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A Chimeric Respiratory Syncytial Virus Fusion Protein Functionally Replaces the F and HN Glycoproteins in Recombinant Sendai Virus

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Entry of most paramyxoviruses is accomplished by separate attachment and fusion proteins that function in a cooperative manner. Because of this close interdependence, it was not possible with most paramyxoviruses to replace either of the two protagonists by envelope glycoproteins from related paramyxoviruses. By using reverse genetics of Sendai virus (SeV), we demonstrate that chimeric respiratory syncytial virus (RSV) fusion proteins containing either the cytoplasmic domain of the SeV fusion protein or in addition the transmembrane domain were efficiently incorporated into SeV particles provided the homotypic SeV-F was deleted. In the presence of SeV-F, the chimeric glycoproteins were incorporated with significantly lower efficiency, indicating that determinants in the SeV-F ectodomain exist that contribute to glycoprotein uptake. Recombinant SeV in which the homotypic fusion protein was replaced with chimeric RSV fusion protein replicated in a trypsin-independent manner and was neutralized by antibodies directed to RSV-F. However, replication of this virus also relied on the hemagglutinin-neuraminidase (HN) as pretreatment of cells with neuraminidase significantly reduced the infection rate. Finally, recombinant SeV was generated with chimeric RSV-F as the only envelope glycoprotein. This virus was not neutralized by antibodies to SeV and did not use sialic acids for attachment. It replicated more slowly than hybrid virus containing HN and produced lower virus titers. Thus, on the one hand RSV-F can mediate infection in an autonomous way while on the other hand it accepts support by a heterologous attachment protein.

The membrane glycoproteins of enveloped viruses play a crucial role in the infection process. They mediate virus binding to cellular receptors and induce fusion between the viral and cellular membranes. For several viruses, including members of the families *Orthomyxoviridae* and *Retroviridae*, binding and fusion are functions of the same envelope glycoprotein. A different strategy is accomplished by members of the family *Paramyxoviridae* which have separate attachment and fusion proteins.

The fusion (F) proteins of paramyxoviruses are typical type I fusion proteins. They are synthesized as inactive precursor proteins that have to be proteolytically cleaved into disulfide-linked subunits to be biologically active (19). For most paramyxoviruses, this cleavage occurs at a multibasic cleavage motif that is recognized by the ubiquitous endoprotease furin in the trans-Golgi network. An example for this type of activation is the F protein of respiratory syncytial virus (RSV) (49). In contrast, Sendai virus (SeV) and some apathogenic Newcastle disease virus strains contain a single arginine residue at the cleavage sites and are activated by trypsin-like proteases that are secreted by a restricted subset of cells or by coinfecting bacteria (40, 41). Viruses of this category usually require addition of trypsin for multistep replication in cell culture. Whatever protease is involved in the processing of the fusion protein, in either case a stretch of hydrophobic amino acids, the so-called fusion peptide, is located at the N terminus of the membrane-anchored subunit. The fusion peptide is probably buried in the native protein unless a conformational change leads to its exposure at the surface of the molecule. Only in this position it interacts with the target membrane and initiates the fusion process (23). For the influenza virus hemagglutinin and other viral fusion proteins, the conformational change required for this process has been shown to occur in response to acidic pH (7, 9, 44). In contrast, fusion by paramyxoviruses takes place at neutral pH and therefore a different trigger for the conformational change must exist. A large body of evidence indicates that the majority of paramyxovirus fusion proteins lack any detectable fusion activity in the absence of the viral attachment protein (1, 6, 11, 14, 26–28, 39). In addition, attempts to replace the homologous attachment protein with one from a related virus were mostly not successful (6, 14, 39, 46). These findings led to the hypothesis that the two proteins act in a cooperative manner. It is believed that binding of the attachment protein to the cellular receptor induces conformational changes in this protein, which in turn trigger conformational changes in the fusion protein (23). Two members of the *Paramyxoviridae* family apparently do not follow this rule. The fusion proteins of simian virus 5 and RSV show fusion activity in the absence of the respective attachment protein (1, 10, 31, 50). In addition, RSV attachment protein G was demonstrated to be dispensable for virus replication in cell culture, indicating

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TABLE 1. Oligonucleotides used for construction of chimeric genes

Primer	5′-sequence-3′[a]
hF-S1	TTTGAATTCACGCGTACA**ATGGAGTTGCCAATCCTCAAAGC**
RSV/SeV-F-AS	GATCGTAATCACAGT**ATTTGTGGTTGATTTACCAGC**
RSV/SeV-F-S	AAATCAACCACAAAT**ACTGTGATTACGATCATAGTAG**
SeV-F-AS	AAAGAATTCGCGCGC**TCATCTTTTCTCAGCCATCGCATCAAACC**
Tail-AS	CATTGACCTTTTGAGTCT**ACAGTATAGGAGCAGTCCAACAGCAATTA**
Tail-S	GGACTGCTCCTATACTGT**AGACTCAAAAGGTCAATGCTAATGGGTAAT**
hF-S2	TTTTGTCGAC**ATGGAGTTGCCAATCCTCAAAGCAAATGC**
SeV-F-AS2	TTTTCTCGAGTTC**TCATCTTTTCTCAGCCATCGCATCAAACCC**

[a] Cleavage sites of restriction endonucleases are underlined. Nucleotides that are part of either the RSV-F or the SeV-F open reading frame are shown in bold.

that RSV-F also has a receptor-binding function (18, 42). Further evidence for this assumption comes from the observation that RSV-F can bind to glycosaminoglycans such as heparin (12). Nevertheless, the G glycoprotein was found to support virus attachment to the cell surface (17, 31, 43).

In this study, we used reverse genetics to analyze RSV-F protein function in the context of infectious SeV. We show that chimeric RSV-F is incorporated into SeV virions and can functionally substitute for the SeV fusion protein and the hemagglutinin-neuraminidase (HN). However, in the presence of HN the intrinsic receptor-binding activity of RSV-F is dominated by the SeV attachment protein.

MATERIALS AND METHODS

Cells. Vero and HeLa cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% fetal bovine serum (FBS). Baby hamster kidney cells (BHK-21) were maintained in Earle's minimal essential medium (EMEM) containing nonessential amino acids and 5% FBS.

Primary antibodies. Purified and fluorescein isothiocyanate (FITC)-conjugated goat sera directed to human parainfluenza virus type 1 (anti-PIV1) were purchased from Acris (Hiddenhausen, Germany). Monoclonal antibodies directed to RSV-F protein (anti-RSV-F) were purchased from Serotec (Düsseldorf, Germany; clone RSV3216) and Acris (Mab9C5). Monoclonal antibodies Mab19 and Mab13 were kindly provided by Geraldine Taylor (Institute for Animal Health, Compton, United Kingdom). A polyclonal antiserum against SeV (anti-SeV) was produced in rabbits by immunization with sucrose gradient-purified virus. For generation of a monospecific antiserum directed against the SeV-F cytoplasmic domain (anti-SeFtail), the peptide CRHMYTNGGFDAM AEKR was covalently linked by an *N*-(-α-maleimide)-succinimide ester to keyhole limpet hemocyanin (Sigma) as carrier protein and used for immunization of rabbits with ABM-ZK (Linaris GmbH, Bettingen, Germany) as adjuvant.

Construction of chimeric RSV-F genes. Plasmids pTM1-hF, containing the F gene of human RSV (HRSV; Long strain) (50), and pCDNA3.1-SeVF, containing the F gene of SeV (Fushimi strain), were used as templates for construction of chimeric RSV-F gene cassettes by an overlapping-PCR technique. Oligonucleotides used for the construction of the chimeric genes are listed in Table 1. The RSV-F protein ectodomain (amino acids 1 to 524) was amplified from pTM1-hF using *Pfu* DNA polymerase (MBI Fermentas) and the primer pair hF-S1 and RSV/SeV-F-AS. The part of the SeV-F gene encoding the transmembrane and cytoplasmic domains (amino acids 500 to 565) was amplified from pCDNA3.1-SeV-F using the oligonucleotides RSV/SeV-F-S and SeV-F-AS. The two PCR products were purified (QIAGEN PCR purification kit), mixed at a molar ratio of 1:1, and heated for 2 min at 95°C for denaturation. The mixture was then incubated at 60°C for 30 s to allow the two fragments to anneal to each other. Hybridization was mediated by overlapping complementary sequences at the 5′ ends of the oligonucleotides RSV/SeV-F-S and RSV/SeV-F-AS, respectively. A complete double-stranded DNA hybrid was obtained after incubating the mixture with *Pfu* polymerase at 72°C for 4 min. The chimeric F gene (F_{ch}MC) was subsequently amplified with the primers hF-S and SeV-F-AS and cloned into the EcoRI site of pCDNA3.1 (Invitrogen). The same technique was used to generate the F_{ch}C chimera where the cytoplasmic domain of the RSV-F protein was replaced with the corresponding domain of the SeV-F protein. The RSV-F protein ectodomain and the transmembrane domain (amino acids 1 to 550) were amplified from pTM1-hF using primers hF-S and Tail-AS. The region of the SeV-F gene encoding the cytoplasmic domain of the protein (amino acids 524 to 565) was amplified from pCDNA3.1-SeV-F with the oligonucleotides Tail-S and SeV-F-AS. All other steps for generation and cloning of the hybrid gene were performed as described above.

Generation of recombinant SeV. For generation of SeV(RSV-F), SeV(F_{ch}C), and SeV(F_{ch}MC), authentic or chimeric RSV-F genes were inserted into an additional transcription unit located between the P and M genes of a modified SeV genome using MluI and BssHII restriction sites (5). SeV-ΔF(F_{ch}C), SeV-ΔF(F_{ch}MC), SeV-ΔFΔHN(F_{ch}C/GFP), and SeV-ΔFΔHN(F_{ch}MC/GFP) genomes were constructed using a genomic cDNA plasmid where the original F and HN open reading frames had been replaced with unique restriction sites (SalI instead of F and XhoI instead of HN), each flanked by the original transcription start and stop signals (4). The F_{ch}C and F_{ch}MC chimeras (see previous section) were amplified by PCR using oligonucleotides hF-S2 and SeV-F-AS2 and inserted into the SalI and XhoI sites of the plasmid pTM1. The authenticity of the chimeric genes was verified by DNA sequencing, and the chimeric genes were then inserted as SalI-XhoI fragments into the compatible SalI site (F position) of the modified SeV genome. Subsequently, the XhoI site (HN position) in the modified genome was filled with the open reading frame of either the enhanced green fluorescent protein (GFP) gene or the HN gene. All resulting recombinant SeV genomes were designed to follow the "rule of six" (8). Recombinant viruses were recovered from transfected HeLa cells according to the previously described procedure (24). To facilitate initial virus rescue, expression plasmids encoding the autologous SeV-F and HN proteins were included in the plasmid mixture used for transfection. Recovered virus was propagated on Vero cells. Virus stocks were prepared from the fifth and sixth passages and used for all following experiments.

Replication kinetics and virus titration. Confluent Vero cell monolayers in six-well plates were inoculated with virus at a multiplicity of infection (MOI) of 0.05. Three wells were infected in parallel with each virus. After adsorption (3 h at 37°C), the inoculum was removed and the cells were washed three times before 2.5 ml of medium (without FBS) was added. In some experiments, acetylated trypsin (Sigma) was added to the medium (1.0 μg/ml). At 0, 24, 48, and 72 h postinfection, 500-μl aliquots were taken and replaced with the same volume of fresh medium. The aliquots were flash-frozen in liquid nitrogen and stored at −80°C. Viruses were titrated in duplicate on Vero cells grown in 96-well microtiter plates. The cells were inoculated with 10-fold dilutions of virus (40 μl/well) for 2 h at 37°C and overlaid with medium containing 2% FBS and 0.9% methylcellulose (Sigma). At day 2 postinfection, the medium was removed and the cells washed twice with phosphate-buffered saline (PBS) and fixed with 3% paraformaldehyde for 20 min at room temperature. Excess paraformaldehyde was quenched with 0.1 M glycine in PBS for 5 min. The cells were incubated for 90 min at room temperature with anti-PIV1 (1:500 in PBS), washed three times with PBS, and incubated for 1 h at room temperature with a horseradish peroxidase-linked rabbit antiserum directed to goat immunoglobulin (1:500 in PBS; Dako). Following three washing steps, the cells were incubated for 15 min with AEC peroxidase substrate (1.7 mM 3-amino-9-ethylcarbazole, 0.1% H_2O_2 in 50 mM sodium acetate buffer, pH 5.0). Immunostained cells were counted under an inverse light microscope. Titers of infectious recombinant SeV expressing GFP were determined on Vero cells by end point dilution and counting of GFP-expressing cells under a fluorescence microscope.

Plaque reduction assay. Serial dilutions of polyclonal anti-SeV serum or anti-RSV-F monoclonal antibody mixture (Mab13, Mab19, RSV3216) were prepared in DMEM, and 100 μl of each dilution was incubated with 100 μl of virus (2×10^4 PFU/ml) for 30 min at 4°C and then added to confluent Vero cells in 96-well microtiter plates (50 μl/well, three parallels). Following incubation for 90 min at 37°C, 200 μl of DMEM containing 0.9% methylcellulose was added to each well. The cells were stained 48 h postinfection as described above. The antibody concentration that produced 50% focus reduction was calculated.

Neuraminidase treatment of cells. BHK-21 cells were seeded into 24-well plates (150,000 cells/well). The following day, the cells were incubated for 60 min at 37°C with 250 μl of EMEM containing 40 mU of affinity-purified neuraminidase from *Clostridium perfringens* (N-2133; Sigma) (NA^+ cells). Control cells were left untreated (NA^- cells). The cells were washed twice and infected with

recombinant SeV (MOI of 10) for 60 min at room temperature, washed three times, and incubated for 20 h at 37°C with EMEM containing 2% FBS and 0.9% methylcellulose. The cells were stained with FITC-conjugated anti-PIV1 (1:200) for 60 min at 4°C, suspended in PBS, and analyzed by flow cytometry. Infection by SeV-ΔFΔHN(F_{ch}C/GFP) and SeV-ΔFΔHN(F_{ch}MC/GFP) was directly monitored taking advantage of the GFP reporter.

Western blot analysis of SeV particles. Confluent monolayers of Vero cells grown in 75-cm^2 flasks were inoculated with recombinant SeV (MOI of 0.05) for 3 h at 37°C. Thereafter, the cells were washed and maintained for 72 h at 37°C. For SeV(DsRed), SeV(RSV-F), SeV(F_{ch}C), and SeV(F_{ch}MC), medium lacking FBS and supplemented with 1 μg of acetylated trypsin/ml was used. For all other recombinant SeVs, the cells were incubated with medium containing 2% FBS. The cell culture supernatants were harvested and subjected to low-speed centrifugation (2,000 × *g*, 15 min, 4°C) to remove cell debris. An aliquot of each supernatant was taken and titrated by plaque assay as described above. Viruses in the remaining supernatant (about 20 ml) were pelleted through a 25% sucrose cushion by ultracentrifugation (105,000 × *g*, 60 min, 4°C) and dissolved in 400 μl of SDS sample buffer. The volume was adjusted corresponding to the calculated virus titers. Aliquots (5 μl, corresponding to 5×10^6 PFU) of the samples were run on sodium dodecyl sulfate (SDS)-polyacrylamide gels under nonreducing conditions and transferred to nitrocellulose by the semidry blotting technique. The nitrocellulose membranes were incubated for 60 min at 4°C with either anti-SeV (1:10,000), anti-hPIV1 (1:1,000), anti-SeV-Ftail (1:500), or anti-RSV-F (1:1,000 each). The blots were washed and subsequently incubated with biotinylated secondary antibodies that were specific for the immunoglobulin G (IgG) fraction of the respective species (1:1,000; Sigma). Finally, the blots were incubated for 60 min with a streptavidin-peroxidase complex (1:1,000; Amersham Biosciences) and the antigens were visualized by enhanced chemiluminescence (Roche Diagnostics).

Immunofluorescence analysis. Vero cells grown on 12-mm-diameter coverslips were infected with recombinant SeVs at an MOI of 10 PFU/cell for 60 min at 37°C, washed once, and maintained in medium with 5% FBS. Twenty-four hours postinfection, the cells were fixed with 3% paraformaldehyde in PBS for 20 min at room temperature and subsequently incubated for 5 min at room temperature with PBS containing 0.1 M glycine. The cells were stained by incubation with a 1:2,000 dilution of anti-SeV serum followed by incubation in the dark with a 1:500 dilution of FITC-conjugated goat anti-rabbit IgG serum (Sigma). Both incubations were performed at room temperature for 60 min. For detection of RSV-F antigen, the cells were subsequently incubated with anti-RSV-F monoclonal antibody (1:200; Serotec) and sheep Cy3-conjugated anti-mouse IgG serum (1:500; Sigma). Conventional epifluorescence was performed with a Zeiss Axioplan 2 microscope.

Electron microscopy. Immunoelectron microscopic analysis of virus particles was performed as described previously (20, 21), with small modifications. Briefly, a drop of virus suspension in DMEM containing 3% paraformaldehyde was deposited on a Formvar carbon-coated nickel grid for 1 min. Excess fluid was absorbed with filter paper, and the grids were floated for 10 min on a drop of PBS containing 2% bovine serum albumin and 0.2% Tween 20. Indirect immunostaining was performed by incubation of the samples for 60 min either with a mouse monoclonal anti-RSV-F antibody (Mab9C5; 1:50) or with a goat polyclonal anti-PIV1 antibody (1:200) in PBS containing 2% bovine serum albumin and 0.2% Tween 20. The bound antibodies were detected with a donkey anti-mouse IgG antibody conjugated with 12-nm-diameter gold particles (Dianova) and with a rabbit anti-goat IgG antibody conjugated with 10-nm-diameter gold particles (BBInternational), respectively. The samples were washed with PBS, negatively stained with 2% phosphotungstic acid, and examined with a Zeiss 109 electron microscope. Micrographs were scanned using the Leafscan 45 software, and the surface area of the virus particles was determined using the Raytest TINA software. The number of gold particles bound to the virions was calculated from 10 to 20 micrographs at a primary magnification of ×50,000. The labeling density (mean ± standard deviation) was expressed as the number of gold particles per 10,000 nm^2 of virus particle area. Differences in labeling between different virus specimens were statistically evaluated by Student's *t* test.

RESULTS

Generation of recombinant SeV encoding native or chimeric RSV-F. The modular organization of negative-strand RNA virus genomes allows both deletion and insertion of genes. To study the functions of the HRSV fusion protein (RSV-F) in the context of SeV infection, we generated a series of modified SeV genomes based on the Fushimi strain (Fig. 1). First, an additional transcription unit was assembled into the intergenic region between the P and M genes. Into this transcription unit, four different transgenes were inserted taking into account the "rule of six" (8). We obtained the following virus genomes: (i) SeV(RSV-F) with an unmodified version of the RSV-F open reading frame, (ii) SeV(F_{ch}C) with a chimeric gene encoding the ectodomain and transmembrane domain of RSV-F and the cytoplasmic domain (C) of the SeV fusion protein (SeV-F), (iii) SeV(F_{ch}MC) with a chimeric F gene encoding the RSV-F ectodomain and the SeV-F transmembrane (M) and cytoplasmic domains, and (iv) SeV(DsRed) containing the cDNA of the red fluorescent protein DsRed originating from the marine organism *Discosoma* sp. As DsRed is unrelated to RSV-F and shows no fusion activity, SeV(DsRed) was regarded as a control construct.

The insertion of native and chimeric RSV-F genes into the SeV genome was expected to result in virus particles with two fusion proteins provided the heterologous glycoproteins would be incorporated into the viral envelope (see below). In order to determine whether RSV-F can functionally substitute for SeV-F, we constructed additional recombinant SeV genomes by replacing the SeV-F gene with either of the two chimeric F proteins, resulting in SeV-ΔF(F_{ch}C) and SeV-ΔF(F_{ch}MC) (Fig. 1B). It should be noted that these genomes do not contain an additional transcription unit and are therefore shorter than the modified SeV genomes mentioned above. However, they correspond approximately to the length of an unmodified SeV genome. Since RSV-F has been reported to have receptor-binding activity (12), we wanted to know whether the chimeric F proteins would also substitute for the HN glycoprotein of SeV. To address this question, the HN gene in SeV-ΔF(F_{ch}C) and SeV-ΔF(F_{ch}MC) was replaced with the enhanced GFP, resulting in the genomes SeV-ΔFΔHN(F_{ch}C/GFP) and SeV-ΔFΔHN(F_{ch}MC/GFP), respectively. Using a previously published procedure (24), all recombinant SeVs were successfully recovered from the cell culture supernatant of transfected HeLa cells. To monitor RSV-F expression in infected BHK-21 cells, an immunofluorescence analysis using anti-SeV and anti-RSV-F antibodies was performed (Fig. 2). RSV-F was detected at the cell surface of SeV(RSV-F), SeV(F_{ch}C), SeV(F_{ch}MC), and SeV-ΔF(F_{ch}MC) but not in cells that had been infected with SeV(DsRed). Similar results were obtained with SeV-ΔF(F_{ch}C), SeV-ΔFΔHN(F_{ch}C/GFP), and SeV-ΔFΔHN (F_{ch}MC/GFP) (data not shown).

Deletion of SeV-F allows chimeric RSV-F protein to be efficiently incorporated into SeV virions. An important aspect of recombinant enveloped viruses that encode heterologous viral glycoproteins is the possibility that these glycoproteins might be incorporated into the virus envelope. To address this question, we purified the recombinant SeV particles from the supernatant of infected Vero cells by ultracentrifugation through a 25% sucrose cushion and analyzed them by immunogold electron microscopy using two different antibodies. The polyclonal anti-PIV1 serum was characterized by Western blot analysis and was found to predominantly react with SeV-F and SeV-HN (for comparison, see Fig. 5c). Figure 3 shows that this antibody strongly reacted with SeV(DsRed), SeV(RSV-F), SeV(F_{ch}MC), and SeV-ΔF(F_{ch}MC) particles, all containing at least one SeV glycoprotein. In contrast, SeV-ΔFΔHN(F_{ch}MC/

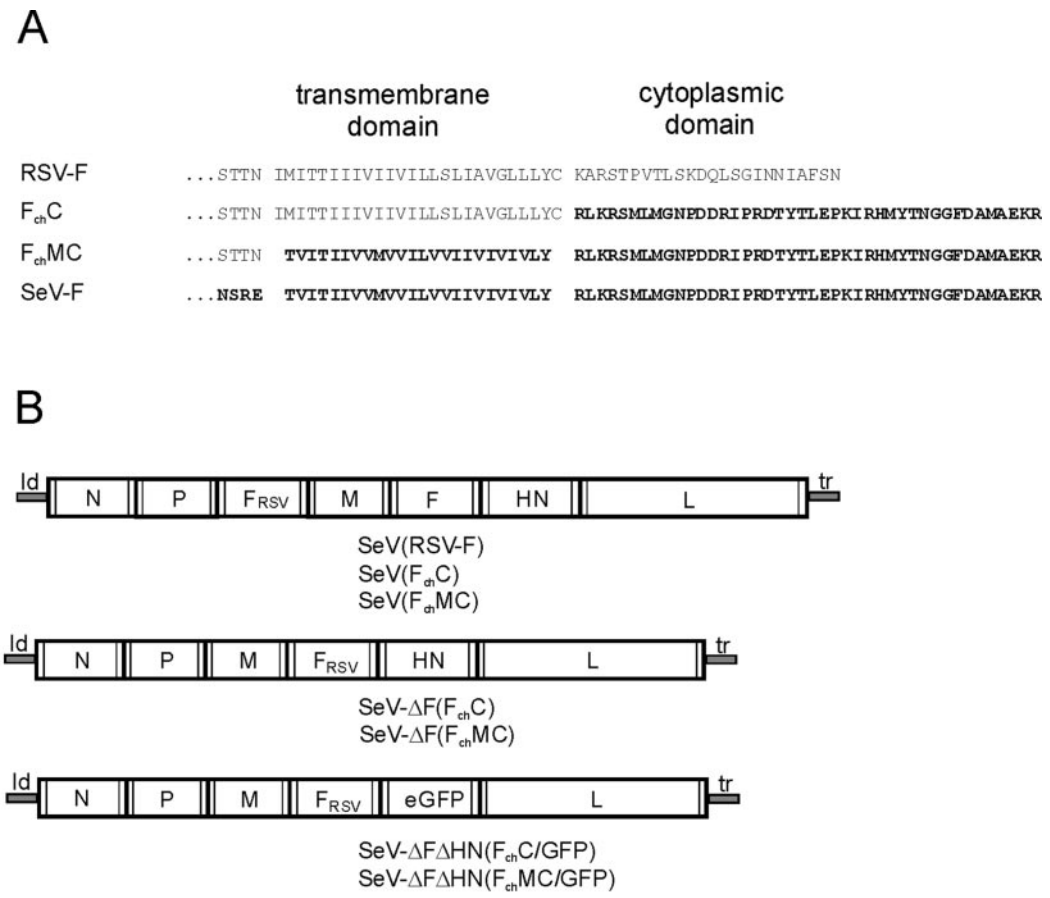

FIG. 1. Generation of recombinant SeV using authentic and chimeric RSV-F genes. (A) The predicted transmembrane and cytoplasmic domains of the fusion proteins from HRSV (Long strain) and SeV (Fushimi strain) and the chimeric fusion proteins $F_{ch}C$ and $F_{ch}MC$ are shown. Sequences derived from SeV-F are in bold type. (B) Schematic representation of the recombinant SeV genomes. The designations of the recombinant viruses that are based on the modified genomes are shown below the schemes.

GFP) lacking both SeV-F and SeV-HN was rarely recognized by anti-PIV1. The gold labeling of some virions is supposed to be due to a weak reaction of the antibody with the SeV nucleoprotein. HRSV was not stained with anti-PIV1 at all. Monoclonal antibody Mab9C5 recognizes an epitope of the RSV-F ectodomain (data not shown). It showed a strong reaction with HRSV virions, while staining of SeV(DsRed) particles was not observed. SeV(RSV-F) was rarely labeled by Mab9C5, and only single gold particles were found bound to some virions. Increased labeling was observed with SeV ($F_{ch}MC$), which expresses a chimeric RSV-F protein, indicating that the SeV-F derived C-terminal sequences of $F_{ch}MC$

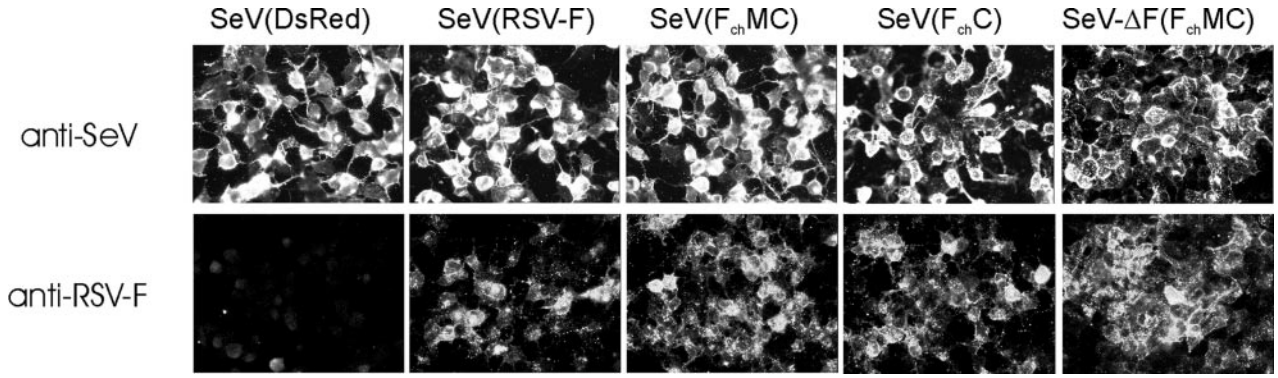

FIG. 2. Indirect immunofluorescence analysis of infected BHK-21 cells. Cells were infected with the indicated recombinant SeV (MOI = 10) and fixed with paraformaldehyde 20 h postinfection. The cells were stained either with a rabbit anti-SeV serum or with a mouse anti-RSV-F antibody as indicated on the left. FITC-conjugated antibodies directed to rabbit IgG or mouse IgG were used as secondary antibodies.

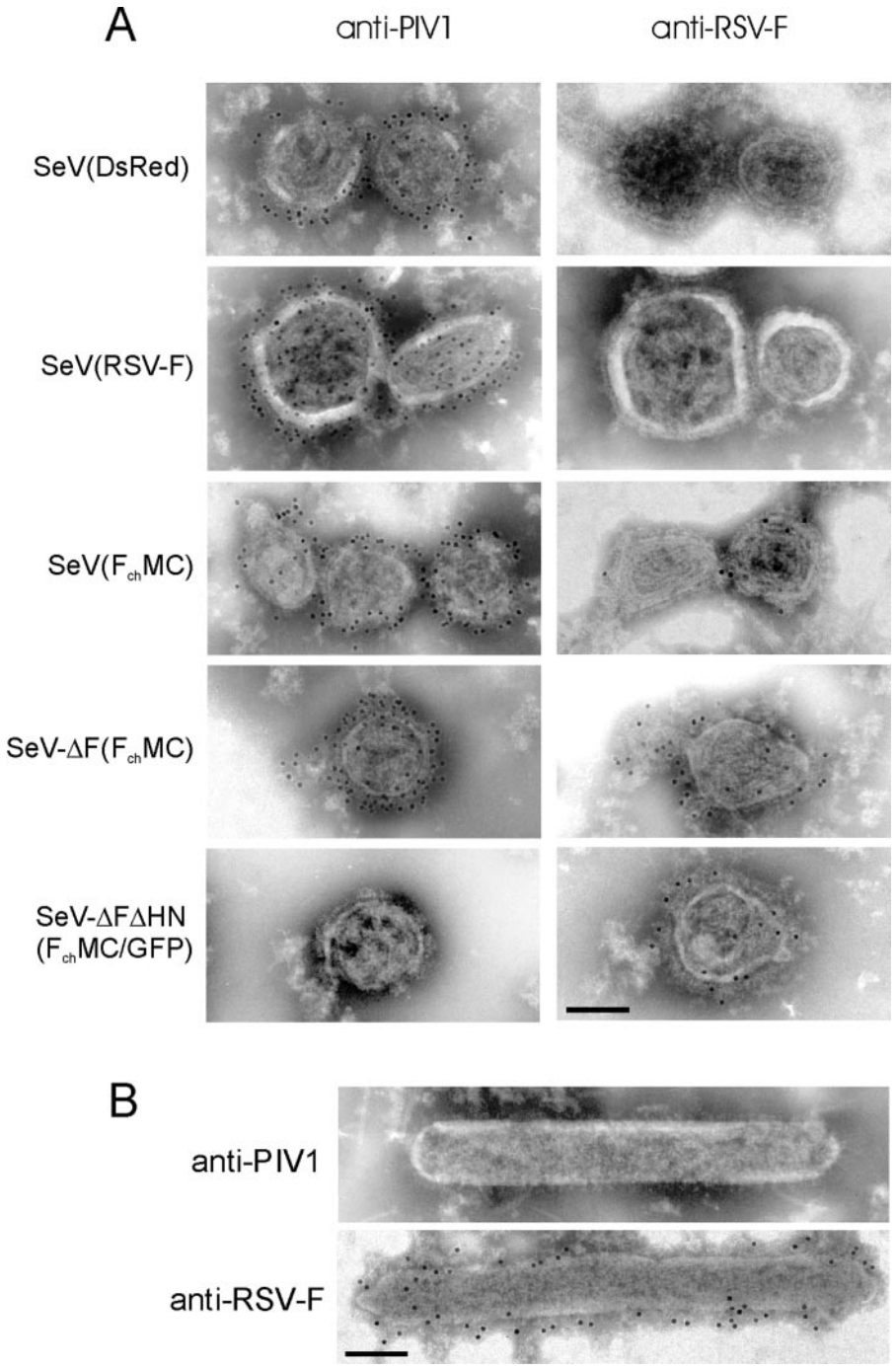

FIG. 3. Immunogold labeling of viruses. The indicated viruses were concentrated from the culture supernatant of infected Vero cells and fixed with 3% paraformaldehyde. The viruses were labeled with the indicated primary antibodies and gold-labeled secondary antibodies as described in Materials and Methods. (A) Labeling of recombinant SeV. (B) Staining of HRSV (Long strain). Bars, 100 nm.

contributed to glycoprotein incorporation. In the absence of SeV-F, the chimeric glycoprotein was most efficiently incorporated, as indicated by the very strong labeling of SeV-ΔF (F_{ch}MC) and SeV-ΔFΔHN(F_{ch}MC/GFP). Figure 4 shows a quantification of the immunogold labeling analysis. A significant difference between SeV-ΔF(F_{ch}MC) and SeV-ΔFΔHN (F_{ch}MC/GFP) was not observed, indicating that the deletion of SeV-HN did not further improve the incorporation of the chimeric fusion proteins.

Western blot analysis was performed to confirm the results obtained by the immunogold labeling approach. After the concentrated virions had been dissolved in SDS sample buffer, the viral proteins were separated by SDS-polyacrylamide gel electrophoresis under nonreducing conditions, transferred to ni-

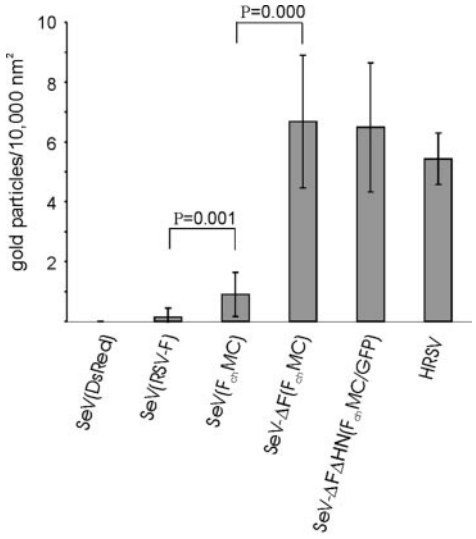

FIG. 4. Quantification of RSV-F antigen in HRSV and recombinant SeV particles. Following staining with a monoclonal anti-RSV-F antibody, the number of gold particles bound to the virions was calculated from 10 to 20 micrographs. The labeling density (mean ± standard deviation) was expressed as the number of gold particles per 10,000 nm^2 of virus particle area. Mean values and standard deviations are shown. Significant differences in the labeling were statistically evaluated by Student's *t* test.

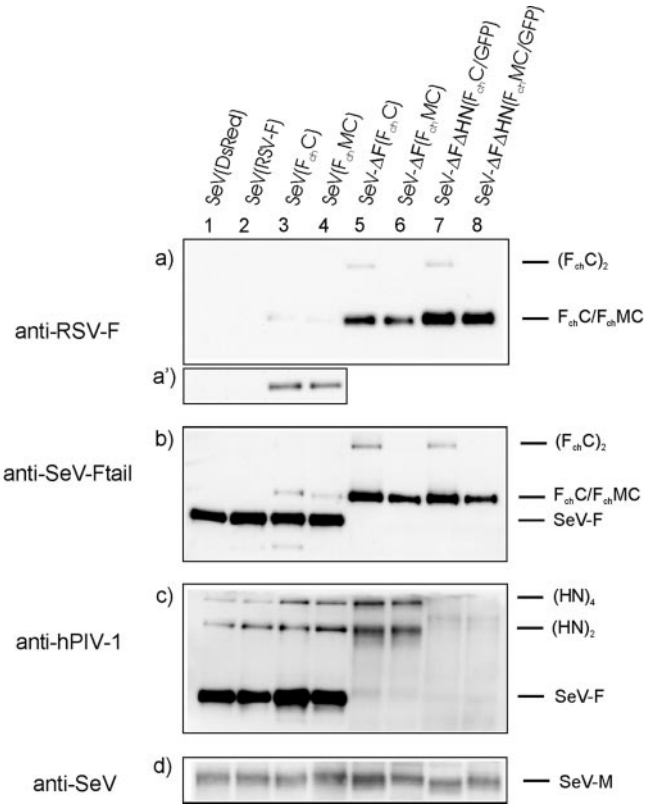

FIG. 5. Incorporation of chimeric RSV-F into SeV particles. Vero cells were infected with the indicated viruses (0.05 PFU/cell) and incubated at 37°C with serum-deficient medium. Virions released into the cell culture supernatant were pelleted through a sucrose cushion by ultracentrifugation, separated by SDS-polyacrylamide gel electrophoresis, and analyzed by Western blot assay using the indicated antibodies. The amount of sample applied to each gel was normalized to infectious virus titers. (Panel a′) To allow detection of minor bands, the amount of the samples applied to the gel was scaled up (threefold) and the chemiluminescence signal was collected over a longer time interval. The virus antigens detected are indicated on the right.

trocellulose membranes, and probed with several antibodies (Fig. 5). When a mixture of monoclonal antibodies directed to the RSV-F ectodomain was used (panels a and a′), a small amount of antigen was detected in SeV(F_{ch}C) and SeV (F_{ch}MC) but not in SeV(DsRed) and SeV(RSV-F) particles. The chimeric glycoproteins F_{ch}C and F_{ch}MC were incorporated into the recombinant particles with similar efficiency, suggesting that the SeV-F transmembrane domain in SeV (F_{ch}MC) exerts no promoting effect on glycoprotein uptake (panel a′, compare lane 3 with lane 4; panel a, compare lane 5 with lane 6 and lane 7 with lane 8). In agreement with the immunogold labeling presented above, incorporation of either chimeric RSV-F glycoprotein was dramatically improved when the recombinant viruses lacked SeV-F (panel a, lanes 5 to 8). This suggests that in SeV(F_{ch}C) and SeV(F_{ch}MC), SeV-F competes with the chimeric RSV-F proteins for incorporation into the virus envelope. The lower signal obtained with chimeric RSV-F protein in SeV-ΔF(F_{ch}C) and SeV-ΔF(F_{ch}MC) (panel a, lanes 5 and 6) compared to the stronger signals detected with SeV-ΔFΔHN(F_{ch}C/GFP) and SeV-ΔFΔHN(F_{ch}MC/GFP) (lanes 7 and 8) is probably due to the differential capacity of the antigens to renature on the nitrocellulose membrane following SDS-polyacrylamide gel electrophoresis. In contrast to chimeric RSV-F of SeV-ΔFΔHN(F_{ch}C/GFP) and SeV-ΔFΔHN(F_{ch}MC/GFP), chimeric RSV-F in SeV-ΔF(F_{ch}C) and SeV-ΔF(F_{ch}MC) is desialylated by the action of SeV-HN. The lack of sialic acids probably makes renaturation of the glycoproteins more difficult and hampers recognition by the monoclonal antibodies that act in a conformation-dependent manner. In SeV(F_{ch}C) (lane 5) and SeV-ΔFΔHN(F_{ch}C/GFP) (lane 7), an additional band of approximately 140 kDa was detected that probably represents a dimeric form of chimeric RSV-F. The band was not observed with SeV(F_{ch}MC) (lane 6) and SeV-ΔFΔHN(F_{ch}MC/GFP) (lane 8).

As the use of different antibodies for detection of SeV-F on the one hand and the chimeric F proteins on the other hand did not allow us to directly compare the uptake of these glycoproteins, we employed a monospecific antibody that was directed to a linear epitope of the SeV-F cytoplasmic domain (Fig. 5b). In SeV(DsRed), SeV(RSV-F), SeV(F_{ch}C), and SeV(F_{ch}MC) particles (lanes 1 to 4), this antibody recognized a band of 65 kDa representing SeV-F, which was absent in SeV-ΔF(F_{ch}C), SeV-ΔF(F_{ch}MC), SeV-ΔFΔHN(F_{ch}C/GFP), and SeV-ΔFΔHN(F_{ch}MC/GFP) (lanes 5 to 8). In addition to SeV-F, a band of 75 kDa representing chimeric F protein was detected in SeV(F_{ch}C) and SeV(F_{ch}MC) particles (lanes 3 and 4). Compared to SeV-F, these glycoproteins were less efficiently incorporated into the SeV envelope, suggesting that also determinants in the SeV-F ectodomain must exist that are involved in glycoprotein uptake. Like the antibody directed to the RSV-F ectodomain (panel a), the anti-SeV-Ftail antibody strongly reacted with the chimeric F proteins incorporated into SeV-ΔF(F_{ch}C), SeV-ΔF(F_{ch}MC), SeV-ΔFΔHN(F_{ch}C/GFP),

and SeV-ΔFΔHN(F_{ch}MC/GFP) (panel b, lanes 5 to 8), while only a faint band was detectable in SeV(F_{ch}C) and SeV(F_{ch}MC) (lanes 3 and 4). This indicates that incorporation of the chimeric glycoproteins is significantly enhanced in the absence of SeV-F. As already observed with anti-RSV-F (panel a), a dimeric form of the chimeric F_{ch}C glycoprotein was also detected with anti-SeV-Ftail (panel b, lanes 5 and 7).

A polyclonal goat antibody directed to human parainfluenza virus type I (hPIV1) was found to primarily react in Western blot assays with the two SeV glycoproteins, SeV-F in its monomeric form with an apparent molecular mass of about 65 kDa and SeV-HN in its dimeric and tetrameric forms with relative molecular masses of about 140 and 280 kDa, respectively. HN was found to be absent in SeV-ΔFΔHN(F_{ch}C/GFP) and SeV-ΔFΔHN(F_{ch}MC/GFP) particles (Fig. 5c, lanes 7 and 8). These two viruses, as well as SeV-ΔF(F_{ch}C) and SeV-ΔF(F_{ch}MC), were also proven in this way to contain no SeV-F antigen (panel c, lanes 5 to 8). A very faint band comigrating with SeV-F represents the SeV nucleoprotein, which is also recognized to some extent by the anti-hPIV1 antibody (panel c, lanes 5 to 8). The samples loaded on the gels were normalized to equal infectious titers (approximately 5×10^6 PFU/lane). As a control for the amount of virus particles applied, the matrix protein of SeV was detected using a polyclonal rabbit antibody directed to purified SeV particles (panel d).

Chimeric RSV-F protein is functional and mediates trypsin-independent replication of SeV. Each of the recombinant viruses SeV(RSV-F), SeV(F_{ch}MC), and SeV(F_{ch}C) contains two genes that encode different fusion proteins. SeV-F is proteolytically activated by secreted trypsin-like enzymes. As many established cell lines do not produce such enzymes, SeV normally requires the addition of a protease to the medium to be infectious. In contrast, RSV-F is cleaved and activated intracellularly by the ubiquitous Golgi protease furin and hence does not require the addition of trypsin to be fusion active. As the chimeric F proteins contain the RSV-F ectodomain, they are also expected to act independently of trypsin. Thus, on the basis of trypsin requirement it should be possible to discriminate between virus infections mediated by one or the other fusion protein. To analyze the function of the heterologous fusion protein in the context of SeV infection, the kinetics of virus replication in the presence or absence of trypsin were studied. Vero cells were infected with the recombinant SeV using an MOI of 0.05 PFU/cell, and aliquots of cell culture supernatant were collected over a 3-day period at 24-h intervals. The virus titers were quantified by a plaque assay facilitated by immunological staining of SeV antigens. Figure 6A shows that SeV-DsRed and SeV-RSV-F replicated well if the medium was supplemented with acetylated trypsin (1 μg/ml) whereas no significant replication was detected in the absence of trypsin, indicating that expression of native RSV-F does not lead to trypsin-independent replication of recombinant SeV. This finding agrees well with our observation that unmodified RSV-F is not incorporated into SeV particles (see above). Though chimeric fusion proteins were incorporated into SeV particles in addition to SeV-F (see above), analysis of SeV(F_{ch}MC) and SeV(F_{ch}C) revealed only very slow trypsin-independent replication (Fig. 6B), suggesting that the fraction of chimeric F protein in the viral envelope is probably too small to support replication. In the presence of trypsin, SeV-F was activated and both viruses showed replication kinetics similar to SeV(DsRed) (compare Fig. 6B with A). In contrast to these viruses, addition of trypsin to the cell culture medium did not affect SeV-ΔF(F_{ch}MC) replication as this virus lacked the SeV-F gene. Nevertheless, this virus replicated as efficiently as trypsin-activated SeV(F_{ch}MC) and SeV(F_{ch}C). When replication of SeV-ΔF(F_{ch}MC) was compared with SeV-ΔF(F_{ch}C), it was found that SeV-ΔF(F_{ch}MC) replicated more efficiently, producing titers that were approximately 1 order of magnitude

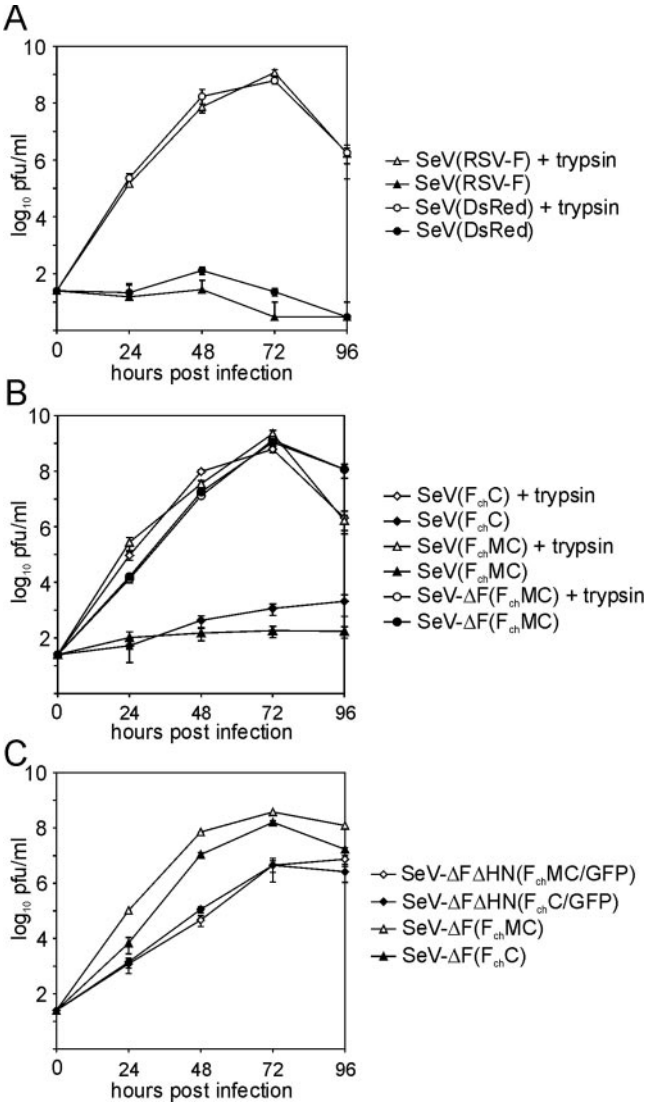

FIG. 6. Multistep replication of recombinant SeV in Vero cells. Triplicate cell monolayers in six-well dishes were infected with the indicated viruses at an MOI of 0.05 and incubated at 37°C with medium in either the absence or the presence of trypsin as indicated on the right-hand side of the figure. Aliquots were taken at the indicated times and titrated in duplicate by plaque assay on Vero cells. Each point shown represents the mean titer from three wells of infected cells. Standard deviations are indicated. For reasons of presentation, the figure has been divided into three parts. (A) Comparison of SeV(DsRed) with SeV(RSV-F); (B) comparison of SeV(F_{ch}C) and SeV(F_{ch}MC) with SeV-ΔF(F_{ch}MC); (C) comparison of SeV-ΔF(F_{ch}MC) with SeV-ΔF(F_{ch}C) and SeV-ΔFΔHN(F_{ch}MC/GFP) and SeV-ΔFΔHN(F_{ch}C/GFP).

TABLE 2. Neutralization of recombinant SeV

Virus	Neutralizing antibody titer[a]	
	Anti-SeV	Anti-RSVF
SeV(DsRed)	2,400	<200
SeV(RSV-F)	2,400	<200
SeV(F_{ch}C)	2,400	<200
SeV(F_{ch}MC)	2,400	<200
SeV-ΔF(F_{ch}C)	2,400	25,600
SeV-ΔF(F_{ch}MC)	2,400	12,800
SeV-ΔF-ΔHN (F_{ch}C/GFP)	<200	51,200
SeV-ΔF-ΔHN (F_{ch}M/GFP)	<200	51,200
HRSV	<200	51,200

[a] A plaque reduction assay was performed to determine the neutralizing titer of polyclonal anti-SeV serum or anti-RSV-F monoclonal antibody mixture. Following preincubation of virus with serial antibody dilutions, the mixture was inoculated with Vero cells and then overlaid with medium containing 0.9% methylcellulose. The cells were stained 48 h postinfection as described in Materials and Methods. The neutralization titers were determined as the reciprocal of the antibody dilution at which 50% focus reduction was observed.

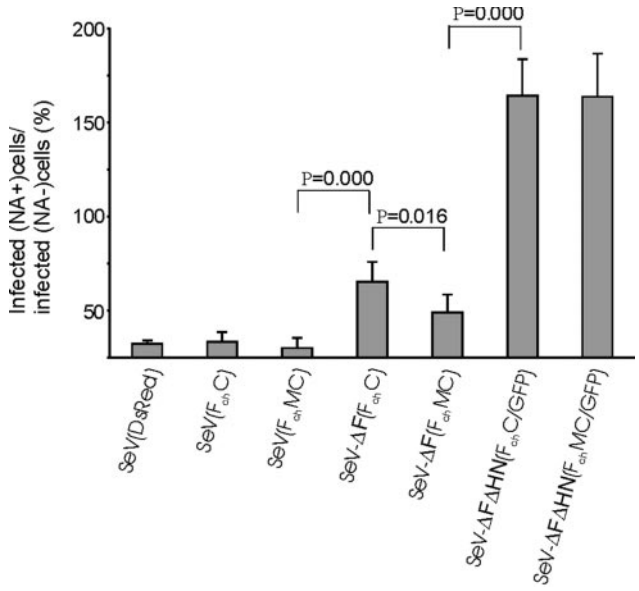

FIG. 7. Importance of sialic acids for infection with recombinant SeV. BHK-21 cells were treated with *C. perfringens* neuraminidase (NA^+ cells) or left untreated (NA^- cells) prior to inoculation with the indicated viruses. Eighteen hours postinfection, the cells were stained with FITC-conjugated anti-PIV1, suspended, and analyzed by flow cytometry (5,000 cells of each sample). Infection with SeV-ΔFΔHN(F_{ch}MC/GFP) and SeV-ΔFΔF HN(F_{ch}C/GFP) was monitored by taking advantage of the GFP reporter. The infection rate of (NA^-) cells was 10% to 20%. The ratio of infected (NA^+) cells to infected (NA^-) cells was calculated. Mean values and standard deviations of three to four experiments are shown.

higher than the titers produced by SeV-ΔF(F_{ch}C) (Fig. 6C). In general, the infectious virus titers reached a maximum at 72 h postinfection. From that time on, the titers decreased, probably due to the increasing cytopathic effect.

SeV with chimeric RSV-F protein as the only envelope glycoprotein is infectious. The results presented above demonstrated that a modified RSV-F protein is capable to functionally replace the fusion protein of SeV. Because recent reports have shown that RSV mutants or recombinant RSV lacking attachment protein G are infectious and are able to replicate in cell culture (18, 42), we asked whether chimeric RSV-F will also substitute for SeV attachment protein HN. SeV-ΔFΔHN(F_{ch}C/GFP) and SeV-ΔFΔHN(F_{ch}MC/GFP) were used to test this hypothesis. Trypsin-independent replication of these viruses in the presence of FBS showed that the amount of chimeric fusion protein incorporated into the virions is sufficient to support both functions, receptor binding and fusion (Fig. 6C). In this viral background, we did not detect any significant differences between the replication of SeV-ΔFΔHN(F_{ch}C/GFP) and SeV-ΔFΔHN(F_{ch}MC/GFP). However, replication of these viruses resulted in significantly lower virus titers compared to SeV-ΔF(F_{ch}MC), indicating that the HN glycoprotein in the latter viruses supports virus replication.

Neutralizing antibodies differentially affect recombinant SeV. Use of neutralizing antibodies directed to RSV-F provides an additional approach to estimate the contribution of the heterologous fusion protein to SeV replication. Vice versa, recombinant SeV infection dependence on or independence from SeV glycoproteins can be determined using neutralizing antibodies that bind to the SeV envelope glycoproteins. We used a plaque reduction assay to study the effect of serial antibody dilutions on the infection of Vero cells by the recombinant SeVs. The results are summarized in Table 2. Infection with SeV(DsRed) was inhibited by a polyclonal anti-SeV serum, while an antibody mixture containing three different monoclonal antibodies directed against RSV-F had no effect on this virus. The neutralizing potential of anti-RSV-F was demonstrated with HRSV, which was completely inhibited even at very high dilutions of this antibody mixture. As expected, anti-SeV serum showed no inhibitory activity toward HRSV. Recombinant viruses SeV(RSV-F), SeV(F_{ch}MC), and SeV(F_{ch}C) showed behavior similar to that of SeV(DsRed); i.e., anti-RSV-F had no apparent inhibitory effect. With recombinant SeV lacking both SeV-F and SeV-HN, a phenotype similar to that of HRSV was observed: SeV-ΔFΔHN(F_{ch}C/GFP) and SeV-ΔFΔHN(F_{ch}MC/GFP) were neutralized by anti-RSV-F but not by anti-SeV. An interesting phenotype was found with SeV-ΔF(F_{ch}C) and SeV-ΔF(F_{ch}MC) since these recombinants were sensitive to both anti-RSV-F and anti-SeV. A possible explanation for this phenotype is that the polyclonal antibodies bound to HN in the envelope and thereby also blocked RSV-F function by steric hindrance.

SeV-HN dominates over the receptor-binding properties of chimeric RSV-F. Our data provided evidence that RSV-F acts in an autonomous way, mediating attachment and fusion of a heterologous virus in the absence of any other viral glycoprotein. On the other hand, our results also indicate that if HN is present it contributes to replication as the recombinants SeV-ΔF(F_{ch}C) and SeV-ΔF(F_{ch}MC) proliferated much more efficiently than SeV-ΔFΔHN(F_{ch}C/GFP) and SeV-ΔFΔHN (F_{ch}MC/GFP) (Fig. 6C). To further assess the role of HN in these hybrid viruses, we studied whether removal of the receptor determinant for HN from the cell surface affects infection and therefore treated BHK-21 cells with bacterial neuraminidase prior to infection with recombinant SeV. Twenty hours later, the cells were analyzed for SeV antigen or GFP expression by flow cytometry (Fig. 7). It turned out that neuraminidase treatment reduced infection with SeV(DsRed), SeV(F-

$_{ch}$C), and SeV(F_{ch}MC) by more than 90%. On the other hand, the recombinants SeV-ΔFΔHN(F_{ch}C/GFP) and SeV-ΔFΔHN(F_{ch}MC/GFP), which lacked HN, did not require the presence of sialic acids for infection. Rather, an enhanced infection rate of about 140% was detected following neuraminidase treatment, indicating that sialic acids might have a masking effect on the recognition of cellular receptors by RSV-F. In striking contrast to the latter viruses, removal of sialic acids from cell surface glycoconjugates reduced infection with SeV-ΔF(F_{ch}C) and SeV-ΔF(F_{ch}MC) by 60% and 76%, respectively. Taking into account that about 10% of the remaining receptor-binding activity was still due to HN binding to sialic acids that were not removed, a significant portion of the sialic acid-independent receptor-binding activity was probably mediated by the chimeric RSV-F protein. However, it should be considered that the binding of RSV-F to cellular receptors would probably have been less pronounced if the cells had not been treated with neuraminidase. Together, these findings indicate that the HN protein of SeV-ΔF(F_{ch}C) and SeV-ΔF(F_{ch}MC) contributed significantly to virus attachment.

DISCUSSION

Virus morphogenesis. Cytoplasmic domains of viral glycoproteins have been shown to play an important role in assembly and infectivity of many enveloped negative-strand RNA viruses (15, 32, 35, 36, 38, 47). It is generally believed that during the budding process these domains interact with cytoplasmic viral components, in particular with the matrix proteins, which provide a link between the viral envelope and the nucleocapsid. Interaction between the glycoprotein tail and the matrix protein has been shown for some viruses to be very specific with the consequence that heterologous glycoproteins are not incorporated into the virus particle (36). In some cases, replacement of the cytoplasmic domain with sequences from a homologous virus glycoprotein was found to result in significantly improved uptake of the foreign glycoprotein (25, 36, 48). On the other hand, there are also viruses that incorporate heterologous membrane proteins in a rather unspecific manner. For example, unmodified RSV-F was found to be incorporated into vesicular stomatitis virus with an efficiency similar to that of a chimeric RSV-F glycoprotein that contained the cytoplasmic domain from the vesicular stomatitis virus G glycoprotein (16). In the present work, we found that the RSV-F protein was not incorporated into SeV virions unless its cytoplasmic domain was replaced with the corresponding domain from SeV-F. In agreement with previous reports (13, 37), this result provides further evidence for a crucial role of the SeV-F cytoplasmic domain in SeV particle formation. We found that the presence of the homotypic transmembrane domain did not further improve the uptake of chimeric RSV-F. However, there must be determinants in the SeV-F ectodomain that are involved in glycoprotein uptake. Analysis of SeV(F_{ch}C) and SeV(F_{ch}MC) showed that chimeric RSV-F was incorporated into SeV virions with low efficiency in the presence of SeV-F, whereas its uptake was dramatically enhanced if SeV-F was deleted. Since SeV-F and F_{ch}MC share the same cytoplasmic and transmembrane domains, we conclude that the ectodomains of the glycoproteins account for these differences. From the vesicular stomatitis virus glycoprotein, it is known that a membrane-proximal region in the ectodomain contributes to assembly of this rhabdovirus (30). We cannot exclude the possibility that there is a similar domain present in SeV-F. Besides, it has to be considered that the homotypic parainfluenza virus glycoproteins F and HN are physically associated with each other (6, 14, 33, 39, 46). Therefore, it is possible that SeV-HN contributes to SeV-F uptake by interacting with its ectodomain whereas it does not bind to the unrelated RSV-F ectodomain. However, in the absence of SeV-F these possible recruitment mechanisms cannot compete with uptake of chimeric RSV-F.

Role of chimeric RSV-F in SeV infection. Chimeric RSV-F was shown in this study to mediate trypsin-independent replication of recombinant SeV. This finding indicates that modifications at the RSV-F C terminus are fully compatible with the functions of this glycoprotein. This is remarkable as many other fusion proteins have been found not to tolerate any modifications of their cytoplasmic domains like elongations, truncations, or heterologous sequences (2, 29, 34, 45). Chimeric RSV-F allowed the generation of infectious SeV lacking both SeV-F and SeV-HN, confirming the notion that RSV-F exhibits fusion and receptor-binding activities (18, 42). It is evident that the amount of incorporated chimeric fusion protein was sufficient to mediate attachment of these viruses to cell surface receptors. However, recombinant SeV combining chimeric RSV-F with HN produced significantly higher titers than SeV containing only chimeric RSV-F though both virus types were found to contain similar amounts of chimeric RSV-F. This observation suggests that HN acted as an additional attachment protein and contributed to virus entry and replication. In accordance with this view, infection by SeV-ΔF(F_{ch}C) and SeV-ΔF(F_{ch}MC) for the most part required the presence of sialic acids on the host cell. Based on these findings, we speculate that in the context of SeV-ΔF(F_{ch}C) and SeV-ΔF(F_{ch}MC) the function of chimeric RSV-F differs from its autonomous function in SeV-ΔFΔHN(F_{ch}C/GFP) and SeV-ΔFΔHN(F_{ch}MC/GFP). The reduced receptor-binding activity of chimeric RSV-F in SeV-ΔF(F_{ch}C) and SeV-ΔF(F_{ch}MC) is supposed to be due to the presence of HN, which might lead to a different spatial distribution of the chimeric fusion protein in the viral envelope compared to the situation in SeV-ΔFΔHN. In the absence of HN, the chimeric RSV-F molecules probably can more easily cooperate with each other, favoring a multivalent interaction with cellular receptors. In addition, we cannot exclude the possibility that the SeV-F transmembrane and/or cytoplasmic domain in chimeric RSV-F mediates an interaction with HN, which in turn might mask the RSV-F receptor-binding domain. Interestingly, compared to SeV-ΔF(F_{ch}C), infection by SeV-ΔF(F_{ch}MC) appeared to be more dependent on HN (Fig. 7), favoring the idea that the transmembrane anchor contributes to this binding. Such an intimate interaction between the two glycoproteins would also explain why anti-SeV polyclonal antiserum showed neutralizing activity toward SeV-ΔF(F_{ch}C) and SeV-ΔF(F_{ch}MC). When the antibodies bound to HN, they might have blocked the low receptor-binding activity of RSV-F due to steric hindrance. Altogether, it appears that HN performs an accessory function in SeV-ΔF(F_{ch}C) and SeV-ΔF(F_{ch}MC) similar to that of the RSV-G protein, which supports RSV attachment but nevertheless is dispensable for virus replication in cell culture (31).

Assistance by HN appeared to be more pronounced with SeV-ΔF(F_{ch}MC) compared to SeV-ΔF(F_{ch}C), which replicated to significantly lower titers. This difference was only apparent in the presence of HN, as replication of SeV-ΔFΔHN(F_{ch}MC) and SeV-ΔFΔHN(F_{ch}C) did not significantly differ from each other. The higher fitness of SeV-ΔF(F_{ch}MC) compared to SeV-ΔF(F_{ch}C) is supposed to be related to the transmembrane region in F_{ch}MC. The presence of the homotypic transmembrane domain may provide enhanced virion stability by, for example, supporting interaction of chimeric RSV-F with the HN glycoprotein. However, this phenomenon requires further analysis to confirm this assumption.

In contrast to many other paramyxoviruses, RSV does not use sialic acid residues for attachment to the host cell surface and does not express a neuraminidase. Rather, RSV was found to bind to glycosaminoglycans (22) by virtue of both the attachment protein RSV-G and the fusion protein RSV-F (43). In this study, we found that neuraminidase treatment of target cells rendered them more susceptible to infection by SeV-ΔFΔHN(F_{ch}MC) and SeV-ΔFΔHN(F_{ch}C) while infection by all other SeV recombinants was negatively affected. A similar effect of neuraminidase treatment on RSV fusion and infection was reported by Barretto et al., though they found that infectivity was increased by treating virions with neuraminidase, but not by treating target cells (3). Though the reason for this discrepancy is not known at the moment, recombinant SeV containing chimeric RSV-F as the only glycoprotein represents a useful tool to further analyze the role of RSV-F in virus entry.

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