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In Vivo Assembly of a Biological Membrane of Defined Size, Shape, and Lipid Composition

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At restrictive temperature, mutant *ts1* of bacteriophage PM2 makes membrane vesicles inside infected *Alteromonas espejiana*. A shift from restrictive to permissive temperature resulted in rapid maturation to infectious virions. The membrane vesicles were isolated from cellular membranes by sucrose density gradient centrifugation. Analysis of the unique peak at $\rho = 1.190$ g/cm^3 showed spheres of two diameters, 50 nm and 54 nm. The wild-type virus is icosahedral with an average diameter of 60 nm. Gel electrophoresis indicated the absence in the vesicles of the coat and spike proteins, sp27 and sp43, respectively, and the presence of only one viral structural protein, sp6.6. DNA was also present. The lipid in the vesicles was composed of phosphatidylglycerol and phosphatidylethanolamine in a proportion similar to that of the wild-type virus, whose ratio is nearly the inverse of that found in the host membrane. Thus, membrane vesicles made by mutant *ts1* resembled the membrane of the wild-type virus in size, shape, and lipid composition, but contained only one of the four structural proteins of the virus. This hydrophobic protein, sp6.6, may be responsible for stimulating membrane morphogenesis.

Recently, much progress has been made in elucidating the pathways for synthesis of the components of biological membranes, namely lipids and proteins. In studying membrane biogenesis, much less attention has been successfully directed toward understanding the assembly of these components. Specifically, we address the question of what controls the size, shape, and composition of a biological membrane.

In the case of the enveloped animal viruses, the viral nucleocapsid is apposed to a region of the host cytoplasmic membrane already containing other viral proteins (23, 35, 38). The size and shape of the virus membrane appears to be determined by molding around the nucleocapsid core. There is little if any control over lipid composition of the virus (23). In the case of subcellular membranes of eucaryotic organelles, it is not evident what controls their size, shape, and composition.

We have chosen to study control of newly assembled intracellular membranes of bacteriophage PM2 because of their simplicity. PM2 infects *Alteromonas espejiana* (8), formerly *Pseudomonas* BAL-31 (15), causing the assembly of new membranes and mature phage particles, followed by lysis of the host (10, 14). PM2 has four major structural proteins (5, 13, 33). Exterior to the spherical phospholipid bilayer (17), the vertices of the icosahedral capsid are composed of sp43 (43,000 daltons), and sp27 completely encapsulates the bilayer (5, 19). The other two structural proteins of the virus, sp13 and sp6.6, appear to have an as yet unspecified association with the viral membrane and possibly with the DNA of the virus (5, 19). Temperature-sensitive mutants of PM2 have been isolated to dissect the process of assembly of the membrane (4). One of these mutants, *ts1*, is of interest because empty, virus-size membrane vesicles are made inside infected cells (3). In this report we describe the isolation and characterization of vesicles made by *ts1* and implications for controlled assembly of a membrane.

MATERIALS AND METHODS

Cells and virus. Culturing of PM2 and its host *A. espejiana* (formerly designated *Pseudomonas* BAL-31) have been described (3). Mutant *ts1* of PM2 was isolated by its ability to replicate at 20°C but not at 30°C (4).

Temperature shift. Cells were grown in Q medium (34) at 30°C to an optical density at 660 nm of 0.3 (3×10^8 cells per ml). At this restrictive temperature, a portion of this culture was infected with *ts1* at a multiplicity of 0.01. A plaque assay at 20 min postinfection was performed to determine the number of infected cells. This and subsequent assays were conducted at 20°C. After 60 min, the culture was transferred to 20°C and assayed as indicated. Another portion of the original culture was transferred to 20°C,

immediately infected, and assayed as indicated.

Electron microscopy. Cells were grown in minimal medium containing glucose and Casamino Acids (3) (10 ml) and were infected with *ts1* virus at a multiplicity of 5 at 30°C. After 60 min, 10 μl of the culture was treated with lysis medium (4) and found to contain 1.2×10^8 PFU/ml (0.5% of a parallel infection with wild-type virus). The remainder of the culture was transferred to 20°C. An 83-fold increase in virus was found. The infected cells were prepared for electron microscopy by addition of 10% glutaraldehyde to equal 0.5%. After 30 min, the culture was centrifuged (5,000 × *g*, 5 min) and let sit another 30 min. The pellet was gently washed with Michaelis buffer (22), followed by overnight fixation and prestaining in 1% OsO_4–2% uranyl acetate in Michaelis buffer. After dehydration through a graded series of ethanol and propylene oxide, the specimen was impregnated and embedded in Epon 812. Sections were stained for 30 s with 0.5% uranyl acetate in 95% ethanol, washed, and stained for 3 min with lead citrate (31).

For negative staining, peak fractions of *ts1* vesicles and wild-type virus from gradients were dialyzed against VSM, concentrated by centrifugation (Ti 50 rotor, 105,000 × *g*, 4°C, 2 h), and resuspended in a small volume of VSM (4). Samples of 5 μl were placed on Formvar–carbon-coated copper grids and fixed by the addition, with mixing, of 1 μl of 10% glutaraldehyde. After 5 min, excess solution was removed with filter paper, and the grid was washed two times with distilled water. Negative staining was with 0.5% aqueous uranyl acetate for 5 s.

Isolation of vesicles. Cells were grown in 30 ml of minimal medium plus glucose at 30°C to 3×10^8 cells per ml, divided, and infected at a multiplicity of 10 with wild-type PM2 (10-ml culture) and *ts1* (20 ml). At 20 min postinfection, [^{35}S]methionine was added to 10 μCi/ml (1.5 μM), conditions found to result in linear incorporation. At 90 min postinfection, unlysed cells were collected by centrifugation (8,000 × *g*, 5 min, 30°C) and treated with 0.5 culture volume of lysis medium (4) for 5 min at 30°C, followed by addition of $MgSO_4$ to 4 mM. Released virus products were separated from unlysed cells by another centrifugation. The supernatants from the two centrifugations were pooled. Subsequent plaque assay at 20°C indicated wild-type virus at 1.6×10^{10} PFU/ml and *ts1* at 3% of this level. The supernatants were centrifuged in a Ti 45 rotor (105,000 × *g*, 4°C, 2 h). The pellets obtained were resuspended in 0.1 ml of VSM (4) and applied to 37 to 47% sucrose gradients made in VSM in tubes for an SW40 rotor. Centrifugation proceeded for 64 h (184,000 × *g*, 4°C). Fractions of 0.4 ml were collected through an absorbance monitor (ISCO model UA-5) set for 280 nm with a gradient fractionator (ISCO models 185 and 568) operated in the peak separation mode. Samples of 5 μl were removed for determination of radioactivity. Samples of 10 μl were taken to determine densities by refractometry in comparison to gravimetric standards.

Polyacrylamide gel electrophoresis of vesicles and quantitation of bands. Cells were grown overnight in $^{32}PO_4$ (5 μCi/ml) in minimal medium plus glucose to constant specific activity. The culture was diluted to 3×10^8 cells per ml (mid-log phase), maintaining the specific activity of ^{32}P, infected, and labeled with [^{35}S]methionine as described above. During the course of infection, small samples were precipitated with trichloroacetic acid and counted to insure identical kinetics and steady-state levels of incorporation for the two cultures. After isolation of vesicles, peak fractions were dialyzed against VSM and concentrated to dryness by filtration through a Millipore PSAC 13-mm-diameter filter (nominal cutoff 2,000 daltons). The residue on the filter was dissolved in gel sample buffer (62.5 mM Tris-hydrochloride, pH 6.8–2% sodium dodecyl sulfate–10% glycerol–0.001% bromophenol blue–5% β-mercaptoethanol) and heated to 100°C for 1 min. Another sample (not shown) was treated with DNase before filtration; after electrophoresis, the bands at the top of the gel were not seen, thus identifying them as DNA. Samples were applied to a slab gel (13 cm wide by 14 cm long by 1 mm thick) composed of a stacking gel (3% acrylamide–0.08% *N'*-*N'*-bis-methylene-acrylamide–125 mM Tris-hydrochloride, pH 6.8–0.1% sodium dodecyl sulfate) and a separating gradient gel (linear from 10% to 17.5% acrylamide with cross-linker 2.67% of the monomer concentration–375 mM Tris-hydrochloride, pH 8.8–0.1% sodium dodecyl sulfate). Gels were polymerized with 0.05% *N,N,N',N'*-tetramethylethylenediamine and 0.1% ammonium persulfate. Electrophoresis was performed at room temperature at 3 W constant power for approximately 3 h. To retain lipid, the gel including the stack was not fixed but was immediately dried under vacuum. Autoradiography was performed with Kodak XR-5 X-ray film. The location of bands was traced onto the dried gel. Bands were cut into small pieces. Radioactivity was eluted with 10 ml of scintillation fluid (95% toluene–Omni-Fluor [New England Nuclear], 5% Protosol [New England Nuclear], 0.75% water) at 37°C for 15 h. Samples were counted by liquid scintillation in a Beckman LS 8000 using automatic quench control. Two-channel counting allowed resolution of ^{35}S and ^{32}P disintegrations. Counts per minute were corrected for background and for spillover by using samples containing a single isotope.

Lipid analysis. Cells were labeled with $^{32}PO_4$ at 2 μCi/ml to uniform specific activity (>15 generations) and either were left uninfected or were infected with *ts1* or wild-type PM2 at 30°C. Uninfected cells, purified virus, and *ts1* vesicles were separately extracted with chloroform–methanol (16). The lipid extracts were applied to silica gel thin-layer plates, developed in chloroform–methanol–water (65:25:4, vol/vol), and autoradiographed. The major spots corresponding to phosphatidylglycerol and phosphatidylethanolamine were scraped into scintillation fluid and counted.

RESULTS

Numerous intracellular membrane vesicles are seen upon examination in the electron microscope of *A. espejiana* infected at restrictive temperature with the *ts1* mutant of bacteriophage PM2 (3). These vesicles are roughly the size and shape of the membrane of the wild-type virus. In contrast to cells infected with wild-type virus, these vesicles appear to lack the internal core of DNA and possibly the protein capsid,

which is external to the membrane. In the absence of pulse-chase evidence, a rapid production of infectious virions after shift to permissive conditions would suggest that these vesicles can mature to infectious virions and that they are not aberrant structures. In Fig. 1A, cells were infected at restrictive temperature with *ts1* virus at a low multiplicity and cultured for 60 min, a period allowing full maturation of wild-type PM2. At this time the temperature sensitivity of *ts1* is manifested by the production of less progeny virus than was used to infect the cells and by lack of lysis of the host. Upon transfer of the culture to permissive temperature, rapid production and release of infectious virus began after only 2 min. Fifty percent of the ultimate progeny were released by 22 min. Progeny were released from secondary infection after 90 min. In a control culture of cells infected at permissive temperature directly (Fig. 1B), 50% of the ultimate progeny were released in 70 min after a lag of >45 min. In the electron microscope, sections of infected cells show the mature virions (Fig. 2) and a few unfilled vesicles. These results suggest that mutant *ts1* vesicles can mature to infectious virions.

(To rigorously prove conversion of these vesicles to mature virions, it is necessary to pulse-label their membranes and shift to permissive temperature. The specific activity of the membranes of isolated virions should be the same regardless of a chase imposed with the shift. However, two factors considerably complicate this demonstration. [i] Labeled lipids in the membrane pool are not chased rapidly. In the case of ^{32}P, a chase was not effective until 20 min after it was applied. [ii] In a wild-type virus infection, the viral membrane is derived one-third from lipids present before infection and two-thirds from lipids synthesized during the course of infection [36]. Nevertheless, we do have preliminary pulse-chase evidence consistent with conversion of vesicles to virus [Brewer, manuscript in preparation].)

Isolation of vesicles. If these vesicles were isolated and compared to the composition of the membrane of the wild-type virus, the minimum components needed to assemble such a membrane might be determined. To this end, cells infected with *ts1* in the presence of [^{35}S]methionine were lysed by treatment with lysozyme and EDTA. Total membranes were collected by ultracentrifugation and applied to a density gradient of sucrose. After centrifugation to apparent equilibrium density, a major peak was found at $\rho = 1.190$ g/cm^3 that was absent in the preparation from cells infected with wild-type virus (Fig. 3). Under these conditions, wild-type virions band at $\rho = 1.216$ g/cm^3. The density position of the peak shown here at $\rho = 1.190$ g/cm^3 did not change with centrifugation for 88 h, whereas the wild-type virions reached a heavier density under these conditions. The infectivity of the $\rho = 1.90$ g/cm^3 peak was 0.05% that of the wild-type peak. Samples from the other *ts1* peak fractions were negatively stained and in the electron microscope were observed to contain ribosomes (fractions 24 to 26) and large bacterial membranes (fractions 12 and 13, 16 and 17) (not shown). Ribosomes appear to be degraded in wild-type infections. The label at the top of the gradient (fraction 1) is unincorporated [^{35}S]methionine.

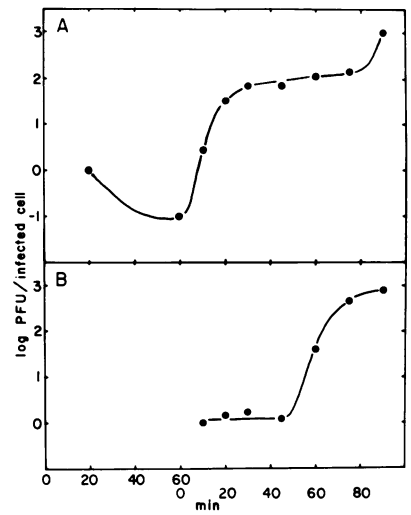

FIG. 1. *Maturation of ts1 upon shift to permissive temperature. (A) cells infected at restrictive temperature and transferred to permissive temperature at 60 min postinfection. (B) Cells infected at permissive temperature.*

Vesicle size. The diameter of native PM2 is 60 nm, and that of the virus membrane as interpreted from X-ray diffraction is 48 nm (17). Examination of the material in the $\rho = 1.190$ g/cm^3 peak by negative staining revealed a population of vesicles relatively homogeneous in size (Fig. 4A). In comparison to wild-type virus (Fig. 4B), their appearance is smaller and swollen with some of them darkened, either penetrable by the stain or collapsed to form a cup that collects stain. The mean diameter of the dark-centered vesicles was 49.8 ± 2.8 nm, whereas that of the uniformly stained vesicles was 54.1 ± 2.7 nm (uncalibrated, accurate to ±5%). In other preparations, the distribution of vesicle size was less uniform, e.g., mean 93 ± 30 nm in diameter. Vesicle fusion from uniform progenitors is suggested by the observation of putative

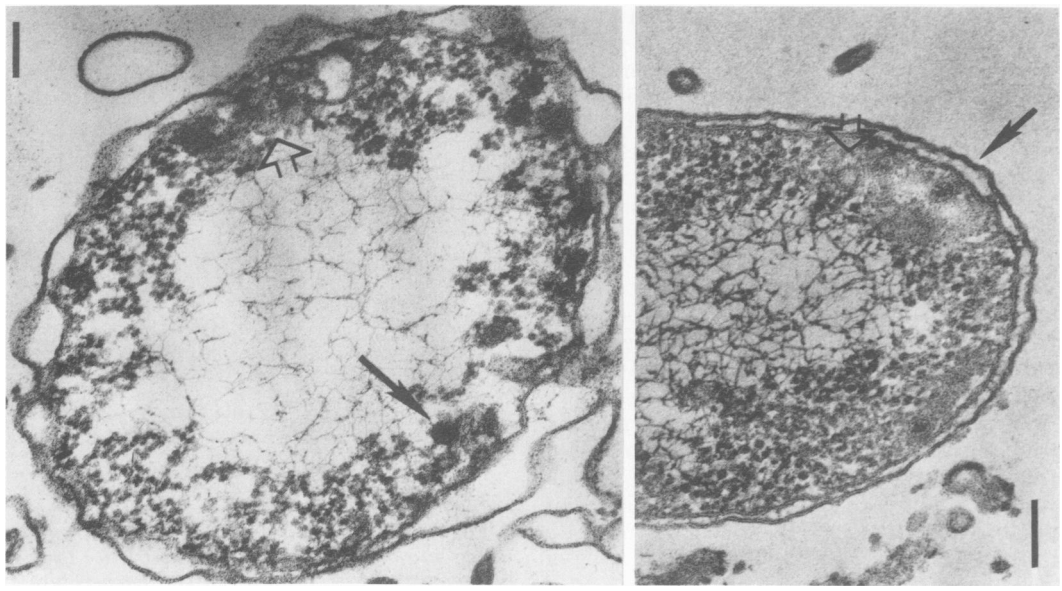

FIG. 2. *Electron micrographs of cells infected with ts1 after shift to permissive temperature. Note virions with dense cores (filled arrows) and a few vesicles (open arrows). Cell on right has not yet lysed; cell on left has begun to break open. Bar equals 100 nm.*

intermediate forms and modes in the distribution of vesicle diameters.

Composition. Radioactive precursors were biosynthetically incorporated into wild-type or mutant particles during infection to label protein ([^{35}S]methionine) and lipid plus DNA (^{32}P). This permitted comparison of the composition of mutant vesicles to that of native virus. After purification as described above, the *ts1* vesicle peak was found to contain both ^{35}S and ^{32}P counts. Molecular fractionation of these vesicles was performed by sodium dodecyl sulfate-polyacrylamide slab gel electrophoresis (Fig. 5). DNA remained in the stacking gel. The proteins in the middle region were resolved from the lipid at the bottom of the gel. In comparison to wild-type virus, it is apparent that *ts1* vesicles contain DNA, phospholipid, and predominantly one protein, the viral sp6.6. In these gradient gels of superior resolution, sp43 and sp6.6 are resolved into doublets. The significance of these doublets is currently under investigation. Quantitative comparisons based on the autoradiogram must be made with caution due to the nonlinear response of the film. Quantitation based on scintillation counting is able to discriminate ^{32}P from ^{35}S disintegrations. Less than 4% of the counts in the regions labeled o and st are due to ^{35}S; they can also be labeled by [*methyl*-^{3}H]-thymidine, confirming their nonprotein, DNA identity. Analysis indicates that *ts1* vesicles have the same proportion of lipid to DNA as does wild-type PM2 (Table 1). However, relative to

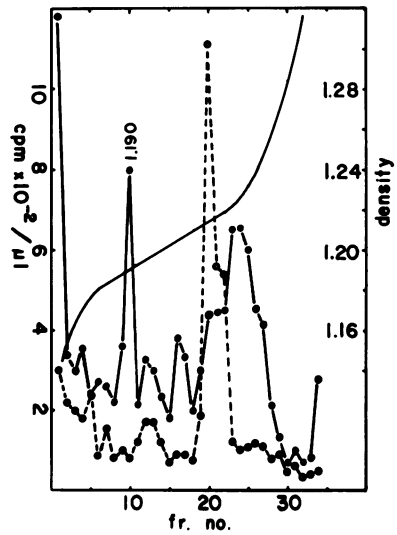

FIG. 3. *Density gradients of lysates from cells infected with wild-type PM2* (○---○) *and ts1* (●——●) *at 30°C. Distribution of [^{35}S]methionine from two gradients are superimposed.*

either lipid or DNA, *ts1* vesicles have only 20% of the wild-type complement of sp6.6.

Surprisingly, ^{32}P is associated with the sp6.6 band, well resolved from phospholipid seen in the control lane of Fig. 5. When *ts1* vesicles or wild-type PM2 were prepared in the presence of [*methyl*-^{3}H]thymidine, no label was observed in this region, whereas the band was labeled when

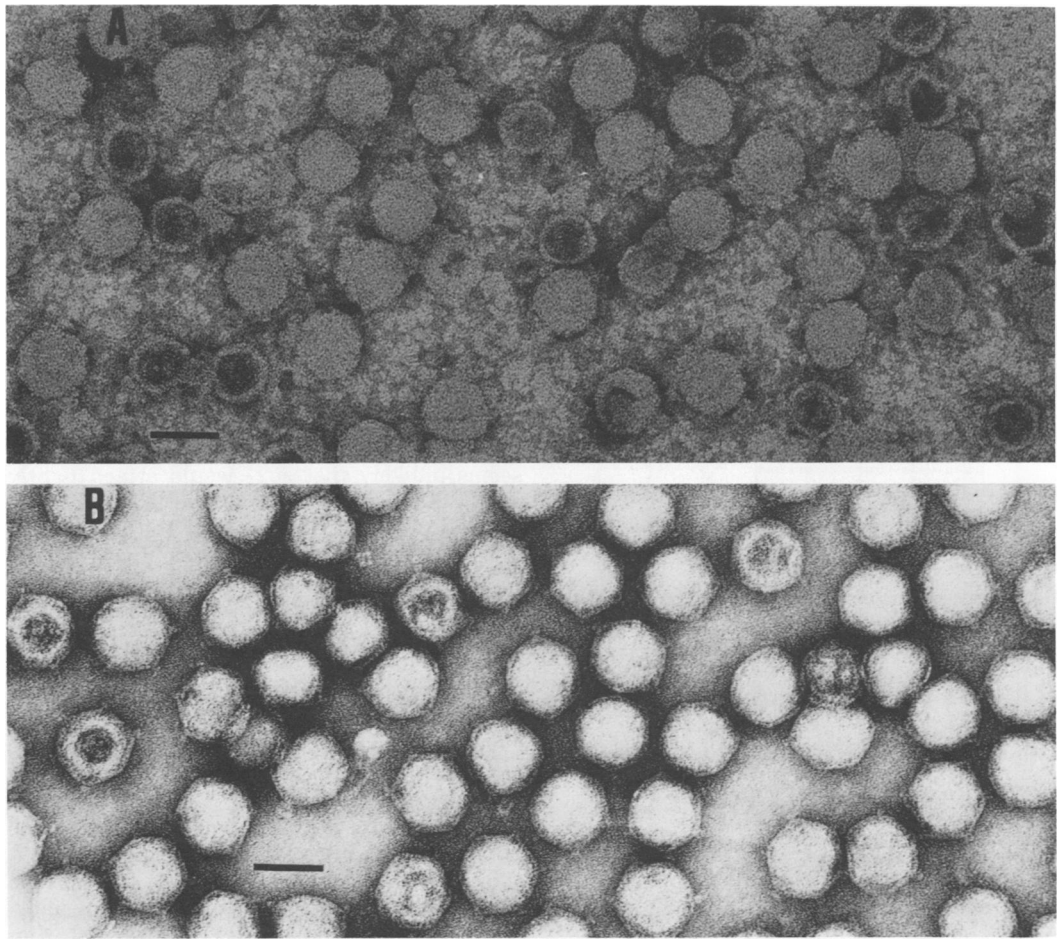

FIG. 4. *Electron micrograph of negatively stained ts1 vesicles (A) and wild-type PM2 (B). Magnification is the same for both samples; bar equals 60 nm.*

cultures were grown in the presence of [9,10-^{3}H]palmitate. The implication that phospholipid remains associated with sp6.6 in the presence of sodium dodecyl sulfate and that sp6.6 is not simply a phosphoprotein will be the subject of a future publication. From Table 1, for wild-type PM2, the amount of ^{32}P associated with sp6.6 is 30% of the ^{32}P in the isolated lipid band. For *ts1* vesicles, a larger proportion comigrates with sp6.6.

The lipid of the virus membranes was analyzed by thin-layer chromatography for the relative amounts of phosphatidylglycerol and phosphatidylethanolamine. In uninfected cells, the ^{32}P was distributed 34% in phosphatidylglycerol and 65% in phosphatidylethanolamine. The ratio of wild-type virus is nearly the inverse of that found in the host (2), 63% phosphatidylglycerol and 37% phosphatidylethanolamine. The proportion of these phospholipids in the *ts1* vesicles was similar to that found in the virus (61% and 39%, respectively).

DISCUSSION

We have investigated the *ts1* mutant of bacteriophage PM2 to determine the factors that control the assembly of its membrane. The membrane vesicles made by this mutant have been isolated. These vesicles may be minimal biological membranes with respect to directed supramolecular assembly. Criteria to assess structural fidelity are size, shape, and lipid composition, all of which are faithful reproductions of the membrane of the wild-type virus. It is pertinent that cells infected by wild-type virus produce small numbers of similar vesicles (10, 12). During the course of infection, the proportion of empty vesicles decreases while the relative proportion of mature-looking full particles

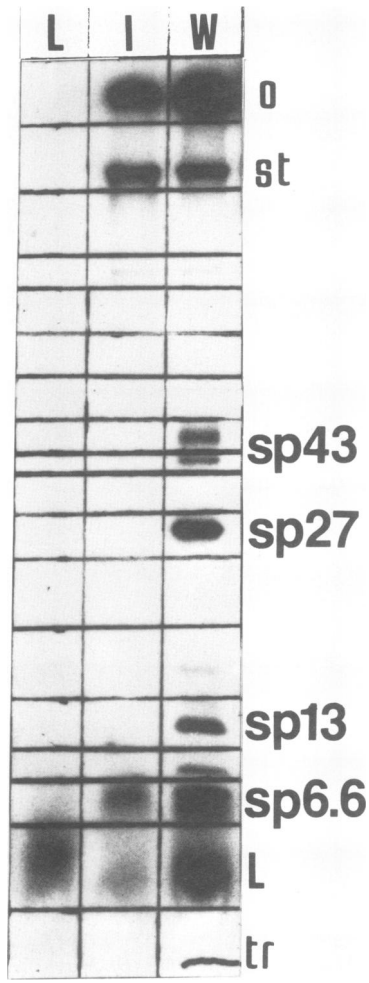

FIG. 5. *Autoradiogram of [^{35}S]methionine, $^{32}PO_4$-labeled ts1 vesicles, and wild-type PM2 after polyacrylamide gel electrophoresis. Samples: ts1 vesicles (I); wild-type PM2 (W); lipid extract from cells grown in $^{32}PO_4$, dried, and dissolved in gel sample buffer (L). Origin (o); boundary of stacking and separating get (st), both containing DNA; viral structural proteins sp43 (10^3 daltons), sp27, sp13, and sp6.6; lipid (L); position of tracking dye (tr).*

increases (12). Therefore, these vesicles are likely to be normal precursors which in the mutant are blocked in assembly at restrictive temperature. Upon relieving the temperature block, they rapidly mature to infectious virions.

Intracytoplasmic membranes have been observed in a number of bacteria (30). The vesicles stimulated by mutant *ts1* of PM2 are more regular than the intra-cytoplasmic membranes of *Gluconobacter oxydans* (1, 9), *Azotobacter vinelandii* (28, 29), a thermosensitive mutant of *Escherichia coli* (20), or *E. coli* infected with an amber mutant of bacteriophage fd (27). They are smaller than those of *Pseudomonas aeruginosa* (7) or *E. coli* O111a$_1$ (37), but similar to those of *E. coli* starved for magnesium (11). To my knowledge, none of these vesicles have been isolated, and mechanisms for their formation are unknown.

Two assembly mechanisms can now be eliminated for determination of the size and shape of the PM2 membrane. First, the membrane does not appear to form inside a shell of capsid proteins (sp43 and sp27), since mutant *ts1* fails to associate capsid with its membrane under restrictive conditions, yet makes a stable membrane. Additionally, another mutant of PM2, *ts5*, appears defective in its ability to attach capsid protein sp27 to the membrane (3). It seems unlikely that sp27 would attach tightly enough to specify the membrane characteristics, yet fall off during isolation of the membrane. This conclusion is in apparent conflict with the remarkable dissociation and reconstitution of PM2 by Schafer and Franklin (32). By disruption of PM2 in 4.5 M urea, nucleocapsid cores can be isolated that contain DNA and sp6.6 and sp13 (19). Upon addition to these cores of phospholipid, serum albumin, sp27, and sp43, particles formed which were similar in appearance and composition to the native virus; their specific infectivity was only 10^{-7} PFU per genome (32). An incorrect phospholipid composition was obtained by in vitro assembly of particles with sp6.6 and sp13 alone. Furthermore, in a biphasic mixture, sp27 causes the partitioning of phosphatidylglycerol out of chloroform into an aqueous urea phase. This has been interpreted as specific affinity of sp27 for phosphatidylglyc-

TABLE 1. *Distribution of radioactivity among DNA, sp6.6, and lipid in ts1 vesicles and wild-type PM2*[a]

Sample	Ratio of counts per minute			
	^{32}P-lipid/ ^{32}P-DNA[b]	^{35}S-sp6.6/ ^{32}P-DNA	^{35}S-sp6.6/ ^{32}P-lipid	^{32}P-sp6.6/ ^{32}P-lipid
ts1 vesicles	0.5	0.4	0.8	0.7
Wild-type PM2	0.5	2.0	3.8	0.3
Ratio[c]	1.0	5.1	4.9	0.4

[a] Samples were cut from the dried gel as seen in Fig. 5 and processed as described in the text. Note that ^{32}P is more energetic than ^{35}S so that counts per minute do not correlate directly with spot intensity on the autoradiogram.

[b] Bands o and st from Fig. 5.

[c] Wild-type PM2/*ts1* vesicles; numbers are correct even though rounding may cause them to appear to be in error.

erol (32). The important question is: is the affinity of this basic protein for an acidic lipid causative in selecting that lipid for assembly into the viral membrane? Many basic proteins would be expected to bind acidic molecules. The failure to show aqueous partitioning of the lipid with sp6.6 is equivocal since sp6.6 itself partitions into chloroform (5, 33). We must conclude that either the *ts1* vesicles or the interactions in urea and organic solvents are due to aberrant associations. It seems more likely that the structure formed in vivo would be genuine. Resolution of these apparent contradictions must await identification of the defective gene product of *ts1* and further physical chemistry.

A second possible mechanism of membrane morphogenesis has precedent in the budding of the enveloped animal viruses. The shape of the viral membrane is determined by the shape of the nucleocapsid, which is apposed to the plasma membrane of the host (24, 35). In mutant *ts1* of PM2, preliminary experiments indicate that the DNA associated with the vesicle is not packaged inside the membrane; treatment of lysates or purified vesicles with DNase results in large reductions in sedimentation velocity. Furthermore, the DNA that is present appears to be full size (Brewer, manuscript in preparation). From these data, in conjunction with the empty appearance of these vesicles in electron microscopy, the size and shape of the membrane of PM2 does not appear to be determined by formation around a core of DNA. The topological problem of packaging DNA inside a preformed shell has precedent with the T-even phages, for example (21, 25, 26).

The most striking feature of the *ts1* vesicles is the presence of only one protein, the viral sp6.6. According to a third mechanism for membrane assembly, this protein, sp6.6, specifically interacts with phospholipid in the host membrane to determine membrane size, shape, and composition. Several previous observations of PM2 support this model. The presence of protein in the virus membrane has been inferred from the increased electron density across the lipid bilayer as seen by X-ray diffraction (17). Second, there is insufficient phospholipid in the native virus to account for the entire volume of the membrane (6). As reported here, the possibility that phospholipid is bound to sp6.6 even in the presence of sodium dodecyl sulfate supports an integral role of sp6.6 in the membrane. Failure to observe such an association (18) may reflect the transient nature of this complex during viral maturation. Since sp6.6 can be cross-linked to the viral DNA by treatment with 1 M $Na_2S_2O_5$ (19), and phosphatidylglycerol is asymmetrically localized in the outside of the bilayer (33; our unpublished data), I propose that sp6.6 spans the viral membrane. We are currently using membrane-impermeable surface labeling techniques to confirm this hypothesis. At this time, it is unclear why *ts1* vesicles should have less sp6.6 than wild-type virus. More work is needed to support the hypothesis that one integral membrane protein is able to mobilize specific lipids into a newly assembled membrane of the desired size, shape, and lipid composition.

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