Identification of the Fusion Peptide-Containing Region in Betacoronavirus Spike Glycoproteins

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ABSTRACT

The fusion peptides (FP) play an essential role in fusion of viral envelope with cellular membranes. The location and properties of the FPs in the spike (S) glycoproteins of different coronaviruses (CoV) have not yet been determined. Through amino acid sequence analysis of S proteins of representative CoVs, we identified a common region as a possible FP (pFP) that shares the characteristics of FPs of class I viral fusion proteins, including high Ala/Gly content, intermediate hydrophobicity, and few charged residues. To test the hypothesis that this region contains the CoV FP, we systemically mutated every residue in the pFP of Middle East respiratory syndrome betacoronavirus (MERS-CoV) and found that 11 of the 22 residues in the pFP (from G953 to L964, except for A956) were essential for S protein-mediated cell-cell fusion and virus entry. The synthetic MERS-CoV pFP core peptide (₉₅₅IAGVGWTAGL₉₆₄) induced extensive fusion of liposome membranes, while mutant peptide failed to induce any lipid mixing. We also selectively mutated residues in pFPs of two other β -CoVs, severe acute respiratory syndrome coronavirus (SARS-CoV) and mouse hepatitis virus (MHV). Although the amino acid sequences of these two pFPs differed significantly from that of MERS-CoV and each other, most of the pFP mutants of SARS-CoV and MHV also failed to mediate membrane fusion, suggesting that these pFPs are also the functional FPs. Thus, the FPs of 3 different lineages of β -CoVs are conserved in location within the S glycoproteins and in their functions, although their amino acid sequences have diverged significantly during CoV evolution.

IMPORTANCE

Within the class I viral fusion proteins of many enveloped viruses, the FP is the critical mediator of fusion of the viral envelope with host cell membranes leading to virus infection. FPs from within a virus family, like influenza viruses or human immunodeficiency viruses (HIV), tend to share high amino acid sequence identity. In this study, we determined the location and amino acid sequences of the FPs of S glycoproteins of 3 β -CoVs, MERS-CoV, SARS-CoV, and MHV, and demonstrated that they were essential for mediating cell-cell fusion and virus entry. Interestingly, in marked contrast to the FPs of influenza and HIV, the primary amino acid sequences of the FPs of β -CoVs in 3 different lineages differed significantly. Thus, during evolution the FPs of β -CoVs have diverged significantly in their primary sequences while maintaining the same essential biological functions. Our findings identify a potential new target for development of drugs against CoVs.

Viruses are obligate intracellular parasites, and host cell membranes act as a barrier to virus entry. Enveloped viruses initiate infection of cells through fusion of the viral and cellular membranes. CoVs are enveloped and single-stranded plus-sense RNA viruses that cause a variety of diseases among many different species (1). Phylogenetically, CoVs are divided into four genera: alphacoronavirus (α -CoV), betacoronavirus (β -CoV), gammacoronavirus (γ -CoV), and deltacoronavirus (δ -CoV) (2).

CoVs enter cells through the interactions of the viral S proteins with host receptors. Several cellular proteins have been identified as receptors for their respective CoVs. Specific examples include human angiotensin-converting enzyme 2 (hACE2) for severe acute respiratory syndrome coronavirus (SARS-CoV) and human CoV NL63 (3, 4), human dipeptidyl peptidase IV (hDPP4) for Middle East respiratory syndrome betacoronavirus (MERS-CoV) (5), bat DPP4 for bat CoV HKU4 (6), human aminopeptidase N (hAPN) for human CoV 229E (7), and mouse carcinoembryonic antigen-related cell adhesion molecule 1a (mCEACAM1a) for mouse hepatitis virus (MHV) (8).

The CoV S protein is a class I viral fusion protein. On the CoV virions, the 180- to 200-kDa S proteins are found as trimers. S monomers contain two subunits, called S1 and S2. S1 contains the

receptor binding domain (RBD) and is responsible for receptor recognition and binding, whereas S2 possesses the membrane fusion machinery (9, 10), including a fusion peptide (FP), two heptad repeat domains (called the N-terminal and C-terminal heptad repeats, HR-N and HR-C), the juxtamembrane domain (JMD), and a transmembrane domain (TMD) (Fig. 1A).

To mediate membrane fusion, S protein must be activated, which requires both proteolytic cleavage (priming) and receptor binding with or without pH change (triggering) (11–13). Several host priming proteases are important for S protein-mediated CoV

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entry, including cathepsin B and L, serine proteases TMPRSS2 and TMPRSS4, trypsin, elastase, human airway trypsin-like protease (HAT), and furin (14–20). S protein activation leads to a series of conformational changes and insertion of a putative FP into target membrane, an essential step in membrane fusion and virus infection. Class I viral fusion proteins generally contain one FP, located either internally, like the FPs of the glycoprotein (GP) of Ebola virus and the envelope protein (Env) of avian sarcoma leukosis virus (ASLV) (21–24), or immediately downstream of the “priming” site, as seen in the hemagglutinin (HA) of influenza virus and the Env protein of HIV (25, 26). Although the primary sequences and lengths of FPs vary significantly among different class I viral fusion proteins, they share several common features. Most are rich in Ala and/or Gly, have an intermediate level of hydrophobicity with membrane binding potential, form helical structures in the presence of trifluoroethanol (TFE), and contain very few charged residues in the middle of their sequences (13, 25, 27).

Although significant efforts have been made to locate the FPs of different CoVs (28–35), the exact locations and sequences of CoV FPs remains controversial. While Chambers et al. predicted that the CoV FP likely was adjacent to HR-N (35), Madu et al. proposed that the sequence immediately following a critical and conserved trypsin cleavage site at the arginine of position 797 (R797) of SARS-CoV S protein, SFIEDLLFNKVTADAGF, may be the FP of SARS-CoV S protein (32). In this study, we used bioinformatics to identify a 17- to 22-amino-acid-long region, just upstream of HR-N, in S2 of different CoVs with characteristic features of the FPs of other class I viral fusion proteins. Using mutational, biochemical, and biophysical analyses of this region of the S proteins of 3 β -CoVs, MERS-CoV, SARS-CoV, and MHV, we provide data to support this region as the functional FP of CoV S proteins.

MATERIALS AND METHODS

Cell culture. HEK-293, 293T, HEK-293 cells stably expressing hACE2 (293/hACE2), HeLa cells stably expressing hDPP4 (HeLa/hDPP4), and HeLa cells stably expressing mouse CEACAM1a (HeLa/mCEACAM1a) were maintained in Dulbecco’s modified Eagle’s medium (DMEM) (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS) and 2% penicillin-streptomycin-amphotericin B (Fungizone; Invitrogen) at 37°C with 5% CO₂.

Constructs and mutagenesis. The constructs, pcDNA-SARS-CoV S Δ 19 (36), pcDNA-MERS-CoV S Δ 16 (37), and pcDNA-MHV S (38), have been described previously. Briefly, DNA encoding codon-optimized SARS-CoV S protein lacking the last 19 amino acids (aa), MERS-CoV S protein lacking the last 16 aa but with a FLAG tag at the C terminus, or full-length MHV S protein was cloned between BamHI and NotI sites of pcDNA3.1. All SARS-CoV, MERS-CoV, and MHV S mutants were derived from the plasmids pcDNA-SARS-CoV S Δ 19, pcDNA-MERS-CoV S Δ 16, and pcDNA-MHV S, respectively. All mutagenesis experiments were carried out using a Q5 mutagenesis kit (NEB, MA, USA). After the entire coding sequences were verified by sequencing, the BamHI- and

FIG 1 pFPs of CoVs. (A) Diagram of CoV spike protein. NTD, N-terminal domain; C-domain, C-terminal domain; Cleavage site, protease cleavage site separating S1 and S2; pFP, possible fusion peptide; HR-N, N-terminal heptad repeat; HR-C, C-terminal heptad repeat; JMD, juxtamembrane domain; TMD, transmembrane domain. (B) Locations of pFPs and TMDs of S proteins of representative CoVs predicted by TMPred. (C) Amino acid sequence alignment of the pFPs of different CoVs. (D) Summary of the amino acid substitutions made in the pFP of MERS-CoV S protein.

NotI-containing mutated S gene was cloned back into pcDNA3.1. A plasmid encoding full-length hACE2 (pACE2-cq) was kindly provided by M. Farzan (Scripps Research Institute, Florida campus). A plasmid encoding full-length human DPP4 (pcDNA-hDPP4) was purchased from Sino Biological Inc. (Beijing, China). A plasmid encoding full-length mouse CEACAM1a (mCEACAM1a) has been described previously (39). To express soluble human ACE2 (shACE2) and soluble human DPP4 (shDPP4), DNA fragments encoding residues 19 to 615 of human hACE2 with N-terminal 6His and FLAG tags and residues 40 to 766 of human DPP4 with C-terminal 6His and AVI tags were cloned between SalI and HindIII and between BamHI and XhoI of modified pFASTBac1 vector with gp67 signal peptide, respectively. To express soluble mouse CEACAM1a (smCEACAM1a), residues 1 to 236 of mCEACAM1a with C-terminal 6His and AVI tags were cloned into EcoRI and NotI of pFASTBac1. These soluble receptors were expressed in High Five insect cells using the Bac-to-Bac system (Invitrogen) and purified using nickel affinity and ion-exchange chromatography.

Analysis of S protein expression on cell surfaces. Briefly, HEK-293T cells were transfected with 2 μ g of either wild-type (WT) or mutant S protein-expressing plasmid using polyethylenimine (PEI) (Polysciences Inc., Warrington, PA, USA). Forty hours later, cells were detached from plates by incubating with phosphate-buffered saline (PBS) plus 1 mM EDTA for 5 min at 37°C. After washing, cells were incubated with the respective primary anti-S antibody for 1 h on ice. The primary antibodies for SARS-CoV SΔ19, MERS-CoV SΔ16, and MHV S protein were rabbit polyclonal anti-SARS S1 antibody (1:300 dilution) (Sinobiological Inc., Beijing, China), mouse monoclonal anti-MERS S antibody (1:300 dilution) (Sinobiological Inc., Beijing, China), and goat polyclonal anti-MHV S antibody (AO4) (1:200 dilution), respectively. After washing, cells were stained with Alexa Fluor 488-conjugated goat anti-rabbit IgG (1:200) (ZSGB-Bio LLC, Beijing, China) for SARS S, goat anti-mouse IgG (1:200) (ZSGB-Bio LLC, Beijing, China) for MERS S, or rabbit anti-goat IgG (1:200) (ZSGB-Bio LLC, Beijing, China) for MHV S. After washing, cells were fixed with 1% paraformaldehyde and analyzed by flow cytometry.

Binding of soluble receptor. HEK-293T cells were transfected with plasmids encoding either wild-type or mutant S proteins with PEI. After 40 h, cells were lifted with PBS plus 1 mM EDTA and immediately washed twice with PBS plus 2% normal donkey serum (NDS). About 2×10^5 cells were incubated with 1 μ g of shACE2, shDPP4, or smCEACAM1a for 1 h on ice. After washing, cells were incubated with mouse monoclonal anti-FLAG M2 antibody (1:1,000 dilution) (Sigma, St. Louis, MO, USA) for shACE2, followed by Alexa Fluor 488-conjugated goat anti-mouse IgG (1:200) or rabbit polyclonal anti-AVI antibody (1:200 dilution) (Shanghai Enzyme-Linked Biotechnology Co., Shanghai, China) for shDPP4 and smCEACAM1a, and finally with Alexa Fluor 488-conjugated goat anti-rabbit IgG (1:200). Cells were fixed with 1% paraformaldehyde and analyzed by flow cytometry.

Production and transduction of S protein-pseudotyped lentiviruses. Pseudovirions with spike proteins were produced as described previously (40), with minor modifications. Briefly, plasmids encoding either wild-type or mutant S proteins were cotransfected into 293T cells with pLenti-Luc-green fluorescent protein (GFP) (a gift from Fang Li, Duke University) and psPAX2 (Addgene, Cambridge, MA) at a molar ratio of 1:1:1 by using PEI. The following day, the cells were fed with fresh medium. After 24 h of incubation, the supernatant media containing pseudovirions were centrifuged at $800 \times g$ for 5 min to remove debris and passed through a 0.45- μ m filter. To quantify S protein-mediated entry of pseudovirions, susceptible cells were seeded at about 25 to 30% confluence in 24-well plates. The following day, cells were inoculated with 500 μ l of 1:1 diluted viruses. At 40 h postinoculation (p.i.), cells were lysed at room temperature with 120 μ l of medium with an equal volume of Steady-Glo (Promega, Madison, WI). Transduction efficiency was monitored by quantitation of luciferase activity using a Modulus II microplate reader (Turner Biosystems, Sunnyvale, CA). All experiments were done in triplicate and repeated at least three times.

Detection of viral spike glycoproteins by Western blotting. To evaluate S protein expression in cells, HEK 293T cells were transfected with plasmids encoding either wild-type or mutant S proteins by using PEI. Forty hours later, cells were lysed with lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 2 mM EDTA, 1% Triton X-100, 0.1% SDS) containing protease inhibitors (Roche, USA). To determine S protein incorporation into pseudotype virions, the virion-containing supernatant was pelleted through a 20% sucrose cushion at 30,000 rpm at 4°C for 2 h in a Beckman SW41 rotor (40). Viral pellets were resuspended in PBS. Cell lysates and pseudovirion pellets were separated on a 4 to 15% SDS-PAGE and transferred to a nitrocellulose blot. The SARS-CoV SΔ19, MERS-CoV SΔ16, and MHV S proteins were detected with polyclonal rabbit anti-SARS S1 antibodies (1:2,000), monoclonal mouse anti-MERS S antibody (1:1,000), and polyclonal goat anti-MHV S antibody (1:2,000), respectively. The blots were further stained with the horseradish peroxidase-conjugated antibodies goat anti-rabbit IgG (1:10,000), goat anti-mouse IgG (1:10,000), and rabbit anti-goat IgG (1:10,000), respectively, and visualized with Clarity Western ECL substrate (Bio-Rad, Hercules, CA, USA). The β -actin and HIV capsid protein (p24) were detected using mouse monoclonal anti- β -actin antibody (1:5,000) (Sigma, St. Louis, MO, USA) and rabbit polyclonal anti-p24 antibody (1:5,000) (Sinobiological Inc., Beijing, China), respectively.

Cell-cell fusion assays. Cell-cell fusion assays were performed as previously described (37), with modifications. Briefly, 293T cells were cotransfected with plasmids encoding CoV S glycoprotein and GFP. Forty hours later, cells were detached with trypsin (0.25%) and overlaid on a 70% confluent monolayer of 293/hACE2, HeLa/hDPP4, or HeLa/mCEACAM1a cells at a ratio of approximately one S-expressing cell to two receptor-expressing cells. After overnight incubation, images of syncytia were captured with a Nikon TE2000 epifluorescence microscope running MetaMorph software (Molecular Devices). To quantify S protein-mediated cell-cell fusion, 293T cells cotransfected with pFR-Luc, which contains a synthetic promoter with five tandem repeats of the yeast GAL4 binding sites that controls expression of the luciferase gene, plasmid encoding S protein, and the receptor-expressing cells (293/hACE2, HeLa/hDPP4, or HeLa/mCEACAM1a) were transfected with pBD-NF- κ B, which encodes a fusion protein with the DNA binding domain of GAL4 and transcription activation domain of NF- κ B. The following day, S-expressing 293T cells were lifted with trypsin and overlaid onto receptor-expressing cells at a ratio of about one S-expressing cell to two receptor-expressing cells. When cell-cell fusion occurred, luciferase expression would be activated through binding of the GAL4-NF- κ B fusion protein to GAL4 binding sites at the promoter of the luciferase gene. After 24 h of incubation, cells were lysed by adding 120 μ l of medium with an equal volume of Steady-Glo, and luciferase activity was measured with a Modulus II microplate reader. All experiments were done in triplicate and repeated at least three times.

Peptide synthesis. All peptides were synthesized using a standard solid-phase Fmoc (9-fluorenylmethoxy carbonyl) method by Scilight Biotechnology LLC (Shanghai, China). Purification was carried out by reverse-phase high-performance liquid chromatography (HPLC) and verified by mass spectrometry. An Ahx-KKK linker was added to all peptides used in circular dichroism (CD) spectroscopy analysis to increase peptide solubility in PBS. The following peptides were used for CD analysis: CTRL, KWGQYTNSPFLTKGF-Ahx-KKK (a control peptide from a previous SARS study [33]); HIV FP (41), AVGIGALFLGLGAAG-Ahx-KKK; and MERS pFP, SSLGSIAGVGWGTAGLSSFAAI-Ahx-KKK. The following peptides were used for the lipid mixing study: CTRL, KWGQYTNSPFLTKGF; HIV FP, AVGIGALFLGLGAAG; MERS short FP (sFP), IAGVGWGTAGL; MERS mutant FP (mFP), IAGRGRTAGL (letters in bold indicate mutations).

CD spectroscopy. CD spectroscopy analysis was performed to study the secondary structure of fusion peptides in increasing trifluoroethanol (TFE) concentrations. CD spectra were acquired on a Jasco J-815 spectropolarimeter (Jasco, Tokyo, Japan) using a 1-nm bandwidth with a 1-nm

step resolution from 195 to 260 nm at room temperature. Spectra were corrected by the subtraction of its respective solvent. The sample spectrum was smoothed with a Savitsky-Golay filter. The α -helical content was estimated from the ellipticity value at 222 nm, $[\theta]_{222}$, according to the empirical equation of Chen et al. (42): percent helical content = $100 \times \{[\theta]_{222}/-395,000 \times (1 - 2.57/n)\}$, where n is the number of peptide bonds.

Preparation of liposomes. Equimolar amounts of egg phosphatidylethanolamine (PE), egg phosphatidylcholine (PC), and cholesterol (Avanti Polar Lipids, Alabaster, AL, USA) were dried from chloroform into a thin film by constant flow of nitrogen gas and rehydrated in Tris buffer (10 mM Tris, 150 mM NaCl, 0.1 mM EDTA, pH 7.2) at a concentration of 10 mM. Large unilamellar vesicles (LUV) were prepared by the extrusion procedure (43). Briefly, after 10 freeze-thaw cycles, liposomes were extruded 21 times through two stacked polycarbonate membranes with a pore size of 0.1 μ m using an Avanti mini-extruder. Liposome with 0.6% (molar ratio) fluorescent resonance energy transfer (FRET) pairs Rho-PE and NBD-PE (Thermo Fisher) were prepared in the same way.

Lipid mixing. Lipid mixing was determined using the resonance energy transfer assay as described by Struck et al. (44), with minor modifications. Briefly, Rho-PE- and NBD-PE-labeled liposomes were mixed with unlabeled liposomes at a ratio of 1:9. The final lipid concentration was 300 μ M. Specified amounts of various peptides were added to initiate fusion, and changes in fluorescence were monitored at 535 nm with the excitation wavelength set at 465 nm and a slit width of 4 nm using Fluoromax-4 (Horiba, Paris, France). The initial residual fluorescence of the labeled and unlabeled vesicles was set up as the baseline for 0% fluorescence value (f_0); 100% fluorescence value (f_{100}) was achieved by addition of Triton X-100 to a final concentration of 0.2%. The extent of lipid mixing was calculated using the following formula: $\%f_t = (f_t - f_0)/(f_{100} - f_0) \times 100$, where f_t is the fluorescence value observed after addition of fusion peptide at time t .

RESULTS

During membrane fusion, the FP of S protein inserts into the host membranes. We reasoned that CoV FPs might share some common properties with the transmembrane domains (TMD) and that the location of the FP within the S protein might be predictable by using TMD prediction software programs. The FPs of HIV-1 Env and influenza virus HA have been studied extensively, and their locations and amino acid sequences are known. As a proof of concept, we first tested whether TM software programs could accurately identify the FPs of HIV-1 Env and influenza H1N1 HA proteins. Both the FPs and TMDs of HIV-1 Env and influenza HA were accurately identified by two software programs, TMpred (http://www.ch.embnet.org/software/TMPRED_form.html) and TMHMM (<http://www.cbs.dtu.dk/services/TMHMM/>) (data not shown). Subsequently, we applied these two software programs to analyze S proteins of a wide variety of CoVs. The positions of the TMDs of the S proteins of all CoVs studied were correctly identified by both software programs (Fig. 1B). In addition, both of these TMD prediction programs identified another region consistently flanked by YT at the N terminus and PF at the C terminus in all of the S proteins of the CoVs tested (Fig. 1B and C). Although the primary amino acid sequences of this region were not conserved in all of the CoVs studied, they were all Ala or Gly rich, relatively hydrophobic, and contained no charged residues, characteristics shared by the FPs of other class I viral fusion proteins (Fig. 1C). We named this region in CoV S proteins the possible FP (pFP).

To investigate if the pFP is the functional fusion peptide of CoVs, we selected the S protein of MERS-CoV, a lineage C β -CoV, as an example. The MERS-CoV pFP contains amino acids 949 to

970 (Fig. 1C). Individual and occasionally double amino acid substitutions were introduced at each position of pFP (Fig. 1D). First, we determined if any of the mutations altered the expression of S protein in 293T cells. Consistent with our previous report (37), two bands around 200 kDa were detected in the cell lysate of 293T cells expressing wild-type (WT) S protein, likely reflecting the different glycosylation of full-length S proteins during transport through the Golgi apparatus. However, the cell lysate also contained a significant proportion of S protein cleaved between S1 and S2, around 100 kDa, which was absent from our previous report but previously reported by the Pohlmann laboratory (45). The difference between that study and our earlier report likely resulted from different culture conditions, especially sera and media from different vendors. Among the 44 total G, A, V, or R substitutions, 30 (S949G, S950G, L952A, G953A, G953R, S954G, S954R, I955G, A956V, A956R, G957A, G957R, V958G, V958R, I955G/V958G, G959A, G959R, W960G, W960R, V958G/W960G, T961A, A962V, A962R, G963A, G963R, L964G, L964R, S965G, S966G, and A968V) showed no or minor effects on S protein expression or processing compared to the WT (Fig. 2A and Table 1). In contrast, 14 substitutions (L951G, L952G, L951G/L952G, S965R, S966R, F967G, L964F/F967G, A968R, A969V, A969R, I970G, P971V, F972G, and I970G/F972G) showed significant reductions in S protein expression and changes in patterns of S protein processing (Fig. 2A and Table 1). The cleaved S protein species were almost absent from corresponding cell lysates, suggesting that these residues (L951, L952, S965, S966, F967, A968, A969, I970, P971, and F972) are important for S protein folding and processing.

We then investigated if any amino acid substitutions in the pFP influenced transport of the S protein to the cell surface. The 293T cells expressing WT or mutant S proteins were incubated on ice with mouse monoclonal anti-MERS-CoV S protein antibody and analyzed by flow cytometry. The same 30 mutants that showed WT levels of S protein expression in cell lysates also showed WT levels of S protein on the cell surface (Fig. 2B and Table 1). As expected, the mutants with defects in S protein expression and processing also showed only low levels of S proteins on the cell surface.

Although the pFP is located within the MERS-CoV S2 subunit, amino acid substitutions in pFP might affect S protein binding to its cognate receptor, hDPP4, by altering the overall conformation of the S protein. To determine whether or not any amino acid substitution in pFP changed S protein binding to hDPP4, we used V5-tagged soluble hDPP4 (shDPP4) to bind 293T cells transiently expressing WT or pFP mutant S proteins of MERS-CoV. The percentage of cells that bound shDPP4 and the level of shDPP4 bound to S protein were quantitated by flow cytometry. The same 30 mutant S proteins that showed WT levels of expression on cell surfaces also bound to shDPP4 at levels similar to those of WT S protein (Fig. 3 and Table 1), indicating that these pFP mutations had no effect on receptor binding.

Because the fusion peptide is essential for S protein-mediated membrane fusion, we then explored whether any mutation in pFP altered MERS-CoV S protein-mediated cell-cell fusion. To more easily visualize cell-cell fusion or syncytia, the 293T cells expressing S protein were cotransfected with a GFP-expressing plasmid and then overlaid with HeLa/hDPP4 cells in the presence of trypsin. Consistent with our previous report (37), WT MERS-CoV S protein induced very large syncytia (Fig. 4), and syncytium for-

FIG 2 Analysis of expression of pFP mutants of MERS-CoV S protein in 293T cells. (A) Western blot analysis of expression of WT or mutant MERS S protein in cell lysate. The MERS S protein was detected by using mouse monoclonal anti-MERS S antibody; β -actin was detected with mouse monoclonal anti-actin antibody. FL, full length. (B) Analysis of surface expression of mutant MERS-CoV S protein by flow cytometry. MERS-CoV S protein-expressing 293T cells were stained with mouse monoclonal anti-MERS S antibody. The amount of wild-type S protein on cell surfaces was set as 100%. All of the experiments shown were repeated at least three times.

mation depended on the availability of hDPP4 (data not shown). Among 30 pFP S protein mutants that were expressed well, transported to the cell surface efficiently, and bound to hDPP4 at levels similar to the WT, 14 mutants (S949G, S950G, G953A, S954G, A956V, A956R, G957A, V958G, G959A, A962V, G963A, L964G, S965G, and A968V) induced large syncytia in HeLa/hDPP4 cells similar to the WT, 12 mutants (G953R, S954R, I955G, G957R, V958R, I955G/V958G, W960R, V958G/W960G, T961G, A962R, G963R, and L964R) induced little or no syncytium formation, and 4 mutants (L952A, G959R, W960G, and S966G) induced syncytia of much smaller size than the WT (Fig. 4). These results indicate

that these 13 residues, L952, G953, S954, I955, G957, V958, G959, W960, T961, A962, G963, L964, and S966, in MERS-CoV S protein are critical for S protein-mediated, receptor-dependent membrane fusion that would lead to virus infection.

To quantify the effect of amino acid substitutions on S protein-mediated syncytium formation, we utilized a luciferase-based quantification assay from a yeast two-hybrid system from Stratagene-Agilent Technologies, Inc. Compared to mock transfection and parental HeLa cell controls, fusion of 293T cells expressing WT MERS-CoV S proteins with HeLa/hDPP4 cells increased luciferase activity by about 1,000-fold (Fig. 5). The overall pattern of

TABLE 1 Summary of pFP mutants of betacoronaviruses

β -CoV	Expression in cell lysate ^a	Expression on cell surface ^b	Incorporation in virion ^c	Receptor binding	Cell-cell fusion ^d	Pseudovirion transduction ^d
MERS wild type	+++	+++	+++	+++	+++	+++
S949A	+++	+++	+++	+++	+++	+++
S950G	+++	+++	+++	+++	+++	+++
L951G	+	—	—	—	—	—
L952G	+	—	—	—	—	—
L952A	++	+++	++	+++	++	+
L951G/L952G	+	—	—	—	—	—
G953A	+++	+++	++	+++	+++	+
G953R	+++	+++	++	+++	—	—
S954G	+++	+++	+++	+++	+++	+++
S954R	+++	+++	+++	+++	—	—
I955G	+++	+++	+++	+++	—	—
A956V	+++	+++	+++	+++	++	+++
A956R	+++	+++	+++	+++	+++	+++
G957A	+++	+++	+++	+++	+++	++
G957R	+++	+++	+++	+++	—	—
V958G	+++	+++	+++	+++	+++	+++
V958R	+++	+++	+++	+++	—	—
I955G/V958G	+++	+++	+++	+++	—	—
G959A	+++	+++	+++	+++	+++	+++
G959R	+++	+++	+++	+++	+	—
W960G	+++	+++	+++	+++	+	+
W960R	+++	+++	+++	+++	—	—
V958G/W960G	+++	+++	+++	+++	—	—
T961G	+++	+++	+++	+++	—	—
A962V	+++	+++	+++	+++	+++	+++
A962R	+++	+++	+++	+++	+	—
G963A	+++	+++	+++	+++	++	++
G963R	+++	+++	++	+++	—	—
L964G	+++	+++	+++	+++	+++	++
L964R	+++	+++	+++	+++	—	—
S965G	+++	+++	+++	+++	+++	+++
S965R	+	+	+	+	—	—
S966G	++	+++	+	++	+++	+
S966R	+	—	—	+	—	—
F967G	+	+	—	+	—	—
L964G/F967G	+	+	—	+	—	—
A968V	+++	+++	+++	++	+++	++
A968R	+	+	—	+	—	—
A969V	+	+	—	+	+	—
A969R	+	+	+	+	—	—
I970G	+	+	+	+	—	—
P971V	+	+	—	—	—	—
F972G	+	+	—	—	—	—
I970G/F972G	+	+	—	—	—	—
SARS wild type	+++	+++	+++	+++	+++	+++
W868R	+++	ND	+++	ND	+	—
W868G	+++	+++	+++	+++	++	+++
F870R	+++	ND	+++	ND	+	—
F870G	+++	+++	+++	+++	++	+++
W868G/F870G	+++	+++	+++	+++	—	—
L876R	+++	ND	+++	ND	—	—
L876G	+++	+++	+++	+++	++	+
I878R	++	ND	++	ND	—	—
I878G	++	+++	++	+++	+	—
L876G/I878G	++	+++	++	+++	—	—
MHV wild type	+++	+++	+++	+++	+++	+++
M963R	+	+	++	++	+	—

(Continued on following page)

TABLE 1 (Continued)

β-CoV	Expression in cell lysate ^a	Expression on cell surface ^b	Incorporation in virion ^c	Receptor binding	Cell-cell fusion ^d	Pseudovirion transduction ^d
F937R	+++	+++	+++	+++	—	—
P938R	++	++	+++	+++	—	—
P939R	+++	+++	+++	+++	+	—
W940R	+++	+++	+++	+++	—	—

^a Western blot analysis on S protein expression in cell lysate: +++, very strong; ++, strong; +, weak; —, absent.
^b S protein expression on cell surface and receptor binding: +++, >70% of WT; ++, 46 to 70% of WT; +, 20 to 45% of WT; —, <20% of WT. ND, not done.
^c Western blot analysis on S protein expression incorporation in virion: +++, strong; ++, weak; +, very weak; —, absent.
^d Cell-cell fusion and pseudovirion transduction: +++, >70% of WT; ++, 31 to 70% of WT; +, 5 to 30% of WT; —, <5% of WT. ND, not done.

cell-cell fusion induced by pFP mutants in this quantification assay was very similar to our visual method (Fig. 4 and 5 and Table 1). Among the same 30 mutants showing WT levels of expression and receptor binding, 16 mutants (S949G, S950G, L952A, G953A, S954G, A956V, A956R, G957A, V958G, G959A, A962V, G963A, L964G, S965G, S966G, and A968V) retained 50 to 110% of WT-level fusion activity, but 14 mutants (G953R, S964R, I955G, G957R, V958R, I955G/V958G, G959R, W960G, W960R, V958G/W960G, T961G, A962R, G963R, and L964R) reduced S protein-mediated cell-cell fusion by more than 85%, indicating that these residues (G953, S954, I955, G957, V958, G959, W960, T961, A962, G963, and L964) are essential for membrane fusion.

To determine whether or not any mutation in the pFP of the S protein of MERS-CoV also affected virus entry, we measured transduction of HeLa/hDPP4 cells by lentiviral pseudovirions with envelopes containing either WT or pFP mutant MERS-CoV S proteins. Compared to the mock-treated control (pseudovirions without any S protein), the luciferase activity in HeLa/hDPP4 cells increased by more than 10,000-fold following transduction by pseudovirions with WT MERS-CoV S proteins (Fig. 6A). Among the same 30 mutants that showed little or no effect on S protein expression or receptor binding (Fig. 2A and B, 3, and Table 1), 5 mutants (L952A, G953A, G953R, G963R, and S966G) showed

marked reduction in S protein incorporation into pseudovirions, whereas the S proteins of the other 25 mutants were incorporated into pseudovirions as well as WT S protein (Fig. 6B). Ten out of these 25 amino acid substitutions, S954R, I955G, G957R, V958R, I955G/V958G, W960R, V958G/W960G, T961G, A962R, and L964R, almost abolished MERS-CoV S protein-mediated, receptor-dependent pseudovirion entry (Fig. 6A and Table 1), suggesting that S954, I955, G957, V958, W960, T961, A962, and L964 are essential for virus entry. In addition, G959R mutation also reduced the transduction by more than 95%, indicating that G959 also is critical for virus entry (Fig. 6A). Interestingly, although G953A, G953R, and G963R mutants showed reduced but similar levels of S protein incorporation into pseudovirions (Fig. 6B), the infectivity of the pseudovirions differed drastically. While G953A results in only 30% of the WT level of pseudovirion entry, the G953R and G963R mutations almost abrogated S protein-mediated pseudovirion entry, indicating that G953 and G963 also are important for virus entry.

Because the FPs of most class I viral fusion proteins fold predominantly in an α-helix structure in the presence of TFE (13), we used circular dichroism (CD) spectroscopy analysis to investigate whether our MERS-CoV pFP also adapts an α-helical fold. A scrambled peptide from a previous SARS-CoV study (33) was chosen as the negative control, and the FP of HIV-1 was selected as the positive control (46). To facilitate the synthesis of the peptides and increase their solubility, an aminocaproic acid (Ahx) linker followed by 3 Lys residues (Ahx-KKK) was added to the C termini of the peptides. Consistent with previous reports (46), while the FP of HIV-1 folded as a random coil in Tris-salt buffer, it formed an α-helix in the presence of TFE (Fig. 7A), a solvent known to stabilize the α-helical formation (47). Similarly, in the absence of TFE, the pFP of MERS-CoV (SSLGSIAGVGVGTAGLSSFAAI) folded as a random coil, but with the addition of TFE it folded as an α-helix. At 95% TFE, the helical population accounted for more than 64% (Fig. 7A).

FPs of class I viral fusion proteins also promote membrane fusion when mixed with liposomes. Accordingly, we investigated whether the pFP of MERS-CoV S protein could mediate liposome fusion using a FRET-based assay. To rule out any possible effect of the Ahx-KKK tag, we decided to use peptides without any tag. However, because of the technical difficulty of synthesizing the full-length pFP without the AHX-KKK tag, we decided to use instead the core sequence of pFP (₉₅₅IAGVGVGTAGL₉₆₄), called short pFP or sFP) in this study, in which almost all of the residues were shown to be essential for cell-cell fusion and virus entry. As shown in Fig. 7B, both the FP of HIV-1 and the sFP of MERS-CoV induced membrane fusion of liposome in a concentration-depen-

FIG 3 Receptor binding by mutant MERS S proteins. MERS-CoV S protein-expressing 293T cells were incubated with soluble AVI-tagged hDPP4, followed by polyclonal rabbit anti-AVI antibody and fluorescein isothiocyanate-conjugated goat anti-rabbit IgG. The results from the wild type were set as 100%.

FIG 4 Cell-cell fusion mediated by WT or mutant MERS-CoV S protein. MERS-CoV S protein-expressing 293T cells were transiently transfected with eGFP and then incubated with HeLa/hDPP4 cells overnight in the presence of trypsin.

FIG 6 Entry of pseudotype virions with wild-type or mutant MERS S protein. (A) Entry of pseudovirions with wild-type or mutant MERS-CoV S proteins into HeLa/hDPP4 cells. Pseudovirus entry was quantitated by luciferase activity at 40 h postinoculation. A typical transduction by wild-type S protein pseudoviruses resulted in a more than 10,000-fold increase of luciferase activity. The experiments were repeated at least three times, and an average from three experiments is shown. (B) Detection of wild-type or mutant S protein incorporation into pseudovirions by Western blot analysis. MERS S protein was detected using mouse monoclonal anti-MERS S antibody; p24, a gag protein of HIV, was detected using rabbit polyclonal anti-p24 antibodies. FL S, full-length S protein. The experiments were repeated twice and a representative is shown.

pFP of β -CoV S protein is the functional viral fusion peptide. This conclusion is further strengthened by our findings that the synthetic pFP of MERS-CoV S protein formed an α -helix in the presence of TFE and its core short sequence, called sFP, mediated membrane fusion of liposome efficiently (Fig. 7), which are characteristics of FPs of other class I viral fusion proteins (13). Our results are also consistent with previous biophysical studies on synthetic peptides from SARS-CoV S protein (29, 33) and previous studies in MHV showing that P939 may be critical for membrane fusion and virus infection (54, 55).

About one-third of the residues located at the C terminus of the pFP of MERS-CoV S protein appear to play important roles in the stability and processing of the S protein, since the introduction of amino acid substitutions into these positions significantly reduced S protein expression, processing, and incorporation into pseudotyped virions. Residues close to the C terminus of the pFP of the SARS-CoV S protein also appear to be important for S protein folding, as replacement of I878 with R or G also decreased S protein expression and incorporation into virions. However, this region might also be important for membrane fusion mediated by S

FIG 7 Biophysical analysis of synthetic pFP peptide of MERS-CoV. (A) CD analysis of secondary structure of pFP of MERS-CoV S protein. CTRL, KWGQYTNSPFLTKGF-Ahx-KKK (a control peptide from a previous

protein. A recent study on SARS-CoV by Liao et al. (56) raised the possibility that this region has direct interactions with the JMD in S protein during membrane fusion.

Among the amino acid substitutions that we introduced into the pFP of MERS-CoV S protein, Arg had a more profound effect on the function of the pFP of MERS-CoV S protein than Gly, Ala, or Val. Compared to Gly, Ala, and Val, Arg is positively charged and its side chain is significantly longer than those of Gly, Ala, or Val; hence, Arg substitution represents a more dramatic change than these amino acids. Moreover, Arg substitution may cause a higher free-energy barrier for insertion of FP into membrane (57). Although the exact mechanism(s) of how these substitutions in the pFP abrogate membrane fusion requires further investigation, there are several possibilities. Introduction of mutation(s) into the pFP of MERS-CoV S protein might distort the structure of FP required for membrane fusion similar to G1V and W14A mutations of the FP of influenza HA (58–60). Alternatively, the substitutions might change how the FP inserts into membranes (61–63) or affect the oligomerization of the FPs that is important for membrane fusion (64, 65).

Recent studies in influenza virus HA (66), paramyxovirus F protein (67), and HIV Env (68) reveal that many viral FPs interact and oligomerize with their TMDs in the lipid, which promotes lipid mixing and membrane fusion. Whether the FP and TMD of CoV S protein interact with each other during membrane fusion remains to be determined. Interestingly, the primary amino acid sequences of the TMDs among different CoVs also do not share high identity (Fig. 9). Of note, there is a GXXXG or (small)XXX(small) motif (G, Gly; small, Ala, Gly, or Ser; X, any residue) present in all of the pFPs of CoVs. These motifs were initially discovered in human glycophorin A and subsequently have been implicated in TMD interactions of more than 20 proteins (69). Recent studies in influenza HA and HIV Env have suggested that such GXXXG motifs also play an important role in FP-FP or FP-TMD interaction (66, 68, 70). There are two GXXXG motifs, GSIAG and GWTAG, within the FP of MERS-CoV. Replacement in MERS-CoV S protein of any one of these four Gly residues (G953, G957, G959, or G963) with Arg abrogated the membrane fusion activity of the viral protein. However, whether these GXXXG motifs in the pFP of MERS S protein are essential for oligomerization or interaction with the TMD requires further investigation.

FPS of some class I viral fusion proteins, like HIV Env and influenza HA, share high identity in primary amino acid sequence within each virus family. In marked contrast, this study found no strong amino acid sequence identity among the pFPs of MERS-CoV, SARS-CoV, and MHV. The lengths of the FPs of these three different lineages of β -CoVs also differ significantly, ranging from

SARS-CoV peptide study [33]); HIV FP, AVGIGALFLGFLGAAG-Ahx-KKK; MERS pFP, SLLGSIAGVGWTAGLSSFAAI-Ahx-KKK. All peptides were dissolved in PBS, and their CD spectra were measured in the presence of the indicated concentration of TFE. Experiments were done twice, and one representative is shown. (B) Lipid mixing induced by synthetic pFP of MERS-CoV S protein. LUVs were made with equimolar amounts of PE, PC, and cholesterol. The extent of lipid mixing was determined by monitoring the changes in fluorescence intensity at 535 nm at 37°C upon addition of peptide. Each data point is averaged from three independent experiments, and error bars represent standard deviations of the means. CTRL, KWGQYTNSPFLTKGF; HIV FP, AVGIGALFLGFLGAAG; MERS sFP, IAGVGWTAGL; MERS mFP, IAGRGRTAGL.

FIG 8 Effects of mutations at the pFPs of SARS-CoV and MHV on pseudovirus transduction and cell-cell fusion. (A and D) Entry of wild-type or mutant SARS-CoV S protein pseudovirions into 293/hACE2 cells (A) or MHV S protein pseudovirions into HeLa/mCEACAM1a cells (D). Pseudovirus entry was quantitated by luciferase activity at 40 h postinoculation. The experiments were repeated at least three times, and averages from three experiments are shown. (B and E) Detection of wild-type or mutant S protein of SARS-CoV (B) or MHV (E) incorporation into pseudovirions by Western blot analysis. SARS S protein was detected using rabbit polyclonal anti-SARS S1 antibody, MHV S protein was detected using goat polyclonal anti-MHV S antibody AO4, and p24, a gag protein of HIV, was detected using rabbit polyclonal anti-p24 antibodies. The experiments were repeated at least three times, and one representative is shown. (C and F). Cell-cell fusion mediated by mutant SARS (C) or MHV (F) S proteins. Experiments were performed as described in the legend to Fig. 3B, except that 293/hACE2 cells were used as targets for SARS-CoV S protein (C) and HeLa/mCEACAM1a cells were used as targets for MHV S protein (F). An average from three experiments is shown.

18 amino acids for MHV to 22 amino acids for MERS-CoV and SARS-CoV (Fig. 1B). Within each lineage of β -CoVs, the pFPs appear to be better conserved (Fig. 1B). Although underlying mechanism(s) causing the amino acid sequence divergence of FPs of different lineages of β -CoVs remains to be determined, CoV RNA-dependent RNA polymerase error, recombination, and selective pressure during evolution likely contribute to these changes. Previous study of MHV persistent infection in DBT cells showed that accumulation of mutations in fusion peptide and HR-N could lead to extending host range (55). The lack of conservation of the pFP amino acid sequences, however, is not unique for CoVs, as FPs from different paramyxoviruses also lack high identity in their primary amino acid sequences (67).

As an internal fusion peptide, how does the activated FP of CoVs fold and mediate membrane fusion? Recent studies have demonstrated that FPs from different class I viral fusion proteins

might adapt different conformations to mediate membrane fusion. Depending on the lipid composition, the FPs of HIV-1 Envs and parainfluenza virus F proteins can fold as either an α -helix (67, 71) or β -sheet (65, 72), and both can be fusigenic. In contrast, the overall conformation of the FPs of Ebola virus GP and influenza virus HA is α -helical in the presence of TFE, but they fold as hairpin-like structures or “knuckle” conformations when they insert into their target membranes (63, 73). Sequence analysis of the S proteins of different CoVs (Fig. 1B) shows the presence in the pFPs of a Gly-Gly (GG) motif in α -, γ -, and δ -CoVs or a Pro-Pro (PP) motif in β -CoVs in lineage A. GG and PP motifs favor the formation of turn or hairpin structures, which suggests that the FPs of some CoVs also adapt a hairpin-like structure when inserting into host membranes. In the FPs of SARS-CoV and MERS-CoV in β -CoV groups b and c, respectively, however, neither a GG nor a PP motif is present. Of note, the FP from group 2

FIG 9 Alignment of TMDs of S proteins of representative CoVs.

influenza virus HA also lacks a central GG or PP motif but instead forms a hairpin-like structure with G13 at the turn with a Trp and a hydrophilic residue immediately following G13 (74). Interestingly, a similar motif is also present in the pFPs of SARS-CoV and MERS-CoV (Fig. 1B).

While all known class III viral fusion proteins have two fusion loops, all known class I viral fusion proteins except for CoV S protein only have a single fusion peptide. In the case of CoVs, in addition to pFP found in this study, Madu et al. previously found a highly conserved region in SARS-CoV S protein essential for membrane fusion and proposed it as the fusion peptide (32), although this sequence lacks some common features of FPs of other class I viral fusion proteins, including high Ala/Gly content. Their proposed FP is about 80 amino acids away from the N terminus of HR-N (50, 52) and about 40 amino acids upstream of the N terminus of our pFP. The possibility of the presence of two fusion peptides in the S protein of CoV is very intriguing. How these two possible fusion peptides collaborate to mediate membrane fusion requires further investigation.

In summary, using a bioinformatics approach we have identified a region in the S proteins of CoVs that has several properties of the FPs of several classical class I viral fusion proteins. Further molecular biological, biochemical, and biophysical analyses demonstrated that this region is essential for receptor-dependent membrane fusion mediated by S proteins of several β -CoVs in different lineages, strongly suggesting that it is the functional FP of these and likely other CoVs. These findings will provide significant clues for future studies of the membrane fusion mechanism of CoVs and may provide a new target for drugs against CoV infections.

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