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Mapping Regions of the Matrix Protein of Vesicular Stomatitis Virus Which Bind to Ribonucleocapsids, Liposomes, and Monoclonal Antibodies

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The matrix (M) protein of vesicular stomatitis virus (VSV) appears to function as a bridge between the ribonucleocapsid (RNP) core and the envelope in assembly of the virion. Two such properties would necessitate at least one site for interaction with the nucleocapsid and one with the envelope. In this study M protein was found to mediate the in vitro binding to RNP cores of phospholipid vesicles, representing membrane structures. The M protein could bind initially to either the vesicles or the RNP cores to promote RNP-vesicle association. A trypsin-resistant fragment (M_T) of M protein, missing the initial 43 amino acids from its amino terminus, reconstituted with acidic phospholipid vesicles with the same binding efficiency as did whole M protein, suggesting that the carboxy-terminal 81% retained those regions of the M protein which interact with a lipid bilayer. The M_T protein, however, was considerably less efficient than intact M protein as an inhibitor of in vitro virus transcription; almost 2.5-fold more M_T protein than intact M protein was required for 50% inhibition of VSV transcription, indicating that a site for interaction with the RNP core may have been lost. A monoclonal antibody which is able to reverse the in vitro inhibition of transcription by M protein did not react by immunoblotting with M_T protein. Partial tryptic digests of the M protein probed with this monoclonal antibody indicated that epitope 1 lies between amino acid residues 18 and 43. This region appears to be a site that promotes interaction of the M protein with the RNP core of VSV. Monoclonal antibodies to epitopes 2 and 3, which exhibit some overlap in binding to M protein but do not reverse transcription inhibition, were mapped by cleavage with *N*-chlorosuccinimide at regions in a carboxy direction from epitope 1.

Vesicular stomatitis virus (VSV) is an enveloped, negative-strand RNA virus with five virus-coded structural proteins (51). The N, L, and NS proteins are tightly associated with the ribonucleoprotein (RNP) core (51), whereas the G protein is an integral membrane protein that comprises the glycoprotein spikes on the surface of the viral envelope (4, 48, 58). The matrix (M) protein is associated with both the envelope and the RNP core structure (58); it is, however, not exposed on the exterior surface of the virion, since it is refractory to protease digestion and lactoperoxidase iodination (4, 30, 31, 36, 48).

In vitro studies indicate that M protein reconstitutes as a peripheral membrane protein with vesicles containing acidic phospholipid components (54, 57). Studies with lipophilic monofunctional and bifunctional cross-linking reagents in intact virions demonstrate that M protein lies in close proximity to the inner surface of the virus envelope but penetrates little, if at all, into the membrane bilayer (11, 33, 43, 50, 52, 58). The highly positively charged (pI $\simeq$ 9.1) M protein (13) is capable of phase separating acidic phospholipids into regional domains in a bilayer to which it has bound (54a). This ability to recruit acidic phospholipids might explain the high level of phosphatidylserine in the VSV envelope as well as its asymmetric bilayer distribution; ~85% of virion membrane phosphatidylserine is found in the inner leaflet (32, 41). In addition to this close association with the envelope, M protein is electrostatically bound to the virion RNP core, causing it to be tightly coiled (37, 38).

After synthesis, the major structural proteins of VSV (N, G, and M) take independent paths to the site of virus assembly at the plasma membrane of the infected cell (1, 2, 6, 16, 22, 23, 34); synthesis of M protein is the rate-limiting step in this maturation process (53). Removal of M protein from the RNP core yields a relaxed, highly extended nucleocapsid structure, while condensation of the RNP into a tightly coiled skeleton is mediated through the association of M protein with the nucleocapsid (20, 37, 38). Appearance of M protein at the cell membrane causes decreased mobility of the already-inserted G protein (46). Protein cross-linking studies with intact virions revealed the formation of G-M and M-N but not G-N heterodimers (18). These studies suggest that M protein functions as a bridge between the cytoplasmic RNP structures and those regions of the cellular membrane into which the G protein has been inserted.

The strong interaction between M protein and the RNP core enables M protein to act as an endogenous inhibitor of viral transcription. Transcription reactions with detergent-disrupted whole virions demonstrate a virus-concentration-dependent regulatory effect which was attributed to an internal viral protein (5, 12, 44). Group III temperature-sensitive mutants with a lesion in the M protein did not exhibit this transcription-inhibitory activity (12, 14, 25). Reconstitution of purified wild-type M protein with RNP cores has been shown to inhibit in vitro viral transcription reactions at the level of RNA chain elongation (13, 15, 17, 39, 42, 55, 56).

These studies collectively suggest that M protein must have at least two sites of interaction: one with the envelope and one with the RNP of the virion. In this study we present data to show that M protein can mediate the binding of RNP structures to acidic phospholipid bilayers. We also present preliminary data to pinpoint a specific antigenic determinant

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as the possible site of interaction of M protein with the RNP core. Two other antigenic domains of the M protein map at different regions. Further study of the sites of interaction of the M protein with the viral RNP and envelope should be useful in elucidating the role of M protein in both the maturation and the transcriptional control of VSV.

MATERIALS AND METHODS

Cells and virus. 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 PFU per cell. The viral proteins were radioactively labeled by the addition of 1 μCi/ml of a ^{3}H-labeled amino acid mixture (New England Nuclear Corp., Boston, Mass.) to the infection medium. After incubation for 21 h at 37°C, virions were collected from the medium and purified to homogeneity by differential, rate zonal, and equilibrium centrifugations (18). Purified virions were stored at −70°C until used.

Preparation of ribonucleocapsid RNP and RNP-M cores. As previously described (39), ribonucleocapsid protein cores associated with M protein (RNP-M) were prepared by disrupting purified VSV virions in the absence of salt at a final virus concentration of 1 mg/ml in 10 mM Tricine (pH 7.5) containing 1% Triton X-100. Ribonucleocapsid protein cores from which most of the M protein has been removed (RNP) were prepared by disrupting the virions at a final virus concentration of 0.5 mg/ml in 10 mM Tricine (pH 8.0) containing 1% Triton X-100, 250 mM NaCl, and 0.2 mg of dithiothreitol per ml. The disrupted virions were centrifuged at 150,000 × *g* for 90 min over a 0.5-ml 50% glycerol pad. The pelleted RNP or RNP-M cores were then suspended in 10 mM Tricine (pH 7.5) and stored at 4°C until used (for not more than 2 days). The RNP cores for in vitro transcription reactions were prepared in the same manner.

Purification of VSV M protein. M protein was isolated from purified VSV virions disrupted in 10 mM Tricine (pH 8.0) containing 1% Triton X-100, 0.25 M NaCl, and 0.2 mg of dithiothreitol per ml and purified by column chromatography on Whatman P11 phosphocellulose exactly as described by Zakowski et al. (57). M protein purified in this manner is >98% pure. Protein concentrations were determined by the method of Lowry et al. (28) and by the specific radioactivity of the M protein labeled with ^{3}H-amino acid. The mole percentage of M protein was determined with a molecular weight value of 26,000.

Preparation of M_T protein. As described previously (40), purified M protein in 10 mM Tricine (pH 7.5) containing 0.6 M NaCl and 10% glycerol was treated with acetylated trypsin in the same buffer. The enzyme-to-protein ratio was 20 μg of trypsin per mg of M protein. The reaction was allowed to proceed at 37°C. Trypsin was removed by passing the reaction mixture through a soybean, trypsin inhibitor, Sepharose column. Small enzymatic products were removed by dialysis against 10 mM Tricine (pH 7.5) containing 0.6 M NaCl. The mole percentage of the trypsin-resistant fragment of M protein (M_T protein) was determined by assuming a molecular weight of 21,000.

Preparation of small SUV. 1,2-Dipalmitoyl-3-sn-phosphatidylcholine (DPPC), 1,2-dimyristoyl-3-sn-phosphatidylcholine (DMPC), and bovine brain phosphatidylserine (BPS) were obtained from Avanti Polar Lipids, Birmingham, Ala., and used without further purification. Stock lipid preparations were assayed before each experiment for total phospholipid phosphorous content by a modification of the Bartlett procedure (29). Small sonicated unilamellar vesicles (SUV) were prepared by the method of Barenholz et al. (3). Appropriate molar amounts of the lipids were mixed with trace amounts of [^{14}C]DPPC (New England Nuclear) and dried under a stream of N_2 with subsequent lyophilization for 18 h in the dark. The dried lipid mixture was then suspended in 4 ml of 10 mM Tricine (pH 7.5) containing 0.6 M NaCl, bubbled with N_2, and gently vortexed at 37°C to ensure that all lipid was suspended. The lipid solutions were sonicated with a Bronson tip sonicator under N_2 at 37°C in 3-min bursts until the solution became clear. Total sonication time never exeeded 9 min. The sonicated lipid suspensions were then centrifuged at 150,000 × *g* for 90 min at 37°C to pellet any multilamellar vesicles. The upper two-thirds of the supernatant fluid was carefully removed and used as the SUV source. Vesicles ranged in size from 30 to 50 nm in diameter (3).

Reconstitution of protein with preformed SUV. Reconstitution of purified M or M_T protein with preformed SUV was performed as previously described (45, 57). Protein was added to a known quantity of SUV to give the desired protein-to-lipid input molar ratio (mole percentage of protein). The mixture was then dialyzed extensively against 10 mM Tricine (pH 7.5) at 5°C above the melting temperature of the lipid mixture. Protein-vesicle reconstitutions performed in this manner give a single peak on sucrose density gradients with essentially all of the protein bound to lipid. The molar ratios of protein to lipid in these peak fractions were then used to calculate the output mole percentage of protein.

Reconstitution of RNP or RNP-M cores with preformed SUV. Purified RNP or RNP-M cores in 10 mM Tricine (pH 7.5) containing 0.14 M NaCl were mixed at appropriate ratios with preformed SUV in 10 mM Tricine (pH 7.5) containing 0.14 M NaCl. The reaction mixtures were placed in the dark at 37°C under N_2 for 6 h with intermittent shaking. The reconstituted suspensions were then subjected to buoyant density analysis in a continuous 0 to 66% (wt/wt) sucrose gradient centrifuged at 150,000 × *g* for 18 h. Densities were determined by refractometry. RNP and RNP-M cores were also reconstituted with phospholipid vesicles previously saturated with M protein.

NCS cleavage of the VSV M and M_T proteins. Proteins were cleaved with *N*-chlorosuccinimide (NCS) by the method of Brown and Prevec (7, 8). Purified M or M_T protein in 10 mM Tricine (pH 7.5) containing 0.5 M NaCl and 10% glycerol was precipitated at −70°C by the addition of 3 volumes of acetone. The precipitated protein was then suspended in 80 μl of 50% acetic acid. To this was added 20 μl of 75 mM *N*-chlorosuccinimide. The solution was mixed, and the reaction was allowed to incubate at room temperature for 2 to 4 h with intermittent shaking. The protein was then precipitated once again by the addition of 3 volumes of acetone and stored at −70°C. This precipitate was used as the protein source for analysis by polyacrylamide gel electrophoresis and Western blots.

In vitro VSV transcription reaction assay. In vitro virus transcription was assayed by a modification of the procedure of Carroll and Wagner (12, 13). Briefly, ~5 μg of purified RNP cores were added to a reaction mixture consisting of 1 mM dithiothreitol, 80 mM NaCl, 7.5 mM $MgCl_2$, 1 mM each of ATP, CTP, and GTP, and 0.1 mM UTP containing [α-^{32}P]UTP in 10 mM Tris (pH 8.0) and various amounts of M or M_T protein. After 3 h of incubation at 31°C, the reaction was stopped by the addition of sodium pyrophosphate, and the incorporated counts were determined by trichloroacetic acid precipitation and Cerenkov counting. A reaction mix-

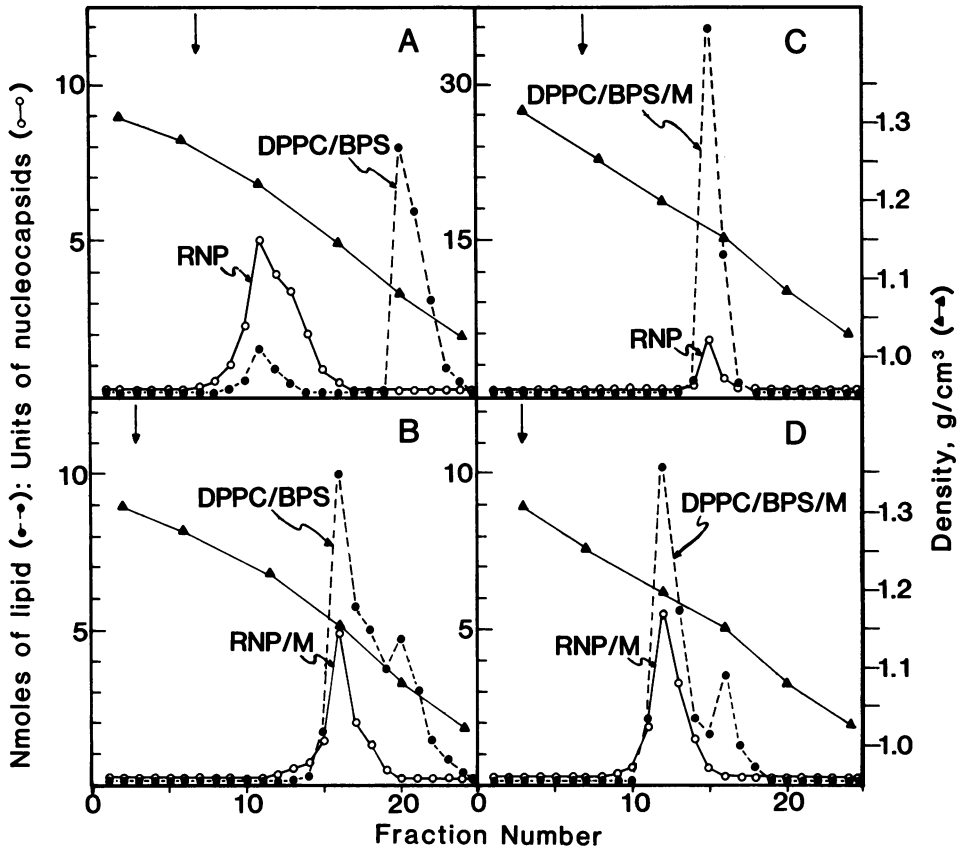

FIG. 1. Sucrose density gradient analysis of reaction mixtures of DPPC/BPS (1:1) SUV or DPPC/BPS/M SUV and VSV ribonucleocapsid protein structures with (RNP/M) or without (RNP) M protein. (A) DPPC/BPS SUV reconstituted with RNP; (B) DPPC/BPS SUV reconstituted with RNP/M; (C) DPPC/PBS SUV previously saturated with M protein (DPPC/BPS/M) reconstituted with RNP; (D) DPPC/BPS SUV previously saturated with M protein (DPPC/BPS/M) reconstituted with RNP-M. At 6 h after reconstitution each mixture was layered on top of a continuous 0 to 66% (wt/wt) sucrose gradient in 10 mM Tricine (pH 7.5) containing 0.14 M NaCl and centrifuged at 150,000 × *g* for 18 h at 4°C. Fractions were collected and analyzed for [^{14}C]DPPC or ^{3}H-protein or both. The specific activities of the reactants were used to calculate the nanomoles of lipid and units of ribonucleocapsids in each fraction. One unit of ribonucleocapsids represents the amount of RNP extracted from 1 μg of whole VSV virions. Densities of the gradient fractions were determined by refractometry. The arrows indicate where lipid-free RNP or RNP-M structures would band in the gradient.

ture without any added matrix protein was used as the uninhibited control.

Monoclonal antibodies to the VSV M protein. Monoclonal antibodies to the VSV (Indiana serotype) M protein were prepared as described elsewhere (40). Western blot analyses of the M and M_T proteins with monoclonal antibodies were performed as described elsewhere (39, 40). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was performed by a slight modification of the procedure of Laemmli and Favre (24).

RESULTS

Role of M protein in mediating the binding of phospholipid vesicles to ribonucleocapsid structures. We have developed an in vitro model system to study the M-protein requirement for the binding of VSV ribonucleocapsid core structures to membrane bilayers. RNP-M and RNP were allowed to bind SUV composed of neutral phospholipids or combined acidic and neutral phospholipids. Core structures were also allowed to react with negatively charged SUV previously saturated with M protein. The results of these experiments are shown in Fig. 1 and summarized in Table 1.

In a control experiment, purified RNP and RNP-M cores were mixed with SUV composed of 100 mol% of the neutral phospholipid DMPC. Density gradient analysis of the reaction mixtures indicated only a very low level of M-protein binding, accompanied by a slight density shift for the DMPC vesicles bound to nucleocapsid cores (Table 1). The presence of M protein on the nucleocapsid core had no significant effect on the level of binding to neutral phospholipid vesicles.

Mixed SUV consisting of 50 mol% DPPC and 50 mol% BPS, a negatively charged phospholipid, were then reconstituted with RNP and RNP-M cores. Figure 1A and B presents the sucrose density gradient profiles of these reaction mixtures. RNP structures bound the same low level of vesicles containing acidic phospholipid as they did vesicles containing only DPPC (Fig. 1A and Table 1). The RNP-M structures, on the other hand, were able to bind much greater amounts of negatively charged DPPC/BPS vesicles than neutral DPPC vesicles (Fig. 1B and Table 1). Clearly, the presence of M protein on the nucleocapsid core greatly increased its ability to bind BPS-containing acidic vesicles; a marked decrease in buoyant density accompanied this increased liposome binding. Removal of salt from the reconstitution mixture eliminated the low-level binding between RNP and acidic vesicles but had no effect on the RNP-M-binding capability (data not shown). This suggests that the

TABLE 1. Buoyant densities and phospholipid-binding capacities of VSV ribonucleocapsid cores reconstituted with liposomes of different composition[a]

Reconstituted sample	Density (g/cm³)	Lipid bound (pmol)/unit of nucleocapsid[b]
RNP alone	1.26	NA[c]
RNP-M alone	1.30	NA
RNP + DMPC SUV	1.17	417
RNP-M + DMPC SUV	1.23	307
RNP + DPPC/BPS SUV	1.22	313
RNP-M + DPPC/BPS SUV	1.15	1,988
RNP + DPPC/BPS/M SUV	1.16	6,203
RNP-M + DPPC/BPS/M SUV	1.19	1,892

[a] As described in Materials and Methods, ribonucleocapsid cores essentially devoid of M protein (RNP) or with a full complement of associated M protein (RNP-M) were mixed with preformed SUV containing DMPC alone or in 1:1 molar ratio of DPPC/BPS without or with saturating M protein (DPPC/BPS/M). The reconstituted suspensions were then subject to equilibrium centrifugation in a 0 to 66% sucrose gradient for measurement of buoyant density of vesicles labeled with [^{14}C]DPPC and M protein labeled with ^{3}H-amino acids.

[b] One unit of nucleocapsid is the amount released from 1 μg of whole VSV virion preparation.

[c] NA, Not applicable.

low level of binding between RNP and acidic phospholipid vesicles is nonspecific and can be easily overcome by altering the repulsion energy between negatively charged vesicles.

Purified RNP and RNP-M cores were next allowed to react with DPPC/BPS SUV previously reconstituted with M protein (DPPC/BPS/M) at a molar ratio of 1:100, ensuring surface saturation of the vesicles with M protein (54). Figure 1C and D illustrates the sucrose density gradient profiles of these reaction mixtures. The presence of M protein on the vesicles greatly enhanced the ability of RNP cores to bind acidic phospholipid vesicles (compare Fig. 1A and C). The RNP structures were able to bind three times as many DPPC/BPS/M vesicles (Fig. 1C) as the RNP-M structures (Fig. 1D and Table 1). This may be due to competition for BPS-binding sites already saturated with M protein or to the increased area for binding afforded by the extended structure of the RNP cores. Whether M protein-M protein interactions were involved in binding of RNP-M to DPPC/BPS/M vesicles could not be determined. As before, a large density change accompanied the binding of phospholipid vesicles to RNP and RNP-M core structures (Table 1).

M_T protein retains the ability to reconstitute with vesicles containing acidic phospholipid. The M protein reconstitutes as a peripheral membrane protein with vesicles containing acidic phospholipids (54, 57). Prolonged trypsinization of M protein yields a 21,000-dalton (21 K) M_T protein (35) missing 43 amino acids from the amino terminus (40). We wished to determine whether this M_T protein retained the ability to reconstitute with phospholipid vesicles.

In a control experiment, purified M and M_T proteins were reconstituted with DMPC SUV; neither protein showed significant binding to the neutral vesicles (data not shown). The M and M_T proteins were then allowed to bind to DPPC/BPS SUV. Figure 2A and B illustrates the sucrose density gradient analyses of the reconstitution mixtures. Reactants were mixed at a protein-to-lipid molar ratio of 1:200. In both cases, all of the protein and lipid were found in the same peak fractions. In control experiments, the unbound vesicles banded at fraction 20 and unbound protein pelleted. The binding of M or M_T proteins to DPPC/BPS SUV resulted in an identical increase in vesicle density. The

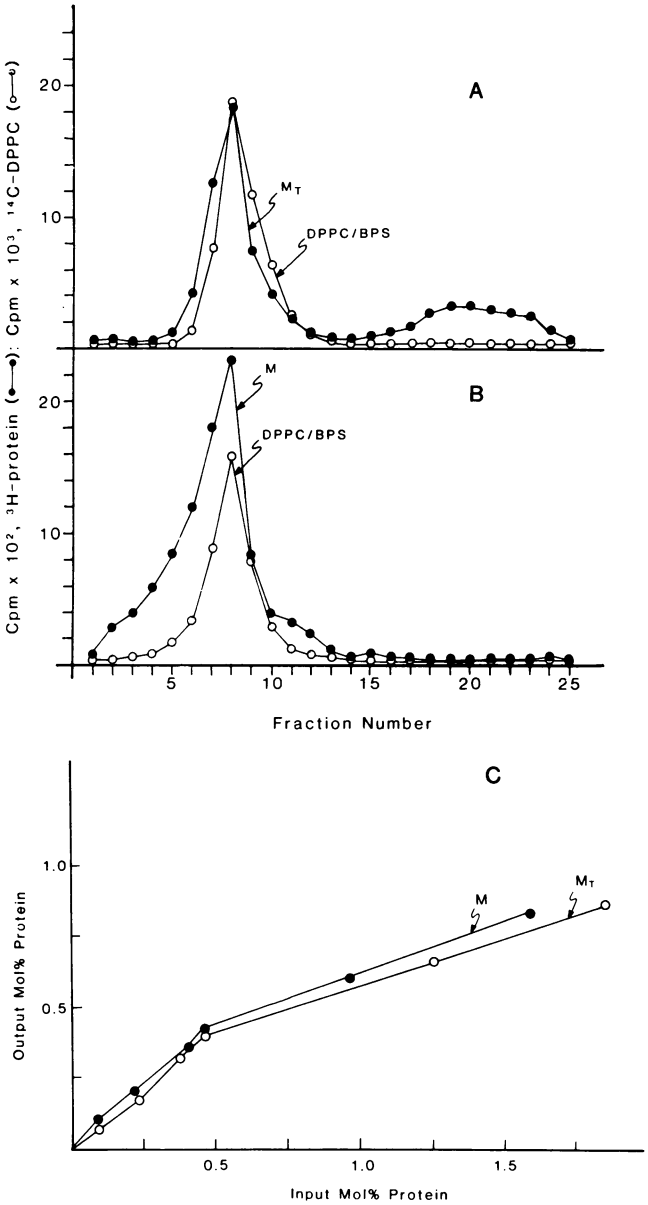

FIG. 2. Density gradient analysis and comparative binding efficiency of VSV M and M_T proteins reconstituted with small unilamellar acidic phospholipid vesicles. The proteins and vesicles were mixed in specific input molar ratios expressed as mole percentage of protein [(moles of protein)/(moles of lipid) × 100)]. After dialysis to remove salt and to allow reconstitution, the various reconstitution mixtures were layered on top of continuous 0 to 30% (wt/wt) sucrose gradients and centrifuged at 150,000 × *g* for 18 h at 4°C. Control protein-free vesicles banded at fraction 20, and unbound protein pelleted. Sucrose density gradient profiles are shown for the reconstitution of DPPC/BPS SUV with M_T protein (A) and M protein (B) at molar ratios of 200:1. (C) The comparative binding efficiency curves are shown for the reconstitution of the M protein and the M_T protein with preformed small DPPC/BPS SUV. The peak fractions containing both protein and lipid were analyzed for output mole percentage of protein. The mole percentage of protein was determined with 26,000 as the molecular weight for M protein and 21,000 as the molecular weight for M_T protein.

two proteins seemed to reconstitute similarly with vesicles containing acidic phospholipid.

Figure 2C illustrates a comparison of the binding efficienciesof the M and M_T proteins reconstituted with DPPC/BPS SUV. Reactants were mixed in increasing protein-to-lipid molar ratios, expressed as mole percentage of protein [(moles of protein)/(moles of lipid) × 100]. After buoyant density analysis, output mole percentage of protein was determined. The almost identical curves indicate that the input-to-output molar ratios for the two proteins are very similar, suggesting that the binding efficiencies of the M and M_T proteins for acidic phospholipid vesicles are the same.

Comparative transcription-inhibitory activity of M and M_T proteins. M protein is recognized as an endogenous virus transcription inhibitor that is able to depress in vitro virus transcription in a reconstituted system (13–15, 17). Moreover, it has been postulated that the lysine-rich N-terminal region of the basic M protein may be responsible for binding to the acidic nucleocapsid (47). Therefore, it was of interest to determine whether the M_T protein, missing the first 43 N-terminal amino acids, retained its transcriptional inhibitory ability. An in vitro VSV transcription system with RNP cores devoid of M protein was set up, and various amounts of M or M_T protein were added. The level of transcription was measured by the incorporation of [^{32}P]UMP into TCA-precipitable counts.

Figure 3 illustrates that intact purified M protein is a potent inhibitor of in vitro virus transcription; inhibition begins at low concentrations of M protein, and the degree increases rapidly, reaching almost 95% inhibition at 0.7 nmol

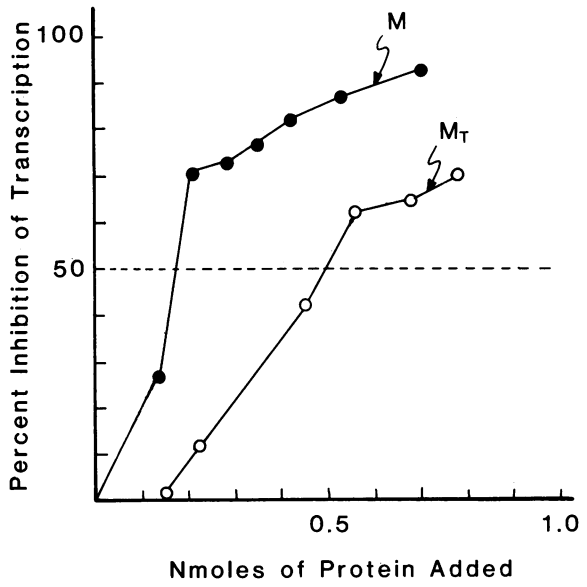

FIG. 3. Comparative inhibitory effects of M and M_T proteins on in vitro transcription by VSV (Indiana serotype) RNP cores. The inhibition was assayed by a modification of the procedure of Carroll and Wagner (13) as described in Materials and Methods. Briefly, ~5 μg of purified RNP cores was added to a reaction mixture consisting of 1 mM dithiothreitol, 80 mM NaCl, 7.5 mM $MgCl_2$, 1 mM each of ATP, CTP, and GTP, and 0.1 mM UTP containing [α-^{32}P]UTP in 10 mM Tris (pH 8.0) and various amounts of M or M_T protein. After 3 h of incubation at 31°C, the reaction was stopped by the addition of EDTA, and the incorporated counts were determined by trichloroacetic acid precipitation and Cerenkov counting. A reaction mixture without added matrix protein was used as the uninhibited control.

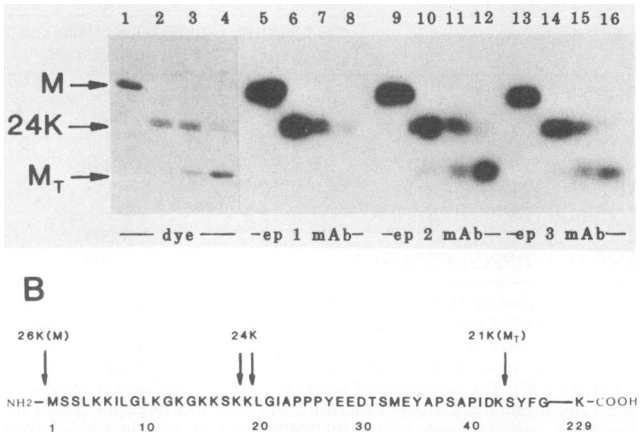

FIG. 4. Western blot of a progressive trypsin proteolysis of VSV (Indiana serotype) M protein (A) and schematic representation of the amino acid sequence of the amino-terminal end of the M protein with tryptic digest sites indicated by arrows (B). Purified M protein in 10 mM Tricine (pH 7.5) containing 10% glycerol and 0.6 M NaCl was added to acetylated trypsin (1 mg of M protein per 20 μg of trypsin) and incubated at 37°C. At various times, samples were taken as shown in the following lanes: 1, 5, 9, and 13, 0 min; 2, 6, 10, and 14, 15 min; 3, 7, 11, and 15, 45 min; 4, 8, 12, and 16, 120 min. After being subjected to 12.5% polyacrylamide gel electrophoresis, the separated proteins were then transferred to nitrocellulose paper by electrophoresis. Lanes 1 through 4 were not transferred to nitrocellulose, but rather the gel was stained directly with Coomassie blue. The nitrocellulose-bound proteins were flooded with purified monoclonal antibodies representing three epitopes: antibody to epitope 1 (lanes 5 to 8), antibody to epitope 2 (lanes 9 to 12), antibody to epitope 3 (lanes 13 to 16). After extensive washing, ^{125}I-labeled *Staphylococcus* protein A was allowed to react with the immobilized antibodies. The bands were visualized by autoradiography. Protein markers are shown at the left of panel A.

of M protein. Purified M_T protein is much less potent as an inhibitor of VSV transcription. Much more M_T protein must be added to initiate any inhibition, and more than 2.5 times as much M_T protein as M protein is required to achieve 50% inhibition. Even at high concentrations of M_T protein, the overall level of inhibition achieved is not as great as that with intact M protein.

Comparative binding of monoclonal antibodies to intact M protein and selectively degraded tryptic fragments. We had previously identified four epitopes on the M protein of VSV (Indiana serotype) and analyzed the competitive binding affinities of three representative monoclonal antibodies (40). A monoclonal antibody to epitope 1 (mAb2) was found to be capable of reversing the transcriptional inhibitory activity of the M protein, whereas monoclonal antibodies to epitopes 2 and 3 (mAb3 and mAb25, respectively) did not reverse the transcriptional inhibitory effect of M protein (39). mAb2 did react slightly with the M_T protein by enzyme-linked immunosorbent assay, but mAb3 and mAb25 reacted equally well with M and M_T proteins (40). With these data in mind, it was of interest to compare the antigenic determinants of M and M_T by immunoblotting with these monoclonal antibodies. Short exposure of M protein to trypsin also generates by partial cleavage a fragment of higher molecular weight than the M_T fragment (40); we also examined this partial cleavage fragment for its capacity to bind monoclonal antibodies and determined its N-terminal amino acid sequences.

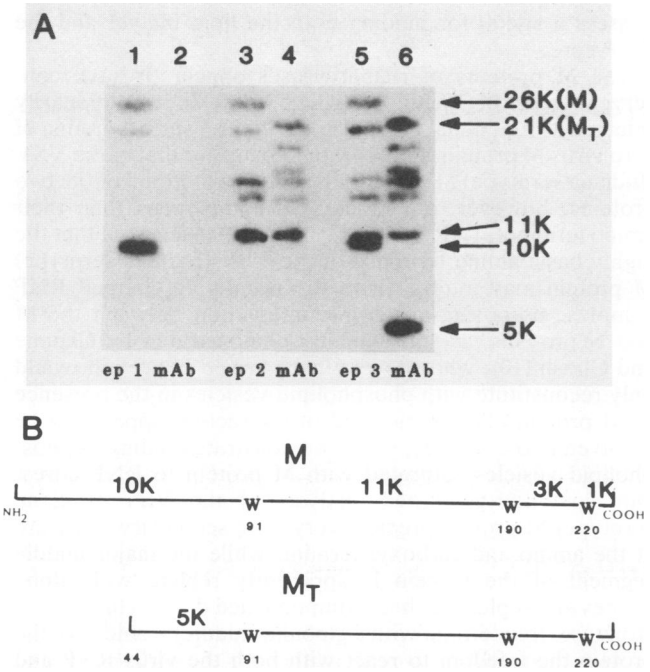

FIG. 5. Cleavage of M and M_T proteins by NCS. (A) Western blot autoradiogram of M and M_T proteins partially cleaved with NCS and reacted with monoclonal antibodies against the three recognized epitopes of the M protein. Purified M (lanes 1, 3, and 5) and M_T (lanes 2, 4, and 6) proteins were treated with NCS as described in Materials and Methods, separated in a 17% sodium dodecyl sulfate-polyacrylamide gel containing 7 M urea, transferred to nitrocellulose paper, flooded with antibody against one of the three epitopes of the protein (lanes 1 and 2 with antibody to epitope 1, lanes 3 and 4 with antibody to epitope 2, and lanes 5 and 6 with antibody to epitope 3), and visualized by autoradiography of bands which subsequently bound ^{125}I-labeled protein A. (B) Schematic representation of the M and M_T proteins, with tryptophan residues indicated by a W. Cleavage at these sites with NCS yields protein fragments ranging from 1,000 to 26,000 daltons and all possible combinations resulting from partial cleavages.

In these experiments, the M_T fragment was generated by treatment of purified M protein with acetylated trypsin at a ratio of 20 μg of enzyme per 1 mg of protein, as outlined in Materials and Methods. Virtually all the M protein was converted to M_T ($M_r \simeq 21{,}000$) after incubation with trypsin for 120 min at 37°C. Exposure of M protein ($M_r \simeq 26{,}000$) to trypsin for 15 or 45 min resulted in production of a cleavage product ($M_r \simeq 24{,}000$), intermediate between the M protein and the M_T product. The uncleaved and trypsin-cleaved M proteins were subjected to electrophoresis on a 12.5% sodium dodecyl sulfate-polyacrylamide gel. Proteins separated by electrophoresis were then transferred by electroblotting to nitrocellulose paper and then exposed to monoclonal antibodies directed against three different epitopes, the binding of which was visualized by reactivity with ^{125}I-labeled *Staphylococcus* protein A and autoradiography as previously described (39, 40). Comparable electrophoresed preparations were not transferred to nitrocellulose but were stained with Coomassie blue.

Figure 4 compares the electrophoretic profiles and monoclonal antibody Western blots of M protein and its partially and completely trypsin-cleaved products, as well as the amino acid sequences at the trypsin-sensitive amino terminus. As indicated by Coomassie blue staining in lanes 1 through 4, exposure of M protein to trypsin generated a 24K polypeptide in 15 min (lane 2), both 24K and 21K M_T products in 45 min, and only the M_T trypsin-resistant polypeptide in 120 min. When transferred to nitrocellulose, the intact 26K M protein and its 24K partial cleavage product and 21K M_T product all reacted strongly with the monoclonal antibody to epitope 2 (Figure 4A, lanes 9 through 12) and with the monoclonal antibody to epitope 3 (Fig. 4A, lanes 13 through 16). In sharp contrast, the monoclonal antibody to epitope 1 (Fig. 4A, lanes 5 through 8) reacted strongly with the intact M protein (lane 5) and the 24K partial cleavage product (lane 6) but not at all with the 21K M_T trypsin-resistant product (lanes 8 and 7). These data strongly suggest that removal by trypsin of the first 43 amino acids of M protein results in complete loss of epitope 1 but not epitopes 2 and 3. Moreover, partial trypsin cleavage resulting in a 24K oligopeptide conserves epitope 1. It is of considerable interest that monoclonal antibodies to epitope 1 also reverse VSV transcription inhibition by M protein.

Figure 4B illustrates the amino acid sequence of the amino-terminal end of the M protein with trypsinization sites indicated by arrows after amino acid residues 18, 19, and 43 (all lysines). The sequence of the M_T protein was determined previously and found to lack the first 43 amino acid residues (40). The sequence of the first eight amino acids from the N terminus of the intermediate trypsin-resistant fragment (24K fragment) was determined by Jay Fox of the University of Virginia Sequencing Center and is as follows: Lys-Leu-Gly-Ile-Ala-Pro-Pro-Pro. Comparison of this sequence with that derived from the cDNA clone of the M protein gene (47) indicated that the intermediate fragment had lost the first 18 amino acids from its amino terminus. A second fragment was also present in the preparation; this fragment had lost its first 19 amino acids, including the second lysine before the double arrow (Fig. 4B). This second fragment, however, made up <5% of the total protein. It seems likely that the region of the M protein between amino acids 18 and 43 contributes to epitope 1 and may be a possible site of interaction for M protein with the RNP core.

Use of NCS to map the antigenic determinants of M protein. The foregoing experiments revealed that the monoclonal antibody to epitope 1 binds to a region of the M protein between N-terminal amino acids 18 to 43, whereas monoclonal antibodies to epitopes 2 and 3 react at regions distal to amino acid 43. To locate somewhat more precisely the antigenic domains of the M protein, we took advantage of the technique described by Brown and Prevec (7, 8), who used partial cleavage by NCS to compare M proteins of various members of the genus *Vesiculovirus*. NCS cleaves proteins only at tryptophan residues, which are generally highly conserved evolutionarily. The M protein of VSV (Indiana serotype) contains only three tryptophans, at amino acid residues 91, 190, and 220 (47); owing to partial cleavage, NCS gives rise to more peptides than the four expected. Since the two N-terminal NCS peptides contain 90 and 99 amino acids and are not easily separable on polyacrylamide gels, we took advantage of the fact that the trypsin-resistant M_T protein provides a 5K peptide (46 amino acids in length) proximal to the first tryptophan residue. Thus, NCS cleavage of M and M_T enables us to map epitopes by Western blotting. NCS oligopeptides generated by the technique of Brown and Prevec (7, 8) outlined in Materials and Methods were separated by electrophoresis on a 17% sodium dodecyl sulfate-polyacrylamide gel containing 7 M urea, transferred to nitrocellulose paper, and exposed to each of the three monoclonal antibodies before binding ^{125}I-labeled protein A for visualization by autoradiography.

Figure 5 shows the autoradiographs of NCS-cleaved M and M_T proteins reacted with monoclonal antibodies to each of the three epitopes and labeled with ^{125}I-labeled protein A; lanes 1, 3, and 5 depict M protein cleaved by NCS, while lanes 2, 4, and 6 depict M_T protein cleaved by NCS. Lanes 1 and 2 were reacted with monoclonal antibody to epitope 1, lanes 3 and 4 were antibody to epitope 2, and lanes 5 and 6 were with antibody to epitope 3. Figure 5B is a schematic representation of the M and M_T proteins, showing positions of tryptophan residues. Cleavage at these sites by NCS yields protein fragments ranging in molecular weight from 1,000 to 25,000 as well as uncleaved 26K M protein and all possible intermediate combinations. No antibodies ever bound to fragments smaller than 5,000 daltons, and so this area of the autoradiogram is not shown in Figure 5A. Control proteins smaller than 5,000 daltons were transferred to nitrocellulose and stained to ensure that short polypeptides could be successfully transferred and trapped (results not shown).

Monoclonal antibody to epitope 1 reacted only with uncleaved 26K M protein, and the NCS-cleaved M protein fragments corresponded to molecular weights of ~10,000 and ~21,000 (Fig. 5A, lane 1). The fact that no binding of the monoclonal antibody to epitope 1 occurred with NCS-cleaved fragments of the M_T protein (lane 2) is another indication that epitope 1 lies somewhere between amino acid residues 18 and 43 (Fig. 4).

When only intact M protein was cleaved and blotted, it was very difficult to determine any binding differences between monoclonal antibodies to epitopes 2 and 3 (Fig. 5A, lanes 3 and 5). Only when the M_T cleaved protein was examined could epitope 2 be pinpointed (lane 4). Since the monoclonal antibody to epitope 2 consistently reacts with a band at ~11,000 daltons and with no smaller fragments, it can be deduced that epitope 2 must lie within the 11K fragment spanning amino acids 92 to 189. The number and location of the larger-molecular-weight bands in lanes 3 and 4 are consistent with this conclusion.

Epitope 3 is much more difficult to pinpoint. The 5K band in lane 6 that binds the monoclonal antibody to epitope 3 corresponds to the 47-amino acid remnant at the amino end of the M_T protein. This would indicate that epitope 3 resides between amino acid residues 44 and 91. However, monoclonal antibodies to epitope 3 also react with protein fragments which correspond to the 11K peptide containing epitope 2. Several possible explanations for this will be considered in the discussion.

DISCUSSION

It is clear from numerous studies that M protein is essential for assembly of VSV virions (26, 27, 49). Maturation of the condensed RNP-M complex at the infected cell membrane can take place in the absence of G protein, suggesting that interaction of M protein with the cell membrane is sufficient to promote envelopment and budding of the VSV virion. Reconstitution experiments presented here demonstrate that M protein can function as the bridge between virus nucleocapsid core structures and simulated membranes in the form of acidic phospholipid vesicles. In these reconstitution experiments, the M protein can bind initially to either RNP cores or the liposomes and still result in formation of a nucleocapsid-membrane complex. As in the case of isolated M protein reconstitution with vesicles (54, 57), the RNP-M core complex bound far more efficiently to vesicles containing an acidic phospholipid component. These studies illustrate, therefore, that the M protein must possess a site(s) for binding both the lipid bilayer and the RNP core.

The M proteins of rhabdoviruses appear to have conserved areas of homology which may be evolutionarily retained as functional binding sites (9). The spring viremia of carp virus M protein is ~28% homologous to that of the VSV (Indiana serotype) M protein; the carboxyl termini of the two proteins, however, are much less homologous than their amino termini (21). Rose and Gallione (47) suggested that the highly basic amino terminus of the VSV (Indiana serotype) M protein may interact with the negatively charged RNP complex, proposing a possible interaction between the M and N proteins, as subsequently demonstrated by Capone and Ghosh (10), who showed that purified N protein would only reconstitute with phospholipid vesicles in the presence of M protein. This same kind of interaction appears to be involved in our studies, which demonstrate binding of phospholipid vesicles saturated with M protein to RNP cores. Our computer-generated analysis of the VSV (Indiana serotype) M protein predicts very little secondary structure at the amino and carboxyl termini, while the major middle segment of the protein is apparently replete with alpha helices and β-pleated sheets (unpublished data). This kind of structure (free termini with a globular middle) would give the protein the freedom to react with both the virus RNP and envelope. In this communication we have at least identified a region at the amino terminus of the M protein which interacts with the RNP core.

Prolonged trypsinization of M protein yields a 21K fragment (M_T) missing the first 43 amino acids from its amino terminus. This protein can be purified to ~95% homogeneity and was compared with intact M protein to investigate the functions of its amino terminus. The M_T protein does not inhibit viral transcription nearly as well as whole M. If sufficient M_T protein is added, however, a 70% inhibition of transcription can be achieved. This may indicate that the amino terminus may not be the only possible site for interaction of M protein with the RNP core. It was not possible to quantitate the comparative RNP-binding activities of M and M_T proteins because they are insoluble at the salt concentrations required for reconstitution experiments (data not shown). Monoclonal antibodies to epitope 1 are able to reverse the in vitro transcriptional inhibitory activity of the whole M protein (41). This M_T protein has lost its capacity to react by immunoblotting with antibodies to epitope 1, even though a low reading with the enzyme-linked immunosorbent assay occurs (40), in all likelihood owing to contamination with uncleaved protein. Generation by progressive proteolysis of 24K and 21K fragments of M protein demonstrated that this epitope lies somewhere between amino acid residues 18 and 43.

Comparative nucleotide sequences of M genes derived from several group III temperature-sensitive mutants of VSV (19) provide important data that bear on these findings. Of particular interest is mutant *ts*023, the M protein of which has lost its ability to inhibit in vitro transcription of VSV (39). A single-point mutation at position 21, changing a glycine residue to a glutamate, is responsible for this phenotypic alteration (19). The *ts*023 M protein was also found to have lost epitope 1 and will not bind monoclonal antibody to this epitope (39). Other group III temperature-sensitive mutants have also lost much of their ability to inhibit VSV transcription but have retained epitope 1 (39); two of these mutants have amino acid changes at the extreme carboxyl terminus of the M protein (19). These data seem to imply that mutations extending over 85% of the coding region of the

M-protein gene can lead to drastic alterations in secondary structure that affect the transcriptional inhibitory activity (19) and, to various degrees, the accessibility to monoclonal antibody of epitope 1 (39).

Epitopes 2 and 3 have been identified by immunoblotting NCS-cleaved fragments of the M and M_T proteins. Epitope 2 was located in an 11K stretch of amino acids from residue 92 to 189. This portion of the protein contains many predicted secondary structures, both alpha helices and β-pleated sheets (unpublished computer analysis). Epitope 3 is more difficult to pinpoint. The monoclonal antibody to epitope 3 binds both to the 11K fragment, containing epitope 2, and to the 5K fragment between amino acid residues 44 and 90. Epitope 3 may either be made up of two linearly noncontiguous portions of the protein which are brought into close proximity by the secondary structure of the protein or may straddle the NCS tryptophan cleavage site at amino acid 90. Monoclonal antibodies to epitopes 2 and 3 showed considerable overlap by competition binding assays, which also indicate their proximity to one another (40).

We plan to prepare synthetic peptides homologous to various regions of M protein from amino acid residues 18 to 43 to locate more precisely the amino acid sequences that bind the monoclonal antibody to epitope 1 and that inhibit VSV transcription. Elucidation of the regions of the protein which interact with the RNP should aid in understanding the roles of M proteins in virus assembly and transcription control. More evidence is also required to locate the region of the M protein responsible for binding to membrane components preparatory to budding of the virus.

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