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An S101P Substitution in the Putative Cleavage Motif of the Human Metapneumovirus Fusion Protein Is a Major Determinant for Trypsin-Independent Growth in Vero Cells and Does Not Alter Tissue Tropism in Hamsters

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Human metapneumovirus (hMPV), a recently described paramyxovirus, is a major etiological agent for lower respiratory tract disease in young children that can manifest with severe cough, bronchiolitis, and pneumonia. The hMPV fusion glycoprotein (F) shares conserved functional domains with other paramyxovirus F proteins that are important for virus entry and spread. For other paramyxovirus F proteins, cleavage of a precursor protein (F_0) into F_1 and F_2 exposes a fusion peptide at the N terminus of the F_1 fragment, a likely prerequisite for fusion activity. Many hMPV strains have been reported to require trypsin for growth in tissue culture. The majority of these strains contain RQSR at the putative cleavage site. However, strains hMPV/NL/1/00 and hMPV/NL/1/99 expanded in our laboratory contain the sequence RQPR and do not require trypsin for growth in Vero cells. The contribution of this single amino acid change was verified directly by generating recombinant virus (rhMPV/NL/1/00) with either proline or serine at position 101 in F. These results suggested that cleavage of F protein in Vero cells could be achieved by trypsin or S101P amino acid substitution in the putative cleavage site motif. Moreover, trypsin-independent cleavage of hMPV F containing 101P was enhanced by the amino acid substitution E93K. In hamsters, rhMPV/93K/101S and rhMPV/93K/101P grew to equivalent titers in the respiratory tract and replication was restricted to respiratory tissues. The ability of these hMPV strains to replicate efficiently in the absence of trypsin should greatly facilitate the generation, preclinical testing, and manufacturing of attenuated hMPV vaccine candidates.

Human metapneumovirus (hMPV) is a recently identified respiratory virus that was initially isolated from children in The Netherlands experiencing symptoms of acute respiratory disease with undetermined etiology. hMPV causes respiratory illness ranging from mild upper respiratory symptoms to severe lower respiratory disease such as bronchiolitis and pneumonia (6, 60, 63). Depending on the patient population sampled, between 5 and 15% of respiratory infections in young children may be attributable to hMPV infection (7, 62, 65). hMPV is also associated with 12 to 50% of otitis media in children (7, 36, 62). In The Netherlands, 55% of tested individuals were seropositive for hMPV by age 2, and almost all individuals 5 years and older were seropositive (59). The distribution of hMPV is world wide, with reports from Europe, North America, Australia, Africa, Israel, Japan, and Hong Kong (4, 18, 20, 21, 30, 34, 36, 38, 45, 66). Testing of archived serum samples indicated that hMPV has been circulating in the population for at least 50 years (60). One reason why it has only been recently identified is that it grows poorly in cell culture with minimal cytopathic effects (13, 18, 60).

hMPV is an enveloped single-stranded negative-sense RNA virus of the *Pneumovirinae* subfamily in the *Paramyxoviridae* family that also includes respiratory syncytial virus (RSV), avian pneumovirus (APV), and pneumovirus of mice (5, 60). Based on homology with other pneumoviruses, eight transcription units have been identified in the following order: 3′-N-P-M-F-M2-SH-G-L-5′ (5, 56, 59). Phylogenetic analysis divides the hMPV strains into two genetic clusters, designated subgroups A and B, that are distinct from APV viruses (3–5, 37, 38, 59). The hMPV A and B subgroups can be further divided into four subtypes, A1, A2, B1, and B2 (59, 61).

The hMPV fusion glycoprotein (F), which is highly conserved between subgroups A and B, shares homology with F proteins of other paramyxoviruses (59). F proteins of pneumoviruses such as RSV and APV are synthesized as full-length precursors (F_0) that are subsequently cleaved at a polybasic furin-like cleavage site to form F_1 and F_2. Cleavage of F_0 exposes a fusion peptide at the N terminus of F_1 which mediates virus penetration and syncytium formation (12, 28, 31, 39, 64). Unlike RSV and APV, the putative cleavage site of hMPV (RQSR) does not conform to the furin-like motif (2, 59).

Reports in the literature indicate that trypsin is required for isolation of hMPV from clinical samples in cell culture (3–6, 13, 18, 37, 38, 43, 60, 61). Therefore, it was unexpected that two isolates of hMPV, strains hMPV/NL/1/00 and hMPV/NL/1/99 (A1 and B1 subtypes, respectively), grew in Vero cells without addition of trypsin. Equally high titers were achieved in the absence or presence of trypsin. We sequenced reverse transcription (RT)-PCR products of wild-type (wt) hMPV/NL/1/00 and wt hMPV/NL/1/99 and found a single nucleotide change that encodes the amino acid substitution S101P in the RQSR motif at the putative cleavage site of F protein compared to the

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previously published sequences GI:20150834 and GI:50059145 (GenBank). In the results reported here, we demonstrated that for both strains hMPV/NL/1/00 and hMPV/NL/1/99, viruses harboring an RQPR motif at the putative cleavage site of the F glycoprotein were able to replicate in Vero cells without exogenously added trypsin. In contrast, hMPV that contained RQSR in the cleavage motif of the F protein required the addition of a protease such as trypsin for viral growth. We evaluated in vitro growth properties, syncytium formation, and cleavage of F glycoproteins of hMPV viruses with amino acid substitutions within and near the putative cleavage motif in the absence and presence of trypsin. S101P in hMPV F was found to be the major genetic determinant that enhanced the cleavage efficiency of F and increased its fusion activity, both of which likely contributed to efficient growth of wt hMPV/NL/1/00 and wt hMPV/NL/1/99 in Vero cells in the absence of trypsin. We also evaluated a trypsin-independent hMPV strain in Syrian Golden hamsters, and the enhanced cleavage of F protein did not alter the replication titers in respiratory tissues, nor did it extend the tissue tropism. Trypsin-independent growth properties offer potential advantages for generating viral stocks for study, preclinical testing, and vaccine manufacture.

MATERIALS AND METHODS

Cells and viruses. Vero cells (American Type Culture Collection; not more than passage 148) were maintained in minimal essential medium (JHR Biosciences) supplemented with 10% fetal bovine serum (HyClone), 2 mM L-glutamine (Gibco BRL), nonessential amino acids (Gibco BRL), and 100 U/ml penicillin G sodium with 100 μg/ml streptomycin sulfate (Biowhittaker). BSR/T7 cells (kindly provided by K. Conzelmann) were maintained in Glasgow minimal essential medium (Gibco BRL) supplemented with 10% fetal bovine serum, 5% tryptose phosphate broth (Sigma), nonessential amino acids, 1 mg/ml G418 (Gibco BRL), and 100 U/ml penicillin G sodium with 100 μg/ml streptomycin sulfate. hMPV and chimeric bovine/human (b/h) parainfluenza virus type 3 (PIV3) were propagated in Vero cells with optiMEM (Gibco BRL) and 100 U/ml penicillin G sodium with 100 μg/ml streptomycin sulfate. Some viruses were propagated with 0.2 μg/ml L-(tosylamido-2-phenyl) ethyl chloromethyl ketone (TPCK) trypsin (Worthington), equivalent to 40 mU/ml. Virus stocks were harvested by scraping the cells and supernatant together with 10× SPG (10× SPG is 2.18 M sucrose, 0.038 M KH_2PO_4, 0.072 M K_2HPO_4, 0.054 M L-glutamate at pH 7.1) to a final concentration of 1× SPG and frozen at −70°C.

The virus isolates wt hMPV/NL/1/93, wt hMPV/NL/1/94, wt hMPV/NL/1/99 and wt hMPV/NL/1/00 were described previously (19, 60). The following recombinant viruses were generated by reverse genetics from full-length cDNA plasmids: rhMPV/NL/1/00/101P, rhMPV/NL/1/00/101S, rhMPV/NL/1/99/101S, rhMPV/93K/101S, rhMPV/93K/101P, b/h PIV3/hMPV F/101P, and b/h PIV3/hMPV F/101S. The variant viruses vhMPV/93K/101P and vhMPV/100K/101P were derived from rhMPV/NL/1/00/101P after growth in Vero cells.

Titer by immunostaining of hMPV plaques. Virus titers (PFU/milliliter) were determined by plaque assay in Vero cells. Plaques were immunostained with anti-hMPV ferret polyclonal antisera (MedImmune Vaccines, Inc.) diluted 1:500 in phosphate-buffered saline (PBS) containing 5% powdered milk (wt/vol) (PBS-milk). The cells were then incubated with horseradish peroxidase-conjugated goat anti-ferret antibody (Ab) (Dako) diluted 1:1,000 followed by incubation with 3-amino-9-ethylcarbazole (Dako) to visualize plaques.

Construction of full-length hMPV cDNA plasmids. cDNAs of hMPV/NL/1/00 (containing 101S) and hMPV/NL/1/99 (containing 101S) were constructed as previously described and used to recover the recombinant viruses named rhMPV/NL/1/00/101S and rhMPV/NL/1/99/101S (19). T3367C and G3343A that encode S101P and E93K, respectively, in the predicted amino acid sequence of hMPV F were introduced using a Quik-change site-directed mutagenesis kit (Stratagene). All cDNAs were sequenced to confirm that no other nucleotide changes were introduced.

Construction of b/h PIV3/hMPV F2 full-length cDNA. b/h PIV3/hMPV F2 (expressing hMPV F containing 101S) was previously described (48). Briefly, the hMPV F gene was inserted between the N and P genes of a chimeric b/h PIV3 cDNA (16, 17). The nucleotide change corresponding to T3367C in the hMPV/NL/1/00 genome was introduced in the hMPV F gene of b/h PIV3/hMPV F2 using a Quik-change mutagenesis kit (Stratagene), resulting in b/h PIV3/hMPV F/101P that expresses hMPV F with proline at amino acid 101.

Generation of recombinant hMPV viruses by reverse genetics. Recombinant viruses were generated from cDNA as described previously (19). Briefly, 1.2 μg of pCITE hMPV N, 1.2 μg of pCITE hMPV P, 0.9 μg of pCITE hMPV M2, 0.6 μg pCITE hMPV L, and 5 μg of full-length hMPV cDNA plasmid in 500 μl optiMEM containing 10 μl Lipofectamine 2000 (Invitrogen) were applied to a monolayer of 10^6 BSR/T7 cells. The medium was replaced with optiMEM 15 h posttransfection and incubated at 35°C for 2 to 3 days. Both scraped cells and supernatant from the transfection were used to infect Vero cells, and virus recovery was verified by positive immunostaining with ferret polyclonal Ab directed to hMPV 6 days postinoculation. Recovered viruses were further amplified in Vero cells by inoculating at a multiplicity of infection (MOI) of 0.1 PFU/cell. Some transfections and growth were performed in the presence of 0.2 μg/ml TPCK trypsin.

RT-PCR of recovered viruses for nucleotide sequence analysis. Total RNA was extracted from hMPV-infected cells using TRizol reagent according to the manufacturer's instructions. RT-PCR products were generated using a one-step RT-PCR kit (Invitrogen). Chromatograms were generated using DNA isolated from agarose gels using a gel extraction kit (QIAGEN).

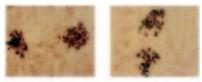
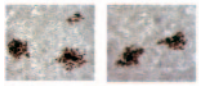
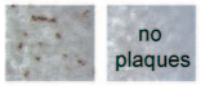
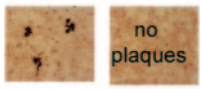
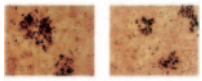
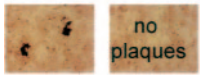
Multicycle growth of hMPV viruses in Vero cells. Subconfluent monolayers of Vero cells in tissue culture-treated 6-well plates were inoculated at an MOI of 0.1 PFU/cell with hMPV virus diluted in optiMEM in either the absence or presence of 0.2 μg/ml TPCK trypsin. After 1 h of adsorption at 35°C, the viral inoculum was replaced with 2 ml per well of optiMEM with or without 0.2 μg/ml TPCK trypsin. Cells combined with supernatant were collected at 24-h intervals for 6 days and frozen at −70°C with SPG. Virus titers were determined by plaque assay in Vero cells with or without 0.2 μg/ml TPCK trypsin.

Surface expression of hMPV F glycoprotein by indirect immunofluorescence. Subconfluent monolayers of Vero cells on glass coverslips were inoculated at an MOI of 5 PFU/cell. Following incubation at 35°C for 3 days, the cells were fixed in PBS containing 3% paraformaldehyde for 10 min. The monolayers were blocked in PBS-milk and immunostained with anti-hMPV F hamster monoclonal Ab (MAb) 121-1017-133 (MedImmune, Inc.) diluted 1:250 in PBS-milk followed by fluorescein isothiocyanate-conjugated anti-Armenian hamster Ab (Jackson Laboratories) diluted 1:1,000 in PBS-milk.

Western blot analysis of hMPV F protein. Subconfluent monolayers of Vero cells were infected with hMPV viruses at an MOI of 0.1 PFU/cell and incubated at 35°C. Four to 6 days postinfection, cells plus supernatants were collected and frozen at −70°C. Samples were prepared by the addition of an equal volume of 2× Laemmli sample buffer (Bio-Rad) containing 5% β-mercaptoethanol (Sigma). Proteins were separated on a 12% polyacrylamide Tris-HCl Ready gel (Bio-Rad) and transferred to a Hybond-P polyvinylidene difluoride membrane (Amersham Biosciences) using a wet transfer cell (Bio-Rad). Membranes were probed with anti-hMPV F MAb 121-1017-133 diluted 1:2,000 in PBS-milk, followed by incubation with horseradish peroxidase-conjugated anti-hamster MAb diluted 1:1,000 in PBS-milk. Membranes were developed with a chemiluminescence substrate (Amersham Biosciences) and exposed to Biomax MR film (Kodak) for visualization of hMPV F protein.

Syncytium formation of hMPV-infected Vero cells. Monolayers of confluent Vero cells in tissue culture-treated 6-well plates were inoculated, in duplicate, at an MOI of 3 PFU/cell or mock infected. Cells were overlaid with 2% methyl cellulose mixed 1:1 with optiMEM with or without 0.2 μg/ml TPCK trypsin. At 48 h or 72 h, the cells were fixed with methanol for 15 min, washed with PBS, and incubated with Hoechst stain solution (0.25 μg/ml of bisbenzimide H 33258 [Sigma] in PBS). Aggregated nuclei of fused cells and single nuclei of unfused cells in 10 randomly selected fields of view (totaling more than 2,000 nuclei) were counted and reported as the percentage of fused cells.

Replication of rhMPV/93K/101S and rhMPV/93K/101P in Syrian Golden hamsters. Five-week-old Syrian Golden hamsters (eight animals per group) were infected intranasally with 10^6 PFU/animal of virus or placebo medium in 100 μl. Four days postinfection, the nasal turbinates, lungs, brains, hearts, kidneys, livers, and spleens were harvested from four animals per group, homogenized, and titered by plaque assay in Vero cells. On day 28 postinfection, sera were obtained immediately prior to challenge for determining neutralization titers by 50% plaque reduction assay as described previously (46). For the challenge, immunized hamsters were inoculated intranasally with 10^6 PFU/animal of wt hMPV/NL/1/00. Four days postchallenge, the nasal turbinates and lungs were harvested and assayed for challenge virus replication by plaque assay.

hMPV strain	subtype	Titer (log_{10} PFU/ml) + trypsin	Titer (log_{10} PFU/ml) - trypsin	Plaque Morphology in Vero cells + trypsin	Plaque Morphology in Vero cells - trypsin
wt hMPV/NL/1/00	A1	7 .4	7.2		
rhMPV/NL/1/00/101P		7.8	7.6		
rhMPV/NL/1/00/101S		7.0	no growth		no plaques
wt hMPV/NL/1/93	A2	6.7	no growth		no plaques
wt hMPV/NL1/99	B1	6.1	6.4		
wt hMPV/NL/1/94	B2	5.7	no growth		no plaques

1.5 mm

FIG. 1. Titers and plaques of four subtypes of wt hMPV and recombinant hMPV. Subconfluent monolayers of Vero cells were inoculated with each of the indicated biologically derived viruses, rhMPV/NL/1/00/101P or rhMPV/NL/1/00/101S, at an MOI of 0.1 PFU/cell with or without 0.2 μg/ml TPCK trypsin. The cells and supernatants were collected 6 days postinoculation and frozen at −70°C. Titers, expressed as log_{10} PFU/milliliter, were determined by plaque assay in Vero cells. Plaques were visualized by incubating infected Vero cell monolayers under 1% methylcellulose for 6 days, immunostaining with ferret anti-hMPV polyclonal Ab, and treating with 3-amino-9-ethylcarbazole.

RESULTS

Trypsin requirement for growth in Vero cells differs among the four representative subtypes of wt hMPV. Biologically derived strains of hMPV representing all four subtypes, A1, A2, B1, and B2, were grown in Vero cells. As expected, wt hMPV/NL/1/93 and wt hMPV/NL/1/94, representative of subtypes A2 and B2, respectively, grew only when trypsin was present in the medium. wt hMPV/NL/1/93 grew to a titer of 6.7 log_{10} PFU/ml, while wt hMPV/NL/1/94 achieved a titer of 5.7 log_{10}. No plaques were detected when trypsin was not present in the medium overlay (Fig. 1). In marked contrast, wt hMPV/NL/1/00 and wt hMPV/NL/1/99, representative of subtypes A1 and B1, respectively, grew to high titers of 6.1 to 7.4 log_{10} PFU/ml in the absence as well as in the presence of trypsin. The plaques, as visualized by immunostaining, were roughly 0.3 to 0.5 mm in diameter after 6 days of growth in Vero cells under 1% methylcellulose (Fig. 1). The diameters of plaques produced in the presence or absence of trypsin by wt hMPV/NL/1/00 and wt hMPV/NL/1/99 were markedly larger than plaques produced by wt hMPV/NL/1/93 or wt hMPV/NL/1/94 with trypsin. Similar titers, plaque morphologies, and trypsin requirements were observed in LLC-MK2 cells for all hMPV subtypes (data not shown). Thus, the two strains of hMPV that did not require trypsin grew to higher titers and had significantly larger plaque sizes than the two strains that required trypsin.

The trypsin requirement for growth in tissue culture may reflect a dependence upon trypsin for cleavage of the fusion protein, suggesting that the cleavage site may differ between these hMPV viruses. The published sequences of the F glycoproteins of all four hMPV subtypes predict an RQSR motif at the putative cleavage site. Sequencing of the F gene confirmed that wt hMPV/NL/1/93 and wt hMPV/NL/1/94 had the predicted RQSR sequence. However, the sequences of wt hMPV/NL/1/00 and wt hMPV/NL/1/99 had a T3367C change that resulted in a predicted S101P amino acid substitution in F protein such that the putative cleavage site was now RQPR. The effect of S101P substitution on trypsin-independent growth of hMPV was further characterized.

Comparison of rhMPV/NL/1/00/101S and rhMPV/NL/1/00/101P replication in Vero cells. To determine if the S101P amino acid substitution in hMPV F contributed to trypsin-independent growth of wt hMPV/NL/1/00, we introduced a T at nucleotide (nt) 3367 into full-length cDNA of hMPV/NL/1/00 to generate rhMPV/NL/1/00/101S or a C at nt 3367 to generate rhMPV/NL/1/00/101P. rhMPV/NL/1/00/101P was readily recovered in the absence of trypsin and formed plaques comparable to those of wt hMPV/NL/1/00 (Fig. 1). In marked contrast, rhMPV/NL/1/00/101S was recovered only in the presence of trypsin and formed plaques significantly smaller than those of rhMPV/NL/1/00/101P (Fig. 1).

Multicycle growth curves were performed in the presence or absence of trypsin. Quantification of infectious virus at each time point was carried out by plaque assays in either the presence or absence of trypsin (Fig. 2A). In the presence of trypsin, both rhMPV/NL/1/00/101S and rhMPV/NL/1/00/101P grew ef-

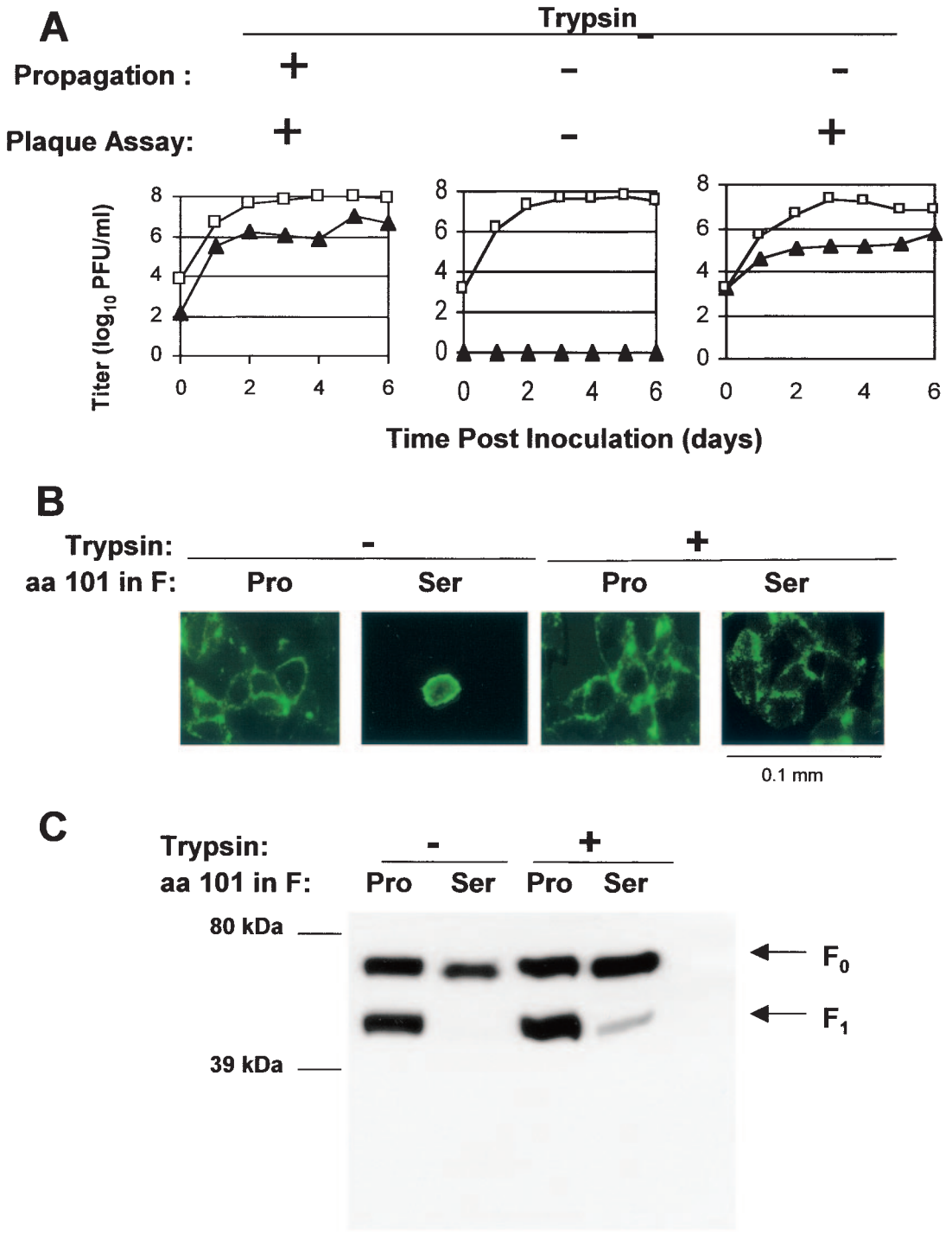


FIG. 2. Comparison of growth properties of rhMPV/NL/1/00/101P and rhMPV/NL/1/00/101S (subtype A1 hMPV). (A) Multicycle growth curves of rhMPV/NL/1/00/101P (open squares) and rhMPV/NL/1/00/101S (closed triangles) were performed in Vero cells at an MOI of 0.1 PFU/cell with or without 0.2 μg/ml trypsin. Titers were determined by plaque assay in Vero cells with or without 0.2 μg/ml trypsin. (B) Surface expression of hMPV F was detected in Vero cells infected with either rhMPV/NL/1/00/101P or rhMPV/NL/1/00/101S with or without 0.2 μg/ml trypsin. Infected cell monolayers were fixed in 3% paraformaldehyde and immunostained with hamster MAb 121-1017-133 directed to hMPV F followed by fluorescein isothiocyanate-conjugated anti-hamster Ab. (C) Western blot analysis of hMPV F protein was performed on viral harvests of Vero cells infected with either rhMPV/NL/1/00/101P or rhMPV/NL/1/00/101S with or without 0.2 μg/ml trypsin. The Western blots were probed with hamster MAb 121-1017-133 directed to hMPV F. The arrows indicate positions of bands corresponding to the predicted sizes of full-length precursor hMPV F (F_0) and cleavage fragment hMPV F_1.

ficiently. rhMPV/NL/1/00/101P reached a titer of 7.8 $\log_{10}$ PFU/ml on day 3, while rhMPV/NL/1/00/101S achieved a titer of 7.0 $\log_{10}$ PFU/ml on day 5 (Fig. 2A). In the absence of trypsin, only rhMPV/NL/1/00/101P underwent multicycle growth, reaching a titer of 7.6 $\log_{10}$ on day 3, similar to growth in the presence of trypsin. No rhMPV/NL/1/00/101S was detected when trypsin was omitted in the plaque assay (Fig. 2A). Interestingly, rhMPV/NL/1/00/101S virus particles that had been propagated without trypsin formed plaques when trypsin was present during the plaque assay. The peak titer of rhMPV/NL/1/00/101S propagated without trypsin was about 2 $\log_{10}$ lower than that of rhMPV/NL/1/00/101P (Fig. 2A). The presence of infectious virus suggested that rhMPV/NL/1/00/101S virions were generated during replication without trypsin; however, they were not infectious until activated by trypsin. These data suggested that the predicted 101P in hMPV F protein contributed significantly to the trypsin-independent growth of rhMPV/NL/1/00/101P.

Effect of S101P on surface expression of hMPV F protein in rhMPV-infected cells. During viral infection, paramyxovirus fusion proteins are transported to the plasma membrane where virus budding occurs. To determine whether the poor growth of rhMPV/NL/1/00/101S was caused by impaired surface expression of hMPV F, infected monolayers were fixed in paraformaldehyde, and surface expression of hMPV F protein was visualized by immunofluorescence assay. hMPV viruses that had been collected after propagation in the absence of trypsin, as in Fig. 2A, were used to inoculate Vero cell monolayers in either the absence or presence of trypsin. In the presence of trypsin, F protein was readily detected on the plasma membranes of almost 100% of the cells infected with either rhMPV/NL/1/00/101P or rhMPV/NL/1/00/101S, suggesting that hMPV F/101S and hMPV/101P were both efficiently expressed on the plasma membrane (Fig. 2B). In the absence of trypsin, only cells infected with rhMPV/NL/1/00/101P resulted in robust surface expression of hMPV F, and it was at levels comparable to inoculation without trypsin. In marked contrast, there were only a few F-positive cells in the monolayer inoculated with rhMPV/NL/1/00/101S in the absence of trypsin, suggesting that there were a few infectious virus particles in the inoculum. Surface expression of hMPV F in these few infected cells did not appear to be impaired, and the fluorescence intensity of the individual infected cells was comparable to the intensity seen in the cells infected in the presence of trypsin (Fig. 2B). We also probed for internal expression of hMPV F in cells similarly infected with rhMPV/NL/1/00/101S in the absence of trypsin, and, as in the immunostaining for surface expression, only a very few cells in the monolayer expressed hMPV F in the absence of trypsin (data not shown). These data suggest that hMPV F with 101S was able to be expressed on the plasma membrane but was unable to promote spread of rhMPV/NL/1/00/101S infection in the absence of trypsin.

Cleavage of hMPV F protein of rhMPV/NL/1/00/101S compared to that of rhMPV/NL/1/00/101P. For other paramyxovirus F proteins, it is well documented that cleavage of the F_0 precursor into the F_1 and F_2 fragments exposes the fusion peptide at the N terminus of the F_1 fragment, a requirement for fusion and infectivity. Amino acid 101 lies within the putative cleavage motif of hMPV F protein, and trypsin-dependent cleavage of the hMPV F/101S precursor would explain the trypsin requirement for growth of rhMPV/NL/1/00/101S. Therefore, we next investigated the cleavage of the hMPV F protein.

To determine the effect of the S101P substitution on F cleavage with or without trypsin, hMPV-infected Vero cell lysates were analyzed by Western blot. For F protein containing 101P, approximately half the amount of the precursor hMPV F protein (F_0) was cleaved to form an F species that corresponded to the predicted size of the putative F_1 fragment. The efficiency of processing for F protein containing 101P was comparable with or without trypsin (Fig. 2C). In contrast, hMPV F containing 101S was cleaved only when exposed to trypsin, and the relative efficiency of cleavage in the presence of trypsin was significantly less than that of hMPV F/101P (Fig. 2C). The relative amount of trypsin-induced cleavage of F protein containing 101S was found to vary among experiments due to differences in the specific activity of the trypsin added (data not shown). However, the relative cleavage of hMPV F/101S was consistently less than that of hMPV F/101P. Overall, these data indicate that the 101P contributed to trypsin-independent cleavage of the F protein, thereby exposing the fusion peptide and enabling fusion activity in the absence of trypsin.

The experiments described above were performed using hMPV/NL/1/00, the representative A1 subtype. Biologically derived wt hMPV/NL/1/99, the representative B1 subtype, was also found to have an S101P substitution in the predicted RQSR cleavage motif of its F protein. Therefore, a recombinant virus, rhMPV/NL/1/00/101S, was utilized to assess the contribution of the S101P substitution on hMPV trypsin independence in the B1 subtype. Growth curves in the presence and absence of trypsin, surface expression of hMPV F in infected Vero cells, and the extent of cleavage as determined by Western blot analysis with either wt hMPV/NL/1/99 or rhMPV/NL/1/99/101S were analogous to the results seen with rhMPV/NL/1/00/101P and rhMPV/NL/1/00/101S (data not shown). These results indicated that the S101P substitution contributed to trypsin-independent growth in the B1 subtype as well as the A1 subtype.

Cleavage of F of hMPV/101S compared to that of hMPV/101P when expressed from a heterologous viral vector. To investigate whether hMPV F cleavage was dependent upon the native viral context provided by the other hMPV viral proteins, the level of hMPV F-protein cleavage was determined for hMPV F protein expressed from a heterologous virus. The hMPV F gene coding for either a predicted 101S or 101P was cloned into a chimeric b/h PIV3 full-length cDNA so that the viruses generated from these cDNAs would express the desired hMPV F protein from the second transcription unit as described previously (44, 46–48). Without exogenously added trypsin, vectored hMPV F/101P protein was partially cleaved in Vero cells while hMPV F/101S protein was uncleaved as determined by Western blot analysis of infected cell lysates (Fig. 3). However, the relative cleavage of vectored hMPV F/101P protein was reduced compared to that of hMPV F/101P in wt hMPV-infected cells (Fig. 3). This difference was no longer apparent when trypsin was added during infection. In the presence of trypsin, the vectored F proteins of both hMPV/NL/1/00/101P and hMPV/NL/1/00/101S were partially cleaved to the same extent as the F protein expressed from wt hMPV/NL/1/00

FIG. 3. Expression of hMPV F vectored in b/h PIV3 as detected by Western blot analysis. Subconfluent monolayers of Vero cells were inoculated with wt hMPV/NL/1/00, b/h PIV3/hMPV F/101P, b/h PIV3/hMPV F/101S, or b/h PIV3 with or without 0.2 μg/ml trypsin. Western blot analysis was performed on viral harvests using MAb 121-1017-133 directed to hMPV F. The arrows indicate positions of bands corresponding to the predicted sizes of full-length precursor hMPV F (F_0) and cleavage fragment hMPV F_1. aa, amino acid.

(Fig. 3). These results indicate that trypsin-independent cleavage occurred without other hMPV proteins, although trypsin-independent cleavage was less efficient than during wt hMPV/NL/1/00 infection.

Spontaneous hMPV F variants of hMPV/NL/1/00. Sequence analysis of independently generated stocks of rhMPV/NL/1/00/101P indicated that this virus rapidly developed other codon changes in or upstream of the putative cleavage site of the fusion protein. One stock of rhMPV/NL/1/00/101P spontaneously developed the mutation G3343A, encoding a predicted E93K amino acid substitution in F. A second stock developed the mutation C3364A, encoding a predicted Q100K substitution in F. These mutations remained genetically stable for 10 additional passages in Vero cells. One of these variant viruses, vhMPV/93K/101P, was sequenced in its entirety (excluding 30 nucleotides at the extreme 3′ and 5′ ends of the genome), and G3343A was the only mutation detected, suggesting that replication of the hMPV genome by the polymerase complex was not inherently error prone.

Five other partial polymorphisms at nucleotides upstream of the putative cleavage site were found in five different virus stocks of rhMPV/NL/1/00/101P. These polymorphisms were G3340A, A3344T, T3350G, G3352A, and A3355C that encoded predicted amino acid substitutions E92K, E93V, I95S, E96K, and N97H, respectively (Table 1). Each virus stock of rhMPV/NL/1/00/101P that developed one of these polymorphisms did so within the first six passages, and never was more than one of these changes observed. These data suggested that any one of these additional mutations might have provided a growth advantage in Vero cells.

To determine whether growth without trypsin was asserting selective pressure for spontaneous mutations in rhMPV/NL/1/00/101P fusion protein, 10 independent transfections were done with trypsin and 10 were done without trypsin. Recovery of virus was equally efficient with or without trypsin. However, after the third passage without trypsin, 7 out of 10 virus stocks had developed a subpopulation with a G3343A or C3364A mutation, while only 1 out of 10 virus stocks grown with trypsin had developed a mutation, and it was G3343A (data not shown). Similarly, for rhMPV/NL/1/00/101S, 10 independent transfections were done with trypsin and 10 were done without trypsin. As expected, no virus was recovered in the absence of trypsin. For the rhMPV/NL/1/00/101S stocks that were recovered and amplified with trypsin, no mutations occurred in the F gene in 10 serial passages. It would appear that in the presence of trypsin, the function of hMPV F cleavage was assumed by the applied exogenous protease, obviating the selection of cleavage-enhancing mutations. In the absence of trypsin, we saw rapid development of secondary mutations that suggested that G3343A or C3364A variants of rhMPV/NL/1/00/101P arose because they facilitated more efficient cleavage of the fusion protein in the absence of trypsin.

These observations led us to conduct a thorough analysis of nucleotide polymorphisms in the fusion gene of biologically derived wt hMPV/NL/1/00, which was derived from three passages in tertiary monkey kidney cells and further passaged three times in Vero cells. Polymorphisms at nucleotides 3343 and 3364 in the F gene were observed (Fig. 4A). To further analyze the sequences found within this viral population, RT-PCR fragments spanning nt 2938 to nt 3896 were cloned into a plasmid vector, and 20 individual clones were sequenced from nt 3200 to nt 3500. In addition to the primary mutation T3367C, encoding the substitution 101P, nine clones had the C3364A mutation (Q100K) and four clones had the G3343A mutation (E93K). These two mutations were identical to the predominant mutations that arose after passaging rhMPV/NL/1/00/101P in Vero cells. Of the remaining clones, three had A3344T, one had A3348C, and one had T3357A, encoding E93V, Q94H, and N97K, respectively (Table 1). No clone

TABLE 1. Mutations and polymorphisms in hMPV F gene of rhMPV/NL/1/00/101P, wt hMPV/NL/1/00, and wt hMPV/NL/1/99[a]

Virus	Trypsin	Mutation										
		E92K, G3340A	E93K, G3343A	E93V, A3344T	Q94K, C3346A	Q94H, A3348C	I95S, T3350G	E96K, G3352A	N97H, A3355C	N97K, T3357A	Q100K, C3364A	S101P, T3367C
rhMPV/NL/1/00/101P	−		X	X			X	X	X		X	X
	+	X	X									X
wt hMPV/NL/1/00	−		X	X		X				X	X	X
wt hMPV/NL/1/99	−				X			X				X

[a] Stocks of the indicated hMPV viruses developed polymorphisms in the F gene in less than six passages in Vero cells. The mutations and consequent predicted amino acid substitutions in hMPV F protein are indicated above each column. All of the stocks of rhMPV/NL/1/00/101P, wt hMPV/NL/1/00 and wt hMPV/NL/1/99 contained the mutation T3367C encoding the substitution S101P. For each stock of rhMPV/NL/1/00/101P that developed a nucleotide polymorphism, only one polymorphism in addition to S101P was found. The wt hMPV stocks contained mixed populations of viruses with each subpopulation containing S101P plus one of the nucleotide polymorphisms shown in the table.

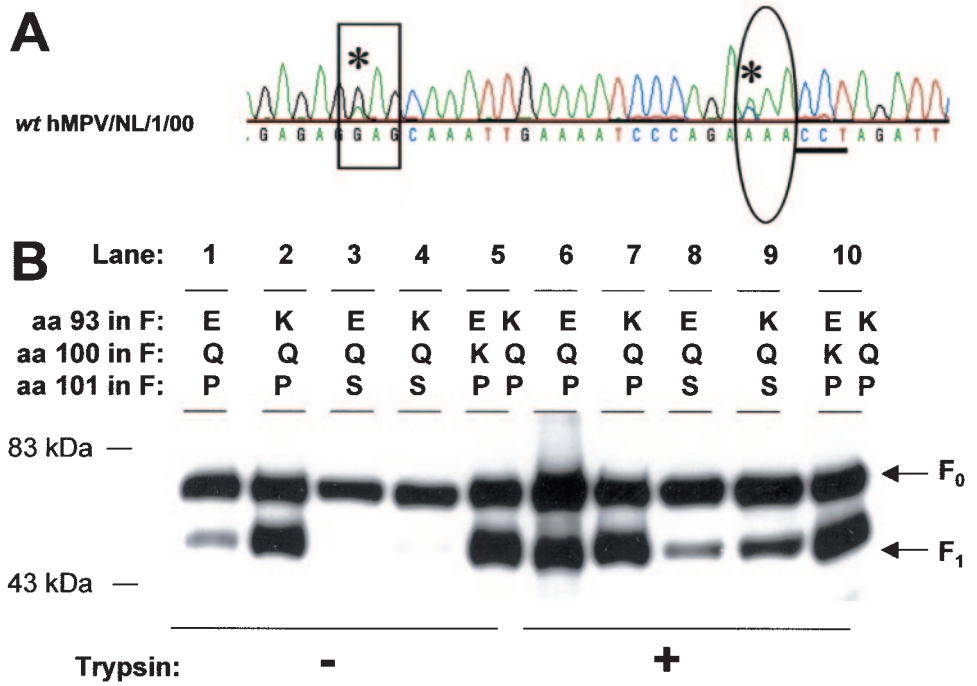


FIG. 4. Chromatogram of nucleotide sequence derived from wild-type hMPV/NL/1/00 and relative cleavage efficiencies of hMPV F protein by Western blot analysis. (A) Chromatogram spanning nt 3348 to 3373 obtained from RT-PCR fragment of wt hMPV/NL/1/00. The codons corresponding to predicted amino acids 93 (rectangle), 100 (oval), and 101 (underlined) of hMPV F glycoprotein are indicated. The asterisks indicate nucleotide polymorphisms. (B) Vero cells were inoculated with the indicated hMPV viruses at an MOI of 0.1 PFU/cell with or without 0.2 μg/ml trypsin. Six days postinoculation, cells and supernatants were harvested and Western blot analysis was performed with MAb 121-1017-133 directed to hMPV F. Shown are rhMPV/NL/1/00/101P (lanes 1 and 6), rhMPV/93K/101P (lanes 2 and 7), rhMPV/NL/1/00/101S (lanes 3 and 8), rhMPV/93K/101S (lanes 4 and 9), and wt hMPV/NL/1/00 (lanes 5, 10). wt hMPV/NL/1/00 is a mixture of hMPV with either a predicted E93K or Q100K in the fusion protein. Arrows indicate bands corresponding to the predicted sizes of full-length precursor hMPV F (F_0) and cleavage fragment hMPV F_1.

contained more than one of these secondary mutations. Attempts to plaque purify wt hMPV/NL/1/00 were not successful due to the minimal cytopathic effect and poor growth of hMPV in Vero cells. These results suggested that wt hMPV/NL/1/00 expanded to P6 was a mixed population that contained two predominant quasispecies. Thus, both biologically derived and recombinant hMPV readily acquired mutations in the hMPV F gene that facilitated their trypsin-independent growth in tissue culture.

Sequencing of RT-PCR fragments derived from the F gene of wt hMPV/NL/1/99 confirmed the primary mutation T3367C, encoding the predicted substitution S101P, and identified two additional nucleotide polymorphisms, C3346A and G3352A, encoding predicted Q94K and E96K amino acid substitutions in F, respectively (Table 1). The secondary mutation, E96K, was also seen in some stocks of rhMPV/NL/1/00/101P, but the Q94K secondary mutation was seen only in wt hMPV/NL/1/99 (Table 1). These data for both A1 and B1 subtypes suggest that an S101P substitution in the RQSR motif of the hMPV F-protein cleavage site alters the protease specificity, resulting in efficient hMPV growth in the absence of trypsin in vitro.

E93K enhanced trypsin-independent cleavage of hMPV F/101P but did not confer trypsin-independent cleavage of hMPV F/101S. E93K was the most frequently observed secondary mutation in rhMPV/NL/1/00/101P. Since this substitution did not arise during passaging of rhMPV/NL/1/00/101S, we investigated the cleavage-enhancing ability of the E93K substitution in the context of both rhMPV/NL/1/00/101S and rhMPV/NL/1/00/101P. Western blot analysis showed that in the absence of trypsin, the presence of E93K greatly enhanced the efficiency of hMPV F/101P cleavage (Fig. 4B, lanes 1 and 2), suggesting a synergistic effect between 101P and 93K on hMPV F-protein processing in Vero cells. However, hMPV F/101S was not cleaved in the absence of trypsin even in conjunction with E93K (Fig. 4B, lanes 3 and 4). In the presence of trypsin, hMPV F/101S was partially cleaved as expected, and the addition of E93K only minimally increased the cleavage efficiency (Fig. 4B, lanes 8 and 9).

E93K and Q100K enhance trypsin-independent cleavage of hMPV F/101P but do not increase titers during multicycle growth. The rapid emergence of the predicted E93K and Q100K substitutions suggested that each of these secondary mutations might confer a growth advantage by enhancing hMPV F cleavage. Western blot analysis of variant viruses containing either predicted E93K or Q100K in the hMPV F sequence showed that approximately half of the hMPV F_0 precursor had been cleaved in the absence of trypsin, suggesting that enhanced cleavage of F had indeed been selected for in Vero cell growth (data not shown).

To determine if enhanced trypsin-independent cleavage of F protein would increase viral titers during multicycle growth of hMPV, Vero cells were inoculated with rhMPV/NL/1/00/101P,

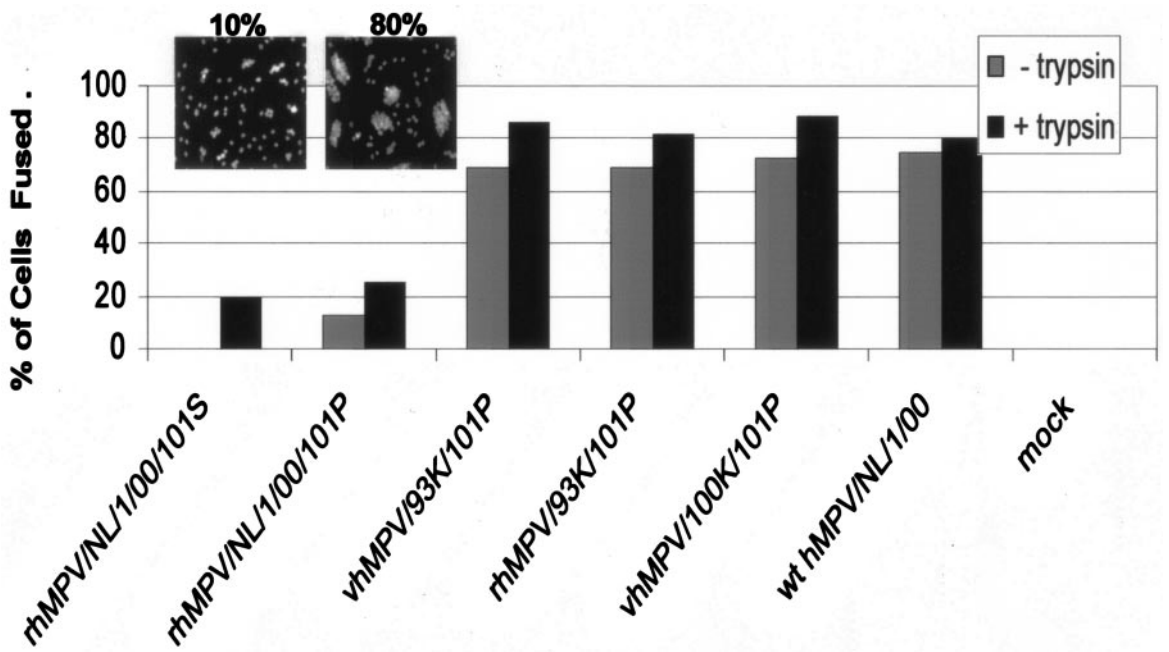


FIG. 5. Relative fusion of Vero cells infected with hMPV viruses. Confluent monolayers of Vero cells were inoculated with the indicated hMPV viruses at an MOI of 3 PFU/cell with or without 0.2 μg/ml trypsin and incubated under 1% methyl cellulose. The monolayers were fixed in methanol at 48 h postinfection. The nuclei were visualized by incubation with Hoechst stain. The inset pictures are representative fields of view. Aggregated nuclei of fused cells and isolated nuclei of unfused cells were counted in 10 fields of view, and the percentage of fused cells was graphed. The data shown are from one of three experiments.

vhMPV/93K/101P, rhMPV/93K/101P, vhMPV/100K/101P, or wt hMPV/NL/1/00 at an MOI of 0.1 PFU/cell in the absence of trypsin. The growth curves for each of the trypsin-independent viruses were comparable, indicating that there was no enhancement in the viral peak titers or growth kinetics with increased cleavage efficiency of the hMPV F that resulted from acquisition of E93K or Q100K (data not shown).

Enhanced hMPV F cleavage correlates with increased fusion activity in hMPV-infected Vero cells. Analogous to other paramyxoviruses, cleavage of full-length precursor hMPV F protein may expose a fusion peptide at the N terminus of the F_1 fragment that can promote fusion between cells (31, 64). Visual inspection of wt hMPV/NL/1/00-infected Vero cell monolayers showed that by day 2 to 3, most of the cells had fused to form large syncytia, whereas rhMPV/NL/1/00/101S-infected cells showed fewer and smaller syncytia. To evaluate if an increase in cell-to-cell fusion activity correlated with enhanced cleavage of F protein, confluent monolayers of Vero cells were inoculated with wild-type, recombinant, and variant hMPV/NL/1/00 viruses with or without trypsin. Fusion activity was quantified at 48 h postinfection by calculating the percentage of DAPI (4′,6′-diamidino-2-phenylindole)-stained nuclei incorporated into syncytia in 10 randomly selected fields of view. For one representative experiment, in the absence of trypsin, 65% to 75% of the Vero cells infected with vhMPV/93K/101P, rhMPV/93K/101P, vhMPV/100K/101P, or wt hMPV/NL/1/00 had fused to form syncytia, and in the presence of trypsin, 80% to 90% of the cells had formed syncytia (Fig. 5). For rhMPV/NL/1/00/101P that did not contain hMPV F-cleavage-enhancing mutations, syncytium formation was considerably reduced: the percentage of cells fused into syncytia was 13% without trypsin and 25% with trypsin. For rhMPV/NL/1/00/101S, formation of small syncytia was observed only in the presence of trypsin, with 20% of the cells fused to form syncytia (Fig. 5).

The enhanced syncytium formation could formally be caused by either enhanced efficiency of cleavage of hMPV F or enhanced replication of hMPV. Since growth curves showed that enhancement of hMPV F cleavage did not increase the replication titers of hMPV, it is likely that enhanced efficiency of hMPV F cleavage caused the increase in fusion activity as demonstrated by the formation of larger and greater numbers of syncytia.

Replication and protection from wt hMPV challenge in Syrian Golden hamsters. To determine if the trypsin-independent growth properties of hMPV containing hMPV F/101P would confer an ability to replicate in tissues outside of the respiratory tract, Syrian Golden hamsters were inoculated with rhMPV/93K/101P and rhMPV/93K/101S. The Syrian Golden hamster model has been previously shown to be permissive for wt hMPV/NL/1/00 replication (29). Replication titers at 4 days postinfection were comparable for rhMPV/93K/101P and rhMPV/93K/101S in the nasal turbinates and lungs (Table 2). Virus titers from brain, heart, kidney, liver, or spleen were below the limit of detection of the plaque assay. rhMPV/93K/101P and rhMPV/93K/101S gave similar neutralization titers and levels of protection upon challenge with wt hMPV/NL/1/00 (Table 2). These results suggested that the predicted S101P substitution in hMPV F did not alter tissue tropism or increase replication titers but showed the same level of immunogenicity and protection compared to rhMPV/93K/101S in the hamster model.

TABLE 2. rhMPV/93K/101S and rhMPV/93K/101P showed similar levels of replication and protection in hamsters[a]

Virus	Replication titer (log_{10} PFU/g)		Neutralization titer (reciprocal log_2)		Titer after challenge (log_{10} PFU/g)	
	Lungs	NT	wt hMPV/NL/1/00	wt hMPV/NL/1/99	Lungs	NT
rhMPV/93K/101P	5.4 ± 0.2	6.2 ± 0.2	9.7 ± 1.2	6.5 ± 0.6	<0.06 ± 0.1	<0.4 ± 0.1
rhMPV/93K/101S	5.3 ± 0.2	6.3 ± 0.3	9.4 ± 1.0	4.8 ± 1.6	<0.09 ± 0.1	<0.4 ± 0.1
Placebo	ND	ND	0.8 ± 0.1	1.5 ± 0.2	4.4 ± 0.7	5.9 ± 0.8

[a] Eight hamsters per group were inoculated with 10^6 PFU per animal. Four hamsters per group were harvested 4 days postimmunization for replication titers. Four hamsters per group were bled for serum neutralization titers and challenged 28 days postimmunization with 10^6 PFU wt hMPV/NL/1/00. Challenge titers were obtained 4 days postinfection. NT, nasal turbinates; ND, not determined.

DISCUSSION

hMPV has been reported to require trypsin for growth (3–6, 13, 18, 37, 38, 43, 60, 62). However, we observed that hMPV/NL/1/00 (subtype A1) and hMPV/NL/1/99 (subtype B1), which had been passaged three times in tertiary monkey kidney cells and three times in Vero cells, exhibited comparable growth kinetics and peak titers in the presence or absence of trypsin. To determine the genetic basis for trypsin-independent growth of hMPV/NL/1/00 and hMPV/NL/1/99, sequences of the hMPV fusion gene of these viruses were compared to sequences recently deposited in GenBank (3, 5, 59, 60). Several nucleotides near and one nucleotide in the RQSR motif at the putative F_1/F_2 cleavage site were found to display nucleotide polymorphisms. One of these nucleotide changes encoded an S-to-P substitution in the RQSR motif at position 101. By analogy with other paramyxovirus fusion proteins, cleavage at the RQS/PR motif likely exposes the fusion domain located at the N terminus of the F_1 fragment that is required for fusion with host cell membrane, syncytium formation, and efficient virus amplification (31, 40, 41).

To investigate the role of an S101P substitution in hMPV F in trypsin-independent growth, recombinant hMPV/NL/1/00 viruses that contained serine or proline at position 101 in the RQSR motif were generated. We found that hMPV that expressed a fusion protein with 101S was incapable of initiating multicycle growth without the addition of trypsin, in marked contrast to rhMPV/NL/1/00/101P. rhMPV/NL/1/00/101P had similar growth kinetics and mean peak titers during infections with or without exogenous trypsin, and this correlated with comparable hMPV F/101P cleavage efficiency in the presence and absence of trypsin. In contrast, rhMPV/NL/1/00/101S was able to initiate multicycle growth only once hMPV F/101S was cleaved by the addition of exogenous trypsin. Thus, the S101P substitution at the RQSR motif is a major determinant of trypsin-independent growth phenotype and plays a major role in promoting the hMPV F_1/F_2 cleavage.

hMPV expressing hMPV F/101P rapidly acquired mutations at other amino acid positions in the putative F_2 fragment but not the F_1 fragment. Most of these secondary mutations were adjacent to the RQPR motif, although one mutation, Q100K, was located in the motif. Of the F_2 mutations that occurred outside the RQPR motif, E93K was identified most frequently, and hMPV engineered to express hMPV F/93K/101P showed enhanced F_0 processing and cell fusion activity. While we did not examine the effects of the other F_2 mutations, we postulate that they also act to enhance the cleavage of hMPV F. The rapidity with which secondary mutations that enhanced hMPV F cleavage arose suggested that they might confer a growth advantage in Vero cell culture, but this was not apparent from the comparative multicycle growth curves done at an MOI of 0.1. Certainly, increased efficiency of hMPV F cleavage did result in the production of more infectious virus when the growth of rhMPV/NL/1/00/101P was compared to that of rhMPV/NL/1/00/101S in the presence of trypsin. However, the growth of rhMPV/NL/1/00/101P may have been sufficiently efficient such that further enhancement in hMPV F cleavage efficiency was unlikely to significantly increase the peak titers.

The trypsin-independent phenotype was also observed for hMPV/NL/1/99, a subtype B1 hMPV. The F proteins of subtypes A1 and B1 share amino acid identity of 94%, and most of the nonhomologous amino acids are located at the C terminus of the hMPV F protein that includes the putative transmembrane domain (61). While an S-to-P substitution at position 101 of the fusion protein also resulted in trypsin-independent growth of hMPV/NL/1/99, sequencing of the P6 stock revealed that the major F_2 polymorphisms were at amino acids 94 and 96, in contrast to 93 and 100 for subtype A1 hMPV F. Since the F proteins of the two subtypes are highly conserved around the F_1/F_2 cleavage site, it was surprising to find different cleavage-enhancing mutations. Passaging of newly generated hMPV/NL/1/99/F 101P may result in amino acid substitutions similar to those found in the subtype A1 F_2 fragment. However, the differences in the F_2 mutations may reflect flexibility in the binding of the protease that catalyzed hMPV F cleavage or higher-order conformational differences in this region of the hMPV F A1 and B1 glycoproteins.

The S101P substitution also increased the cleavage efficiency of hMPV F following expression from a chimeric bovine/human PIV3 virus vector, indicating that cleavage of the hMPV fusion protein occurred in the absence of interaction with other hMPV proteins. However, the amount of hMPV F_1 fragment derived from PIV3-infected cells was relatively less than that observed in hMPV-infected cells, suggesting that interactions with other hMPV proteins resulted in more cleavage activity. Other possibilities include inhibitory effects of PIV3 proteins or differences in cellular states induced by hMPV infections compared to PIV3 infections. Nonetheless, these observations serve as further confirmation that the S101P substitution in the RQSR motif of hMPV F is an important determinant of cleavage activity in Vero cells.

The surface expression of hMPV F/101S suggested that the uncleaved hMPV F_0 precursor was trafficked to the cell surface. We note that in Vero cells, a substantial amount of the hMPV F_0 precursor was protected from cleavage even in the presence of trypsin, in contrast to the processing of RSV fusion proteins (11, 15). This result suggested that the processing of

hMPV F precursor is inefficient and/or that hMPV F_0 has a functional role in the replication cycle of hMPV in vitro. hMPV F/101S appeared to be cleaved extracellularly after exposure to exogeneously added trypsin. However, it is unclear whether hMPV F/101P is cleaved intra- or extracellularly. Other paramyxovirus virus fusion proteins that contain multiple basic residues at the cleavage site are thought to be cleaved by an intracellular protease such as furin (8, 23, 26, 27). If there is an intracellular protease capable of cleaving hMPV F protein, it was surprising that we did not detect any S101P substitutions during multiple attempts to grow rhMPV/NL/1/00/101S in the absence of trypsin. Extensive passaging of rhMPV/NL/1/00/101S in the presence of trypsin also did not generate any virus with an S101P substitution in the fusion protein. However, under more stringent growth conditions, such as neutralization of trypsin after initiation of infection, populations of rhMPV/NL/1/00/101S with the S101P amino acid substitution in the fusion protein may arise.

There remains the formal possibility that the S101P mutation that enhanced cleavage of F in wt hMPV/NL/1/00 and wt hMPV/NL/1/99 could have originated as subpopulations in the original clinical isolates that were enriched after passage in tissue culture. While most hMPV clinical isolates have been reported to contain the RQSR cleavage motif, 3 of 46 clinical isolates of nasal swabs from a study at Vanderbilt University were found to contain RQPR at the F cleavage site (Chin-Fen Yang and Jim Crowe, unpublished data). These isolates represented both subtypes A1 and B1 hMPV. Thus, the RQPR motif occurred in a small population of naturally occurring hMPV and was not necessarily induced during tissue culture expansion.

Cleavage of the F_0 precursor is a prerequisite for infectivity and pathogenicity of paramyxoviruses (24, 26). For some respiratory viruses such as influenza, Newcastle disease virus, PIV2, and Sendai virus, changes in the F protein that altered recognition by a tissue-specific protease to a nonspecific ubiquitous protease such as furin have given rise to host range differences or an increase in virulence (1, 8–10, 12, 14, 23, 24, 26, 27, 32, 33, 42, 57, 58). For example, Sendai virus that contained PQSR at the F cleavage site was found to require a protease such as exogenous trypsin in tissue culture or tryptase Clara in lung cells for F cleavage and growth (22, 24, 25, 49, 51, 55). However, Sendai virus mutants that expressed fusion protein with the PQPR cleavage motif can replicate without trypsin in tissue culture (51, 54). The altered protease specificity conferred by the PQPR cleavage motif enabled the mutant Sendai virus to replicate outside the respiratory tract, although the pantropic phenotype was dependent on an additional mutation in the matrix protein (35, 50, 52, 53).

Preliminary studies in hamsters suggested that the S101P mutation in the hMPV F protein that obviated the need for trypsin during tissue culture growth did not generate a pantropic phenotype for hMPV. It is also noteworthy that hMPV clinical strains that contained RQPR were isolated from secretions from three pediatric subjects with uncomplicated upper respiratory tract illness (Jim Crowe, personal communication).

The identification of hMPV strains that replicate efficiently in Vero cells without exogenous trypsin will facilitate the generation, characterization, preclinical testing, and assay development of potential live attenuated hMPV vaccine candidates. Our results in the hamster model demonstrated that hMPV containing hMPV F/101P replicated, induced neutralizing antibodies, and protected hamsters from challenge to the same extent as hMPV expressing F/101S and did not extend the tissue tropism. However, the important correlations between differences in protease specificity and viral pathogenicity have to be further addressed experimentally before further development of these hMPV strains as live attenuated vaccine candidates.

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REFERENCES

1. **Bando, H., M. Kawano, K. Kondo, M. Tsurudome, H. Komada, M. Nishio, and Y. Ito.** 1991. Growth properties and F protein cleavage site sequences of naturally occurring human parainfluenza type 2 viruses. Virology **184:**87–92.
2. **Barr, P. J.** 1991. Mammalian subtilisins: the long-sought dibasic processing endoproteases. Cell **66:**1–3.
3. **Bastien, N., S. Normand, T. Taylor, D. Ward, T. C. T. Peret, G. Boivin, L. J. Anderson, and Y. Li.** 2003. Sequence analysis of the N, P, M and F genes of Canadian human metapneumovirus strains. Virus Res. **93:**51–62.
4. **Bastien, N., D. Ward, P. Van Caeseele, K. Brandt, S. H. Lee, G. McNabb, B. Klisko, E. Chan, and Y. Li.** 2003. Human metapneumovirus infection in the Canadian population. J. Clin. Microbiol. **41:**4642–4646.
5. **Biacchesi, S., M. H. Skiadopoulos, G. Boivin, C. T. Hanson, B. R. Murphy, P. L. Collins, and U. J. Buchholz.** 2003. Genetic diversity between human metapneumovirus subgroups. Virology **315:**1–9.
6. **Boivin, G., Y. Abed, G. Pelletier, L. Ruel, D. Moisan, S. Cote, T. C. T. Peret, D. D. Erdman, and L. J. Anderson.** 2002. Virological features and clinical manifestations associated with human metapneumovirus: a new paramyxovirus responsible for acute respiratory-tract infections in all age groups. J. Infect. Dis. **186:**1330–1334.
7. **Boivin, G., G. De Serres, S. Cote, R. Gilca, Y. Abed, L. Rochette, M. G. Bergeron, and P. Dery.** 2003. Human metapneumovirus infections in hospitalized children. Emerg. Infect. Dis. **9:**634–640.
8. **Bosch, F. S., W. Garten, H.-D. Klenk, and R. Rott.** 1981. Proteolytic cleavage of influenza virus hemagglutinin. Primary structure of the connecting peptide between HA1 and HA2 determines proteolytic cleavability and pathogenicity of avian influenza viruses. Virology **113:**725–735.
9. **Chen, Y., M. Shiota, M. Ohuchi, T. Towatari, J. Tashiro, M. Murakami, M. Yano, B. Yang, and H. Kido.** 2000. Mast cell tryptase from pig lungs triggers infection by pneumotropic Sendai and influenza A viruses. Eur. J. Biochem. **267:**3189–3197.
10. **Collins, M. S., J. B. Bashiruddin, and D. J. Alexander.** 1993. Deduced amino acid sequences at the fusion protein cleavage site of Newcastle disease viruses showing variation in antigenicity and pathogenicity. Arch. Virol. **128:**363–370.
11. **Collins, P. L., and G. Mottett.** 1991. Post-translational processing and oligomerization of the fusion glycoprotein of human respiratory syncytial virus. J. Gen. Virol. **72:**3095–4101.
12. **Collins, P. L., R. M. Chanock, and B. R. Murphy.** 2001. Respiratory syncytial virus, p. 1443–1486. *In* D. M. Knipe, P. M. Howley, D. E. Griffin, R. A. Lamb, M. A. Martin, B. Roizman, and S. E. Straus (ed.), Fields virology, 4th ed. Lippincott Williams & Wilkins, Philadelphia, Pa.
13. **Deffrasnes, C., S. Cote, and G. Boivin.** 2005. Analysis of replication kinetics of the human metapneumovirus in different cell lines by real-time PCR. J. Clin. Microbiol. **43:**488–490.
14. **Glickman, R. L., R. J. Syddall, R. M. Iorio, J. P. Sheehan, and M. A. Bratt.** 1988. Quantitative basic residue requirements in the cleavage-activation site of the fusion glycoprotein as a determinant of virulence for Newcastle disease virus. J. Virol. **62:**354–356.
15. **Gonzalez-Reyes, L., M. B. Ruiz-Arguello, B. Garcia-Barreno, L. Calder, J. A. Lopez, J. P. Albar, J. J. Skehel, D. C. Wiley, and J. A. Melero.** 2001. Cleavage of the human respiratory syncytial virus fusion protein at two distinct sites is required for activation of membrane fusion. Proc. Natl. Acad. Sci. USA **98:**9859–9864.
16. **Haller, A. A., M. MacPhail, M. Mitiku, and R. S. Tang.** 2001. A single amino

acid substitution in the viral polymerase creates a temperature-sensitive and attenuated recombinant bovine parainfluenza virus type 3. Virology **288:**342–350.
17. **Haller, A. A., T. Miller, M. Mitiku, and K. Coelingh.** 2000. Expression of the surface glycoproteins of human parainfluenza virus type 3 by bovine parainfluenza virus type 3, a novel attenuated virus vaccine vector. J. Virol. **74:**11626–11635.
18. **Hamelin, M.-E., Y. Abed, and G. Boivin.** 2004. Human metapneumovirus: a new player among respiratory viruses. Clin. Infect. Dis. **38:**983–990.
19. **Herfst, S., M. de Graaf, J. H. Schickli, R. S. Tang, J. Kaur, C.-F. Yang, R. R. Spaete, A. A. Haller, B. G. van den Hoogen, A. D. M. E. Osterhaus, and R. A. M. Fouchier.** 2004. Recovery of human metapneumovirus genetic lineages A and B from cloned cDNA. J. Virol. **78:**8264–8270.
20. **Howe, M.** 2002. Australian find suggests worldwide reach for metapneumovirus. Lancet Infect. Dis. **2:**202.
21. **IJpma, F. F., D. Beekhuis, M. F. Cotton, C. H. Pieper, J. L. Kimpen, B. G. van den Hoogen, G. J. van Doornum, and D. M. Osterhaus.** 2004. Human metapneumovirus infection in hospital referred South African children. J. Med. Virol. **73:**486.
22. **Ishida, N., and M. Homma.** 1978. Sendai virus. Adv. Virus Res. **23:**349–383.
23. **Kawaoka, T., C. W. Naeve, and R. G. Webster.** 1984. Is virulence of H5N2 influenza viruses in chickens associated with loss of carbohydrate from the hemagglutinin? Virology **139:**303–316.
24. **Kido, H., Y. Niwa, Y. Beppu, and T. Towatari.** 1996. Cellular proteases involved in the pathogenicity of enveloped animal viruses, human immunodeficiency virus, influenza virus A and Sendai virus. Adv. Enzyme Regul. **36:**325–347.
25. **Kido, H., T. Yokogoshi, K. Sakai, M. Tashiro, Y. A. Kishino, A. Fukutomi, and N. Katunuma.** 1992. Isolation and characterization of a novel trypsin-like protease found in rat bronchiolar epithelial Clara cells: a possible activator of the viral fusion glycoprotein. J. Biol. Chem. **267:**13573–13579.
26. **Klenk, H.-D., and W. Garten.** 1994. Host cell proteases controlling virus pathogenicity. Trends Microbiol. *2*(2)**:**39–43.
27. **Klenk, H.-D., and R. Rott.** 1988. The molecular biology of influenza virus pathogenicity. Adv. Virus Res. **34:**247–281.
28. **Lamb, R. A.** 1993. Paramyxovirus fusion: a hypothesis for changes. Virology **197:**1–11.
29. **MacPhail, M., J. H. Schickli, R. S. Tang, J. Kaur, C. Robinson, R. A. M. Fouchier, A. D. M. E. Osterhaus, R. R. Spaete, and A. A. Haller.** 2004. Identification of small-animal and primate models for evaluation of vaccine candidates for human metapneumovirus (hMPV) and implications for hMPV vaccine design. J. Gen. Virol. **85:**1655–1663.
30. **Maggi, F., M. Pifferi, M. Vatteroni, C. Fornai, E. Tempestini, S. Anzilotti, L. Lanini, E Andreoli, V. Ragazzo, M. Pistello, S. Specter, and M. Bendinelli.** 2003. Human metapneumovirus associated with respiratory tract infections in a 3-year study of nasal swabs from infants in Italy. J. Clin. Microbiol. **41:**2987–2991.
31. **Morrison, T. G.** 2003. Structure and function of a paramyxovirus fusion protein. Biochim. Biophys. Acta **1614:**73–84.
32. **Nagai, Y., M. Hamaguchi, and T. Toyoda.** 1989. Molecular biology of Newcastle disease virus. Prog. Vet. Microbiol. **5:**16–64.
33. **Nagai, Y., H.-D. Klenk, and R. Rott.** 1976. Proteolytic cleavage of the viral glycoproteins and its significance for the virulence of Newcastle disease virus. Virology **72:**494–508.
34. **Nissen, M. D., D. J. Siebert, I. M. Mackay, T. P. Sloots, and S. J. Withers.** 2002. Evidence of human metapneumovirus in Australian children. Med. J. Aust. **176**:188.
35. **Okada, H., J. T. Seto, N. L. McQueen, H.-D. Klenk, R. Rott, and M. Tashiro.** 1998. Determinants of pantropism of the F1-R mutant of Sendai virus: specific mutations involved are in the F and M genes. Arch. Virol. **143:**2342–2352.
36. **Peiris, J. S., W. H. Tang, K. H. Chan, P. L. Khong, Y. Guan, Y. L. Lau, and S. S. Chiu.** 2003. Children with respiratory disease associated with metapneumovirus in Hong Kong. Emerg. Infect. Dis. **9:**628–633.
37. **Peret, T. C. T., Y. Abed, L. J. Anderson, D. D. Erdman, and G. Boivin.** 2004. Sequence polymorphism of the predicted human metapneumovirus G glycoprotein. J. Gen. Virol. **85:**679–686.
38. **Peret, T. C. T., G. Boivin, Y. Li, M. Couillard, C. Humphrey, A. D. M. E. Osterhaus, D. D. Erdman, and L. J. Anderson.** 2002. Characterization of human metapneumoviruses isolated from patients in North America. J. Infect. Dis. **185:**1660–1663.
39. **Russell, C. J., T. S. Jardertzky, and R. A. Lamb.** 2001. Membrane fusion machines of paramyxoviruses: capture of intermediates of fusion. EMBO J. **20:**4024–4034.
40. **Scheid, A., and P. W. Choppin.** 1974. Identification of the biological activities of paramyxovirus glycoproteins. Activation of cell fusion, hemolysis and infectivity by proteolytic cleavage of an inactive precursor protein of Sendaivirus. Virology **57:**475–490.
41. **Scheid, A., and P. W. Choppin.** 1977. Two disulfide linked polypeptide chains constitute the active F protein of paramyxoviruses. Virology **80:**54–66.
42. **Seal, B. S., H. S. Sellers, and R. J. Meinersmann.** 2000. Fusion protein predicted amino acid sequence of the first US avian pneumovirus isolate and lack of heterogeneity among other US isolates. Virus. Res. **66:**139–147.
43. **Skiadopoulos, M. H., S. Biacchesi, U. J. Buchholz, J. M. Riggs, S. R. Surman, E. Amaro-Carambot, J. M. McAuliffe, W. R. Elkins, M. St. Claire, P. L. Collins, and B. R. Murphy.** 2004. The two major human metapneumovirus genetic lineages are highly related antigenically, and the fusion (F) protein is a major contributor to this antigenic relatedness. J. Virol. **78:**6927–6937.
44. **Skiadopoulos, M. H., S. R. Surman, J. M. Riggs, C. Orvell, P. L. Collins, and B. R. Murphy.** 2002. Evaluation of the replication and immunogenicity of recombinant human parainfluenza virus type 3 vectors expressing up to three foreign glycoproteins. Virology **297:**136–152.
45. **Stockton, J., I. Stephenson, D. Fleming, and M. Zambon.** 2002. Human metapneumovirus as a cause of community-acquired respiratory illness. Emerg. Infect. Dis. **8:**897–901.
46. **Tang, R. S., M. MacPhail, J. H. Schickli, J. Kaur, C. L. Robinson, H. A. Lawlor, J. M. Guzzetta, R. R. Spaete, and A. A. Haller.** 2004. Parainfluenza virus type 3 expressing the native or soluble fusion (F) protein of respiratory syncytial virus (RSV) confers protection from RSV infection in African green monkeys. J. Virol. **78:**11198–11207.
47. **Tang, R. S., K. Mahmood, M. MacPhail, J. M. Guzzetta, A. A. Haller, H. Liu, J. Kaur, H. A. Lawlor, E. A. Stillman, J. H. Schickli, R. A. M. Fouchier, A. D. M. E. Osterhaus, and R. R. Spaete.** 2005. A host-range restricted parainfluenza virus type 3 (PIV3) expressing the human metapneumovirus (hMPV) fusion protein elicits protective immunity in African green monkeys. Vaccine **23:**1657–1667.
48. **Tang, R. S., J. H. Schickli, M. MacPhail, F. Fernandes, L. Bicha, J. Spaete, R. A. M. Fouchier, A. D. M. E. Osterhaus, R. Spaete, and A. A. Haller.** 2003. Effects of human metapneumovirus and respiratory syncytial virus antigen insertion in two 3′ proximal genome positions of bovine/human parainfluenza virus type 3 on virus replication and immunogenicity. J. Virol. **77:**10819–10828.
49. **Tashiro, M., and M. Homma.** 1983. Pneumotropism of Sendai virus in relation to protease-mediated activation in mouse lungs. Infect. Immun. **39:**879–888.
50. **Tashiro, M., N. L. McQueen, J. T. Seto, H.-D. Klenk, and R. Rott.** 1996. Involvement of the mutated M protein in altered budding polarity of a pantropic mutant, F1-R, of Sendai virus. J. Virol. **70:**5990–5997.
51. **Tashiro, M., E. Pritzer, M. A. Khoshnan, M. Yamakawa, K. Kuroda, H.-D. Klenk, R. Rott, and J. T. Seto.** 1988. Characterization of a pantropic variant of Sendai virus derived from a host-range mutant. Virology **165:**577–583.
52. **Tashiro, M., J. T. Seto, S. Choosakul, M. Yamakawa, H.-D. Klenk, and R. Rott.** 1992. Budding site of Sendai virus in polarized epithelial cells is one of the determinants for tropism and pathogenicity in mice. Virology **187:**413–422.
53. **Tashiro, M., J. T. Seto, H.-D. Klenk, and R. Rott.** 1993. Possible involvement of microtubule disruption in bipolar budding of a Sendai virus mutant, F1-R, in epithelial MDCK cells. J. Virol. **67:**5902–5910.
54. **Tashiro, M., M. Yamakawa, K. Tobita, H.-D. Klenk, R. Rott, and J. T. Seto.** 1990. Organ tropism of Sendai virus in mice: proteolytic activation of the fusion glycoprotein in mouse organs and budding site at the bronchial epithelium. J. Virol. **64:**3627–3634.
55. **Tashiro, M., Y. Yokogoshi, K. Tobita, J. T. Seto, R. Rott, and H. Kondo.** 1992. Tryptase Clara: an activating protease for Sendai virus in rat lungs that is involved in pneumopathogenicity. J. Virol. **66:**7211–7216.
56. **Toquin, D., C. de Boisseson, V. Beven, D. A. Senne, and N. Eterradossi.** 2003. Subgroup C avian metapneumovirus (MPV) and the recently isolated human MPV exhibit a common organization but have extensive sequence divergence in their putative SH and G genes. J. Gen. Virol. **84**:2169–2178.
57. **Towatari, T., M. Ide, K. Ohba, Y. Chiba, M. Murakami, M. Shiota, M. Kawachi, H. Yamada, and H. Kido.** 2002. Identification of ectopic anionic trypsin I in rat lungs potentiating pneumotropic virus infectivity and increased enzyme level after virus infection. Eur. J. Biochem. **269:**2613–2621.
58. **Toyoda, T., T. Sakaguchi, K. Imai, N. M. Inocencio, B. Gotoh, M. Hamaguchi, and T. Nagai.** 1987. Structural comparison of the cleavage-activation site of the fusion glycoprotein between virulent and avirulent strains of Newcastle disease virus. Virology **158:**242–247.
59. **van den Hoogen, B. G., T. M. Bestebroer, A. D. M. E. Osterhaus, and R. A. M. Fouchier.** 2002. Analysis of the genomic sequence of human metapneumovirus. Virology **295:**119–132.
60. **van den Hoogen, B. G., J. C. de Jong, J. Groen, T. Kuiken, R. de Groot, R. A. M. Fouchier, and A. D. M. E. Osterhaus.** 2001. A newly discovered human pneumovirus isolated from young children with respiratory tract disease. Nat. Med. **7:**719–724.
61. **van den Hoogen, B. G., S. Herfst, L. Sprong, P. A. Cane, E. Forleo-Neto, R. L. de Swart, A. D. M. E. Osterhaus, and R. A. M. Fouchier.** 2004. Antigenic and genetic variability of human metapneumoviruses. Emerg. Infect. Dis. **10:**658–666.
62. **van den Hoogen, B. G., A. D. M. E. Osterhaus, and R. A. M. Fouchier.** 2004. Clinical impact and diagnosis of hMPV infections. Pediatr. Infect. Dis. J. **23:**S25–S32.
63. **van den Hoogen, B. G., G. J. van Doornum, J. C. Fockens, J. J. Cornelissen,**

W. E. Beyer, R. deGroot, A. D. Osterhaus, and R. A. Fouchier. 2003. Prevalence and clinical symptoms of human metapneumovirus infection in hospitalized patients. J. Infect. Dis. **188:**1571–1577.

64. **White, J. M.** 1990. Viral and cellular membrane fusion proteins. Annu. Rev. Physiol. **52:**675–697.
65. **Williams, J. V., P. A. Harris, S. J. Tollefson, L. L. Halburnt-Rush, J. M. Pingsterhaus, K. M. Edwards, P. F. Wright, and J. E. Crowe, Jr.** 2004. Human metapneumovirus and lower respiratory tract disease in otherwise healthy infants and children. N. Engl. J. Med. **350:**443–450.
66. **Wolf, D., Z. Zakay-Rones, A. Fadeela, D. Greenberg, and R. Dagan.** 2003. High seroprevalence of human metapneumovirus among young children in Israel. J. Infect. Dis. **188:**1865–1867.