Identification and Characterization of Cell Lines with a Defect in a Post-adsorption Stage of Sendai Virus-mediated Membrane Fusion*

Received for publication, December 14, 1999, and in revised form, February 24, 2000
Published, JBC Papers in Press, March 27, 2000, DOI 10.1074/jbc.M910004199

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In the early stage of infection, Sendai virus delivers its genome into the cytoplasm by fusing the viral envelope with the cell membrane. Although the adsorption of virus particles to cell surface receptors has been characterized in detail, the ensuing complex process that leads to the fusion between the lipid bilayers remains mostly obscure. In the present study, we identified and characterized cell lines with a defect in the Sendai virus-mediated membrane fusion, using fusion-mediated delivery of fragment A of diphtheria toxin as an index. These cells, persistently infected with the temperature-sensitive variant Sendai virus, had primary viral receptors indistinguishable in number and affinity from those of parental susceptible cells. However, they proved to be thoroughly defective in the Sendai virus-mediated membrane fusion. We also found that viral HN protein expressed in the defective cells was responsible for the interference with membrane fusion. These results suggest the presence of a previously uncharacterized, HN-dependent intermediate stage in the Sendai virus-mediated membrane fusion.

Sendai virus (SV) belongs to the Paramyxoviridae, carrying a nonsegmented, negative strand genomic RNA (15, 384 nucleotides). The viral envelope, made up of a lipid bilayer and two glycoproteins (F and HN), encapsulates the nucleocapsid, consisting of the genomic RNA and three nucleocapsid proteins (NP, P, and L). In the early stage of infection, SV delivers the nucleocapsid into the cytoplasm by fusing its envelope with the cell membrane at neutral pH. SV also induces cell-to-cell fusion under appropriate conditions. These phenomena have been investigated intensively as a model of fusion between biological membranes (2).

SV-mediated membrane fusion consists of two distinguishable stages: the binding of the viral envelope to the cell membrane, and the subsequent fusion between the lipid bilayers. The former stage, mediated by the interaction between viral HN protein and the cell surface receptors containing sialic acid (N-acetylneuraminic acid), is essential for subsequent membrane fusion (3, 4). A number of molecules have been assigned to SV receptors, including glycoproteins (5–9) and gangliosides (3, 10–15). In cultured mammalian cells, gangliosides with terminal sialic acid residues, such as GD1a, GT1b, and GQ1b, have been reported to serve as functional receptors (10, 11).

The second stage of fusion between the lipid bilayers is triggered by the viral F protein. This protein is initially synthesized as a precursor form (F0), which is then processed by proteolytic cleavage to a mature form consisting of two polypeptides (F1 and F2) (16). The cleavage exposes a well conserved hydrophobic domain at the N terminus of the F1 subunit (17), which binds cholesterol (18) and plays a principal role in the fusion between lipid bilayers.

This simple two-step model has been thought to represent most types of membrane fusion induced by various envelope viruses (19). However, the recent finding that human immunodeficiency virus (HIV) and herpes simplex virus (HSV) require certain cellular factors for infection in addition to specific cell surface receptors (20, 21) offers a new perspective on this process. These cellular factors (co-receptors) are not involved in the primary binding, but their interaction with viral components is essential for membrane fusion.

Several lines of evidence suggest that SV may also depend on one or more unidentified cellular factors for membrane fusion, in addition to the primary receptor. For example, Okada and Tadokoro (22) reported that SV could aggregate but not fuse human and murine primary lymphocytes. We also found that SV could bind to but not fuse with human primary B cells and the cell lines derived from B cells (23). However, the nature of the hypothetical SV co-receptor, if any, is still unknown.

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To investigate the mechanism of the virus-mediated membrane fusion further, it is important to establish a simple but quantitative fusion assay. In the case of HIV and HSV, expression of Escherichia coli β-galactosidase (20) and firefly luciferase (24) encoded in the recombinant virus genome was used as the index of membrane fusion. In this study, we used the delivery of fragment A of the diphtheria toxin (DTA) as the index. Although DTA is nontoxic as long as it is present in the culture medium (25), just one molecule of DTA in the cytoplasm is sufficient to kill the cell through inactivation of elongation factor 2 (26). Therefore, if we can deliver DTA into the cytoplasm through membrane fusion, cytotoxicity becomes a good index of the fusion. For delivery of DTA, we used a unique delivery system named fusogenic liposome (FL). FL has a unilamellar membrane associated with SV envelope glycoproteins and fuses with the cell membrane by the same mechanism as SV (27). As the empty FL is not toxic, we can estimate the efficiency of SV-mediated membrane fusion from the toxicity of FL encapsulating DTA (FL-DTA) (28).

We report here on the establishment and biochemical characterization of cell lines that bound SV particles efficiently but that had a defect in SV-mediated membrane fusion. Our results suggest that there is a novel, HN-dependent intermediate stage in SV-mediated membrane fusion.

**EXPERIMENTAL PROCEDURES**

**Viruses**—Sendai virus Z strain was prepared as described previously (28), except that the virus was further purified by sucrose step centrifugation (20%/50%) and suspended in buffered salt solution (BSS) (150 mM NaCl, 10 mM Tris-HCl, pH 7.6). SV cl.151 strain, a temperature-sensitive variant, was prepared using fertilized chicken eggs at 32 °C, as described previously (29, 30).

**Cells**—All the cells used in this study were cultured in the presence of antibiotics (100 units/ml penicillin and 100 μg/ml streptomycin) at 37 °C under 5% CO2. HeLa cells, LLCMK2 cells (rhesus monkey kidney cell line) and their derivatives were cultured in Eagle’s minimum essential medium (MEM) supplemented with 10% newborn calf serum. COS-1 cells (31) were cultured in Dulbecco’s modified MEM supplemented with 10% fetal calf serum. Cell lines infected by SV cl.151 strain persistently (HeLa cl.151 and cl.152) were established as follows. Parental cells (1 × 105 cells) were placed in 100-mm dishes on day 0. On day 1, the cells were exposed to the virus (multiplicity of infection = 10) for 30 min at 4 °C and then for 30 min at 37 °C. The cells were harvested once with the medium harvested, and reseeded in 100-mm culture dishes at 10 cells/dish. The cells were cultured at 37 °C to isolate the colonies on day 10. To confirm the uniformity of infection, the cells were examined by indirect immunofluorescence microscopy using anti-NP mouse mAb.

Cell lines carrying a defective interfering SV genome (HeLa DI and LL DI cells) were established as follows. Five million parental cells were infected with the virus as described above, except that SV Z strain containing DI particles, prepared by undiluted passage in fertilized chicken eggs (32), was used at a multiplicity of infection of 1,000. After infection, the cells were cultured for 7 days without passage. On day 8, surviving colonies were isolated, cultured, and subcloned once. Uninfected LLCMK2 cells infected with rc-SV during the initial 60-min exposure would die. Protein synthesis was determined by colony formation, as described previously (28). Briefly, 500 cells were seeded in 35-mm dishes on day 0. After 8 h, the cells were washed once with ice-cold BSS (+) (150 mM NaCl, 2 mM CaCl2, 10 mM Tris-HCl, pH 7.6) and incubated with various concentrations of FL-DTA at 4 °C for 30 min and then at 37 °C for 30 min. The cells were washed once with medium and then refed with fresh medium. The number of surviving colonies was determined on day 7. For the experiment shown in Fig. 6, the cells were treated with anti-SV RN rabbit polyclonal Ig4 affinity-purified on protein A-Sepharose (Amersham Pharmacia Biotech) or with control rabbit Ig for 1 h at 37 °C in MEM (serum free) just before exposure to FL-DTA.

Susceptibility to replication-competent SV (rc-SV) was also determined by colony formation, because progeny Sendai viruses produced by cultured cells were inactive (16) and therefore only the cells infected with rc-SV during the initial 60-min exposure would die. Protein synthesis was determined as described previously (42), except that [35S]Sime-thionine (37 TBq/mmol; NEN Life Science Products, Inc., Boston, MA) was used in place of [3H]leucine.

**Binding Assay**—We labeled 50 μg of Sendai virus with 18.5 MBq of 125I-SV using Bolton-Hunter labeled Bolton-Hunter (81.4 TBq/mmol) (NEN Life Science Products, Inc., Boston, MA) and purified it by gel filtration according to the protocol provided by the supplier. Specific activity of the labeled sample was 1.1–7.0 × 106 cpm/μg. For the experiment shown in Fig. 4, we mixed 50 μg of labeled virus with 34 μg of cold virus to adjust the specific activity to 1.8 × 107 cpm/μg.

For the experiment shown in Fig. 4, cells were seeded at 5 × 104 cells/well in 96-well plates on day 6. On day 1, the cells were washed once with BSS (+) containing 0.1% gelatin and then incubated with 190 μl of gelatin-BSS (+) containing 5.0 × 106 to 3.5 × 109 cpm of 125I-SV for 60 min at 4 °C with gentle shaking. The cells were then washed three times with gelatin-BSS (+), and radioactivity associated with the cells was determined with a γ counter. Radioactivity associated with the cells in the presence of 13 μM dithiothreitol (DTT) was subtracted as nonspecific binding. The results were analyzed to determine the affinity constant and the number of binding sites and to examine the homogeneity of the binding site, as described previously (43).

For the experiments shown in Table 1, a binding assay was performed as described above except that 0.12 μg of 125I-SV (1.1–7.0 × 107 cpm/μg) was used for each well. To determine the effect of sialidase treatment, the cells were treated with 50 units of Clostridium perfringens sialidase (New England Biolabs, Inc., Beverly, MA) in 200 μl of MEM containing 20 mM MES-NaOH (pH 5.5) at 37 °C for 90 min. To determine the effect of DTT, 125I-SV was incubated with 5 μM DTT at 37 °C for 20 min. In both of these experiments, the radioactivity associated with the cells in the presence of 5,000-fold excess cold SV was subtracted as nonspecific binding. To determine the effect of antibodies, 125I-SV was incubated at 25 °C for 90 min with 30 μg/ml of mouse IgG purified on protein A-Sepharose. In this experiment, radioactivity associated with the cells treated with 50 units of sialidase as described above was subtracted as nonspecific binding.

2 N. Miura, unpublished observations.

3 M. Nakashima, unpublished observations.
RESULTS

Cell Lines Persistently Infected with Temperature-sensitive Variant Sendai Virus Had the Defects in Membrane Fusion—One of the efficacious approaches for investigating the virus-cell interaction is to compare the characteristics of the susceptible cells with those of unsusceptible cells, which lack the susceptibility naturally or lose the susceptibility after infection by syngeneic or heterogeneous viruses (interference). Among these unsusceptible cells we may find the cells defective in membrane fusion, which would be useful sources for analyzing the mechanism of membrane fusion. As the cells infected with SV persistently became unsusceptible to further challenge by rc-SV (44), we examined whether these cells were defective in SV-mediated membrane fusion.

SV can establish persistent infection in cultured cells by two different mechanisms: the temperature-sensitive (ts) defects in the viral protein function (29, 30, 45), and the suppression of viral replication by a defective interfering (DI) genome with a large internal deletion (46). Hence, we established several cell clones infected with SV persistently by one of these two mechanisms and examined their ability to fuse with the SV envelope.

After confirming the uniformity of infection by examining the expression of major nucleocapsid (NP) protein (Fig. 1), we challenged these cells with rc-SV and examined the susceptibility by colony formation. As shown in Fig. 2, the cells persistently infected with ts variant virus (29, 30) (LL cl.151 and HeLa cl.151 cells; open circles) were extremely resistant to the challenge by rc-SV, indicating that these cells blocked the replication of rc-SV efficiently. The cells persistently infected with DI-containing virus (LL DI and HeLa DI cells; closed squares) were also resistant to rc-SV compared with parental susceptible cells (LLCMK2 and HeLa cells; closed circles). The same phenomenon was observed in several cell clones that had been established independently (data not shown).

We then examined whether these unsusceptible cells had a defect in fusion with the SV envelope by assessing the cytotoxicity of FL-DTA. As shown in Fig. 3, susceptible cells (LLCMK2 and HeLa cells; closed circles) were extremely sensitive to FL-DTA, as expected; FL-DTA containing 1 ng/ml of DTA is
sufficient to kill all the cells in the culture. In contrast, the cells infected with ts variant SV (open circles) were highly resistant to FL-DTA; even at the highest concentration (containing 50 ng/ml of DTA), FL-DTA did not affect the growth of these unsusceptible cells at all. These data clearly demonstrated that the latter cells had some defect in SV-mediated membrane fusion. LL DI and HeLa DI cells were moderately resistant to FL-DTA (closed squares), indicating that these cells had a defect in a later stage of the replication cycle in addition to the primary membrane fusion.

Because other envelope viruses (measles virus and vaccinia virus) could replicate in these three types of cells at almost equal efficiency (data not shown), the defect in membrane fusion should not result from general cellular responses to suppress viral replication. Rather, the defect in membrane fusion was caused by the Sendai virus-specific mechanism. Moreover, the defect in membrane fusion should not result from selecting genetically altered cells resistant to virus infection, because this ts variant virus (SV cl.151 strain) could readily establish persistent infection in cultured cells without selection because of the temperature-sensitive defect of the matrix protein (33). Therefore, we further investigated the SV-mediated membrane fusion by using one of the cell lines persistently infected with SV cl.151 strain (LL cl.151 cells).

**Cells Defective in Membrane Fusion Can Bind Sendai Virus Particles as Efficiently as Parental Susceptible Cells**—There were two possibilities that could account for the defect of LL cl.151 cells in SV-mediated membrane fusion: the cells lacked the receptors for SV or the cells had some defect in the critical stage following viral adsorption. To examine these possibilities, we first characterized the adsorption of SV particles on the cell surface using 125I-labeled SV as a probe.

As shown in Fig. 4a, we found that both susceptible cells (closed circles) and defective LL cl.151 cells (open circles) could bind the same amount of SV in a saturable manner. Further analysis of the results by Scatchard plotting (Fig. 4b) showed that both cell types had the same number of receptors (about 2,000 binding sites/cell) with the same high affinity ($K_d = 3 \times 10^{-10}$ M). Analysis by Hill plotting (Fig. 4c) also showed that both cell types had only a single kind of receptor. These results demonstrate that defective LL cl.151 cells have the specific receptors for SV indistinguishable in number and affinity from those on parental susceptible cells.

We further characterized the binding of SV to LL cl.151 cells by examining the effects of various interfering reagents. We found that the treatment of the defective cells with C. perfringens sialidase abolished the binding with SV completely (Table I). We also found that treating SV with DTT (5 mM) and}

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**TABLE I**

<table>
<thead>
<tr>
<th>Treating Reagent</th>
<th>[125I]-Sendai virus bound</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>LLCMK2</td>
</tr>
<tr>
<td>Cell</td>
<td></td>
</tr>
<tr>
<td>Mock</td>
<td>20,887 (100.0)</td>
</tr>
<tr>
<td>Sialidase</td>
<td>-252 (-1.2)</td>
</tr>
<tr>
<td>Virus</td>
<td></td>
</tr>
<tr>
<td>Mock</td>
<td>5,621 (100.0)</td>
</tr>
<tr>
<td>DTT</td>
<td>-44 (-0.8)</td>
</tr>
<tr>
<td>Virus</td>
<td></td>
</tr>
<tr>
<td>Mock</td>
<td>4,213 (100.0)</td>
</tr>
<tr>
<td>Control IgG</td>
<td>4,199 (99.7)</td>
</tr>
<tr>
<td>Anti-HN (HN1)</td>
<td>245 (5.8)</td>
</tr>
</tbody>
</table>

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anti-HN mAb (HN-1), both of which interfere in the receptor-binding activity of HN (40, 47), impaired the binding of SV to the defective cells severely (Table I). These results clearly demonstrate that the binding of SV to the defective LL cl.151 cells is dependent on the interaction between viral HN protein and the receptor containing sialic acid. Therefore, we concluded that LL cl.151 cells have a normal capacity to bind SV in sialic acid- and HN-dependent manner and that this cell line has the defect in membrane fusion at a later stage following virus adsorption to SV-specific receptors.

**HN Protein on the Cell Surface Interfered with the Sendai Virus-mediated Membrane Fusion**—We next investigated the mechanism underlying the defect in membrane fusion observed in LL cl.151 cells. Because this defect was specific to SV, we hypothesized that some SV protein, probably either of two SV glycoproteins (F and HN) expressed on the cell surface, was involved in the interference. Hence we estimated the amount of F and HN proteins on the surface of thoroughly defective LL cl.151 cells and of partially defective LL DI cells by indirect immunofluorescence microscopy and found that LL cl.151 cells expressed larger amounts of F and HN proteins than did LL DI cells (Fig. 1). These results suggest strongly that the presence of either F or HN proteins on the cell surface interferes with SV-mediated membrane fusion.

We next produced the cells expressing either F or HN protein by gene transfer to examine the role of these proteins in the interference with membrane fusion separately. We first established a cell line (F-LL cells) expressing a large amount of F protein stably as LL cl.151 cells (Fig. 5a) and found that they could fuse with FL-DTA (Fig. 5b, closed squares) as efficiently...
Cells with Defects in a Novel Stage of Virus-mediated Fusion

Membrane fusion induced by paramyxoviruses has been investigated using various biological and physical approaches. Analysis of the syncytium formation induced by expressing envelope glycoproteins from cloned cDNAs is one of the most popular of these techniques (19). This assay has greatly contributed to revealing the function of envelope glycoproteins but has contributed little to uncovering the nature of cellular factors required for the fusion. Therefore, we need another quantitative assay for determining the efficiency of the fusion between the viral envelope and the cell membrane to obtain the information about critical cellular factors.

The fusogenic liposome encapsulating fragment A of diphtheria toxin (FL-DTA) is one of the most effective quantitative tools to assess the efficiency of the fusion between the Sendai virus envelope and the cell membrane. FL is a unique delivery system with an envelope-like unilamellar structure associated with the SV glycoproteins (27). FL is prepared by fusing a simple lipid vesicle and a SV particle, the genome RNA of which was inactivated in advance, and can deliver its content (proteins and DNA preloaded into the simple liposome) into the cell membrane was necessary but insufficient for inducing membrane fusion, even when functional F protein was present on the cell surface (27). Therefore, it is quite rational to use FL-DTA as the representative of an SV particle in a membrane fusion assay.

In the present study, we report that cells infected persistently with ts variant SV were fully resistant to FL-DTA (Fig. 3). We carefully characterized the SV binding property of parental susceptible cells and these defective cells and found that both types of cells could bind the same amount of SV with an indistinguishable high affinity (Fig. 4). These results are consistent with our previous finding that a majority of human peripheral B lymphocytes and T lymphocytes are fully resistant to FL-DTA and that these cells nevertheless can bind FL on their surface efficiently (23). Combining these results, we concluded that primary binding of the SV particle to the cell membrane was necessary but insufficient for inducing membrane fusion, even when functional F protein was present on the virus particles. This conclusion suggested strongly that in SV-mediated membrane fusion there is a critical intermediate stage that occurs immediately after the adsorption of the SV...
particle to the cell surface.

In the present study, we also found that HIV protein, but not F protein, on the cell surface was involved in the interference with SV-mediated membrane fusion. We should note that LL cl.151 cells have primary SV receptors indistinguishable from those of susceptible cells, despite the presence of HIV protein with sialidase activity. Based on these observations, we hypothesized that HIV protein would interact with some unidentified critical non-receptor molecules (co-receptor) and that the expression of HIV protein on the cell surface would interfere with the membrane fusion by suppressing this hypothetical co-receptor. This phenomenon may correspond to the previous finding in HSV that the cell lines expressing glycoprotein D of HSV can bind, but not fuse with, HSV (48, 49). In HSV, glycoproteins B and C were assigned roles as ligands to a primary receptor (glycosaminoglycan), and glycoprotein D was assigned the role as a ligand to co-receptor molecules (50). HIV protein might be a ligand to both the primary receptor and the hypothetical co-receptor, as is envelope glycoprotein gp120/gp41 of HIV (21).

There is accumulating evidence suggesting that paramyxovirus HIV protein is not only a binding ligand to cell surface primary receptors, but is also directly involved in membrane fusion. First, several anti-HN monoclonal antibodies interfere in the virus-mediated membrane fusion without affecting the binding of the virus to the cell surface (40, 51–53). Second, successful syncytium formation usually requires the expression of F protein and the homotypic HIV protein in the same cell (54–56), suggesting that the specific interaction between F and HIV is essential for fusion (57). The region required for this hypothetical interaction was assigned in the primary structure (58–60), suggesting that the specific interaction between F and some HN protein (58–60). Third, analysis of variant HN proteins revealed that the fusion promotion activity can be found in HSV that the cell lines expressing glycoprotein D of HSV can bind, but not fuse with, HSV (48, 49). In HSV, glycoproteins B and C were assigned roles as ligands to a primary receptor (glycosaminoglycan), and glycoprotein D was assigned the role as a ligand to co-receptor molecules (50). HIV protein might be a ligand to both the primary receptor and the hypothetical co-receptor, as is envelope glycoprotein gp120/gp41 of HIV (21).


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J. Biol. Chem. 2000, 275:17549-17555,
doi: 10.1074/jbc.M910004199 originally published online March 27, 2000

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