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Membrane Association of Functional Vesicular Stomatitis Virus Matrix Protein In Vivo

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The matrix (M) protein of vesicular stomatitis virus (VSV) is a major structural component of the virion which is generally believed to bridge between the membrane envelope and the ribonucleocapsid (RNP) core. To investigate the interaction of M protein with cellular membranes in the absence of other VSV proteins, we examined its distribution by subcellular fractionation after expression in HeLa cells. Approximately 90% of M protein, expressed without other viral proteins, was soluble, whereas the remaining 10% was tightly associated with membranes. A similar distribution in VSV-infected cells has been observed previously. Conditions known to release peripherally associated membrane proteins did not detach M protein from isolated membranes. Membrane-associated M protein was soluble in the detergent Triton X-114, whereas soluble M protein was not, suggesting a chemical or conformational difference between the two forms. Membranes containing associated M protein were able to bind RNP cores, whereas membranes lacking M protein were not. We suggest that this membrane-bound M fraction constitutes a functional subset of M protein molecules required for the attachment of RNP cores to membranes during normal virus budding.

The molecular mechanisms that govern the complex process by which enveloped viruses bud from the plasma membranes of infected cells are not well understood. Many enveloped viruses contain an internal matrix protein which is thought to lie between the viral membrane and nucleocapsid core. Vesicular stomatitis virus (VSV), the prototype rhabdovirus, is an enveloped, negative-strand RNA virus containing a single membrane-spanning glycoprotein (G), a nucleocapsid (N) protein tightly associated with the viral RNA, and two proteins, L and NS, which form the RNA-dependent RNA polymerase. In addition, virions contain a matrix (M) protein which is essential to the budding process (26, 40, 48). VSV has therefore been a useful model for studying the roles of M protein in virion morphogenesis.

VSV M protein is a 26-kDa nonglycosylated protein constituting a major fraction of the total viral protein (46). Its function as an inhibitor of viral transcription, as well as its ability to condense helical nucleocapsid cores into tight coils, has been well documented elsewhere (9, 11, 36, 37, 52). M protein has also been shown to assemble at the inner surface of the plasma membranes of infected cells (3, 31). Hence, by interacting with both internal and envelope components of the virus, M protein could initiate the process of VSV assembly. Although M protein is known to bind electrostatically to the nucleocapsid core (52), the nature of its interaction with the viral envelope remains unclear. An interaction between G and M at the plasma membrane has been suggested from cross-linking experiments with intact virions (16, 30, 35) and from the ability of purified M protein to enhance the association of detergent-solubilized G protein subunits (29). In addition, G protein mobility in the plasma membrane is reduced in the presence of M protein (41).

Other data also suggest that M protein can interact with lipid bilayers in the absence of G protein. Purified M protein can associate with artificial liposomes devoid of G protein (8, 39, 51, 53), and soluble M protein can also bind to the plasma membrane vesicles of uninfected cells (12). Since the rate of M association with plasma membrane is faster than that of G protein in infected cells (2, 13, 25), it has been suggested that M protein is the first VSV protein to modify the plasma membrane during the process of assembly (1). Indeed, an intimate association between M protein and the lipid bilayer has been implicated through labeling studies of M protein with various hydrophobic probes in intact virions (28, 30) and by the ability of M protein to cause the lateral reorganization of phospholipids in artificial liposomes (50).

Here, we have studied the membrane binding and function of M protein expressed in vivo in the absence of other VSV proteins. We report that a fraction of M protein is capable of associating with cellular membranes in the absence of other VSV proteins in vivo, and we characterize this stable membrane interaction. We also demonstrate that membrane-associated M protein can bind viral nucleocapsids in vitro. On the basis of these findings, we discuss a model of VSV assembly.

MATERIALS AND METHODS

Viruses and cell culture. Plaque-purified VSV of the Indiana serotype (San Juan strain) was used to infect baby hamster kidney (BHK-21) cell monolayers at a multiplicity of infection of 0.1. Virions were radioactively labeled at 2 h postinfection with 200 μCi of [^{35}S]methionine (New England Nuclear Corp., Boston, Mass.) per ml in methionine-free, serum-free Dulbecco's modified Eagle's medium (DMEM). After 6 h at 37°C, labeling medium was replaced with DMEM containing 5% fetal calf serum. After 24 h, virions were purified from the medium by differential, rate-zonal, and equilibrium centrifugations (16). Purified virions were stored at −70°C at a concentration of 1 mg/ml. The recombinant vaccinia virus vTF7-3 (18) was prepared as previously described (49).

Plasmid construction. The DNAs encoding the M, G, and N proteins of VSV (Indiana serotype, San Juan strain) were obtained from the plasmids pMZ10 (17a), pARG (49), and

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pJS223 (44), respectively, by digestion with *Xho*I. Fragments were cloned into the unique *Xho*I site of pBS-SK(+) (Stratagene, La Jolla, Calif.) to generate the plasmids pBSM, pBSG, and pBSN.

Expression, radiolabeling, and immunoprecipitation of proteins. HeLa cells (10^6 cells per 3.5-cm plate) were infected for 30 min at 37°C with vTF7-3 at a multiplicity of infection of 10 in 100 μl of DMEM. The inoculum was removed, and the cells were transfected with 5 μg of plasmid DNA per plate in 1 μl of DMEM with the cationic liposome reagent TransfectACE (Bethesda Research Laboratories, Gaithersburg, Md.). After 3 h at 37°C, the cells were supplemented with 5% fetal calf serum and the mixture was incubated for an additional hour. The medium was removed and replaced with 1 ml of methione-free DMEM containing 50 μCi of [^{35}S]methionine per ml for 1 h at 37°C. When indicated, labeling medium was then replaced with 2 ml of DMEM supplemented with 2.5 mM unlabeled methionine and 5% fetal calf serum for a 2-h chase period. Proteins were immunoprecipitated from cell lysates with anti-VSV serum as previously described (42). Labeled proteins were resolved by polyacrylamide gel electrophoresis (PAGE) on 10% polyacrylamide gels containing sodium dodecyl sulfate (SDS) (27) and visualized by fluorography (4).

Analysis of membrane-associated VSV proteins. (i) Transfection and metabolic labeling. Two plates of HeLa cells (2.5×10^6 cells per 6.0-cm plate) were transfected and radiolabeled as described above, with some modifications. Cells were transfected with 10 μg of plasmid DNA(s) per plate in 2 ml of DMEM. [^{35}S]methionine labeling was done in 2 ml of methionine-free medium followed by a 2-h chase period in 4 ml of medium supplemented with methionine. When more than one plasmid was cotransfected into cells, the amounts of DNA added were adjusted to give equal expression of the encoded proteins.

(ii) Sucrose flotation gradient centrifugation and immunoprecipitation. Transfected and radiolabeled cells (5×10^6 cells) were harvested and fractionated essentially as described by Bergmann and Fusco (3), with some modifications. Plates were first rinsed with phosphate-buffered saline (PBS) and then scraped into an ice-cold 10% (wt/wt) sucrose homogenization buffer containing 10 mM Tris-hydrochloride (Tris-HCl; pH 7.4), 1 mM EDTA, and 100 kallikrein units of aprotinin per ml. Cells were disrupted with 60 strokes of a Dounce homogenizer on ice. Nuclei and debris were removed from the cell lysate by centrifugation at 1,000 rpm for 4 min at 4°C. The resulting supernatant was made to 80% (wt/wt) sucrose, placed at the bottom of a Beckman SW41 centrifuge tube, and overlaid with 65% (5 ml) and 10% (2.5 ml) sucrose. The step gradient was then centrifuged to equilibrium at 35,000 rpm for 18 h at 4°C. Fractions were collected from the top, diluted with detergent solution, and immunoprecipitated with rabbit anti-VSV serum as previously described (42). Labeled proteins were analyzed by SDS-PAGE (10% polyacrylamide gel).

Preparation of cellular membranes. Total cellular membranes were prepared from the indicated numbers of transfected and radiolabeled HeLa cells by the sucrose flotation gradient method described above. The visible membrane band at the 65-10% sucrose interface was removed by side puncturing the centrifuge tube. Membranes were diluted to 10 ml with 10 mM Tris-HCl (pH 7.4) and pelleted by centrifugation at 35,000 rpm for 1 h at 4°C. The membrane pellet was resuspended in 100 μl of 10 mM Tris-HCl (pH 7.4) and kept on ice.

Preparation of cytosolic M protein. Cytosol containing radiolabeled M protein was prepared from transfected HeLa cells (5×10^6 cells) according to the method described by McCreedy et al. (32). Cells were rinsed with ice-cold PBS and Dounce homogenized in ice-cold hypotonic buffer containing 10 mM Tris-HCl (pH 7.5), 1.5 mM MgCl, 10 mM NaCl, and 100 kallikrein units of aprotinin per ml. The postnuclear supernatant was subjected to centrifugation at 110,000 × *g* for 30 min at 4°C to pellet membranes, and approximately 0.5 ml of cytosol was kept on ice.

Reconstitution of RNP cores with membranes. Binding studies were conducted according to the method described by Ogden et al. (39). Total cellular membranes from transfected HeLa cells (1.5×10^7 cells) were isolated by the sucrose flotation gradient method, except that the final membrane pellet was resuspended in 10 mM Tris-HCl (pH 7.5) containing 0.14 M NaCl. Ribonucleoprotein (RNP) cores were prepared from purified, radiolabeled VSV virions as described by Ogden et al. (39). RNP cores were resuspended in 10 mM Tris-HCl (pH 7.5)–0.14 M NaCl buffer at a volume corresponding to that of the original suspension and kept at 4°C. One unit of RNP cores represents the amount of RNP cores extracted from 1 μg of whole VSV virions as defined by Ogden et al. (39). One unit of purified RNP cores was resuspended with the indicated membranes for 1 h at 37°C, with intermittent shaking. The suspension was then subjected to buoyant-density analysis by centrifugation on a continuous 10 to 70% (wt/wt) sucrose gradient at 150,000 × *g* for 45 min at 4°C. Fractions were collected from the bottom, diluted with detergent solution, and immunoprecipitated with anti-VSV serum as described above.

RESULTS

M protein associates with cellular membranes in the absence of other VSV proteins. To obtain a high level of M protein expression in the absence of other VSV proteins, we used a transient expression system in which HeLa cells were first infected with a recombinant vaccinia virus encoding the bacteriophage T7 RNA polymerase (18). Cells were then transfected with a plasmid-encoding M protein under the control of the T7 promoter. When cell lysates were immunoprecipitated with anti-VSV serum and analyzed by SDS-PAGE, a 26-kDa protein corresponding to the VSV M protein was detected (Fig. 1, lane 1). The expressed protein comigrated with the M protein marker from solubilized VSV virions (Fig. 1, lane 3).

To examine the distribution of M protein in transfected HeLa cells, we employed a subcellular fractionation scheme previously used to demonstrate the membrane association of M protein in VSV-infected cells (3). Total-cell lysates were prepared by disrupting cells with a Dounce homogenizer. Samples were adjusted to 80% sucrose and then placed at the bottom of a sucrose gradient. Membranes were fractionated by equilibrium flotation during ultracentrifugation.

When the VSV transmembrane glycoprotein G or the cytosolic nucleocapsid N protein was expressed in HeLa cells and analyzed for membrane association by this fractionation scheme, all of the G protein was localized to the 65-10% sucrose interface near the top of the gradient, whereas all of the cytosolic N protein remained at the bottom of the gradient, demonstrating a clear separation of membranes from cytosolic material by this procedure (Fig. 2A). Under these conditions, approximately 10% of the total M protein expressed in HeLa cells colocalized with membranes at the 65-10% sucrose interface whereas the majority of M protein remained with cytosolic proteins at the gradient

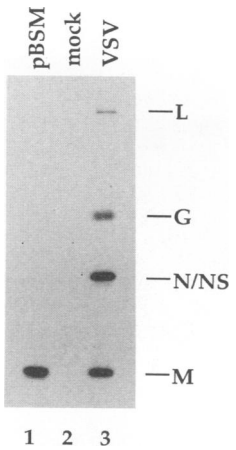

FIG. 1. Expression of matrix protein. HeLa cells were infected with a recombinant vaccinia virus (vTF7-3). After 30 min, the infected cells were transfected with 5 μg of pBSM DNA (lane 1) or left untransfected (lane 2). At 4 h postinfection, the cells were labeled for 1 h with 50 μCi of [^{35}S]methionine in 1 ml of methionine-free medium and lysed in a detergent solution, and cell lysates were immunoprecipitated with anti-VSV serum. Immunoprecipitated proteins were analyzed by SDS-PAGE followed by fluorography. VSV protein markers from solubilized virions are shown to the right (lane 3). Letters indicate the protein designations.

bottom (Fig. 2A). In addition, a 10% membrane association of M protein was also observed when cells were homogenized in the presence of *N*-ethylmaleimide, indicating that disulfide bond formation after lysis between the single cysteine residue of M protein and those of integral-membrane proteins was not responsible for membrane association. Although Bergmann and Fusco (3) reported that about 80% of total M protein associated with cellular membranes in VSV-infected MDCK cells, we rarely detected more than a 10% membrane association. Even in VSV (*ts*045)-infected MDCK cells, under the conditions described by Bergmann and Fusco, we observed a 10% membrane association of M protein. We have no explanation for this difference in results. However, our results are similar to those obtained by Knipe et al. (25) for VSV-infected CHO cells.

The subcellular distribution pattern of M protein remained unaltered when M protein was coexpressed with G protein (Fig. 2B) or when M protein was coexpressed with G protein and N protein (Fig. 2C). In these cases, separate plasmids encoding these proteins were transfected together into HeLa cells and coexpression was verified by immunofluorescence microscopy (data not shown). Because G protein expression at the plasma membrane did not enhance the membrane association of M protein (Fig. 2B and C), we conclude that binding of M protein to G protein must be a low-affinity interaction or might require other virion components. Autoradiographs from two experiments of cells expressing M, G, or N protein separately and from three experiments of cells expressing M, G, and N protein together were analyzed by scanning densitometry, and average values representing the percentages of total M, G, or N protein expressed were determined for each gradient fraction (Fig. 2D).

Stable interaction of M protein with cellular membranes. Membrane-associated proteins have been defined operationally as peripheral or integral on the basis of the conditions required to detach them from membranes. Peripheral proteins are characteristically removed by altered pH, by ionic strength, or after chelation of divalent cations. They are therefore thought to associate through electrostatic interactions with lipid headgroups or other membrane proteins (20, 20a). To assess the basis of M protein interaction with cellular membranes, we isolated membranes containing M protein and subjected them to three different conditions: high salt, EDTA, or high pH. High-salt extraction (2 M KCl) is expected to shield charges and weaken ionic interactions which bind peripheral proteins to membranes either directly or indirectly through other membrane proteins. Membrane association mediated by divalent cation bridge formation can be disrupted by the addition of EDTA. Vesicles can also be transformed into membrane sheets when treated with carbonate buffer (pH > 11), which consequently should release soluble or peripheral proteins trapped in vesicles (19). Membranes containing M protein were isolated from transfected HeLa cells; treated with 2 M KCl, 50 mM EDTA, or carbonate buffer (pH 11.0); and then subjected to sucrose flotation gradient analysis to determine whether any of these conditions dislodged M protein from membranes. All of these conditions failed to release M protein from the membrane fraction, as shown in Fig. 3. Even when membranes containing M protein were isolated, washed, and rehomogenized in the appropriate disrupting agent and then subjected to sucrose flotation gradient analysis in the presence of the same buffer, all of the M protein still remained membrane associated. This finding is consistent with the stable nature of membrane-associated M protein reported for VSV-infected cells (34).

To further characterize the M protein associated with membranes, we conducted Triton X-114 detergent extractions on membrane fractions containing M or G protein. This type of extraction is commonly used to characterize integral-membrane or lipid-anchored proteins by providing a convenient phase partitioning of hydrophobic from hydrophilic proteins (5). Membrane-associated G protein was found in the detergent phase, as expected for an integral-membrane protein, and, interestingly, most of the membrane-associated M protein partitioned into the detergent phase as well (data not shown).

To determine whether detergent partitioning was specific to membrane-associated M protein, we performed the experiment shown in Fig. 5. HeLa cells expressing M protein were fractionated by the sucrose flotation gradient procedure before phase separation with Triton X-114. Interestingly, soluble M protein at the bottom of the gradient partitioned into the aqueous phase, whereas M protein near the top of the gradient partitioned into the detergent phase. This result shows that membrane-associated M has chemical properties different from those of soluble M. We considered the possibility that the partitioning of M into detergent is the result of membranes present in the fraction. In the experiment shown, therefore, we added a membrane fraction to soluble M fractions before phase partitioning. This addition did not result in M partitioning into the detergent phase (Fig. 4, lanes 5 to 11).

Membrane-associated M protein binds RNP cores and cytosolic M protein. Studies have shown that M protein interacts with the nucleocapsid core and inhibits viral transcription (15). This inhibitory effect was found to be salt dependent, in agreement with previous reports of the electrostatic nature of M interaction with nucleocapsids (52). Also, it is known that M protein condenses extended nucleocapsids into compact structures in vitro similar to the virus-like skeletons seen when the viral envelope is removed with detergent (36). To determine whether membrane-bound

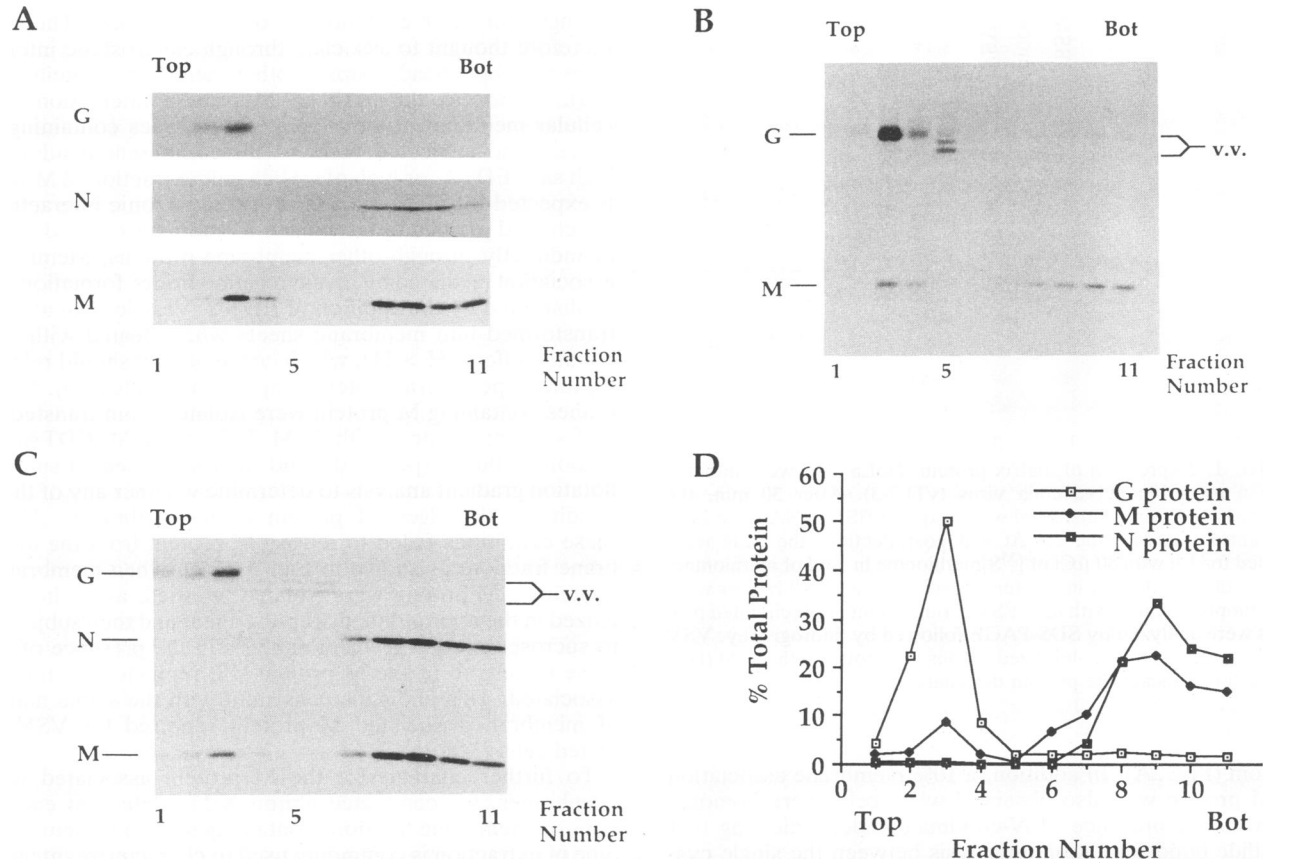

FIG. 2. Analysis of membrane-associated VSV proteins by sucrose flotation gradients. The assay was performed as described in Materials and Methods. HeLa cells were first infected with vTF7-3 for 30 min and then transfected with the appropriate plasmid DNA(s). Cells were pulse-labeled with 50 μCi of [^{35}S]methionine for 1 h and then chased in medium containing excess cold methionine for 2 h. Cells were then broken with a Dounce homogenizer in a sucrose homogenization buffer, made to 80% (wt/wt) sucrose, and overlaid with 65 and 10% sucrose layers. The step gradient was centrifuged at 35,000 rpm for 18 h at 4°C, and fractions were collected from the top. Fractions were diluted with detergent solution, immunoprecipitated with rabbit anti-VSV serum, and analyzed by SDS-PAGE. Shown are gradients from cells expressing G protein, N protein, or M protein individually (A), cells coexpressing G and M proteins (B), and cells coexpressing G, M, and N proteins (C). Fractions are numbered from the top (fraction 1) to the bottom (Bot; fraction 11). Vaccinia virus (v.v.) background proteins are bracketed. Quantitation of the amount of labeled protein in each fraction was carried out by scanning densitometry of autoradiographs. (D) Graph generated from data collected from two experiments with cells expressing G, M, or N protein separately and three experiments with cells coexpressing G, M, and N proteins.

M protein also bound nucleocapsids, we performed the following experiment. RNP cores stripped of M protein were prepared by detergent and salt extraction of radiolabeled virus (39). These cores contain L, N, and NS proteins and only trace amounts of M protein (data not shown). RNP cores were incubated with purified membranes containing either M or G protein or lacking any VSV proteins, and binding was assayed by colocalization of radiolabeled RNPs with membranes on sucrose density gradients. RNP cores associated with membranes containing M protein (Fig. 5A, lanes 7, 8, and 9) but not with membranes containing only G protein or lacking VSV proteins (Fig. 5B and C). Clearly, the presence of M protein on membranes facilitated the binding of RNP cores in vitro. Apparently, the residual M protein tightly associated with RNP cores could not facilitate membrane association (Fig. 5C).

To determine whether soluble M protein could bind to membranes, purified HeLa cell membranes were incubated with cytosol containing soluble, radiolabeled M protein (32). Samples were then subjected to sucrose flotation gradient analysis. Soluble M protein did not associate with membranes in vitro (Fig. 6B), supporting our earlier finding that membrane association was occurring prior to cell lysis. However, some soluble M protein did bind to membranes containing previously bound M protein (Fig. 6A). To distinguish a binding interaction from an exchange between soluble M protein and membrane-associated M protein, the converse experiment, in which radiolabeled, membrane-associated M protein was incubated with cytosol containing unlabeled M protein or unlabeled cytosol, was done. We did not detect any radiolabeled M protein at the bottom of the gradients, showing that the radiolabeled, membrane-associated M protein did not exchange with cytosolic M protein or any other cytosolic components (data not shown). It is therefore likely that an M protein-M protein interaction facilitated the binding of soluble M protein to membranes in Fig. 6A.

DISCUSSION

We have shown that a functional population of M protein can stably associate with cellular membranes in the absence

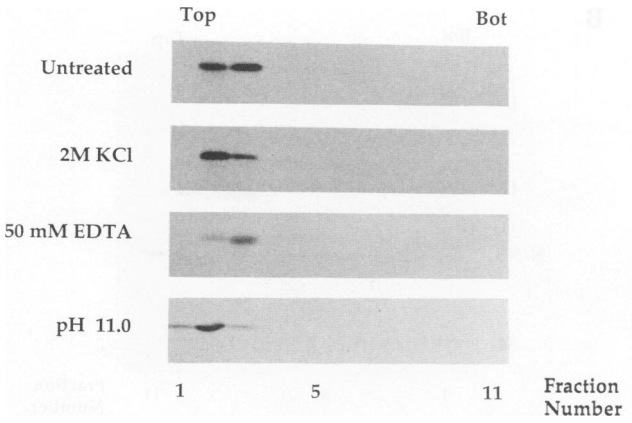

FIG. 3. Stability of M protein association with membranes. Total cellular membranes containing associated M protein from transfected and [^{35}S]methionine-labeled HeLa cells (1.5×10^7 cells) were prepared by the sucrose flotation method as described in Materials and Methods. Membranes were treated with either 2 M KCl–10 mM Tris-HCl (pH 7.4) or 50 mM EDTA–10 mM Tris-HCl (pH 7.4) for 1 h at 25°C or extracted with carbonate buffer (pH 11.0) for 30 min at 0°C (19) or left untreated. Samples were made to 80% (wt/wt) sucrose, and membranes were reisolated on a second sucrose flotation gradient. Fractions were collected, diluted with detergent solution, immunoprecipitated with rabbit anti-VSV serum, and analyzed by SDS-PAGE. Fractions are numbered from the top (fraction 1) to the bottom (Bot; fraction 11).

of other viral components in vivo and have demonstrated the nonperipheral nature of its membrane interaction. On the basis of its ability to interact with virus nucleocapsid cores, soluble M protein, and the lipid bilayer, we suggest that this subset of M protein is critical for orchestrating the assembly of VSV.

Although previous studies involving subcellular fractionation, stereoimaging, and immunofluorescence analysis of VSV-infected cells showed that M protein could associate with cellular membranes with or without concomitant association to nucleocapsids (2, 14, 25, 31, 38), the question of a requirement for G protein remained open. An attempt to address this issue with a temperature-sensitive G protein mutant demonstrated membrane association of M protein (3); however, these results are not entirely conclusive, since transmembrane fragments of G protein containing the cytoplasmic tail are known to exist at the plasma membrane and are capable of assembling into noninfectious particles at the nonpermissive temperature (10, 33). In our study, expression of VSV M protein in transfected HeLa cells was chosen to examine its membrane association in the absolute absence of all other VSV components. Our subcellular fractionation studies demonstrated that 10% of the total M protein expressed in cells associated with membranes in vivo.

It has been suggested that once M protein and G protein are localized to the plasma membrane in infected cells, a subsequent G-M interaction may induce the clustering of G protein and specific host cell glycoproteins into areas active in virus assembly (23). Although an interaction between M protein and solubilized G protein has been demonstrated in vitro (29), we did not observe enhancement of M protein binding to membranes containing G protein. This result suggests that the M-G binding is of low affinity.

In these studies, we have concentrated on characterizing M protein bound to membranes in vivo. We observed no binding in vitro of soluble M protein to membranes from mock-transfected cells, suggesting that the binding we ob-

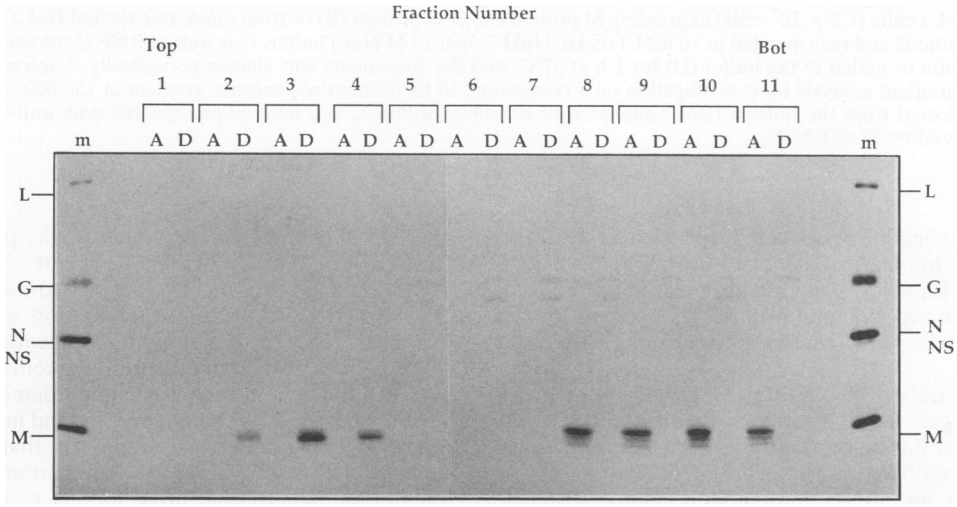

FIG. 4. Triton X-114 phase partition analysis of membrane-associated and soluble M protein. [^{35}S]methionine-labeled HeLa cells expressing M protein were lysed, and the lysates were subjected to sucrose flotation gradient fractionation as described in Materials and Methods. Cellular membranes were isolated from 5×10^6 mock-transfected and radiolabeled HeLa cells by the sucrose flotation gradient method and then added to each of the bottom seven fractions (fractions 5 to 11) of the M gradient. Fractions were then dialyzed in 10 mM Tris-HCl (pH 7.4), lyophilized, and extracted with a 1% Triton X-114–TN buffer (10 mM Tris-HCl [pH 7.4]–150 mM NaCl) at 0°C by using a modified version of the method described by Bordier (5). Aqueous (A) and detergent (D) phases of each fraction were separated by being warmed to 37°C and centrifugation through a 6% (wt/wt) sucrose cushion containing 0.06% Triton X-114–TN buffer at $300 \times g$ for 3 min at room temperature. The aqueous phase was removed from the detergent pellet and reextracted twice more with 1% Triton X-114–TN buffer. All of the aqueous and detergent phases were combined and adjusted to the same final volume and the same detergent and salt concentrations by the addition of Triton X-114 and TN buffer, respectively. Samples were immunoprecipitated by the addition of detergent solution and anti-VSV serum, and labeled proteins were resolved by SDS-PAGE. Fractions are numbered from the top (fraction 1) to the bottom (Bot; fraction 11). Labeled VSV protein markers from solubilized virions are shown to the left and right (m). Protein designations are at the sides.

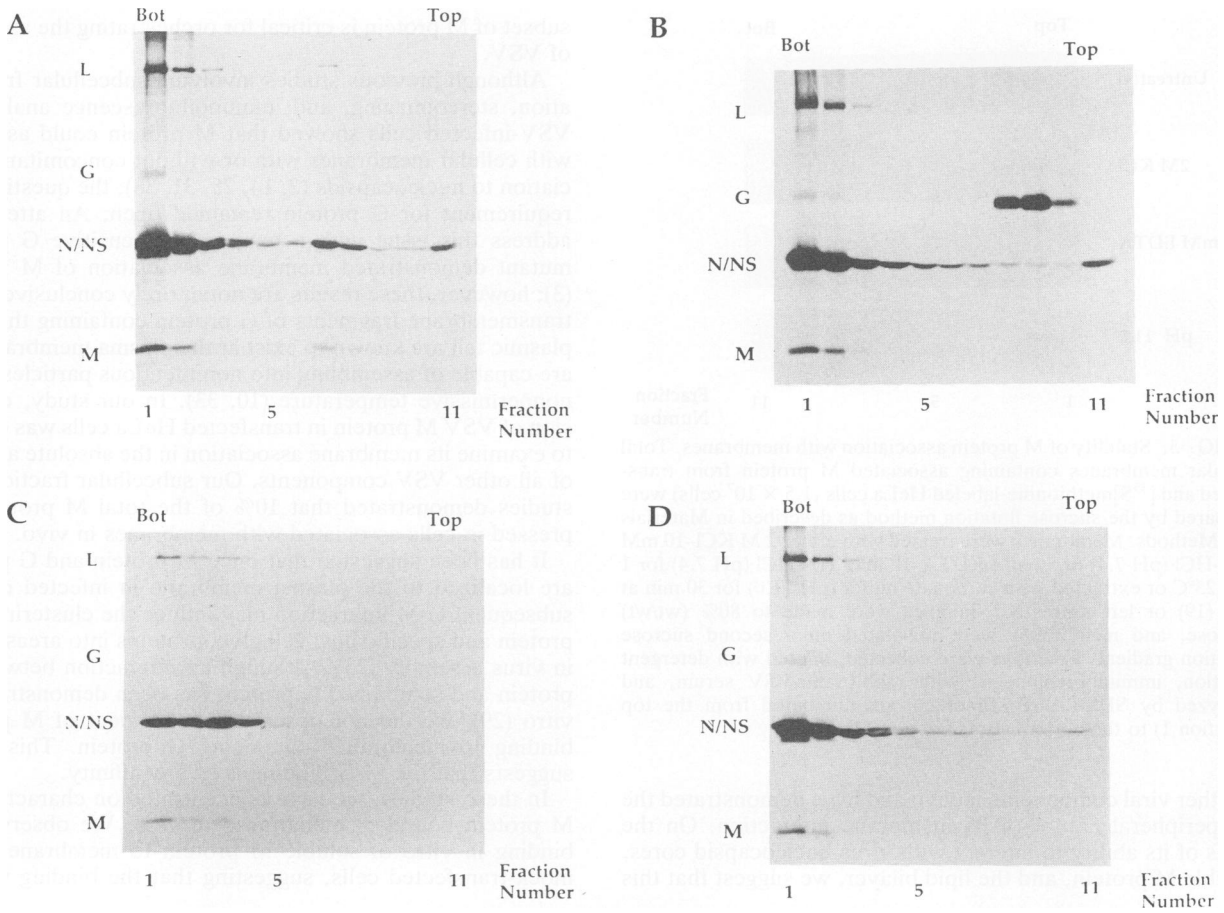

FIG. 5. Sucrose density gradient analysis of membrane-bound M protein and RNP interaction. Membranes were isolated from transfected and radiolabeled HeLa cells (1.5×10^7 cells) expressing M protein (A) or G protein (B) or from mock-transfected HeLa cells (C) as described in Materials and Methods and resuspended in 10 mM Tris-HCl (pH 7.5)–0.14 M NaCl buffer. One unit of RNP cores was incubated with each membrane preparation or added to the buffer (D) for 1 h at 37°C, and the suspension was shaken periodically. Each mixture was subjected to buoyant-density gradient analysis by centrifugation on a continuous 10 to 70% (wt/wt) sucrose gradient at 150,000 × *g* for 45 min at 0°C. Fractions were collected from the bottom (Bot), diluted with detergent solution, and immunoprecipitated with anti-VSV serum. Labeled proteins were resolved by SDS-PAGE.

served was occurring prior to cell lysis. Others have reported M protein binding to artificial phospholipid vesicles (39, 51, 53) or HeLa cell plasma membranes (12). It is likely that differences in experimental procedures account for our inability to see binding in vitro, but we have not pursued this in any detail.

Association of only 10% of total M protein with membranes in vivo suggested that interaction with lipid bilayers may actually occur during or shortly after protein synthesis. One attractive possibility is that during the folding of the polypeptide chain, an intermediate molten globule structure exposes a hydrophobic surface which might normally be hidden in the fully folded molecule (22, 45, 47). This hydrophobic region could then insert into the lipid bilayer. We are currently trying to address this model by setting up an in vitro synthesis and membrane association system for M protein.

Although the M protein structure has not been determined, analysis of the primary amino acid sequence of M does not reveal any stretches of hydrophobic residues indicative of a transmembrane domain. Computer models of M protein predict an internal core of alpha-helices and beta-pleated sheets, either of which might promote membrane association. In addition, we have not observed any palmitoylation of M protein (43), and the known sequence motifs for myristoylation and isoprenylation are absent from M protein. Hence, although lipid modifications of a fraction of M protein cannot be ruled out, they seem unlikely at present.

The stable membrane association demonstrated here suggests that part of M protein may extend into the lipid bilayer, rendering it resistant to conditions that characteristically remove peripherally associated membrane proteins. Several membrane-penetrating, cross-linking reagents have labeled M protein in intact virions, and there is evidence that the amino terminus of M protein is embedded in the viral envelope, probably as an amphipathic helix (28, 30, 54). Furthermore, the ability of M protein to cause the lateral reorganization of lipids is highly suggestive of a protein which is at least partially embedded in the bilayer (50). Such proteins are believed to introduce strain into the otherwise fluid lipid bilayer and induce lateral curvature, which can promote protein-protein interactions as well as cause membrane rigidity (20a).

Interestingly, the matrix proteins of Sendai virus and

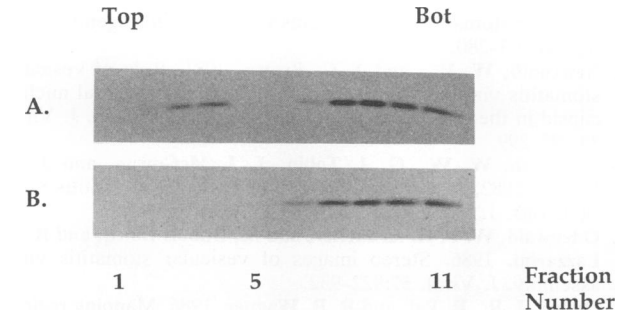

FIG. 6. Interaction of soluble M protein with membranes containing M protein. Membranes were prepared from transfected HeLa cells (1.5×10^7 cells) expressing M protein (A) or mock-transfected cells (B) by the sucrose flotation gradient method as described in Materials and Methods. Membranes were resuspended in 10 mM Tris-HCl (pH 7.5), and the suspension was incubated with 0.25 ml of cytosol containing soluble M protein from transfected and radiolabeled HeLa cells for 1 h at 37°C and was shaken periodically. Suspensions were then subjected to flotation on a second sucrose gradient, and fractions were collected from the top of the gradient, diluted with detergent solution, and immunoprecipitated with anti-VSV serum. Labeled proteins were resolved by SDS-PAGE. Fractions are numbered from the top (fraction 1) to the bottom (Bot; fraction 11).

Newcastle disease virus of the paramyxovirus family and the M1 protein of influenza virus of the orthomyxovirus family have also been shown to interact to artificial liposomes in a nonelectrostatic manner (6, 7, 17, 21). The identification of a domain(s) which is responsible for the membrane interaction of VSV M protein may provide a clearer understanding of the mechanism by which matrix proteins in general interact with the lipid bilayer.

The finding that membrane-associated M protein was functionally competent and essential to bind viral nucleocapsids to membranes in vitro is consistent with earlier reports which demonstrated that RNP cores bound more efficiently to phospholipid vesicles that were first reconstituted with M protein (39). It has been suggested that M protein may interact with RNP cores at the membrane prior to or at the time of budding (36, 38), and it is possible that the amount of membrane-associated M protein is limiting to the assembly process (24).

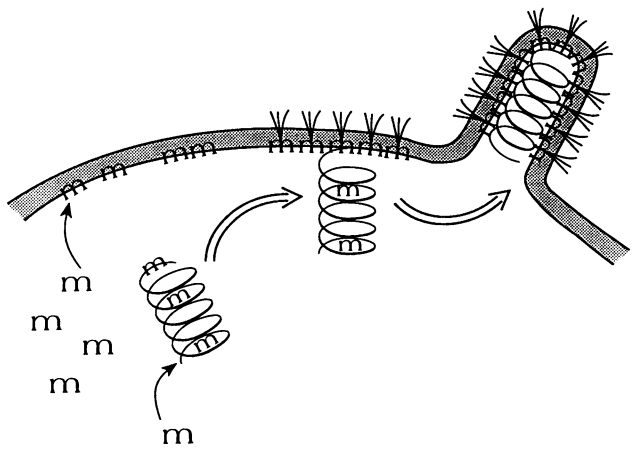

FIG. 7. Proposed model for the assembly of VSV.

In our studies, membranes containing associated M protein were also able to bind soluble M protein in vitro. Taken together, our data and data from previous studies are suggestive of the virus assembly model diagrammed in Fig. 7. We envisage that approximately 10% of newly synthesized M protein binds to the plasma membrane and that another fraction of M protein binds to and facilitates assembly (condensation) of nucleocapsids. Condensed nucleocapsids would then bind to regions of the membrane containing M (and presumably G) protein. In this model, M protein would exist in a cytosolic, nucleocapsid-associated, or membrane-associated state. Possibly, homotypic interactions between membrane-bound and nucleocapsid-bound M protein would facilitate the budding process at the host cell plasma membrane.

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