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Noninfectious Vesicular Stomatitis Virus Particles Deficient in the Viral Nucleocapsid

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Several temperature-sensitive mutants of vesicular stomatitis virus in complementation group III produce, at nonpermissive temperature, noninfectious particles which contain the viral M (matrix) and G (glycoprotein) proteins but less than 10% of the normal proportion of N protein or RNA. Since group III mutants are thought to be defective in the structural gene for the virus M protein, these findings demonstrate that an interaction between M and the nucleocapsid is of importance in virus budding. Taken together with earlier results, they suggest that M is the key protein in bud formation.

A vesicular stomatitis virus (VSV) particle is formed by budding from the plasma membrane of infected cells. This complex process is not well understood but is thought to involve the concerted interaction of the transmembrane viral glycoprotein (G) imbedded in the plasma membrane; the viral matrix protein (M); and the viral nucleocapsid, consisting of one molecule of viral RNA and the other three virus-encoded proteins, N, NS, and L (1, 4, 5, 15). The M protein is localized to the inner surface of the virus membrane and may serve as a "bridge" between the nucleocapsid and G proteins (3, 11, 16).

Some information concerning the interactions involved in virus budding have come from studies of VSV temperature-sensitive (*ts*) mutants. Mutants in complementation group V are defective in maturation of the virus G protein (7, 9, 13); the glycoprotein is synthesized and inserted into the rough endoplasmic reticulum, and the nascent G chain is glycosylated with the normal "core" sugars *N*-acetyl glucosamine and mannose. However, the mutant G does not mature to the plasma membrane and does not receive normal amounts of the "terminal" sugars galactose and sialic acid (7). Budding from these mutant-infected cells are VSV particles containing no glycoprotein but normal amounts of the other viral structural proteins and of RNA (2, 10; T. J. Schnitzer, C. Dickson, and R. A. Weiss, submitted for publication). Thus, there is no absolute requirement for G in the formation of a budding particle.

In this paper we show that certain *ts* mutants in group III, the structural gene for the M protein (7, 9, 13), produce at nonpermissive temperature small numbers of particles which contain G and M but are deficient in N protein and RNA. This result pinpoints an interaction between M proteins and the nucleocapsid as being of importance in the assembly of VSV.

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MATERIALS AND METHODS

Cells and virus. Vero cells were obtained and maintained as previously described (10). Wild-type VSV (Indiana strain) and the temperature-sensitive mutant *ts*O45 (V) have been recently described (10). Six group III mutants were utilized in this study: *ts*G31 (III) isolated by Pringle (12); G32 (III) and G33 (III), isolated by Pringle and Duncan (14), and *ts*M301 (III), M302 (III), and M303 (III), the latter three selected at M.I.T. but probably representing accidental reisolates of *ts*G33 (III) (7).

Infection of cells and metabolic labeling of viral products. Vero cells were maintained as confluent monolayers in plastic petri dishes. Cells were washed once with phosphate-buffered saline (PBS) and then infected with indicated virus at a multiplicity of infection of 3 to 5 PFU/cell. After adsorption for 60 min at room temperature, the inoculum was removed, and the cells were washed twice in PBS. Metabolic labeling of viral products was accomplished by overlaying cells after viral adsorption either with Dulbecco-modified Eagle medium modified to contain 10% of the normal concentration of methionine and supplemented with 50 μCi of [^{35}S]methionine per ml or with Dulbecco-modified Eagle medium supplemented with 50 μCi of [^{3}H]uridine per ml. All media used in labeling experiments contained 2% dialyzed fetal calf serum. Virus and supernatants were harvested 16 h postadsorption. Medium from infected cells was clarified by centrifugation at 2,000 rpm for 10 min in an MSE bench top centrifuge. From the clarified supernatants virus was pelleted through 20% sucrose by centrifugation for 60 min at 135,000 $\times g$ and 4°C using a SW50.1 rotor in a Beckman L5-64 ultracentrifuge. The viral pellets were then either taken up in electrophoresis dissociation buffer or resuspended in PBS.

Equilibrium and velocity gradient sedimenta-

tion. For comparison of buoyant density, virus was grown and pelleted as described above. The viral pellets were suspended in PBS and layered onto a continuous 20 to 50% sucrose gradient containing 0.01 M Tris-hydrochloride (pH 7.5) and centrifuged for 16 h at 40,000 × *g* using a SW50.1 rotor in a Beckman L5-65 ultracentrifuge. Fractions were collected by pumping from the bottom of the tube, and samples were taken for determination of refractive index. The remainder of the samples was made 10% in trichloroacetic acid, allowed to stand at 4°C for 1 h, filtered, and washed twice in cold 5% trichloroacetic acid. Filters were then placed into toluene-2,5-diphenyloxazole and counted on a Tri-Carb scintillation counter set for double-channel counting. For comparison of sedimentation rate of gradient-purified virus, those fractions containing labeled particles were combined, diluted with PBS, and pelleted by centrifugation at 135,000 × *g* for 60 min in a SW50.1 rotor. The virus pellet was resuspended in PBS, and samples were then layered over a preformed 10 to 30% sucrose gradient and centrifuged at 135,000 × *g* for 80 min in a SW50.1 rotor. Fractions were collected, and radioactivity was determined as described above.

Polyacrylamide gel electrophoresis. The virion preparations were dissociated and separated on slab gels using the procedure of sodium dodecyl sulfate-polyacrylamide gel electrophoresis described by Laemmli (8). 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). For quantitation, the autoradiogram was scanned in a Joyce-Loebl microdensitometer, and the areas under the peaks were determined by integration.

RESULTS

To examine the formation of defective virus particles, cells were labeled with [^{35}S]methionine after VSV infection. The clarified cell supernatant was subsequently layered atop a cushion of 20% sucrose and centrifuged, and the pellet was examined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Cells infected at 39.5°C by *ts*M301 (III) (Fig. 1A) produced particles containing M and G protein in the normal proportion but only 1/10 the relative amount of N characteristic of normal virions (Fig. 1C). The amount of radioactive virus proteins in the particulate fraction from *ts*M301-infected cells was about 0.3% that produced by cells infected with wild-type VSV. This material is essentially noninfectious, since the yield of infectious particles (PFU) from these cells was only 0.01% that from cells infected with wild-type VSV. Similar results were obtained after infection of cells at 39°C by *ts*G33 (III), *ts*M302 (III), and *ts*M303 (III) (not shown). However, no proteins were found in the particulate fraction produced by cells infected at 39.5°C by *ts*G31 (III). Under identical conditions, cells infected with *ts*O45, a group V mutant, produced particles lacking detectable G protein but containing all other viral proteins, as has been previously reported (2, 10; Schnitzer et al., submitted for publication). At the permissive temperature (32°C), cells infected by all group III mutants produced normal amounts of infectious particles having the normal composition of virus polypeptides (data not shown).

Figure 2 shows that the particles produced by cells infected at 39.5°C by *ts*M301 (III) have a sedimentation velocity about 61% of that of wild-type VSV. These particles also band in an equilibrium sucrose gradient at a much lower density than do wild-type VSV: 1.149 g/cm^3 versus 1.176 g/cm^3 (Fig. 3). Thus these particles have a slightly greater density than plasma membranes of VSV-infected cells (4). Sodium dodecyl sulfate gel analysis of the peak fractions from these gradients indicates a polypeptide composition identical to that of the crude particulate fraction layered on the gradient (Fig. 1A).

In several experiments attempts were made to label these defective particles with [^{3}H]uridine. However, no radioactivity could be found banding with these particles in gradients such as those of Fig. 2. We calculate that these particles contain less than 10% the amount of viral RNA, relative to total VSV protein, compared to normal VSV virions.

Thus, the physical and analytical properties of the particles produced by *ts*M301 are those of a phospholipid membrane containing the VSV G and M proteins, but lacking the internal nucleocapsid of N protein and RNA. The resolution and sensitivity of our gel analysis does not allow accurate quantitation of NS and L proteins in these particles, but no amount of L could be detected. NS, in particular, comigrates with N; the material migrating at 5 cm (Fig. 1A and C) apparently is a contaminating host protein.

DISCUSSION

Several lines of evidence indicate that mutants in complementation group III contain a defect in the structural gene for the matrix (M) protein. Lafay (9) showed that M protein synthesized in cells infected with *ts*O89 (III) at 39°C was defective because it did not become incorporated into virions after transfer to 31°C. Pringle (13) detected differences relative to wild-type VSV in the electrophoretic mobility of the M polypeptide synthesized at 39°C by three different group III mutants. Finally, Knipe et al. (7) showed that the M protein synthesized at 39°C by three group III mutants [*ts*G33 (III), *ts*M301 (III), *ts*M303 (III)] was unstable at 39°C and was degraded considerably faster than was M

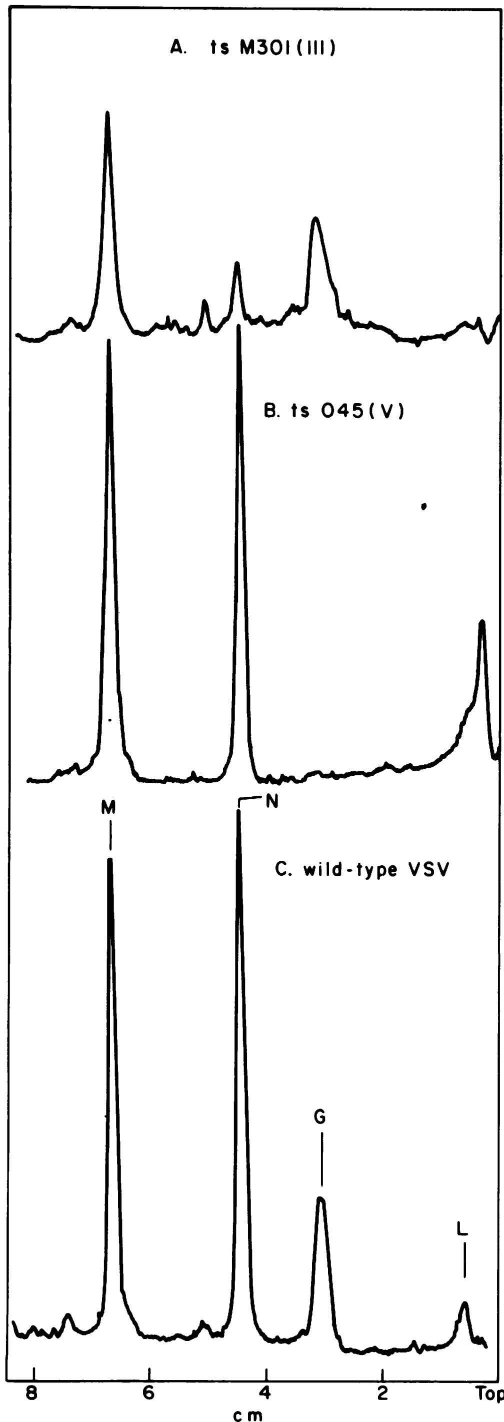

FIG. 1. *Comparison of viral proteins in the particulate fraction of cells infected at 39.5°C with tsM301 (III) (A), tsO45 (III) (B), or wild-type VSV (C). The particulate fraction was obtained and prepared for polyacrylamide gel electrophoresis as described. The radiolabeled proteins were located by autoradiography and quantitated by scanning on a Joyce-Loebl microdensitometer with a full-scale pen deflection of 1.0 optical density unit, within the linear range of the X-ray film.*

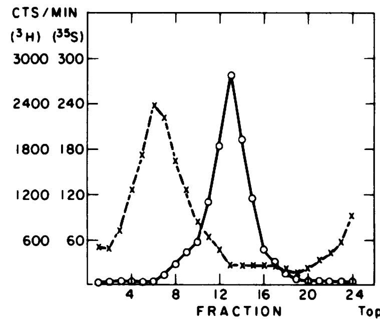

FIG. 2. *Comparison of sedimentation rate of gradient-purified particles produced at 39.5°C by VSV ts mutant M301 (III) with VSV wild-type particles. Virus particles were grown and purified by equilibrium density centrifugation as described above. Fractions containing labeled particles were combined, diluted with PBS, and pelleted by centrifugation at 135,000 × g for 45 min in a SW50.1 rotor. The virus pellet was suspended in 100 μl of PBS and samples were then layered over a preformed 10 to 30% sucrose gradient and centrifuged at 135,000 × g for 80 min in a SW50.1 rotor. Fractions were collected, and radioactivity was determined as described above.* (×) *[³H]uridine-labeled wild-type VSV;* (O) *[³⁵S]methionine-labeled tsM301 (III) particles.*

protein synthesized after infection by wild-type VSV or by mutants in other complementation groups. This instability of M could not be rescued upon simultaneous infection by wild-type VSV.

Cells infected by five of the six group III mutants tested produce, at 39.5°C, particles containing G and M proteins in the normal proportion, but 10% or less of the normal amount of N and VSV RNA. These noninfectious particles all have the density of plasma membranes or smooth membranes from VSV-infected cells. In cells infected at 39.5°C by group III mutants, viral G protein matures normally and accumulates in the plasma membrane (5, 7). Three lines of evidence suggest that the defective particles are derived by budding from the plasma membrane and are not fragments of intracellular membranes released from lysed cells: (i) the particles sediment homogenously in a velocity gradient; (ii) no free viral nucleocapsid can be found in the cell supernatant, as might be expected if lysed cells were releasing their contents; (iii) cells infected at 39°C by *ts*G31 (III), *ts*L411 (IV), and *ts*L412 (IV) also lyse, but produce no virus-like particles of the sort described here (H. F. Lodish and R. A. Weiss, submitted for publication).

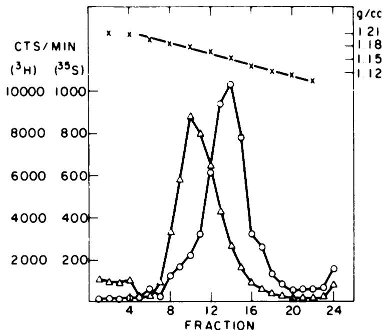

FIG. 3. *Comparison of buoyant density of particles produced at 39.5°C by VSV ts mutant M301 (III) with VSV wild-type particles. Virus was inoculated onto a confluent monolayer of Vero cells at a multiplicity of infection of 3 to 5 and allowed to adsorb for 1 h at room temperature. The inoculum was removed, and the cells were washed once with Dulbecco-modified Eagle medium and then fed with either Eagle medium + 50 μCi of [^{3}H]uridine per ml (wild-type VSV infected) or Eagle medium with 10% normal methionine concentration + 50 μCi of [^{35}S]methionine per ml [tsM301 (III) infected] and incubated at 39.5°C for 16 h. Supernatant fluids were removed, clarified by centrifugation at 2,000 rpm for 10 min in an MSE bench top centrifuge, layered over 20% sucrose, and virus pelleted by centrifugation in a Beckman SW50.1 rotor at 135,000 × g for 45 min. The virus pellet was suspended in 100 μl of PBS and run on a 20 to 50% sucrose gradient in the same rotor overnight at 40,000 × g. Fractions were collected by pumping from the bottom of the tube, and samples were taken for determination of refractive index. The remainder of the samples was then precipitated and counted (see the text). (△) [^{3}H]uridine-labeled wild-type VSV; (○) [^{35}S]methionine-labeled tsM301 (III) particles; (×) density.*

It is somewhat difficult to interpret the result that no particles are formed after infection at 39.5°C by *ts*G31 (III). Stocks of this mutant have never yielded plaques at 39.5°C, in contrast to cloned stocks of *ts*G33 (III), *ts*M301 (III), *ts*O45 (V), and most other VSV ts mutants which produce revertant plaques at a frequency of 10^{-5} to 10^{-4}. It seems possible that *ts*G31 (III) is a double mutant with two lesions in the gene for M protein.

Although the defective particles contain essentially only the two virus-encoded membrane proteins G and M, a small amount of N (or NS) is invariably present. This result might suggest that a small amount of N protein—presumably a piece of viral nucleocapsid—is essential for the budding process. Alternatively, 10% of the defective particles could have a normal complement of N, and the remainder could have none. This would imply that a virus bud could form in the total absence of N. What is clear is that some interaction between M and N is essential for normal budding to take place, and that the lesions in the M polypeptide in *ts*G32 (III), *ts*G33 (III), *ts*M301 (III), *ts*M302 (III), and *ts*M303 (III) prevent, to a large extent, this interaction. These results suggest that M and G are sufficient for some virus budding to occur.

Taken together with earlier studies of ts mutants in group V, which demonstrated that particles can be formed lacking G but containing M, N, NS, and L in normal proportions (2, 10; Schnitzer et al., submitted for publication), it appears that M itself is the key polypeptide in forming a virion. Possibly it can self-aggregate under the plasma membrane in a two-dimensional polypeptide network. This alone might be sufficient to induce a bud in the overlying plasma membrane, or a small amount of nucleocapsid might also be essential.

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LITERATURE CITED

1. **Atkinson, P. H., S. A. Moyer, and D. F. Summers.** 1976. Assembly of vesicular stomatitis virus glycoprotein and matrix protein in HeLa cell plasma membranes. J. Mol. Biol. **102:**613–631.
2. **Deutsch, V.** 1976. Parental G protein reincorporation by a vesicular stomatitis virus temperature-sensitive mutant of complementation group V at nonpermissive temperature. Virology **69:**607–616.
3. **Eger, R., R. W. Compans, and D. B. Rifkin.** 1975. The organization of the proteins of vesicular stomatitis virions: labeling with pyridoxal phosphate. Virology **66:** 610–615.
4. **Knipe, D. M., D. Baltimore, and H. F. Lodish.** 1977. Separate pathways of maturation of the major strutural proteins of vesicular stomatitis virus. J. Virol. **21:** 1128–1139.
5. **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.
6. **Knipe, D. M., H. F. Lodish, and D. Baltimore.** 1977. Localization of two cellular forms of the vesicular stomatitis viral glycoprotein. J. Virol. **21:**1121–1127.
7. **Knipe, D. M., 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.
8. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227:**680–685.
9. **Lafay, F.** 1974. Envelope proteins of vesicular stomatitis virus: effect of temperature-sensitive mutants in complementation groups III and V. J. Virol. **14:**1220–1228.
10. **Little, S. P., and A. S. Huang.** 1977. Synthesis and distribution of vesicular stomatitis virus-specific polypeptides in the absence of progeny production. Virology **81:**37–47.
11. **Nakai, T., and A. F. Howatson.** 1968. The fine structure of vesicular stomatitis virus. Virology **35:**268–281.

12. **Pringle, C. R.** 1970. Genetic characteristics of conditional lethal mutants of vesicular stomatitis virus induced by 5-fluorouracil, 5-azacytidine, and ethyl methane sulfonate. J. Virol. **5**:559-567.
13. **Pringle, C. R.** 1977. P. 239-289. *In* R. R. Wagner, and H. Fraenkel-Conrat (ed.), Comprehensive virology, vol. 9. Plenum Press, N.Y.
14. **Pringle, C. R., and I. B. Duncan.** 1971. Preliminary physiologic characterization of temperature-sensitive mutants of vesicular stomatitis virus. J. Virol. **8**:56-61.
15. **Wagner, R. R., M. P. Kiley, R. M. Snyder, and C. A. Schnaitman.** 1972. Cytoplasmic compartmentalization of the protein and ribonucleic acid species of vesicular stomatitis virus. J. Virol. **9**:672-683.
16. **Wagner, R. R., L. Prevec, F. Brown, D. F. Summers, F. Sokol, and R. MacLeod.** 1972. Classification of rhabdovirus proteins: a proposal. J. Virol. **10**:1228-1230.