Polycaryocyte Formation Mediated by Sindbis Virus Glycoproteins

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The process of cell fusion mediated by Sindbis virus membrane proteins synthesized after infection was examined. At the times after infection at which virus proteins were detectable on the cell surface, Sindbis virus-infected BHK-21 cells were found to express a fusion function after brief treatment at acid pH. In studies employing wild-type virus and temperature-sensitive mutants and testing drug or protease inhibition of virus production, we made the following observations on Sindbis virus-mediated fusion from within. (i) Fusion requires the synthesis of virus glycoproteins and their transport to the cell surface. (ii) Modification of the cell plasma membrane by polypeptides PE₂ and E₁ alone is not sufficient for expression of the fusion function. (iii) The proteolytic conversion of plasma membrane-associated PE₂ to E₂ is not essential for fusion. (iv) Glycosylation of virus plasma membrane proteins is essential for fusion. (v) The lesions of Sindbis virus temperature-sensitive mutants do not affect their ability to fuse cells.

Many families of enveloped viruses are capable of inducing fusion activity in cell cultures. Such fusion activity can be induced directly by virus particles (fusion from without [FFWO]) or after the synthesis of virus-specific products (fusion from within [FFWI]). FFWO occurs at a high input multiplicity and takes place immediately after adsorption, before the synthesis of virus-coded components. FFWI occurs at a low input multiplicity and, due to an absolute requirement for synthesis of virus-coded components, takes place several hours or even days after adsorption. (For complete discussions of virus-induced fusion, see references (10, 14, and 15). Previous studies have shown FFWO for the alphaviruses Sindbis virus (21) and Semliki Forest virus (22). In this paper, we report experiments investigating the conditions required for FFWI of Sindbis virus-infected BHK-21 cells.

Sindbis virus is a structurally simple virus composed of single-stranded, plus-polarity 42S RNA, which, together with multiple copies of virus capsid protein, forms an icosahedral nucleocapsid (3, 18, 19). The capsid is in turn enclosed within a host-derived lipid bilayer containing equimolar ratios of the virus-specified, host-glycosylated proteins E₁ and E₂ (3). In a Sindbis virus-infected cell, virus structural polypeptides are synthesized from a 26S subgenomic RNA derived from the 3' end of the viral 42S RNA (18). Virus structural proteins are produced as a single polypeptide; capsid protein is cleaved from this polypeptide and associates

with 42S RNA to form an icosahedral nucleocapsid. The remainder of the polypeptide is integrated into cellular membranes and undergoes cleavage and glycosylation; the resultant products, E₁ and PE₂ (the precursor to E₂), are transported to the host plasma membrane (9, 17). E₁, PE₂, and the capsid protein interact, so that PE₂ is cleaved to E₂, and envelopment of the nucleocapsid occurs.

Although the early events leading to the infection of a cell are currently the subject of some controversy, it is generally agreed that infection involves the fusion of host and virus membranes, releasing the nucleocapsid into the cytoplasm for uncoating (5, 6, 11).

Although fusion activity may be required for infection of cells, the process would be unnecessary and possibly detrimental during envelopment. For this reason, it has been proposed that fusion activity is only exposed as a result of the protein interactions leading to cleavage of PE₂ to E₂ and envelopment of the nucleocapsid (3, 20). White et al. (22) have shown FFWO for alphaviruses by a procedure that involves lowering the pH. By a similar technique, we have undertaken an examination of the macromolecular events associated with FFWI for Sindbis virus-infected cells with wild-type virus and the temperature-sensitive mutants *ts20* and *ts23*.

Previous studies have shown that at nonpermissive temperatures, mutants *ts20* (complementation group E) and *ts23* (complementation group D) produce intact nucleocapsids and viral

glycoproteins PE₂ and E₁ (2, 4, 12, 20). In cells infected at the nonpermissive temperature with mutant *ts20*, polypeptides E₁ and PE₂ are transported to the cell surface, but cleavage of PE₂ to E₂ does not occur (9). In cells infected at the nonpermissive temperature with mutant *ts23*, virus glycoproteins are not transported beyond the Golgi membranes (9). These mutants are well suited for a study of the expression of the virus fusion functions at the cell surface.

MATERIALS AND METHODS

Cells, virus, and media. BHK-21 cells were grown in Eagle minimal essential medium (MEM) (7) supplemented with 10% fetal calf serum (FCS), 2 mM glutamine, and 10% tryptose phosphate broth as described previously (9). Heat-resistant wild-type Sindbis virus (SVHR) and Sindbis virus temperature-sensitive mutants *ts20* and *ts23* were originally provided by E. Pfefferkorn (Dartmouth Medical College) and propagated as previously described (16).

Fusion of Sindbis virus-infected cells. Infected cells in MEM plus 1% FCS were washed twice with phosphate-buffered saline (PBS) plus 3% FCS. Fusion medium at the appropriate temperature was added to the cells for 1 min. The fusion medium was RPMI 1640 without bicarbonate, containing 0.2% FCS, 10 mM 2(*N*-morpholino)ethanesulfonic acid, and 10 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid), essentially as described by White et al. (22). The pH of the medium was adjusted to 5.3 (fusion medium) or 7.2 (control medium) by adding NaOH.

Cells treated with fusion or control medium were returned to growth medium (MEM, pH 7.2) and incubated for 1 h at the appropriate temperature, after which they were evaluated with a phase-contrast light microscope. Fusion did not occur when cells were retained at low pH. The degree of fusion of the monolayers was scored by the convention described by Gallaher et al. (10). The number of nuclei in a standard field was counted and divided by the number of cells counted in the same field. The values obtained in monolayers treated at low pH were compared with those for identical untreated monolayers. Fusion was scored as follows: -, no fusion; +, less than 10 nuclei per polycaryon, less than 25% of cells fused; ++, more than 10 nuclei per polycaryon, less than 50% of cells fused; +++, more than 10 nuclei per polycaryon, more than 50% of cells fused; +++, more than 95% of cells fused (see Fig. 1).

Other procedures. Polyacrylamide gel electrophoresis of virus polypeptides, density gradient purification of labeled virus, and trichloroacetic acid precipitation of protein were carried out as described previously (1). Antisera preparation and ¹²⁵I insoluble labeling procedures were exactly as described previously (9).

RESULTS

Criteria for evaluating FFWI. Figure 1 shows monolayers of Sindbis virus-infected BHK-21 cells fused after exposure to low-pH medium. The extent to which a monolayer fused depended upon experimental conditions and was scored from + for very little fusion (Fig. 1B) to +++,

representing the fusion of over 95% of those cells in the monolayer into a single polycaryon (Fig. 1E). Intermediate values are represented as ++ or +++ (Fig. 1C and D). Monolayers of BHK-21 cells infected for a period of 5 h with SVHR readily fused after treatment with low-pH medium (Fig. 1E). Fusion of the infected monolayers occurred between pH 5.1 and 5.7, with optimal fusion occurring between pH 5.3 and 5.5. Although the following studies were carried out in RPMI 1640, fusion could be carried out efficiently in physiological saline, PBS, MEM, or 2(*N*-morpholino)ethanesulfonic acid buffer at the appropriate pH.

We have found that Sindbis virus-mediated FFWO can take place in the presence of cycloheximide (100 µg/ml) and requires multiplicities in excess of 1,000 PFU/cell (particle-to-PFU ratio, 6:10 [B. Riedel, unpublished data]). However, when cells were infected at high multiplicities, FFWO could be prevented by washing the cells two times with cold PBS plus 3% FCS (as described above) before treating them with low-pH medium. This washing treatment was used in the experiments that follow to remove any newly synthesized intact virus particles that, by accumulating at the cell surface, could have caused FFWO. Fusion occurring after the cells were washed must therefore be caused by cell-associated virus proteins (FFWI).

FFWI mediated by SVHR and temperature-sensitive mutants. The protocols described above were used to qualitatively evaluate the ability of cells infected with SVHR and temperature-sensitive Sindbis virus mutants to form polycaryocytes (Table 1). The extent of FFWI induced by SVHR was found to depend on the time after infection and correlated with the appearance of virus protein on the surface of the infected cells. We showed previously that Sindbis virus antigens can be detected on the cell surface in moderate amounts at 2 h postinfection. We found that the amount of detectable protein on the cell surface increased until it reached a maximum at 5 h postinfection (9). Limited FFWI can be obtained at 2 h postinfection, when the amount of protein detected on the cell surface is about one fourth of the maximum amount detected at 5 h (9) (Table 2).

The fusion of SVHR-infected cell monolayers at 5 h postinfection was proportional to the input multiplicity of virus at multiplicities of infection less than 1. For example, at an input multiplicity of 0.05 PFU/cell, patches of fused cells (similar to those shown in Fig. 1B) were seen when low-pH treatment was carried out at 5 h postinfection. The number of areas of fused cells correlated with the input multiplicity (5 viruses per 100 cells). This observation supports the hypothesis of Poste and Pasternak (15) that an infected cell

FIG. 1. Qualitative comparison of low-pH-fused SVHR-infected BHK-21 cells. Cells were infected with SVHR, *ts20*, or *ts23* and incubated at appropriate temperatures and times (see text). Micrographs were taken 1 h after low pH treatment. (A) Uninfected, low-pH-treated monolayer (– score [control]). Infected, low-pH-treated monolayers scored as (B) +, (C) ++, (D) +++, and (E) +++++. See the text for definitions of scores.

is capable of fusing with noninfected cells in the monolayer.

FFWI was found to depend on the synthesis of virus protein. Cells treated with cyclohexi-

imide (Table 1) before being infected with 50 to 100 PFU of Sindbis virus per cell would not fuse at any time tested (Table 1). Although virus protein synthesis seems to be essential for estab-

TABLE 1. Low-pH-induced fusion of SVHR- and temperature-sensitive mutant-infected cells

Expt conditions ^a	Fusion score for cells infected with:		
	SVHR	<i>ts23</i>	<i>ts20</i>
Low MOI, ^b low-pH treatment at 5 h p.i.	++++	++++ ^d	++++ ^d
High MOI, ^c low-pH treatment at zero time	++++	++++	++++
CX pretreatment, ^e low MOI, low-pH treatment at 5 h p.i.	—	— ^d	— ^d
CX pretreatment, ^e high MOI, low-pH treatment at zero time	++++	++++	++++
Low MOI, NPT, low-pH treatment at 7 h p.i.	++++	—	++
Low MOI, PT, low-pH treatment at 7 h p.i.	++++	++++	++++
Low MOI, NPT, shift to PT at 5 h p.i., low-pH treatment at 7 h p.i.	++++	++	+++
Low MOI, NPT, CX treatment ^f at 4.5 h p.i. shift to PT at 5 h p.i., low-pH treatment at 7 h p.i.	++++	—	++
Low MOI, PT, trypsin treatment ^g at 3 h p.i., low-pH treatment at 7 h p.i.	++++	ND ^h	++++
Low MOI, PT, tunicamycin treatment ⁱ at zero time, low-pH treatment at 7 h p.i.	—	—	ND

^a Abbreviations: MOI, multiplicity of infection; CX, cycloheximide; NPT, nonpermissive temperature (39°C); PT, permissive temperature (32°C); p.i., postinfection.

^b Low MOI, <100 PFU/cell.

^c High MOI, >1,000 PFU/cell.

^d Incubated at the PT.

^e CX concentration was 100 µg/ml in the appropriate medium 1 h before adsorption and maintained during the incubation steps after adsorption.

^f CX concentration was 100 µg/ml in MEM plus 1% FCS.

^g Trypsin (50 µg/ml in PBS) treatment was for 2 min, followed by soybean trypsin inhibitor (200 µg/ml in PBS deficient in calcium and magnesium).

^h ND, Not determined.

ⁱ Tunicamycin concentration was 1 µg/ml in MEM plus 1% FCS.

lishing the requisite conditions for infected cells to fuse, experiments with the temperature-sensitive mutant *ts23* showed that the production of virus protein alone is not adequate for fusion (Table 1). Mutant *ts23* produces virus glycoproteins PE₂ and E₁ at nonpermissive temperatures; however, we have previously shown that these proteins do not appear in significant amounts on the cell surface (9) and, in fact, are not transported beyond the Golgi membranes (9, 10). Cells infected with *ts23* at the permissive temperature fuse readily when treated with low-pH medium at 7 h postinfection (Table 1). Mutant *ts23*-infected cells maintained at the nonpermissive temperature (39.5°C) produced no detectable fusion when similarly treated. Mutant *ts23*-infected cells maintained at 39.5°C for 4 h could be fused if they were shifted to 32°C for an additional 3 h. This fusion could be prevented if the shift was carried out in the presence of cycloheximide, confirming the nonreversible nature of the defect in this mutant (9, 20).

Sindbis virus mutant *ts20* (complementation group E) is similar to mutant *ts23* (complementation group D) in that it allows the production of polypeptides PE₂ and E₁ (2, 9, 12, 20). It differs from *ts23* in that these proteins are transported to the cell plasma membrane and that these modified membranes bind capsid protein (4, 20). We have previously shown that at 5 h postinfection

at the nonpermissive temperature, the surface of the *ts20*-infected cells has 95% of the protein detectable by immunological techniques found in the SVHR-infected cell (9). This mutant is ideal for determining whether modification of the plasma membrane in the absence of virus production is adequate for the expression of fusion activity. Whereas cells infected with *ts20* at the permissive temperature were readily fused by low-pH treatment at 7 h postinfection, only ++ fusion could be achieved when the experiment was conducted with cells infected at the nonpermissive temperature (Table 1). As in cells infected with mutant *ts23*, the virus defect in cells infected with *ts20* was found to be irreversible after a shift to the permissive temperature in the presence of cycloheximide (Table 1). These results show that the presence of virus glycoproteins on the cell surface is not adequate for the expression of fusion activity. The limited amount of fusion seen in this experiment may reflect the production of some nondefective virus proteins in cells infected for extended periods of time with mutant *ts20*. We have previously shown that in 8 h, small amounts of Sindbis virus polypeptide E₂ can be detected at the cell surface by lactoperoxidase iodination in cells infected with *ts20* at the nonpermissive temperature (20). Cells infected with *ts20* or *ts23* at the nonpermissive temperature could be fused from

TABLE 2. Immunological detection of Sindbis virus proteins on the surface of infected BHK cells in the presence of tunicamycin^a

Expt conditions	¹²⁵ I incorporated (mcpm) ^b
Uninfected cells	2.0
Cells infected with SVHR (5 h), ¹²⁵ I-protein A ^c	1.8
Infected cells (2 h) and anti-Sindbis virus serum, ¹²⁵ I-protein A	10.5
Infected cells (5 h) and anti-Sindbis virus serum, ¹²⁵ I-protein A	32.4
Infected cells (5 h) in tunicamycin ^d and anti-Sindbis virus serum, ¹²⁵ I-protein A	23.0

^a Cells were infected and labeled with anti-Sindbis virus serum and ¹²⁵I-labeled staphylococcal protein A, as described previously (9). The antiviral serum immune-precipitated only virus proteins from infected cells (data not shown).

^b Data were obtained from monolayers containing equal numbers of cells treated with equal quantities of antibody and staphylococcal protein A. Antibody was preadsorbed against uninfected BHK-21 cells.

^c SVHR-infected cells were treated with ¹²⁵I-labeled staphylococcal protein A as a control for nonspecific binding.

^d Tunicamycin (1.0 µg/ml) was added at 1 h postinfection and remained in the culture throughout the experiment.

without by the addition of a high multiplicity of virus (ca. 1,000 PFU/cell) and treatment with low-pH medium, indicating that the higher temperature and level of infection with these mutants did not render the cells incapable of fusion.

The lack of fusion activity in *ts23*- or *ts20*-infected cells at the nonpermissive temperature led us to test these virus mutants by themselves for the presence of temperature-sensitive fusion activity. However, neither transient exposure of *ts20* or *ts23* virions to nonpermissive conditions (5 min at 41°C) nor a shift of *ts23*- or *ts20*-infected monolayers from permissive to nonpermissive conditions (5 h at 32°C, followed by 2.5 h at 39°C) had any effect on fusion activity. Thus, in mutants *ts23* and *ts20* the fusion factor itself is not temperature sensitive.

The results presented above suggest that (i) fusion requires significant production of polypeptide E₂ at the cell surface or (ii) if fusion can be mediated by virus polypeptides PE₂ and E₁ on the cell surface, the defect in *ts20*-coded membrane glycoproteins prevents the proteins from achieving an aggregation state or conformation essential for the expression of fusion activity.

It is particularly interesting that fusion of virus-infected cells could be obtained at any

time postinfection when the cells were maintained in low-pH growth medium. Fusion only occurred when cells were returned to medium having a neutral pH. Exposure of an infected monolayer to pH 5.3 for as little as 30 s followed by a shift to neutral pH was sufficient to produce maximum fusion. When cells were maintained at low pH for longer periods of time (up to 30 min), fusion still occurred after their return to neutral pH. Extended exposure to low pH eliminated the capacity of cells to fuse.

FFWI mediated by SVHR-infected cells blocked in conversion of polypeptide PE₂ to E₂. We have previously shown that the plasma membranes of BHK-21 cells infected with SVHR contain virus polypeptides PE₂, E₁, and E₂ (9, 17). Capsid protein is attached to the plasma membrane through contact with the PE₂ polypeptide (17). We have also shown that BHK-21 cell plasma membranes can be modified by virus glycoproteins PE₂ and E₁ without the conversion of PE₂ to E₂ when the infected cells are either (i) grown in the presence of the drug tunicamycin (17) or (ii) treated briefly with trypsin after virus infection (1). Cells infected and treated by either of these procedures incorporate virus glycoproteins into cell plasma membranes at levels equivalent to those seen in untreated SVHR-infected cells. Cells infected with Sindbis virus in the presence of tunicamycin have 70% of the virus antigen detectable on the surface of untreated cells at 5 h postinfection; this exceeds the amount required to produce fusion in untreated cells (Table 2). Tunicamycin-treated Sindbis virus-infected cell plasma membranes differ from untreated cell plasma membranes in that proteins PE₂ and E₁ are not glycosylated (13, 17); capsid protein still associates with the PE₂ polypeptide (17). We have previously shown that trypsin treatment of Sindbis virus-infected cells, carried out as employed in this study, does not affect the transport of protein to the cell surface. Trypsin-treated, Sindbis virus-infected cell plasma membranes contain glycosylated PE₂ and E₁ but bind little capsid protein, and conversion of PE₂ to E₂ does not occur (1). When infected cells treated by either of these two procedures were tested for their ability to be fused by low-pH treatment, we found that tunicamycin-treated, virus-infected cells did not fuse at all, whereas trypsin-treated, virus-infected cells fused as efficiently as untreated cells did (Table 1). Trypsin treatment of uninfected cells did not establish conditions for fusion.

These data suggest that glycosylation of virus proteins is essential for Sindbis virus-infected cells to express FFWI, but that the conversion of PE₂ to E₂ or the binding of capsid to the inner surface of the modified membrane is not essen-

FIG. 2. Effect of low pH treatment on maturation of Sindbis virus proteins. BHK-21 cell monolayers were pretreated with 4 μ g of actinomycin D per ml, infected with 100 PFU of SVHR per cell and incubated for 4.5 or 5 h at 37°C. (A) Cells were starved for 30 min at 4.5 h postinfection in methionine-free medium before being labeled with 50 μ Ci of [³⁵S]methionine per ml. After a 5-min pulse, cells were washed for 5 min with 37°C chase medium (MEM plus 75 μ g of cycloheximide per ml and 2 mM cold methionine). Cells were treated for 1 min at 37°C with (○) fusion (pH 5.3) or (●) control medium (pH 7.2) and returned to chase medium for 3 h at 37°C. The medium was collected, and labeled virus was detected by density gradient

tial for the expression of the virus-associated fusion activity.

Effect of low-pH treatment on the maturation of Sindbis virus proteins. In an accompanying report (8), we show that exposure of intact, purified Sindbis virus to low pH produces a conformational change in the virus structural proteins and rapidly inactivates virus infectivity. It seemed necessary to determine whether low-pH treatment of infected cells, i.e., conditions leading to cell fusion, could affect the maturation of virus proteins. The production of physical virus particles by cells exposed to low-pH medium was evaluated in two ways. Cells were either labeled with [³⁵S]methionine and treated with low-pH medium or treated with low-pH medium and then labeled with [³⁵S]methionine. In the former, cycloheximide (75 μ g/ml) was added to the medium before and during the exposure to low pH to ensure that no synthesis of virus proteins occurred after the low-pH treatment. Surprisingly, low-pH treatment had no effect on the ability of proteins labeled before treatment to be matured into physical virus particles (Fig. 2). These cells also released amounts of infectious virus equivalent to those released by control cells (data not shown). Proteins synthesized after the shift to low pH were not efficiently matured. The arrest of new virus production after low-pH treatment seemed to result from a reduction in total virus proteins synthesized relative to synthesis in untreated cells (only 25% as determined by net counts incorporated into trichloroacetic acid-insoluble material under identical conditions of labeling with [³⁵S]methionine). We further found that the inhibitory effect on virus production of low-pH treatment was not observed when the treatment was carried out early in infections (before 3 to 4 h postinfection). The reason for this delayed sensitivity is not known. Polyacrylamide gel electrophoresis of the intracellular virus proteins produced in the above experiments revealed typical profiles for PE₂, E₁, E₂, and the capsid protein (data not shown).

DISCUSSION

Virus-induced syncytia formation or FFWI has long been used as a criterion for characterization of groups of viruses, in particular the paramyxoviruses (14, 15). The data presented above add Sindbis virus to the growing list of viral agents that, under appropriate conditions, can induce syncytia formation. We found that

centrifugation as described in the text. (B) Cells treated at 5 h postinfection with 37°C fusion medium (○) or PBS (●) (control) for 1 min, washed with methionine-free medium, and then labeled for 3 h with 25 μ Ci of [³⁵S]methionine per ml.

the alphavirus Sindbis readily and efficiently induces cell fusion, leading to the production of polycaryocytes in which over 95% of the cells in a monolayer participate. The data presented above, although qualitative rather than quantitative in nature, show that Sindbis virus-induced polycaryocyte formation (i) requires modification of the cell surface with glycosylated virus membrane proteins, (ii) does not require the conversion of PE₂ to E₂ or extensive membrane association of the capsid protein, and (iii) does not affect the maturation of protein synthesized before fusion into mature virus. As to the last point, we found that a polycaryocyte produced from a monolayer of 10⁶ cells matured an amount of virus equivalent to that matured by an equal number of nonfused cells (data not shown). Thus, the process of fusion at low pH does not alter the ability of the cell to produce new virus from existing proteins, although at certain times after infection low-pH treatment did interfere with net protein synthesis.

Gallaher et al. (10) pointed out that although the data indicate that virus-induced fusion activity is under control of the viral genome, no one has shown the most convincing evidence for genetic identification of the fusion factor, i.e., a fusion factor produced by virus under permissive conditions that loses its fusion activity when shifted to nonpermissive conditions. Tests in our lab (see above) have shown that the fusion factor itself in mutants *ts23* and *ts20* is not temperature sensitive.

The data presented here suggest that the combination of PE₂ and E₁ on the surface of a Sindbis virus-infected cell can mediate cell fusion after brief exposure to low pH. This observation implies that a conformational change in one or both of these two proteins exposes a previously buried protein domain capable of producing membrane fusion without altering the ability of the proteins to be matured into virus. In an accompanying communication (8), we used purified virus to demonstrate that the conformational change leading to the fusion of virus membranes with cell membranes is in the E₂ polypeptide.

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