Two mutations were critical for bat-to-human transmission of MERS coronavirus

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Abstract

To understand how MERS coronavirus (MERS-CoV) transmitted from bats to humans, we compared the virus-surface spikes of MERS-CoV and a related bat coronavirus HKU4. Although HKU4 spike cannot mediate viral entry into human cells, two mutations enabled it to do so by allowing it to be activated by human proteases. These mutations are present in MERS-CoV spike, explaining why MERS-CoV infects human cells. These mutations therefore played critical roles in the bat-to-human transmission of MERS-CoV.

Text

Since its emergence in 2012, Middle East respiratory syndrome coronavirus (MERS-CoV) has infected over 1100 people with a case fatality rate of ~38% (1, 2). Bats are considered the natural reservoir of MERS-CoV because several coronaviruses, including HKU4, have been isolated from bats and are genetically related to MERS-CoV (3-5). An envelope-anchored spike protein mediates coronavirus entry into host cells. It first binds a host receptor through its S1 subunit and then fuses membranes through its S2 subunit (6-8). For membrane fusion, the spike must be cleaved at the S1/S2 boundary by one or more host proteases (9-13). We recently showed that HKU4 and MERS-CoV spikes both recognize host receptor dipeptidyl peptidase 4 (DPP4) (14). Furthermore, only MERS-CoV spike, but not HKU4 spike, mediates viral entry into human cells because the former, but not the latter, can be activated by human endogenous proteases (14). Here we further identified two residue differences between the two spikes that account for their different capability to mediate viral entry into human cells. Our results

have revealed a likely evolutionary pathway for the emergence of MERS-CoV as a human pathogen.

By comparing the sequences of MERS-CoV and HKU4 spikes, we identified two regions in MERS-CoV spike that may serve as human protease target motifs, but differ from the corresponding regions in HKU4 spike (Fig.1). The first region is a motif for human proprotein convertases (hPPC motif) (15, 16), but the critical Arg748 in MERS-CoV spike corresponds to Ser746 in HKU4 spike, which deviates from the hPPC motif. The second region is a motif for human endosomal cysteine proteases (hECP motif) (17, 18), but the motif Ala763-Phe764-Asn765 in MERS-CoV spike corresponds to Asn762-Tyr763-Thr764 in HKU4 spike, which likely introduces an N-linked glycosylation site and blocks the access of human endosomal cysteine proteases. These residue differences in the two human protease motifs between MERS-CoV and HKU4 spikes may affect the spikes' capability to mediate viral entry into human cells.

To evaluate the potential genetic changes required for HKU4 to infect human cells, we re-engineered HKU4 spike, aiming to build its capacity to mediate viral entry into human cells. To this end, we introduced two single mutations, S746R and N762A, into HKU4 spike. The S746R mutation was expected to restore the hPPC motif in HKU4 spike, whereas the N762A mutation likely disrupted the potential N-linked glycosylation site in the hECP motif in HKU4 spike. To confirm that the S746R mutation restored the hPPC motif, we produced retroviruses pseudotyped with HKU4 spike (hereinafter referred to as HKU4 pseudoviruses) in human cells, and showed that HKU4 spike containing the S746R mutation was partially cleaved during the molecular maturation

process, whereas wild type HKU4 spike remained intact (Fig.2A). Confirming that the N762A mutation disrupted the N-linked glycosylation site in the hECP motif was technically challenging because of the large size and heavy glycosylation of HKU4 spike (trimeric HKU4 spike has 78 predicted N-linked glycosylate sites and a total molecular weight of ~530 kDa). Nevertheless, we managed to identify a slight downward shift of HKU4 spike with N762A mutation using Western blot analysis (Fig.2B), consistent with successful removal of the N-linked glycosylation site. Currently we do not have direct evidence to show that the spikes are cleaved right at the hPPC and hECP motifs by human proteases. However, both of the hPPC and hECP motifs in the spikes have been well documented to be the cleavage sites for human proteases (16-18). Moreover, mutations in these motifs in coronavirus spikes have demonstrated dramatic effects on viral entry into human cells (see below). Thus, the identified hPPC and hECP motifs are likely to be cleaved by human proteases, although we cannot completely rule out the possibility that alteration of these motifs may affect protease cleavages at a spatially adjacent site on the spikes. In either case, our study reveals that both of these motifs play critical roles in HKU4- and MERS-CoV-spike mediated entry into human cells (see below).

We examined the capability of mutant HKU4 spike to mediate viral entry into three types of human cells (Fig.3A for HEK293T cells; data not shown for Huh-7 and MRC-5 cells), using pseudovirus entry assay as previously described (14). In the absence of exogenous protease trypsin, HKU4 pseudoviruses bearing either the re-engineered hPPC motif or the re-engineered hECP motif were able to enter human cells, whereas HKU4 pseudoviruses bearing both of the re-engineered human protease motifs entered

97 human cells as efficiently as when activated by exogenous trypsin (Fig.3A). In contrast,
98 wild type HKU4 pseudoviruses failed to enter human cells. Therefore, the re-engineered
99 hPPC and hECP motifs enabled HKU4 spike to be activated by human endogenous
100 proteases, and thereby allowed HKU4 pseudoviruses to bypass the need for exogenous
101 proteases to enter human cells. These results reveal that HKU4 spike only needs two
102 single mutations at the S1/S2 boundary to gain the full capacity to mediate viral entry
103 into human cells.

104 To confirm the functions of the re-engineered human protease motifs in HKU4
105 spike, we used protease inhibitors to probe the human proteases that activate HKU4-
106 spike-mediated viral entry into human cells. Human proprotein convertase (PPC)
107 inhibitor and endosomal cysteine protease (ECP) inhibitor both blocked human cell entry
108 of HKU4 pseudoviruses bearing the re-engineered hPPC motif and the re-engineered
109 hECP motif, respectively (Fig.3B). This result verified that the gained capability of
110 HKU4 pseudoviruses to enter human cells was due to the re-engineered human protease
111 motifs in HKU4 spike.

112 To investigate the functions of the two human protease motifs in MERS-CoV-
113 spike-mediated viral entry into human cells, we re-engineered MERS-CoV spike in the
114 opposite way, aiming to reduce or eliminate its capability to mediate viral entry into
115 human cells. To this end, we introduced a single mutation R748S and a triple mutation
116 A763N/F764Y/N765T into MERS-CoV spike (Fig.1). The R748S mutation destroyed the
117 hPPC motif in MERS-CoV spike (Fig.2A), whereas the triple mutation
118 A763N/F764Y/N765T successfully added an N-linked glycosylation site to the hECP

motif in MERS-CoV spike (Fig.2C). MERS-CoV pseudoviruses bearing either the mutated hPPC motif or the mutated hECP motif demonstrated decreased capability to enter three types of human cells (Fig.3C for HEK293T cells; data not shown for Huh-7 and MRC-5 cells). MERS-CoV pseudoviruses bearing both of the mutated human protease motifs were unable to enter human cells. Exogenous trypsin was able to fully rescue the capability of mutant MERS-CoV pseudoviruses to enter human cells. Moreover, PPC inhibitor and ECP inhibitor both blocked human cell entry of wild type MERS-CoV pseudoviruses, but had no effect on human cell entry of MERS-CoV pseudoviruses bearing the mutant hPPC motif or the mutant hECP motif, respectively (Fig.3D). Thus, the mutations in the hPPC and hECP motifs together eliminate MERS-CoV-spike-mediated viral entry into human cells. These results demonstrate that the two functional human protease motifs in MERS-CoV spike played a critical role in the bat-to-human transmission of MERS-CoV.

After examining HKU4- and MERS-CoV-spike mediated viral entry into human cells, we investigated how these spikes mediate viral entry into bat cells. Because of the low transfection efficiency of bat cells, we were unable to package pseudoviruses in bat cells. Instead, we packaged HKU4 and MERS-CoV pseudoviruses in HEK293T cells and subsequently performed HKU4- and MERS-CoV-spike-mediated pseudovirus entry into two types of bat cells: RSKT and Tb1-Lu cells (Fig.4). Wild type HKU4 pseudoviruses entered bat cells efficiently, whereas HKU4 pseudoviruses bearing the re-engineered hECP motif (which removed the N-linked glycosylation site) demonstrated enhanced capability to enter bat cells (Fig.4A, 4B). On the other hand, wild type MERS-CoV pseudoviruses entered bat cells efficiently, whereas MERS-CoV pseudoviruses bearing

the mutant hECP motif (which added the N-linked glycosylation site) demonstrated reduced, but still significant, capability to enter bat cells (Fig.4C, 4D). Moreover, most of HKU4 and MERS-CoV-spike-mediated pseudovirus entry into bat cells could be blocked by the ECP inhibitor, also suggesting that bat endosomal cysteine proteases activate coronavirus spikes bearing a glycosylated hECP motif (Fig.4). These results indicate that unlike human endosomal cysteine proteases, bat endosomal cysteine proteases are capable of recognizing and cleaving efficiently the hECP motif containing a glycosylation site. The molecular and structural differences between human and bat hECPs accounting for their functional differences will be investigated in future research.

Understanding the molecular mechanisms for cross-species transmissions of viruses is critical for evaluating their emerging disease potentials and for preventing and controlling their spread in human populations. Here we examined the different cell entry activities of the spike proteins from human-infecting MERS-CoV and a closely related bat coronavirus HKU4. Although MERS-CoV and HKU4 spikes share a high sequence homology and recognize the same host DPP4 receptor, only MERS-CoV spike, but not HKU4 spike, mediates viral entry into human cells. Our study revealed that introduction of two single mutations, S746R and N762A, into HKU4 spike at the S1/S2 boundary fully instilled its capability to mediate viral entry into human cells. MERS-CoV spike already contained both of these mutations, explaining why MERS-CoV is capable of infecting human cells. Thus these two mutations in the spike are essential for MERS-CoV to transmit from bats to humans by allowing MERS-CoV spike to be activated by human cellular proteases. Viral adaptation to human cellular proteases is critical for viral infection of human cells because human cellular proteases, particularly endosomal

proteases, are more reliable sources than some extracellular proteases to activate viral entry. Previous research also identified two mutations in SARS-CoV spike that led SARS-CoV to transmit from palm civets to humans (19-22). These mutations increased SARS-CoV spike's capability to bind human receptor angiotensin-converting enzyme 2. Thus, different entry factors appear to have played the most critical roles in the cross-species transmission of MERS-CoV and SARS-CoV: adaption to human cellular proteases by MERS-CoV and adaption to human receptor by SARS-CoV. Although MERS-CoV spike might also need to adapt to human DPP4 receptor upon infecting human cells (14, 23), such adaptations might only have incremental effects on the infectivity of MERS-CoV in human cells. In contrast, the two mutations adaptive to human cellular proteases transformed MERS-CoV spike from completely lacking to fully owning the capacity to mediate viral entry into human cells, and thus they likely played the most critical role in the bat-to-human transmission of MERS-CoV.

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268 **Figure legends**

269 **Figure 1. Domain structure of MERS-CoV and HKU4 spike proteins.** The spikes
270 contain a receptor-binding S1 subunit, a membrane-fusion S2 subunit, a transmembrane
271 anchor (TM), and an intracellular tail (IC). S1 contains the receptor-binding domain
272 (RBD) that binds DPP4 receptor. S2 contains the fusion peptide (FP), heptad repeat 1
273 (HR1), and heptad repeat 2 (HR2), all of which are essential structural elements for the
274 membrane fusion process. The S1/S2 boundary in MERS-CoV spike (defined as the
275 region between the RBD and the fusion peptide) contains one established human protease
276 motif that is recognized by proprotein convertases (hPPC) (15, 16); it also contains one
277 established human protease motif that is recognized by endosomal cysteine proteases
278 (hECP) (17, 18). Sequence alignments of these regions in MERS-CoV and HKU4 spikes
279 (Genbank accession codes: AFS88936.1 for MERS-CoV spike; ABN10839.1 for HKU4
280 spike) are shown, with the critical residue differences labeled in red. Arrows indicate the
281 predicted cleavage sites in MERS-CoV spike by human proteases.
282
283 **Figure 2. Characterization of two protease motifs in MERS-CoV and HKU4 spike**
284 **proteins.** (A) Western blot analysis of HKU4 and MERS-CoV spikes in pseudovirus
285 particles. Retroviruses pseudotyped with HKU4 spike (i.e., HKU4 pseudoviruses) or
286 MERS-CoV spike (i.e., MERS-CoV pseudoviruses) were prepared in HEK293T cells
287 (human embryonic kidney) as previously described (14). The incorporations of wild type
288 (WT) and mutant HKU4 and MERS-CoV spikes into pseudovirus particles were
289 measured by Western blot using antibody against their C-terminal C9 tags. Plots below
290 the Western blot images correspond to quantifications of the band intensities from the

cleaved and uncleaved spikes combined. The numbers below the plots indicate the relative amount of spikes incorporated into pseudovirus particles compared to wild type HKU4 and MERS-CoV spikes, respectively. All quantifications were done using ImageJ software (National Institutes of Health). (B) Glycosylation state of HKU4 spike at the hECP motifs. HEK293T cells exogenously expressing wild type (WT) and mutant HKU4 spikes were lysed and subjected to western blot analysis. To improve the separation of large molecular weight spikes, 3-8% NuPAGE Tris-Acetate Gels (Life Technologies) were used for gel electrophoresis. To improve the accuracy of the result, each of the mutant and wild type spikes was run in two lanes that alternated between samples. The experiment was also repeated in a separate gel. Compared with wild type HKU4 spike, the downward shift in the band of HKU4 spike bearing the mutated hECP motif (i.e., mutation N762A) is consistent with the removal of glycosylation. (C) Glycosylation state of MERS-CoV spike at the hECP motifs. Compared with MERS-CoV spike bearing the re-engineered hPPC motif (i.e., mutation R748S), the upward shift in the band of MERS-CoV spike bearing both the re-engineered hPPC and hECP motifs (i.e., mutations R748S/A763N/F764Y/N765T) indicates the introduction of glycosylation.

Figure 3. HKU4 and MERS-CoV-spike-mediated pseudovirus entry in human cells. (A) HKU4 pseudoviruses bearing no mutation, re-engineered hPPC motif (S746R), re-engineered hECP motif (N762A), or both of the re-engineered motifs (S746R/N762A) were prepared in HEK293T cells (human embryonic kidney), and then were used to infect HEK293T cells exogenously expressing human DPP4 (GenBank accession code NP_001926.2). The infections were carried out in the presence or absence of exogenous

trypsin. The pseudovirus entry efficiencies were characterized as luciferase activity accompanying the entry, and normalized against the relative amount of spikes incorporated into pseudovirus particles (Fig.2A). The pseudovirus entry mediated by HKU4 spike bearing both of the re-engineered motifs in the absence of exogenous trypsin was taken as 100%. (B) Pseudovirus-producing HEK293T cells were treated with proprotein convertase (PPC) inhibitor (dec-RVKR-CMK) 5 hours after transfection of plasmids that encode HKU4 spike containing the re-engineered hPPC motif (S746R). Pseudovirus-targeting HEK293T cells were treated with endosomal cysteine protease (ECP) inhibitor (E-64d) before being infected by HKU4 pseudoviruses bearing the re-engineered hECP motif (N762A). Both of the inhibitors were used for HKU4 pseudoviruses bearing both of the mutations. (C) MERS-CoV pseudoviruses bearing no mutation, mutated hPPC motif (R748S), mutated hECP motif (A763N/F764Y/N765T), or both of the mutated motifs (R748S/A763N/F764Y/N765T) were used to infect HEK293T cells exogenously expressing human DPP4. The pseudovirus entry mediated by wild type MERS-CoV spike in the absence of exogenous trypsin was taken as 100%. (D) Pseudovirus-producing HEK293T cells were treated with dec-RVKR-CMK 5 hours after transfection of plasmids that encode MERS-CoV spike containing the mutated hPPC motif (R748S). Pseudovirus-targeting HEK293T cells were treated with E-64d before being infected by MERS-CoV pseudoviruses bearing the mutated hECP motif (A763N/F764Y/N765T). Both of the inhibitors were used for MERS-CoV pseudoviruses bearing both of the mutations. Error bars indicate SEM (n = 4).

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Figure 4. HKU4 and MERS-CoV-spike-mediated pseudovirus entry into bat cells. (A) HKU4 pseudoviruses bearing no mutation or re-engineered hECP motif (N762A) and were prepared in HEK293T cells, and then used to infect RSKT cells endogenously expressing bat DPP4 (*Rhinolophus sinicus* bat kidney cells (24)). The cells were pre-treated with indicated concentrations of ECP inhibitor (E-64d) before being infected by pseudoviruses. (B) The same HKU4 pseudoviruses were used to infect Tb1-Lu cells (*Tadarida brasiliensis* bat lung cells) exogenously expressing bat DPP4 (GenBank accession number KC249974). (C) MERS-CoV pseudoviruses bearing no mutation or the mutant hECP motif (A763N/F764Y/N765T) were prepared in HEK293T cells, and then used to infect RSKT cells. (D) The same MERS-CoV pseudoviruses were used to infect Tb1-Lu cells. The pseudovirus entry efficiencies were characterized as luciferase activity accompanying the entry, and normalized against the relative amount of spike proteins incorporated into pseudovirus particles. The pseudovirus entry mediated by wild type HKU4 spike (for panels A and B) or wild type MERS-CoV spike (for panels C and D) in the absence of the inhibitor was taken as 100%. Error bars indicate SEM (n = 4).