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Heterogeneity of Vesicular Stomatitis Virus Particles: Implications for Virion Assembly

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Vesicular stomatitis virus (VSV) particles formed at early times after infection contain only one-third the amount of viral glycoprotein (G protein), relative to the major internal structural proteins M and N, as is found in particles released later. These "early" particles also have a lower density in equilibrium sucrose gradients than do those formed later; however, the sedimentation velocity and specific infectivity of these two classes of particles are the same. VSV-infected cells also release virus-like particles which sediment considerably faster than authentic virions and contain a higher-than-normal proportion of the VSV G protein relative to internal VSV proteins. These particles have a reduced specific infectivity but a normal density in sucrose gradients. All classes of VSV virions contain a constant proportion of M and N polypeptides. The ratio of G protein to M or N protein, in contrast, can vary over a sixfold range; this implies that an interaction between a precise number of surface G proteins with either of the underlying M and N proteins is not a prerequisite for budding of infectious viral particles from the cell surface.

A vesicular stomatitis virus (VSV) particle, like those of most lipid-containing animal viruses, is formed by budding from the plasma membrane of an infected cell (reviewed in references 3, 11, 12, 18, and 21). This complex process is not well understood, but must reflect the structural organization of the viral proteins in the infected cell and in the virion. The transmembrane (5, 6, 13, 16, 20) viral glycoprotein (G protein) is imbedded in the plasma membrane; it becomes, by far, the major protein exposed on the surfaces of infected cells and on the surface of the virion (2, 3, 7, 21). The M protein is apparently localized in the inner surface of the virus membrane and may serve as a "bridge" between the G protein and the viral nucleocapsid, which consists of one molecule of viral RNA and the other three virus-encoded proteins, N, NS, and L (11, 12, 17, 21, 22).

The VSV budding process is not totally specific for the VSV G protein, since budding VSV cores can incorporate into virion glycoproteins of other, unrelated viruses, such as retroviruses, and possibly also specific cell surface antigens (4, 15, 23, 24). Cellular glycolipids and various enzymes have also been identified in VSV particles (reviewed in reference 21). Studies of VSV pseudotypes indicated that the VSV M protein is essential for the formation of a virus particle containing the VSV nucleocapsid (15; R. A. Weiss and P. L. Bennett, submitted for publication). In contrast, cells infected at nonpermissive temperatures by VSV mutants defective in maturation of the G protein release, at low levels, noninfectious particles with the shape and physical properties of normal VSV. These particles contain no G protein but contain VSV RNA and all of the other VSV proteins in normal proportions (14, 19). This result implies that the VSV G protein may not be essential for the formation of a budding particle.

We show here that among various classes of released VSV particles the ratio of surface viral glycoproteins M and N can vary considerably, although the ratio of M to N proteins is constant. Our results suggest that an interaction between a precise number of surface G proteins with either the M or N proteins is not an essential prerequisite for VSV maturation.

MATERIALS AND METHODS

Cells and viruses. Monkey (Vero) cells were maintained as monolayers in minimal essential medium (GIBCO Laboratories, Grand Island, N.Y.; no. F10-1100) containing 10% decomplemented fetal calf serum. The Indiana serotype of VSV was originally obtained from C. Pringle (Institute of Virology, Glasgow, United Kingdom). The procedures for plaque assays with Vero cells were described previously (14, 23).

For infection, 10-cm-diameter dishes containing about 8×10^6 cells were infected with 10 PFU of VSV per cell as described previously (14). Each dish was then incubated in 4 ml of medium.

Labeling of infected cells. For labeling with [^{35}S]methionine, the medium contained no methionine and 10% dialyzed fetal calf serum. At 2 h postinfection,

cells were washed twice in this medium; to each dish was added 4 ml of medium containing 50 μCi of [^{35}S]methionine (700,000 mCi/mmol; Radiochemical Centre, Amersham, England) per ml. In some experiments, the labeled medium was removed at intervals after infection and replaced with an equal volume of the medium containing the same concentration of [^{35}S]methionine.

Fractionation of virus. The medium was harvested postinfection at times indicated in the individual experiments, centrifuged at 2,000 × *g* for 10 min, and filtered through a membrane filter (0.22-μm-diameter pore size; Millipore Corp., Bedford, Mass.). Generally, the medium from several plates was pooled; 8 ml was layered on top of a 2-ml cushion of 20% (wt/wt) sucrose in phosphate-buffered saline and centrifuged for 2.5 h at 37,000 rpm and 4°C in a Beckman SW40 rotor. Each pellet was suspended in 1 ml of Tris-saline (NaCl, 8 g/liter; KCl, 0.3 g/liter; Na_2HPO_4, 1 g/liter; dextrose, 1 g/liter; Trizma base, 30 g/liter; pH to 7.4) overnight and then subjected to four 30-min pulses in a model G112SPIT water bath sonicator (Laboratory Supplies Co., Hicksville, N.Y.).

For velocity gradient analysis (see Fig. 1), the solutions were layered on an 11-ml, 15 to 40% (wt/wt) linear sucrose gradient made up in phosphate-buffered saline and centrifuged for 40 min at 37,000 rpm in the Beckman SW40 rotor. Fractions were collected by pumping from the bottom. For analysis by gel electrophoresis, samples were diluted 10-fold with phosphate-buffered saline; the particulate matter was recovered by ultracentrifugation and was dissolved directly in the sample buffer for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (14, 19). For analysis by equilibrium gradient centrifugation (see Fig. 4), the sample was diluted with 2 volumes of phosphate-buffered saline and layered on top of an 11-ml, 20 to 50% (wt/wt) linear sucrose gradient made up in phosphate-buffered saline; centrifugation in the SW40 rotor was for 18 h at 37,000 rpm and 4°C.

Gel electrophoresis. Conditions for analysis of viral or cellular proteins by electrophoresis through 10% polyacrylamide gels containing sodium dodecyl sulfate were detailed previously (10, 14).

RESULTS

VSV particles released at different times after infection. The heterogeneity of VSV particles can be shown in at least two ways; perhaps the most dramatic is a comparison of the different proportions of structural proteins found in virions released at different times after infection. In these studies, cells were labeled with [^{35}S]-methionine beginning at 2 h postinfection; at hourly intervals starting at 5 h, the medium was removed and replaced with fresh medium containing the same concentration of [^{35}S]methionine. The particulate matter in the extracellular medium was recovered by centrifugation through a cushion of sucrose and analyzed by velocity gradient centrifugation.

Figure 1 shows that the labeled virions released at all times after infection had the same sedimentation profile: a major peak of virions, together with a considerable amount of faster-sedimenting material. Also, the specific infectivities (PFU/counts per minute) of virions released at different times were not significantly different (Fig. 2); if anything, the virus released at early times had a higher specific infectivity. In contrast, VSV released at early times (5 h) after infection contained only one-third the amount of labeled G protein, relative to M or N protein, as did VSV released at later times (Fig. 2 and 3). The ratio of M to N protein, on the other hand, was the same in virions released at all times after infection.

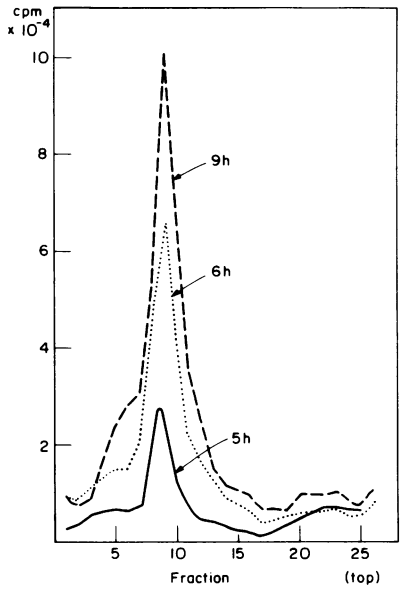

FIG. 1. *Velocity gradient analysis of virions released at different times after infection. As detailed in the text, six plates of cells were labeled with [^{35}S]methionine beginning at 2 h postinfection. Beginning at 4 h, the medium was harvested at hourly intervals and replaced with fresh medium containing [^{35}S]methionine. Shown here are velocity gradient profiles of the particulate matter released into the medium between 4 and 5 h (solid line), 5 and 6 h (dotted line), and 8 and 9 h (dashed line). Centrifugation is from right to left; plotted is the ^{35}S radioactivity in 50 μl of the 0.5-ml gradient fractions.*

These differences did not result from differential action of proteases released into the medium on budded virus. The medium from cells labeled at 8 to 9 h after infection was mixed with the medium harvested at 5 h from unlabeled cells and incubated for 1 h at 37°C. There was no change in the amount of labeled G protein in purified virus, relative to M or N protein, com-

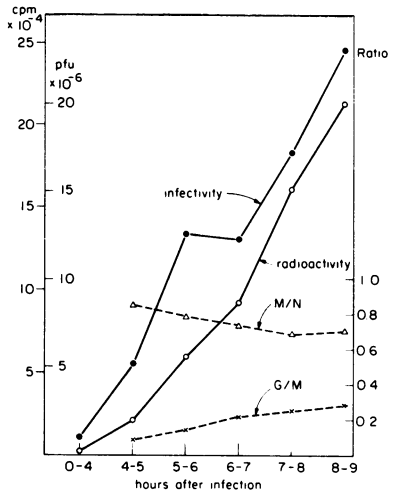

FIG. 2. *Properties of VSV particles released at different times after infection. As described in the legend to Fig. 1, infected cells were labeled with [^{35}S]methionine at 2 h, the medium was harvested from cells at hourly intervals and replaced with fresh medium containing [^{35}S]methionine. Shown here are the PFU per milliliter of medium (●) and the amount of ^{35}S radioactivity in the peak of virions purified by velocity gradient sedimentation (○) (see Fig. 1). Virions from the velocity gradient were purified further by equilibrium gradient centrifugation (see Fig. 4), recovered by centrifugation dissolved in buffer containing sodium dodecyl sulfate, and analyzed by polyacrylamide gel electrophoresis as described in the legend to Fig. 3. Shown here are the ratios of the areas under the peaks of G relative to M protein (×) and M relative to N protein (△); these were determined by densitometer analysis of the radioautograms of the dried gels.*

pared with unincubated samples. Similarly, the medium which was exposed to unlabeled cells at 8 to 9 h after infection was mixed with the medium harvested from cells labeled at 2 to 5 h after infection; again, there was no effect on the amount of the labeled virus proteins in purified virions (data not shown).

Noninfectious virus-like particles which lack the viral G protein (produced in cells infected at the nonpermissive temperature by temperature-sensitive [*ts*] mutants defective in maturation of the G protein) have a lighter density (1.16 versus 1.18 g/cm^3) in sucrose than do normal, infectious particles (14, 19). Presumably, this difference is due to the absence of the G protein. Thus, one might expect that wild-type VSV particles released early after infection and containing a reduced proportion of the G protein might have a lighter density in sucrose than do particles released at later times. Figure 4 shows that indeed this was the case: as measured either by infectivity or by [^{35}S]methionine radioactivity, the average density of particles released at 5 h was about 0.005 g/cm^3 lighter than that of particles released at 9 h. The differences in the profiles of radioactivity and infectivity observed in the 9-h virus preparation (Fig. 4b) were not consistently observed, but the differences between the 9- and 5-h virus preparations were completely reproducible. Note that the ratio of G to M or M to N proteins across each of the peaks of virions was roughly constant, but the average ratio of G to M proteins in the particles

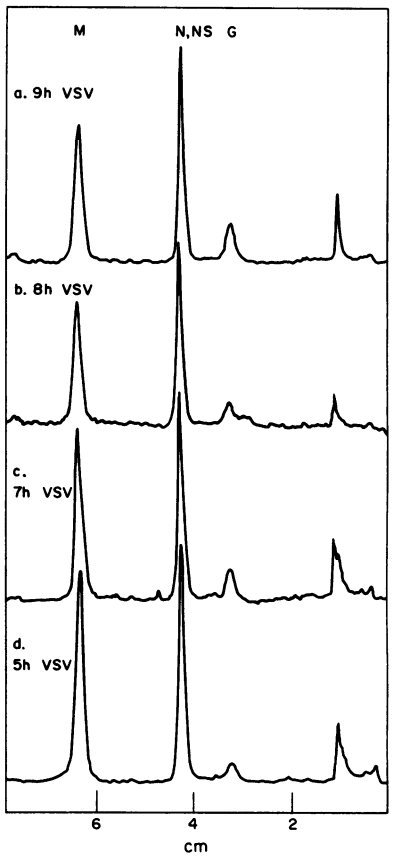

FIG. 3. *Polyacrylamide gel analysis of [^{35}S]methionine-labeled virions released at different times after infection. Labeling was done as described in the legends to Fig. 1 and 2; virions were purified by both velocity and equilibrium gradient centrifugations. Scanning of the radioautograms of the dried gels was done with a Joyce-Lobel microdensitometer with a full-scale pen deflection with an optical density of 1.16, the film exposure was within the linear range of both the film and the densitometer. Calculations of the data from this experiment are presented in Fig. 2. (a) Particles released from 8 to 9 h; (b) particles released from 7 to 8 h; (c) particles released from 6 to 7 h; (d) particles released from 4 to 5 h.*

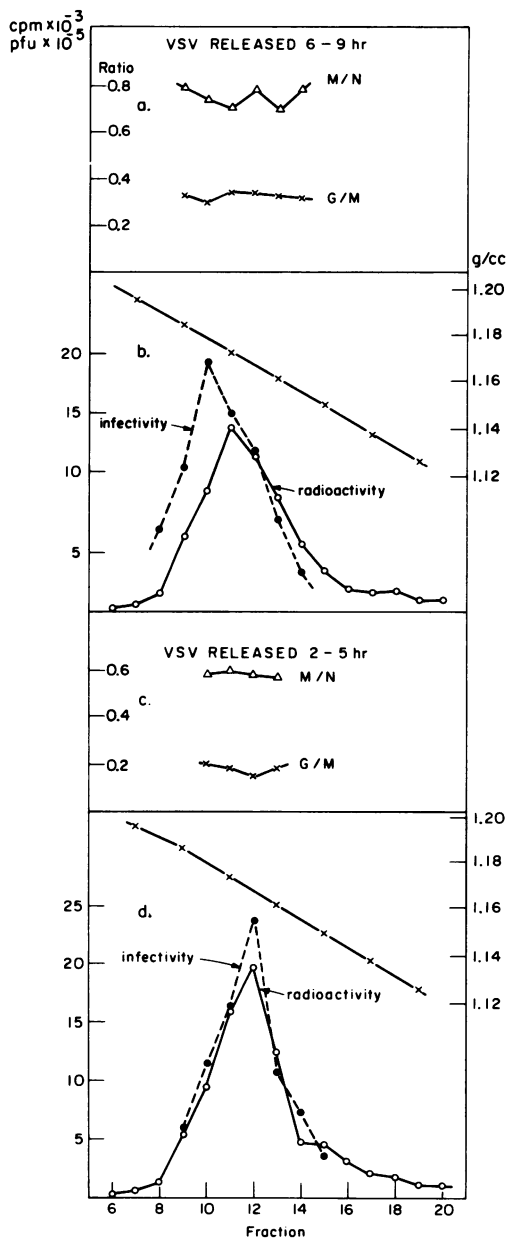

FIG. 4. *Equilibrium gradient centrifugation of VSV released at two times after infection. Labeling with [^{35}S]methionine was done as described in the legends to Fig. 1 and 2. Virus released from 2 to 5 h after infection (c and d) and virus released from 6 to 9 h after infection (a and b) were purified by velocity sedimentation. As detailed in the text, they were then centrifuged to equilibrium in a 20 to 50% (wt/wt) sucrose gradient. Shown in (b) and (d) are the radioactivity per 100 μl of the 500-μl gradient fractions (○) and the PFU per milliliter (●). The density of alternate fractions (×) was determined from the refractive index. The remainder of the virus particles in the peak fractions was recovered by centrifugation, and the virion proteins were analyzed by polyacrylamide gel electrophoresis as described in the legend to Fig. 3. Shown in (a) and (c) are the ratios of radioactivity in G relative to M protein (×) and in M relative to N protein (△).*

released at 5 h was, again, about 40% that of the particles released at 9 h.

Fast-sedimenting VSV particles with abnormal proportions of viral proteins. Vero cells labeled with [^{35}S]methionine after infection released labeled particles which sedimented considerably faster than did normal infectious VSV virions (Fig. 1 and 5). The specific infectivities (PFU/radioactivity) of these particles were one-fifth or less than those of normal virions. Several lines of evidence suggest that these may not have been aggregates of VSV with cellular debris, as we had first imagined, but rather VSV particles with an abnormal composition of proteins.

First, the proportion of these fast-sedimenting particles was unaffected by prolonged ultrasonic treatment, which might be expected to disrupt aggregates. Sonication for 4 min (see above) began to destroy authentic virions, but did not otherwise alter the gradient profiles such as those of Fig. 1 and 5 (data not shown).

Second, the density in sucrose gradients of these fast-sedimenting particles was the same as, or very slightly greater than, that of infectious virions (Fig. 6). Aggregation of virions with cellular membranes might be expected to alter the density of VSV particles.

Finally, and most importantly, these fast-sedimenting particles contained a much higher proportion of the VSV G protein, relative to the internal proteins M and N, than did the slower-sedimenting infectious particles. The ratio of M to N proteins in these particles, on the other hand, was normal. These properties, particularly the last, were difficult to reconcile with the hypothesis that there are aggregates of normal VSV particles with cellular membranes or other debris. Rather, they appear to represent a class of virus-like particles with a higher-than-normal proportion of viral surface glycoproteins.

DISCUSSION

VSV particles with varying proportions of the G protein. The present experiments define two normal classes of VSV particles released from infected cells. One class of particles is released at early times after infection. It has a normal specific infectivity and sedimentation velocity, but a lighter density than do particles

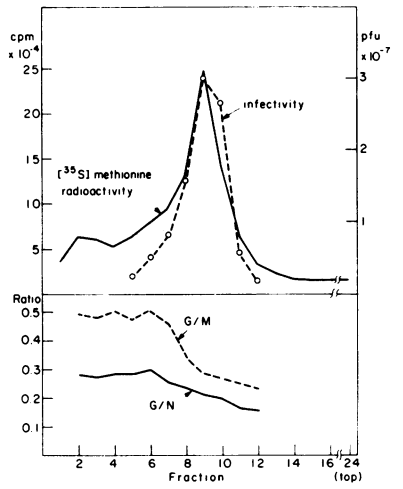

FIG. 5. *Fast-sedimenting VSV particles with abnormal proportions of VSV G proteins. Vero cells were infected with VSV and labeled with [35S]methionine at 2 to 9 h. As described in the legend to Fig. 1, the particulate fraction released into the medium at 9 h was analyzed by velocity gradient centrifugation. Infectivity was assayed directly on the gradient fractions. Material in each gradient fraction was also recovered by centrifugation and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The only bands visible on the radioautogram were those of the five VSV polypeptides. The ratios of G to M and G to N proteins were calculated from the areas of the respective peaks on the microdensitometer scans of these radioautograms. (Top) (—) radioactivity (counts per minute per 100-μl aliquot of the 500-μl fraction); (----) PFU per milliliter. (Bottom) (—) ratio of G to N; (----) ratio of G to M.*

released later, and contains only one-third the normal proportion of the G protein relative to M or N protein. The second class is released at all times after infection. It has a normal density in sucrose gradients, but a faster-than-normal sedimentation velocity. It has a reduced specific infectivity and a higher-than-normal ratio of G to M proteins, but a normal proportion of the internal structural proteins M and N. Little more is known of the structure of biosynthesis of these latter particles, but arguments advanced above suggest that they are not mere aggregates of VSV with cellular debris.

With respect to a consideration of the process of assembly of a VSV virion, the first class of particles appears to be the more significant. The existence of these particles establishes that the ratio of surface glycoproteins to internal structural proteins M and N in budding particles can be one-third that characteristic of normal (late-released) particles. The infectivities of these particles are at least the same as of particles released later, and the ratio of M to N proteins and, presumably, N protein to VSV RNA, is normal. Thus, the budding process does not require an interaction between a precise number of surface glycoprotein molecules and the internal N-M protein complex.

Molecules of the G protein are not found on the cell surface or in budded VSV particles until 35 to 45 min after their synthesis, whereas encapsulation of M and N proteins begins with 10 min after their formation (8, 9). Also, the relative synthesis of the five different VSV proteins does not change significantly during infection. Thus, at some time early after infection, one would expect there to be a pool of intracellular nucleocapsids and M protein, but a much lower relative amount of the G protein on the surface; this would account for the lower proportion of the G protein in the particles released at early times.

The present results provide at least a partial explanation for a paradoxical class of particles produced at the nonpermissive temperatures by cells infected with certain VSV *ts* mutants in complementation group IV, which corresponds to the gene for the viral N protein (14). Like normal virions produced at early times after infection, these particles contain one-third to one-half the normal proportion of the G protein and are of lighter-than-normal density. We presume that the primary defects in these mutant-

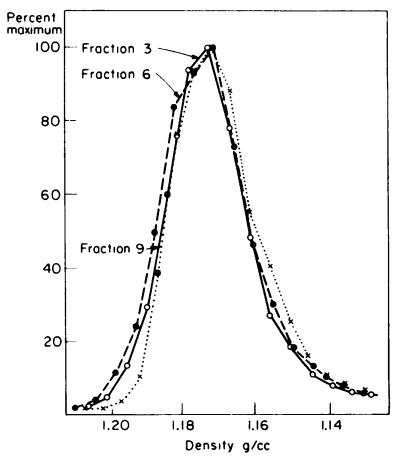

FIG. 6. *Density of fast-sedimenting VSV particles. Material from fractions 3, 6, and 9 from the gradient depicted in Fig. 5 was diluted and then centrifuged to equilibrium in a sucrose gradient as described in the legend to Fig. 4. The peak samples of radioactivity contained, per 50 μl of the 500-μl fraction, 11,500 cpm (gradient from fraction 3) (—), 17,200 cpm (gradient from fraction 6) (-----), and 51,000 cpm (gradient from fraction 9) (·····). Plotted here is the percentage of this peak radioactivity found in the different fractions as a function of the density of these fractions. Gradient fractions not plotted here contained less than 2% of the radioactivity of the peak sample.*

infected cells are replication and transcription of VSV mRNA. These would result in a delayed synthesis of all classes of VSV proteins and, in particular, in a delayed appearance of the G protein on the cell surface. As at early times after infection by wild-type VSV, there would be a lower-than-normal ratio of the surface G protein to intracellular M and N proteins; this could result in the formation of particles, even at late times after infection, which contain a reduced proportion of the G protein. It should be pointed out, though, that the particles produced by the mutant-infected cells do have a reduced specific infectivity (14). As discussed previously, the proper lateral clustering or conformation of the surface G protein could be dependent in some manner on a functional viral nucleocapsid, and this interaction could also be defective in cells infected by this class of *ts* mutants (14).

Recent work has shown that budding wild-type VSV particles incorporate a discrete subset of normal host cell surface proteins (H. F. Lodish, D. W. Wirth, and M. Porter, Ann. N.Y. Acad. Sci., in press; H. F. Lodish and M. Porter, submitted for publication). As might be expected, the proportion of these host proteins is much higher in virions released early (5 h) after infection than late (9 h), but at any time, the amount of these surface proteins is never more than 10% of the G protein. Thus, there does not seem to be a simple replacement of host cell surface proteins with the G protein in virions formed at different times.

Formation of a VSV particle. Beyond the above results, it is difficult to formulate an exact mechanism for the formation of a budding VSV particle, since we are ignorant of many key aspects of VSV structure. It seems clear that the G protein spans the surface membrane; about 30 amino acids at the COOH terminus face the inner (cytoplasmic) surface, and another 20 to 30 are buried within the phospholipid bilayer. Most of the remainder of the polypeptide, including the two Asn-linked oligosaccharides, face the outer surface (3, 5, 6, 11, 13). Where the M protein is located is not as clear. No large region of the M protein is apparently exposed on the outer surface of the virion, but the existing experiments do not eliminate the possibility of a small extracytoplasmic segment (2). It is also not known whether the M protein binds directly to the lipid membrane; the NH_2-terminal region of the M protein is hydrophobic and could bind directly to the phospholipid membrane (D. Sabatini, personal communication), nor is it clear whether there is a direct interaction between parts of the M and G polypeptides. Chemical cross-linking experiments have yielded no convincing evidence for such interactions either in VSV or in influenza virus (1; D. Wiley, personal communication).

Our current view is that M-M and M-N protein interactions are of primary importance in inducing the formation of a budding particle. M polypeptides, either soluble or, more likely, associated with the plasma membrane, would polymerize to yield a skeletal structure of the general shape of VSV. The length of this particle would be determined by the size of the underlying nucleocapsid. In some manner the budding N-M. protein complex would select certain cellular and viral (G) plasma membrane proteins for incorporation into a virion particle. Clearly there is some selectivity as to which cellular or viral proteins are incorporated (Lodish et al., in press), but as is shown by the present data, virus budding does not require a fixed number or proportion of these surface proteins.

Whether any cell or virus surface proteins are required to form a VSV particle cannot be answered at present. Virions of normal morphology, but totally lacking the VSV G protein, bud from cells infected at nonpermissive temperatures with all VSV *ts* mutants in complementation group V (14, 19). These particles are noninfectious, but the particles can be made infectious if the mutant is grown at nonpermissive temperatures in a cell producing an appropriate retrovirus glycoprotein (14, Weiss and Benne, submitted for publication). The retrovirus surface glycoprotein is incorporated into VSV pseudotype particles which contain the *ts* (V) VSV genome and M protein but no surface G protein. The surface retrovirus glycoprotein is the determinant of the pseudotype host range. The formation of these *ts* (V) leukosis virus pseudotypes is totally resistant to complement-induced lysis with anti-VSV serum, so there is apparently no functional VSV G protein on the surface of the infected cells. Thus, no surface G protein is essential for the formation of these G protein-defective particles and pseudotypes (Weiss and Bennett, submitted for publication). One cannot conclude, however, that the surface G protein has no role in the formation of a budding particle since the amount of G protein-deficient particles produced in these mutant-infected cells is only 1 to 10% that formed after infection with wild-type VSV. The presence of the G protein on the cell surface may facilitate the rate or extent of virus budding. Also, some cell surface proteins may fulfill any crucial role normally played by the G protein.

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