Complementation of M Gene Mutants of Vesicular Stomatitis Virus by Plasmid-Derived M Protein Converts Spherical Extracellular Particles into Native Bullet Shapes

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The matrix (M) protein of vesicular stomatitis virus (VSV) binds the nucleocapsid to the cytoplasmic surface of the host plasma membrane during virus assembly by budding. It also condenses the nucleocapsid into a tightly coiled nucleocapsid-M protein complex that appears to give the virion its bullet-like shape. As described here, temperature-sensitive (ts) M mutants produced two classes of membrane-containing extracellular particles at the nonpermissive temperature. These could be distinguished by sedimentation in sucrose gradients and by electron microscopy. One class contained nucleocapsids and envelope glycoprotein, but very little M protein. The other class was devoid of nucleocapsids. Most of these particles were spherical or pleomorphic in shape as determined by electron microscopy. Expression of wild-type (wt) M protein from plasmid DNA using the vaccinia/T7 virus system did not enhance the incorporation of nucleocapsids into extracellular particles from cells coinfected with the ts M mutants but did enhance the incorporation of M protein into these particles. Electron microscopy showed that wt M protein served to impart the bullet-like shape typical of VSV virions to what would otherwise be spherical or pleomorphic virus-like particles. These data suggest that there are two distinct processes in VSV envelope biogenesis. One process involves envelopment of the nucleocapsid and can be accomplished by the ts M mutants at the nonpermissive temperature, albeit at a low level compared to wt VSV. The other process involves conversion of virion components into the bullet-like shape and requires a function provided by wt M protein.

INTRODUCTION

Matrix (M) proteins of enveloped viruses play a central role in virus assembly by mediating the binding of the viral nucleocapsid to the cytoplasmic surface of the host plasma membrane during the budding process. Thus M proteins of most viruses have the ability to interact both with nucleocapsids and with cellular membranes in order to function in virus assembly. In the case of vesicular stomatitis virus (VSV), the prototype rhabdovirus, the virion contains approximately 2000 copies of M protein (Thomas et al., 1985). The nucleocapsid consists of 1300 copies of N protein, the major nucleocapsid protein associated with the 11-kb negative-strand RNA genome, and lesser amounts of two polymerase-associated proteins, P and L. The envelope contains a single species of transmembrane glycoprotein (G protein). Binding of the M protein to the nucleocapsid serves to condense the nucleocapsid into a tightly coiled helical complex that appears to be responsible for the characteristic bullet-like shape of virions (Newcomb and Brown, 1981; Newcomb et al., 1982). A considerable body of evidence suggests that in virions, M protein is associated both with the nucleocapsid and with the envelope (reviewed by Pal et al., 1987), although it is possible that separate populations of M protein molecules are associated with the nucleocapsid versus the envelope (Barge et al., 1993).

VSV mutants containing temperature-sensitive (ts) lesions in their M genes (complementation group III) are blocked in the late stages of virus assembly at the nonpermissive temperature. Although most of the mutants express similar amounts of M protein at both temperatures, binding of viral nucleocapsids to the host plasma membrane and binding of M protein to nucleocapsids are dramatically reduced compared to that observed at the permissive temperature (Knipe et al., 1977a; McCreedy and Lyles, 1989; Ono et al., 1987). In contrast to the apparent block in nucleocapsid binding, the ability of the ts M protein to bind to the cytoplasmic surface of host plasma membranes in areas devoid of nucleocapsids is not impaired at the nonpermissive temperature (McCreedy and Lyles, 1989), suggesting that the mutant M proteins might retain some biological activity.

Complementation of the growth of VSV ts M mutants by M proteins expressed from plasmid DNA using vaccinia vectors that produce T7 RNA polymerase (vaccinia/T7 virus; Fuerst et al., 1987) has been used to assess the function of plasmid-derived wild-type (wt) and mutant M proteins (Black et al., 1993; Kaptur et al., 1995; Li et al.,
MATERIALS AND METHODS

Viruses

Working stocks of ts M mutants tsO23 and tsM301 were prepared from isolated plaques using BHK cells. Titers of virus stocks were determined by plaque assays performed at 31 and 39°C. Only those stocks were used in which the titer at 39°C was approximately 3 × 10^5 times that at 31°C, corresponding to literature values for these mutants (Flamand, 1970; Knipe et al., 1977b; Morita et al., 1987). Original stocks of tsO23 were obtained from John Lenard (University of Medicine and Dentistry of New Jersey) in 1989. Original stocks of wt VSV (San Juan strain) and tsM301 were obtained from Harvey Lodish (Massachusetts Institute of Technology) indirectly through M. J. Ruebush (Bowman Gray School of Medicine) in 1979. The vaccinia virus recombinant VTF7.3 (vaccinia/T7 virus), which expresses the bacteriophage T7 RNA polymerase (Fuerst et al., 1987), was provided by Bernard Moss (National Institutes of Health). Vaccinia/T7 virus stocks were prepared in CV-1 cells and assayed as described previously (Black et al., 1993). Unless otherwise noted, cells were grown in Dulbecco modified Eagle medium (DMEM) containing 10% fetal bovine serum.

Complementation assay

The plasmid containing the wt M gene of VSV (Orsay strain, Indiana serotype) cloned downstream of the T7 promoter in the vector pT7/T3×19 (GIBCO BRL) has been described (Black et al., 1993). The details of transfection with plasmid DNA and infection with vaccinia/T7 and tsO23 viruses have been described previously (Kaptur et al., 1995). Briefly, BHK cells were infected with vaccinia/T7 virus at a multiplicity of infection (m.o.i.) of 5–10 for 2 hr at 37°C. Cells were transfected with plasmid DNA containing either wt M gene or no M gene as a control using Lipofectin reagent (GIBCO BRL) and incubated at 37°C. At 5 hr posttransfection, cells were washed and then coinfected with tsO23 or tsM301 virus or wt VSV (m.o.i. 20). After a 1-hr incubation at 39°C, the cells were washed and incubated at 39°C for 16 hr in DMEM containing [35S]-methionine (25 μCi/ml; Dupont, NEN, Research Products). Unless otherwise noted, all media contained cytosine arabinoside (araC, 40 μg/ml, Sigma Chemical Co.) except the vaccinia/T7 virus inoculum. Culture supernates were collected, and cellular debris was removed by low-speed centrifugation. Yield of infectious virus was determined by plaque assay at 31°C. Alternatively, extracellular particles were pelleted by ultracentrifugation of supernates over a 15% sucrose cushion. Pellets were then resuspended for analysis by SDS-PAGE, sedimentation analysis, or electron microscopy.

Sucrose gradient sedimentation of extracellular particles and nucleocapsids

35S-labeled extracellular particles released by tsO23 virus-infected BHK cells at 39°C in the presence or absence of plasmid-derived M protein were prepared as described above and resuspended in 0.5 ml of PBS. The samples were layered over a 25–45% sucrose gradient (wt/wt in PBS) and centrifuged at 35,000 rpm for 90 min in a Beckman SW50.1 rotor at 5°C. Gradients were collected into 10 fractions and an aliquot of each fraction was analyzed by SDS-PAGE and fluorography.

Nucleocapsids were released from extracellular particles by resuspending pellets in 0.5 ml buffer containing 10 mM Tris, 10 mM NaCl, 50 mM octyl glucoside, pH 8.1, to solubilize the virus envelope. Samples were layered over a 4-ml 10–35% sucrose gradient (wt/wt in the same buffer) over a 0.5-ml cushion of 60% sucrose. Gradients were spun at 35,000 rpm for 90 min in a Beckman SW50.1 rotor at 5°C. Gradients were collected into 13 fractions, and an aliquot of each was analyzed by SDS-PAGE and fluorography. 35S-labeled nucleocapsids were isolated from wt VSV by solubilization of the envelope in the presence of 0.25 M NaCl (to dissociate the M protein) as described by Newcomb et al. (1982). The nucleocapsids were analyzed by sedimentation in a sucrose gradient, and the amount of nucleocapsid protein in each fraction was quantitated by liquid scintillation counting.

Electron microscopy

Electron microscopy was described (Black et al., 1993). The absence of nucleocapsid and envelope glycoprotein, but very little M protein. The other class was devoid of nucleocapsids. Most of these particles had a spherical or pleomorphic shape as determined by electron microscopy. Expression of wt M protein from plasmid DNA enhanced the incorporation of M protein into extracellular particles but did not enhance the incorporation of nucleocapsids into particles from cells coinfected with the ts M mutants. Electron microscopy showed that expression of wt M protein served to impart the bullet-like shape typical of VSV virions to what would otherwise be spherical or pleomorphic virus-like particles.
samples were dehydrated through a graded series of ethanols and embedded in Epon 812. Thin sections were obtained with a diamond knife in a Sorvall MT-2B ultramicrotome, collected on carbon-stabilized, Formvar-coated copper grids, and stained with lead citrate and uranyl acetate. Sections approximately 50 nm thick were viewed with a Philips EM-400 transmission electron microscopy (100 keV). Sections approximately 500 nm thick were viewed with a Philips CM-30 transmission electron microscope (300 keV).

Indirect immunolabeling and negative staining with phosphotungstic acid were performed essentially as described by Whitt et al. (1989). The primary antibody was monoclonal antibody I1 against the G protein (Lefrancois and Lyles, 1982) or an isotype-matched negative control antibody. The secondary antibody was goat anti-mouse IgG adsorbed to 15-nm colloidal gold particles (Amer sham). Micrographs were obtained using the Philips EM-400 electron microscope.

RESULTS
Release of extracellular particles from cells infected with tsO23 virus at the nonpermissive temperature in the presence and absence of wt M protein

Complementation of the growth of VSV ts M mutants by M proteins expressed from plasmid DNA using vaccinia/T7 vectors has been used to assess the function of plasmid-derived wild-type and mutant M proteins (Black et al., 1993; Kaptur et al., 1995; Li et al., 1988a,b; Sun et al., 1994). The goal of this study was to determine the protein composition and morphology of the extracellular particles produced by tsO23 virus at the nonpermissive temperature under conditions of complementation by plasmid-derived M protein. BHK cells were infected with vaccinia/T7 virus and then transfected with T7 expression plasmids encoding either wt M protein (M, lanes 1, 3, 5, and 8) or no M protein as a control (C, lanes 2, 4, 6, 7, and 9), and then superinfected with tsO23 virus (ts, lanes 1, 2, 7, 8, and 9), wt VSV (wt, lanes 3, 4, and 11), or no VSV (lanes 5, 6, 9, and 12) and incubated in medium containing [35S]methionine and either 40 μg/ml araC (lanes 1–6) or no araC (lanes 7–12) for 16 hr at 39°C. Extracellular particles were collected from the supernates by ultracentrifugation through a sucrose cushion and analyzed by SDS-PAGE and fluorography (A). Cell lysates were prepared in SDS sample buffer, and 1/10 of the sample was analyzed (B). Lanes 1a, 2a, 7a, 8a, and 9a are from a 10-fold longer exposure of the gel in (A).

FIG. 1. Assembly of extracellular particles at the nonpermissive temperature for tsO23 virus growth. BHK cells were infected with vaccinia/T7 virus (vac/T7, lanes 1–9) or were mock-infected (lanes 10–12), transfected with T7 expression plasmids encoding either wt M protein (M, lanes 1, 3, 5, and 8) or no M protein as a control (C, lanes 2, 4, 6, 7, and 9), and then superinfected with tsO23 virus (ts, lanes 1, 2, 7, 8, and 9), wt VSV (wt, lanes 3, 4, and 11), or no VSV (lanes 5, 6, 9, and 12) and incubated in medium containing [35S]methionine and either 40 μg/ml araC (lanes 1–6) or no araC (lanes 7–12) for 16 hr at 39°C. Extracellular particles were collected from the supernates by ultracentrifugation through a sucrose cushion, and the pellet was analyzed by SDS-PAGE and fluorography (A). Cell lysates were prepared in SDS sample buffer, and 1/10 of the sample was analyzed (B). Lanes 1a, 2a, 7a, 8a, and 9a are from a 10-fold longer exposure of the gel in (A).
The key result in Fig. 1A is shown in lanes 1–4. It was anticipated that expression of wt M protein from plasmid DNA would enhance the envelopment and release of nucleocapsids from tsO23 virus-infected cells. However, similar amounts of extracellular particles containing nucleocapsids were released from cells infected with tsO23 virus in the presence (lane 1) and absence (lane 2) of plasmid-derived M protein, as shown by the amount of N protein. In both cases, the amount of extracellular particles produced by tsO23 virus was well below that produced by wt VSV (lanes 3 and 4). The particles released in the absence of plasmid-derived wt M protein were deficient in M protein, as might be expected from the nature of the ts mutant. However, the release of similar amounts of N protein in these particles in the presence and absence of plasmid-derived M protein was particularly surprising in light of the evidence that M protein functions to bind the nucleocapsid to the host plasma membrane during virus assembly by budding (Pal et al., 1987). The extracellular particles released from tsO23 virus-infected cells in the absence of wt M protein do contain detectable amounts of mutant M protein, which is barely visible in Fig. 1A, lane 2, but is readily apparent in a 10-fold longer exposure (lane 2a). Cells that expressed plasmid-derived wt M protein in the absence of VSV coinfection released particles containing M protein as the only major protein (lane 5). This has been observed previously with M protein expressed from baculovirus and vaccinia virus vectors and has been interpreted to indicate that M protein is capable of budding in the absence of other VSV proteins (Li et al., 1993; Justice et al., 1995).

Results of experiments similar to that in Fig. 1A, lanes 1–4, were quantitated by densitometry and are summarized in Table 1. The amount of M protein in extracellular particles released from virus-infected cells was expressed as a ratio of M protein to N protein. In the absence of plasmid-derived M protein, particles released from tsO23 virus-infected cells contained about 14% as much M protein as wt VSV (M/N ratio of 0.144 versus 1.029). In the presence of plasmid-derived M protein, the M/N ratio of extracellular particles was about 76% of that of wt VSV. Also shown in Table 1 is the ratio of the amount of N protein released by tsO23 virus-infected cells in the presence versus the absence of plasmid-derived M protein, which averaged about 0.88. Thus, there was no enhancement of nucleocapsid release by the plasmid-derived M protein over that produced by tsO23 virus alone. Despite the fact that similar amounts of N protein-containing extracellular particles were produced, the infectivity of particles assembled in the presence of wt M protein was considerably greater than those assembled in the absence of wt M protein. The ratio of tsO23 virus yields in the presence versus the absence of plasmid-derived wt M protein (complementation ratio), determined by plaque assay at 31°C, averaged 14.4 (range, 4.0–51.8; N = 18) when performed under the same conditions as in Table 1 and Fig. 1 (lanes 1 and 2). Temperature-stable revertants contributed very little to these titers, since titers obtained at 39°C were 10- to 100-fold less, as previously reported (Li et al., 1988a).

As shown in Fig. 1B, similar amounts of VSV proteins were synthesized in cells infected with tsO23 virus versus wt VSV, and expression of plasmid-derived M protein neither had little effect or slightly reduced the level of expression of the other VSV proteins (lanes 1–4). This indicates that the differences in extracellular particles released from tsO23 or wt virus-infected cells in the presence versus the absence of wt M protein cannot be accounted for by differences in amounts of viral proteins present in infected cells. However, the effect of araC in suppressing vaccinia-mediated interference with VSV replication is readily apparent in Fig. 1B. In the absence of araC, very little VSV-specific protein synthesis occurred in cells coinfected with tsO23 virus (Fig. 1B, lanes 7 and 8) compared to those incubated in the presence of araC (lanes 1 and 2). Small amounts of extracellular particles were released from these cells in the absence of araC, which are visible in the 10-fold longer exposure of the gel in Fig. 1A (lanes 7a and 8a). These particles
are similar to those produced in the presence of araC. When assembled in the presence of wt M protein (lane 8a), the M/N ratio was 0.45, as determined by densitometry, but when assembled in the absence of wt M protein (lane 7a), the M/N ratio was 0.10.

Also shown in Fig. 1 are the results obtained with tsO23-, wt VSV-, or mock-infected cells in the absence of coinfection with vaccinia/T7 virus (lanes 10, 11, and 12, respectively). The amount of G protein released by tsO23 virus-infected cells was considerably greater in the absence versus the presence of coinfection with vaccinia/T7 virus (lane 10 versus lane 2). In contrast, the amount of N protein released from cells infected with tsO23 virus alone was similar to that released from cells coinfectcd with both tsO23 and vaccinia/T7 viruses. In repeated experiments, there was no significant difference in the amount of N protein released into extracellular particles in the presence versus the absence of vaccinia/T7 virus (ratio, 1.13; Table 1). These data indicate that release of N protein-containing particles is not affected by coinfection with vaccinia/T7 virus, although the amount of G protein in extracellular particles is reduced by coinfection with vaccinia/T7 virus.

Additional data (not shown) indicated that results similar to those in Fig. 1 were obtained under a variety of experimental conditions: (1) The same results were obtained at two different nonpermissive temperatures (39 and 41°C) and with three different clonal isolates of tsO23 virus, which displayed a degree of temperature sensitivity similar to published values (Flamand, 1970; Morita et al., 1987). Virus stocks were derived from the initial passage following plaque isolation to reduce as far as possible the potential contribution of temperature-stable revertants. (2) Results similar to those in Fig. 1 were obtained with a different ts M protein mutant, tsM301, isolated in a different laboratory (Knipe et al., 1977b), but belonging to the same complementation group (group III). Similar results were also obtained using CV-1 cells or Vero cells instead of BHK cells, indicating that these results were not unique to a particular virus–cell combination. (3) Although the amount of transfected plasmid DNA and the multiplicities of infection of vaccinia and tsO23 viruses used in Fig. 1 were optimal for the production of virions, similar results were obtained with threefold higher and threefold lower amounts of each reagent. (4) Results similar to those in Fig. 1 were obtained by harvesting extracellular particles at 5 hr postinfection with tsO23 virus rather than 16 hr, indicating that their release was not confined to late times postinfection but instead occurred throughout the normal course of VSV infection.

Heterogeneity of extracellular particles produced by tsO23 virus at the nonpermissive temperature

The extracellular particles produced by tsO23 virus at the nonpermissive temperature are actually heterogeneous in composition as shown by sucrose gradient centrifugation. 35S-labeled extracellular particles assembled by tsO23 virus at 39°C in the presence or absence of wt M protein were harvested as in the experiment in Fig. 1 and then were analyzed by sedimentation in sucrose gradients. The gradient fractions were analyzed by SDS-PAGE and fluorography (Fig. 2). In the case of particles produced in the absence of wt M protein (Fig. 2A), the major population of G protein-containing particles was in fractions 2 and 3, while most of the particles containing both G and N proteins were found in fractions 4–6. The small amount of ts M protein assembled into these particles was approximately evenly distributed between these two populations. In the case of particles assembled by tsO23 virus in the presence of wt M protein (Fig. 2B), fractions 2 and 3 again contained particles with G and M proteins, while most of the N protein-containing particles were in fractions 4–6. However, in contrast to particles assembled in the absence of wt M protein, most of the M protein was also found in fractions 4–6. In Fig. 2B,
the M protein band appears as a doublet resulting from resolution of the tsO23 M protein from wt M protein. The results in Fig. 2 indicate that extracellular particles produced by tsO23 virus-infected cells consist of a mixture of particles containing G, M, and N proteins and those containing G and M proteins but devoid of nucleocapsids.

Extracellular particles produced by tsO23 virus at the nonpermissive temperature contain intact nucleocapsids

35S-labeled extracellular particles assembled by tsO23 virus at 39°C in the presence or absence of wt M protein were harvested as in the experiment in Fig. 1, solubilized with the detergent octyl glucoside, and analyzed by sedimentation in sucrose gradients to determine whether the N protein found in these particles was incorporated into intact nucleocapsids. The gradient fractions were analyzed by SDS–PAGE and fluorography, and the amount of N protein in each fraction was quantitated by densitometry (Fig. 3A). In a separate experiment, 35S-labeled nucleocapsids were isolated from wt VSV by solubilization of the envelope in the presence of 0.25 M NaCl (to dissociate the M protein) as described by Newcomb et al. (1982). The nucleocapsids were analyzed by sedimentation in a sucrose gradient, and the amount of nucleocapsid protein was quantitated by liquid scintillation counting (Fig. 3B). The sedimentation of N protein from extracellular particles produced by tsO23 virus-infected cells in the presence or absence of wt M protein was similar to that of nucleocapsids isolated from wt VSV, indicating that these particles contained intact nucleocapsids.

The nucleocapsids were enveloped in a membrane, as expected for particles assembled by budding. Treatment of extracellular particles with trypsin (not shown) resulted in proteolysis of the G protein, but the N and M proteins were protected from proteolysis. In contrast, after solubilization of the envelope with detergent, all three major structural proteins were susceptible to proteolysis. This indicates that the N and M proteins released were incorporated into enveloped particles and were not the result of cellular debris from a small number of lysed cells.

Morphology of extracellular particles produced by tsO23 virus at the nonpermissive temperature

Extracellular particles assembled by tsO23 virus at 39°C in the presence and absence of wt M protein were harvested as in the experiments in Fig. 1, and the pellets were fixed, embedded, and sectioned for electron microscopy. Representative examples of the types of particles observed are shown in Fig. 4. Cells infected with tsO23 virus in the absence of wt M protein produced two types of membranous particles that were clearly distinct from material produced by control uninfected cells. The first type was spherical enveloped particles approximately 200 nm in diameter whose internal contents had the appearance of viral nucleocapsids (Fig. 4A). The bilaminar appearance of the envelope, which was slightly thicker than a typical cell membrane, is clearly visible in Fig. 4A. The viral nucleocapsid appears less distinct except in areas where it lies along the focal plane of the micrograph (arrowhead in Fig. 4A). The spherical shape of these particles was confirmed by examining thick sections (500 nm) in a 300-keV electron microscope in which the entire particle could be visualized (not shown).

The second type of particles produced by tsO23 virus-infected cells in the absence of wt M protein was membranous particles that were largely devoid of electron-
dense material (Fig. 4B). These particles tended to be variable in size and shape, but were usually between 200 and 300 nm in diameter. The membrane of these particles was markedly thickened by an electron-dense layer on the interior surface.

In the presence of wt M protein, tsO23 virus-infected cells produced particles similar to those assembled in the absence of wt M protein (Figs. 4A and 4B), but in addition, many of the particles had the bullet-shaped morphology typical of VSV virions (Fig. 4C). These electron microscopic data are consistent with the biochemical data obtained from sucrose gradient centrifugation (Fig. 2). They show that tsO23 virus-infected cells produced two classes of membranous particles at 39°C—those that contain nucleocapsids and those that do not. Complementation by plasmid-derived M protein lead to the additional production of particles with the composition and morphology of wt VSV.

The "empty" particles produced by tsO23 virus-infected cells (Fig. 4B) were similar in appearance to membranous particles generated by cells expressing wt M protein in the absence of other VSV gene products (Li et al., 1993). Figure 4D shows electron micrographs of a cell transfected with plasmid DNA encoding wt M protein in the absence of coinfection with tsO23 virus. Numerous "empty" particles with the characteristic thickened membrane could be seen budding from the surface of the cells. In nearly all cases, the cellular cytoplasmic contents such as ribosomes and cytoskeletal elements were largely excluded from the budding particles. This was distinctly different from membranous debris released by control cells transfected with plasmid DNA lacking an M gene, which inevitably contained these cytoplasmic components (not shown). The production of these "empty" particles as a result of M protein expression has been taken as evidence that M protein can mediate the budding process in the absence of other viral gene products (Li et al., 1993; Justice et al., 1995). The similarity of these particles to those produced by tsO23 virus-infected cells (Figs. 4A and 4B) suggests that the tsO23 M protein retains some of its ability to mediate budding at 39°C.

Electron microscopy of negatively stained preparations was used to quantitate the different morphological types of extracellular particles produced by tsO23 virus-infected cells. Extracellular particles assembled by tsO23 virus at 39°C in the presence and absence of wt M protein were harvested as in the experiments in Fig. 1 and then immunolabeled with colloidal gold and negatively stained for electron microscopy. The particles were labeled using an antibody against the VSV G protein in order to distinguish virus-specific particles in the preparation. Representative examples of the types of labeled particles are shown in Fig. 5. In the case of extracellular particles assembled by tsO23 virus at 39°C in the absence of wt M protein, most of the labeled particles were approximately spherical (Fig. 5a), and some were pleomorphic (Fig. 5b). Very few had the bullet-shaped morphology typical of VSV virions. In the case of tsO23 virus particles assembled at 39°C in the presence of wt M protein, spherical particles similar to those assembled in the absence of wt M protein were often observed, but in addition, many of the particles had the bullet-shaped morphology typical of VSV virions (Fig. 5c). In many cases, penetration of the negative stain into the bullet-shaped particles, as in Fig. 5c, revealed the tightly coiled helical structure of the nucleocapsid. In some cases, the bullet-shaped particles contained extensions from their ends ('a bullet with a tail'), which can be seen in the lower particle in Fig. 5c. These membrane blebs are sometimes produced upon negative staining of wt VSV after freezing and thawing of virus. However, they were rarely observed in our preparations of wt VSV and may represent particles whose contents were either incompletely condensed or more fragile compared to those produced by wt VSV. Also shown in Fig. 5 for comparison are examples of wt VSV virions labeled with anti-G protein antibody (Fig. 5d) or incubated with an isotype-matched control antibody as a negative control for the immunolabeling (Fig. 5e).

The relative frequency of the different types of tsO23 virus particles assembled in the presence or absence of wt M protein was determined by counting approximately 100 randomly observed labeled particles (Fig. 6). The labeled particles were included in one of four categories: bullets (B), bullets with tails (B + T), pleomorphic (P), or spherical (S). While these categories actually represent a continuum of shapes, in most cases members of each category were easily distinguished, as shown above in Fig. 5. Spherical particles were the predominant type released by tsO23 virus-infected cells at 39°C in the absence of wt M protein (62% of the total). The few particles that had the bullet-like shape typical of VSV virions probably represent the level of leakiness of the ts mutant. In

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**Fig. 4.** Electron microscopy of extracellular particles produced by tsO23 virus or by expression of wt M protein at 39°C. Extracellular particles released by tsO23 virus-infected BHK cells at 39°C in the absence (A, B) or presence (C) of plasmid-derived M protein were prepared as described in Fig. 1, and the pellets were fixed, embedded, and sectioned for electron microscopy. (A) A spherical enveloped particle is shown. The internal contents include strands that have the appearance of viral nucleocapsids. This is most clearly seen when they lie along the focal plane of the micrograph (arrowhead). (B) Membranous particles largely devoid of electron-dense material are shown. (C) Bullet-shaped particles produced by tsO23 virus in the presence of wt M protein are shown. This sample also contained particles similar to those in (A) and (B). For example, two particles largely devoid of electron-dense material can also be seen in this micrograph. (D) A cell transfected with plasmid encoding wt M protein in the absence of coinfection with tsO23 virus is shown. (D1) Lower magnification of the same cell as in (D1) to show the production of numerous particles lacking electron density. Bar, 100 nm.
the presence of wt M protein, the number of particles that resembled bullets or bullets with tails (combined, 39% of the total) was approximately the same as the number of spherical particles (37% of the total), while the number of pleiomorphic particles was unchanged (24% of the total).

These data show a marked shift in the types of particles produced by tsO23 virus-infected cells in the presence of plasmid-derived wt M protein. This shift is especially significant since at the multiplicity used in these experiments (m.o.i. 20), >95% of the cells were infected with tsO23 virus, but only 25–30% express plasmid-derived M protein (Black and Lyles, 1992). Thus the untransfected cells would continue to produce predomi-
be spherical or pleomorphic particles. M protein-induced condensation of the VSV nucleocapsid into a tightly coiled helix has been demonstrated by in vitro disassembly and reassembly experiments with detergent-solubilized virions (Barge et al., 1993; Newcomb and Brown, 1981; Newcomb et al., 1982) and by immunoelectron microscopy experiments that demonstrated tightly coiled nucleocapsids associated with the cytoplasmic surface of the plasma membrane of infected cells (Odenwald et al., 1986; Ono et al., 1987). Thus the conversion of spherical extracellular particles produced by tsO23 virus into the native bullet-like shape most likely reflects the condensation of the nucleocapsid by wt M protein.

In immunofluorescence experiments, M protein appears to be diffusely distributed throughout the cytoplasm and nucleus of infected cells and is not colocalized with nucleocapsids (Lyles et al., 1988; Ohno and Ohtake, 1987; Ono et al., 1987). The only site in infected cells at which colocalization of M protein and nucleocapsids has been observed is at the sites of budding on the cytoplasmic surface of the plasma membrane (McCreedy and Lyles, 1989; Odenwald et al., 1986; Ono et al., 1987). In addition to the M protein associated with nucleocapsids, M protein is diffusely distributed on the cytoplasmic surface of the host plasma membrane in areas that are devoid of nucleocapsids (McCreedy and Lyles, 1989). In fact, 10–25% of M protein expressed in the absence of other VSV gene products is associated with cellular membranes (Chong and Rose, 1993, 1994; Ye et al., 1994). It has been suggested that this membrane-bound form of M protein might act as an initiation signal for binding of nucleocapsids to the cytoplasmic surface of the plasma membrane followed by coalescence of M protein from the cytosol or membrane to form the tightly coiled nucleocapsid–M protein complex (Chong and Rose, 1993; McCreedy and Lyles, 1989). The ability of the ts M protein to bind to the cytoplasmic surface of host plasma membranes in areas devoid of nucleocapsids is not impaired at the nonpermissive temperature (McCreedy and Lyles, 1989). Thus the production of extracellular empty particles or particles containing nucleocapsids by ts M mutants probably reflects the residual ability of the membrane-bound ts M protein to initiate envelope biogenesis. This would be consistent with the observation that these extracellular particles contain a small amount of the ts M protein (Figs. 1 and 2 and Table 1).

It was expected that expression of wt M protein would promote binding of tsO23 virus nucleocapsids to the plasma membrane. This should have increased the amount of viral nucleocapsids incorporated into extracellular particles over that seen in the absence of wt M protein. Instead, similar amounts of nucleocapsids were released from cells infected with ts M protein mutants at the nonpermissive temperature in the presence and
absence of wt M protein (Fig. 1 and Table 1). This observation cannot be accounted for by low levels of synthesis of wt M protein, since it was expressed from plasmid DNA at levels similar to those expressed by wt VSV in a coinfection with vaccinia virus (Fig. 1B). A more likely possibility is that ts M protein interfered with the function of wt M protein in cells that coexpress the two proteins. The ability of ts mutants of many different viruses to interfere with the growth of wt viruses at the nonpermissive temperature has been widely documented (reviewed by Whitaker-Dowling and Youngner, 1987) and has been observed previously for VSV ts M mutants (Youngner et al., 1986). A possible mechanism of this effect might be that ts M protein, which binds nucleocapsids inefficiently, competes effectively with wt M protein for the available membrane binding sites in cells that coexpress ts and wt M protein. This might reflect a higher affinity of the ts M protein for membranes compared to wt M protein, as originally suggested by Mancarella and Lenard (1981). The effect would be to reduce the ability of wt M protein to mediate association of nucleocapsids with the membrane, particularly if the association requires multivalent binding between the nucleocapsid and membrane-bound M protein.

It has been reported previously that ts M mutants release membranous particles containing G and M proteins but deficient in nucleocapsids at the nonpermissive temperature (Schnittzer and Lodish, 1979). Similar “empty” particles were also observed in the present study (Fig. 2) and probably correspond to the membranous particles that lack electron-dense contents observed by electron microscopy (Fig. 4B). Similar particles are produced when wt M protein is expressed in the absence of other VSV gene products (Fig. 4; Li et al., 1993; Justice et al., 1995), suggesting that the “empty” particles produced by ts M mutants at the nonpermissive temperature reflect the residual membrane-binding activity of the ts M protein in the absence of binding to nucleocapsids.

Extracellular particles containing G and M proteins but lacking nucleocapsids are the predominant type of extracellular particle released by cells infected with ts M mutants in the absence of vaccinia/T7 virus coinfection (Schnittzer and Lodish, 1979; Fig. 1). The release of these “empty” particles was considerably reduced as a result of coinfection with vaccinia/T7 virus as shown by the reduced amount of G protein in extracellular particles in the presence versus the absence of vaccinia/T7 virus coinfection (Fig. 1). In contrast, release of particles containing nucleocapsids was not affected by vaccinia/T7 virus coinfection (Fig. 1 and Table 1). This result makes it unlikely that envelopment of nucleocapsids results from nonspecific entrapment in membrane vesicles formed by the activity of G protein alone, as recently described for G protein expressed from an alphavirus vector (Rolls et al., 1994). Also, the vesicles produced by G protein alone are considerably smaller (50-100 nm, Rolls et al., 1994) than the particles containing nucleocapsids observed here (approximately 200 nm, Figs. 4 and 5).

Clearly cells expressing VSV proteins are capable of releasing a variety of membranous virus-like particles. The individual activities of both the G and M proteins promote their incorporation into extracellular particles (Li et al., 1993; Justice et al., 1995; Rolls et al., 1994). The production of the variety of virus-like particles by VSV proteins either individually or in combinations where one or more proteins are in limiting amounts has been very useful in dissecting the individual steps in virus assembly. In the present case, the release of virus-like particles from cells infected with ts M mutants in the presence versus the absence of wt M protein provided a means to separate the process of nucleocapsid envelopment from conversion of virion components into the bullet-like shape. However, during wt VSV infection, the potential heterogeneity of extracellular particles that could be produced by the limited activity of one or more viral proteins is vastly overshadowed by the efficient production of virions by all of the viral proteins acting in concert.

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