Lysosomal Proteases Are a Determinant of Coronavirus Tropism

Yuan Zheng,[a] Jian Shang,[a] Yang Yang,[a] Chang Liu,[a] Yushun Wan,[a] Qibin Geng,[a] Michelle Wang,[a] Ralph Baric,[b] Fang Li[a]

[a]Department of Veterinary and Biomedical Sciences, College of Veterinary Medicine, University of Minnesota, Saint Paul, Minnesota, USA

[b]Department of Epidemiology, University of North Carolina, Chapel Hill, North Carolina, USA

ABSTRACT Cell entry by coronaviruses involves two principal steps, receptor binding and membrane fusion; the latter requires activation by host proteases, particularly lysosomal proteases. Despite the importance of lysosomal proteases in both coronavirus entry and cell metabolism, the correlation between lysosomal proteases and cell tropism of coronaviruses has not been established. Here, we examined the roles of lysosomal proteases in activating coronavirus surface spike proteins for membrane fusion, using the spike proteins from severe acute respiratory syndrome coronavirus (SARS-CoV) and Middle East respiratory syndrome coronavirus (MERS-CoV) as the model system. To this end, we controlled the contributions from receptor binding and other host proteases, thereby attributing coronavirus entry solely or mainly to the efficiency of lysosomal proteases in activating coronavirus spike-mediated membrane fusion. Our results showed that lysosomal proteases from bat cells support coronavirus spike-mediated pseudovirus entry and cell-cell fusion more effectively than their counterparts from human cells. Moreover, purified lysosomal extracts from bat cells cleave cell surface-expressed coronavirus spikes more efficiently than their counterparts from human cells. Overall, our study suggests that different lysosomal protease activities from different host species and tissue cells are an important determinant of the species and tissue tropism of coronaviruses.

IMPORTANCE Coronaviruses are capable of colonizing new species, as evidenced by the recent emergence of SARS and MERS coronaviruses; they can also infect multiple tissues in the same species. Lysosomal proteases play critical roles in coronavirus entry by cleaving coronavirus surface spike proteins and activating the fusion of host and viral membranes; they also play critical roles in cell physiology by processing cellular products. How do different lysosomal protease activities from different cells impact coronavirus entry? Here, we controlled the contributions from known factors that function in coronavirus entry so that lysosomal protease activities became the only or the main determinant of coronavirus entry. Using pseudovirus entry, cell-cell fusion, and biochemical assays, we showed that lysosomal proteases from bat cells activate coronavirus spike-mediated membrane fusion more efficiently than their counterparts from human cells. Our study provides the first direct evidence supporting lysosomal proteases as a determinant of the species and tissue tropisms of coronaviruses.

KEYWORDS coronavirus spike protein, lysosomal proteases, species tropism, tissue tropism

One of the most outstanding features of viruses is their tropism, including species and tissue tropism (1). Viral entry into host cells is among the most important determinants of viral tropism (2–4). Entry of enveloped viruses involves two steps: receptor binding and membrane fusion. Enveloped viruses often hijack the endocytosis

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Address correspondence to Fang Li, lifang@umn.edu.

Y.Z., J.S., and Y.Y. contributed equally to this work.

pathway: they enter endosomes, proceed to lysosomes, and then fuse the viral and lysosomal membranes. The lysosomes play critical roles in cell metabolism by breaking down biomolecules and cellular debris and also by providing nutrients for other cellular functions (5, 6). The lysosomal protease activities are central to the functions of lysosomes (7). They are also required to activate the membrane fusion of a variety of viruses, including coronaviruses and filoviruses (8–11). Understanding the correlation between lysosomal protease activities and viral tropism has important implications for investigating viral pathogenesis, developing antiviral strategies, and identifying zoonotic strains with prepandemic potential.

Coronaviruses are large, enveloped, single-stranded RNA viruses (12, 13). They pose significant health threats to humans and other animals. Severe acute respiratory syndrome coronavirus (SARS-CoV) was responsible for the SARS epidemic in 2002 and 2003, causing over 8,000 infections with an ~10% fatality rate in humans (14, 15). Middle East respiratory syndrome coronavirus (MERS-CoV) was identified in 2012 and has so far caused over 2,200 infections with an ~35% fatality rate in humans (16, 17). An envelope-anchored spike protein guides coronavirus entry into host cells (18, 19). It first binds to a receptor on the host cell surface for viral attachment through its S1 subunit and then fuses viral and host membranes through its S2 subunit. The membrane fusion step by coronavirus spikes requires two prior cleavages by host proteases, the first at the S1-S2 boundary (i.e., the S1-S2 site) and the second within S2 (i.e., the S2′ site) (8, 19–21). Depending on the virus, the spike-processing proteases may come from different stages of the coronavirus infection cycle. For MERS-CoV, the spike can be processed by proprotein convertases (e.g., furin) during the molecular maturation process in virus-producing cells, by cell surface proteases (e.g., transmembrane protease serine 2 [TMPRSS2]) after viral attachment, and by lysosomal proteases (e.g., cathepsins) after endocytosis in virus-targeted cells (22–26). It was previously reported that MERS-CoV spike could be processed by furin after viral endocytosis in virus-targeted cells (21), but this finding was not supported by a recent study (27). The protease activation pattern of SARS-CoV entry is similar to that of MERS-CoV, except that SARS-CoV spike can also be processed by extracellular proteases (e.g., elastase) after virus release (20, 28–30). It has been suggested that the tissue tropisms of MERS-CoV and SARS-CoV are correlated with the tissue distributions of proprotein convertases, extracellular proteases, and cell surface proteases in the host (22, 23, 26, 29–31). For example, the availability of trypsin-like proteases in the respiratory tract has been suggested to be a determinant of the respiratory tropism of SARS-CoV (29, 30). However, although coronavirus entry also depends on lysosomal proteases, it is not clear whether the species and tissue tropisms of coronaviruses are correlated with different lysosomal protease activities from different hosts or tissue cells.

Both MERS-CoV and SARS-CoV are thought to have originated from bats. SARS-like coronaviruses isolated from bats and SARS-CoV isolated from humans are genetically highly similar to each other; some of the bat SARS-like coronaviruses recognize the same receptor, angiotensin-converting enzyme 2 (ACE2), as human SARS-CoV (32–35). MERS-like coronaviruses isolated from bats and MERS-CoV isolated from humans so far are also genetically similar to each other, albeit not as similar as between bat SARS-like coronaviruses and human SARS-CoV (36–39). Several MERS-like coronaviruses from bats, including HKU4, recognize the same receptor, dipeptidyl peptidase 4 (DPP4), as MERS-CoV (24, 40–43). Moreover, human lysosomal proteases activate only MERS-CoV spike, but not HKU4 spike, for viral entry into human cells, while bat lysosomal proteases activate both MERS-CoV and HKU4 spikes for viral entry into bat cells (44). Furthermore, the expression level of lysosomal proteases in human lung cells is lower than in human liver cells, leading to inefficient activation of MERS-CoV spike by lysosomal proteases in human lung cells (45). These results point to the possibility that lysosomal protease activities differ among cells from different hosts or even among cells from the same host species, restricting coronavirus entry and their tropism. However, these studies did not control the contribution from host receptors, despite the fact that receptor homologues from different host species may differ in their

functions as coronavirus receptors or that the same receptor protein may be expressed at different levels in different tissues within one host species. Moreover, these studies were carried out at the cellular level and did not provide direct biochemical evidence to demonstrate that lysosomal proteases from human and bat cells process coronavirus spikes differently. Therefore, factor-controlled viral entry data and direct biochemical data are both needed to establish the correlation between lysosomal protease activities and coronavirus tropism.

In this study, we controlled the contributions of receptor binding and other proteases, and our data support the hypothesis that different lysosomal protease activities of bat and human cells impact the efficiency of coronavirus entry into these cells. We also purified lysosomal extracts from bat and human cells and showed that bat and human lysosomal proteases process coronavirus spikes differently and activate coronavirus entry differently. Overall, this study provides the first direct evidence supporting the notion that different lysosomal protease activities are an important determinant of the species and tissue tropisms of coronaviruses.

RESULTS

Screening for cells that are suitable for studying lysosomal protease-activated coronavirus entry. To study lysosomal protease-activated coronavirus entry, we had to carefully control the contributions from the host receptor and other intracellular and extracellular proteases so that coronavirus spike-mediated viral entry would be solely or mainly dependent on the contributions from lysosomal proteases. In other words, we partitioned the membrane fusion process from the receptor binding step and also separated the effects of lysosomal proteases from those of the other proteases that may participate in coronavirus entry. To this end, we screened for cell lines that met the following three criteria: (i) the cells from different species or tissues had to endogenously express no or low levels of receptor protein for the coronavirus of interest so that they could be controlled to exogenously express the receptor protein from a single host species, (ii) the cells had to express no or low levels of cell surface proteases so that lysosomal proteases from the cells were the only or the main cellular proteases that activated the membrane fusion process for the coronavirus of interest (proprotein convertases were not a factor here because the same batch of viruses, which had gone through the same molecular maturation process, would be used to infect different cells), and (iii) the cells could be transfected easily so that cells from different origins could be controlled to express similar levels of the receptor protein from a single host species. In sum, we were looking for cells that were both "naked" (i.e., not expressing or expressing low levels of coronavirus receptor or cell surface proteases) and easily transfectable.

To identify and exclude cells that endogenously expressed coronavirus receptors, we performed coronavirus spike-mediated pseudovirus entry in a number of human, monkey, and bat cell lines. To this end, retroviruses pseudotyped with MERS-CoV or SARS-CoV spike (i.e., MERS-CoV pseudoviruses or SARS-CoV pseudoviruses, respectively) were used to test the endogenous levels of receptor expression from different cell lines, including human kidney (HEK293T) cells, human cervix (HeLa) cells, human liver (Huh7) cells, human lung (A549 and MRC5) cells, monkey kidney (Vero) cells, bat kidney (RSKT and BKD9) cells, and bat lung (PESU-B5L and Tb1-Lu) cells. The results showed that among these cells, Huh7 cells, Vero cells, MRC5 cells, PESU-B5L cells, and RSKT cells all supported significant levels of MERS-CoV pseudovirus entry, suggesting that the cells endogenously express significant levels of DPP4 (either human, monkey, or bat DPP4, depending on the cell origin) (Fig. 1A). In contrast, only Vero cells and RSKT cells supported significant levels of SARS-CoV pseudovirus entry, suggesting that the cells endogenously express significant levels of ACE2 (monkey and bat ACE2, respectively) (Fig. 1B). These results are largely consistent with previous studies, with two exceptions: previous studies showed that PESU-B5L cells do not support the infection of MERS-CoV and that Huh7 cells support the infection of SARS-CoV (35, 40, 44, 46–48). Overall, the cells that endogenously expressed significant levels of DPP4 or ACE2 were not suitable

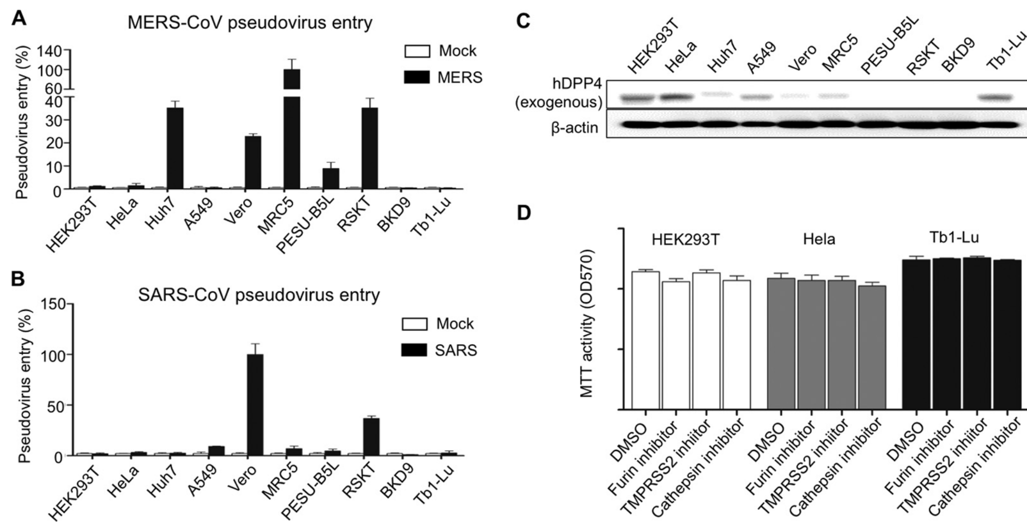

FIG 1 Screening for cell lines that are suitable for studying lysosomal protease-activated coronavirus entry. To screen for cell lines that endogenously express no or low levels of receptor protein for the coronavirus of interest, MERS-CoV pseudoviruses (A) or SARS-CoV pseudoviruses (B) were used to enter a number of cells from different tissues of different host species (human, monkey, and bat). Entry efficiency was characterized using luciferase activity accompanying entry and calibrated against the highest entry efficiency (MERS-CoV entry into MRC5 cells was taken as 100% in panel A, whereas SARS-CoV entry into Vero cells was taken as 100% in panel B). Mock, no pseudoviruses were added. The error bars indicate standard errors of the mean (SEM) ($n = 5$). (C) To screen for cell lines that could be easily transfected and hence controlled to exogenously express receptor protein for the coronavirus of interest, different cells were transfected with a plasmid encoding human DPP4 (hDPP4); subsequently, the expression level of human DPP4 in each of the cell lines was detected through Western blotting using an antibody recognizing its C-terminal C9 tag. The expression level of β-actin in each of the cell lines was used as a positive control. (D) MTT cell viability assay, showing that the viabilities of three types of cells were not affected by the presence of different protease inhibitors. The error bars indicate SEM ($n = 5$). OD570, optical density at 570 nm. There was no statistical significance under different conditions within each cell group ($P > 0.05$, based on a two-tailed t test).

for studying the roles of lysosomal proteases in coronavirus entry and hence were excluded from subsequent studies.

To investigate which of the cells could be controlled to exogenously express significant levels of coronavirus receptors, we transfected the cells with a plasmid encoding human DPP4 and then performed Western blotting using an antibody recognizing the C-terminal C9 tag of exogenously expressed human DPP4 in the cells (Fig. 1C). The results showed that (i) HEK293T cells, HeLa cells, and Tb1-Lu cells exogenously express significant levels of human DPP4; (ii) Huh7 cells, A549 cells, Vero cells, and MRC5 cells exogenously express low levels of human DPP4; and (iii) PESU-B5L cells, RSKT cells, and BKD9 cells do not exogenously express human DPP4. Therefore, HEK293T, HeLa, and Tb1-Lu cells were selected for downstream studies designed to evaluate the roles of lysosomal proteases in coronavirus entry because they met two of the three above-mentioned criteria: they are naked without endogenously expressing coronavirus receptors, and they are easily transfectable and hence can be controlled to exogenously express coronavirus receptors. In addition, an MTT [3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide] cell viability assay showed that the viability of these three types of cells was not affected by the presence of different protease inhibitors, allowing the use of the protease inhibitors in characterizing the roles of different proteases in coronavirus entry (Fig. 1D). Furthermore, as shown below, they are also naked, with no or low endogenous expression of cell surface proteases. Characterization and selection of these cells laid the foundation for defining the roles of lysosomal proteases in coronavirus entry.

Lysosomal proteases from human and bat cells activate coronavirus spike-mediated membrane fusion differently. To examine the roles of lysosomal proteases in MERS-CoV spike-mediated membrane fusion, we performed MERS-CoV pseudovirus entry in the three model cell lines where exogenous expression of human DPP4 can be

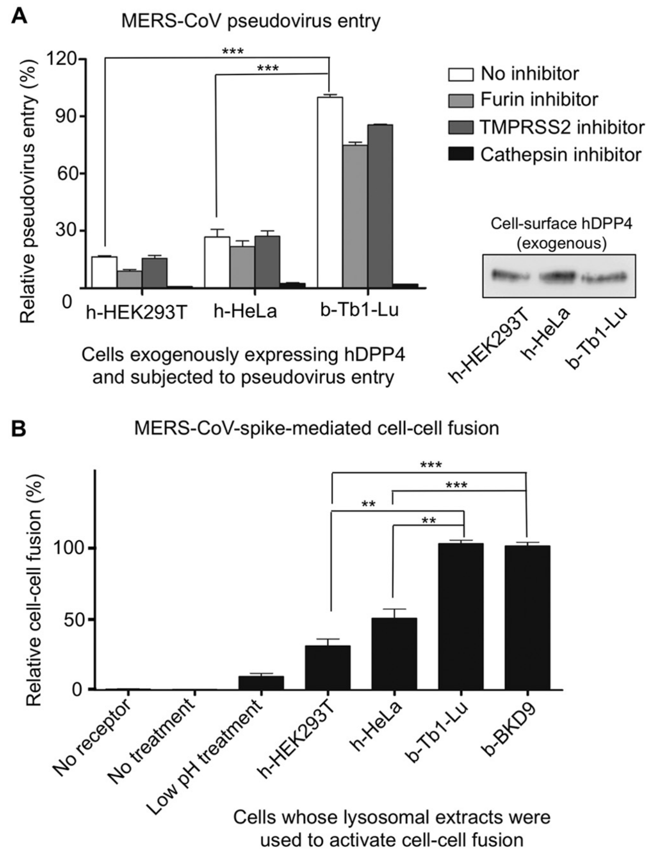

FIG 2 Roles of lysosomal proteases in MERS-CoV spike-mediated membrane fusion. (A) Roles of lysosomal proteases in MERS-CoV pseudovirus entry. Three types of cells, h-HEK293T, h-HeLa, and b-Tb1-Lu, were controlled to exogenously express human DPP4 as shown in Fig. 1C and then subjected to MERS-CoV pseudovirus entry as shown in Fig. 1A. The furin inhibitor chloromethyl ketone, the cell surface protease (i.e., TMPRSS2) inhibitor camostat, and the lysosomal protease (i.e., cathepsin) inhibitor E64d were used in parallel experiments to investigate the relative contributions of the different proteases to MERS-CoV pseudovirus entry. The expression levels of cell surface-associated C9-tagged human DPP4 were measured through Western blot analysis using an anti-C9 tag monoclonal antibody and were further calibrated across the three types of cells. (B) MERS-CoV spike-mediated cell-cell fusion in the presence of lysosomal extracts. h-HEK293T cells exogenously expressing MERS-CoV spike and h-HEK293T cells exogenously expressing human DPP4 were mixed at pH 5.6 in the presence of lysosomal extracts from h-HEK293T cells, h-HeLa cells, b-Tb1-Lu cells, or b-BKD9 cells. Cell-cell fusion efficiency was characterized using luciferase activity accompanying fusion and calibrated against the highest fusion efficiency (i.e., in the presence of lysosomal extracts from b-Tb1-Lu cells). Three negative controls were used: (i) cells not expressing human DPP4 were used (No receptor); (ii) no lysosomal proteases were added to the medium, and the medium was at neutral pH (No treatment); (iii) no lysosomal proteases were added, but the medium was at pH 5.6 (Low pH treatment). Statistical analyses were performed using a two-tailed *t* test. The error bars indicate SEM ($n = 4$). ***, $P < 0.001$; **, $P < 0.01$.

measured and calibrated: human HEK293T (h-HEK293T) cells, human HeLa (h-HeLa) cells, and bat Tb1-Lu (b-Tb1-Lu) cells. The results showed that all three types of cells supported MERS-CoV pseudovirus entry at significant levels when they exogenously expressed human DPP4 (Fig. 2A). When the expression levels of cell surface-associated human DPP4 were measured and calibrated across the three types of cells (Fig. 2A), b-Tb1-Lu cells supported MERS-CoV pseudovirus entry more efficiently than both h-HEK293T cells and h-Hela cells. Because no extracellular protease was added to the pseudovirus entry assay mixture, these data suggest that cellular proteases were responsible for the high efficiency of b-Tb1-Lu cells in activating MERS-CoV pseudovirus entry. MERS-CoV pseudovirus entry in the presence of different cellular protease inhibitors showed that lysosomal protease (i.e., cathepsin) inhibitor almost completely inhibited MERS-CoV pseudovirus entry into the cells, whereas proprotein convertase (i.e., furin) inhibitor and cell surface protease (i.e., TMPRSS2) inhibitor had much less

impact on the efficiency of the cells in supporting MERS-CoV pseudovirus entry (Fig. 2A). Thus, lysosomal proteases were mainly responsible for MERS-CoV pseudovirus entry into the cells. Therefore, after the contributions from host receptor and other proteases were controlled, lysosomal proteases from b-Tb1-Lu cells supported MERS-CoV spike-mediated membrane fusion more efficiently than their counterparts from h-HEK293T cells and h-HeLa cells.

To further demonstrate that different lysosomal protease activities directly impact MERS-CoV spike-mediated membrane fusion, we performed MERS-CoV spike-mediated cell-cell fusion in the presence of purified lysosomal extracts from different cells. To this end, we purified lysosomal extracts from h-HEK293T cells, h-HeLa cells, b-Tb1-Lu cells, and bat BKD9 (b-BKD9) cells. Subsequently, we mixed one batch of h-HEK293T cells exogenously expressing MERS-CoV spike and another batch of h-HEK293T cells exogenously expressing human DPP4. Then, we added the same amount (i.e., mass) of each of the lysosomal extracts to the mixture of the above-mentioned h-HEK293T cells while reducing the pH of the cell culture medium to the level at which lysosomal proteases were active (i.e., pH 5.6). As we showed earlier, h-HEK293T cells do not endogenously express significant amounts of cell surface proteases (Fig. 2A). Hence, the efficiency of cell-cell fusion likely reflects the activation of MERS-CoV spike-mediated membrane fusion by purified lysosomal extracts from different types of cells. The results showed that lysosomal extracts from b-Tb1-Lu cells and b-BKD9 cells both activate MERS-CoV spike-mediated cell-cell fusion more efficiently than their counterparts from h-HEK293T cells and h-HeLa cells (Fig. 2B). In comparison, in the absence of any lysosomal extracts, there was no significant cell-cell fusion at neutral pH and only low levels of cell-cell fusion at low pH, suggesting that pH alone has little or no effect on MERS-CoV spike-mediated cell-cell fusion (Fig. 2B). Therefore, consistent with the MERS-CoV pseudovirus entry assay results, the cell-cell fusion assay also revealed that lysosomal extracts from bat cells support MERS-CoV spike-mediated membrane fusion more efficiently than their counterparts from human cells.

To examine the purity of the lysosomal extracts, we investigated potential contamination of the lysosomal extracts by proteins from plasma or the endoplasmic reticulum (ER). Because alkaline phosphatase (ALP) and cytochrome P450 reductase (CPR) are markers of plasma enzymes and ER enzymes, respectively, their activities in lysosomal extracts are commonly used as indicators of the purity of lysosomal extracts (49). Hence, we measured the ALP and CPR activities of the lysosomal extracts from different cell lines (Fig. 3A and B). The results showed that, compared to the whole-cell lysates, the ALP and CPR activities in the lysosomal extracts were low (for some unknown reason, the ALP activities of BKD9 cells were very low). Thus, based on the indicator proteins, the contamination of the lysosomal extracts by plasma and ER proteins was low.

To extend the above-described findings from MERS-CoV to other coronaviruses, we investigated whether lysosomal proteases from human and bat cells activate SARS-CoV spike-mediated membrane fusion differently, also after controlling the contributions from host receptor and other proteases. To this end, we performed SARS-CoV pseudovirus entry into h-HEK293T cells, h-HeLa cells, and b-Tb1-Lu cells, all of which were controlled to exogenously express human ACE2. The results showed that, like MERS-CoV pseudoviruses, SARS-CoV pseudoviruses entered b-Tb1-Lu cells more efficiently than they did h-HEK293T and h-HeLa cells (Fig. 4A). Lysosomal protease inhibitor almost completely inhibited SARS-CoV pseudovirus entry into these cells, while proprotein convertase inhibitor and cell surface protease inhibitor had much less impact on SARS-CoV pseudovirus entry into the cells. Hence, lysosomal proteases were the main contributor to SARS-CoV pseudovirus entry into the cells. Moreover, we carried out SARS-CoV spike-mediated cell-cell fusion in the presence of lysosomal extracts from h-HEK293T cells, h-HeLa cells, b-Tb1-Lu cells, or b-BKD9 cells. The results showed that lysosomal extracts from bat cells activated SARS-CoV spike-mediated cell-cell fusion more efficiently than their counterparts from human cells (Fig. 4B). Taken together, our data support the hypothesis that lysosomal proteases from bat cells support SARS-CoV

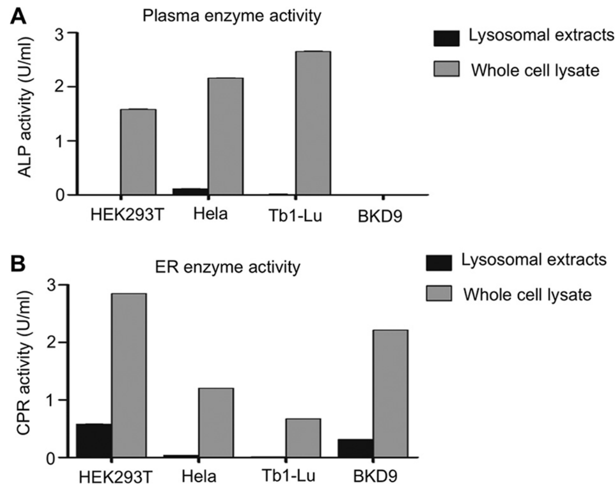

FIG 3 Characterization of the purity of lysosomal extracts from different cell lines. Because ALP and CPR are enzymatic markers of plasma and the ER, respectively, the purified lysosomal extracts and whole-cell lysates from different cell lines (for each cell line, lysosomal extracts and whole-cell lysates were at equal concentrations) were assayed for their ALP activities (A) and CPR activities (B) to evaluate potential contaminants from other cell organelles. The error bars indicate SEM ($n = 3$; some of the error bars may be too small to be seen).

spike-mediated membrane fusion, in the forms of both pseudovirus entry and cell-cell fusion, more efficiently than their counterparts from human cells.

Lysosomal proteases from human and bat cells process MERS-CoV spike differently. To provide direct biochemical evidence supporting the notion that lysosomal

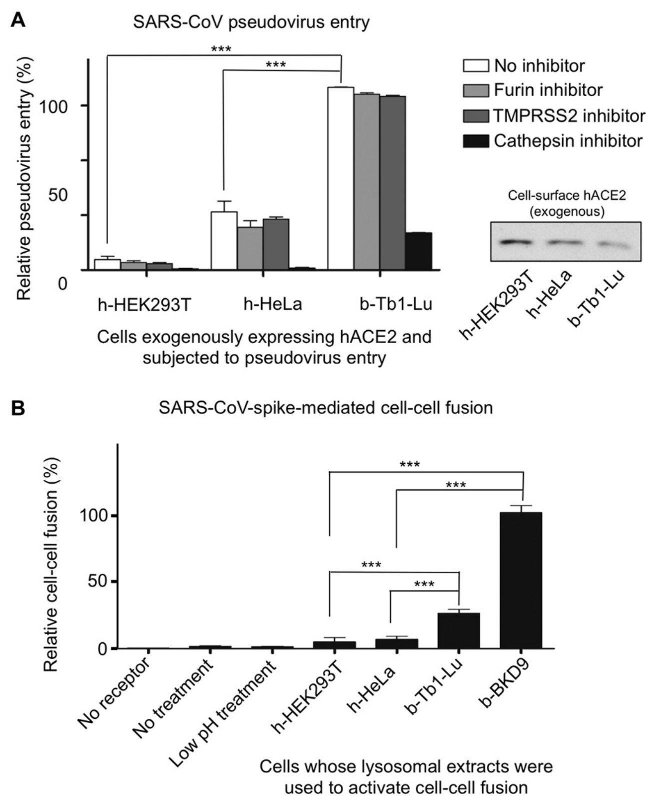

FIG 4 Roles of lysosomal proteases in SARS-CoV spike-mediated membrane fusion. The experiments were performed in the same way as for Fig. 2, except that SARS-CoV spike and its receptor, human ACE2 (hACE2), replaced MERS-CoV spike and human DPP4, respectively.

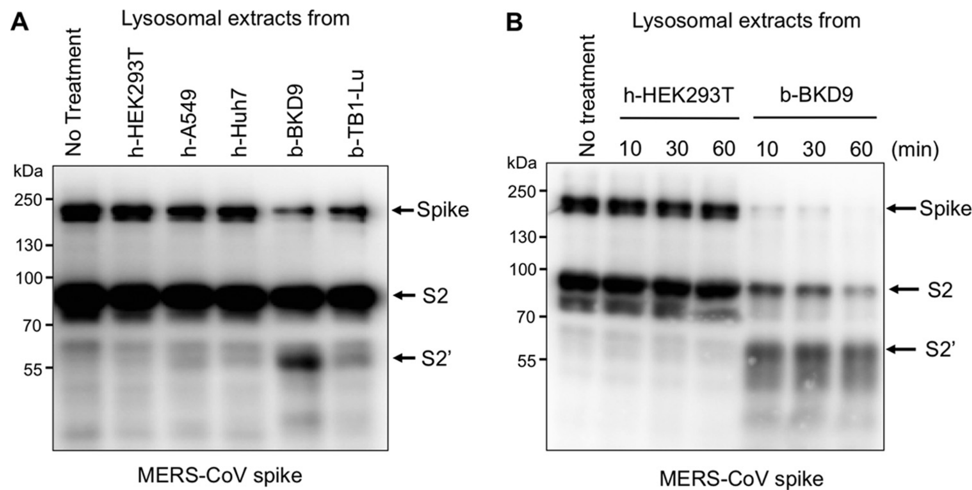

FIG 5 Cleavage of cell surface-expressed MERS-CoV spike using purified lysosomal extracts. (A) Cleavage of cell surface-expressed MERS-CoV spike using lysosomal extracts from a number of cell lines. MERS-CoV spike was exogenously expressed on the surfaces of h-HEK293T cells and then treated with 50 μg/ml lysosomal extracts (from different types of cells) at pH 5.6 for 30 min. The cleavage state of MERS-CoV spike was detected through Western blotting using an antibody recognizing its C-terminal C9 tag. (B) Cleavage of cell surface-expressed MERS-CoV spike using 100 μg/ml lysosomal extracts (from two types of cells) at pH 5.6 in a time-dependent manner (10, 30, and 60 min). The experiments were repeated five times, and representative results are shown.

proteases from human and bat cells process MERS-CoV spike differently, we digested cell surface-expressed MERS-CoV spike using lysosomal extracts from human and bat cells. To this end, we exogenously expressed MERS-CoV spike on the surfaces of h-HEK293T cells. Meanwhile, we purified lysosomal extracts from different types of human and bat cells. Then, we incubated the cell surface-expressed MERS-CoV spike with the same amount of lysosomal extracts from each type of cell, and we performed Western blotting to detect the cleavage state of MERS-CoV spike. The results showed that more than half of the MERS-CoV spike molecules had been cleaved to S2 by proprotein convertases during the molecular maturation process and that lysosomal extracts from bat cells were more efficient than their counterparts from human cells in further cleaving MERS-CoV spike to produce S2′ fragments (Fig. 5A). Between the two types of bat cells, lysosomal extracts from b-BKD9 cells processed MERS-CoV spike more efficiently than their counterparts from b-Tb1-Lu cells. We further compared the lysosomal extracts from b-BKD9 cells and their counterparts from h-HEK293T cells; lysosomal extracts from b-BKD9 cells processed MERS-CoV spike much more efficiently than their counterparts from h-HEK293T cells in a time-dependent manner (Fig. 5B). Overall, lysosomal extracts from bat cells demonstrated higher efficiency in processing MERS-CoV spike than their counterparts from human cells.

To further compare the coronavirus spike-processing activities of human and bat lysosomal proteases, we examined whether lysosomal extracts from human and bat cells process the spike from a MERS-like bat coronavirus, HKU4, differently. Previously, we showed that HKU4 spike contains a glycosylated lysosomal protease site at the S1-S2 boundary and that it mediates virus entry into bat cells, but not human cells (44). Here, we investigated direct biochemical evidence for the different HKU4 spike-processing activities of human and bat lysosomal proteases. To this end, we purified lysosomal extracts from h-HEK293T cells and b-Tb1-Lu cells and incubated them individually with HKU4 spike expressed on the surfaces of h-HEK293T cells. The results showed that lysosomal extracts from b-Tb1-Lu cells, but not their counterparts from h-HEK293T cells, cleaved HKU4 spike containing a glycosylated lysosomal protease motif to produce S2 (Fig. 6A). Next, we introduced an N762A mutation into HKU4 spike; the mutation had been shown to remove the glycosylation from the lysosomal protease motif in HKU4 spike (44). The result showed that lysosomal extracts from both h-HEK293T cells and b-Tb1-Lu cells cleaved the mutant HKU4 spike to produce S2

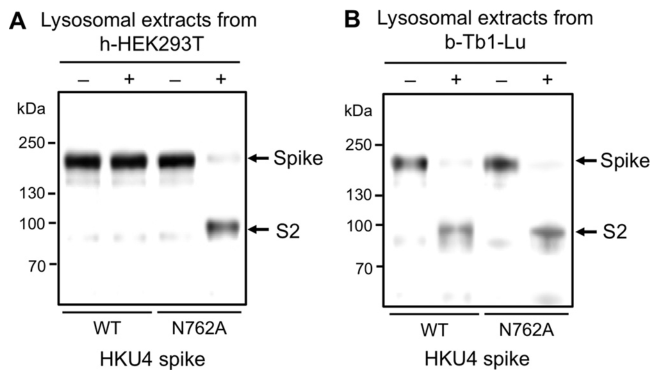

FIG 6 Cleavage of cell surface-expressed HKU4 spike using purified lysosomal extracts. The experiments were performed in the same way as for Fig. 5A, except that HKU4 spike (either wild type [WT] or containing an N762 mutation that removed a glycosylation site from the lysosomal protease motif) replaced MERS-CoV spike. The experiments were repeated five times, and representative results are shown.

(Fig. 6B). These results provided direct biochemical evidence demonstrating that lysosomal extracts from b-Tb1-Lu cells, but not their counterparts from h-HEK293T cells, can process the glycosylated lysosomal protease motif in HKU4 spike, whereas lysosomal extracts from both h-HEK293T cells and b-Tb1-Lu cells can process the unglycosylated lysosomal protease motif in HKU4 spike.

DISCUSSION

The tropism of coronaviruses includes species and tissue tropisms (1). Lysosomal proteases play a critical role in coronavirus entry (8, 10, 11), but their roles in coronavirus tropism have not been established. In contrast, extracellular proteases and other cellular proteases have been shown to be important determinants of coronavirus tropism (22, 23, 26, 29–31). We and others previously showed that a MERS-like coronavirus from bats, HKU4, uses the same host receptor, DPP4, as MERS-CoV (24, 41), and we also showed that cellular proteases from bat and human cells support HKU4 entry differently (24, 44). However, two factors can complicate the roles of lysosomal proteases in coronavirus tropism: human and bat DPP4 molecules have different activities as coronavirus receptors, and other proteases may also play significant roles in the cell entry process of coronaviruses. In the current study, we quantified and controlled the contributions from host receptor and other proteases to coronavirus entry so that the roles of lysosomal proteases in coronavirus entry into cells from different origins could be clearly defined. To this end, we screened a number of cell lines originating from different tissues and host species and found three types of cells that were suitable for studying the roles of lysosomal proteases in coronavirus tropism: human HEK293T cells, human HeLa cells, and bat Tb1-Lu cells. These three types of cells share the following common features: they are naked for endogenously expressing very low levels of coronavirus receptor or cell surface proteases, and they can be easily transfected to exogenously express the coronavirus receptor from a single host species. As a result, lysosomal proteases likely function as the only or the main contributor to coronavirus spike-mediated entry. The above-described approach and findings may be extended to study the roles of lysosomal proteases in the entry of other viruses.

The current study investigated the roles of lysosomal proteases from the above-mentioned human and bat cells in coronavirus entry using a combination of pseudovirus entry, cell-cell fusion, and biochemical assays. To this end, we exogenously expressed human DPP4 in different types of cells and performed MERS-CoV spike-mediated pseudovirus entry and cell-cell fusion. In the presence of DPP4 from the same species and in the absence of extracellular proteases and other cellular proteases, lysosomal proteases and lysosomal extracts from bat cells supported MERS-CoV spike-mediated membrane fusion more efficiently than their counterparts from human cells.

These observations were then extended to SARS-CoV spike-mediated pseudovirus entry and cell-cell fusion. Moreover, we prepared lysosomal extracts from human and bat cells and showed that lysosomal extracts from bat cells cleaved MERS-CoV spike more efficiently than their counterparts from human cells. We also showed that lysosomal extracts from bat cells cleaved HKU4 spike, which contains a glycosylated lysosomal protease motif, more efficiently than their counterparts from human cells. These results demonstrated that the spikes from MERS-CoV, SARS-CoV, and HKU4 all mediated viral entry into bat cells at higher efficiency than into human cells, due to or mainly due to the higher coronavirus spike-processing activities of bat lysosomal proteases.

The correlation between lysosomal protease activities and coronavirus tropism is a novel finding in virology. Previous studies had already shown that the expression levels of lysosomal proteases vary among different tissues within the same host species due to the different physiological functions of the tissue cells (7, 45). Our study demonstrates that lysosomal protease activities may also vary among different mammalian species, indicating that adaptation of coronaviruses to new species may occur through adaptation to different lysosomal protease activities. The physiological reason for different lysosomal protease activities among mammalian species is not clear, but it could be due to the different lifestyles of these species. For instance, bats are the only flying mammals, and hence, the enhanced lysosomal protease activities of bat cells may provide fast turnover of metabolic products and also produce high levels of nutrients, although this is speculative. In this sense, supporting coronavirus entry efficiently could be a by-product of the enhanced lysosomal protease activities of bat cells. It is worth noting that, due to the difficulty in culturing bat tissue cells, this study was performed using bat cell lines. Although cell lines usually maintain many features of original tissue cells, these findings need to be confirmed using bat tissue cells. Our study suggests that no matter whether cells are from different host species or from different tissues of the same host species, cells with higher lysosomal protease activities in general support coronavirus entry more efficiently than cells with lower lysosomal protease activities. It remains to be further investigated whether the higher lysosomal protease activities in some cells are due to enhanced enzymatic activity, elevated expression levels, or other changes to their lysosomal proteases. Nevertheless, our study has established that different lysosomal proteases from different types of cells have direct impacts on coronavirus entry, which has implications for the tissue and species tropism of coronaviruses.

MATERIALS AND METHODS

Cell lines and plasmids. HEK293T cells (human embryonic kidney cells), HeLa cells (human cervical epithelial cells), A549 cells (human alveolar epithelial cells), Vero cells (monkey kidney cells), MRC5 cells (human lung cells), and Tb1-Lu cells (*Triatoma brasiliensis* bat lung cells) were obtained from the ATCC (American Type Culture Collection). RSKT cells (*Rhinolophus sinicus* bat kidney cells), PESU-B5L cells (*Perimyotis subflavus* bat lung cells), and BKD9 cells (*Myotis davidii* bat kidney cells) were purchased from Sigma-Aldrich. Huh-7 cells (human hepatoma cells) were kindly provided by Charles M. Rice (Rockefeller University). All the cells were cultured in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 2 mM L-GLUTAMINE, 100 units/ml penicillin, and 100 μg/ml streptomycin (Life Technologies). The full-length genes of MERS-CoV spike (GenBank accession number AFS88936.1), SARS-CoV spike (GenBank accession number AFR58742), human DDP4 (GenBank accession number NM_001935.3) and human ACE2 (GenBank accession number NM_021804) were synthesized (GenScript Biotech) and subcloned into the pcDNA3.1(+) vector (Life Technologies) with a C-terminal C9 tag (i.e., a tag corresponding to the 9 amino acids at the C terminus of human complement component C9 protein). Plasmids (pFR-Luc and pBD-NF-κB) for cell-cell fusion were kindly provided by Zhaohui Qian (Chinese Academy of Medical Sciences and Peking Union Medical College).

Coronavirus spike-mediated pseudovirus entry into human and bat cells. Retroviruses pseudotyped with MERS-CoV or SARS-CoV spike (i.e., MERS-CoV pseudoviruses or SARS-CoV pseudoviruses, respectively) were generated as described previously (24). Briefly, HEK293T cells were cotransfected with a plasmid carrying an Env-defective, luciferase-expressing HIV-1 genome (pNL4–3.luc.R-E-) and a pcDNA3.1(+) plasmid encoding MERS-CoV or SARS-CoV spike. Pseudoviruses were harvested 72 h after transfection and used to enter human and bat cells. For screening of cell lines expressing no or low levels of coronavirus receptor, different types of cells were seeded in 96-well plates and infected immediately with pseudoviruses. To study the roles of lysosomal proteases in coronavirus entry, cells were transfected with the pcDNA3.1(+) plasmid encoding human DPP4 or human ACE2; 24 h after the transfection, the

cells expressing the receptor were seeded in 96-well plates and then infected with pseudoviruses. After incubation at 37°C for 6 h, the medium was replaced with fresh DMEM. After another 60 h, the cells were washed with phosphate-buffered saline (PBS) and lysed. Aliquots of the cell lysates were transferred to an Optiplate-96 plate (PerkinElmer Life Sciences), and then luciferase substrate (Promega) was added. Relative luciferase units were measured using an EnSpire plate reader (PerkinElmer Life Sciences) and normalized for exogenous expression levels of the corresponding receptor in the cell membranes (see below).

Inhibition of pseudovirus entry using various protease inhibitors was carried out as described previously (50). Briefly, target cells were preincubated with medium containing a final concentration of 50 μM camostat mesylate (Sigma-Aldrich), 50 μM E-64d (Sigma-Aldrich), 50 μM chloromethyl ketone (Enzo), or DMSO (dimethyl sulfoxide) (negative control) at 37°C for 1 h. The cells were subsequently infected with pseudoviruses. The cells were incubated at 37°C for 6 to 8 h, and then the medium was replaced with fresh DMEM. After another 48 h, the cells were lysed and measured for luciferase activity.

Exogenous expression of coronavirus receptor in cells and on cell surfaces. To examine the exogenous expression level of coronavirus receptor in whole-cell lysates, cells were transfected with pcDNA3.1(+) plasmid encoding human DPP4 or human ACE2 containing a C-terminal C9 tag; 48 h after transfection, the cells were lysed using ultrasonication, and aliquots of cell lysates were subjected to Western blotting. The C9-tagged coronavirus receptors were detected using an anti-C9 tag monoclonal antibody (Santa Cruz Biotechnology). The current assay measures the total expression level of coronavirus receptor in a certain number of cells without specifying how many of the cells were transfected or how much protein was expressed in each transfected cell.

To examine the exogenous expression level of coronavirus receptor in cell membranes, the cells expressing the receptor were harvested as described above, and all membrane-associated proteins were extracted using a membrane protein extraction kit (Thermo Fisher Scientific). Briefly, cells were centrifuged at 300 $\times$ *g* for 5 min and washed with cell wash solution twice. The cell pellets were resuspended in 0.75 ml permeabilization buffer and incubated at 4°C for 10 min. The supernatant containing cytosolic proteins was removed after centrifugation at 16,000 $\times$ *g* for 15 min. The pellets containing membrane-associated proteins were resuspended in 0.5 ml solubilization buffer and incubated at 4°C for 30 min. After centrifugation at 16,000 $\times$ *g* for 15 min, the membrane-associated proteins from the supernatant were transferred to a new tube. The expression level of membrane-associated C9-tagged coronavirus receptor among the membrane-associated proteins was then measured using Western blot analysis as described above and used for normalizing the results from pseudovirus entry assays. Although the current assay could not differentiate between plasma membrane-associated proteins and internal-membrane-associated proteins, ACE2 and DPP4 are known to be strongly associated with plasma membranes due to their respective plasma membrane-targeting signal peptides (51, 52).

MTT assay. Cells were seeded in 96-well plates and treated with DMSO or protease inhibitors dissolved in DMSO at 37°C. After incubation for 6 h, the medium was replaced with fresh DMEM. After incubation for 70 h at 37°C, 10 μl MTT solution (Biotium) was added to each well and mixed with the medium. After incubation at 37°C for 2 h, 200 μl DMSO or protein inhibitor dissolved in DMSO was added to each well and mixed with the medium. The MTT signal was measured as absorbance at 570 nm using a Synergy 2 multimode microplate reader (BioTek Instruments).

Preparation of lysosomal extracts. Lysosomal extracts from human or bat cells were prepared according to the lysosome isolation kit procedure (Sigma-Aldrich). Briefly, cells were harvested and washed with PBS buffer and then resuspended in 2.7 packed cell volumes (PCV) of extraction buffer. The cells were broken in a 7-ml Dounce homogenizer using a loose pestle (i.e., pestle B) until 80% to 85% of the cells were broken (protease inhibitors from the kit were omitted in our procedure). The samples were centrifuged at 1,000 $\times$ *g* for 10 min, and the supernatants were transferred to a new centrifuge tube and centrifuged at 20,000 $\times$ *g* for another 20 min. The supernatants were removed, and the pellets were resuspended in extraction buffer as the crude lysosomal fraction (CLF). The CLF was diluted in buffer containing 19% Optiprep density gradient medium solution and further purified using density gradient centrifugation at 150,000 $\times$ *g* for 4 h to yield lysosomal extracts. The concentrations of the lysosomal extracts were measured using a NanoDrop 8000 (Thermo Fisher Scientific) and calculated according to their absorbance at 280 nm. The purities of the lysosomal extracts were examined using the assays described below.

CPR is an ER marker. For evaluation of the potential contamination of the purified lysosomal extracts by ER proteins, the cytochrome P450 reductase activities of the purified lysosomal extracts were measured using a cytochrome P450 reductase assay kit (Biovision). Briefly, a glucose-6-phosphate (G6P) standard curve was first calculated by mixing a series of volumes of 1 mM G6P standard solution with 5 μl NADPH substrate and 5 μl G6P standard developer to make the final volume 100 μl/well. The well contents were then mixed and incubated at room temperature for 30 min (protected from light). Absorbance at 460 nm was measured. Then, 5 μl lysosomal extracts from different cell lines was mixed with 55 μl CPR assay buffer. After adding 30 μl of the assay reaction mixture to each well and incubating the solutions at room temperature for 5 min, 10 μl of the 20 mM G6P solution was added to each well. Absorbance at 460 nm was measured immediately in kinetic mode at 25°C for 25 min using a Synergy 2 multimode microplate reader (BioTek Instruments). Calculation of the cytochrome P450 reductase activity was performed according to the manufacturer's manual.

ALP is a plasma enzyme marker. For evaluation of the potential contamination of the purified lysosomal extracts by plasma proteins, the alkaline phosphatase activity of the purified lysosomal extracts was measured using an alkaline phosphatase assay kit (Abnova). Briefly, a standard curve was first calculated by mixing a series of concentrations of 4-methylumbelliferyl phosphate disodium salt

(MUP) standard with 10 μl ALP enzyme solution. The reaction mixtures were incubated at 25°C for 30 min (protected from light). The ALP enzyme can convert MUP substrate to an equal molar amount of fluorescent 4-methylumbelliferone (4-MU). Hence, 20 μl 0.5 mM MUP substrate solution was added to each well containing 5 μl lysosomal extracts from different cell lines. After mixing and incubating at 25°C for 30 min (protected from light), all the reactions were stopped by adding 20 μl stop solution to each reaction mixture. Then, the fluorescence intensities at excitation and emission wavelengths of 360 and 440 nm, respectively, were measured using a Synergy 2 multimode microplate reader (BioTek Instruments). Calculation of the alkaline phosphatase activity was performed according to the manufacturer's manual.

Coronavirus spike-mediated cell-cell fusion. Cell-cell fusion was performed as described previously (53). Briefly, to produce cells expressing one of the coronavirus spikes, HEK293T cells were cotransfected with the plasmid pFR-Luc, which contains a synthetic promoter with five tandem repeats of the *Saccharomyces cerevisiae* GAL4 binding sites that controls expression of the luciferase gene, and the pcDNA3.1(+) plasmid encoding one of the coronavirus spikes. To produce cells expressing one of the corresponding coronavirus receptor proteins, HEK293T cells were cotransfected with pBD-NF-κB, which encodes a fusion protein with the DNA binding domain of GAL4 and the transcription activation domain of NF-κB, and a pcDNA3.1(+) plasmid encoding one of the corresponding coronavirus receptor proteins. After culturing for 24 h, the spike-expressing HEK293T cells were lifted, centrifuged, and then resuspended in low-pH medium containing 10 mM sodium citrate, pH 5.6. Subsequently, the spike-expressing HEK293T cells were treated with purified lysosomal extracts (100 μg/ml) in the low-pH medium. After incubation at 37°C for 30 min, the spike-expressing cells were centrifuged, resuspended in fresh neutral pH medium, and then overlaid onto receptor-expressing HEK293T cells at a ratio of 1:2. When cell-cell fusion occurred, the expression of the luciferase gene was activated through binding of the GAL4–NF-κB fusion protein to GAL4 binding sites at the promoter of the luciferase gene. After incubation for 24 h, the cells were lysed, aliquots of the cell lysates were transferred to an Optiplate-96 plate (PerkinElmer Life Sciences), and then luciferase substrate (Promega) was added. Relative luciferase units were measured using an EnSpire plate reader (PerkinElmer Life Sciences).

Cleavage of coronavirus spikes using purified lysosomal extracts. HEK293T cells were transfected with pcDNA3.1(+) plasmid encoding MERS-CoV spike or HKU4 spike; 48 h after transfection, the cells were harvested and washed with PBS buffer. The cells were then treated with 50 μg/ml purified lysosomal extracts at pH 5.6 for 30 min or 100 μg/ml purified lysosomal extracts at pH 5.6 for different periods of time (i.e., 10, 30, or 60 min). After treatment, the cells were lysed and boiled for Western blotting. The C9-tagged spikes were detected using an anti-C9 tag monoclonal antibody (Santa Cruz Biotechnology).

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