Role of Matrix Protein in Assembling the Membrane of Vesicular Stomatitis Virus: Reconstitution of Matrix Protein with Negatively Charged Phospholipid Vesicles†

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ABSTRACT: The matrix (M) protein of vesicular stomatitis virus (VSV) was reconstituted into phospholipid vesicles by detergent dialysis. Reconstitution of the positively charged M protein occurred only in the presence of negatively charged phospholipids such as phosphatidylserine, phosphatidic acid, or phosphatidylinositol. Preformed vesicles containing negatively charged phospholipids also bound free M protein. Derivatization of the positively charged lysines in M protein with acetic anhydride or succinic anhydride prevented M protein reconstitution but did not affect the biological property of M protein to inhibit in vitro VSV transcription. An additional indication of the electrostatic nature of the M protein binding to the vesicles was that M protein could not be reconstituted in the presence of 0.5 M NaCl. Nonelectrostatic forces also appear to be involved in the association of the M protein with vesicles, since previously reconstituted M protein remained associated with the vesicles upon subsequent exposure to 0.5 M NaCl.

Vesicular stomatitis virus (VSV)[1] is an RNA-containing rhabdovirus which is enveloped by a membrane derived from the plasma membrane of the infected host cell (Wagner, 1975). The virion membrane contains only two proteins, both of which are coded by the viral genome, the glycoprotein (G) and the matrix (M) protein (Patzer et al., 1979). The G protein forms the spikes seen on the viral surface by negative-stain electron microscopy, has a molecular weight of ~69 000 (Wagner, 1975), and is anchored in the viral membrane by a protease-resistant tail fragment (Mudd, 1974; Schloemer & Wagner, 1975). The nucleotide sequence of a cDNA clone representing the 3′ end of G mRNA reveals a region of the G protein 29 amino acids from the carboxy terminus that contains 20 consecutive hydrophobic amino acids, by which the G protein presumably spans the viral membrane (Rose et al., 1980).

The viral M protein, which is the most abundant protein in the VS virion (Wagner, 1975), is not exposed on the exterior surface of the VSV membrane as demonstrated by its resistance to both protease digestion (Mudd, 1974; Schloemer & Wagner, 1975) and lactoperoxidase labeling with ^{125}I of intact virus (McSharry, 1977). The proximity of the M protein to the inner monolayer of VSV has been determined by the use of monofunctional lipophilic photoactivatible probes and bifunctional cross-linking reagents. Photoactivation of 16-azido[9,10-$^{3}H_2$]palmitate metabolically incorporated into the viral phospholipids, followed by polyacrylamide gel electrophoresis, demonstrated labeling of only the G protein (Stoffel et al., 1978) with the M protein not penetrating far enough into the membrane to be labeled with this probe. The M protein lies in close enough proximity to the inner monolayer of the VSV membrane to be cross-linked to phosphatidylethanolamine (PE) with the free amino cross-linking reagent dimethylsuberimidate (Pepinsky & Vogt, 1979). Recently, the M and G proteins have been cross-linked to viral phospholipids with the photoactivatible nitrenes tartryl diazide and dithiobis(phenyl azide), confirming the close proximity of the M protein to the lipid envelope (Zakowski & Wagner, 1980). The interaction of the M protein with the membrane is at least partly hydrophobic, since the hydrophobic aryl azide probes 5-[^{125}I]iodonaphthyl 1-azide and [^{3}H]pyrenesulfonyl azide labeled the M protein of intact virus (Zakowski & Wagner, 1980). We present here evidence for the reconstitution of the M protein into phospholipid vesicles and demonstrate that net negatively charged phospholipids are required for the M protein to associate with the reconstituted vesicle membrane and that the lysine residues of the M protein are responsible for the initial attraction of M protein to lipid.

Experimental Procedures

Materials. Egg PC and egg PE were purchased from Makor Chemicals, Ltd., Jerusalem, Israel; thermolysin (EC 3.4.24.4) and brain PS were from Sigma Chemical Co., St. Louis, MO; PA and PI were from Avanti Polar-Lipids, Inc., Birmingham, AL; β-D-octyl glucoside was obtained from Calbiochem, La Jolla, CA. Acetic anhydride and succinic anhydride were purchased from Aldrich Chemical Co., Milwaukee, WI. A ^{3}H-labeled L-amino acid mixture was purchased from New England Nuclear Corp., Boston, MA; [^{14}C]PC (58 mCi/mmol) and [α-^{32}P]UTP (410 Ci/mmol) were obtained from Amersham Corp., Arlington Heights, IL.

Purification and Derivatization of ^{3}H-Labeled VSV M Protein. VSV Indiana serotype was grown on BHK-21 cells at 31 °C in the presence of a 5 μCi/mL ^{3}H-labeled amino acid mixture and purified from the medium according to the procedure of Barenholz et al. (1976). Protein concentration was estimated by the procedure of Lowry et al. (1951).

Purification of ^{3}H-labeled M protein was based on the procedure of Carroll & Wagner (1979). In brief, VSV was suspended to a final concentration of 0.5 mg/mL in 10 mM Tris (pH 8.0) containing 1% Triton X-100, 0.25 M NaCl, and

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[1] Abbreviations used: VSV, vesicular stomatitis virus; PE, phosphatidylethanolamine; PC, phosphatidylcholine; PS, phosphatidylserine; PA, phosphatidic acid; PI, phosphatidylinositol; RNA, ribonucleic acid; cDNA complementary deoxyribonucleic acid; mRNA, messenger RNA; Tris, tris(hydroxymethyl)aminomethane; Hepes, 4-(2-hydroxymethyl)-1-piperazineethanesulfonic acid; PPO, 2,5-diphenyloxazole; $NaDodSO_4$, sodium dodecyl sulfate.

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0.2 mg/mL dithiothreitol. After 30 min at room temperature, the sample was centrifuged in a Beckman SW50.1 rotor at 200000*g* for 2 h, and the supernatant, containing mostly the G and M proteins, was removed. An equal volume of 10% glycerol and 10 mM Tris (pH 7.5) was added to this supernatant, the pH was adjusted, and the sample was then applied onto a 6-mL column of Whatman P11 phosphocellulose which had been equilibrated in 0.1 M NaCl, 10% glycerol, and 10 mM Tris (pH 7.5). After the sample was applied, the column was washed with the column equilibration buffer and the ^{3}H-labeled M protein then eluted with 0.65 M NaCl, 10% glycerol, and 10 mM Tris (pH 7.5). This procedure yields ^{3}H-labeled M protein which is >98% pure as determined by the polyacrylamide gel electrophoresis system used by Zakowski & Wagner (1980).

Derivatization of ^{3}H-labeled M protein with acetic anhydride was adapted from the method of Riordan & Vallee (1972), and derivatization with succinic anhydride was adapted from the method of Klapper & Klotz (1972). ^{3}H-Labeled M protein (0.5 mg) in 0.65 M NaCl, 10% glycerol, and 10 mM Tris (pH 7.5) was stirred at 0 °C in a fume hood, and either 1.6 μL of acetic anhydride or 1.7 mg of succinic anhydride was added. The pH was maintained at 7.5–8.0 for 30 min, and the sample was then dialyzed overnight against 1 L of 0.1 M NaCl, 10% glycerol, and 10 mM sodium phosphate (pH 7.5) at 4 °C. The extent of derivatization was determined by assay for primary amino groups by the fluorescamine method of Udenfriend et al. (1972).

Reconstitution of ^{3}H-Labeled M Protein into Vesicles. Reconstitution of purified ^{3}H-labeled M protein into phospholipid vesicles was performed by the procedure of Petri & Wagner (1979). To mixtures of 100 μg of phospholipid in chloroform containing 0.025 μCi of [^{14}C]PC was added sufficient β-D-octyl glucoside in chloroform so that the final concentration of octyl glucoside would be 30 mM after ^{3}H-labeled M protein addition. The chloroform was taken to dryness under N_2 and the phospholipid–octyl glucoside mixture resuspended in 0.6 M NaCl, 10% glycerol, and 10 mM Tris (pH 7.5) containing 50 μg of ^{3}H-labeled M protein. The sample was then dialyzed against 1 L of 10 mM Tris (pH 7.5) which had previously had N_2 bubbled through it to remove O_2. Dialysis was continued for 24 h at 4 °C with one change of buffer. The sample was then mixed with an equal volume of 65% sucrose, 0.15 M NaCl, and 10 mM Tris (pH 7.5) and overlaid with a gradient of 0–30% sucrose in 0.15 M NaCl and 10 mM Tris (pH 7.5) before centrifugation in a Beckman SW50.1 rotor at 200000*g* for 16 h. Gradients were then fractionated, and 50-μL aliquots were assayed for ^{3}H and ^{14}C radioactivity by mixing with 10 mL of Ready-Solv EP and counting in a Beckman LS-230 liquid scintillation counter. Densities of fractionated gradients were determined by refractometry.

Vesicles reconstituted with ^{3}H-labeled M protein were dialyzed to remove the sucrose after flotation centrifugation and then digested with the protease thermolysin at a concentration of 75 units/mg of M protein at 37 °C for 1 h. Samples were then lyophilized and electrophoresed on 17.5% polyacrylamide gels by the procedure of Petri & Wagner (1979). After Coomassie Blue staining, the bands of ^{3}H-labeled M protein were excised and added to 1 mL of Nuclear Chicago Solubilizer and 10 mL of PPO–toluene, and radioactivity was determined.

Association of ^{3}H-labeled M protein with preformed vesicles was determined by preparing 50% PC–50% PS vesicles as described above, but in the absence of ^{3}H-labeled M protein, and then mixing them with 50 μg of free ^{3}H-labeled M protein in 0.6 M NaCl, 10% glycerol, and 10 mM Tris (pH 7.5). Dialysis and flotation centrifugation was performed as described above.

Determination of Individual Phospholipids in Vesicles. Vesicles of 50% PC–50% PS were made in the presence and absence of ^{3}H-labeled M protein as described above. The phospholipid from each sample was extracted with 4 volumes of chloroform–methanol (2:1 v/v) and applied onto a 250-μm silica gel G thin-layer chromatography plate (Analtech, Newark, DE). Chromatography was by the one-dimensional, one-solvent system of Pal et al. (1980) using chloroform–methanol–30% ammonia (65:25:5 v/v/v). Spots of individual phospholipid (PC or PS) were visualized by I_2 vapor and scraped from the plate, and concentrations were determined by phosphate analysis according to the procedure of Marinetti (1962).

Assay for Inhibition of Viral Transcription by M Protein. Transcription activity of virions was assayed by a modification of the procedure of Carroll & Wagner (1978) in a final reaction mixture consisting of 1 mg/mL VSV, 1 mM dithiothreitol, 140 mM NaCl, 0.2% Triton X-100, 7.5 mM $MgCl_2$, 1.2 mM each of ATP, CTP, and GTP, and 0.12 mM [α-^{32}P]UTP in 10 mM Hepes (pH 7.8) in a total volume of 16 μL. Assay mixtures measuring inhibition of transcription by M protein contained 0.4 mg/mL ^{3}H-labeled M protein. Reactions were incubated at 31 °C for 1 h. Duplicate 2-μL samples were removed from each reaction onto nitrocellulose filters (GA-6, Gelman Instrument Co., Ann Arbor, MI). Radioactivity was determined after batch-washing of the disks with three changes of cold trichloroacetic acid and 3% sodium pyrophosphate by counting of Cerenkov radiation.

Results

Phospholipid Requirements for Reconstitution of M Protein. The interaction of membrane-associated VSV M protein with phospholipids was investigated by attempting to reconstitute the protein into vesicles of defined phospholipid composition by the octyl glucoside detergent dialysis method. ^{3}H-Labeled M protein was purified free of viral lipid, mixed with octyl glucoside and phospholipid containing [^{14}C]PC, dialyzed 24 h against 10 mM Tris (pH 7.5), and centrifuged on flotation gradients of 0–30% sucrose.

Figure 1 shows the results of this attempted reconstitution of the ^{3}H-labeled M protein with vesicles of different phospholipid compositions. M protein not associated with vesicles remains at the bottom of the centrifuge tubes. With phospholipid vesicles made of 100% PC or 50% PC–50% PE there was no significant reconstitution of the ^{3}H-labeled M protein with the vesicles (Figure 1A,B). When vesicles were made of 50% PC–50% PS, 50% PC–50% PA, or 50% PC–50% PI, reconstitution of the ^{3}H-labeled M protein was observed (parts C, D, and E, respectively, of Figure 1). Typically, 25–30% of the ^{3}H-labeled M protein was recoverable in the vesicles with the remainder having precipitated and pelleted to the bottom of the centrifuge tube. Vesicles with M protein reconstituted in them had densities ~0.02 g/mL heavier than similar vesicles made in the absence of protein. These data are summarized in Table I.

PC–PS vesicles reconstituted with ^{3}H-labeled M protein were isolated and treated with the protease thermolysin. Subsequent 17.5% polyacrylamide gel electrophoresis showed no small molecular weight fragments of the M protein to be protected from proteolysis. Radioactivity of intact M protein was only 5% of that present in the initial sample, revealing that 95% of the reconstituted M protein was located on the

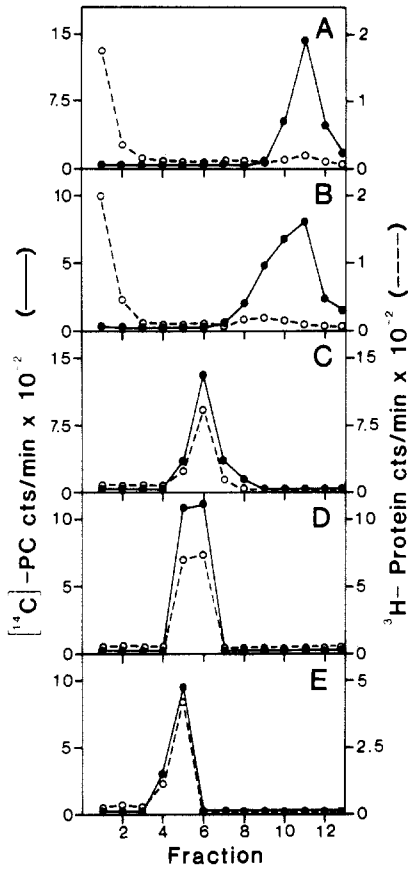

FIGURE 1: Reconstitution of ^{3}H-labeled M protein into phospholipid vesicles by detergent dialysis. Phospholipid (100 μg) containing [^{14}C]phosphatidylcholine was mixed with 2.0 mg of β-D-octyl glucoside and dried. ^{3}H-Labeled M protein (50 μg) purified free of detergent and viral lipids was added and the phospholipid redissolved by vortex mixing. The sample was dialyzed against 1 L of 10 mM Tris (pH 7.5) for 24 h at 4 °C in the absence of O_2 with one change of buffer, then made 35% in sucrose, and floated upward through a gradient of 0–30% sucrose in 0.15 M NaCl and 10 mM Tris (pH 7.5) by centrifugation in a Beckman SW50.1 rotor at 45000 rpm for 16 h. Gradients were fractionated and aliquots counted for ^{3}H and ^{14}C. Vesicles were made from (A) 100 μg of phosphatidylcholine, (B) 50 μg of phosphatidylcholine plus 50 μg of phosphatidylethanolamine, (C) 50 μg of phosphatidylcholine plus 50 μg of phosphatidylserine, (D) 50 μg of phosphatidylcholine plus 50 μg phosphatidic acid, and (E) 50 μg of phosphatidylcholine plus 50 μg of phosphatidylinositol.

Table I: Densities of Vesicles Made in the Presence or Absence of VSV M Protein

vesicle composition[a]	density (g/mL)	
	−M protein	+M protein
PC only	1.02	NR[b]
PC-PE (1:1)	1.02	NR
PC-PS (1:1)	1.06	1.08
PC-PA (1:1)	1.07	1.09
PC-PI (1:1)	1.07	1.09

[a] Abbreviations used for phospholipids: PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine; PA, phosphatidic acid; PI, phosphatidylinositol. [b] Not reconstitutable with M protein.

exterior surface of the vesicles and 5% was trapped in vesicles (data not shown).

The possibility that association with M protein was selecting for certain phospholipids and altering the concentrations of phospholipid in the vesicles was investigated. The PC–PS vesicles reconstituted with M protein (Figure 1C) were isolated as were PC–PS vesicles made identically but in the absence of protein. These samples were extracted with 4 volumes of chloroform–methanol (2:1) and run on thin-layer chromatography. Spots visualized by I_2 vapor were scraped from the plate and phosphate analyses performed to determine the concentrations of the individual phospholipids. The relative concentrations of PC and PS as determined by the phosphate analyses were identical in vesicles that had ^{3}H-labeled M protein associated and in those that did not. Association of PC–PS vesicles with ^{3}H-labeled M protein did not apparently alter the phospholipid ratios in the vesicles. Negative-stain electron microscopy also failed to reveal any structural or size differences or aggregation of vesicles reconstituted with M protein compared with those without M protein (data not shown).

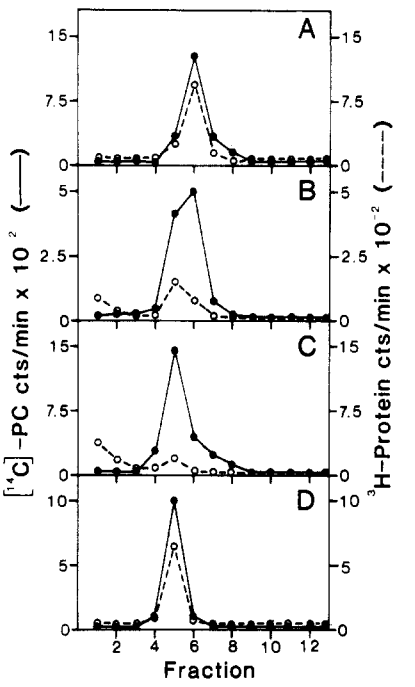

FIGURE 2: Effect of salt on reconstitution of ^{3}H-labeled M protein into vesicles of phosphatidylcholine–phosphtidylserine. ^{3}H-Labeled M protein was reconstituted into 50% phosphatidylcholine–50% phosphatidylserine vesicles as described in Figure 1 but with varying amounts of salt added to the dialysis buffer: (A) no added salt; (B) 0.15 M NaCl; (C) 0.5 M NaCl. (D) Vesicles from panel A reconstituted in the absence of salt were isolated, dialyzed against 1 L of 0.5 M NaCl and 10 mM Tris (pH 7.5) for 24 h at 4 °C in the absence of O_2, and then centrifuged again on a flotation gradient as described in Figure 1.

Salt Requirements of Vesicle Reconstitution of M Protein. The inability of M protein to reconstitute with PC or PC–PE vesicles while retaining the ability to reconstitute with PC–PS, PC–PA, and PC–PI vesicles raised the question of the mechanism by which M protein is reconstituted with phospholipid vesicles. PC and PE have a net neutral electric charge while PS, PA, and PI all have a net negative electric charge. M protein has an isoelectric point of 9.1 and is positively charged at neutral pH (Carroll & Wagner, 1979). This raised the possibility that the association of M protein with these phospholipid vesicles is an electrostatic phenomenon which could be destroyed by high salt concentrations. In order for this hypothesis to be tested, ^{3}H-labeled M protein was reconstituted into 50% PC–50% PS vesicles as described above, but NaCl was added to the normal dialysis buffer of 10 mM Tris (pH 7.5). In the absence of added salt, the flotation gradient shown in Figure 2A was obtained. When 0.15 or 0.5 M NaCl was added to the dialysis buffer, the patterns shown in parts B and C, respectively, of Figure 2 were obtained. The presence of high salt during the detergent dialysis appears to inhibit reconstitution of the M protein with the vesicles.

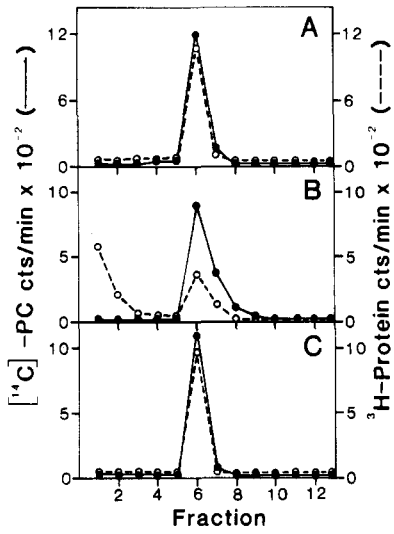

FIGURE 3: Association of ^{3}H-labeled M protein with preformed vesicles. Vesicles of 50% phosphatidylcholine–50% phosphatidylserine were prepared as described in Figure 1 but in the absence of ^{3}H-labeled M protein. These vesicles were isolated, mixed with 50 μg of ^{3}H-labeled M protein, and dialyzed for 24 h at 4 °C in the absence of O_2 against 1 L of (A) 10 mM Tris (pH 7.5) or (B) 0.5 M NaCl and 10 mM Tris (pH 7.5). Flotation gradients were then run normally. (C) M protein associated vesicles made in the absence of salt as in panel A were isolated and dialyzed against 1 L of 0.5 M NaCl and 10 mM Tris (pH 7.5) for 24 h at 4 °C in the absence of O_2, and flotation gradients were run normally.

However, the reconstitution of ^{3}H-labeled M protein with vesicles was found not to be a strictly electrostatic phenomenon. When M protein was reconstituted with PC–PS vesicles in the absence of salt (Figure 2A) and then dialyzed against 0.5 M NaCl and 10 mM Tris (pH 7.5) for 20 h, centrifugation in a 0–30% sucrose and 0.5 M NaCl flotation gradient gave the pattern shown in Figure 2D. Thus, high salt concentrations can prevent the reconstitution of ^{3}H-labeled M protein into PC–PS vesicles, but once the M protein becomes reconstituted, high salt cannot dissociate it from the vesicles. This implies a two-step mechanism of M protein reconstitution with phospholipid vesicles. The first step, an electrostatic attraction, is inhibited by high salt while the subsequent binding of M protein to vesicles, possibly a hydrophobic interaction, is not reversible by high salt.

Association of M Protein with Preformed PC–PS Vesicles. The association of M protein with preformed PC–PS vesicles was also investigated. Vesicles of 50% PC–50% PS were prepared in the absence of M protein, isolated, and then mixed with ^{3}H-labeled M protein. The samples were dialyzed for 24 h against 10 mM Tris (pH 7.5), and flotation centrifugation was performed again. Figure 3A shows that M protein can be associated with these preformed vesicles. When this same procedure is performed but 0.5 M NaCl is added to the dialysis buffer, Figure 3B shows that high salt can inhibit the association of M to preformed vesicles just as it can the reconstitution of M protein into vesicles as described above (Figure 2C). Once the M protein becomes associated with the preformed PC–PS vesicles, addition of 0.5 M NaCl cannot dissociate it (Figure 3C) which is also similar to the results seen during reconstitution (Figure 2D).

Reconstitution with Derivatized M Protein. The M protein has a high isoelectric point of 9.1 and thus has a large number of positively charged functional groups which could represent the domain that is initially electrostatically attracted to the net negatively charged phospholipids during protein–lipid association. To test this hypothesis, we derivatized the M protein lysine ϵ-amino groups with either succinic anhydride

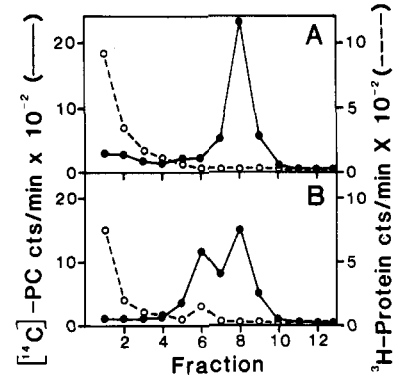

FIGURE 4: Reconstitution of derivatized ^{3}H-labeled M protein into vesicles. Reconstitution with 50% phosphatidylcholine–50% phosphatidylserine was performed normally as described in Figure 1 except that the ^{3}H-labeled M protein had been previously derivatized with (A) acetic anhydride or (B) succinic anhydride.

or acetic anhydride to chemically block the positively charged lysine residues. The extent of derivatization of the M protein was estimated by a fluorescamine assay of remaining primary amine groups. Succinic anhydride derivatized M protein had 50% and acetic anhydride derivatized M protein had 75% of their primary amino groups derivatized as determined by loss of reactivity in the fluorescamine assay. These samples were assayed for ability to inhibit viral transcription in an in vitro system (Carroll & Wagner, 1979) in order to determine if they still retained this biological activity after blocking of the lysine residues. Underivatized M protein, at a concentration of 0.4 mg/mL in the in vitro transcription assay mixture, was found to inhibit transcription by 97%. At identical concentrations, the succinylated and acetylated M protein samples produced inhibition of 92% and 94%, respectively (data not shown). These derivatization procedures thus do not affect this property of the M protein and presumably do not denature the protein.

The succinylated and acetylated M protein samples were used in a detergent dialysis reconstitution procedure with 50 PC–50% PS as described above, and the results from the flotation gradient are shown in Figure 4. The acetic anhydride derivatized M protein showed no association with the PC–PS vesicles (Figure 4A). The results with the succinic anhydride derivatized M protein (Figure 4B) showed two peaks of ^{14}C-labeled PC–PS vesicles. The 1.06 g/mL density of the upper peak is indicative of vesicles not associated with protein, and there was no ^{3}H-labeled M protein radioactivity observed associated with this peak. The 1.08 g/mL density of the lower peak is characteristic of vesicles with bound M protein, and there is a small amount of associated ^{3}H-labeled M protein radioactivity. The 75% derivatization of M protein with acetic anhydride is seen to effectively block the ability of the protein to reconstitute with PC–PS vesicles while not affecting its ability to inhibit viral transcription. The 50% derivatization of the M protein with succinic anhydride is not as complete as the derivatization with acetic anhydride, and a low ability to reconstitute with PC–PS vesicles remains.

Discussion

Recent studies with membrane probes have indicated that the matrix M protein of VSV is normally a membrane-associated protein but that it probably does not extend very deeply into the interior of the virion membrane (Zakowski & Wagner, 1980; Pepinsky & Vogt, 1979; Stoffel et al., 1978). More detailed studies of this protein–membrane association and of the M protein itself have been difficult because of its inaccessible location on the interior surface of the viral membrane. The ability to reconstitute the M protein into phospholipid

vesicles, where it might be located on the external surface of the lipid bilayer, would put it in a position where its interaction with membranes could be studied.

We have reconstituted the VSV M protein into vesicles of defined phospholipid composition by the detergent dialysis method of Petri & Wagner (1979). Reconstitution occurs only when the vesicles contain phospholipids which are net negatively charged such as PS, PA, or PI. PA and PI are only minor constituents of the VSV membrane in vivo, but PS makes up 18% of the viral membrane phospholipid, and most of this PS is oriented toward the interior surface of the VSV membrane (Patzer et al., 1978). It can be postulated, therefore, that in vivo the M protein binds to the inner PS of the membrane since it is the only negatively charged phospholipid present in significant amounts. This observation may also be a factor in accounting for the high concentration of PS in the viral membrane as opposed to its concentration in the plasma membrane of the host cells from which the virus buds (McSharry & Wagner, 1971).

The VSV M protein has an isoelectric point of 9.1 (Carroll & Wagner, 1979) and is very positively charged at neutral pH. The cDNA nucleic acid sequence of the mRNA for M protein has been determined (Rose, 1980) and indicates the presence of 21 lysine and 15 arginine residues out of 231 amino acids. The amino-terminal 19 amino acids contain 8 lysine residues constituting a very positively charged domain. The reconstitution of the positively charged M protein with the negatively charged phospholipid is not, however, a strictly electrostatic interaction. Although high salt concentrations (0.5 M NaCl) can inhibit both the reconstitution of M protein into vesicles during detergent dialysis and the association of the protein with preformed vesicles, it cannot strip the M protein off of the vesicles once the association is made. Thus, the initial attraction of M protein to membrane is electrostatic, but the forces binding the protein to the membrane must also include nonelectrostatic ones. Studies of intact VSV have shown that the virion M protein is membrane associated and that it extends into the viral membrane to a limited extent (Zakowski & Wagner, 1980; Pepinsky & Vogt, 1979; Stoffel et al., 1978). This association is presumably a hydrophobic one.

The possibility that the M protein is not in true association with the phospholipid vesicles but is simply physically trapped within the vesicles was discounted by the results of the thermolysin experiment. Proteolysis of isolated vesicles reconstituted with M protein indicated that 95% of the protein was accessible to and degradable by the thermolysin. Only 5% or less of the M protein was resistant to proteolysis, and this figure presumably represents the amount of M protein physically trapped within the vesicles. $NaDodSO_4$–polyacrylamide gel electrophoresis of these thermolysin-treated vesicles also showed that no small molecular weight fragments of the M protein were protected from proteolysis by association with the vesicles and, thus, that the protein is not buried deeply within the lipid bilayer. A protease-resistant tail fragment has been observed for intrinsic membrane proteins such as the VSV G protein (Petri & Wagner, 1979) and cytochrome b_5 (Enoch et al., 1979) reconstituted into vesicles.

In order to further investigate the initial electrostatic interaction of M protein with vesicles, we derivatized the protein with either succinic anhydride or acetic anhydride. These reagents specifically block the ϵ-amino group of the positively charged lysine residues which make up such a large percentage of the M protein. We were only able to achieve 75% derivatization with the acetic anhydride and 50% derivatization with the succinic anhydride. This was sufficient, however, to show that chemical blocking of the lysine residues can abolish or greatly diminish binding of M protein to phospholipid vesicles. Acetylated M protein totally lost its ability to reconstitute, and succinylated M protein retained only very little ability. Acetylation of lysine yields a neutrally charged functional group while succinylation yields a negatively charged one. In neither case was reconstitution with positively charged 100% PC vesicles observed. Lowering the isoelectric point of the protein is thus not a sufficient condition to induce association with positively charged vesicles, and other factors must be involved. That the M protein was not denatured by the derivatization procedures was demonstrated by retention of all of its ability to inhibit viral transcription (Carroll & Wagner, 1979).

Influenza virus also has a matrix M protein which may be related to VSV M protein in terms of their location within the virion and their presumed functions in virion assembly. The influenza M protein has recently been reconstituted into phospholipid vesicles by procedures similar to those described here (Gregoriades, 1980; Bucher et al., 1980), but many differences are apparent from the results presented here. Reconstitution of influenza M protein can be performed with phospholipid vesicles of any net electrical charge and is impervious to the effects of salt; the vesicles show a larger increase in density when the protein is associated, and there exists a protease-resistant fragment of 5000 molecular weight. These comparisons to VSV M protein stress the fact that analogies should not be drawn too closely in comparing features of apparently related systems. In fact, it is interesting to speculate that these differences in the membrane-binding properties of the M proteins from influenza virus and VSV may be responsible for the different sites of budding of these viruses from epithelial cell monolayers (Rodriguez Boulan & Sabatini, 1978).

The ability to reconstitute VSV M protein on the surface of phospholipid vesicles provides a model system for the detailed investigation in a direct manner of protein–lipid interactions which was not available heretofore. It can also provide an opportunity for study of the interactions of viral components with each other and with the lipid bilayer which may shed light on questions concerning viral maturation, budding, and the defects present in certain mutants that are restricted in budding.

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Epidermal Growth Factor: Relationship between Receptor Down Regulation in Cultured NRK Cells and Epidermal Growth Factor Enhancement of Phosphorylation of a 170 000 Molecular Weight Membrane Protein in Vitro†

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ABSTRACT: Incubation of confluent nondividing NRK cells in serum-free media with unlabeled epidermal growth factor (EGF) leads to a reduction in the specific binding capacity for ^{125}I-labeled EGF. This modulation of the binding capacity for ^{125}I-labeled EGF by unlabeled EGF, termed receptor down regulation, was dependent on EGF concentration and time. Membranes from untreated NRK cells have a phosphorylating system which catalyzed in vitro the phosphorylation of numerous membrane components; this phosphorylating system was stimulated by EGF. Although EGF enhanced the phosphorylation of many membrane proteins, one major component with M_r 170K and a minor band of M_r 150K were primarily affected. A comparison of the membrane phosphoproteins of untreated and down-regulated cells by in vitro phosphorylation and $NaDodSO_4$ gel electrophoresis revealed that down regulation of EGF receptors results in a specific decrease in ^{32}P phosphorylation of the 170K- and 150K-dalton components to subsequent stimulation with EGF in vitro. We further characterized the modulation of phosphorylation of the 170K protein by down regulation with EGF and found it to be dependent on EGF concentration and time. These studies demonstrated a correlation between the loss of ^{125}I-labeled EGF binding activity by the cells and the loss of the vitro EGF-dependent ^{32}P phosphorylation of the 170K-dalton membrane protein. In addition, the results suggest that the major 170K M_r phosphoprotein band is a component of the receptor for EGF which is a substrate of the phosphorylation reaction.

Epidermal growth factor (EGF),[1] a low molecular weight peptide hormone isolated from mouse submaxillary glands, is a potent mitogen for a variety of cell types including fibroblasts and epidermal cells (Carpenter & Cohen, 1979). The initial event in EGF-mediated stimulation of DNA synthesis is the interaction between EGF and its specific plasma membrane receptor (Carpenter & Cohen, 1979; Fox & Das, 1979; Fox et al., 1979). Specific saturable binding sites have been found on cell surfaces of a wide variety of mammalian cells, including NRK cells (Carpenter & Cohen, 1979; Guinivan & Ladda, 1979). Specifically bound EGF is internalized by the cells and subsequently degraded in the lysosomes (Fox & Das, 1979). Incubation of high concentrations of EGF with cells induces a loss in the binding capacity for the hormone; this phenomenon has been termed receptor down regulation (Fox et al., 1979; Wrann & Fox, 1979). The loss of binding activity which parallels EGF internalization and degradation has been attributed to internalization and degradation of EGF receptors (Carpenter & Cohen, 1979; Fox & Das, 1979). This phenomenon has been shown to correlate with the mitogenic activity of EGF (Fox et al., 1979). The interaction of EGF with its membrane receptors in vitro also results in specific stimulation of phosphorylation of endogenous membrane proteins in the presence of [γ-^{32}P]ATP (Carpenter et al., 1979; King et al., 1980). Although EGF stimulated the phosphorylation

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[1] Abbreviations used: EGF, epidermal growth factor; DME, Dulbecco–Vogt modified Eagle's medium; BSA, bovine serum albumin; PBS, phosphate-buffered saline; $NaDodSO_4$, sodium dodecyl sulfate; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; ATP, adenosine 5′-triphosphate; EDTA, ethylenediaminetetraacetic acid.