Receptor usage and cell entry of bat coronavirus HKU4 provide insight into bat-to-human transmission of MERS coronavirus

Yang Yang[a,1], Lanying Du[b,1], Chang Liu[a,1], Lili Wang[b], Cuiqing Ma[b], Jian Tang[b], Ralph S. Baric[c], Shibo Jiang[b,d,2], and Fang Li[a,2]

[a]Department of Pharmacology, University of Minnesota Medical School, Minneapolis, MN 55455; [b]Lindsley F. Kimball Research Institute, New York Blood Center, New York, NY 10065; [c]Department of Epidemiology, University of North Carolina, Chapel Hill, NC 27559; and [d]Key Laboratory of Medical Molecular Virology of Ministries of Education and Health, Shanghai Medical College and Institute of Medical Microbiology, Fudan University, Shanghai 200032, China

Edited by Michael Farzan, The Scripps Research Institute, Jupiter, Florida, and accepted by the Editorial Board July 10, 2014 (received for review March 29, 2014)

Middle East respiratory syndrome coronavirus (MERS-CoV) currently spreads in humans and causes ∼36% fatality in infected patients. Believed to have originated from bats, MERS-CoV is genetically related to bat coronaviruses HKU4 and HKU5. To understand how bat coronaviruses transmit to humans, we investigated the receptor usage and cell entry activity of the virus-surface spike proteins of HKU4 and HKU5. We found that dipeptidyl peptidase 4 (DPP4), the receptor for MERS-CoV, is also the receptor for HKU4, but not HKU5. Despite sharing a common receptor, MERS-CoV and HKU4 spikes demonstrated functional differences. First, whereas MERS-CoV prefers human DPP4 over bat DPP4 as its receptor, HKU4 shows the opposite trend. Second, in the absence of exogenous proteases, both MERS-CoV and HKU4 spikes mediate pseudovirus entry into bat cells, whereas only MERS-CoV spike, but not HKU4 spike, mediates pseudovirus entry into human cells. Thus, MERS-CoV, but not HKU4, has adapted to use human DPP4 and human cellular proteases for efficient human cell entry, contributing to the enhanced pathogenesis of MERS-CoV in humans. These results establish DPP4 as a functional receptor for HKU4 and host cellular proteases as a host range determinant for HKU4. They also suggest that DPP4-recognizing bat coronaviruses threaten human health because of their spikes' capability to adapt to human cells for cross-species transmissions.

As of June 16, 2014, the recently emerged Middle East respiratory syndrome coronavirus (MERS-CoV) had infected 701 people, with a fatality rate of ∼36% (www.who.int/csr/don/2014_06_16_mers/en/), and had demonstrated the capability for human-to-human transmission (1, 2). Alarmingly, coronavirus surveillance studies have suggested that MERS-CoV originated from animals, with bats as the likely natural reservoir and camels as the likely intermediate hosts (3–6). Hence, cross-species transmission of MERS-CoV from bats to humans, either directly or through camels, poses a constant and long-term threat to human health. Phylogenetic analysis has revealed that MERS-CoV is genetically related to two bat coronaviruses, HKU4 and HKU5 (7-9). Understanding the pathogenesis and potential cross-species transmissibility of these bat coronaviruses is critical for evaluating long-term emerging disease potentials and for preventing and controlling the spread of bat-originated coronaviruses in humans. This study investigates the receptor usage and cell entry mechanisms of HKU4 and HKU5, providing insight into how MERS-CoV and MERS-related bat coronaviruses can cross species barriers, adapt to human cells, and gain infectivity in humans.

Receptor recognition has been established as an important determinant of the host range and tropism of coronaviruses (10, 11). An envelope-anchored spike protein mediates coronavirus entry into host cells by first binding to a host receptor through its S1 subunit and then fusing the host and viral membranes via its S2 subunit. Coronaviruses recognize a wide range of receptors, including proteins and sialic acids (12). MERS-CoV uses dipeptidyl peptidase 4 (DPP4) as its receptor (13). A defined receptor-binding domain (RBD) in MERS-CoV spike S1 subunit binds human DPP4 with high affinity (14–18). MERS-CoV RBD shares 56% and 54% sequence similarity with the corresponding S1 domain in HKU4 and HKU5, respectively (Fig. S1*A*) (9), raising the possibility that HKU4 and HKU5 may also use DPP4 as their receptor. However, previous studies showed that receptor recognition by coronaviruses is sensitive to residue changes in coronavirus RBDs (19). For example, one or two residue changes in the RBD of another bat-originated coronavirus, severe acute respiratory syndrome coronavirus (SARS-CoV), can dictate whether SARS-CoV uses human angiotensin-converting enzyme 2 as its receptor for efficient human infections (19–22). Therefore, investigating the receptors of HKU4 and HKU5 is important for understanding their potential threats to human health and for delineating the causes of MERS-CoV infections in humans.

Coronavirus entry into host cells is limited not only by receptor recognition but also by membrane fusion. To fuse host and viral membranes, coronavirus spikes need to be cleaved at the S1/S2 boundary by host proteases (23–27). The availability of these host proteases to coronaviruses and the specificities of these host proteases on coronavirus spikes can contribute to the host range

Significance

A constant and long-term threat to human health is cross-species transmission of Middle East respiratory syndrome coronavirus (MERS-CoV) from bats to humans. However, this process is poorly understood. Examining the cross-species transmissibility of bat coronavirus HKU4, which is genetically related to MERS-CoV, can provide critical information about the likely causes of MERS-CoV infections in humans. Here we investigate the receptor usage and cell entry mechanism of HKU4 compared with MERS-CoV. Our results reveal that MERS-CoV has adapted to use human receptor and cellular proteases for efficient human cell entry, whereas HKU4 can potentially follow-up and also infect human cells. These findings are critical for evaluating emerging disease potentials of bat coronaviruses and for preventing and controlling their spread in humans.

Author contributions: Y.Y., L.D., C.L., S.J., and F.L. designed research; Y.Y., L.D., C.L., L.W., C.M., and J.T. performed research; R.S.B. contributed new reagents/analytic tools; Y.Y., L.D., C.L., L.W., C.M., J.T., S.J., and F.L. analyzed data; and F.L. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission. M.F. is a guest editor invited by the Editorial Board.

[1]Y.Y., L.D., and C.L. contributed equally to this work.

[2]To whom correspondence may be addressed. E-mail: lifang@umn.edu or sjiang@nybloodcenter.org.

This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1405889111/-/DCSupplemental.

and tropism of coronaviruses. Coronavirus-spike-processing host proteases may include proprotein convertases from the virus-producing cells (e.g., furin), proteases from the extracellular environment (e.g., elastase), proteases on the cell surface of virus-targeting cells (e.g., type 2 transmembrane serine protease, or TMPRSS2), and proteases in the endosomes of virus-targeting cells (e.g., cathepsin L) (23, 24). Bat-originated and human-infecting coronaviruses MERS-CoV and SARS-CoV both use cell-surface protease TMPRSS2 and endosomal protease cathepsins L for human cell entry, although proteases from the extracellular environment may also facilitate the cell entry process (23–30). However, the mechanisms for human cell entry by bat coronaviruses are poorly understood, leaving a critical missing link in the bat-to-human transmission of coronaviruses. Therefore, examining the cell entry of HKU4 and HKU5 can provide novel knowledge of the causes of MERS-CoV (and SARS-CoV) infections in humans.

In this study, we have elucidated the receptor usage and cell entry activity of HKU4 and HKU5 spikes compared with MERS-CoV spike. Our study illuminates the mechanisms by which bat coronaviruses adapt to human cells during cross-species transmission events and provides valuable knowledge that can be used to evaluate emerging disease potentials of bat coronaviruses and to prevent and control the future spread of bat coronaviruses in humans.

Results

We expressed and purified the S1 domains of four viruses (HKU4, HKU5, MERS-CoV, and SARS-CoV), as well as DPP4 ectodomain from two species (human and bat), using procedures described previously (14, 22). Here the S1 domains of HKU4 (residues 372–593) and HKU5 (residues 375–586) correspond to the DPP4-binding RBD in MERS-CoV spike (residues 367–588) (Fig. S1*A*). The two bat species from which HKU4 and HKU5 were isolated belong to the same family, but different genera (Fig. S1*B*) (7–9). To understand coronavirus spike/DPP4 interactions, the DPP4 and coronavirus spike genes under investigation need to come from the same or similar bat species. Because the DPP4 genes from the above two bat species are unavailable, we chose the DPP4 gene from another bat species in the same genus as the one from which HKU5 was isolated (Fig. S1*B*). All of the recombinant proteins were purified to high homogeneity (Fig. S2) and subsequently used in the following biochemical studies.

Using three alternative approaches, we characterized the binding interactions between the S1 domain of each virus and DPP4 from each species. AlphaScreen protein–protein binding assay showed that whereas the MERS-CoV S1 domain bound human DPP4 (hDPP4) with significantly higher affinity than it bound bat DPP4 (bDPP4), the corresponding HKU4 S1 domain bound bDPP4 slightly better than it bound hDPP4 (Fig. 1*A*). In contrast, the HKU5 S1 domain did not bind hDPP4 or bDPP4. As a control, the SARS-CoV S1 domain only bound its own receptor human angiotensin-converting enzyme 2, but not hDPP4 or bDPP4. Pull-down assay revealed that hDPP4 was more efficient than bDPP4 at pulling down the MERS-CoV S1 domain from solution, whereas both hDPP4 and bDPP4 pulled down the HKU4 S1 domain efficiently (Fig. 1*B*). In contrast, neither hDPP4 nor bDPP4 pulled down the SARS-CoV or HKU5 S1 domain. Pseudovirus inhibition assay demonstrated that the HKU4 S1 domain, but not the HKU5 or SARS-CoV S1 domain, inhibited MERS-CoV-spike-mediated pseudovirus entry into HEK293T cells exogenously expressing hDPP4 or bDPP4 [HEK293T cells are human embryonic kidney cells that do not endogenously express DPP4 (31)]. This indicates that the HKU4 S1 domain and MERS-CoV-spike-packaged pseudoviruses competed for the same DPP4 receptor on HEK293T cell surface (Fig. 1*C*). Taken together, these results showed that the HKU4 S1 domain, but not the HKU5 S1 domain, binds both

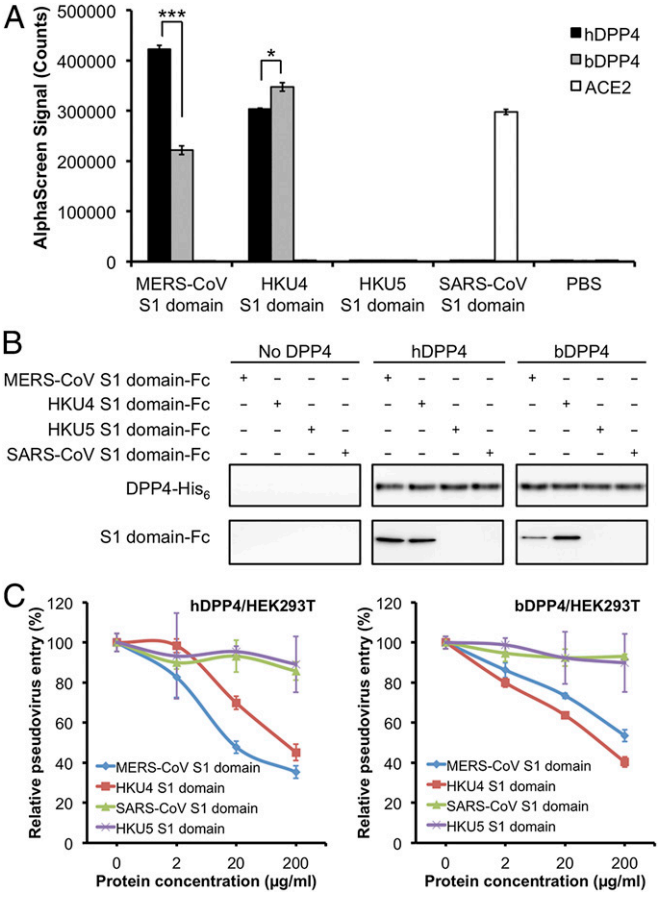

Fig. 1. Binding interactions between HKU4 spike and DPP4. (*A*) AlphaScreen assay showing the direct binding interactions between the coronavirus spike S1 domains and hDPP4 or bDPP4. Binding affinity was characterized as AlphaScreen counts. Error bars indicate SEM (two-tailed *t* test, $*P < 0.05$, $***P < 0.001$; $n = 3$). (*B*) Pull-down assay showing the direct binding interactions between the coronavirus S1 domains and hDPP4 or bDPP4. His_6-tagged hDPP4 or bDPP4 was incubated with each of the Fc-tagged coronavirus S1 domains. The S1 domain/DPP4 complex was then precipitated with nickel-nitrilotriacetic acid beads. Binding affinity was characterized as Western blotting on the precipitated Fc-tagged coronavirus S1 domains. (*C*) Inhibition of MERS-CoV-spike-mediated pseudovirus entry by the HKU4 S1 domain. HEK293T cells expressing hDPP4 or bDPP4 were preincubated with gradient concentrations of purified coronavirus S1 domains and then infected by MERS-CoV-spike-packaged pseudoviruses expressing luciferase. The pseudovirus entry efficiency was characterized as luciferase activity accompanying the entry and normalized relative to the entry in the absence of any coronavirus S1 domain. Error bars indicate SEM ($n = 3$).

hDPP4 and bDPP4, and that the MERS-CoV S1 domain binds hDPP4 significantly better than it binds bDPP4, whereas the HKU4 S1 domain binds bDPP4 slightly better than it binds hDPP4. These results strongly suggest that HKU4 spike, but not HKU5 spike, uses DPP4 as its receptor and that the HKU4 S1 domain is the DPP4-binding RBD.

To confirm that DPP4 is the receptor for HKU4 spike, we investigated whether HKU4 spike could mediate viral entry into DPP4-expressing human cells. Because live HKU4 virus has never been successfully cultured, it is not an option to use live HKU4 virus in this study. Instead, we performed an HKU4-spike-mediated pseudovirus entry assay. To this end, retroviruses pseudotyped with HKU4 spike were used to enter HEK293T cells exogenously expressing either hDPP4 or bDPP4 on their surface. Surprisingly, HKU4 spike failed to mediate pseudovirus entry into these DPP4-expressing HEK293T cells (Fig. 2*A*). As

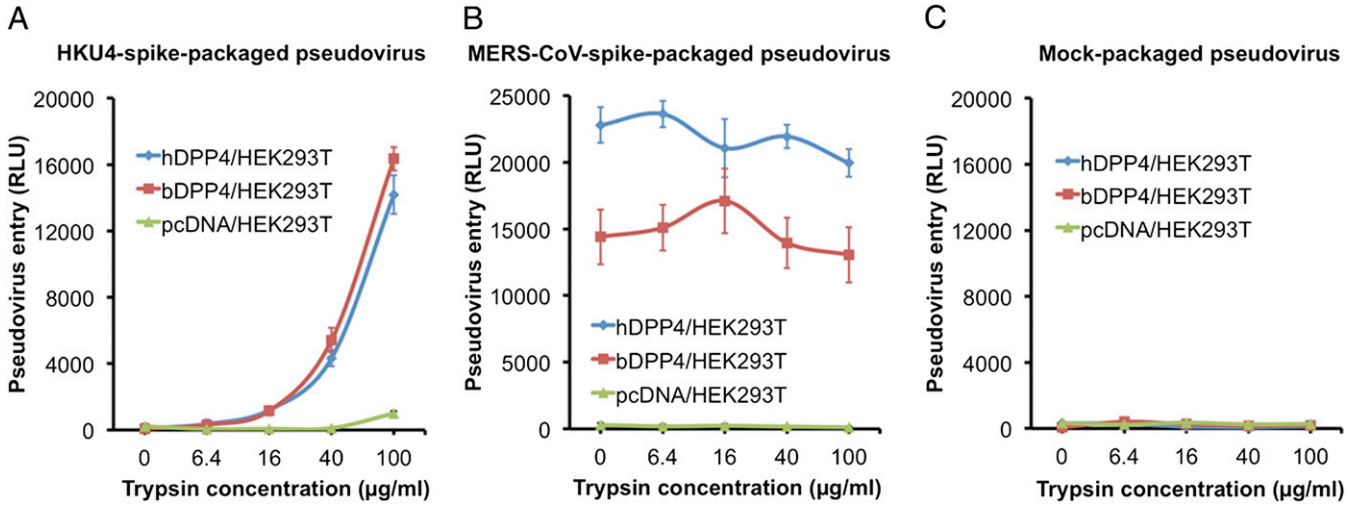

Fig. 2. HKU4- and MERS-CoV-spike-mediated pseudovirus entry into human cells. Retroviruses pseudotyped with HKU4 spike (*A*), MERS-CoV spike (*B*), or no spike (mock) (*C*) were incubated with gradient concentrations of trypsin and used to enter HEK293T cells (exogenously expressing hDPP4, bDPP4, or no DPP4, respectively). The pseudovirus entry efficiency was characterized as luciferase activity accompanying the entry. Error bars indicate SEM ($n = 4$).

a comparison, MERS-CoV spike efficiently mediated pseudovirus entry into hDPP4-expressing HEK293T cells, but less efficiently into bDPP4-expressing HEK293T cells (Fig. 2*B*). However, when treated with trypsin, HKU4-spike-packaged pseudoviruses entered both hDPP4- and bDPP4-expressing HEK293T efficiently. As a comparison, trypsin treatment had little effect on MERS-CoV-spike-mediated pseudovirus entry into DPP4-expressing HEK293T cells. To validate these findings, we performed two control experiments to ensure that HKU4-spike-mediated entry depended on both HKU4 spike and DPP4. First, after trypsin treatment, HKU4-spike-pseudotyped retroviruses could not enter HEK293T cells not expressing DPP4 (Fig. 2*A*). Second, retroviruses not pseudotyped with HKU4 spike could not enter DPP4-expressing HEK293T cells (Fig. 2*C*). Overall, these results support two conclusions: DPP4 is the functional receptor for HKU4 spike, and exogenous trypsin is needed for HKU4-spike-mediated, but not MERS-CoV-spike-mediated, pseudovirus entry into human cells.

Further confirmation that DPP4 is the receptor for HKU4 spike came from an examination of whether anti-hDPP4 polyclonal antibodies could competitively block the interactions between HKU4 spike and hDPP4. First, dot blot hybridization assay showed that the antibodies almost completely blocked the binding between HKU4 RBD and hDPP4 and significantly inhibited the binding between MERS-CoV RBD and hDPP4 (Fig. S3*A*). In contrast, the antibodies did not block the binding between HKU4 or MERS-CoV RBD and bDPP4, suggesting that the antibodies did not target bDPP4. Second, pseudovirus neutralization assay revealed that the antibodies efficiently blocked both HKU4- and MERS-CoV-spike-mediated entry into hDPP4-expressing HEK293T cells, but not bDPP4-expressing cells (Fig. S3*B*). These results demonstrated that anti-hDPP4 antibodies competed with HKU4 spike for the binding sites on hDPP4, further confirming that DPP4 is the functional receptor for HKU4 spike.

To further investigate the cell entry mechanism of HKU4, we repeated the pseudovirus entry assay using Huh-7 cells (human liver cells), Calu-3 cells (human lung cells), and MRC-5 cells (human lung cells), all of which endogenously express hDPP4 (30-32). The results again showed that exogenous trypsin was

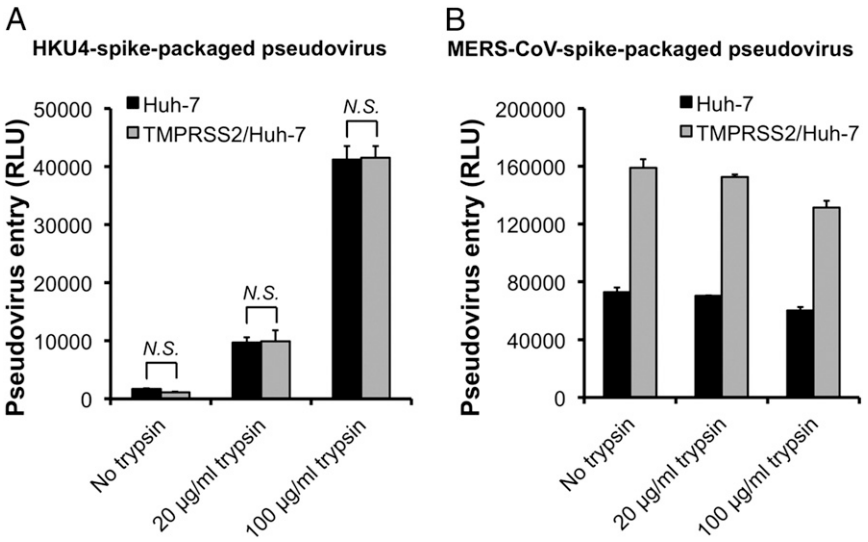

Fig. 3. Effects of human TMPRSS2 on HKU4- or MERS-CoV-spike-mediated pseudovirus entry into human cells. Retroviruses pseudotyped with HKU4 spike or MERS-CoV spike were pretreated with no trypsin, 20 μg/mL trypsin, or 100 μg/mL trypsin and used to enter Huh-7 cells or Huh-7 cells exogenously expressing human TMPRSS2. The pseudovirus entry efficiency was characterized as luciferase activity accompanying the entry. Error bars indicate SEM (two-tailed *t* test, *N.S.*, $P > 0.05$; $n = 4$).

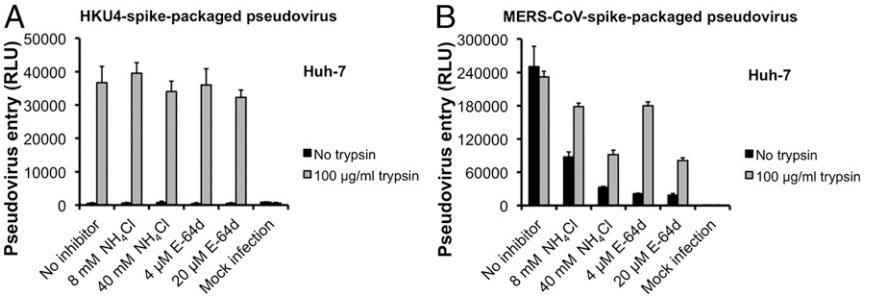

Fig. 4. Role of human endosomal proteases in HKU4- and MERS-CoV-spike-mediated entry into human cells. Huh-7 cells were first preincubated with endosomal acidification inhibitor NH_4Cl or endosomal protease inhibitor E-64d at the indicated concentrations. Then the cells were infected by HKU4- or MERS-CoV-spike-pseudotyped retroviruses that had been pretreated or not pretreated with 100 μg/mL trypsin. The pseudovirus entry efficiency was characterized as luciferase activity accompanying the entry. Error bars indicate SEM ($n = 3$).

needed for HKU4-spike-mediated, but not MERS-CoV-spike-mediated, pseudovirus entry into Huh-7, Calu-3, and MRC-5 cells (Fig. S4). Thus, our finding on the effects of trypsin on HKU4- and MERS-CoV-spike-mediated entry can be extended to different types of human cells.

What human cellular proteases process MERS-CoV spike, but not HKU4 spike? To address this question, we looked into all three stages in which human cellular proteases may play a role in coronavirus-spike-mediated cell entry. First, by performing Western blot on MERS-CoV- and HKU4-spike-packaged pseudoviruses, we found that MERS-CoV spike, but not HKU4 spike, had been partially cleaved coming out of virus-producing HEK293T cells (Fig. S5). Moreover, HKU4 spike was cleaved by exogenous trypsin (Fig. S5). Thus, MERS-CoV spike, but not HKU4 spike, can be partially processed in virus-producing cells by human endogenous proteases. The processing may be achieved potentially by human proprotein convertases. Second, we carried out the pseudovirus entry assay using Huh-7 cells exogenously expressing human TMPRSS2 [Huh-7 cells do not endogenously express TMPRSS2 (33)]. The results showed that human TMPRSS2 enhanced MERS-CoV-spike-mediated, but not HKU4-spike-mediated, pseudovirus entry (Fig. 3). Third, we explored the role of human endosomal proteases in MERS-CoV- and HKU4-spike-mediated pseudovirus entry into Huh-7 cells, using two types of inhibitors: endosomal acidification inhibitor NH_4Cl and endosomal protease inhibitor E-64d [which inhibits endosomal cathepsins L, B, H, etc (34)] (Fig. 4). MERS-CoV-spike-mediated entry was significantly inhibited by both NH_4Cl and E-64d, revealing the critical role of endosomal proteases in MERS-CoV-spike-mediated entry (the higher-than-baseline level of pseudovirus entry in the presence of NH_4Cl or E-64d is probably a result of the prior partial processing of MERS-CoV spike during virus packaging and release). In contrast, these inhibitors showed little effect on HKU4-spike-mediated entry. Because HKU4 spike fails to transduce the same cell line whose endosomal proteases activate MERS-CoV spike, HKU4 spike most likely cannot be activated by human endosomal proteases. Overall, these results have demonstrated that MERS-CoV spike, but not HKU4 spike, can be processed by human cellular proteases from three different stages of virus infection: virus packaging and release, viral attachment to human cell surface, and viral endocytosis.

Finally, we investigated whether HKU4 and MERS-CoV spikes can mediate pseudovirus entry into bat cells. We repeated pseudovirus entry assay, using Tb1-Lu cells (bat lung cells) that exogenously express hDPP4 or bDPP4. The results show that in the absence of trypsin, both HKU4 and MERS-CoV spikes efficiently mediated pseudovirus entry into Tb1-Lu cells, even though trypsin could further enhance HKU4-spike-mediated pseudovirus entry into Tb1-Lu cells (Fig. 5). In a control experiment, Tb1-Lu cells without exogenously expressed hDPP4 or bDPP4 failed to support MERS-CoV- or HKU4-spike-mediated pseudovirus entry, indicating a lack of endogenously expressed DPP4 in Tb1-Lu cells. These results revealed that in the presence of an appropriate receptor, activation by exogenous proteases is not a requirement for HKU4- or MERS-CoV-spike-mediated pseudovirus entry into bat cells.

Discussion

This study has characterized the receptor usage and cell entry of MERS-related bat coronaviruses HKU4 and HKU5 compared with bat-originated but human-infecting MERS-CoV. Because HKU4 and HKU5 had never infected human cells, this study provided an opportunity to understand human cell adaptations by MERS-CoV. Through comparative analysis of these viruses, this study not only has identified the functional receptor for HKU4 but also has revealed the functional differences between MERS-CoV and HKU4 spikes in their adaptations to use human receptor and cellular proteases for human cell entry.

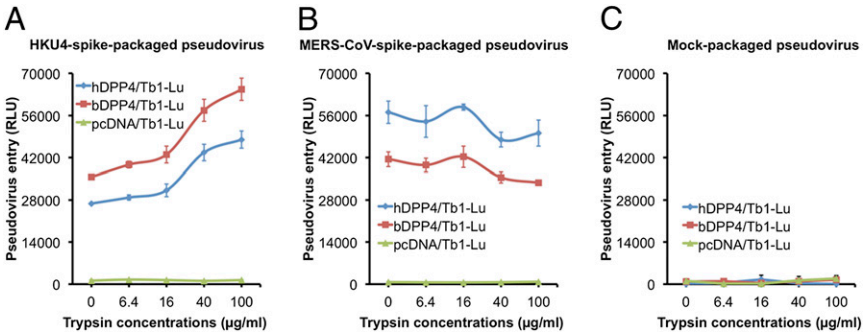

Fig. 5. HKU4- and MERS-CoV-spike-mediated pseudovirus entry into bat cells. Retroviruses pseudotyped with HKU4 spike (*A*), MERS-CoV spike (*B*), or no spike (mock) (*C*) were incubated with gradient concentrations of trypsin and used to enter Tb1-Lu bat cells (exogenously expressing hDPP4, bDPP4, or no DPP4, respectively). The pseudovirus entry efficiency was characterized as luciferase activity accompanying the entry. Error bars indicate SEM ($n = 4$).

First, using a combination of experimental approaches including AlphaScreen, protein pull down, dot blot hybridization, pseudovirus inhibition, pseudovirus entry, and antibody neutralization, this study has established DPP4 as the functional receptor for HKU4. Although HKU4 spike and bDPP4 are from two different bat species (Fig. S1*B*), the positive interaction between the two proteins suggests that HKU4 spike should also interact positively with bDPP4 from its own bat species. Indeed, our study shows that both hDPP4 and bDPP4 are efficient receptors for HKU4 spike, although humans and bats are only remotely related species. In contrast, HKU5 spike does not use DPP4 as its receptor, despite sharing sequence similarity with MERS-CoV and HKU4 spikes. Again, this is unlikely because of the different bat species from which HKU5 and bDPP4 were isolated, but is a result of two deletions in the presumable DPP4-binding region of the HKU5 S1 domain (Fig. S1*A*). Moreover, whereas MERS-CoV spike binds hDPP4 with significantly higher affinity than it binds bDPP4, HKU4 spike slightly prefers bDPP4 over hDPP4. MERS-CoV's preference for hDPP4 over bDPP4 as its receptor is consistent with a recent finding (35). Thus, it is likely that MERS-CoV spike has evolved to use hDPP4 efficiently but has become less effective in recognizing bDPP4, whereas HKU4 spike has not evolved adaptive mutations to promote efficient hDPP4 usage.

Second, using exogenous trypsin, endosomal acidification and protease inhibitors, and pseudovirus entry into various types of human and bat cells, this study has revealed that human cellular proteases can activate MERS-CoV-spike-mediated, but not HKU4-spike-mediated, pseudovirus entry into human cells. These human cellular proteases act on different stages of MERS-CoV-spike-mediated pseudovirus entry and include human endogenous proteases (e.g., proprotein convertases) acting during virus packaging and release, human TMPRSS2 acting during viral attachment to human cell surface, and human endosomal cathepsins acting during viral endocytosis. Exogenous proteases contribute to, but are not essential for, MERS-CoV entry into human cells exogenously expressing low-affinity DPP4 homologs (35). In contrast, an exogenous protease is essential for HKU4-spike-mediated entry into human cells. However, both MERS-CoV and HKU4 spikes can mediate efficient pseudovirus entry into bat cells, suggesting that bat cellular proteases can process both MERS-CoV and HKU4 spikes. Because at this time, little is known about the functions of bat cellular proteases, future research will be needed to understand how coronavirus spikes mediate virus entry into bat cells. Nevertheless, this study indicates that MERS-CoV spike, but not HKU4 spike, has adapted to use human cellular proteases and thus has evolved the ability to mediate virus entry into human cells.

These findings provide critical insight into the current MERS-CoV infections and raises concerns over potential future bat coronavirus infections in humans. DPP4-recognizing bat coronaviruses such as HKU4 pose a threat to human health because their spikes can potentially adapt to use human DPP4 and human cellular proteases for human cell entry. Viral adaptation to human receptors enhances viral attachment to human cells, whereas viral adaptation to human cellular proteases reduces viral dependence on the extracellular environment. Both adaptation processes can facilitate coronaviruses to expand their host ranges and tropisms. These evolutionary changes may have already taken place during the bat-to-human transmission of MERS-CoV. Recent findings suggest that MERS-CoV has been circulating in camels for some time (5, 6, 36, 37). Viral adaptations to camel cells could have been an intermediate step toward viral adaptations to human cells. Regardless of whether the transmission to humans came directly from bats or indirectly through camels, the eventual viral adaptations to human cells may have contributed to the increased replication, transmission, and pathogenesis of MERS-CoV in humans. Our findings enhance understanding of the likely causes of MERS-CoV infections in humans, the potential health risks associated with DPP4-recognizing bat coronaviruses, and the mechanisms of cross-species transmissions of animal viruses in general.

Materials and Methods

Cell Lines. The HEK293T (human embryonic kidney), MRC-5 (human lung), and Tb1-Lu (*Triatoma brasiliensis* bat lung) cells were obtained from ATCC (www.atcc.org). Huh-7 (human liver) and Calu-3 (human lung) cells were kindly provided by Charles M. Rice at Rockefeller University and Chien-Te K. Tseng at the University of Texas Medical Branch, respectively. These cell lines were maintained in Dulbecco's modified Eagle medium, supplemented with 10% FBS, 2 mM L-glutamine, and 1% penicillin/streptomycin (Life Technologies Inc, Grand Island, NY).

Protein Expression and Purification. The S1 domains of MERS-CoV (residues 367–588; GenBank accession no. AFS88936.1), HKU4 (residues 372–593; GenBank accession no. ABN10839.1), HKU5 (residues 375–586; GenBank accession no. ABN10875.1), and SARS-CoV (residues 306–527; GenBank accession no. NC_004718) were expressed and purified as previously described (14, 22). Briefly, the coronavirus S1 domains containing an N-terminal honey bee melittin signal peptide and a C-terminal His_6 tag were expressed in insect cells using the Bac-to-Bac expression system (Life Technologies Inc), secreted to cell culture medium, and purified sequentially on HiTrap nickel-chelating HP column and Superdex 200 gel filtration column (GE Healthcare, Pittsburgh, PA). Fc-tagged coronavirus S1 domains were obtained by fusion of the human IgG_4 Fc region to the C terminus of the proteins and were expressed using the same procedure as for the His_6-tagged S1 domain. The Fc-tagged S1 domains were purified sequentially on a HiTrap Protein G HP column and Superdex 200 gel filtration column.

hDPP4 ectodomain (residues 39–766; GenBank accession no. NP_001926.2) and bDPP4 ectodomain (residues 36–760; GenBank accession no. KC249974) containing an N-terminal human CD5 signal peptide and a C-terminal His_6 tag were expressed and purified using the same procedure as for His_6-tagged coronavirus S1 domains.

AlphaScreen Protein–Protein Binding Assay. The interactions between different coronavirus S1 domains and hDPP4 or bDPP4 were measured using AlphaScreen, as previously described (38, 39). Briefly, each of the Fc-tagged coronavirus S1 domains at 3 nM final concentrations was mixed with 100 nM His_6-tagged hDPP4 or bDPP4 in 1/2 AreaPlate (PerkinElmer, Waltham, MA) for 1 h at room temperature. AlphaScreen Nickel Chelate Donor Beads and AlphaScreen protein A acceptor beads (PerkinElmer) were added to the mixtures at final concentrations of 5 μg/mL The mixtures were incubated at room temperature for 1 h and protected from light. The assay plates were read in an EnSpire plate reader (PerkinElmer).

Pull-Down Assay. Protein pull-down assay was carried out as previously described (14). Briefly, 5 μg His_6-tagged hDPP4 or bDPP4 was mixed with each of the 5 μg Fc-tagged coronavirus S1 domains. The formed complex was precipitated using nickel-nitrilotriacetic acid agarose (Thermo Scientific, Waltham, MA). The DPP4 and coronavirus S1 domains were separated by SDS/PAGE and detected by anti-His_6 and anti-human IgG_4 Fc antibodies (Santa Cruz Biotechnology, Dallas, TX), respectively.

Dot Blot Hybridization Assay. Dot blot hybridization assay was carried out as previously described (40). Briefly, 10 μg each of the Fc-tagged coronavirus S1 domains was dotted onto a nitrocellulose membrane. The membranes were dried completely and blocked with 5% skim milk at 37 °C for 1 h. Next, 25 μg/mL His_6-tagged hDPP4 or bDPP4 was preincubated alone or with 20 μg/mL goat anti-hDPP4 polyclonal antibodies (R&D Systems, Minneapolis, MN) at 37 °C for 1 h, added to the membrane, and incubated at 4 °C overnight. The membrane was then washed five times with phosphate-buffered saline with Tween-20 (PBST), incubated with anti-His_6 mouse monoclonal IgG_1 HRP conjugate antibody (Santa Cruz biotechnology) at 37 °C for 2 h, and washed five times with PBST. Finally, the bound proteins were detected using ECL plus (GE Healthcare).

Inhibition of MERS-CoV-Spike-Mediated Pseudovirus Entry by HKU4 RBD. Pseudovirus cell entry assay was carried out as previously described (31). Briefly, MERS-CoV-spike-pseudotyped retroviruses expressing a luciferase reporter gene were prepared by cotranfecting HEK293T cells with a plasmid carrying Env-defective, luciferase-expressing HIV-1 genome (pNL4-3.luc.R-E-) and a plasmid encoding MERS-CoV spike protein. The produced pseudovirus

particles were harvested 72 h after transfection. HEK293T cells transiently expressing hDPP4 or bDPP4 were preincubated with gradient concentrations of each of the purified coronavirus S1 domains for 1 h at 37 °C and then infected by equal amounts of MERS-CoV-spike-pseudotyped retrovirus particles. After incubation for 5 h at 37 °C, medium was changed and cells were incubated for an additional 60 h. Cells were then washed with PBS and lysed. Aliquots of cell lysates were transferred to Optiplate-96 (PerkinElmer), followed by addition of luciferase substrate. Relative light units were measured using EnSpire plate reader (PerkinElmer).

HKU4-Spike-Mediated Pseudovirus Entry into Human and Bat Cells. Retroviruses pseudotyped with MERS-CoV spike, HKU4 spike, or no spike (mock) were incubated with gradient concentrations of TPCK-treated trypsin (Sigma-Aldrich, St. Louis, MO) for 10 min at 25 °C, and 100 μg/mL soybean trypsin inhibitor (Sigma-Aldrich) was then added to stop the proteolysis reactions. Trypsin-treated pseudoviruses were then used to spin-infect HKE293T cells (transiently expressing hDPP4, bDPP4, or no DPP4), Huh-7 cells (transiently expressing TMPRSS2 or no TMPRSS2), Calu-3 cells, MRC-5 cells, or Tb1-Lu cells (transiently expressing hDPP4, bDPP4, or no DPP4) in 96-well plates at 1200 × *g* for 2 h at 4 °C. After incubation for 5 h at 37 °C, medium was changed. Cells were incubated for an additional 60 h. The cells were then lysed and measured for luciferase activity.

Neutralization of HKU4-Spike-Mediated Pseudovirus Entry by Anti-hDPP4 Antibodies. HEK293T cells expressing hDPP4, bDPP4, or no DPP4 were preincubated with 0, 2 μg/mL, or 20 μg/mL goat anti-hDPP4 polyclonal antibodies (R&D Systems) at 37 °C for 1 h and then infected by equal amounts of MERS-CoV-spike-pseudotyped retroviruses with no trypsin treatment or HKU4-spike-pseudotyped retroviruses after trypsin treatment. The infection procedure was the same as described earlier.

Protease Processing of MERS-CoV- or HKU4-Spike-Packaged Pseudoviruses. Retroviruses pseudotyped with MERS-CoV spike, HKU4 spike, or no spike (mock) were incubated with gradient concentrations of TPCK-treated trypsin (Sigma-Aldrich) for 10 min at 25 °C, and 100 μg/mL soybean trypsin inhibitor (Sigma-Aldrich) was then added to stop the proteolysis reactions. Trypsin-treated pseudoviruses were concentrated in an Amicon Ultracentrifuge filter unit with 100-kDa cutoff (EMD Millipore, Billerica, MA). The pseudovirus samples were then subjected to Western blot analysis. The His_6-tagged spikes were detected by antibody against their C-terminal His_6 tag.

Inhibition of Pseudovirus Entry into Human Cells by Endosome Inhibitors. Huh-7 cells were preincubated with 8 or 40 mM NH_4Cl (Sigma-Aldrich) or 4 or 20 μM E-64d (Sigma-Aldrich) at 37 °C for 1 h and then infected by HKU4- or MERS-CoV-spike-pseudotyped retroviruses that had been pretreated or not pretreated with trypsin. The infection procedure was the same as described earlier. Huh-7 cells infected by retroviruses pseudotyped with no spike (mock) were used as control.

ACKNOWLEDGMENTS. We thank Dr. Tom Gallagher, Dr. Gary Whittaker, Dr. Charles M. Rice, and Dr. Chien-Te K. Tseng for plasmids and cells. This work was supported by National Institutes of Health Grant R01AI089728 (to F.L.) and National Institutes of Health Grant R21AI109094 (to L.D.).

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