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Oligomerization Is Essential for Transport of Vesicular Stomatitis Viral Glycoprotein to the Cell Surface

Thomas E. Kreis,*‡ and Harvey F. Lodish†‡
*European Molecular Biology Laboratory
Postfach 10.2209
D-6900 Heidelberg, FRG
†Whitehead Institute for Biomedical Research
Nine Cambridge Center
Cambridge, Massachusetts 02142
‡Department of Biology
Massachusetts Institute of Technology
Cambridge, Massachusetts 02139

Summary

Using ts045, a temperature sensitive strain of Vesicular stomatitis virus, we show that oligomerization of G protein is a prerequisite for its transport from RER to the Golgi apparatus and for its subsequent maturation. While wild-type G forms an oligomer in the RER, ts045 G synthesized at the nonpermissive temperature does not. When the permissive temperature is reinstated, ts045 G forms an oligomer and moves to the Golgi. The state of oligomerization was determined by chemical cross-linking and by the ability of a microinjected monoclonal antibody specific for the carboxy-terminal five amino acids of the cytoplasmic tail of G to cause patching of G in intracellular membranes. We conclude that formation of an oligomer of G protein, probably a trimer, is necessary for G protein maturation.

Introduction

The vesicular stomatitis viral glycoprotein, G, like cell-surface glycoproteins, is cotranslationally glycosylated and inserted into the rough endoplasmic reticulum membrane (RER, reviewed by Sabatini et al., 1982; Wickner and Lodish, 1985). From the RER it is transported first to the Golgi complex (Bergmann et al., 1981; Wehland et al., 1982) and then to the plasma membrane, where assembly and budding of viral particles occurs (reviewed by Lenard and Compans, 1974; Atkinson, 1978; Lodish et al., 1980). In the same cell, different membrane and secreted proteins can take very different times for transport to the cell surface; the rate-limiting and distinctive step appears to be transport from the RER to an early (or medial) Golgi compartment (Fitting and Kabat, 1982; Lodish et al., 1983; Ledford and Davis, 1983; Williams et al., 1985). Also, many temperature-sensitive mutations of the G protein, as well as in other viral glycoproteins, caused accumulation of the glycoprotein at the nonpermissive temperature in the RER (Knipe et al., 1977; Bergmann et al., 1981; Lodish and Weiss, 1979). In at least the case of two G mutations, ts045 and tsL513, this block in maturation is reversible; following a shift down to the permissive temperature, G rapidly leaves the RER and migrates through the Golgi apparatus to the cell surface (Bergmann et al., 1981; Lodish and Kong, 1983). The single mutation in ts045 that blocks maturation is located in the large exoplasmic domain (Gallione and Rose, 1985).

In this report we use ts045 to show that formation of a dimer or higher oligomer of G is a prerequisite for maturation of G from the RER. Wild-type G forms an oligomer in the RER, while ts045 G synthesized at the nonpermissive temperature does not; when the temperature is lowered, ts045 G forms an oligomer and subsequently moves to the Golgi. While formation of an appropriate quaternary structure may be critical in regulation of intracellular transport, there has been no detailed analysis of the oligomerization of G during its biosynthesis. G remains fully soluble in nonionic detergent during maturation (Chatterjee et al., 1984) and therefore a tight association with the cytoskeletal framework is unlikely. Contradictory results have been obtained on the quaternary organization of G in virions. Dubovi and Wagner (1977) used chemical cross-linking of intact virions to show significant amounts of homo-oligomers of G as well as heterodimers of G with the viral matrix protein M. Crimmins et al. (1983) reported, on the other hand, that intact G, extracted from virus by octyl-β-D-glucoside had the physical properties of a monomer. Also, an aqueous soluble form of G, obtained by cathepsin D digestion of VSV, formed a monomeric molecule (Crimmins et al., 1983).

Specific alterations of the amino acid sequence in the cytoplasmic domain of G, introduced by genetic engineering, profoundly affect rate and extent of transport of G from the RER to the Golgi (Rose and Bergmann, 1983). It is unclear how such altered cytoplasmic domains reduced the rate of transport of G to the cell surface. For example, a specific interaction of that domain with another cytoplasmic component could be blocked, or the formation of a particular quaternary structure of G (i.e., oligomerization) could be obstructed. At least one asn-linked carbohydrate is essential for proper maturation of the G protein (Machamer et al., 1985), but how or why is not known. It is important to understand the role of the cytoplasmic tail as well as the other segments of the protein for proper folding and also intracellular transport of G. Our results suggest that proper quaternary folding of G is a prerequisite for its normal maturation.

Results

Antibodies against the Cytoplasmic Domain of G

Affinity-purified polyclonal (αP4) and monoclonal (P5D4) antibodies were prepared against a synthetic peptide containing the 15 carboxy-terminal amino acids of G (Kreis, 1986). P5D4 reacted with one epitope, containing the 5 carboxy-terminal amino acids (507–511) of G; and αP4 reacted with all epitopes tested within the carboxy-terminal cytoplasmic segment of G (Kreis, 1986). Significant cross-reaction of the antibodies with endogenous cellular proteins was not observed.

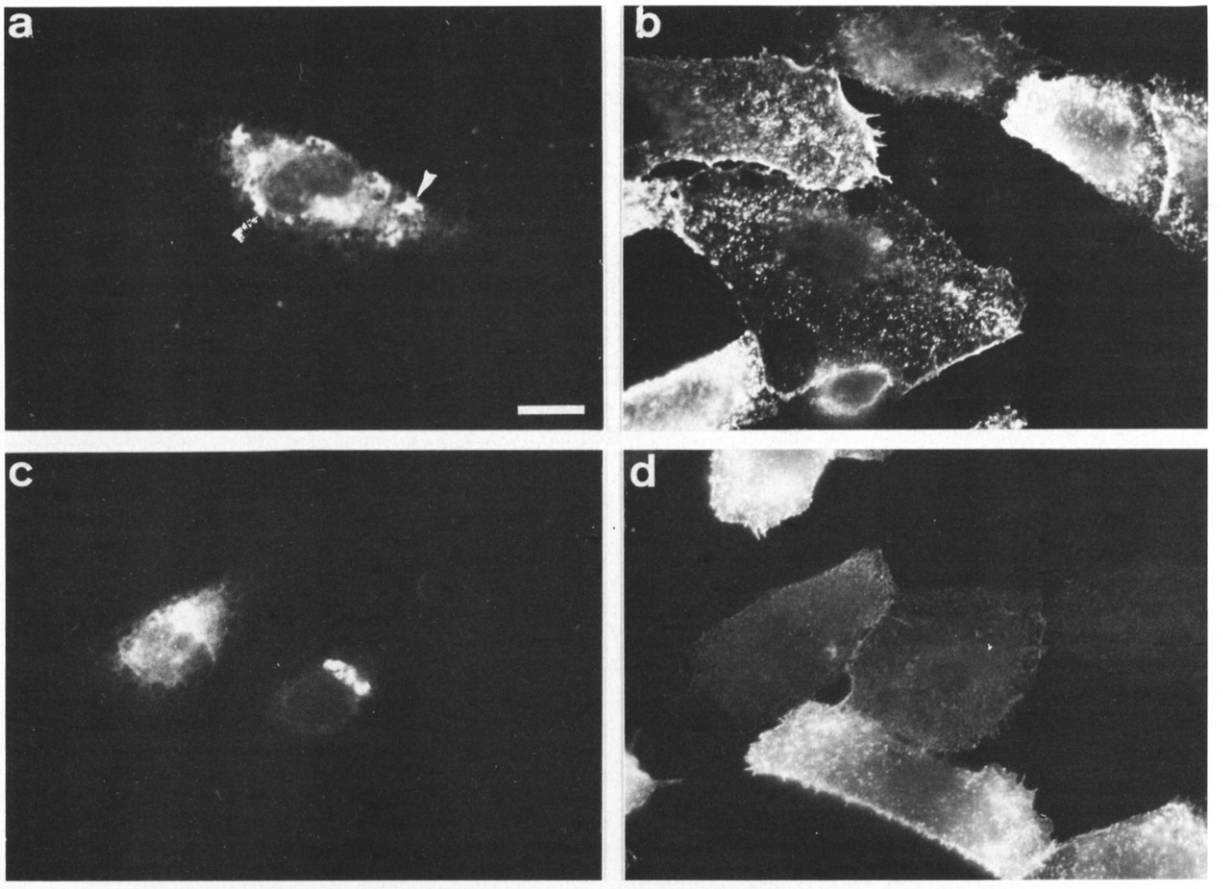

Figure 1. Effect of Microinjected Antipeptide Antibodies on Transport of G to the Cell Surface

Two hours after infection (at 39.5°C) with ts045-VSV, Vero cells were microinjected at 0°C to 4°C with (a and b) P5D4 (1 mg/ml) or (c and d) P5D4-Fabs (0.8 mg/ml). At 4.5 hr after infection at 39.5°C (for details, see Experimental Procedures), the cells were transferred into medium containing 10 μg/ml cycloheximide and incubated for 45 min at 31°C. The distribution of the microinjected P5D4 was visualized after fixation and permeabilization by rhodamine-conjugated second antibodies (a and c). Appearance of G at the surface of the same cells was monitored, prior to permeabilization with detergent, with rabbit anti-G and fluorescein-coupled second antibodies (b and d). The same cells are viewed in a and b, and in c and d. Arrowheads point to patches of aggregated G. Bar = 20 μm.

P5D4 remained homogeneously distributed following microinjection into uninfected Vero cells. When P5D4 or αP4 was microinjected into cells that contain G protein en route from the RER to the cell surface, however, both bound to all forms of VSV-G (Kreis, 1986).

Microinjected Antipeptide Antibodies Block Transport of G Protein to the Cell Surface

To study the effect of the antipeptide antibodies P5D4 and αP4 on transport of G to the cell surface, Vero or PtK2 cells were microinjected at 2 hr after infection by ts045 under nonpermissive conditions. Microinjection was performed after transferring the infected cells from culture medium at 39.5°C directly into Hank's buffered saline kept at 0°C–4°C. The coverslips with injected cells were transferred back to culture medium at 39.5°C immediately after microinjection was completed, usually within less than 10 min. G remained tightly arrested with the RER despite this 2-fold shift in temperature (cf. Figures 3a and 3b). During the subsequent 2 hr incubation at 39.5°C and 30 min at 31°C, divalent P5D4 completely blocked maturation and transport of G to the surface of the recipient cells (Figures 1a and 1b). Under similar conditions Fab fragments of P5D4, however, had no significant effect upon transport of G (Figures 1c and 1d), though the microinjected Fab become localized to discrete regions of the cytoplasm containing VSV-G (Figure 1c).

Aggregation of Intracellular ts045-G by Microinjected Monoclonal Antipeptide Occurs Only at the Permissive Temperature

In about 30% of ts045-infected cells microinjected with divalent P5D4, patches of aggregated G were observed, but only if the cells were shifted to the permissive temperature for 30–45 min (arrowheads in Figure 1, Figures 2d–2f, and Figure 3). We never observed patching or aggregation of G when cells infected with ts045-VSV and microinjected with P5D4 were kept at the nonpermissive temperature, where G is retained in the RER (Figures 2a–2c). Patching of ts045 G also occurred at 31°C, when rhodamine-coupled P5D4 was microinjected and no further labeling with second antibodies was required (data

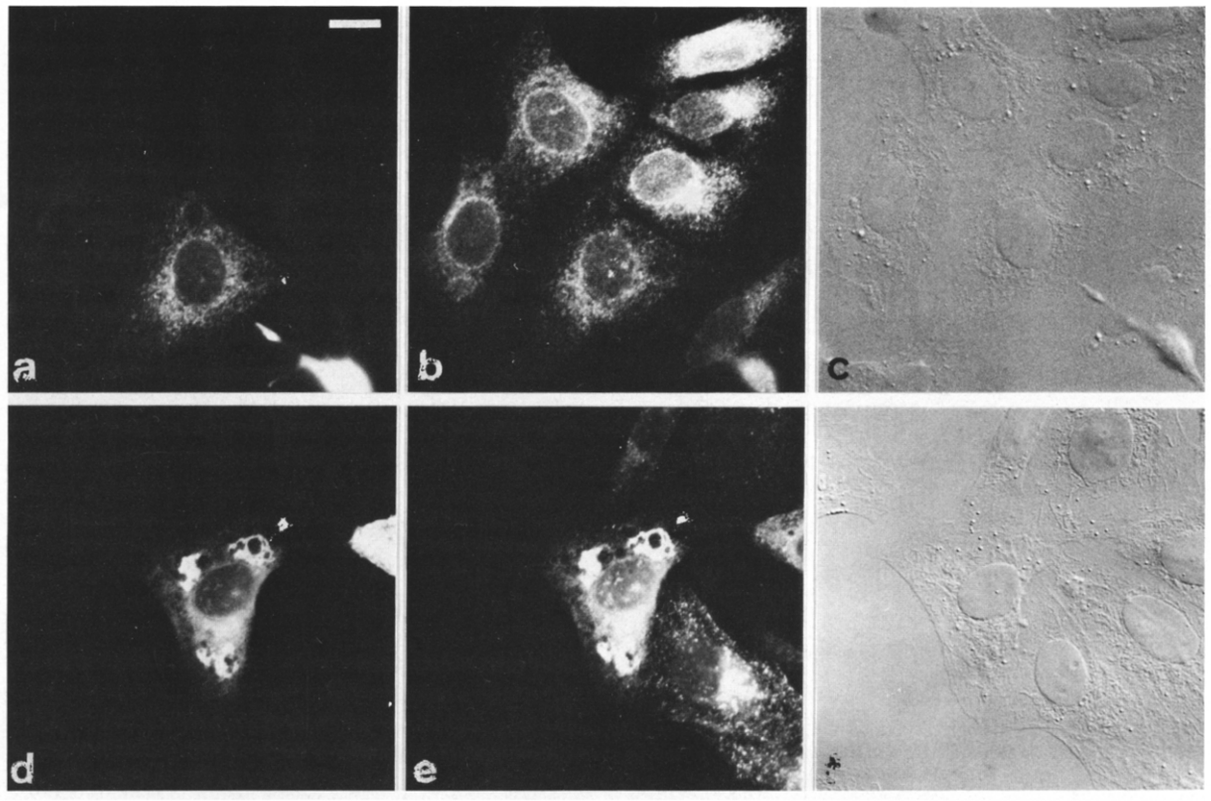

Figure 2. Aggregation of ts045-G by Microinjected Antipeptide Antibodies Occurs Only at Permissive Temperature

Verro cells were microinjected at 0°C to 4°C with P5D4 2 hr after infection (at 39.5°C) with ts045-VSV. Recipient cells were shifted for 45 min to 31°C 4.5 hr after infection (d, e, and f) or were kept at 39.5°C (a, b, and c). Microinjected P5D4 was visualized with rhodamine-coupled second antibodies (a and d), and G was monitored in the same cells after permeabilization with rabbit anti-G and fluorescein-coupled anti-rabbit antibodies (b and e). The same areas were also photographed with Nomarski optics (c and f). Arrowheads indicate patches of aggregated G. Bar = 20 μm.

not shown). Similar patches as those induced by P5D4 were also detected in cells microinjected with the polyclonal antibodies αP4 (data not shown). Patching of G was never observed in cells microinjected with Fab fragments of P5D4 or αP4 (data not shown). Patching of G was observed in various VSV-infected tissue culture cells, including Vero cells (Figures 1–3), PtK_2 cells (data not shown), and BHK cells (Arnheiter et al., 1984) and thus, seemed not to be a cell-specific phenomenon. Patching proceeded only when ts045-infected cells were shifted to the permissive temperature (Figures 2d–2f). We feel that, at 39.5°C, ts045 G protein is in a monomeric form and becomes oligomeric at 31°C. This conclusion is supported by the chemical cross-linking studies (below).

Aggregation of G Occurs in a Late or Post-RER Compartment

Double immunolabeling was performed in an attempt to localize the intracellular compartment where patching of G was induced by the microinjected antipeptide antibodies. Only weak staining of G patches could be detected with antibodies reacting with RER (Louvard et al., 1982; Figures 3a–3c) or antidocking protein (provided by M. Hortsch and D. Meyer, EMBL, not shown). Thus patching of G probably occurred in a late or post-RER compartment. Usually there was no overlapping labeling of patches of G with Golgi-specific markers like fluorescein-conjugated wheat germ agglutinin (Figures 3d–3f) or antibodies recognizing galactosyltransferase (Roth and Berger, 1982; not shown). Furthermore, no significant transport of G to the Golgi complex seemed to have proceeded in cells microinjected with P5D4 or αP4 (cf. Figure 2 with Figure 3). Very little G, visualized by the microinjected antipeptide antibodies, could be detected in the perinuclear area of the Golgi complex (cf. Figure 2d with Figure 2e). Therefore, patching of G by microinjected P5D4 or αP4 most likely occurred in a late RER or early Golgi compartment, or in between.

Disulfide-Linked G Oligomers and Chemical Cross-Linking of G with DTSP

Chemical cross-linking with 10^{-4} M DTSP was performed on living cells infected with VSV in order to retain the native, endogenous membrane organization (Figure 4). Trypan blue exclusion during the reaction showed that the plasma membrane of cross-linked cells remained intact. The concentration of the DTSP cross-linker was kept low to prevent nonspecific coupling. Cross-linking of wild-type G clearly occurred (Figure 4b, 4c, 4f, and Figure 5) and cross-linking of G to other viral proteins did not (Figures 5g and 5h). Increasing the concentration of DTSP above 2×10^{-4} yielded a higher proportion of cross-linked G

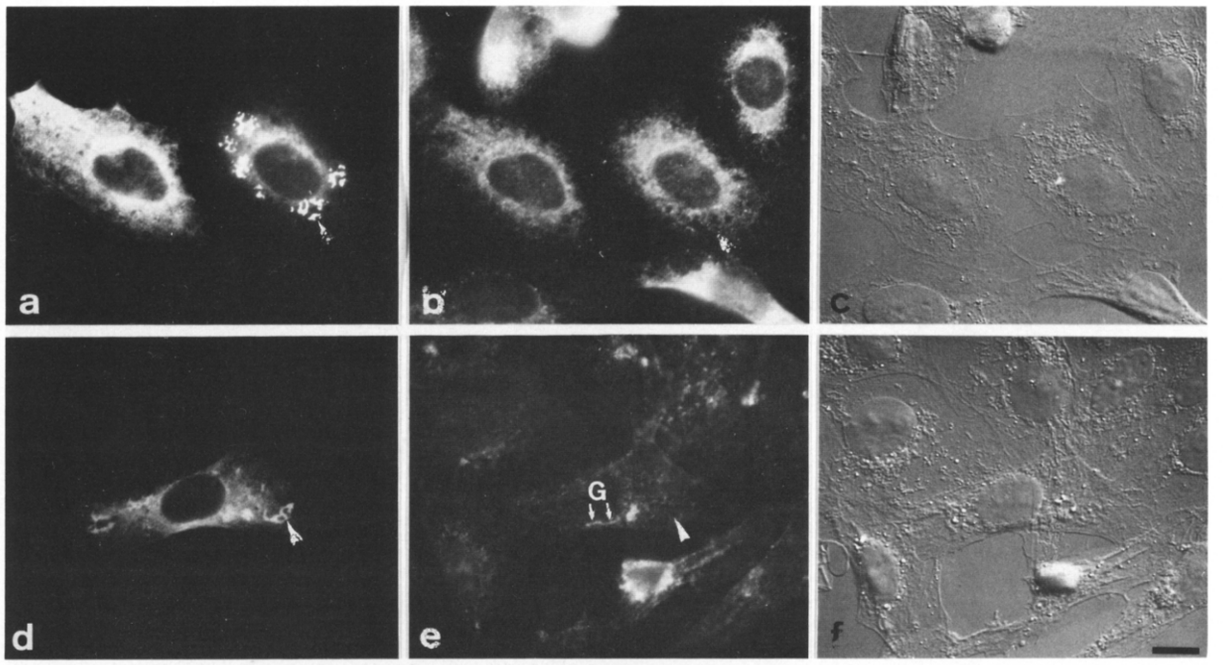

Figure 3. Localization of G Patches by Double Labeling with Specific Markers for RER and Golgi Complex

Microinjection of ts045-VSV-Infected Vero cells with P5D4 and subsequent culture at 31°C was performed as described in Figure. 1. Cells microinjected with P5D4 and labeled with second rhodamine-conjugated antibodies (a and d) were double-stained with fluorescein with anti-RER antibodies (b; Louvard et al., 1982); or (e) with fluorescein-conjugated wheat germ agglutinin (1 μg/ml). (a, b, and c) and (d, e, and f) show the same cells as in c and f visualized by Normarski optics. Arrowheads point to patches of aggregated G, and small arrows indicate the area of the Golgi complex. Bar = 20 μm.

oligomers; however, cross-linking of G to other viral proteins, which were considered to be nonspecific, occurred (data not shown). The appearance of a similar pattern of oligomers of viral nucleocapsid protein N (data not shown), as reported by Chatterjee et al. (1984), further verified that under these conditions cross-linking of protein located within the cytoplasm could occur.

In wild-type-infected cells, following a 10 min pulse with [^{35}S]methionine and a 10 min chase, over 70% of labeled G is sensitive to Endoglycosidase H, and thus is still within the RER or possibly in an early Golgi compartment. Reaction of such cells with DTSP generated radiolabeled proteins, immunoprecipitated with anti-G antibodies, that migrated on nonreduced SDS-PAGE with apparent molecular weights corresponding to dimers and trimers of G (Figures 4b and 4c). The various bands in the region of oligomeric G (G_2 and G_3, Figures 4b and 4c) may reflect coupling of G with increasing levels of intramolecular cross-links. By cross-linking infected cells pulse-chased with [^{3}H]mannose, a pattern of oligomers of G was generated that was virtually identical with that obtained by [^{35}S]methionine labeling (Figures 4e and 4f). Furthermore, when partially purified VSV particles labeled with [^{35}S]methionine were cross-linked, predominantly bands indicative of G dimers, but not trimers, were generated (Figure 4d).

Analysis of immunoprecipitated cross-linked G on two-dimensional SDS-PAGE, nonreduced in the first dimension and under reducing conditions in the second, cleaving DTSP-induced cross-links, demonstrated the absence of labeled proteins other than G in the oligomers (data not shown). Furthermore, when cross-linking was performed on cells metabolically labeled for 15 hr with [^{35}S]methionine prior to infection with VSV, which then proceeded in the absence of [^{35}S]methionine, material immunoprecipitated with αP4 antibodies did not contain significant amounts of labeled cellular protein (data not shown).

To test whether occurrence of cross-linked G was due to random collision of G by diffusion in the membrane, infected cells were cross-linked after a 3 min solubilization with a 1% solution of the nonionic detergent NP-40 in PBS (Figure 4c). There was no significant difference in the pattern of G oligomers whether or not detergent was present (Figures 4b and 4c).

We conclude that a significant fraction of wild-type G protein, both shortly after synthesis and in virions, is part of a G oligomer. Because we deliberately used a low concentration of DTSP, we cannot be sure of the proportion of G in oligomers. When ts045-VSV-infected Vero cells were pulsed for 10 min with [^{35}S]methionine at 39.5°C 4.5 hr after infection, chased for 5 min at 39.5°C and then for 7 min at 31°C, were cross-linked with 10^{-4} DTSP, a significant fraction of labeled G was in oligomers (Figure 5d). Densitometric scanning of the autoradiographs indicated about 20% and 12% of dimeric and trimeric G, respectively. At that period of chase more than 95% of VSV was present in an Endo H-sensitive form.

Importantly, in anti-G immunoprecipitates of samples

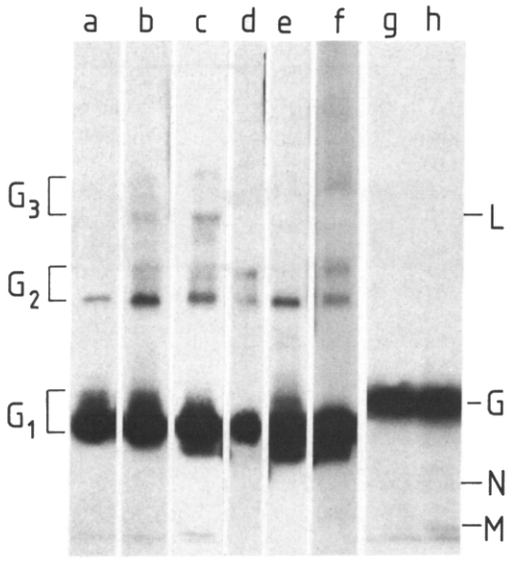

Figure 4. Cross-Linking of Viral Proteins in Infected Vero Cells and Virus Particles

Vero cells were pulsed for 10 min with [^{35}S]methionine 4.5 hr after infection with wild-type VSV and subsequently chased for 10 min (a–c, g, and h). Isolation of radiolabeled VSV particles (d) and pulse-labeling of infected cells with [^{3}H]mannose (e and f) were performed as described in Experimental Procedures. Intact cells or virus particles were cross-linked with 10^{-4} M DTSP (b, c, d, f, and h) or kept as the non-cross-linked reference (a and e). In (c) cells were permeabilized for 3 min with 1% NP-40 in PBS prior to cross-linking. Lysates of cross-linked cells or virus particles were immunoprecipitated with αP4 (a–c, g, and h) or αG2 (d–f). Precipitated material was run nonreduced (a–f) or in the presence of β-mercaptoethanol as reducing agent (g and h). Thus g and h are the parallel reduced samples of a and b, respectively. G protein in its reduced, denatured form migrates significantly slower than in the nonreduced form (compare a and g for example). L, G, N, and M indicate the viral proteins. G_1, G_2, and G_3 indicate monomeric, dimeric, and trimeric G, respectively.

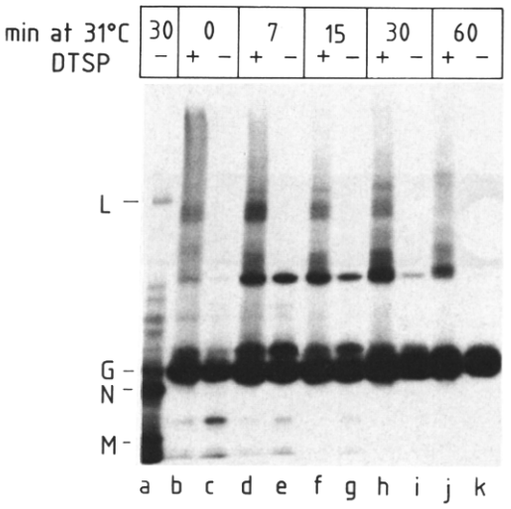

Figure 5. Cross-Linking of [^{35}S]Methionine-Labeled ts045 G Protein during Maturation

ts045-VSV-infected Vero cells were pulsed with [^{35}S]methionine for 10 min at 39.5°C (for details see Experimental Procedures). After a chase in normal culture medium for 60 min at 39.5°C (b and c); 7 min (d and e); 15 min (f and g); 30 min (h and i); or 60 min (j and k) at 31°C, one-half of the cells were cross-linked with 10^{-4} M DTSP (b, d, f, h, and j) or were kept on ice as the non-cross-linked reference (c, e, g, i, and k). The cells were then lysed and immunoprecipitated with αP4, and the precipitated material was run on SDS-PAGE under nonreducing conditions. (a) The total cell lysate at 30 min after the [^{35}S]methionine pulse; L, G, N, and M indicate the respective viral proteins.

not treated with DTSP, but analyzed under nonreducing conditions, about 11% of the G radioactivity migrated with an apparent molecular weight corresponding to dimeric G (Figure 5e). This probably represents a disulfide-linked "native" dimer of G, since addition of reducing agent quantitatively yielded monomeric G (Figure 4g).

Formation of Oligomers during Maturation of ts045 G Protein

When [^{35}S]methionine-labeled ts045-infected cells were chased and maintained at 39.5°C, no native disulfide-linked dimers of G could be detected in anti-G immunoprecipitates (Figure 5c). This is additional evidence that ts045 G does not form normal (disulfide-linked) oligomers at 39.5°C. Disulfide-linked dimers were formed within 7 min after the pulse-chase labeled cells were placed at 31°C (Figure 5c). This dimeric form of G gradually disappeared with increasing time periods of incubation at 31°C (Figures 5g, 5i, and 5k), as G protein is incorporated into virions. It is noteworthy that this dimeric form of G remained completely sensitive to Endo H (not shown) and may represent an intracellular, disulfide-linked G oligomer that is an intermediate in G biosynthesis.

Additionally, when [^{35}S]methionine-labeled ts045-infected cells, maintained at 39.5°C, were cross-linked with DTSP, little (<7%) of G protein was recovered in oligomers (Figure 5b). Residual aggregation may have occurred when the temperature was shifted down from 39.5°C to 0°C during the DTSP reaction. Within 7 min after shifting the cells to 31°C, over 30% of the labeled (at 39.5°C) G was found in cross-linked oligomers (Figure 5d). At this time, all of the labeled G is sensitive to Endo H (not shown), and thus has not yet passed through the medial Golgi. We conclude that, at 39.5°C, ts045 G is in a monomeric state. It forms an oligomer, probably a trimer, within minutes after shifting to 31°C, prior to Golgi modification of its oligosaccharides.

The amounts of cross-linked oligomers of G gradually decreased with prolonged incubation at 31°C (Figures 5f, 5h, and 5j) as G acquired resistance towards Endo H. After a 60 min chase at 31°C predominantly DTSP-cross-linked dimers of G remained associated with the host cells (Figure 5j). Interestingly only DTSP-cross-linked G dimers, not native disulfide-linked dimers could be detected in isolated virions of VSV (Figure 4d). Each of the DTSP-cross-linked, anti-G precipitates lacked viral proteins other than G when run after reduction on SDS-PAGE (not shown). Moreover, virtually no G_s was detected in immunoprecipitates of either cross-linked cells or virions.

Discussion

The ts045 mutant of VSV has proven particularly useful for study of maturation of cell-surface glycoproteins. At

39.5°C, the G protein is inserted normally as a transmembrane protein into the membrane of the RER, and receives normally the two asn-linked oligosaccharides (Zilberstein et al., 1980; Lodish and Kong, 1983). Maturation of ts045 G is blocked in the RER at 39.5°C. Gibson et al. (1978) detected a change in detergent extractability of ts045 G from cells at 39°C, relative to 31°C, suggesting some abnormality in G conformation. Soon after the temperature is lowered to 31°C, the G protein rapidly moves to the Golgi, where the oligosaccharides are modified, and thence to the cell surface.

Our principal conclusion is that wild-type G protein forms an oligomer, probably a trimer, soon after synthesis. It is also an oligomer in virions, but our cross-linking studies only allow us to conclude that it is at least dimeric (Figure 4d). By contrast, ts045 G at 39.5°C is a monomer. It forms an oligomer within minutes after shifting the temperature to 31°C, prior to transport through the Golgi. We conclude that formation of an oligomer of G, probably a trimer, occurs in the RER, and is essential for maturation of G to or through the Golgi. Our studies use microinjection of specific antibodies and also chemical cross-linking with a cleavable bifunctional reagent.

Microinjection of a monoclonal antibody, specific for the carboxy-terminal 5 amino acids of G, into ts045-infected cells at 39.5°C did not lead to patching of G protein (Figures 2a and 2b). Importantly, however, the microinjected monoclonal antibody did induce aggregation of the viral glycoprotein into large patches upon shift-down of ts045-infected cells to the permissive temperature (Figures 2d and 2e; Figures 3a and 3d). Recently, Arnheiter et al. (1984) showed that polyclonal antibodies directed against the 22 carboxy-terminal amino acids of G induced aggregation of G in intracellular membranes. It is not surprising that microinjected polyclonal antibodies could, in situ, lead to an immunoprecipitation reaction, given the right ratio of antibodies to antigen. Aggregation of G induced by microinjected polyclonal antibodies against the cytoplasmic tail of G (αP4) could also have occurred if G had been present exclusively in a monomeric form, since the polyclonal αP4 reacts with several epitopes present in that domain of the transmembrane protein (Kreis, 1986). By definition, however, divalent monoclonal antibodies, reacting with only one epitope on a protein, can induce patching only if the protein is present in an oligomeric form. Since the epitope recognized by P5D4 was confined to the five very carboxy-terminal amino acids of the cytoplasmic domain of G (Kreis, 1986), oligomerization of G must have occurred during normal maturation. Conversely, the failure of microinjected P5D4 to aggregate ts045 G protein at 39.5°C suggests that it is a monomer at this temperature.

We have corroborated the presence of oligomers (predominantly dimers and trimers) of G by chemical cross-linking with DTSP according to the protocol of Lomant and Fairbanks (1976). In order to keep membrane topology undisturbed, DTSP was added to intact virus-infected cells. Virtually identical patterns of cross-linked proteins were observed whether DTSP was added to non-permeabilized or to detergent-extracted cells. Under our reaction conditions we found virtually no cross-linking of G with either viral N- nor M-protein (Figure 4 and Figure 5). Dubovi and Wagner (1977) found homo-oligomers of G, as well as hetero-dimers of G with M-protein when they cross-linked purified vesicular stomatitis virions. This difference with our results may be explained by their use of isolated virus and also of a 5-fold higher concentration of DTSP in their cross-linking experiments. We kept the concentration of DTSP low on purpose, since we tried to avoid unspecific coupling reactions (for a critical appraisal of chemical cross-linking, see Ji, 1979). Obviously, this strategy yielded reduced cross-linking efficiency. Nevertheless, the cross-linking experiments (Figure 4 and Figure 5) and the microinjection studies, taken together, provide strong evidence for the presence of oligomers of G within the intact host cells. Crimmins et al. (1983) analyzed the physical properties of G after solubilization of virions with octyl-β-D-glucoside or cathepsin D digestion and found predominantly monomeric G. These results together with ours indicate that the transmembrane and/or cytoplasmic domains are involved in maintaining the quaternary structure of G.

The membrane glycoproteins of VSV and influenza virus always appear as straight spikes when virions are analyzed by electron microscopy (cf. Wilson et al., 1981; Adrian et al., 1984). Clearly a trimeric or tetrameric form of a viral spike protein can contain a higher degree of rigidity than can a monomer that is anchored to the membrane by a single transmembrane α-helix. (A stool must have a minimum of three legs!) X-ray crystallography of influenza virus hemagglutinin and neuraminidase membrane glycoproteins showed that both are assembled into a quaternary structure corresponding to a trimer and tetramer, respectively (Wilson et al., 1981; Varghese et al., 1983). By analogy to influenza virus, the native spike glycoprotein of VSV may also be oligomeric.

Analysis of genetically engineered mutant membrane proteins has not revealed a strict requirement for an intact cytoplasmic domain for proper transport to the plasma membrane. Considerable deletions can be introduced into the cytoplasmic domains of the LDL receptor, H-2L^d transplantation antigen, and Semliki Forest virus glycoprotein E2 without any significant effects upon maturation of these transmembrane proteins (Garoff et al., 1983; Zuniga et al., 1983; Murre et al., 1984; Lehrman et al., 1985). Analysis of several mutants of the VSV G protein and of the influenza hemagglutinin with altered cytoplasmic tails has shown that this domain may play an important role in the transport to the plasma membrane (Rose and Bergmann, 1983; Doyle et al., 1985). Yet, no specific sequence in the cytoplasmic domain appeared to be required for proper transport. These workers did not analyze whether the introduced alterations affected the state of oligomerization of the glycoproteins. An altered primary structure within the cytoplasmic tail, for instance, may interfere with close apposition of these domains and result in an inhibition of oligomerization of the membrane proteins. Our hypothesis that oligomerization is essential for proper transport of G to the plasma membrane is compatible with the properties of these mutant viral membrane proteins.

Maturation of other membrane proteins from the RER requires that they fold into oligomers. The MHC H2 and HLA-A heavy chains, for instance, do not exit the RER unless combined with β2-microglobulin (Ploegh et al., 1979; Owen et al., 1980).

Arnheiter et al. (1984) observed patches of G, induced by microinjected polyclonal antibodies recognizing the 22 carboxy-terminal amino acids of G, in large vacuoles, which they tentatively identified as dilated cisternae of the endoplasmic reticulum. We tried in two independent ways to localize the intracellular compartment where patching of G occurred: double labeling immunocytochemistry with RER- and Golgi-specific markers and following with chemical cross-linking the maturation of [^{35}S]methionine pulse-labeled ts045 G. Consistently, we found no patching at 39.5°C, where ts045 G was arrested in the RER. Also, cross-linking with DTSP revealed only low amounts of G oligomers as long as the ts045-infected cells were kept at the nonpermissive temperature (Figure 5b). Nor were native, disulfide-linked G dimers observed in (non-cross-linked) ts045-infected cells at 39.5°C (Figure 5c). Thus, we concluded that oligomerization of G did not occur at 39.5°C. Gallione and Rose (1985) suggested that reversible denaturation of ts045 G occurs at 39.5°C. Partial denaturation might interfere with oligomerization of G, either by restriction of lateral mobility within the membrane bilayer or by inducing a conformational change in its tertiary structure that prevents self-association.

Oligomerization of ts045 G into dimers and trimers occurred within 7 min after shifting the host cells to the permissive temperature. At that time, virtually all G was completely sensitive to Endo H. It may, however, already have entered the cis-cisternae of the Golgi complex (Bergmann et al., 1981). Double labeling of P5D4-induced G patches revealed weak staining with RER-specific antibodies, but showed exclusion of Golgi markers (Figure 3). Since we never observed accumulation of G in the perinuclear area where the Golgi complex usually is located, we concluded that G patching and oligomerization must have occurred in a pre-Golgi compartment (cf. also Arnheiter et al., 1984). Most likely oligomerization occurred within the RER membrane immediately after the shift to 31°C. By analogy to BHK cells infected with Semliki Forest virus, where the density of the E1 glycoprotein in the RER membrane is only 93 molecules per μm^2 (Quinn et al., 1984), the density of G molecules in the RER may have been too low to allow patching. Oligomerization and patching of G probably occurred in a compartment located in between RER and Golgi where initial sorting and accumulation of G proceeds (Palade, 1975; Saraste and Kuismanen, 1984; Tooze et al., 1984). Further experiments, such as double immunolocalization at the electronmicroscopic level of the G-P5D4 complex, together with specific intracellular membrane markers, are required to localize more precisely the compartment where oligomerization of G occurs.

Concomitant with the temperature shift-down to 31°C, a disulfide-linked dimeric form of ts045G was formed (Figure 5e). This homodimer was not detected when ts045-infected cells were kept at the nonpermissive temperature. An enzyme involved in formation of disulfide bonds, protein disulfide isomerase, has been characterized, and is thought to be localized to the RER (Brockway et al., 1980; Roth and Koshland, 1981; Edman et al., 1985). Thus, the native disulfide-linked G dimer may form in a late RER compartment.

Microinjected divalent antipeptide antibodies completely inhibited transport of G to the plasma membrane. Although not all these microinjected cells exhibited patches of G, submicroscopal aggregation might have occurred that effectively interfered with the delivery of G to the plasma membrane. Furthermore, polyclonal αP4-Fabs blocked transport of G to the cell surface as well, whereas monoclonal P5D4-Fabs had no effect (Kreis, 1986). Thus αP4-Fabs could bind to a domain on the cytoplasmic tail of G, which is essential for oligomerization. Whether the cytoplasmic domain of G is directly involved in guiding transport of G to the plasma membrane remains an open question. Specific regions located within this domain of the transmembrane protein, however, may be essential for oligomerization and thus for G maturation. Further experiments are required to analyze whether oligomerization of G is sufficient for specific delivery of G from the RER to the plasma membrane.

Experimental Procedures

Cells and Viruses

Vero cells (African green monkey kidney cells) or PtK_2 cells (potoroo kidney cells) were grown in MEM containing 5% fetal calf serum (FCS), 1% nonessential amino acids, and 1% L-glutamine. For microinjection experiments, cells were grown on No. 1 glass coverslips.

Wild-type and ts045-VSV (Indiana serotype) were plaque-purified on monolayer Vero or PtK_2 cultures. Stock viruses were diluted into PBS containing 1% FCS and adsorbed to cells at a multiplicity of 10 for 1 hr at room temperature. Unadsorbed virus was removed, and cells were incubated in regular cell culture medium at the temperature indicated.

Microinjection of Antibodies and Immunolabeling

Polyclonal and monoclonal antibodies against a synthetic peptide containing the 15 carboxy-terminal amino acids of G (Rose and Gallione, 1981) were prepared and purified as described (Kreis, 1986).

Microinjection of cells grown on glass coverslips was performed as described (Kreis and Birchmeier, 1982; Kreis et al., 1983). Immunofluorescence on microinjected cells was performed by fixation in 3% paraformaldehyde (PFA) followed by permeabilization of membranes with 0.1% Triton X-100 in PBS and incubation with specific antibodies as described (Geiger and Singer, 1979). For staining of G at the cell surface, cells fixed with PFA were incubated for 20 min with antibodies directed against extracellular epitopes of G, then rinsed with PBS to wash away unbound antibodies, and fixed again with 3% PFA to prevent diffusion of extracellular antibodies into cytoplasmic domains. These cells were subsequently permeabilized with Triton X-100 as described above and labeled by double immunofluorescence. Sheep anti-rabbit IgG and goat anti-mouse IgG antibodies (Cappel Laboratories, West Chester) were coupled with rhodamine or fluorescein (Bradtzaeg, 1973) and used as second antibodies. Fluorescence microscopy was performed as described (Kreis et al., 1982). Immunoblotting was performed as described (Burnette, 1981; Burke et al., 1982).

Anti-N protein was a generous gift of Dr. A. Huang (Children's Hospital Medical Center, Boston). Polyclonal and monoclonal antibodies directed against extracellular epitopes of G protein were kindly provided by Dr. K. Simons (EMBL, Heidelberg) and Dr. M. Paternak (MIT, Cambridge). Anti-docking protein and antibodies to the RER were obtained from Drs. M. Hortsch, D. Meyer, and G. Warren (EMBL, Heidelberg). Anti-galactosyltransferase was a generous gift of Dr. E. Berger (University of Bern). Fluorescein-labeled wheat germ agglutinin was purchased from Behring Diagnostics (San Diego, CA).

Labeling of Infected Cells with [^{35}S]Methionine or [^{3}H]Mannose

Cells infected with either wild-type or ts045-VSV were pulsed for 10 min at 37°C or 39.5°C, respectively, 4 to 4.5 hr after infection in methionine-deficient medium with 100 μCi [^{35}S]methionine/ml, or for 30 min with 100 μCi [^{3}H]mannose/ml of medium with reduced glucose concentration, essentially as described (Zilberstein et al., 1980), except that no actinomycin D was added.

Partially purified virions were prepared from [^{35}S]methionine or [^{35}H]mannose-labeled VSV wild-type-infected Vero cells as described (Zilberstein et al., 1980). Cells were labeled by addition of radioactive isotopes at 100 μCi/ml at 3 hr and 5 hr after infection, and the culture supernatant was harvested 9 hr after infection.

Chemical Cross-Linking of Living Cells

Chemical cross-linking of radiolabeled, infected Vero cells was performed about 4.5 hr after infection, when cytopathic effects due to virus infection were still low. Cross-linking was performed with the cleavable, bifunctional reagent dithiobis(succinimidyl)propionate (DTSP; Pierce, Rockford) on intact, living cells. DTSP was diluted 100-fold with PBS from a 100 mM stock in DMSO immediately before reaction. Twenty-five microliters of 1 mM DTSP in PBS was added to 225 μl PBS containing about 10^6 living Vero cells in suspension (cells were detached from the cell culture dish with a disposable cell scraper, washed, and resuspended appropriately into ice-cold PBS). This reaction suspension was vortexed gently, and cross-linking was allowed to proceed for 30 min at 0°C. Remaining cross-linker was then inactivated by addition of 2 mM glycine. Another sample of cells from the same stock was run in parallel as the non-cross-linked control. Intactness of cells exposed to this treatment, with or without addition of DTSP, was tested by Trypan blue exclusion.

Immunoprecipitation of Viral Proteins

Cells were lysed for immunoprecipitation into an equal volume of 2× lysis buffer B (2% NP-40; 20 mM Tris-HCl; 300 mM NaCl; and 2 mM EDTA, pH 7.4) containing 0.2 mM PMSF and 2 mg/ml BSA for 30 min on ice. Nuclei and aggregated material were removed by a 15 min centrifugation at 4°C and 10,000 × g. The supernatant was then incubated overnight with 5 to 20 μg of specific antibodies. Antigen–antibody complexes were bound to 100 μl of a 1:1 slurry of protein-A Sepharose 4B (Pharmacia, Uppsala) in buffer B and subjected to a 30 min incubation at 4°C. The beads were washed with each of the following buffers in turn: buffer B containing 0.6 M NaCl and 5 mg BSA/ml; buffer B with 0.6 M NaCl; and finally buffer B. Antigen was released by boiling the last pellet in a minimal volume of gel sample buffer. For Endo H treatment, antigen was released by boiling the last pellet for 5 min in 50 μl of 0.3 M citrate and 0.1% SDS (pH 5.5). After cooling down the suspension, Endo H was added (Zilberstein et al., 1980) and, after incubation overnight at 37°C, gel sample buffer was added.

Proteins were analyzed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) according to the method of Laemmli (1970) using 5% to 8% linear gradients of acrylamide. Fluorography was performed with 1 M Na salicylate as described (Chamberlain, 1979).

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