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# Matriptase, HAT, and TMPRSS2 Activate the Hemagglutinin of H9N2 Influenza A Viruses

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**Influenza A viruses of the subtype H9N2 circulate worldwide and have become highly prevalent in poultry in many countries. Moreover, they are occasionally transmitted to humans, raising concern about their pandemic potential. Influenza virus infectivity requires cleavage of the surface glycoprotein hemagglutinin (HA) at a distinct cleavage site by host cell proteases. H9N2 viruses vary remarkably in the amino acid sequence at the cleavage site, and many isolates from Asia and the Middle East possess the multibasic motifs R-S-S-R and R-S-R-R, but are not activated by furin. Here, we investigated proteolytic activation of the early H9N2 isolate A/turkey/Wisconsin/1/66 (H9-Wisc) and two recent Asian isolates, A/quail/Shantou/782/00 (H9-782) and A/quail/Shantou/2061/00 (H9-2061), containing mono-, di-, and tribasic HA cleavage sites, respectively. All H9N2 isolates were activated by human proteases TMPRSS2 (transmembrane protease, serine S1 member 2) and HAT (human airway trypsin-like protease). Interestingly, H9-782 and H9-2061 were also activated by matriptase, a protease widely expressed in most epithelia with high expression levels in the kidney. Nephrotropism of H9N2 viruses has been observed in chickens, and here we found that H9-782 and H9-2061 were proteolytically activated in canine kidney (MDCK-II) and chicken embryo kidney (CEK) cells, whereas H9-Wisc was not. Virus activation was inhibited by peptide-mimetic inhibitors of matriptase, strongly suggesting that matriptase is responsible for HA cleavage in these kidney cells. Our data demonstrate that H9N2 viruses with R-S-S-R or R-S-R-R cleavage sites are activated by matriptase in addition to HAT and TMPRSS2 and, therefore, can be activated in a wide range of tissues what may affect virus spread, tissue tropism and pathogenicity.**

**H**uman influenza A viruses cause acute respiratory illness that affects millions of people during seasonal outbreaks and occasional pandemics and are therefore of major public health concern. Avian influenza A viruses are responsible for recurrent outbreaks in chickens and turkeys that may be connected with high morbidity and mortality and lead to serious economic losses in the poultry industry. Influenza A viruses belong to the family of *Orthomyxoviridae* and contain a segmented single-stranded RNA genome of negative polarity that codes for 11 to 13 proteins (1). Based on antigenic criteria of the two surface glycoproteins hemagglutinin (HA) and neuraminidase (NA), influenza A viruses are divided into 17 HA (H1 to H17) and 10 NA (N1 to N10) subtypes (2). Most subtypes circulate in wild aquatic birds, their natural reservoir, and are occasionally transmitted to other species, including poultry, pigs, and humans. Avian influenza viruses (AIV) differ in their pathogenicity and are classified as either low- or high-pathogenicity avian influenza viruses (LPAIV or HPAIV, respectively). LPAIV replicate primarily in the intestinal and also in the respiratory tract of birds, cause mild or asymptomatic infections, and spread via the fecal-oral route. In contrast, HPAIV cause systemic infections in poultry, with mortality rates up to 100%. All HPAIV belong to the subtypes H5 and H7, but not all H5 and H7 viruses are highly pathogenic (3, 4).

Influenza virus replication is initiated by the major viral surface glycoprotein hemagglutinin (HA), which binds to sialic acid-containing receptors and mediates fusion of the viral envelope with the endosomal membrane in order to release the virus genome into the target cell. HA is synthesized as a precursor protein, HA0, and has to be cleaved at a distinct arginine-glycine peptide bond by a host cell protease into the subunits HA1 and HA2 to gain its fusion capacity. Cleavage of HA0 is a prerequisite for a

conformational change at low pH in the endosome that triggers membrane fusion and is, therefore, essential for viral infectivity and spread. Depending on the amino acid sequence at the cleavage site, HAs vary in their susceptibility to different host cell proteases. Most LPAIV and mammalian viruses, including seasonal and pandemic human viruses, contain a single arginine (R) or rarely a lysine (K) at the HA cleavage site and are cleaved by trypsin *in vitro* (5). Relevant trypsin-like proteases are present in a restricted number of tissues, such as the respiratory or intestinal tract. We identified the type II transmembrane serine proteases (TTSPs) HAT (human airway trypsin-like protease) and TMPRSS2 (transmembrane protease, serine S1 member 2) as HA-activating enzymes in the human airway epithelium (6). More recently, the related protease TMPRSS4 was shown to cleave HA with a monobasic cleavage site too (7). In contrast, HPAIV of subtypes H5 and H7 possess a multibasic HA cleavage site of the consensus sequence R-X-R/K-R that is activated by ubiquitous proteases furin and proprotein convertase 5/6 (PC5/6), supporting systemic infections (4, 8, 9).

Within the last few years, influenza viruses of the subtype H9N2 have attracted particular attention. H9N2 viruses were first

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TABLE 1 Virus isolates and HA cleavage sites<sup>a</sup>

| Virus isolate                  | Abbreviation | HA1 ↓ HA2 cleavage site <sup>b</sup> |                                          | Accession no. |
|--------------------------------|--------------|--------------------------------------|------------------------------------------|---------------|
| A/quail/Shantou/782/00 (H9N2)  | H9-782       | P-A-                                 | <b>R</b> -S-S- <b>R</b> ↓ G-L-F          | EF154908      |
| A/quail/Shantou/2061/00 (H9N2) | H9-2061      | P-A-                                 | <b>R</b> -S- <b>R</b> - <b>R</b> ↓ G-L-F | EF154912      |
| A/turkey/Wisconsin/1/66 (H9N2) | H9-Wisc      | P-A-                                 | V-S-S- <b>R</b> ↓ G-L-F                  | CY087824      |
| A/SouthCarolina/1/18 (H1N1)    | H1-SC18      | P-A-                                 | I-Q-S- <b>R</b> ↓ G-L-F                  | AF117241      |
| A/Hamburg/5/09 (H1N1)          | H1-HH09      | P-A-                                 | I-Q-S- <b>R</b> ↓ G-L-F                  | HQ111364      |
| A/HongKong/1/68 (H3N2)         | H3-HK68      | P-A-                                 | K-Q-T- <b>R</b> ↓ G-L-F                  | CY112249      |
| A/ostrich/Italy/984/00 (H7N1)  | H7-Italy     | P-E-I-P-K-G-S-                       | <b>R</b> -V- <b>R</b> - <b>R</b> ↓ G-L-F | DQ991336      |
| A/chicken/Germany/N/49 (H10N7) | H10-Germany  | P-E-V-                               | V-Q-G- <b>R</b> ↓ G-L-F                  | GQ176136      |

<sup>a</sup> Abbreviations of viruses and NCBI database accession numbers of each HA are given.

<sup>b</sup> Amino acid sequences at the HA0 cleavage sites were aligned. The cleavage site between HA1 and HA2 is indicated by a downward arrow. Arginines at the cleavage site are shown in boldface.

isolated from turkeys in the United States in 1966 (10). However, since the mid-1990s, H9N2 viruses have become endemic in poultry in many countries of Asia and the Middle East, and vaccines have been deployed to bring the disease under control (3, 11, 12). Moreover, H9N2 viruses have been repeatedly isolated from pigs and humans with influenza-like illness, and seroepidemiological studies indicate that asymptomatic human H9N2 infections are not uncommon (11, 13, 14). H9N2 viruses show a high genetic compatibility with other subtypes and have undergone extensive reassortments and rapid evolution in diverse host species worldwide (15–18). Many H9N2 isolates have acquired human virus-like receptor specificity with preferential binding to  $\alpha$ 2,6-linked sialic acids (19) and are able to replicate efficiently in human airway epithelial cells, mice, and ferrets without prior adaptation, indicating that they have already adapted to mammalian hosts (11, 20–22). In general, H9N2 viruses are defined as LPAIV. However, they have been responsible for widespread and severe disease in poultry farms in several countries of Asia and the Middle East within the last decade (3, 11, 23, 24). Mortality has been associated with virus replication in various organs, including the trachea, lung, and kidney. In addition, coinfections with bacteria or infectious bronchitis virus (IBV) have been shown to exacerbate disease in chickens (23, 25–27). Moreover, some H9N2 strains cause significant morbidity and mortality in experimentally infected mice (11, 20).

H9N2 influenza viruses vary remarkably in the amino acid sequence at the HA cleavage site. While H9N2 viruses in America, Europe, and Africa contain diverse monobasic HA cleavage site motifs, many recent H9N2 isolates from Asia and the Middle East possess di- or tribasic HA cleavage sites of the sequence R-S-S-R or R-S-R-R. Importantly, in contrast to the cleavage sites of HPAIV of subtypes H5 and H7, the di- or tribasic cleavage sites of H9N2 viruses evolved by substitution and not by insertion of basic amino acids (Table 1) and are not cleaved by furin (28, 29).

In the present study, we investigated proteolytic activation of the early H9N2 isolate A/turkey/Wisconsin/1/66 with a monobasic cleavage site and two more recent H9N2 isolates from quails with R-S-S-R and R-S-R-R cleavage motifs (Table 1). We show that all H9N2 viruses were activated by HAT and TMPRSS2. Interestingly, H9N2 isolates with an R-S-S-R or R-S-R-R cleavage site were also activated by matriptase. Matriptase is a member of the TTSP family that is expressed in epithelial cells of most tissues (30, 31). Our data suggest that cleavage of HA by matriptase supports proteolytic activation of H9N2 viruses in a wider range of tissues and may affect organ tropism, virus spread, and pathogenicity.

## MATERIALS AND METHODS

**Cells.** 293T human embryonic kidney cells and Madin-Darby canine kidney (MDCK) cell lines MDCK(H) (28, 32) and MDCK-II (originally provided by K. Simons, Max-Planck-Institute of Molecular Cell Biology and Genetics, Dresden, Germany) were cultivated in Dulbecco's modified Eagle medium (DMEM; Gibco) supplemented with 10% fetal calf serum (FCS; Gibco), glutamine, penicillin, and streptomycin. MDCK-HAT and MDCK-TMPRSS2 cells, which express HAT and TMPRSS2, respectively, under doxycycline-dependent transcriptional activation have been described elsewhere (33) and were maintained in DMEM as described above, supplemented with 0.3 mg/ml Geneticin (Gibco) and 2  $\mu$ g/ml puromycin (InvivoGen). Expression of either protease was induced by the addition of 0.2  $\mu$ g/ml doxycycline (InvivoGen) to the medium. All cell growth and incubations occurred at 37°C and 5% CO<sub>2</sub>.

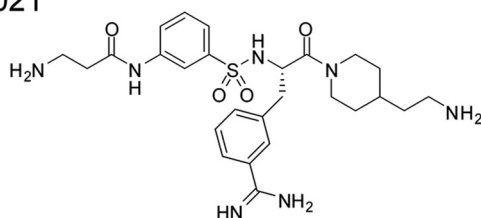
Chicken embryo kidney (CEK) cells were prepared from kidneys of 15-day-old specific-pathogen-free (SPF) chicken embryos (VALO Bio-Media). Kidneys were isolated and digested with 0.25% trypsin (Gibco) in glucose-potassium-sodium (GPS) buffer (1.0 g/liter D-glucose, 0.4 g/liter KCl, 8.0 g/liter NaCl, 0.12 g/liter NaH<sub>2</sub>PO<sub>4</sub> × 2H<sub>2</sub>O, 0.24 g/liter NaH<sub>2</sub>PO<sub>4</sub> × H<sub>2</sub>O) for 2 h at room temperature. The cell suspension was pelleted by centrifugation (3 min at 900 rpm) and resuspended in growth medium. Cells were seeded in collagen (BD Biosciences)-coated tissue plates or on collagen-coated coverslips and incubated at 37°C and 5% CO<sub>2</sub>. At 24 h after seeding, confluent CEK monolayers were used for virus infection studies or isolation of total RNA.

**Viruses.** The influenza viruses used in this study were A/quail/Shantou/2061/00 (H9N2), A/quail/Shantou/782/00 (H9N2) (34), A/turkey/Wisconsin/1/66 (H9N2), A/ostrich/Italy/984/00 (H7N1) (kindly provided by I. Capua, Istituto Zooprofilattico Sperimentale delle Venezie, Viale dell'Università, Legnaro, Italy), A/Hamburg/5/09 (H1N1), and A/chicken/Germany/N/49 (H10N7). Avian influenza viruses were propagated in 11-day-old embryonated chicken eggs. The pandemic virus A/Hamburg/5/09 (H1N1) and mutant virus H9-2061mut were propagated in MDCK(H) cells in infection medium containing 1  $\mu$ g/ml tosyl phenylalanyl chloromethyl ketone (TPCK)-treated trypsin (Sigma). Cell supernatants and allantoic fluid were cleared by low-speed centrifugation and stored at –80°C.

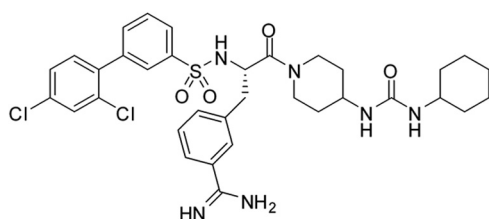
**Antibodies.** Polyclonal antibodies against A/quail/Shantou/2061/00 (H9N2), A/Hamburg/5/09 (H1N1), A/chicken/Rostock/34 (H7N1), and A/Aichi/2/68 (H3N2) were derived from rabbits. A monoclonal antibody against the influenza A virus nucleoprotein (NP) was provided by A. Klimov (Centers for Disease Control and Prevention, Atlanta, GA). Species-specific horseradish peroxidase (HRP)-conjugated secondary antibodies were purchased from Dako.

**Plasmids, mutagenesis, and transfection.** The mammalian expression plasmid pCAGGS-H3-HK68, coding for the HA of A/HongKong/1/68 (H3N2), has been described previously (6). To generate pHW2000 expression plasmids encoding the HA of H9-782 or H9-2061, viral RNA was isolated using the QIAamp viral RNA minikit (Qiagen) and reverse transcribed and amplified by using the OneStep reverse transcription

## MI-021



## MI-462



## MI-701

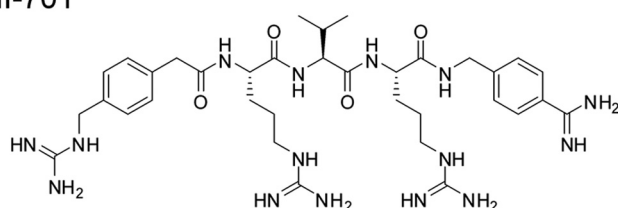


FIG 1 Structures of the matriptase (MI-021 and MI-462) and furin (MI-701) inhibitors used.

(RT)-PCR kit (Qiagen) with universal primers described elsewhere (35). The cDNAs were cloned into the pHW2000 plasmid using BsmBI restriction sites. To generate pCAGGS expression plasmids encoding the HA of H9-Wisc or H1-HH09, the cDNAs were synthesized as described above by using HA-specific primers (primer sequences are available on request). The cDNAs of H9-Wisc and H1-HH09 were subcloned into pCAGGS plasmid using XhoI and NotI or EcoRI and NotI restriction sites, respectively. To generate the plasmid pCAGGS-H1-SC18, encoding the HA of A/South Carolina/1/18 (H1N1), the HA cDNA was synthesized by GenScript Corporation (Piscataway, NJ) and inserted into the pCAGGS plasmid using XmaI and NheI restriction sites. Mutagenesis at the HA cleavage site of H9-2061mut and H1-HH09mut was performed using the QuikChange II site-directed mutagenesis kit (Stratagene) according to the protocol of the manufacturer. Primer sequences are available on request. All transfections were performed using Lipofectamine 2000 (Invitrogen) according to the manufacturers' protocol.

**Generation of recombinant H9N2 virus.** Recombinant A/quail/Shantou/2061/00 (H9N2) virus with an A-S-R-R cleavage site (H9-2061mut) was generated by reverse genetics using the pHW2000-based eight-plasmid system described by Hoffmann et al. (36). Briefly, 293T cells were cotransfected with eight pHW2000 plasmids containing all eight influenza virus gene segments. At 48 h after transfection, the supernatant was treated with 1  $\mu$ g/ml TPCK-trypsin for 1 h at 37°C and then incubated on MDCK(H) cells for 1 h. Afterwards, cells were washed and maintained in infection medium containing 1  $\mu$ g/ml TPCK-trypsin for 2 to 3 days. Virus-containing supernatants were analyzed by plaque assay and sequencing, and stock virus was propagated on MDCK(H) cells.

**Matriptase and protease inhibitors.** The serine protease domain of human matriptase was expressed in *Escherichia coli* and purified and activated as described previously (37). The matriptase inhibitors MI-021 (37) and MI-462 (38) and the furin inhibitor MI-701 (39) have been described previously. The structures of the inhibitors are shown in Fig. 1.

**Cleavage of HA by matriptase and trypsin.** At 48 h after transfection with HA-expressing plasmids, the cells were washed and resuspended in phosphate-buffered saline (PBS), split into three aliquots, and pelleted by centrifugation. Subsequently, cell pellets were incubated with either 3  $\mu$ g/ml of recombinant soluble catalytic domain of matriptase in reaction buffer (25 mM Tris, 150 mM NaCl, 0.05% Brij 58 [pH 7.5]) for 3 h at 37°C or 1  $\mu$ g/ml TPCK-trypsin in reaction buffer for 1 h at 37°C to allow cleavage of HA expressed on the surface of the cells. The third cell pellet was mock treated by incubation in protease reaction buffer for 3 h at 37°C. Afterwards, cleavage of HA was analyzed by SDS-PAGE and Western blotting using HA-specific antibodies.

**SDS-PAGE and Western blotting.** To analyze cleavage of HA in transfected or infected cells, cell lysates were subjected to SDS-PAGE and Western blot analysis as described previously (40). To analyze released virus, the supernatants of infected cells were cleared from cell debris by low-speed centrifugation, and virus was pelleted by ultracentrifugation (28,000 rpm, 2 h, 4°C). Pellets were resuspended in reducing SDS sample buffer, heated at 95°C for 5 min, and subjected to SDS-PAGE and Western blot analysis.

**Infection of cells and multicycle viral replication.** Infection studies were performed using infection medium (DMEM supplemented with 0.1% bovine serum albumin [BSA], glutamine, and antibiotics). For analysis of multicycle viral replication kinetics, MDCK-II, MDCK-HAT, or MDCK-TMPRSS2 cells were seeded in 6-well plates and grown to confluence. MDCK-HAT and MDCK-TMPRSS2 cells were cultivated with or without doxycycline. Cells were inoculated with virus at a multiplicity of infection (MOI) of 0.0001 for 1 h, and then the inoculum was removed and the cells were incubated in infection medium for 48 h. As indicated, the medium was supplemented with 3  $\mu$ g/ml matriptase, 1  $\mu$ g/ml TPCK-trypsin, or 50  $\mu$ M peptide-mimetic inhibitor MI-021, MI-462, or MI-701. At the indicated time points postinfection (p.i.), virus titers in the supernatants were determined by plaque assay.

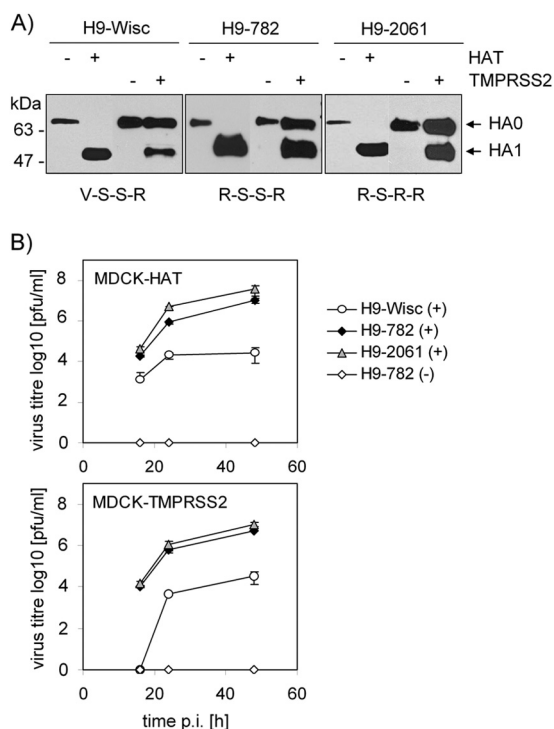
To analyze HA cleavage in chicken embryo kidney (CEK) epithelial cells, confluent monolayers in 6-well plates were inoculated with virus at an MOI of 0.1 to 0.01 for 1 h and then incubated in the absence or presence of TPCK-trypsin (0.5  $\mu$ g/ml) and with or without protease inhibitors for 24 h. Cell lysates were subjected to SDS-PAGE and immunoblotting as described above. To analyze virus spread in CEK cells, cells seeded on coverslips were infected with the indicated viruses at an MOI of 0.01 to 0.001 and incubated in the absence or presence of 0.5  $\mu$ g/ml TPCK-trypsin for 24 h. Afterwards, cells were washed with PBS, fixed, and immunostained as described below.

**Plaque assay.** Virus titers were determined by plaque assay with Avicel overlay as described elsewhere (41). Briefly, MDCK(H) cells were inoculated with 10-fold serial dilutions of each sample and incubated with Avicel overlay containing 1  $\mu$ g/ml TPCK-trypsin or without trypsin for 24 to 48 h. Afterwards, cells were fixed, permeabilized, and immunostained using an antibody against NP, peroxidase-conjugated secondary antibodies, and subsequent incubation with the peroxidase substrate TrueBlue (KPL).

**Immunostaining of infected CEK cells.** Infected CEK cells were fixed and permeabilized by incubation with methanol-acetone (1:1) for 20 min on ice and afterwards blocked by incubation with 10% normal horse serum (Sigma) and 0.1% Tween 80 in PBS for 1 h. The cells then were incubated with a monoclonal antibody against NP and a fluorescein isothiocyanate (FITC)-labeled secondary antibody. Cell nuclei were counterstained using DAPI (4',6-diamidino-2-phenylindole). Cells were mounted on microscopic slides using Fluoprep (bioMérieux). Microscopic analysis was performed with a fluorescence microscope (Zeiss Axiophot).

## RESULTS

**HAT and TMPRSS2 activate H9N2 viruses with mono-, di-, and tribasic HA cleavage sites.** In the present study, we wished to identify host cell proteases that are capable of activating the HAs of



**FIG 2** Proteolytic activation of H9N2 viruses with mono-, di-, and tribasic cleavage sites by TMPRSS2 and HAT. (A) MDCK-HAT and MDCK-TMPRSS2 cells with doxycycline-induced expression (+) of either HAT or TMPRSS2 were infected with H9N2 isolate H9-Wisc, H9-782, or H9-2061 at an MOI of 0.1. Cells infected in the absence of doxycycline (–) were used as a control. At 24 h p.i., virus-containing supernatants were analyzed by SDS-PAGE and Western blotting using a polyclonal H9N2 serum that recognizes HA0 and HA1. The amino acid sequence at the HA cleavage site of each virus is indicated in the one-letter code. (B) MDCK-HAT and MDCK-TMPRSS2 cells were infected with the indicated H9N2 viruses at an MOI of 0.0001 and incubated in the presence (+) or absence (–) of doxycycline. At indicated time points p.i., viral titers were determined by plaque assay. Results are the mean values from three independent experiments.

different H9N2 viruses with mono-, di-, and tribasic cleavage sites. The early H9N2 isolate H9-Wisc contains a single arginine at the HA cleavage site, while the two current Asian isolates H9-782 and H9-2061 possess the motifs R-S-S-R and R-S-R-R, respectively (Table 1). Two recent studies demonstrated that H9N2 with such a di- or tribasic cleavage site is not activated by furin, although they match the minimal consensus sequence (28, 29).

First, we investigated whether HAT and TMPRSS2 that were identified as HA-activating proteases in the human airway epithelium (6) cleave H9 at different cleavage sites. For that purpose, we used MDCK-HAT and MDCK-TMPRSS2 cells that express either protease under doxycycline-induced transcriptional activation (33). The cells were infected with H9-Wisc, H9-782, or H9-2061 and incubated in the presence or absence of doxycycline for 24 h. Subsequently, HA cleavage of progeny virus was analyzed by SDS-PAGE and Western blotting. As shown in Fig. 2A, both HAT and TMPRSS2 were capable of cleaving the HA0 of all three H9N2 isolates into HA1 and HA2 (not detected by the antiserum). In the presence of HAT and TMPRSS2, large amounts of viral proteins were detected in cell supernatants, indicating multicycle viral replication (Fig. 2A). In contrast, progeny virus released from cells without expression of either HAT or TMPRSS2 contained only

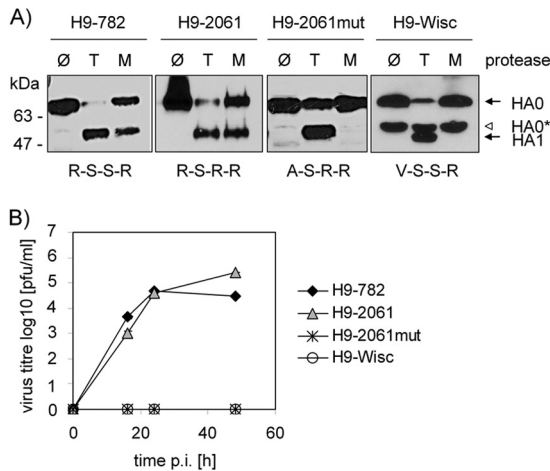
noncleaved HA0 and smaller amounts of viral protein were detected due to single-cycle virus replication. In HAT-expressing cells, the HA0 of all three viruses was completely cleaved, whereas cleavage of HA0 in TMPRSS2-expressing cells was less efficient. The growth curves of H9-Wisc, H9-782, and H9-2061 in MDCK-HAT and MDCK-TMPRSS2 cells are shown in Fig. 2B. The results confirm that the three H9N2 viruses were able to perform multicycle replication in these cells, providing further evidence for proteolytic activation of HA. Virus replication in the absence of doxycycline-induced protease expression was used as a control. None of the viruses was able to grow in cells lacking HAT or TMPRSS2 expression, as shown for H9-782 (Fig. 2B). Taken together, our data demonstrate that HAT and TMPRSS2 support proteolytic activation of H9N2 viruses at mono-, di-, and tribasic cleavage sites.

**Matriptase activates H9N2 viruses at the HA cleavage motifs R-S-S-R and R-S-R-R.** The preferred cleavage site of the TTSP matriptase shows the dibasic amino acid motif R-X-S-R or K-X-S-R, although natural substrates may also be cleaved at other sites (42–44). It was therefore of interest to find out if matriptase cleaves H9 with R-S-S-R and R-S-R-R motifs. 293T cells were transfected with expression plasmids encoding the HA of H9-782 or H9-2061 or H9-Wisc as a control. In addition, an HA cleavage site mutant of H9-2061 that contains the motif A-S-R-R instead of R-S-R-R was generated by site-directed mutagenesis and analyzed for cleavage by matriptase. At 24 h posttransfection, the cells were harvested and incubated with a recombinant, soluble catalytic domain of matriptase to allow cleavage of HA at the surface of cells. Matriptase activity has been demonstrated to depend solely on the catalytic domain of matriptase (37, 45). As controls, HA-expressing cells were either incubated with trypsin or remained untreated. Subsequently, cells were lysed and analyzed by SDS-PAGE and immunoblotting. As shown in Fig. 3A, H9-782 and H9-2061 were cleaved by matriptase. In contrast, neither H9-Wisc nor H9-2061mut was cleaved by matriptase, indicating that the arginine in position P4 is crucial for recognition by this protease. As expected, all HAs were cleaved by trypsin. The prominent band between HA0 and HA1 always detected after transient overexpression of H9-Wisc represents nonglycosylated HA0 (HA0\*), as indicated by treatment with *N*-glycosidase F (data not shown).

We next investigated whether cleavage by matriptase activates the fusogenic potential of HA. For that purpose, MDCK(H) cells that do not support activation of H9 by endogenous proteases (Fig. 4A) were infected with H9-782, H9-2061, H9-Wisc, or H9-2061mut at an MOI of 0.0001 and incubated in the presence of exogenous matriptase for 48 h. At the indicated time points, virus titers were determined by plaque assay. Multicycle replication of H9-782 and H9-2061 was observed in the presence of matriptase, while H9-Wisc and H9-2061mut were not able to replicate under these conditions (Fig. 3B).

In conclusion, our data demonstrate that matriptase supports proteolytic activation of H9N2 viruses with cleavage site motifs R-S-S-R and R-S-R-R, but not that of those containing a single arginine or the motif A-S-R-R.

**Endogenous matriptase supports multicycle replication of H9N2 viruses in MDCK-II and CEK cells.** To further elucidate matriptase as an HA-activating protease, we examined proteolytic activation of H9-782 and H9-2061 in cell lines that express endogenous matriptase. Since MDCK cells have been reported to express matriptase (46), we analyzed plaque formation of H9N2

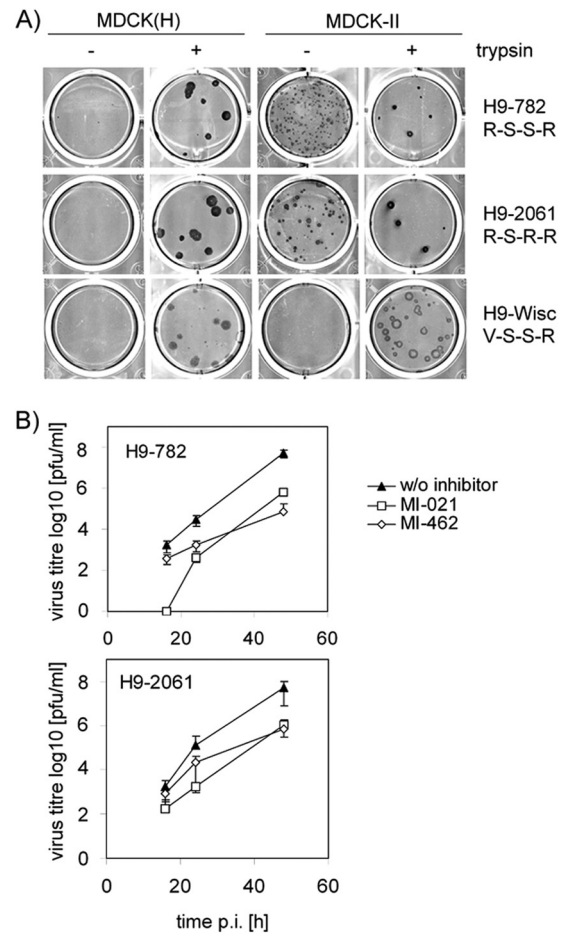


**FIG 3** Matriptase activates H9 with R-S-S-R and R-S-R-R cleavage sites. (A) 293T cells were transfected with plasmids encoding the HA of H9-782, H9-2061, H9-2061mut, or H9-Wisc. At 24 h posttransfection, cells were harvested and either treated with soluble matriptase (M) or trypsin (T) or remained untreated (Ø). Cells were lysed and subjected to SDS-PAGE under reducing conditions and Western blot analysis using antibodies against H9N2. The additional band in H9-Wisc-expressing cells represents nonglycosylated HA0 (HA0\*). The amino acid sequence at the HA cleavage site of each virus is indicated in the one-letter code. (B) MDCK(H) cells were infected with H9-Wisc, H9-782, H9-2061, or H9-2061mut at an MOI of 0.0001 and incubated in the presence of soluble matriptase (3 µg/ml) for 48 h. At 16, 24, and 48 h p.i., virus propagation was analyzed by plaque titration. The results shown are the mean values from two independent experiments.

viruses in two different MDCK cell lines: MDCK-II cells, which are the most common MDCK cell line, and MDCK(H) cells, a subset of MDCK cells that has been described previously (28, 32). It has to be mentioned that MDCK-HAT and MDCK-TMPRSS2 cells are derived from MDCK(H) cells. Interestingly, H9-782 and H9-2061 were able to form plaques in the absence of exogenous trypsin due to activation by an endogenous protease in MDCK-II cells, but not in MDCK(H) cells (Fig. 4A). In contrast, H9-Wisc required trypsin for plaque formation in both MDCK cell lines. The cleavage specificity of the endogenous HA-activating protease in MDCK-II cells strongly suggested matriptase as the relevant protease.

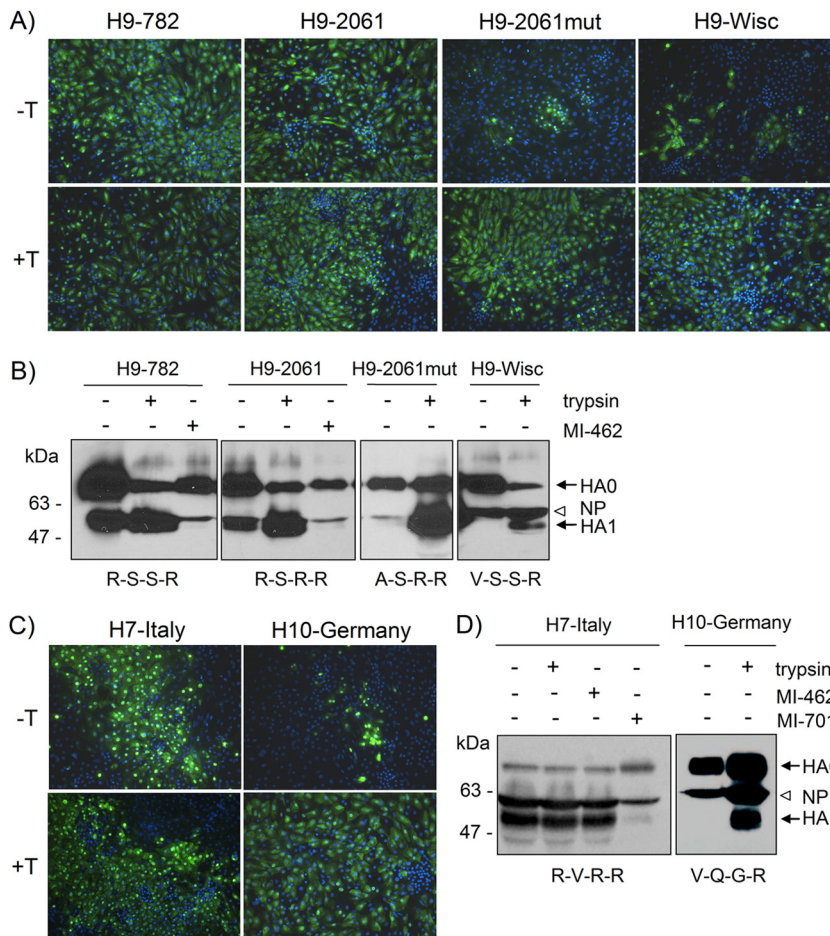
To further examine whether matriptase may be responsible for proteolytic activation of H9-782 and H9-2061 in MDCK-II cells, the cells were infected and virus propagation was determined in the presence of synthetic inhibitor MI-021 or MI-462, which inhibit matriptase with  $K_i$  values of 3.8 and 5.1 nM, respectively (37, 38). As shown in Fig. 4B, virus titers of H9-782 and H9-2061 were reduced 100- to 1,000-fold at 48 h p.i. in the presence of 50 µM MI-021 and MI-462 compared to the level in nontreated cells. These data strongly suggest that matriptase is responsible for activation of H9 with R-S-S-R and R-S-R-R cleavage sites in MDCK-II cells.

In recent H9N2 outbreaks in chicken farms and in experimentally infected chickens, nephrotropism of the virus was observed (23, 26, 27). Matriptase is expressed in the kidney (31, 47), and we therefore examined whether matriptase may play a role in virus replication in primary chicken embryo kidney (CEK) epithelial cells. By using primers specific for chicken matriptase (accession no. XM\_417872), we were able to amplify matriptase mRNA from total RNA of CEK cells, indicating that matriptase is likely ex-



**FIG 4** Proteolytic activation of H9-782 and H9-2061 in MDCK-II cells and its suppression by matriptase inhibitors. (A) MDCK(H) and MDCK-II cells were infected with either H9-782, H9-2061, or H9-Wisc and incubated with an Avicel overlay with (+) or without (-) trypsin for 48 h. Infected cells were fixed and immunostained using antibodies against H9N2. (B) MDCK-II cells were infected with H9N2 virus H9-782 or H9-2061 at a low MOI and incubated in the presence of matriptase inhibitor MI-021 or MI-462 (each at 50 µM) for 48 h. At the indicated time points, virus titers were determined by plaque assay. The growth curves shown are the mean values from three independent experiments.

pressed in CEK cells (data not shown). Next, CEK cells were infected with H9-782, H9-2061, H9-Wisc, and H9-2061mut and incubated in the absence or presence of trypsin for 24 h. Immunostaining of infected cells revealed that H9-782 and H9-2061 were able to replicate and spread efficiently in CEK cells in the absence of trypsin, while H9-Wisc and H9-2061mut again required trypsin for efficient multicycle replication (Fig. 5A). Western blot analysis demonstrated cleavage of HA of H9-782 and H9-2061 independent of exogenous trypsin in CEK cells, and cleavage was inhibited by treatment with the matriptase inhibitor MI-462 (50 µM) (Fig. 5B). In contrast, HA0 of H9-Wisc and H9-2061mut was only cleaved in trypsin-treated cells. These data strongly suggest that matriptase is responsible for the activation and spread of H9-782 and H9-2061 in CEK cells. As additional controls, CEK cells were infected with HPAIV H7-Italy and the LPAIV H10-Germany, which are activated by furin- and trypsin-like proteases, respectively. As expected, H7-Italy was proteolyti-



**FIG 5** Proteolytic activation of H9-2061 and H9-782 in chicken embryo kidney (CEK) epithelial cells. (A) Primary CEK cells were infected with the indicated viruses at an MOI of 0.01 to 0.001 and incubated in the absence (–T) or presence (+T) of trypsin (0.5 µg/ml) for 24 h. Cells were fixed and immunostained using an antibody against NP and an FITC-conjugated secondary antibody. Cell nuclei were counterstained with DAPI. (B) Primary CEK cells were infected with the indicated virus and incubated in either the absence or presence of trypsin (0.5 µg/ml) and with or without matriptase inhibitor MI-462 (50 µM). At 24 h p.i., cell lysates were subjected to SDS-PAGE and immunoblotting using antibodies against H9N2. The amino acid sequences of the HA cleavage sites are indicated in the one-letter code. (C) As controls, CEK cells were infected with HPAIV H7-Italy and LPAIV H10-Germany in the absence or presence of trypsin for 24 h and immunostained against NP as described. (D) CEK cells were infected with the indicated virus at an MOI of 0.01 and incubated for 24 h with or without trypsin and in the presence or absence of peptide-mimetic inhibitors of matriptase (MI-462) or furin (MI-701) (each at 50 µM). Cell lysates were analyzed by SDS-PAGE and immunoblotting using virus-specific antibodies. The amino acid sequence at the HA cleavage site of each virus is indicated in the one-letter code.

cally activated in CEK cells and HA0 cleavage was inhibited by the furin inhibitor MI-701 but not by the matriptase inhibitor MI-462 (Fig. 5C and D). In contrast, H10-Germany required exogenous trypsin for proteolytic activation and spread in CEK cells, similar to H9-Wisc and H9-2061mut.

Our data demonstrate that matriptase can activate the HA of current H9N2 viruses with di- or tribasic cleavage site motifs R-S-S-R and R-S-R-R and thereby support proteolytic activation of HA and multicycle viral replication in kidney cells.

**Restriction of matriptase as an HA-activating protease to current H9N2 viruses.** Most influenza A viruses contain conserved amino acid sequences with a single arginine at the HA cleavage site (Table 2). Although the HA cleavage motifs I-Q-S-R and K-Q-T-R of human H1N1 and H3N2 viruses, respectively, do not fit the preferred substrate specificity of matriptase, we examined whether matriptase cleaves HA of these influenza viruses. To test this, 293T cells were transfected with expression plasmids encoding the HA of pandemic viruses from 1918 (H1-SC18), 1968

(H3-HK68), or the recent 2009 pandemic (H1-HH09) and were treated with matriptase or trypsin or remained untreated. In addition, the cleavage site R-S-S-R was introduced into the HA of H1-HH09 by amino acid substitution, and cleavage of HA by matriptase was analyzed too. None of the HAs was cleaved by matriptase, while efficient cleavage of each HA by trypsin was observed (Fig. 6). Interestingly, also the H1-HH09mut strain with the R-S-S-R cleavage site was not cleaved by matriptase, indicating that the cleavage site is not accessible to matriptase in H1, most likely due to steric hindrances.

Our data indicate that matriptase may be an HA-activating protease specific for H9N2 viruses with cleavage motifs R-S-S-R and R-S-R-R due to its cleavage specificity.

## DISCUSSION

Influenza viruses of subtype H9N2 are considered candidates that may cause the next pandemic. They have become established worldwide in several avian species and since the mid-1990s have

**TABLE 2** Amino acid consensus sequence at the HA cleavage site of LPAIV and mammalian influenza viruses of subtypes H1 to H17

| Subtype | Amino acid sequence at HA cleavage site <sup>a</sup> |
|---------|------------------------------------------------------|
| H1      | P-S-I-Q-S- <b>R</b> ↓ G-L-F                          |
| H2      | P-Q-I-E-S- <b>R</b> ↓ G-L-F                          |
| H3      | P-E-K-Q-T- <b>R</b> ↓ G-L-F                          |
| H4      | P-E-K-A-S/T- <b>R</b> ↓ G-L-F                        |
| H5      | P-Q-R-E-T- <b>R</b> ↓ G-L-F                          |
| H6      | P-Q-I-E-T- <b>R</b> ↓ G-L-F                          |
| H7      | P-E-I/N-P-K-G/T- <b>R</b> ↓ G-L-F                    |
| H8      | P-S-I/V-E-P- <b>R/K</b> ↓ G-L-F                      |
| H9      | P-A-R/K/X-S/L-R/X- <b>R</b> ↓ G-L-F                  |
| H10     | P-E-I/V-M/V-Q-G/E- <b>R</b> ↓ G-L-F                  |
| H11     | P-A-I-A-T/S- <b>R</b> ↓ G-L-F                        |
| H12     | P-Q-V-Q-N- <b>R</b> ↓ G-L-F                          |
| H13     | P-A-I-S-N/T- <b>R</b> ↓ G-L-F                        |
| H14     | P-G-K-Q-A- <b>R</b> ↓ G-L-F                          |
| H15     | P-E-K-I-R/H-T- <b>R</b> ↓ G-L-F                      |
| H16     | P-S-I-G-E- <b>R</b> ↓ G-L-F                          |
| H17     | P-Q-M-E-G- <b>R</b> ↓ G-L-F                          |

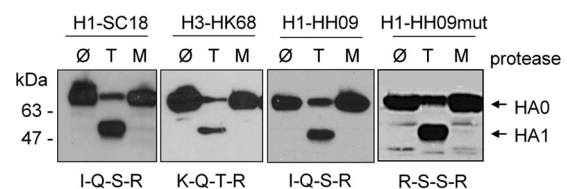
<sup>a</sup> The cleavage site is indicated by an arrow. All sequences were obtained from the NCBI database. The basic amino acid at the cleavage site is shown in boldface.

been responsible for widespread outbreaks in poultry farms in many countries (3). Moreover, H9N2 viruses have been repeatedly transmitted to humans and pigs in Asia (13, 14). In 2011, the WHO included the development of H9N2 vaccines as an important element of pandemic preparedness.

Proteolytic cleavage of the HA is essential for influenza virus infectivity and spread. H9N2 viruses vary in the amino acid sequence at the HA cleavage site, and many H9N2 isolates from Asia and the Middle East have attracted attention because they contain di- and tribasic HA cleavage motifs R-S-S-R and R-S-R-R typical for HPAIV. However, two recent studies demonstrated that these basic motifs are not cleaved by ubiquitous furin unless further basic amino acids are inserted at the cleavage site (28, 29). In the present study, we demonstrate that the Asian isolates H9-782 and H9-2061, containing R-S-S-R and R-S-R-R cleavage sites, respectively, can be activated by matriptase in addition to HAT and TMPRSS2. The TTSPs HAT and TMPRSS2 were identified as HA-activating proteases in the human airway epithelium and have been shown to activate different human H1N1 and H3N2 viruses at monobasic cleavage sites (6, 7, 40). HAT expression is prominent in the trachea and bronchi and was also detected in the gastrointestinal tract and in the skin. TMPRSS2 is expressed in epithelial cells of the respiratory, gastrointestinal, and urogenital tracts, with high expression levels in prostate and colon (47, 48). Recently, TMPRSS2 was shown to support proteolytic activation of HA with a monobasic cleavage site in human Calu-3 airway epithelial cells and Caco-2 intestinal epithelial cells (49, 50). Matriptase (also known as epithin, ST14, and MT-SP1) is one of the best-studied members of the TTSP family and plays essential roles in the formation and integrity of the oral and intestinal epithelium and the epidermis (30, 47, 51). Matriptase is widely expressed in multiple epithelial tissues, with high expression levels in the gastrointestinal tract and the kidney (31), and has also been demonstrated to be expressed in neural progenitor cells and neurons and in monocytes and macrophages (47, 52). Thus, matriptase may support proteolytic activation of HA in a wide range of tissues.

We found that H9-782 and H9-2061 were proteolytically activated in canine kidney (MDCK-II) and chicken embryo kidney (CEK) cells by an endogenous protease. In contrast, H9-Wisc and H9-2061mut required addition of exogenous trypsin for HA activation in both cell lines, similar to LPAIV H10-Germany. Virus propagation of H9-782 and H9-2061 in MDCK-II cells was suppressed about 100- to 1,000-fold by treatment with matriptase inhibitors MI-021 and MI-462, and MI-462 inhibited cleavage of H9 and virus replication in CEK cells. These data strongly suggest that matriptase is responsible for activation of H9-782 and H9-2061 in MDCK-II and CEK cells. In recent H9N2 outbreaks on chicken farms, virus replication was demonstrated in various organs, including trachea, lung, spleen, and kidney, and nephrotropism and longer persistence of the virus in the kidney were associated with increased mortality in some studies (23, 26, 27). The data presented here suggest that matriptase supports proteolytic activation and virus replication of H9N2 in the kidney and thereby may contribute to increased pathogenicity in chickens. Orthologs of matriptase have been identified in all vertebrate genomes examined to date (47), and proteolytic activation of H9-782 and H9-2061 in MDCK-II cells suggests that matriptase may support HA activation in several hosts. Some H9N2 strains were reported to cause lethal infections in mice with virus replication in the lung and the brain (11, 20). Matriptase has recently been shown to be expressed in neural progenitor cells and neurons (52). Thus, matriptase may also support proteolytic activation of H9N2 in the brain.

In the present study, we observed differences in proteolytic activation of H9-782 and H9-2061 in MDCK(H) and MDCK-II cells, indicating that matriptase activity is present in MDCK-II but not MDCK(H) cells. Matriptase is an 80- to 90-kDa transmembrane protease that is synthesized as an inactive zymogen, and activation, subcellular localization, and enzymatic activity are highly complex and tightly regulated by the endogenous inhibitors hepatocyte growth factor inhibitor 1 (HAI-1) and HAI-2 under physiological conditions (30, 53, 54). Furthermore, the catalytic domain of matriptase can be shed from the cell surface, in general complexed with HAI-1, and deregulated matriptase activity or the imbalance of the matriptase/HAI-1 ratio has been implicated in a variety of human cancers (44, 54). It remains unknown so far whether higher expression levels of matriptase or additional differences in the expression of HAI-1 and HAI-2 contribute to activation of H9 in MDCK-II cells in contrast to MDCK(H) cells.



**FIG 6** HA of human H1 and H3 viruses is not cleaved by matriptase. 293T cells were transfected with pCAGGS plasmids encoding the HA of pandemic viruses H1-SC18, H3-HK68, and H1-HH09 (Table 1) or the HA cleavage site mutant H1-HH09mut. The amino acid sequence at the cleavage site of each HA is indicated in the one-letter code. At 24 h posttransfection (p.t.), cells were incubated with soluble matriptase (M) or trypsin (T) or were mock treated (Ø), and subsequently cell lysates were subjected to SDS-PAGE and Western blotting using H1- or H3-specific antibodies.

Our data demonstrate that influenza viruses can possess cleavage site motifs that facilitate recognition of HA by additional host cell proteases and thereby support virus activation in a wider range of tissues. The influenza virus A/WSN/33 (H1N1), which is neurotropic in mice, contains the unusual HA cleavage site I-Q-Y-R with a tyrosine (Y) in P2 that was shown to facilitate efficient cleavage by plasmin, supporting proteolytic activation and virus spread to other tissues (55, 56). The HPAIV A/chicken/Pennsylvania/1370/83 (H5N2) contains the multibasic cleavage site K-K-K-R, which is not cleaved by furin because of the lysine (K) in P4. In addition, a carbohydrate side chain at Asn-11 of HA1 has been demonstrated to interfere with cleavage of this HA by steric hindrance, and loss of the glycosylation was associated with ubiquitous HA cleavage and virulence (57). Recently, the type II transmembrane protease MSPL/TMPRSS13, which is expressed in several tissues, has been shown to cleave the HA at such a motif and to enable systemic infection independent of furin (58). Therefore, the strict separation into HA with monobasic and multibasic cleavage sites that are activated by trypsin-like proteases and furin, respectively, does not apply to all influenza virus isolates, and attention should be drawn to unusual cleavage sites.

It is of particular interest to find out in future studies if other HAs are also susceptible to cleavage by matriptase. Matriptase prefers R or K in position P4, a nonbasic or basic amino acid at P3, Ser at P2, and R at P1 (R/K-X-S-R ↓) (42–44). According to this, substitution for the H9-2061 cleavage site R-S-R-R with A-S-R-R abolished cleavage by matriptase. The amino acid consensus sequence at the HA cleavage site of subtypes H1 to H17 indicates that besides some H9 viruses, only H3, H4, and LPAIV H5 and H14 subtypes possess an additional basic amino acid in position P4 (Table 2). The H5 of LPAIV contains the motif R-E-T-R. By using internally quenched fluorescent peptides, however, E in P3 has been reported to abolish cleavage by matriptase (42), indicating that H5 with this motif is not cleaved, too. The subtypes H3, H4, and H14 contain a K in position P4. Here, we observed that H3-HK68 with the K-Q-T-R motif is not activated by matriptase. Cleavage of H4 and H14 remains to be investigated. Interestingly, introduction of the R-S-S-R motif into the H1 of H1-HH09 did not facilitate cleavage by matriptase, although it was efficiently cleaved by trypsin, providing evidence that the HA *per se* was cleavable. Therefore, other factors together with the R-S-S-R motif, such as steric access of the cleavage site to the substrate-binding pocket, seem to facilitate cleavage of H9-782 and H9-2061 but not of H1-HH09mut by matriptase. The cleavage site of HA0 is located in a prominent surface loop (59), and the conformation and exposure of the loop or the presence of carbohydrate side chains may interfere with access of different proteases to different HA subtypes. During preparation of this article, a study by Hamilton et al. was published that investigated activation of HA of human influenza viruses of subtypes H1 and H3 by recombinant soluble matriptase (60). In agreement with our results, the HA of H1-SC18 and the HA of a 1968 H3N2 pandemic isolate were not cleaved by matriptase. However, Hamilton et al. found that the HA of a few H1N1 strains was activated by matriptase, including the 2009 H1N1 pandemic isolate A/California/04/09 with the monobasic cleavage site motif I-Q-S-R. This is in contrast to what we have observed for H1-HH09, and what contributes to the differences in cleavage of these two 2009 H1N1 isolates by matriptase remains to be investigated.

In conclusion, we demonstrated that H9N2 viruses with di-

and tribasic cleavage sites endemic in many countries of Asia and the Middle East can be proteolytically activated by matriptase, in addition to HAT and TMPRSS2, supporting HA activation in a wider range of tissues. To date, H9N2 viruses have caused only mild respiratory disease in humans. However, cocirculation of H9N2 viruses with other subtypes, including H5N1, H1N1, and H3N2 viruses, carries the permanent risk of the emergence of novel reassortants with increased pathogenicity. Activation of H9 by matriptase may contribute to the pathogenicity of such reassortant viruses. The prevalence of R-S-S-R and R-S-R-R cleavage sites therefore should be monitored in influenza virus surveillance of poultry, pigs, and humans.

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