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Morphological and Biochemical Characterization of Viral Particles Produced by the *tsO45* Mutant of Vesicular Stomatitis Virus at Restrictive Temperature

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The growth at restrictive temperature of *tsO45*, a group V (glycoprotein) conditional lethal mutant of vesicular stomatitis virus (VSV), was demonstrated to result in the production of large numbers of noninfectious viral particles. The infectivity of these *tsO45* particles could be enhanced by procedures known to promote membrane fusion. Morphologically and biochemically these particles differed from wild-type VSV by their lack of viral glycoprotein. The other structural proteins of VSV were present and indistinguishable by size and relative proportion from those of virus grown at the permissive temperature. Examination of glycoprotein maturation at the restrictive temperature (39.5°C) in *tsO45*-infected cells demonstrated the synthesis of normal viral glycoprotein but failed to demonstrate the presence of this glycoprotein in either the cell membrane or the envelope of free virions. The further absence of soluble viral glycoprotein from the supernatants of such cells strongly suggests that viral glycoprotein may not be necessary for the successful budding of VSV.

Genetic studies of vesicular stomatitis virus (VSV), a member of the rhabdovirus group, have delineated six complementation groups (2, 7, 8, 24, 25, 27), and five virion-coded proteins have been biochemically defined (11, 38). *tsO45*, a temperature-sensitive mutant of VSV selected on the basis of its inability to produce a normal number of infectious progeny virus at restrictive temperatures (7), has been assigned to complementation group V (7, 8) and has been demonstrated to be defective in the assembly of the viral envelope glycoprotein (14, 15, 18). Studies from a number of laboratories examining the synthesis and processing of the envelope glycoprotein (G) of *tsO45* have demonstrated that G is synthesized and initially glycosylated in a normal fashion in cells infected by *tsO45* at restrictive temperatures (14, 15, 18, 20, 26), but that a block exists in the normal maturation of G from its site of synthesis in the endoplasmic reticulum to the plasma membrane (14, 15, 18). The production of *tsO45* viral particles under these conditions, however, has been shown to occur (5, 20, 26), and the virus produced has been partially characterized (5, 20). The mechanism by which the absence of G from the cell surface could result in the loss of viral infectivity while still permitting virus production was not further investigated. Little and Huang (20), however, demonstrated in the supernatant of *tsO45*-infected cells the presence at restrictive temperature both of noninfectious viral particles lacking G and of soluble viral glycoprotein. They suggested that G synthesized under such conditions was only transiently and weakly associated with the cell surface, this association being essential for the successful budding of viral particles but not for the stable incorporation of G into the particles themselves. A similar conclusion, based on results of pseudotype studies employing *tsO45* at restrictive temperatures, has been forwarded by Witte and Baltimore (41). Further studies reported here were therefore undertaken to examine the mechanism of virus budding and to characterize morphologically and biochemically the virus particles produced by cells infected with *tsO45* at restrictive temperature.

MATERIALS AND METHODS

Cells and viruses. Vero cells were obtained and grown as previously described (32). Primary cultures of chicken embryo cells derived from Leghorn flocks (C/E) were used after one passage and were maintained in Dulbecco-modified Eagle medium containing antibiotics and supplemented with 10% tryptose phosphate broth, 1% heat-inactivated chick serum, and 1% heat-inactivated fetal calf serum. Standard conditions for cell maintenance during VSV plaque assays were employed (44).

Our clones of wild-type VSV (Indiana strain), and the temperature-sensitive mutants, *tsO45* and *tsM501* (kindly provided by C. Pringle and D. Baltimore),

have been recently described (R. A. Weiss and P. Bennett, submitted for publication).

Assay of viral infectivity. Infectious titer of virus was determined by a plaque assay using Vero cells as previously described (44). In experiments utilizing Sendai virus, the assay cells were washed twice with phosphate-buffered saline (PBS) at 4°C, overlaid with cold PBS containing 2,000 hemagglutinating units of β-propriolactone-inactivated Sendai virus, and incubated at 4°C. After 30 min the cells were washed again with cold PBS and then infected with serial dilutions of VSV. Control cells were treated in an identical fashion but incubated at 4°C in the absence of Sendai virus. The remainder of the assay proceeded under standard conditions.

Infection of cells and metabolic labeling of viral products. Both Vero and chick cells were maintained as confluent monolayers in 90-mm plastic petri dishes. Cells were washed once with PBS and then infected with indicated virus at a multiplicity of infection of 5 to 10 PFU/cell. After adsorption for 60 min at room temperature the inoculum was removed, and the cells were washed twice in PBS, then fed with either maintenance medium or labeling medium containing radioactive precursors and incubated at either 32 or 39.5°C.

Metabolic labeling of viral products was accomplished by overlaying cells after viral adsorption with either Dulbecco-modified Eagle medium modified to contain 10% of the normal concentration of methionine and supplemented with 20 μCi of [^{35}S]methionine per ml or with Dulbecco-modified Eagle medium supplemented with 5 μCi of [^{3}H]uridine per ml. All media used in labeling experiments contained 2% dialyzed fetal calf serum. Virus and supernatants were then harvested at 16 h postadsorption. Medium from infected cells was clarified by centrifugation at 3,000 rpm for 10 min in an MSE bench-top centrifuge. Virus was pelleted from the clarified supernatants by centrifugation for 90 min at 50,000 × *g*. The viral pellets were then suspended in PBS, layered onto a continuous 20 to 50% sucrose gradient containing 0.01 M Tris-hydrochloride (pH 7.5), and centrifuged for 4 h at 135,000 × *g* using an SW50.1 rotor in a Beckman L5-65 ultracentrifuge. Fractions were collected by puncture of the bottom of the tubes, and samples were measured for both radioactivity and refractive index. Appropriate fractions were then pooled, diluted by the addition of PBS, and virus pelleted by centrifugation as above.

Labeling of intracellular viral products was accomplished by addition of labeling medium 4 h postadsorption. After 30 min, cells were washed once and then disrupted directly by addition of the electrophoresis dissociation buffer.

Techniques for the labeling of both virion and cell surface glycoproteins using tritium-labeled potassium borohydride (KBH_4) followed Critchley's method (3).

Preparation and immunoprecipitation of viral and cell extracts. Samples of VSV to be immunoprecipitated were diluted to 0.1 ml with buffer (0.01 M Tris-hydrochloride [pH 7.0], 0.025 M KCl, 0.005 M $MgCl_2$) and made 1% with respect to Triton X-100 and sodium deoxycholate.

Cell cultures were prepared by homogenizing the washed cells in 1 ml of buffer containing 1% Triton X-100 and 1% sodium deoxycholate. The nuclei were removed by centrifugation at 1,000 × *g* for 5 min, and the supernatant was clarified by centrifugation at 100,000 × *g* for 40 min. These samples were immunoprecipitated as described by Kessler (13). Briefly, to each sample 5 μl of anti-VSV immunoglobulin G (IgG) or control serum was added and incubated at room temperature for 1 h. A 10% suspension of fixed *Staphylococcus aureus* was added as an IgG adsorbent for a further hour. The bacterial suspension was washed three times in buffer, and the IgG complexes were recovered by the addition of 50 μl of electrophoresis dissociation buffer, heated at 100°C for 1 min. The bacteria were removed by centrifugation, and the samples were directly loaded onto a sodium dodecyl sulfate-polyacrylamide gel. The efficacy of the procedure, and the specificity of the anti-VSV IgG, was tested using purified disrupted VSV. The anti-VSV IgG efficiently immunoprecipitated all the VSV structural proteins (see Fig. 6).

Polyacrylamide gel electrophoresis. The immunoprecipitates or whole virion preparations were dissociated and separated on slab gels using the procedure of SDS-polyacrylamide gel electrophoresis described by Laemmli (17). After electrophoresis the gels were fixed in 10% trichloroacetic acid at 4°C for 1 h, washed in water for 2 h, and dried under vacuum. The labeled proteins were located by autoradiography of the gel using Kodak X-ray film (X-Omat H1).

Preparation of virus for electron microscopy. Gradient-purified virus was resuspended in PBS, and 1 drop was placed on a dental wax plate. A carbon-coated grid was placed on the viral suspension for 20 s, then stained with 2% phosphotungstic acid (pH 7.0) for 5 s, followed by a second treatment with the same stain for 20 s. The grids were then air dried and viewed on a Siemens 1A Elmiskop electron microscope with an accelerating voltage of 80 kV and an objective aperture of 20 μm.

RESULTS

Particle production at restrictive temperature. The *tsO45* mutant of VSV had been originally selected for the inability to produce infectious virus particles at restrictive temperatures (7). However, viral infectivity was shown to be "rescuable" at high efficiency by production of phenotypically mixed particles (pseudotypes of *tsO45*) from cells doubly infected with an avian retrovirus and *tsO45* and maintained at the restrictive temperature (43, 45). The inability to find biologically active (infectious) virus produced under similar conditions in control cells devoid of retrovirus expression was believed to be due to the failure of *tsO45* to bud from the cell surface as a consequence of the defect in the assembly of the VSV glycoprotein (14, 15, 18). These observations could also be explained, however, by mechanisms that would not preclude the viral budding process itself, which has been reported to occur (5, 20, 26), but would result in the production of VSV particles struc-

turally altered so as to make them noninfectious and hence not apparent when assayed by biological means. To examine this possibility a series of investigations was initiated to search for and characterize VSV particles produced under restrictive conditions.

Vero cells believed to be free of known endogenous virus expression were infected at both permissive and restrictive temperatures with the *tsO45* mutant of VSV in the presence of medium containing either [^{35}S]methionine or [^{3}H]uridine as described. Released virus was detected by determining the incorporation of these radiolabeled compounds into materials capable of being pelleted from the clarified supernatants of the infected cells. This material was characterized by equilibrium density centrifugation and then compared to similarly labeled *tsO45* particles produced at 32°C or VSV wild-type particles by co-sedimentation in a linear 20 to 50% sucrose gradient. Cells infected with *tsO45* at 32°C incorporated [^{3}H]uridine into particles that banded at the same density (1.17 to 1.18 g/ml) as [^{35}S]methionine-labeled wild-type particles produced at the permissive or the restrictive temperature (Fig. 1). *tsO45* viral particles produced at restrictive temperature labeled with either [^{3}H]uridine or [^{35}S]methionine had a lower density (1.16 g/ml) than wild-type particles produced at either 32 or 39.5°C. This small but reproducible difference in density would be consistent with the absence of the viral glycoprotein from *tsO45* particles produced at 39.5°C.

Characterization of *tsO45* virus particles produced at restrictive temperature. Although the noninfectious nature of *tsO45* particles released at restrictive temperature could be due entirely to the absence of viral glycoprotein, as suggested by others (5, 20), investigations to characterize these noninfectious particles further were necessary to preclude other defects that could contribute to or explain their biological inactivity. To analyze the structural proteins of these particles, cells were infected at both permissive and restrictive temperatures with the *tsO45* mutant of VSV in the presence of medium containing [^{35}S]methionine and released virus purified by sucrose gradient centrifugation. Examination of the polypeptide composition of this material was then undertaken by SDS-polyacrylamide gel electrophoresis. Examined in this manner, the viral proteins of *tsO45* virus particles produced at permissive temperature appeared identical to those of the wild-type virus grown at either 32 or 39.5°C (Fig. 2). Examination of material pelleted from the supernatant of cells infected in an identical manner with *tsO45* at restrictive temperature also demonstrated the incorporation of [^{35}S]methionine into VSV-specific bands, but in an amount only 5 to 10% of that found with wild-type virus grown and analyzed under identical conditions. That these bands did not represent labeling of "leaky" or revertant virus produced at restrictive temperature was shown by the fact that the biological activity of the particles produced at restrictive temperature remained at a level at least 100,000-fold lower than that seen with either particles from cells infected with *tsO45* at permissive temperature or wild-type virus at restrictive temperature (Table 1). Furthermore, although relative molar amounts of the other viral structural proteins appeared normal, no incorporation of [^{35}S]methionine into the protein band corresponding to VSV G could be demonstrated in

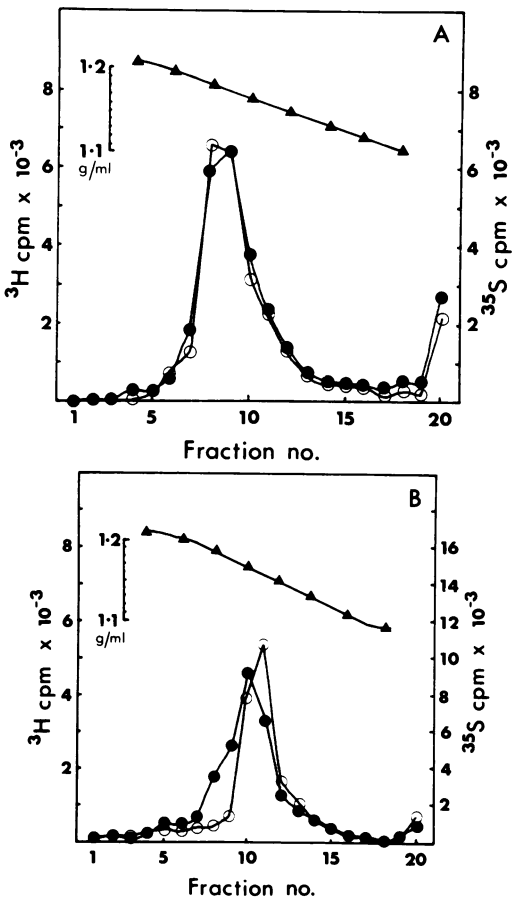

FIG. 1. *Sucrose density gradients of [^{35}S]methionine-labeled wild-type VSV centrifuged to equilibrium in the presence of [^{3}H]uridine-labeled tsO45 produced at (A) 32°C, the permissive temperature, and (B) 39.5°C, the restrictive temperature. The wild-type VSV (●) and tsO45 (○) produced at 32°C bands at a density of 1.17 to 1.18 g/ml, but tsO45 produced at 39.5°C bands at a lighter density of 1.16 to 1.17 g/ml.*

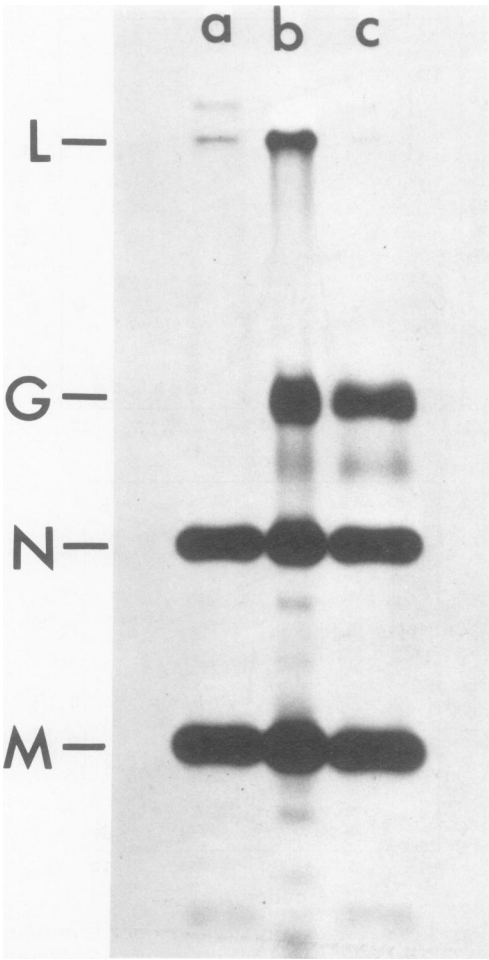

FIG. 2. *Autoradiogram of sucrose density gradient-purified [^{35}S]methionine-labeled tsO45 and wild-type VSV separated on a 10% SDS-polyacrylamide gel. (a) Proteins found in tsO45 particles grown at 39.5°C; (b) tsO45 grown at 32°C; (c) wild-type VSV grown at 39.5°C.*

tsO45 viral particles produced at restrictive temperature (Fig. 2). Under these conditions of electrophoresis the protein NS could not be resolved from N. These data strongly suggested that VSV particles were being released in large numbers from cells infected at restrictive temperature by *tsO45*, but that such particles lacked G and were not biologically active.

Further evidence supporting the absence of viral glycoprotein from the virus envelope was derived from electron microscopic examination of *tsO45* particles produced at permissive and restrictive temperatures. Previous investigations of the ultrastructure of VSV had demonstrated the presence of "spikes" on the surface of wild-type viral particles when examined by negative staining techniques (21). Treatment of these particles with proteases such as Pronase, known to digest the surface proteins without rupturing the lipid bilayer, results in not only the removal of the spikes but also the concomitant production of noninfectious viral particles (21). Electron microscopic examination of *tsO45* particles produced at 39.5°C, however, showed a significantly different appearance, lacking spikes and closely resembling Pronase-treated wild-type particles (Fig. 3).

To determine whether the production at restrictive temperature of noninfectious VSV particles lacking viral glycoprotein could be demonstrated with other temperature-sensitive mutants of group V, *tsM501* was studied in a manner similar to *tsO45*. Analysis by SDS-polyacrylamide gel electrophoresis of material released from cells infected by this mutant at 39.5°C in the presence of [^{35}S]methionine demonstrated a pattern of protein labeling indistinguishable from that seen with *tsO45* grown under similar conditions (data not shown). Other group V mutants have not been examined, but the finding of at least one other mutant of this group capable of producing noninfectious particles lacking viral glycoprotein at restrictive temperature argues against *tsO45* being a unique type of mutant.

Metabolism of viral glycoprotein at restrictive temperature. The finding of VSV *tsO45* particles lacking glycoprotein stimulated further studies aimed at examining the intracellular metabolism of the viral glycoprotein in the hope of elucidating its role in particle production. That normal VSV glycoprotein is synthesized at restrictive temperature in *tsO45*-infected cells had been previously reported by others (14, 15, 18, 20, 42) and was demonstrated in these studies by the incorporation of [^{35}S]methionine into all viral proteins, including viral glycoprotein, in an identical manner in *tsO45*-infected cells maintained at either 32 or 39.5°C (Fig. 4). The subsequent fate of the synthesized viral

TABLE 1. *Rescue of tsO45 infectivity by pretreatment of assay cells with inactivated Sendai virus*

Virus	Growth temp (°C)	Titer (PFU/ml × $\log_{10}$) after pretreatment of assay cells with:	
		PBS	Sendai virus
tsO45	32	8.7	8.6
	39.5	2.7	3.9
VSV wild type	32	8.8	8.7
	39.5	8.3	8.3

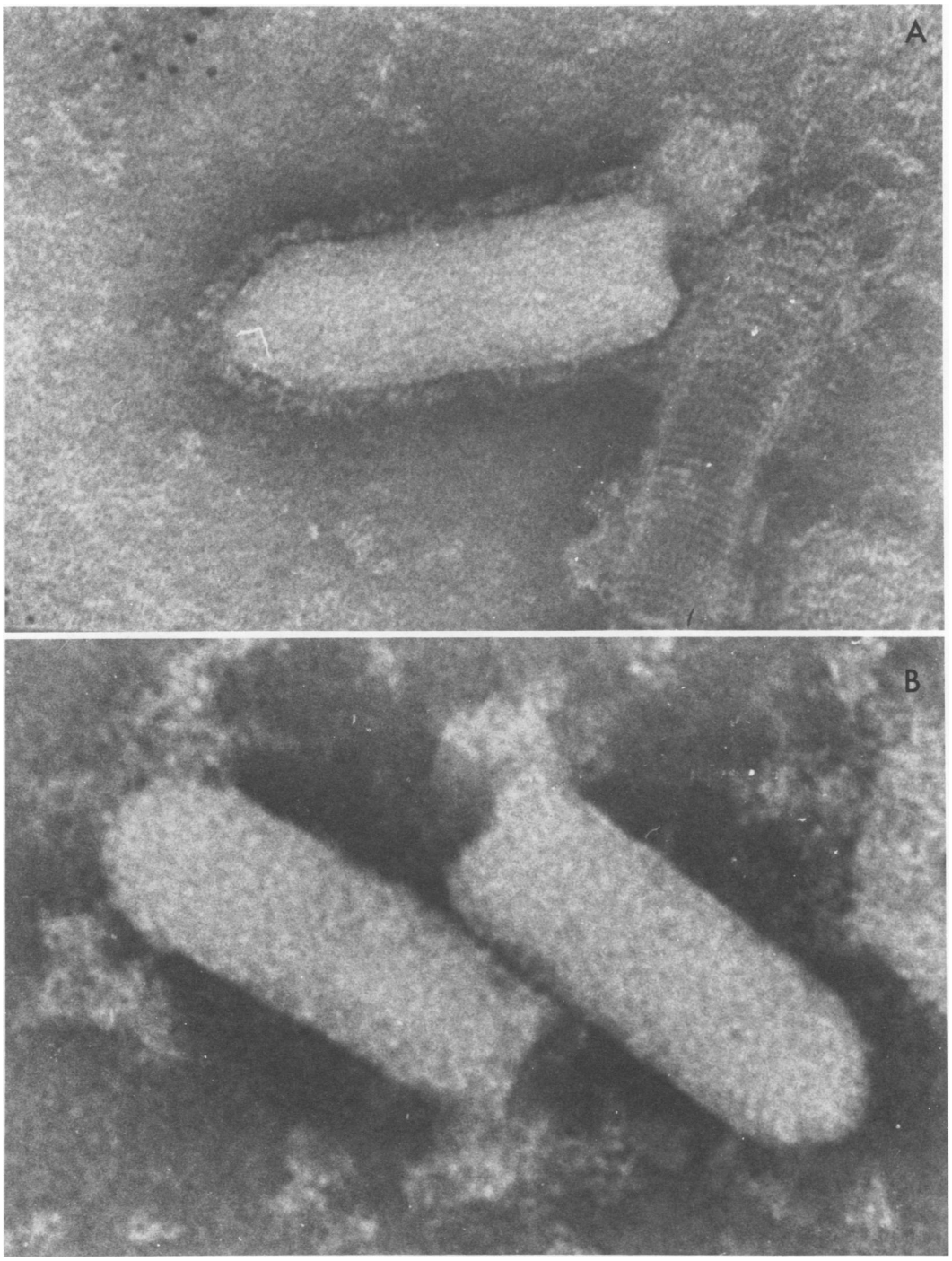

FIG. 3. *Electron micrographs of negatively stained (2% phosphotungstic acid), gradient-purified preparations of tsO45 grown at (A) 32°C and (B) 39.5°C. The absence of spikes can be clearly seen on the 39.5°C-grown virus (magnification, ×400,000).*

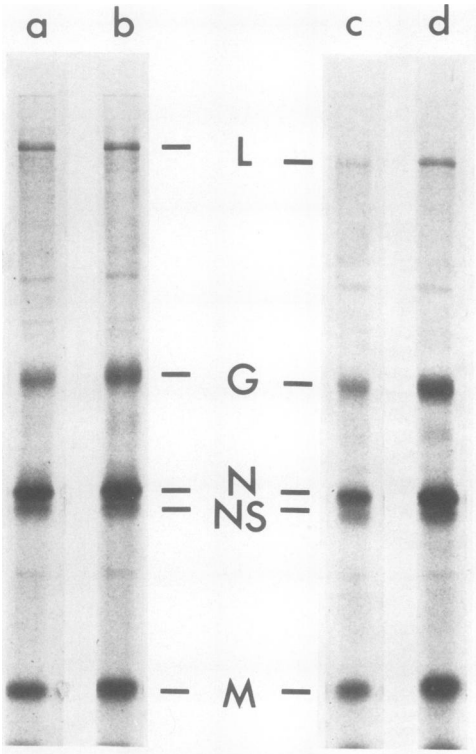

FIG. 4. *Autoradiogram of a polyacrylamide gel showing labeled cells infected with tsO45 (a and c) or wild-type VSV (b and d). Four hours postinfection, the cells were pulsed for 30 min with [^{35}S]methionine. Cells were incubated at 32°C (a and b) or 39.5°C (c and d).*

glycoprotein was investigated in these studies by procedures directed towards identifying G either on the surface or in the supernatant of infected cells. ^{3}H labeling of surface glycoproteins was accomplished by means of a galactose oxidase-KBH_4 technique (3). Subsequent examination of labeled glycoproteins was accomplished by preparation of an extract, using detergent to solubilize the membrane proteins from cells infected with *tsO45* at permissive and restrictive temperatures, which was then immunoprecipitated as described, and the immunoprecipitates were separated in a 10% polyacrylamide gel. Employing these techniques, it was possible to demonstrate (Fig. 5) the presence, on the surface of cells infected with *tsO45* at permissive temperature, of material which had the same mobility on gels as the structural virion glycoprotein of VSV analyzed in a similar fashion. No viral glycoprotein was detected by these techniques on the surface of cells infected with *tsO45* at restrictive temperature despite the fact that the surface of wild-type-infected cells could be labeled successfully at 39.5°C, suggesting that under these conditions the *tsO45* viral glycoprotein, although synthesized normally, did not reach, or was not inserted in a normal fashion into, the cell surface. Similar investigations directed at trying to demonstrate the presence of the viral glycoprotein on the surface of the virion particles themselves confirmed the earlier metabolic labeling experiments. No viral-specific glycoprotein could be demonstrated on the surface of *tsO45* particles produced at restrictive temperature, whereas wild-type particles produced under these conditions and *tsO45* particles produced at 32°C were shown to manifest VSV glycoprotein as expected (Fig. 5).

Because viral glycoprotein could not be demonstrated on the surface of either *tsO45*-infected cells at restrictive temperature or the progeny virus particles themselves, the finding by Little and Huang (20) of an increased level of a soluble form of VSV glycoprotein in the supernatant of *tsO45*-infected CHO cells maintained at 39.5°C was explored by similar investigations employing the Vero and avian cells used in these studies. Supernatants from cells infected by *tsO45* in the presence of [^{35}S]methionine at 32 and 39.5°C were harvested 16 h postinfection and, after low-speed clarification, subjected to centrifugation at 45,000 rpm for 90 min to remove all particulate matter including virus particles. The resulting supernatants were then treated with anti-VSV serum to immunoprecipitate virus-specific products, and the pellets were maintained for examination. The material thus prepared was solubilized in SDS and analyzed in a standard polyacrylamide gel electrophoresis.

At the permissive temperature, labeled amino acid was incorporated primarily into a protein, Gs, which migrated slightly faster than the glycoprotein found in virions (Fig. 6). This band probably represented the smaller, soluble form of the VSV glycoprotein described by Kang and Prevec (10) and thought to occur as a consequence of proteolytic cleavage of the glycoprotein from the cell surface (20). The presence of any soluble viral glycoprotein, however, could not be detected by immunoprecipitation in culture fluids of cells infected at the nonpermissive temperatures. An examination of the high-speed pellets (data not presented) showed the expected viral proteins seen in Fig. 2. These findings indicate that the VSV glycoprotein, although synthesized in an apparently normal fashion in *tsO45*-infected Vero cells at restrictive temperature, failed to undergo the normal maturation process and was unable to reach the cell surface, either to be incorporated in a normal manner into cellular membranes or to be capable of

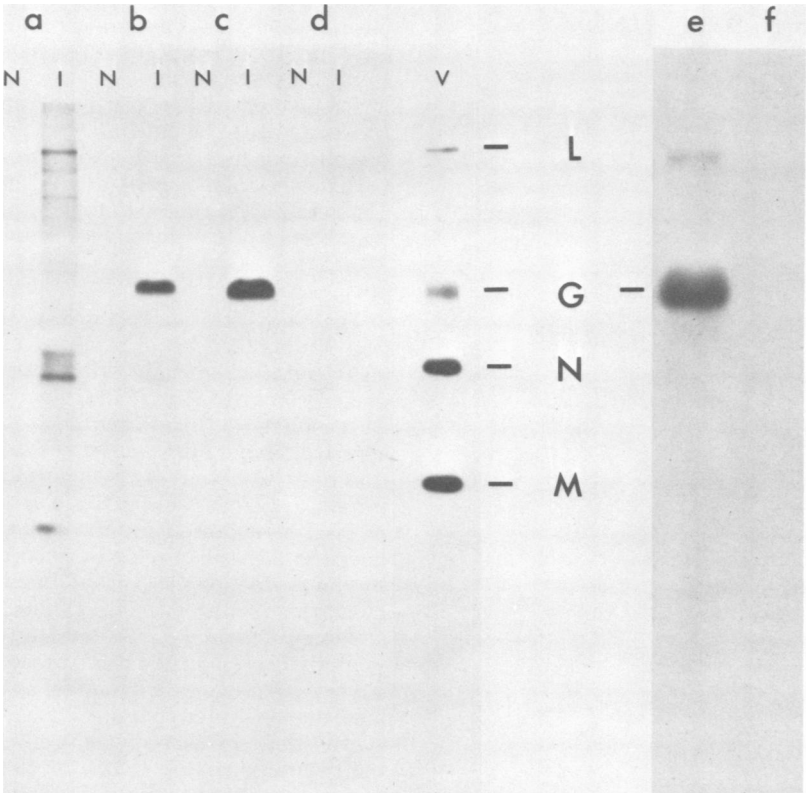

FIG. 5. *Fluorogram showing a polyacrylamide gel of the labeling of VSV glycoprotein on virus and infected cells. Cultures were infected with tsO45 (c and d) or wild-type VSV (b), or left uninfected (a). After several hours the surface of the cells was labeled using the KBH_4-galactose oxidase procedure. Cell extracts were prepared and immunoprecipitated with either anti-VSV IgG (I) or a normal rabbit serum (N). A control sample of virus (V) was run in parallel. A similar procedure was performed on isolated tsO45 preparations obtained from cultures incubated at the permissive (e) and nonpermissive temperatures (f).*

being cleaved into the soluble form with subsequent release into the cell supernatant.

These studies have demonstrated that *tsO45* particles produced at 39.5°C appeared to lack viral glycoprotein; however, the noninfectious nature of these particles could nevertheless have been the consequence of their containing subgenomic-length RNA rather than of their being altered structurally, although this would seem unlikely since *ts* mutations usually result from a base change event. To preclude this explanation, RNA was extracted from gradient-purified *tsO45* particles produced at 32 and 39.5°C and compared by velocity sedimentation in a linear 10 to 30% sucrose gradient. The RNAs were found to cosediment with RNA from wild-type VSV (data not shown).

Rescue of viral infectivity. The infectivity of enveloped viral particles is thought to require the interaction between viral glycoproteins and specific cell surface receptors for these molecules (1, 23). The production of viral particles that are noninfectious because of alterations in their envelope glycoproteins has been described with many different groups of viruses, and in several instances production of noninfectious virus particles lacking all the major envelope glycoprotein has been reported (4, 9, 12, 28, 39). In studies of defective Rous sarcoma virus, rescue of viral infectivity was demonstrated by procedures that nonspecifically caused fusion of the viral envelope with the cell membrane (9, 39). To determine whether the lack of infectivity of *tsO45* particles produced at restrictive temperature could have been a consequence of the absence of the viral glycoprotein required for successful interaction with target cells, methods similar to those previously described were employed.

Cells infected with *tsO45* were incubated at restrictive temperature, and virus was harvested from the supernatants 16 h later. The titer of infectious virus when assayed directly on any of

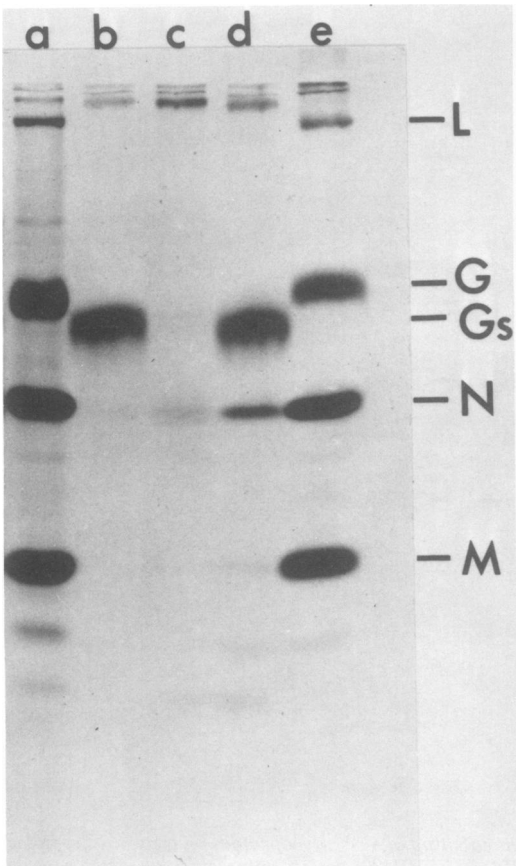

FIG. 6. *Autoradiogram showing a polyacrylamide gel of the immunoprecipitates from the culture fluids of tsO45- and wild-type-infected cells. The immunoprecipitates were obtained with the culture fluids (4 ml) of the following infected cells: (b) wild-type VSV; (c) and (d) tsO45 grown at 39.5 and 32°C, respectively. Track (a) is marker virus run directly, and track (e) is detergent-disrupted virus immunoprecipitated with the anti-VSV IgG under conditions similar to the culture fluids.*

a wide variety of cell types always ranged between 10^2 and 10^3 PFU/ml, this level of infectivity representing the "leakiness" of the *tsO45* mutant. Pretreatment of the assay cells with β-propriolactone-inactivated Sendai virus resulted in a 10-fold increase in the infectious titer of the *tsO45* virus grown at 39.5°C but had no effect on the titer of virus grown at 32°C (Table 1). The procedure of Sendai treatment to rescue infectivity of VSV or Rous sarcoma virus by promoting membrane fusion has been shown to be a relatively inefficient one (40), and hence the demonstration of only a 10-fold increase in infectious titer may be a result of limitations of the procedure rather than the absolute number of potentially rescuable particles present. It is clear, however, that a significant number of particles produced by *tsO45* at restrictive temperature do retain full biological activity once allowed to fuse with host cell membranes, a finding consistent with the data of Deutsch (5) and with the previous investigations demonstrating virus particles morphologically and biochemically indistinguishable from wild-type virus, except for the absence of envelope glycoprotein.

DISCUSSION

The demonstration of a high level of production of noninfectious virus particles, morphologically and biochemically differing from wild-type particles by their lack of viral glycoprotein, from cells infected by *tsO45* mutants of VSV at restrictive temperature was an unexpected finding that has also been recently reported by Deutsch (5) and by Little and Huang (20). The absence of viral glycoprotein from virion particles believed to have been released from infected cells by the normal process of budding from the cell membrane raises questions about the role of, and requirement for, the viral glycoprotein in viral maturation at the cell surface.

Previous studies by a number of investigators have demonstrated that the VSV glycoprotein is synthesized in *tsO45*-infected cells at restrictive temperatures (14, 15, 18, 20, 26, 42). The failure to find this glycoprotein in released virions may be explained on the basis of several different mechanisms. First and most simply, the glycoprotein may not be required for viral budding and therefore need not be demonstrable at the cell surface or be found free in soluble form in the supernatant of cells infected at 39.5°C. This interpretation is consistent with both the data of Knipe et al. (14, 15), demonstrating the failure of VSV glycoprotein to reach the cell membrane under these conditions, and the report by Deutsch (5) of the absence of viral glycoprotein in the supernatant of infected cells at restrictive conditions. In addition, this interpretation has precedent in the avian retrovirus system, in which the production of noninfectious virus particles lacking gp85 by cells infected with avian sarcoma virus has been known for some time (4, 9, 12, 28, 39).

Alternatively, the virion glycoprotein may be required to be present to initiate the budding process but not be required for its successful completion. If this were the case, the viral glycoprotein could be hypothesized to be transiently or weakly associated with the cell/virus membrane, being shed either during or immediately after the budding process and therefore absent in the final released virus particles them-

selves. Data from Little and Huang (20) demonstrating a soluble form of VSV glycoprotein in the supernatant of CHO cells infected at 39.5°C with *tsO45* provides indirect support for this interpretation. However, the fact that their studies did not demonstrate the actual presence of glycoprotein on the cell surface raises the possibility that the glycoprotein found in the supernatant could have been released as a consequence of mechanisms unrelated to budding or the requirement for glycoprotein on the infected cell surface.

Data obtained from the studies reported here confirm the findings of Deutsch (5) and Knipe et al. (14, 15) and are consistent with the interpretation that the VSV glycoprotein is not required to be present at the cell surface for the successful production and release of virion particles, although it may increase the efficiency of the budding process. Following surface labeling of the glycoproteins of cells infected with *tsO45* at the restrictive temperature, immunologically recognizable VSV glycoprotein could not be detected, nor could this material be found free in the supernatant under conditions of metabolic labeling of virion peptides at restrictive temperatures. The possibility that VSV glycoprotein was present at the cell surface but in a state that rendered it immunologically altered or nonreactive with the surface labeling reagents seems unlikely in the light of data from the metabolic labeling experiments, which failed to detect by immune precipitation any evidence of virion glycoproteins either in the virions themselves or free in the supernatants of cells infected by *tsO45* at restrictive temperature. We cannot rule out the possibility that the particles studied were derived from released nucleocapsids that had sequestered lipid membranes from cell debris. This seems highly unlikely, since the particles appeared to be individually wrapped by an uninterrupted membrane containing no demonstrable viral glycoprotein.

The data of Little and Huang (20) and of Witte and Baltimore (41) have led them to propose the requirement for VSV glycoprotein on the cell surface for successful budding and release of viral particles. The reason for the differences between their data and those reported here is not known. In the studies reported by Little and Huang, however, it is possible that the VSV glycoprotein may have been metabolized in the CHO cells in a manner different from its metabolism in the Vero or avian cells employed in this report. Since glycosylation of virion peptides has been demonstrated (30) to be mediated by cellular glycosidases, and since cleavage of peptides from the cell surface may also be a function of cellular enzymes (proteases), differences in the expression of viral functions in the different cell types may very well be a function of the host cell (6, 22, 29, 30), particularly in the case of viral mutants having alterations in proteins or functions subject to cellular modification. The soluble form of the viral glycoprotein found in the supernatant of infected cells could therefore simply represent a by-product of normal CHO cell glycoprotein metabolism, and the elevated levels of this glycoprotein could be a consequence of its unstable association with the endoplasmic reticulum or of the increased enzymatic activity at 39.5°C of cellular proteases responsible for the cleavage of glycoprotein from sites other than the cell surface.

The fact that inhibitors of glycosylation such as tunicamycin (19, 36, 37), glucosamine (31, 34), and 2-deoxy-D-glucose (31, 33) have been shown to reduce the yield of enveloped viruses from infected cells has also suggested that competent viral glycoprotein is required for successful viral budding. However, many of these studies have not examined the possibility of noninfectious-particle production; furthermore, these inhibitors of glycosylation have been shown to alter not only viral glycoproteins but also the glycosylation pattern of normal cellular proteins (16, 35). It may be the presence of specific cellular glycoproteins, rather than viral glycoproteins, that is important for the successful budding of viral particles from cell surfaces. Alterations of these cellular elements would therefore be expected to substantially inhibit virus particle production. Indeed, the presence of viral glycoproteins on the cell surface may merely serve to increase the efficiency of a process already inherent in the infected cell membrane itself. Further studies with group III (M protein) mutants of VSV have suggested that M protein interactions may be critical for the successful budding of VSV (T. J. Schnitzer and H. F. Lodish, manuscript in preparation).

In future studies aimed at more clearly defining the role of the VSV glycoprotein in viral budding, it will be important to produce and examine a larger number of glycoprotein mutants of VSV. Results from experiments with *tsM501*, another group V mutant of VSV, demonstrated that noninfectious virus particle production by mutants of this group was not a feature unique to *tsO45*. Although the specific step(s) at which viral glycoprotein synthesis and/or assembly is altered has not been identified in any group V mutant, investigations of further mutants of this group might be expected to demonstrate examples of viruses defective in

the production of particles themselves. The failure to find such examples among a larger number of group V mutants, particularly among mutants in which the actual synthesis of complete viral glycoprotein was blocked or altered, would argue strongly that VSV glycoprotein was not required for viral release.

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