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# Insertion of Influenza M Protein into the Viral Lipid Bilayer and Localization of Site of Insertion

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Recent studies with isolated M protein from influenza virus have shown that the protein has a high affinity for lipid. The ability of M to partition into lipid vesicles merely by shaking vesicles and M together is suggestive evidence that the protein could be interacting with the lipid in the virus particle. A more direct analysis was carried out here to determine whether M is in contact with the viral lipid in situ, by using the photoactivatable hydrophobic probe, pyrenesulfonyl azide. Covalent linkage of this probe to M indicated that a segment of M resides within the virus membrane in contact with the lipid bilayer. M inserted into lipid vesicles at two locations on the molecule. A major insertion into lipid occurred in the middle of the molecule where a large cluster of 20 hydrophobic and neutral amino acids occurs. A second insertion occurred approximately one fourth in from the amino terminus, where a smaller segment of 13 uncharged amino acids is found. Confirmation that M inserted into lipid at these locations came also from results with cyanogen bromide fragments of M. Of the 12 to 13 fragments produced, 3 specifically bound to lipid vesicles. These were the first, second, and third contiguous segments beginning at the amino terminus and containing the two hydrophobic areas noted above.

The major component of influenza is the membrane or matrix (M) protein, which is situated internally in the virus (11, 33). Its interaction with the viral lipid or with other viral proteins remained ambiguous. Then two reports indicated that purified M has a marked affinity for lipid and is easily incorporated into lipid vesicles when these are prepared (9, 18), so that the protein can be visualized on the vesicles by freeze-fracture techniques (9). The high affinity of isolated M for lipid is clear since aggregated M readily partitions into preformed vesicles merely by shaking the vesicles and protein together (18). Different methods can be used to isolate M without materially altering its ability to associate with lipid. The property of partitioning into vesicles is exhibited by known membrane components. The neuraminidase and hemagglutinin of influenza, for example, partition into preformed vesicles by sonication (3), whereas the G protein of vesicular stomatitis virus partitions into vesicles merely by incubating the vesicles and protein together at 37°C (30). These proteins are anchored to the lipid by a stretch of hydrophobic amino acids by which they insert into the bilayer of their respective viruses (8, 26, 29, 30, 34, 38). In this respect, the M protein appears to be anchored to lipid vesicles by a fragment of approximately 5,000 daltons. That a discrete area of the molecule has the capacity to bind to lipid is also suggested by the fact that three specific fragments bound to vesicles although 12 to 13 were produced after cleaving the methionine residues with cyanogen bromide (CNBr) (18).

The work reported below presents evidence that M in situ appears to lie within the lipid bilayer of the virus. The sites of insertion of M into bilayers were localized.

Pyrenesulfonyl azide (PySA), which is a hydrophobic probe that partitions into lipid bilayers, was used to localize M in situ. The probe covalently reacts with the lipid and components lying in the lipid when it is exposed to UV light and thus provides evidence that a protein segment sits in the bilayer (4, 8, 32, 35, 39). The probe is relatively easy to use, since components which covalently bind PySA emit a bright-blue fluorescence. Proteins can thus be detected as fluorescent bands on polyacrylamide gels. The WSN strain of influenza virus was grown on chicken embryo fibroblasts and purified as previously described (19). PySA (Molecular Probes, Plano, Tex.) was resuspended in acetone at a concentration of 300 to 600 μg/20 μl, and WSN virus was at a concentration of 0.5 mg/ml in 0.1

M NaCl-0.01 M Tris (pH 7.6)-$10^{-3}$ M EDTA. All procedures were carried out in the dark, using red-lamp illumination. A total of 20 $\mu$l of PySA was added to 2 ml of virus, and the acetone was removed by evacuation for several seconds. The material was left at room temperature 20 min and then irradiated with long-range UV light (UV SL58 lamp; Ultra Violet Products, San Gabriel, Calif.) at a distance of 3 cm from the source. The reaction was stopped by transferring the material into 5 ml of cold acetone (39). The tubes were left on ice several minutes, and the protein was collected by centrifugation. The pellets were reprecipitated with 5 ml of cold acetone, collected by sedimentation at 2,000 rpm for 10 min, and solubilized with 1% sodium dodecyl sulfate and mercaptoethanol. M was extracted from PySA-treated virus by making the preparation 0.4 M with respect to NaCl immediately after irradiation and transferring to 2 volumes of chloroform-methanol (2:1). The material was then processed as described (16). Samples were analyzed for fluorescence after separation on 10% polyacrylamide gels (23) with a Chromato-Vue Transilluminator C-60 (Ultra Violet Products, Inc.).

Figure 1 (lane 1) indicates that no fluorescence of any viral proteins occurred when PySA was introduced into the virus and the sample was not irradiated with UV light. PySA did associate with all viral proteins when the virus was disrupted with sodium dodecyl sulfate to expose all components before UV irradiation (Fig. 1, lane 2). When PySA was introduced into native virus and the preparation was then exposed to UV light, the external proteins which are anchored to the lipid by hydrophobic stretches (10, 26, 34, 38) and the M became fluorescent. However, the internal proteins, the nucleoprotein and the polymerase (P1, P2, P3), did not. The bands below the hemagglutinin $HA_2$ polypeptide are due to PySA-linked lipid. The M protein was extracted into chloroform-methanol from one half of the preparation depicted in Fig. 1, lane 3, and analyzed on lane 4 of Fig. 1. The M protein was clearly fluorescent, indicating covalent linkage of PySA. Bovine serum albumin, added to the virus at a concentration of 0.2 mg/ml, did not alter the fluorescent pattern of WSN virus and did not itself become labeled under these conditions (Fig. 1, lane 5), indicating that the probe had partitioned into the lipid. These data suggested that M is part of the lipid bilayer of the virus and could be classified as an integral membrane protein.

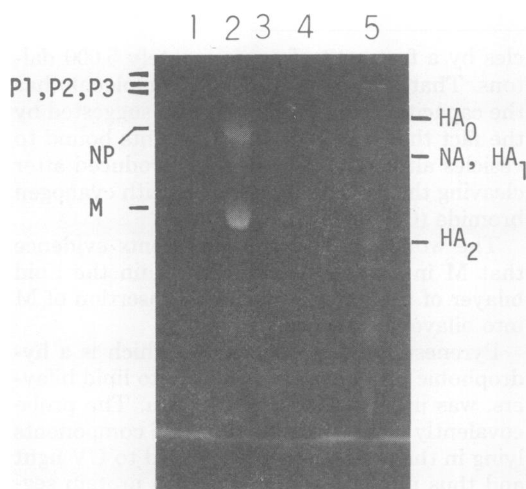


FIG. 1. *Polyacrylamide gel analysis of WSN virus treated with PySA. Lane 1, WSN treated with PySA, no UV irradiation. Lane 2, WSN disrupted with 1% sodium dodecyl sulfate and treated with PySA and UV light. Lane 3, whole virus treated with PySA and UV light. Lane 4, M extracted from the sample shown in lane 3 into acidic chloroform-methanol. Lane 5, WSN virus treated with PySA and UV light in the presence of 200 µg of bovine serum albumin. P1, P2, P3, Polymerase; NP, nucleoprotein; $HA_0$, uncleaved hemagglutinin; $HA_1$, $HA_2$, cleaved hemagglutinin; NA, neuraminidase; M, matrix or membrane protein.*

When M is incorporated into lipid vesicles made of viral lipid or egg phosphatidylcholine, most of the protein can be removed by proteolytic enzymes and is externally situated (18). The assumption was made in this study that the point of insertion of M into lipid in the in vitro studies is identical to that in the virus particle and that the only distinction is that, in one case, M is outside on the bilayer whereas, in the other, it is within the bilayer and inside of the virus. We proceeded to determine the location on M where insertion into bilayers occurs by two methods. Localization was possible since the complete nucleotide sequence of the RNA coding for the M protein of the PR8 strain (37) and a recombinant of the PR8 strain (2) has been published and the amino acid sequence has been deduced in its entirety.

The first method localized the area of the molecule where insertion into lipid took place by sequencing the part of the protein which remained associated with vesicles after trypsinization. The M protein was incorporated into lipid vesicles made with egg phosphatidylcholine (P-L Biochemicals, Inc., Milwaukee, Wis.) by the detergent dialysis method (18), and vesicles were treated with trypsin (50 to 100%, 2 h at 37°C) and collected after flotation through 35% sucrose. Protein material bound to vesicles was delipidated by shaking with 2 volumes of chloroform-methanol and centrifuging at 2,000 rpm for 10 min. The aqueous and organic solvents

were discarded, and the interface containing the protein was resuspended in formic acid and lyophilized. Sequence analysis was then carried out with a Beckman sequencer. As described above, this procedure was unproductive, and no amino terminal was detected. When, however, the material was delipidated and resuspended in 0.01 M ammonium bicarbonate (pH 8.6) instead of formic acid and then lyophilized, an amino terminal, glutamic acid, was available. Exposure of material to acid may have cyclized glutamic to pyrrolidonecarboxylic acid, thus making an amino terminal unavailable (6), or the inability to get an amino terminal may have been due to the marked insolubility of the final material. The sequence of the first seven amino acids of this insertion is given in Table 1 (M insertion 1). This series of amino acids can be placed at position 114 in the sequence of M, where a cluster of 20 hydrophobic and neutral amino acids occurs uninterrupted (Fig. 2). It is the longest hydrophobic stretch in M.

The major problem encountered with the protein material remaining associated with lipid vesicles after trypsinization was its remarkable insolubility. Chromatographic procedures had indicated that one component was present, although some material remained at the origin when analysis was carried out in two dimensions on silica gel plates (18). However, when sequence analysis was carried out with material that had been digested extensively with trypsin (18) and which was then exposed to acid, making the glutamic amino terminus unavailable, a second sequence could be detected. This fragment was probably produced by the chymotryptic activity present in the high concentration of trypsin used. The sequence of the first four amino acids of this component is given in Table 1 (M insertion 2). This series of amino acids is found at position 62 on the whole protein (Fig. 2) and is part of the second longest stretch of hydrophobic and neutral amino acids found in the molecule. The conclusion from these data was that M protein interacted with the lipid bilayer in two positions: one major insertion occurred approximately midway in the molecule, and a second insertion was one fourth in from the amino terminus.

The second method used to localize the area of M which inserted into lipid utilized CNBr fragments of M. It was possible that the three CNBr fragments that bound to lipid when the protein was cleaved at its methionine residues represented, in part, the same area of the molecule which partitioned into vesicles when the whole M protein was used. To determine definitively which parts of the molecule these fragments represented, we treated M with CNBr, added lipid, and separated the fragments which associated with lipid from the rest by flotation through sucrose (18). The fragments were delipidated after the fractions were collected from the gradient by adding 2 volumes of chloroform-methanol (2:1). The material was agitated, and the tubes were centrifuged at 2,500 rpm for 20 min. The aqueous and organic phases were discarded, and the interface containing the fragments was resuspended in 50% formic acid and separated on a column of Bio-Gel P-10 (1.5 by 115 cm), using 50% formic acid. Samples were removed, the formic acid was evaporated in a vacuum oven, and the radioactivity was determined after shaking vials with 0.1% sodium dodecyl sulfate in water for 1 h. The total mixture of CNBr fragments distributed themselves among seven to eight peaks on Bio-Gel P-10 (Fig. 3A; 31). CNBr fragments which associated with lipid separated into three peaks (Fig. 3B). The fractions under each peak of Fig. 3B were pooled, lyophilized, and analyzed on a Beckman sequencer.

TABLE 1. *Partial amino acid sequence of fragments that interact with lipid vesicles*[a]

| Step | Peptide | | | | |
|---|---|---|---|---|---|
| | M insertion 1 | M insertion 2 | CNBr fragment of Bio-Gel column | | |
| | | | Peak 1 | Peak 2 | Peak 3 |
| 1 | Glu | Val | Asp | Glu | Gly* |
| 2 | Ile | Phe | Lys | Lys | Leu |
| 3 | Ala* | Thr | Ala | Thr | Leu |
| 4 | Leu | Leu | Val | Arg | Thr |
| 5 | [ ] | | Lys | Pro | Glu |
| 6 | Tyr | | Leu | Ile | Val |
| 7 | Ala* | | Tyr | Leu | Glu |
| 8 | | | [ ] | | [ ] |
| 9 | | | Lys | | Tyr |
| 10 | | | Leu | | Val |
| 11 | | | Lys | | Leu |
| 12 | | | [ ] | | |
| 13 | | | Glu | | |
| 14 | | | Ile | | |

[a] Automatic amino acid sequence analysis was carried out with a Beckman model 890C sequencer with 0.1 M Quadrol buffer (13). Fragments were resuspended in 30% formic acid, and Polybrene was added (22, 36). Identification of the phenylthiohydantoin amino acids was carried out by high-pressure liquid chromatography with a Waters HPLC (model ALC/GPC-204) equipped with a Bondapak $C_{18}$ column (3.9 mm by 30 cm; Waters Associates, Milford, Mass.) and by amino acid analysis after back-hydrolysis in 0.2 ml of 6 N HCl containing 5 μl of 5% $SnCl_2$ in vacuo (24). Amino terminal analysis of fragments was performed by the dansyl chloride method (14) or by the manual Edman degradation technique (15). *, Indicates difference with the DNA sequence; [ ], indicates residues not identified.

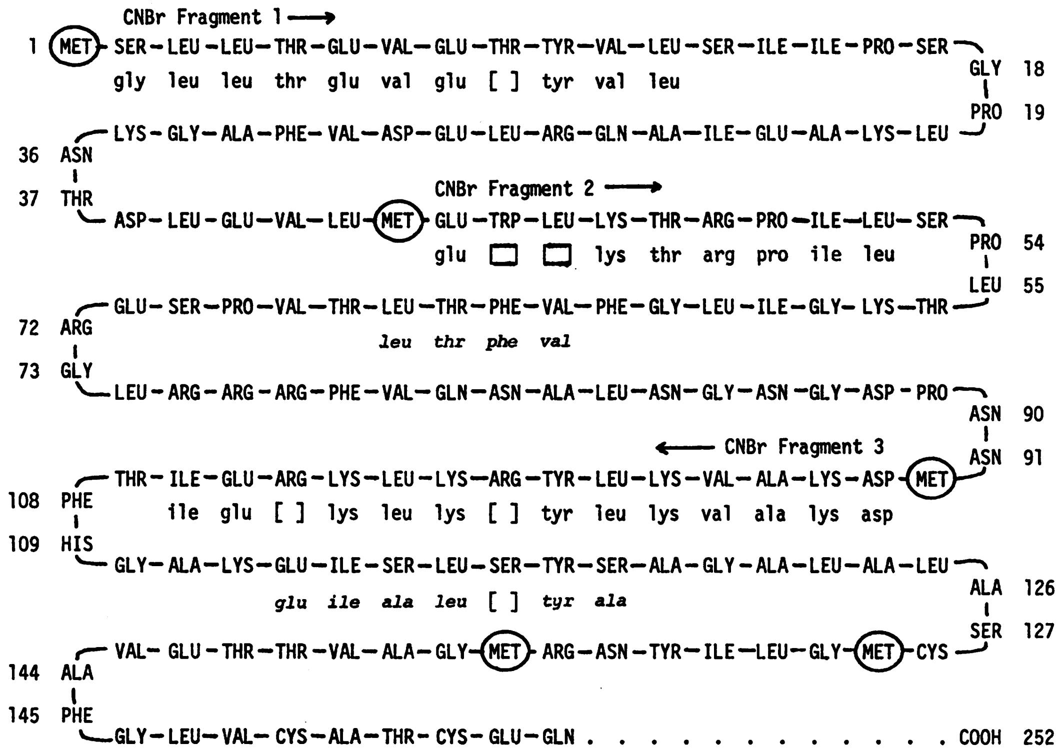


FIG. 2. *Deduced amino acid sequence of the M protein of the PR8 strain of influenza virus (37) from nucleotide analysis and localization of the segments of M which bound to lipid. The type in italics represents the insertion of whole M into vesicles. [ ] represents unidentified amino acid residues.* □, *Indicates that no residues were found in the WSN strain.*

The sequence of the first 12 amino acids of the protein in peak 1 of the Bio-Gel column is given in Table 1 and was part of the third CNBr fragment from the amino terminus of the molecule (Fig. 2). This fragment contains the major stretch of 20 uncharged and hydrophobic amino acids, which remained attached to the lipid vesicles when whole M was used (M insertion 1). The peptide present in the second peak of Bio-Gel P-10 represented the second fragment from the amino terminus (Table 1) and contained the sequence of the second insertion of whole M described above and depicted in Fig. 2. Finally, the sequence of the first 11 amino acids of the component in the third Bio-Gel peak (Table 2) was part of the first fragment containing the amino terminus of the molecule. This fragment houses the third longest hydrophobic stretch contained in M.

M may be classified as a proteolipid (12) on the basis of its solubility in organic solvents. The overall amino acid composition, however, is not particularly hydrophobic (16, 25), which could account for this solubility. It is now apparent from sequence analysis of the nucleic acid coding for M (2, 37), that there are, in fact, areas of the molecule where clusters of hydrophobic and neutral amino acids occur which may contribute to the solubility of M in organic solvents. The major cluster occurs in the middle of the molecule at position 114 of the sequence, where 20 amino acids of hydrophobic and neutral character occur uninterrupted (Fig. 2). A second cluster of 13 occurs at position 58, and a third cluster occurs at position 9, where 12 such amino acids can be found. A fourth cluster of 10 residues occurs at position 143. There are thus a number of areas in M which would not only contribute to such solubility properties, but where insertion into lipid bilayers could occur.

Although M and specific fragments of M could insert into lipid in vitro, interaction of M with lipid in virus remained unknown. Fluorescent probes embedded in the virus bilayer suggested that M could be interacting with the lipid (27). However, this conclusion was partly complicated by the presence of the hydrophobic tails of the hemagglutinin and neuraminidase still present in the lipid bilayer after proteolytic treatment of the virus (10, 26, 34, 38). PySA, which prefer-

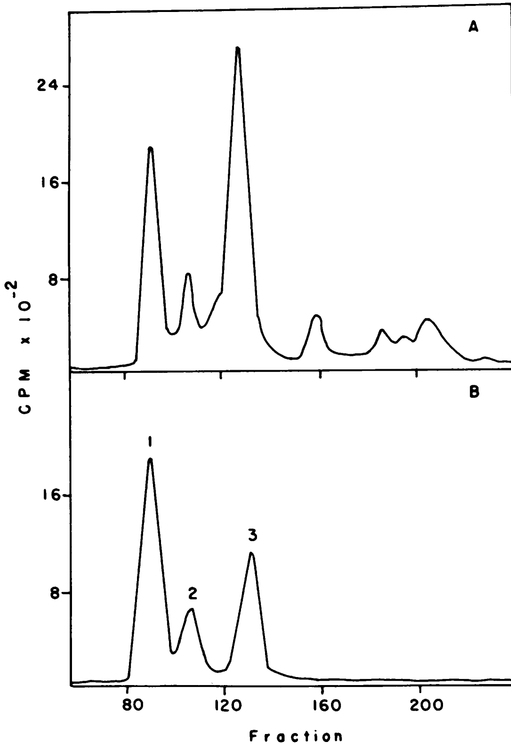


FIG. 3. *Fractionation of CNBr fragments of M on a column of Bio-Gel P-10 (1.5 by 115 cm) equilibrated with 50% formic acid. (A) Total mixture of CNBr fragments. (B) Fragments which floated upwards through discontinuous gradients which had associated with phosphatidylcholine vesicles.*

entially partitions into lipid and binds to components lying in lipid bilayers, was used here to determine the location of M in situ in the virus particle. Covalent linkage of PySA to M suggests that M is in contact with the viral lipid bilayer. A study of the interaction of isolated M with lipid vesicles in vitro indicated that whole M inserts into lipid bilayers at two sites. One site occurs at the major hydrophobic cluster located in the middle of the molecule, and a second occurs at the second longest stretch of hydrophobic and neutral amino acids found in M.

The ability of M to partition with ease into lipid may be a consideration in the overall process of assembly of the subviral components into virus particles. The protein is found on smooth and rough endoplasmic reticulum and never free in the cytoplasm (20). It may possibly partition into lipid immediately after its synthesis. It is also present in high concentrations in the nucleus even early after infection (17, 21, 28) and on the surface of infected cells (1, 5, 7). The protein may serve a number of functions. Its insertion into the lipid bilayer would have a stabilizing effect on the virus membrane. Secondly, positively charged areas on the molecule could interact with the ribonucleoprotein to facilitate assembly of the genome into virus particles.

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