Fusogenic Variants of a Noncytopathic Paramyxovirus

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SER virus is a type 5 parainfluenza virus that does not exhibit syncytium formation, in contrast to most other paramyxoviruses. This property has been attributed, at least in part, to the presence of an extension of the cytoplasmic tail (CT) of the SER F protein, as truncations or mutations of this region resulted in enhanced fusion. In this study we used repeated passage to select for mutant SER viruses, which were found to be fusogenic. The mutant viruses replicated at levels comparable to or higher than the wild-type SER virus and caused plaque formation, in contrast to the wild-type virus which does not form plaques. The mutants differed strikingly in their plaque sizes. The F genes of mutant viruses were cloned and sequenced and shared some mutations, including a proline-to-leucine change at position 22 and an isoleucine-to-leucine substitution at position 191; other changes that were specific to each mutant were also found. The HN proteins of mutant viruses also showed mutations spanning the length of the protein whereas the M protein showed a consistent mutation, threonine to isoleucine, at position 129. The structure of the F protein was used to identify residues involved in the mutant phenotypes in terms of their location and proximity to heptad repeat domains.

SER virus, an isolate of parainfluenza virus type 5, belongs to the family Paramyxoviridae and was isolated from aborted pigs. Simian virus 5 (SV5) is a prototype type 5 parainfluenza virus which has been shown to induce syncytium formation (fusion) in most cell types. SER virus is closely related to SV5 serologically and in its protein profile and its nucleotide sequence but replicates without causing cytopathology or syncytium formation (31). Sequence comparisons of SER with SV5 revealed the presence of an extended cytoplasmic tail (CT) in the SER F protein. Truncations or mutations in this domain resulted in enhanced syncytium formation and indicated that the elongated CT interferes with membrane fusion in a sequence-dependent manner (28, 31). In addition to the extended CT, the SER F protein differs from SV5 F by nine amino acids, six in the ectodomain, one in the transmembrane, and two in the CT domain. Mutation of some of these residues enhanced SER F-induced fusion dramatically, demonstrating that changes in multiple domains in the SER F protein can trigger enhanced fusion (26).

Cell entry by paramyxoviruses is mediated by two surface glycoproteins, the hemagglutinin-neuraminidase (HN) and the fusion (F) proteins. The F protein is synthesized as an inactive precursor, F0, which is assembled into a trimer that is posttranslationally cleaved by a host protease into two disulfide-linked subunits, designated F1 and F2. The cleavage of the F protein is essential for virus-cell and cell-cell fusion (12), but the exact mechanism of viral glycoprotein-induced membrane fusion is not yet completely understood. Viral entry is a multistep process that requires viral attachment, merger of the two membranes, pore formation and pore expansion, and subsequent fusion to the host cell (2, 11). Extensive studies of many F proteins implicate multiple domains in the fusion process. A hydrophobic sequence designated the fusion peptide is present at the amino-terminal regions of F1 polypeptides of all paramyxoviruses and may insert into target membranes, causing disorder of the bilayer to bring about the fusion of two interacting membranes (19). Several heptad repeat regions are also known to play a significant role in fusion (4, 8). Heptad repeat 1 (HR1) is located carboxyl to the fusion peptide, and HR2 is located proximal to the transmembrane domain. It has been suggested that another heptad repeat (HR3) is important for fusion activity in Newcastle disease virus (NDV) and Sendai virus F proteins (8, 9). The role of heptad repeats in fusion activity has been shown by mutational analysis as well as the inhibitory activity of peptides with sequences matching these two domains (10, 22, 25). Structural analysis shows that, in the postfusion F structure, the heptad repeats are complexed to form a six-stranded helical bundle with an interior core trimer of HR1 domains and the HR2 bound in the grooves of the trimer in an antiparallel fashion (2, 6, 38). Recently, Yin et al. demonstrated that the structure of a secreted but uncleaved anchor-negative ectodomain of the paramyxovirus type 3 F protein was present in a postfusion conformation (36). The metastable prefusion structure of the paramyxovirus type 5 F protein was also recently determined (37). The F trimer was shown to have a globular head comprising three domains, DI, DII, and DIII; the head is attached to a three-helix coiled-coil stalk formed by the C-terminal HR-B region. DIII contains the fusion peptide, HR-C helix, and C terminus of h4, a component of HR-A. The core components of DIII prevent the folding of HR-A into a postfusion conformation. DI and DII are rigid domains unlike DIII, which undergoes major structural reorganization during the conformational change that occurs to facilitate fusion (37).

The CT domains of several viral fusion proteins have also been shown to play a role in membrane fusion. Truncations in
the CT domains of F proteins of parainfluenza virus types 3 and 5 abolished fusion activity, whereas CT truncation of the parainfluenza virus type 2 F protein had no effect on fusion (1, 35). Continued passage of simian immunodeficiency virus (SIV) SIVmac239 in HUT78 cells resulted in the appearance of a virus encoding a transmembrane (TM) protein with a truncated CT domain, which showed multiple effects including increased incorporation of Env proteins into virions or virus-like particles (33), enhanced fusion activity of the Env protein, and alteration of the conformation of the external domain (23, 29, 30). It was also shown that alteration of the CT domain influences SIV replication efficiency and neutralization sensitivity (32). Naturally occurring truncation of the transmembrane protein has also been observed with isolates of human immunodeficiency virus type 2 (HIV-2) in which the 154-amino-acid CT domain was truncated to 17 amino acids (13, 16). The length of the CT of HIV-2 glycoprotein was shown to modulate the fusion activity of the glycoprotein complex in a cell-specific manner (18).

In this study, we serially passaged SER virus to determine if fusogenic or cytopathic virus variants could be recovered. We characterized the properties of the resulting viruses and their F proteins to identify differences compared to the wild-type (WT) noncytopathic SER virus.

MATERIALS AND METHODS

Cells and viruses. MDBK (Madin-Darby bovine kidney), BHK21, CV-1, and HeLa T4 cells were maintained in Dulbecco’s modified minimal essential medium (DMEM) supplemented with 10% fetal bovine serum (HyClone Laboratories, UT). Rabbit anti-SV5 antibody was a kind gift from R. A. Lamb (Northwestern University). SER virus was obtained from H.-D. Klenk (University of Marburg, Germany) and was propagated in MDBK cells as described previously (31).

Isolation of fusogenic SER virus variants. SER virus was serially passaged in MDBK cells in the presence of a 50 nM concentration of brefeldin A1 (BFA1), and each passage was tested for its ability to show fusion in BHK21 cells. After five passages, syncytium formation was observed in BHK21 cells infected with the passaged virus. End point dilution was performed to recover biological clones of SER virus mutants. Subsequently, plaque purification was also carried out to recover individual clones of mutant fusogenic viruses.

Plaque assay and virus titration. CV1 cells were grown in 12-well plates and inoculated for 1 h at 37°C with serial dilutions of WT or mutant SER virus in DMEM. Virus dilutions were washed off and subconfluent cells were overlaid with 2× DMEM–2% fetal bovine serum and white agar (1:1). After 4 to 5 days, cell monolayers were stained with crystal violet (0.1%). Immunostaining to titrate WT SER F proteins were expressed by using the vaccinia virus–bacteriophage T7 RNA polymerase transient-expression system (20). Briefly, 35-mm dishes of subconfluent cells were infected with vTF7-3 (recombinant vaccinia virus expressing T7 RNA polymerase) at a MOI of 10 for 1 h and then transfected with 3 μg of plasmid DNA using Lipofectin (Invitrogen, Carlsbad, CA). At 18 h posttransfection, the transfected cells were starved in DMEM lacking methionine and cysteine for 45 min, pulse-labeled with 100 μCi/ml of [35S]methionine/cysteine for 30 min at 37°C, and then chased with DMEM containing 10% fetal calf serum for 2 h. The cells were washed thrice and then lysed with cell dissociation buffer (10 mM Tris-HCl, pH 8.0, 250 mM NaCl, 0.5% Triton X-100, and 0.5% sodium deoxycholate). The cell lysate was incubated with SV5 hyperimmune serum for 2 h at 4°C, followed by precipitation using protein A-agarose (Immunopure; Pierce Chemical, Rockford, IL) for 2 h at 4°C. The beads were washed extensively and resuspended in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer and boiled at 95°C for 5 min. Proteins were characterized by SDS-8% PAGE and subsequently by autoradiography.

Cell surface biotinylation assay. Expression of proteins at the cell surface was detected by a biotinylation assay as described earlier (17). At 18 h posttransfection, the transfected cells were starved in DMEM lacking methionine and cysteine for 45 min, pulse-labeled with 100 μCi/ml of [35S]methionine/cysteine for 30 min at 37°C, and then chased with DMEM containing 10% fetal calf serum for 2 h. Cells were washed thrice with ice-cold phosphate-buffered saline (PBS) containing 10 mM CaCl2, PBS-CM, and incubated with 1 ml of a 0.5-mg/ml solution of sulfo-NHS-LC-biotin (Pierce) in 0.1% SDS and diluted with 1 ml lysis buffer. The biotinylated proteins in the supernatant from the protein A-agarose beads were recovered by incubation with streptavidin-agarose beads for 2 h at 4°C. The beads were washed extensively, resuspended in SDS-PAGE sample buffer, and boiled at 95°C for 5 min. Proteins were then characterized by SDS-8% PAGE and autoradiography.

Cell fusion assays. Thirty-five-millimeter dishes of subconfluent HeLa T4 cells were infected for 1 h with vTF7-3 at a MOI of 10 and then transfected with 3 μg of the WT or the mutant SER F construct with or without cotransfection of the WT SER HN plasmid using Lipofectin. At 16 to 20 h posttransfection, the cells were monitored for syncytium formation under a light microscope. The syncytia were defined as multinucleated cells with at least four nuclei per microscopic field (magnification, ×200). Five such random fields were selected, and the number of fusion of each mutant was expressed as a percentage of syncytia observed in cells coexpressing WT SER F and HN.

Modeling of the SER F protein structure. The SER F protein postfusion structure was modeled based on the existing structural information available for the F protein from NDV, PDB file 1g5g (6), and SV5, PDB file 1svf (2). The alignment of SER F to NDV F was based on the sequence homology analyzed by NCBI-BlastP (http://www.ncbi.nlm.nih.gov/BLAST), Pfam (http://pfam.sanger.ac.uk/Software/Pfam); ProDom (http://prodom.prabi.fr/prodom/current/index.html/home.php); InterPro (http://www.ebi.ac.uk/interpro); Blocks (http://blocks.thrcr.org/), and Psisie (http://us.expasy.org). All programs showed approximately 30% similarity across residues 102 to 517 (SER F). The helical extensions were modeled based on a chimeric structure of NDV F (residues 33 to 105 and 171 to 454) and SV5 F (residues 122 to 180 and 440 to 477). Modeler 7.7 was used to model SER F against the NDV and SV5 F proteins. The alignment was optimized manually after multiple rounds of modeling and minimization. To visualize the chimeric SER F model, Sybyl 7.0 software and Protein Explorer were used. Prefusion SV5 F protein structure information was obtained from T. Jardetzky (Northwestern University, IL) (PDB file 2B9B), and the mutated residues in SER F fusion variants were incorporated using Sybyl software.

RESULTS

Isolation of fusogenic SER mutants. SER virus, which does not induce any cytopathic effect, was serially passaged in the
FIG. 1. Polykaryon formation by BHK21 cells infected with WT or mutant SER virus. BHK21 cells in six-well plates were infected with WT or mutant SER virus at a MOI of 5 for 1 h at 37°C. At 24 to 48 h p.i., cells were observed for syncytium formation under a light microscope and photographed.
presence of bafilomycin as described in Materials and Methods, the passaged virus was subcloned, and the resulting clones were analyzed for fusion activity as shown in Fig. 1. We observed a number of clones which were fusogenic and subcloned seven SER mutants which were further characterized; no mutant viruses were recovered when SER virus was similarly passaged in the absence of bafilomycin. We categorized mutants as highly fusogenic (A mutants), including 9-1N, 6-1N, and 8-1N, and less fusogenic (B mutants), including 9-2N, 6-4N, 7-1, and 7-2N (Tables 1 and 2).

The mutants 9-1N, 6-1N, and 8-1N caused the formation of large multinucleate cells in pronounced syncytia; the other mutant viruses mediated syncytium formation to various degrees (Fig. 1). The mutant viruses were also assayed for kinetics of syncytium formation at intervals up to 48 h p.i. The mutants that were highly fusogenic, such as 9-1N, showed fusion with a faster kinetics than other less fusogenic mutant SER viruses (Table 1). The mutant viruses 9-1N and 6-1N showed larger multinucleate cells, and

### TABLE 1. Fusion kinetics of SER mutants in comparison to SER and SV5 viruses

<table>
<thead>
<tr>
<th>Virus</th>
<th>12 h</th>
<th>24 h</th>
<th>36 h</th>
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<tr>
<td><strong>Highly fusogenic mutants</strong></td>
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<tr>
<td>9-1N</td>
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<td>+</td>
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<td>-</td>
<td>+</td>
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<td>+</td>
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<td><strong>Less-fusogenic mutants</strong></td>
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<tr>
<td>9-2N</td>
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<td>+</td>
<td>+</td>
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<td>+</td>
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<tr>
<td>SER</td>
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* CV1 cells infected with SER (WT) or mutants and SV5 (MOI, 5) were assayed for syncytium formation up to 48 h posttransfection. The syncytia were defined as multinucleated cells with at least four nuclei per microscopic field. Five such random fields were selected, and the extent of fusion of each mutant virus was expressed as the percentage of syncytia observed in cells infected with WT SV5. D, detached; +, 1 to 25%; ++, 25 to 50%; +++, 50 to 75%; ++++, 75 to 100%; –, no fusion.

Within 48 h p.i. the entire monolayer of cells consisted of large aggregates of nuclei, indicating that extensive cell-cell fusion had occurred. Other mutants showed slower kinetics for syncytium formation and produced only moderate-size syncytia at 48 h p.i.
Growth properties of mutant SER viruses. Most of the mutant viruses replicated to similar titers as the WT SER virus (Table 2). To further investigate the growth properties of the mutant viruses, a multistep growth curve assay was performed. At various time points p.i., the culture medium was harvested, and virus titers were determined by plaque and HA assay. The growth kinetics of most of the mutant viruses were faster by about 12 h than those of the WT SER virus (Fig. 2A). The faster growth kinetics of the fusogenic viruses may be related to their fusogenic F proteins, leading to a more-rapid cell-to-cell spread. Mutant viruses were also found to grow in the presence of BFLA1, whereas the WT SER virus was not observed to grow in the presence of BFLA1 (Fig. 2B and C).

Plaque formation by mutant SER virus. The fusogenic mutant viruses were found to form plaques on CV1 cells, unlike the WT SER virus, which formed microscopic plaques that were detected only by immunostaining (Fig. 3). The mutant SER viruses displayed plaques of strikingly different sizes; mutant 8-1N formed plaques larger than those of SV5 virus; mutants 9-1N, 6-1N, 9-2N, 7-1, and 7-2N formed medium-size plaques similar to those of SV5. The mutant viruses also formed plaques (as observed by immunostaining) in the presence of bafilomycin (Fig. 4). In contrast, WT SER virus showed no plaques under these conditions. The basis for the differences in the plaque size for the fusogenic mutants is of interest for further study.

Sequence analysis and fusion activity of mutant F proteins. RNA was isolated from the cells infected with various mutant viruses, RT-PCR was performed using gene-specific primers as described in Materials and Methods, and the amplified genes were cloned into the pCR2.1 vector (Promega) and sequenced using both vector-specific and gene-specific internal primers (Fig. 5). We observed that two consistent mutations, resulting in a Pro-to-Leu change at amino acid position 22 and an Ile-to-Leu change at amino acid position 191, were present in all the mutant F genes. Sequencing results also showed the presence of other mutations unique to specific mutants, spanning the length of the mutant F protein. In only one case, the 6-1N mutant, a mutation was found in the CT region of the mutant F proteins, Arg to Gly at position 551. These results together with previous studies indicate that changes in the external and CT domains can modulate the fusion activity of the SER F protein.

To study the activity of these mutant F proteins in inducing cell fusion, we transiently expressed the proteins, together with SER HN, using the T7-vaccinia virus expression system; the mutant proteins were found to be expressed and transported to the cell surface as shown in cell surface biotinylation experiments (Fig. 6A and B). The fusion properties of the expressed mutant F proteins were studied using syncytium formation assays. Whereas the WT SER and mutant 6-4N, 7-2N, and 7-1 F proteins induced no cell fusion, the mutant 9-1N, 9-2N, 8-1N, and 6-1N F proteins coexpressed with WT SER HN showed fusion activity, but to a lesser extent than when they were coexpressed with the HN protein of the respective mutant virus (Fig. 6C). No fusion activity was observed in the absence of HN (data not shown). This suggests that, in addition to the mutations in the F glycoproteins, mutations in other SER proteins
also play a role in enhancing fusion activity in the context of the mutant viruses.

**Sequence analysis of HN and M proteins.** The fusion activity of most paramyxoviruses is known to involve a cooperative interaction of the F and HN proteins. Also, the assembly of paramyxoviruses requires the coalescence of viral components at the budding site (the plasma membrane of the infected cells), including the HN and M (matrix) proteins. Since we

<table>
<thead>
<tr>
<th>Mutant</th>
<th>FUSION(%)</th>
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<tbody>
<tr>
<td>9-1N</td>
<td>+</td>
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<tr>
<td>9-2N</td>
<td>+</td>
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<td>6-1N</td>
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<td>8-1N</td>
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<td>7-2N</td>
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<td>7-1</td>
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</tr>
<tr>
<td>6-4N</td>
<td>+</td>
</tr>
<tr>
<td>SV5F</td>
<td>+++++</td>
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![A: WT SER-HN](image1)

![B: WT SER-M](image2)

![C: Mutant FUSION(%)](image3)

**FIG. 5.** Sequence analysis of F proteins of fusogenic variants. RT-PCR was carried out on RNA isolated from mutant infected cells at 24 h p.i. using F-specific primers as described in Materials and Methods. The PCR-amplified genes were then cloned into pCR2.1 and sequenced. The positions of observed amino acid changes in the deduced sequences are indicated. aa, amino acids.

**FIG. 6.** Expression of mutant SER F proteins. (A and B) Surface expression of mutant F proteins. HeLa T4 cells were infected with vTF7-3 at a MOI of 10 for 1 h at 37°C and then transfected with plasmids carrying the mutant SER F constructs. At 18 h posttransfection, cells were metabolically labeled with methionine and cysteine as described in Materials and Methods. Labeling was followed by biotinylation and immunoprecipitation using anti-SV5 antisera and protein A-agarose beads at 4°C for 2 h. The samples were prepared in reducing sample buffer and analyzed by SDS–8% PAGE and autoradiography. (C) Cell fusion assay with mutant SER F proteins coexpressed with SER HN. HeLa T4 cells were infected with vTF7-3 at a MOI of 10 for 1 h at 37°C and then cotransfected with the mutant SER F and SER HN plasmids using Lipofectin as described in Materials and Methods. At 16 to 20 h posttransfection, cells were observed for syncytium formation using an inverted phase-contrast microscope. +, 1 to 25%; +++++, 75 to 100%; −, no fusion.

**FIG. 7.** Sequence analysis of HN and M proteins of fusogenic variants. RT-PCR was carried out on RNA isolated from mutant infected cells at 24 h p.i. using HN-specific or M-specific primers as described in Materials and Methods. The PCR-amplified mutant HN and M genes were then cloned into pCR2.1 and sequenced, and the positions of mutated amino acids are shown. aa, amino acids.
FIG. 8. Structural model showing positions of SER F mutations. (A) Structural comparison of mutated residues on pre- and postfusion conformations of SER F protein. Prefusion SV5 F structure information was obtained from the protein databank (PDB file 2B9B [37]). SER F was modeled using a modeler program and existing molecular structure information available for the NDV F ectodomain and SV5 F coiled-coil domain, which served as the postfusion SER F model. Residues mutated in the fusogenic mutants are shown as space-filling models in color. The three polypeptide chains of the F trimer are depicted as cyan, green, and yellow. (B) Analysis of Pro22Leu using Protein Explorer and Sybyl 7.0. Left panel, prefusion F conformation and residue Leu at position 22 (cyan); right panel, postfusion conformation from Pro (white) at position 22 to Leu (green) and its atomic overlap at 40Å (dot surface plot). (C) Analysis of Ile191Leu using Protein Explorer. Shown is the prefusion F conformation and residue Leu at position 191. Interacting residues are shown in white. (D) Analysis of Leu447Trp using Protein Explorer and Sybyl 7.0. Top panel, prefusion conformation and residue Trp at position 447 (purple) interacting with neighboring residues (white); bottom panel, postfusion conformation, in which Leu at 447 to Trp may overlap with Gln at 166, Val at 168, and Gly at 162 on chain C (yellow) and Gln at 169 and Val at positions 165 and 168 on the other monomer (white). (E) Analysis of Asn431Ser using Protein Explorer and Sybyl 7.0. Top panel, prefusion conformation and residue Ser at position 431 (purple); bottom panel, postfusion conformation and the mutation Asn at position 431 to Ser on chain A (cyan), which is shown to overlap with Val at position 50 and Met at position 54 on chain C (yellow). (F) Analysis of Ala246Thr using Protein Explorer and Sybyl 7.0. Prefusion (top panel) and postfusion (bottom panel) conformations are shown. Residues interacting with Thr at position 246 are shown in white in the prefusion conformation.
observed changes in viral replication profiles and fusion kinetics, we also determined if any sequence changes were observed in the HN and M proteins of the fusogenic mutant viruses. As shown in Fig. 7A, the HN proteins of the fusogenic variants showed substitutions specific for each mutant. The M proteins of the fusogenic variants showed a conserved mutation of threonine to isoleucine at position 129 in addition to other mutations (Fig. 7B). These mutations may play a role in enhancing fusion and/or virus replication of the fusogenic parainfluenza virus variants.

**Structural analysis of mutant F proteins.** We modeled the structure of the SER F protein using a program (Bimcore; Emory University) which compared the existing structure information available for F proteins from related paramyxoviruses NDV and SV5. The postfusion SER F structure was initially modeled based on a chimeric model in which the residues from HR-A (122 to 185) and HR-B (440 to 477) of SV5 F were linked to other residues from NDV F. Also Yin et al. recently described the structure of the parainfluenza virus 5 F protein in its metastable, prefusion conformation (37). The SV5 F protein in its prefusion conformation contains a globular head attached to a trimeric coiled-coil stalk formed by the C-terminal HR-B region. The globular head has three domains, DI, DII, and DIII. The DIII domain prevents the folding of HR-A in its prefusion conformation, and the intersubunit interactions seem to differ between the pre- and postfusion conformations. For several of the mutants (Fig. 8A), the mutated residues are compared on the initial SER F model that was based on the postfusion conformation and on the SV5 F prefusion conformation. The difference between the pre- and postfusion conformations can be observed by comparison of mutated residues between the two structures. Most
of the residues involved in mutations show different intra- as well as intersubunit interactions. In the mutant 8-1N F protein, the two residues Pro22 and Asn431 are in close proximity to each other structurally in the prefusion conformation as opposed to the SER F postfusion model. Similarly, other residues in the mutant F proteins show striking differences between the pre- and postfusion states.

**DISCUSSION**

In the present study, we characterized a set of mutant SER viruses which were found to be fusogenic, in contrast to the WT virus, which lacks fusion activity. Most of the mutant viruses replicated more rapidly than WT virus. The virus mutants were selected by serial passage in the presence of bafilomycin, which was previously observed to suppress production of SER virus but not the closely related fusogenic virus SV5 (27). We observed that, in comparison to SER virus, the fusogenic variants are able to replicate more effectively in the presence of bafilomycin. Recent studies have shown that early steps in SER virus replication were not inhibited by bafilomycin (3). It therefore seems likely that the enhanced replication of the fusogenic viruses may be related to their more rapid replication kinetics, enabling higher levels of virus production.
under conditions where some cytopathic effects occur as a result of drug treatment.

Unlike the WT SER virus, the mutants caused plaque formation in cells. Strikingly, we observed significant differences in the plaque sizes for the mutants. The mutant 8-1N formed large-size plaques but grew to similar titers as WT SER virus. Other mutants, such as 6-1N, 9-1N, 9-2N, 6-4N, 7-1, and 7-2N, showed medium-size plaques similar to SV5. The mutant viruses also differed in their fusion activities. The highly fusogenic mutants, such as 9-1N, 6-1N, and 8-1N, had faster fusion kinetics, forming large multinucleated cells in less than 48 h p.i.; other mutants showed medium-size syncytia. We observed differences in fusogenic potential of the mutant viruses versus the expressed F proteins, indicating that mutations not only in the F protein but also in the HK protein, and possibly other proteins, contribute to the mutant phenotype.

The F proteins of all the fusogenic mutants possessed two consistent mutations, Pro22Leu and Ile191Leu. In the SV5 W3a strain F protein, a Pro residue at position 22 was demonstrated to be important for conferring HK-independent fusion as well as faster kinetics and a lower-temperature requirement for fusion activity (21). We have further analyzed the positions of residues which were found to be mutated in the fusogenic SER F proteins (pre- and postfusion conformations) based on the SV5 F prefusion structure (37) and the SER F model (a chimeric model based on the structural information available from NDV F and SV5 F which depicts the postfusion conformation). There are striking differences between pre- and postfusion conformations. In the prefusion conformation, Pro 22 resides near the beginning of the F2 subunit and is proximal to a helical domain (residues 30 to 40; Fig. 8B and E). It interacts with other DI residues, Gln 350 and Gly 351, located on the loop region (residues 350 to 359). Upon mutation to Leu 22, interaction with Leu 349 was observed in addition to other interacting neighbors Gln 350 and Gly 351 in the short helical region in the DI domain (Fig. 8B and E). A mutation of proline to leucine might change the overall conformation of F2, altering flexibility in this domain. In the postfusion conformation, Pro 22 does not appear to interact with other residues as it faces outwards from the F protein surface (Fig. 8B, postfusion).

In the prefusion conformation, Ile at position 191 (another consistent mutation) resides in the h4 helical region of HR-A (top of the globular head domain), which interacts with residues in h3 of the same chain (Val 175) and the adjacent chain (Ser 174) (Fig. 8C). A mutation at this position can lead to conformational changes as HR-A surrounds the DIII core domain, which prevents it from forming a six-helix bundle structure. The postfusion conformation model suggests that the amino acid at position 191 interacts with residues on other monomers at the trimer interface, including Leu at position 436 and Asp at position 437 on chain B and Thr at position 67 on chain C (data not shown). This mutation may destabilize the association between monomers of the SER F trimer in the prefusion conformation or strengthen the interactions with its neighbors in the postfusion state.

In the 6-1N F protein, two mutations were observed in addition to the two consistent mutations. The mutated residues are Leu at position 447 and Arg at position 551 in the CT domain, for which structural information is not available. In the structure of the prefusion SV5 F protein, Yin et al. showed that Leu 447 resides in a hydrophobic pocket and mutations of Leu 447 and Ile 449 can destabilize the F protein. It was also reported by Russell et al. (24) that mutation of Ser 443 to Pro and mutation of Leu 447 and Ile 449 to aromatic residues (residues proximal to heptad repeat B) in the SV5 F protein resulted in increased levels of fusion as well as fusion promotion at lower temperatures. Previous studies showed that mutations Leu140Met and Leu161Met (residues in the heptad repeat A) abolish the α-helical structure required for the formation of a coiled-coil domain but enhance syncytium formation (34). In the 6-1N mutant, Leu 447 can interact with residues Ile 444, Thr 357, and Gln 304 of the neighboring chain (data not shown) but, when mutated to Trp, as shown in Fig. 8D (upper panel, prefusion model), it has more potential intrasubunit interactions with Thr 345, Asp 344, and Ser 342 of the adjacent chain, possibly causing a decrease in the stability of (and enhanced fusion by) the mutant F protein. In the postfusion model (left bottom panel), Leu 447 lies proximal to the HR-B domain in the F monomer, and mutation of this residue to Trp could alter its possible interactions with residues in the HR-A (160 to 170) domain of other monomers (chains A and C, right bottom panel) that might affect the stability of the F trimer. This suggests that altered intrasubunit or intersubunit interactions in the SER mutants can lead to destabilization and enhance the fusion activity of the F protein.

In the 8-1N mutant (prefusion conformation), residue Asn 431 is located on the HR-B linker region and shows interaction with Gln 27 in the region preceding HR-C (Fig. 8E, upper panel). This region was shown to be highly flexible (37), but specific mutations in the HR-B linker can also destabilize the threefold-symmetric conformation, leading to a more fusogenic F protein. Modeling of the 8-1N mutant F protein in the postfusion conformation revealed that Asn at position 431 might have an atomic overlap with residues on other monomers, Val at position 50 and Met at position 54 (Fig. 8E, bottom panel), and mutation to Ser might affect these intermolecular interactions in the F trimer.

In the prefusion conformation of mutant 7-1, Thr 246 is located on the helical domain in DIII and interacts with Glu 237 on a neighboring helix and Thr 75 on the HR-C of the adjacent monomer (Fig. 8F, upper panel). Another mutated residue, Leu 341, interacts very closely with Ile 316 located on the adjoining β-strand of the three-stranded β-sheet in the DI domain (data not shown). The Asn 329 residue is located on the loop region in the DI domain, and it seems to interact with Arg 36, located on the β-strand of a three-stranded β-sheet domain. In the postfusion conformation, Ala at position 246 was found to have an atomic overlap with Leu 87 (the putative heptad repeat C domain) in other monomers of the F trimer (Fig. 8F, lower panel). The Asn at position 329 connects the two antiparallel β-strands within the β-barrel assembly of the head region of F protein, as described by Chen et al. (6). Mutation of this residue may alter the interactions in the hydrophobic core of the F trimer. The other mutated residue, Leu at position 341, precedes a two-turn helix which forms the wall of the radial channel, and mutation of this residue might destabilize the hydrophobic core of the F trimer.

It has been observed that individual residues contribute differently to the stability of the proteins. In the hydrophobic
model of stability, hydrophobic interactions are believed to be the main driving force that can stabilize the protein structure. Earlier work by Zhou and Zhou (39) suggests that the contributions of hydrophilic residues to the stability could be marginal compared to those of the hydrophobic residues that are buried in the interior of the protein, which leads to a large gain in stability. The effects of amino acid sequence on stability can be compared by measuring the free energies of the native, WT sequence and its mutant sequences. If a mutation increases the free energy of the folded state of the protein, the mutation is considered destabilizing, whereas if the mutation decreases the free energy of the protein, it is considered stabilizing. We performed free-energy calculations for some of the mutant F proteins to study their overall free energies according to the stability scales provided by Zhou and Zhou (39). We found that the F proteins of mutants 9-1N and 8-1N, which are the most fusogenic mutants, have higher overall Gibbs free energies of ~3.75 and 2.64 kcal/mol, respectively, than the WT-SER F protein, suggesting that the mutations are destabilizing. The F protein of the less-fusogenic mutant 7-2N has a free-energy level of ~0.21 kcal/mol, suggesting that these mutations are less destabilizing than those of the 9-1N and 8-1N mutants. Since the biological function of a protein is sensitive to its conformation, activity serves as a phenotype for the mutated form.

We also investigated the possible effect of these mutations on the pathogenicity of the mutant SER viruses in comparison to the WT SER and SV5 viruses in the lungs of mice (Swiss C57BL/6). Studies of experimental infection with SV5 in Swiss mice, hamsters, and monkeys have been described previously (5, 14). At 2, 3, 5, and 7 days postinoculation via the intranasal route, the mice did not show any disease symptoms such as change in body weight or ruffling of fur. No virus replication was detected in lung extracts of SER- or SV5-infected mice. We observed the presence of low levels of antibodies, as estimated with quantitative enzyme-linked immunosorbent assay, in sera collected at intervals after inoculation against SER (WT or mutants) or SV5, suggesting some degree of humoral immune responses against both SV5 and SER viruses (data not shown).

In conclusion, we have observed that various mutations in the external domains of fusogenic SER mutant F proteins can significantly enhance fusion activity. Thus, the fusion-suppressive effect of the extended CT domain can be compensated by changes in ectodomain residues. The structural positions of the substitutions observed in the mutants may affect the conformation of the metastable prefusion form of the SER F protein by altering either the intra- or intermolecular interactions in the SER F trimer, causing the F protein to acquire a lower energy state that can be activated to induce fusion upon triggering. In addition, changes in the postfusion conformation can strengthen the associations between neighboring residues, which may also enhance fusion activity.

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