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Further Characterization of the Vesicular Stomatitis Virus Temperature-Sensitive O45 Mutant: Intracellular Conversion of the Glycoprotein to a Soluble Form

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Reexamination of the viral products of *ts*O45, a glycoprotein mutant of vesicular stomatitis virus, showed that at 39°C there was a conversion of the glycoprotein (G) to a truncated, soluble form, G_s, which subsequently appeared in the extracellular medium. The half-life for this intracellular conversion and extracellular appearance was about 2 h at 39°C. G_s was precipitated by a monoclonal antibody to the ektodomain but not by an antipeptide serum made against the first 15 amino acids at the carboxy terminus of G. G_s was also resistant to endoglycosidase H digestion. On the basis of pulse-chase experiments, the generation of G_s most probably occurred in the rough endoplasmic reticulum. This additional phenotype of the *ts*O45 mutant provides another approach for studying the generation and subsequent transport of a secreted protein in fibroblast cells.

The glycoprotein (G) of vesicular stomatitis virus (VSV) is a potent viral immunogen (15; C. S. Reiss, S. L. Chen, A. S. Huang, and R. Doherty, Microbial Pathol., in press) as well as an excellent tool for dissecting the synthetic and transport steps of integral membrane glycoproteins (14, 27, 34). From the sequencing of the gene (25) and biochemical analyses of the protein (2, 3, 5, 24, 26, 28, 29) the mature VSV G can be divided into the following three functional domains: (i) the ektodomain, containing 90% of the protein, with two asparagine-linked complex carbohydrate chains and a hemolytic six-amino-acid peptide at the amino terminus; (ii) the hydrophobic transmembrane region, containing 20 amino acids; and (iii) the cytoplasmic domain, containing 29 amino acids at the carboxy terminus of the protein, with a cysteine residue near the transmembrane region for covalent palmitic acid attachment.

During virus infection a truncated, soluble form of G called G_s accumulates in the extracellular fluids (13). It is thought to consist of only the ektodomain with intact carbohydrate side chains (4, 12, 21). Although it was assumed to be a specific degradation product of G at the cell surface, G_s was recently detected intracellularly in association with the microsomal membrane fraction (9; R. Doherty and A. S. Huang, unpublished observations).

To learn more about viral glycoproteins as well as their transport mechanisms, conditional lethal mutants may be useful. *ts*O45 is a well-characterized temperature-sensitive mutant of VSV with a lesion in the glycoprotein (7, 19). At the nonpermissive temperature of 39°C, G is synthesized but remains in an endoglycosidase H-sensitive state, presumably in the rough endoplasmic reticulum (1, 16, 19). At 39°C the protein gradually degrades completely (16, 19). Although little to no infectious virus is produced, extracellular virions are found which appear bald or spikeless by electron microscopy (30). Gel electrophoresis of these particles shows the absence of G (20). Gallione and Rose (8) sequenced the gene of the *ts*O45 glycoprotein mutant and found many differences between this and the Orsay wild type. Use of a series of DNA recombinants showed that the relevant lesion was found to be a substitution of the single polar amino acid Ser for a nonpolar Phe at position 204 in the ektodomain. This suggests that the ektodomain as well as the other two domains may contribute to the transport and targeting of G to the plasma membrane. Moreover, *ts*O45-infected cells were reported to shed G_s at 39° (20); this finding, however, was not confirmed (4, 30).

Because of the potential of this mutant for the study of functional glycoprotein domains, particularly during intracellular transport, the *ts*O45 phenotype was reexamined. This became feasible because of better serologic reagents for immunoprecipitation. Reported here is the conversion of G to G_s at 39°C. The failure of some investigators to detect G_s at 39°C was probably due to the pH dependence of G-to-G_s cleavage. The significance of these findings is discussed.

MATERIALS AND METHODS

Cells and viruses. Chinese hamster ovary (CHO) cells and the Indiana serotype, San Juan strain, of VSV have been described in detail (31). *ts*O45, a temperature-sensitive mutant with a lesion in G, was kindly provided by Craig Pringle, Medical Research Council Institute of Virology, Glasgow, Scotland. Purified and cloned virus preparations were used throughout.

Infections. Confluent monolayers in 60-mm petri plates were infected at a multiplicity of infection of 20 and incubated in 2 ml of Joklik modified minimum essential medium containing 2.5% fetal calf serum and nonessential amino acids. Attachment and further incubation for 3.5 h were carried out at 31°C. At 3.5 h after the initiation of infection the medium was replaced with 50 to 100 μCi of [^{35}S]methionine per ml in 1 ml of methionine-free Joklik modified minimal essential medium containing 1% dialyzed fetal calf serum. After incorporation of radioactivity at the temperature and time indicated for each experiment, unlabeled methionine at 5 times the normal concentration found in Joklik minimal essential medium was added to initiate the chase period.

To harvest the infected cell culture we centrifuged the

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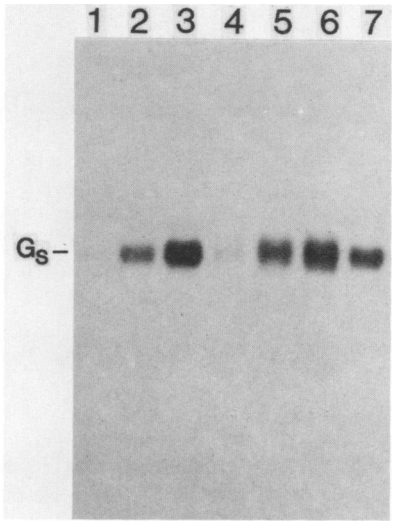

FIG. 1. Gel electrophoresis of VSV G_s from the total extracellular fraction of *ts*O45-infected cultures incubated at 31 and 39°C. Infected CHO cells were labeled with [^{35}S]methionine for 1, 2, or 3 h at 31 or 39°C, after which the total extracellular fractions were harvested, immunoprecipitated with anti-G(ekto), and electrophoresed. Marker G_s obtained from San Juan VSV-infected cultures at 37°C was also electrophoresed. Lanes: 1 through 3, *ts*O45 at 31°C for 1, 2, and 3 h, respectively; 4 through 6, *ts*O45 at 39°C for 1, 2, and 3 h, respectively; 7, marker G_s.

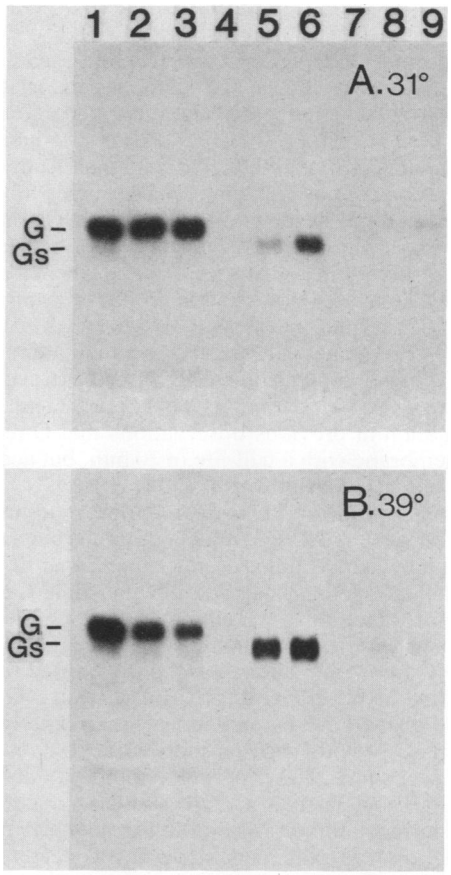

FIG. 2. Gel electrophoresis of pulse-chased radiolabeled VSV glycoproteins from the cell-associated and extracellular fractions of *ts*O45-infected cultures. Infected CHO cells were labeled for 30 min with [^{35}S]methionine at 31 or 39°C, after which excess unlabeled methionine was added and the cells were further incubated at the respective temperatures for 1 or 2 h. Then the cell-associated, extracellular soluble, and extracellular particulate virion fractions were prepared, immunoprecipitated with anti-G(ekto), and electrophoresed. Lanes: 1, cell-associated, pulse; 2, cell-associated, chase 1 h; 3, cell-associated, chase 2 h; 4, soluble, pulse; 5, soluble, chase 1 h; 6, soluble, chase 2 h; 7, virion, pulse; 8, virion, chase 1 h; 9, virion, chase 2 h.

medium at 10,000 × *g* in the Eppendorf microfuge for 3 min at 4°C to precipitate unattached cells. The supernatant extracellular fraction was sometimes further separated into extracellular particulate or soluble fractions by centrifugation at 30,500 × *g* for 100 min. The particulate virus pellet was solubilized in 0.5 M NaCl in RIPA buffer (10 mM Tris hydrochloride [pH 7.2], 1% Triton X-100, 0.1% sodium dodecyl sulfate, 1% sodium deoxycholate, 5 mM EDTA), whereas the soluble supernatant fraction was adjusted to contain 1% Nonidet P-40 and 1% sodium deoxycholate before immunoprecipitation. The cells were extracted by the method of Strous and Lodish (32). Briefly, they were washed twice with ice-cold phosphate-buffered saline containing 2 mM phenylmethylsulfonyl fluoride and then lysed by the addition of 0.6 ml of phosphate-buffered saline containing 1% sodium deoxycholate, 1% Nonidet P-40, and 2 mM phenylmethylsulfonyl fluoride. After 5 min at 4°C, the lysates were aspirated, leaving behind the nuclei, which could be observed to adhere to the plate. The lysates were further cleared by centrifugation at 10,000 × *g* for 10 min at 4°C.

Immunoprecipitations. Two different antibody preparations were used. The mouse monoclonal cell line 217-G2 was prepared by Mark Pasternak and Herman Eisen, Massachusetts Institute of Technology, Cambridge, and its anti-G(ekto) antibody was prepared by Doug Faller, Dana Farber Cancer Institute, Boston, Mass. Anti-G(ekto) recognizes an epitope in the extracellular domain of G; it neutralizes VSV weakly, but in precipitations and in enzyme-linked immunosorbent assays it binds G strongly (E. J. O'Rourke and A. S. Huang, unpublished observations). A C-terminal 15-amino acid portion of the cytoplasmic domain of G, as predicted by Rose and Gallione (25), was used to raise a rabbit monospecific antiserum, which was kindly supplied by Thomas Kreis and Harvey Lodish, Massachusetts Institute of Technology, Cambridge. In this manuscript it is named anti-G(COOH). The peptide was synthesized at the Children's Hospital Peptide Synthesis Facility, Boston, Mass.

Equivalent portions were mixed with either 0.1 ml of anti-G(ekto) or 2 μl of anti-G(COOH) and incubated at 4°C for 2 to 3 h. After incubation of the mixture, the complexes were precipitated with prewashed staphylococcal protein A (IgSorb, The Enzyme Center, Inc., Boston, Mass.); they were then washed three times in RIPA buffer containing 0.5 M NaCl and three times in RIPA buffer containing 0.15 M NaCl and boiled in Laemmli buffer (18). The proteins were separated on 7.5% sodium dodecyl sulfate-polyacrylamide gels as detailed previously (35). Molecular weight markers accompanied every run. Gels were fluorographed by adding Enlightening (New England Nuclear Corp.-Dupont, Boston, Mass.) as specified by the manufacturer. Dried gels were exposed to X-ray films (Kodak XAR-5 or Dupont Cronex). Radioactivity in the gels was quantitated by scintillation spectroscopy, after excision of G or G_s bands, by using the autoradiogram as template and immersing the excised pieces in Biofluor (New England Nuclear).

RESULTS

Synthesis and shedding of G_s by *ts*O45-infected cells at the nonpermissive temperature. To demonstrate that G_s was synthesized by *ts*O45-infected cells and found extracellularly at 39 as well as 31°C, infected cells were labeled with [^{35}S]methionine and the total extracellular fractions were immunoprecipitated at 1, 2, and 3 h after being labeled. The immunoprecipitates were compared with a G_s marker obtained from a San Juan VSV infection. Ample G_s was produced at both temperatures by *ts*O45. The accumulation of G_s at 39°C indicated that a stable product was produced by *ts*O45 (Fig. 1, lanes 4 through 6). In fact, as reported previously (20), somewhat more G_s accumulated at 39 than at 31°C. G, however, was only faintly seen extracellularly at 31°C after a 2- or 3-h continuous label (lanes 2 and 3). This is in confirmation of previous observations that G reaches the plasma membrane with a half-life of 20 min, but accumulates slowly in extracellular virions (13, 17, 20).

Degradation of intracellular G and appearance of extracellular G_s. Because *ts*O45 G is blocked in the rough endoplasmic reticulum, a pulse-chase experiment was done to see whether the intracellular degradation of G at 39°C accounted for the appearance of extracellular G_s. *ts*O45-infected cells were labeled with [^{35}S]methionine at 3.5 h after infection at 31 and 39°C for 30 min and subsequently chased for 1 or 2 h at the same two temperatures. Intracellular G declined during the chase, and G_s appeared extracellularly (Fig. 2). Longer chase periods showed more G degradation. Again, within these short chase periods, more G_s accumulated extracellularly at 39 than at 31°C, with a concomitant decrease of intracellular G. When the extracellular particulate virion fraction was examined, some G was detected at 31°C only after 2 h of chase (Fig. 2A, lane 8 and 9).

Kinetics of the G-to-G_s conversion with *ts*O45 at 39°C. To further examine the kinetics of the G-to-G_s conversion with *ts*O45 at 39°C, several samples were harvested during the chase period, fractionated into cell-associated and extracellular compartments, and quantitatively examined by gel electrophoresis. The intracellular G band and the region just below it was excised and quantitated and are shown in Fig. 2. This faster-migrating band contained some radioactive material, especially at the end of the pulse-labeling period. This region is known to contain some nascent, underglycosylated G as well as completed G_s by differential immunoprecipitation (Doherty and Huang, unpublished observations).

Cell-associated G increased within the first 30 min of the chase and then gradually declined (Fig. 3). This increase was seen on repeated experiments. It may be that not all nascent chains of G were immunoprecipitated, but reacted with the anti-G(ekto) serum upon completion. Material migrating ahead of G gradually declined, consistent with the idea that some of the material may be chased into G and some may form a cytoplasmic pool of G_s. This pool was most probably fed by the degradation of G and reduced by the secretion of G_s. Extracellularly, G_s accumulated steadily. By about 2 h, 50% of labeled G was quantitatively converted to extracellular G_s.

Differential immunoprecipitation of G_s synthesized by *ts*O45 at 39°C. Although G_s produced by *ts*O45 under nonpermissive conditions comigrated with authentic San Juan G_s, to ensure that it was authentic G_s we used antibody made against the first 15 amino acids at the carboxy terminus of G, anti-G(COOH). Extracellular G_s produced by *ts*O45 at either temperature was precipitated by anti-G(ekto) but not by

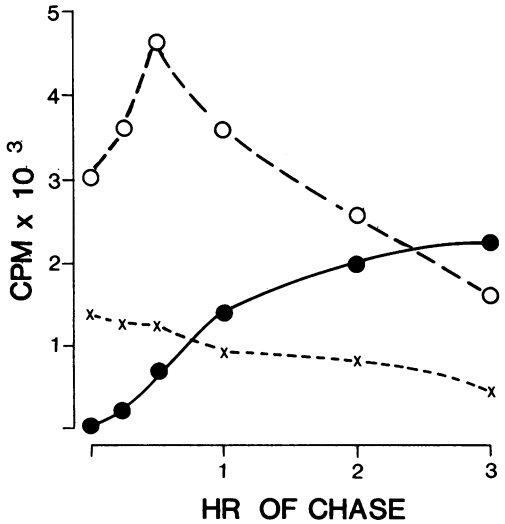

FIG. 3. Kinetics of conversion of intracellular VSV glycoproteins into extracellular G_s from *ts*O45-infected cells at 39°C. Infected CHO cells were labeled with [^{35}S]methionine for 30 min at 39°C, and excess unlabeled methionine was then added. After further incubation at 39°C, cell cultures were harvested at the indicated times and separated into cell-associated and total extracellular fractions. The proteins in the fractions were immunoprecipitated with anti-G(ekto) and electrophoresed. Radioactivity was quantitated as described in Materials and Methods. Symbols: ○, cell-associated G; ●, extracellular G_s; ×, intracellular species migrating faster than G.

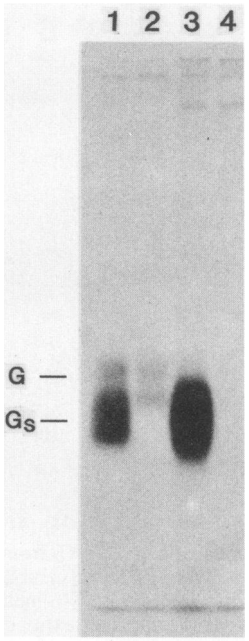

FIG. 4. Gel electrophoresis of G_s synthesized by *ts*O45-infected cells after immunoprecipitation with antibodies directed to the ektodomain or the carboxy terminus. Extracellular fluids from *ts*O45-infected cultures that had been labeled with [^{35}S]methionine for 30 min and chased with excessive cold methionine for 3 h, either at 31 or at 39°C, were immunoprecipitated with anti-G(ekto) or anti-G(COOH), followed by electrophoresis. Lanes: 1, 31°C, anti-G(ekto); 2, 31°C, anti-G(COOH); 3, 39°C, anti-G(ekto); 4, 39°C, anti-G(COOH).

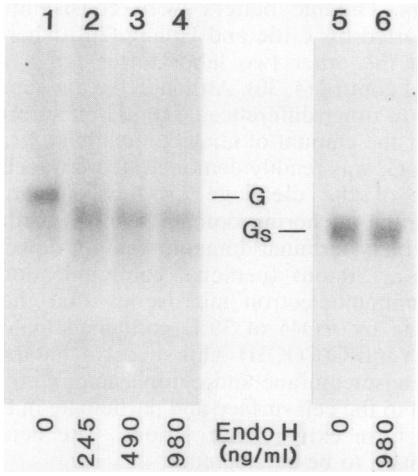

FIG. 5. Gel electrophoresis of G_s synthesized by *ts*O45-infected cells before and after treatment with endoglycosidase H. Extracellular G_s was prepared from *ts*O45-infected cells incubated at 39°C by the protocol described in the legend to Fig. 4. After G_s was immunoprecipitated with anti-G(ekto), the immunocomplex was eluted from protein A by boiling in 40 μl of 50 mM Tris hydrochloride (pH 6.8) containing 1% sodium dodecyl sulfate. To 20-μl aliquots of the immunocomplex, 3 μl of Endo H in 0.3 M sodium citrate buffer (pH 5.5) was added to yield final concentrations of 980 ng/ml. Samples without enzyme were similarly constituted and incubated for 18 h at 37°C. Then the samples were mixed 1:1 with 2× Laemmli buffer and electrophoresed. As a positive control for the Endo H digestion, cell-associated G from San Juan VSV-infected cells were labeled for only 10 min with [^{35}S]methionine and were similarly digested with different concentrations of Endo H as indicated. Lanes: 1 through 4, G from San Juan; 5 and 6, G_s from *ts*O45.

anti-G(COOH) (Fig. 4). G, as expected, was precipitated by both antisera (lanes 1 and 2). These results suggest that mutant G_s was similar to San Juan G_s in missing the carboxy-terminal domain.

Endoglycosidase H-resistance of G_s made by *ts*O45 at 39°C. Previous studies on San Juan G_s showed that it was as fully glycosylated as G and contained terminal sialic acids (12, 21). Another approach to characterization of the carbohydrate residues on G_s, either as the high-mannose or as the complex type, is to determine its sensitivity to endoglycosidase (Endo) H (33). Extracellular G_s synthesized by *ts*O45 at 39°C was compared with immature intracellular G from San Juan VSV-infected cells labeled for only 10 min. The latter provided a positive control for enzyme activity.

Figure 5 shows that Endo H treatment at 245 to 980 ng/ml was able to alter the migration of immature G (lanes 1 through 4), whereas extracellular G_s produced by the mutant remained fully resistant to the highest concentration (lane 6). This demonstrated that mutant G_s contained complex-type carbohydrates and most probably was as fully glycosylated as the San Juan G_s. Parenthetically, Endo H digestion of cell-associated G synthesized by *ts*O45 at 39°C showed that over 90% of G remained Endo H sensitive throughout a 3-h chase period (data not shown). This confirms previous reports (1, 16, 19).

Effects of pH on generation and shedding of G_s. Because of the inability of workers in other laboratories (4, 30) to confirm our finding that *ts*O45-infected cells shed G_s at 39°C (20), pH was tested as a possible source of discrepancy. An organic buffer was used to adjust the medium at four different pHs between 6.6 and 7.6. When the cell-associated

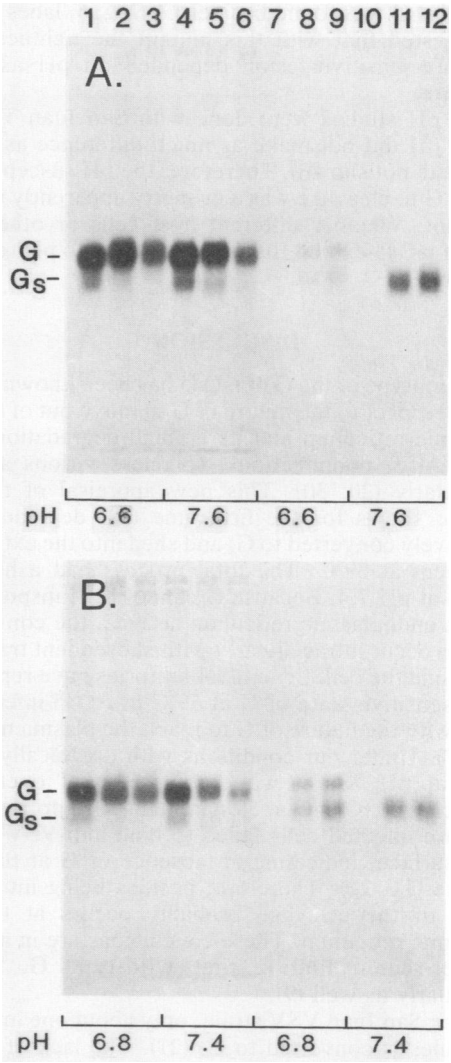

FIG. 6. Gel electrophoresis of VSV glycoproteins produced by *ts*O45-infected cells at different pHs. Infected cell cultures were maintained in media buffered at pH 6.6, 6.8, 7.4, or 7.6 with 25 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES). At 3.5 h after infection, [^{35}S]methionine was added for 30 min and then unlabeled excess methionine was added. At different times of chase the cultures were separated into cell-associated and total extracellular fractions. The fractions were immunoprecipitated with anti-G(ekto) and processed for electrophoresis. Each set of three lanes labeled underneath with a different pH contains the immunoprecipitates obtained at 0, 1.5, and 3 h of chase. Lanes: 1 through 6, cell-associated fraction; 7 through 12, total extracellular fraction.

and total extracellular immunoprecipitable glycoproteins were examined (Fig. 6), extracellular G_s was more readily detected at the higher pH. This effect of pH appeared to act during the cleavage step more so than during the transport of G_s to the extracellular environment. Much less G was cleaved to G_s in 3 h at pH 6.6 compared with pH 7.6 as well as at pH 6.8 compared with pH 7.4 (Fig. 6A and B, lanes 3 and 6). Cytoplasmic material migrating ahead of the G band was chased away at all pHs. It is unknown how extracellular pH affects a cleavage step that presumably occurs at the rough endoplasmic reticulum.

An unexpected result was obtained when the extracellular glycoproteins were examined. At pH 6.6 or 6.8, some

extracellular G was found besides G_s (Fig. 6, lanes 8 and 9). This suggested that with this mutant the tightness of the temperature-sensitive lesion depended on pH as well as temperature.

Similar pH studies were done with San Juan VSV. The effects of pH did not make as much difference as with the mutant (data not shown). Therefore, the pH susceptibility of the *ts*O45 G to cleavage was a property apparently unique to this mutant. Whether different host cells or other cloned isolates of *ts*O45 exhibit the same response to pH remains to be examined.

DISCUSSION

The phenotype of the G of *ts*O45 has been known for some time with respect to the failure of G to move out of the rough endoplasmic reticulum and its gradual degradation at 39°C (16, 19). Also, noninfectious, spikeless virions are found extracellularly (20, 30). This new appraisal of the *ts*O45 phenotype shows for the first time that degraded G was quantitatively converted to G_s and shed into the extracellular environment at 39°C. The total process had a half-life of about 2 h at pH 7.4. Because G cannot be transported from the rough endoplasmic reticulum at 39°C, the conversion is thought to occur intracellularly with subsequent transport of G_s to outside the cell. Several laboratories have reported the Endo H-sensitive state of G at 39°C in *ts*O45-infected cells together with the failure of G to reach the plasma membrane (3, 16, 27). Under our conditions with organically buffered medium at pH 7.4, it was confirmed that over 90% of cell-associated *ts*O45 G at 39°C remained Endo H sensitive. Also, these infected cells failed to bind anti-VSV serum to the cell surface, indicating an absence of G at the plasma membrane (11, 22). Therefore, besides being intracellular, the bulk of the cleavage probably occurs at the rough endoplasmic reticulum. These conclusions are in agreement with the recent finding that wild-type G_s is found intracellularly as well (9).

With the San Juan VSV strain, only about one in six of the G molecules is converted to G_s (21). The lack of complete conversion of wild-type G to G_s may be due to the transit time of G in the rough endoplasmic reticulum. Therefore, methods which keep G longer at its site of conversion may increase the ratio of G_s to G. With *ts*O45, the increased G_s production at 39 compared with 31°C supports such a hypothesis. However, if the transport of G_s is rapid, a small amount of G-to-G_s conversion at other intracellular locations or even at the plasma membrane may go undetected. Current studies with inhibitors of transport will further define the location of G-to-G_s conversion.

Given the generation of G_s and its transport intracellularly, its pathway can now be compared with the pathway taken by mature G to see whether secretion is an active targeted function or a passive integral glycoprotein-linked mechanism in fibroblast cells (6, 10, 32). With *ts*O45 a comparison can be made of two naturally occurring related glycoproteins which end up at different cellular sites. The differential effects of temperature and pH on the appearance of G and G_s extracellularly with this mutant suggest that the transport of G_s may differ from that of G. Further studies with both *ts*O45 and other strains of VSV are needed to determine these pathways.

The effect of external pH on the cleavage of G and extracellular appearance of G_s is especially interesting because it helps to explain the discrepancies found between results obtained in our laboratory and results of other investigators. Organic buffers were consistently used in studies reported by Little and Huang (20), whereas results obtained in the other two laboratories failed to indicate stringent pH control (4, 30). Although the discrepancies may still be due to other differences, the pH effect provides one way to alter the amount of G_s accumulation extracellularly.

Although G_s was readily demonstrated intracellularly, the other part of the cleavage product, presumably the transmembrane anchoring domain together with the cytoplasmic carboxy-terminal domain, was not detected by our methods. Kai Simons (personal communication) recently found by immunoelectron microscopy that the spikeless virions made by *ts*O45 at 39°C contained material which reacted with anti-G(COOH). This suggests that the other two domains, transmembrane and cytoplasmic, were able to be transported to the cell surface and participate in the budding process to form extracellular virions. The details of this process remain to be elucidated.

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