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Lysosomal proteases are a determinant of coronavirus tropism

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Abstract

Cell entry of coronaviruses involves two principal steps: receptor binding and membrane fusion, the latter of which 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 tropisms of coronaviruses has not been critically established. Here we examined the roles of lysosomal proteases in activating coronavirus-surface spike proteins for membrane fusion, using the spike proteins from SARS and MERS coronaviruses 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 spike proteins more efficiently than their counterparts from human cells. Overall, our study suggests that differential lysosomal protease activities from different host species and tissue cells are an important determinant of the species and tissue tropism of coronaviruses.

Significance

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 differential lysosomal protease activities from different cells impact coronavirus entry? Here we controlled the contributions from known factors that function in coronavirus entry, such that lysosomal protease activities became the only or 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 tropism of coronaviruses.

Introduction

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 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 strategy, and identifying zoonotic strains with prepandemic potential.

Coronaviruses are large, enveloped, and single-stranded RNA viruses (12, 13). They pose significant health threat to humans and other animals. Severe acute respiratory syndrome coronavirus (SARS-CoV) was responsible for the SARS epidemic in 2002-2003, causing over 8000 infections and ~10% fatality rate in humans (14, 15). Middle East respiratory syndrome coronavirus (MERS-CoV) was identified in 2012 and has so far caused over 2200 infections and ~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 host cell surface for viral attachment through its S1 subunit, and then fuses

83 viral and host membranes through its S2 subunit. The membrane fusion step by
84 coronavirus spikes requires two prior cleavages by host proteases: the first at the S1/S2
85 boundary (i.e., S1/S2 site) and the second within S2 (i.e., S2' site) (8, 19-21). Depending
86 on the virus, the spike-processing proteases may come from different stages of the
87 coronavirus infection cycle. For MERS-CoV, its spike can be processed by proprotein
88 convertases (e.g., furin) during the molecular maturation process in virus-producing cells,
89 by cell-surface proteases (e.g., transmembrane protease serine 2 or TMPRSS2) after viral
90 attachment, and by lysosomal proteases (e.g., cathepsins) after endocytosis in virus-
91 targeted cells (22-26). It was previously reported that MERS-CoV spike could be
92 processed by furin after viral endocytosis in virus-targeted cells (21), but this finding was
93 not supported by a recent study (27). The protease activation pattern of SARS-CoV entry
94 is similar to that of MERS-CoV, except that SARS-CoV spike can also be processed by
95 extracellular proteases (e.g., elastase) after viral release (20, 28-30). It has been suggested
96 that the tissue tropisms of MERS-CoV and SARS-CoV are correlated with the tissue
97 distributions of proprotein convertases, extracellular proteases, and cell-surface proteases
98 in the host (22, 23, 26, 29-31). For example, the availability of trypsin-like proteases in
99 the respiratory tracts has been suggested to be a determinant of the respiratory tropism of
100 SARS-CoV (29, 30). However, although coronavirus entry also depends on lysosomal
101 proteases, it is not clear whether the species and tissue tropism of coronaviruses are
102 correlated with differential lysosomal protease activities from different hosts or tissue
103 cells.

104 Both MERS-CoV and SARS-CoV are thought to have originated from bats.
105 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 only activate the MERS-CoV spike, but not the 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 differentially. Therefore, factor-controlled viral entry data and direct biochemical data are both needed to critically and directly establish the correlation between lysosomal protease activities and coronavirus tropism.

In this study, we controlled the contributions from receptor binding and other proteases, and our data support the hypothesis that differential lysosomal protease activities from 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 differentially process coronavirus spikes and activate coronavirus entry. Overall, this study provides the first direct evidence supporting the notion that differential lysosomal protease activities are an important determinant of the species and tissue tropism of coronaviruses.

Results

Screening for cells that are suitable for studying lysosomal-proteases-activated coronavirus entry

To study lysosomal-proteases-activated coronavirus entry, we must carefully control for the contributions from the host receptor and other intracellular and extracellular proteases, such that coronavirus-spike-mediated viral entry would be solely or mainly dependent on the contribution from lysosomal proteases. In other words, we partition the membrane fusion process from the receptor binding step and also separate the effects of lysosomal proteases from 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 endogenously must express no or low levels of receptor protein for the coronavirus of interest, such that they can be controlled to exogenously express the receptor protein from a single host species; (ii) The cells must express no or low level of cell-surface proteases, such that lysosomal proteases

152 from these cells are the only or main cellular proteases that activate the membrane fusion
153 process for the coronavirus of interest (proprotein convertases are not a factor here
154 because the same batch of viruses, which had gone through the same molecular
155 maturation process, would be used to infect different cells); (iii) The cells can be
156 transfected easily, such that the cells from different origins can be controlled to express
157 similar levels of the receptor protein from a single host species. In sum, we were looking
158 for cells that are both "naked" (not expressing or expressing low levels of coronavirus
159 receptor or cell-surface proteases) and "easily transfectable".

160 To identify and exclude those cells that endogenously express coronavirus
161 receptors, we performed coronavirus-spike-mediated pseudovirus entry in a number of
162 human, monkey and bat cell lines. To this end, retroviruses pseudotyped with the MERS-
163 CoV or SARS-CoV spike (i.e., MERS-CoV pseudoviruses or SARS-CoV pseudoviruses,
164 respectively) were used to test the endogenous levels of receptor expression from
165 different cell lines including human kidney cells (HEK293T), human cervix cells (HeLa),
166 human liver cells (Huh7), human lung cells (A549 and MRC5), monkey kidney cells
167 (Vero), bat kidney cells (RSKT and BKD9), and bat lung cells (PESU-B5L and Tb1-Lu).
168 The results showed that among these cells, Huh7 cells, Vero cells, MRC5 cells, PESU-
169 B5L cells, and RSKT cells all supported significant levels of MERS-CoV pseudovirus
170 entry, suggesting that these cells endogenously express significant levels of DPP4 (either
171 human, monkey, or bat DPP4, depending on the cell origin) (Fig. 1A). In contrast, only
172 Vero cells and RSKT cells supported significant levels of SARS-CoV pseudovirus entry,
173 suggesting that these cells endogenously express significant levels of ACE2 (monkey and
174 bat ACE2, respectively) (Fig. 1B). These results are largely consistent with previous

175 studies with two exceptions: previous studies showed that PESU-B5L cells do not
176 support the infection of MERS-CoV and that Huh7 cells support the infection of SARS-
177 CoV (35, 40, 44, 46-48). Overall, the cells that endogenously express significant levels of
178 DPP4 or ACE2 were not suitable for studying the roles of lysosomal proteases in
179 coronavirus entry and hence were excluded from downstream studies.

180 To investigate which of the cells can be controlled to exogenously express
181 significant levels of coronavirus receptors, we transfected these cells with a plasmid
182 encoding human DPP4. We then performed Western blotting using an antibody
183 recognizing the C-terminal C9 tag of exogenously expressed human DPP4 in these cells
184 (Fig. 1C). The result showed that: (i) HEK293T cells, HeLa cells, and Tb1-Lu cells
185 exogenously express significant levels of human DPP4; (ii) Huh7 cells, A549 cells, Vero
186 cells, and MRC5 cells exogenously express low levels of human DPP4; (iii) PESU-B5L
187 cells, RSKT cells, and BKD9 cells do not exogenously express human DPP4. Therefore,
188 HEK293T, HeLa cells, and Tb1-Lu cells were selected for downstream studies designed
189 to evaluate the roles of lysosomal proteases in coronavirus entry because they met two of
190 the three aforementioned criteria: they are naked without endogenously expressing
191 coronavirus receptors, and they are easily transfectable and hence can be controlled to
192 exogenously express coronavirus receptors. In addition, an MTT cell viability assay
193 showed that the viabilities of these three types of cells were not affected by the presence
194 of different protease inhibitors, allowing the use of these protease inhibitors in
195 characterizing the roles of different proteases in coronavirus entry (Fig. 1D).
196 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 differentially

To examine the role 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 measured and calibrated: human HEK293T cells (h-HEK293T), human HeLa cells (h-HeLa), and bat Tb1-Lu cells (b-Tb1-Lu). 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). After 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-HeLa cells and b-Tb1-Lu cells. Because no extracellular protease was added to the pseudovirus entry assay, there data suggest that cellular proteases were responsible for the highest 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., cathepsins) inhibitor almost completely inhibited MERS-CoV pseudovirus entry into these cells, whereas proprotein convertase (i.e., furin) inhibitor and cell-surface protease (i.e., TMPRSS2) inhibitor had much less impact on the efficiency of these cells in supporting MERS-CoV pseudovirus entry (Fig. 2A). Thus, lysosomal proteases were mainly responsible for MERS-CoV pseudovirus entry into

these 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 differential 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 the MERS-CoV spike and another batch of h-HEK293T cells exogenously expressing human DPP4. Then we added the same amount (in mass) of each of the lysosomal extracts to the mixture of the above h-HEK293T cells, while reducing the pH of the cell culture medium to where lysosomal proteases were active (i.e., pH 5.6). As we showed earlier, h-HEK293T cells do not endogenously express significant amount 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 result 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 level of cell-cell fusion at low pH, suggesting that pH alone has no or little effect on MERS-CoV-spike-mediated cell-cell fusion (Fig. 2B). Therefore, consistent with MERS-CoV pseudovirus

entry assay, 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 these lysosomal extracts, we investigated potential contaminations of the lysosomal extracts by proteins from plasma or 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 purities of lysosomal extracts (49). Hence we measured the ALP and CPR activities of the lysosomal extracts from different cell lines (Fig. 3A, 3B). 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 these indicator proteins, the contaminations of the lysosomal extracts by plasma and ER proteins are low.

To extend the above findings from MERS-CoV to other coronaviruses, we investigated whether lysosomal proteases from human and bat cells activate SARS-CoV-spike-mediated membrane fusion differentially, 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 result 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 convertease inhibitor and cell-surface protease inhibitor had much less impact on SARS-CoV pseudovirus entry into these cells. Hence, lysosomal proteases were the main contributor to SARS-CoV pseudovirus entry into these 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 result 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-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 differentially

To provide direct biochemical evidence supporting that lysosomal proteases from human and bat cells process MERS-CoV spike differentially, 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 surface of h-HEK293T cells. In the 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 the cells, and we performed Western blotting analysis to detect the cleavage state of MERS-CoV spike. The result 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

286 bat cells were more efficient than their counterparts from human cells in further cleaving
287 MERS-CoV spike to produce S2' fragments (Fig. 5A). Between the two types of bat
288 cells, lysosomal extracts from b-BKD9 cells processed MERS-CoV spike more
289 efficiently than their counterparts from b-Tb1-Lu cells. We further compared the
290 lysosomal extracts from b-BKD9 cells and their counterparts from h-HEK293T cells:
291 lysosomal extracts from b-BKD9 cells processed MERS-CoV spike much more
292 efficiently than their counterparts from h-HEK293T cells in a time-dependent manner
293 (Fig. 5B). Overall, lysosomal extracts from bat cells demonstrated higher efficiency in
294 processing MERS-CoV spike than their counterparts from human cells.

295 To further compare the coronavirus-spike-processing activities of human and bat
296 lysosomal proteases, we examined whether lysosomal extracts from human and bat cells
297 process the spike protein from a MERS-like bat coronavirus HKU4 differentially.
298 Previously we showed that HKU4 spike contains a glycosylated lysosomal protease site
299 at the S1/S2 boundary and it mediates virus entry into bat cells, but not human cells (44).
300 Here we investigated direct biochemical evidence for the differential HKU4-spike-
301 processing activities of human and bat lysosomal proteases. To this end, we purified
302 lysosomal extracts from h-HEK293T cells and b-Tb1-Lu cells, and incubated them
303 individually with HKU4 spike expressed on the surface of h-HEK293T cells. The result
304 showed that lysosomal extrats from b-Tb1-Lu cells, but not their counterparts from h-
305 HEK293T cells, cleaved HKU4 spike containing a glycosylated lysosomal protease motif
306 to produce S2 (Fig. 6A). Next we introduced an N762A mutation into HKU4 spike; the
307 mutation had been shown to remove the glycosylation from the lysosomal protease motif
308 in HKU4 spike (44). The result showed that lysosomal extracts from both h-HEK293T

309 cells and b-Tb1-Lu cells cleaved the mutant HKU4 spike to produce S2 (Fig. 6B). These
310 results provided direct biochemical evidence demonstrating that lysosomal extracts from
311 b-Tb1-Lu cells, but not their counterparts from h-HEK293T cells, can process the
312 glycosylated lysosomal protease motif in HKU4 spike, whereas lysosomal extracts from
313 both h-HEK293T cells and b-Tb1-Lu cells can process the unglycosylated lysosomal
314 protease motif in HKU4 spike.

315 **Discussion**

316 The tropism of coronaviruses includes their species and tissue tropism (1).
317 Lysosomal proteases play a critical role in coronavirus entry (8, 10, 11), but their roles in
318 coronavirus tropism have not been critically established. In contrast, extracellular
319 proteases and other cellular proteases have been shown to be important determinants of
320 coronavirus tropism (22, 23, 26, 29-31). We and others previously showed that a MERS-
321 like coronavirus from bats, HKU4, uses the same host receptor DPP4 as MERS-CoV (24,
322 41), and we also showed that cellular proteases from bat and human cells differentially
323 support HKU4 entry (24, 44). However, two factors can complicate the roles of
324 lysosomal proteases in coronavirus tropism: human and bat DPP4 molecules have
325 different activities as coronavirus receptors, and other proteases may also play significant
326 roles in the cell entry process of coronaviruses. In the current study, we quantified and
327 controlled the contributions from host receptor and other proteases to coronavirus entry,
328 such that the role of lysosomal proteases could be clearly defined in coronavirus entry
329 into cells from different origins. To this end, we screened a number of cell lines
330 originated 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 main contributor to coronavirus-spike-mediated entry. The above 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 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 spike proteins from MERS-CoV, SARS-CoV, and HKU4 all mediated viral entry into bat

354 cells at higher efficiency than into human cells, due to or mainly due to the higher
355 coronavirus-spike-processing activities of bat lysosomal proteases.

356 The correlation between lysosomal protease activities and coronavirus tropism is
357 a novel finding in virology. Previous studies already showed that the expression levels of
358 lysosomal proteases vary among different tissues within the same host species, due to the
359 different physiological functions of tissue cells (7, 45). Our study demonstrates that
360 lysosomal protease activities may also vary among different mammalian species,
361 indicating that adaptation of coronaviruses to new species may occur through adaptation
362 to different lysosomal protease activities. The physiological reason behind different
363 lysosomal protease activities among mammalian species is not clear, but it could be due
364 to different lifestyles of these species. For instance, although speculative, bats are the
365 only flying mammals and hence the enhanced lysosomal protease activities of bat cells
366 may provide fast turnover of metabolic products and also produce high levels of
367 nutrients. In this sense, supporting coronavirus entry efficiently could be a byproduct of
368 the enhanced lysosomal protease activities of bat cells. It is worth noting that due to the
369 difficulty in culturing bat tissue cells, this study was performed using bat cell lines.
370 Although cell lines usually maintain many features of original tissue cells, these findings
371 will need to be confirmed using bat tissue cells. Our study suggests that no matter
372 whether cells are from different host species or from different tissues of the same host
373 species, those cells with higher lysosomal protease activities in general support
374 coronavirus entry more efficiently than the cells with lower lysosomal proteases do. It
375 remains to be further investigated whether the higher lysosomal protease activities in
376 some cells are due to enhanced enzymatic activities, elevated expression levels, or some

other changes that happened to their lysosomal proteases. Nevertheless, our study has established that differential lysosomal proteases from different types of cells have direct impact on coronavirus entry, which has implications for the tissue and species tropism of coronaviruses.

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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), Tb1-Lu cells (*Triatoma brasiliensis* bat lung cells) were obtained from ATCC (the 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 Dr. Charles M. Rice at Rockefeller University. All cells were cultured in DMEM supplemented with 10% 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. Plasmids (pFR-Luc and pBD-NFκB) for cell-cell fusion are kindly provided by Dr. Zhaohui Qian at the 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 protein (i.e., MERS-CoV pseudoviruses or SARS-CoV pseudoviruses, respectively) were generated as described previously (24). Briefly, HEK293T cells were co-transfected with a plasmid carrying an Env-defective, luciferase-expressing HIV-1 genome (pNL4–3.luc.R-E-) and

412 pcDNA3.1(+) plasmid encoding MERS-CoV or SARS-CoV spike. Pseudoviruses were
413 harvested 72 hours after transfection, and were used to enter human and bat cells. For
414 screening of cell lines expressing no or low levels of coronavirus receptor, different types
415 of cells were seeded in 96-well plates and infected immediately by pseudoviruses. For
416 studying the roles of lysosomal proteases in coronavirus entry, cells were transfected with
417 pcDNA3.1(+) plasmid encoding human DPP4 or human ACE2. 24 hours after the
418 transfection, the cells expressing the receptor were seeded in 96-well plates and then
419 infected by pseudoviruses. After incubation at 37 °C for 6 hours, the medium was
420 replaced with fresh DMEM. After another 60 hours, cells were washed with PBS and
421 lysed. Aliquots of cell lysates were transferred to an Optiplate-96 plate (PerkinElmer Life
422 Sciences), and then luciferase substrate (Promega) was added. Relative luciferase units
423 were measured using EnSpire plate reader (PerkinElmer Life Sciences), and normalized
424 for exogenous expression levels of the corresponding receptor in cell membranes (see
425 below).

426 Inhibition of pseudovirus entry using various protease inhibitors was carried out
427 as described previously (50). Briefly, target cells were preincubated with medium
428 containing a final concentration of 50 μM camostat mesylate (Sigma-Aldrich), 50 μM E-
429 64d (Sigma-Aldrich), 50 μM Chloromethylketone (Enzo), or DMSO (negative control) at
430 37 °C for 1 hour. The cells were subsequently infected by pseudoviruses. The cells were
431 incubated at 37 °C for 6 to 8 hours, and then the medium was replaced with fresh
432 DMEM. After another 48 hours, the cells were lysed and measured for luciferase activity.

433 *Exogenous expression of coronavirus receptor in cells and 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 hours after transfection, the cells were lysed using ultrasonication, and aliquots of cell lysates were subjected to Western blotting analysis. 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 amount of cells, without specifying how many of these 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 above and all membrane-associated proteins were extracted using the Membrane Protein Extraction Kit (Thermo Fisher Scientific). Briefly, cells were centrifuged at 300 × *g* for 5 minutes 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 minutes. The supernatant containing cytosolic proteins was removed after centrifugation at 16,000 × *g* for 15 minutes. The pellets containing membrane-associated proteins were resuspended in 0.5 mL Solubilization Buffer and incubated at 4°C for 30 minutes. After centrifugation at 16,000 × *g* for 15 minutes, 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 above, and further 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 peptide (51, 52).

MTT assay

Cells were seeded in 96-well plates and treated with DMSO or DMSO-dissolved protease inhibitors at 37 °C. After incubation for 6 hours, the medium was replaced with fresh DMEM. After incubation for 70 hours 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 hours, 200 μL DMSO or DMSO-dissolved protein inhibitor was added to each well and mixed with the medium. The MTT signal was measured as absorbance at 570 nm using Synergy 2 multi-mode 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 by PBS buffer, and then resuspended by 2.7 PCV (i.e., packed cell volume) extraction buffer. Cells were broken in a 7 ml Dounce homogenizer using a loose pestle (i.e., Pestle B) until 80%-85% of cells were broken (protease inhibitors from the kit were omitted in our procedure). The samples were centrifuged at 1,000xg for 10 min, and the supernatants were transferred to a new centrifuge tube and centrifuged at 20,000xg for another 20 min. The supernatants were removed, and then the pellets were resuspended in extraction buffer as CLF (crude lysosomal fraction). The CLF was diluted in buffer containing 19% Optiprep Density Gradient Medium Solution and further purified using density gradient centrifugation at 150,000xg for 4 hours 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 following assays.

Cytochrome P450 reductase (CPR) is an endoplasmic reticulum (ER) marker. For evaluation of the potential contamination of the purified lysosomal extracts by ER proteins, cytochrome P450 reductase activity of the purified lysosomal extracts was measured using the cytochrome P450 reductase assay kit (Biovision). Briefly, a glucose-6-phosphate (G6P) standard curve was first calculated through 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 at least 30 min (protected from light). Absorbance at 460 nm was measured. Then 5 µl lysosomal extracts from different cell lines were 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 Synergy 2 multi-mode microplate reader (BioTek Instruments). Calculation of the cytochrome P450 reductase activity was performed according to the manufacturer's manual.

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

503 (MUP) standard with 10 µl ALP enzyme solution. The reactions were incubated at 25 °C
504 for 30 min (protected from light). The ALP enzyme can convert MUP substrate to equal
505 molar amount of fluorescent 4-Methylumbelliferone (4-MU). Hence 20 µl 0.5 mM MUP
506 substrate solution was added to each well containing 5 µl lysosomal extracts from
507 different cell lines. After mixing and incubating at 25 °C for 30 min (protected from
508 light), all reactions were stopped through adding 20 µL stop solution into each reaction.
509 Then fluorescence intensities at Ex/Em 360/440 nm were measured using Synergy 2
510 multi-mode microplate reader (BioTek Instruments). Calculation of the alkaline
511 phosphatase activity was performed according to the manufacturer's manual.

512 *Coronavirus-spike-mediated cell-cell fusion*

513 Cell-cell fusion was performed as described previously (53). Briefly, to produce
514 cells expressing one of the coronavirus spikes, HEK293T cells were co-transfected with
515 plasmid pFR-Luc, which contains a synthetic promoter with five tandem repeats of the
516 yeast GAL4 binding sites that controls expression of the luciferase gene, and
517 pcDNA3.1(+) plasmid encoding one of the coronavirus spikes. To produce cells
518 expressing one of the corresponding coronavirus receptor proteins, HEK293T cells were
519 co-transfected with pBD-NF-kappaB, which encodes a fusion protein with the DNA
520 binding domain of GAL4 and transcription activation domain of NF-kappaB, and
521 pcDNA3.1(+) plasmid encoding one of the corresponding coronavirus receptor proteins.
522 After culturing for 24 hours, the spike-expressing HEK293T cells were lifted,
523 centrifuged, and then resuspended in low pH medium containing 10 mM sodium citrate
524 pH 5.6. Subsequently the spike-expressing HEK293T cells were treated with purified
525 lysosomal extracts (100 µg/ml) in the low pH medium. After incubation at 37 °C for 30

minutes, 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 would be activated through binding of the GAL4-NF-kappaB fusion protein to GAL4 binding sites at the promoter of the luciferase gene. After incubation for 24 hours, the cells were lysed, the aliquots of 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 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 hours after transfection, the cells were harvested and washed by PBS buffer. The cells were then treated with 50 µg/ml purified lysosomal extracts at pH 5.6 for 30 minutes or 100 µg/ml purified lysosomal extracts at pH 5.6 for different periods of time (i.e., 10, 30, 60 minutes). After treatment, the cells were lysed and boiled for Western blotting analysis. The C9-tagged spikes were detected using an anti-C9 tag monoclonal antibody (Santa Cruz Biotechnology).

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Figure legends

Figure 1. Screening for cells lines that are suitable for studying lysosomal-proteases-activated coronavirus entry. To screen for cell lines that endogenously express no or low level 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 by luciferase activity accompanying entry, and calibrated against the highest entry efficiency (i.e., 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. Error bars indicate S.E.M. (n=5). (C) To screen for cell lines that can 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; subsequently, the expression level of human DPP4 in each of the cell lines was detected through Western blotting analysis using an antibody recognizing its C-terminal C9 tag. The expression level of β-actin in each of the cell lines was used as positive controls. (D) MTT cell viability assay showing that the viabilities of three types of cells were not affected by the presence of different protease inhibitors. Error bars indicate S.E.M. (n=5). There is no statistical significance for each cell group (i.e., $P>0.05$ based on two-tailed *t* test).

Figure 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, human HEK293T (h-HEK293T), human HeLa (h-HeLa) and bat Tb1-Lu (b-Tb1-Lu),

were controlled to exogenously express human DPP4 as described in Fig. 1C, and then subjected to MERS-CoV pseudovirus entry as described in Fig. 1A. Furin inhibitor chloromethylketone, cell-surface protease (i.e., TMPRSS2) inhibitor camostat, or lysosomal protease (i.e., cathepsins) inhibitor E64d was used in parallel experiments to investigate the relative contributions of 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 together at pH 5.6 in the presence of lysosomal extracts from h-HEK293T cells, h-HeLa cells, b-Tb1-Lu cells, or bat BKD9 (b-BKD9) cells. Cell-cell fusion efficiency was characterized by 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 for fusion (i.e., no receptor); (ii) no lysosomal proteases were added to the medium and the medium was at neutral pH (i.e., no treatment); (iii) no lysosomal proteases were added, but the medium was at pH 5.6 (i.e., low pH treatment). For both panels, statistic analyses were performed using two-tailed *t*-test. Error bars indicate S.E.M. (n=4). *** $P<0.001$. ** $P<0.01$.

Figure 3. Characterization of the purity of lysosomal extracts from different cell lines. Because alkaline phosphatase (ALP) and cytochrome P450 reductase (CPR) are

783 enzymatic markers of plasma and endoplasmic reticulum (ER), respectively, the purified
784 lysosomal extracts and whole cell lysates from different cell lines (for each cell line,
785 lysosomal extracts and whole cell lysates were in equal concentrations) were assayed for
786 their ALP activities (panel A) and CPR activities (panel B) as evaluation of potential
787 contaminants from other cell organelles. Error bars indicate S.E.M. (n=3) (some of the
788 error bars may be too small to be seen).
789
790 **Figure 4.** Roles of lysosomal proteases in SARS-CoV-spike-mediated membrane fusion.
791 The experiments were performed in the same way as in Fig. 2, except that SARS-CoV
792 spike and its receptor human ACE2 replaced MERS-CoV spike and human DPP4,
793 respectively.
794
795 **Figure 5.** Cleavage of cell-surface-expressed MERS-CoV spike using purified lysosomal
796 extracts. (A) Cleavage of cell-surface-expressed MERS-CoV spike using lysosomal
797 extracts from a number of cell lines. MERS-CoV spike was exogenously expressed on
798 the surface of h-HEK293T cells, and then treated with 50 µg/ml lysosomal extracts (from
799 different types of cells) at pH 5.6 for 30 minutes. The cleavage state of MERS-CoV spike
800 was detected through Western blotting analysis using an antibody recognizing its C-
801 terminal C9 tag. (B) Cleavage of cell-surface-expressed MERS-CoV spike using 100
802 µg/ml lysosomal extracts (from two types of cells) at pH 5.6 in a time-dependent manner
803 (i.e., 10, 30, and 60 minutes). These experiments were repeated five times, and
804 representative results are shown here.
805

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