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MAJOR ARTICLE

Inhibition of Proprotein Convertases Abrogates Processing of the Middle Eastern Respiratory Syndrome Coronavirus Spike Protein in Infected Cells but Does Not Reduce Viral Infectivity

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Middle East respiratory syndrome coronavirus (MERS-CoV) infection is associated with a high case-fatality rate, and the potential pandemic spread of the virus is a public health concern. The spike protein of MERS-CoV (MERS-S) facilitates viral entry into host cells, which depends on activation of MERS-S by cellular proteases. Proteolytic activation of MERS-S during viral uptake into target cells has been demonstrated. However, it is unclear whether MERS-S is also cleaved during S protein synthesis in infected cells and whether cleavage is required for MERS-CoV infectivity. Here, we show that MERS-S is processed by proprotein convertases in MERS-S–transfected and MERS-CoV–infected cells and that several RXXR motifs located at the border between the surface and transmembrane subunit of MERS-S are required for efficient proteolysis. However, blockade of proprotein convertases did not impact MERS-S–dependent transduction of target cells expressing high amounts of the viral receptor, DPP4, and did not modulate MERS-CoV infectivity. These results show that MERS-S is a substrate for proprotein convertases and demonstrate that processing by these enzymes is dispensable for S protein activation. Efforts to inhibit MERS-CoV infection by targeting host cell proteases should therefore focus on enzymes that process MERS-S during viral uptake into target cells.

Keywords. MERS-coronavirus; protease; TMPRSS2; trypsin; proprotein convertase; spike; activation.

The emergence and subsequent pandemic spread of the severe acute respiratory syndrome (SARS) coronavirus in 2002–2003 caused almost 800 deaths and wreaked enormous economic havoc [1, 2]. The virus was transmitted from bats, potentially via intermediate hosts, to humans, demonstrating that the zoonotic transmission of novel coronaviruses from animal reservoirs to humans can pose a significant threat to public health [3, 4]. A similar outbreak scenario unfolded in 2012, when a novel coronavirus, initially named human coronavirus EMC and now termed Middle East respiratory syndrome coronavirus (MERS-CoV), was detected in a patient from Jordan hospitalized with a severe and ultimately fatal pneumonia [5]. Subsequently, the virus spread within the Middle East and, through travel activity, occasionally to Europe, Africa, Asia, and North America [6–9]. The outbreak has, as of 4 July 2014, entailed 827 laboratory-confirmed infections and 287 deaths [10], and adaptation of the virus to more-efficient human-to-human spread is a public health concern. Therefore, it is imperative to identify and explore novel targets for antiviral therapy.

The viral surface protein spike (S), a type I transmembrane protein synthesized in the constitutive secretory pathway of infected cells, mediates coronavirus entry into target cells [11, 12]. For this, the MERS-CoV spike protein (MERS-S) binds to its receptor dipeptidyl-peptidase-4 (DPP4, CD26) on the surface of target cells [13] and drives

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fusion of the viral envelope with a target cell membrane, which allows delivery of viral proteins and RNA into the host cell cytoplasm, the site of viral replication. However, MERS-S and other coronavirus S proteins are synthesized as inactive precursors in infected cells and only acquire the ability to drive membrane fusion upon processing into the surface unit (S1) and the transmembrane unit (S2) by host cell proteases [14, 15]. The activity of the responsible proteases is essential for viral infectivity, which makes these enzymes potential targets for antiviral intervention.

It has been demonstrated that the cysteine protease cathepsin L [14, 16, 17] and the type II transmembrane serine protease TMPRSS2 [14, 16, 17] can activate MERS-S during viral binding and uptake into target cells. However, activation of viral glycoproteins, including activation of the S protein of certain strains of the coronavirus infectious bronchitis virus (IBV) [18], may also proceed in the constitutive secretory pathway of infected cells and is often accomplished by furin and other proprotein convertases [19–22]. Whether MERS-S is also cleaved during the passage of the secretory pathway and whether these cleavage events contribute to S protein activation is at present unknown.

Here, we show that proprotein convertases process MERS-S in transfected and infected cells, and we demonstrate that the integrity of several RXXR motifs located at the border of the S1 and S2 subunit is required for S protein processing. Treatment of MERS-CoV–infected cells with a proprotein convertase inhibitor (PCI) abrogated S protein cleavage but did not alter viral infectivity, indicating that S protein processing in infected cells is dispensable for MERS-S activation.

MATERIALS AND METHODS

Plasmids

Expression plasmids encoding MERS-S [14], vesicular stomatitis virus glycoprotein (VSV-G) [23], Zaire ebolavirus glycoprotein (EBOV-GP) [23], Lassa virus glycoprotein (LASV-GPC) [23], DPP4 [13], and TMPRSS2 [24] have been described previously. Plasmids pGAL4-VP16 [25], pGAL5-luc [25], pNL4-3-Luc-R$^-$E- [26], and p96ZM651gag-opt [27], as well as plasmids encoding MERS-S [14] and EBOV-GP [28] with a C-terminal V5 tag have also been described. A Constructs expressing LASV-GPC and MERS-S potential cleavage site mutants (PCM) 1 (626-AXXA-629), PCM2 (691-AXXA-694), PCM3 (748-AXXA-751), and PCM4 (884-AXXA-887) with a C-terminal V5 tag were generated by polymerase chain reaction (PCR)–based mutagenesis. The integrity of all PCR-amplified sequences was confirmed by automated sequence analysis.

Cell Culture

293T cells were propagated in Dulbecco's modified Eagle's medium (DMEM; Life Technologies) supplemented with 10% fetal bovine serum (FBS; PAN-Biotech), penicillin, and streptomycin. Caco-2 cells were cultured in DMEM-GlutaMAX medium (Invitrogen) supplemented with 10% FBS, penicillin, and streptomycin. Vero B4 cells were cultured in DMEM supplemented with 5% FBS, 1% L-glutamine, 1% sodium pyruvate, 1% nonessential amino acids (all from Life Technologies), and antibiotics as stated above. All cell lines were grown in humidified atmosphere at 37°C and 5% CO_2.

Analysis of MERS-S Expression and Cleavage

For the detection of MERS-S expression in transfected cells, 293T cells underwent calcium phosphate transfection with the respective plasmids encoding S proteins with a C-terminal V5 antigenic tag. For immunoblotting, the lysates were separated by sodium dodecyl sulfate gel electrophoresis and transferred onto nitrocellulose membranes (Hartenstein). MERS-S expression was detected using a monoclonal antibody directed against the V5 tag (Invitrogen) or a polyclonal antibody directed against the S2 subunit of the MERS-S protein (Sino Biological). For detection of MERS-S protein in infected cells, Caco-2 and Vero B4 cells were infected with MERS-CoV (human betacoronavirus 2c EMC/2012) at a multiplicity of infection (MOI) of 0.01 and 5, respectively. At 24 hours after infection, the cells were harvested and treated with NuPAGE LDS sample buffer (Invitrogen), boiled for 20 minutes at 95°C, and analyzed by Western blot as described above. As a loading control, the membranes were incubated with anti-β-actin antibody (Sigma).

Inhibition of Proprotein Convertases

To assess the role of proprotein convertase activity in MERS-S processing in transfected 293T cells, the cells were transfected with plasmids encoding MERS-S, EBOV-GP, and LASV-GPC or with empty plasmid. The medium was changed 6 hours after transfection, and PCI (Merck) was added to the fresh medium at the indicated concentrations. Medium was replaced again 32 hours after transfection, and inhibitor was replenished. Cells were lysed 48 hours after transfection, and cleavage of viral glycoproteins was detected by Western blot, using a monoclonal antibody recognizing the V5 tag. As a loading control, an anti-β-actin antibody was used.

To assess whether proprotein convertase activity is required for MERS-S–driven cell-cell fusion, a previously described cell-cell fusion assay was used [24], which is based on mixing effectors cells expressing the trans-activator VP16 with target cells expressing luciferase under the control of a VP16-responsive promoter. Specifically, 293T target cells that expressed MERS-S or no glycoprotein and effector cells that were transfected to express DPP4 and/or TMPRSS2 were incubated with 1 μM PCI. One day after transfection, the cells were mixed to allow cell-cell fusion, and medium was supplemented with PCI at 1 μM final concentration. Cell-cell fusion was quantified by determination of luciferase activity in cell lysates at 48 hours after cocultivation, using a commercially available kit (PJK).

For analysis of the importance of proprotein convertase activity for MERS-S–driven virus-cell fusion, a previously established pseudotyping strategy was used [14]: pNL4-3-Luc-R$^-$E$^-$ [26] vectors bearing MERS-S, LASV-GPC, EBOV-GP, or VSV-G or no glycoprotein were generated in the presence or absence of 1 μM PCI. As target cells, 293T cells transfected with DPP4 encoding plasmid or empty plasmid, were seeded into 96-well plates and pretreated with 0.5 μM PCI for 60 minutes at 37°C. The cells were then incubated with pseudotypes for 8 hours, followed by replacement of infection medium with culture medium containing inhibitor. Transduction efficiency was measured after 72 hours by determining luciferase activities in cell lysates.

To determine the role of proprotein convertase activity in infection with authentic MERS-CoV, Caco-2 cells seeded in 24-well plates were incubated with dimethyl sulfoxide or rising concentrations of PCI for 1 hour at 37°C and were then inoculated with MERS-CoV (MOI 0.01 and 0.001 in quadruplicates) in the presence of inhibitor. After incubation for 30 minutes at 4°C, the cells were washed and again incubated in culture medium with the inhibitor. At 24 hours after infection, the cells were washed and harvested, and the pellet was lysed with RIPA lysis buffer, supplemented with 4xNuPAGE (Invitrogen) and boiled for 20 minutes at 95°C. S protein expression in lysates was detected by Western blot, using a polyclonal antibody directed against the S2 subunit of MERS-S (Sino Biological). In parallel, for quantification of viral RNA, 50 μL of the cell supernatant was dissolved in RAV1 buffer (Macherey-Nagel) for RNA extraction, followed by quantitative reverse-transcription PCR analysis, using the upE assay as previously described [29]. Quantification of infectious particles was done by plaque assay, using Vero B4 cells as published elsewhere [6]. Briefly, 10-fold dilutions of supernatants were tested in duplicates, using cell monolayers of Vero B4 cells. After 1 hour of virus adsorption, cells were washed and overlayed with a 1.2% avicel resin. After 3 days, the plates were fixed with 7% paraformaldehyde and stained with crystal violet solution. To investigate the influence of PCI on the formation of MERS-CoV–induced cytopathogenic effects, Vero B4 cells were infected at an MOI of 0.1 and fixed with 7% paraformaldehyde 42 hours after infection. MERS-CoV antigen detection was performed by incubation with a serum specimen from a patient with MERS as described elsewhere [30]. Bound antibodies were detected with a cyanine 2–labeled goat-anti human immunoglobulin G secondary antibody (Dianova). Nuclei were stained by mounting the slides with DAPI containing ProLong Gold antifade mounting medium (Life technologies).

RESULTS

MERS-S Is Proteolytically Processed in Transfected and Infected Cells

To investigate MERS-S cleavage in virus producing cells, we determined whether the S protein is cleaved in transfected and infected cells. Western blot analysis of S protein transfected 293T cells and MERS-CoV–infected Vero B4 cells with an antibody specific to the S2 subunit of MERS-S revealed 2 prominent S protein bands with molecular weights of 170 kDa and 90 kDa (Figure 1), in keeping with our previous results [14]. The 170k-Da band corresponds to uncleaved MERS-S, while the presence of the 90-kDa band indicates efficient processing of MERS-S into an N-terminal S1 subunit (not detected) and a C-terminal S2 subunit (Figure 1).

Several RXXR Motifs Located at the Border of the S1 and S2 Subunits Are Required for Processing of MERS-S

Inspection of the sequences located at the border of the S1 and S2 subunits of MERS-S revealed the presence of 4 RXXR sequences (Figure 2*A*), which might represent recognition sites for proprotein convertases [22]. To determine the importance of these motifs for MERS-S cleavage, we changed the arginine residues in 626-RXXR-629, 691-RXXR-694, 748-RXXR-751, and 884-RXXR-887 to alanine residues, creating the potential cleavage site mutants PCM1, PCM2, PCM3, and PCM4 (Figure 2*A*). All S protein mutants were expressed with the same efficiency as wild-type MERS-S in transfected cells (Figure 2*B*). The only exception was PCM2, for which consistently no expression was detected (not shown). Importantly, mutation of potential cleavage sites 3 and 4 alone impacted S protein processing: the presence of the 90-kDa band was reduced in cells expressing PCM3, and a band with a molecular weight slightly

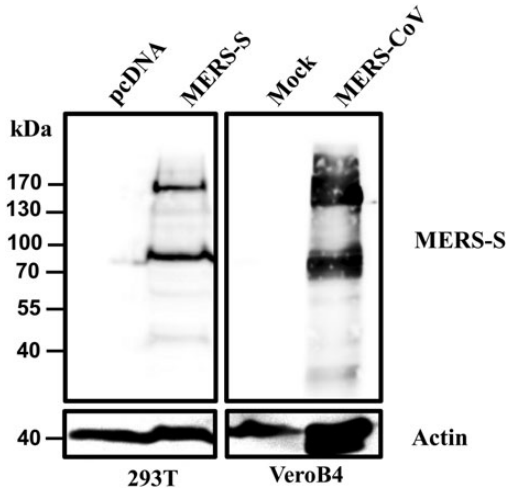

Figure 1. The Middle East respiratory syndrome coronavirus (MERS-CoV) spike protein (MERS-S) is cleaved in transfected and infected cells. 293T cells were transfected with a plasmid encoding the MERS-S protein or with empty plasmid (pcDNA). Vero B4 cells were either infected with MERS-CoV at a multiplicity of infection of 5 or mock infected. Subsequently, the cells were lysed and analyzed by Western blot, using a polyclonal antibody directed against the S2 subunit of MERS-S. A β-actin antibody served as a loading control. Similar results were obtained in 2 separate experiments.

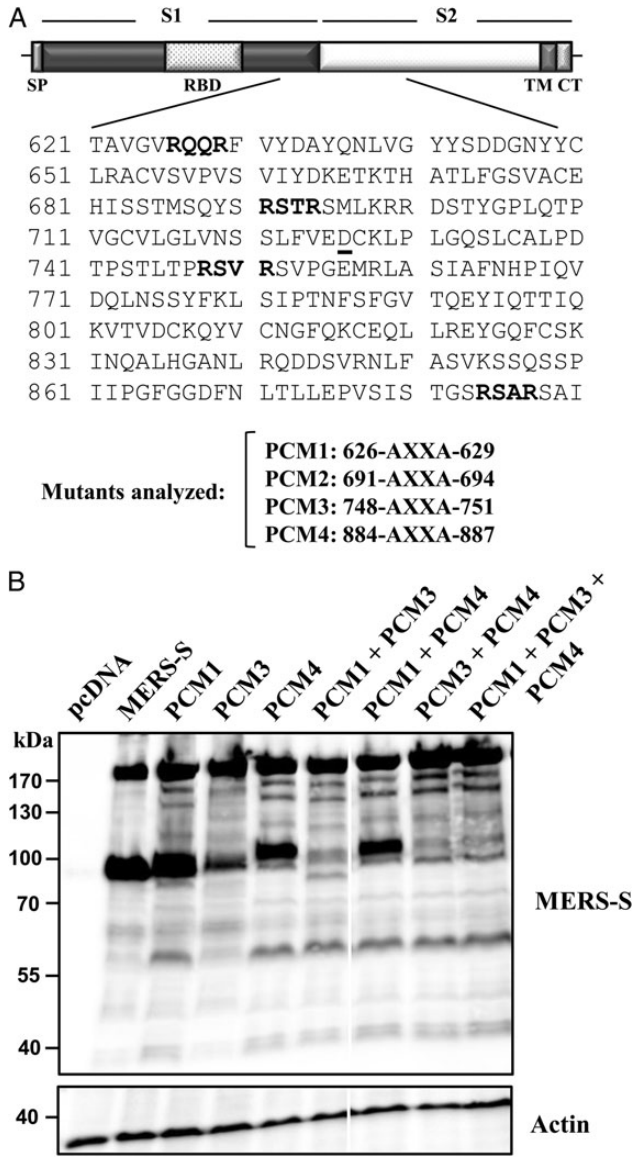

Figure 2. RXXR motifs located at the border between S1 and S2 are required for efficient processing of the Middle East respiratory syndrome coronavirus spike protein (MERS-S). *A*, The domain organization of the MERS-S protein is schematically depicted. The MERS-S sequence at the border between the S1 and S2 subunits is shown. RXXR motifs, which constitute potential cleavage sites, are highlighted, and the predicted start of the S2 subunit is underlined. The mutations introduced into the potential cleavage sites in MERS-S are shown. *B*, 293T cells were transfected with expression plasmids coding for MERS-S wild type and the indicated MERS-S mutants equipped with a C-terminal V5 tag. Transfection of empty plasmid (pcDNA) served as negative control. Expression of S proteins in cell lysates was determined by Western blot, using a V5 tag–specific monoclonal antibody. Expression of β-actin in cell lysates was assessed as a loading control. The results shown are representative for at least 3 independent experiments. Abbreviations: CT, cytoplasmic tail; PCM, potential cleavage site mutant; RBD, receptor binding domain; SP, signal peptide; TM, transmembrane domain.

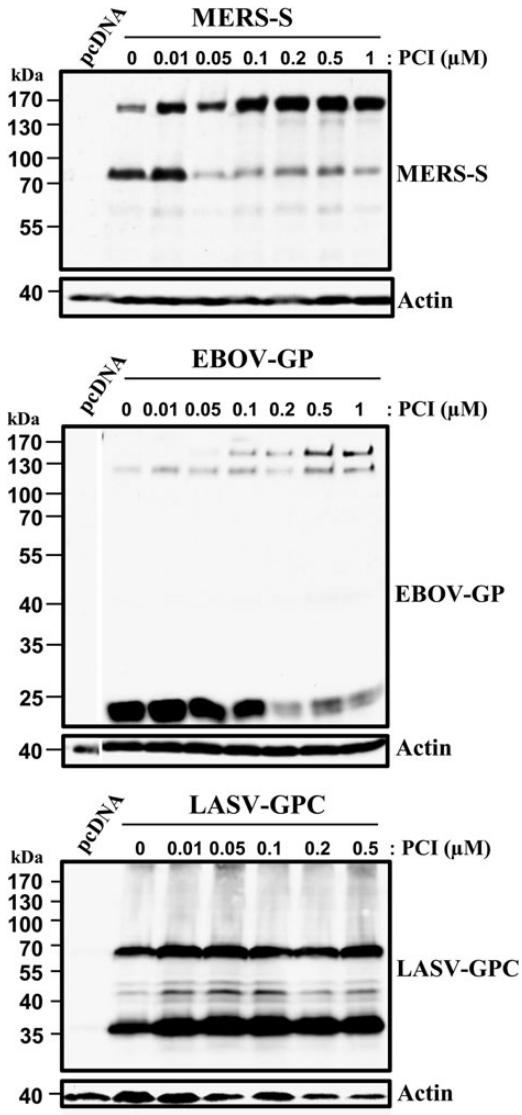

Figure 3. The Middle East respiratory syndrome coronavirus spike protein (MERS-S) is cleaved by proprotein convertases. 293T cells were transfected with expression plasmids encoding MERS-S, Zaire ebolavirus glycoprotein (EBOV-GP), or Lassa virus glycoprotein (LASV-GPC), all equipped with a C-terminal V5 tag. Cells transfected with empty plasmid (pcDNA) served as negative control. Subsequently, cells were incubated with the indicated concentrations of the proprotein convertase inhibitor (PCI). At 48 hours after transfection, glycoprotein expression was analyzed by Western blot, using a V5 tag–specific monoclonal antibody. Detection of β-actin served as loading control. The results shown are representative of three independent experiments.

higher than 90 kDa was observed upon expression of PCM4 (Figure 2*B*). Processing of PCM1 was comparable to that seen for MERS-S wild type. However, combined mutation of potential cleavage sites 1 and 3 (PCM1 + PCM3) abrogated S protein processing (Figure 2*B*), suggesting that also potential cleavage site 1 contributes to S protein cleavage in this experimental setting. Similar effects were seen when potential cleavage sites 3 and 4 were simultaneously altered (PCM3 + PCM4) or when all 3 potential cleavage sites were mutated in parallel

(PCM1 + PCM3 + PCM4). Collectively, these results indicate that MERS-S cleavage is reduced or altered upon mutation of potential cleavage sites 1, 3, and 4.

MERS-S Is Processed by Proprotein Convertases

We next sought to identify the proteases responsible for MERS-S cleavage. For this, we investigated whether S protein cleavage can be blocked by a cell-permeable tripeptide derivative containing an Arg-X-Arg motive, which is known to inhibit several proprotein convertases, including furin [31]. Treatment with this PCI reduced the production of the 90-kDa band in cells transfected to express MERS-S (Figure 3). Moreover, the presence of PCI diminished processing of the Zaire ebolavirus glycoprotein (EBOV-GP) into GP1 and GP2 (Figure 3), which depends on the activity of the proprotein convertase furin [32]. In contrast, processing of the glycoprotein of Lassa virus (LASV-GPC), which is mediated by the proprotein convertase SKI-1/S1P [33], was not suppressed (Figure 3). This finding is in keeping with the known differences in substrate specificity (and thus inhibitor sensitivity) of SKI-I/SIP, compared with proprotein convertases with specificity for basic amino acids, like furin [34]. Collectively, these results show that the activity of proprotein convertases is essential for MERS-S processing in transfected cells.

Proprotein Convertase Activity Is Not Essential for Efficient MERS-S–Driven Cell-Cell and Virus-Cell Fusion

We next assessed whether proprotein convertase activity is required for S protein–driven cell-cell fusion. For this, we used a previously described assay [24], which is based on directed expression of S protein and receptor/protease in effector and target 293T cells, respectively. Expression of MERS-S in effector cells allowed inefficient fusion with control-transfected 293T target cells, which are known to express low amounts of endogenous DPP4 [35], and the efficiency of cell-cell fusion was markedly increased when target cells were transfected with DPP4 and/or TMPRSS2 encoding plasmid (Figure 4*A*). However, the continuous presence of PCI in target and effector cell cultures, before, during, and after mixing had no appreciable effect on fusion efficiency (Figure 4*A*), suggesting that proprotein convertase activity is dispensable for MERS-S–driven cell-cell fusion.

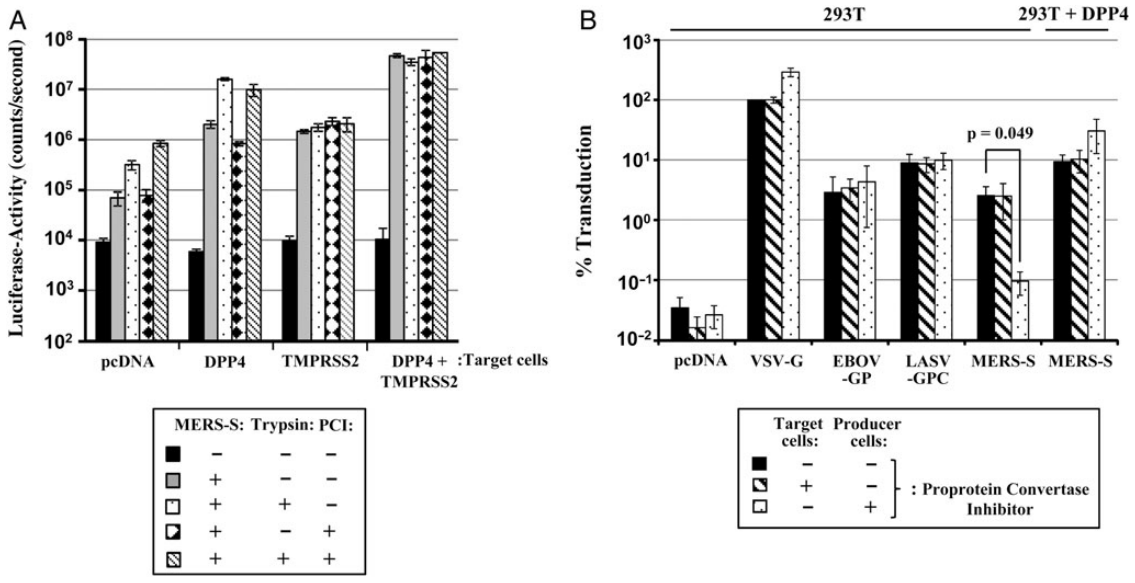

Figure 4. Proprotein convertase activity is dispensable for Middle East respiratory syndrome coronavirus spike protein (MERS-S)–driven cell-cell and virus-cell fusion. *A*, Fusion of 293T effector cells transfected to express MERS-S with target cells transfected to express DPP4 and/or TMPRSS2 or control transfected with empty plasmid (pcDNA) was assessed. Both effector and target cells were incubated with 1 μM of proprotein convertase inhibitor (PCI) as indicated and, 1 day later, were mixed for cocultivation. The effector/target cell mixtures were incubated with phosphate-buffered saline (PBS) or PCI, and cell-cell fusion was quantified by determination of luciferase activities in cell lysates. The results of a representative experiment performed with triplicate samples are shown. Error bars indicate standard deviation (SD). Two separate experiments yielded similar results. *B*, Lentiviral pseudotypes carrying MERS-S, Lassa virus glycoprotein (LASV-GPC), Zaire ebolavirus glycoprotein (EBOV-GP), or the glycoprotein of vesicular stomatitis virus (VSV-G), as well as pseudotypes bearing no viral glycoprotein (pcDNA), were generated in the presence or absence of PCI (1 μM). Subsequently, target 293T cells transfected with empty plasmid or DPP4 expression plasmid were preincubated with dimethyl sulfoxide (DMSO), PBS, or PCI at a final concentration of 0.5 μM for 30–60 minutes, followed by transduction with the pseudotypes specified above. At 72 hours after transduction, luciferase activities in cell lysates were measured. The average of 5–7 independent experiments performed with triplicate samples is shown. Transduction with pseudotypes bearing VSV-G in the absence of inhibitor was set as 100%. Error bars indicate standard error of the mean (SEM). A 2-tailed Student *t* test was used to assess statistical significance.

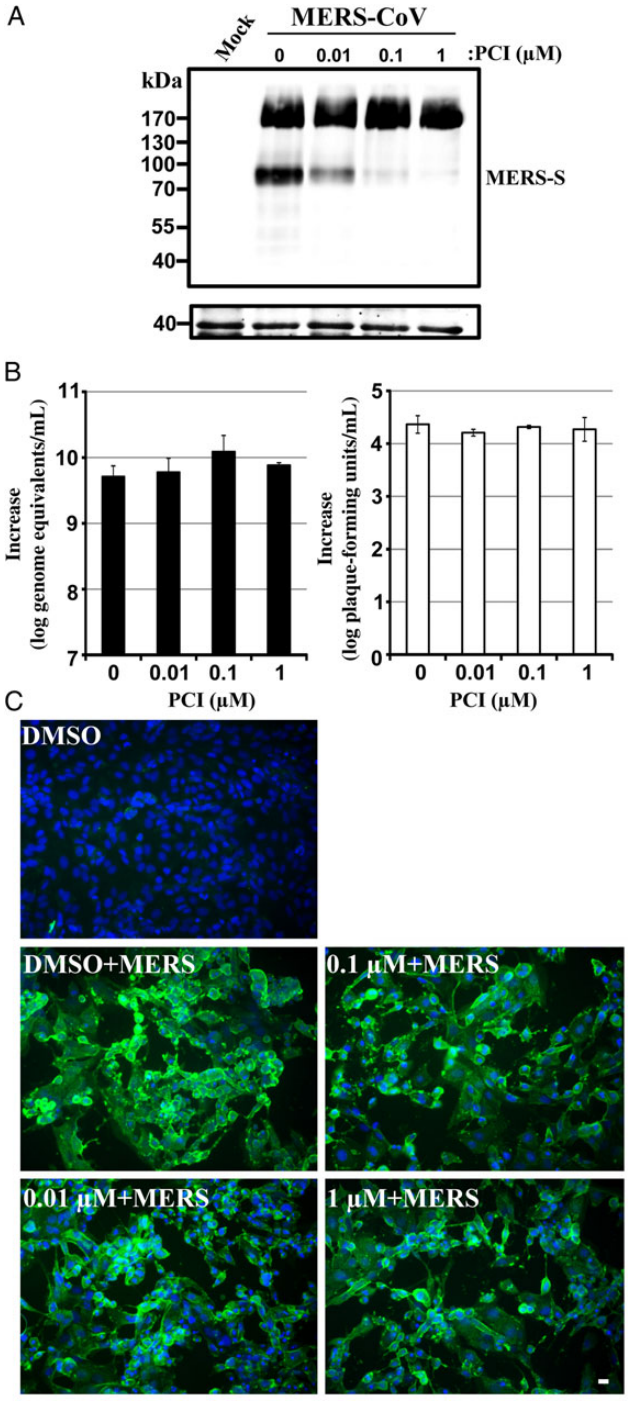

Figure 5. Processing of Middle East respiratory syndrome coronavirus (MERS-CoV) spike protein (MERS-S) by proprotein convertases is dispensable for MERS-CoV infectivity. Caco-2 cells were incubated with the indicated concentrations of proprotein convertase inhibitor (PCI) for 1 hour and then inoculated with MERS-CoV at a multiplicity of infection (MOI) of 0.01 and 0.001 for 30 minutes at 4°C. Thereafter, virus was removed, inhibitor was replenished, and cells were lysed 24 hours after infection for S protein detection by Western blot analysis (*A*, shown only for the MOI of 0.01). In parallel, viral genomic RNA copies (*B*, left panel) or infectious MERS-CoV particles (*B*, right panel) in supernatants of MERS-CoV–infected Caco-2 cells (with and without PCI treatment) were determined by quantitative reverse-transcription polymerase chain reaction (upE assay) or plaque assay.

To determine whether proprotein convertase activity is required for MERS-S–dependent virus-cell fusion, we used a previously reported vector system [14]. Notably, the addition of PCI to cells producing MERS-S–harboring lentiviral particles reduced transduction of 293T target cells by roughly 100-fold (Figure 4*B*). In contrast, infectivity of particles bearing LASV-GPC or EBOV-GP was not affected (Figure 4*B*), in keeping with the findings that processing of LASV-GPC is not inhibited by PCI (Figure 3) and that processing of EBOV-GP by proprotein convertases is dispensable for GP-driven virus-cell fusion [36, 37]. Of note, no inhibitory effect was detected when 293T cells transfected to express DPP4 were chosen as targets (Figure 4*B*), indicating that S protein processing in virus-producing cells might be dispensable for infectivity when target cells express robust amounts of DPP4. In sum, these results suggest that proprotein convertase activity is largely dispensable for MERS-S–driven cell-cell and virus-cell fusion, at least when target cells produce high levels of DPP4.

Proprotein Convertase Activity Is Not Required for MERS-CoV Infectivity

The lack of a prominent inhibitory effect of PCI on MERS-S–driven cell-cell and virus-cell fusion might be due to high levels of directed MERS-S and DPP4 expression in these experimental systems. Therefore, we assessed whether PCI inhibits MERS-CoV spread in target cells expressing endogenous DPP4. PCI treatment of Caco-2 and Vero B4 cells infected with MERS-CoV did not inhibit total MERS-S expression but reduced S protein cleavage efficiently and in a concentration-dependent manner (Figure 5*A* and data not shown), confirming that MERS-S is a substrate of proprotein convertases in infected cells. However, PCI treatment did not reduce MERS-CoV propagation, as determined by the amount of viral RNA (Figure 5*B* and data not shown) or infectious units (Figure 5*B* and data not shown) present in culture supernatants. Similarly, treatment of MERS-CoV–infected cultures had little if any effect on the formation of cytopathic effects, as demonstrated by comparable destruction of the cell monolayer (Figure 5*C*). Thus, processing of MERS-S by proprotein convertases is dispensable for MERS-CoV infectivity in cell cultures.

Figure 5 continued. The experiment was performed in quadruplicates and repeated twice with 2 different MOIs, giving comparable results. Shown is 1 representative experiment (MOI, 0.001). *C*, Cytopathogenic effects of MERS-CoV (MOI, 0.1) infected Vero B4 cells 42 hours after infection in the presence and absence of PCI. Viral antigen was detected with a specimen from a patient with MERS (diluted 1:100), followed by a cyanine 2–labeled goat-anti human immunoglobulin G. Nuclei were stained with DAPI-containing mounting medium. Samples were analyzed by immunofluorescence microscopy (Zeiss), and pictures were taken at the same microscopic settings at a magnification of 200×. Similar results were obtained in 2 independent experiments performed with duplicate samples.

DISCUSSION

The processing of the glycoproteins of human immunodeficiency virus (HIV) [19] and highly pathogenic avian influenza viruses [20] by proprotein convertases is essential for viral infectivity. As a consequence, antiviral strategies aiming at the inhibition of these enzymes are being developed [31]. Our results add MERS-S to the list of proprotein convertase substrates and show that several RXXR motifs within MERS-S are required for efficient S protein processing. However, activity of proprotein convertases was dispensable for infectivity of MERS-CoV, indicating that, in the context of MERS-CoV infection, proprotein convertases do not constitute targets for antiviral intervention.

The observations that TMPRSS2 activates MERS-CoV [14, 16, 17] and other coronaviruses [38–40] and that knock out of TMPRSS2 has no phenotype in the absence of infection [41] defined TMPRSS2 as a target for therapeutic intervention. However, TMPRSS2 might not be the only cellular protease that constitutes a potential therapeutic target in the context of coronavirus infection. Thus, cleavage of MERS-S during biogenesis in infected cells might be required for subsequent cleavage and activation of the S protein by TMPRSS2 during viral uptake into target cells. Such a scenario would not be unprecedented, given that entry mediated by the glycoproteins of certain bunyaviruses depends on glycoprotein processing during synthesis in the constitutive secretory pathway and, as suggested by a recent report, during viral uptake into target cells [42]. Alternatively, it is conceivable that processing of MERS-S in infected cells is dispensable for virus-cell but required for cell-cell fusion, which results in formation of syncytia, a feature of MERS-CoV pathogenesis [5].

We found that MERS-S is efficiently although not completely cleaved in S protein–transfected 293T cells and MERS-CoV–infected Vero B4 and Caco-2 cells, and inhibition experiments showed that proprotein convertase activity is essential for MERS-S cleavage. The finding that MERS-S is efficiently cleaved in transfected or MERS-CoV–infected cells is in keeping with several studies examining MERS-S expression for other purposes [16, 43, 44]. One report showed that MERS-S is not cleaved in transfected cells [17]; however, a MERS-S variant with a truncated cytoplasmic tail was examined, and it is conceivable that this deletion interfered with S protein exposure to or recognition by proprotein convertases. Alternatively, the cells used for S protein expression might have produced only low amounts of proprotein convertases. Robust S protein processing by proprotein convertases has also been reported for mouse hepatitis virus strain A59 [45, 46] and IBV [18, 47], while cleavage of SARS-S by these proteases is inefficient [48–50]. Thus, MERS-S belongs to the subgroup of coronavirus S proteins that are substrates of proprotein convertases, and MERS-S processing in different cellular systems, including the colon-derived cell line Caco-2, is robust.

Most proprotein convertases cleave the following motif: (R/K)-2nX-R↓, with n standing for 0–3 amino acids [34]. The finding that RXXR motifs located at the predicted border between S1 and S2 subunit are important for MERS-S processing by proprotein convertases might therefore not be unexpected. Nevertheless, it is noteworthy that at least 2 RXXR motifs had to be mutated in parallel to markedly reduce S protein processing. This suggests that the processing enzyme(s) can recognize >1 cleavage site. Indeed, the S2 fragment produced upon cleavage of PCM4 showed a slightly increased molecular weight relative to the fragment generated from MERS-S wild type, which would be in keeping with use of an upstream cleavage site, most likely potential cleavage site 3. Alternatively, the integrity of the RXXR motifs located at the border of the S1 and S2 subunit might be required to present a single cleavage site in a protease sensitive configuration.

Interference with ebolavirus glycoprotein processing by proprotein convertases is compatible with robust viral spread in vitro and in vivo [36, 37]. In contrast, proprotein convertase activity is required for full activity of certain MHV and IBV S proteins in cell-cell and virus-cell fusion reactions [18, 45–47]. Moreover, processing of the HIV envelope protein by proprotein convertases is essential for viral infectivity, and it has been suggested that processing of SARS-S, although being inefficient, is still required for full viral spread and for the establishment of cytopathic effects in infected cultures [48]. The present study provides evidence that efficient blockade of MERS-S processing by PCI has no appreciable effects on S protein activity in cell-cell fusion assay and does not modulate MERS-CoV spread in susceptible cells. In contrast, inhibition of MERS-S processing markedly reduced MERS-S–driven fusion of virions with 293 T cells expressing low amounts of endogenous DPP4. It is therefore conceivable that processing of MERS-S by proprotein convertases is required for optimal spread of MERS-CoV in target cells expressing low levels of DPP4. Such a scenario would be in keeping with the observation that inefficient SARS-S–driven cell-cell fusion due to limited receptor expression can be rescued by directed expression of S protein–activating proteases and vice versa [50].

In sum, our results identify MERS-S as a substrate of proprotein convertases but indicate that the activity of these enzymes is dispensable for MERS-CoV spread in target cell lines and potentially also in the infected human host. Furthermore, our findings suggest that the mode of S protein activation might depend on the level of receptor expression, a scenario that warrants further analyses.

Notes

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All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

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