JOURNAL OF VIROLOGY, Nov. 1994, p. 7386–7396
0022-538X/94/$04.00+0

Vol. 68, No. 11

Membrane-Binding Domains and Cytopathogenesis of the Matrix Protein of Vesicular Stomatitis Virus

ZHIPING YE, WEI SUN, KALACHAR SURYANARAYANA, PETER JUSTICE, DRUEN ROBINSON, AND ROBERT R. WAGNER*

Department of Microbiology and Cancer Center, University of Virginia School of Medicine, Charlottesville, Virginia 22908

Received 3 June 1994/Accepted 8 August 1994

The membrane-binding affinity of the matrix (M) protein of vesicular stomatitis virus (VSV) was examined by comparing the cellular distribution of wild-type (wt) virus M protein with that of temperature-sensitive (*ts*) and deletion mutants probed by indirect fluorescent-antibody staining and fractionation of infected or plasmid-transfected CV1 cells. The M-gene mutant *ts*O23 caused cytopathic rounding of cells infected at permissive temperature but not of cells at the nonpermissive temperature; wt VSV also causes rounding, which prohibits study of M protein distribution by fluorescent-antibody staining. Little or no M protein can be detected in the plasma membrane of cells infected with *ts*O23 at the nonpermissive temperature, whereas ~20% of the M protein colocalized with the membrane fraction of cells infected with *ts*O23 at the permissive temperature. Cells transfected with a plasmid expressing intact 229-amino-acid wt M protein (M1-229) exhibited cytopathic cell rounding and actin filament dissolution, whereas cells retained normal polygonal morphology and actin filaments when transfected with plasmids expressing M proteins truncated to the first 74 N-terminal amino acids (M1-74) or deleted of the first 50 amino acids (M51-229) or amino acids 1 to 50 and 75 to 106 (M51-74/107-229). Truncated proteins M1-74 and M51-229 were readily detectable in the plasma membrane and cytosol of transfected cells as determined by both fluorescent-antibody staining and cell fractionation, as was the plasmid-expressed intact wt M protein. However, the expressed doubly deleted protein M51-74/107-229 could not be detected in plasma membrane by fluorescent-antibody staining or by cell fractionation, suggesting the presence of two membrane-binding sites spanning the region of amino acids 1 to 50 and amino acids 75 to 106 of the VSV M protein. These in vivo data were confirmed by an in vitro binding assay in which intact M protein and its deletion mutants were reconstituted in high- or low-ionic-strength buffers with synthetic membranes in the form of sonicated unilammelar vesicles. The results of these experiments appear to confirm the presence of two membrane-binding sites on the VSV M protein, one binding peripherally by electrostatic forces at the highly charged NH_2 terminus and the other stably binding membrane integration of hydrophobic amino acids and located by a hydropathy plot between amino acids 88 and 119.

The matrix (M) protein of vesicular stomatitis virus (VSV) is the most abundant structural protein of the virus (25), and it apparently plays the central role in assembly of the virion (22, 26). A major portion of the M protein binds to nascent VSV ribonucleoprotein (RNP) cores, possibly at the inner surface, resulting in condensation of RNP cores into tight coils (2, 20). Binding of M protein also results in down-regulation of transcription by RNP cores (6). The M protein is quite basic and has a pI of ~9.1 (6), largely because of a plethora of lysines and arginines at the amino terminus (23). The positively charged amino terminus of the M protein appears to provide the site for binding of M protein to RNP cores (10, 24).

The affinity in vivo of M protein for the plasma membrane was demonstrated conclusively by the recent finding that at least 10% of all vector-expressed M protein migrates to plasma membrane, where it is tightly bound (7). Moreover, expression of M protein alone results in evagination of M-protein vesicles from the plasma membrane surface (13). Extensive studies of the membrane-binding capacity of the VSV M protein showed that wild-type (wt) M protein could bind in vitro to synthetic lipid vesicles only if the phospholipids contained acidic headgroups (27, 31). Affinity labeling and protease digestion revealed the presence of a membrane-binding site of the M protein at its basic 19 N-terminal amino acids (12). In another study, it was found that M protein missing the first 43 N-terminal amino acids retained some capacity to bind phospholipid vesicles, suggesting the presence of another membrane-binding site distal to the highly charged N terminus (21). Chong and Rose (8) very recently found that a truncated expressed M protein, missing the first 15 N-terminal amino acids, retained its capacity to associate with plasma membrane of transfected cells but was less tightly bound than plasma membrane-associated intact M protein or truncated M protein devoid of its 14 C-terminal amino acids. The evidence to date thus suggests the possibility of two membrane-binding sites on the M protein of VSV.

In an effort to identify more precisely the existence and location of membrane-binding sites on VSV M protein, we studied its distribution in CV1 cells infected with wt VSV and a temperature-sensitive VSV mutant or transfected with deletion mutants of the M gene. We also compared the ionic binding stabilities on phospholipid vesicles of wt and mutant M proteins. The data suggest the presence of two distinct membrane-binding sites with differing affinities on the VSV M protein.

* Corresponding author. Mailing address: Department of Microbiology, School of Medicine, Box 441, University of Virginia, Charlottesville, VA 22908. Phone: (804) 924-1948 or (804) 924-5111. Fax: (804) 982-1071. Electronic mail address: rrw@virginia.edu.

MATERIALS AND METHODS

Viruses and cell culture. The wt Orsay strain of VSV-Indiana and its group III (M gene) temperature-sensitive

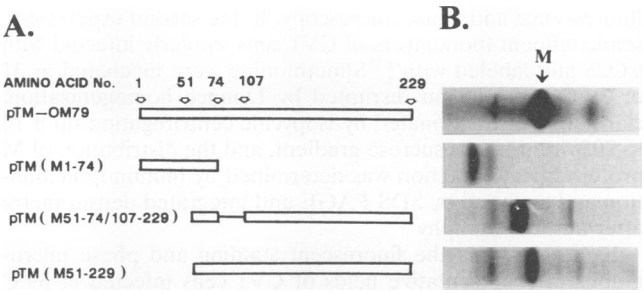

FIG. 1. Stick model (A) of wt and deletion mutants of the VSV-Indiana cDNA M gene cloned in vector pTM1 and autoradiographed electropherogram (B) showing migration of ^{35}S-labeled wt and deleted M proteins by SDS-PAGE. The numbers in parentheses for the deletion mutants refer to their amino acid sequences, which are also indicated by the arrows above the wt M protein showing relevant amino acid sites for each deleted protein.

mutant *ts*O23 were obtained from Anne Flamand of the Université de Paris-Sud, Orsay, France. Viruses were grown at 31°C on cultures of BHK-21 cells and titrated by plaque assay on monolayers of L cells at permissive (31°C) and restrictive (39°C) temperatures (24). The vaccinia virus recombinant (vTF1-6,2) expressing the T7 bacteriophage DNA-dependent RNA polymerase was a gift of Bernard Moss of the National Institutes of Health. VSV infection and plasmid transfection/vTF1-6,2 coinfection were performed by using CV1 cells grown to 95% confluence at 37°C in Dulbecco modified Eagle medium supplemented with 10% tryptose-phosphate broth, 10% fetal bovine serum, and antibiotics.

Complete and deleted M-gene expression vectors. The wt M gene used in these studies was a cDNA coding for all 229 amino acids of the M protein; it was obtained from the mRNA of the VSV-Indiana Orsay strain and was cloned in this laboratory into a vector designated pYL-OM79 flanked by the promoter and terminator sequences for bacteriophage T7 RNA polymerase (14). A truncated M gene coding for the first 74 N-terminal amino acids, designated pYL(M1-74), was created by digestion of pYL-OM79 with *Bgl*II at M-gene nucleotide 258 and with *Kpn*I, which cleaves at a vector site immediately downstream from the M gene; the gap was sealed with a synthetic oligonucleotide linker, providing an in-frame stop codon. A truncated M-gene clone in which the first 50 N-terminal amino acids are deleted, designated pYL(M51-229), was constructed by PCR using an upstream primer providing an *Nco*I cleavage site at an internal in-frame ATG start codon corresponding to M-gene nucleotides 249 to 251; a downstream primer provided an *Eco*RI cleavage site. A double deletion of the M gene, designated pYL(M51-74/107-229), was also constructed by PCR using an upstream primer providing an *Nco*I cleavage site at an internal in-frame ATG start codon corresponding to M-gene nucleotides 249 to 251 and a downstream primer providing an *Eco*RI cleavage site to delete the first 50 amino acids and by cleaving at nucleotides 258 and 358 with *Bgl*II to delete amino acids 75 to 106.

To provide maximum expression of M proteins in vitro and in vivo, the PCR products of pYL(M1-74), pYL(M51-229), and pYL(M51-74/107-229) were excised with *Nco*I and *Eco*RI and inserted between the *Nco*I and *Eco*RI sites of the expression vector pTM1, kindly provided by Bernard Moss (19). The pTM1 vector contains the T7 bacteriophage polymerase promoter and terminator sequences, as well as the cap-independent translation property. Figure 1 shows stick models for the M-gene deletion plasmids pTM(M1-74), pTM(M51-74/107-229), and pTM(M51-229), as well as the complete M gene cloned in pTM1 and designated pTM-OM79 (Fig. 1A). Also shown by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) are the [^{35}S]methionine-labeled proteins expressed by these four plasmids in a TnT in vitro coupled transcription-translation system (Fig. 1B). These ^{35}S-labeled complete and truncated M proteins were purified by affinity chromatography on a column of Sepharose coupled with monospecific polyclonal antibody made in rabbits immunized with purified M protein.

Immunofluorescence studies. CV1 cells were grown on glass coverslips for 2 days prior to infection with wt or *ts*O23 virus (multiplicity of infection of ~5 PFU per cell) or to plasmid transfection. Infected or transfected cells were incubated for various periods at temperatures chosen to provide optimal permissive (31°C) or nonpermissive (39°C) conditions for the mutant *ts*O23. For transient expression of mutant or wt M proteins cloned in plasmids, the CV1 cells were first infected with the vaccinia virus recombinant vTF1-6,2 expressing the T7 polymerase (multiplicity of infection of 5 PFU per cell) and then transfected with plasmids containing M-gene mutants (0.01 mg) in the presence of Lipofectin. After incubation for various periods, the infected or transfected CV1 cells were washed in cold phosphate-buffered saline (PBS), fixed for 20 min in 3% formaldehyde, and permeabilized by exposure to 0.4% Triton X-100 for 3 min. Nonspecific staining was blocked by incubation of the cells for 30 min in 3% bovine serum albumin in PBS. The infected or transfected cells were then incubated at room temperature for 40 min with rabbit anti-M protein serum (immunoglobulin G [IgG]) at a concentration of 0.005 mg/ml in PBS and with rhodamine-phalloidin (to stain actin filaments). Each coverslip was then flooded with donkey anti-rabbit IgG conjugated with fluorescein isothiocyanate (FITC) and incubated for 40 min at room temperature. Washed coverslips were then mounted in 90% glycerol and 10% PBS in 3,4,5-trihydroxybenzoic acid *N*-propylester to prevent photobleaching. Cellular distribution of immunofluorescence was determined with a transmission microscope under an epifluorescent UV light source. Cellular morphology of the same field was examined by using phase-contrast micrographs under a regular light source.

Fractionation of infected and transfected cells. Subconfluent monolayers of CV1 cells in 60-mm-diameter plates were first infected with VSV or the T7 polymerase recombinant vTF1-6,2 by 30 min of adsorption at a multiplicity of infection of 5 PFU per cell. The vTF1-6,2-infected cells were then transfected with 10 μg of M-gene recombinant plasmid cDNA assisted by Lipofectin for 2 h at 37°C. After being washed with PBS, the cells were incubated for 1 h at 37°C in 2 ml of methionine-free minimum essential medium with 5% fetal bovine serum and 0.05 mCi of [^{35}S]methionine. The labeling medium was then replaced with 2 ml of minimum essential medium containing 5% fetal bovine serum and 5 mM unlabeled methionine. After further incubation, the ^{35}S-labeled cells were washed with PBS and harvested by scraping the monolayers in homogenization buffer (HB; 10 mM Tris-HCl [pH 7.4], 10% sucrose, 1 mM EDTA, 1 μg of aprotinin per ml). Cells were disrupted by 20 strokes in a tight-fitting Dounce homogenizer (Thomas; 0.1-nm clearance) cooled by ice, and the lysates were clarified by centrifugation at 2,000 × *g* for 5 min at 4°C. The clarified lysates were then layered on a 4.5-ml 10 to 70% continuous sucrose gradient for centrifugation at 100,000 × *g* for 20 h at 4°C. The fractions were collected from the bottom, diluted with equal volumes of 2× NET-gel buffer (50 mM Tris-HCl [pH 7.5], 150 mM NaCl, 0.1% Nonidet P-40,

1 mM EDTA, 0.25% gelatin, 0.02% sodium azide), and immunoprecipitated with rabbit anti-M protein polyclonal serum (1:500 dilution of serum). The ^{35}S-labeled immunoprecipitated proteins in each fraction were analyzed by SDS-PAGE and scored by integrated densitometry.

In vitro reconstitution of M proteins with sonicated vesicles. Complete and deleted VSV M proteins labeled with [^{35}S]methionine were synthesized in vitro from M-gene recombinant pTM1 plasmids by using the Promega TnT coupled transcription-translation kit driven by phage T7 polymerase and translated in a reticulocyte lysate. The efficiency of [^{35}S]methionine incorporation into newly translated proteins was 22%, with background incorporation of 0.2%. All translated M proteins were purified by affinity chromatography on a column of Sepharose conjugated with polyclonal antibody specific for M protein. The sonicated vesicles were prepared as previously described (29). In short, 500 nmol of dipalmitoylphosphatidylcholine (DPPC) was mixed with 500 nmol of dipalmitoylphosphatidylglycerol (DPPG); this 1:1 molar ratio of DPPC/DPPG was dried under nitrogen and lyophilized overnight. The lipid samples were then suspended in 2 ml of 10 mM Tricine buffer (pH 7.4) containing no salt, 0.15 M NaCl, or 0.65 M NaCl and sonicated at 42°C for 3 to 5 min under nitrogen with a Heat-Systems W-350 sonicator. Multilamellar vesicles were removed by centrifuging the sonicated lipid samples at 150,000 $\times$ *g* for 3 h. The resulting small unilamellar vesicles (about 25 nmol) were mixed with wt or truncated ^{35}S-labeled M proteins (150,000 cpm) and were then dialyzed for 24 h against 10 mM Tricine containing the desired NaCl concentrations. The vesicles reconstituted with M protein were then subjected to buoyant-density (isopycnic) centrifugation for 16 h at 200,000 $\times$ *g* at 46°C in a 0 to 30% sucrose gradient as previously described (27). Samples (0.4 ml each) were analyzed for buoyant density by refractometry, and the gradient distribution of ^{35}S-M proteins was determined by SDS-PAGE and autoradiography and integrated by densitometry.

RESULTS

Cellular distribution of *ts*O23 M protein at permissive and restrictive temperatures. It has been known for some time that the VSV M protein lines the inner surface of the virion envelope and binds to the inner surface of the plasma membrane, as well as the RNP cores, in VSV-infected cells (26). Chong and Rose (7) have shown that, in the absence of other VSV proteins, 90% of the plasmid-expressed M protein is distributed in the cytosol and 10% is associated with the plasma membranes of transfected cells. It was of interest to determine whether at nonpermissive temperatures the VSV M-gene conditional mutant *ts*O23, which is defective in virion assembly, synthesizes M protein restricted in binding to plasma membrane. Because of the cytopathic effect of cell rounding caused by M protein synthesized in cells infected with wt VSV or its M-gene mutant *ts*G33 at permissive temperatures (5), fluorescent-antibody labeling studies were likely to be of limited use in determining the distribution of M protein in infected cells. For this reason, cell fractionation studies were also performed. It was also of interest to compare the cell rounding induced by *ts*O23 with that induced by the different M-gene mutant *ts*G33 tested previously (5).

In these experiments, CV1 cells were infected with wt VSV or *ts*O23 and incubated for 4 h at 31° (permissive temperature) or 39°C (restrictive temperature). As described in Materials and Methods, infected CV1 cells grown on coverslips were exposed to monospecific rabbit anti-M serum and labeled with FITC-conjugated donkey anti-rabbit IgG for examination by fluorescence and phase microscopy. In the second experiment, semiconfluent monolayers of CV1 cells similarly infected with *ts*O23 and labeled with [^{35}S]methionine were incubated at 31 or 39°C for 4 h and disrupted by Dounce homogenization, clarified, and fractionated by isopycnic centrifugation on a 10 to 70% continuous sucrose gradient, and the distribution of M protein in each fraction was determined by immunoprecipitation and analyzed by SDS-PAGE and integrated densitometry after autoradiography.

Figure 2 shows the fluorescent staining and phase micrographs of representative fields of CV1 cells infected at 39°C with wt VSV and at 31 or 39°C with *ts*O23. Clearly, the cells were rounded and shrunken after infection with wt virus at 39°C (Fig. 2A and B) or with *ts*O23 at 31°C (Fig. 2E and F). The M protein was uniformly distributed and could not be localized to any region of the cells infected with wt virus (Fig. 2A) or with *ts*O23 at 31°C (Fig. 2E). In sharp contrast, CV1 cells infected with *ts*O23 at 39°C retained their large size and typical polygonal morphology when examined by fluorescence microscopy (Fig. 2C) or by phase microscopy (Fig. 2D). The *ts*O23 M protein appeared to be widely distributed throughout the cytoplasm of cells infected at 39°C, with no particular localization to the surface membrane (Fig. 2C). Quite obviously, the membrane localization, if any, in cells infected with wt VSV or *ts*O23 at 31°C cannot be determined because of the cytopathic effect. Of some interest is the finding that *ts*O23 causes the same cell rounding at permissive but not nonpermissive temperatures as does another M-gene mutant, *ts*G33 (5).

Figure 3 shows the distribution of ^{35}S-labeled M protein synthesized in CV1 cells infected at 31°C (Fig. 3A) or 39°C (Fig. 3B) with *ts*O23 after disruption, fractionation by isopycnic centrifugation, immunoprecipitation, SDS-PAGE, autoradiography, and integrated densitometry. As noted, ~75% of the M protein synthesized at 31°C banded at a mean density of ~1.234 g/cm^3 and ~25% of the M protein banded at a peak of ~1.130 g/cm^3 (Fig. 3A). The radioactivity at high density is consistent with the presence of free M protein in the cytoplasm or of M protein in association with nascent RNP cores. The lower density peak at 1.142 g/cm^3 indicates the presence of M protein associated with plasma membrane, which bands at this density in such a sucrose gradient (1). By comparison, virtually all the *ts*O23 M protein synthesized at 39°C banded at a density of 1.234 g/cm^3, except for a trailing decreasing amount of radioactivity near the top of the gradient (Fig. 3B).

The question arises whether the *ts*O23 M protein fails to bind to plasma membrane at 39°C because it can be sequestered on the perinuclear membrane or cytoskeleton at the nonpermissive temperature. This is particularly relevant because of the studies of Ono et al. (21a), who found that the M protein of the phenotypically similar VSV mutant *ts*G33 forms perinuclear aggregates at nonpermissive temperatures but is diffusely distributed in the cytoplasm at permissive temperatures. Therefore, we analyzed by the same method (21a) the distribution of M protein in the nuclei, cytoskeletons, and cytoplasm of CV1 cells infected with *ts*O23 at 31 and 39°C in the presence of [^{35}S]methionine. Briefly, *ts*O23-infected CV1 cells washed at 6 h postinfection at 31 and 39°C were suspended in a HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid) buffer and disrupted by adding 0.5% Triton X-100 for 30 s. After centrifugation of the disrupted cells at 1,000 $\times$ *g* for 5 min, the soluble supernatant fluid was removed for analysis. The pelleted material was separated into nuclei and cytoskeleton by homogenization in lysis buffer containing 1% Tween 20 and 0.5% deoxycholate, and then it was centrifuged at 1,000 $\times$ *g* for 5 min. The ^{35}S-labeled M protein in each

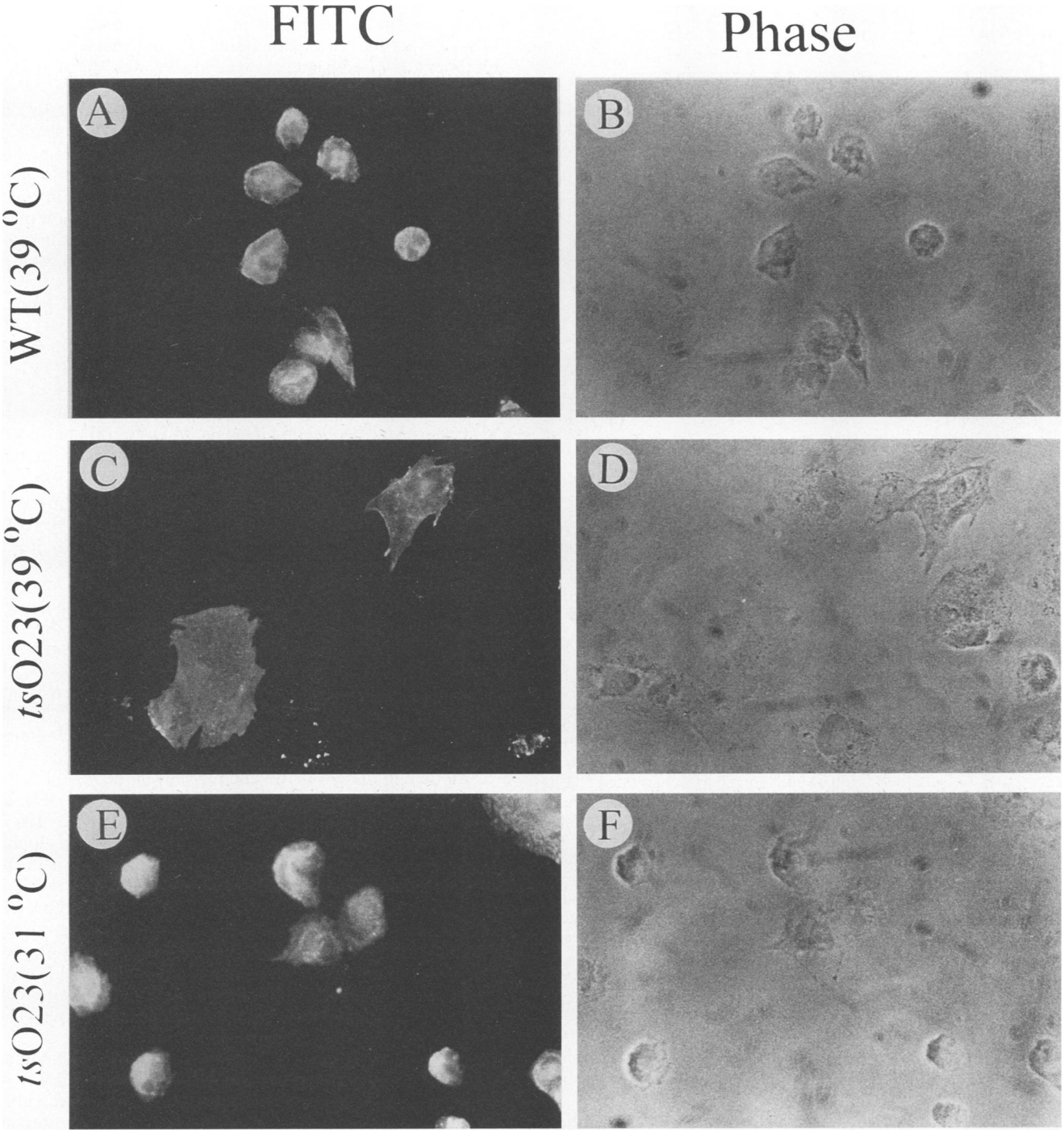

FIG. 2. Indirect fluorescent-antibody staining of M protein and phase microscopy of CV1 cells infected 4 h previously with wt VSV-Indiana at 39°C (A and B) or with its M-gene mutant *ts*O23 at the nonpermissive temperature of 39°C (C and D) or the permissive temperature of 31°C (E and F). As described in Materials and Methods, infected CV1 cells were fixed, permeabilized, exposed to rabbit anti-M monospecific polyclonal antibody, and labeled with FITC-conjugated donkey anti-rabbit IgG. Photomicrographs show cells in the same fields examined by fluorescence microscopy and phase microscopy at a magnification of ×472.

fraction was immunoprecipitated and quantitated by SDS-PAGE, autoradiography, and integrated densitometry. The results showed that the distribution of *ts*O23 M protein synthesized in CV1 cells at the permissive temperature was as follows: 49% in the cytosol, 20% associated with nuclei, and 31% associated with the cytoskeleton fraction. By comparison, *ts*O23 M protein synthesized at the nonpermissive temperature was distributed in CV1 cells as follows: 30% in the cytosol, 40% in the nuclear fraction, and 30% in the cytoskeletal compartment (data not shown). These data indicate that the sequestration of *ts*O23 M protein in perinuclear or cytoskeletal fractions is not responsible for its failure to bind to plasma

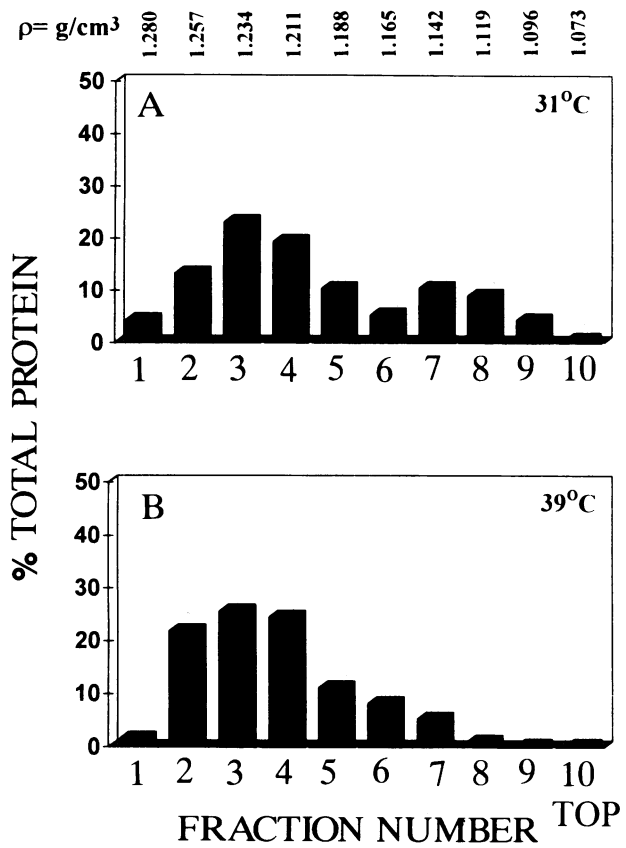

FIG. 3. Distribution of M protein in cytoplasm and membrane of fractionated CV1 cells infected with VSV mutant *ts*O23 at the permissive temperature of 31°C (A) or the nonpermissive temperature of 39°C (B). Cells labeled with [^{35}S]methionine were harvested at 4 h after infection and disrupted by Dounce homogenization. As described in Materials and Methods, disrupted cells were fractionated by isopycnic centrifugation in a 10 to 70% sucrose gradient. The ^{35}S-labeled M protein in each of the ten fractions was immunoprecipitated by adding rabbit anti-M serum in the presence of 0.4% Triton X-100 and subjected to SDS-PAGE and autoradiography. The relative proportions of ^{35}S-M protein in each fraction were determined by integrated densitometry, and the buoyant density (ρ) was determined by refractometry.

membrane at the nonpermissive temperature, since almost as much *ts*O23 M protein was soluble in the cytoplasm and available for membrane binding at the permissive temperature as at the nonpermissive temperature.

These data indicate that mutant *ts*O23 M protein synthesized in cells at the restrictive temperature binds only minimally, if at all, to plasma membrane, suggesting that there is a conditional defect in membrane association of the mutant M protein. The distribution of 75% cytoplasmic and 25% membrane-associated *ts*O23 M protein at the permissive temperature is not significantly different from the 10% binding to plasma membrane of vector-expressed wt M protein reported by Chong and Rose (7).

Cellular distribution of plasmid-expressed complete and truncated M proteins. It was of interest to determine whether the intact wt M protein and some of its deletion mutants were associated with cell membrane in the absence of other intracellular VSV proteins. Therefore, CV1 cells were transfected with pTM1 recombinants expressing the entire wt M protein or its deletion mutants depicted in Fig. 1 and coinfected with vTF1-6,2 vaccinia virus expressing T7 polymerase. In one experiment, CV1 cells grown on coverslips were fixed at 16 h posttransfection and exposed to polyclonal rabbit anti-M serum followed by FITC-conjugated donkey anti-rabbit IgG or by rhodamine-phalloidin to stain actin filaments. The shape of these same fluorescently labeled cells was also determined by phase microscopy. In the second series of experiments, plasmid-transfected CV1 cells expressing intact M protein or its deletion mutants were fractionated and the distribution of M proteins between cytosol and membrane was determined by equilibrium (isopycnic) centrifugation as described in Materials and Methods.

Figure 4 shows the cellular distribution of intact M protein and its deletion mutants, the presence of actin filaments, and the shape of the cells at 16 h after transfection with pTM-OM79, pTM(M1-74), pTM(M51-229), or pTM(M51-74/107-229). As indicated in Fig. 4A through C, expression of the entire wt M protein resulted in marked rounding of transfected CV1 cells, a paucity of actin filaments, and uniform fluorescent staining of M protein, in a manner quite similar to that observed for CV1 cells infected with wt VSV or with *ts*O23 at 31°C (Fig. 2). In sharp contrast, CV1 cells transfected with the three M-gene deletion mutants retained their polygonal shape and actin filaments as judged by fluorescence and phase microscopy despite ample expression of FITC-stainable M protein. The distribution of FITC-stainable M protein and the presence of actin filaments reactive with rhodamine-phalloidin are clearly visible in cells transfected with all three deletion mutants. The M protein truncated to the first 74 amino acids, expressed in cells transfected with pTM(M1-74), was clearly found at the cell surface, presumably at the plasma membrane, as well as in the cytoplasm (Fig. 4D). Similarly, CV1 cells transfected with pTM(M51-229), in which the first 50 amino acids were deleted, expressed this truncated M protein, which is distributed in the cytoplasm and partially in the region of the surface plasma membrane as well as the nucleus (Fig. 4G). By comparison, deletion of amino acids 1 to 50 and 75 to 106 in pTM(M51-74/107-229) resulted in the synthesis of a doubly deleted M protein detectable only as punctate clusters in the cytoplasm, and not the surface membrane, of transfected CV1 cells (Fig. 4J). These findings indicate that the entire M protein is required for causing a cytopathic effect resulting in cell rounding and dissolution of actin filaments. In addition, two deletions are required to prevent the association of M protein with plasma membrane, suggesting the presence of two membrane-binding sites on M protein located in the regions of amino acids 1 to 50 and 75 to 106.

Figure 5 provides additional data concerning the membrane and cytosol distribution of ^{35}S-labeled M protein expressed in CV1 cells transfected with pTM-OM79 (expressing the entire wt M protein), pTM(M1-74), the double-deletion mutant pTM(M51-74/107-229), and pTM(M51-229), as determined by cell fractionation, equilibrium density centrifugation, and SDS-PAGE. As noted, ~90% of the wt M protein expressed by pTM-OM79 banded at a mean density of ~1.131 g/cm^3, equivalent to the density expected for membrane (Fig. 5A). Much lower yields of M protein truncated to the first 74 N-terminal amino acids were recoverable from pTM(M1-74)-transfected CV1 cells, but cell fractionation revealed that ~70% of this protein was present in cytosol at a mean density of 1.211 g/cm^3 and ~30% was present in a putative membrane fraction at a density of 1.119 g/cm^3 (Fig. 5B). The doubly deleted M protein expressed by pTM(51-74/107-229) expressed in CV1 cells fractionated by isopycnic centrifugation banded entirely at a mean density of 1.234 g/cm^3, i.e., at a

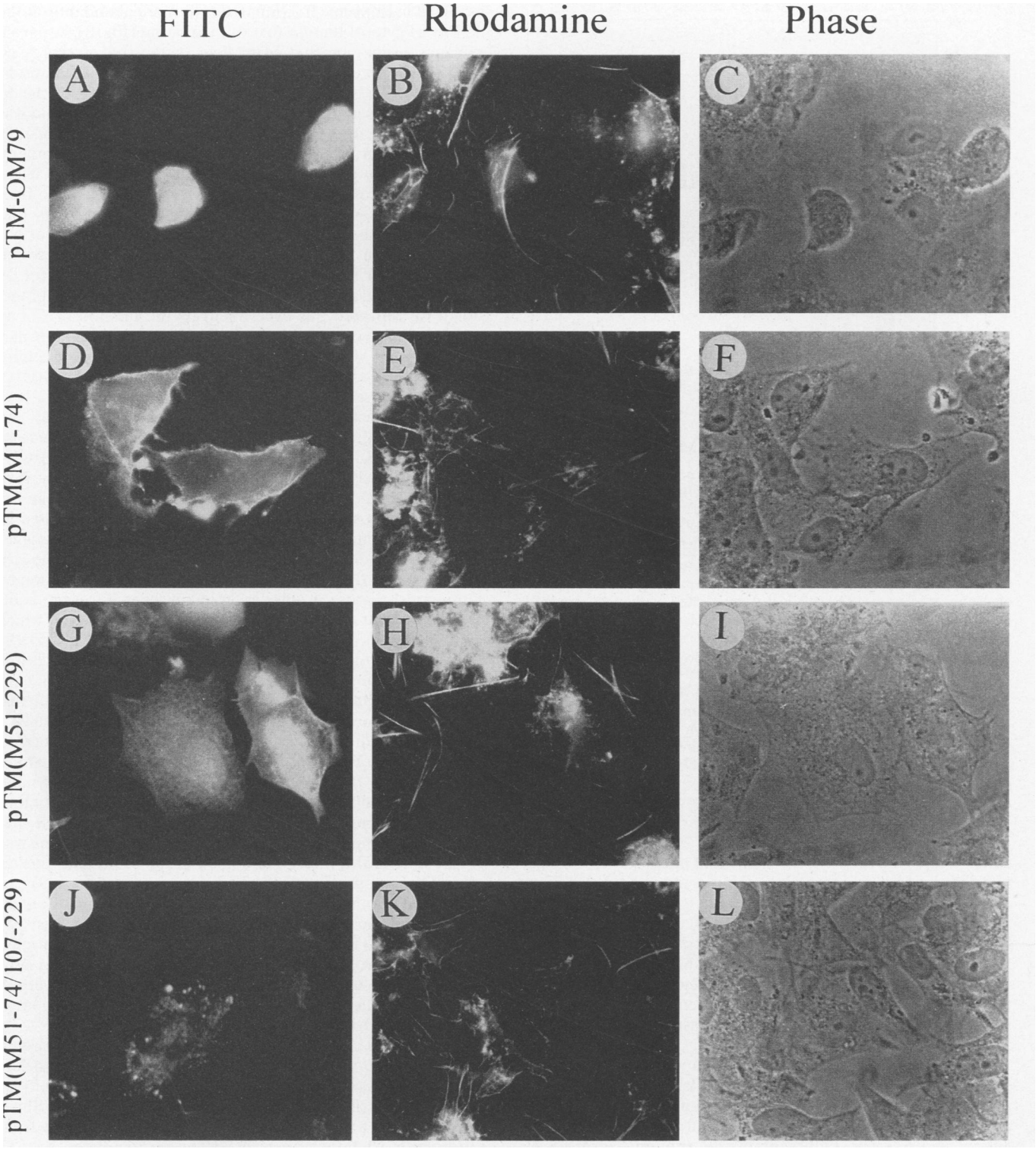

FIG. 4. Indirect immunofluorescent-antibody staining of M protein, actin filament labeling, and phase microscopy of CV1 cells transfected with plasmids expressing the entire VSV M gene, pTM-OM79 (A through C), or plasmids expressing deleted M genes, pTM(M1-74) (D through F), pTM(M51-229) (G through I), or pTM(M51-74/107-229) (J through L). As described in Materials and Methods, CV1 cells were infected with T7 polymerase-expressing vTF1-6,2 and transfected with recombinant M-gene plasmids in the presence of Lipofectin. After incubation at 37°C for 16 h after transfection, cells were fixed, permeabilized with 0.4% Triton X-100, exposed to rabbit anti-M serum, and stained with FITC-conjugated donkey anti-rabbit IgG and rhodamine-phalloidin. The photomicrographs show representative fields of the same cells examined for distribution of M protein by fluorescein labeling (A, D, G, and J), for actin filaments by rhodamine fluorescence (B, E, H, and K), or for shape by phase microscopy (C, F, I, and L). Magnification, ×458.

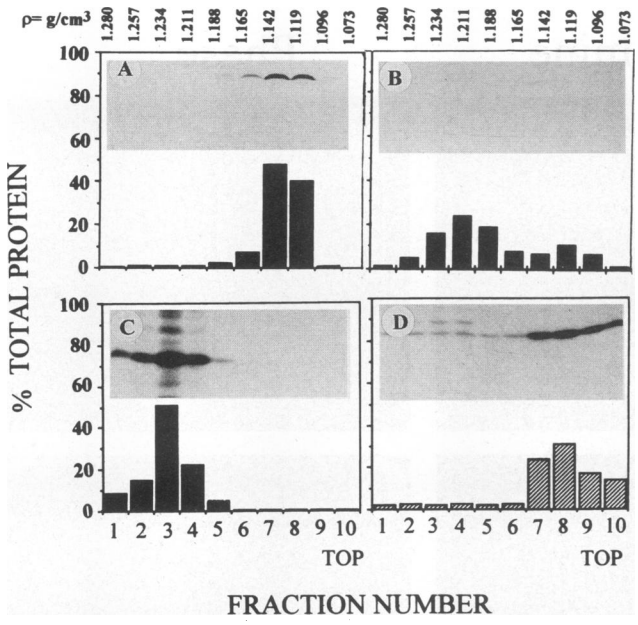

FIG. 5. Distribution of M protein between the cytoplasm and plasma membrane of CV1 cells transfected with pTM-OM79 expressing the entire M protein (A), pTM(M1-74) expressing M protein truncated to the first 74 amino acids (B), pTM(M51-74/107-229) expressing M protein deleted of amino acids 1 to 50 and 75 to 106 (C), or pTM(M51-229) expressing M protein deleted of the first 50 amino acids (D). As described in Materials and Methods, CV1 cells previously infected with vTF1-6,2 were transfected with each plasmid, labeled with [^{35}S]methionine, and disrupted by Dounce homogenization after incubation for 16 h. After removal of debris and nuclei by low-speed centrifugation, the disrupted cells were fractionated by isopycnic centrifugation on a 10 to 70% sucrose gradient. The ^{35}S-labeled M protein in each of the 10 gradient fractions was immunoprecipitated with rabbit anti-M serum and analyzed by SDS-PAGE. Autoradiographs of M proteins (shown above bar graphs) were then integrated by densitometry, and buoyant density (ρ) was determined by refractometry. The results are plotted as the percentage of ^{35}S-M protein in each gradient fraction.

density expected for cytosol devoid of membrane; virtually none of the M protein missing amino acids 1 to 50 and 75 to 106 could be detected at a density expected for plasma membrane (Fig. 5C). In marked contrast, when CV1 cells were fractionated by equilibrium density centrifugation after transfection with pTM(M51-2229) expressing M protein from which only the first 50 amino acids were deleted, ~80% of the truncated M protein banded at a mean density of ~1.119 g/cm^3 in presumptive association with cell plasma membrane (Fig. 5D).

These findings by cell fractionation appear to confirm the data obtained by fluorescent-antibody staining, indicating the presence of membrane-binding sites on M protein in the region of the first 74 amino acids as well as a region distal to amino acid 51. The failure of the M protein expressed by pTM(M51-74/107-229) to bind to low-density cell fractions indicates the presence of two membrane-binding sites on M protein located between amino acids 1 to 50 and between amino acids 75 to 106.

It is incumbent upon us to comment on why M protein expressed by pTM-OM79 and by pTM(M51-229) should be found so abundantly at isopycnic densities consistent with that of membranes, whereas the M protein expressed during infection with wt VSV or *ts*O23 at the permissive temperature is localized in isopycnic fractionated cells more abundantly at the density of cytosol than at that of membrane (Fig. 3). A partial explanation may be that M protein synthesized during VSV infection is predominantly associated with nucleocapsids, which band at high densities. However, only 10% of the M protein expressed in HeLa cells by a recombinant plasmid was found to colocalize with plasma membrane at the 65%-10% interface of a discontinuous isopycnic sucrose gradient, whereas ~90% of the expressed M protein was found at the 80%-65% sucrose interface thought to be in a cytosolic fraction (7). A possible explanation for this discrepancy is that expression of the M-gene construct in HeLa cells was for 6 h (7), whereas in our studies the M-gene constructs were expressed for 16 h, ostensibly providing a longer time for M proteins to migrate from the cytosol to the plasma membrane. When we repeated this experiment by the method of Chong and Rose (7, 8), using isopycnic flotation centrifugation in a discontinuous 80%-65%-10% sucrose gradient for fractionation of cells transfected with pTM-OM79, we found that 21% of the expressed M protein colocalized at the 65%-10% sucrose interface (presumably with plasma membrane) and 79% was at the 80%-65% (presumably cytosolic) sucrose interface (data not shown). The same experiment with CV1 cells transfected with pTM(M51-229) revealed that only 38% of the truncated M protein colocalized with cytosol at the 80%-65% sucrose interface, whereas 62% of the truncated M protein was found in the presumptive membrane-associated fraction at the 65%-10% sucrose interface (data not shown). The question arises as to the reliability of cell fractionation by continuous sucrose isopycnic gradient centrifugation compared with that of a discontinuous sucrose gradient in which buoyant density cannot be measured. Regardless, the doubly deleted M protein expressed by pTM(M51-74/107-229) is clearly not membrane associated (Fig. 5C).

Vesicle binding affinity of intact and deleted M proteins. The preceding experiments suggest the presence of two in vivo membrane-binding sites on the M protein of VSV, one located within the first 50 amino acids and the other between amino acids 75 and 106. The existence of a similar N-terminal membrane-binding site on M protein was recently demonstrated by Chong and Rose (8), who also demonstrated that stability of membrane binding under high-salt conditions was attributable to the C-terminal region of the M protein. Earlier studies from our laboratory had revealed that wt VSV M protein binds in vitro to synthetic phospholipid vesicles only if they contain acidic headgroups that recognize the basic M protein (27, 31). It seemed logical therefore to determine whether the two putative membrane-binding sites on M protein contribute to its binding affinity for acidic phospholipids, as well as whether ionic and/or hydrophobic bonds contributed to the two putative binding sites.

In these experiments unilamellar vesicles containing equal concentrations of DPPC and DPPG (DPPC/DPPG vesicles) were prepared by sonication and purified to homogeneity as previously described (27, 29). M proteins labeled with [^{35}S]methionine were prepared in vitro with the TnT coupled transcription-translation system by using as templates the T7 polymerase-driven plasmids pTM-OM79, expressing the entire wt M protein, and pTM(M1-74), pTM(M51-229), and pTM (M51-74/107-229), each of which expresses the relevant truncated M protein (Fig. 1; see Materials and Methods). Sonicated DPPC/DPPG unilamellar vesicles were incubated with ^{35}S-labeled entire or truncated M proteins in buffers containing no salt, 0.15 M NaCl, or 0.65 M NaCl. As described in Materials and Methods, the interacting vesicles and M proteins

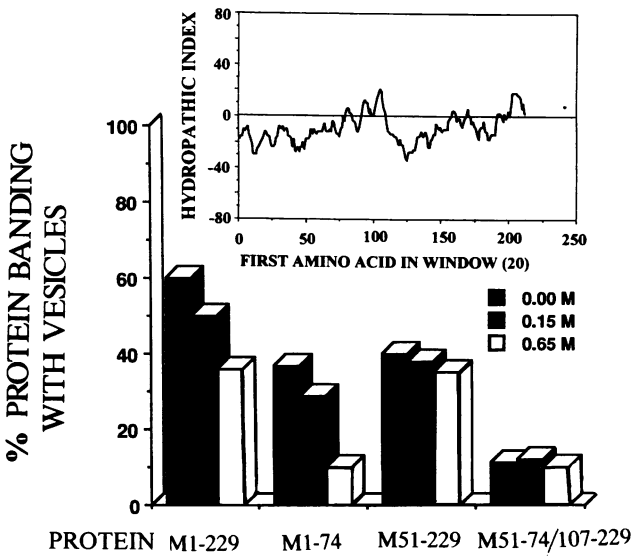

FIG. 6. Salt-dependent affinity for in vitro binding of M protein and its deletion mutants to sonicated unilamellar phospholipid vesicles and hydropathy plot of wt VSV M protein. Complete M protein containing all 229 amino acids (M1-229) and deletion mutants of M protein truncated to the first 74 amino acids (M1-74), deleted of the first 50 amino acids (M51-229), or deleted of amino acids 1 to 50 and 75 to 106 (M51-74/107-229) were synthesized in vitro and labeled with [^{35}S] methionine in the TnT coupled transcription-translation system. Unilamellar vesicles were prepared by sonication of equimolar concentrations of DPPC and DPPG. Mixtures of liposomes and ^{35}S-labeled complete or truncated M proteins were reacted at 42°C for 24 h during dialysis against Tricine buffer with no salt or 0.15 or 0.65 M NaCl. Vesicles banded by buoyant-density centrifugation in a continuous 0 to 30% sucrose gradient were tested for their concentrations of bound ^{35}S-M protein by SDS-PAGE, autoradiography, and integrated densitometry. The percentage of M protein bound to vesicles at each salt concentration was calculated from the total ^{35}S-protein throughout the gradient. The inset shows the hydropathy index of wt VSV-Indiana M protein calculated by moving segments of 20 amino acid residues according to the values assigned by Kyte and Doolittle (11).

were subjected to isopycnic centrifugation in a 0 to 30% continuous sucrose gradient and the percentage of ^{35}S-M protein banding with lipid vesicles was measured by SDS-PAGE, autoradiography, and integrated densitometry.

Figure 6 shows bar graphs of the percentages of ^{35}S-M protein associated with DPPC/DPPG vesicles after interaction in buffer containing no NaCl or 0.15 or 0.65 M NaCl followed by isopycnic centrifugation. Also shown in Fig. 6 for reference is a hydropathy plot of the entire wt M protein. As indicated, in a reaction mixture containing no salt, 60% of the whole M protein bound to DPPC/DPPG vesicles, compared with 37% of the M protein truncated to the first 74 amino acids, 40% of the M protein extending from amino acid 51 to 229, and only 11% of the double-deletion M51-74/107-229 protein; 10 to 12% of the binding can be assumed to represent background nonspecific binding of M protein. We interpret this last result as indicating that there was essentially no binding of the mutant M protein deleted in two regions (from amino acids 1 to 50 and 75 to 106) to DPPC/DPPG vesicles; these data appear to support the preceding studies indicating the complete loss of membrane binding in vivo of this doubly deleted mutant M protein. When the wt and truncated M proteins were reconstituted with DPPC/DPPG vesicles at physiological salt concentrations (0.15 M NaCl), their binding affinities decreased slightly: there was a 10% decrease for the complete M protein (M1-229), an 8% decrease for truncated protein M1-74, and an insignificant 2% decrease for truncated protein M51-229. Increasing the salt concentration in the vesicle-M protein binding medium to 0.65 M NaCl resulted in decreased binding of the entire M protein (M1-229), from 50 to 60% to 29%, and of the M protein truncated to the first 74 amino acids (M1-74), from 29 to 37% to 10%, essentially to the background level for this procedure. In contrast, the binding reaction performed at 0.65 M NaCl reduced the binding to vesicles of the M protein from which the first 50 amino acids were deleted (M51-229) from a level of 38 to 40% to 35%, an insignificant decline in binding affinity at a high salt concentration. Quite similar binding affinities were observed when ^{14}C-labeled wt and mutant M proteins were interacted with sonicated ^{3}H-DPPC/DPPG unilammelar vesicles in the presence of no NaCl or 0.15 or 0.65 M NaCl before isopycnic centrifugation. All but the double mutant ^{14}C-M51-74/107-229 colocalized with ^{3}H-DPPC/DPPG vesicles at low salt concentrations, and ^{14}C-M1-74 showed no affinity for binding to ^{3}H-DPPC/DPPG vesicles in the presence of 0.65 M NaCl (data not shown).

These data indicate that the M protein truncated to the first 74 amino acids (M1-74) binds to DPPC/DPPG vesicles by ionic bonds, whereas the M protein in which only the first 50 amino acids are deleted (M51-229) appears to bind DPPC/DPPG vesicles by nonionic bonds, since it is not significantly impeded in binding in the presence of 0.65 M NaCl. In the case of the entire M protein (M1-229), both ionic and nonionic binding appear to be operative, with the greater binding force apparently being nonionic in character. This finding is consistent with the study reported by Chong and Rose (8), who found that membrane-prebound M protein lacking the first 15 amino acids was only slightly more dissociable by high salt concentrations than was the membrane-prebound intact M protein, indicating the presence of a carboxy-distal nonionic membrane-binding site.

It is well known that the N-terminal region of VSV M protein is highly charged and rich in basic lysine and arginine residues (23, 24), thus explaining its binding affinity for vesicles containing phospholipids with acidic headgroups (27, 31). Figure 6 (inset) shows a hydropathy plot, created according to the method of Kyte and Doolittle (11), indicating the regions of the wt VSV M protein possessing significant stretches of hydrophobic amino acids. As indicated, the region between amino acids 88 and 119 can be considered to be hydrophobic. This could explain why the deletion of amino acids 75 to 106 in this region (along with amino acids 1 to 50) results in a loss of capacity to bind cell membrane and lipid vesicles. This could also explain why binding at this region is not prevented by high salt concentrations as is binding at the other putative binding site spanning the first 50 amino acids, which quite obviously binds to membranes through electrostatic forces. The other hydrophobic region at the extreme C-terminal end (amino acids 202 to 208) of the M protein apparently is not likely to play a role in stable membrane association; deletion of 14 C-terminal amino acids did not disrupt binding of this truncated M protein (8), and it would not be expected to do so.

Finally, we set out to determine the levels of binding stability of the complete M protein and the truncated M proteins complexed with vesicles. In these experiments pTM-OM79, pTM(M1-74), and pTM(M51-229) were expressed in the TnT coupled transcription-translation system, and proteins M1-229, M1-74, and M51-229, respectively, were heavily labeled with [^{35}S]methionine. After purification, these ^{35}S-labeled M proteins were incubated with DPPC/DPPG vesicles in the salt-free Tricine buffer described in Materials and Methods. These

vesicles, complexed with whole M protein or with the two truncated M proteins, were dialyzed overnight against Tricine buffer without salt or with 0.65 M NaCl. The vesicles were then purified by isopycnic centrifugation banding, and the levels of ^{35}S-M protein associated with vesicles exposed to no NaCl or 0.65 M NaCl were compared by radioactivity determined by scintillation spectrometry. The results revealed that, after exposure to 0.65 M NaCl, 83% of the whole M protein and 78% of the M protein truncated to amino acids 51 to 229 remained associated with vesicles, respectively. By comparison, only 12% of the M protein truncated to amino acids 1 to 74 remained associated with vesicles exposed to 0.65 M NaCl (data not shown). These results are quite consistent with the effect of salt on the binding of M protein and its truncations to vesicles (Fig. 6). These data further support the hypothesis that the N-terminal region of M protein binds to membrane electrostatically and that the C-terminal region, possibly amino acids 75 to 106, binds to membrane more stably by hydrophobic bonds.

DISCUSSION

It is generally assumed that the VSV M protein is essential for virion assembly in serving as the bridge that joins the RNP to the G-protein-containing plasma membrane. M protein appears to be associated with RNP cores, as judged by electron microscopic immunogold labeling (2, 20), as well as by chemical cross-linking studies (9, 17). The M protein also appears to line the inner surface of the virion membrane in close proximity to the integral membrane G protein (9), the C-terminal region of which has been considered to be a docking site for the M protein-RNP complex (15). However, plasmid-expressed M protein, in the absence of other viral proteins, is transported to and binds to plasma membrane (3, 7), and it is capable on its own of causing evagination of plasma membrane, resulting in budded vesicles (13). Intact M protein and truncated M proteins interact with and bind to isolated plasma membranes (8) and to phospholipid vesicles (21, 27). In a reconstitution system the M protein shows considerable affinity for binding RNP cores (28). The exact RNP-binding site(s) on VSV M protein is not known, but studies with *ts* mutants and revertants, as well as studies using protease protection of M protein complexed with RNP cores, indicated that three-dimensional structures markedly influence the stability of endogenous M protein bound to RNP cores isolated from virions (10). Recent studies revealed that plasma membrane-associated intact M protein binds to RNP cores in a reconstitution assay, but truncated M protein missing the first 14 N-terminal amino acids does not bind RNP cores, suggesting that the RNP-binding site is located at the N terminus of M protein (8). In unpublished studies using M proteins expressed by the constructs described in this paper, it was found that RNP cores free of endogenous M protein were able to bind M proteins truncated to the first 74 amino acids (M1-74) and missing the first 50 amino acids (M51-229) but that they could bind these somewhat less efficiently than they could bind complete M protein (M1-229), indicating the possible existence of two sites on M protein capable of binding RNP cores (30).

Evidence that the M protein binds to membranes and lies in close proximity to the VSV virion envelope was obtained with protein-phospholipid cross-linking reagents (32). Similar cross-linking studies revealed that a membrane-binding site on the virion M protein was localized to its first 19 N-terminal amino acids (12). In vitro reconstitution studies revealed that soluble M protein binds to sonicated unilamellar phospholipid vesicles but that it does so far more efficiently if the vesicles contain negatively charged phospholipids; binding of M protein to vesicles with acidic phospholipids was markedly reduced by derivatization of lysine residues or by the presence of 0.5 M NaCl, indicating the occurrence of primarily electrostatic binding to the lysine-rich N terminus of the M protein (31). However, subsequent studies showed that removal of the first 43 N-terminal amino acids by trypsin resulted in a truncated M protein that retained vesicle-binding activity but at considerably reduced efficiency (21), suggesting the presence of two independent membrane-binding sites on the VSV M protein. About 90% of the plasmid-expressed M protein in transfected cells was distributed in cytosol, and 10% was tightly bound to plasma membrane, not dissociable except by detergents (7). Expression in transfected cells of a truncated M protein lacking the first 19 amino acids also migrated to plasma membrane, but this protein was less tightly bound than was intact M protein; removal of the carboxy-terminal 14 amino acids has no effect on membrane or nucleocapsid binding of this truncated M protein (8).

The studies reported here confirm the theory that intracellularly synthesized wt M protein migrates to and binds to plasma membrane of cells infected with wt VSV or transfected with a recombinant plasmid expressing only wt M protein. Transfecting deletion mutants revealed that expressed M proteins truncated to the first 74 amino acids or to amino acids 51 to 229 bind to plasma membrane as determined by indirect fluorescent-antibody staining or by cell fractionation. Most importantly, deletion of amino acids 1 to 50 and 75 to 106 results in an expressed M protein that remains in the cytosol and cannot be detected in plasma membrane either by fluorescent-antibody staining or by cell fractionation. These results collectively indicate the presence of two membrane-binding sites on wt M protein, one at the N terminus in the neighborhood of amino acids 1 to 50 and a second between amino acids 75 and 106. These in vivo studies were confirmed by in vitro experiments in which the entire M protein and its three deletion mutants were tested for their capacity to bind sonicated phospholipid vesicles. The double-deletion mutant (M51-74/107-229) showed only background binding to vesicles even in the absence of salt or in a 0.15 M NaCl buffer. The vesicle binding exhibited by the two single-deletion mutants (M1-74 and M51-229) was somewhat less than that exhibited by the complete M protein (M1-229) at physiological salt concentrations. The M protein truncated to the first 74 amino acids (M1-74) did not bind to vesicles beyond the background level in a reaction buffer containing 0.65 M NaCl, whereas the M protein deleted of the first 50 amino acids (M51-229) showed no significant decline in its capacity to bind vesicles at high salt concentrations. Moreover, after prebinding to vesicles in a low-salt environment, truncated protein M1-74 was readily dissociated from vesicles by exposure to 0.65 M NaCl, whereas truncated protein M51-229 remained stably associated with vesicles exposed to 0.65 M NaCl. These data appear to confirm the presence of two membrane-binding sites on VSV M protein. (i) The N-terminal site, rich in charged amino acids, appears to bind to cellular or vesicle membranes by electrostatic forces. (ii) The region on M protein spanning amino acids 75 to 106 is rich in hydrophobic amino acids and is stably bound to cellular or vesicle membranes in an integrative manner. Quite clearly, the loss of membrane-binding activity of the double-deletion mutant could be due to a conformational change and to aberrant folding of the M protein, folding which was more extensive than that which occurred in the case of each of the single deletions alone.

The M protein synthesized at the nonpermissive tempera-

ture in cells infected with the VSV mutant *ts*O23 was also not detectable, or barely detectable, in plasma membrane either by fluorescent-antibody staining or by cell fractionation. This mutant has amino acid substitutions at residues 21, 111, and 227 (18). On the basis of studies with spontaneous revertants, the major mutation resulting in markedly reduced assembly and yield of infectious progeny at the nonpermissive temperature is a leucine→phenylalanine substitution at amino acid 111 (18); supporting this conclusion was the finding that expression of mutant M protein with a Phe→Leu reversion at residue 111 complements growth of *ts*O23 at the nonpermissive temperature (14). Studies with similar M-gene mutants and revertants led Kaptur et al. (10) to conclude that the temperature-sensitive phenotype affected the RNP-binding affinity of M protein because of altered conformation. It seems entirely likely that the reduced binding to plasma membrane of the *ts*O23 mutant M protein in experiments reported here is also due to altered conformation.

Another property of the VSV M protein is its capacity to cause a cytopathic effect evidenced by cell rounding, probably due to dissolution of actin filaments, in cells infected with wt VSV or its mutant *ts*G33 at permissive temperatures (5). Another, probably unrelated, effect of M protein is the inhibition of cell-directed transcription of target genes (4) when the M protein is transported to the nucleus (16). Cells infected with the VSV M-gene mutant *ts*G33 at the restrictive temperature do not exhibit the cytopathic effect of cell rounding (5). We have obtained, as reported here, identical results with the VSV M-gene mutant *ts*O23, which causes cell rounding and actin filament dissolution at permissive temperatures but not at nonpermissive temperatures. It seems entirely likely that the cell-rounding effect also depends on correct conformation of the M protein only at permissive temperatures. We found that expression of wt M protein by pTM-OM79 also results in cell rounding and actin filament dissolution, whereas none of the three deletion mutant M proteins caused cell rounding or actin filament dissolution. It seems likely that altered conformation of *ts*O23 mutant M protein produced at the nonpermissive temperature or caused by deletion mutants compromises its capacity to produce this cytopathic effect in infected or transfected cells. It is tempting to speculate that the same conformation of wt, but not mutant, M protein is responsible for both the affinity for membrane binding and the cell-rounding cytopathic effect. Conceivably, intracellularly synthesized M protein in the correct conformation binds to the inner surface of the plasma membrane, resulting in detachment of actin filaments and subsequent loss of normal polygonal cell morphology.

The data presented here, and those presented previously by us and others, suggest a possible model testable by future experimentation. In cells infected with wt VSV, most of the M protein attaches to and tightly coils newly synthesized RNP cores, which eventually migrate and bind to virus-converted plasma membrane. The remainder of the M protein migrates independently and binds to plasma membrane by a route different from that of G protein (7). The M protein, alone or complexed with RNP cores, binds to the inner surface of the plasma membrane by electrostatic forces generated by the positively charged N terminus of the M protein and the negatively charged headgroup of phosphatidylserine (27). This electrostatic binding possibly results in a conformational change that exposes the hydrophobic domain of M protein in the region of amino acids 88 to 119, which then could partition into the lipid bilayer, forming a stable complex not dissociable by high-ionic-strength buffers. The stable M protein-plasma membrane complex conceivably detaches the ends of actin filaments, resulting in the loss of the characteristic polygonal shape and in the subsequent rounding of the cell. The final steps of envelopment of RNP-M protein cores by the G-protein-containing plasma membrane and budding of the progeny virions are beyond the scope of this paper.

ACKNOWLEDGMENT

This research was supported by Public Health Service grant R37 AI-11112 from the National Institute of Allergy and Infectious Diseases.

REFERENCES

1. **Barenholz, Y., N. J. Moore, and R. R. Wagner.** 1976. Enveloped viruses as model membrane systems: microviscosity of vesicular stomatitis virus and host cell membranes. Biochemistry **15:**3563–3570.
2. **Barge, A., Y. Gaudin, P. Coulon, and W. H. Ruigrok.** 1993. Vesicular stomatitis virus M protein may be inside the ribonucleocapsid coil. J. Virol. **67:**7246–7253.
3. **Bergmann, J. E., and P. J. Fusco.** 1988. The M protein of vesicular stomatitis virus associates specifically with the basolateral membranes of polarized epithelial cells independently of the G protein. J. Cell Biol. **107:**1707–1715.
4. **Black, B. L., and D. S. Lyles.** 1992. Vesicular stomatitis virus matrix protein inhibits host cell-directed transcription of target genes in vivo. J. Virol. **66:**4058–4064.
5. **Blondel, D., G. G. Harmisom, and M. Schubert.** 1990. Role of matrix protein in cytopathogenesis of vesicular stomatitis virus. J. Virol. **64:**1716–1725.
6. **Carroll, A. R., and R. R. Wagner.** 1979. Role of the membrane (M) protein in endogenous inhibition of in vitro transcription of vesicular stomatitis virus. J. Virol. **29:**134–142.
7. **Chong, L. D., and J. K. Rose.** 1993. Membrane association of functional vesicular stomatitis virus matrix protein in vivo. J. Virol. **67:**407–414.
8. **Chong, L. D., and J. K. Rose.** 1994. Interaction of normal and mutant vesicular stomatitis virus matrix proteins with the plasma membrane and nucleocapsids. J. Virol. **68:**441–447.
9. **Dubovi, E. J., and R. R. Wagner.** 1977. Spatial relationships of the proteins of vesicular stomatitis virus: induction of reversible oligomers by cleavable protein cross-linkers and oxidation. J. Virol. **22:**500–509.
10. **Kaptur, P. E., R. B. Rhodes, and D. S. Lyles.** 1991. Sequences of the vesicular stomatitis virus matrix protein involved in binding to nucleocapsids. J. Virol. **65:**1057–1065.
11. **Kyte, J., and R. F. Doolittle.** 1982. A simple method for displaying the hydropathic character of a protein. J. Mol. Biol. **157:**105–132.
12. **Lenard, J., and R. Vanderoef.** 1990. Localization of the membrane-associated region of vesicular stomatitis virus M protein at the N terminus, using the hydrophobic, photoreactive probe ^{125}I-TID. J. Virol. **64:**3486–3491.
13. **Li, Y., L. Luo, M. Schubert, R. R. Wagner, and C.-Y. Kang.** 1993. Viral liposomes released from insect cells infected with recombinant baculovirus expressing the M protein of vesicular stomatitis virus. J. Virol. **67:**4415–4420.
14. **Li, Y., L. Luo, and R. R. Wagner.** 1989. Transcription inhibition site on the M protein of vesicular stomatitis virus located by marker rescue of mutant *ts*O23(III) with M-gene expression vectors. J. Virol. **63:**2841–2843.
15. **Lyles, D. S., M. McKenzie, and W. Parce.** 1992. Subunit interactions of vesicular stomatitis virus envelope glycoprotein stabilized by binding to viral matrix protein. J. Virol. **66:**349–358.
16. **Lyles, D. S., L. Puddington, and B. J. McCreedy, Jr.** 1988. Vesicular stomatitis virus M protein in the nuclei of infected cells. J. Virol. **62:**4387–4392.
17. **Mancarella, D. A., and J. Lenard.** 1981. Interaction of wild-type and mutant M protein of vesicular stomatitis virus with viral nucleocapsid and envelope in intact virions. Evidence from $^{[125]}$I iodonaphthyl azide labeling and specific cross-linking. Biochemistry **20:**6872–6877.
18. **Morita, J., R. Vanderoef, and J. Lenard.** 1987. Phenotypic revertants of temperature-sensitive M protein mutants of vesicular

stomatitis virus: sequence analysis and functional characterization. J. Virol. **61:**256–263.

19. **Moss, B., O. Elroy-Stein, T. Mizukami, W. A. Alexander, and T. R. Fuerst.** 1990. New mammalian vector. Nature (London) **348:**91–92.
20. **Newcomb, W. W., and J. C. Brown.** 1981. Role of the vesicular stomatitis virus matrix protein in maintaining the viral nucleocapsid in the condensed form found in native virions. J. Virol. **39:** 295–299.
21. **Ogden, J. O., R. Pal, and R. R. Wagner.** 1986. Mapping regions of the matrix protein of vesicular stomatitis virus which bind to ribonucleocapsids, liposomes, and monoclonal antibodies. J. Virol. **58:**860–868.

21a. **Ono, K., M. E. Dubois-Dalcq, M. Schubert, and R. A. Lazzarini.** 1987. A mutated membrane protein of vesicular stomatitis virus has an abnormal distribution within the infected cell and causes defective budding. J. Virol. **61:**1332–1341.

22. **Pal, R., and R. R. Wagner.** 1987. Rhabdovirus membrane and maturation, p. 75–128. *In* R. R. Wagner (ed.), The rhabdoviruses. Plenum Publishing Corp., New York.
23. **Rose, J. K., and C. J. Gallione.** 1981. Nucleotide sequences of the mRNAs encoding the vesicular stomatitis virus G and M proteins determined from nucleotide sequences containing the complete coding regions. J. Virol. **39:**519–528.
24. **Shipley, J. B., R. Pal, and R. R. Wagner.** 1988. Antigenicity, function, and conformation of synthetic oligopeptide corresponding to amino-terminal sequences of wild-type and mutant matrix proteins of vesicular stomatitis virus. J. Virol. **62:**2569–2577.
25. **Thomas, D., W. W. Newcomb, J. C. Brown, J. S. Wall, J. E. Hainfeld, B. L. Trus, and A. D. Stevens.** 1985. Mass and molecular composition of vesicular stomatitis virus: a scanning transmission electron microscopy analysis. J. Virol. **54:**598–607.
26. **Wagner, R. R.** 1987. Rhabdovirus biology and infection: an overview, p. 9–74. *In* R. R. Wagner (ed.), The rhabdoviruses. Plenum Publishing Corp., New York.
27. **Wiener, J. R., R. Pal, Y. Barenholz, and R. R. Wagner.** 1985. Effect of the vesicular stomatitis virus matrix protein on the lateral organization of lipid bilayers containing phosphatidylglycerol: use of fluorescent phospholipid analogues. Biochemistry **24:**7651–7658.
28. **Wilson, T., and J. Lenard.** 1981. Interaction of wild-type and mutant M protein of vesicular stomatitis virus with nucleocapsids *in vitro*. Biochemistry **20:**1349–1354.
29. **Ye, Z., R. Pal, J. W. Fox, and R. R. Wagner.** 1987. Functional and antigenic domains of the matrix (M_1) protein of influenza A virus. J. Virol. **61:**239–246.
30. **Ye, Z., W. Sun, and R. R. Wagner.** Unpublished data.
31. **Zakowski, J. J., W. A. Petri, Jr., and R. R. Wagner.** 1981. Role of matrix protein in assembling the membrane of vesicular stomatitis virus: reconstitution of matrix protein with negatively charged phospholipid vesicles. Biochemistry **20:**3902–3907.
32. **Zakowski, J. J., and R. R. Wagner.** 1980. Localization of membrane-associated proteins in vesicular stomatitis virus by use of hydrophobic membrane probes and cross-linking reagents. J. Virol. **36:**93–102.