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Recombinant Sendai Viruses Expressing Fusion Proteins with Two Furin Cleavage Sites Mimic the Syncytial and Receptor-Independent Infection Properties of Respiratory Syncytial Virus▽

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Cell entry by paramyxoviruses requires fusion between viral and cellular membranes. Paramyxovirus infection also gives rise to the formation of multinuclear, fused cells (syncytia). Both types of fusion are mediated by the viral fusion (F) protein, which requires proteolytic processing at a basic cleavage site in order to be active for fusion. In common with most paramyxoviruses, fusion mediated by Sendai virus F protein (F_{SeV}) requires coexpression of the homologous attachment (hemagglutinin-neuraminidase [HN]) protein, which binds to cell surface sialic acid receptors. In contrast, respiratory syncytial virus fusion protein (F_{RSV}) is capable of fusing membranes in the absence of the viral attachment (G) protein. Moreover, F_{RSV} is unique among paramyxovirus fusion proteins since F_{RSV} possesses two multibasic cleavage sites, which are separated by an intervening region of 27 amino acids. We have previously shown that insertion of both F_{RSV} cleavage sites in F_{SeV} decreases dependency on the HN attachment protein for syncytium formation in transfected cells. We now describe recombinant Sendai viruses (rSeV) that express mutant F proteins containing one or both F_{RSV} cleavage sites. All cleavage-site mutant viruses displayed reduced thermostability, with double-cleavage-site mutants exhibiting a hyperfusogenic phenotype in infected cells. Furthermore, insertion of both F_{RSV} cleavage sites in F_{SeV} reduced dependency on the interaction of HN with sialic acid for infection, thus mimicking the unique ability of RSV to fuse and infect cells in the absence of a separate attachment protein.

Human respiratory syncytial virus (RSV) and Sendai virus (SeV) are enveloped, negative-strand RNA viruses that belong to the *Pneumovirinae* and *Paramyxovirinae* subfamilies, respectively, of the *Paramyxoviridae* (6). Cell entry by paramyxoviruses requires virus binding to target cell receptors, followed by fusion of the viral envelope with the cellular plasma membrane. Cells infected by paramyxoviruses may also fuse with adjacent cells to form syncytia (multinucleated cells). Both virus-cell and cell-cell fusion processes are mediated by the viral fusion (F) protein, a trimeric type I integral membrane glycoprotein.

For members of the *Paramyxovirinae*, including SeV, fusion mediated by the F glycoprotein requires participation of the homologous attachment protein (glycoprotein [G], hemagglutinin [H], or hemagglutinin-neuraminidase [HN]). It has been hypothesized that conformational changes occurring in the attachment protein following binding to target cell receptors are transduced to the F protein to activate it for fusion "at the right time and in the right place" (26–28). Sialic acid-containing gangliosides act as the cellular receptor for the Sendai virus HN attachment protein (31, 32). While the specific cell surface receptor for pneumoviruses is unknown, RSV has been shown to interact with cell surface glycosaminoglycans (GAGs), in particular, with heparan sulfate (12, 13, 18, 20, 25, 33). Furthermore, the fusion protein of both human and bovine RSV (BRSV), as well as human metapneumovirus (MPV) is sufficient to mediate attachment and fusion in the absence of the G attachment protein (2, 16, 21, 43, 44, 47, 48, 52). Thus, the role of pneumovirus attachment proteins in triggering fusion is currently unclear. Since RSV F protein (F_{RSV}) also binds to GAGs, the interaction between F_{RSV} and heparan sulfate on the target cell surface may mediate virus attachment in the absence of the G protein (14, 20, 48).

The paramyxovirus F protein is synthesized as an inactive precursor (F_0), which is cleaved at a mono- or multibasic cleavage site by cellular proteases to produce a fusion-competent, disulfide-linked F_2-F_1 complex (for a review, see reference 28). Cleavage is an absolute requirement for fusion since the newly formed F_1 N terminus contains a hydrophobic fusion peptide that is inserted into the cell membrane during fusion (40). Furthermore, the nature of the cleavage motif is a major determinant of virus pathogenicity (23). Sendai virus F protein (F_{SeV}) is cleaved at a monobasic cleavage site (R116) and thus requires the addition of trypsin to fuse cells in culture (22, 42). In contrast, RSV F protein (F_{RSV}) possesses two conserved multibasic furin-dependent cleavage sites (site I, RARR109; site II, KKRKRR136), separated by a region of 27 amino acids (pep27). The presence of two cleavage sites in F_{RSV} is unique among paramyxoviruses although two cleavage sites have been observed in the severe acute respiratory syndrome (SARS) coronavirus spike protein (1). Interestingly, while cell-cell fusion directed by F_{RSV} does not depend on coexpression of an attachment protein, there is a requirement for proteolytic processing at both cleavage sites, accompanied by removal of the

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intervening pep27, which is secreted into the culture medium (16, 39, 52). Furthermore, completion of cleavage at both sites is associated with a change in shape of the RSV F protein (34, 39). However, while double cleavage of F_{RSV} is an essential requirement for cell-cell fusion, its role in infection is less clear. For instance, recombinant bovine RSV (rBRSV) expressing F protein with an intact cleavage site II, but lacking both site I and the intervening pep27, replicated with kinetics similar to that of the parental virus, despite displaying drastically reduced syncytium formation (53).

We have previously constructed a series of plasmids encoding chimeric F_{SeV} mutants by inserting one or both F_{RSV} cleavage sites and various regions of pep27 into F_{SeV} (38). Inclusion of both F_{RSV} cleavage sites in F_{SeV} resulted in a dramatic increase in fusion of transfected cells, as well as a decreased dependency on the HN protein for cell-cell fusion. It was hypothesized that the presence of two multibasic cleavage sites might represent a strategy to activate the RSV F protein for cell-cell fusion in the absence of an attachment protein. However, mutations found to destabilize the envelope (Env) glycoprotein of murine leukemia virus (MLV) and enhance syncytium formation in transfected cells were found to be detrimental for viral growth (29). The explanation offered for these findings was that unstable Env proteins at the cell membrane are continuously replaced by new molecules, which are able to mediate cell-cell fusion. In contrast, such unstable proteins may be prematurely activated in the virus particle before virus-cell contact takes place, thereby inactivating the virus. Moreover, it has been suggested that cell-cell and virus-cell fusion processes have different structural requirements (7).

Therefore, as a natural continuation of our previous work, it was of interest to generate recombinant Sendai viruses (rSeV) that express mutant F proteins containing one or both F_{RSV} cleavage sites, separated by either a partial or complete intervening pep27 sequence. We found that rSeV expressing cleavage site F protein mutants displayed reduced thermostability compared to wild-type (wt) rSeV. Moreover, rSeV expressing F protein containing both F_{RSV} cleavage sites displayed enhanced cell-to-cell fusion and a decreased dependency on receptor recognition for viral infection. Therefore, insertion of the double F_{RSV} cleavage sites in F_{SeV} in the context of rSeV reproduced the ability of RSV to infect cells in the absence of an attachment protein. The RSV F protein double cleavage sites may thus represent an alternative mechanism to regulate fusion, which is independent of the viral attachment protein.

MATERIALS AND METHODS

Cell lines. BSR-T7/5 (4) (a gift from K.-K. Conzelmann, Munich, Germany) and LLC-MK2 cells were maintained in Dulbecco's modified Eagle's medium (DMEM; Bio Whittaker), supplemented with 10% fetal calf serum (FCS). Alternate passages of BSR-T7/5 cells were supplemented with 1 mg/ml G418 sulfate (Sigma) in order to select for T7 polymerase expression. CHO cell derivatives (45), Pro-5 (ATCC CRL-1781), and sialic acid-deficient Lec-2 cells (ATCC CRL-1736) were obtained from the American Type Culture Collection and maintained in alpha minimum essential medium (Sigma) supplemented with 10% FCS.

Plasmids and mutagenesis. Construction of the pTM1 plasmids encoding Sendai virus F protein (F_{SeV}) cleavage site mutants detailed in Fig. 1 has been described previously (38). The genes encoding wild-type F_{SeV} from the Harris strain (F_H) and cleavage mutants (Fc, containing F_{RSV} cleavage site II, and F117 and F130, both containing F_{RSV} cleavage sites I and II and a partial [F117] or complete [F130] intervening sequence [see Fig. 1]) were subcloned from pTM1 into the FL5 plasmid. FL5 is an 18.3-kb plasmid that contains the full-length SeV cDNA (Z strain) and MluI and RsrII unique restriction sites, which flank the F gene. Sendai virus F genes were initially amplified from pTM1 plasmids by PCR using the following oligonucleotides containing MluI and RsrII restriction sites (italicized): AACTTAGGGATAAAGTCCCTTGTGA*ACGCGT*GGTTGCAA AACTCTCCCCTTGGGAAACATGACAGCATATATCCAGAGGTCACA GTGC (positive sense) and TAAGTTTTTCTTATTATATACAGAT*CGGACC G*AATACCATGCCTGCTTTACAAGACATCTGATAATGGTCGTGA**AGT**T CATCGTTTCTCAGCCATCGCATCAAACC (negative sense). According to the "rule of six," replication of the SeV genome occurs efficiently only when the genome is an exact multiple of six nucleotides (5). Thus, on replacement of the wild type F_{SeV} gene with the cleavage site mutants, insertion of 3 nucleotides in the noncoding region of SeV (shown in bold) was required to ensure that the length of SeV remained a multiple of six. The resulting PCR products were digested with MluI and RsrII enzymes (Roche) and subcloned into plasmid FL5 in order to replace the wild-type SeV F gene (Z strain) present in FL5.

Recovery of recombinant Sendai virus. BSR-T7/5 cells that constitutively express T7 polymerase were cotransfected using FuGENE HD (Roche) with FL5 plasmids containing either wild-type F_H or cleavage site F mutants, along with three pTM1 plasmids expressing the nucleoprotein (N), phosphoprotein (P), and large RNA-dependent RNA polymerase (L) proteins under transcriptional control of the T7 promoter. At 24 h posttransfection, cells were washed with serum-free DMEM, and 0.5 μg/ml trypsin was added to the culture medium. Cells were incubated for a further 24 h at 33°C to allow virus growth, and fresh trypsin was added at 48 h posttransfection. Cells were collected at 72 h posttransfection and injected into 9-day-old embryonated chicken eggs, which permit the growth of SeV to high titers (15). Following incubation for 48 h at 34°C, allantoic fluid was collected and clarified by low-speed centrifugation. In order to confirm virus rescue, allantoic fluid was pelleted through a 25% glycerol cushion for 30 min at 15,000 rpm and loaded onto a 10% SDS-PAGE gel to detect viral proteins. Alternatively, clarified allantoic fluid was purified through a 4-ml 20% sucrose cushion in Beckman SW28 tubes by centrifugation for 2 h at 26,000 rpm, as previously described (30).

Virus titration and growth kinetics. In order to follow replication of SeV over multiple replication cycles, LLC-MK2 cells in six-well plates were inoculated with rSeV at a multiplicity of infection (MOI) of 0.05 and incubated at 37°C for 2 h in DMEM containing 2.5% fetal calf serum (DMEM–2.5% FCS). Following adsorption, cells were washed and incubated at 33°C or 37°C for a further 96 h in serum-free DMEM with or without 0.3 μg/ml trypsin. Aliquots of cell culture supernatant (500 μl) were taken at 24-h intervals and replaced with fresh medium with or without trypsin. Viruses were subsequently titrated in duplicate by the method of Borisevich et al. (3). Briefly, LLC-MK2 cells in 96-well plates were infected with serial dilutions of virus in DMEM–2.5% FCS. Cells were incubated at 37°C for 2 h, washed in serum-free DMEM, and incubated in serum-free medium supplemented with 0.3 μg/ml trypsin. At 18 h postinfection, cells were washed with phosphate-buffered saline (PBS) containing 1% bovine serum albumin (PBS–1% BSA) and fixed for 30 min at 4°C with methanol containing 2% H_2O_2. Fixed cells were blocked with PBS–1% BSA and stained for expression of SeV F protein with monoclonal antibody (MAb) GB5 (a gift from S. Wharton and J. J Skehel, London, United Kingdom) for 1 h at room temperature, followed by incubation with anti-mouse immunoglobulin conjugated to horseradish peroxidase (Amersham Biosciences UK Ltd.) for 30 min at room temperature. The resulting immune complexes were developed using the substrate 3-amino-9-ethylcarbazole (AEC; Sigma) dissolved in dimethyl sulfoxide (DMSO; 3.3 mg/ml), 0.1 M citrate, 0.2 M phosphate, pH 5.5, and 0.06% H_2O_2. Immunostained (red) cells were counted using a light microscope.

Western blotting. Proteins separated on a 10% SDS-PAGE gel were transferred to an Immobilon membrane (Millipore) and subjected to Western blotting using polyclonal rabbit serum raised against amino acids 104 to 117 of RSV F protein [F_{RSV}(104–117)] (16) or the cytoplasmic tail of SeV F protein [F_{SeV}(CT)] (a gift from L. Roux, Geneva, Switzerland). Membranes were subsequently incubated with anti-mouse immunoglobulin conjugated to peroxidase. Bands were visualized using an Amersham ECL Advance Western Blotting Detection Kit (GE Healthcare UK, Ltd.) and imaged using a Kodak Gel Logic 1500 Imaging System camera and Kodak Molecular Imaging software. The net intensity of protein bands indicated in the legend to Fig. 3 was measured using Kodak Molecular Imaging software, and the percentage of F protein cleavage to F_1 was calculated by dividing the intensity of F_1 bands by the total net intensity.

Hemolysis. Purified SeV (5×10^8 focus-forming units [FFU]) was incubated at 33°C for 45 min with 0.5 ml of washed guinea pig erythrocytes (1%, vol/vol, in PBS). Hemolysis was measured by determining the optical density of the erythrocyte supernatant at 520 nm.

Immunofluorescence. Infected LLC-MK2 or CHO cells growing in microchamber culture slides in the absence or presence of 0.3 μg/ml trypsin were fixed with cold (−20°C) methanol for 5 min, followed by cold acetone for 30 s. Fixed cells were subsequently immunostained using MAb GB5 directed against F_{SeV}, followed by incubation with anti-mouse fluorescein-linked antibody (GE Healthcare UK, Ltd.). Cells were examined using a Zeiss microscope and photographed using an AxioCam HRc digital camera and Axiovision, version 3.1, software. The sizes of syncytia for LLC-MK2 cells were measured by counting the number of nuclei in 10 syncytia selected at random from distinct ×20 magnification fields. For the experiment shown in Fig. 7, LLC-MK2 cells in monolayers were pretreated prior to infection with 120 mU of neuraminidase (NA) from *Clostridium perfringens* (Sigma) in OptiMEM medium (Gibco) for 90 min at 37°C. The level of infection in the absence or presence of neuraminidase was determined by counting the number of infected cells in five ×20 magnification fields selected at random.

Luciferase reporter gene cell-cell fusion assay. BSR-T7/5 cells were infected in DMEM–2.5% FCS at an MOI of 5 and incubated at 37°C for 2 h. BSR-T7/5 cells were subsequently washed in serum-free DMEM and incubated for 14 h in serum-free medium supplemented with or without 0.25 μg/ml trypsin. LLC-MK2 cells in 100-cm dishes were transfected (FuGENE HD; Roche) with 30 μg of a plasmid encoding the luciferase gene (pTM1-Luc) and incubated at 37°C for 14 h in DMEM–2.5% FCS. For the cell-cell fusion assay, LLC-MK2 cells were detached 14 h posttransfection using 1 mM EDTA in Ca^{2+}- and Mg^{2+}-free PBS, resuspended at a density of 1×10^6 cells/ml in serum-free medium, and overlaid at a 1:1 ratio onto BSR-T7/5 cells, either in the presence or absence of 0.25 μg/ml trypsin (Sigma). The mixed donor and target cells were incubated for 3 h at 33°C or 37°C to allow fusion. Cells were subsequently washed with PBS and lysed in 1× passive lysis buffer (Promega Inc.), according to the manufacturer's instructions. Ten microliters of clarified cell extract was mixed with 100 μl of luciferase assay substrate (Promega Inc.) and assayed for luciferase activity over a 10-s measurement read using a Turner Biosystems 20/20n Luminometer instrument.

Enzyme-linked immunosorbent assay (ELISA). Infected Pro-5 or Lec-2 cells (MOI of 1) growing in 96-well microtiter plates were washed at 20 h postinfection with PBS containing 0.05% Tween 20 (Bio-Rad Laboratories) (PBS-Tween) and fixed in 80% acetone. The wells were blocked for 30 min at room temperature with 5% pig serum in 0.05% PBS-Tween. Cells were subsequently stained for expression of SeV F protein with MAb GB5 for 1 h at room temperature, followed by incubation with anti-mouse immunoglobulin conjugated to horseradish peroxidase (Amersham Biosciences UK, Ltd.) for 45 min at room temperature. The optical density at 490 nm was measured after the addition of the substrate *O*-phenyl-diamine (OPD), dissolved in a buffer containing 0.1 M citrate, 0.2 M phosphate, pH 5.5, and 0.06% H_2O_2.

RESULTS

Generation and expression of recombinant Sendai viruses containing mutations at the fusion protein cleavage site. Recombinant Sendai viruses (rSeV) containing F_{SeV} cleavage site mutations were generated from a cDNA clone (FL5). FL5 is an 18.3-kb plasmid that contains the full-length SeV cDNA (Z strain) and various unique restriction enzyme sites. The genes encoding wild-type SeV F from the Harris strain (F_H) and SeV cleavage mutants Fc, F117, and F130 (38) (Fig. 1A) were subcloned into the FL5 plasmid between MluI and RsrII restriction sites, replacing the wild-type SeV F gene (Z strain) present in FL5.

BSR-T7/5 cells that constitutively express T7 RNA polymerase were cotransfected with FL5 cDNA and pTM1 plasmids encoding the nucleoprotein (N), the phosphoprotein (P), and the large RNA-dependent RNA polymerase (L) proteins under transcriptional control of the T7 promoter. Transfected BSR-T7/5 cells were incubated in the absence or presence of trypsin at 33°C for 72 h, and the cell culture supernatant was subsequently passaged in 9-day-old embryonated chicken eggs to increase viral yield (15). In order to detect the presence of viral proteins, allantoic fluid was pelleted through a 25% glycerol cushion for 30 min at 15,000 rpm and loaded onto a 10% SDS-PAGE gel. As seen in Fig. 1B, all SeV recombinants (SeV-F_H, SeV-Fc, SeV-F117, and SeV-F130) were successfully rescued. Titration of the allantoic fluid by the method of Borisevich et al. (3) revealed that double-cleavage-site mutants SeV-F117 and SeV-F130 were attained at lower titers than single-cleavage-site mutants SeV-FH and SeV-Fc, as detailed in the legend of Fig. 1B. Whether these differences reflect lower replication or lower efficiency of rescue of the double-cleavage-site viruses compared with single-cleavage-site viruses is not known at present. The band representing the M protein appears fainter, and additional bands are present for the double-cleavage-site mutant proteins, in particular, for F117. This may reflect degradation of purified virus on SDS-PAGE sample preparation or the presence of contaminating bands from the allantoic fluid on virus purification. Sequencing of RT-PCR products confirmed that the F and HN genes had not acquired spurious mutations during the rescue (data not shown).

F protein expression by rSeV was confirmed by Western blotting with a polyclonal serum directed against the cytoplasmic tail (CT) of F_{SeV} (Fig. 1C). Faint bands migrating with a size corresponding to F_0 were observed only for single-cleavage-site F_H and Fc mutants, suggesting that almost complete cleavage of the F proteins to F_1 had taken place. It is likely that trypsin-like endoproteases present in embryonated chicken eggs are responsible for cleavage of the F protein (17, 35).

Multicycle replication of recombinant Sendai viruses. LLC-MK2 cells were infected with the rSeV at a low multiplicity of infection (MOI) of 0.05 and incubated in the absence or presence of trypsin for 96 h. At 24-h intervals, aliquots of cell culture supernatant were taken and replaced with fresh medium, with or without trypsin. Titration of the culture supernatant was performed by immunohistochemical staining of infected LLC-MK2 cell foci in 96-well plates (3). As can be seen from Fig. 2, wt SeV-F_H virus required the presence of trypsin in order to replicate, consistent with previously published findings that SeV requires trypsin for F protein cleavage (42). In contrast, all of the F protein cleavage mutant strains replicated to high titers in both the absence and presence of trypsin although viruses grew to higher titers (at least one logarithm) in the presence of trypsin (Fig. 2B). While all of the mutants reached titers comparable to the titer of wt SeV-F_H at 96 h postinfection, the double-cleavage-site mutants displayed slightly faster replication kinetics during the first 48 h of infection. This difference in replication kinetics was further enhanced when virus was grown at 33°C, with double-cleavage-site mutants replicating to titers approximately 1 logarithm higher than single-cleavage-site viruses during the first 48 h of infection (Table 1). Therefore, insertion of both F_{RSV} cleavage sites in SeV F protein leads to faster replication kinetics in LLC-MK2 cells during the first 48 h of replication, in particular at 33°C, but does not alter the overall titers obtained after 96 h.

Proteolytic cleavage of recombinant Sendai viruses. The extent of proteolytic cleavage of mutant F proteins from virus present in cell culture supernatants was examined by Western blotting. LLC-MK2 cells were infected at an MOI of 0.05 and incubated in the absence or presence of trypsin (added every 24 h) for 4 days. Cell culture supernatant was analyzed by Western blotting with polyclonal serum directed against the cytoplasmic tail of F_{SeV} (Fig. 3, upper right panel). A band

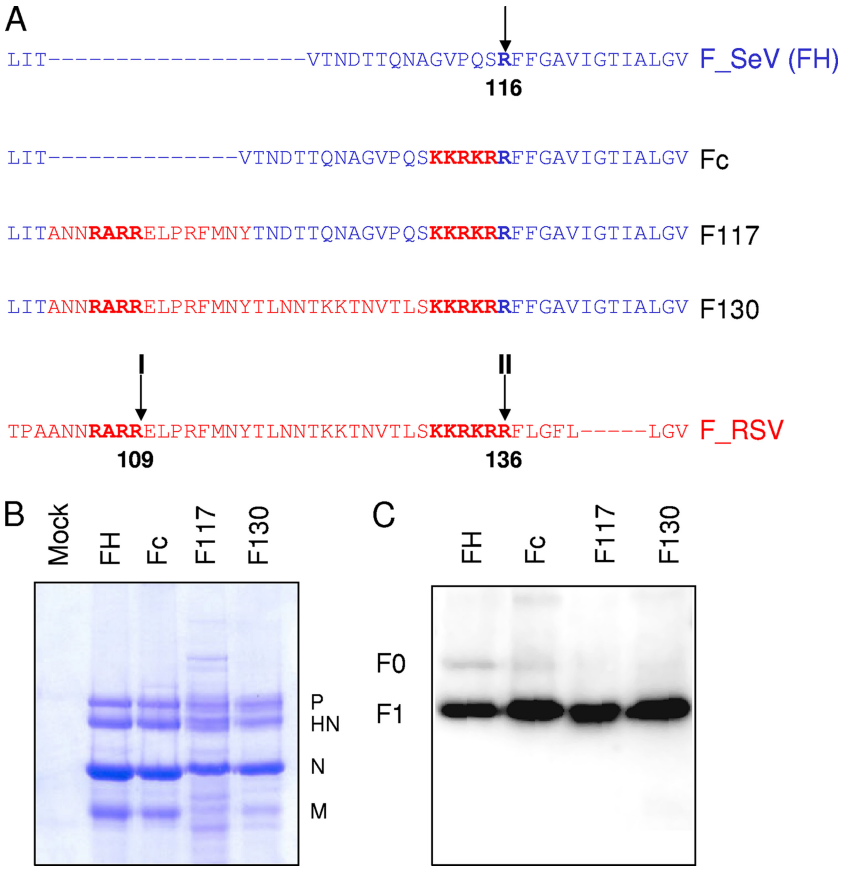

FIG. 1. Rescue of recombinant Sendai viruses (rSeV). (A) Alignment of SeV fusion protein (F_{SeV}) (amino acids 99 to 129; blue) and RSV fusion protein (F_{RSV}) (amino acids 100 to 144; red). Cleavage sites of F_{RSV} (site I, RARR109, and site II, KKRKRR136) and F_{SeV} (R116) are shown in bold and indicated by arrows. Residues from F_{RSV} that were inserted into the F_{SeV} backbone to produce cleavage mutants are shown in red. The numbering assigned to F_{SeV} mutants produced by mutation of Fc refers to the last residue of F_{RSV} inserted, excluding cleavage site II. Recombinant Sendai virus expressing the F protein mutants was rescued by cotransfection of BSR-T7/5 cells with the FL5 plasmid containing the complete SeV cDNA and pTM1 plasmids encoding SeV N, P, and L genes. Rescued virus was subsequently passaged three times in embryonated chicken eggs. Titration of the allantoic fluid revealed that double-cleavage-site viruses were obtained at lower titers (FFU/ml) than single-cleavage-site viruses (SeV-F_H titer, 5×10^{11}; SeV-Fc, 9×10^{11}; SeV-F117, 9×10^{10}; SeV-F130, 5×10^{10}). Allantoic fluid from the third passage in eggs was clarified and centrifuged through a 25% glycerol cushion, and equivalent titers of SeV-F_H, SeV-Fc, SeV-F117, and SeV-F130 viruses were analyzed on a 10% SDS-PAGE gel stained with Coomassie blue (B) or by Western blotting (C) using the polyclonal rabbit antiserum that recognizes the cytoplasmic tail of F_{SeV}. P, phosphoprotein; HN, hemagglutinin-neuraminidase; N, nucleoprotein; M, matrix protein.

representing wild-type F_H was not observed in the absence of trypsin (Fig. 3, upper left panel), reflecting the inability of SeV-F_H to grow in the absence of trypsin (Fig. 2). In contrast, the single-cleavage-site mutant Fc was present in uncleaved F_0 and cleaved F_1 forms in a ratio of 34%/66%, as determined by densitometry. Faint bands representing the F_0 precursor were seen for the double-cleavage-site mutants F117 and F130 in the absence of trypsin (Fig. 3, upper left panel). In contrast, prominent bands representing partially cleaved intermediates (F_1+) were observed for both F117 and F130, which result from cleavage at site I but not at site II (16, 39). The presence of cleavage intermediates was confirmed by the use of a polyclonal serum (Fig. 3, αF_104–117), which recognizes amino acids 104 to 117 of F_{RSV} (an epitope included in the intervening region between the two cleavage sites that is also present in the F117 and F130 mutant proteins). The difference in migration between intermediate cleavage products for F117 and F130 may reflect differential glycosylation of pep27 since F117 contains two potential N-glycosylation sites within pep27, whereas F130 possesses three potential sites (Fig. 1A). It has previously been estimated that at least two of the three sites are N-glycosylated for F_{RSV} (52). Indeed, the presence of an additional band for F130 F_1+ that migrates with the same rate as F117 F_1+ (Fig. 3, upper left panel) may result from variable glycosylation of pep27. The proportion of fully cleaved F_1 protein was approximately the same for single-cleavage-site mutant Fc (66%) as double-cleavage-site mutants F117 (64%) and F130 (66%), suggesting that the insertion of a second, upstream cleavage site does not significantly affect cleavage of site II. In the presence of trypsin, the F proteins of all rSeV were completely cleaved to F_1 (Fig. 3, upper right panel). Completion of cleavage of F117 and F130 at both sites was confirmed by loss of reactivity with the F_{RSV}(104-117) serum (Fig. 3, lower right panel). Thus, both cleavage sites I and II are functional in double-cleavage-site mutants SeV-F117 and SeV-F130.

Insertion of one or both RSV cleavage sites in the SeV fusion protein leads to decreased thermostability. Given the in-

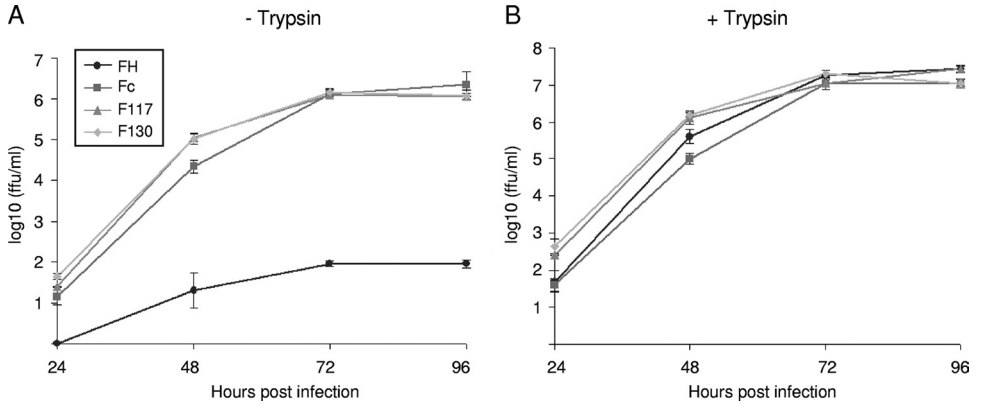

FIG. 2. Multistep growth curve. LLC-MK2 cells were infected at an MOI of 0.05 and incubated in the absence (A) or presence (B) of 0.3 μg/ml trypsin for 96 h. At 24-h intervals, aliquots of the cell culture supernatant were collected and replaced with fresh medium with or without trypsin. Titration of the culture supernatant was performed by immunohistochemical staining of infected LLC-MK2 cell foci in 96-well plates. Results represent the mean of two independent infections, which were titrated in duplicate. FFU, focus forming units.

creased virus titers seen at 33°C (Table 1), it was hypothesized that double-cleavage-site mutants may have decreased thermostability. rSeV were purified from the allantoic fluid of embryonated eggs by sucrose gradient centrifugation and preincubated at 37°C for 2, 4, or 6 h. Heat-treated rSeV were subsequently tested for their ability to mediate hemolysis of guinea pig erythrocytes. rSeV was incubated at 33°C for 45 min with 0.5 ml of guinea pig erythrocytes (1%), and the extent of hemolysis was determined by measuring the optical density at 520 nm. Hemolysis results from changes in membrane permeability of erythrocytes following viral fusion and can therefore be used as a measure of virus-cell fusion. Hemolysis directed by the wt SeV-F_H strain was significantly more resistant to heat treatment than the cleavage site mutant viruses (Fig. 4A), retaining approximately 40% hemolytic activity even after 6 h of heat treatment, in agreement with previously published results for the fusion of SeV with liposomes (51). In contrast, all cleavage site mutants were more readily inactivated by heat treatment. In particular, hemolysis directed by the double-cleavage-site mutant SeV-F130 strain, which contains the complete pep27 region of F_{RSV}, was reduced to approximately 20% following just 4 h of incubation at 37°C. We subsequently tested the ability of heat-treated virus obtained from cell culture supernatants to infect LLC-MK2 cells. As shown in Fig. 4B, the titer of heat-inactivated viruses reflects the hemolysis results since wt SeV-F_H was more resistant to heat treatment than the cleavage site mutants. Following 6 h of incubation at 37°C, the titer of wt SeV-F_H virus remained approximately 2 logarithms higher than that of the cleavage site mutant viruses, with SeV-F130 displaying the lowest titer following heat treatment. Thus, insertion of one or both F_{RSV} cleavage sites in F_{SeV} leads to reduced thermostability since cleavage site mutants display reduced hemolytic activity and replicate to lower titers than wt SeV-F_H following heat treatment. Moreover, the double-cleavage-site F130 mutant that contains the complete pep27 region, and thus most closely resembles F_{RSV}, was the least thermostable virus.

Insertion of both RSV cleavage sites in the SeV fusion protein results in increased cell-cell fusion in recombinant SeV-infected cells. In order to compare the cell-cell fusion activity

TABLE 1. Titration of rSeV at 48 h postinfection

Virus	Titer (FFU/ml) at:[a]	
	37°C	33°C
SeV-F_H	$4.3 \times 10^5 \pm 1.8$	$8.9 \times 10^3 \pm 2.5$
SeV-Fc	$1.1 \times 10^5 \pm 0.4$	$3.3 \times 10^3 \pm 0.9$
SeV-F117	$1.4 \times 10^6 \pm 0.6$	$1.1 \times 10^5 \pm 0.6$
SeV-F130	$1.5 \times 10^6 \pm 0.2$	$1.0 \times 10^5 \pm 0.4$

[a] Titers were determined at 48 h postinfection of culture supernatant from LLC-MK2 cells infected with rSeV at 37°C or 33°C.

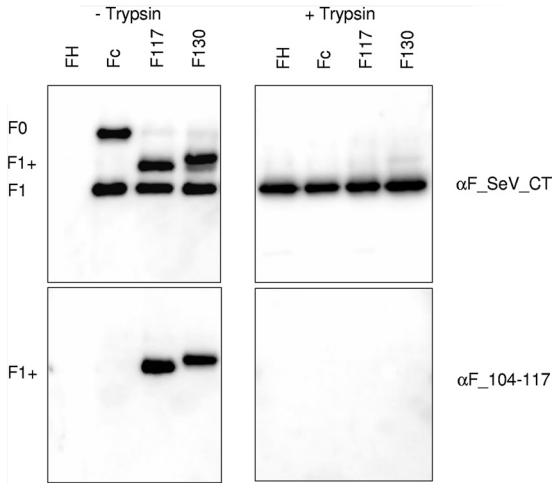

FIG. 3. Proteolytic cleavage of rSeV. Western blotting was employed to analyze cell culture supernatant from infected LLC-MK2 cells (MOI of 0.05) incubated for 96 h in the absence (−) or presence (+) of 0.3 μg/ml trypsin. Western blots were developed using polyclonal rabbit serum αF_SeV_CT, which recognizes the CT of SeV F protein, or αF_104–117, which recognizes amino acids 104 to 117 of the RSV F protein. Also indicated are bands representing uncleaved F protein precursor (F_0), the fully cleaved F_1 chain (F_1), and partially cleaved intermediate (F_1+), which results from cleavage at site I in the absence of cleavage at site II. The percentage of F protein cleavage to F_1 in the absence of trypsin (upper left panel) was determined by measuring the densitometry of bands, as detailed in Materials and Methods (Fc, 66%; F117, 64%; F130, 66%).

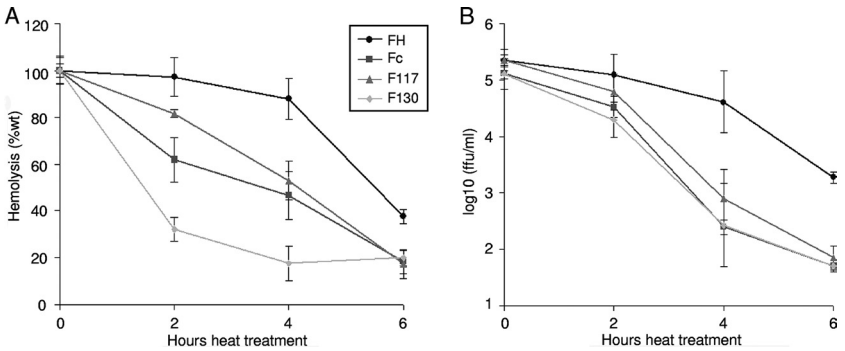

FIG. 4. Thermostability of recombinant Sendai viruses. (A) Purified SeV was preincubated at 37°C for 2, 4, or 6 h and subsequently incubated at 33°C for 45 min with 0.5 ml of washed guinea pig erythrocytes (1%, vol/vol, in PBS). Hemolysis was measured by determining the optical density of the erythrocyte supernatant at 520 nm. Results represent the mean of two independent experiments performed in duplicate. (B) Supernatant from infected LLC-MK2 cells was preincubated at 37°C for 2, 4, or 6 h and subsequently titrated on LLC-MK2 cells in 96-well plates by immunohistochemical staining. Results represent the mean of three independent experiments.

of cleavage site mutant viruses, LLC-MK2 cells grown in microchamber culture slides were infected at an MOI of 0.5 and incubated in the absence or presence of trypsin at 37°C. Cells were fixed at 30 h postinfection and stained with monoclonal antibody (MAb) GB5 that recognizes F_{SeV} (Fig. 5A). In order to quantitate the extent of syncytium formation by rSeV, the number of nuclei per syncytium was counted (Fig. 5B). The presence of trypsin was essential for the formation of syncytia by SeV-F_H, whereas all SeV cleavage mutant viruses formed syncytia in the absence of trypsin. Cleavage site mutant viruses also formed larger syncytia than SeV-F_H. In particular, double-cleavage-site mutant viruses SeV-F117 and SeV-F130 formed syncytia that were approximately four times the size of the syncytia formed by SeV-F_H virus in the presence of trypsin. Similar results were observed in BSR-T7/5 cells (data not shown).

Further quantitation of cell-cell fusion by recombinant viruses was carried out by analysis of cell content mixing using a luciferase reporter gene assay. BSR-T7/5 cells were infected with rSeV (MOI of 5) and subsequently mixed (14 h postinfection) with LLC-MK2 cells, which had been previously transfected with a plasmid encoding the luciferase gene (pTM1-Luc). The two populations of cells were left to fuse for 3 h at 37°C (Fig. 6A) or 33°C (Fig. 6B) in the absence or presence of

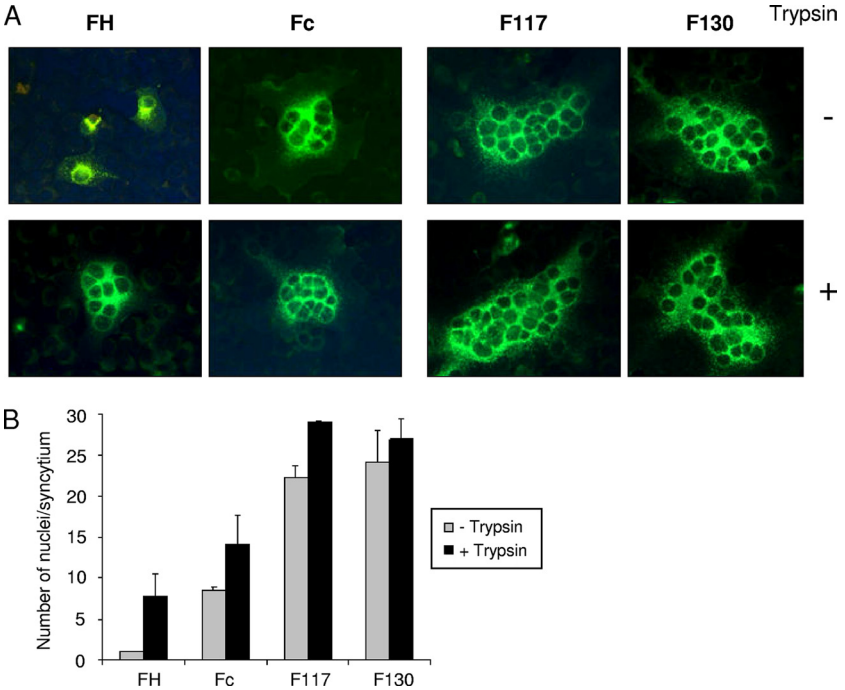

FIG. 5. Syncytium formation assay. (A) LLC-MK2 cells growing in microchamber wells were infected with SeV-F_H, SeV-Fc, SeV-F117, or SeV-F130 virus at an MOI of 0.5 in the absence or presence of 0.3 μg/ml trypsin. Infected cells were processed for syncytium formation and immunostaining at 30 h postinfection. (B) The number of nuclei in 10 syncytia (chosen at random) in duplicate infections were counted using an AxioCam HRc digital camera (×20 magnification). Results are representative of three independent experiments.

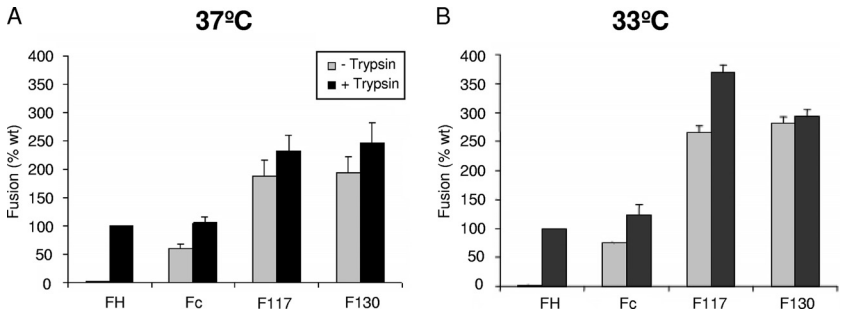

FIG. 6. Quantitative cell-cell fusion assay. BSR-T7/5 cells were infected with SeV-F_H, SeV-Fc, SeV-F117, or SeV-F130 virus at an MOI of 5 and incubated for 14 h in the absence or presence of 0.25 μg/ml trypsin. LLC-MK2 cells, which had previously been transfected with pTM1-Luc, were overlaid onto infected BSR-T7/5 cells at 14 h postinfection and incubated in the absence or presence of 0.25 μg/ml trypsin for 3 h at 37°C (A) or 33°C (B) to allow fusion. Cells were subsequently lysed and analyzed for luciferase activity. Results (as relative light units) are expressed as a percentage of wild-type (wt) fusion (SeV-F_H with trypsin), with mean values from at least two independent experiments shown.

trypsin. The results are expressed as a percentage of wild-type fusion (fusion of SeV-F_H in the presence of trypsin). Cell-cell fusion mediated by the single-cleavage-site SeV-Fc mutant virus was not significantly increased with respect to wt SeV-F_H at either 33°C or 37°C (Fig. 5). In contrast, double-cleavage-site mutants SeV-F117 and SeV-F130 displayed increased cell-cell fusion, particularly at 33°C, with SeV-F117 producing the highest level of cell-cell fusion (approximately 3.5 times the level of wt cell fusion at 33°C). The increased titers seen for double-cleavage-site mutant viruses, particularly at 33°C (Fig. 2 and Table 1), may reflect increased cell-cell spread (Fig. 5 and 6). In summary, insertion of double F_{RSV} cleavage sites in F_{SeV} leads to an increase in both syncytia size and the extent of cell content mixing.

Infection by double-cleavage-site rSeV mutants is less dependent on sialic acid receptor recognition. It has previously been reported that RSV infection can occur in the absence of the attachment protein (21). It was therefore of interest to examine the dependency of recombinant Sendai viruses expressing cleavage site mutant proteins on sialic acid recognition by HN for infection. Two alternative approaches were employed to address this question.

In the first approach, LLC-MK2 cells growing in microchamber culture slides were treated with 120 mU of NA for 90 min at 37°C. Cells were subsequently infected with rSeV at an MOI of 1 and incubated in the absence of trypsin. The extent of infection was revealed by immunofluorescence of fixed cells with MAb GB5 at 20 h postinfection. The level of infection in response to neuraminidase treatment was determined by counting the number of infected cells. As shown in Fig. 7A, both single-cleavage-site viruses SeV-F_H and SeV-Fc failed to produce significant infection in cells pretreated with neuraminidase. In contrast, double-cleavage-site mutant viruses SeV-F117 and SeV-F130 retained the ability to infect neuraminidase-treated cells, producing approximately 40% infection following neuraminidase treatment, compared to approximately 10% infection produced by single-cleavage-site mutants (Fig. 7B). Similar results were seen when cells were incubated at 33°C (results not shown).

In the second approach, we infected a CHO cell line (Lec2) that is defective in sialic acid expression (45). Lec2 cells are a derivative of Pro-5 cells and display a 90% reduction in the sialylation of glycoproteins due to defective transport of CMP-sialic acid into the *trans*-Golgi compartment (11, 46). Lec2 and Pro-5 cells were infected in microchamber culture slides at an MOI of 1 and subjected to immunofluorescence staining at 20 h postinfection. Whereas the single-cleavage-site viruses SeV-F_H and SeV-Fc were unable to produce significant infection of Lec-2 cells, both double-cleavage-site viruses SeV-F117 and SeV-F130 were able to infect Lec2 cells, albeit at a reduced level compared to the level of infection observed in Pro-5 cells (Fig. 8A). Syncytium formation was not observed in Lec2 cells, which may reflect the lower level of infection by F117 and F130 in this cell type. In order to quantify these results, an ELISA was carried out on infected Pro-5 and Lec2 cells at 20 h postinfection using the MAb GB5 directed against SeV F protein (Fig. 8B). Single cleavage mutants produced only a low level of infection in Lec2 cells (approximately 4% the level of infection of wt Pro-5 cells), which probably reflects the background level of sialic acid expression in Lec2 cells (46). However, double cleavage mutants SeV-F117 and SeV-F130 produced a higher level of infection in Lec2 cells (26% and 28%, respectively). Thus, while infection by double-cleavage-site mutants is enhanced by conditions that favor an interaction between HN and sialic acid, the presence of two cleavage sites reduces dependency on the HN protein for infection.

DISCUSSION

RSV is unique among paramyxoviruses since the RSV fusion protein (F_{RSV}) contains two multibasic cleavage sites and is able to fuse with target cell membranes in the absence of a separate attachment protein. It has previously been reported that insertion of both multibasic F_{RSV} cleavage sites in the SeV fusion protein (F_{SeV}) leads to enhanced cell-cell fusion and decreased dependence on the attachment HN protein for syncytium formation in transfected cells (38). We have extended this study to recombinant SeV (rSeV) that express either wild-type F protein (F_H) or F protein cleavage mutants containing F_{RSV} cleavage site II (Fc) or both F_{RSV} cleavage sites I and II and a partial or complete intervening pep27 sequence (Fig. 1A, F117 and F130). The presence of both F_{RSV} cleavage sites was found to increase the fusogenicity of rSeV (Fig. 5 and 6) and decrease the dependency on a separate receptor binding protein for infection by rSeV (Fig. 7 and 8), thereby mimicking the

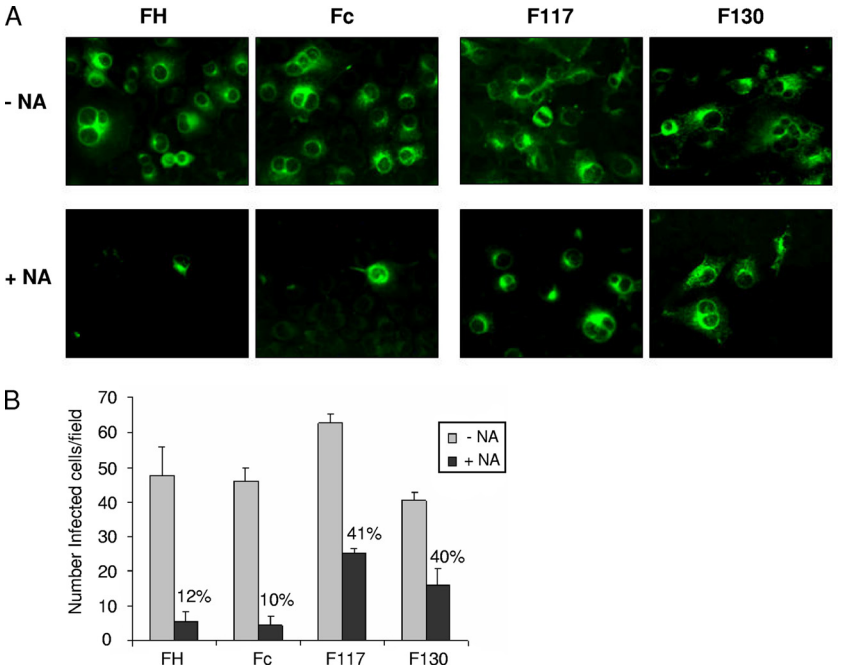

FIG. 7. Effect of neuraminidase treatment on rSeV infection. (A) LLC-MK2 cells growing in microchamber wells were pretreated with 120 mU of neuraminidase (NA) for 90 min at 37°C and subsequently infected with SeV-F_H, SeV-Fc, SeV-F117, or SeV-F130 virus at an MOI of 1. Cells were processed for immunostaining at 20 h postinfection. (B) The number of infected cells in five fields (chosen at random) in duplicate infections were counted using an AxioCam HRc digital camera (×20 magnification). Also indicated is the percent infection in NA-treated (+) cells compared to untreated (−) cells. Results are representative of three independent experiments.

unique properties of RSV to fuse and infect cells in the absence of an attachment protein.

Cleavage site mutants, in particular, the double-cleavage-site mutant F130, were found to be less thermostable than wt SeV-F_H virus (Fig. 4). Previous studies have reported that mutations in the parainfluenza virus type 5 (PIV5) F protein (W3A strain) that result in hyperfusogenicity and HN-independent syncytium formation (19) act by destabilizing the prefusion conformation of the F protein (37, 41, 50). Such destabilizing mutations may lower the activation energy required to trigger the F protein for fusion, thus reducing dependency on the HN protein for fusion (8, 37). Similarly, the presence of double cleavage sites may reduce the thermostability of the F protein, thereby facilitating F activation for fusion by lowering the energy threshold required to trigger conformational changes involved in fusion. Indeed, our results indicated that the least thermostable mutant, SeV-F130, displayed the greatest ability to infect cells in an HN-independent manner. However, there was no strict correlation between thermostability (Fig. 4) and HN independence for infection since the single-cleavage-site mutant Fc was less thermostable than the wild type but still required sialic acid receptors for infection (Fig. 7 and 8).

Destabilizing mutations that result in increased cell-cell fusion of the MLV Env glycoprotein were previously shown to be disadvantageous for virus growth (29). In contrast, hyperfusogenic double-cleavage-site mutant rSeV were able to infect cells devoid of sialic acid receptors, reproducing the ability of recombinant RSV expressing the F protein in the absence of other cell surface glycoproteins to fuse and infect cells (21, 47, 48). The infectivity of recombinant RSV that lacks attachment G protein expression suggests that the RSV F protein alone is capable of binding to target cells in order to mediate fusion. It has been shown that F_{RSV} binds to cell surface glycosaminoglycans (GAGs), although less efficiently than the attachment G glycoprotein (14, 20, 48). Indeed, infection by recombinant RSV is enhanced approximately 3-fold by the presence of the G protein (47). Similarly, infection by the double-cleavage-site rSeV mutants was enhanced by the presence of sialic acid receptors on target cells (Fig. 7 and 8). While the precise region of the F protein involved in the interaction with GAGs has not been mapped, a heparin-binding peptide corresponding to F_{RSV} cleavage site II and part of the fusion peptide was able to inhibit both attachment and infection by RSV (9), suggesting that the cleavage site II region of F_{RSV} interacts with cell surface GAGs.

The furin-dependent cleavage site of a number of other viruses has also been suggested as a candidate heparan sulfate binding domain (10, 24, 36). In particular, binding of Sindbis virus E2 protein to heparan sulfate correlates with the amount of uncleaved E2 present in the virion (24). The presence of partially cleaved F protein from rSeV grown in the absence of trypsin indicates that the uncleaved, multibasic cleavage site II is present on approximately one-third of F molecules (Fig. 3) and would thus be available to bind to cell surface GAGs. Binding of cleavage site II to GAGs may promote fusion in the absence of a separate attachment protein by facilitating either completion of cleavage of all F molecules within the trimer by furin present at the plasma membrane (49) or the fusion of adjacent, F protein trimers that are already fully cleaved. How-

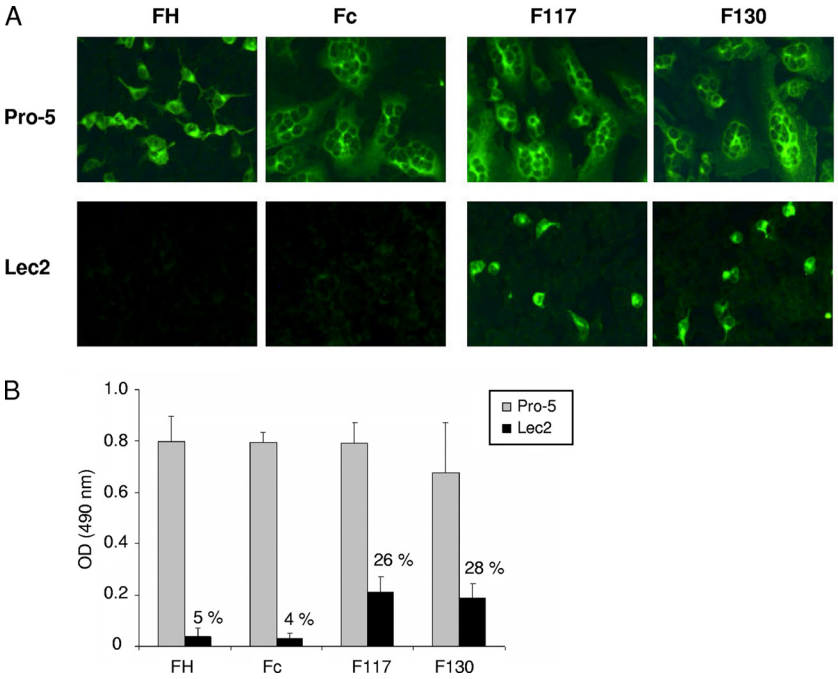

FIG. 8. Infection of sialic acid-deficient cells. (A) Pro-5 or sialic acid-deficient Lec2 cells growing in microchamber wells were infected with SeV-F_H, SeV-Fc, SeV-F117, or SeV-F130 virus at an MOI of 1. Cells were processed for immunostaining at 20 h postinfection. (B) Pro-5 or Lec2 cells growing in 96-well plates were infected with SeV-F_H, SeV-Fc, SeV-F117, or SeV-F130 virus at an MOI of 1 and fixed at 20 h postinfection. Cells were subsequently stained for expression of SeV F protein with MAb GB5, and the optical density (OD) at 490 nm was measured in order to quantitate infection. The percent infection in Lec2 cells relative to Pro-5 cells is indicated. Results represent the mean of three independent experiments performed in duplicate.

ever, only double-cleavage-site rSeV mutants were able to infect sialic acid-deficient cells, suggesting that furin cleavage site I is also involved in the binding to GAGs and/or the triggering of fusion.

While mutations of F_{RSV} cleavage site I and the intervening peptide in the context of recombinant bovine RSV (rBRSV) affect syncytium formation, they still permit virus replication in cell culture (53). Given the potential role of double cleavage in attachment protein-independent fusion, it would be of interest to investigate the phenotype of F_{RSV} cleavage site mutations in the context of a recombinant RSV lacking the attachment G protein.

In addition to the role of HN in binding to target cell receptors, it is also possible that the HN protein played a role in the stabilization of the rSeV cleavage mutants by interacting with mutant F proteins. In order to confirm whether double cleavage of F_{SeV} permits infection in the complete absence of the HN protein, we are currently investigating if rSeV expressing the double-cleavage-site F protein mutants can be rescued in the absence of the HN attachment protein or in the presence of an HN protein with altered receptor binding properties. rSeV in which both the SeV F and HN proteins were replaced by RSV F protein has previously been rescued (54). Therefore, although other regions of F_{RSV} could also contribute to attachment protein-independent fusion, the results presented here indicate that the double-cleavage region of F_{RSV} may play a crucial role in the unique ability of RSV to fuse and infect cells in the absence of a separate attachment protein.

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