Middle East respiratory syndrome coronavirus (MERS-CoV) infects humans from zoonotic sources and causes severe pulmonary disease. Virions require spike (S) glycoproteins for binding to cell receptors and for catalyzing virus–cell membrane fusion. Fusion occurs only after S proteins are cleaved sequentially, first during their secretion through the exocytic organelles of virus-producing cells, and second after virus binding to target-cell receptors. To more precisely determine how sequential proteolysis contributes to CoV infection, we introduced S mutations obstructing the first cleavages. These mutations severely compromised MERS-CoV infection into human lung-derived cells, but had little effect on infection into several other cell types. These cell type-specific requirements for proteolysis correlated with S conformations during cell entry. Without the first cleavages, S proteins resisted cell receptor-induced conformational changes, which restricted the second, fusion-activating cleavages. Consistent with these findings, precleaved MERS viruses used receptor-proximal, cell-surface proteases to effect the second fusion-activating cleavages during cell entry, whereas the more rigid uncleaved MERS viruses trafficked past these cell-surface proteases and into endosomes. Uncleaved viruses were less infectious to human airway epithelial and Calu3 cell cultures because they lacked sufficient endosomal fusion-activating proteases. Thus, by sensitizing viruses to receptor-induced conformational changes, the first S cleavages expand virus tropism to cell types that are relevant to lung infection, and therefore may be significant determinants of MERS-CoV virulence.

Proteolytic processing of Middle East respiratory syndrome coronavirus spikes expands virus tropism

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nveloped viruses deposit their genomes into host cells by coalescing their membranes with the cell. These functions are executed by virion envelope-anchored glycoprotein trimers termed “membrane-fusion proteins.” In virus-infected cells, these proteins are synthesized as inactive forms, structured such that they can maintain their membrane-fusion potential throughout their residence on extracellular virus particles. The proteins then transit into fusion-competent forms during virus-cell entry. Various environmental stimuli control these cell entry-related structural transitions. Proteolysis is central, as fusion proteins cleaved by host proteases are frequently liberated to undergo transitions into fusion-competent forms (1–3). Knowledge of the proteolytic cleavages and host proteases regulating virus infections can be used to predict viral tropism and pathogenesis (4, 5), and can also reveal antiviral strategies (6).

Coronaviruses (CoVs) are enveloped, positive-stranded RNA viruses in the order \textit{Nidovirales}. These viruses infect mammals and birds, and are mainly associated with respiratory and enteric tract disorders (7). Of six known human CoVs, the severe acute respiratory syndrome (SARS)-CoV and Middle East respiratory syndrome (MERS)-CoV are the most recent to have emerged from zoonotic reservoirs, which include bats (8–10), Himalayan palm civets and other animals found in wet markets in China (11, 12), and Dromedary camels (13). Infection can cause acute respiratory distress with high mortality, particularly in elderly individuals and those with underlying pulmonary dysfunctions (14, 15). It is unknown whether the spread of these highly pathogenic CoVs between animals and humans, as well as within the infected respiratory system, relates to their propensity to use particular lung proteases as entry factors.

CoV fusion proteins, called spike (S) proteins, are integral-membrane ~500-kDa trimers that project ~20 nm from viral envelopes (Fig. 1A). Atomic resolution structures of two CoV S protein ectodomains have recently been obtained (16, 17). These structures can be broadly divided into receptor-binding domains (RBDDs in Fig. 1A), and fusion-catalyzing domains (FDs in Fig. 1A). For MERS-CoV, the S RBDDs bind to dipeptidyl peptidase 4 (DPP4/CBD2) receptors (18). Following reV infection binding, the FDs are revealed through unfolding transitions, such that viral fusion peptides (FP in Fig. 1B) intercalate into host cell membranes. Subsequent refolding transitions bring viral and cellular membranes so close that they coalesce.

The unfolding of the FDS is thought to require S protein cleavage by host proteases (19). Although the MERS-CoV S proteins have several proteolytic cleavage sites between the RBDDs and FDs, a scission at the S1/S2 site (Fig. 1B) takes place in virus-producing cells, by furin or related proprotein convertases (20). Subsequent proteolyses at more C-terminal sites, notably the S2′ site (Fig. 1B), takes place later in the virus transmission process, after virions have been released from producing cells and bound to target-cell receptors (1, 21, 22). These proteases include members of type II transmembrane serine proteases (TTSPs) (23, 24), furin/proprotein convertases (21, 25), and cathepsins (23, 26).

Proteolysis at S2′ is thought to trigger S-mediated membrane fusion. Therefore, the abundance and subcellular distribution of the TTSPs, furin/proprotein convertases, and cathepsins quite likely determines host cell susceptibility to CoV infection, as well as the tempo and site of CoV fusion into cells (19). In contrast, the relevance of the S1/S2 proteolysis to CoV infection is more ambiguous. For example, several infectious CoVs lack S1/S2

Significance

Middle East respiratory syndrome coronavirus (MERS-CoV) can cause lethal pneumonia by infecting lung epithelial cells. Infection requires viral “spike” proteins, which catalyze virus–cell membrane fusion during cell entry. Fusion catalysis requires that spikes be first cleaved by cellular proteases. MERS-CoV spikes are cleaved in virus-producing cells before subsequent virus-cell entry, whereas other human coronaviruses have spikes that are cleaved in virus-infecting cells during virus-cell entry. Here, we found that the early MERS-CoV cleavages are required for the subsequent infection of human lung-like cells, but are dispensable for infection of several other cell types. Our findings demonstrate that the spike cleavage status of MERS-CoV dictates cell tropism, and points to spike proteolytic processing as a correlate of MERS-CoV virulence.
proteolytic processing motifs and therefore secrete as uncleaved forms but remain susceptible to extracellular serine protease cleavage at S1/S2 sites.

These mutations were incorporated into the MERS-CoV genome and recombinant viruses were generated (32). WT and mutant MERS-CoVs, produced in Vero81 cells, were infected into several different target cells, including Vero81, Huh7, and Calu3. These target cells have differing amounts and types of cellular proteases (23, 25, 30, 33), and therefore each cell type might uniquely process MERS-CoV S proteins, generating differential infection. At 5-h postinfection, total RNAs were extracted from the infected cells and the relative amounts of subgenomic nucleocapsid (N) mRNA were quantified as a measure of infection. In Vero81 and Huh7 cells, infections by WT and mutant MERS-CoVs were comparable, with only a modestly reduced infection of the SSVR mutant into Huh7 cells (Fig. 1C). However, in Calu3 cells, the mutants produced ∼4 log10 less viral mRNA than WT (Fig. 1C). Consistently, WT and mutant MERS-CoVs produced comparable amounts of progeny viruses in Vero81 and Huh7 cells, whereas in Calu3 cells, mutant virus titers were nearly 3 log10 less virus than those of the WT (Fig. 1D). Calu3 are human lung adenocarcinoma cells (34). To assess susceptibility of normal human respiratory cells, viruses were infected into primary cultures of well-differentiated human airway epithelia (HAEs). Average progeny virus yields from HAEs were ∼10^3 for WT and mutant SSVR viruses, but were ∼10^5 lower at ∼10^1 for mutant MERS-CoV viruses (Fig. 1D).

These findings demonstrated that the engineered mutations specifically disabled infection into lung-derived Calu3 cells, with replacement of all basic S1/S2 residues also compromising infection into HAE cultures.

Infected cells were also evaluated for cleaved S proteins by Western blotting (35). Partial WT S1/S2 cleavage was evident in all cell types, but the mutant S proteins were either uncleaved or absent (Fig. 1F). Collectively, these findings suggested that viruses must undergo a minimal extent of S1/S2 cleavage in producer Vero81 cells to be infectious to target Calu3 cells.

S1/S2 Cleavage Increases MERS S-Mediated Virus-Cell Entry. To discern the mechanisms by which preliminary S1/S2 cleavage increases MERS-CoV infection, we produced HIV- and vesicular stomatitis virus (VSV)-based MERS pseudoparticles (pps) in 293T cells. Upon S-mediated transduction of target cells, these pps express firefly luciferase (Fluc), with Fluc levels providing a quantitative measure of virus-cell entry.

First, the pps were pelleted and particle-associated S proteins were evaluated by detecting C-terminal epitopes on Western blots. WT MERS-CoV S proteins were mostly cleaved, whereas both mutant S proteins were uncleaved (Fig. 2A). When inoculated at equivalent input multiplicities, the uncleaved mutants were specifically compromised for transduction into lung-derived Calu3 cells (Fig. 2A) and HAE cells (Fig. 2B). These results were comparable to those obtained with infectious viruses, demonstrating that the MERS pseudoparticle (pp) transductions reflect authentic virus entry, and indicating that the S1/S2 cleavages affect infection at the level of virus-cell entry.

Second, the MERS pps were prepared in serum-free 293T media (SFM) and incubated with trypsin before transduction. Trypsin processed the uncleaved fraction of WT S proteins, as well as the uncleaved SSVR mutant S proteins, into fragments at ∼95 kDa (Fig. 2C), consistent with scission at the RST751/S cleavage site. In contrast, SSAS S proteins were cleaved at an alternative more N-terminal cleavage site, most likely RST694/S (30), as inferred from the ∼100-kDa C-terminal fragments (Fig. 2C). On transduction into Calu3 cells, this trypsin processing was associated with a >100-fold increase in SSVR pp transduction (Fig. 2C). In contrast, the WT and SSAS pp transductions were not affected by trypsin (Fig. 2C).

The fact that the SSAS pps remained incompetent for transduction even after trypsin proteolysis to an ∼100 kDa form suggested that cleavage at a precisely defined S1/S2 site is a prerequisite for Calu3 infection.

Third, MERS pps were produced in the presence of a pro-protein convertase inhibitor (PCI, also known as a dec-RVKR-cmk)
SARS-CoV S cleavage by trypsin facilitates entry into Calu3 cells. (A) MERS-pp transductions in the presence of WT, S1/S2 mutant (YSAS and SVSR) MERS pps, or with pp-lacking S proteins (Bald). (B) Three HAE cultures were inoculated with VSVV-based WT and S1/S2 mutant MERS pseudoparticles. (C) MERS pps were pretreated with trypsin and then used to inoculate Calu3 cells. (D) MERS pps were produced in the presence of PCI, cleared free of residual PCI, and used to inoculate Calu3 cells. In all experiments, virus entry was quantified by measuring luciferase levels at 18 h (B) or at 48 h (A, C, D) posttransduction. Lower panels depict S proteins on MERS pps after Western blotting. Uncleaved (S1′) and cleaved (S2′) positions are indicated. The numbers at the left indicate molecular mass in kilodaltons. Error bars present SD from the mean (n = 3). Statistical significance was assessed by Student’s t test. *P < 0.05; †P < 0.01; ‡P < 0.001; ns, not significant.

Fig. 2. S1/S2 cleavage is required for MERS pp entry into Calu3 cells. (A) Vero81, HuH7, and Calu3 cells were inoculated with WT, S1/S2 mutant (YSAS and SVSR) MERS pps, or with pp-lacking S proteins (Bald). (B) Three HAE cultures were inoculated with VSVV-based WT and S1/S2 mutant MERS pseudoparticles. (C) MERS pps were pretreated with trypsin and then used to inoculate Calu3 cells. (D) MERS pps were produced in the presence of PCI, cleared free of residual PCI, and used to inoculate Calu3 cells. In all experiments, virus entry was quantified by measuring luciferase levels at 18 h (B) or at 48 h (A, C, D) posttransduction. Lower panels depict S proteins on MERS pps after Western blotting. Uncleaved (S1′) and cleaved (S2′) positions are indicated. The numbers at the left indicate molecular mass in kilodaltons. Error bars present SD from the mean (n = 3). Statistical significance was assessed by Student’s t test. *P < 0.05; †P < 0.01; ‡P < 0.001; ns, not significant.

Fig. 3. SARS-CoV S cleavage by trypsin facilitates entry into Calu3 cells. (A) SARS and HCoV-229E pps were treated with trypsin. S proteins on pps were analyzed by Western blot. Uncleaved (S1′) and cleaved (S2′) positions are indicated. The numbers at the left indicate molecular mass in kilodaltons. (B) Calu3 cells were incubated with trypsin pretreated pps. Virus entry was quantified by measuring luciferase levels at 48 h posttransduction. Error bars present SD from the mean (n = 4). Statistical significance was assessed by Student’s t test. *P < 0.001.
In determining whether uncleaved MERS pps were differentially sensitive to these protease inhibitors, we chose to use Huh7 cells, as they supported WT and uncleaved mutant MERS-CoVs nearly equally (Figs. 1 and 2). In these cells, the WT and uncleaved mutant MERS-CoVs were similar in their resistance to camostat, and in their modest sensitivity to PCI, but were set apart by the mutants’ hyper-sensitivity to E64d (Fig. 5C). This pattern, in which E64d potently blocked the mutant pp transductions, was also observed in Caco2 cells (Fig. S2). These findings indicated that the uncleaved viruses required a late endosomal cell entry pathway. We considered whether the uncleaved mutant pps might use early-acting proteases if they were provided in abundance. To this end, we overexpressed the TTSP family TMPR prerin 2 (TMPPRSS2) in Huh7 cells, and then evaluated MERS pp transductions. TMPPRSS2 overexpression increased Flue accumulation (about eightfold) and made all MERS pp transductions slightly sensitive to camostat, and also rendered WT MERS pp transduction completely resistant to E64d (Fig. 5C), indicating ample supplies of the TMPPRSS2 proteases. However, the overexpressed TMPPRSS2 did not render the uncleaved YSAS and SSVR MERS pp transductions resistant to E64d (Fig. 5C), indicating that the uncleaved MERS-CoVs are dependent on late-acting cathepsin proteases even when early-acting proteases are abundantly available.

Cathepsin L Sensitizes Calu3 Cells to Uncleaved MERS Viruses. Because uncleaved MERS viruses require late proteases and do not infect Calu3 cells, we inferred that Calu3 cells are depleted in endosomal cathepsins, at least relative to infectable Huh7 cells. This inference was validated by measuring protease-encoding transcripts by quantitative RT-PCR. Huh7 cells contained DPP4, furin, cathepsin L, and cathepsin B, but very few TMPPRSS2 transcripts (Fig. 6A). Relative to the Huh7 cells, the lung and airway-derived Calu3 and HAE cultures had far more TMPPRSS2 transcripts (Fig. 6A). To determine whether the relatively low levels of late-acting proteases in Calu3 cells accounted for virus resistance, we transduced the cells with human cathepsin L genes and then evaluated MERS pp entry. Transduced cells were highly sensitized to uncleaved MERS pp entry, and were made resistant by E64d (Fig. 6C). Similar augmentation of uncleaved MERS transduction was achieved by exposing cell-bound MERS pps to purified human cathepsin L (Fig. 6D). These results indicate that uncleaved viruses can only infect cells containing sufficient late-acting endosomal proteases, and they make it clear that CoV-cell tropism is related to the abundance and distribution of proteases in both virus-producing and virus-targeting cells.

Discussion

CoV–cell entry can be viewed in the context of a proteolytic cascade that includes at least two cleavages in S proteins, first at S1/S2, then at S2'. For MERS-CoV, the cascade can begin shortly after virus morphogenesis in virus-producing cells. Extensive S1/S2 cleavage at this beginning stage allows for a similarly extensive S2' cleavage shortly after virus binding to receptors in virus-targeted cells, and the cascade ends when a sufficient number of adjacent S proteins are triggered for fusion activation by early-acting proteases. However, for several CoVs, and for some variant forms of MERS-CoV, the cascade begins when viruses bind target cells, and then ends much later after virus endocytosis and late-acting intraendosomal proteolysis to activate a sufficient number of adjacent S proteins into fusion competence. These variations in the beginning and ending stages of the CoV infection-related proteolytic cascade are illustrated in Fig. 7.

The beginning and ending points of this proteolytic cascade correlated with cell tropism. MERS-CoVs that began proteolysis at S1/S2 in virus-producing cells could infect Calu3 cells, but mutant MERS-CoVs that remained uncleaved could not (Figs. 1 and 2). Uncleaved MERS viruses bypassed early proteases (Fig. 5C) and required late proteases (Fig. 6D), making it clear that S1/S2 cleavage promotes early entry. Of note, uncleaved viruses were able to infect the more cathepsin protease-enriched Huh7 and Vero81 cells (Figs. 1 and 2). Earlier reports used similar cathepsin-enriched target cell types in their experiments, and thus came to conclusions that the preliminary S1/S2 cleavages had a limited relevance to infection (20, 29, 30). It was the paucity of virus-activating endosomal proteases in Calu3 cells that revealed a key importance for the S1/S2 cleavages in cell tropism.

The relevance of the S1/S2 cleavages extended to infection of primary HAE cultures, as evidenced by the poor growth of the YSAS uncleaved mutant MERS-CoVs (Fig. 1E) and the relatively inefficient entry of the YSAS and SSVR uncleaved pseudoviruses (Fig. 2B). The WT-level growth of the SSVR mutants might argue against the importance of S1/S2 cleavages in HAE infections; however, we note that HAE are frequently collected from patients with underlying pulmonary diseases (e.g., chronic obstructive pulmonary disease, cystic fibrosis, pulmonary fibrosis, α-1 antitrypsin deficiency), conditions associated with increased levels of mucus-associated proteases (40–42). Airway mucus-associated proteases are known to cleave SARS-CoV S proteins (43), and therefore we suggest that the SSVR mutant MERS-CoVs are similarly cleaved by extracellular proteases, making their infection resemble that of WT viruses at the point of entry into some HAE cells.

Once on Calu3 or HAE target cells, the precleaved viruses are presumably processed to fusion-ready S2' products by early cell-surface serine proteases (Fig. S4) (23). That uncleaved viruses are not similarly processed by these proteases is best explained by their failure to respond to receptor binding, which limits downstream S2' processing. MERS S protein interactions with
DPP4 were necessary to reveal a proteolytic cleavage site at or posttransduction. Error bars present SD from the mean (n = 3). Statistical significance was assessed by Student’s t test. *P < 0.01.

The recent 4A resolution structures of murine CoV (MHV) and human CoV (HKU-1) S proteins depict uncleaved ectodomain trimers, without ligation to receptors (16, 17). Although images of S proteins in complex with cellular receptors will add important insights, the current structures do suggest that receptor binding locks relatively dynamic S proteins into conformations that expose cleavage sites to host proteases. S1/S2 cleavage may increase the dynamic properties of S proteins, giving them more opportunities to assume locked receptor-bound conformations, making the S2 sites more rapidly available to proteases. A theme here is that S protein dynamics, achieved through proteolysis or through destabilizing mutations (44), may generally allow for early, cell-surface cleavage at the activating S2 sites. Of note, such destabilizing mutations are liable to be counter-selected during in vitro virus growth in many cell cultures. In vitro conditions typically select for extracellular viroin stability, which maintains infectivity in culture fluids. Therefore, the adaptive mutations fixed into CoV S proteins during in vitro virus propagation may restrict dynamic structural transitions (45). These in vitro mutations are frequently attenuating in in vivo CoV infection models (46). We suggest that in vivo attenuation arises because the stabilized viruses have reduced capacities to assume the receptor-bound conformations enabling S2 proteolysis and fusion activation. The stabilized, cell culture-adapted CoVs are therefore directed to the late endosomal entry routes, which may correlate with diminished capacities to infect many of the target cells found in in vivo environments.

Several CoVs, including SARS- and human 229E-CoVs, remain uncleaved throughout their morphogenesis and secretion from virus-producing cells (27, 28). Thus, several CoV S proteins remain in their more rigidly structured uncleaved forms until target cell entry. For these less flexible viruses to transit into protease-sensitive and fusion-competent forms, they may need the energy derived from high-affinity interactions with receptors. Indeed the affinity of SARS-CoV S with its receptor angiotensin converting enzyme 2 (ACE2) is 10–20-fold higher than that of MERS-CoV S with its receptor DPP4 (47, 48). Thus, one can suggest that S1/S2-cleavages may reduce the need for high-specificity receptor interactions, and in doing so, may allow CoVs to bind adaptably to receptor orthologs, fostering zoonoses. Consistent with this hypothesis, the human circulating MERS-CoVs have not undergone significant adaptations in their RBDs, at least not toward higher-affinity binding to DPP4. In fact, one lineage of human MERS-CoVs acquired reduced affinity to DPP4 (49). This finding contrasts with the SARS-CoV epidemics of 2003–2004, where adaptive changes increased binding affinities to ACE2 (50). Receptor utilization in relation to S proteolytic processing will inform us on CoV transmissions.

Recent excellent reports have made connections between CoV S proteolytic processing and CoV virulence. For example, CoV S protein proteolytic processing, principally by TMPRSS2, was suggested to increase viral pathogenesis by generating S “decoy” fragments that bind and sterically inactivate antiviral antibodies (24). Additionally, S protein proteolysis, again by TMPRSS2, was suggested to increase pathogenesis by allowing viruses to bypass IFN-induced transmembrane protein 3, an innate antiviral effector that blocks virus-endosome membrane fusions (51, 52). Here we claim that MERS-CoVs, and other CoVs, will preferentially use TMPRSS2 if they have been precleaved; that is, preliminary S1/S2 proteolysis gives infecting viruses the facility to use TMPRSS2 and other early-acting proteases for fusion activation. Thus, the preliminary S1/S2 cleavages may be the more proximal determinants of pathogenesis. Of the six known human CoVs, only MERS-CoV secretes from virus-producing cells with cleaved S proteins, and SARS-CoV, secreted uncleaved, can be processed by extracellular proteases before encountering target cells (Fig. 3) (1). Therefore, these viruses have special facility for using early-acting entry proteases. Preliminary S cleavages may also contribute to the tissue tropism and pathogenesis of feline and murine CoVs (4). As human CoVs continue to infect humans, it will be important to consider possible adaptive changes in their S protein proteolytic processing cascades, as these may contribute to CoV disease.

**Fig. 6.** Uncleaved MERS entry depends on endosomal cathepsins. (A) DPP4, TMPRSS2, furin, cathepsin L (Cat. L), cathepsin B (Cat. B), and hypoxanthine phosphoribosyltransferase (HPRT) mRNA levels were measured in Huh7 cell extracts by quantitative RT-PCR, and plotted relative to HPRT. (B) The same mRNA levels were measured in Calu3 and HAE cell extracts and plotted relative to those in Huh7 cells. (C) Calu3 cells were transfected with lentiviral vectors encoding human cathepsin L or GFP. After 3 d, cells were treated with or without 10 μM E64d and then transduced with WT or S1/S2 mutant MERS pps. (D) Calu3 cells were incubated with WT or YSAS MERS pps at 4 °C, and then treated with cathepsin L. Virus entry was quantified by measuring luciferase levels at 48-h posttransduction. Error bars present SD from the mean (n = 3). Statistical significance was assessed by Student’s t test. *P < 0.01.

**Fig. 7.** MERS-CoV–cell entry model. In some producer cell types, MERS-CoV S proteins are cleaved by furin/proprotein convertases in the exocytic pathway. Cleaved MERS-CoV S proteins change their conformations rapidly after receptor binding, exposing subsequent proteolytic cleavage sites, which are processed by proteases (i.e., TMPRSS, found at or near cell surfaces). Early cell-surface entry is achieved when several adjacent S proteins are processed. In other producer cell types, MERS-CoV S proteins are not cleaved. Uncleaved MERS-CoV S proteins slowly change their conformations after receptor binding. MERS-CoVs having uncleaved S proteins traffic to the late endosomes/lysosomes and late endosomal entry is achieved when several adjacent S proteins are eventually processed by cathepsins.
Experimental Procedures

Recombinant MERS-CoV Production and Infection. Recombinant WT and mutant MERS-CoVs were generated from bacterial artificial chromosomes and infected into Vero81, HuH7, and Calu3 cells or HAE cultures. HAE cultures were obtained from the University of Iowa Cell Culture Facility, which acquired tissue by informed consent under an Institutional Review Board–approved organ research donation protocol. Virus infectivities were analyzed by real-time RT-PCR and by plaque assay.

HIV and VSV Pseudoparticle Transduction. Viral pseudoparticles were transduced into Vero81, HuH7, Calu3, or HAE cultures, with or without prior protease or protease inhibitor exposures. Flu levels were measured posttransduction.

In Vitro S Fragmentation Assay. MERS pps and DPP4 pps were incubated in 1.5 M ratios, digested with graded doses of trypsin, and 3 fragments visualized by Western blotting.

For additional information, see SI Experimental Procedures. See Tables S1-S3 for primers used for MERS-CoV, real-time PCR, and mutant MERS pps.

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