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Sendai Virus Efficiently Infects Cells via the Asialoglycoprotein Receptor and Requires the Presence of Cleaved F_0 Precursor Proteins for This Alternative Route of Cell Entry

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Biochemical evidence suggests that the asialoglycoprotein receptor (ASGP-R) can be used as an alternative receptor for a temperature-sensitive Sendai virus (SV) mutant. We now have investigated this possible alternative route of infection for SV wild-type (SV-wt) strain *Fushimi* by using a pair of cell lines which differ only with regard to ASGP-R expression. Infection studies after enzymatic destruction of conventional sialic acid-containing SV receptors (SA-R) revealed that only ASGP-R-expressing cells could be infected by SV-wt. This alternative route of cell entry could be completely blocked by incubation of cells with ASGP-R-specific antibodies prior to infection. Furthermore, cleavage of SV-F_0 precursor protein into the subunits F_1 and F_2 was necessary to establish infection via ASGP-R, suggesting a fusion-mediated cell entry after binding of SV-wt to the ASGP-R on host cells. Interestingly, infection via ASGP-R was found to be nearly as efficient as infection via conventional sialic acid-containing SV receptors. A possible physiological role of the ASGP-R-mediated route of SV infection is discussed.

In many cases, members of the same genus of a virus family have been found to select different chemical entities for cell attachment and invasion of host cells. Moreover, a few viruses have been described that can use more than one distinct receptor population, e.g., human immunodeficiency virus, coxsackievirus A9, and encephalomyocarditis virus (reviewed in reference 31).

The host cell tropism of Sendai virus (SV), a well-characterized member of the paramyxovirus family, is determined by its two surface glycoproteins: hemagglutinin-neuraminidase (HN) binds to sialic acid-containing ganglioside receptors (SA-R) ubiquitously expressed on the surface of all eucaryotic cells (reviewed in reference 14), followed by F protein-mediated fusion of the viral envelope with the cell membrane (reviewed in reference 11). Interestingly, an alternative route of infection has been suggested for the temperature-sensitive SV mutant *ts*271 (13). The unique feature of this mutant is a dramatically reduced HN incorporation in the viral lipid bilayer when propagated at rising temperatures, resulting in a loss of infectivity concerning all conventional host cells (17, 18, 29). As an exception, the human hepatoma cell line HepG2 could still be infected by these viral particles (13). Inhibition studies indicated that the remaining F protein—beyond its well-characterized membrane fusion property—also functions as a ligand for the hepatocyte-specific asialoglycoprotein receptor (ASGP-R). However, a more consistent proof that the ASGP-R alone is sufficient for SV adsorption and cell entry (e.g., by SV wild-type [SV-wt] infection studies after complete destruction of all conventional SA-R receptors on a cell pair differing only with regard to ASGP-R expression) has not been described so far. Moreover, because of the missing characterization of the *ts*271-F protein, it is unclear whether the ASGP-R–F interaction is a special feature of this mutant or whether the ASGP-R can be used in general as an alternative receptor for SV-wt strains.

The ASGP-R is a hepatic receptor and uptake system for desialylated glycoproteins (reviewed in references 9 and 26). Ligands are specifically bound, internalized, and delivered to lysosomes, where they are proteolytically degraded. The specificity and hepatic location of this receptor make it an attractive tool for liver-specific targeting of chemotherapeutic agents (1) and therapeutic genes (32, 33). Unfortunately, the efficiency of the ASGP-R-mediated endocytosis of complexed DNA is limited as a consequence of endosomal uptake and subsequent lysosomal degradation. Prolonged gene expression of genes delivered to the liver in vivo in this manner required a partial hepatectomy (4). To circumvent these problems, reconstituted SV envelopes containing exclusively fusion-active F proteins on their surfaces have been generated and were found to release their virosomal contents directly to the cytosol when tested on HepG2 cells (2). From this finding, direct uptake of ASGP-R-bound SV particles could also be hypothesized.

Here we report that the ASGP-R can be used efficiently by SV-wt strain *Fushimi* as an alternative receptor for the infection of host cells. Moreover, after complete enzymatic destruction of SA-R, we demonstrate that the ASGP-R alone is sufficient for SV-wt infection. Concerning the mode of cell entry, the infection via this alternative route is dependent on the cleavage of SV-F_0 precursor protein into the subunits F_1 and F_2, suggesting fusion-mediated cell entry.

MATERIALS AND METHODS

Reagents. Polyclonal goat anti-human ASGP-R serum was kindly provided by Gilbert Ashwell (National Institutes of Health, Bethesda, Md.). Neuraminidase from *Vibrio cholerae* and Nutridoma SR were purchased from Boehringer (Mannheim, Germany).

Virus and cells. Sendai virus (strain *Fushimi*) was either grown in 9-day-old embryonated chicken eggs as described previously (22) or propagated in CV-1 cells. In brief, CV-1 cells were infected at a multiplicity of infection (MOI) of 1 and overlaid with medium M199 (Gibco-BRL, Eggenstein, Germany) containing Nutridoma SR instead of fetal calf serum. Forty-eight hours postinfection, su-

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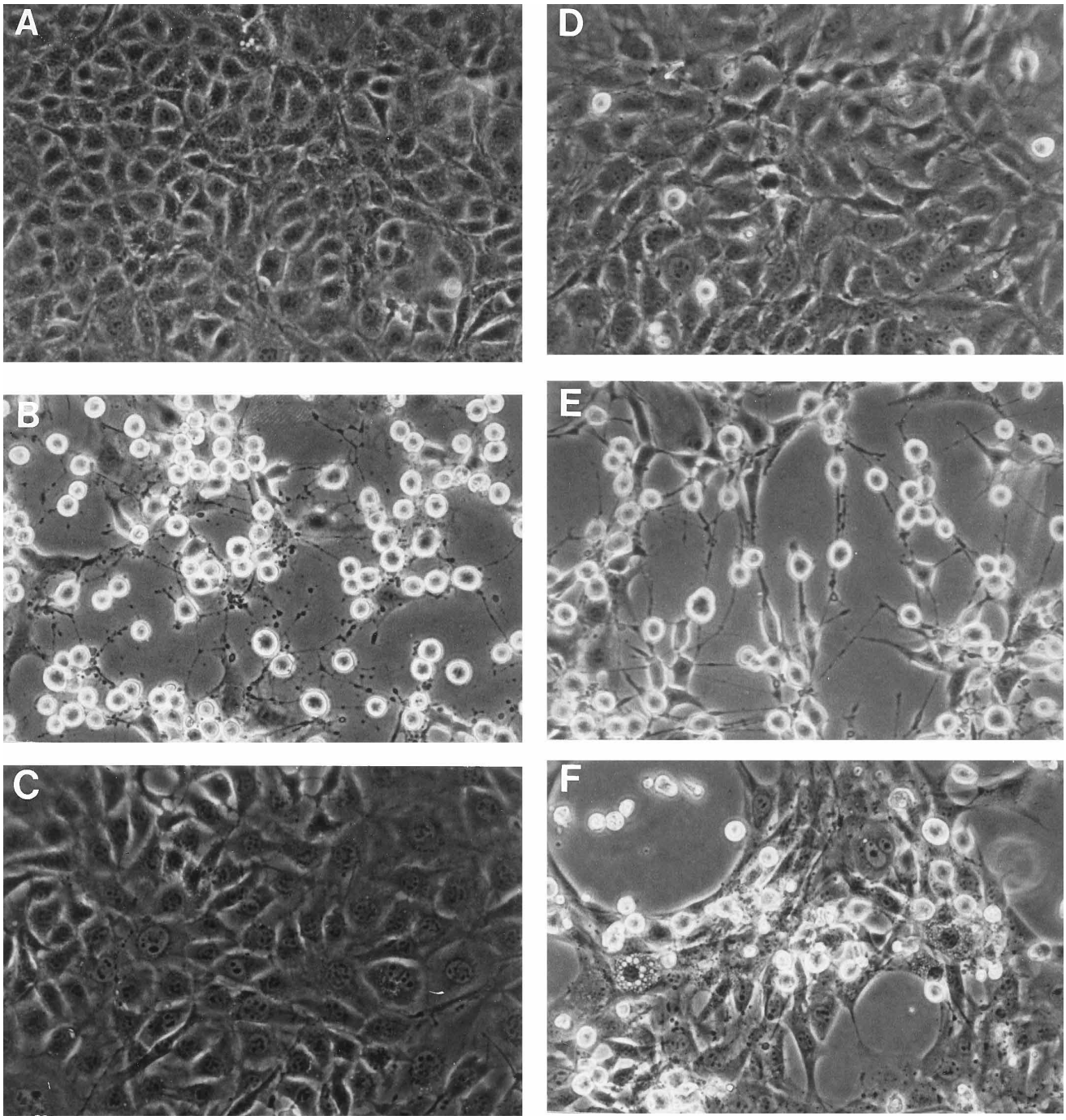

FIG. 1. Morphological changes induced by SV-wt infection of target cells. (A and D) Uninfected NIH 3T3 (A) and 1-7-1 (D) cells (negative controls). (B and E) CPE observed in NIH 3T3 (B) and 1-7-1 (E) cells 24 h after SV-wt infection. (C and F) NIH 3T3 (C) and 1-7-1 (F) cell morphology 24 h after destruction of SA-R followed by SV-wt infection.

pernatants were harvested and incubated at 37°C for a further 30 min either with or without 4 μg of acetylated trypsin per ml for cleavage of the F_0 precursor protein. Trypsin activity was stopped by addition of 1% fetal calf serum. The 50% tissue culture infectivity dose ($TCID_{50}$/ml) was determined by limiting dilution as described previously (16). One $TCID_{50}$/ml was equivalent to 5,000 PFU/ml in our setting.

NIH 3T3 Swiss mouse embryo cells were maintained in Dulbecco's modified Eagle's medium (Gibco), HuH-7 human hepatoma cells were maintained in medium M199 (Gibco), and Madin-Darby canine kidney (MDCK) cells were maintained in minimal essential medium (Gibco), all containing 10% fetal calf serum. NIH 3T3 and MDCK cells stably transfected with the ASGP-R cDNA—thereby constituting the new cell lines 1-7-1 (21) and M12 (7), respectively—were kind gifts from Martin Spiess (University of Basel) and were maintained as described previously (7, 21).

Infection of cells and neuraminidase treatment. For infection and neuraminidase treatment, cells were used when monolayers reached 85 to 90% confluency in 35-mm-diameter dishes. As a standard inoculation procedure, monolayers were washed twice with medium lacking fetal calf serum (washing medium) and overlaid with phosphate-buffered saline containing SV-wt at a moi of 5. After incubation for 15 min at 37°C, unadsorbed virus was removed by repeated washing of the cells. Medium containing Nutridoma SR (growth medium) was added, and the cells were incubated for 48 h at 37°C. (Replacement of fetal calf

TABLE 1. SV infection of sialidase-treated cells

Cells	Sialidase treatment[a]	Result by assay of:		
		CPE[b]	HAD[c]	HA (HA units/ml)[d]
NIH 3T3	−	+++	++++	320
	+	−	−	<5
1-7-1	−	+++	++++	320
	+	+	++	40

[a] Cells underwent neuraminidase treatment (7.5 mU per 35-mm-diameter dish) at 37°C for 60 min prior to infection.

[b] +++, more than 90% of cells; ++, more than 50% of cells; +, more than 20% of cells; −, no CPE.

[c] ++++, 90 to 100% of cells; +++, 50 to 89% of cells; ++, 20 to 49% of cells; +, 2 to 19% of cells; (+), single cells; −, no HAD.

[d] Number of HA units per milliliter in supernatant 48 h after inoculation. The limit of progeny virus detection was 5 HA units/ml.

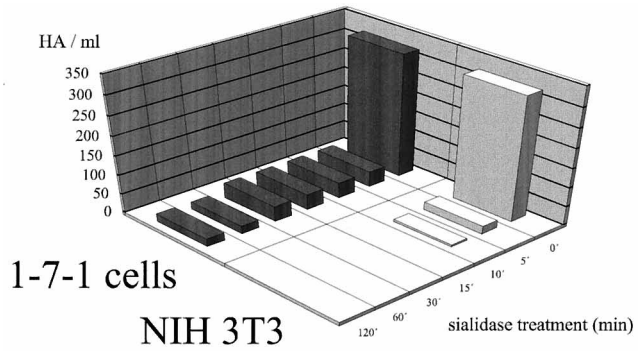

FIG. 2. SA-R destruction by neuraminidase treatment over various periods of time, followed by subsequent infection challenge of target cells. Progeny virus release of infected cells was quantitated by HA assay of supernatants sampled 48 h after infection. Missing bars indicate sample activity of <5 HA units/ml.

serum with Nutridoma SR was found to increase the sensitivity of the subsequent hemagglutination (HA) assay on the order of fourfold.) Neuraminidase treatment prior to infection was carried out according to the method described in reference 12. In brief, monolayers were washed twice, overlaid with 500 μl of medium containing 7.5 mU of neuraminidase, and incubated at 37°C. After 60 min, cells were washed twice and immediately infected as described above.

HA and HAD assays. Virus yield was quantitated by HA assay with supernatants of infected cells. To determine HA titers, serial dilutions of 100 μl of culture fluids were made in 96-well plates and incubated with 25 μl of a 0.5% human erythrocyte (group 0) suspension for 2 h at 4°C. Hemadsorption (HAD) assays were performed as described previously (30).

Inhibition experiments. After neuraminidase treatment, cells were washed twice with medium and incubated with ASGP-R-specific antiserum with rising concentrations of up to 2 mg of protein/ml at 4°C for 20 min. SV was then added at an MOI of 5, and incubation was continued for 15 min at 4°C. Cells were washed three times subsequent to infection. Growth medium was added, followed by incubation at 37°C for 48 h.

RESULTS

ASGP-R expression does not alter SV-wt replication in NIH 3T3 cells. Previous competitive inhibition experiments by ASGP-R ligands suggested that the SV mutant *ts*271 could use the ASGP-R as an alternative receptor for cell entry (13). Now, the availability of two cell lines which differ only with regard to ASGP-R expression (ASGP-R-negative NIH 3T3 cells and 1-7-1 cells generated by stable ASGP-R cDNA transfection of NIH 3T3 cells) enables a detailed analysis of the ASGP-R as a potential SV receptor. First, we compared both cell lines with regard to infection with SV-wt strain *Fushimi*. Beginning 14 to 16 h postinfection (MOI of 5), both cell lines showed a cytopathic effect (CPE) spreading over the culture 20 to 24 h postinfection (Fig. 1B and E). Infection and replication were further confirmed by adsorption of erythrocytes to infected cells (HAD) and by quantitation of progeny virus in the supernatant of infected cells (HA) 48 h postinfection (Table 1). As a result, both cell lines were found equal concerning CPE, percentage of infected cells (HAD), and progeny virus synthesis (HA) (Table 1), indicating that the ASGP-R-expressing cell line 1-7-1 does not differ from the cell line NIH 3T3 with regard to SV-wt infection and subsequent replication.

SA-R destruction in NIH 3T3 cells. To characterize the alternative route of virus entry via ASGP-R, a complete inactivation of conventional SA-R, which was present on both cell lines, was required. In the first step, the ability of *V. cholerae* neuraminidase to inactivate SA-R was tested by enzymatic treatment of NIH 3T3 cells for 60 min at 37°C prior to infection with SV-wt (MOI of 5). Complete receptor inactivation was demonstrated by a lack of CPE for NIH 3T3 cells (Fig. 1C), as well as by negative HAD and HA tests (Table 1). Subsequently, to investigate the capacity of neuraminidase-treated cells for endogenous receptor recovery, infection was carried out at different time intervals after enzymatic treatment, and virus progeny release into the supernatant of infected cells was measured 48 h postinfection by the HA assay. Interestingly, the time interval between neuraminidase treatment and infection had to be at least 60 min for the detection of small amounts of progeny virus (5 HA units/ml), and full susceptibility to SV-wt infection (320 HA units/ml) was regained after a 3-h recovery period prior to infection. These experiments showed that there is no detectable infection via SA-R and subsequent progeny virus release during a certain time interval after neuraminidase treatment, allowing infection studies with ASGP-R-positive 1-7-1 cells without interference of SA-R.

Alternative route of SV-wt infection in 1-7-1 cells. After enzymatic destruction of SA-R molecules in both cell lines, we investigated the susceptibility to SV-wt infection. In contrast to NIH 3T3, 1-7-1 cells were found to be still infected (MOI of 5) (Fig. 1F and Table 1). This result refers to a second receptor population in 1-7-1 cells not being inactivated by incubation with neuraminidase. To further distinguish between these alternative routes of infection, the kinetics of SA-R destruction on both cell lines were monitored and compared by restricting neuraminidase treatment to defined time intervals prior to infection (Fig. 2). We found that both cell lines showed a rapid linear decrease in the level of SV-wt infection during the first 5 min, as demonstrated by virus progeny release 48 h after infection. Whereas 15 min of enzymatic treatment completely blocked SV-wt infection in NIH 3T3 cells (<5 HA units/ml; HAD negative), 1-7-1 cells could be infected even after prolonged enzymatic treatment (Fig. 2). These kinetics of receptor inactivation are consistent with virus entry by different sets of receptors in 1-7-1 cells. Furthermore, these results demonstrate that the ASGP-R alone is sufficient for SV-wt cell entry and subsequent replication in host cells.

To further confirm the role of the ASGP-R as an SV-wt receptor, a second pair of cell lines, MDCK (ASGP-R negative) and the corresponding derivative M12 (ASGP-R positive), as well as the human hepatoma cell line HuH-7, known to express functional ASGP-R (25), were treated with neuraminidase prior to infection (Table 2). As a result, only ASGP-R-expressing cell lines (M12 and HuH-7) could be infected even after a sustained enzymatic treatment for 90 min, whereas 15 min was sufficient to render MDCK cells resistant to SV-wt infection.

Specific inhibition of ASGP-R-mediated route of infection. ASGP-R expression is known to be the only difference between

TABLE 2. Impact of neuraminidase treatment for various periods of time prior to infection

Cells	ASGP-R	Effect of neuraminidase incubation on virus progeny release (HA units/ml) for[a]:		
		0 min	15 min	90 min
MDCK	−	40	<5	<5
M12	+	40	40	40
HuH-7	+	80	40	40

[a] Eighty to 90% confluent monolayers were incubated with neuraminidase at 37°C for different time intervals prior to infection. Forty-eight hours after infection, virus progeny were quantitated with the supernatant of the infected cells for the HA assay. The limit of detection of virus progeny in the supernatant was 5 HA units/ml.

TABLE 3. Role of SV-F fusion activity in ASGP-R-mediated infection

Viral protein[a]	Neuraminidase treatment[b]	Effect of neuraminidase treatment on virus progeny release (HA units/ml)[c]	
		NIH 3T3	1-7-1
F_0	−	<5	<5
	+	ND[d]	<5
F_1/F_2	−	160	160
	+	<5	80

[a] F_0, uncleaved, fusion-inactive SV-F; F_1/F_2, cleaved, fusion-active F_0 protein.
[b] Sixty minutes of neuraminidase treatment prior to infection experiments.
[c] Virus progeny in the supernatant were determined 48 h postinfection; the limit of detection was 5 HA units/ml.
[d] ND, not determined.

1-7-1 and NIH 3T3 cells. Therefore, inhibition of the ASGP-R-mediated route of cell entry should block infection of neuraminidase-treated 1-7-1 cells. Subsequently, we tried to block the alternative route of SV-wt infection by using a polyclonal anti-human ASGP-R goat antiserum. Incubation of neuraminidase-treated 1-7-1 cells with rising concentrations of the ASGP-R antiserum resulted in complete blocking of SV-wt infection at a 2-mg of protein/ml antiserum concentration, as demonstrated by the absence of both detectable progeny virus release (Fig. 3) and HAD to these cells 48 h after infection (data not shown). This result identifies for the first time the ASGP-R as an SV-wt receptor and further excludes other membrane components from being responsible for cell attachment and subsequent replication after neuraminidase treatment. To rule out unspecific inhibition of SV-wt infection by this antiserum, native NIH 3T3 cells were incubated with different concentrations of antiserum prior to SV-wt infection. No inhibition could be detected (Fig. 3).

ASGP-R-mediated cell entry requires cleaved SV-wt fusion glycoprotein. After the confirmation of the ASGP-R as an SV-wt receptor, it became of interest to investigate the mode of cell entry after receptor binding. ASGP-R ligands usually enter cells by receptor-mediated endocytosis and continue along the endocytic pathway to lysosomes (9). In contrast, SV-wt cell entry via conventional SA-R occurs by fusion of the viral envelope with cell membranes to release the viral nucleocapsid directly into the cytoplasm of host cells. This fusion process is known to require the cleavage of the viral F_0 precursor protein (F_0) into the subunits F_1 and F_2 (10, 19). Therefore, theoretically at least two different entry mechanisms after SV-wt binding to the ASGP-R are possible: endocytosis, or penetration by F-mediated fusion of membranes.

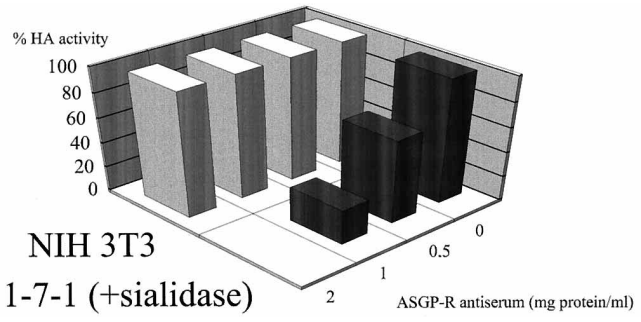

FIG. 3. Specific inhibition of ASGP-R-mediated cell entry by ASGP-R antiserum. Target cells were preincubated with ASGP-R-specific antiserum; subsequent progeny virus release was quantitated by HA assay of supernatants sampled 48 h later. Values represent the percentage of HA activity of sample cells in relation to cells not incubated with antibodies prior to infection. The missing bar indicates relative HA activity of <1% (limit of detection).

To address the need of fusion-active virions for infection via ASGP-R, we infected NIH 3T3 and 1-7-1 cells with equal amounts of either cell-grown SV-F_0 or trypsin-activated cell-grown SV-F_1 and -F_2 virions (Table 3). As a result, both cell lines as well as neuraminidase-treated 1-7-1 cells could be infected by SV-$F_{1/2}$ particles. These results correspond to our experiments with egg-grown virions (see above). In contrast to these observations, SV-F_0 infected neither NIH 3T3 nor 1-7-1 cells, independent of neuraminidase treatment prior to infection. This clearly demonstrates that infection via ASGP-R depends on the presence of the fusion-active subunits F_1 and F_2, indicating a fusion-mediated cell entry of SV after binding to the ASGP-R.

DISCUSSION

We have characterized the interaction of SV-wt strain *Fushimi* with the hepatocyte-specific ASGP-R by infection and specific inhibition assays. Our results clearly demonstrate that SV-wt is able to efficiently use two distinct receptors (i.e., sialic acid-containing gangliosides and the ASGP-R).

The first data that suggested that SV-wt might be able to bind to and infect cells via the ASGP-R came from a study with the temperature-sensitive SV mutant *ts*271 (13). It was thought that terminal galactose residues of F (34) could be responsible for the binding of *ts*271 to the ASGP-R. However, the efficiency of this alternative route of infection has not been compared to that of infection via SA-R, and although there was good biochemical evidence for the ASGP-R as an alternative receptor, the participation of other membrane components in *ts*271-infection could not be ruled out. We now clearly demonstrate by infection studies with two closely related cell lines, NIH 3T3 (ASGP-R negative) and 1-7-1 (ASGP-R positive), which differ only with regard to ASGP-R expression, that SV-wt strain *Fushimi* can indeed use the ASGP-R efficiently. A quantitative comparison revealed that at an MOI of 5, about 30 to 40% of 1-7-1 cells with SA-R destroyed were still found to be infected via the ASGP-R (HAD assay). This reduced infectivity compared to that of the conventional SA-R-mediated infection could be explained in part by the observation that the molecular structure of F protein (34) is not consistent with those of the high-affinity ligands of the ASGP-R (26). Furthermore, the ASGP-R itself contains carbohydrate side chains which display terminal galactose residues after neuraminidase incubation, leading to a competition with exogenous ligands for the ASGP-R binding sites (24). With these limitations

taken into account, we found that SV-wt infection via ASGP-R is nearly as efficient as infection via conventional SA-R. Moreover, infection of cells after SA-R destruction demonstrated that the ASGP-R alone is sufficient for SV-wt infection and subsequent replication and does not depend on the SA-R as a possible cofactor.

Concerning the mode of SV-wt entry in neuraminidase-treated ASGP-R-positive cells, there are theoretically at least two possibilities: endocytosis and fusion-mediated penetration. Indirect evidence has suggested that ASGP-R-bound *ts*271 particles are able to promote membrane fusion (13). We now can demonstrate for SV-wt that cleavage of the F_0 precursor molecule leading to fusion-active particles is necessary to establish infection via the ASGP-R. This indicates that SV-F might protect the nucleocapsid from endosomal-lysosomal degradation by fusion of the cell membrane with the viral envelope. Interestingly, inhibition of lysosomal degradation processes by incubation of 1-7-1 cells with 500 μM chloroquine (20) prior to infection increased the susceptibility to SV-wt infection in sialidase-treated 1-7-1 cells twofold (virus progeny release of 640 versus 320 HA units/ml), whereas NIH 3T3 cells were not affected (320 HA units/ml). These observations correspond to those of previous experiments showing that the contents of reconstituted SV envelopes which contain only F protein can be internalized in ASGP-R-expressing HepG2 cells through both endocytosis and membrane fusion (2). The question of whether penetration and endocytosis are actually competing events after the binding of SV-wt particles to the ASGP-R will be further examined with cell separation experiments.

Other viruses have been reported recently to interact with the ASGP-R (e.g., Marburg virus [MBG]) (3). As a prerequisite for ASGP-R binding, the MBG glycoprotein GP contains terminal galactose residues when grown in certain cell lines, e.g., E6 cells (6). Interestingly, the presence of terminal galactose residues of SV-F is thought to be independent of the propagation cell line, but dependent on the neuraminidase activity of the second viral glycoprotein, HN (5). Thus, there seems to be no common strategy among different viruses for generating the structural requirements for ASGP-R binding. Remarkably, concerning disease manifestations in MBG infection, there is a marked hepatic tropism, which could be explained in part by enhanced hepatic uptake of MBG mediated by the ASGP-R (3). With regard to SV-wt airborne infections, detailed studies of the presence of SV-wt in organs other than the lungs (e.g., the liver) have not been undertaken yet. So far, published data indicate that acute infections are limited primarily to the surface epithelium of the respiratory tract (15, 28). However, a previously described pantropic SV mutant causing systemic infection in mice (27) suggests that at least some SV strains and mutants get access to the hepatic ASGP-R in vivo. The ASGP-R might exert an important physiological role as an alternative SV receptor in settings in which SA-R inactivation on host cells takes place, i.e., after SA-R destruction in infected cells by SV-HN activity. This viral enzymatic activity is known to destroy SA-R molecules on the surface of infected cells, which is thought to facilitate the release of progeny virions (5). However, after SV-wt infection of NIH 3T3 and 1-7-1 cells in our experiments, there was no difference with regard to virus progeny release, suggesting that ASGP-R expression does not affect the virus yield. In infected cells, ASGP-R molecules might substitute for destroyed SA-R molecules, allowing superinfection of cells despite missing conventional SV receptors.

The observation of the specific interaction of SV-wt with the human ASGP-R is of major importance for the development of new hepatotropic vectors required for in vivo liver gene therapy applications. So far, the ASGP-R has been employed in generating hepatotropic non-viral gene transfer systems such as ASGP-polylysine-DNA conjugates (32, 33). Although genes have been selectively targeted to hepatocytes both in vitro and in vivo, the efficiency of this method was found to be partly limited because of the lysosomal degradation of endocytosed ligands, requiring a 66% hepatectomy for prolonged gene expression (4). Interestingly, in this context, the SV-F protein acts in a bifunctional way, i.e., binding to the hepatocyte-specific lectin followed by fusion-mediated direct release of the SV particle content to the target cell cytoplasm. To further explore the capacity of F protein as a target protein for hepatocytes, we are currently developing pseudotyped Moloney murine leukemia viruses containing chimeric F proteins in the ecotropic retroviral envelope (23). Moreover, in the meantime, a new generation of hepatotropic gene transfer systems could be generated based on recombinant SV vectors, which have been described recently (8).

In conclusion, we can show that SV-wt strain *Fushimi* can infect cells via the ASGP-R efficiently. Furthermore, this alternative route of cell entry is dependent on SV fusion properties, suggesting fusion-mediated cell entry after receptor binding.

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