JOURNAL OF VIROLOGY, June 2006, p. 5798–5806 Vol. 80, No. 12
0022-538X/06/$08.00+0 doi:10.1128/JVI.00294-06

Modification of the Trypsin-Dependent Cleavage Activation Site of the Human Metapneumovirus Fusion Protein To Be Trypsin Independent Does Not Increase Replication or Spread in Rodents or Nonhuman Primates

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Received 9 February 2006/Accepted 31 March 2006

The contribution of cleavage activation of the fusion F protein of human metapneumovirus (HMPV) to replication and pathogenicity in rodents and nonhuman primates was investigated. Recombinant HMPVs were generated in which the naturally occurring trypsin-dependent cleavage sequence (R-Q-S-R ↓) was replaced by each of three sequences whose cleavage in vitro does not depend upon added trypsin. Two of these were multibasic sequences derived from avian metapneumovirus type A (R-R-R-R) or type C (R-K-A-R), with the former containing the consensus furin protease cleavage motif (R-X-R/K-R ↓). The third one (R-Q-P-R) was derived from a recently described trypsin independent HMPV isolate (J. H. Schickli, J. Kaur, N. Ulbrandt, R. R. Spaete, and R. S. Tang, J. Virol. 79:10678–10689, 2005). To preclude the possibility of conferring even greater virulence to this significant human pathogen, the modifications were done in an HMPV variant that was attenuated by the deletion of two of the three envelope glycoproteins, SH and G. Each of the introduced cleavage sequences conferred trypsin independent F cleavage and growth to HMPV in vitro. However, they differed in the efficiency of trypsin independent growth and plaque formation in vitro: R-R-R-R > R-K-A-R > R-Q-P-R > R-Q-S-R. The R-R-R-R mutant was the only one whose growth in vitro was not augmented by added trypsin, indicative of highly efficient trypsin independent cleavage. When inoculated intranasally into hamsters, there was essentially no difference in the magnitude of replication in the upper or lower respiratory tract between the mutants, and virus was not detected in organs outside of the respiratory tract. Evaluation of the most cleavage-efficient mutant, R-R-R-R, in African green monkeys showed that there was no detectable change in the magnitude of replication in the upper and lower respiratory tract or in immunogenicity and protective efficacy against HMPV challenge. These results suggest that cleavage activation is not a major determinant of HMPV virulence.

Human metapneumovirus (HMPV) is a major etiologic agent for lower respiratory tract disease in children that was first reported in 2001 (33), and subsequently it was recognized to be of worldwide prevalence (15, 18, 34). Retrospective studies showed that HMPV is not a newly emerging agent but rather one that had previously been overlooked (8, 33). HMPV is a member of the *Metapneumovirus* genus of the subfamily *Pneumovirinae*, family *Paramyxoviridae*, order *Mononegavirales*.

The HMPV genome is a single strand of negative-sense RNA that ranges in length from 13,280 to 13,335 nucleotides (nt) for the available sequences (4, 32). The genome contains eight genes in the order 3′-N-P-M-F-M2-SH-G-L-5′ and encodes nine proteins (4, 32). By analogy to human respiratory syncytial virus (HRSV), the HMPV proteins are the following: N, nucleoprotein; P, phosphoprotein; M, matrix protein; F, fusion protein; M2-1, putative transcription factor; M2-2, RNA synthesis regulatory factor; SH, small hydrophobic protein of unknown function; G, attachment glycoprotein; and L, viral polymerase.

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The F glycoproteins of HMPV and HRSV of *Pneumovirinae* appear to be similar in overall structure and function to those of the more highly characterized prototypic members of *Paramyxovirinae*, such as Newcastle disease virus (NDV) and Sendai virus (SeV), although there has been extensive amino acid sequence divergence (28). The prototypic *Paramyxoviridae* F protein is a type I glycoprotein, with an N-terminal cleaved signal peptide and a C-proximal membrane anchor. It is synthesized as an inactive precursor, F_0, that is converted into the fusogenic form by cleavage by host endoproteases to yield two subunits that remain linked by a disulfide bond: NH_2-F_2-S-S-F_1-COOH. The cleaved form of the F protein mediates fusion involved in viral entry and syncytium formation (for a review, see reference 14).

Studies with prototypic viruses such as NDV and SeV have shown that cleavage of F_0 is a prerequisite for infectivity and is an important determinant of virulence. For many members of *Paramyxoviridae*, the F_0 protein is cleaved intracellularly by host proteases of the exocytic pathway, most notably furin (22). Cleavage occurs on the C-terminal side of a sequence that typically is rich in basic amino acids; the consensus motif for furin is R-X-R/K-R ↓ (25). Furin is present in most cell types; consequently, viruses with furin cleavage sites have a potential for growth in a wide range of tissues. There also are cases of

cleavage sites that are not highly basic but nonetheless are cleaved intracellularly, such as certain F protein mutants of SeV (20, 30) as well as the F protein of Nipah virus (19). In contrast, the F_0 proteins of some viruses are insensitive to intracellular proteases and depend on extracellular cleavage by secretory proteases. Typically, these F proteins have a cleavage sequence that contains fewer basic residues, often with only a single arginine residue immediately upstream of the cleavage site. Because of their dependence on secreted protease, these viruses are restricted for replication in vivo to the lumen of the respiratory tract and, in some cases, the enteric tract where these proteases occur. In vitro, cleavage depends on the addition of trypsin to the cell culture medium.

Every direct clinical isolate of HMPV described to date has the cleavage site R-Q-S-R and is dependent on added trypsin for growth in vitro (4, 9, 24, 32). We were interested in determining whether this cleavage phenotype affects HMPV virulence. We therefore used reverse genetics to replace the naturally occurring HMPV cleavage sequence with three sequences known to be associated with intracellular cleavability; two were derived from avian metapneumovirus (AMPV), and the third is a version of the HMPV cleavage sequence that was identified in HMPV variants that arose during passage in vitro and were identified as being trypsin independent (26). To preclude the possibility of creating a more virulent form of HMPV, we created the cleavage site mutants in an HMPV mutant background that had been attenuated by deletion of its G and SH genes (rHMVΔSH/G) (6). The resulting mutant viruses were evaluated for replication in vitro in hamsters and in African green monkeys (AGMs).

MATERIALS AND METHODS

Cells and viruses. Vero cells (ATCC CCL 7.1) were maintained in Opti-ProSFM (Invitrogen) supplemented with 4 mM L-glutamine. BSR T7/5 cells are baby hamster kidney 21 cells that constitutively express T7 RNA polymerase (11). They were maintained in Glasgow minimal essential medium (MEM) supplemented with 2 mM L-glutamine and amino acids (1× final MEM amino acids solution; Invitrogen), 10% fetal bovine serum, and 1 mg/ml of Geneticin. The recombinant HMPVs (all derived from HMPV CAN97-83 [24]) were propagated in Vero cells at 32°C in the absence of serum and in the presence or absence of trypsin as noted. In all experiments in this work in which trypsin was present, its concentration was 5 μg/ml (Cambrex). Virus titers were determined by plaque assay on Vero cells under methylcellulose overlay containing trypsin as described previously (5). Plaques were visualized 6 days later by immunostaining with an anti-HMPV polyclonal rabbit antiserum (5), and the final titer was expressed in PFU per milliliter.

Mutation of the F protein cleavage site. We previously constructed plasmid pHMPV encoding the antigenomic RNA of HMPV clinical isolate CAN97-83 (8). The sequence of the encoded RNA is identical to the CAN97-83 consensus sequence (GenBank accession number AY297749 [4]), except for four substitutions in the M-F intergenic region that created a unique NheI site (nt 3038) (5). In addition, pHMPV had previously been modified by three nucleotide substitutions in the M2-SH intergenic region that create a BsiWI site (6). To modify the F protein cleavage site, the NheI/Acc65I fragment of pHMPV(BsiWI), containing the F, M2, SH, and G genes (Fig. 1), was subcloned, and site-directed mutagenesis was performed using the QuickChange kit (Stratagene). The naturally occurring HMPV CAN97-83 F cleavage site (R-Q-S-R) was replaced by that of (i) AMPV-A (R-R-R-R), (ii) AMPV-C (R-K-A-R), or (iii) the variant HMPV (R-Q-P-R) using the following pairs of complementary primers, respectively (mutated nucleotides are in boldface): (i) GAGAGGAACAAATTGAG AATCCCAGAC**GCCGC**AGGTTTGTTCTAGGAGCAATAGC and GCTATT GCTCCTAGAACAAACCT**GCGGC**GTCTGGGATTCTCAATTTGTTCCT CTC, (ii) GAGAGGAACAAATTGAGAATCCCAGA**AA**G**G**C*C*AGGTTTG TTCTAGGAGCAATAGC and GCTATTGCTCCTAGAACAAACCT**GGC CTT**CTGGGATTCTCAATTTGTTCCTCTC, or (iii) GAGAGGAACAAA TTGAGAATCCCAGACAA**C**CTAGGTTTGTTCTAGGAGCAATAGC and GCTATTGCTCCTAGAACAAACCTAG**G**TTGTCTGGGATTCTCAATTTG TTCCTCTC. The mutated subclones were then digested with NheI/BsiWI, which cuts upstream of the F gene and downstream of the M2 gene and in effect deletes the SH and G genes. Each resulting NheI/BsiWI fragment (bearing the F and M2 genes) was cloned into the NheI/Acc65I window (the sticky ends of BsiWI and Acc65I are compatible but do not regenerate either site) of pHMPV to create plasmids pHMPVΔSH/G-$F_{AMPV\text{-}A}$, pHMPVΔSH/G-$F_{AMPV\text{-}A}$, and pHMPVΔSH/G-F_{S101P}. The sequence of the NheI/Acc65I insert of each modified pHMPV was confirmed by nucleotide sequencing performed with an ABI 3730 automatic sequencer using the Big-Dye terminator ready reaction kit v1.1 (Applied Biosystems) and specific primers. The pHMPVΔSH/G-F_{WT} plasmid had been constructed previously (6).

Recombinant HMPV (rHMPV) recovery. As described previously (5), confluent BSR T7/5 cells in 6-well dishes were transfected with 5 μg of antigenome plasmid pHMPVΔSH/G, pHMPVΔSH/G-$F_{AMPV\text{-}A}$, pHMPVΔSH/G-$F_{AMPV\text{-}C}$, or pHMPVΔSH/G-F_{S101P}, together with 2 μg each of pT7-N and pT7-P and 1 μg each of pT7-M2-1 and pT7-L per well; these are support plasmids that encode the HMPV N, P, M2-1, and L proteins, respectively. Transfections were done with Lipofectamine 2000 (Invitrogen) in OptiMEM without trypsin or serum (day 1) and maintained overnight at 32°C. The Lipofectamine transfection medium was removed (day 2) and discarded and replaced with Glasgow MEM without trypsin or serum. Trypsin was added on day 3, and on day 4 cells were scraped. Cell-medium mixtures were passaged onto fresh Vero cells and incubated at 32°C for 10 days in the presence of trypsin. The medium supernatants were harvested, and the rHMPVΔSH/G-F_{WT} (F_{WT}), rHMPVΔSH/G-$F_{AMPV\text{-}A}$ ($F_{AMPV\text{-}A}$), rHMPVΔSH/G-$F_{AMPV\text{-}C}$ ($F_{AMPV\text{-}C}$), and rHMPVΔSH/G-F_{S101P} (F_{S101P}) viruses were then amplified by two passages in Vero cells in the presence of trypsin.

Verification of recombinant viral genome structure. Viral RNA was extracted from the virus stocks of the second passage of the F_{WT}, $F_{AMPV\text{-}A}$, $F_{AMPV\text{-}C}$, and F_{S101P} viruses (QIAamp viral RNA kit; QIAGEN). The viral RNA was copied and amplified by reverse transcriptase PCR (RT-PCR) with a positive-sense primer designed to hybridize within the M gene (CAN97-83 nt 2719 to 2738) and a negative-sense primer designed to hybridize within the L gene (CAN97-83 nt 7499 to 7479). PCR products were analyzed on a 1% agarose gel and were also subjected to direct nucleotide sequencing in their entirety (Fig. 1). Control RT-PCRs in which the reverse transcriptase was omitted did not yield detectable products, confirming that the products that were sequenced were generated from RNA templates.

Protein electrophoresis and Western blot assay. Vero cells were infected at an input multiplicity of infection (MOI) of 5 PFU per cell with each F mutant and incubated at 32°C in the presence or absence of trypsin. At 48 h postinfection, the cells were collected and lysates were prepared, and the cell culture medium was collected and clarified by centrifugation at 2,000 × *g* for 15 min. The cell lysates and clarified medium supernatants were reduced and denatured at 99°C for 5 min and analyzed on a 4 to 12% polyacrylamide Bis-Tris gel (NuPAGE Novex Bis-Tris; Invitrogen). The separated proteins were electrotransferred onto a polyvinylidene difluoride membrane (Invitrolon; Invitrogen). The membrane was treated for 2 h at room temperature in Western Blocking Reagent (Roche) and then incubated with a hamster antiserum raised against a recombinant human parainfluenza virus type 1 (HPIV1) expressing the HMPV CAN97-83 F protein (27) or with a rabbit antiserum raised against sucrose-purified CAN97-83 virions (5). Bound antibodies were visualized by binding with horseradish peroxidase-conjugated goat anti-rabbit or anti-hamster immunoglobulin G antibodies (KPL, Gaithersburg, MD) followed by chemiluminescence (SuperSignal West Pico Chemiluminescent Substrate; Pierce).

Replication of F_{WT}, $F_{AMPV\text{-}A}$, $F_{AMPV\text{-}C}$, and F_{S101P} viruses in hamsters. Groups of 18-week-old Golden Syrian hamsters were inoculated intranasally under light methoxyflurane anesthesia with 0.1 ml per animal of L15 medium containing 10^6 PFU of F_{WT}, $F_{AMPV\text{-}A}$, $F_{AMPV\text{-}C}$, or F_{S101P} virus. On days 3, 5, and 7 postinfection, six animals from each group were sacrificed, and the lungs, nasal turbinates, brains, livers, spleens, kidneys, thymus, and intestines (duodenum) were harvested. Tissue homogenates were prepared and analyzed for infectious virus by plaque assay.

Evaluation of F_{WT} and $F_{AMPV\text{-}A}$ in AGMs. Using previously described methods (3), young adult AGMs were confirmed to be negative for serum-neutralizing antibodies to HMPV and were inoculated simultaneously by the intranasal and intratracheal routes with a 1-ml inoculum per site containing 10^6 PFU of rHMPV, F_{WT}, or $F_{AMPV\text{-}A}$ in L15 medium. A mock infection control group received L15 medium alone. Each group contained four animals except for the mock infection and the rHMPV group, which contained two animals. Clinical observations were made daily on days 0 to 12 following inoculation. Nasopha-

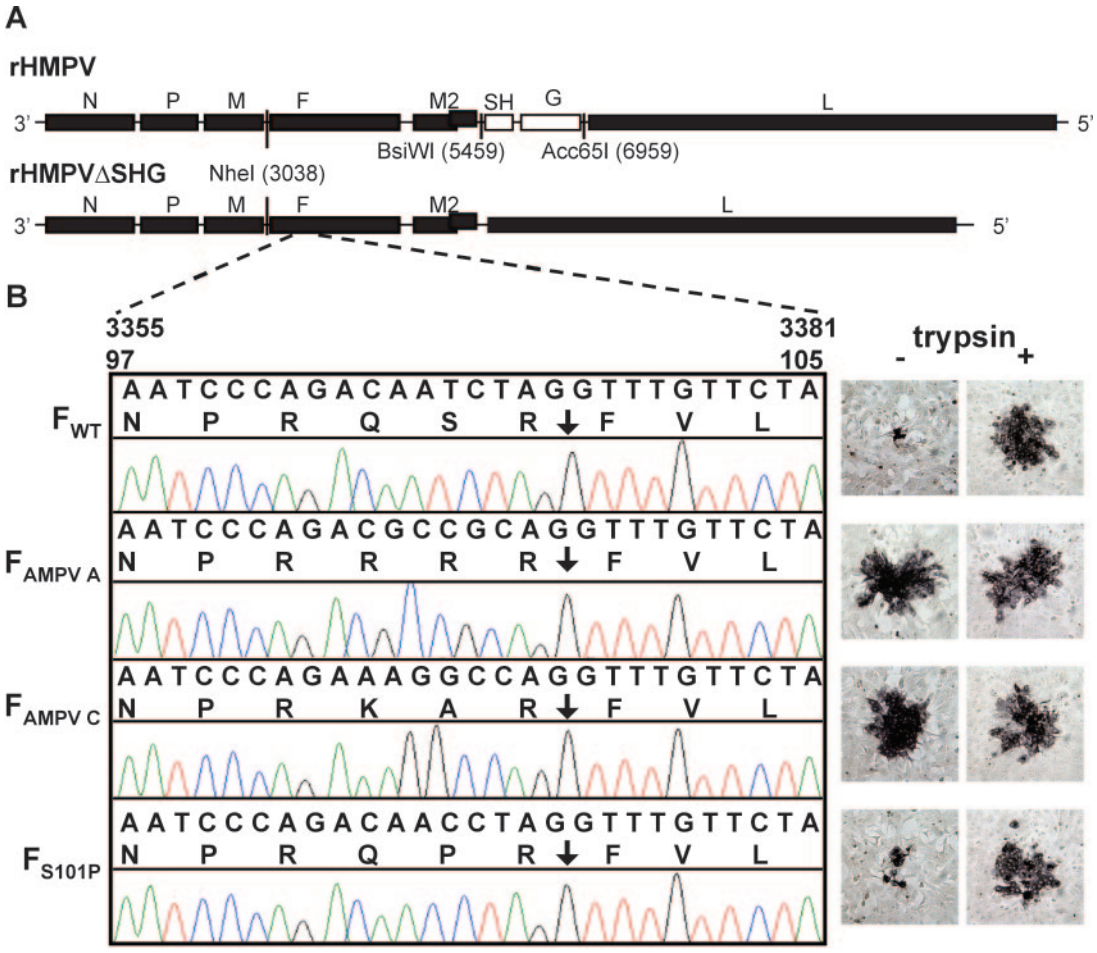

FIG. 1. Construction, recovery, and plaque morphologies of the HMPV F cleavage site mutants in the ΔSH/G background. (A) The complete rHMPV genome is shown at the top, drawn approximately to scale. The NheI and BsiWI sites that had been added (5, 6) are shown, as well as a naturally occurring unique Acc65I site. Underneath is the rHMPVΔSH/G genome in which the sequence of the F cleavage site was manipulated. (B) Sequences of the F cleavage sites in recovered ΔSH/G viruses and plaque size in Vero cells. The electropherograms show the nucleotide and amino acid sequences surrounding the F protein cleavage site (nt 3355 to 3381 of the HMPV genome and amino acids 97 to 105 of the F protein). The red letters indicate mutated nucleotides and amino acids, and the arrow shows the presumed cleavage site. On the right are photomicrographs showing the plaque sizes of the recovered viruses in Vero cells in the presence or absence of added trypsin in methylcellulose culture medium taken on day 6 postinfection, after fixation and immunostaining with polyclonal antibodies raised against gradient-purified HMPV.

ryngeal (NP) swabs were collected daily for 10 days following inoculation and on day 12. Tracheal lavage (TL) samples were collected on days 2, 4, 6, 10, and 12 postinfection. The amount of virus present in NP and TL samples was quantified by plaque assay. On day 28, each monkey was challenged by the intranasal and intratracheal routes with 10^6 PFU of rHMPV in a 1.0-ml volume per site. Virus shedding was examined by plaque assay of NP and TL samples collected on days 2, 4, 6, and 8 postchallenge. Serum samples were collected on days 0, 28, and 56 (28 days postchallenge), and the titers of HMPV-neutralizing antibodies were determined using an endpoint dilution neutralization assay as described previously (7).

RESULTS

Construction and recovery of rHMPVΔSH/G with altered F protein cleavage sequences. We constructed F cleavage site mutants using a derivative of HMPV that was attenuated by deletion of the G and SH genes (6). This was done to avoid the possibility of conferring increased virulence to a wild-type version of this human pathogen. Four rHMPVΔSH/G-based viruses were constructed, namely, F_{WT} bearing the wild-type F cleavage sequence (R-Q-S-R); $F_{AMPV\text{-}A}$ bearing the multibasic sequence derived from AMPV-A (R-R-R-R) that contains the consensus furin motif; $F_{AMPV\text{-}C}$ bearing the cleavage sequence from AMPV-C (R-K-A-R) that, while not conforming to the consensus furin motif, is multibasic and contains a minimal furin motif (R-X-X-R); and F_{S101P} bearing the spontaneously derived cleavage sequence (R-Q-P-R; the difference underlined) from recently described variants of HMPV strains NL/1/00 and NL/1/99, a substitution that also was involved in the gain of intracellular cleavability by SeV mutants (26).

These viruses were recovered from cDNA and amplified by two passages in Vero cells. The recovery assays and passages were performed using culture medium containing trypsin to preclude selective pressure for cleavability. Following the final passage, the mutants were subjected to RT-PCR using primers specific to the end of the M gene and the beginning of the L gene. In each case, the uncloned RT-PCR product was completely sequenced, which in each case confirmed the presence

of the expected cleavage sequence (Fig. 1B), the absence of the SH and G genes, and the absence of adventitious mutations.

In vitro replication and trypsin independence of the F mutant viruses. The F_{AMPV-A}, F_{AMPV-C}, and F_{S101P} mutants were compared to F_{WT} (each in the rHMPVΔG/SH background) with regard to the plaques produced in the presence or absence of trypsin (Fig. 1B). Following a 6-day incubation in Vero cells under a methylcellulose overlay and in the presence of trypsin, the F_{WT} virus and the three F mutant viruses produced plaques that were similar in size. In parallel cultures lacking trypsin, the plaques formed by the F_{AMPV-A} and F_{AMPV-C} mutants were comparable in size to those formed in the presence of trypsin, whereas those produced by the F_{S101P} mutant each consisted of only a few contiguous infected cells. In comparison, the F_{WT} virus produced only single infected cells in the absence of trypsin, indicating a lack of cell-to-cell fusion activity as previously described with a recombinant rHMPV-GFP expressing the green fluorescent protein (5). Thus, introduction of the multibasic R-R-R-R and R-K-A-R cleavage sequences into HMPV resulted in the acquisition of efficient trypsin independent cell-to-cell fusion, whereas the R-Q-P-R site derived from the trypsin independent HMPV variants conferred limited trypsin independent cell-to-cell fusion.

The F mutant viruses also were examined for the ability to execute multicycle replication in Vero cells. Replicate monolayers were inoculated with an input MOI of 0.01 PFU per cell, samples from the overlying medium were collected daily, and the titers of released virus were determined by plaque titration. This experiment was done in three ways: (i) both the multicycle growth and the plaque assay were performed in the presence of trypsin; (ii) growth was performed without trypsin, but the plaque assay was performed with trypsin in order to detect progeny virus independent of whether the F protein was activated during growth; and (iii) both growth and the plaque assay were performed without trypsin in order to detect only virus containing active F protein. In the presence of trypsin, the F_{WT} and F mutant viruses replicated with similar efficiencies (Fig. 2A). When the F_{WT} virus was grown in the absence of trypsin, no cytopathic effect was evident and the virus titer detected by plaque assay with trypsin was reduced 100-fold (Fig. 2B). The presence of this virus at moderate titers throughout the 7-day period likely reflects continued release of this moderately cytopathic virus from the cells infected by the initial inoculum, progeny that remained noninfectious unless activated by trypsin. F_{WT} virus was not detected when growth and the plaque assay were both performed in the absence of trypsin (Fig. 2C), indicative of a strong trypsin dependence. When the F_{S101P} virus was grown in the absence of trypsin and assayed in its presence, its titer was approximately 10-fold lower compared to that when growth was performed in the presence of trypsin (Fig. 2B). Its titer was further reduced when growth and plaque assay were both in the absence of trypsin (Fig. 2C). This indicates that this virus was only partially trypsin independent. In contrast, the titers of the F_{AMPV-C} and F_{AMPV-A} viruses were largely independent of whether trypsin was present during growth and plaque assay. In the case of the F_{AMPV-C} virus, the titers were slightly reduced in the absence of trypsin, suggesting that this virus might not be fully trypsin independent. In the case of the F_{AMPV-A} virus, the titers with and without trypsin were essentially identical in repeat experiments, indicating that replication of this virus was unaffected by and independent of trypsin.

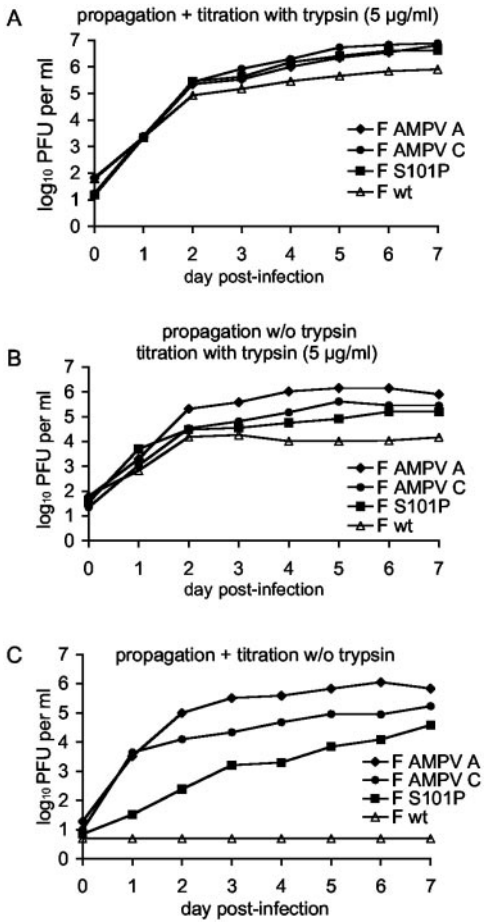

FIG. 2. Comparison of the multistep growth kinetics of the F cleavage site mutations in the presence or absence of trypsin. Monolayer cultures of Vero cells were infected at an input MOI of 0.01 PFU per cell with the indicated HMPVΔSH/G virus. Three conditions were evaluated: the multicycle growth and plaque assay were both performed in the presence of trypsin (A); multicycle growth was in the absence of trypsin, and the plaque assay was performed with trypsin (B); and multicycle growth and the plaque assay were both performed in the absence of trypsin (C). Supernatant aliquots (0.5 ml out of a total medium volume of 2 ml per well) were taken on the indicated days postinfection and replaced by an equivalent volume of fresh medium with or without trypsin as appropriate. The samples were flash frozen and analyzed later in parallel by plaque assay. Each time point was represented by two wells, and each virus titration was done in duplicate. Means are shown. The standard errors were calculated but the bars are not shown, because the errors were very small and the bars were obscured by the symbols. w/o, without.

Proteolytic processing of the F protein. The processing of the F protein mutants in vitro also was evaluated by Western blot analysis. Vero cells were infected at an input MOI of 5 PFU per cell and were incubated in the presence or the absence of trypsin. At 48 h postinfection, the cells were collected and lysates were prepared, and the overlying cell culture medium was collected and subjected to a low-speed clarification to remove cell debris. The cell lysates and clarified medium supernatants were separately denatured, reduced, and subjected to Western blot analysis.

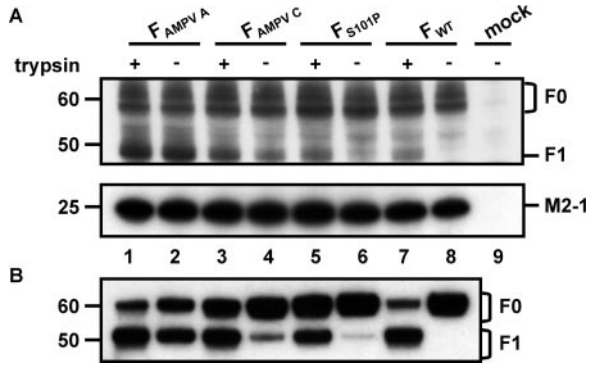

FIG. 3. Western blot analysis of F protein expressed in Vero cells infected with the rHMPVΔSH/G F cleavage site mutants in the presence or absence of trypsin. Cell lysates (A) or clarified medium supernatants (B) were analyzed to detect intracellular and virion-associated F protein, respectively. The samples were denatured, reduced, and analyzed on 4 to 12% polyacrylamide gels. Following electrotransfer, membranes were incubated with a hamster antiserum raised against an HPIV1 vector expressing the HMPV F protein (27) to detect the F protein (A, upper panel, and B) or with a rabbit antiserum raised against sucrose-purified HMPV virions (A, lower panel) to detect the M2-1 protein as a loading control. Bound antibodies were detected with a peroxidase-conjugated goat anti-rabbit or anti-hamster immunoglobulin G and were visualized by chemiluminescence. The HMPV proteins are indicated on the right, and the positions of molecular markers (in kilodaltons) are shown on the left.

In the Western blot of cell-associated proteins (Fig. 3A), upper and lower segments of the membrane were excised and reacted with two different antisera. The lower segment, containing the M2-1 protein, was incubated with a hamster hyperimmune serum raised against infectious HMPV, which showed that a comparable amount of this control protein was present for each virus (Fig. 3A, lower panel). The upper segment of the membrane, containing F_0 and the F_1 subunit, was incubated with an antiserum against the F protein that had been prepared by infecting hamsters with a recombinant human parainfluenza type 1 virus expressing the HMPV F protein from an added gene (Fig. 3A, upper panel). This showed that the F_{wt} protein was not detectably cleaved when growth was performed in the absence of trypsin (Fig. 3A, upper panel, lane 8), whereas some F_1 cleavage product was detected in its presence (lane 7). For the F protein mutants, in each case some F_1 cleavage product was detected in the absence of trypsin, with the amount increasing in the order $F_{S101P} < F_{AMPV\text{-}C} < F_{AMPV\text{-}A}$ (Fig. 3A, upper panel, lanes 6, 4, and 2, respectively). For the F_{S101P} and $F_{AMPV\text{-}C}$ mutants, the accumulation of F_1 was augmented when growth was performed in the presence of trypsin (Fig. 3A, upper panel, lanes 5 and 3, respectively), whereas in the case of $F_{AMPV\text{-}A}$ the amount of F_1 cleavage product was greater than that observed for the other viruses and did not appear to be significantly increased when growth was in the presence of trypsin (compare lanes 1 and 2).

The clarified cell culture medium supernatants also were subjected to Western blot analysis with the F-specific antiserum (Fig. 3B). In the absence of trypsin, all of the F_{WT} protein was in the form of the F_0 precursor, whereas in the presence of trypsin this was mostly in the form of the F_1 cleavage product (Fig. 3B, lanes 8 and 7, respectively). For the F protein mutants, some F_1 cleavage product was detected in the absence of trypsin, with the amount increasing in the order $F_{S101P} < F_{AMPV\text{-}C} < F_{AMPV\text{-}A}$ (Fig. 3B, lanes 6, 4, and 2, respectively). In each case, the amount of F_1 cleavage product was augmented in the presence of trypsin, although the increase for the $F_{AMPV\text{-}A}$ mutant was marginal. These data showed that, for each of the F mutants, some cleavage activation of F occurred in the absence of trypsin, with the amount being greater for the two mutants containing the greater number of basic residues, $F_{AMPV\text{-}C}$ and $F_{AMPV\text{-}A}$.

Replication in hamsters. Golden Syrian hamsters in groups of 18 were infected intranasally with 6 $\log_{10}$ PFU per animal of $F_{AMPV\text{-}A}$, $F_{AMPV\text{-}C}$, F_{S101P}, or F_{WT} virus (Table 1). Six animals from each group were sacrificed 3, 5, and 7 days later, the nasal turbinates and lungs were harvested, and viral titers were determined (Table 1). In addition, samples of the brain, liver, spleen, kidneys, thymus, and duodenum were harvested and analyzed by plaque assay for the presence of infectious virus. There were no significant differences ($P \leq 0.05$) in the virus titers in the nasal turbinates or lungs for the F mutants compared to those of F_{WT}, indicating that there were no significant differences in the efficiency of replication. In addition, virus was not detected in any of the six other organs that were analyzed. Thus, there was no evidence that any of the viruses replicated significantly outside of the respiratory tract. These results demonstrate that the acquisition of increased intracellular cleavability by the HMPV F protein was not associated with increased or prolonged replication in the respiratory tract or detectable dissemination from the respiratory tract and replication in other organs, at least in hamsters.

Replication, immunogenicity, and protective efficacy of the F_{WT} and $F_{AMPV\text{-}A}$ viruses in nonhuman primates. We also evaluated the F mutants in AGMs, an experimental animal that is reasonably permissive to HMPV infection and is more closely related anatomically and phylogenetically to the natural human host. Since growth in vitro of the $F_{AMPV\text{-}A}$ mutant was independent of trypsin, it was chosen for evaluation in parallel with rHMPVΔSH/G-F_{WT} and rHMPV; the latter served as a control virus that replicates to the level of biologically derived HMPV. Twelve AGMs that were negative for serum-neutralizing antibodies to HMPV (<1.5 reciprocal $\log_2$) were inoculated simultaneously by the intranasal and intratracheal routes

TABLE 1. Level of replication in the upper and lower respiratory tracts of hamsters of rHMPVΔSH/G mutants bearing the indicated F cleavage sites

Virus[a]	Mean peak titer[b] ($\log_{10}$ PFU/g ± SE) and % shedding[c] from:				
	Nasal turbinates			Lungs	
	Day 3	Day 5	Day 7	Day 3	Day 5
F_{WT}	3.6 ± 0.3	4.2 ± 0.2	1.6 ± 0.4 (50%)	2.6 ± 0.1	2.2 ± 0.3 (83%)
$F_{AMPV\text{-}A}$	3.7 ± 0.1	3.6 ± 0.2	1.2 ± 0.2 (66%)	2.6 ± 0.0	1.9 ± 0.4 (83%)
$F_{AMPV\text{-}C}$	3.7 ± 0.2	4.0 ± 0.1	0.9 ± 0.2 (50%)	2.7 ± 0.1	2.9 ± 0.1
F_{S101P}	3.4 ± 0.2	3.4 ± 0.3	1.1 ± 0.3 (50%)	2.5 ± 0.1	2.1 ± 0.2 (83%)

[a] Hamsters in groups of 18 were inoculated on day 0 by the intranasal route using a 0.1-ml inoculum containing 6.0 $\log_{10}$ PFU of the indicated virus.

[b] The level of virus replication is expressed as the geometric mean of the peak virus titers ($\log_{10}$ PFU/gram ± standard errors) for the animals in each group. The lower limit of detection is 0.7 $\log_{10}$ PFU/g. A value of 0.7 $\log_{10}$ PFU/g is assigned to samples with no detectable virus; samples from lungs on day 7 had no detectable virus.

[c] The percentage of shedding is indicated for each group when different from 0 or 100%.

TABLE 2. Level of replication of rHMPV and ΔSH/G-F_{WT} and ΔSH/G-F_{AMPV-A} derivatives in the upper and lower respiratory tracts of AGMs

Virus[a]	Nasopharyngeal swab			Tracheal lavage		
	Duration of shedding (days ± SE)	Mean peak titer[b] (log_{10} PFU/ml ± SE) and statistical grouping[c]	Reduction of mean peak titer[d] (log_{10} PFU/ml)	Duration of shedding (days ± SE)	Mean peak titer[b] (log_{10} PFU/ml ± SE) and statistical grouping[c]	Reduction of mean peak titer[d] (log_{10} PFU/ml)
rHMPV	9.3 ± 0.6	3.8 ± 0.1, A		8.5 ± 1.0	5.4 ± 0.2, A	
F_{WT}	3.5 ± 0.9	2.1 ± 0.3, B	1.7	4.0 ± 1.0	3.1 ± 0.1, B	2.3
F_{AMPV-A}	4.3 ± 1.2	1.3 ± 0.3, B	2.5	3.0 ± 0.0	2.6 ± 0.1, B	2.8

[a] AGMs in groups of four were inoculated on day 0 simultaneously by the intranasal and intratracheal routes by using a 1-ml inoculum per site containing 6.0 log_{10} PFU of F_{WT} or F_{AMPV-A}. Two animals were inoculated with 6.0 log_{10} PFU of rHMPV in parallel; results from 10 additional animals that were inoculated with rHMPV under identical conditions in previous experiments were included.

[b] The level of virus replication is expressed as the geometric mean of the peak virus titers (log_{10} PFU/milliliter ± standard errors) for the animals in each group irrespective of sampling day. The lower limit of detection was 0.7 log_{10} PFU/ml. A value of 0.7 log_{10} PFU/ml was assigned to samples with no detectable virus.

[c] Mean peak virus titers were assigned to statistically similar groups by the Tukey-Kramer post hoc test. Values within a column that share a common letter are not significantly different; values with different letters are significantly different ($P < 0.05$).

[d] Reduction of mean peak titer compared to that of rHMPV.

with 6.0 log_{10} PFU of virus per site. The groups inoculated with F_{AMPV-A} or F_{WT} contained four animals each, while the rHMPV and mock immunization control (L15 medium) groups each contained two animals; additionally, results from 10 AGMs that had been inoculated under identical conditions with 6.0 log_{10} PFU per site of rHMPV in previous studies were included. NP swabs and TL samples were collected at intervals over 12 days postinfection to monitor virus replication in the upper and lower respiratory tracts, respectively, and virus titers were determined subsequently by plaque assay. The AGMs were monitored daily for clinical symptoms (body temperature, feeding behavior, weight loss, and nasal discharge).

Compared to rHMPV, replication of the SH/G deletion mutants F_{WT} and F_{AMPV-A} was reduced about 50- and 310-fold in the upper respiratory tract and about 200-fold and 600-fold in the lower respiratory tract, respectively (Table 2). Furthermore, the peak of virus shedding in the upper respiratory tract was delayed 1 or 2 days, and the duration of shedding in the upper and lower respiratory tract was reduced (Fig. 4; Table 2). Compared with F_{WT}, replication by F_{AMPV-A} was somewhat reduced in the upper respiratory tract and also slightly reduced in the lower respiratory tract (Fig. 4; Table 2). Thus, there was no evidence of increased replication associated with the altered F cleavage sequence. None of the AGMs had disease signs during the experiment.

We also compared the immunogenicity and protective efficacy of F_{WT} and F_{AMPV-A}. This was done on the possibility that the F_{AMPV-A} virus might have increased spread to subluminal structures or to immune cells such as macrophages or dendritic cells that might not be detected by virus shedding but might result in enhanced immunogenicity. The levels of serum HMPV-neutralizing antibodies induced by infection with F_{wt} or F_{AMPV-A} were similar to each other and slightly (but with no statistical significance) reduced compared to that of rHMPV (Table 3). Twenty-eight days postimmunization, the AGMs were challenged by the intranasal and intratracheal routes with 6.0 log_{10} PFU of rHMPV per site. Each of the animals that had been previously infected with one of the ΔSH/G mutants was completely protected against HMPV challenge, as assayed by virus titration of NP and TL samples. Also, there were no significant differences in the titers of HMPV-neutralizing antibodies in sera collected 28 days postchallenge (Table 3). Thus, there was no evidence of a change in immunogenicity and protective efficacy associated with the altered F cleavage sequence.

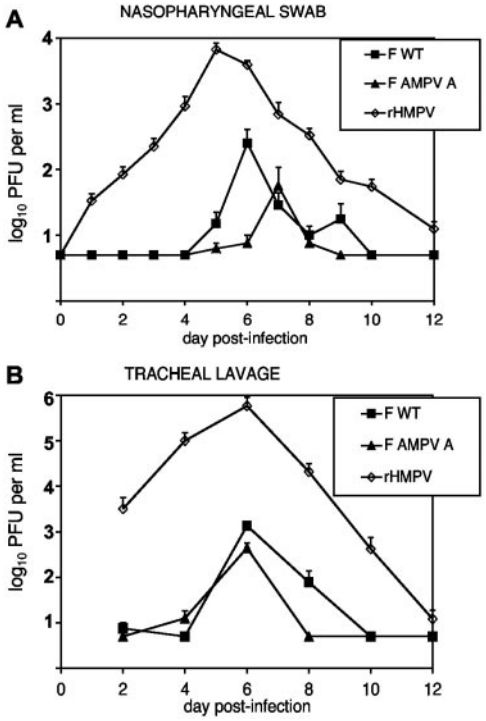

FIG. 4. Kinetics of replication of rHMPV and the ΔSH/G-F_{WT} and ΔSH/G-F_{AMPV-A} mutants in the upper and lower respiratory tracts of African green monkeys. Two animals (rHMPV group) or four animals (ΔSH/G-F_{WT} and ΔSH/G-F_{AMPV-A} groups) were each inoculated on day 0 by the combined intranasal and intratracheal routes with a 1-ml inoculum per site containing 10^6 PFU of the indicated virus. Results from 10 additional animals that were inoculated with rHMPV under identical conditions in previous experiments were included. The nasopharyngeal swab (A) and tracheal lavage (B) specimens were taken on the indicated days, and the titers of shed virus were quantified by plaque assay. The detection limit was 0.7 log_{10} PFU/ml.

TABLE 3. Immunogenicity and protective efficiency of rHMPV, rHMPVΔSH/G-F_{WT}, and F_{AMPV-A} in AGMs

Virus[a]	Mean serum-neutralizing antibody titer[b] (reciprocal $\log_2$ ± SE)		Challenge virus replication after challenge[c] from:			
			Nasopharyngeal swab		Tracheal lavage	
	28 Days postimmunization	28 Days after challenge	Duration of shedding (days ± SE)	Mean peak titer ($\log_{10}$ PFU/ml ± SE)	Duration of shedding (days ± SE)	Mean peak titer ($\log_{10}$ PFU/ml ± SE)
Mock	≤1.5	9.2 ± 0.5	8.0 ± 1.0	4.0 ± 0.1	7.0 ± 0.0	4.9 ± 0.6
rHMPV	8.1 ± 0.4[d]	8.3 ± 0.3	1.9 ± 0.8	0.9 ± 0.1	0.1 ± 0.1	0.7 ± 0.0
F_{WT}	7.3 ± 0.2[d]	7.0 ± 0.7	0.0 ± 0.0	≤0.7	0.0 ± 0.0	≤0.7
F_{AMPV-A}	6.7 ± 0.6[d]	8.2 ± 0.5	0.0 ± 0.0	≤0.7	0.0 ± 0.0	≤0.7

[a] AGMs in groups of four (F_{wt} and F_{AMPV-A}) or two (mock) animals were immunized by intranasal and intratracheal infections using a 1-ml inoculum per site containing 6.0 $\log_{10}$ PFU of the indicated virus or L15 medium (mock). Two animals were inoculated with rHMPV in parallel; results from 10 animals which were inoculated with rHMPV under identical conditions in previous experiments were included.

[b] Sera were collected on days 0 and 28 following the first infection, and the neutralizing antibody titer against HMPV was determined. The preinfection anti-HMPV serum titers were ≤1.5 (reciprocal $\log_2$) for each animal in the study.

[c] On day 28, AGMs from each group were challenged intranasally and intratracheally with 6.0 $\log_{10}$ PFU of rHMPV. The level of virus replication is expressed as the geometric mean of the peak virus titers ($\log_{10}$ PFU/illiliter ± standard errors) for the animals in each group irrespective of sampling day. The lower limit of detection is 0.7 $\log_{10}$ PFU/ml. A value of 0.7 $\log_{10}$ PFU/ml is assigned to samples with no detectable virus.

[d] Not statistically different ($P < 0.05$) by the analysis of variance/Tukey-Kramer post-hoc test.

DISCUSSION

HMPV appears to be associated with severe disease much less frequently than is the case for HRSV. The clinical isolates described to date for HMPV require the addition of trypsin for growth in vitro, raising the possibility that HMPV virulence is restricted by the inefficient cleavage phenotype of its F protein. It also raised the possibility that the characteristically inefficient, slow growth of HMPV in vitro might be due to the cleavage phenotype of its F protein. Therefore, we replaced the naturally occurring HMPV cleavage sequence with three different ones derived from F proteins that are cleaved intracellularly, namely, those of AMPV-A, AMPV-C, and a variant of HMPV that acquired trypsin independence during passage in vitro (26). Each of the three inserted sequences conferred trypsin independent growth in vitro with an efficiency that increased in the order $F_{S101P} < F_{AMPV-C} < F_{AMPV-A}$. The trypsin independence of HMPV-F_{S101P} and F_{AMPV-C} was incomplete, since cleavage of the F protein and the titer of infectious virus were increased in the presence of added trypsin. In contrast, F_{AMPV-A} appeared to be fully trypsin independent.

Evaluation of the panel of F mutants in hamsters and, for F_{AMPV-A}, in AGMs showed that the F cleavage sequence substitutions made no detectable difference in the ability of the mutant viruses to replicate in the upper and lower respiratory tract of hamsters and AGMs. Also, there was no detectable spread in hamsters of virus from the respiratory tract. Thus, the F mutants had no detectable effect on the ability of HMPV to spread within or beyond the respiratory tract. This supported the interpretation that the virulence of HMPV is not greatly affected by the cleavage phenotype of its F protein. HMPV is avirulent in hamsters or African green monkeys, and thus virulence could not be evaluated directly by disease and mortality; those properties are reliably observed only in seronegative human infants. Therefore, the magnitude of replication was used as a marker of virulence. This premise is supported by clinical studies of vaccine candidates for HRSV and parainfluenza virus type 3 in which respiratory disease signs were associated with a higher level of virus replication (2, 17, 35). Also, the magnitude of disease associated with infection of paramyxoviruses such as bovine respiratory syncytial virus, pneumonia virus of mice, SeV, and NDV in their respective animal hosts has a positive correlation with the level of virus replication.

The conclusion that the gain of function of intracellular cleavability was not associated with increased virulence is offered with the caveat that this study involved an attenuated HMPV backbone. Attenuation was achieved by deleting the G and SH proteins, with deletion of G conferring most of the attenuation (6). One obvious issue is the possibility that increased spread within or beyond the respiratory tract might have been more possible or more easily detected with mutants based on wild-type HMPV because of its more efficient replication. We agree but maintain that it was necessary and appropriate to perform this initial study in an attenuated background for biosafety concerns. As already noted, quantitative studies in humans and/or mammals with respiratory viruses indicate that virulence is, in large part, a function of the level of replication of the virus in the host. In any event, even wild-type HMPV does not reliably cause disease in these experimental animals and thus in that regard offers no advantage. Although the ΔSH/G virus is attenuated, it replicated detectably in the infected hamsters out to at least day 7 and achieved a titer of 4.0 $\log_{10}$. Given the lack of enhanced replication or spread observed in the present study, it would now be reasonable to evaluate the R-R-R-R cleavage sequence in a wild-type HMPV background, perhaps under biosafety level 3 conditions. Such a study could include direct evaluation of lung histopathology to monitor virus localization more directly.

Another issue is whether the absence of SH and G might have affected the outcome of the study due to functional considerations. We chose this specific method of attenuation mostly because our options were limited. We wanted to use a gene deletion because of its presumed stability, and in any event these were the only available HMPV-attenuating mutations. There was the alternative possibility of deleting the M2-2 open reading frame, but that deletion reduced replication in hamsters below the threshold of detection (10) and thus would be overattenuated for the purposes of this study. For most members of *Paramyxovirinae*, fusion depends on an interaction between the F protein and its cognate attachment protein (12).

However, there is no evidence that cleavage is dependent on such an interaction, and every *Paramyxovirinae* F protein that has been expressed by itself by a heterologous vector has been found to be processed and cleaved without any apparent deficiency. Furthermore, in the case of *Pneumovirinae*, the F proteins do not depend upon their cognate attachment proteins for fusion function and are processed efficiently and correctly into a biologically active form when expressed in the absence of the other viral proteins (16, 21, 29). Thus, we believe that the absence of SH and G did not significantly affect the outcome of this study.

The most compelling evidence of a relationship between intracellular cleavability of F and virulence comes from naturally occurring strains of NDV, which exhibit a wide spectrum of virulence that is determined in large part by the cleavability of the F protein (12, 23). This association also was demonstrated for SeV, which normally is dependent on extracellular cleavage and is strictly pneumotropic, but for which mutants have been identified that have gained intracellular cleavability and the ability to infect and spread to other tissues (20, 30). A positive correlation between intracellular cleavability and virulence also is well known for the hemagglutinin protein of avian influenza A viruses (36). Also, the ability of the reconstructed 1918 pandemic influenza virus hemagglutinin to be cleaved intracellularly, albeit by a mechanism that does not involve a multibasic cleavage sequence, makes an important contribution to its virulence (31). Evidence for a comparable relationship between intracellular cleavability and virulence is less clear for the human paramyxoviruses. Modification of the cleavage site of recombinant measles virus (MeV) from the naturally occurring intracellularly cleaved form (R-H-K-R) was associated with a loss of intracellular cleavage and reduced virulence in a mouse model (17a), but results involving loss-of-function mutations should be interpreted with caution because of the possibility of nonspecific effects. Variation in cleavability has not been reported in naturally occurring strains of MeV and is not involved in the attenuated MeV vaccine strains. Among the HPIVs, HPIV3 and HPIV2 are cleaved intracellularly whereas HPIV1 and HPIV4 are not, but it is not clear what impact this has on virulence. Differences in the cleavage sequence have been noted for strains of HPIV2 and HPIV3, for which some strains conform to the consensus furin motif R-X-K/R-R and others conform to the minimal motif R-X-X-R (1, 13). In the case of HPIV2, the minimal motif was associated with reduced intracellular cleavability in Vero cells but not in primary simian cells, whereas for HPIV3 there was no evidence of restriction in vitro or in vivo. Thus, there is no evidence of naturally occurring differences in virulence associated with cleavability for the HPIVs, although this has not been investigated exhaustively.

The present paper, together with the recent report of Schickli et al. (26), are the first studies to investigate a possible relationship between intracellular cleavage activation of F and virulence for a member of *Pneumovirinae*. The report of Schickli et al. involved a point mutation that confers intracellular cleavability without introducing a furin motif. It is not known what protease mediates this cleavage or whether this necessarily would confer pantropism. In contrast, the present study involves cleavage sequences that contain the furin motif, albeit in an attenuated background. Taken together, these studies suggest that F cleavability is not an important determinant of virulence for these viruses.

ACKNOWLEDGMENTS

This research was supported by the Intramural Research Program of the NIH, NIAID.

We thank Jens Fricke and Cindy Luongo for helpful discussions and Guy Boivin (Laval University, Quebec, Canada), Teresa Peret, Dean Erdman, and Larry Anderson (CDC, Atlanta, GA) for providing an initial inoculum of HMPV83.

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