Fusion of Semliki Forest Virus with the Plasma Membrane Can be Induced by Low pH

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ABSTRACT When BHK-21 cells with Semliki Forest virus (SFV) bound at the plasma membrane are briefly treated with low pH medium (pH 5–6), fusion between the viral membrane and the plasma membrane occurs, releasing the viral nucleocapsid into the cytoplasm. The fusion reaction resembles that described previously for Sendai virus but with one fundamental difference; it is strictly dependent on low pH. The fusion reaction is highly efficient. Up to 86% of bound viruses fuse, and 6×10^6 virus spike proteins can be inserted into the plasma membrane of each cell. The process is very rapid (full activity is observed after 5 s) and it occurs over a wide temperature range and equally well with all five cell lines tested (BHK-21, HeLa B, HeLa suspension, Raji, and 3T3).

Low pH-induced fusion of the virus at the plasma membrane can lead to infection of susceptible cells. The artificial nature of this infection pathway is, however, demonstrated by the facts that infection through the plasma membrane occurs only at subphysiological pH and that it is insensitive to inhibitors of the normal entry route. Nevertheless, these results indicate that low pH membrane fusion introduces the viral genome into the cytoplasm in a form suitable for replication.

Semliki Forest virus (SFV), an alphavirus, enters cells by adsorptive endocytosis and is subsequently delivered to intracellular vacuoles and lysosomes (1, 2). Our previous results indicate that the final and critical step in the penetration of the viral genome into the cytoplasm is a low pH-induced membrane fusion event between the viral and lysosomal membranes (1–3). That SFV possesses membrane fusion activity, similar to that observed for paramyxoviruses (4, 5), has recently been confirmed by studies with erythrocytes (6, 7) and liposomes (1, 8). SFV causes hemolysis and erythrocyte fusion (6, 7), and it fuses with artificial lipid bilayers (liposomes), introducing the viral nucleocapsid into the internal space of the liposomes (8). The fusion is very rapid, up to 96% efficient, and nonleaky (i.e., the internal contents of the virus and liposomes do not mix with the external phase) (8). Cholesterol is required in the target membranes, and the virus spike glycoproteins must be intact. In contrast to the membrane fusion activity of paramyxoviruses, the fusion events observed with SFV are strictly dependent on a pH of 6 or below (6–8).

Here we show that low pH can trigger efficient fusion between the SFV membrane and the plasma membranes of a variety of tissue culture cells. The fusion inserts viral spike glycoproteins into the plasma membrane and the nucleocapsid into the cytoplasm. In some of the cell lines, the fusion results in infection. However, because this method of infection has features clearly different from those observed during normal infection, fusion at the plasma membrane can be ruled out as the physiological mechanism of SFV entry into host cells.

MATERIALS AND METHODS

Virus and Cells

A prototype strain of SFV was grown in BHK-21 cells and purified as described (9). SFV was labeled with either [^{35}S]methionine (Radiochemical Centre, Amersham, England) (0.4 mCi/ml culture medium) or [^{3}H]uridine (0.1 mCi/ml culture medium) (Radiochemical Centre), and stored as described previously (1, 10). The specific activities were 3×10^3 virus particles per cpm ^{35}S and 8.1×10^4 particles per cpm ^{3}H. BHK-21 cells were grown in Glasgow MEM medium as described (1). HeLa B and 3T3 cells were grown in Eagle's MEM with Earle's salts, 10% fetal calf serum, 2 mM glutamine, 100 U/ml penicillin, and 0.1 mg/ml streptomycin sulfate. The cells were grown in a 37°C, 5% CO_2 incubator (Forma Scientific, Inc., Mallinckrodt, Inc., Marietta, Ohio) and passaged twice weekly. HeLa suspension cells were grown in Eagle's S-MEM containing 1 g of sodium bicarbonate/liter, 5% fetal calf serum, 5% newborn calf serum, 100 U/ml penicillin, and 0.1 mg/ml streptomycin sulfate in Spinner flasks at 37°C without CO_2 and split twice weekly at a ratio of 1:3 or 1:4. Raji cells

THE JOURNAL OF CELL BIOLOGY · VOLUME 87 OCTOBER 1980 264-272

(lymphoblastoid-like cells from a Burkitt lymphoma) were grown in RPMI 1640 medium containing 10% newborn calf serum, fungizone (0.5 μg/ml), and kanamycin (100 μg/ml) in falcon flasks (Falcon Labware, Div. of Becton, Dickson & Co., Oxnard, Calif.) in a 5% CO_2 incubator at 37°C. All of the reagents for tissue culture were obtained from the Grand Island Biological Co., (Grand Island, N. Y.). Plastic cell culture bottles (75 cm^2, Falcon Labware) petri dishes (3.5 × 1.0 cm, Falcon Labware), 24-well Linbro trays (Linbro Div., Flow Laboratories, Hamden, Conn.), and glass coverslips (1.1 cm diameter) were used.

Fusion of SFV with the Cell Plasma Membrane

SFV in RPMI 1640 medium containing 0.2% bovine serum albumin (BSA, fraction V, Armour Pharmaceutical, Co., Chicago, Ill.) and 10 mM HEPES (Serva, W. Germany), pH 6.8 (binding medium) was allowed to bind to the cells for 1.5 h at 4°C. The binding medium was removed and the cells were incubated for the indicated time at 37°C in 1 ml of RPMI 1640 medium containing 0.2% BSA, and 10 mM (2-[morpholino]ethane sulfonic acid, (MES, Serva) at varying pH values. The medium was removed and replaced with RPMI medium at pH 7.2 and 4°C. Controls included cells that had not been treated with virus and cells treated with virus but maintained at pH 7.2 throughout.

Proteinase K Treatment to Remove Bound SFV

Surface-bound SFV was removed from the monolayer cells (BHK-21, 3T3, and HeLa B) using proteinase K as described previously (1, 3). The amount of virus bound was determined as described in reference 3. A slightly modified procedure was employed to remove surface-bound [^{35}S]methionine or [^{3}H]uridine-labeled SFV from suspension cells. The cells (2×10^6 in 1.5 ml Eppendorf tubes [Hamburg, W. Germany]) were washed twice with binding medium, and radioactively labeled SFV (50 μl in binding medium) was allowed to bind to the cells for 1 h on ice with occasional vortexing. The cells were pelleted, washed once with binding medium, and then 1 ml of 37°C, pH 5.5 medium (RPMI, 10 mM MES, 0.2% BSA) was added. The cells were quickly resuspended by vortexing, placed in a 37°C water bath for 30 s, and then chilled. After pelleting (5,500 rpm, 4°C, in a table-top centrifuge [Wifug, Stockholm, Sweden]), the cells were resuspended and incubated for 45 min at 4°C in 0.5 ml of a phosphate-buffered saline (PBS) solution containing 0.5 mg of proteinase K (Boehringer, Mannheim, W. Germany) per ml. Then 0.5 ml of PBS containing 30 mg of BSA/ml (PBS-BSA) and 4 mM phenylmethylsulfonyl fluoride (PMSF, Sigma Chemical Co., St. Louis, Mo.) was added. The cells were pelleted as above, and washed two times with 1 ml of PBS-BSA, and transferred to scintillation vials with 3 ml Triton X-100/toluol scintillation fluid (Rotiszint 22 (containing 800 ml H_2O/10 liter), Roth Chemical Co., Karlsruhe, W. Germany). An additional 6 ml of scintillation fluid was added to each vial, and the radioactivity was determined in a Mark III scintillation counter (Searle Analytic Inc., Des Plaines, Ill.). To determine the amount of SFV bound, the cells (after the 1-h binding period in the cold) were washed twice with cold binding medium and analyzed for cell-associated radioactivity as above.

Electron Microscopy

For thin-section electron microscopy, confluent BHK-21 cells on coverslips were incubated for 1 h with 8 μg SFV in 50 μl binding medium on ice. The coverslips were dipped rapidly into 37°C RPMI medium of the indicated pH value for 2 or 10 s, then into ice-cold RPMI medium, pH 7.2, and then immediately fixed for 30 min with ice-cold 2.5% glutaraldehyde in 0.05 M cacodylate buffer, pH 7.2. Staining with ferritin-labeled antibodies to visualize the viral glycoprotein spikes was conducted as follows: After the indicated treatments, the cells were fixed for 20 min at room temperature with 0.75% glutaraldehyde in 0.05 M cacodylate buffer, pH 7.2, treated with 2% BSA in PBS for 40 min at room temperature, washed two times with PBS, and labeled for 30 min with 50 μl of rabbit anti-SFV antiserum diluted 1:4 in PBS. The cells were then washed twice with PBS, labeled for 30 min at room temperature with 50 μl of ferritin-conjugated goat anti-rabbit IgG (Miles Laboratories Inc., Miles Research Products, Elkart, Ind.), diluted 1:10 in PBS, washed thoroughly in PBS, and then fixed for 30 min in 2.5% glutaraldehyde in 0.05 M cacodylate buffer, pH 7.2 on ice. All antibody solutions were centrifuged at 5,500 rpm to remove aggregated material before use. Dehydration, embedding, sectioning, and staining with uranyl acetate were performed as described previously (1, 11). A Philips 400 electron microscope was employed.

[^{3}H]Uridine Incorporation Assay

The incorporation of [^{3}H]uridine into viral RNA under different conditions of infection was determined by a modification of the method of Miller et al. (12), for which a detailed description will be presented elsewhere.[1] Briefly, BHK-21 cells were grown as confluent monolayers on 11-mm diameter glass coverslips in 24-well Linbro trays. After washing with cold binding medium, 2×10^7 plaque-forming units (PFU) of SFV (20 PFU/cell) were added to each well in 0.25 ml cold binding medium. The virus was allowed to bind to the cells at 4°C for 10 min and, after the indicated treatments, the medium was replaced with 37°C medium adjusted to pH 7.2. After 1.5 h at 37°C in a 5% CO_2 incubator, actinomycin D was added to a final concentration of 2 μg/ml to inhibit cellular RNA synthesis, and after an additional 30 min at 37°C, [^{3}H]uridine (2.5 μCi in 0.25 ml medium containing 2 μg/ml actinomycin D) was added to each well, and the cells incubated for a further 2.5 h. The coverslips were then removed, washed thoroughly with cold PBS containing 10 mM uridine, transferred to new trays containing 1 ml of PBS/well for 15 min on ice, and then treated for 60 min with 10% TCA at 4°C. The coverslips were then removed, placed in scintillation vials, and the radioactivity was determined after addition of 7 ml of Rotiszint 22. Samples were prepared in triplicate. The background radioactivity in the presence of actinomycin D but in the absence of virus was <5% of the maximum incorporation and was subtracted from the other values.

Other Methods

SDS polyacrylamide gel electrophoresis was conducted using a modified Laemmli system (13). Samples of cellular material were prepared for analysis as follows: Pelleted cells from one 35-mm petri dish were resuspended in 200 μl of buffer containing 2% SDS, 50 mM Tris, pH 8.0, 2 mM PMSF. The solution was then passed up and down 20 times in a syringe fitted with a 22-gauge needle, heated to 95°C for 5 min, and then 5–10-μl samples were reduced and alkylated as described in reference 13. Fluorography was conducted as described previously (8). Indirect immunofluorescence was conducted as described (1) using rabbit anti-SFV-spike protein serum (14) and affinity-purified goat anti-rabbit IgG labeled with 3 mol rhodamine/mol IgG. Antibody solutions were centrifuged to remove aggregated material before use.

RESULTS

Effect of Lysosomotropic Weak Bases on SFV Infection

Chloroquine, a lysosomotropic weak base, inhibits SFV infection by preventing the penetration of the viral genome into the cytoplasm (1, 3, and footnote 1). We observed previously that this inhibition could be bypassed if BHK-21 cells with adsorbed virus were briefly treated with pH 5.5 medium at 37°C (1). We have confirmed and extended this finding using three lysosomotropic weak bases (chloroquine, ammonium chloride, and methylamine) and a convenient [^{3}H]uridine incorporation assay to determine the extent of infection. Viruses were allowed to bind to cells in the cold in neutral pH medium which was replaced after 10 min with 37°C medium at different specified pH values for 90 s. The cells were returned to neutral medium and placed in a 37°C CO_2 incubator. Infection was assayed after 4.5 h by determining the amount of [^{3}H]uridine incorporated into viral RNA. The indicated lysosomotropic inhibitors were present in all of the solutions.

The results in Fig. 1 show that, at neutrality, SFV infection was inhibited by 80–95% by the lysosomotropic weak bases. In each case, however, the inhibition could be by-passed at pH 6 or below. The extent of infection at pH 5.5 in the presence of chloroquine was reproducibly higher than in the control (Fig. 1). Considering the similarity in the pH dependence of infection (Fig. 1) and the virus fusion activity (6–8), the most likely explanation for the bypass of inhibition was that the low pH triggered fusion between the viral membrane and the plasma membrane and thereby released the nucleocapsid directly into the cytoplasm for replication.

[1] Helenius, A., M. Marsh, and J. White. Manuscript in preparation.

Fusion of SFV with the Plasma Membrane

Thin-section electron microscopy was used to determine whether fusion occurred at the plasma membrane. When cells with virus bound at the surface were warmed up at neutrality, no fusion was observed; instead, viruses were seen entering the cell in coated pits and coated vesicles as described previously (1). If the warming was performed at pH 5.5 as described above for the inhibitor experiments and the cells fixed after 2–10 s, fusion was observed (Fig. 2). It occurred mainly at the microvilli which are the preferential binding sites for SFV on BHK-21 cells (1) and was also often seen at the edges of coated pits but never within the coated regions (Fig. 3).

Several stages of fusion could be seen in samples fixed after 10 s of warning. Apparently the fusion begins by a close approach of the viral membrane and the plasma membrane. The distance between the two membranes (9–12 nm at neutrality, Fig. 2*a*) is reduced to 5 nm or less (Fig. 2*b*). A narrow neck region is formed between the membranes (Fig. 2*c*) which widens (Fig. 2*d–f*), revealing the continuity between the membranes (Fig. 2*h* and *k*). The densely stained spherical nucleocapsid (diameter 33 nm) becomes clearly visible underlying the membrane (Fig. 2*k*) and, in the next stage, it dissociates from the membrane (Fig. 2*i–k*). After a 5-min further incubation at pH 7.2, intracellular nucleocapsids were only occasionally observed, and after 20 min they could no longer be seen. However, the viral spike proteins could still be identified in the plasma membrane by indirect immunoferritin staining. Initially, the spike proteins are restricted to the fusion site (Fig. 4) but, at later times, when the nucleocapsids are no longer discernible, the spike proteins appear diffusely distributed along the plasma membrane surface. The fusion process, although reversed in direction, is morphologically quite similar to the budding of virus particles from infected cells. The nucleocapsid size (33 nm) and shape are the same and similar intermediate stages seem to be involved. Whereas fusion occurs mainly on the microvilli, the budding does not display such preference (15).

Quantitative Assay for Fusion

To determine the fusion quantitatively, we took advantage of the fact that the cell surface receptors for SFV on BHK-21 cells are sensitive to proteinase K digestion in the cold. The enzyme removes 97% of viruses bound to the cell surface with little damage to the virus itself or to cell viability (1, 3). Morphological and biochemical methods were employed to show that proteinase K could be utilized to differentiate between bound and fused viruses.

SFV was allowed to bind to confluent cell monolayers at 4°C, at which temperature no endocytosis occurs (3). The cells were then incubated for 30 s at 37°C in medium of pH 7.2 or 5.5, chilled to 4°C, and digested with proteinase K in the cold. Indirect immunofluorescence using rabbit anti-SFV-spike protein antiserum and rhodamine-labeled goat anti-rabbit IgG was used to detect viral spike protein antigens that remained on the cell surface. The results in Fig. 5 show that the cells kept at pH 7.2 throughout (Fig. 5 *c* and *d*) lost all viral surface proteins during the proteinase K treatment, whereas the cells subjected to low pH treatment were brightly stained (Fig. 5 *b*). The stain occurred in patches, and in many cases only one segment of the cell surface (presumably the part originally facing the bulk solution) was stained (Fig. 5*a* and *b*). Thin-section electron microscopy confirmed that neither cell preparation contained virus particles on the cell surface.

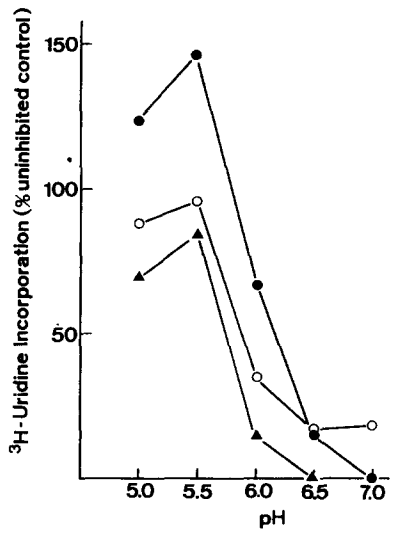

FIGURE 1 pH dependence of SFV infection in the presence of lysosomotropic weak bases. Virus (20 PFU/cell) was allowed to bind to BHK-21 cells in Linbro trays for 10 min on ice. The cells were then warmed by addition of 2 ml of serum-free MEM (37°C) containing 0.2% BSA and 10 mM MES adjusted to the indicated pH. After 90 s, the media were removed, and replaced with 1 ml of pH 7.2 serum-free medium containing 0.2% BSA and 10 mM HEPES and the cells were incubated in a 37°C, CO_2 incubator for 1.5 h. The incorporation of [^{3}H]uridine during a 3-h labeling period in the presence of actinomycin D was then determined. All solutions except the uninhibited controls contained lysosomotropic agents: (●) 0.2 mM chloroquine, (○) 20 mM NH_4Cl, and (▲) 20 mM methylamine.

These results were confirmed biochemically with [^{35}S]methionine-labeled SFV. After virus binding in the cold, cells were warmed up at pH 7.2 or pH 5.5 for 30 s and chilled to 4°C. One plate of cells treated at pH 7.2 and another treated at pH 5.5 were digested with proteinase K in the cold, washed, and solubilized in SDS. Fig. 6 shows a fluorogram obtained after SDS gel electrophoresis. It is clear (see lanes 1 and 2) that proteinase K removes virtually all of the virus proteins originally bound to the cells which were kept at neutral pH. The barely visible traces of E1/E2 (the membrane glycoproteins) and the capsid (C) protein (the only protein component of the nucleocapsid) seen in lanes 2 and 4 probably represent the small amount of endocytosed viruses. During a 30-s warming period, only 5% of the surface-bound SFV is endocytosed (3, see Fig. 9, below). In agreement with the immunofluorescence results, the cells warmed at pH 5.5 retained most of the viral proteins in a proteinase K resistant form (lane 3). Proteinase K could also be used to show that after low pH treatment the spike glycopolypeptides E1 and E2 were at the plasma membrane of the cell. We have previously observed that the viral surface proteins are sensitive to proteinase K if digestion is performed at 37°C instead of on ice (8). Lane 5 shows that when the cells that had been incubated at pH 5.5 were digested at 37°C, the spike proteins were digested but the C protein was not. This provides biochemical evidence that the nucleocapsid has been internalized and that the spike glycoproteins remain at the plasma membrane accessible to the bulk solution.

These results show that proteinase K digestion in the cold can be used to quantitate the amount of radioactively labeled virus that has fused at the cell surface.

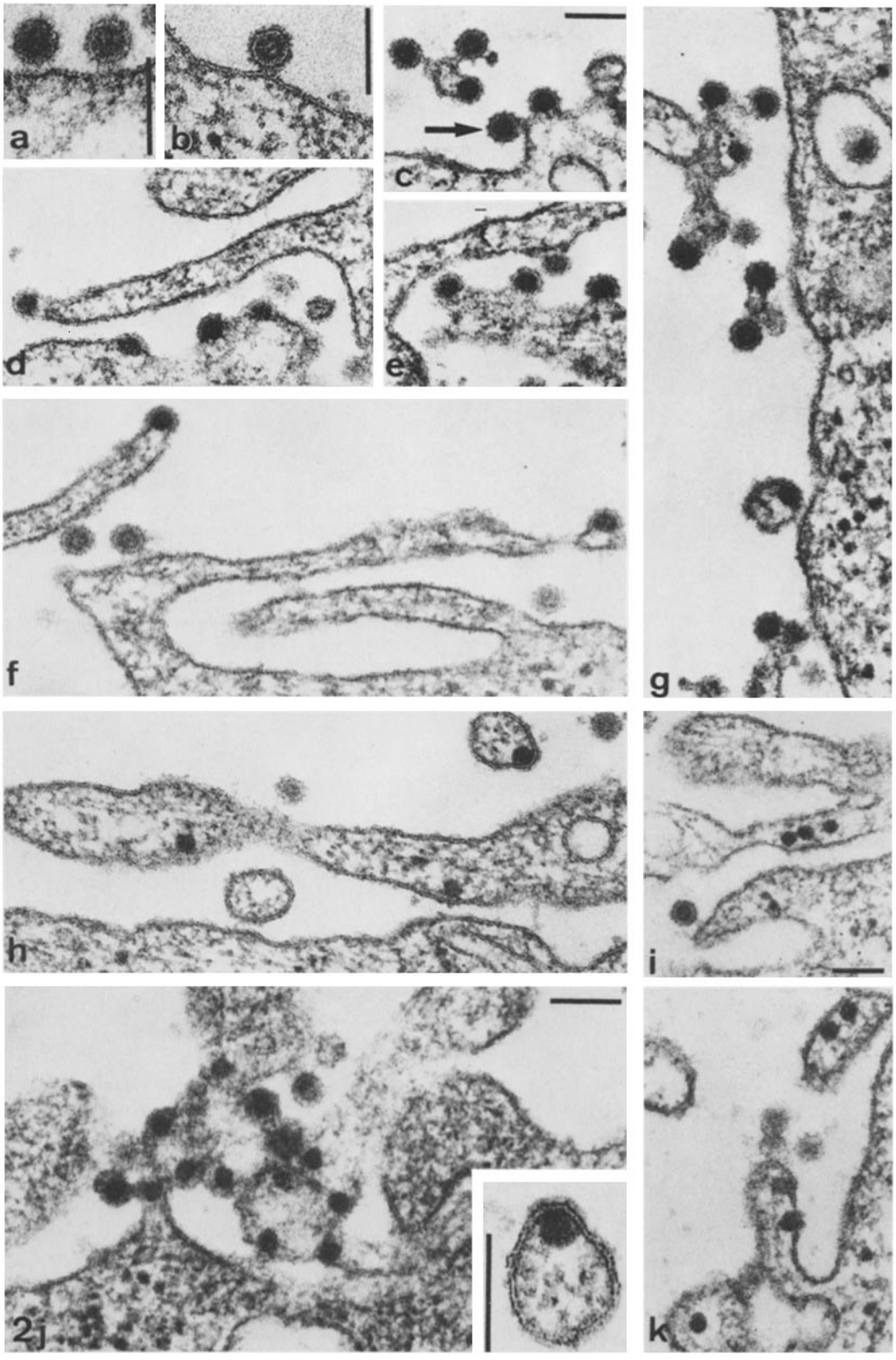

FIGURE 2 Interaction of SFV with the plasma membrane at pH 5.5. Viruses were allowed to bind to cells in the cold, whereafter the cells were either fixed directly (*a*), or after warming to 37°C in pH 5.5 medium for 2 s (*a–g*) or 10 s (*h–k*). Fusion occurs preferentially at the microvilli. The initial stage in the fusion is the close approach of the viral and plasma membranes (*b*). The next stage is characterized by the formation of a neck region between the two membranes (arrow in *c*) which widens (e.g., *d–f*), revealing the continuity between the viral and plasma membranes (e.g., upper right in *h*, *inset* in *j*). At later time points, the nucleocapsids can be seen either underlying the plasma membrane (*h*, *j*, and *k*) or freely dissociated in the cytoplasm (*i* and *k*). Bars, 0.1 μm; (*a*) × 150,000; (*b*) × 135,000; (*c–h* and *k*) × 93,000; (*i*) × 80,000; (*j*) × 110,000; *inset* × 185,000.

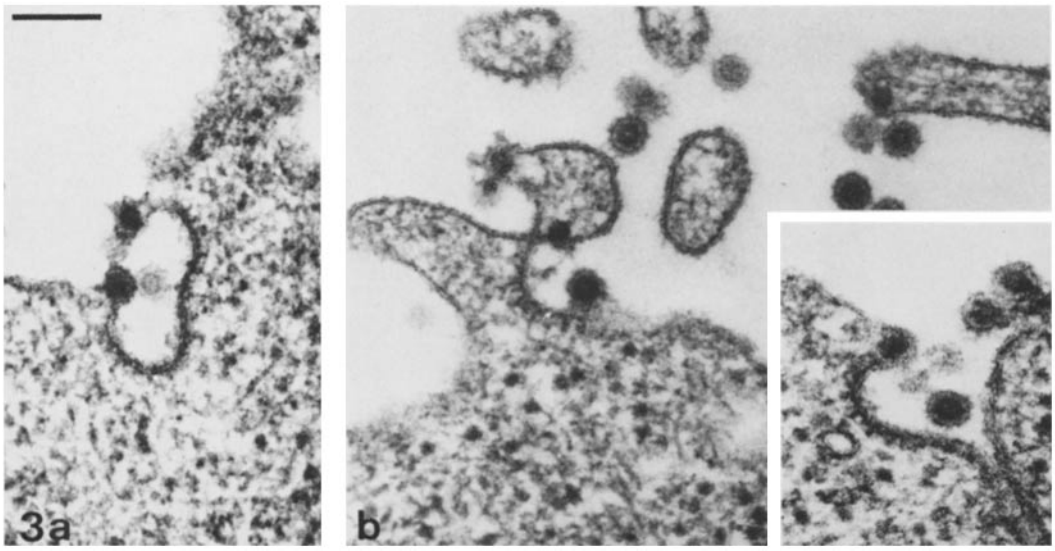

FIGURE 3 Fusion of SFV does not occur in coated pits. Fusion is, however, seen at the edges of the coated region. Virus was allowed to bind to cells in the cold, after which the cells were warmed to 37°C in pH 5.5 medium for 10 s before fixation. Bars, 0.1 μm. × 115,000.

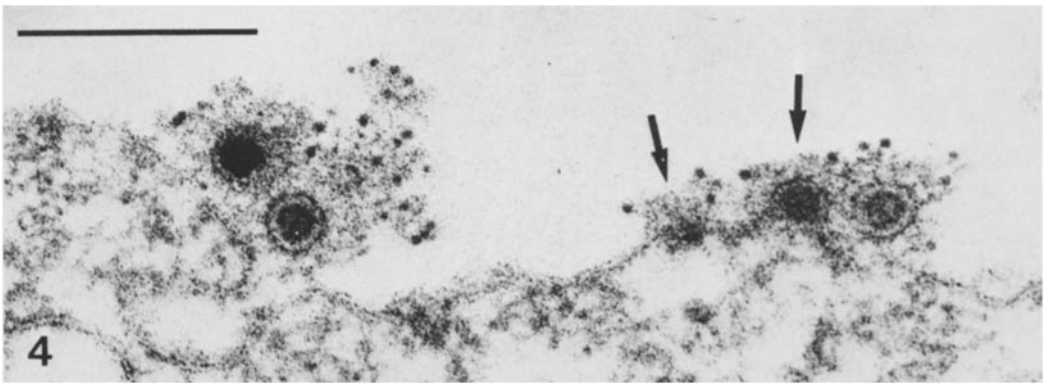

FIGURE 4 Visualization of SFV spike proteins on the BHK-21 cell surface as detected by indirect immune electron microscopy using ferritin-labeled antibody. Virus was allowed to bind to cells in the cold. The cells were then warmed to 37°C for 30 s in pH 5.5 medium and then further incubated in pH 7.2 medium for 5 min. The spike proteins are initially seen localized at the fusion site (arrows). Bar, 0.2 μm. × 165,000.

Properties of the Fusion Reaction

Using resistance to proteinase K as an assay, we characterized the efficiency of fusion between SFV and the plasma membrane of BHK-21 cells under different conditions. When trace amounts of ^{35}S-methionine-labeled virus were used, we found that 84% of the cell-bound viruses fused with the plasma membrane during a 30 s warming period with pH 5.5 medium. As the amount of virus was increased, the relative efficiency of fusion decreased (Table I). At the highest virus concentration, 2.5×10^4 viruses were fused per cell. Because there are 240 spike proteins (16) and 3.5×10^4 lipid molecules (17) per virus, this amounts to the incorporation of 6×10^6 spike proteins and 8.7×10^8 lipid molecules into the plasma membrane. Using 8.4×10^{-3} μm^2 as the membrane area of a virus particle (18) and 5×10^3 μm^2 as the surface area of a BHK-21 cell (1), this insertion increases the plasma membrane surface area by 4%.

Fig. 7 shows that fusion at the plasma membrane is strictly dependent on low pH. The pH dependence curve is identical to that previously observed for fusion between SFV and liposomes (8), but slightly shifted compared to the pH dependence of infection seen in the presence of lysosomotropic agents (Fig. 1).

Although maximal between 25° and 35°C, fusion was observed over a wide temperature range (Fig. 8). Considerable fusion (25% of maximal) was observed at 0°C, in agreement with our results using liposomes as target membranes (8). When cells with prebound virus were treated at 37°C with pH 5.5 medium, the virus became proteinase K resistant extremely rapidly with full effect at 5 s (Fig. 9). In controls where the pH was kept at 7.2, there was a slow increase in protection, owing to the adsorptive endocytosis of viruses (3).

To determine whether lysosomotropic weak bases affect the fusion reaction, chloroquine (0.2 mM), amantadine (0.5 mM), and ammonium chloride (20 mM) were included in the binding and the fusion media. Fusion was induced by a 30-s drop in pH to 5.5 at 37°C, and the extent of fusion was determined using proteinase K in the cold. The lysosomotropic agents had no significant effect on fusion.

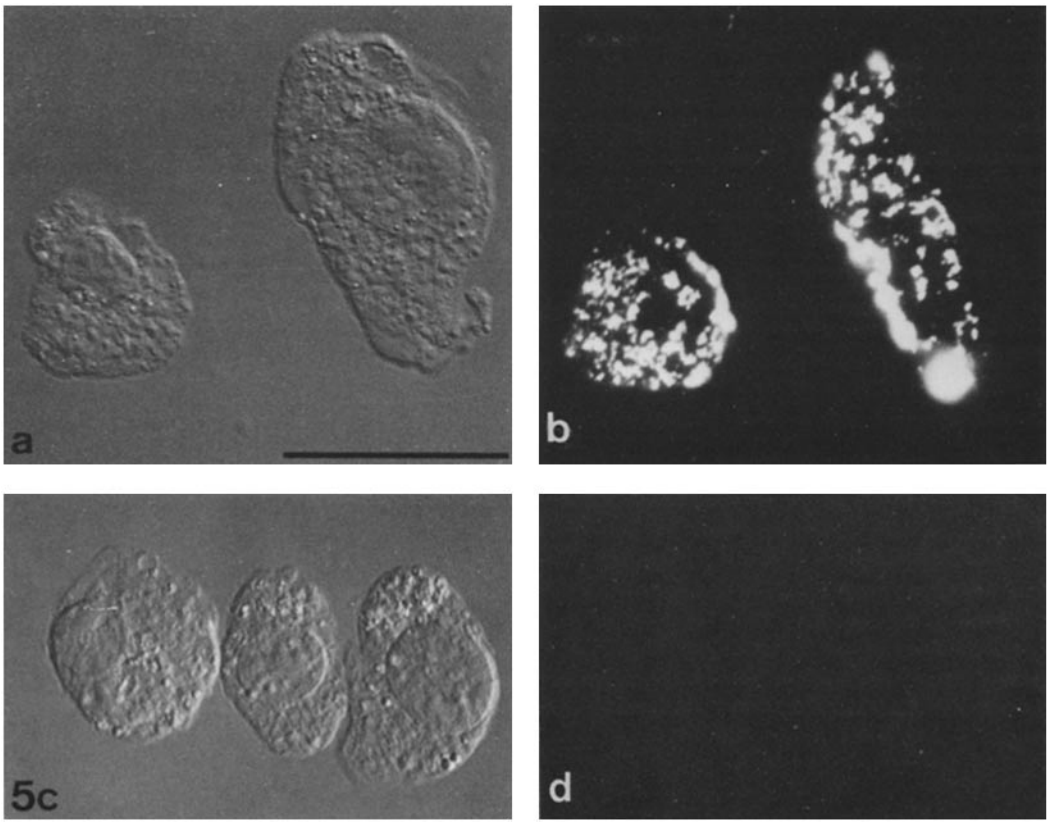

FIGURE 5 Distribution of SFV spike glycoproteins on the surface of proteinase K treated BHK-21 cells. Virus was allowed to bind to cells at 4°C. The cells were warmed to 37°C with pH 5.5 (*a* and *b*) or pH 7.2 (*c* and *d*) media for 30 s, treated with proteinase K for 45 min in the cold, and stained for spike proteins by indirect immunofluorescence. Often, only one part of the cells treated at low pH (probably the part originally facing the medium) was fluorescent. Nomarsky optics (*a* and *c*). Fluorescence optics (*b* and *d*). Bars, 30 μm. × 1,000.

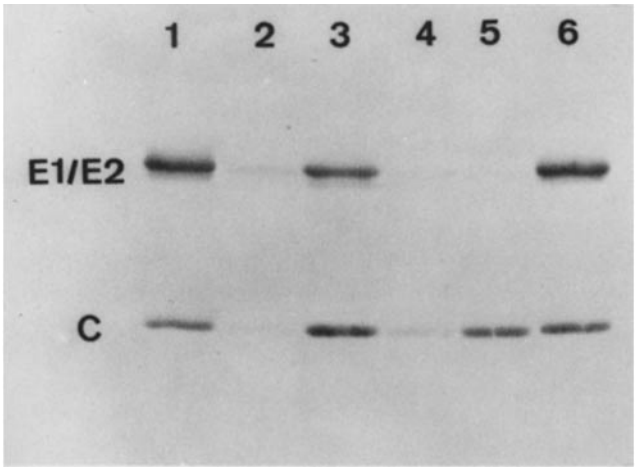

FIGURE 6 Proteinase K sensitivity of SFV proteins after binding and fusion with the BHK-21 plasma membrane. [^{35}S]Methionine-labeled SFV was bound to cells in the cold. The cells were warmed to 37°C in pH 7.2 (lanes *2* and *4*) or pH 5.5 (lanes *3* and *5*) media for 30 s, incubated with proteinase K for 45 min on ice (lanes *2* and *3*) or at 37°C (lanes *4* and *5*), and prepared for SDS gel electrophoresis. Untreated SFV (lanes *1* and *6*).

TABLE I

Efficiency of Fusion between SFV and the BHK-21 Cell Plasma Membrane

No SFV particles bound per cell ($\times 10^{-4}$)	No SFV particles fused per cell ($\times 10^{-4}$)	% Fusion
0.043	0.036	84
4.2	3.5	83
5.7	2.7	47
5.8	2.6	45

[^{35}S]Methionine-labeled SFV was allowed to bind to BHK-21 cells for 1 h in the cold in RPMI medium containing 0.2% BSA and 10 mM HEPES, pH 6.8. The number of viruses bound was determined directly and the number of viruses fused by the proteinase K assay after a 30-s warming period at pH 5.5, 37°C.

Fusion and Infection with Various Cell Lines

We determined the extent of SFV fusion with confluent 3T3 cell monolayers and with Raji and HeLa cells in suspension. The cells varied in their virus binding capacity, but with all of them, the relative fusion efficiency, as determined by proteinase K resistance, was equally high (≥70%) (Table II). These results were confirmed by indirect immunofluorescence after proteinase K treatment by the method described in Fig. 5.

To determine whether the cells were infected by the fusion, a test similar to that described in Fig. 1 was repeated for the 3T3, HeLa B, BHK-21, Raji, and HeLa suspension cells. In this case, virus (10 PFU/cell) was allowed to bind to the cells for 1 h in the cold and, after a brief (90 s) treatment at 37°C with pH 7.2 or pH 5.5 medium (with or without chloroquine as indicated), the cells were returned to neutral pH and further

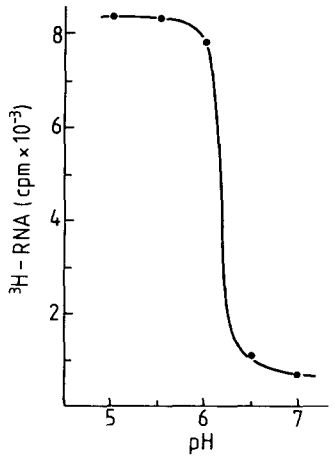

FIGURE 7 pH dependence of SFV fusion with the plasma membrane of BHK-21 cells. [^{3}H]Uridine-labeled SFV was bound to BHK-21 cells for 1 h in the cold in RPMI 1640 medium containing 0.2% BSA and 10 mM HEPES, pH 6.8. The cells were warmed to 37°C for 30 s by adding 1 ml of 37°C RPMI 1640 medium (containing 0.2% BSA and 10 mM MES) at the indicated pH values. These media were then removed, the cells were chilled, subjected to proteinase K treatment in the cold, and analyzed for proteinase K resistant [^{3}H]RNA.

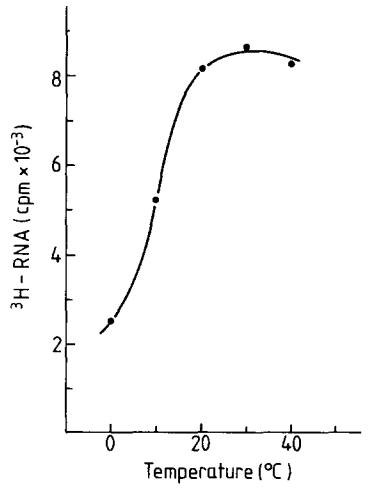

FIGURE 8 Temperature dependence of fusion between SFV and the plasma membrane. [^{3}H]Uridine-labeled SFV was bound to cells in the cold as described in the legend to Fig. 7. The cells were then incubated in a thermostated water bath for 30 s in 1 ml of pH 5.5 medium at the indicated temperature, after which they were chilled to 2°C, treated with proteinase K, and assayed for proteinase K resistant [^{3}H]RNA.

incubated for 9 h in a 37°C, 5% CO_2 incubator. At this time, the amount of virus in the culture medium was determined using the [^{3}H]uridine incorporation assay. In the absence of chloroquine, the 3T3, HeLa-B and BHK-21 cells were infected, whereas the Raji and HeLa suspension cells were not. None of the cell lines was infected in the presence of chloroquine at neutral pH (Table III). The chloroquine block could, however, be circumvented by a brief (90 s) low pH treatment of the 3T3, HeLa-B, and BHK-21 cells, suggesting that fusion of SFV at the plasma membrane of these cells introduced the viral genome into the cytoplasm in a replicable form and resulted in the production of virus particles. However, the Raji and HeLa suspension cells could not be infected by this procedure. For these cells, it is apparently not sufficient to introduce the viral nucleocapsid directly into the cytoplasm. Their resistance to SFV infection cannot therefore be attributed to a block in the entry pathway of the viral genome.

DISCUSSION

Two pathways are generally considered in the entry of enveloped animal viruses into cells (19, 20). In the first, viruses may fuse with the plasma membrane and thereby insert their genomes directly into the cytoplasm. Such fusion is best documented for Sendai and other paramyxoviruses (4, 5, 21). These viruses possess a specific membrane glycoprotein, the fusion protein, which mediates the process (22–24). The second route, which appears to be employed by most other enveloped viruses, is endocytosis of the particle into the cell, after which the viral genome is liberated into the cytoplasm by unknown mechanisms (19, 20).

Under normal tissue culture conditions, SFV enters cells by endocytosis (1–3). After binding to the cell surface, the virus particles are internalized by adsorptive endocytosis in coated vesicles and routed into intracellular vacuoles and lysosomes. How the genome finally enters the cytoplasm is not known in detail, but our previous studies strongly suggest that fusion between the viral membrane and the membrane of the lysosome into which the virus has been delivered might occur,

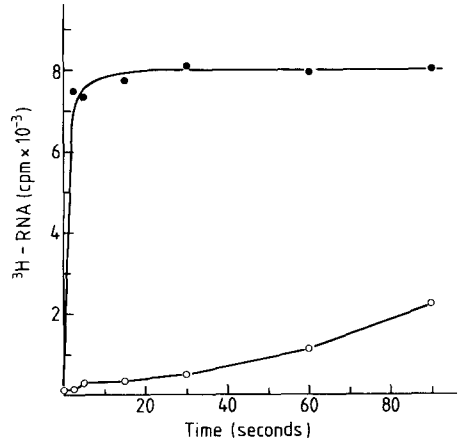

FIGURE 9 Time dependence of fusion between SFV and the plasma membrane. [^{3}H]Uridine-labeled SFV was bound to cells in the cold as described in the legend to Fig. 7. The cells were then warmed to 37°C by submersion in pH 5.5 (●) or pH 7.2 (○) RPMI 1640 medium. After the indicated times, the media were removed and the cells were immediately chilled, treated with proteinase K, and analyzed for proteinase K resistant [^{3}H]RNA.

TABLE II

Fusion of SFV with Various Cells

Cell	SFV bound*	Proteinase K resistant		% Fusion
		pH 7.2	pH 5.5	
	cpm × 10^{-3}	cpm × 10^{-3}		
3T3	47.0	2.6	41.0	87
Raji	6.6	1.1	4.6	70
HeLa suspension	37.0	4.0	27.0	73
BHK-21	5.8	0.3	5.0	86

* [^{35}S]Methionine-labeled SFV was bound to the cells for 1 h at 4°C.

TABLE III

SFV Infection by Fusion with the Plasma Membrane *

Cell	SFV Titer		
	Chloroquine (−), pH 7.2	Chloroquine (+), pH 7.2	Chloroquine (+), pH 5.5
		PFU/ml × 10^{-6}	
BHK-21	45.2	1.0	77.3
3T3	15.3	1.0	16.4
HeLa B	472.0	24.0	235.0
HeLa suspension	0.1	0.3	0.3
Raji	2.0	1.6	2.0

* SFV (10 PFU/cell) was allowed to bind to the various cells for 1 h on ice in serum-free MEM containing 0.2% BSA and 10 mM HEPES, pH 6.8. The cells were then warmed for 90 s to 37°C in pH 7.2 or pH 5.5 media and then incubated in MEM containing 10% fetal calf serum in a 37°C, 5% CO_2 incubator for 2 h. The medium was then replaced with 1.2 ml serum-free MEM containing 0.2% BSA and 10 mM HEPES, pH 7.2, and after an additional 7 h in the 37°C CO_2 incubator, the cell medium was centrifuged to remove cellular debris and then 0.25-ml samples were assayed for virus by the [^{3}H]uridine incorporation assay. A standard curve was prepared using virus samples of known titer (determined by plaque titration). In the chloroquine (+) samples, 0.2 mM chloroquine was present in all but the final incubation media.

thereby transferring the nucleocapsid into the cytoplasm. The fusion is probably triggered by the low lysosomal pH, because agents that raise the pH in the lysosomes inhibit release of the viral genome into the cytoplasm (1–3, and footnote 1).

The results described in this paper show that SFV can also infect susceptible cells after fusion with the plasma membrane. As in the liposome system, which we have studied in some detail (8), fusion requires low pH. Because the pH must be 6 or lower, fusion at the plasma membrane is unlikely, as the entry mechanism under normal tissue culture conditions. The artificial nature of this pathway is also demonstrated by the fact that it is completely insensitive to lysosomotropic agents which are potent inhibitors of the normal route of SFV penetration (1). Moreover, fusion of the virus with the plasma membrane at neutral pH is never observed in the electron microscope, and, unlike Sendai virus, viral spike proteins are not left in the plasma membrane after normal infections (25, K. Simons, unpublished results).

Despite the artificial nature of SFV penetration through the plasma membrane, the results described in this paper are relevant to our understanding of the normal route of entry of SFV into host cells. First, they emphasize that fusion triggered by low pH is an effective way of introducing the SFV nucleocapsid into the cytoplasm in an infective form. This is compatible with our suggestion that fusion induced by the low pH in lysosomes may be the final step in SFV penetration under physiological conditions (1, 2). Second, the lack of inhibition of fusion and infection by weak bases after low pH treatment shows that fusion, uncoating, and the rest of the viral replication cycle are not directly affected by these agents. This agrees with our proposal that the inhibition by lysosomotropic agents is caused by elevation of the intralysosomal pH above the critical value required for fusion (1, 2). Third, the demonstration that low pH-induced fusion through the plasma membrane leads to infection of susceptible cells supports the idea that lysosomal modification of the virus is not required for fusion (i.e., that the low pH in the lysosomes may be the only factor needed to trigger fusion). Fourth, the results show that after dropping the pH in the medium, fusion occurs very rapidly, and that the nucleocapsid as a recognizable morphological unit is short-lived in the cytoplasm. The rapidity of fusion may explain why we have not been able to recognize fusion in the lysosomes by electron microscopy. The reason why fusion is readily visualized at the plasma membrane is that the process can be synchronized by a drop in pH. The speed of fusion also makes it easier to understand how the virus might escape degradation in the lysosomes. Because the viral spike glycoproteins must be intact for fusion to occur (8), degradation by lysosomal proteases would prevent fusion and hence the penetration of the viral genome into the cytoplasm.

Fusion could be demonstrated with all five cell lines tested. The reaction thus appears to be nonselective. Apparently, the cell's plasma membrane must only possess binding sites for the virus and, by analogy to the liposome system (8), it must contain cholesterol. In practice, neither of these requirements is very limiting, as SFV binds to most tissue culture cells (26), and because cholesterol is a major component of the plasma membrane (27). The fusion reaction was maximally 86% efficient and it provides a convenient mechanism for introducing the viral membrane proteins and lipids into the plasma membrane of a variety of cells. In this way, the plasma membrane can be specifically labeled, facilitating studies of the turnover, recycling, lateral diffusion, and polarity of the cell surface and its components.

Another potential use for low pH-induced fusion is the possibility of artificially infecting cells. When the normal route of entry into susceptible cells is blocked by lysosomotropic agents, infection can be induced by fusion at the plasma membrane. However, the two cell lines so far tested, which are refractory to infection under normal conditions, could not be infected by this procedure. The reason for this result is not clear, and more cell lines will have to be tested to verify the generality of the effect. It may be relevant that the Raji cells are also refractory to infection by vesicular stomatitis virus, another enveloped RNA virus, and that this resistance cannot apparently be attributed to the production of interferon (28, 29). It will be interesting to elucidate the step at which infection is blocked in the refractory cell lines. Using fusion through the plasma membrane, it may also be possible to identify cells which possess a block in the entry pathway.

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REFERENCES

1. Helenius, A., J. Kartenbeck, K. Simons, and E. Fries. 1980. On the entry of Semliki Forest virus into BHK-21 cells. *J. Cell Biol.* 84:404–420.
2. Helenius, A., M. Marsh, and J. White. 1980. The entry of viruses into animal cells. *Trends Biochem. Sci.* 5:104–106.
3. Marsh, M., and A. Helenius. 1980. Adsorptive endocytosis of Semliki Forest virus. *J. Mol. Biol.* In press.
4. Choppin, P. W., and R. W. Compans. 1975. Replication of paramyxoviruses. *In* Comprehensive Virology. Vol. 4, H. Fraenkel-Conrat and R. Wagner, editors, Plenum Press, New York. 95–178.
5. Poste, G., and C. A. Pasternak. 1978. Virus induced cell fusion. *In* Cell Surface Reviews. Vol. 5. G. Poste and G. L. Nicolson, editors. North-Holland Publishing Co., Amsterdam. 305–367.
6. Väänänen, P., and L. Kääriäinen. 1979. Haemolysis by two alphaviruses: Semliki Forest and Sindbis virus. *J. Gen. Virol.* 43:593–601.
7. Väänänen, P., and L. Kääriäinen. 1980. Fusion and haemolysis of erythrocytes caused by

three togaviruses: Semliki Forest, Sindbis and Rubella. *J. Gen. Virol.* 46:467–475.
8. White, J., and A. Helenius. 1980. pH dependent fusion between the Semliki Forest virus membrane and liposomes. *Proc. Natl. Acad. Sci. U. S. A* 77:3273–3277.
9. Kääriäinen, L., K. Simons, and C.-H. von Bonsdorff. 1969. Studies of Semliki Forest virus subviral components. *Ann. Med. Exp. Biol. Fenn.* 47:235–248.
10. Kääriäinen, L., and H. Soderlund. 1971. Properties of Semliki Forest virus nucleocapsid. I. Sensitivity to pancreatic ribonuclease. *Virology.* 43:291–299.
11. Helenius, A., E. Fries, and J. Kartenbeck. 1977. Reconstitution of Semliki Forest virus membrane. *J. Cell Biol.* 75:866–880.
12. Miller, D., B. Feuer, R. Vanderoef, and J. Lenard. 1980. Reconstituted G protein-lipid vesicles from vesicular stomatitis virus and their inhibition of VSV infection. *J. Cell Biol.* 84:421–429.
13. Blobel, G., and B. Dobberstein. 1975. Transfer of proteins across membranes. I. Presence of proteolytically processed and unprocessed nascent immunoglobulin light chains on membrane-bound ribosomes of murine myeloma. *J. Cell Biol.* 67:835–851.
14. Ziemiecki, A., and H. Garoff. 1978. Subunit composition of the membrane glycoprotein complex of Semliki Forest virus. *J. Mol. Biol.* 122:259–269.
15. Acheson, N. H., and I. Tamm. 1967. Replication of Semliki Forest virus: an electron microscopic study. *Virology.* 32:128–143.
16. von Bonsdorff, C.-H, and S. C. Harrison. 1975. Sindbis virus glycoproteins form a regular icosahedral surface lattice. *J. Virol.* 16:141–145.
17. Laine, R., H. Soderlund, and O. Renkonen. 1973. Chemical composition of Semliki Forest virus. *Intervirology* 1:110–118.
18. Harrison, S., A. David, J. Jumblatt, and J. Darnell. 1971. Lipid and protein organisation in Sindbis virus. *J. Mol. Biol.* 60:523–528.
19. Dales, S. 1973. Early events in cell-animal virus interactions. *Bacteriol. Rev.* 37:103–135.
20. Lonberg-Holm, K., and L. Philipson. 1974. Early interaction between animal viruses and cells. *Monogr. Virol.* 9:1–149.
21. Morgan, C., and C. Howe. 1968. Structure and development of viruses as observed in the electron microscope. IX. Entry of parainfluenza 1 (Sendai) virus. *J. Virol.* 2:1122–1132.
22. Scheid, A., and P. W. Choppin. 1974. Identification of biological activities of paramyxovirus glycoproteins. Activation of cell fusion, hemolysis and infectivity by proteolytic cleavage of an inactive precursor protein of Sendai virus. *Virology.* 57:475–490.
23. Homma, M., and M. Ohuchi. 1973. Trypsin action on the growth of Sendai virus in tissue culture cells. III. Structural differences of Sendai virus grown in eggs and tissue culture cells. *J. Virol.* 12:1457–1465.
24. Gething, M. J., J. White, and M. D. Waterfield. 1978. Purification of the fusion protein of Sendai virus: analysis of the NH_2-terminal sequence generated. *Proc. Natl. Acad. Sci. U. S. A.* 75:2737–2770.
25. Fan, D. P., and B. M. Sefton. 1978. The entry into host cells of Sindbis virus, vesicular stomatitis virus and Sendai virus. *Cell.* 15: 985–992.
26. Fries, E., and A. Helenius. 1979. Binding of Semliki Forest virus and its isolated glycoproteins to cells. *Eur. J. Biochem.* 97:213–220.
27. Demel, R. A., and B. De Kruyff. 1976. The function of sterols in membranes. *Biochim. Biophys. Acta.* 457:109–132.
28. Epstein, M. A., B. G. Achong, Y. M. Barr, B. Zajac, G. Henle, and W. Henle. 1966. Morphological and virological investigations on cultured Burkitt tumor lymphoblasts (Strain Raji). *J. Natl. Cancer Inst.* 37:547–559.
29. Henle, G., and W. Henle. 1966. Studies on cell lines derived from Burkitts lymphoma. *Trans. N. Y. Acad. Sci.* 29:71–79.