in the pellet fraction, indicating that it was part of an aggregate (see Figs. 6–8; 0 min of chase). The major fraction of G protein was recovered in the aggregate regardless of whether cells were solubilized at acid or neutral pH, or whether centrifugation was performed at acid or neutral pH. Partial dissociation (<20%) to monomers could be detected during centrifugation on neutral gradients, suggesting that acid gradients provided enhanced stability of

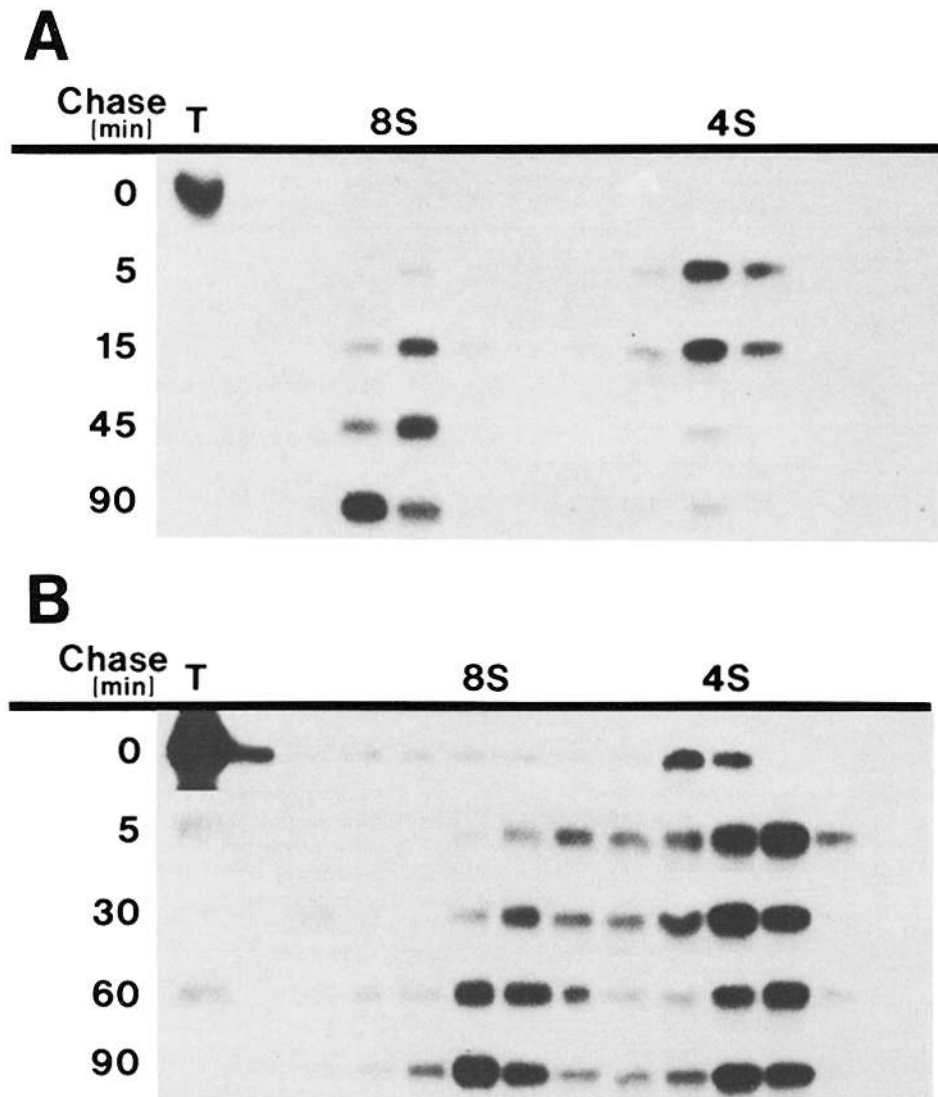


Figure 7. Kinetics of ts045 VSV G trimerization at 15°C and on ice. (A) CHO clone 15B cells infected with ts045 were labeled at 39°C as described in Materials and Methods. The cells were then rapidly shifted to 15°C by a brief incubation in ice water, and aliquots were solubilized at the indicated time (minutes) in pH 5.5 lysis buffer at 15°C for 1 min before transfer to ice. The lysates were analyzed on acid sucrose-density gradients as described in Fig. 5. (B) Cells labeled at 39°C were diluted into a 100-fold volume of ice-cold labeling medium to rapidly reduce the temperature. Aliquots were solubilized at the indicated time (minutes) in pH 5.5 lysis buffer on ice, and analyzed on acid sucrose-density gradients as described in Fig. 5.

the aggregate to the effects of centrifugation. The aggregated form of G protein did not pellet during a brief (5 min) 10,000-g spin in a microfuge, nor was it detected in the pellet after 1 h at 100,000 g, suggesting that it was not part of a large detergent-insoluble matrix or precipitate. The aggregate was not dissociated by the addition of 50 mM dithiothreitol (DTT) to the lysis buffer or to the sucrose-density gradients. The appearance of the aggregated form of G was, furthermore, independent of the infection conditions. G protein was recovered in the aggregate whether the cells were infected for 4 h at 32°C or for 4 h at 39°C. Thus, the aggregate was not a consequence of the long-term accumulation of ts045 G protein in the ER. Taken together the results suggest that ts045 G protein is not transported out of the ER at nonpermissive temperature because it is trapped in an aggregate.

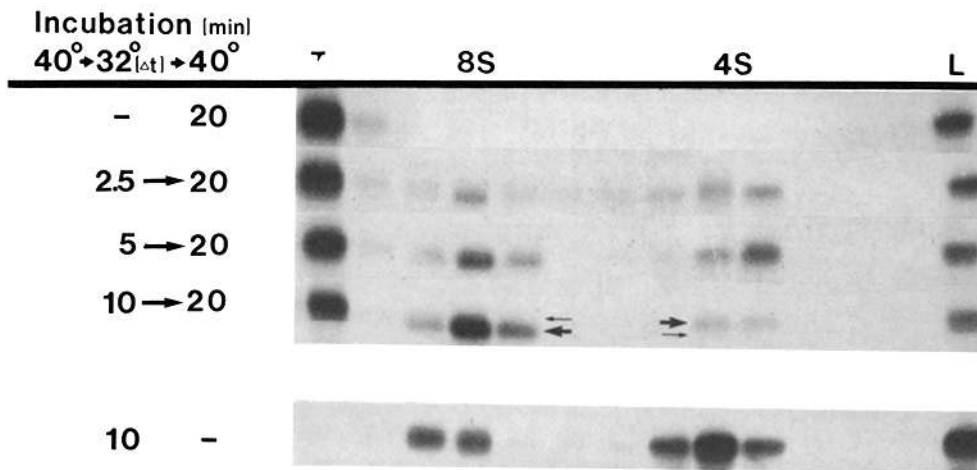
When cells labeled at the nonpermissive temperature were shifted to the permissive temperature for different time periods before solubilization and analysis using acid pH gradients, the ts045 G protein aggregate was rapidly ($t_{1/2} < 1$ min) converted to the monomeric species (Fig. 6, A and B). Upon further incubation at the permissive temperature the

monomers proceeded to trimerize ($t_{1/2} = 5$ min), and move to the *cis*-Golgi compartment at a rate nearly identical to that observed for wild-type G protein. The reversal of the temperature block thus involved rapid dissociation of ts045 G protein from the aggregate to the monomer followed by trimerization. We conclude that disassembly of the ts045 aggregate is the phenotypic event essential to initiate further steps in export.

Trimerization Occurs before Export from the ER

To further define the intracellular site of trimerization, we took advantage of the well-established temperature blocks in the transport of G protein from ER to the Golgi complex. Incubation at 15°C prevents the transport of G protein to the *cis*-Golgi, presumably resulting in its accumulation in pre-Golgi vesicles (Saraste and Kuismanen, 1984). After [³⁵S]methionine labeling of ts045 G protein at 39°C, cells were rapidly cooled to 15°C and incubated for variable time periods at this temperature. As shown in Fig. 7 A, the aggregates present at the nonpermissive temperature rapidly

A



B

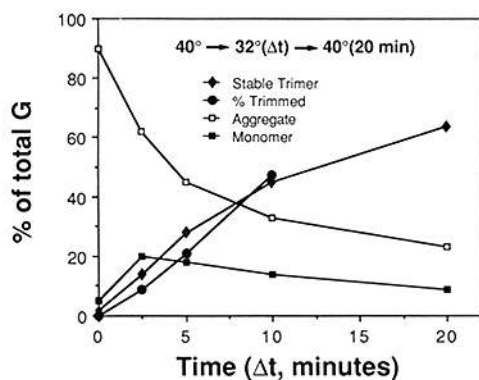


Figure 8. Thermostable trimers are found in the Golgi compartment. (A) Ts045-infected clone 15B cells were labeled at 39°C as described in Materials and Methods. Cells were transferred to the permissive temperature for the indicated time (Δt). The cells were then reincubated at 39°C for 20 min to inhibit exit from the ER as well as to allow the G protein released from the ER during the 32°C incubation to be trimmed to the $\text{man}_5\text{GlcNAc}_2$ form in the *cis*-Golgi compartment (Balch et al., 1986). After the 20-min incubation at 39°C, the cells were lysed at 39°C, and incubated 1 min before transfer to ice. The distribution of G protein was analyzed by centrifugation on acid sucrose gradients as described in Fig. 5. (B) The amount of G protein that sedimented as the aggregate, trimer, or monomer was determined from the data presented in A. Arrows show the positions of trimmed and untrimmed G protein.

dissociated to monomers at 15°C. Monomers trimerized, albeit at a considerably slower rate than at higher temperatures ($t_{1/2} \sim 30$ min). All of the trimeric G protein was untrimmed, confirming that it had not reached the *cis*-Golgi compartment. This result indicated that trimerization did not require transport to the *cis*-Golgi.

Trimerization also occurred when cells were incubated on ice (Fig. 7B). When cells were rapidly mixed with a 100-fold excess of cold chase medium, aggregates disappeared rapidly, monomers were formed, and trimerization occurred with a $t_{1/2}$ of ~ 70 min. Because incubation on ice inhibits all membrane transport, the result clearly indicated that trimerization can occur in the ER. Very slow trimerization of wild-type G protein was also observed during prolonged incubation on ice. These results are to be distinguished from the previous experiments (Figs. 4–6) in which the cells were solubilized at 32°C or 39°C before transfer to ice, stabilizing G protein in its various oligomeric forms for subsequent gradient analysis.

The above experiments established that trimerization of ts045 G protein occurred in the ER at reduced temperature, and that trimerization is complete before delivery to the *cis*-Golgi. However, it was still conceivable that trimerization at the permissive temperature was a post-ER event. To determine the site of trimerization at the permissive temperature,

we took advantage of the thermoreversibility of ts045 G protein export from the ER. We have previously shown that when ts045-infected cells at 39°C are incubated at the permissive temperature and then returned to the nonpermissive temperature, only a portion of the G protein exits the ER and enters the *cis*-Golgi where it is trimmed to the $\text{man}_5\text{GlcNAc}_2$ form (Balch and Keller, 1986). The amount of G protein delivered to the Golgi compartment is directly proportional to the time of incubation at the permissive temperature. To relate oligomerization to this thermoreversible defect in transport, infected cells were labeled at the nonpermissive temperature, shifted to 32°C for increasing time periods, and then reincubated at the restrictive temperature for an additional 20 min in order to block further export from the ER and chase any G protein that had escaped from the ER to the *cis*-Golgi compartment. The distribution of G protein among monomers, trimers, and the aggregate was then analyzed on acid pH gradients and SDS-PAGE. The results are shown in Fig. 8.

When the cells were kept at the permissive temperature for only a short period of time (Fig. 8A, 2.5 min) before return to 39°C, ~ 85 –90% of the untrimmed G protein was recovered in the aggregated pellet fraction. The remaining untrimmed G protein representing 10–15% of the total was detected in the monomer peak. In contrast, G protein existed

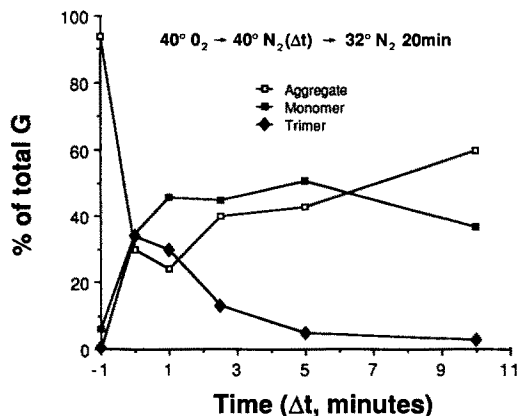


Figure 9. ATP depletion inhibits ts045 G trimerization. Ts045-infected clone 15B cells were labeled at 39°C, and cells were injected into an oxygen-free, nitrogen atmosphere and held for the indicated time (Δt) prior to incubation at 32°C for 20 min, also under nitrogen, as described in Materials and Methods. The cells were lysed at 32°C. After 1 min cells were transferred to ice, and the G protein was analyzed on acid sucrose-density gradients as described in Fig. 5. Cells at the -1 min time point were labeled and lysed at 39°C prior to exposure to N₂.

solely as monomers and trimers in control cells shifted to 32°C for the same short chase times but not returned to 39°C (see Fig. 6). Thus, most of the G monomers and all of the trimers remaining in the ER after 2.5, 5, and 10 min (Fig. 8 A) incubation at the permissive temperature were induced to reaggregate when the cells were returned to the nonpermissive temperature.

In contrast, G protein trimers that had escaped the ER and become trimmed in the *cis*-Golgi compartment did not reaggregate when returned to the nonpermissive temperature (Fig. 8 A; see 8S peak). Instead, stable trimmed trimers accumulated in the 8S peak. These results suggested that either a difference exists in the thermostability of ER and Golgi trimers, or that the conditions prevailing in the lumen of the ER and subsequent compartments of the secretory pathway can modulate the oligomerization profile of G differentially.

The extent of stable trimerization was proportional to the time of incubation at the permissive temperature. The kinetics with which thermostable trimers accumulated defined a line which extrapolated to the origin (Fig. 8 B). This result indicated that trimerization is kinetically indistinguishable from the temperature and ATP-sensitive step required to initiate export from the ER described previously (Balch et al., 1986; Balch and Keller, 1986), providing direct evidence that trimerization at 32°C must be an ER-associated step preceding export.

ATP Dependence of Quaternary Structure Changes in G Protein

It has been shown on numerous occasions that transport of secretory membrane proteins from the ER to the Golgi complex requires metabolic energy in the form of ATP (Jamieson and Palade, 1968). One way to reversibly deplete cells of ATP is to immerse them in a N₂ atmosphere. We have previously shown that the ATP level in 15B cells under N₂ is halved in 3 min and that ts045 G protein transport from the ER stops within 1 min (Balch et al., 1986; Balch and Keller, 1986).

To determine whether metabolic energy was required for any of the ER steps preceding transport, ts045-infected cells were first labeled with [³⁵S]methionine at the nonpermissive temperature. The ATP pool was then reduced by injecting cells into a vial containing N₂ atmosphere while keeping the temperature at 39°C. After a variable period of time under N₂, the cells were shifted to the permissive temperature and incubated for an additional 20 min in the presence or absence of N₂. The cells were then solubilized and the oligomeric state of G was analyzed to determine to what extent a decrease in ATP levels affected the dissociation of the ts045 aggregate, the trimerization of G from monomers, and the transport of G from the ER to the Golgi complex.

The results, shown in Fig. 9, indicated that ATP depletion affected all of these steps. If ATP depletion was initiated 1 min before the temperature downshift and maintained throughout the 20-min incubation at 32°C, it was found that the G protein was equally distributed among monomers, trimers, and aggregates. All of the G was, moreover, in the untrimmed form indicating that it had not reached the *cis*-Golgi. In the control sample, which had been restored to air (and to normal ATP levels) after the 1-min ATP depletion, only trimers were seen and all the G protein was trimmed (data not shown). The difference indicated that the ATP depletion resulted in inefficient disassembly of the aggregated form of G, inefficient trimerization of monomers, and a total block in the transport of trimers to the *cis*-Golgi complex. The results were even more dramatic if the ATP depletion was allowed to proceed for 2.5 min or longer before the downshift to permissive temperature. Under these conditions, G was nearly equally divided between the aggregate and monomer forms, with <10% being found in trimers. The inhibitory effects of ATP depletion on assembly and transport (Balch et al., 1986) were found to be completely reversible at all time points by restoring the cells to air at the permissive temperature (data not shown).

These findings indicated that metabolic energy is needed for the conversion of G protein from aggregate to monomer and from monomer to trimer, as well as transport of ts045 G protein from the ER to the Golgi complex. Whether ATP per se or an indirect effect related to cellular ATP depletion inhibits oligomerization and transport of G protein from the ER remains to be determined.

Discussion

Quaternary Structure of G Protein

Noncovalent protein-protein interactions are frequently sensitive to detergent solubilization and other manipulations, making it difficult to assess the quaternary structure of membrane proteins. Dissociation of oligomers during sucrose-gradient centrifugation in the presence of nonionic detergent is not uncommon. The viral spike glycoproteins of the Semliki Forest virus (Vogel et al., 1986; Helenius et al., 1976) and an intracellular trimeric form of influenza HA (Cope land et al., 1986) are both sensitive to disruption during gradient analysis. This problem is also illustrated by the VSV G protein. Hydrodynamic analyses of detergent-solubilized G protein and a cathepsin-cleaved ectodomain fragment have suggested that the G protein is monomeric (Crimmins et al.,

1983). Cross-linking in the presence of DMS and other chemical cross-linkers has, in contrast, suggested that G protein can be present as dimers or higher-order oligomers (Dubovi and Wagner, 1977; Kreis and Lodish, 1986). Our use of mildly acidic sucrose gradients resulted for the first time in quantitative recovery of G protein trimers.

Several lines of evidence showed that low pH stabilized the quaternary structure of the native G protein against the dissociative effects of detergent and sucrose-gradient centrifugation. The pH stabilization of G trimers on sucrose gradients correlated approximately with the pH dependence of fusion activity of the G protein and therefore, by analogy with other viral fusion proteins (Doms et al., 1985; Kielian and Helenius, 1986), most likely reflected an acid-induced conformational change. Because acid stabilization is reversible, G protein may undergo a reversible acid-activated conformational change, making it unique among acid-sensitive viral fusion factors such as the influenza virus HA (Doms et al., 1985) and the toga (alpha) virus spike glycoproteins (Kielian and Helenius, 1985) which undergo irreversible conformational changes.

Assembly of G Protein Trimers

Using the acid gradient assay for trimerization and monitoring carbohydrate side-chain trimming for arrival of G protein to the *cis*-Golgi compartment, we were able to establish the overall properties of wild-type G protein assembly in relation to early steps of the secretory pathway. Before the formation of the trimers, newly synthesized G proteins sedimented as monomers, suggesting that trimer assembly occurs from a freely mobile monomeric G protein pool. In support of this interpretation, we have recently shown that influenza HA monomers randomly assemble to form trimers in the ER of cells coinfecting with antigenically distinct virus strains (F. Boulay, R. W. Doms, R. G. Webster, and A. Helenius, manuscript submitted for publication).

Trimerization was a posttranslational event which occurred with a $t_{1/2}$ of 6–8 min after synthesis, but trimers could be detected as early as 1–2 min after synthesis. In contrast, delivery to the *cis*-Golgi compartment was detected only after 5–10 min. Only G protein trimers reached the *cis*-Golgi where they were trimmed to the $\text{man}_5\text{GlcNAc}_2$ species. The results suggested that trimerization was an ER-associated event and that the monomers were not transported to the Golgi complex. Two other lines of evidence clearly established that trimerization preceded export from the ER. G protein not only formed trimers during incubation on ice, but a direct kinetic analysis of trimerization of ts045 G protein identified the site of trimerization to be indistinguishable from the initial temperature-sensitive, ATP-dependent export step essential for exit of G protein from the ER (Balch et al., 1986).

No evidence for the formation of G protein dimers during assembly was obtained, suggesting that if such intermediates occur, they must either be short-lived or unstable to centrifugation. The trimerization pathway of wild-type G protein reported here is similar to the assembly of the influenza HA0 (Gething et al., 1986; Copeland et al., 1986). HA0 trimers are formed with similar kinetics as G, and the assembly probably occurs as a late ER event (Copeland and Helenius, unpublished results). The ER has also been implicated as the site of assembly for numerous other membrane and secretory

proteins (Kvist et al., 1982; Merlie, 1984; Minami et al., 1987).

Studies on the Ts045 G Protein Aggregate

To address the properties of G trimerization in more detail, we took advantage of the extensive knowledge of ts045 G protein that has been acquired over the last few years (Bergmann and Singer, 1981; Balch et al., 1986; Balch and Keller, 1986). The most surprising result was that ts045 G protein, at the nonpermissive temperature, pelleted in sucrose gradients. This was unexpected because a recent report by Kreis and Lodish (1986) concluded, on the basis of cross-linking studies, that the ts045 G protein was a monomer at the nonpermissive temperature. The inability of G protein monomers to trimerize at the nonpermissive temperature was thought to prevent transport to the Golgi complex. Some of the results of Kreis and Lodish might be readily explained by the temperature (on ice) at which some experiments were carried out; we have found that incubation of intact cells on ice induced rapid ($t_{1/2} < 1$ min) and quantitative conversion of the aggregated form of ts045 G protein to a monomer. The aggregated form can only be detected when the cells are solubilized at the nonpermissive temperature. Our results suggest that ts045 G protein is not transported from the ER because it is present in an aggregate.

The composition of the aggregate is presently unclear. It is apparent that G becomes associated with the aggregate immediately after synthesis at the nonpermissive temperature. G is not covalently associated in the aggregate by disulfide bonds, and the aggregate may be of relatively small size because it does not pellet with the Triton X-100 insoluble cellular components after 1 h at 100,000 g. Aggregation is not simply a consequence of prolonged accumulation of G protein in the ER during infection at the nonpermissive temperature; even brief (5-min) incubation of ts045-infected cells at the nonpermissive temperature to radiolabel G protein in the ER resulted in the recovery of G protein in the aggregate. Aggregation of G protein has been observed when wild-type infected cells are grown in the presence of tunicamycin (Gibson et al., 1979), an inhibitor of N-linked glycosylation (Takatsuki et al., 1975; Tkacz and Lampen, 1975). The unglycosylated G protein was also prevented from transport to the cell surface, but unlike the temperature-sensitive ts045 G protein, the aggregation and transport block were irreversible. A similar result has been obtained when the G protein glycosylation sites are removed by site-directed mutagenesis (Machamer et al., 1985). It is also possible that aggregation occurred only after solubilization of the cells with Triton X-100. The aggregate was, however, also observed when cells were solubilized with β -octylglucoside or Nikkol, so artifactual aggregation due to Triton solubilization per se is unlikely.

Several other observations are pertinent to our current understanding of the composition of the aggregate. First, morphologic studies using an antibody directed against the cytoplasmic tail of ts045 G protein have suggested that G protein is evenly distributed throughout the reticular network of the ER at the nonpermissive temperature (Kreis, 1986; Kreis and Lodish, 1986). This result can be interpreted to mean that the aggregate is not simply a precipitated, clumped form of G. Secondly, it is important to note that ts045 G, even after it has been allowed to form free monomers and trimerize in

the ER, can be recaptured in the aggregate by returning to the nonpermissive temperature. Whereas monomers and trimers present in the ER reaggregate at the nonpermissive temperature, trimers that have reached the Golgi complex proved stable under these conditions. The thermoreversibility of the association of G protein with the ER aggregate, and the contrasting stability of assembled trimers that have escaped the ER, explains our previous observation that the ts045 G protein is able to continue its transit to the cell surface at the nonpermissive temperature provided it is first allowed to reach the stage at which it is trimmed in the *cis*-Golgi compartment (Balch and Keller, 1986). The factors regulating aggregation are presently unclear. The ER may provide a unique environment (or contain unique components) that modulate the oligomeric composition of G protein. Alternatively, G protein that has reached the Golgi apparatus may have undergone modification(s) such as fatty acylation or carbohydrate side-chain trimming which preclude trimer dissociation and reaggregation at the nonpermissive temperature.

Whether the aggregate constitutes a homopolymer of G protein, or is a complex of G protein with resident ER proteins remains to be determined. In preliminary experiments we have failed to detect the immunoglobulin binding protein in the aggregate (D. Bole, R. W. Doms, and W. Balch, unpublished results). This protein, a ubiquitous and prominent ER-associated protein, is believed to play a role in the retention of the immunoglobulin heavy chain in the ER before and during assembly of the IgG molecule in lymphocytes (Bole et al., 1986), and to associate with aberrant forms of influenza HA0 and possibly other incorrectly assembled proteins in the ER (Gething et al., 1986; Copeland et al., 1986). A number of resident proteins in the ER membrane remain associated when dissolved with Triton X-100. The docking protein, the ribophorins, and the signal peptidase are thought to be a part of such a complex (Hortsch et al., 1987). It is intriguing to speculate that the ts045 G protein aggregate reflects the association of G with this or other ER protein complexes. Studies are currently underway to examine this possibility.

Role of ATP in Oligomerization

It is well known that ATP depletion blocks the transport of secretory proteins and membrane proteins from the ER to the Golgi complex (Jamieson and Palade, 1968). In previous studies we have shown that ts045 G protein requires a minimum of 60–70% of the normal cellular ATP level to support transport at this stage. A reduced requirement is observed for subsequent stages in delivery of G protein to the plasma membrane (Balch and Keller, 1986). The first ATP-dependent step was postulated to be required for an initial "export" step, possibly associated with the formation of a transport vesicle (Balch et al., 1986).

Our results on the assembly of ts045 G throw new light on the role of ATP in export of protein from the ER. They indicate that ATP depletion disrupts the dissociation of the mutant G protein from an ER aggregate after the down shift to the permissive temperature. Similarly, trimerization may be ATP sensitive, assuming that the peak of monomer observed in gradients after ATP depletion is a consequence of incomplete trimerization, and not simply reflecting an instability of this aggregate to the conditions of gradient centrifugation.

Whether ATP per se, or some indirect consequence of cellular ATP depletion influences oligomerization remains to be determined.

Do these findings imply that export and transfer from the ER to the Golgi complex does not require ATP? Transport to the *cis*-Golgi compartment (trimming) was not observed at ATP levels in which ts045 G protein was found to be distributed equally among the aggregate, monomeric, and trimeric forms. This result illustrates that an additional requirement beyond trimerization is sensitive to cellular ATP. This step must precede or be coincident with the temperature-sensitive site, in that transfer to the Golgi complex is less ATP sensitive during subsequent delivery step(s) (Balch et al., 1986). A synergistic interaction between these two ATP-sensitive steps may play a role in the remarkably small decrease in cellular ATP required to inhibit ts045 G protein export, but not wild-type protein. We have observed that export of wild-type G protein requires a lower threshold (20–40% of normal cellular ATP) to efficiently inhibit ER export (W. E. Balch, unpublished).

ER Pathway

Our results provide an approximate itinerary for the assembly of ts045 G protein in the ER and transport to the early Golgi compartment shown schematically in Fig. 10. An important question illustrated in the model is whether the aggregated form observed at the nonpermissive temperature reflects an aberrant side pathway specific for ts045 G protein, or whether it represents an intermediate in the normal pathway of wild-type G protein processing in the ER. A small but reproducible amount of wild-type G protein could be detected as an aggregate during very early times of chase (Fig. 5). In addition, we have also detected an aggregated form of wild-type G protein under conditions of ATP depletion (R. W. Doms and W. E. Balch, unpublished data). These observations suggest that newly synthesized wild-type G protein may also be transiently associated in a complex before it is released as a monomer. Whereas ts045 G protein remains permanently trapped at the nonpermissive temperature, wild-type G protein may rapidly dissociate from the complex and trimerize. Cellular ATP may also directly or indirectly influence the release of monomers and trimerization of wild-type G protein. In the case of ts045, the disassembly of the aggregate can only be triggered by a conformational change associated with shift to the permissive temperature in the presence of ATP. For both wild-type and ts045 G protein, trimers are selectively transported to the *cis*-Golgi compartment by a step that is sensitive to ATP, and is inhibited at 15°C.

It is clear that the quality control system that regulates transport from the ER works on a large variety of unrelated cellular and viral proteins and must therefore operate according to some very general principles. This and other studies show that the process of proper folding and successful attainment of correct quaternary structure is one general prerequisite for transport. Monomeric or incompletely assembled subunits of immunoglobulins (Mains and Sibley, 1983; Bole et al., 1986), retinal binding protein (Ronne et al., 1983), influenza HA (Gething et al., 1986) and the major histocompatibility complex (MHC) class I antigens (Severinsson and Peterson, 1984) are generally not transported. However, assembly in and of itself is not the only controlling signal for

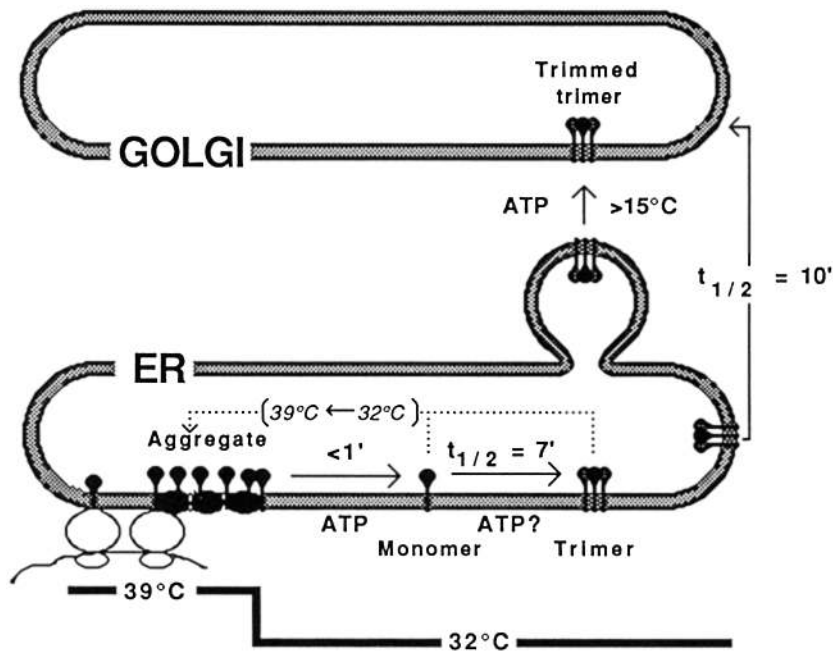


Figure 10. Model for trimerization and transport of G protein from the ER to the Golgi complex. Newly synthesized ts045 G protein monomers are associated with an aggregate at the nonpermissive temperature. Disassembly of the aggregate occurs with a reduction in temperature to 32°C and requires the presence of ATP. ATP may also be required for assembly of monomers to trimers, which occurs with a $t_{1/2}$ of ~ 7 min. Only G protein trimers are transported to the Golgi complex in an ATP and temperature-dependent step. Return to the nonpermissive temperature causes ER monomers and trimers, but not Golgi trimers, to reaggregate. Wild-type G protein may be transiently associated with an aggregate in an ATP-dependent fashion as well. Like ts045, only wild-type trimers are successfully transported from the ER.

transport. Recent studies have shown that mutant G proteins with sequence modifications in the COOH-terminal cytoplasmic domain (R. W. Doms and J. K. Rose, unpublished results) which are transported to the plasma membrane slowly or not at all will trimerize with wild-type kinetics. Thus, additional information resident in the cytoplasmic domain may also be required for transport (Rose and Bergmann, 1983; Doyle et al., 1985). Further studies using viral proteins such as the G protein and its mutants should prove useful in elucidating the signals inherent in the structure of a protein that allow its transport, as well as the cellular components involved in this complicated process. Our recent success in reconstitution of ER to Golgi transport *in vitro* using semi-intact cells should provide direct insight into this problem (Balch et al., 1987; Beckers et al., 1987).

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References

- Balch, W. E., M. Elliot, and D. S. Keller. 1986. ATP-coupled transport of vesicular stomatitis virus G protein between the endoplasmic reticulum and the Golgi. *J. Biol. Chem.* 261:14681-14689.
- Balch, W. E., and D. S. Keller. 1986. ATP-coupled transport of vesicular stomatitis virus G protein. Functional boundaries of secretory compartments. *J. Biol. Chem.* 261:14690-14696.
- Balch, W. E., K. R. Wagner and D. S. Keller. 1987. Reconstitution of transport of vesicular stomatitis virus G protein from the endoplasmic reticulum to the Golgi complex using a cell-free system. *J. Cell Biol.* 104:749-760.
- Beckers, C. J. M., D. S. Keller, and W. E. Balch. 1987. Semi-intact cells permeable to macromolecules: use in reconstitution of protein transport from the endoplasmic reticulum to the Golgi complex. *Cell.* 50:523-534.
- Bergmann, J. E., and S. J. Singer. 1983. Immunoelectron microscopic studies of the intracellular transport of the membrane glycoprotein (G) of vesicular stomatitis virus in infected Chinese hamster ovary cells. *J. Cell Biol.* 97:1777-1787.

- Bole, D. G., L. M. Hendershot, and J. F. Kearney. 1986. Posttranslational association of immunoglobulin heavy chain binding protein with nascent heavy chains in nonsecreting and secreting hybridomas. *J. Cell Biol.* 102:1558-1566.
- Copeland, C. S., R. W. Doms, E. M. Bolzau, R. G. Webster, and A. Helenius. 1986. Assembly of influenza hemagglutinin trimers and its role in intracellular transport. *J. Cell Biol.* 103:1179-1191.
- Crimmins, D. L., W. B. Mehard, and S. Schlesinger. 1983. Physical properties of a soluble form of the glycoprotein of vesicular stomatitis virus at neutral and acidic pH. *Biochemistry.* 22:5790-5796.
- Doms, R. W., and A. Helenius. 1986. Quaternary structure of influenza virus hemagglutinin after acid treatment. *J. Virol.* 60:833-839.
- Doms, R. W., A. H. Helenius, and J. M. White. 1985. Membrane fusion activity of the influenza virus hemagglutinin: the low pH-induced conformational change. *J. Biol. Chem.* 260:2973-2981.
- Doyle, C., M. G. Roth, J. Sambrook, and M.-J. Gething. 1985. Mutations in the cytoplasmic domain of the influenza virus hemagglutinin affect different stages of intracellular transport. *J. Cell Biol.* 100:704-714.
- Dubovi, E. J., and R. R. Wagner. 1977. Spatial relationships of the proteins of vesicular stomatitis virus: induction of reversible oligomers by cleavable protein cross-linkers and oxidation. *J. Virol.* 22:500-509.
- Dunphy, W. G., R. Brands, and J. E. Rothman. 1985. Attachment of terminal *N*-acetylglucosamine to asparagine-linked oligosaccharides occurs in central cisternae of the Golgi stack. *Cell.* 40:463-472.
- Flammond, A. 1970. Etude génétique du virus de la stomatite vésiculaire: classement de mutants thermosensibles spontanés engroupe de complémentation. *J. Gen. Virol.* 8:187-195.
- Gallione, C. J., and J. K. Rose. 1985. A single amino acid substitution in a hydrophobic domain causes temperature sensitive cell-surface transport of a mutant viral glycoprotein. *J. Virol.* 54:374-382.
- Gething, M.-J., K. McCammon, and J. Sambrook. 1986. Expression of wild-type and mutant forms of influenza hemagglutinin: the role of folding in intracellular transport. *Cell.* 46:939-950.
- Gibson, R., S. Schlesinger, and S. Kornfeld. 1979. The nonglycosylated glycoprotein of vesicular stomatitis virus is temperature sensitive and undergoes intracellular aggregation at elevated temperatures. *J. Biol. Chem.* 254:3600-3607.
- Gottlieb, C., J. Baenziger, and S. Kornfeld. 1975. Deficient uridine diphosphate-*N*-acetylglucosamine: glycoprotein *N*-acetylglucosaminyltransferase activity in a clone of Chinese hamster ovary cells with altered surface glycoproteins. *J. Biol. Chem.* 250:3303-3309.
- Helenius, A., E. Fries, H. Garoff, and K. Simons. 1976. Solubilization of the Semliki Forest virus membrane with sodium deoxycholate. *Biochem. Biophys. Acta.* 436:319-334.
- Hortsch, M., D. Avossa, and D. I. Meyer. 1986. Characterization of secretory protein translocation: ribosome-membrane interaction in endoplasmic reticulum. *J. Cell Biol.* 103:241-253.
- Hortsch, M., C. Crimmaudo, and D. I. Meyer. 1987. Structure and function of the rough endoplasmic reticulum. In Proceedings of the Fourth FAOB Congress: Symposium on Integration and Control of Metabolic Processes: Pure

- and Applied Aspects. ICSU Press, Miami, FL. In press.
- Jamieson, J. D., and G. E. Palade. 1968. Intracellular transport of secretory proteins in the pancreatic exocrine cell. IV. Metabolic requirements. *J. Cell Biol.* 39:589-603.
- Kielian, M., and A. Helenius. 1985. pH-induced alterations in the fusogenic spike protein of Semliki Forest virus. *J. Cell Biol.* 98:139-145.
- Knipe, D. M., D. Baltimore, and H. F. Lodish. 1977. Separate pathways of maturation of the major structural proteins of vesicular stomatitis virus. *Virology.* 21:1149-1158.
- Kreis, T. E. 1986. Microinjected antibodies against the cytoplasmic domain of vesicular stomatitis virus glycoprotein block its transport to the cell surface. *EMBO (Eur. Mol. Biol. Organ.) J.* 5:931-941.
- Kreis, T. E., and H. F. Lodish. 1986. Oligomerization is essential for transport of vesicular stomatitis viral glycoprotein to the cell surface. *Cell.* 46:929-937.
- Kvist, S., K. Wiman, L. Claesson, P. Peterson, and B. Dommerstein. 1982. Membrane insertion and oligomeric assembly of HLA DR histocompatibility antigens. *Cell.* 29:61-69.
- Lafay, F. 1974. Envelope proteins of vesicular stomatitis virus: effect of temperature sensitive mutations in complementation groups III and V. *J. Virol.* 14:1220-1228.
- Li, E., I. Tabas, and S. Kornfeld. 1978. The synthesis of complex oligosaccharides. I. Structure of the lipid-linked oligosaccharide precursor of the complex-type oligosaccharide of the vesicular stomatitis virus G protein. *J. Biol. Chem.* 253:7762-7770.
- Machamer, C. E., R. Z. Florkiewicz, and J. K. Rose. 1985. A single N-linked oligosaccharide at either of the two normal sites is sufficient for transport of vesicular stomatitis virus G protein to the cell surface. *Mol. Cell Biol.* 5:3074-3083.
- Mains, P. E., and C. H. Sibley. 1983. The requirement of light chain for the surface deposition of the heavy chain of immunoglobulin M. *J. Biol. Chem.* 258:5027-5033.
- Merlie, J. P. 1984. Biogenesis of the acetylcholine receptor, a multisubunit integral membrane protein. *Cell.* 36:573-575.
- Merlie, J. P., and J. Lindstrom. 1983. Assembly in vivo of mouse muscle acetylcholine receptor: identification of an alpha subunit species that may be an assembly intermediate. *Cell.* 34:747-757.
- Minami, Y., A. M. Weissman, L. E. Samelson, and R. D. Klausner. 1987. Building a multichain receptor: synthesis, degradation, and assembly of the T-cell antigen receptor. *Proc. Natl. Acad. Sci. USA.* 84:2688-2692.
- Ronne, H., C. Ocklind, K. Wiman, L. Rask, B. Obrink, and P. A. Peterson. 1983. Ligand-dependent regulation of intracellular protein transport: effect of vitamin A on the secretion of the retinol-binding protein. *J. Cell Biol.* 96:907-910.
- Rose, J. K., and J. E. Bergmann. 1983. Altered cytoplasmic domains affect intracellular transport of the vesicular stomatitis virus glycoprotein. *Cell.* 34:513-524.
- Rose, J. K., and C. Gallione. 1981. Nucleotide sequences of the mRNAs encoding the VSV G and M proteins as determined from cDNA clones containing the complete coding regions. *J. Virol.* 39:519-528.
- Rose, J. K., W. J. Welch, B. M. Sefton, F. S. Esch, and N. C. Ling. 1980. Vesicular stomatitis virus glycoprotein is anchored in the viral membrane by a hydrophobic domain near the COOH terminus. *Proc. Natl. Acad. Sci. USA.* 77:3884-3888.
- Rothman, J. E., and H. F. Lodish. 1977. Synchronized transmembrane insertion and glycosylation of a nascent membrane protein. *Nature (Lond.)*. 269:775-780.
- Saraste, J., and E. Kuismanen. 1984. Pre- and post-Golgi vacuoles operate in the transport of Semliki Forest virus membrane glycoproteins to the cell surface. *Cell.* 38:535-549.
- Schnitzer, T., C. Dickson, and R. A. Weiss. 1979. Morphological and biochemical characterization of viral particles produced by the ts045 mutant of vesicular stomatitis virus at restrictive temperature. *J. Virol.* 29:185-195.
- Severinsson, L., and P. A. Peterson. 1984. β_2 -Microglobulin induces intracellular transport of human class I transplantation antigen heavy chains in *Xenopus laevis* oocytes. *J. Cell Biol.* 99:226-232.
- Silverberg, M., and V. T. Marchesi. 1978. The anomalous electrophoretic behavior of the major sialoglycoprotein from the human erythrocyte. *J. Biol. Chem.* 253:95-98.
- Takatsuki, A., K. Kohno, and G. Tamura. 1975. Inhibition of biosynthesis of polyisoprenol sugars in chick embryo microsomes by tunicamycin. *Agric. Biol. Chem.* 39:2089-2091.
- Tkacz, J. S., and J. O. Lampen. 1975. Tunicamycin inhibition of polyisoprenyl N-acetylglucosaminyl pyrophosphate formation in calf liver microsomes. *Biochem. Biophys. Res. Commun.* 65:248-257.
- Vogel, R. H., S. W. Provencher, C.-M. Bonsdorff, M. Adrian, and J. Dubochet. 1986. Envelope structure of Semliki Forest virus reconstructed from cryo-electron micrographs. *Nature (Lond.)*. 320:533-535.
- White, J., M. Kielian, and A. Helenius. 1983. Membrane fusion proteins of enveloped animal viruses. *Q. Rev. Biophys.* 16:151-195.
- White, J., K. Matlin, and A. Helenius. 1981. Cell fusion by Semliki Forest, influenza and vesicular stomatitis viruses. *J. Cell Biol.* 89:674-679.