Membrane Fusion Mutants of Semliki Forest Virus

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Abstract Previous reports have indicated that the entry of Semliki Forest virus (SFV) into cells depends on a membrane fusion reaction catalyzed by the viral spike glycoproteins and triggered by the low pH prevailing in the endosomal compartment. In this study the in vitro pH-dependent fusion of SFV with nuclease-filled liposomes has been used to select for a new class of virus mutants that have a pH-conditional fusion defect. The mutants obtained had a threshold for fusion of pH 5.5 as compared with the wild-type threshold of 6.2, when assayed by polykaryon formation, fusion with liposomes, or fusion at the plasma membrane. They were fully capable of infecting cells under standard infection conditions but were more sensitive to lysosomotropic agents that increase the pH in acidic vacuoles of the endocytic pathway. The mutants were, moreover, able to penetrate and infect baby hamster kidney–21 cells at 20°C, indicating that the endosomes have a pH below 5.5. The results confirm the involvement of pH-triggered fusion in SFV entry, emphasize the central role played by acidic endosomal vacuoles in this reaction, shed further light on the mechanism of SFV inhibition by lysosomotropic weak bases, and demonstrate the usefulness of mutant viruses as biological pH probes of the endocytic pathway.

Semliki Forest virus (SFV),1 a simple, well characterized animal virus of the toga (alpha) virus family, infects cells in culture via an endocytic pathway (13, 29). After internalization by receptor-mediated endocytosis in coated vesicles, the virus particles are delivered into prelysosomal vacuoles (endosomes). The acidic pH in the endosomes apparently triggers a change in the virus spike glycoproteins that initiates the fusion of the viral membrane with the endosomal membrane. This fusion reaction is thought to release the viral genome into the cytoplasm, and result in infection.

The fusion of SFV with cellular and artificial target membranes provides an attractive system to study the penetration of enveloped animal viruses into their host cells, and the mechanism of protein-catalyzed membrane fusion in biological systems. The general features of the fusion reaction have been characterized in some detail (48, 49); it shows a sharp threshold at pH 6.2–5.8, requires the presence of cholesterol in the target membrane, and is efficient, rapid, and nonleaky. The integrity of the virus spike proteins, consisting of three glycopolypeptide chains (E1, E2, and E3, molecular weights 50,786, 52,855 and 11,369, respectively [10]), is critical. Although the central role of the spike glycoproteins in catalyzing the fusion reaction is well established (22, 28), the actual mechanism remains unclear.

Here we describe our first results from a genetic approach to the problem of SFV membrane fusion. Using a positive selection based on the in vitro fusion activity of SFV, virus mutants have been isolated that have a modified pH threshold for fusion. The phenotype of this novel type of "pH-conditional" mutant is described with respect to its fusion properties and its entry into cultured cells.

Materials and Methods

Viruses and Cells: Virus was propagated, as previously described, either in baby hamster kidney (BHK) cells (16), or in fibroblasts from specified pathogen-free chick embryos (CEF) (19). Plaque assays used overlays containing either 0.5% carboxymethylcellulose or 0.9% bactoagar. Stock virus was grown at a low multiplicity of infection (~0.01 plaque-forming units [pfu] per cell) and was stored as aliquots in growth medium (Eagle's minimum essential medium + 0.2% bovine serum albumin + 10 mM HEPES) at −70°C after centrifugation at 10,000 g for 30 min at 4°C to remove cell debris. Virus labeled with [3H]thymidine or with [35S]methionine was prepared essentially as described before (13, 17), except that the lower multiplicity of infection of 1 pfu/cell was used for 35S. Radioisotope was not added until 4–5 h after infection and the length of labeling time was increased to 12 h. 35S-labeled virus polypeptides were resolved by SDS PAGE (2).

As wild-type stock virus, either a prototype strain of SFV from the virology department in Helsinki or a plaque-purified stock derived from this (19) was
used. The wild-type (wt) virus used for individual experiments was the plaque-purified stock unless otherwise indicated in the figure legends. Some differences between the plaque-purified and original virus stocks were observed, and these are currently under investigation.

**Mutagenesis and Selection:** Plaque-purified wt stock SFV (1.5 x 10^6 pfu/ml in growth medium) was mutagenized by adding 50 μg/ml N-methyl-N'-nitro-N-nitrosoguanidine and incubating 15 min at room temperature. The pellet was then dialyzed for 20 h at 4°C in PBS. The nitrosoguanidine treatment resulted in an 80% reduction in virus titer.

Mutagenized virus was grown for 6 h on CEF at low multiplicity, centrifuged to remove cell debris, and pelleted at 200,000 g for 60 min at 4°C. The pellet was resuspended in 250 μl of MBS buffer (20 mM 2,4-N-morpholinopropionic acid, 0.3% bovine serum albumin, 0.13 M NaCl, pH 7.0). RNase-containing liposomes prepared as previously described (48) were added to a final concentration of 1 mM lipid. The pH of this suspension was dropped to 5.5 by the addition of 0.5 N acetic acid, and it was incubated for 20 min at 37°C to allow virus-liposome fusion and the degradation of viral RNA. The mixture was then neutralized with 0.5 N NaOH, diluted to the appropriate virus concentration in growth medium, and added to CEF monolayers for a second round of virus replication. The progeny virus released was concentrated and fused with liposomes as described above.

The growth and selection protocol was first tested with untreated virus to give an estimate of the virus yield and the efficiency of virus fusion with liposomes. Each selection of mutagenized virus by fusion caused a reduction in the virus titer by a factor of 1-4 x 10^3.

The surviving virus from the second fusion was plated on CEF under agar. Well isolated plaques were picked, eluted in 1 ml of growth medium, and scored as described below. Working stock of the mutants were then prepared for third or fourth passage after two plaque purifications. fus+1, -2, and -3 were originally designated C77, C18, and C11 (20).

**Cell Fusion:** Cell fusion of SFV-infected cells was routinely tested using confluent monolayers of BHK cells in 96-well microtiter plates. Each well was infected with 10^4-10^5 pfu from a plaque cluate or stock. Virus was allowed to replicate for from 12 to 21 h. The cell monolayers were then washed once with Hank's saline, "pH medium" (PBS containing 10 mM Hepes and 10 mM MES at the desired pH was added, and the cells were incubated for 3-4 min at 37°C. pH medium was then replaced with growth medium of neutral pH, and the cells were returned to culture for 1 h, fixed, stained with Giemsa's, and evaluated for cell-cell fusion by light microscopy (50).

Plaque fusion assays were performed by a similar treatment of 2-d plaques formed under carboxymethylcellulose in 50-mm petri plates. The final staining was done with crystal violet.

**Uridine Incorporation Assay:** The penetration and infection of SFV in BHK cells was assayed by measuring the incorporation of [H]uridine into viral RNA, as has been previously described (14). Since, in the present study, cells were infected at a lower multiplicity, the labeling time with [H]uridine was lengthened from 2.5 h as described for individual experiments. Also, in most experiments, cells were infected in R-medium (RPMI containing 10 mM Hepes and 10 mM MES at the desired pH was added, and the cells were incubated for 3-4 min at 37°C. pH medium was then replaced with growth medium of neutral pH, and the cells were returned to culture for 1 h, fixed, stained with Giemsa's, and evaluated for cell-cell fusion by light microscopy (50).

Uridine incorporation was assayed by a similar treatment of 2-d plaques formed under carboxymethylcellulose in 50-mm petri plates. The final staining was done with crystal violet.

**Results**

**Selection of pH-Conditional Mutants**

The pH threshold for wt SFV fusion is 6.2, while the pH in endosomes and lysosomes is at least half a pH unit lower than this value (32, 46, 47). An SFV mutant with a more acidic fusion pH than wt thus should encounter pH values <6.2 in the endocytic vacuolar system. Such a mutant, therefore, might not necessarily exhibit impaired infectivity. By utilizing the efficient in vitro fusion properties of SFV with liposomes containing trapped ribonuclease (RNase), we were able to select for such "pH-conditional" mutants as follows (see Materials and Methods for details).

SFV was first mutagenized with nitrosoguanidine, a mutagen previously used by several groups for the isolation of temperature-sensitive togavirus mutants (44). The surviving viruses were propagated for a single growth cycle at low multiplicity in either BHK cells or chick embryo fibroblasts. Progeny virus was then mixed with RNase-containing liposomes at pH 5.5, which resulted in fusion and concomitant destruction of virtually all of the input viruses judging by the 1,000-fold reduction in infectivity. From the remaining viruses, single plaque isolates were used to infect BHK cell monolayers, and the pH needed to induce polykaryon formation was determined.

Three separate selections, each including two liposome-fusion steps, were performed in all, and a total of 370 plaque isolates were screened for cell fusion. Among these, three were found that caused cell fusion only at pH 5.6 or below (as shown in Fig. 1). They also displayed a different plaque morphology after pH-5.5 treatment of the culture dish. Whereas wt plaques acquired a halo of fused cells, the mutant plaques showed no cell fusion (data not shown).

The pH-conditional fusion mutants were isolated with a frequency of approximately one in 10^7 and will be referred to as fus-1, -2, and -3. They were all derived from the same selection and thus it is possible that they are progeny of the same original mutant. No clear-cut differences in the phenotype have been observed between them to date.

**General Characteristics of the Fusion Mutants**

Replication of fus-1, -2 and -3 in BHK cells at low multiplicities of infection (moi) was somewhat slower than wt, but, at 25 h postinfection, comparable titers could be obtained (Fig. 2A). Efficient incorporation of [35S]methionine (and other radioactive precursors) and adequate propagation and isolation of mutant particles were therefore feasible. To detect any major defects in the mutant virus structural proteins and their precursors, we analyzed isolated [35S]methionine-labeled mutants and extracts of mutant-infected [35S]methionine-labeled cells by SDS PAGE and autoradiography. No differences were seen in the migration or relative amounts of the viral structural proteins, E1, E2, E3, and capsid, or in the p62 cellular precursor when compared with the wt (data not shown). Thin-section electron micrographs of mutant-infected and wild-type-infected cells were also indistinguishable.

8 h after infecting BHK cells at low multiplicity, virus particles were seen in the process of budding at the plasma membrane. Progeny viruses were bound to the cell surface on microvilli and in coated pits, and others were seen inside coated vesicles and large endocytic vacuoles (data not shown). Both fus-1 and wt-infected cells gave strong positive reactions
when examined by indirect immunofluorescence using affinity-purified rabbit antibodies directed against the SFV spike. The intracellular and surface staining appeared similar between the two samples.

Surprisingly, the growth of fus-1 and fus-2 at high multiplicities of infection was much less efficient than that of wt. This is shown in Fig. 2B where the incorporation of [3H]uridine into fus-1 or wt RNA has been determined over a range of 0.01–10 pfu/cell. At multiplicities of infection >1 pfu/cell, fus-1 and -2 infected cells show a striking decrease in [3H]uridine incorporation. The drop in infectivity at high multiplicity correlated with reduced production of virus particles as confirmed by electron microscopic examination of thin-sectioned cells. In mixed infections, fus-1 was able to interfere with wt virus replication but only when both viruses were present at multiplicities >1. In all subsequent studies, multiplicities ≤1 pfu/cell were used.

Another difference between fus-1 and wt was the pH dependence of attachment to the BHK cell surface at 0-4°C (Fig. 3; see also reference 8). In contrast to wt, [3H]uridine-labeled fus-1 bound quite inefficiently at pH values >7.5, with maximum binding (50% of added virus bound during 2.5 h) observed at pH 6.4. In subsequent experiments in which prebinding was required, media of pH 6.5 were used. However, the standard infection of cells at higher temperatures occurred quite efficiently in a range of pH values from 6.8 to 7.8. The pH dependence of binding to BHK cells was also assayed for fus-2 and -3, and was similar to that shown for fus-1.

In Vitro Fusion Activity

To characterize the modified membrane fusion properties of the mutant viruses, we employed two quantitative assays. Surface-bound wt SFV can be fused with the plasma membrane of tissue culture cells when the pH in the medium is briefly lowered ≤6.2 (49). Fusion can be quantitated using radio-labeled virus and proteinase K digestion at 0°C, which selectively removes unfused virus particles. Fig. 4 shows an experiment in which the pH dependence of fus-1 and wt fusion have been compared. [3H]uridine-labeled fus-1 and [35S]methionine-labeled wt SFV were mixed and added to
FIGURE 4  Fusion of $[^3]H$uridine- (○) and $[^35]S$methionine- (●) labeled SFV with the plasma membrane of BHK cells. A mixture of $[^3]H$-labeled fus-1 and $[^35]S$-labeled wt SFV was bound to BHK cells in R-medium, pH 6.5, for 2 h on ice. Duplicate plates were then treated for 3 min at 37°C with medium of various pHs, after which unfused virus was removed by proteinase K digestion at 4°C. Bound virus was determined on an untreated set of plates as described for Fig. 3. wt was the original stock.

**The Mode of Action of Lysosomotropic Inhibitors**

Having established that the mutants differ from the wt in fusion pH, we proceeded to use them as tools to confirm the mode of action of lysosomotropic amines on virus penetration and to measure the pH inside endosomal vacuoles. Weak bases such as chloroquine, ammonium chloride, methyl amine, and amantadine constitute a group of agents known to inhibit the entry of numerous animal viruses (14). This effect has been credited either to a generalized block in endocytic uptake of viruses and other receptor-bound ligands (39), to processes involving the uncoating of the genome from the nucleocapsids (11, 18, 24), or to a block in the intracellular penetration step (reviewed in reference 14). In previous studies we have shown that prebound SFV is endocytosed normally and routed through the endocytic pathway in BHK cells in the presence of these agents but that the intracellular penetration step appears to be blocked (13, 14). Since the agents do not affect the fusion activity in vitro, we have proposed that they inhibit the reaction indirectly by raising the pH in endocytic vacuoles above the threshold required to trigger fusion. The lipophilic weak bases used have been shown to accumulate in lysosomes (5) and to increase the pH in endosomes and lysosomes in a concentration-dependent fashion (31, 32). If the proposed mechanism were correct, we expected to find that the fusion mutants would be more sensitive to lysosomotropic weak bases than the wild type.

Cells were infected with fus-1 or wt SFV in the presence of different concentrations of ammonium chloride, chloroquine, or amantadine. The resulting infection was assayed by measuring $[^3]H$uridine incorporation into viral RNA (Fig. 6) under conditions (15 mM NH$_4$Cl) where further infection was blocked. Both fus-1 and the wt virus were sensitive to the three agents, but in each case the mutant was affected at lower concentrations. The concentrations of ammonium chloride which gave 50% inhibition were 6.5 mM for the wild type and 3.5 mM for the mutant. For chloroquine the values were 0.08 mM and 0.02 mM respectively, and for amantadine, 1.1 mM and 0.6 mM. fus-2 and fus-3 were also found to be more sensitive (data not shown). We conclude that virus sensitivity to weak bases correlates with the pH needed to trigger their fusion. The result strongly supports the argument that the

cells, and the cells were treated with buffers at different pH for 3 min. Whereas the wt SFV fused with 50% efficiency over the 4.8–6.0 pH range, the mutant showed little fusion until pH values below 5.3. At pH 4.8, the fusion activity was 70% of cell-bound virus. The background seen in the 5.3–6.5 range is due to endocytosis of some of the surface-bound virus that occurs during the pH treatment. fus-2 and -3 gave similar results (data not shown).

A difference in the threshold pH of fus-1 and wt SFV fusion was also seen when RNase-containing liposomes were used as target membranes (Fig. 5). In this assay the RNase-resistant, $[^3]H$uridine-labeled RNA is determined after pH-induced fusion of viruses with the liposomes (48). Maximal fusion of the wt SFV occurred at pH 5.9 while efficient fusion of fus-1 required pH values <5.5 (with maximal fusion observed at pH 4.8).

Taken together the fusion results indicate that the overall fusion activity of fus-1 is of efficiency comparable to that of the wt SFV but it requires a pH <5.5 (and preferably <5.0) to be efficiently expressed. Like the wild-type, the mutant fusion seems relatively independent of the composition and origin of the target membranes.

FIGURE 5  pH dependence of wt and fus-1 fusion with liposomes. $[^3]H$uridine-labeled SFV and liposomes (0.75 mM lipid) containing trapped RNase were mixed at pH 7.0. Aliquots were adjusted to lower pH by the addition of dilute HCl. Acid-precipitable $[^3]H$uridine was then determined. Background counts (~7% of total) in samples solubilized with 1% Triton X-100 before incubation have been subtracted. wt was the original stock.

FIGURE 6  Sensitivity of fus-1 and wt infection to weak bases. BHK cells were preincubated for 15 min in R-medium, pH 7.4 (NH$_4$Cl and chloroquine) or 7.8 (amantadine), at 37°C. Cells were then infected with fus-1 or wt-SFV (0.5 pfu/cell) in these media for 90 min at 37°C. After infection, actinomycin D and $[^3]H$uridine were added as usual, in the presence of 15 mM NH$_4$Cl throughout to prevent further infection and ensure equivalent labeling conditions. Labeling time with $[^3]H$uridine was 3 h.

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inhibitory effect of lysosomotropic weak bases on SFV infection depends on the elevated pH in the vacuoles of the endocytic pathway.

The Endosomal pH

We have previously shown that SFV penetration occurs mainly in the endosomal compartment (29). Given the pH threshold for the wt, this indicates that the endosomal pH must be \(<6.2\). The fact that fus-1 has a considerably lower fusion pH than the wt provided a means of determining whether the endosomal pH is \(<6.2\).

BHK cells were exposed to either fus-1 or wt at 20°C. At this temperature the viruses are endocytosed and delivered into endosomes but transport into lysosomes is inhibited (29). After 2 h, ammonium chloride was added to some of the cultures to elevate the endosomal and lysosomal pH (and thus block any further penetration). The temperature of the cultures was then raised to 37°C to allow replication of any virus that had penetrated during the 20°C incubation. The extent of infection was assayed by measuring [3H]uridine incorporation into viral RNA. The results, shown in Fig. 7, indicate that wt and fus-1 were equally infectious at 20°C (i.e., 43-44% of non-NH4CI treated controls). Hence, both fus-1 and wt viruses reach sufficiently acidic compartments at 20°C to trigger penetration. The pH in the prelysosomal vacuoles (or a subpopulation of them) must therefore be \(<5.5\).

DISCUSSION

fus-1, -2, and -3 represent a novel type of mutant with many potential uses in the study of membrane fusion, virus entry, and the endocytic pathway. Unlike most other animal virus mutants available, they were obtained by a rigorous, positive selection protocol that did not depend on temperature as a conditional parameter. The selection resulted in mutants that replicate relatively normally under standard infection conditions, and whose main altered properties are revealed only by in vitro assays, or when the host cells are treated with lysosomotropic drugs.

The mutants isolated so far have pleiotropic phenotypes. fus-1, the best characterized, differs from the wild type in the following ways: (a) The pH threshold for fusion activity is lower. Maximal fusion activity is observed at values \(<5.0\), compared with 6.0 for the wt. Fusion starts at pH 5.3–5.5 depending on the assay used, compared with 6.2 for the wt. (b) The pH dependence of binding to BHK cells at 0–4°C is shifted to lower values. (c) The growth is somewhat slower than that of wt. (d) It expresses an infectivity block at multiplicities of infection >1.

Whether all of these traits result from a single mutation is not clear. Cloning and sequencing of fus-1 structural genes are presently in progress. Sequence analysis of complementary DNA from the wt and from several recently isolated revertants and the use of an expression system for complementary DNA genes for the SFV structural proteins (21) should enable the assignment of the fusion pH alteration to specific structural proteins, and to specific domains within them. Such detailed structural studies should provide relevant information for understanding the mechanism of fusion in the toga (alpha) virus system. It is already clear that the spike glycoproteins (which consist of E1, E2, and E3 in SFV, and E1 and E2 in the closely related Sindbis virus) are necessary and sufficient for fusion activity (22, 28), but it is unknown which of the individual glycopolypeptides is the fusion factor. The identification of E1 as the low pH–dependent hemagglutinin (4, 12), the localization of a highly conserved hydrophobic sequence in the E1 external domain of SFV and Sindbis (10, 36), and the recent observation that cell fusion is only expressed if E1 is present on the cell surface (22) indicate that E1 is central. On the other hand, it has been observed that low pH treatment of Sindbis virus leads to the exposure of tryptic cleavage sites in E2, and this has been interpreted as evidence for E2 involvement in fusion (6). In influenza virus, where information is presently more complete, acid-dependent fusion is catalyzed by a conformational change in the hemagglutinin molecule (41). This change exposes a previously hidden hydrophobic peptide segment (the HA-2 terminal segment) which probably inserts into the target membrane. After low pH treatment, the hemagglutinin interacts with both the viral bilayer in which it is anchored and the target membrane. This dual interaction probably helps to overcome the intrinsic repulsion between the membranes and cause fusion (51). No fusion mutants are yet available for influenza virus, but the threshold pH for fusion in different natural strains varies within the pH 5–6 range (51), and it has been demonstrated that the pH dependence is conferred by the hemagglutinin gene (23). In addition, influenza virus mutants have been described that have altered sensitivity to amantadine and its analogues (1, 11, 24, 40). Whether these mutants have increased sensitivity to other lysosomotropic agents and whether they display differences in fusion pH remain to be determined.

The increased sensitivity of fus-1, -2, and -3 to chloroquine, amantadine, and ammonium chloride confirms that the inhibitory effect of weak bases in SFV infection involves the fusion reaction and that inhibition results from an elevation of pH in endocytic vacuoles. Reports by Pastan and co-workers (38, 39) have suggested that a variety of lysosomotropic agents, including amantadine, block endocytic uptake.

| NH4Cl | Incorporation | % Uninhibited |
|-------|--------------|---------------|
| wt    | +            | 18,191        | 43%           |
|       | –            | 42,549        |               |
| fus-1 | +            | 11,701        | 44%           |
|       | –            | 26,325        |               |

**FIGURE 7** Effect of 20°C on infection by fus-1 and wt. BHK cells were preincubated for 30 min in R-medium, pH 7.4, at 20°C, and then infected for 90 min with fus-1 or wt virus (0.5 pfu/cell) in the same medium. 15 mM NH4Cl was then added to some cultures, and all were shifted to 37°C for 1 h, after which actinomycin D and [3H]uridine were added for a 3-h labeling period. The indicated [3H]uridine incorporations are the counts above infected cell background in which 15 mM NH4Cl was present throughout.
of vesicular stomatitis virus and that this explains the block in infection. Our previous endocytosis assays (13, 14) and the data presented here are not consistent with such a mechanism for SFV inhibition. The increased sensitivity of the mutants to weak bases provides proof that the low pH-dependent fusion activity of SFV is, indeed, relevant to the process of productive infection. If the fusion activity at low pH were an experimental artefact, as has been recently suggested by Brown and co-workers (6, 25), the pH in vacuoles of the endocytic pathway and the pH dependence of fusion would be irrelevant for entry and infection. Viruses of many families (orthomyxov-, rhabo-, toga-, retro-, and herpes viruses) have been reported to be inhibited by weak bases (14, 18, 30, 35, 37, 39, 45), and by carboxylic ionophores (27, 38), which elevate lysosomal and endosomal pH (31, 32). Of these, the first three virus families have now been shown to possess low pH-dependent membrane fusion activity. The sensitivity of infection by these viruses to lysosomotropic agents suggests the involvement of an intracellular low pH-dependent step in entry as well. An increase in endosomal/lysosomal pH thus could explain the prophylactic effect of amantadine and its analogues against influenza. However, careful studies with various influenza strains having different pHs of fusion are needed.

Whereas the low pH prevailing in lysosomes is well documented, acidity of endosomes has only recently been demonstrated. Fluorescein-conjugated endocytic markers have been used as pH probes by Tycko and Maxfield (46) and van Renswoude et al. (47). The use of wt SFV has provided an independent bioassay of endosomal acidity (29). The acidity of the prelysosomal vacuoles is now recognized as an important factor in determining the dissociation of ligand-receptor complexes and the control of membrane recycling (3, 15, 42). As in lysosomes, the low pH in endosomes apparently depends on an ATP-driven proton pump, and inhibitor studies in vitro indicate that the lysosomal and endosomal acidification mechanisms are quite similar (9, 34). A pump of similar mechanism on an ATP-driven proton pump, and inhibitor studies in vitro indicate that the lysosomal and endosomal acidification mechanisms are quite similar (9, 34). A pump of similar mechanism

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