

## Mutational Changes in the Vesicular Stomatitis Virus Glycoprotein Affect the Requirement of Carbohydrate in Morphogenesis

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The role of carbohydrate in the morphogenesis of vesicular stomatitis virus was studied, using the antibiotic tunicamycin to inhibit glycosylation. It has been reported previously (Gibson et al., *J. Biol. Chem.* **254**:3600-3607, 1979) that the San Juan strain of vesicular stomatitis virus requires carbohydrate for efficient migration of the glycoprotein (G) to the cell surface and for virion formation, whereas the prototype or Orsay strain of vesicular stomatitis virus is less stringent in its carbohydrate requirement at 30°C. However, there are many differences between the two strains. We found that mutational changes within the G protein of the same strain of virus (prototype or Orsay) alters the requirement for carbohydrate at 30°C. Group V or G protein mutants *tsO45* and *tsO44*, like their prototype parent, did not require carbohydrate for efficient morphogenesis. In contrast, the G protein of another group V mutant, *tsO110*, was totally dependent upon carbohydrate addition for migration to the cell surface. Furthermore, no *tsO110* particles were released in the absence of glycosylation. The wild-type prototype strain did require carbohydrate at 39.5°C for insertion of the G protein into the plasma membrane and virion formation. However, a pseudorevertant of *tsO44* (*tsO44R*), unlike the prototype parent, no longer exhibited this temperature-sensitive requirement for carbohydrate. At 39.5°C in the presence of tunicamycin, *tsO44R*-infected cells released normal yields of particles and the unglycosylated G reached the cell surface very efficiently. In contrast to *tsO110*, which absolutely requires carbohydrate, mutational change in the *tsO44R* G protein has eliminated the requirement for carbohydrate. Thus, simple mutational changes, as opposed to many changes in the molecule, are sufficient to alter the carbohydrate requirement.

On the outside surface of enveloped viruses are spike structures composed of glycoproteins. These glycoproteins are polypeptides with one or several covalently linked oligosaccharide chains (28). The precise role(s) of carbohydrate found on membrane glycoproteins is still debated. It has been proposed that the carbohydrate may play a role in the (i) stability of the protein, (ii) intracellular transport of the glycoprotein to the appropriate membrane, or (iii) biological activity of the glycoprotein (6, 21, 24, 28).

The role of carbohydrate in the life cycle of vesicular stomatitis virus (VSV), an enveloped RNA virus (28), has been most thoroughly studied, using the antibiotic tunicamycin. Tunicamycin is a glucosamine-containing antibiotic which inhibits glycosylation of nascent glycoproteins without significantly affecting the synthesis of the protein or other aspects of cellular metabolism (26, 27).

In 1977, Leavitt et al. (18) showed that in the

presence of tunicamycin no detectable glycoprotein (G) was found at the surface of cells infected with the San Juan strain of VSV, and the release of VSV particles was inhibited 99.0 to 99.9% at 37°C and 90 to 95% at 30°C. This result suggested that carbohydrate may play a role in intracellular transport of the glycoprotein. However, Gibson et al. (9, 10) subsequently reported that the morphogenesis of another isolate of the Indiana serotype of VSV, the Orsay or prototype strain (3), was much less stringent in its requirement for carbohydrate addition at 30°C: tunicamycin inhibited the release of particles from cells infected with this strain by 30 to 55%. At higher temperatures (37 to 38°C) the requirement for carbohydrate addition in morphogenesis was still quite stringent. This result led to the suggestion that carbohydrate plays a role in determining the conformation of the protein and that different glycoproteins may have different requirements for oligosaccharide addition.

Different strains of VSV may have many un-

defined differences. To eliminate these variables, we examined the effect of tunicamycin on the morphogenesis of a number of glycoprotein mutants and one membrane protein mutant. These mutants were all isolated from the prototype strain of VSV (3).

We have found that mutants derived from the same strain of VSV, the Orsay or prototype (the term prototype will be used hereafter) strain, and which fall into complementation group V function differently in the absence of glycosylation. Our results not only confirm that different glycoproteins have different carbohydrate requirements for plasma membrane insertion, but also that simple mutational changes in a protein can affect this requirement.

### MATERIALS AND METHODS

**Cells and virus.** Cells used were Chinese hamster ovary cells. The San Juan strain of VSV and *tsO45* were obtained from H. L. Lodish. The prototype strain of VSV was obtained from Donald Summers. *tsO110*, *tsO23*, and *tsO44* were kindly provided by Phillip I. Marcus and Margaret Sekellick. *tsO44R* was isolated as a spontaneous revertant from the *tsO44* stock. Virus stocks were grown and purified as described previously (25).

**Preparation of cytoplasmic extracts.** Chinese hamster ovary cells ( $4 \times 10^6$ ) were infected with VSV at a multiplicity of 5 PFU per cell. Tunicamycin (gift to Donald J. Tipper from G. Tamura), when used, was added at 1 h post infection at a concentration of  $1 \mu\text{g}/\text{ml}$ . At 6 h postinfection at  $30^\circ\text{C}$ , the cells were harvested by centrifugation, washed once, and resuspended in 0.5 ml of modified Eagle minimal essential medium with no added methionine (supplemented with nonessential amino acids and 7.5% dialyzed fetal calf serum). [ $^{35}\text{S}$ ]methionine ( $25 \mu\text{Ci}/\text{ml}$ , 750 Ci/mmol; Amersham Corp.) was added to the cells. After incubation at  $30^\circ\text{C}$ , the cells were harvested by centrifugation and washed in cold 5% sucrose. A total of  $2 \times 10^7$  uninfected cells resuspended in 5% sucrose were added as carrier. The cells were disrupted with a tight-fitting Dounce homogenizer, and the nuclei were removed from the cytoplasmic extract by centrifugation. The nuclei were washed once in 5% sucrose, and the resulting supernatant was combined with the cytoplasmic extract.

**Growth, labeling, and purification of virus.** Confluent monolayers ( $2 \times 10^6$ ) of Chinese hamster ovary cells, pretreated with tunicamycin ( $0.5 \mu\text{g}/\text{ml}$ ) for 2 h, were infected with virus at a multiplicity of 5 PFU per cell. After 1 h of incubation at  $30^\circ\text{C}$  the medium was removed, and the monolayers were washed three times with methionine-free minimal essential medium supplemented with nonessential amino acids and 7.5% dialyzed fetal calf serum. [ $^{35}\text{S}$ ]methionine ( $50 \mu\text{Ci}/\text{ml}$ , 500 Ci/mmol; Amersham Corp.) and tunicamycin ( $1.0 \mu\text{g}/\text{ml}$ ) were added to the monolayers, and incubation was continued at  $30$  or  $39.5^\circ\text{C}$  for 17 or 11 h, respectively. These times were determined to be optimal for virion yield at the respective temperatures. A  $5\text{-}\mu\text{l}$  portion of supplemented

minimal essential medium containing nonradioactive methionine was added to each monolayer after 8.5 h ( $30^\circ\text{C}$ ) or 5.5 h ( $39.5^\circ\text{C}$ ). The medium was then removed, and virus in the supernatants was gradient purified in 9 to 40% (wt/vol) continuous sucrose gradients with an 80% sucrose pad (sucrose solutions were made in Dulbecco phosphate-buffered saline (5)). The gradient was centrifuged for 1 h in a Beckman SW27 rotor at 22,000 rpm and  $4^\circ\text{C}$ .

**Cell-free translation and extraction of VSV-specific RNA.** VSV RNA was prepared according to the procedure of Clinkscates et al. (4). Wheat germ extracts were prepared as described by Roberts et al. (22). Reaction mixtures ( $50 \mu\text{l}$ ) contained, per milliliter,  $20 \mu\text{mol}$  of *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (pH 7.8),  $2 \mu\text{mol}$  of dithiothreitol,  $1 \mu\text{mol}$  of ATP,  $0.02 \mu\text{mol}$  of GTP,  $8 \mu\text{mol}$  of creatine phosphate,  $40 \mu\text{g}$  of creatine phosphokinase,  $0.02 \mu\text{mol}$  of amino acids (minus methionine),  $56 \mu\text{mol}$  of potassium acetate,  $3 \mu\text{mol}$  of magnesium acetate,  $0.3 \text{ ml}$  of S-30,  $60 \mu\text{mol}$  of spermidine-free base, and  $250 \mu\text{l}$  of [ $^{35}\text{S}$ ]methionine ( $1,000 \text{ Ci}/\text{mmol}$ ,  $5 \text{ mCi}/\text{ml}$ ; Amersham Corp.).

**Lactoperoxidase iodination of cell surfaces and cytoplasmic extracts. (i) Surface labeling.** Infected Chinese hamster ovary cells ( $2 \times 10^6$ ) were washed three times in phosphate-buffered saline and resuspended in  $0.4 \text{ ml}$  of ice-cold phosphate-buffered saline containing lactoperoxidase ( $20 \mu\text{g}$ ,  $50 \text{ U}/\text{mg}$ ; Miles Laboratories), NaI ( $2.0 \mu\text{M}$ ), and  $\text{Na}^{125}\text{I}$  ( $100 \mu\text{Ci}/0.1 \text{ ml}$ ; New England Nuclear Corp.). Samples of hydrogen peroxide ( $50 \mu\text{M}$ ) were added at 1-min intervals for 10 min. The reaction was then allowed to proceed for 5 min at  $4^\circ\text{C}$  and was made  $2.5 \text{ mM}$  in NaI. The cells were then washed three times with cold phosphate-buffered saline and resuspended in  $100 \mu\text{l}$  of NET buffer ( $150 \text{ mM NaCl}$ ,  $5 \text{ mM EDTA}$ ,  $50 \text{ mM Tris-hydrochloride}$  [pH 7.4]) containing 1% Triton X-100. The samples were then immunoprecipitated as described below.

**(ii) Cytoplasmic extracts.** A  $40\text{-}\mu\text{l}$  amount of VSV-infected cytoplasmic extract (prepared as described above) was iodinated as described above. VSV proteins were immunoprecipitated as described below.

**Immunoprecipitation.** Cell extracts were incubated with rabbit anti-VSV antiserum, and immune complexes were precipitated with Immunobeads (Bio-Rad Laboratories) to which goat anti-rabbit antisera were coupled. Before use, Immunobeads were washed once in NET containing 1% Nonidet P-40 and  $5 \text{ mg}$  of bovine serum albumin per ml and twice in NET-1% Nonidet P-40 containing  $1 \text{ mg}$  of bovine serum albumin per ml. Concentrations of antibody and Immunobeads used in the immunoprecipitation reactions were those necessary to precipitate all VSV proteins from the cell extract.

**Polyacrylamide gel electrophoresis.** Polypeptides were resolved in 10% polyacrylamide slab gels ( $14$  by  $22$  by  $0.15 \text{ cm}$ ), prepared and run as described by Laemmli (14). The gels were then fixed and stained with Coomassie brilliant blue as described by Clinkscates et al. (4), dried, and subjected to autoradiography (X-ray film X-Omat R, Eastman Kodak Co.). The resulting autoradiograms were scanned with an Ortec microdensitometer. The gels containing  $^3\text{H}$ -labeled

proteins were impregnated with the water-soluble fluor sodium salicylate (1 M; Mallinckrodt) and exposed to preflashed film and stored at  $-70^{\circ}\text{C}$  as described by Chamberlain (2).

## RESULTS

**Release of particles in the presence of tunicamycin.** To determine the effect of tunicamycin on the assembly of different strains and mutants of VSV, radioactively labeled virus particles released from infected cells were purified in sucrose gradients. The total trichloroacetic acid-precipitable [ $^{35}\text{S}$ ]methionine-labeled material in each gradient fraction was determined (Fig. 1). As has been reported by Gibson et al. (9), at  $30^{\circ}\text{C}$  tunicamycin-treated cells infected with the wild-type prototype strain or the San Juan strain released, respectively, 30 and 5.0% the yield of particles from untreated infected cells (Fig. 1A and C, Table 1). At  $39.5^{\circ}\text{C}$ , in the presence of tunicamycin, prototype and San Juan strain-infected cells released only small

amounts of particles: 4 and 3% the yield of particles from untreated cells (Fig. 1B and D, Table 1).

Due to the temperature-sensitive lesion, the group V mutants *tsO110*, *tsO45*, and *tsO44* and the group III mutant *tsO23* do not release virus particles at  $39.5^{\circ}\text{C}$  (the nonpermissive temperature; 12, 13, 15–17). However, at  $30^{\circ}\text{C}$ , these mutants release normal yields of virus particles. To determine whether these mutants exhibit differences in morphogenesis in the absence of glycosylation at  $30^{\circ}\text{C}$ , the yield of particles released from tunicamycin-treated infected cells was compared with untreated infected cells. Tunicamycin-treated cells infected with *tsO45* and *tsO44* released 33 and 48% the yields of untreated infected cells, respectively (Fig. 1F and G, Table 1). In contrast, *tsO110*-infected, tunicamycin-treated cells did not release any detectable [ $^{35}\text{S}$ ]methionine-labeled particles (Fig. 1E, Table 1).

*tsO23*, the group III mutant, released 46% the

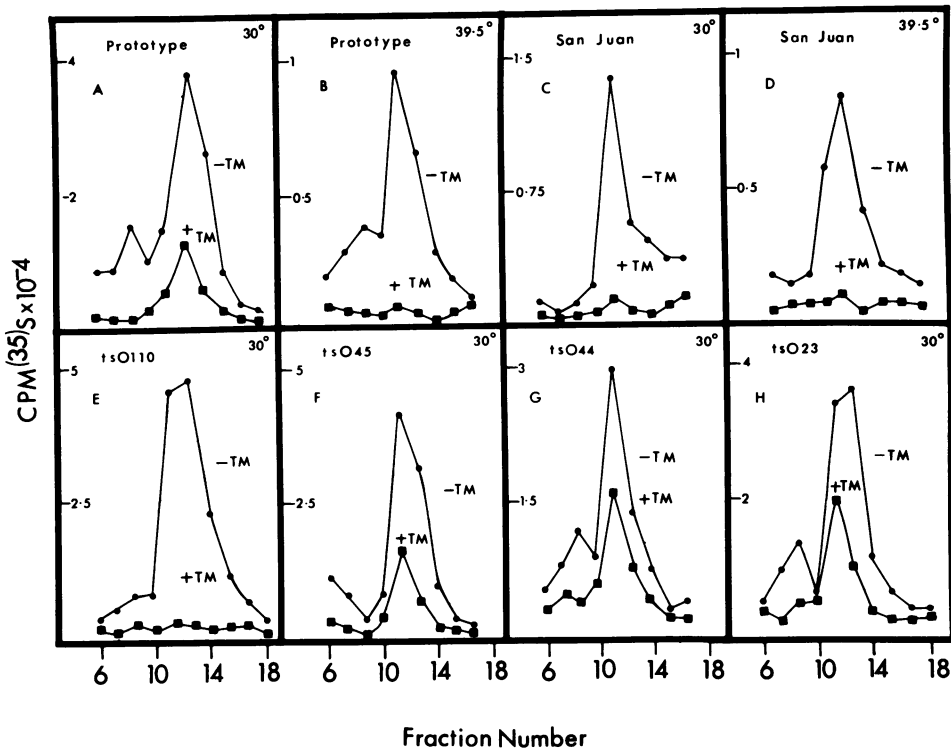


FIG. 1. [ $^{35}\text{S}$ ]methionine-labeled particles released from tunicamycin-treated cells. Tunicamycin (TM) (1.0  $\mu\text{g}/\text{ml}$ ) was added 2 h before infection. [ $^{35}\text{S}$ ]methionine was added 1 h postinfection, and the media from the cultures were removed after 12.5 and 18 h at  $39.5$  and  $30^{\circ}\text{C}$ , respectively. Virus in the supernatant released from  $2 \times 10^6$  cells was gradient purified as described in the text. The figure shows trichloroacetic acid-insoluble radioactivity across each gradient. (A and B) Prototype strain at  $30$  and  $39.5^{\circ}\text{C}$ , respectively. (C and D) San Juan strain at  $30$  and  $39.5^{\circ}\text{C}$ , respectively. (E, F, and G) Group V mutants *tsO110*, *tsO45*, and *tsO44*, respectively, at  $30^{\circ}\text{C}$ . (H) Group III mutant *tsO23* at  $30^{\circ}\text{C}$ . Symbols:  $\bullet$ , untreated;  $\blacksquare$ , tunicamycin treated.

TABLE 1. Properties of temperature-sensitive mutants in the presence of tunicamycin<sup>a</sup>

Virus strain/ mutant	Group	Tunica- mycin (1 μg/ml)	<sup>35</sup> S (cpm × 10 <sup>-4</sup> )		% Particle formation		% G at cell surface	
			30°C	39.5°C	30°C	39.5°C	30°C	39.5°C
San Juan	<i>ts</i> <sup>+</sup>	-	2.30	1.70	100	100	100	100
		+	0.12	0.06	5	3	1	<1
Prototype	<i>ts</i> <sup>+</sup>	-	7.39	2.16	100	100	100	100
		+	2.22	0.09	30	4	90.4	<1
O45	V	-	3.99		100		100	
		+	1.34		33		52	
O44	V	-	2.49		100		100	
		+	1.21		48		86	
O110	V	-	5.96		100		100	
		+	0.07		1		4 <sup>b</sup>	
O23	III	-	8.09		100		100	
		+	3.73		46		88	
O44R	V/R	-	2.60	2.24	100	100	100	100
		+	1.40	0.95	50-80	43	98	99

<sup>a</sup> Values shown in columns 4 and 5 represent total trichloroacetic acid-precipitable [<sup>35</sup>S]-methionine radioactivity present in gradient-purified virions released from 2 × 10<sup>6</sup> cells. The amount of radioactively labeled particles released in the absence of tunicamycin was taken as 100% (columns 6 and 7). Values given are an average of three independent experiments (±5%). The amount of <sup>125</sup>I-labeled G protein at the cell surface (columns 8 and 9) was determined by scanning autoradiograms of the polyacrylamide gels shown in Fig. 3 and 5 and comparable gels containing San Juan G protein, using an Ortec densitometer. Concentrations of rabbit anti-VSV antisera and Immunobeads necessary for complete precipitation of the VSV protein in cell extracts was determined in preliminary experiments. In addition, the immunoprecipitation procedure was shown to be highly reproducible within a given sample: iodinated samples were aliquoted into four fractions; each was immunoprecipitated separately. The total counts and the amount of protein precipitated in each reaction were identical. Furthermore, preliminary experiments established that degradation of the glycoproteins, including *ts*O110 G protein, during the immunoprecipitation did not occur. The amount of radioactively labeled G protein at the cell surface in the absence of tunicamycin was taken as 100%. The results of the iodination presented, with the exception of *ts*O110, are the averages from three independent experiments (±8%).

<sup>b</sup> Iodination of the *ts*O110-infected cell surface was carried out six times. Two of the six times we detected a small amount of G at the cell surface (~12%); the remaining four times we did not detect any G at the surface.

yield of [<sup>35</sup>S]methionine-labeled particles from untreated infected cells (Fig. 1H, Table 1).

**Characterization of particles released in the presence of tunicamycin.** To verify that particles released from tunicamycin-treated cells contained the unglycosylated G protein, proteins present in gradient-purified [<sup>35</sup>S]methionine-labeled particles were separated by polyacrylamide gel electrophoresis. Particles released in the presence of tunicamycin at either 30 or 39.5°C contained the unglycosylated G (Fig. 2, slots 2, 6, 8, and 10). Furthermore, normal amounts of glycoprotein were present in particles released from tunicamycin-treated cells.

**Unglycosylated G at the cell surface.** Since a distinct difference in particle yield was found between *ts*O110 and the other temperature-sensitive mutants in the presence of tunicamycin, we determined whether the decrease in particle formation was accompanied by a de-

crease in the amount of unglycosylated G found at the cell surface. Cell surface glycoprotein was detected by lactoperoxidase-catalyzed iodination. Iodination of untreated cells infected with the prototype strain of VSV and the temperature-sensitive mutants resulted in the labeling of only one major polypeptide (Fig. 3, slots 1, 3, 5, 7, 9, 11, and 13) which comigrated with glycosylated G. Iodination of infected cells treated with tunicamycin also resulted in the labeling of one polypeptide (Fig. 3, slots 2, 6, 10, 12, and 14) which comigrated with the unglycosylated G. Compared with the glycosylated form of the G protein, the amount of <sup>125</sup>I label in the unglycosylated G protein at the surface of cells infected with prototype, *ts*O45, *ts*O44, and *ts*O23 is only slightly reduced (Table 1). The unglycosylated G of *ts*O110, in contrast, was barely detectable at the cell surface.

Infection of group V mutants at 39.5°C results

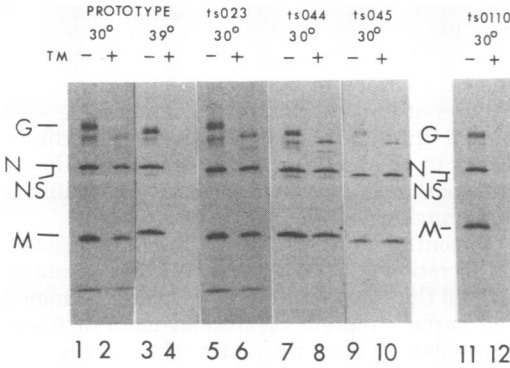


FIG. 2. Proteins present in particles released from untreated and tunicamycin-treated cells. Gradient-purified [ $^{35}$ S]methionine-labeled virus particles were acetone precipitated, and the proteins were resuspended in gel sample buffer and analyzed by polyacrylamide gel electrophoresis as described in the text. Similar amounts of radioactivity was loaded in slots 1 through 3 and 5 through 11 (this represents virus-associated material released from  $2.5 \times 10^5$  to  $5.0 \times 10^5$  cells). Total virion-sized material released from  $2 \times 10^6$  cells was loaded in slots 4 and 12. Proteins in particles released from untreated cells are shown in slots 1, 3, 5, 7, 9, and 11. Proteins in particles released from tunicamycin-treated cells are shown in slots 2, 4, 6, 8, 10, and 12. The extra protein bands which migrate slightly faster than the G proteins appear variably and may be the result of degradation during virion purification.

in the failure of G to reach the cell surface (12, 13, 16), whereas iodination of cells infected with prototype and *tsO23* resulted in the labeling of G at the cell surface. However, in the presence of tunicamycin at  $39.5^\circ\text{C}$ , neither the unglycosylated G of the wild type nor *tsO23* was detectable at the cell surface (Fig. 3, slots 4 and 8) by iodination.

At  $30^\circ\text{C}$  all of the mutants behaved as the wild type in the presence of tunicamycin, with the exception of *tsO110*. It is possible that the unglycosylated *tsO110* G protein is merely slow to reach the cell surface. However, even at late times after infection (12 h), the unglycosylated G protein was found at the cell surface in very small amounts (<1%; Table 2).

It is also possible that the unglycosylated *tsO110* G is not found at the cell surface because it is more susceptible to intracellular proteases, and therefore unstable. However, we have found that the unglycosylated G protein is as stable as the glycosylated form of the G protein (Table 2).

Alternatively, the *tsO110* unglycosylated G may be at the cell surface but in an altered conformation such that efficient iodination of the molecule is prevented. However, when cytoplasmic extracts from *tsO110*-infected, tunicamycin-treated cells were iodinated, the intracellular form of the *tsO110* unglycosylated G protein was iodinated as efficiently as the unglycosylated form of G derived from *tsO23* (Table 2).

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**Properties of *tsO44R*.** We have isolated a revertant of *tsO44*, *tsO44R*, which releases infectious virus particles at  $39.5^\circ\text{C}$  (Fig. 4B and Fig. 5, slot 4) after infection. At  $30^\circ\text{C}$  in the presence of tunicamycin, *tsO44R*-infected cells released between 50 and 80% of the yield of particles released from untreated cells (Fig. 4A and Fig. 5, slot 2; Table 1), and the unglycosylated G protein reached the cell surface very efficiently (Fig. 5, slot 6). At  $39.5^\circ\text{C}$ , in contrast to the wild-type VSV (prototype), *tsO44R* released 43% of the yield of particles from untreated cells (Fig. 4B and Fig. 5, slot 4; Table 1), and the unglycosylated G protein reached the cell surface as efficiently as the glycosylated G protein (Fig. 5, slot 8).

**Inhibition of glycosylation by tunicamycin.** To draw valid conclusions from the results presented above, it is critical that all G protein synthesized in the presence of tunicamycin be totally unglycosylated. This problem was explored in two ways.

First, after infection with *tsO44R*, the virus which most efficiently releases particles in the presence of tunicamycin, the incorporation of [ $^3\text{H}$ ]glucosamine and [ $^3\text{H}$ ]mannose into virion glycoprotein in the presence of tunicamycin was determined to be less than 0.1% (Fig. 6, slots 7

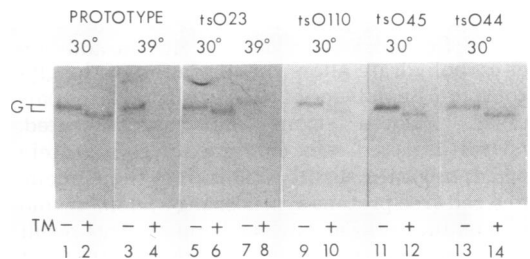


FIG. 3. Lactoperoxidase-catalyzed iodination of infected Chinese hamster ovary cells. Infected Chinese hamster ovary cells ( $2 \times 10^6$ ) were iodinated as described in the text. The [ $^{125}\text{I}$ ]labeled polypeptides were immunoprecipitated, resuspended in gel sample buffer, and subjected to electrophoresis on 10% polyacrylamide gels as described in the text. [ $^{125}\text{I}$ ]labeled immunoprecipitated polypeptides from  $10^6$  cells were loaded in each slot. The figure shows an autoradiogram of the fixed dried gel containing cell surface radiolabeled G protein in the absence of tunicamycin (-TM; slots 1, 3, 5, 7, 9, 11, and 13) and in the presence of tunicamycin (+TM; slots 2, 4, 6, 8, 10, 12, and 14). No radioactive material was precipitated with anti-VSV antisera after iodination of uninfected cell surfaces.

TABLE 2. Properties of *tsO110* unglycosylated G

Mutant	Presence of tunicamycin	% Cell surface glycoprotein at time postinfection <sup>a</sup>		% Remaining of unglycosylated G after chase of: <sup>b</sup>			% Iodination of intracellular unglycosylated G <sup>c</sup>
		8 h	12 h	0 min	30 min	60 min	
		<i>tsO110</i>	-	100	100	100	
	+	<1	<1	100	94.0	92.0	95
<i>tsO23</i>	+						100

<sup>a</sup> Infected cell surfaces were iodinated at 8 and 12 h postinfection as described in the legend to Fig. 3. The values given for the unglycosylated *tsO110* G protein (+ tunicamycin) and the glycosylated G protein (- tunicamycin) are averages of two independent experiments ( $\pm 1\%$ ). The amount of glycosylated G protein found at the cell surface at 12 h was determined by scanning an autoradiogram and is taken as 100%.

<sup>b</sup> Cells infected with *tsO110* in the absence and presence of tunicamycin were labeled with [<sup>35</sup>S]methionine at 6 h postinfection for 5 min. An excess of unlabeled methionine was added. Portions of the culture were removed at 0, 30, and 60 min after the onset of the nonradioactive chase. The cells were pelleted and resuspended in gel sample buffer. Proteins present were resolved by polyacrylamide gel electrophoresis. The resulting autoradiogram was scanned with an Ortec densitometer. The amount of G protein in each sample was determined and expressed as a percentage of the amount present at the beginning of the chase period. The effectiveness of the chase was monitored by assaying trichloroacetic acid-precipitable counts from samples removed from the culture during the chase period.

<sup>c</sup> Cytoplasmic extracts of *tsO23*- and *tsO110*-infected cells were iodinated. Samples were immunoprecipitated, and proteins present were resolved by polyacrylamide gel electrophoresis and exposed to X-ray film. The autoradiogram was scanned, and the amount of unglycosylated *tsO23* G protein labeled with <sup>125</sup>I was taken as 100%.

and 9). Second, it is well known that the addition of carbohydrate alters the migration of the glycoprotein on polyacrylamide gels (23). Virus particles isolated from tunicamycin-treated, *tsO44R*-infected cells contained a glycoprotein which migrates slightly faster than the G made in a cell-free protein-synthesizing system devoid of membranes (Fig. 6, slots 3 and 4). This result not only suggests that G is unglycosylated but also that a signal sequence has been removed. Thus, by two criteria we estimate the level of glycosylation after tunicamycin treatment to be less than 0.1%.

## DISCUSSION

Carbohydrate moieties are ubiquitous components of surface membrane proteins, secreted proteins, and viral envelope proteins. The precise function of the oligosaccharide has been a subject of speculation and investigation for a number of years. It has been suggested that carbohydrate may serve to direct the migration of the glycoprotein to its ultimate location in the

cell (6). Therefore, any failure to glycosylate a membrane or secreted protein would result in the inability of the glycoprotein to migrate to the cell surface or be secreted from the cell. Studies of immunoglobulin secretion have supported this idea (11). Carbohydrate addition seems to be absolutely necessary in subcellular transport and secretion of immunoglobulin A and immunoglobulin E from plasma cells.

In contrast to the studies with immunoglobulin secretion (11), Olden et al. (21) have demonstrated that the failure to glycosylate the major cell surface protein of chicken embryo fibroblasts did not alter the ability of the protein to reach the cell surface. However, the intracellular degradation rate of the major cell surface protein was increased two- to threefold in the absence of glycosylation. Thus, carbohydrate stabilizes this protein against proteolytic degradation.

Studies dealing with the role of carbohydrate in the life cycle of enveloped RNA viruses have also produced various results. Leavitt et al. (18) have demonstrated that a failure to add carbohydrate to the viral glycoproteins of both Sindbis virus and the San Juan strain of VSV results in the inability of the glycoproteins to migrate to the cell surface. In addition, in the absence of glycosylation the release of virus particles is inhibited by 99.9%.

In contrast, Nakamura and Compans (20) reported that the absence of carbohydrate does not prevent the efficient insertion of the influenza glycoproteins into the plasma membrane. Thus, carbohydrate may be required for trans-

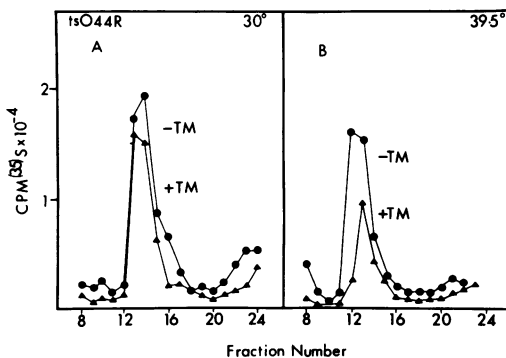


FIG. 4. [<sup>35</sup>S]methionine-labeled *tsO44R* particles released from untreated and tunicamycin (TM)-treated cells. Cells were infected with *tsO44R*, and virus released into the supernatant from  $2 \times 10^6$  cells was gradient purified as described in the legend to Fig. 1. The figure shows trichloroacetic acid-precipitable counts across the gradient. (A) Particles released from *tsO44R*-infected cells at 30°C; (B) particles released from *tsO44R*-infected cells at 39.5°C. Symbols: ●, untreated; ▲, treated with tunicamycin.

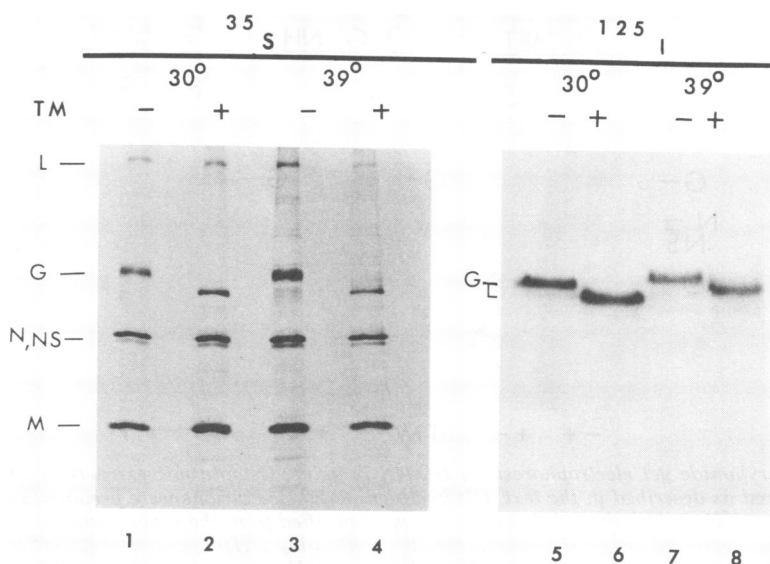


FIG. 5. Proteins present in *tsO44R* particles and iodination of *tsO44R*-infected cell surfaces. Gradient-purified [ $^{35}\text{S}$ ]methionine-labeled particles were analyzed by polyacrylamide gel electrophoresis. *tsO44R*-infected cells were iodinated, and the labeled proteins were immunoprecipitated and analyzed by polyacrylamide gel electrophoresis. The figure shows [ $^{35}\text{S}$ ]methionine-labeled particles released from untreated and tunicamycin (TM)-treated cells at 30 and 39.5°C (lanes 1-4); it also shows immunoprecipitated cell surface radiolabeled G protein in the absence and presence of tunicamycin at 30 and 39.5°C (lanes 5-8).  $^{125}\text{I}$ -labeled immunoprecipitated polypeptides from  $10^6$  cells were loaded in each slot.

port of some viral glycoproteins to the cell surface but not for others.

This conclusion has been supported by Gibson et al. (10), who studied the requirement for carbohydrate in morphogenesis of two different strains of VSV from the Indiana serotype, the San Juan and the prototype. At 30°C, in the presence of the glycosylation inhibitor tunicamycin, cells infected with the San Juan strain release only 5 to 10% of the normal yield of virus particles, whereas the prototype strain releases between 45 and 70%. These data suggest that, unlike the San Juan strain, carbohydrate is not critical for efficient morphogenesis of the prototype strain of VSV at 30°C. We have found similar results with the San Juan and prototype strains of VSV. However, the requirement for carbohydrate for prototype particle release is temperature sensitive. At 39.5°C, prototype-infected cells treated with tunicamycin release only 4% of the normal yield of virus (10).

The glycoproteins of the two strains are apparently quite different. The peptide patterns of the San Juan G protein and prototype G protein were shown to be quite different when compared by partial peptide mapping (1). There are minor differences in the NS protein peptide maps as well. In addition, the RNA fingerprints of the different isolates of the Indiana serotype differ in their oligonucleotide patterns by 50 to 75% for

the G gene, 20% for the NS gene, and 15% for the L gene (8). We were concerned about whether the differences in carbohydrate requirement between the two strains were due to different G proteins or to some other undefined differences between the two strains.

There exists a collection of temperature-sensitive mutants derived from the prototype strain of VSV. These mutants were spontaneous isolates and not the result of mutagenesis. As such, they may represent single mutational changes. However, since the frequency of spontaneous mutations in VSV is high (2.2% at 30°C), these mutants may in fact contain multiple alterations (7). We examined the effect of tunicamycin on the morphogenesis of several different group V or glycoprotein mutants, *tsO110*, *tsO45*, and *tsO44* (7, 15-17) and one group III or membrane protein mutant, *tsO23* (7, 15-17). Cells infected with these mutants do not release particles at 39.5°C, the nonpermissive temperature. However, at 30°C, the permissive temperature, cells infected with these mutants do release normal yields of particles.

At 30°C, we found that tunicamycin has different effects on the yield of [ $^{35}\text{S}$ ]methionine-labeled particles released from mutant-infected cells. Similar to the wild type at 30°C, tunicamycin-treated *tsO45*-infected cells released about 30% of the normal yield of [ $^{35}\text{S}$ ]methio-

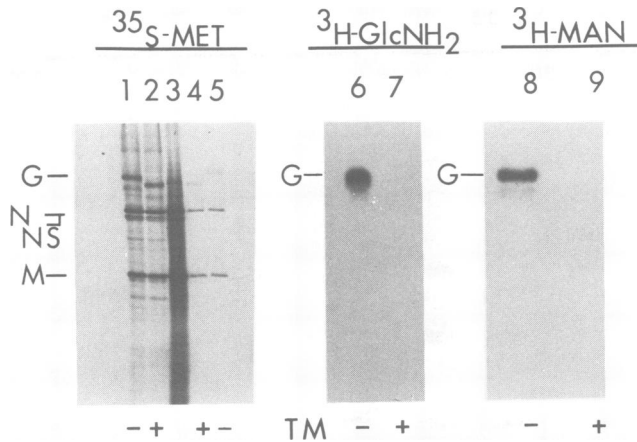


FIG. 6. Polyacrylamide gel electrophoresis of *tsO44R* proteins. Cytoplasmic extracts of *tsO44R*-infected cells were prepared as described in the text. [ $^{35}\text{S}$ ]methionine-labeled virions were prepared as described in the legend to Fig. 1. [ $^3\text{H}$ ]carbohydrate-labeled virions were purified from the supernatant of cells which had been grown on supplemented minimal essential medium containing [ $^3\text{H}$ ]glucosamine (25  $\mu\text{Ci}/\text{ml}$ , 5 to 15 Ci/mmol; New England Nuclear Corp.) and 500  $\mu\text{M}$  D-glucose or [ $^3\text{H}$ ]mannose (25  $\mu\text{Ci}/\text{ml}$ , 200 to 400 Ci/mmol; Amersham Corp.) and 2.5  $\mu\text{M}$  mannose. *tsO44R* proteins were synthesized in a wheat germ cell-free system as described in the text. The figure shows autoradiograms of 12.5% polyacrylamide gels containing [ $^{35}\text{S}$ ]methionine-labeled polypeptides from untreated and tunicamycin (TM)-treated *tsO44R*-infected cells (slots 1 and 2), polypeptides synthesized in a cell-free reaction directed by 12–18S *tsO44R* RNA (slot 3), and polypeptides present in virions released from *tsO44R*-infected cells treated with tunicamycin (slot 4) and in virions released from *tsO44R*-infected cells in the absence of tunicamycin (slot 5). The figure also shows an autoradiogram of a fixed, dried 10% polyacrylamide gel which contained gradient-purified virions radioactively labeled with [ $^3\text{H}$ ]glucosamine in the absence and presence of tunicamycin (slots 6 and 7, respectively) and gradient-purified virions radioactively labeled with [ $^3\text{H}$ ]mannose in the absence and presence of tunicamycin (slots 8 and 9, respectively). Each slot contains virus-associated material released from  $2 \times 10^6$  cells. The gels containing  $^3\text{H}$  label were impregnated with sodium salicylate. The amount of radioactivity present in  $^3\text{H}$ -carbohydrate-labeled G isolated on polyacrylamide gels was quantified with an Ortec densitometer.

nine-labeled particles and *tsO44*-infected cells released 48% of the normal yield of [ $^{35}\text{S}$ ]methionine-labeled particles. Furthermore, the unglycosylated G of *tsO45* and *tsO44* reached the cell surface very efficiently. The differences in particle yield between *tsO45* and *tsO44* are reproducible but not dramatically different from the wild type. Also, like the wild type, *tsO23* (group III)-infected cells released 46% of the normal yield of particles. The results obtained with *tsO110*, another group V mutant, vary significantly from the other mutants and the wild type. Tunicamycin-treated *tsO110*-infected cells do not release any detectable [ $^{35}\text{S}$ ]methionine-labeled particles, and the migration of the unglycosylated G to the cell surface as assayed by cell surface iodination is very inefficient.

The failure to detect the unglycosylated *tsO110* G at the cell surface is not due to an inability of the *tsO110* G to be iodinated. Intracellular *tsO110* unglycosylated G can be iodinated to the same extent as the unglycosylated form of the *tsO23* G protein. Furthermore, failure to detect the unglycosylated *tsO110* G at the cell surface is not due to a slower appearance of

the *tsO110* G at the cell surface. Even at late times after infection, the amount of unglycosylated G at the cell surface is negligible. Finally, the failure to detect the unglycosylated *tsO110* G at the cell surface is not due to a decrease in the stability of the intracellular unglycosylated G. The unglycosylated G is just as stable as its glycosylated counterpart. Thus, the mutational change in the *tsO110* G protein is such that even at 30°C the G cannot migrate efficiently to the cell surface or be incorporated into virus particles without the addition of carbohydrate.

As discussed above, the wild-type prototype strain is temperature sensitive in its requirement for carbohydrate. At 39.5°C, this strain absolutely requires carbohydrate: few particles are released from tunicamycin-treated cells and no detectable unglycosylated G protein can be found at the infected cell surface. The glycoprotein of *tsO23*, the group III or M protein mutant, is also temperature sensitive in its requirement for carbohydrate for plasma membrane insertion.

We have isolated a virus which overcomes this temperature-sensitive requirement for carbohy-



drate. We have isolated a spontaneously occurring revertant of *tsO44* (*tsO44R*) which, like the wild type, is capable of releasing virus at the nonpermissive temperature (39.5°C). However, unlike the wild-type virus, this revertant does not require carbohydrate for migration of the G to the cell surface at 39.5°C or for its efficient incorporation into virus particles. The phenotype displayed by *tsO44R* suggests that this revertant has undergone a second-step mutation and is not a true revertant to the wild-type virus. The second mutation (or pseudoreversion) has eliminated the need for carbohydrate at high temperature.

The results with *tsO44R* support the conclusion that mutational changes in the G protein may influence the protein's requirement for carbohydrate. Thus, we have found a mutational change that causes G protein to be totally dependent upon carbohydrate: *tsO110*. In contrast, a mutational change may occur that eliminates the need for carbohydrate. *tsO44R* does not require carbohydrate for morphogenesis at either low or high temperatures. Our results not only suggest that it is indeed the amino acid sequence of the G protein itself which determines the requirement for carbohydrate, but also that even simple mutational changes in the G protein can dramatically affect the carbohydrate requirement.

Tunicamycin inhibition of virion formation is consistently greater than the inhibition of migration of G to the cell surface (Table 1). The reason for this finding is unclear at present. Perhaps virions containing the unglycosylated G protein are more unstable. Alternatively, carbohydrate may play some small role in the final stages of budding of the virus from the cell.

In conclusion, we have presented evidence that the amino acid sequence of the VSV G protein determines the importance of carbohydrate for insertion of the G protein into plasma membranes. Mutants isolated from the same strain of VSV and which fall into the same complementation group have different requirements for carbohydrate addition for insertion of the G protein into the plasma membrane. Perhaps, as has been suggested by Gibson et al. (10), the conformation of the polypeptide is of ultimate importance in efficient migration through the cell. Some molecules may obtain this conformation in the absence of carbohydrate, whereas others may require carbohydrate to assume the appropriate structure.

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#### LITERATURE CITED

1. Burge, B. W., and A. S. Huang. 1979. Conserved peptides in the proteins of vesicular stomatitis virus. *Virology* **95**:445-453.
2. Chamberlain, J. 1979. Fluorographic detection of radioactivity in polyacrylamide gels with the water-soluble fluor, sodium salicylate. *Anal. Biochem.* **98**:132-135.
3. Clewley, J. P., and D. H. L. Bishop. 1978. Evolution of rhabdovirus genomes, p. 271-283. *In* B. W. J. Mahy and R. D. Barry (ed.), *Negative strand viruses and the host cell*. Academic Press, Inc., New York.
4. Clinkscales, C. W., M. A. Bratt, and T. G. Morrison. 1977. Synthesis of Newcastle disease virus polypeptides in a wheat germ cell-free system. *J. Virol.* **22**:97-101.
5. Dulbecco, R., and M. Vogt. 1954. Plaque formation and isolation of pure lines with poliomyelitis viruses. *J. Exp. Med.* **99**:167-182.
6. Eylar, E. H. 1965. On the biological role of glycoproteins. *J. Theor. Biol.* **10**:89-113.
7. Flamand, A. 1970. Etude genetique du virus de la stomatite vesiculaire: classement de mutants thermosensibles spontanés en groupe de complementation. *J. Gen. Virol.* **8**:187-195.
8. Freeman, G. J., D. D. Rao, and A. S. Huang. 1979. RNA synthesis of vesicular stomatitis virus. VIII. Oligonucleotides of the structural genes and mRNA. *Gene* **5**:141-157.
9. Gibson, R., R. Leavitt, S. Kornfeld, and S. Schlesinger. 1978. Synthesis and infectivity of vesicular stomatitis virus containing nonglycosylated G protein. *Cell* **13**:671-679.
10. 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.
11. Hickman, S., A. Kulczycki, Jr., R. G. Lynch, and S. Kornfeld. 1977. Studies of the mechanism of tunicamycin inhibition of IgA and IgE secretion by plasma cells. *J. Biol. Chem.* **252**:4402-4408.
12. Knipe, D. M., D. Baltimore, and H. F. Lodish. 1977. Maturation of viral proteins in cells infected with temperature-sensitive mutants of vesicular stomatitis virus. *J. Virol.* **21**:1149-1158.
13. Knipe, D. D., H. F. Lodish, and D. Baltimore. 1977. Analysis of the defects of temperature-sensitive mutants of vesicular stomatitis virus: intracellular degradation of specific viral proteins. *J. Virol.* **21**:1140-1148.
14. Laemmli, U. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (London)* **227**:680-685.
15. Lafay, F. 1969. Etude des mutants thermosensibles du virus de la stomatite vesiculaire: classification de quelques mutants d'apres des criteres de fonctionnement. *C.R. Acad. Sci. Ser. D.* **268**:2385-2389.
16. 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.
17. Lafay, F., and A. Berkaloff. 1969. Etude des mutants thermosensibles du virus de la stomatite vesiculaire: mutants de maturation. *C. R. Acad. Sci. Ser. D* **269**:1031-1035.
18. Leavitt, R., S. Schlesinger, and S. Kornfeld. 1977. Tunicamycin inhibits glycosylation and multiplication of Sindbis and vesicular stomatitis viruses. *J. Virol.* **21**:375-385.
19. Marx, J. L. 1980. Newly made proteins zip through the

- cell. *Science* **207**:164-167.
20. Nakamura, K., and R. W. Compans. 1978. Effects of glucosamine, 2-deoxyglucose, and tunicamycin on glycosylation, sulfation, and assembly of influenza viral proteins. *Virology* **84**:303-319.
  21. Olden, K., R. M. Pratt, and K. M. Yamada. 1978. Role of carbohydrates in protein secretion and turnover: effects of tunicamycin on the major cell surface glycoprotein of chick embryo fibroblasts. *Cell* **13**:461-473.
  22. Roberts, B. E., B. M. Patterson, and R. Sperling. 1970. The cell-free synthesis and assembly of viral specific polypeptides into TMV particles. *Virology* **59**:307-313.
  23. Segrest, J. P., and R. L. Jackson. 1972. Molecular weight determination of glycoproteins by polyacrylamide gel electrophoresis in sodium dodecyl sulfate. *Methods Enzymol.* **23**:54-63.
  24. Sharon, N. 1978. *Complex carbohydrates*. Addison-Wesley Publishing Co., Reading, Mass.
  25. Stampfer, M. A., A. Huang, and D. Baltimore. 1969. Ribonucleic acid synthesis of vesicular stomatitis virus. I. Species of ribonucleic acid found in Chinese hamster ovary cells infected with plaque-forming and defective particles. *J. Virol.* **4**:154-161.
  26. Takatsuki, A., K. Arima, and G. Tamukra. 1971. Tunicamycin, a new antibiotic. I. Isolation and characterization of tunicamycin. *J. Antibiot.* **24**:215-223.
  27. Takatsuki, A., K. Kohro, and G. Tamura. 1975. Biosynthesis of polyisoprenol sugars in chick embryo microsomes is inhibited by tunicamycin. *Agric. Biol. Chem.* **39**:2089-2091.
  28. Wagner, R. R. 1975. Reproduction of rhabdoviruses, p. 1-93. *In* H. Frankel-Conrat and R. R. Wagner (ed.), *Comprehensive virology*, vol. 4. Plenum Press, New York.