Localization of a Region in the Fusion Protein of Avian Metapneumovirus That Modulates Cell-Cell Fusion

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The genus *Metapneumovirus* within the subfamily *Pneumovirinae* of the family *Paramyxoviridae* includes two members, human metapneumovirus (hMPV) and avian metapneumovirus (aMPV), causing respiratory tract infections in humans and birds, respectively. Paramyxoviruses enter host cells by fusing the viral envelope with a host cell membrane. Membrane fusion of hMPV appears to be unique, in that fusion of some hMPV strains requires low pH. Here, we show that the fusion (F) proteins of aMPV promote fusion in the absence of the attachment protein and low pH is not required. Furthermore, there are notable differences in cell-cell fusion among aMPV subtypes. Trypsin was required for cell-cell fusion induced by subtype B but not subtypes A and C. The F protein of aMPV subtype A was highly fusogenic, whereas those from subtypes B and C were not. By construction and evaluation of chimeric F proteins composed of domains from the F proteins of subtypes A and B, we localized a region composed of amino acid residues 170 to 338 in the F protein that is responsible for the hyperfusogenic phenotype of the F from subtype A. Further mutagenesis analysis revealed that residues R295, G297, and K323 in this region collectively contributed to the hyperfusogenicity. Taken together, we have identified a region in the aMPV F protein that modulates the extent of membrane fusion. A model for fusion consistent with these data is presented.

The subfamily *Pneumovirinae* of the family *Paramyxoviridae* includes a number of significant human and animal respiratory pathogens, such as human respiratory syncytial virus (hRSV), bovine RSV (bRSV), human metapneumovirus (hMPV), avian metapneumovirus (aMPV), and pneumonia virus of mice (PVM). The major cytopathic effect in paramyxovirus-infected culture cells is the formation of multinucleate syncytia. This is mediated by membrane fusion induced by surface glycoprotein spikes (reviewed in reference 24). For viruses in the *Paramyxovirinae* subfamily, it is firmly established that membrane fusion requires a specific interaction between two glycoproteins, the attachment protein (HN, H, or G) and the fusion (F) protein (1, 9, 14, 15, 23, 39), and such fusion occurs at neutral pH (4, 27). Specifically, it is thought that the binding of the attachment protein to cell surface receptors triggers major conformational changes in F, which in turn activates membrane fusion (1, 27, 29, 30, 39).

However, membrane fusion of pneumoviruses appears to be unique among the paramyxoviruses, in that fusion is accomplished by the F protein alone without help from the attachment glycoprotein (6, 7, 17, 21, 42, 43). This suggests that the F proteins of pneumoviruses possess dual functions, receptor binding and fusion promotion. To date, RSV F is arguably the best characterized F protein within the *Pneumovirus* subfamily. The F protein of RSV is capable of causing membrane fusion and initiating virus infection in the absence of its attachment G glycoprotein at neutral pH (6, 57). Furthermore, it has been demonstrated that RSV F specifically recognizes and interacts with host cell nucleolin that mediates viral entry and infection (46). Recently, it was found that the F protein of hMPV, a member of the genus *Metapneumovirus*, was able to induce fusion without the G protein, but in a trypsin-dependent manner (42, 43). In addition, recombinant aMPV lacking the G protein can be recovered by reverse genetics, suggesting that aMPV G protein is not essential for aMPV entry (19,

35). In contrast to all other paramyxoviruses, fusion of some hMPV strains requires low pH (21, 31, 42, 43). In addition, it has been suggested that hMPV F protein utilizes $\alpha\beta 1$ integrin as a cellular receptor based on the evidence that hMPV F protein possesses an integrin binding motif and integrin promotes hMPV infection (12). Most recently, it was shown that interaction between integrins and hMPV occurs after the initial binding of hMPV F to heparan sulfate proteoglycans, suggesting that heparan sulfate is the primary receptor for hMPV F protein (7). A receptor(s) for aMPV has not been identified. Overall, the detailed mechanism underlying fusion promotion in pneumoviruses is still poorly understood.

Paramyxovirus F proteins are trimeric, type I membrane glycoproteins. Each monomer is first produced as an inactive precursor, F0, which is further proteolytically cleaved into two disulfide-linked polypeptides, F1 and F2. This cleavage generates a hydrophobic fusion peptide (FP), which is directly inserted into the membrane to initiate fusion. Two heptad repeat (HR) regions, HRA and HRB, in the F1 polypeptide have been identified as critical for fusion. HRA is located just at the carboxyl terminal to the fusion peptide, and HRB is located adjacent to the transmembrane (TM) domain. During fusion, HRA and HRB are rearranged to form a highly stable six-helix bundle that brings two

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membranes together to initiate fusion (reviewed in references 25 and 26). Recently, crystal structures of the human parainfluenza virus 3 (hPIV3), PIV5, Newcastle disease virus (NDV), and RSV F proteins in their postfusion conformation and of the PIV5 F protein in its prefusion conformation have been determined (8, 32, 44, 45, 54, 55, 56). Most recently, the partial structure of hMPV F protein composed of three domains (DI-DII-DIII) has been solved (52). However, the conformation (prefusion or postfusion) of this partial F protein remains unknown (52). Upon triggering, the metastable pretriggered F protein undergoes a dramatic and irreversible conformational change (10, 41, 54, 55). In the prefusion trimer, HRB forms a coiled-coil stalk, and HRA forms 11 distinct segments, including four helices, two β -strands, and five loop, kink, or turn segments, and they are located in the globular head (55). In the postfusion trimer, the 11 segments in HRA are refolded into a single, extended α -helical conformation, and HRB helices and linkers pack against an HRA coiled coil, burying the latter in the center of a six-helix bundle (45, 54). Nonetheless, it remains to be further elucidated how F initiates these conformational changes.

The increasing diversity of membrane fusion within the *Paramyxoviridae* family prompted us to characterize the fusion activity of aMPV, the only other member in the genus *metapneumovirus* (16, 50, 51). aMPV, also known as avian pneumovirus or turkey rhinotracheitis, is an economically important pathogen that causes acute respiratory disease in turkeys (2, 16, 18). Based on antigenicity and genetic diversity, four subtypes of aMPV, designated A, B, C, and D, have been defined (16, 20). Subtypes A, B, and D are found mainly in Europe and Asia (16, 20, 36), while subtype C is prevalent in the United States (2, 11, 18, 49). The full-length sequences of the F genes of all subtypes except subtype D have been determined. The F proteins of aMPV subtypes A, B, and C share 70 to 80% amino acid sequence homology. Interestingly, the F protein of aMPV subtype C shares a higher homology with hMPV than other aMPV subtypes. To date, the requirements for fusion have not been characterized for any aMPV subtype.

In this study, we characterized the basic requirements for membrane fusion promoted by different subtypes of aMPV. Our results showed that the aMPV F protein can promote cell-cell fusion in the absence of the attachment protein, and such fusion does not require low pH, distinguishing aMPV from hMPV. Moreover, trypsin was required for cell-cell fusion induced by subtype B, but not subtypes A and C. The F protein of aMPV subtype A was highly fusogenic, whereas that from subtypes B and C was not. By construction of chimeric F proteins between subtypes A and B, we identified a region in the F protein and critical amino acid residues within it that are responsible for the phenotypic differences among the aMPV subtypes.

MATERIALS AND METHODS

Cell lines. Vero E6 cells (ATCC no. CRL-1586) and BHK-SR19-T7 cells (kindly provided by Apath, LLC, Brooklyn, NY) were grown in Dulbecco's modified Eagle's medium (DMEM; Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS). The medium of the BHK-SR19-T7 cells was supplemented with 10 μ g/ml neomycin (Invitrogen) every other passage to select for T7 polymerase-expressing cells.

Recombinant plasmids and site-directed mutagenesis. The F genes of aMPV Tian-Tai strain (subtype A), Shao-Xin strain (subtype B), Minnesota strain (subtype C), and Colorado strain (subtype C) were cloned into the pCAGGS vector at EcoRI and XhoI sites, which resulted in the construction of pCAGGS-A-F, pCAGGS-B-F, pCAGGS-C-F-MN, and

pCAGGS-C-F-CO, respectively. The G gene from each aMPV subtype was also cloned into the pCAGGS vector at EcoRI and XhoI sites, which resulted in the construction of pCAGGS-A-G, pCAGGS-B-G, pCAGGS-C-G-MN, and pCAGGS-C-G-CO. To facilitate the detection of the F protein, a hemagglutinin (HA) tag was engineered into the C terminus of each F gene, which resulted in the construction of pCAGGS-A-F-HA, pCAGGS-B-F-HA, pCAGGS-C-F-MN-HA, and pCAGGS-C-F-CO-HA. All constructs were confirmed by sequencing.

The F gene mutations were generated by site-directed mutagenesis using the QuikChange methodology (Stratagene, La Jolla, CA). The presence of the desired mutation was verified by sequencing. In addition, an HA tag was engineered into the C terminus of each F mutation to facilitate the detection of F proteins.

Construction of chimeric F genes. Using unique, homologous restriction enzyme sites in the F genes of aMPV subtypes A and B, a series of chimeric F genes were constructed.

To exchange F2 and F1 of aMPV subtype A and aMPV subtype B, we took advantage of a unique MfeI site at position 107 in aMPV subtype B-F. Since there are two MfeI sites at positions 107 and 426 in aMPV subtype A-F, the MfeI site at position 426 was removed by site-directed mutagenesis. This did not result in any amino acid changes in aMPV subtype A-F. The two plasmids pCAGGS-A-F and pCAGGS-B-F were digested by MfeI and XhoI, and the C-terminal fragments of the two F genes were isolated. The fragments were ligated into the heterologous F gene previously digested with the same enzymes. This resulted in chimeras CH-A and CH-B, in which the N-terminal 102 residues are derived from the F genes of aMPV subtype A and B, respectively, and the rest of the protein is derived from the heterologous F protein.

To exchange the amino acids between 170 and 441 in aMPV subtype A-F and aMPV subtype B-F, we took advantage of unique PstI (position 170) and SacI (position 441) sites in aMPV subtype B-F. The other PstI at position 35 and SacI at position 81 in aMPV subtype A-F were removed by silent mutations. The two plasmids pCAGGS-A-F and pCAGGS-B-F were digested with PstI and SacI, and the fragments were ligated into the heterologous F gene that had been digested with the same enzymes, resulting in the construction of chimeras CH-C and CH-D.

To exchange the amino acids between residues 170 and 338 in aMPV subtype A-F and aMPV subtype B-F, a PstI site at position 338 was introduced into plasmid CHC. Plasmid CHC was digested by EcoRI and PstI, and the fragment spanning amino acid residues from 170 to 338 was ligated into pCAGGS-B-F previously digested with the same enzymes, which resulted in the construction of chimera CH-E.

To exchange the amino acids between residues 338 and 441 in aMPV subtype A-F and aMPV subtype B-F, plasmid CHC was digested by PstI and XhoI, and the fragment spanning amino acid residues from 338 to 441 was ligated into pCAGGS-B-F previously digested with the same enzymes, which resulted in the construction of chimera CH-F.

Syncytium formation assay. Monolayers of Vero E6 cells in 6-well plates were transfected with 2 μ g of each plasmid using Lipofectamine (Invitrogen) according to the manufacturer's instructions. After transfection, 2 ml of OPTI-MEM (Invitrogen) was added. After 24 h posttransfection, the cells were washed and incubated at 37°C in OPTI-MEM with 0.2 μ g/ml TPCK (L-1-tosylamide-2-phenylethyl chloromethyl ketone)-treated trypsin (TPCK-trypsin) for 1 h. The cells were washed four times with phosphate-buffered saline (PBS; pH 7.0 or 5.0) containing 10 mM HEPES and 5 mM MES (morpholineethanesulfonic acid) for 4 min each at intervals of 2 h. Two ml of fresh OPTI-MEM then was added into each well. At 48 h posttransfection, the cells were fixed with methanol, and syncytia were visualized by Giemsa staining. Digital photographs of syncytia were taken under a Nikon TS100 inverted phase-contrast microscope mounted with a Nikon Coolpix995 camera.

Quantitation of fusion by content-mixing assay. Confluent Vero E6 cells in 6-well plates were cotransfected with 2 μ g each plasmid containing the aMPV F gene and a plasmid (pGINT7 β -gal) encoding a β -galactosidase gene under the control of the T7 promoter. At 24 h posttransfection,

the cells were detached with trypsin, washed twice with DMEM, and re-suspended in DMEM plus 10% FBS. The plasmid-transfected cells were mixed with equal numbers of BHK21 cells, which constitutively express T7 RNA polymerase. After incubation at 37°C for another 24 h, the cells were washed 4 times with PBS (pH 7.0 or 5.0) containing 10 mM HEPES and 5 mM MES. The cells were lysed by Nonidet P40 solution (0.5%). The extent of fusion was quantitated by measuring the β -galactosidase activity using a luminometer plate reader (Molecular Devices, Sunnyvale, CA) (29, 30, 33).

Biotinylation and Western blotting. The F protein expression level on the cell surface was determined by biotinylation of surface proteins with a membrane-impermeable biotinylating agent followed by Western blotting as described previously (34). Vero E6 cells were grown at 37°C in 6-well plates and were transfected with 2 μ g of pCAGGS carrying HA-tagged F genes. At 36 h posttransfection, surface proteins were biotinylated using membrane-impermeable EZ-Link Sulfo-NHS-SS-Biotin (Thermo Scientific, Rockford, IL), and the cells were lysed in DH buffer (50 mM HEPES [pH 7.2], 0.5% dodecyl- β -D-maltoside [Affymetrix, Inc., Maumee, OH], 100 mM NaCl, Complete Mini EDTA-free protease inhibitor cocktail [Roche Diagnostics, Indianapolis, IN]). The lysates were centrifuged at 12,000 $\times$ g for 20 min at 4°C, and the supernatants were collected. The cell surface F proteins were immunoprecipitated using streptavidin beads (Thermo Scientific) followed by Western blotting using an HA monoclonal antibody (Sigma). The immunoprecipitated proteins were separated by electrophoresis in a 12% sodium dodecyl sulfate-polyacrylamide gel (SDS-PAGE) under denaturing and reducing conditions. Separated proteins were transferred to a Hybond ECL nitrocellulose membrane (Amersham) in a Mini Trans-Blot electrophoretic transfer cell (Bio-Rad). The blot was probed with HA monoclonal antibody (Sigma) at a dilution of 1:5,000, followed by horseradish peroxidase-conjugated goat anti-mouse IgG secondary antibody (Santa Cruz) at a dilution of 1:5,000. The blot was visualized with SuperSignal West Pico chemiluminescent substrate (Thermo Scientific) and exposed to Kodak BioMax MR film (Kodak).

Sequence alignment and analysis. Sequence alignment was performed by using the MegAlign program in Lasergene (DNASTAR, Madison, WI). The F genes used in sequence analysis included aMPV subtype A, B, and C and hMPV lineage A and B strains.

Structure modeling of F proteins. Structure prediction of F proteins of aMPV were performed using Modeler 9.0 using the prefusion crystal structure of parainfluenza virus 5 (PIV5) F protein as the template (47).

Statistical analysis. All experiments were performed three to five separate times. Quantitative analysis was performed by either densitometric scanning of autoradiographs or by using a phosphorimager (Typhoon; GE Healthcare) and ImageQuant TL software (GE Healthcare, Piscataway, NJ). Statistical analysis was performed by one-way multiple comparisons using SPSS 8.0 statistical analysis software (SPSS Inc., Chicago, IL). A *P* value of <0.05 was considered statistically significant.

RESULTS

Distinct phenotypes of cell-cell fusion promoted by F proteins of aMPV subtypes A, B, and C. We first investigated syncytium formation by the F proteins of each aMPV subtype at pH 7.0 in the absence of trypsin, conditions under which fusion occurs for most paramyxoviruses. As shown in Fig. 1A, the F protein of aMPV subtype A was highly fusogenic. Numerous large syncytia were observed at 48 h, and the cells were detached at later time points. In contrast, the F protein of aMPV subtype C formed much fewer and smaller syncytia than did subtype A (Fig. 1A). In addition, there is no significant difference between syncytium formation between F proteins of aMPV C-MN and aMPV C-CO strains. Surprisingly, few to no syncytia were observed with the F protein of subtype B in the absence of trypsin (Fig. 1A).

Since we were not able to observe any syncytium formation

with the F protein of aMPV subtype B, we postulated that fusion promotion for this subtype requires trypsin. In fact, trypsin is essential not only for the growth of hMPV (3, 13) but also for fusion promotion induced by its F protein (42, 43). To determine the role of trypsin in aMPV fusion, we analyzed syncytium formation of the aMPV F protein following trypsin treatment. Briefly, the transfected cells were washed 4 times with PBS solution (pH 7.0), and cell monolayers were incubated with 2 ml of OPTI-MEM either with or without TPCK-trypsin (0.2 μ g/ml). Interestingly, small syncytia were observed for the F protein of subtype B when TPCK-trypsin was added, indicating that subtype B requires trypsin for cell-cell fusion (Fig. 1A). For the F proteins of aMPV subtypes A and C, syncytia were significantly enhanced and fusion occurred much earlier with the addition of trypsin (Fig. 1A). Unfortunately, we were unable to quantitatively compare the fusion activity with or without trypsin because we had to detach the cells with trypsin in the quantitative content-mixing fusion assay. To further determine the effect of trypsin on cell-cell fusion, we determined the kinetics of syncytium formation of subtype A-F protein by fixing the cells at different times. Large syncytia were observed at 14 h posttransfection for subtype A with trypsin (Fig. 2). In contrast, syncytia were not observed until 22 h posttransfection without trypsin (Fig. 2). Thus, these results demonstrate that (i) cell-cell fusion of aMPV F protein occurs at neutral pH and is independent of the G protein, and (ii) trypsin is required for aMPV B fusion and is able to enhance it but is not required for the induction of cell-cell fusion by subtypes A and C.

aMPV F proteins do not require low pH for cell-cell fusion.

Although aMPV F proteins promoted cell-cell fusion at pH 7.0, we cannot exclude the possibility that lower pH enhances their fusion-promoting activities. Indeed, the F proteins of some hMPV strains require low pH for cell-cell fusion (21, 31, 42, 43). To further elucidate the role of pH in aMPV fusion, we evaluated the fusion activities of aMPV F proteins at lower pH. Syncytium formation experiments were conducted as described earlier except that, after transfection, the cells were exposed to PBS at pH 5 or 7 four times, each of which lasted 4 min with an interval of 2 h. As shown in Fig. 1A and B, low pH did not significantly enhance the syncytium formation for any aMPV subtype regardless of the presence or absence of trypsin. Subsequently, fusion activities were quantitated by a content-mixing assay at pH 7.0 or 5.0 as described in Materials and Methods. As shown in Fig. 1C, there is no significant difference between the fusion activities of each aMPV F protein at pH 5.0 and 7.0 (*P* > 0.05), indicating that aMPV F protein-mediated cell-cell fusion has no pH preference. In addition, the F protein of aMPV subtype A exhibited fusion activity that was about 5- to 6-fold higher than that of F protein of aMPV subtype B or C, whereas the difference between the F proteins of aMPV subtypes B and C was moderate.

The effect of aMPV G protein on cell-cell fusion. The results described above clearly demonstrated that aMPV G protein is not required for cell-cell fusion. However, whether the presence of the G protein modulates cell-cell fusion is not known. To determine this, Vero E6 cells were cotransfected with 2 μ g of plasmids encoding F and G genes of each aMPV subtype, and the syncytium formation assay was carried out as described previously. As shown in Fig. 3A, cotransfection of a plasmid encoding the aMPV G gene significantly decreased syncytium formation for aMPV subtype A- and B-F proteins but not subtype C-F protein. Quantitative fusion assay showed that coexpression of aMPV G protein reduced fu-

FIG 1 Characterization of syncytium formation induced by F proteins of subtypes A, B, and C. (A) Syncytium formation induced by F proteins of aMPV subtypes at pH 7.0. Vero E6 cells were transfected with 2 μ g of each indicated plasmid. After 24 h, cell monolayers were washed 4 times with PBS solution (pH 7.0) supplemented with 10 mM HEPES and 5 mM MES, each of which lasted 4 min with an interval of 2 h. The cells were incubated with 2 ml of OPTI-MEM either with or without TPCK-trypsin (0.2 μ g/ml). At 48 h, monolayers were fixed with methanol and stained with Giemsa stain. (B) Syncytium formation induced by F proteins of aMPV subtypes at pH 5.0. Experiments were conducted as described for panel A, except that the transfected cells were washed 4 times with low-pH PBS buffer (pH 5.0). (C) The effect of pH on the cell-cell fusion of aMPV F proteins. Vero E6 cells were cotransfected with 2 μ g of plasmid containing the aMPV F gene and a reporter gene plasmid, pGINT7, in which a β -galactosidase gene is under the control of the T7 promoter. At 24 h posttransfection, the cells were detached with trypsin and mixed with equal numbers of BHKT7 cells, which express T7 RNA polymerase. After incubation at 37°C for 24 h, the cells were washed 4 times with PBS at pH 7.0 or 5.0. The cells were lysed by Nonidet P40 solution (0.5%), and then 50 μ l of the lysates was mixed with 50 μ l of the β -galactosidase substrate chlorophenol red- β -D-galactopyranoside (16 mM) and incubated at room temperature for 4 h. The extent of fusion was quantitated by microplate spectrophotometer at an absorbance of 570 nm. Data shown are averages from three independent experiments.

sion to approximately 25 and 22% for subtype A- and B-F proteins, respectively, compared to fusion of the corresponding F protein alone (Fig. 3B and C). However, subtype C-MN G protein did not significantly affect cell-cell fusion by its homologous F protein ($P > 0.05$) (Fig. 3D). Therefore, G protein inhibited cell-cell fusion of aMPV subtypes A- and B-F proteins but not subtype C-F protein.

The effect of HA tag on cell-cell fusion of aMPV F protein.

The F proteins of aMPV subtypes A, B, and C share 70 to 80% amino acid sequence homology. Currently, there is no universal antibody available that can recognize the F proteins of all three aMPV subtypes. Therefore, we engineered an HA tag to the C terminus of each F gene to facilitate the detection of F proteins. Previously, it has been shown that the HA tag may enhance or inhibit the cell-cell fusion of some paramyxovirus F proteins. To determine the effect of the HA tag on cell-cell fusion of aMPV F proteins, the fusion activities of HA-tagged F proteins were compared to each wild-type F protein. As shown in Fig. 4A, no signif-

icant difference in syncytium formation was observed between the HA-tagged and wild-type F proteins. In the content-mixing fusion assay, it was found that A-F-HA and C-F-HA only slightly decreased cell-cell fusion, whereas B-F-HA slightly increased the fusion activity, each relative to the untagged proteins (Fig. 4B to D). However, these differences are not statistically significant ($P > 0.05$). This result demonstrated that the HA tag had no significant effect on the ability of aMPV F proteins to promote cell-cell fusion.

The expression of F proteins on the cell surface and cleavability of the F proteins of aMPV subtypes. To begin to explore the basis for the fusogenic differences among the F proteins of the aMPV subtypes, we first determined whether the F protein of each aMPV subtype was processed and cleaved on the cell surface. The expression of F proteins on the cell surface was determined either with or without TPCK-trypsin by biotinylation of surface F protein followed by Western blotting using an HA-specific monoclonal antibody. The cell surface F0 (native F) and F1 (cleaved F) was

FIG 2 Effect of trypsin on syncytium formation induced by F protein of aMPV subtype A. Vero E6 cells were transfected with 2 μ g of pCAGGS-A-F. After transfection, cell monolayers were incubated with 2 ml of OPTI-MEM with or without TPCK-trypsin (0.2 μ g/ml). At the indicated time, monolayers were fixed with methanol and stained with Giemsa stain.

FIG 3 Effect of G protein on cell-cell fusion of aMPV F protein. (A) Syncytium formation induced by coexpression of aMPV F and G proteins. Vero E6 cells were transfected with 2 μ g of pCAGGS-A-F, pCAGGS-B-F, or pCAGGS-C-MN-F or cotransfected with 2 μ g of pCAGGS-A-G, pCAGGS-B-G, or pCAGGS-C-MN-G. At 24 h posttransfection, cell monolayers were washed 4 times with PBS solution (pH 7.0). Each wash lasted 4 min with an interval of 2 h. The cells then were incubated with 2 ml of OPTI-MEM with TPCK-trypsin (0.2 μ g/ml). At 48 h, monolayers were fixed with methanol and stained with Giemsa stain. (B) Content-mixing fusion assay for aMPV subtype A-F and G proteins. (C) Content-mixing fusion assay for aMPV subtype B-F and G proteins. (D) Content-mixing fusion assay for aMPV subtype C-MN-F and G proteins.

quantitated by densitometry. As shown in Fig. 5A to C, there were striking differences in the expression level of the F protein from each aMPV subtype on the cell surface. First, trypsin increased the levels of all three aMPV F proteins on the cell surface (Fig. 5A and B). Without trypsin, the level of subtype B-, C-MN-, and C-CO-F proteins was approximately 66, 500, and 200% that of subtype A-F protein (100%), respectively. With trypsin, the level was increased to 150, 120, 530, and 230% for F proteins of subtypes A, B, C-MN, and C-CO, respectively. It is likely that trypsin treatment makes more protein available to biotinylation and detection by Western blotting. Second, trypsin enhanced the efficiency of cleavage of the F proteins (Fig. 5C). Without trypsin, approximately 75, 50, 64, and 62% of the F proteins were cleaved for F proteins of subtypes A, B, C-MN-F, and C-CO-F, respectively. With trypsin, the percentage of cleaved F1 was increased to 90, 75, 75, and 78%, respectively. Thus, this result suggests that trypsin treatment increases the intracellular and/or cell surface cleavage of the F protein. Third, the F0 and F1 of subtype C-MN migrated the lower than subtype C-CO-F, although both proteins are composed of 537 amino acids. The mobility and expression difference of C-MN-F and C-CO-F may due to the amino acid difference (98.7% homology) between these two proteins. The fusion activity of the F proteins of each subtype was normalized to the cell surface expression level. The fusion activity of subtype A-F was approximately 3, 5, and 4 times higher than that of subtypes B-F, C-MN-F, and C-CO-F, respectively. Together, these data suggest that (i) the differences in processing and expression level of the F proteins on the cell surface might contribute to, but are not the primary determinants of, the difference in fusogenic ability for each aMPV subtype, and (ii) trypsin increased the expression level and processivity of aMPV F proteins.

Mutations for the conserved histidine residues in aMPV subtype C did not affect fusion activity. One of the unique characteristics of hMPV F-induced membrane fusion is that the F pro-

FIG 4 Effect of HA tag on cell-cell fusion of aMPV F protein. (A) Comparison of syncytium formation induced by wild-type F and HA-tagged F protein. Vero E6 cells were transfected with 2 μ g of each plasmid. At 48 h posttransfection, the monolayers were fixed with methanol and stained with Giemsa stain. (B to D) Fusion activity of wild-type F and HA-tagged F protein. The extent of fusion was quantitated with the content-mixing fusion assay.

teins of some hMPV strains require low pH for fusion (42, 43). The mechanism of low-pH-mediated fusion is thought to involve electrostatic repulsion by protonation of histidine residues (5, 24, 40). Previous mutagenesis analyses of three conserved histidine residues (H332, H368, and H435) in the F protein of the hMPV CAN 97-83 strain showed that mutations at H435 ablated fusion activity without altering the protein expression level on the cell surface (42). Thus, it is thought that protonation of H435 may be involved in triggering the low-pH-dependent hMPV fusion (42). Consistent with the finding that the F proteins of aMPV subtypes A and B do not require low pH for fusion, F protein residue H435 is not even conserved in these two subtypes (Fig. 6A). Interestingly, all three histidine residues are conserved in the F protein of aMPV subtype C (Fig. 6A). Therefore, the corresponding histidine residues in F protein of aMPV subtype C MN strain were replaced with alanine, and the fusion activities of mutant F proteins were quantified. Mutations at these three histidine residues did not significantly affect cell-cell fusion (Fig. 6B) or protein expression level on the cell surface (data not shown), suggesting that these three conserved histidine residues do not contribute to the cell-cell fusion of aMPV subtype C. Thus, these results further support our finding that aMPV-induced cell-cell fusion does not require low pH. Since the histidine residue (H435) responsible for low-pH-mediated fusion of hMPV F protein is not conserved in the F proteins of subtypes A and B, we did not pursue further mutagenesis of the histidine residue in these two F proteins.

FIG 5 Cleavability and expression of aMPV F proteins on the cell surface. (A) Analysis of cell surface expression of aMPV F proteins by biotinylation and Western blotting. Vero E6 cells were transfected with 2 μ g of pCAGGS carrying HA-tagged F genes. At 36 h posttransfection, surface proteins were biotinylated using membrane-impermeable EZ-Link Sulfo-NHS-SS-Biotin. The cell surface F protein was immunoprecipitated using streptavidin beads. Proteins were separated by 12% SDS-PAGE under denaturing and reducing conditions. Protein levels on the cell surface were detected by Western blotting using an HA monoclonal antibody followed by HRP-conjugated goat anti-mouse IgG. F0, native full-length F protein; F1, cleaved F protein. (B) The effect of trypsin on the levels of aMPV F proteins at the cell surface. For densitometric quantitation, all Western blot films were scanned using a Typhoon 9210 scanner, and the intensities of protein bands were determined using ImageQuant TL software. For each protein band, background was subtracted and the intensities of F0 and F1 were normalized to the value for subtype A-F protein (without trypsin). (C) The effect of trypsin on cleavability of aMPV F proteins. The intensities of F0 and F1 were quantitated using ImageQuant TL software. For each aMPV subtype, the percentage of F1 was normalized by the total of proteins (F0 and F1) on the cell surface.

The role of the F cleavage site in fusion promotion of aMPV subtypes A and B. We then attempted to identify specific motifs or amino acid residues in the aMPV F protein that may be responsible for the observed difference in cell-cell fusion activity. Se-

FIG 6 Effect of mutations at the conserved histidine residues on the fusion of aMPV. (A) Amino acid sequence alignment of hMPV and aMPV F proteins. Amino acid residues at positions 294, 332, 368, and 435 that may contribute to the low-pH-mediated fusion are shown. The role of low pH in fusion for each strain is indicated on the right side. (B) Mutations at the conserved histidine residues did not affect fusion activity of aMPV subtype C MN strain. Fusion was quantified by a cell content-mixing assay at pH 7.0. Data shown are averages from three independent experiments.

sequence homology analyses of aMPV and hMPV F proteins reveal notable differences in the amino acid sequence of the cleavage site in the F proteins of aMPV subtypes (Fig. 7A). The F cleavage sites of subtypes A and B are ⁹⁹RRRR¹⁰² and ⁹⁹RKKR¹⁰², respectively, which are composed of four basic amino acids, the preferred motif for recognition by intracellular proteases. In contrast, the F cleavage site of subtype C is ⁹⁹RKAR¹⁰², which contains only three basic amino acids. To determine whether the F cleavage site contributes to the phenotypic difference, the F cleavage site of subtype A was changed to that of subtypes B and C by site-directed mutagenesis, which resulted in construction of AB-F and AC-F, respectively. Similarly, the F cleavage site of subtypes B and C was changed to that of subtype A, which resulted in construction of BA-F and CA-F, respectively. The expression level of these F cleavage site mutants on the cell surface was determined by biotinylation and Western blotting. As shown in Fig. 7B and C, F cleavage site mutants (AB-F and AC-F) of subtype A were expressed at the cell surface at a level similar to that of the wild-type subtype A-F protein. Similarly, BA-F did not significantly alter the protein expression level compared to that of the wild-type subtype B-F protein. However, when the F cleavage site of subtype C was changed to that of subtype A, the resultant F mutant (CA-F) carrying K100R and A101R substitutions had an approximately 2-fold reduction in protein expression level compared to C-F. Interestingly, CA-F exhibited a mobility pattern that is similar to that of the F proteins of subtype C-CO-F proteins. Subsequently, the effect of the F cleavage site mutations on cell-cell fusion was quantified by the

FIG 7 Effect of mutation in F cleavage site on fusion of aMPV F protein. (A) Sequence alignment of F cleavage site of aMPV F proteins. (B) Expression and cleavability of F cleavage site mutants. Cell surface expression of F protein was determined by biotinylation and Western blotting. AB-F, the F cleavage sites of subtype A-F changed to that of subtype B-F. BA-F, the F cleavage sites of subtype B-F changed to that of subtype A-F. AC-F, the F cleavage sites of subtype A-F changed to that of subtype C-F. CA-F, the F cleavage sites of subtype C-F changed to that of subtype A-F. (C) Quantitation of expression of F cleavage site mutants on the cell surface. The intensities of F0 and F1 were quantitated using ImageQuant TL software. The percent expression for each F mutant was normalized to the expression level of A-F. (D) Mutation in F cleavage site did not significantly affect fusion activity of aMPV F protein. Fusion was quantitated by content-mixing fusion assay and normalized by the percentage of expression on the cell surface.

content-mixing fusion assay and normalized to the level of the F protein expression on the cell surface. As shown in Fig. 7D, AB-F and AC-F did not exhibit any significant reduction in cell-cell fusion activity. Similarly, BA-F and CA-F did not exhibit signifi-

FIG 8 Construction of chimeric F proteins and their phenotypes. (A) Diagram of the structure of the F protein. The F protein is proteolytically cleaved to produce the two disulfide-linked polypeptides, F1 and F2. Cleavage generates the hydrophobic fusion peptide (FP). There are two heptad repeats (HRs) in aMPV F: HRA, adjacent to the FP, and HRB, linked to the transmembrane (TM) and cytoplasmic domain (CT). (B) Strategy for construction of F chimeras. Chimeras were constructed between the F gene of subtype A-F (white bars) and that of subtype B-F (gray bars). This was made possible by the use of unique restriction enzyme sites in the two genes corresponding to amino acid residues 107, 170, 338, and 441. (C) Expression level of chimeric F proteins on the cell surface determined by biotinylation and Western blotting. (D) Densitometric quantitation of expression level of chimeric F proteins. Data were expressed as the percentage of subtype A-F protein. (E) Fusion activity of chimeric F proteins. The extent of fusion was quantitated with the content-mixing fusion assay and normalized to the expression level of each F protein.

cantly increased cell-cell fusion. Therefore, the differences in the F cleavage sites did not contribute to the hyperfusion activity of F protein of subtype A.

Localization of a specific region in aMPV F that is responsible for the hyperfusogenic activity of aMPV subtype A. To identify the domains or residues that account for the differences in the phenotypic properties of F proteins of aMPV A and B, we constructed a series of chimeric F proteins exchanging functionally important domains between these two F proteins (Fig. 8B). There are a total of 62 amino acid differences (78.5% homology) between the F proteins of subtypes A and B scattered over the entire length of the protein. We first switched the F1 and F2 fragments of aMPV A and B and generated chimeras CH-A and CH-B. Both chimeras were expressed efficiently on the cell surface (Fig. 8C and D). CH-A has the F2 segment derived from subtype B-F and the F1 segment from subtype A-F protein. This chimera induced a hyperfusion phenotype similar to that of the subtype A-F protein (Fig. 8E). CH-B is the reciprocal chimera with an F2 portion from the subtype A-F protein and an F1 portion from the subtype B-F protein. This chimera exhibited a fusogenic activity similar to that of subtype B-F protein (Fig. 8E). These results indicate that the determinant(s) of

the fusion differences between the F proteins of subtypes A and B reside in the F1 segment of the protein.

To determine which portion of the F1 fragment contributes to these phenotypic differences, we next divided the F1 segment into three regions based on the conserved functional domains. Region 1 (encompassing residues 103 to 170) contains the fusion peptide (F) and heptad repeat A (HR-A). Region 3 (residues 441 to 538) contains heptad repeat B (HR-B), TM, and CT. Region 2 includes the rest of the F1 protein, which is composed of amino acid residues 170 to 441 located between HR-A and HR-B. Chimeras CH-C and CH-D were constructed to address whether region 2 of F protein contributes to the phenotypic differences between the F proteins of subtypes A and B. As shown in Fig. 8E, chimera CH-C with the region 2 derived from subtype A and the rest of the protein from subtype B exhibited a hyperfusion phenotype that was similar to the subtype A-F protein. In contrast, the reciprocal chimera CH-D, with region 2 derived from subtype B and the rest of the protein from subtype A, behaved like the subtype B-F protein. Taken together, these results show that region 2 in the F1 protein is responsible for the phenotypic differences between the subtype A- and B-F proteins.

FIG 9 Sequence alignment of 2a region (amino acids 170 to 338) in the F proteins of aMPV and hMPV. Amino acids that are unique to subtype A-F protein are highlighted by boxes. The three residues responsible for the phenotypic difference between the aMPV subtype A-F and B-F proteins are indicated by arrows. Sequence alignment was performed by using the Megalign program in Lasergene (DNASTAR). The F proteins used for sequence alignment include hMPV lineage A1 strain NL/1/00 (accession number [AF371337](#)), lineage A2 strain CAN 97-83 (accession number [AY297749](#)), lineage B1 strain NL/1/99 (accession number [AY525843](#)), lineage B2 strain NL/1/94 (accession number [AY304362](#)), aMPV subtype A strain Tian-Tai (accession number [JN224984](#)), subtype B strain Shao-Xin (accession number [JN224985](#)), and subtype C strains MN (accession number [FJ977568](#)) and CO (accession number [AY579780](#)).

Since region 2 is quite large (271 amino acids), we decided to further narrow down the specific region that accounts for the hyperfusion activity. To do this, we divided region 2 into two segments, 2a and 2b, which are composed of amino acid residues 170 to 338 and 338 to 441, respectively (Fig. 8A). We have constructed chimeras CH-E and CH-F, in which regions 2a and 2b, respectively, are derived from the subtype A-F protein. On the one hand, CH-E, with the region 2a from subtype A-F and the rest of the protein from subtype B-F, exhibited a phenotype similar to that of subtype A-F (Fig. 8E). On the other hand, CH-F, with the region 2b derived from subtype A and the rest of the protein from subtype B-F, displayed a phenotype similar to that of subtype B-F protein (Fig. 8E). Hence, residues responsible for the phenotypic difference between the F proteins of subtypes A and B are located within region 2a ranging from residues 170 to 338.

Mutagenesis analysis of region 2a of F protein identified amino acid residues essential for the hyperfusion activity. Comparison of the sequences of the region 2a in the F proteins of subtypes A and B reveals nine amino acid differences (Fig. 9). To probe for specific residues responsible for the hyperfusion activity, we limited our selection to residues that are physicochemically distinct between these two F proteins, namely, residues 225, 264, 295, 297, and 323 (Fig. 9). In subtype A-F, these residues are ²²⁵Asp, ²⁶⁴Asp, ²⁹⁵Arg, ²⁹⁷Gly, and ³²³Lys, whereas the corresponding residues in subtype B-F are ²²⁵Ala, ²⁶⁴Gly,

²⁹⁵Glu, ²⁹⁷Glu, and ³²³Glu. To evaluate the contribution of each of these residues to the phenotypic differences between aMPV A and B, we replaced the residues at these positions in subtype A-F with their counterparts in subtype B-F and evaluated the effect on protein expression and fusion promotion. As shown in Fig. 10, D225A- and D264G-mutated A-F proteins did not exhibit any detectable alteration in protein expression level or fusion activity, suggesting that these residues do not contribute to the phenotypic difference between A-F and B-F. In contrast, the R295E, G297E, and K323E substitutions significantly decreased the cell-cell fusion activity while their expression levels on the cell surface were comparable (Fig. 10B and C), suggesting that these three amino acids contribute to the phenotypic differences.

Reciprocally, we replaced the residues at positions 295, 297, and 323 in subtype B-F with their counterparts in subtype A-F and determined the effect on fusion promotion and protein expression level. As shown in Fig. 11, all mutated B-F proteins were expressed efficiently at the cell surface. Interestingly, the E297G substitution increased cell-cell fusion activity by approximately 1.7-fold compared to that of the subtype B-F protein (Fig. 11A and D). Conversely, the E295R and E323K substitutions significantly decreased cell-cell fusion. Subsequently, we constructed two double B-F mutants, E295R + E323K and E297G + E323K. Similar to the E295R single substitution, the combination of E295R and E323K decreased fusion activity.

FIG 10 Mutagenesis analysis in 2a region of subtype A-F protein. (A) Syncytium formation induced by subtype A-F mutants. Vero E6 cells were transfected with 2 μ g of each plasmid. At 22 h posttransfection, the monolayers were fixed with methanol and stained with Giemsa stain. (B) Expression level of F mutants on the cell surface determined by biotinylation and Western blotting. (C) Densitometric quantitation of expression level of F mutants. Data were expressed as the percentage of subtype A-F protein. (D) Fusion-promoting activities of subtype A-F mutants. The extent of fusion was quantitated with the content-mixing fusion assay and normalized by expression level of each F protein on the cell surface.

Interestingly, the combination of E297G and E323K significantly decreased fusion activity compared to the E297G single-amino-acid substitution. However, only when all three mutations were introduced in subtype B-F protein did the protein

acquire the hyperfusogenic phenotype of the subtype A-F protein. This triple mutant (E295R + E297G + E323K) exhibited nearly 2.5 times the fusion activity of the subtype B-F protein (Fig. 11A and D). Therefore, three amino acids at positions

FIG 11 Mutagenesis analysis in 2a region of subtype B-F protein. (A) Syncytium formation induced by subtype B-F mutants. Vero E6 cells were transfected with 2 μ g of each plasmid. At 48 h posttransfection, the monolayers were fixed with methanol and stained with Giemsa stain. (B) Expression level of F mutants on the cell surface determined by biotinylation and Western blotting. (C) Densitometric quantitation of expression level of F mutants. Data were expressed as percentages of subtype B-F protein. (D) Fusion-promoting activities of subtype B-F mutants. The extent of fusion was quantitated with the content-mixing fusion assay and normalized to the expression level of each F protein on the cell surface.

295, 323, and 297 collectively contribute to the phenotypic differences between the two F proteins.

DISCUSSION

Membrane fusion of the *Pneumovirinae* subfamily is less understood than that of the *Paramyxovirinae* subfamily. In the present study, we have demonstrated that none of the F proteins of the three aMPV subtypes requires the homologous attachment protein for cell-cell fusion. Similar observations have been reported with other viruses in the *Pneumovirinae* subfamily, such as RSV and hMPV, indicating that such attachment protein-independent fusion is common among members of the *Pneumovirinae* subfamily. However, there are notable differences in cell-cell fusion among the aMPV subtypes. The F protein of aMPV subtype A is highly fusogenic, whereas the F proteins from subtypes B and C induce cell-cell fusion only at low levels. The F protein of aMPV subtype B, but not A and C, requires trypsin for cell-cell fusion. In addition, the F proteins of all three aMPV subtypes do not require low pH for cell-cell fusion, which is in contrast to the fusion requirement of some hMPV strains (21, 31, 42, 43).

It is generally accepted that the binding of the attachment protein to cell surface receptors triggers *Paramyxovirinae* F proteins, although the detailed molecular mechanism underlying fusion triggering is poorly understood. Since the G attachment protein is not required for F protein triggering in pneumoviruses, it is not clear what triggers the F proteins of pneumoviruses. While it has been proposed that low pH triggers the fusion of hMPV F protein through protonation of H435 (42), emerging evidence indicates that low-pH-induced membrane fusion mediated by the hMPV F protein is a rare and strain-dependent phenomenon (21, 31). Indeed, only 6% of hMPV lineage A strains may require low pH for triggering, whereas all known hMPV lineage B strains and the majority of lineage A strains do not require low pH for fusion, suggesting that low pH is not a general trigger for hMPV F protein. Strain comparison and mutagenesis analysis found that the glycine residue at position 294 is responsible for the low pH requirement among different lineages of hMPV (21). In this study, we found that none of the F proteins of aMPV subtypes requires low pH for cell-cell fusion. Consistent with this, mutations of the conserved histidine residues did not affect cell-cell fusion. It is worthwhile to note that this glycine residue at position 294 is also conserved in aMPV subtype C-F proteins (Fig. 6A), but none of the tested aMPV subtypes requires low pH for cell-cell fusion. This observation further supports the idea that the fusion requirements of aMPV F proteins differ from those of the hMPV F protein. It was also reported that heat was able to trigger many paramyxovirus fusion proteins, such as those of Sendai virus (53) and PIV5 (38). The mechanism probably involves thermal destabilization of the metastable F protein, initiating its conformational change (38, 53). Recently, it was found that a soluble pretriggered F protein of pneumovirus RSV was triggered by exposure to a low molarity buffer (6). Although it is not clear whether this treatment is similar to the physiological triggering of the F protein on the cell or virion surface, it is known that salt concentration plays an important role in protein stability and conformational change. It is likely that decreasing or increasing the salt concentration will destabilize the metastable pretriggered F protein, which in turn would trigger the conformational changes for fusion.

Another interesting observation for aMPV cell-cell fusion is that the aMPV G protein significantly inhibited cell-cell fusion of

the corresponding subtype A- and B-F proteins. Consistent with our observation, it has been shown that recombinant aMPV subtype A virus lacking the G protein formed much larger syncytia than wild-type virus despite the fact that it grew more slowly in cell culture (35). It appears that aMPV G has a negative impact on cell-cell fusion of subtypes A and B. In contrast, we found that aMPV subtype C G protein did not have a significant effect on cell-cell fusion promoted by subtype C-F protein. Govindarajan et al. found that recombinant aMPV subtype C virus lacking the G protein replicated slightly better than did wild-type aMPV in Vero cells (19). In the case of hMPV, it was found that G inhibited cell-cell fusion promoted by hMPV F protein in Vero cells by approximately 50% (43). However, hMPV G appeared to enhance hMPV F-promoted fusion in BHK cells, suggesting that the effect of G is cell type specific (43). For human RSV, bovine RSV, and ovine RSV, it seems that the G protein was able to enhance cell-cell fusion (22, 37). Therefore, the pneumovirus G protein has variable effects on cell-cell fusion that depend on the particular virus via a poorly understood mechanism. It could be related to the competition for receptor, interaction of these two glycoproteins, and/or interference of F conformational changes.

By construction of chimeric F proteins derived from aMPV subtypes A and B, we identified a region (2a) composed of amino acid residues 170 to 338 that is responsible for the hyperfusion activity. Further site-directed mutagenesis studies revealed that residues R295, G297, and K323 in this region play crucial roles in modulating the hyperfusion promoted by aMPV subtype A-F protein. While individual substitution of these residues in subtype A-F with the corresponding residues in subtype B-F significantly reduced cell-cell fusion, we failed to completely restore the hyperfusion phenotype in subtype B-F protein by replacing individual residues at positions 295, 297, and 323 with their counterparts in subtype A-F, suggesting that several amino acids in this region collectively contribute to the hyperfusion activity. Thus, we also cannot rule out the possibility that residues other than R295, G297, and K323 contribute to the hyperfusion phenotype.

To gain possible structural insight into the hyperfusion induced by aMPV subtype A-F protein, we constructed model structures of the F proteins from aMPV subtypes A, B, and C using the atomic coordinates of the crystal structure of the prefusion PIV5 F protein (55). As in PIV5 F protein, each F monomer is composed of five well-structured domains, namely, DI to DIII, HRA, and HRB, and one relatively loose loop connecting the C terminus of DII and HRB, which is named the HRB linker (Fig. 12A and D). As revealed in the crystal structures of prefusion PIV5 F protein and postfusion PIV3 F protein, the HRB linker is relatively flexible, and such flexibility appears to facilitate the transition from pre- to postfusion forms of F protein (54, 55). The structural homology in the HRB linker is very low among aMPV and hMPV F proteins compared to the homology of other domains, such as DIII and DII, and interestingly, the HRB linker in the aMPV subtype A-F protein is the most flexible one (Fig. 12A and D). Thus, it is possible that the difference in the fusion activity among aMPV and hMPV F proteins is partly attributed to the flexibility of the HRB linker.

In the model structure of aMPV F trimer (Fig. 12C and F), the majority of residues in region 2a (residues 170 to 338) are located at the surface of the F trimer. Interestingly, the corresponding region in the F protein of the *Paramyxovirinae* subfamily has been

FIG 12 Comparisons of the model structures of aMPV F proteins. (A) Superposition of F monomers of aMPV subtype A. The model structures of F protein were generated using Modeler 9.0, and the template was the A chain of PIV5 F trimer. The arrow represents a loop present only in the HRB linker of aMPV A-F protein, indicating that aMPV A-F protein is more flexible in the HRB linker than other aMPV F proteins. Residues 170 to 338 are highlighted in magenta. (B) Superposition of F monomers of aMPV A by a 180° rotation. (C) The positioning of residues 170 to 338 in aMPV subtype A F trimer. Only residues 170 to 338 in the A chain of the aMPV F trimer are highlighted in magenta. (D) Superposition of F monomers of aMPV B. (E) Superposition of F monomers of aMPV B by a 180° rotation. (F) The positioning of residues 170 to 338 in aMPV subtype B-F trimer.

shown to specifically interact with the homologous attachment protein for fusion promotion (28, 47, 48). Previous studies using chimeric F proteins derived from hPIV2 and simian virus 41 (SV41) have shown that a region composed of 144 amino acids (from positions 227 to 370) in HPIV2 F protein harbors the site(s) that determines the specificity of the interaction with homologous HN protein (47). Most recently, the HN specificity domains were further localized into two smaller regions, called M1 (amino acids from 234 to 246) and M2 (amino acids from 278 to 285) (48). Of note, these HN specificity domains were located in the surface of the F trimer based on the predicted structure (48). A similar region in the F protein of canine distemper virus (CDV) mediates the interaction with the homologous H protein (28). While the metapneumovirus F proteins do not require the G attachment protein for fusion, one possibility is that this region interacts with putative receptors required for fusion promotion. This hypothesis is supported by the fact that the F proteins of metapneumovirus possess a putative integrin binding motif (RGD, RDD, or RSD) located at positions 329 to 331. In hMPV F, it has been shown that the RGD motif binds to the $\alpha\beta 1$ integrin receptor that is essential for viral infection (12). Although recent evidence suggests that the interaction between integrins and hMPV occurs after the initial binding of hMPV F to heparan sulfate proteoglycans (7), integrins did play an important role in promoting hMPV entry and infec-

tion. In fact, our preliminary data showed that mutations of the RGD motif in the hMPV F protein abolished or significantly diminished fusion activity (Y. Wei and J. Li, unpublished data). Interestingly, we also found that a K323E substitution in aMPV A-F protein abolished its fusion activity, and an E323K mutation in the aMPV B-F protein decreased fusion activity. In the model structures of both F proteins, residue 323 is located in DI and close to the putative integrin binding motif RDD (residues 329 to 331) (Fig. 13A and B). As revealed in the crystal structure of PIV5 F protein, DI forms the bottom of the large cavity present at the base of the head, which may be responsible for binding to this putative receptor. In the predicted trimer structure, the RDD motif is located at the surface of F protein, whereas K323 is buried inside the F trimer (Fig. 13C and D). Even though residue 323 is not directly involved in binding to the receptor, it may facilitate the binding of the receptor to the RDD motif or function to improve the specificity of receptor binding.

In the model structure of the aMPV subtype A-F protein (Fig. 13A), there exists a cluster of positively charged residues in the HRB linker that lie in close proximity to one other, including K294, R295, R296, (G297), K405, R435, and K438. It is possible that the electrostatic repulsion among these positively charged side chains destabilizes the conformation of the HRB linker, which may facilitate the transition of prefusion to

FIG 13 Location of amino acid residues important for hyperfusogenicity in predicted aMPV F protein. (A) aMPV A-F monomer. Residues (R295, G297, and K323) important for hyperfusogenicity, putative integrin binding sites (R329, D330, and D331), positively charged residues (K294, R296, K405, and K438), and two histidine residues (H368, and H396) are highlighted. (B) aMPV B-F monomer. Negatively charged residues (E295, E297, and E323), putative integrin binding sites (R329, D330, and D331), positively charged residues (R296, K405, and K438), and two histidine residues (H368 and H396) are highlighted. (C) aMPV A-F trimer. The corresponding amino acid residues from panel A are highlighted. The bottom left panel indicates the location of amino acid residues K396, K405, R295, G297, and K438. (D) aMPV B-F trimer. The corresponding amino acid residues from panel B are highlighted. The bottom left panel indicates the location of amino acid residues K396, K405, E295, E297, and K438.

postfusion F protein, thus leading to a high fusion activity. This notion is supported by a previous study showing that electrostatic repulsion involving K295, H345, and K438 in the HRB linker region destabilizes its conformation and contributes to the trigger of hMPV F at acidic pH (31, 42). Interestingly, there are notable variations in this group of residues among aMPV and hMPV F proteins. In aMPV subtype B-F protein, the corresponding residues are H294, E295, R296, E297, K405, R435, and K438 (Fig. 13B). Apparently, the presence of the negatively charged E295 and E297 and the near-neutral H294 may decrease the electrostatic repulsion among these residues, thus stabilizing the conformation of the HRB linker, which consequently disfavors the transition from pre- to postfusion. Consistent with this hypothesis, both R295E and G297E substitutions of aMPV subtype A-F protein led to significantly decreased fusion activities, whereas an E297G substitution in the aMPV subtype B-F protein significantly enhanced its fusion activity. However, our results also suggest that there are mechanisms other than the aforementioned electrostatic repulsion underlying the contribution of the HRB linker to fusion. For example, E295R substitution in aMPV subtype B-F protein is assumed to increase the electrostatic repulsion and fusion activity. However, this mutation resulted in a significant decrease

in fusion activity. In the predicted trimer structure, residues R295 and G297 are partially buried inside subtype A-F protein (Fig. 13C), whereas E295 and E297 are exposed at the surface of subtype B-F protein (Fig. 13D). In addition, residues at 295 and 297 do not contact with residues in other monomers based on the structure prediction and thus may not play a role in the interactions among monomers. Understanding the exact roles of these two residues in hyperfusogenic activity of subtype A-F protein will await the determination of the crystal structure of aMPV F protein.

In summary, we have shown that (i) aMPV-induced cell-cell fusion occurs at pH 7.0; (ii) aMPV cell-cell fusion does not require the attachment G protein; (iii) the aMPV G protein inhibits cell-cell fusion promoted by the F proteins of subtypes A and B but not C; (iv) the fusogenic ability of the F protein is strikingly different among aMPV subtypes; (v) trypsin is required for cell-cell fusion mediated by subtype B but not subtypes A and C; and (vi) amino acid residues in the HRB linker region are responsible for the hyperfusion activity of subtype A. Dissection of the basic requirements for membrane fusion in aMPV will contribute to our understanding of the mechanism of metapneumovirus entry in the future.

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