

Location of the Spike Glycoproteins in the Semliki Forest Virus Membrane

(formyl-[³⁵S]methionyl sulfone methylphosphate/dimethylsuberimidate/peptide mapping/
sodium dodecyl sulfate-gel electrophoresis)

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ABSTRACT Labeling experiments with formyl-[³⁵S]-methionyl sulfone methylphosphate and crosslinking studies with dimethylsuberimidate suggest that the spike glycoproteins of Semliki Forest virus extend through the viral membrane into close contact with the nucleocapsid. Based on this finding, we present a mechanism for the formation of virus-specific patches in the host cell plasma membrane during virus assembly.

We have been studying the membrane of Semliki Forest virus (SFV), a group A togavirus, to investigate the structure and assembly of biological membranes (1). SFV is composed of a smooth-surfaced nucleocapsid, which is surrounded by a lipid membrane (2, 3). The nucleocapsid is assembled in the cytoplasm of the host cell (2). It consists of the viral RNA and one lysine-rich protein species [molecular weight (MW) 33,000] (4). The viral membrane is acquired from the host cell plasma membrane by a budding process in the final stage of SFV maturation (2). It has a lipid composition similar to that of the host cell plasma membrane (5), and its lipids seem to be arranged in a bilayer structure (6). Three virus-specific glycosylated proteins have been found in about equimolar ratios in the SFV membrane: E1, E2 (both with a MW of about 50,000), and E3 (MW about 10,000) (H. Garoff, K. Simons, and O. Renkonen, *Virology*, in press). The glycoproteins form spikes on the external surface of the bilayer, and are responsible for the hemagglutinating activity of the virus (7). The spikes can be cleaved off by thermolysin, leaving two hydrophobic peptides in the membrane derived from E1 and E2 (7). The peptides are large enough (MW about 5000 for each) to span the viral membrane like the intramembranous region of the MN glycoprotein (glycophorin) in the human erythrocyte membrane (8).

Since no host cell proteins are found in the viral membrane of the mature virus particle, this membrane must be derived from a segment of the plasma membrane from which all host cell proteins are excluded (1). How such patches of viral membrane are generated is an enigma (9). In this report we present results suggesting that one or both of the viral spike glycoproteins E1 and E2 extend through the SFV membrane into close contact with the nucleocapsid. Based on this finding, a mechanism is presented that could explain the formation of virus-specific patches in the host cell plasma membrane during virus assembly.

Abbreviations: SFV, Semliki Forest virus; MW, molecular weight; NaDodSO₄, sodium dodecyl sulfate; DMS, dimethylsuberimidate; [³⁵S]FMMP, formyl-[³⁵S]methionyl sulfone methylphosphate; NC-E complex, nucleocapsid-membrane protein complex.

We used two different experimental approaches. The first uses a radioactive labeling reagent, formyl-[³⁵S]methionyl sulfone methylphosphate ([³⁵S]FMMP) and the second a crosslinking reagent, dimethylsuberimidate (DMS). Both react with the amino groups of proteins and lipids (10-13). DMS diffuses readily across intact biological membranes (14), whereas [³⁵S]FMMP penetrates poorly (10).

MATERIALS AND METHODS

Reagents. [³⁵S]FMMP was prepared according to Bretscher (10). DMS was synthesized from suberic acid as described (15).

Virus. A prototype strain of SFV was grown in monolayer cultures of baby hamster kidney cells and purified as described (16). Virus was labeled with a mixture of [³H]leucine, [³H]isoleucine, and [³H]valine (33 μCi of each per ml) (16), and with [³²P]orthophosphate (20 μCi/ml) (17).

Labeling with [³⁵S]FMMP and Two-dimensional Peptide Mapping. Intact SFV containing 260 μg of protein in 140 μl of Na₂CO₃ buffer (pH 9.7) was mixed with 40 μl of the [³⁵S]-FMMP reagent and incubated at room temperature for 10 min. Excess reagent was removed by sucrose density gradient centrifugation (16). Viral membranes were released from another virus preparation containing 540 μg of protein by treatment with 170 μg of Triton X-100 in 170 μl of the Na₂CO₃ buffer for 10 min at room temperature as described (17) before the labeling reagent (50 μl of [³⁵S]FMMP) was added. The released membranes were separated from the nucleocapsids by centrifugation in a 15-30% (w/w) sucrose density gradient containing 0.1 M NaCl-0.05 M Tris buffer (pH 7.4) and 0.013% (w/w) Triton X-100, for 150 min at 18° and 39,000 rpm in an SW41 rotor. Fractions (0.3 ml) were collected from below, and radioactivity was measured as described (17). Samples of both labeled preparations were subjected to sodium dodecyl sulfate (NaDodSO₄)-polyacrylamide gel electrophoresis (18). The acrylamide concentration of the gels was 10%. The gels were analyzed for ³⁵S-radioactivity as described (19).

For peptide mapping, sucrose was first removed from the SFV and the membrane preparations by extensive dialysis (48 hr) against 50 mM NH₄HCO₃ containing 0.013% Triton X-100 (to decrease adsorption to the walls of the dialysis bag). The preparations were then lyophilized, taken up in 200 μl of 0.1 M NH₄HCO₃, and digested by addition first of 25 μg of both TPCK-trypsin and chymotrypsin (Worthington) and, after 2 hr, of half this amount of both enzymes. After 12 hr of incubation at 37° the digests were fractionated on

Whatman 3 M paper by chromatography (*n*-butanol-acetic acid-H₂O, 17:5:25, v/v) followed by electrophoresis at pH 3.5 in the second dimension. The peptide maps were autoradiographed on Kodirex film for 2-6 days.

Crosslinking. SFV samples containing 50 μ g of protein and usually trace amounts of radioactively labeled SFV in 50 μ l of 0.15 M NaCl were mixed with an equal volume of freshly prepared 0.2 M triethanolamine solution (pH 8.5 with HCl) containing 0.5-12 mg of DMS per ml and incubated for 2 hr at room temperature as described (20). We terminated the reaction by treating the samples with NaDodSO₄ and 2-mercaptoethanol or by removing excess reagent by sucrose density gradient centrifugation, as described below. The crosslinked virus preparations were analyzed (1) after denaturation by heating in the presence of 1% NaDodSO₄ and 1% 2-mercaptoethanol in 3.5% polyacrylamide gels (20), (2) after Triton X-100 treatment (500 μ g of detergent per 50 μ g of virus protein in a volume of 100 μ l, incubated for 30 min at room temperature) in linear 20-50% (w/w) sucrose density gradients containing NaCl-Tris buffer (pH 7.4) and 0.05% (w/w) Triton X-100, which were centrifuged for 100 min in an SW50 rotor at 18° and 45,000 rpm, and (3) by electron microscopy by negative staining with potassium phosphotungstate at pH 7.4 as described (16). "Nucleocapsids" isolated from crosslinked virus (see Fig. 5B) were also analyzed (1) by precipitation with antisera against SFV membrane protein, prepared in rabbits by immunization three times at 2-week intervals with 300 μ g of the membrane protein fraction isolated from SFV that had been disrupted with Triton X-100 (21), with Freund's complete adjuvant, (2) in hemagglutination assays (16), and (3) by velocity centrifugation for 100 min at 18° and 39,000 rpm in a linear 15-30% (w/w) sucrose gradient containing NaCl-Tris buffer (pH 7.4) and 0.05% (w/w) Triton X-100.

RESULTS

Labeling with [³⁵S]FMMP. The viral membrane was labeled from outside and from both sides, and the labeling of the membrane glycoproteins was compared by peptide mapping. Labeling from outside was performed by treating intact SFV with [³⁵S]FMMP. Electron microscopy showed no morphological changes in the labeled SFV. When analyzed by NaDodSO₄-gel electrophoresis, all three membrane glycoproteins were found to be labeled. Most (86%) of the ³⁵S

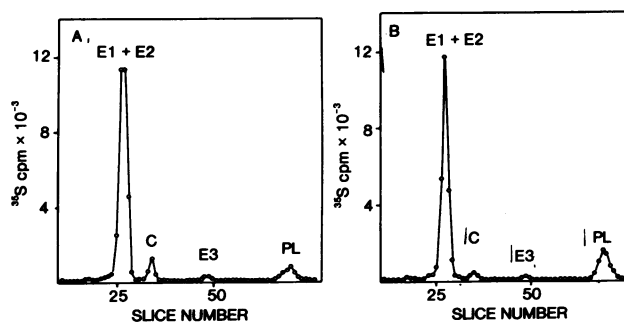


FIG. 1. NaDodSO₄-gel electrophoretic patterns of [³⁵S]-FMMP-labeled intact SFV (A) and SFV membranes (B), which were released from SFV with Triton X-100 treatment. Viral glycoproteins (E1, E2, and E3); nucleocapsid protein (C); phospholipid (PL); ³⁵S radioactivity (○). E1 and E2 are not resolved in this electrophoresis system, but radioautographic analysis of discontinuous NaDodSO₄ gels (22) showed that both were labeled.

label in the virus, was found in the E1 and E2 glycoproteins (Fig. 1A), E3 having only one lysine (H. Garoff, K. Simons, and O. Renkonen, *Virology*, in press). Less than 5% of the ³⁵S label was associated with the lysine-rich nucleocapsid protein. This confirms our earlier findings that the intact SFV membrane is almost impermeable to [³⁵S]FMMP and, thus, is labeled mostly from the outside (23). Viral membranes that had been released from nucleocapsids by treatment of the virus particles with a small amount of Triton X-100 were reacted with [³⁵S]FMMP in order to label the membrane from both sides. This preparation contained membrane sacs and fragments together with nucleocapsids when examined by electron microscopy. When the membranes were separated from the nucleocapsids by sucrose density gradient centrifugation (17), 20% of the ³⁵S label was associated with the nucleocapsids. In NaDodSO₄-gel electrophoresis (Fig. 1B) the isolated SFV membrane showed labeling of the same membrane components as in intact SFV. However, relatively more label was now associated with the phospholipids. These findings indicate that the internal surface of the viral membrane is exposed to [³⁵S]FMMP under these experimental conditions. Trypsin-chymotrypsin fingerprints of labeled intact SFV and labeled Triton X-100 released membranes are shown in Fig. 2A and B, respectively. Most of the ³⁵S-labeled peptides obtained correspond to each other. However, two additional

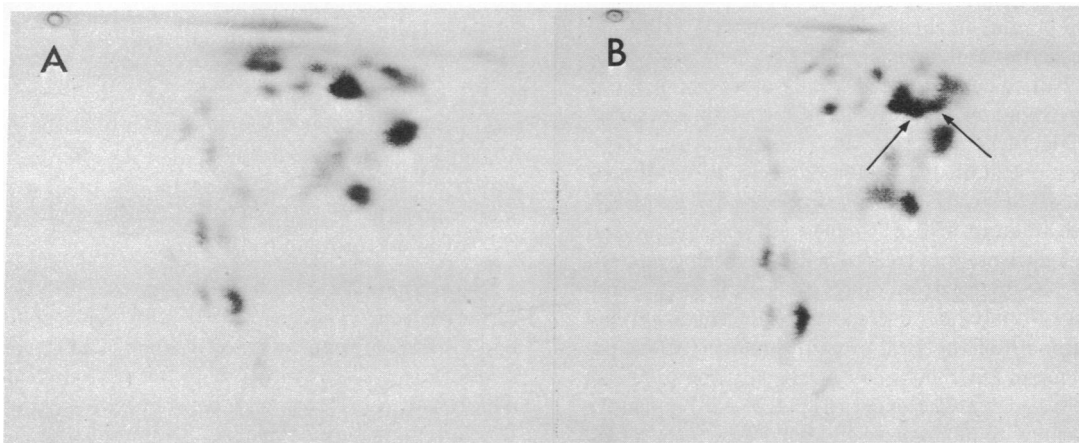


FIG. 2. Fingerprints of intact SFV (A) and SFV membranes released by Triton X-100 (B) labeled with [³⁵S]FMMP. The arrows in (B) indicate the two additional peptides found in [³⁵S]FMMP-labeled membranes.

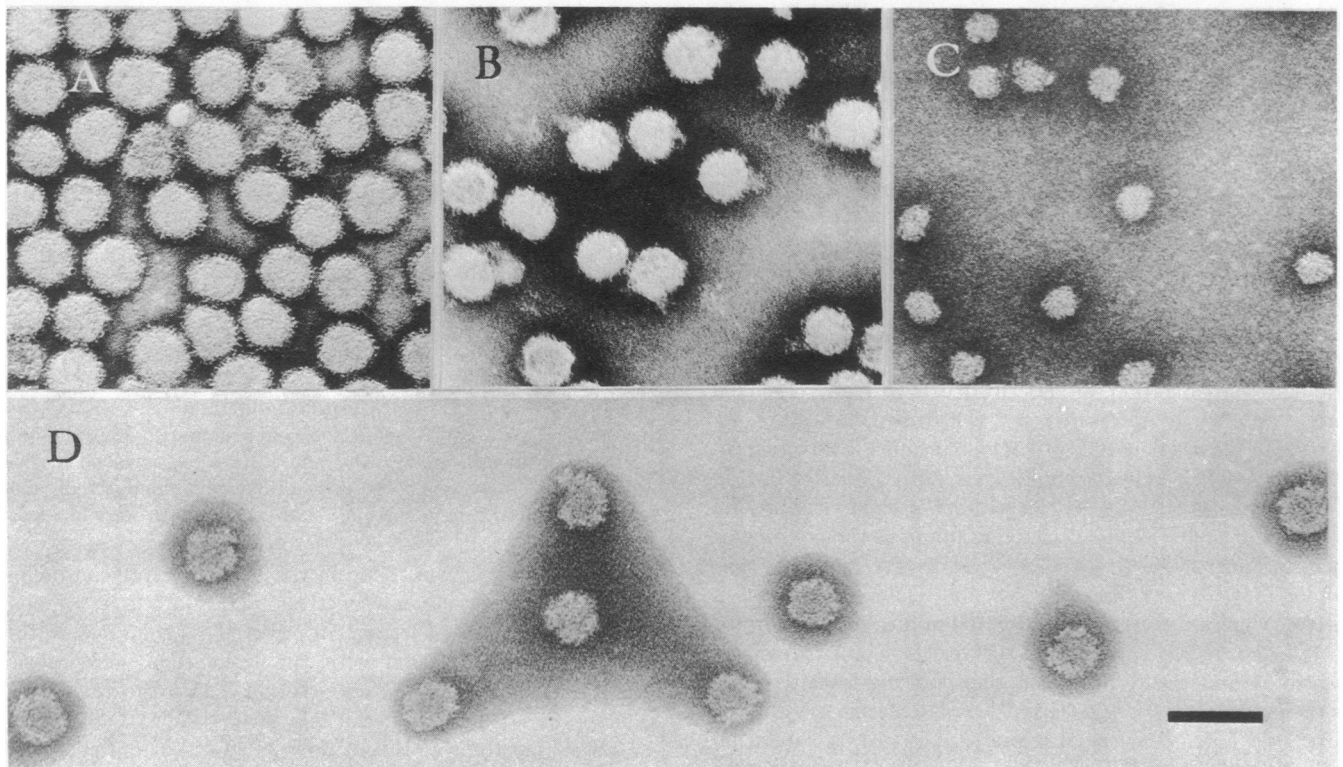


FIG. 3. Electron micrographs of intact SFV particles (A), SFV crosslinked with 3 mg of DMS per ml (B), control nucleocapsids (C), and nucleocapsid-membrane protein complexes (D), isolated as shown in Fig. 5. The bar is 100 nm. Staining was with potassium phosphotungstate at pH 7.4.

heavily labeled peptides, indicated by arrows in Fig. 2B, were found in the fingerprint of detergent-released membranes. No contamination of peptides from the nucleocapsid protein labeled with [35 S]FMMP was seen.

Crosslinking with DMS. SFV was treated with DMS at concentrations varying from 0.25–6 mg/ml. Electron micrographs of the crosslinked SFV preparations showed no morphological changes in the virus particles, except for a slight decrease in their diameter to about 62 nm (Fig. 3B) compared with about 65 nm in the control virus (Fig. 3A).

NaDodSO₄-Gel Electrophoresis. When SFV was treated with 0.25 mg of DMS per ml and analyzed by NaDodSO₄-gel electrophoresis (Fig. 4B), most of the viral polypeptides were still found to be present in their monomeric forms: as 30,000 MW nucleocapsid proteins and 50,000 MW membrane proteins (E1 and E2). The additional bands corresponded (by their molecular weights) to polymeric forms of both the nucleocapsid (Fig. 4A) and the two membrane proteins. [E3, which does not stain for protein in gels, is not cross-linked by DMS. (H. Garoff, *Virology*, in press).] When SFV, containing trace amounts of [3 H]leucine-, [3 H]isoleucine-, and [3 H]valine-labeled virus, was treated with increasing concentrations of DMS before electrophoresis, the bands corresponding to nucleocapsid polymers decreased in concentration and an increasing amount of the viral protein remained at the top of the gel. At 3 mg of DMS per ml or more, only about 50% of the viral protein penetrated the gel (Fig. 4C). All bands seen in the gel correspond to monomeric and polymeric forms of the membrane proteins. Analysis of isolated nucleocapsids that had been treated with 3 mg of DMS per ml showed that the nucleocapsid protein remained almost entirely at the origin

when run in NaDodSO₄ gels, whereas crosslinked (3 mg of DMS per ml) viral membranes (released with Triton X-100) mostly showed bands of the membrane proteins, corresponding to the bands seen in Fig. 4C, with less than 5% remaining at the top of the gel.

Dissociation with Triton X-100. Control virus, containing [3 H]leucine, [3 H]isoleucine, and [3 H]valine, was completely separated into nucleocapsids and a membrane fraction when treated with excess Triton X-100 (500 μ g of detergent per 50 μ g of virus protein) and subjected to density gradient centrifugation (Fig. 5A). The nucleocapsids contained about 17% of the total protein label. However, if the virus was crosslinked with increasing concentrations of DMS before the detergent treatment, increasing amounts of the viral protein label were associated with the nucleocapsids (forming a nucleocapsid-membrane protein complex, NC-E, Fig. 5B). A maximum of about 65% was reached at about 3 mg of DMS per ml (Fig. 6). If SFV was labeled in its RNA and phospholipids with [32 P]orthophosphate, the distribution of 32 P radioactivity between the membrane and the nucleocapsid fraction was found to be the same, within experimental errors, in DMS-treated (3 mg/ml) and control virus.

Characterization of the NC-E Complex. The NC-E complexes (isolated from virus crosslinked with 3 mg of DMS per ml) had a sedimentation constant of about 200 S, compared with nucleocapsids from control SFV, which sedimented at 150 S. The NC-E complexes were pelleted by low-speed centrifugation after antiserum against membrane protein was added. They also had hemagglutinating activity. Control nucleocapsids did not show these properties. The electron micrographs of negatively stained NC-E samples showed a fairly

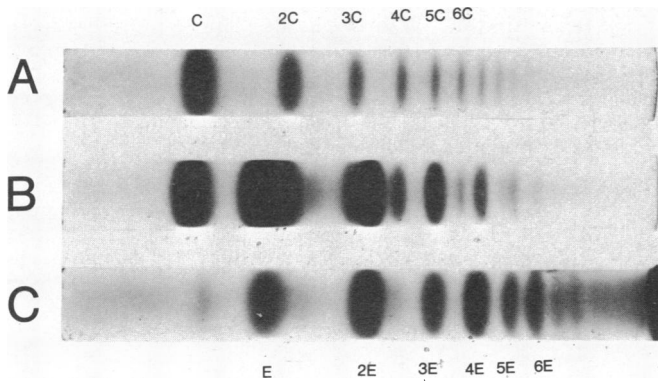


FIG. 4. NaDodSO₄ gel electrophoretic patterns of (A) nucleocapsids (10 µg of protein) crosslinked with 0.25 mg of DMS per ml, (B) SFV (50 µg of protein) crosslinked with 0.25 mg of DMS per ml, and (C) SFV (50 µg of protein) crosslinked with 3.0 mg of DMS per ml. Nucleocapsid protein monomers (C) and polymers (2-6C); 50,000 MW membrane protein monomers (E) and polymers (2-6E). Migration from right to left.

homogeneous population of particles with a rough surface and a diameter of about 56 nm (Fig. 3D). The NC-E particles were clearly larger than the control nucleocapsids, which had a diameter of about 39 nm (Fig. 3C). NC-E particles containing [³H]leucine-, [³H]isoleucine-, and [³H]valine-labeled protein were also denatured by treatment with 1% NaDodSO₄ and 1% 2-mercaptoethanol. Distinct particles were still seen in electron microscopy after such a treatment. However, they were of irregular size (diameter 55-65 nm). Control nucleocapsids were completely disrupted if treated with NaDodSO₄. When the denatured NC-E particles were subjected to sucrose density gradient centrifugation, about 65% of the material sedimented like the untreated NC-E particles or somewhat slower. In NaDodSO₄-gel electrophoresis, about 65% of the denatured NC-E complex remained at the origin; the rest consisted of a heterogeneous population of high-molecular-weight polymers.

DISCUSSION

[³⁵S]FMMP Labeling. The first convincing experiments demonstrating that a membrane protein may extend through

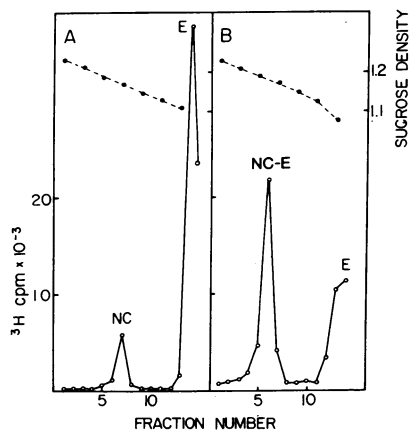


FIG. 5. Sucrose density gradient analyses of crosslinked (B) and control (A) SFV (that had been treated with Triton X-100) labeled with [³H]leucine, [³H]isoleucine, and [³H]valine. Nucleocapsids (NC); nucleocapsid-membrane protein complex (NC-E); membrane fraction (E). O, ³H radioactivity; ●, sucrose density.

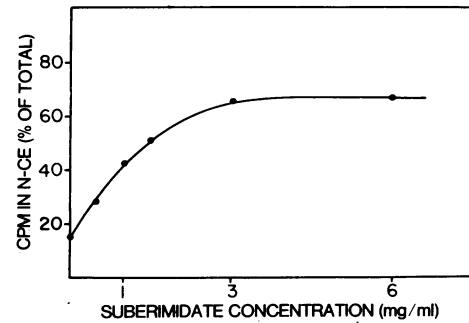


FIG. 6. The amount of nucleocapsid-membrane protein complex (NC-E) obtained as shown in Fig. 5 when SFV (labeled with [³H]leucine, [³H]isoleucine, and [³H]valine) was crosslinked at various concentrations of DMS and treated with Triton X-100.

the cell membrane were the [³⁵S]FMMP labeling studies by Bretscher on the major human erythrocyte glycoproteins (24). We have used [³⁵S]FMMP in a similar way to study whether the SFV membrane glycoproteins span the membrane. To label the viral membrane both from the external and the internal side, we released the membrane by adding small amounts of Triton X-100 to the viral particles. We know from previous studies that viral membranes released in this way still have a lamellar structure (see also ref. 25) and show surface projections similar to the spikes seen on intact virus particles (17). No lipids are extracted from the released membranes (17). The membranes retain the hemagglutinating activity of the virus (17). In general, Triton X-100 is notably mild in its effects on proteins. The detergent does not seem to induce conformational changes in water-soluble proteins (26). Many membrane proteins preserve their biological activities when solubilized with Triton X-100 (see ref. 21). In the SFV membrane, glycoprotein solubilization with (large amounts of) Triton X-100 leads to the formation of lipid-free protein-detergent complexes (21), in which the detergent appears to occupy the same binding sites on the membrane proteins as the lipids in the native membrane (7). Altogether we find it unlikely but not excluded that the small amount of Triton X-100 added would lead to such structural alterations in the SFV membrane that would expose the two additional heavily labeled peptides seen in the fingerprint in Fig. 2B. We consider it more likely that these peptides are derived from parts of the glycoproteins exposed on the internal surface of the viral membrane.

Crosslinking with DMS. Diimidoesters are mild crosslinking reagents that have been used to study intramolecular distances in protein molecules (27), subunit associations in oligomeric proteins (20), arrangement of ribosomal proteins (28, 29), and protein-protein associations in the erythrocyte membrane (12, 14, 30). DMS can crosslink protein amino groups not further apart than 11 Å (28). This corresponds to about one-fourth of the SFV bilayer thickness (6). An extensive crosslinking of the spike glycoproteins to the nucleocapsid would therefore be possible only if the glycoproteins extended through the viral membrane into close contact with the nucleocapsid. All the available evidence suggests that the nucleocapsid does not protrude into the lipid bilayer (6, 17).

When SFV was treated with 3 mg of DMS or more per ml and analyzed in NaDodSO₄ gels, about 50% of the viral protein remained at the top of the gel. This material contained most of the nucleocapsid protein, since only faint bands corresponding to its monomeric and polymeric forms were seen

in NaDodSO₄ gels of both virus and isolated nucleocapsids that had been crosslinked with 3 mg of DMS per ml. However, since the nucleocapsid protein accounts for only about 20% of the total viral protein, most of the material at the top of the gel must be membrane protein. This membrane protein is apparently linked to the nucleocapsid because more than 95% of the membrane protein penetrated the gel when released with Triton X-100 from its contact with the nucleocapsid before crosslinking. We were indeed able to isolate nucleocapsid particles after Triton X-100 treatment of crosslinked virus, with which up to about 65% of the membrane glycoproteins were still associated. All phospholipid was removed from these particles. Most of the associated membrane protein remained attached to the nucleocapsid even after NaDodSO₄ and mercaptoethanol denaturation. The possibility that the membrane protein would be attached to the nucleocapsid not by covalent membrane protein-nucleocapsid linkages, but by the formation of a crosslinked network of membrane protein enclosing the nucleocapsid, seems unlikely. The amount of membrane protein that was attached to the nucleocapsid at low DMS concentrations is insufficient to form such a network. The fact that no low-molecular-weight nucleocapsid protein-membrane protein polymers could be detected by gel electrophoresis of crosslinked SFV is probably explained by the very low concentration of such complexes due to rapid and complete crosslinking of the lysine-rich nucleocapsid proteins into high-molecular-weight complexes, to which the membrane proteins are crosslinked.

A Mechanism for the Budding Process in SFV Assembly. Our results suggest a mechanism for the budding process in SFV assembly, which could explain the generation of virus-specific patches in the host cell plasma membrane during budding. The nucleocapsid and the viral glycoproteins move through different routes to the plasma membrane (9). Several observations indicate that the viral spike glycoproteins arrive at the plasma membrane before the nucleocapsid (9). If the viral glycoproteins E1 and (or) E2 span the plasma membrane in the same way as in the virus, the nucleocapsid may bind to those parts of the spike glycoproteins that are exposed on the cytoplasmic surface of the host cell membrane. The nucleocapsid then acts as a nucleation site for additional glycoproteins moving into the growing patch in the plasma membrane by lateral diffusion. The association of the viral glycoproteins with the nucleocapsid proceeds until all binding sites are filled, which leads to the release of the mature virus particle into the extra-cellular medium. We assume that host cell proteins move out from the viral glycoprotein patch simply for steric reasons.

This model for the budding mechanism of virus assembly is analogous to the patch formation induced by antibody to surface antigens in lymphocytes (31), or more closely to the aggregation of anionic external sites induced by anti-spectrin reacting with the internal surface of erythrocyte ghosts (32). Similar mechanisms may be responsible for the generation of virus specific patches in the host cell membranes during the assembly of other enveloped viruses.

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