

Insertion of the Two Cleavage Sites of the Respiratory Syncytial Virus Fusion Protein in Sendai Virus Fusion Protein Leads to Enhanced Cell-Cell Fusion and a Decreased Dependency on the HN Attachment Protein for Activity[∇]

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Cell entry by paramyxoviruses requires fusion of the viral envelope with the target cell membrane. Fusion is mediated by the viral fusion (F) glycoprotein and usually requires the aid of the attachment glycoprotein (G, H or HN, depending on the virus). Human respiratory syncytial virus F protein (F_{RSV}) is able to mediate membrane fusion in the absence of the attachment G protein and is unique in possessing two multibasic furin cleavage sites, separated by a region of 27 amino acids (pep27). Cleavage at both sites is required for cell-cell fusion. We have investigated the significance of the two cleavage sites and pep27 in the context of Sendai virus F protein (F_{SeV}), which possesses a single monobasic cleavage site and requires both coexpression of the HN attachment protein and trypsin in order to fuse cells. Inclusion of both F_{RSV} cleavage sites in F_{SeV} resulted in a dramatic increase in cell-cell fusion activity in the presence of HN. Furthermore, chimeric F_{SeV} mutants containing both F_{RSV} cleavage sites demonstrated cell-cell fusion in the absence of HN. The presence of two multibasic cleavage sites may therefore represent a strategy to regulate activation of a paramyxovirus F protein for cell-cell fusion in the absence of an attachment protein.

Human respiratory syncytial virus (RSV) and Sendai virus (SeV) are members of the *Pneumovirinae* and *Paramyxovirinae* subfamilies, respectively, within the *Paramyxoviridae* family of negative-stranded RNA viruses (*Mononegavirales*) (5). Cell entry by paramyxoviruses requires fusion of the viral envelope with the target cell membrane at the cell surface. Paramyxovirus-infected cells are also capable of fusing with adjacent cells to form syncytia (multinuclear, giant cells). Fusion in both cases is mediated by viral envelope glycoproteins, namely, the fusion (F) protein, a trimeric type I integral membrane glycoprotein.

The F polypeptide is synthesized as an inactive precursor (F₀), which requires proteolytic cleavage to yield fusion-competent, disulfide-linked F₂-F₁ chains (for a review see reference 21). Cleavage takes place at either a mono- or multibasic (RXK/RR) cleavage site, recognized by trypsin- or furin-like proteases, respectively, immediately upstream of a hydrophobic fusion peptide that is inserted into the cell membrane during fusion (30). Cleavage is an absolute requirement for fusion, and indeed the nature of the cleavage motif has been shown to play a role in the pathogenicity of some paramyxoviruses (17).

Whereas for most paramyxoviruses, fusion mediated by the F glycoprotein requires participation of the homotypic attachment protein (G, H, or HN, depending on the virus), the F

protein of human respiratory syncytial virus is able to fuse membranes in the absence of the attachment G protein. Spontaneous mutants (15) or genetically engineered recombinant RSVs expressing F as the only surface glycoprotein (38, 39) are able to infect cells and form syncytia. Furthermore, expression of the RSV F protein as the only viral protein in transfected cells is sufficient to induce syncytium formation (12, 45).

RSV F protein (F_{RSV}) is unique among the *Paramyxoviridae*, since F_{RSV} possesses two furin cleavage sites (site I, RARR₁₀₉, and site II, KKRKRR₁₃₆), which are separated by a region of 27 amino acids (pep27), as shown in Fig. 1A, below. The two cleavage sites and the length, but not the sequence, of pep27 are highly conserved in all bovine and human RSV strains. Proteolytic processing at both cleavage sites is required by F_{RSV} to form syncytia in transfected cells (12, 45). Thus, while membrane fusion by F_{RSV} does not depend on coexpression of an attachment protein, there is a requirement for double cleavage of the F protein and removal of the intervening pep27, which is secreted into the medium (28). The function of the double cleavage and subsequent pep27 removal are currently unknown, although completion of cleavage is associated with a change in shape of the RSV F protein (25, 28). An immunomodulatory role has been suggested for bovine RSV pep27 (48); however, no such role has been attributed to the human RSV intervening peptide, which does not share sequence similarity with its bovine counterpart.

Despite low sequence identity, paramyxovirus F proteins share structural elements, including the location of cysteine residues, hydrophobic domains, and heptad repeats (HR) A and B, as shown in Fig. 1A, below. Crystallographic data indicate that refolding of the F protein during fusion results in the

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assembly of HRA and HRB into a six-helix bundle, consisting of an internal core of three HRA helices surrounded by three antiparallel HRB helices (2, 44). Such an arrangement positions the fusion peptide and transmembrane domain at the same end of the F protein and is thought to be directly linked to merging of viral and cell membranes, with the free energy released on six-helix bundle formation driving membrane fusion (26, 30). The crystal structures of the pre- and postfusion forms of two paramyxovirus F proteins have recently been solved, revealing substantial conformational differences between the two structures (42, 43). The trigger responsible for activation of the prefusion F protein for fusion is currently unknown. However, in the case of viruses belonging to the *Paramyxovirinae* subfamily, binding of the attachment protein to the corresponding cell surface receptor is thought to play a key role in F activation (for a review see reference 20).

Paramyxovirus attachment proteins are type II integral membrane proteins that differ widely in their structure and receptor usage. An interaction between the F and HN proteins has been demonstrated on the surface of transfected (8) or infected cells (36). This interaction has been shown to decrease under conditions that favor HN attachment (24), suggesting that F and HN interact before the binding of HN to its receptor takes place. The current model of membrane fusion hypothesizes that the HN protein forms a prefusion, metastable complex with F in the virion. Following attachment of the HN protein to the target cell receptor, conformational changes that take place in HN may be transduced to the F protein to activate it for fusion "at the right time and in the right place" (20). However, the fusion protein of human metapneumovirus (HMPV) and human and bovine RSV is sufficient to mediate attachment and fusion in the absence of other surface glycoproteins (3, 15, 33, 34, 38, 39). Thus, the precise role in fusion of attachment proteins belonging to members of the *Pneumovirinae* subfamily is less clear.

In order to explore the significance of the double cleavage of F_{RSV} for fusion, we decided to investigate the effect of the F_{RSV} proteolytic processing region in the context of the Sendai virus F protein (F_{SeV}). Sendai virus is a member of the *Paramyxovirinae* subfamily, and in contrast to the RSV F protein, F_{SeV} is cleaved at a monobasic cleavage site (R_{116}) and requires coexpression of both the HN attachment protein and trypsin in order to fuse cells in culture (16, 32). A series of chimeric mutants of F_{SeV} were constructed by inserting one or both F_{RSV} cleavage sites and various regions of pep27 into F_{SeV} . The ability of these mutants to cause cell-cell fusion was evaluated in the absence and presence of the HN protein and trypsin. Inclusion of both F_{RSV} cleavage sites in F_{SeV} resulted in a dramatic increase in cell-cell fusion activity in the presence of HN. Furthermore, chimeric mutants containing both F_{RSV} cleavage sites also demonstrated cell-cell fusion in the absence of HN. These results suggest that the presence of two multi-basic cleavage sites may represent an alternative strategy to regulate the activation of a paramyxovirus F protein for cell-cell fusion in the absence of an attachment protein.

MATERIALS AND METHODS

Cell lines. BSR-T7/5 cells (4) (a gift from K.-K. Conzelmann, Munich, Germany) and BHK-21 cells were maintained in Dulbecco's modified Eagle's medium (DMEM; BioWhittaker), supplemented with 10% fetal calf serum (FCS).

Alternate passages of BSR cells were supplemented with 1 mg/ml G-418 sulfate (Sigma-Aldrich), in order to select for T7 polymerase expression.

Plasmids and mutagenesis. The pTM1 plasmid (9), carrying the full-length cDNA insert of the RSV F gene under transcriptional control of the T7 promoter (pTM1- F_{RSV}), has been described previously (12). Plasmids carrying the mutations in F_{RSV} shown in Fig. 7A, below, have also been reported (28, 29). The mutant F genes were subsequently subcloned in pTM1 between NcoI and BamHI restriction sites.

The genes encoding Sendai virus F and HN proteins (Harris strain) were amplified from pGEM-F and pGEM-HN plasmids (a gift from L. Roux, Geneva, Switzerland), by PCR, and cloned into the pTM1 plasmid downstream of the T7 promoter, between NcoI and SpeI restriction sites. The resulting pTM1- F_{SeV} plasmid was subjected to mutagenesis, using the QuikChange site-directed mutagenesis kit (Stratagene) to generate the mutants shown in Fig. 2, below. Three (RKR) or five (KKRKR) basic residues from the F_{RSV} cleavage site II were inserted into F_{SeV} using the forward oligonucleotides pTM1-F_RKR, 5'GCCG GTGTTCCACAGTCCGAGGAAAAGAAGATTCTTCGGTGCTGTG, and pTM1-F_KKRKR (Fc), 5'GCCGGTGTTCACAGTCCGAGGAAAAGAAGAAAAGAAG ATTCTTCGGTGCTG, with complementary reverse oligonucleotides. Subsequent mutagenesis of pTM1-F_KKRKR (termed F cleavage, or Fc) was carried out in order to insert F_{RSV} cleavage site I, producing the pTM1-Fc_103-110 mutant (where the numbers refer to intervening F_{RSV} residues), using the following forward oligonucleotide: pTM1-Fc_103-110, 5'GATCTTCAGGAGGCTCTGA TAACTGCAAAACAATCGAGCCAGAAGAGAAGTCCACCAATGATACGA CAAAAATGCC.

Further mutagenesis of pTM1-Fc_103-110 was performed as detailed in Fig. 2, below, using the following forward oligonucleotides to insert various regions of the intervening segment between the two cleavage sites (pep27): pTM1-Fc_103-117, 5'CTGCAAACAATCGAGCCAGAAGAGAACTACCAAGGTTTATGAA TTACACCAATGATACGACACAAAAATGCCGGTG; pTM1-Fc_103-117, 5'G CCAGAAGAGAACTACCAAGGTTTATGAATTACACCTCGAAGAAAAGG AAAAGAAGATTCTTCGGTGC; pTM1-Fc_103-130, 5'GCCAAG AGAACTACCAAGGTTTATGAATTACACCTCAACAATACCAAAAAA CCAATGTAACATTATCGAAGAAAAGGAAAAGAAGATTCTTCGGTGC.

All mutations were confirmed by automated sequencing of the complete F gene. The RSV G protein was similarly cloned into the pTM1 plasmid between NcoI and SpeI restriction sites (pTM1-G), and the gene encoding luciferase was cloned into pTM1 between NcoI and PstI restriction sites (pTM1-Luc).

Syncytium formation assay. BSR-T7/5 cells (a BHK-derived cell line that constitutively expresses the T7 RNA polymerase) were grown in microchamber culture slides to 90 to 100% confluence and transfected in DMEM (2.5% FCS) with 0.5 μ g total DNA, using FuGENE HD (Roche Biochemicals, Inc.). The transfection mixture was removed 7 h posttransfection, and cells were incubated in serum-free medium with or without 0.25 μ g/ml trypsin (Sigma-Aldrich). Cells were fixed for 32 h (F_{SeV}) or 48 h posttransfection (F_{RSV}) with cold methanol for 5 min, followed by cold acetone for 30 s. Fixed cells were subsequently immunostained using monoclonal antibodies directed against F_{SeV} (GB5; a gift from S. Wharton and J. J. Skehel, London, United Kingdom) or F_{RSV} (2F) (11), 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 3.1 software.

Luciferase reporter gene cell-cell fusion assay. BSR-T7/5 cells were transfected in 48-well plates with 0.5 μ g total DNA, as described above. BHK cells in 100-cm dishes were simultaneously transfected with 30 μ g of a plasmid encoding the luciferase gene (pTM1-Luc). For the cell-cell fusion assay, BHK cells were detached 7 h posttransfection using 1 mM EDTA in Ca^{2+} - and Mg^{2+} -free phosphate-buffered saline (PBS), resuspended at a density of 1×10^6 cells/ml in serum-free medium, and overlaid at a 1:1 ratio onto BSR cells, either in the presence or absence of 0.25 μ g/ml trypsin (Sigma-Aldrich). The mixed donor and target cells were subsequently incubated for 22 h to allow fusion. At 30 h posttransfection (or 48 h posttransfection for experiments involving F_{RSV}), cells were washed with PBS and lysed in $1 \times$ passive lysis buffer (Promega, Inc.), according to the manufacturer's instructions. Ten to 20 μ l of clarified cell extract was mixed with 100 μ l luciferase assay substrate (Promega) and immediately assayed for luciferase activity over a 10-second measurement using a Turner Biosystems 20/20n luminometer instrument. Background activity (subtracted from all results shown) was determined by overlaying BHK cells transfected with pTM1-Luc onto BSR cells, which had previously been transfected with the empty pTM1 plasmid.

Flow cytometry. Transfected BSR-T7/5 cells were detached 24 h posttransfection using 1 mM EDTA in Ca^{2+} - and Mg^{2+} -free PBS and resuspended in DMEM (2% FCS). A total of 3×10^5 cells were incubated for 30 min at 4°C with monoclonal antibody GB5 (F_{SeV}) or 2F (F_{RSV}). After pelleting and

washing the cells two times with PBS, cells were stained by subsequent 30-min incubations with biotinylated anti-mouse immunoglobulin (Amersham Biosciences UK Ltd.) and streptavidin-R-phycoerythrin (Southern Biotechnology Associates). Finally, cells were fixed in 1% paraformaldehyde and the fluorescence of 2×10^4 cells was determined using a Becton Dickinson FACSCalibur instrument with CellQuest software. Data were analyzed using FlowJo software (Tree Star, Inc.).

Immunoprecipitation and Western blotting. BSR-T7/5 cells were transfected as previously described. Twenty-four hours posttransfection, cells were washed with PBS and extracts of membrane proteins prepared using the ProteoExtract native membrane protein extraction kit (Calbiochem). For the immunoprecipitation experiments, purified GB5 antibody was bound to CNBr-activated Sepharose (Pharmacia), according to the manufacturer's instructions. One ml of membrane protein extract was incubated at 4°C with 50 μ l of GB5-conjugated Sepharose. Following an overnight incubation, Sepharose beads were washed four times with PBS, resuspended in 50 μ l sample buffer (80 mM Tris, 2% sodium dodecyl sulfate [SDS], 10% glycerol, and 0.01% bromophenol blue), and boiled for 6 min. Following centrifugation to remove beads from the eluted proteins, 5% β -mercaptoethanol was added and the immunoprecipitated proteins were separated on a 10% acrylamide SDS-polyacrylamide gel electrophoresis gel. Proteins were transferred to an Immobilon membrane (Millipore) and subjected to Western blotting in order to detect F_{SeV} proteins using biotinylated GB5 monoclonal antibody and streptavidin-horseradish peroxidase conjugate (Amersham Biosciences UK Ltd.). Bands were visualized using the Amersham ECL Advance Western blotting detection kit (GE Healthcare UK Ltd.) and imaged using a VersaDoc camera and Quantity One 1D analysis software (Bio-Rad Laboratories).

RESULTS

RSV and SeV fusion proteins have different requirements for cell-cell fusion. Qualitative and quantitative assays of cell-cell fusion were carried out in order to compare the requirements for fusion of RSV and SeV F proteins (comparative primary structure diagrams of both proteins are shown in Fig. 1A) and to confirm previous findings. BSR-T7/5 cells, which constitutively express T7 RNA polymerase, were transfected with pTM1 plasmids encoding viral glycoproteins under transcriptional control of the T7 promoter. Cells were fixed 32 h (F_{SeV}) or 48 h (F_{RSV}) posttransfection and immunostained using monoclonal antibodies directed against F_{SeV} or F_{RSV} proteins (Fig. 1B). Formation of syncytia (multinuclear, fused cells) was observed in transfected cells that expressed F_{RSV} as the only viral protein, both in the presence and absence of trypsin. The number and size of syncytia was enhanced by coexpression of F_{RSV} with G and by the presence of trypsin in the culture medium. In contrast, F_{SeV} displayed a strict requirement for both HN coexpression and trypsin in order to form syncytia. Thus, in the absence of HN, only individual, unfused cells expressing F_{SeV} were observed.

These results were confirmed by a luciferase reporter gene assay (Fig. 1C). BSR cells transfected with the genes of interest were mixed with BHK cells, which had been previously transfected with the pTM1-Luc plasmid, and left to fuse in the absence or presence of trypsin. Since pTM1-Luc contains the luciferase reporter gene under transcriptional control of the T7 promoter, expression of luciferase only takes place in BHK cells that have fused with BSR cells, permitting quantitative measurement of cell-cell fusion. While trypsin produced only a moderate enhancement in the level of luciferase activity induced by F_{RSV} , coexpression of G increased cell-cell fusion by approximately twofold. Therefore, while not a strict requirement, G enhances F_{RSV} -mediated cell-cell fusion. In accordance with the syncytium formation assay, F_{SeV} required both

HN coexpression and trypsin in order to produce significant luciferase activity. These results are in good agreement with previously published findings, both for F_{SeV} , expressed with or without the HN protein (37), and for F_{RSV} , expressed in the absence of the G protein (12, 45).

Design and expression of SeV F protein cleavage site mutants. As indicated in Fig. 1A, RSV F protein is cleaved by furin-like proteases at two cleavage sites (site I R_{109} and site II R_{136}), which are separated by a region of 27 amino acids (pep27). In contrast, SeV F protein contains a single arginine residue at its cleavage site (R_{116}) and requires the addition of trypsin to the cell culture medium for cleavage. Alignment of F_{RSV} and F_{SeV} (Fig. 2) revealed that although both proteins possess homologous fusion peptides, the two multibasic cleavage sites and the intervening segment of F_{RSV} cannot be aligned with F_{SeV} . We decided to investigate if the unique characteristics of the F_{RSV} proteolytic processing region play a role in the distinct fusion requirements by F_{RSV} and F_{SeV} . To this aim, mutagenesis was carried out on the F_{SeV} gene (cloned in the pTM1 plasmid) in order to reproduce partially, or totally, the sequences that determine cleavage of F_{RSV} . Initially, part of the second cleavage site of F_{RSV} was inserted directly preceding the single cleavage site of F_{SeV} (R_{116}), producing F_{RKR} or F_{KKRKR} (Fc) mutants, containing a minimal furin recognition sequence (RKRR) or the complete F_{RSV} cleavage site II (KKRKRR), respectively (Fig. 2). Further mutation of the Fc construct was carried out in order to introduce F_{RSV} cleavage site I, producing the $Fc_{103-110}$ mutant (where the numbering refers to F_{RSV} residues). As detailed in Fig. 2, regions of pep27 were subsequently inserted to produce additional mutants, including $Fc_{103-117}$, which contains both F_{RSV} cleavage sites I and II separated by 27 intervening amino acids derived from both F_{RSV} and F_{SeV} . $Fc_{\Delta 103-117}$ was produced from the $Fc_{103-117}$ mutant by a deletion N-terminal to the second cleavage site, resulting in a shortened intervening segment composed entirely of F_{RSV} residues. Finally, the remaining residues of F_{RSV} pep27 were inserted into $Fc_{\Delta 103-117}$ to produce the mutant $Fc_{103-130}$, which contains the complete F_{RSV} pep27 sequence.

In order to confirm cell surface expression, BSR-T7/5 cells were transfected with the chimeric F_{SeV} mutants, and flow cytometry of the cells was performed using a monoclonal antibody directed against F_{SeV} . As shown in Fig. 3A, all F_{SeV} mutants were expressed at the cell surface at levels comparable to the wild-type F protein, although a slight decrease in expression was noted for mutants containing both F_{RSV} cleavage sites.

With the aim of determining whether the F_{SeV} mutants could be cleaved by cellular proteases, GB5-conjugated Sepharose beads were used to immunoprecipitate SeV F proteins from membrane extracts of transfected BSR cells, which were subsequently visualized by Western blotting. As shown in Fig. 3B (upper panel), the SeV wild-type F0 protein precursor remained essentially uncleaved in the absence of trypsin, whereas some cleavage of F0 to F1 was observed for the mutant F_{RKR} . Insertion of the complete F_{RSV} cleavage site II into F_{SeV} led to a greater extent of cleavage, as observed for the Fc mutant. Similarly, mutants $Fc_{103-110}$, $Fc_{103-117}$, and $Fc_{103-130}$, which contain both F_{RSV} cleavage sites I and II, were also present as uncleaved F0 and fully cleaved F1

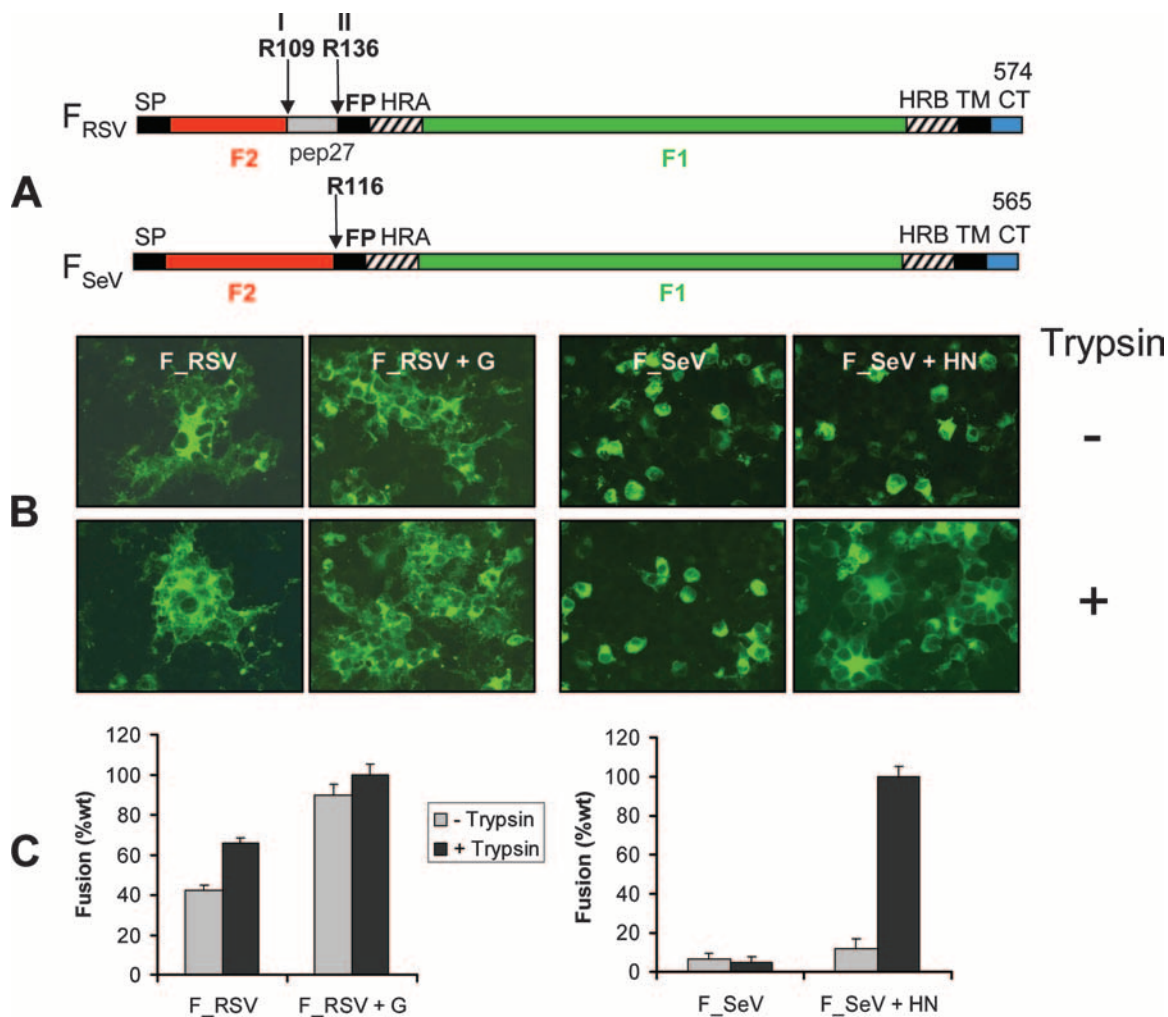


FIG. 1. RSV and SeV fusion proteins have different requirements for cell-cell fusion. (A) Schematic diagram of RSV fusion (F_{RSV}) and SeV fusion (F_{SeV}) proteins. The three principal hydrophobic regions found in paramyxovirus F proteins are shown in black: the N-terminal signal peptide (SP), the fusion peptide (FP), located adjacent to the cleavage site at the N terminus of F1, and the C-terminal transmembrane region (TM). Heptad repeat sequences HRA and HRB (hatched boxes) are located adjacent to the FP and TM regions, respectively. Cleavage sites of F_{RSV} (site I, R₁₀₉, and site II, R₁₃₆) and F_{SeV} (R₁₁₆) are indicated by arrows. Cleavage results in the formation of disulfide-linked F2 and F1 polypeptides, and in the case of F_{RSV} , cleavage at both sites results in the removal of the intervening segment pep27, shown in light gray. (B) BSR-T/7/5 cells were cotransfected in microchamber culture slides with 0.25 μ g of pTM1 plasmids encoding RSV or SeV F genes and 0.25 μ g pTM1-G or pTM1-HN plasmids, as indicated, or 0.25 μ g empty vector pTM1 (not indicated). The transfection mixture was removed 7 h posttransfection and cells were incubated in serum-free medium with (+) or without (–) trypsin. Cells were fixed and immunostained as indicated in Materials and Methods. Immunofluorescence of cells transfected with the empty pTM1 plasmid was conducted in parallel, and the absence of fluorescence confirmed specific antibody staining (data not shown). (C) BSR-T/7/5 cells were transfected in 48-well plates with plasmids encoding RSV or SeV genes. BHK cells were simultaneously transfected with a plasmid encoding the luciferase gene (pTM1-Luc). At 7 h posttransfection, BHK cells were detached using 1 mM EDTA, resuspended at a density of 1×10^6 cells/ml in serum-free medium, and overlaid at a 1:1 ratio onto BSR cells, either in the presence or absence of trypsin. At 30 h posttransfection (or 48 h posttransfection for experiments involving F_{RSV}), cells were lysed and analyzed for luciferase activity. Results (relative light units) are expressed as a percentage of the wild-type (wt) fusion (F_{SeV} plus HN plus trypsin, or F_{RSV} plus G plus trypsin), with background activity subtracted (determined by overlaying BHK cells transfected with pTM1-Luc onto BSR cells transfected with the empty pTM1 plasmid). Mean values from three independent experiments are shown.

forms in approximately equal proportions. A band migrating at a lower molecular weight than F0 was observed for the mutant Fc Δ _{103–117}. Although the identity of this band has not been thoroughly investigated, it may represent the F0 precursor of the Fc Δ _{103–117} mutant, which lacks an N-glycosylation site that has previously been shown to be used in the Z strain of SeV F protein (N104) (35).

When trypsin was added to the medium of the transfected cells (Fig. 3B, lower panel), cleavage of the F0 precursor of

both wild-type F_{SeV} and F_{RKR} to F1 was clearly enhanced. In contrast, only a slight increase in F0 processing was detected when trypsin was added to cells expressing the Fc mutant. However, the extent of F0-to-F1 cleavage was enhanced by trypsin in all four F_{SeV} mutants containing both F_{RSV} cleavage sites, and Fc Δ _{103–130} appeared fully cleaved to F1 in the presence of trypsin. Thus, insertion of an F_{RSV} cleavage site II in the SeV F protein increases the extent of cleavage at this site in the absence of trypsin. This cleavage is further enhanced by

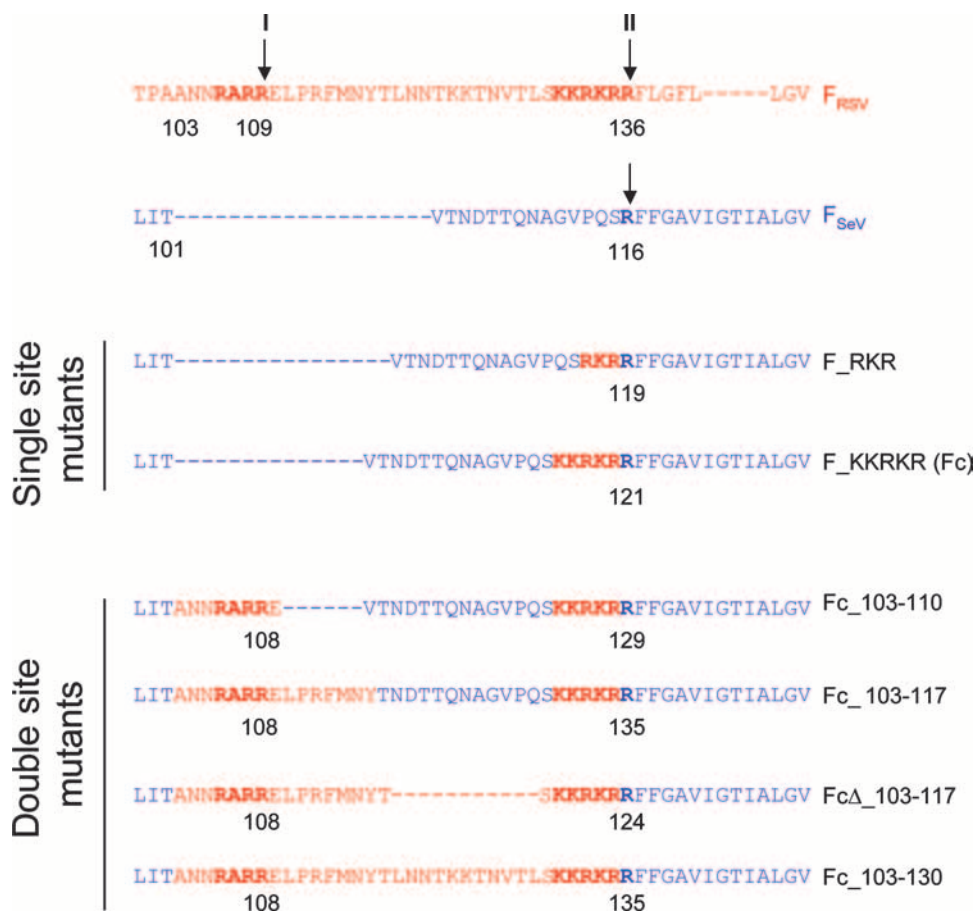


FIG. 2. Design of SeV F protein cleavage site mutants. An alignment of F_{SeV} (amino acids 99 to 129 [blue]) and F_{RSV} (amino acids 100 to 144 [red]) is shown. Cleavage sites of F_{RSV} (site I, RARR₁₀₉, and site II, KKRKRR₁₃₆) and F_{SeV} (R₁₁₆) are shown in bold and indicated by arrows. Residues from F_{RSV} that were inserted into a backbone of F_{SeV} are shown in red. The numbering of F_{SeV} mutants produced by mutation of Fc refers to the inserted F_{RSV} residues.

trypsin, following the insertion of F_{RSV} cleavage site I upstream of site II.

Insertion of RSV F cleavage site II in SeV F leads to trypsin-independent syncytium formation. Cell-cell fusion by the SeV F protein mutants F_{RKR} or Fc, containing part (RKRR) or the complete F_{RSV} cleavage site II (KKRKRR), respectively, was tested in the syncytium formation assay. In the presence of the HN attachment protein, only small syncytia were observed for F_{RKR} when trypsin was excluded from the culture medium (Fig. 4). This reflects the poor cleavage of F_{RKR} in the absence of trypsin (Fig. 3B). In contrast, the Fc mutant formed very large syncytia even in the absence of trypsin (Fig. 4), consistent with the enhanced cleavage of F0 to F1 that was observed for this mutant (Fig. 3B). Fc was also capable of forming more extensive syncytia than wild-type F_{SeV} (compare with Fig. 1B). However, both F_{RKR} and Fc mutants continued to require HN coexpression in order to form syncytia (Fig. 4). Thus, in spite of the large syncytia formed by Fc in the presence of HN, insertion of the complete F_{RSV} cleavage site II in F_{SeV} does not lead to HN-independent fusion. Similar results were obtained with F_{RKR} and Fc mutants in the quantitative luciferase assay, presented comparatively with other mutants in subsequent sections.

Insertion of both RSV F cleavage sites I and II in SeV F protein leads to enhanced membrane fusion in the presence of HN. Mutants containing both F_{RSV} cleavage sites I and II (Fig. 2) were also tested for their ability to fuse cells in the syncytium formation assay. As shown in Fig. 5A, all F_{SeV} chimeras containing the two F_{RSV} cleavage sites formed extremely large syncytia in the presence of HN, without a requirement for trypsin. In order to confirm the results of the syncytium formation assay, all of the mutants detailed in Fig. 2 were coexpressed with HN and analyzed for cell-cell content mixing using the luciferase reporter gene assay (Fig. 5B). Insertion of the complete F_{RSV} cleavage site II (KKRKRR) in F_{SeV} to form the Fc mutant led to a marked increase in cell-cell fusion compared to wild-type F_{SeV}. In the absence of trypsin, Fc resulted in 181% of wild-type F_{SeV} fusion (F_{SeV} coexpressed with HN in the presence of trypsin represents 100% fusion). In contrast, the F_{RKR} mutant containing the partial F_{RSV} cleavage site II produced only 50% of wild-type fusion in the absence of trypsin and fused cells to the same extent as the wild-type F protein on addition of trypsin. Thus, the presence of six basic residues at the single cleavage site of F_{SeV} enhances the fusogenic potential of this protein when coexpressed with HN, even in the absence of trypsin. In the presence of HN, all

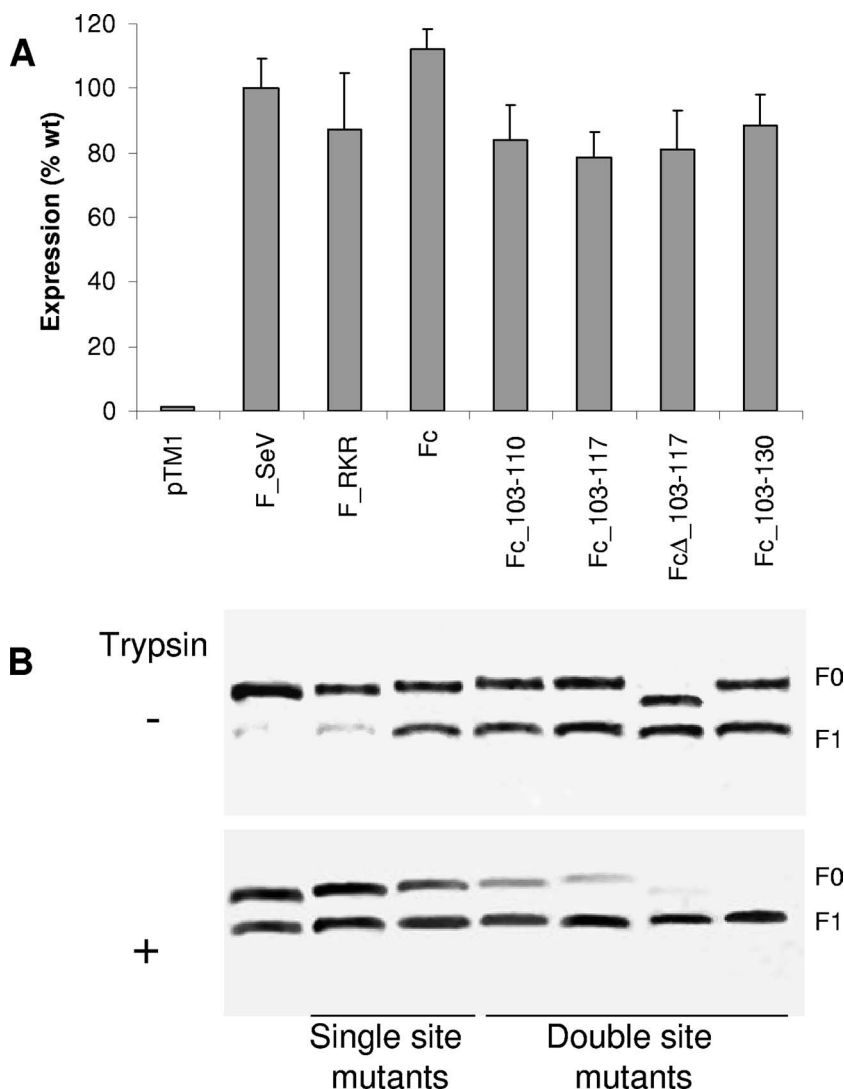


FIG. 3. Cell surface expression and proteolytic cleavage of chimeric SeV F mutants. (A) The cell surface expression levels of wild-type (wt) and mutant F_{SeV} proteins were determined by flow cytometry analysis of transfected BSR-T7/5 cells 24 h posttransfection, using the monoclonal antibody GB5. The mean fluorescent intensity of cells is represented as a percentage of wt F_{SeV} expression, and mean values from three independent experiments are shown. (B) Extracts of membrane proteins were prepared from transfected BSR-T7/5 cells incubated in the absence (upper panel) or presence (lower panel) of trypsin and subjected to immunoprecipitation using purified GB5 antibody bound to Sepharose beads. Following an overnight incubation, immunoprecipitated F_{SeV} proteins were eluted, fractionated by SDS-polyacrylamide gel electrophoresis under reducing conditions, and analyzed by Western blotting using biotinylated GB5 antibody and a streptavidin-horseradish peroxidase conjugate.

mutants containing both F_{RSV} cleavage sites I and II dramatically increased fusion with respect to the wild-type F protein (by up to 364% or 450% in the absence or presence of trypsin, respectively). The intervening sequence between the two cleavage sites appeared to exert a minor modulatory role on fusion in the presence of HN.

No strict correlation was observed between the extent of F0 cleavage to F1 in the absence or presence of trypsin (Fig. 3B) and the relative differences in fusion observed between the mutants (Fig. 5B). However, the enhanced fusogenicity of double cleavage site mutants reflects their increased susceptibility to cleavage when trypsin was added to the culture medium (Fig. 3B, lower panel).

Insertion of both RSV F cleavage sites I and II in SeV F protein leads to a decreased dependence on HN for membrane fusion. In contrast to the Fc mutant that contains only the second F_{RSV} cleavage site, all of the F_{SeV} mutants containing both F_{RSV} cleavage sites were also able to form syncytia in the absence of HN coexpression (Fig. 6A). Therefore, inclusion of the two multibasic F_{RSV} cleavage sites in F_{SeV} facilitates syncytium formation, even in the absence of an attachment protein. Although HN-independent cell-cell fusion was observed in the absence of trypsin, trypsin inclusion did enhance the size and number of syncytia formed. The insertion of both F_{RSV} cleavage sites and not the intervening pep27 sequence per se conveys HN independence for cell-cell fusion, since the

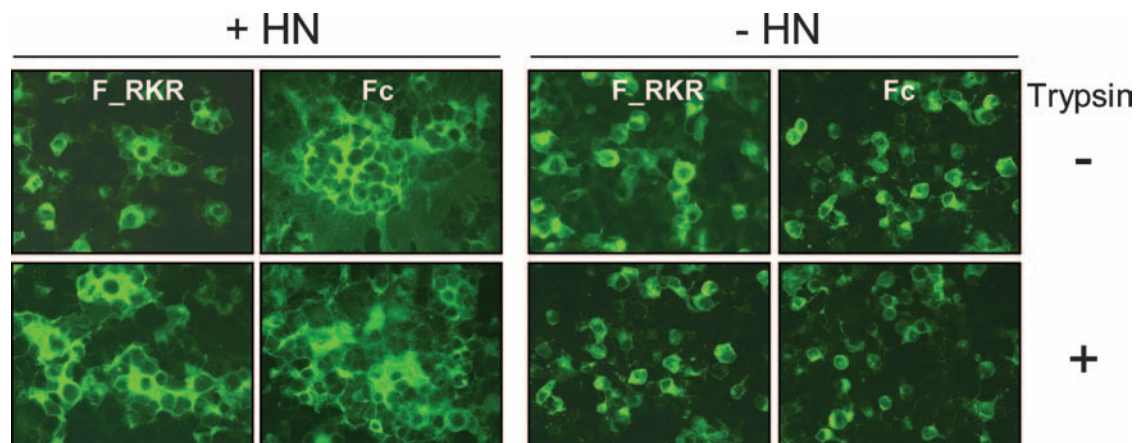


FIG. 4. Insertion of the RSV F cleavage site II in SeV F leads to trypsin-independent syncytium formation. BSR-T7/5 cells growing in microchamber wells were cotransfected as previously described with 0.25 μ g pTM1-F_RKR or pTM1-Fc mutants and 0.25 μ g pTM1-HN plasmid (+HN) or 0.25 μ g empty pTM1 vector (-HN). The transfection mixture was removed 7 h posttransfection, and cells were incubated in serum-free medium with (+) or without (-) trypsin. Cells were fixed 32 h posttransfection and subjected to immunostaining as described in Materials and Methods.

Fc_{103–110} mutant forms syncytia in the absence of HN. However, the nature and/or length of the intervening residues between the two cleavage sites does appear to influence fusion to a greater extent in the absence of HN, since Fc_{103–117} formed large syncytia whereas Fc Δ _{103–117} formed only small syncytia without HN.

In the absence of HN coexpression, three mutants containing both F_{RSV} cleavage sites (Fc_{103–110}, Fc_{103–117}, and Fc_{103–130}) produced fusion activity in the luciferase reporter gene assay in the presence of trypsin (Fig. 6B). As observed in the syncytium formation assay, Fc did not produce significant fusion in the absence of HN. Fc Δ _{103–117} also failed to produce HN-independent fusion, reflecting the small syncytia produced by this mutant in the absence of an attachment protein, in spite of the fact that it contains both F_{RSV} cleavage sites.

Enhanced membrane fusion in the absence of HN by the double cleavage site mutants paralleled the increase in F0-to-F1 cleavage by trypsin, compared with single site mutants (Fig. 3B). However, no precise quantitative correlation between F0 processing and membrane fusion activity in the absence of HN could be found. For instance, while Fc Δ _{103–117} was highly susceptible to trypsin cleavage, this mutant was poorly active in both membrane fusion assays (Fig. 6). Furthermore, although Fc_{103–130} was cleaved by trypsin more efficiently than Fc_{103–110} or Fc_{103–117} (Fig. 3B), it did not produce larger syncytia or induce greater luciferase activity in the absence of HN (Fig. 6).

The Fc_{103–117} mutant produced the greatest fusion activity in the absence of the HN protein and was subjected to a dose-response fusion assay in the presence of trypsin and compared with Fc (Table 1). Increasing quantities of DNA were used to transfect BSR cells, and the corresponding increase in cell surface expression was determined by flow cytometry. An average fusion index was subsequently calculated from the fusion and expression data. While increasing expression of Fc did not enhance fusion, a dose response of fusion was seen for Fc_{103–117}, confirming the ability of this double cleavage site mutant to fuse cells in the absence of HN. The fusion index

estimated for Fc_{103–117} was 0.72, compared to 0.08 for the Fc mutant and 1.0 for wild-type F (F_{SeV} coexpressed with HN in the presence of trypsin). However, higher expression levels of Fc_{103–117} were required in the luciferase reporter gene assay, compared to the syncytium formation assay, in order to observe a level of HN-independent cell-cell fusion comparable to the wild type. This may reflect differences in the requirements of the two assays. For instance, the presence of F protein in both target and donor cells in the syncytium formation assay may facilitate cell-cell fusion with respect to the reporter gene assay, in which F protein is present only in the donor BSR cells. Furthermore, the reporter gene assay involves the interaction between two distinct populations of cells and may depend to a greater extent on the attachment ability of the mutated F proteins.

Coexpression of the attachment G protein fails to restore the reduced fusogenic capacity of RSV F cleavage site mutants. It has previously been shown that cleavage at both sites I and II is a requirement for fusion of the F_{RSV} protein, expressed in transfected cells in the absence of the attachment G protein. Mutations of F_{RSV} cleavage site I abrogated syncytium formation in the absence of trypsin, whereas deletion of F_{RSV} cleavage site II prevented syncytium formation, even in the presence of trypsin (12, 45). In light of our findings that insertion of F_{RSV} cleavage site I in F_{SeV} led to syncytium formation by F_{SeV} in the absence of HN, we aimed to determine whether coexpression of RSV G protein with F_{RSV} cleavage site mutants would restore cell-cell fusion.

A series of F_{RSV} mutants (Fig. 7A), which have been previously described (12, 28, 29), were subcloned in the pTM1 plasmid. These mutations included changes in cleavage site I (RARR) to produce the F_{R108/109N} mutant (RANN) or deletion of four basic residues in cleavage site II (KKRKR), resulting in the F Δ _{131–134} mutant (12). Further deletions in F_{R108/109N} resulted in the RSV F Δ _{108–120} and F Δ _{108–132} mutants (28, 29). All mutant proteins were expressed at the cell surface at a level comparable to F_{RSV} (Fig. 7B), with the exception of the cleavage site II mutant F Δ _{131–134}, which

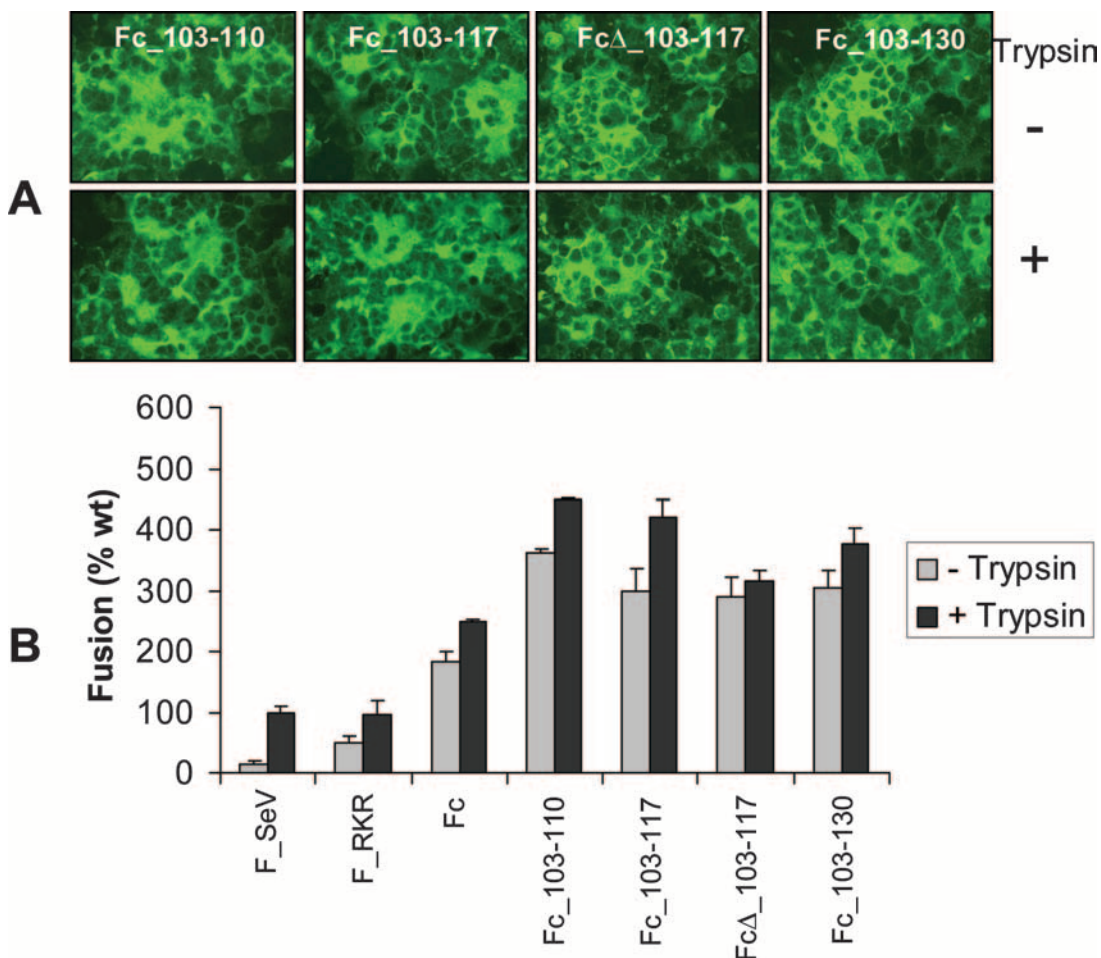


FIG. 5. Insertion of both RSV F cleavage sites I and II in SeV F protein enhances membrane fusion in the presence of HN. (A) BSR-T7/5 cells growing in microchamber wells were cotransfected with 0.25 μ g pTM1-Fc₁₀₃₋₁₁₀, pTM1-Fc₁₀₃₋₁₁₇, pTM1-Fc Δ ₁₀₃₋₁₁₇, or pTM-Fc₁₀₃₋₁₃₀ mutants and 0.25 μ g pTM1-HN plasmid and processed for syncytium formation and immunostaining as described in the legend for Fig. 1. (B) BSR-T7/5 cells were cotransfected in 48-well plates with 0.25 μ g pTM1 plasmids encoding wild-type (wt) or mutant F_{ScV} genes and 0.25 μ g pTM1-HN, as previously described. BHK cells were simultaneously transfected with 30 μ g of pTM1-Luc and overlaid at a 1:1 ratio onto BSR cells, as described in the legend to Fig. 1C. Cells were mixed either in the presence or absence of trypsin and incubated for 22 h to allow fusion. Cells were subsequently lysed and analyzed for luciferase activity at 30 h posttransfection. Results (relative light units) are expressed as a percentage of wt fusion (F_{ScV} plus HN plus trypsin), with mean values from three independent experiments shown.

was expressed at a slightly reduced level (60% of wild-type F_{RSV}). This is consistent with previous findings that mutation of F_{RSV} cleavage site II increases the susceptibility of the protein to degradation (45).

F_{RSV} cleavage mutants were subjected to the luciferase fusion assay described in Fig. 1C. F_{R108/109N} produced a low level of fusion, which was increased by the presence of trypsin but not by coexpression of the G protein. F Δ ₁₃₁₋₁₃₄ failed to produce fusion above background levels, irrespective of the inclusion of trypsin or G protein. Further mutation of F_{R108/109N} by deletion of pep27 to produce F Δ ₁₀₈₋₁₂₀ and F Δ ₁₀₈₋₁₃₂ mutants led to an increase in cell-cell fusion activity, compared to F_{R108/109N}. However, the fusion activity of both F Δ ₁₀₈₋₁₂₀ and F Δ ₁₀₈₋₁₃₂ in the presence of G was approximately half that of the wild type (54% and 49% of wild-type F coexpressed with G in the presence of trypsin, respectively). Therefore, coexpression of the G protein failed

to rescue the reduced fusogenic capacity of the RSV F cleavage site mutants.

DISCUSSION

In the current study, the differences between cell-cell fusion mediated by RSV and SeV fusion proteins were initially highlighted. In addition to confirming previously reported qualitative measurements of cell-cell fusion mediated by F_{RSV}, it was observed that the G protein increased cell-cell fusion by approximately twofold in a quantitative assay (Fig. 1C). Therefore, while not a strict requirement, G enhances F_{RSV}-mediated cell-cell fusion by a mechanism that remains essentially unknown. These results are in good agreement with those of Techaarpornkul et al. (38), who reported a similar level of fusion enhancement by the G protein in

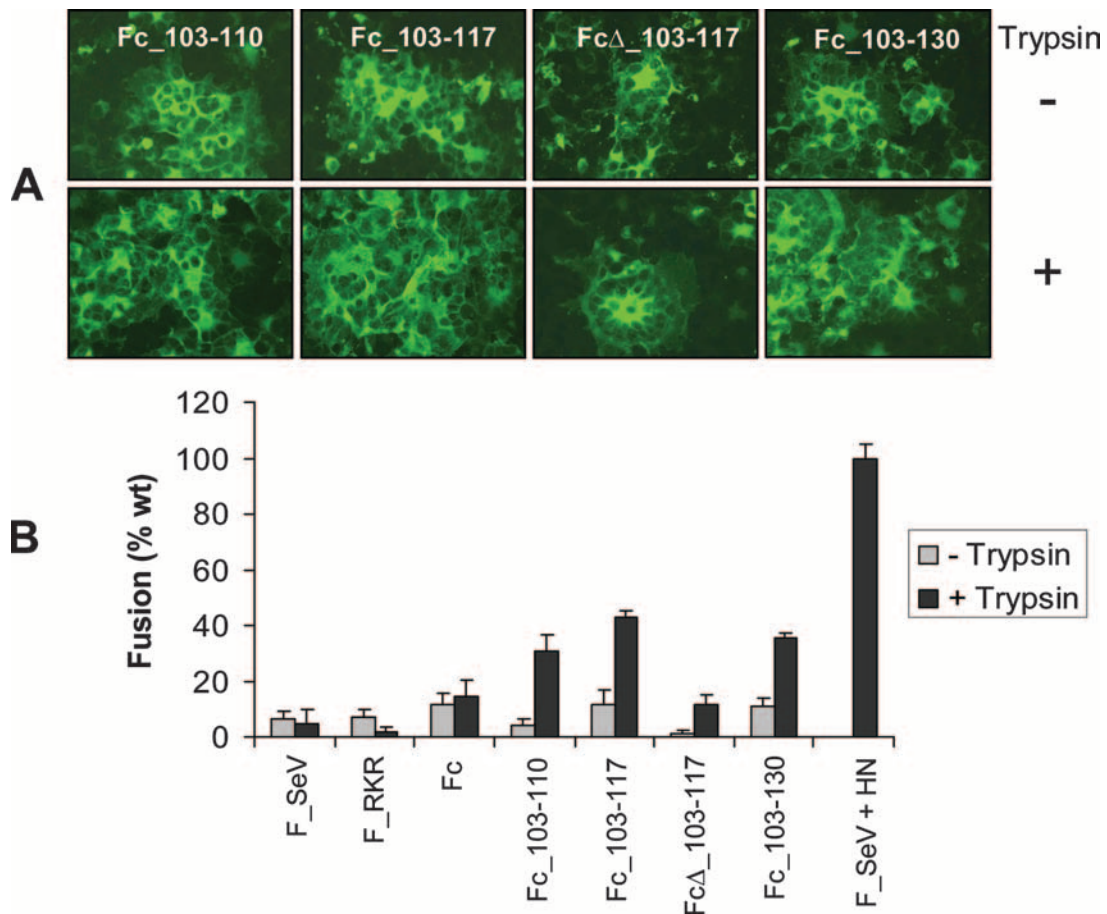


FIG. 6. Insertion of both RSV F cleavage sites I and II in SeV F protein decreases dependency on HN for membrane fusion. (A) BSR-T7/5 cells growing in microchamber wells were cotransfected with 0.25 μ g pTM1-Fc₁₀₃₋₁₁₀, pTM1-Fc₁₀₃₋₁₁₇, pTM1-Fc Δ ₁₀₃₋₁₁₇, or pTM1-Fc₁₀₃₋₁₃₀ mutants and 0.25 μ g empty pTM1 plasmid and processed in parallel to the cultures shown in Fig. 5A. (B) BSR-T7/5 cells were cotransfected in 48-well plates with 0.25 μ g pTM1 plasmids encoding wild-type (wt) or mutant F_{SeV} genes and 0.25 μ g empty pTM1 plasmid. BHK cells were simultaneously transfected with 30 μ g of pTM1-Luc, overlaid at a 1:1 ratio onto BSR cells to allow fusion, and processed in parallel to those in Fig. 5B.

recombinant RSV that expressed either F protein or both F and G proteins.

In contrast, F_{SeV} displayed an absolute requirement for both HN coexpression and trypsin for cell-cell fusion, confirming

previously published results (37). F_{SeV} protein contains a single basic cleavage site (R₁₁₆), whereas F_{RSV} requires cleavage of two furin-dependent cleavage sites (RARR₁₀₉ and KKRKRR₁₃₆) for cell-cell fusion (12, 45). A number of chimeras

TABLE 1. Dose effect of Fc103-117 and Fc on membrane fusion

Plasmid	F DNA (ng) ^a	Fusion (% of wt) ^b	Cell surface expression (% of wt) ^c	Fusion index (fusion/cell surface expression)	Avg fusion index ^d
F _{SeV} + HN	250	100.0 \pm 10.8	100.0 \pm 9.1	1.00	1.0
Fc ₁₀₃₋₁₁₇	250	45.7 \pm 9.2	78.6 \pm 7.7	0.58	0.72 \pm 0.13
	375	72.1 \pm 8.0	85.7 \pm 13.0	0.84	
	500	88.1 \pm 7.0	119.5 \pm 12.6	0.74	
Fc	250	10.7 \pm 9.9	112.0 \pm 6.4	0.10	0.08 \pm 0.04
	375	14.3 \pm 0.4	124.3 \pm 10.9	0.11	
	500	6.0 \pm 1.4	136.8 \pm 2.2	0.04	

^a The total amount of transfected DNA was maintained constant at 500 ng by using empty pTM1 plasmid (Fc₁₀₃₋₁₁₇ and Fc) or pTM1-HN (F_{SeV}).

^b Wild-type (wt) fusion was determined in a luciferase reporter gene assay using 250 ng F_{SeV} coexpressed with HN in the presence of trypsin. Fusion data represent the means of three independent experiments in the presence of trypsin.

^c Wild-type (wt) cell surface expression was determined by flow cytometry of BSR cells transfected with 250 ng F_{SeV}, as previously described. Flow cytometry results represent the means of at least two independent experiments.

^d An average fusion index was calculated for each F protein by averaging the fusion index (fusion/cell surface expression) for each quantity of transfected DNA.

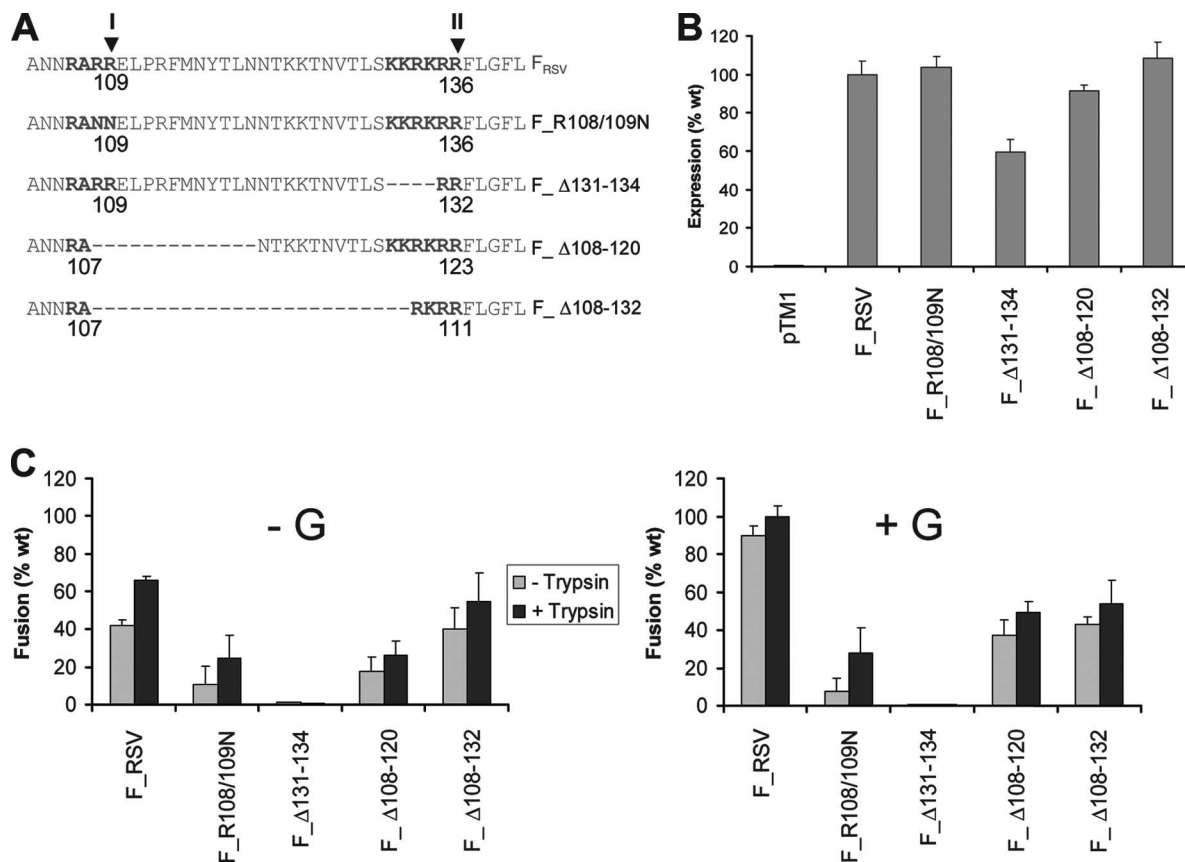


FIG. 7. Coexpression of the attachment G protein fails to restore the reduced fusogenic capacity of RSV F cleavage site mutants. (A) The amino acid sequence surrounding the F_{RSV} proteolytic processing region (amino acids 103 to 141) is shown. Cleavage sites of F_{RSV} (site I, RARR₁₀₉, and site II, KKRKR₁₃₆) are shown in bold. Deleted residues are indicated by a dashed line. (B) The levels of cell surface expression of wild-type (wt) and mutant F_{RSV} proteins were determined by flow cytometry of transfected BSR-T7/5 cells 24 h posttransfection, using the monoclonal antibody 2F. The mean fluorescent intensity of cells is represented as a percentage of wt F_{RSV} expression, and mean values from three independent experiments are shown. (C) BSR-T7/5 cells were cotransfected in 48-well plates with 0.25 μg pTM1 plasmids encoding wt or mutant F_{RSV} genes and 0.25 μg pTM1 (-G) or pTM1-G (+G). BHK cells were simultaneously transfected with 30 μg of pTM1-Luc and overlaid at a 1:1 ratio onto BSR cells, as described in the legend to Fig. 1C. Cells were lysed and analyzed for luciferase activity at 48 h posttransfection. Results (relative light units) are expressed as a percentage of wt fusion (F_{RSV} plus G plus trypsin), with mean values from three independent experiments shown.

between RSV and SeV fusion proteins were constructed in order to determine whether the differential requirements for fusion exhibited by F_{RSV} and F_{SeV} are related to the distinct proteolytic processing pathways of the two proteins.

Insertion of the complete F_{RSV} cleavage site II (KKRKR) in F_{SeV} augmented cell-cell fusion in an HN-dependent manner and decreased the dependence on trypsin for fusion. The presence of six basic residues at the cleavage site of Fc increased cleavage and cell-cell fusion over the minimal furin recognition sequence present in F_{RKR}. However, the Fc mutant was still dependent on the coexpression of HN for cell-cell fusion.

Insertion of the F_{RSV} cleavage site I (RARR) in the backbone of the Fc mutant further augmented trypsin-independent cell-cell fusion in the presence of HN. Although no clear correlation was found between the extent of F0 cleavage in the absence of trypsin and the ability of the differing mutants to mediate cell-cell fusion, mutants with double cleavage sites were more susceptible to proteolytic processing when trypsin was added to the culture medium. The presence of two cleav-

age sites may increase the local level of F protein processing by proteases in the vicinity of the target membrane, increasing the number of F protein molecules available for engagement in membrane fusion. It has previously been shown that cleavage of F_{RSV} at site I, in the absence of cleavage at site II, results in a partially cleaved protein, F_{Δ1-109}, seen as an intermediate band between F0 and F1 (12, 45). In the present study, we did not observe a partially cleaved intermediate for F_{SeV} mutants containing both cleavage sites. This suggests that site I is either not used in the chimeric F_{SeV} mutants or that if cleavage at site I does take place, it is immediately preceded by cleavage at site II to form F1. Interestingly, other studies of F_{RSV} cleavage in transfected (18) or infected (47) cells also failed to observe F_{Δ1-109}, suggesting that the ability to observe this partially cleaved F_{RSV} intermediate is dependent on cell type and/or the expression system.

Insertion of both F_{RSV} cleavage sites in F_{SeV} also resulted in syncytium formation in the absence of the HN protein. All four mutants that possessed two F_{RSV} cleavage sites formed syncytia without a requirement for HN coexpression. The interven-

ing segment between the two cleavage sites slightly modulated the capacity of the F_{SeV} protein to fuse membranes, and this modulation appeared greater in the absence of HN. In particular, while the $Fc_{103-117}$ mutant formed large syncytia without HN, $Fc\Delta_{103-117}$ (containing a shortened intervening segment) produced only small syncytia. $Fc\Delta_{103-117}$ is related to $Fc_{103-117}$ by a deletion N-terminal to the second cleavage site, which results in removal of an N-glycosylation motif that has previously been shown to be glycosylated in the Z strain of SeV F protein (35). There are three N-glycosylation sites present in pep27, and it has been estimated that at least two of the three sites are glycosylated (45). Mutation of one site (N126) resulted in increased F_{RSV} cell-cell fusion, suggesting that differential glycosylation of pep27 may modulate fusion (46, 47).

While the exact mechanism by which introduction of the two F_{RSV} cleavage sites in F_{SeV} leads to HN-independent membrane fusion is not known, there are several possibilities which deserve consideration. The presence of two multibasic cleavage sites may influence F protein activation by altering the energy threshold required to trigger the conformational changes for fusion. Mutations in the parainfluenza virus 5 F protein that resulted in HN-independent syncytium formation are thought to destabilize the prefusion conformation of the F protein, lowering the activation energy barrier required to convert the high-energy prefusion form into the more thermally stable postfusion form (14, 27, 31, 41).

The introduction of basic residues at the F_{SeV} cleavage site may also facilitate attachment of the SeV F protein to the target cell. F_{RSV} has previously been shown to bind to cells in an interaction that is largely dependent on glycosaminoglycans (GAGs) (10, 39). Studies using overlapping peptides of F_{RSV} revealed that a heparin-binding peptide, corresponding to F_{RSV} cleavage site II and part of the fusion peptide, was one of only two F_{RSV} peptides capable of inhibiting both attachment and infection of Vero cells by the RSV A2 strain (7). Since binding of the peptide to cells was dependent on GAGs, it was hypothesized that this region of F_{RSV} is involved in interactions with cell surface proteoglycans. An attractive hypothesis is that the insertion of F_{RSV} cleavage site I directly mediates attachment to cells. Alternatively, the insertion of cleavage site I may alter the local structure of the F_{SeV} proteolytic processing region, thereby facilitating the projection of cleavage site II (KKRKRR) from the surface of the F prefusion structure to bind to target cell GAGs. This hypothesis is supported by previous findings which suggest that the sequence surrounding the F_{SeV} cleavage site is important for fusion (13).

It is currently unknown how activation of the F protein for fusion could be coordinated with attachment so that triggering of the F protein would only occur following binding of the F protein to the target membrane. In support of a role for cellular attachment in F protein activation, F_{SeV} has previously been shown to mediate fusion of virosomes with red blood cells in the absence of the HN protein, via an interaction with the asialoglycoprotein receptor (1). In addition, virus-like particles that expressed SeV protein, but that lacked HN, were able to infect cells via the asialoglycoprotein pathway (23). The ability of F_{SeV} to bind to cells may reduce the requirement of an attachment protein for fusion, as has been observed for F_{RSV} (38, 39). Correct timing of F protein activation is crucial to

ensure activation for fusion "at the right time and in the right place," (19). As shown by Western blotting of transfected cell extracts, intracellular cleavage of F0 to F1 in the F_{SeV} double cleavage site mutants was incomplete. Previous studies have also confirmed incomplete cleavage of F_{RSV} in both transfected and infected cell extracts (12, 45). It is possible that partial cleavage of some, but not all, of the F protein monomers in the trimeric F protein prevents conformational changes to the postfusion form before the F protein reaches the cell surface. The presence of uncleaved F_{RSV} monomers at the cell surface would permit attachment of the proposed heparin binding region at cleavage site II to cell surface glycosaminoglycans. Following attachment, cleavage of all three monomers and removal of the intervening segment (pep27) may be completed by trypsin added to the culture medium, or by furin present at the cell surface as it recycles between the plasma membrane and Golgi network (for a review see reference 40). Completion of cleavage could, in turn, facilitate activation of the F protein for membrane fusion.

We have shown that the RSV F proteolytic processing region may be involved in attachment protein-independent fusion, at least in the context of chimeric SeV F. However, the failure of the RSV G protein to rescue membrane fusion when coexpressed with site I mutants RSV $F_{R108/109N}$ and $F_{\Delta108-120}$ (Fig. 7) contrasts with the high fusion activity displayed by SeV Fc in the presence of HN (Fig. 5), given that all three F proteins share the same polybasic sequence at site II. It therefore appears that the cooperative effect of HN on SeV F-mediated fusion cannot be reproduced by the G protein when coexpressed with RSV F proteins that contain only a single furin recognition sequence (site II).

Interestingly, despite displaying drastically reduced syncytium formation, recombinant bovine RSV expressing F protein with an intact cleavage site II, but lacking both site I and the intervening segment ($\Delta106-130$), replicated with kinetics similar to that of the parental virus (47). Recombinant bovine RSV expressing $F_{R108/109N}$ also replicated in cells, albeit with a growth retardation. Thus, while mutations of F_{RSV} cleavage site I and the intervening peptide affect cell-cell fusion, they still permit virus replication in cell culture. It is possible that the structural requirements for virus-cell and cell-cell fusion are different, as recently suggested for parainfluenza virus 5 (6). Similarly, while certain mutations that destabilize the envelope (Env) glycoprotein of a murine leukemia virus enhanced syncytium formation in transfected cells, the same mutations were detrimental when incorporated into virus particles (22). It is possible that unstable Env proteins at the cell membrane that are continuously being replaced by new molecules facilitate cell-cell fusion. In contrast, such unstable proteins may be prematurely activated in the virus particle before virus-cell contact is able to take place, leading to virus inactivation. It remains to be tested whether the chimeric SeV F proteins reported here could replace SeV F in infectious virus particles.

Further studies are currently under way to investigate the mechanism by which insertion of the F_{RSV} proteolytic processing region in F_{SeV} is able to confer decreased dependence on HN for cell fusion. Although the RSV F protein possesses unique properties among the *Paramyxoviridae* family of viruses, studies of such features may shed light on the mecha-

nism of F protein activation, particularly in the absence of an attachment protein. HMPV F protein has also been shown to mediate cell-cell fusion (34) and virus-cell fusion (3) in the absence of the attachment G protein. Interestingly, syncytium formation by the HMPV F protein expressed alone in transfected cells was dependent on low pH (34). It is therefore possible that members of the *Pneumovirinae* subfamily have evolved distinct mechanisms of F protein activation compared to those of the *Paramyxovirinae* subfamily of paramyxoviruses.

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