JOURNAL OF VIROLOGY, Apr. 1978, p. 115-125
0022-538X/78/0026-0115$02.00/0

Vol. 26, No. 1
Printed in U.S.A.

Assembly of Viral Membranes: Nature of the Association of Vesicular Stomatitis Virus Proteins to Membranes

TRUDY G. MORRISON* AND CATHERINE O. McQUAIN
Department of Microbiology, University of Massachusetts Medical School, Worcester, Massachusetts 01605

Received for publication 27 September 1977

To explore the interaction of vesicular stomatitis virus (VSV) proteins with cellular membranes, we have isolated membranes from infected cells that have been radioactively pulse-labeled. We have found conditions of isolation that result in membrane preparations which contain primarily the VSV membrane protein (M) and glycoprotein (G). Both of these proteins are very firmly attached to membranes: conditions known to release peripherally associated membrane proteins from membranes (S. Razin, Biochim. Biophys. Acta **265**:241-246, 1972; S. J. Singer, Annu. Rev. Biochem. **43**:805-826, 1974; S. J. Singer and G. L. Nicholson, Science **175**:720-731, 1972) are ineffective in detaching either the G or the M protein. The results of trypsin digestion of these membrane fractions suggest that the M protein resides primarily on one side, the cytoplasmic side of cellular membranes, whereas the glycoprotein has been transported to the lumen of the membrane vesicle. However, we present evidence that the glycoprotein is transmembranal and that approximately 3,000 daltons of one end of the molecule is on the cytoplasmic side of the membrane. We have also found that undenatured VSV M protein contains a trypsin-resistant core with a molecular weight of 22,000. This region of the M protein is trypsin resistant regardless of its association with membranes.

Vesicular stomatitis virus (VSV) is an enveloped RNA virus composed of an inner ribonucleoprotein core surrounded by a membrane (8, 9, 17). The membrane of virus particles, derived from plasma membranes of host cells (46), contains two virus-encoded membrane proteins, the glycoprotein (G) (molecular weight, 69,000), which forms the spikes of the virion (8, 9), and the membrane or matrix (M) protein (8, 9, 45) (molecular weight, 29,000). The ribonucleoprotein core is composed of the viral genome, the nucleocapsid protein (N) (molecular weight, 50,000), the NS protein (molecular weight, 45,000), and the L protein (molecular weight, 190,000) (21, 44).

The immediate precursors of mature VS virions are regions of infected-cell plasma membranes, which contain these virion components (46). These modified regions of host cell plasma membrane bud out to form mature virions (42, 46), the inner side of the cellular membrane becoming the inner side of the virion membrane and the external surface of the cell becoming the external surface of the virion (15; E. J. Patzer and R. R. Wagner, Abstr. Annu. Meet. Am. Soc. Microbiol. 1977, S114, p. 298).

How these virion components arrive at the plasma membrane is not understood in detail. Proteins that bind to the cytoplasmic side of membranes may spontaneously assemble with membranes after their synthesis and after they have assumed the appropriate conformation and/or modifications. In contrast, since membranes are thought to be asymmetric, with little random movement of membrane components between the two surfaces of the membrane (39, 40), proteins that exist primarily on the external surface of membranes or that are transmembranal require a special mechanism for their transport across the membrane barrier of the cell.

The signal sequence hypothesis originally proposed by Blobel and Dobberstein (5) to account for the transport of secreted proteins across membranes could, with modifications, also account for the membrane assembly of transmembrane proteins or membrane proteins that exist primarily on the outer surface of cells. The signal sequence hypothesis proposes that proteins destined for the external surface of cells are synthesized with an amino terminus, which directs the transport of the protein across the membrane barrier of the cell.

The VSV glycoprotein is synthesized on membrane-bound polyribosomes (6, 16, 33) on the cytoplasmic side of intracellular membranes. The glycoprotein, however, ends up on the external surface of infected cells (24) and ulti-

mately on the external surface of virions (14, 32, 35). Thus the VSV glycoprotein must be transported across cell membranes at some point in its transit from the rough endoplasmic reticulum to virions.

In contrast, the VSV M protein, although membrane associated, is thought to remain within or on the inner side of viral membranes (14, 32, 35). Similarly, the ribonucleoprotein core, which is thought to be assembled free in the cytoplasm (43), also associates with the inner or cytoplasmic side of the plasma membrane and viral membrane (8, 9, 24). These components of virions may spontaneously associate with membrane once they have assumed the appropriate conformation and/or once the appropriate receptors exist in the cellular membrane.

We have characterized the nature of the association of VSV membrane proteins to cellular membranes in order to explore the steps involved in virion morphogenesis. We have found that the VSV glycoprotein is transported across intracellular membranes. Furthermore, we present evidence that the viral glycoprotein is a transmembrane protein in intracellular membranes. We have also found that the VSV M protein is very firmly attached to the cytoplasmic side of membranes, perhaps as an integral membrane protein.

MATERIALS AND METHODS

Cells and virus. Cells used were Chinese hamster ovary cells. VSV, Indiana serotype, obtained from H. Lodish, was grown and purified as described previously (41).

Preparation of infected cell membranes. Chinese hamster ovary cells (4.0×10^8) growing at 37°C were infected with purified B particles of VSV at a multiplicity of 3 PFU/cell as described by Huang et al. (18), except that 2 μg of actinomycin D per ml (Merck Sharp & Dohme) was added at the beginning of infection. At 4.5 h postinfection, cells were harvested by centrifugation, washed once in modified Eagle medium (minimum essential medium) minus methionine (supplemented with nonessential amino acids and dialyzed fetal calf serum [7.5%]), and resuspended in 2 ml of supplemented Eagle minimum essential medium minus methionine. [^{35}S]methionine (1.5 mCi/ml; 520 Ci/mmol; Amersham/Searle) was added to the infected cells, and incubation was continued at 37°C. At 15 min after the addition of the [^{35}S]methionine, the infected cells were removed and rapidly mixed with 10 ml of frozen 10% sucrose. Cells were harvested by centrifugation, washed once in 10 ml of cold 10% sucrose, and disrupted with a tight-fitting Dounce homogenizer. Nuclei were removed from the cell lysate by centrifugation, resuspended in 10% sucrose, sheared with a Dounce homogenizer, and again pelleted by centrifugation. The resulting supernatant was combined with the cytoplasmic extract. The cytoplasmic extract was layered over a preformed discontinuous sucrose gradient containing a layer of 80% sucrose (wt/vol) and a layer of 20% sucrose (wt/vol). The gradients were centrifuged for 18 h in a Beckman SW27 rotor at 22,000 rpm and 5°C. The membranous material banding at the interface between the 80 and 20% sucrose layers was collected, made 80% (wt/vol) with respect to sucrose, placed in the bottom of a Beckman SW27 centrifuge tube, and overlaid with 65 and 20% sucrose (wt/vol). The gradient was centrifuged to equilibrium (22,000 rpm, 18 h, 5°C). Membranous material floating to the interface of the 65 and 20% sucrose layers was collected and frozen in samples at −70°C.

Tryptic peptide analysis. Trypsin digestion of individual polypeptides was accomplished as described previously (33). The resulting tryptic peptides were resolved by paper electrophoresis at pH 3.5 (29).

Cell-free protein synthesis. VSV proteins were synthesized in cell-free systems derived from rabbit reticulocytes (36). Extracts derived from rabbit reticulocytes were prepared as described by Hunt and Jackson (19). Exogenous RNA-dependent, cell-free protein synthesis was accomplished in these extracts by modification of the procedures of Pelham and Jackson (36). Extracts were digested for 10 min at 20°C with micrococcal nuclease (5 μg/ml; Boehringer/Mannheim) in the presence of added potassium acetate (50 mM), Tris-hydrochloride (50 mM, pH 7.8), and $CaCl_2$ (10 mM). Ethyleneglycol-bis(β-aminoethyl ether)-*N*,*N*′-tetraacetic acid (EGTA; final concentration, 50 mM) was added to terminate the nuclease digestion. As reported by Pelham and Jackson (36), such digestion completely abolished background incorporation of [^{35}S]methionine by these extracts. Cell-free protein synthesis reactions (60 μl) contained, per milliliter: HEPES, 20 μmol, pH 7.8; β-mercaptoethanol, 6 μmol; ATP, 1 μmol; GTP, 0.2 μmol; creatine phosphate, 8 μmol; creatine phosphokinase, 4.0 μg; 19 unlabeled amino acids, 0.02 to 0.03 μmol; potassium acetate, 96 μmol; magnesium acetate, 3 μmol; extract, 0.2 ml; [^{35}S]methionine (520 Ci/mmol; Amersham/Searle), 250 μCi; optimal VSV 13-15S mRNA concentrations; yeast tRNA (gift from A. Hopper), 150 μg; and hemin (prepared as described by Hunt and Jackson [19]), 5 μg. Reactions were incubated at 25°C for 90 min.

Polyacrylamide gel electrophoresis. Polypeptides were resolved on 10 or 15% polyacrylamide slab gels (14 by 12 by 0.15 cm) prepared and run as described by Laemmli (27). The gels were then fixed and stained with Coomassie brilliant blue as described by Clinkscales et al. (10), dried, and subjected to autoradiography (Kodak, X-ray film X-OMAT). The resulting autoradiograms were scanned with an Ortec microdensitometer.

Preparation of VSV 12-15S mRNA. Preparation of VSV mRNA has been described previously (34).

Chemical analysis of membranes. Protein content of membranes was determined by the method of Lowry et al. (31). The phospholipid phosphate content of membranes was determined by the method of Ames and Dubin (3). In calculating the phospholipid concentration, the phosphate concentration was multiplied by 25 (25).

RESULTS

Properties of membranes from VSV-infected cells. To visualize newly made VSV membrane proteins, VSV-infected Chinese hamster ovary cells were radioactively labeled with [^{35}S]methionine for 15 min. Membranes were prepared from cytoplasmic extracts by flotation in discontinuous sucrose gradients. Figure 1 shows that total cytoplasmic membranes isolated from such pulse-labeled cells contain primarily the VSV glycoprotein and M protein, small amounts of the VSV N and NS proteins, and no detectable amounts of L protein.

Polyacrylamide gels of material found in these membrane preparations also contain radioactive material with an apparent molecular weight of 140,000. The methionyl tryptic peptide pattern obtained from this material is identical to that derived from the VSV glycoprotein (not shown). It seems most likely that this material represents aggregates of glycoprotein not disrupted by the gel sample buffer.

Membrane-associated proteins have been defined as peripherally or integrally associated with membranes (39, 40). This classification is based on the ease with which the protein can be detached from membranes (37, 39, 40). To characterize the nature of the binding of newly made VSV M and G proteins to infected cell membranes, VSV-infected cell membranes were subjected to various conditions in an attempt to remove one or both of the VSV proteins. VSV-infected cell membranes were incubated for 1 h at 25°C at 2 M KCl, 50 mM EDTA, pH 11, or pH 3.5 and then reisolated by flotation on discontinuous sucrose gradients. Figure 2 shows the profiles of trichloroacetic acid-precipitable material across the sucrose gradients. Any material that becomes detached from membranes should remain in the bottom of the gradient, whereas membrane-associated material will float to the 70 to 20% sucrose interface. Neither 2 M KCl, 50 mM EDTA, nor pH 3.5 will detach any VSV protein from membranes. Incubation of membranes at pH 11 removed some VSV protein from membranous material. Polyacrylamide gel analysis of the membrane-associated material after incubation at high pH reveals that some detachment of the M protein results. However, chemical analysis of membranes after incubation at pH 11 indicates that some membrane disruption occurs under these conditions (Table 1). Whereas the phospholipid content of membranes remains unchanged, incubation of membranes at pH 11 decreases the total protein content of the infected cell membranes by at least 16% (Table 1).

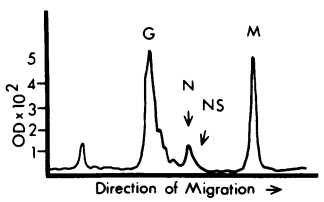

FIG. 1. *VSV proteins present in infected cell membranes. Membranes were prepared from VSV-infected cells radioactively labeled with [^{35}S]methionine for 15 min as described in the text. A sample of the membrane preparation was acetone precipitated, and the precipitate was resuspended in gel sample buffer (1% sodium dodecyl sulfate, 0.125 M Tris-hydrochloride [pH 6.8], 0.7 M β-mercaptoethanol, and 20% glycerol), boiled, and subjected to polyacrylamide gel electrophoresis on a 10% Laemmli gel (27). The figure shows a densitometer scan of the autoradiogram of the fixed dried gel. OD, Optical density.*

Proteolytic digestion of infected cell membranes. Another approach to the characterization of membrane-protein complexes is the use of proteolytic enzymes. Proteins or portions of proteins exposed on the surface of membranes should be susceptible to digestion with proteolytic enzymes, whereas those proteins or portions of proteins embedded within the lipid matrix of membranes or residing inside a sealed membrane vesicle should be protected from digestion. To study the trypsin sensitivity of VSV proteins associated with intracellular membranes, radioactively labeled VSV-infected cell membranes were digested with trypsin at 25°C. The trichloroacetic acid-precipitable material decreased with time for 30 min. Longer incubation did not result in further digestion. After digestion was complete, 54% of the radioactivity was still trichloroacetic acid precipitable. If the detergent deoxycholate is added at concentrations high enough to disrupt membrane structure, 90% of the radioactivity present in these purified membranes becomes trichloroacetic acid soluble.

To ascertain how much of the trichloroacetic acid-precipitable material remaining after proteolytic digestion is still associated with membranes, membranes were reisolated by flotation on discontinuous sucrose gradients after digestion with trypsin. A total of 57% of trichloroacetic acid-precipitable material remaining after proteolytic digestion still remains associated with material that will float in a discontinuous sucrose gradient.

The trypsin-resistant material still associated with membranes, as well as that released from membranes, was characterized by polyacrylamide gel electrophoresis. The material released from membranes by trypsin contained primarily N-sized material (50,000 daltons) and a 22,000-dalton fragment (Fig. 3, channel C). The mate-

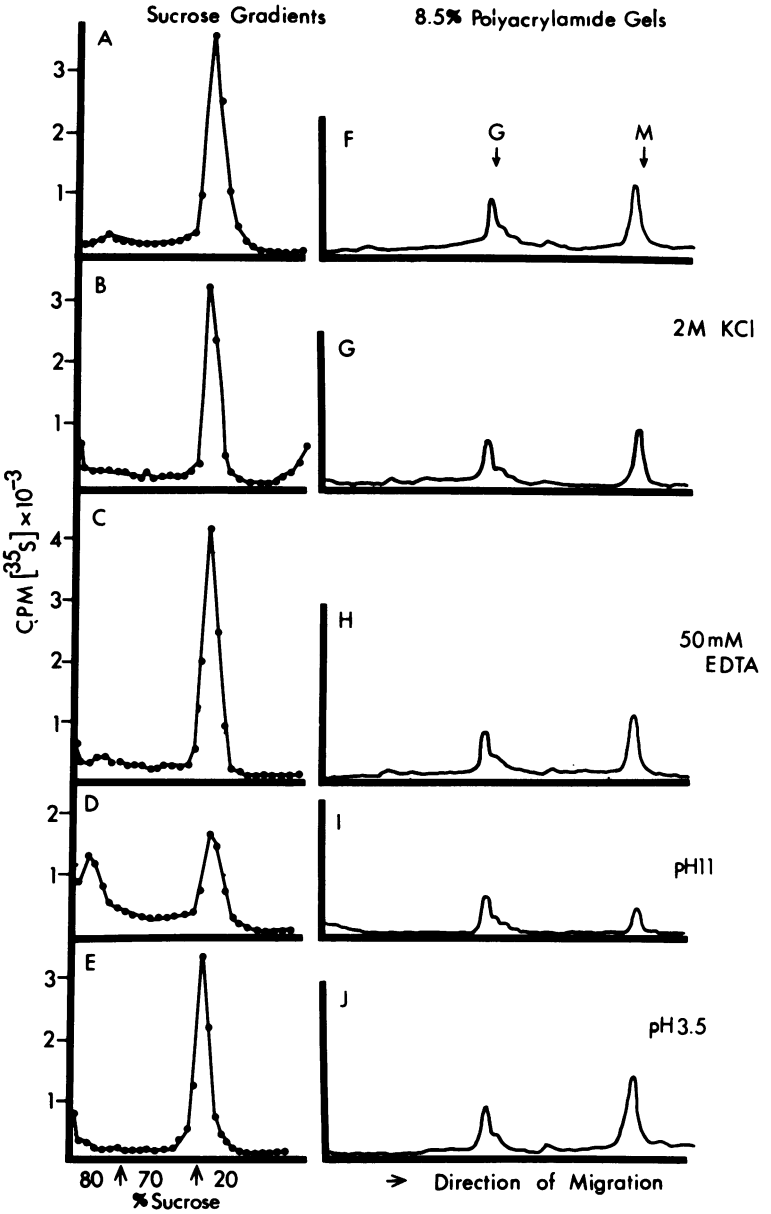

FIG. 2. *Stability of infected cell membrane-protein complexes. VSV-infected cell membranes purified from cells radioactively labeled for 15 min were untreated (A and G); made 3 M with respect to KCl (B and H); made 50 mM with respect to EDTA (C and I); made pH 11 with ammonium hydroxide (D and J); made pH 3.5 with HCl (E and K). After incubation at 25°C for 1 h, the membranes were made 80% (wt/vol) with respect to sucrose (ρ = 1.300 g/cm^3 at 20°C), and the membranes were floated in discontinuous sucrose gradients (2 ml of 80% sucrose; 8 ml of 70% sucrose, ρ = 1.255 g/cm^3; 5 ml of 20% sucrose, ρ = 1.05), which were centrifuged for 18 h at 22,000 rpm in a Spinco SW27.1 rotor. The gradients were collected from the bottom, and samples were digested with 0.1 N NaOH and then precipitated with 10% trichloroacetic acid. (A–F) Pattern of trichloroacetic acid-precipitable counts across sucrose gradients. Material that floated up the gradient was pooled, acetone precipitated, and subjected to electrophoresis on 10% polyacrylamide gels. (G–K) Densitometer scans of autoradiographs of the dried gels.*

TABLE 1. *Chemical analysis of membranes*[a]

Treatment	Protein (mg)[b]	Phospholipid (mg)[b]	Phospholipid/protein
None	551	417	0.757
2 M KCl	575 (104)	429 (103)	0.746
50 mM EDTA	522 (95)	429 (103)	0.822
pH 11	460 (83)	410 (98)	0.891
pH 3.5	510 (93)	415 (99)	0.814

[a] Membranes were incubated and reisolated as described in the legend to Fig. 2. After centrifugation, the protein content and the phospholipid content were analyzed as is described in Materials and Methods.

[b] Numbers in parentheses are percentages.

rial that remains associated with membranes after digestion with trypsin contains primarily three large polypeptides with molecular weights of 22,000, 56,000, and 66,000 (Fig. 3, channels D and E). There were small amounts of polypeptides with molecular weights of 28,000 and 37,000 (Fig. 3).

Figure 4 shows densitometer scans of polyacrylamide gels similar to those shown in Fig. 3. These scans clearly show that the largest protected fragment is not quite the size of undigested glycoprotein but migrates with an apparent molecular weight of 66,000.

To prove that these polypeptides are protected from digestion by membranes, the membranes were chloroform-methanol extracted, and the delipidated proteins were digested with trypsin. The digested material was resolved by electrophoresis on a 15% polyacrylamide gel and found to be very small-molecular-weight material, which is electrophoresed off the 15% polyacrylamide gel (not shown).

To determine the origin of these largest polypeptides generated by trypsin digestion of infected cell membranes, each polypeptide was excised from polyacrylamide gels and further digested with trypsin, and the resulting tryptic peptides were resolved by paper electrophoresis at pH 3.5. Figures 5 and 6 show the pattern of radioactivity across the paper after electrophoresis. Tryptic peptides derived from full-sized M and G proteins were subjected to electrophoresis in parallel. Figure 5 shows clearly that the 22,000-dalton, membrane-associated tryptic fragment is derived from the VSV M protein. This 22,000-dalton fragment is missing at least two of the methionine-labeled tryptic peptides normally found in full-sized M protein. The 22,000-dalton fragment released from membranes after trypsin treatment is also derived from the VSV M protein. This fragment has a tryptic peptide pattern identical to that shown for the membrane-associated, 22,000-dalton fragment (not shown). Figure 6 shows that the 56,000- and the 66,000-dalton fragments are derived from the VSV glycoprotein. These tryptic fragments appear to contain overlapping peptides. The 28,000- and 37,000-dalton fragments are also derived from the VSV G protein (not shown).

These results suggest that significant portions of both the VSV G and M proteins are protected from proteolytic digestion by membranes. Proteins may be protected because they are embedded in the membrane or they are totally, or in part, inside of membrane vesicles. The procedures of Kriebich and Sabatini (26) provide a way to distinguish between these possibilities. They found that material inside vesicles could be rendered susceptible to proteolytic digestion if the membrane vesicles were made porous with very low concentrations of deoxycholate (26). Therefore, VSV-infected cell membranes were treated with increasing concentrations of deoxycholate (0.025 to 0.07%) in the presence of trypsin. The resulting material was subjected to polyacrylamide gel electrophoresis. Figure 7 shows densitometer scans of the resulting autoradiograms. At the lowest detergent concentration the largest polypeptides derived from glycoprotein are now susceptible to proteolytic digestion. Thus, the large glycoprotein fragments obtained after trypsin digestion of membranes appear to be protected from trypsin digestion because they span the membrane and protrude into the inside of membrane vesicles.

As membranes are subjected to increasing amounts of detergent, the fate of the M protein-derived fragment (22,000 molecular weight) appears to be different than the glycoprotein-derived fragments. The M protein fragment does diminish with increasing concentrations of detergent, but not as dramatically as the G-derived fragments. At the lowest concentrations of detergent the M-derived fragment is reduced by only 50%, whereas the largest G-sized fragments are present in less than 10% the amount found in membranes not treated with detergent.

Trypsin sensitivity of the VSV M protein. To test the trypsin sensitivity of VSV proteins that are free of membranes but are in an undenatured state, VSV proteins were synthesized in a cell-free, protein-synthesizing system which is reportedly devoid of membranes. VSV M, N, NS, and G proteins were synthesized in a cell-free system derived from rabbit reticulocytes directed by VSV mRNA (Fig. 8A). These extracts are reported to be deficient in membranous material (30). The products of the cell-free reaction were digested with trypsin, and the resulting digest was resolved on polyacrylamide gels. The VSV proteins were digested to very small-molecular-weight material except for a

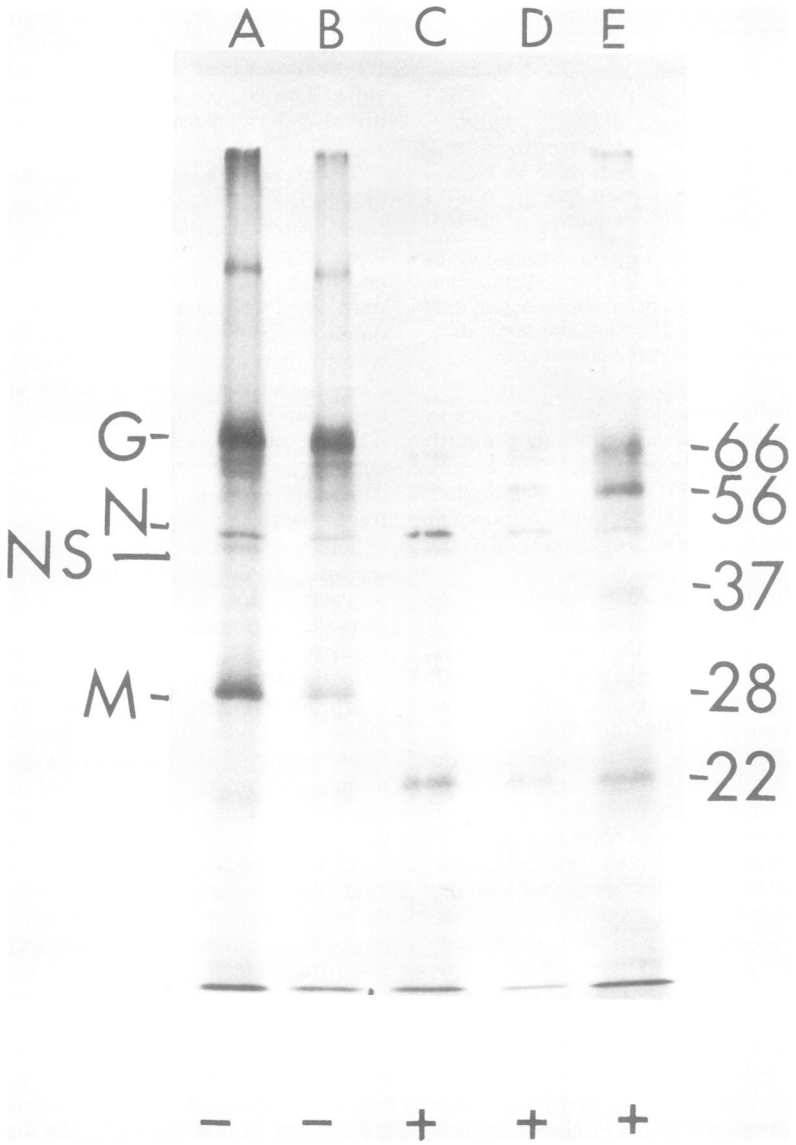

FIG. 3. *Polyacrylamide gel analysis of membrane-associated and released, trypsin-resistant material. VSV-infected cell membranes prepared from cells radioactively labeled for 15 min were digested with trypsin (50 μg/ml at 25°C) for 1 h. TLCK (N-α-tosyl-L-lysine chloromethyl ketone) (150 μg/ml, Sigma) was added, and membranes were made 80% with respect to sucrose, placed in a Spinco SW27.1 tube, overlaid with 2 ml of 65% sucrose (ρ = 1.240 g/cm³), 2 ml of 60% sucrose (ρ = 1.225 g/cm³), 4 ml of 45% sucrose (ρ = 1.180 g/cm³), 2 ml of 40% sucrose (ρ = 1.155 g/cm³), and 2 ml of 30% sucrose (ρ = 1.090 g/cm³), and centrifuged for 18 h at 22,000 rpm (5°C). Untreated membranes were reisolated in parallel with trypsin-treated membranes. Material remaining in the bottom of the gradients (80% sucrose layer) as well as membranous material was acetone precipitated and subjected to polyacrylamide gel electrophoresis on 10% polyacrylamide gels. After trypsin digestion, most of the membrane-associated material floated to the interface of the 40 and 30% sucrose layers, but some membrane material was found in the 65, 60, and 45% sucrose layers. The figure shows an autoradiogram of the fixed, dried gel. Lanes A and B show undigested membrane material: (A) material in heavy density membranes (membranes that floated into the 60% sucrose layer); (B) material in light density membranes (membranes that floated into the 40% sucrose layer). Lanes C through E show trypsin-digested material: (C) material released from membranes (material remaining in the 80% sucrose); (D) membrane-associated material (material found in the 65, 60, and 45% sucrose layers); (E) membrane-associated material (material that floated into the 40 and 30% sucrose layers).*

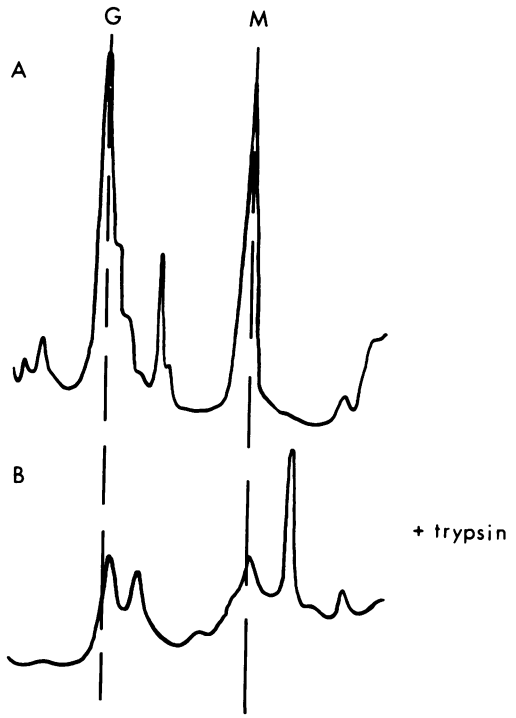

FIG. 4. *Densitometer scan of autoradiograms of 10% polyacrylamide gel containing trypsin-resistant material associated with VSV-infected cell membranes. Membranes prepared from VSV-infected cells radioactively labeled for 15 min were digested with trypsin as described in the legend to Fig. 3. Membrane-associated material was subjected to polyacrylamide gel electrophoresis on 10% gels as described in the legend to Fig. 2. (A) No trypsin; (B) plus trypsin.*

22,000-dalton fragment (Fig. 8B).

After electrophoresis on polyacrylamide gels, this polypeptide was further digested with trypsin, and the resulting tryptic peptides were resolved by ionophoresis at pH 3.5. The pattern of tryptic peptides obtained from this 22,000-dalton polypeptide is identical to the pattern obtained from the M-derived fragment found in infected cell membranes (not shown).

The 22,000-dalton, trypsin-resistant core derived from the M protein becomes susceptible to further digestion by trypsin only in the presence of 0.5% sodium dodecyl sulfate (Fig. 8C) or 0.5% deoxycholate or after denaturation upon chloroform-methanol extraction (not shown). Triton X-100 will not render this fragment susceptible to digestion. Thus, the VSV M protein synthesized in a reticulocyte cell-free reaction is in a conformation such that a 22,000-dalton core is resistant to digestion with trypsin.

DISCUSSION

To explore the interactions of newly made VSV proteins with intracellular membranes, we have pulse-labeled infected cells for 15 min with [^{35}S]methionine. The vast majority of the glycoprotein synthesized during this time interval is present in intracellular compartments of infected cells. An estimate of the time required to incorporate G into virions is 20 min (4, 20, 23). Furthermore, it has been shown that the glycoprotein requires at least 15 min just to begin to be detected at the surface of infected cells (4, 24), and, in fact, we have shown that much of the glycoprotein synthesized during the 15-min labeling period is associated with membranes with a density characteristic of rough endoplasmic reticulum (Fig. 3, channel A).

In contrast, the VSV M protein can be de-

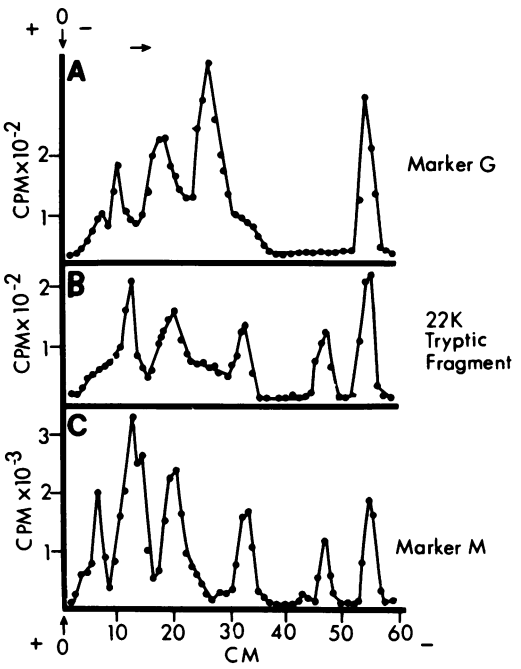

FIG. 5. *Tryptic peptide analysis of material present in membranes after proteolytic digestion. Individual polypeptides were excised from polyacrylamide gels similar to those shown in Fig. 3 and 4 and digested with trypsin as has been previously described (33), and the resulting methionyl tryptic peptides were resolved by paper electrophoresis at pH 3.5 (29). Samples shown together were coelectrophoresed. After electrophoresis the paper was cut into 1-cm strips, and radioactivity present in each strip was determined by liquid scintillation counting. (A and C) Patterns obtained from intact glycoprotein and M protein, respectively; (B) pattern obtained from the 22,000-dalton tryptic fragment.*

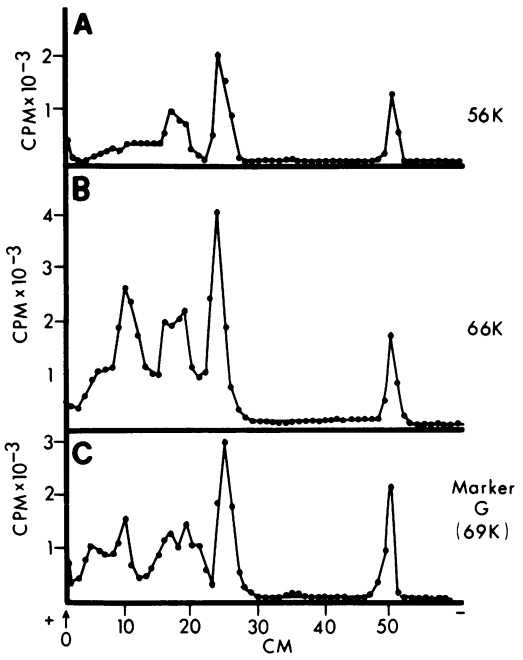

FIG. 6. *Tryptic peptide analysis of material present in membranes after proteolytic digestion. See legend to Fig. 5. (A) 56,000-dalton tryptic fragment; (B) 65,000-dalton tryptic fragment; (C) intact glycoprotein.*

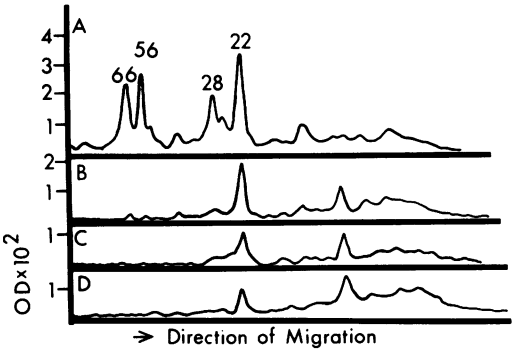

FIG. 7. *Trypsin sensitivity of VSV M and G proteins in membranes treated with low concentrations of deoxycholate. Membranes prepared from VSV-infected cells radioactively labeled for 15 min were digested with trypsin as described in the legend to Fig. 3, except that various concentrations of deoxycholate (DOC) were added with the trypsin. After digestion, polypeptide material associated with membranes was subjected to electrophoresis on 15% polyacrylamide gels. (A) 0% DOC; (B) 0.025% DOC; (C) 0.050% DOC; (D) 0.075% DOC.*

tected in virions within 2 to 5 min of its synthesis (4, 20, 22, 23). Even so, it is unlikely that much of the membrane-associated M protein synthesized in the 15-min pulse is in the form of contaminating virions. First, our membrane preparations appear to have little virion contamination since there is little N protein present even in labeling periods of up to 1 h (unpublished observations). Second, the M protein present in these membrane preparations is susceptible to trypsin. There is no population of M protein molecules that remain undigested, as they should were they inside complete virions (14, 32, 35, 38). Some of the M protein synthesized during the 15-min labeling period is likely to be present in plasma membranes. However, much of the M protein is located in membranes with a density of rough endoplasmic reticulum (Fig. 3, channel A).

Membrane association of the VSV glycoprotein. Virion-associated glycoprotein is likely to be an integral membrane protein. Upon digestion of intact virions with proteolytic enzymes, it has been shown that there is a fragment of the glycoprotein that is embedded in the membrane of the virion (35, 38).

We have characterized the membrane protein association of newly made glycoprotein. First, the intracellular glycoprotein appears to be very firmly bound to intracellular membranes: no treatment of the membrane that does not dis-

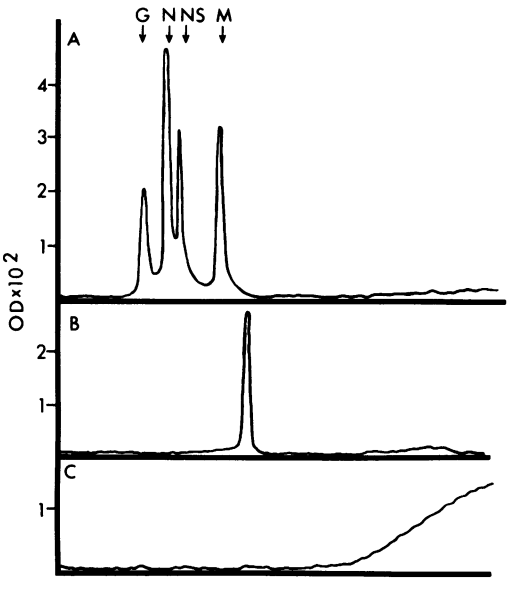

FIG. 8. *Trypsin digestion of VSV proteins synthesized in a cell-free protein-synthesizing system. VSV proteins synthesized in reticulocyte cell extracts directed by VSV 13-15S RNA were digested with trypsin (50 μg/ml) at 25°C for 1 h, diluted with gel sample buffer (27), and subjected to electrophoresis on 15% polyacrylamide gels. (A) VSV proteins; (B) VSV proteins + trypsin; (C) VSV proteins + trypsin + 1% sodium dodecyl sulfate. OD, Optical density.*

rupt membranes will release the glycoprotein from membranous material, which will float up in a sucrose gradient. One percent sodium dodecyl sulfate will release the glycoprotein (unpublished observations).

To further characterize glycoprotein membrane interaction, we subjected these membranes to trypsin. Proteins or portions of proteins that are exposed on the surface of membranes should be susceptible to proteolytic digestion, whereas proteins or portions of proteins that are embedded in the membrane bilayer or are inside a sealed vesicle should be resistant to digestion. Membrane fractions isolated from intracellular membranes are usually in the form of vesicles (1, 2, 5, 26). These vesicles may be inside-out vesicles with the cytoplasmic side of the membrane on the outer surface of the vesicle or outside-out vesicles with the cytoplasmic side inside the vesicle. Membranes with the cytoplasmic side out will have ribosomes on the outside of the vesicle, whereas vesicles with the reverse orientation will have ribosomes inside the vesicle. We have found that our membrane preparations are in a conformation such that upon incubation in high salt and puromycin, conditions known to release ribosomes from membranes (2), 85% of the ribosomes associated with these vesicles are released from the membranes (unpublished observations). Therefore, the vast majority of our membranes are present as cytoplasmic side out or unsealed vesicles.

Upon trypsin digestion of membranes prepared from VSV-infected cells, we have found that the majority of the glycoprotein is found in fragments with molecular weights of 66,000 and 56,000. The proportion of glycoprotein found in these large fragments varied with the membrane preparation. In some preparations 90% of the glycoprotein was present in these fragments, whereas other preparations yielded as low as 50% of the glycoprotein in these large fragments. This variability probably reflects the concentration of sealed membrane vesicles in the preparation.

No full-sized glycoprotein was detected after digestion. Fragments this large are not likely to be protected from digestion, not because they are embedded in the membrane bilayer but rather because they are inside a sealed vesicle. Kriebich and Sabatini (26) have shown that material inside membrane vesicles may be rendered susceptible to proteolytic digestion if the membrane vesicles are made porous with very low concentrations of deoxycholate. We have found that at low concentrations of deoxycholate trypsin digests the glycoprotein to very small-molecular-weight material. These results are consistent with the idea that newly made glycoprotein is, in fact, transported across the membrane of the cell, from the cytoplasmic side of the membrane into the lumen of the endoplasmic reticulum.

It is significant that no full-sized glycoprotein is found after trypsin digestion of membranes but that the largest protected fragment obtained is approximately 3,000 daltons smaller than intact glycoprotein. This result suggests that intracellular glycoprotein is transmembranal and that a small portion of one end of the glycoprotein extrudes from the cytoplasmic side of the membrane and is, therefore, susceptible to trypsin. This portion of the glycoprotein could, in fact, be a site of interaction between the glycoprotein and other VSV proteins.

The finding that there are also smaller glycoprotein-derived fragments resulting from trypsin treatment of membranes may reflect varying degrees of penetration of the membrane vesicle by nascent glycoprotein. The fact that these fragments contain overlapping tryptic peptides supports this idea. This finding is currently under investigation.

The finding that the VSV glycoprotein is a transmembranal protein in intracellular membranes is interesting in light of the report of Brown and Riedel (7) that there are no transmembranal structures in the budding structure found on the surfaces of VSV-infected cells. Perhaps final stages in the morphogenesis of VSV include a minor cleavage of the glycoprotein such that virion-associated glycoprotein assumes a different conformation with respect to the virion membrane.

Membrane association of VSV M protein. The sequence of events involved in the assembly of VSV M protein into virions is obscure. M protein can be found both membrane associated and soluble in infected cell extracts (11, 12, 23). It has been reported that newly made M protein exists in a soluble state briefly and then accumulates in membranes (13).

How M protein binds to membranes in infected cells and in virions is unclear. Results presented above show that M protein found in intracellular membranes is bound very firmly to intracellular membranes. No condition that did not disrupt cell membranes removed the M protein from membranes. Repeated washing of the membrane in the absence of ions and washes in high salt, EDTA, or low pH had little effect on the membrane association of this protein. Incubation at high pH removed some M protein from membranes, but only when some membrane disruption had occurred. These results suggest, although they certainly do not prove, that membrane-associated M protein binds to membranes as an integral membrane protein. The extent to which the M protein might penetrate the membrane bilayer is as yet undefined.

Upon trypsin digestion of infected cell membranes, M protein was digested to a 22,000-dalton trypsin fragment. However, protection of this large fragment from trypsin may be due not to its association with membranes but rather to its conformation in an undenatured state. This conclusion is indicated by the fact that M protein synthesized in rabbit reticulocyte cell extracts, extracts reportedly devoid of membrane (30), also contains a 22,000-dalton, trypsin-resistant core. Alternatively, reticulocyte extracts may, in fact, contain membranous material or lipid which associates with the M protein, thereby protecting the 22,000-dalton core from digestion. This core becomes susceptible to trypsin digestion upon chloroform-methanol extraction or exposure to high concentration of deoxycholate or sodium dodecyl sulfate, conditions that remove lipid as well as alter protein conformation.

In summary, we have isolated membranes from VSV-infected cells that have been radioactively pulse-labeled. We have found conditions of isolation which result in membrane preparations that contain primarily the VSV M and G proteins. We have found that both these proteins are very firmly attached to membranes. The results of proteolytic digestion of these membrane fractions suggest that the M protein resides primarily on one side, the cytoplasmic side of cellular membranes, whereas G protein has been transported to the lumen of the membrane vesicle. However, we have presented evidence that the G protein in intracellular membranes is transmembranal and that approximately 3,000 daltons of one end of the molecule is on the cytoplasmic side of the membrane.

ACKNOWLEDGMENTS

This study was supported by American Cancer Society grant VC-167A and National Science Foundation grant PCM 76-23290.

We thank C. Madansky, M. Bratt, W. Clinkscales, A. Hopper, and G. Willsky for helpful discussions, Patricia Theriault for preparation of the manuscript, and Dwight Simpson for preparation of the figures.

LITERATURE CITED

1. **Adelman, M. R., G. Blobel, and D. D. Sabatini.** 1973. An improved cell fractionation procedure for the preparation of rat liver membrane bound ribosomes. J. Cell Biol. **56**:191-205.
2. **Adelman, M. R., G. Blobel, and D. D. Sabatini.** 1974. Nondestructive separation of rat liver rough microsomes into ribosomal and membrane components. Methods Enzymol. **31**:201-215.
3. **Ames, B. N., and D. Dubin.** 1960. The role of polyamines in the neutralization of bacteriophage deoxyribonucleic acid. J. Biol. Chem. **235**:769-775.
4. **Atkinson, P. H., S. A. Moyer, and D. F. Summers.** 1976. Assembly of vesicular stomatitis virus glycoprotein and matrix protein into Hela cell plasma membranes. J. Mol. Biol. **102**:613-632.
5. **Blobel, G., and B. Dobberstein.** 1975. Transfer of proteins across membranes. II. Reconstruction of functional rough microsomes from heterologous components. J. Cell Biol. **67**:852-862.
6. **Both, G. W., S. A. Moyer, and A. K. Bannerjee.** 1975. Translation and identification of the viral mRNA species isolated from subcellular fractions of vesicular stomatitis virus-infected cells. J. Virol. **15**:1012-1019.
7. **Brown, D. T., and B. Riedel.** 1977. Morphogenesis of vesicular stomatitis virus: electron microscope observation with freeze-fracture techniques. J. Virol. **21**:601-609.
8. **Cartwright, B., S. S. Smale, F. Brown, and R. Hull.** 1972. A model for vesicular stomatitis virus. J. Virol. **10**:256-260.
9. **Cartwright, B., P. Talbot, and F. Brown.** 1970. The proteins of biologically active subunits of vesicular stomatitis virus. J. Gen. Virol. **7**:267-272.
10. **Clinkscales, C. W., M. A. Bratt, and T. G. Morrison.** 1977. Synthesis of Newcastle disease virus polypeptides in a wheat germ cell-free system. J. Virol. **22**:97-101.
11. **Cohen, G. H., P. H. Atkinson, and D. F. Summers.** 1971. Interactions of vesicular stomatitis virus structural proteins with Hela plasma membranes. Nature (London) New Biol. **231**:121-124.
12. **Cohen, G. H., and D. F. Summers.** 1974. In vitro association of vesicular stomatitis virus proteins with purified Hela and erythrocyte plasma membranes. Virology **57**:566-569.
13. **David, A. E.** 1977. Assembly of the vesicular stomatitis virus envelope: transfer of viral polypeptides from polysomes to cellular membranes. Virology **76**:98-108.
14. **Eger, R., R. W. Compans, and D. B. Rifkin.** 1975. The organization of the proteins of vesicular stomatitis virions: labeling with pyridoxal phosphate. Virology **66**:610-615.
15. **Fong, B. S., R. C. Hunt, and J. C. Brown.** 1976. Asymmetric distribution of phosphatidylethanolamine in the membrane of vesicular stomatitis virus. J. Virol. **20**:658-663.
16. **Grubman, M. J., S. A. Moyer, A. K. Bannerjee, and E. Ehrenfeld.** 1975. Sub-cellular localization of vesicular stomatitis virus messenger RNAs. Biochem. Biophys. Res. Commun. **62**:531-538.
17. **Howatson, A.** 1970. Vesicular stomatitis and related viruses. Adv. Virus Res. **16**:195-256.
18. **Huang, A. S., D. Baltimore, and M. Stampfer.** 1970. Ribonucleic acid synthesis of vesicular stomatitis virus III multiple complementary messenger RNA molecules. Virology **42**:946-957.
19. **Hunt, T., and R. J. Jackson.** 1974. The rabbit reticulocyte lysate as a system for studying mRNA, p. 300-307. *In* R. Neth, R. Gallo, S. Spiegelman, and F. Stohlman (ed.), Modern trends in human leukaemia. J. F. Lehmanns Verlag, Munich.
20. **Hunt, L. A., and D. F. Summers.** 1976. Association of vesicular stomatitis virus proteins with Hela cell membranes and released virus. J. Virol. **20**:637-645.
21. **Kang, C. Y., and L. Prevec.** 1969. Proteins of vesicular stomatitis virus. II. Polyacrylamide gel analysis of viral antigens. J. Virol. **3**:403-413.
22. **Kang, C. Y., and L. Prevec.** 1971. Proteins of vesicular stomatitis virus. III. Intracellular synthesis and assembly of virus specific proteins. Virology **46**:678-680.
23. **Knipe, D. M., D. Baltimore, and H. F. Lodish.** 1977. Separate pathways of maturation of the major structural proteins of vesicular stomatitis virus. J. Virol. **11**:1128-1139.
24. **Knipe, D. M., H. F. Lodish, and D. Baltimore.** 1977. Localization of two cellular forms of the vesicular stomatitis viral glycoprotein. J. Virol. **21**:1121-1127.
25. **Kopaczyk, K. C.** 1967. Preparation of properties of the heart mitochondrial electron transporting particles (inner membrane). Methods Enzymol. **10**:253-258.

26. **Kriebich, G., and D. D. Sabatini.** 1973. Microsomal membranes and the translational apparatus of eukaryotic cells. Fed. Proc. **32**:2133–2138.
27. **Laemmli, U.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227**:680–685.
28. **Lafay, F.** 1974. Envelope proteins of vesicular stomatitis virus: effect of temperature sensitive mutations in complementation groups III and V. J. Virol. **14**:1220–1228.
29. **Lodish, H. F.** 1968. Bacteriophage f_2 RNA: control of translation and gene order. Nature (London) **220**:345–349.
30. **Lodish, H. F.** 1973. Biosynthesis of reticulocyte membrane proteins by membrane-free polyribosomes. Proc. Natl. Acad. Sci. U.S.A. **70**:1526–1530.
31. **Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall.** 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193**:265–275.
32. **Moore, N. F., J. M. Kelley, and R. R. Wagner.** 1974. Envelope proteins of vesicular stomatitis virions: accessibility to iodination. Virology **61**:292–296.
33. **Morrison, T. G., and H. F. Lodish.** 1975. Site of synthesis of membrane and non-membrane proteins of vesicular stomatitis virus. J. Biol. Chem. **250**:6955–6962.
34. **Morrison, T. G., and C. O. McQuain.** 1977. Association of vesicular stomatitis virus membrane proteins and membranes in a cell-free system. J. Virol. **1**:451–458.
35. **Mudd, J. A.** 1974. Glycoprotein fragment associated with vesicular stomatitis virus after proteolytic digestion. Virology **62**:573–577.
36. **Pelham, H. R. B., and R. J. Jackson.** 1976. An efficient mRNA-dependent translation system from reticulocyte lysates. Eur. J. Biochem. **67**:247–256.
37. **Razin, S.** 1972. Reconstitution of biological membranes. Biochim. Biophys. Acta **265**:241–246.
38. **Schloemer, R. H., and R. R. Wagner.** 1975. Association of vesicular stomatitis virus glycoprotein with virion membranes: characterization of the lipophilic tail fragment. J. Virol. **16**:237–249.
39. **Singer, S. J.** 1974. The molecular organization of membranes. Annu. Rev. Biochem. **43**:805–826.
40. **Singer, S. J., and G. L. Nicholson.** 1972. The fluid mosaic model of the structure of cell membranes. Science **175**:720–731.
41. **Stampfer, M., A. Huang, and D. Baltimore.** 1969. Ribonucleic acid synthesis of vesicular stomatitis virus I. Species of ribonucleic acid found in Chinese hamster ovary cells infected with plaque-forming and defective particles. J. Virol. **1**:154–161.
42. **Wagner, R. R.** 1975. Reproduction of rhabdoviruses, p. 1–93. *In* H. Frankel-Conrat and R. R. Wagner (ed.), Comprehensive virology, vol. 4. Plenum Press, New York.
43. **Wagner, R. R., M. P. Kiley, R. M. Snyder, and C. A. Schnaitman.** 1972. Cytoplasmic compartmentalization of the proteins and ribonucleic acid species of vesicular stomatitis virus. J. Virol. **9**:672–683.
44. **Wagner, R. R., T. C. Schnaitman, and R. M. Snyder.** 1969. Structural proteins of vesicular stomatitis virus. J. Virol. **3**:395–403.
45. **Wagner, R. R., R. M. Snyder, and S. Yamazaki.** 1970. Proteins of vesicular stomatitis virus: kinetics and cellular sites of synthesis. J. Virol. **5**:548–558.
46. **Zee, Y. C., A. J. Hackett, and L. Talens.** 1970. Vesicular stomatitis virus maturation sites in six different host cells. J. Gen. Virol. **7**:95–102.