Matriptase Proteolytically Activates Influenza Virus and Promotes Multicycle Replication in the Human Airway Epithelium

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Influenza viruses do not encode any proteases and must rely on host proteases for the proteolytic activation of their surface hemagglutinin proteins in order to fuse with the infected host cells. Recent progress in the understanding of human proteases responsible for influenza virus hemagglutinin activation has led to the identification of members of the type II transmembrane serine proteases TMPRSS2 and TMPRSS4 and human airway trypsin-like protease; however, none has proved to be the sole enzyme responsible for hemagglutinin cleavage. In this study, we identify and characterize matriptase as an influenza virus-activating protease capable of supporting multicycle viral replication in the human respiratory epithelium. Using confocal microscopy, we found that matriptase to colocalize with hemagglutinin at the apical surface of human epithelial cells and within endosomes, and we showed that the soluble form of the protease was able to specifically cleave hemagglutinins from H1 virus, but not from H2 and H3 viruses, in a broad pH range. We showed that small interfering RNA (siRNA) knockdown of matriptase in human bronchial epithelial cells significantly blocked influenza virus replication in these cells. Lastly, we provide a selective, slow, tight-binding inhibitor of matriptase that significantly reduces viral replication (by 1.5 log) of H1N1 influenza virus, including the 2009 pandemic virus. Our study establishes a three-pronged model for the action of matriptase: activation of incoming viruses in the extracellular space in its shed form, upon viral attachment or exit in its membrane-bound and/or shed forms at the apical surface of epithelial cells, and within endosomes by its membrane-bound form where viral fusion takes place.

Despite effective worldwide vaccination campaigns, the influenza virus and its complications are still recognized as one of the leading causes of hospitalizations and deaths, with World Health Organization annual estimates of 3 to 5 million cases of severe illness and up to 500,000 deaths worldwide each year (1). Past pandemics (1918, 1957, and 1968), transmission of H5N1 avian influenza virus directly to humans in 1997 (2), and the 2009 H1N1 pandemic underscore the scope and severity of the consequences associated with such infections.

The influenza virus, which belongs to the Orthomyxoviridae family, is enveloped and contains eight single-stranded RNA segments coding for up to 12 proteins (3). It evades the host immune system by undergoing a continuous antigenic evolution through frequent mutations of surface antigens (4, 5) and reassortment of the genetic material of cocirculating strains leading to the emergence of severe epidemics or pandemics (6–8).

Besides vaccination strategies, only two classes of anti-influenza virus drugs are currently available to treat infection: inhibitors of the viral M2 channel (amantadine and rimantadine) and inhibitors of the viral neuraminidase (zanamivir, oseltamivir, and peramivir) (9). Although targeting viral proteins has proven to be effective, both classes of drugs are now plagued by important resistance problems (9, 10), and novel strategies are greatly needed. One of these strategies is to target essential components of the infected host cell that are necessary for viral infectivity or replication. The influenza virus requires the proteolytic activation of its hemagglutinin (HA) fusion protein in order to be infectious (11). Influenza virus HA is synthesized as a single protein precursor, termed HA0, that is then cleaved into subunits HA1 and HA2. This posttranslational cleavage is required for the protein to change conformation under the acidic conditions of the endosome (11, 12), which exposes the hydrophobic fusion peptide located in the HA2 subunit (13, 14), a key step leading to viral fusion with the host cell. Because the virus does not encode any proteases, it must rely on proteases of the host for its activation (12). HAs of pathogenic avian influenza viruses are characterized by multibasic cleavage sites containing RXR furin-like recognition sequences and are activated by ubiquitous proprotein convertases (15–18). Conversely, the HA glycoproteins of nonavian viruses have monobasic cleavage sites more preferentially recognized by extracellular proteases of the host (18). All human influenza A virus HAs have a single arginine residue (except the rare lysine) at the HA cleavage site, making this a particularly attractive therapeutic target for the development of protease inhibitors. Although several proteases, such as trypsin, cleave HA0 and allow epithelial cell infection in vitro, the physiological mechanism of HA0 cleavage in vivo is not well established.

Recent work indicates that certain type II transmembrane serine proteases (TTSPs) are capable of initiating HA0 cleavage and enabling viral activation (19). These proteases efficiently cleave proteases containing an arginine residue at the P1 position, making them ideally suited to process HA0. To date, only a few proteases of the TTSP family, including human airway...
trypsin-like protease (HAT/TMPRSS11d), TMPRSS2, TMPRSS4, and MSPL/TMPRSS13 have been shown to activate influenza virus HA in various kidney and intestinal cell lines (19–22). During the preparation of the manuscript, Hamilton et al. also showed that the catalytic domain of matriptase cleaved hemagglutinins of H1 viruses (23). However, to date only TMPRSS2 has been shown to functionally activate influenza virus in human bronchial epithelial cells (24). Interestingly, among TTSPs, TMPRSS2 has also been shown to activate spike proteins of severe acute respiratory syndrome (SARS) coronavirus and porcine epidemic diarrhea virus (25–27) and has been implicated in the spread of human metapneumovirus (28). This suggests that different viruses may have adapted to use different TTSPs to ensure their amplification in humans (29). Thus, understanding how viruses interact with the host by using these enzymes represents a potential avenue for antiviral drug development.

The TTSP family currently encompasses 20 members in both humans and mice, divided into four subfamilies, namely, the HAT/differentially expressed in squamous cell carcinoma (DESC), hepsin/TMPRSS, matriptase, and corin subfamilies, based on the composition of the stem region, the serine protease domain, and the chromosomal location of their genes (30). Although some members of the TTSP family have been shown to potentially be involved in influenza virus HA activation, matriptase, of the matriptase subfamily, could be of additional importance since it is expressed in human respiratory tract tissues and is important for the maintenance of epithelial integrity (31, 32). Matriptase is bound by hepatocyte growth factor inhibitor-1 (HAI-1) at the plasma membrane, which regulates its activity by inhibiting its proteolytic activity (33), and can be shed into the extracellular space, where it may have additional functions (34). Thus, matriptase is ideally positioned to potentially be involved in influenza virus HA proteolytic activation.

In this study, we characterized the role of matriptase in the proteolytic cleavage/activation of influenza virus HA and, for the first time, its role in multicycle replication in the human respiratory epithelium. We established its expression in normal human lungs and bronchial epithelial cells and determined its subcellular localization. We showed its ability to cleave consensus cleavage sequences of known human influenza viruses. Using small interfering RNA (siRNA) knockdown of matriptase, we showed a significant reduction in viral replication in human airway epithelial cells. Finally, we developed a selective, slow, tight-binding inhibitor of the enzyme with a unique serine trap capable of blocking influenza virus replication in human bronchial epithelial cells.

MATERIALS AND METHODS

Cell culture. Calu-3 and MDCK cells were cultured in Eagle’s minimal essential medium (EMEM) supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin, 100 μg/ml streptomycin, 1 mM sodium pyruvate, 100 μM nonessential amino acids, and 2 mM l-glutamine (Wisent, Inc.). A549 and NCI-H292 cells were cultured in RPMI 1640 medium with the same supplements mentioned above. NHBE primary cells were cultured in bronchial epithelial cell basal medium (BEBM) supplemented with growth factors (SingleQuots, Lonza). For air-liquid culture of Calu-3, cells were seeded in Transwell inserts (0.4-μm pore-size membrane) and cultured initially with EMEM without antibiotics (added in upper and lower chambers). Upper chamber medium was removed 24 h after seeding. Cells were cultured for 10 days to reach confluence and to allow tight-junction formation. Cell lines were cultured at 5% CO₂ and 37°C.

Viruses. Original viral stocks of the mouse-adapted strains A/Puerto Rico/8/34 (H1N1; PR8) and A/Hong Kong/31–3 (H3N2; X-31) were provided by David Topham (University of Rochester Medical Center). X-31 and PR8 viruses were amplified in 10-day-old embryonated hens’ eggs as described previously (35, 36). pH1N1/2009 (A/California/7/2009-like) was an isolate of the 2009 H1N1 pandemic virus that was obtained from Hugues Charest (Institut National de Santé Publique du Québec) and was propagated in MDCK cells using tosylsulfonyl phenylalanyl chloromethyl ketone (TPCK)-treated trypsin (Sigma-Aldrich).

Infection of cells. Cells were washed with Dulbecco’s phosphate-buffered saline (D-PBS) and exposed to influenza virus (diluted in incomplete medium; 0.2% bovine serum albumin [BSA] instead of FBS). After virus adsorption (1 h at 37°C), cells were washed once with D-PBS, and cells were incubated in incomplete culture medium for 24 up to 200 h. For Calu-3 air-liquid cell culture, infection was performed as described above, but no culture medium was added in the upper chamber after viral adsorption. At time points specified in the figure legends, viruses from the apical surface of these cells were obtained by washing the cells with 1.2 ml of incomplete EMEM.

Viral titer determination in samples. Viral titers were determined from clarified cell culture supernatants obtained after centrifugation (at 400 × g and 4°C for 5 min). Serial 10-fold dilutions of samples were prepared in incomplete EMEM and were titrated in MDCK cells according to standard viral plaque assays using an Avicel overlay medium containing TPCK-treated trypsin, as described previously (35, 37). The number of viral plaques was counted to determine viral PFU levels.

Generation of HA viruses. MDCK cells were cultured to confluence and infected either with PR8, X31, or pH1N1/2009 virus at a multiplicity of infection (MOI) of 2 as described above. Viruses were allowed to replicate for 24 h in cells cultured in incomplete medium without TPCK-treated trypsin. Viruses were recovered from supernatants, centrifuged, and stored at −80°C.

Production of matriptase’s catalytic domain. Matriptase (catalytic domain; amino acids 596 to 855) was produced in Escherichia coli M15 competent cells as previously described (38). Matriptase was purified using immobilized metal-chelate affinity chromatography and then using Mono-Q ion-exchange chromatography on an Explorer system (GE Healthcare).

Detection of matriptase and TTSPs in human respiratory epithelial cells. Cellular RNA was extracted using TRIzol reagent. TTSP gene expression (matriptase, TMPRSS2, TMPRSS4, HAT, and MSPL) was determined by reverse transcription-PCR (RT-PCR) assays. Amplicons were visualized by agarose gel electrophoresis. The sequences of the PCR primers used in this study are listed in Table 1. For the detection of the matriptase protein,
cells were lysed in radioimmunoprecipitation assay (RIPA) buffer containing protease inhibitors. Cell lysates were centrifuged at 12,000 × g for 10 min, and supernatants were stored at −20°C. Equal protein amounts (35 μg) were subjected to SDS-PAGE. Matriptase and actin proteins were detected by Western blotting using specific rabbit anti-human matriptase (either from Bethyl Laboratories, Inc., or Abcam) or anti-actin antibodies (Santa Cruz Biotechnology, Inc.).

Cleavage of influenza virus HA consensus cleavage sequences by matriptase. Internally quenched fluorescent peptide (IQFP) mimetics of H1, H2, and H3 consensus cleavage sequences were designed to incorporate four amino acids on the P side of the cleavage site and four amino acids on the P′ side. Fluorescence and quenching pairs were 3-nitrotyrosine and ortho-aminobenzoic acid (Abz). Peptides (Selleck Chemicals, Houston, TX) were as follows: H1, Abz-IQSRGLFG-Tyr(3NO2); H2, Abz-IERSGLFG-Tyr(3NO2); H3, Abz-KQTGRGFG-Tyr(3NO2); control, Abz-IQSR. Cleavage of IQFP by the matriptase catalytic domain and determination of Vmax were done essentially as described by Beliveau et al. (39). Briefly, hydrolysis of the IQF peptide substrates (5 nM) by matriptase (50 μM) was measured at 37°C using an FLX-800 TBE microplate reader. Fluorescence was monitored at an excitation wavelength (Ex:0) of 320 nm and emission wavelength (Em:0) of 420 nm for 30 min to determine Vmax.

Proteolytic cleavage of viral HAs by matriptase. HA0 (0.5 μg) of PR8 and X31 viruses was incubated in the presence or absence of 200 nM or 500 nM matriptase (or 43 nM TPCK-treated trypsin as a control) in Tris buffer at pH 7.4 and 37°C for 1 h. Viruses were then disrupted with Triton X-100 (1% final) for 45 min. Hemagglutinin cleavage was then determined by Western blotting using anti-H1 and -H3 antibodies (from MyBioSource (1% final) for 45 min. Hemagglutinin cleavage was then determined by Western blotting using anti-H1 and -H3 antibodies (from MyBioSource and Abcam, respectively) and quantified by densitometry. Further experiments were conducted at reaction mixture pHs ranging from 5 to 9.

In vitro cleavage of viral HAs by matriptase and multicopy replication of influenza virus in MDCK cells. Confluent MDCK cells were infected with 25 PFU of PR8 virus for 1 h. Cells were then washed once, and medium was replaced with Avicel overlay medium containing 43 nM matriptase or as control 43 nM TPCK-treated trypsin. The number of viral plaques was determined at 48 h postinfection as described above. To determine the state of viral HA in such experiments, cell supernatants were recovered and lysed with Triton X-100, and HA cleavage by matriptase (or trypsin) was determined by Western blotting using specific antibodies.

Multicopy replication of H1N1 influenza virus in human bronchial epithelial cells (Calu-3) and siRNA knockdown of matriptase. Confluent Calu-3 cells were infected as described above. Viral titers were evaluated in the supernatant at the time points postinfection indicated in the figure legends using standard viral plaque assays. To assess the role of matriptase in viral production, matriptase was knocked down using siRNA technology. Briefly, 2.25 × 105 Calu-3 cells were seeded into 24-well plates 24 h before transfection in complete EMEM without antibiotics. A Mission endoribonuclease-prepared siRNA (esiRNA) targeting matriptase (Sigma) or a scrambled siRNA control was used. siRNA transfections (50 nM) were carried out using Lipofectamine RNAiMax (Invitrogen). Cells were incubated for 48 h. At this point, knockdown of matriptase was determined by Western blotting using specific antibodies. Separate wells were infected with PR8 virus at an MOI of 0.0001. Supernatants were collected 92 h later and titrated as described above.

Epifluorescence microscopy. Calu-3 cells were grown on coverslips. Upon confluence, cells were fixed with 1% paraformaldehyde (PFA) (30 min at 4°C). Cells were successively blocked (PBS with 2% BSA; 30 min), incubated with rabbit anti-human matriptase primary antibody (1:100; 2 h at 4°C) (Bethyl Laboratories, Inc.) and incubated with a goat anti-rabbit-Alexa Fluor 488 secondary antibody (1:200; 1 h at RT) (Invitrogen). Nuclei were stained with 4’,6’-diamidino-2-phenylindole (DAPI). Between each step, coverslips were washed with D-PBS–Tween 0.2%. Slides were mounted using Vectashield (Vector Laboratories) and were analyzed using a Zeiss epifluorescence microscope.

Confocal microscopy. Calu-3 cells were seeded at 6 × 104 cells/cm² onto coverslips, and the next day, subconfluent cells were infected with PR8 virus (MOI of 0.5). Cells were fixed at 24 h postinfection with 1% PFA (30 min) and permeabilized with 0.1% saponin, and coverslips were blocked with 0.25% gelatin. Coverslips were incubated with a rabbit anti-H1 antibody for 45 min (1:600) (MBS432021; MyBioSource), washed and fixed again with 1% PFA (20 min). Next, coverslips were incubated with the sheep anti-matriptase antibody (1:25) (AF3946; R&D Systems) and chicken anti-early endosomal antigen 1 (EEA-1) antibody (1:200) (GTX82138; GeneTex, Inc.). Subsequently, coverslips were incubated with secondary antibodies: goat anti-chicken Alexa Fluor 488, donkey anti-rabbit Alexa Fluor 594, and donkey anti-sheep Alexa Fluor 647 (all from Jackson ImmunoResearch Laboratories, Inc.). Cells were stained with DAPI for nuclear staining. Coverslips were mounted as described above. Alternatively, inserts from air-liquid-cultured Calu-3 cells were embedded in paraffin. Five-micrometer sections were deparaffinized, and slides were processed as for the staining of submerged cell cultures.

Cells were viewed using a 63× oil immersion objective on an inverted scanning confocal microscope (FV1000) equipped with an argon laser and laser diodes (Olympus). Fluorescence emission of Alexa Fluor 488 and Alexa Fluor 594 was collected sequentially at wavelengths of 500 to 530 nm and >560 nm, respectively, to avoid fluorescence cross talk. Fluorescence from Alexa Fluor 647 and DAPI was collected at the same time (at wavelengths 645 to 745 and of 425 to 475, respectively) in a second phase. Serial horizontal optical sections of 512 by 512 pixels with two time levels averaging were taken at 0.4-μm intervals through the entire thickness of the cell.

Microscope image analysis. For illustration purposes, images were contrast enhanced, pseudocolored according to the best color match using Fluoview software (Olympus), and then cropped and assembled using Adobe Photoshop software (Adobe Systems). For confocal quantitative analysis, raw fluorescence data were plotted on dot 2D graphs by first determining colocalization of Alexa Fluor 594 (HA) and Alexa Fluor 647 (matriptase) fluorescence. Next, within Alexa Fluor 594/Alexa Fluor 647 colocalizing dots, Alexa Fluor 488 (EEA-1) fluorescence colocalization was determined. For example, to determine colocalization of fluorescent pixels, overlap quadrant ranks (thresholds) were placed forming background (C), Alexa Fluor 594–only (D), Alexa Fluor 647–only (A), and colocalization areas (B). The colocalization index was calculated as (B)/(B + D), and the percentage of colocalization was calculated as (B)/(B + D) × 100, as described by Manders et al. (40). Quantitative analysis was performed on five size-matched cells or cell clusters for each experimental condition, and experiments were performed three times.

Selective, slow, tight-binding inhibitor of matriptase and inhibition of virus production in Calu-3 cells. A selective, slow, tight-binding inhibitor of matriptase containing a ketobenzothiazole serine trap (IN-1) was designed based on matriptase’s auto-catalytic domain (RQAR). Details on the synthesis and characteristics of the inhibitor have been described previously (41). Additional experiments were carried out using compound 21, a generous gift from Drew Rowan (University of Newcastle), described by Steinmetzer et al. (42). Confluent Calu-3 cells were infected with PR8 or pH1N1/2009 virus at an MOI of 0.003 as described above. Virus was left to adsorb for 1 h, and cells were washed once. Cells were then incubated with various concentrations of IN-1 (in water) or compound 21 (in dimethyl sulfoxide [DMSO]) for 48 h. Supernatants were collected and titrated using standard plaque assays as described above.

RESULTS
Matriptase is expressed in the human respiratory tract. In order to determine the expression of matriptase in the human respiratory tract, RNA extracts were obtained from normal human lung (Ambion) as well as from various human bronchial and alveolar cell lines (Calu-3, NCI-H292, and A 549) and primary human epithelial cell cultures (NHBE cells). RNA was reverse transcribed and analyzed by PCR using specific primers. The expression of
matriptase mRNA was compared to that of other known influenza virus-activating TTSPs, TMPRSS2, TMPRSS4, HAT, and MSPL. Our results show that matriptase mRNA is expressed in the normal human lung and by primary normal human bronchial epithelial cells. (Fig. 1A). Moreover, matriptase mRNA is also expressed by human bronchial epithelial cell lines Calu-3 and NCI-H292 but not by the alveolar epithelial cell line A549 (Fig. 1A). Compared to matriptase mRNA, TMPRSS2 was strongly expressed in normal human lung, but expression of TMPRSS2 was weaker than that of matriptase in Calu-3 cells. Very weak expression of TMPRSS2 was detected in NCI-H292 and NHBE cells. Strong expression of TMPRSS4 and MSPL was observed in Calu-3, NCI-H292, NHBE cells and in normal human lung. HAT mRNA was expressed more weakly than matriptase in Calu-3 and NCI-H292 cells; however, strong expression was detected in NHBE cells. Very faint bands for HAT were observed in normal human lung and A549 cells (Fig. 1A). Overall, our data show that matriptase mRNA (and that of other TTSPs) is expressed by epithelial cells of the respiratory tract and that this expression seems to be more characteristic of the bronchial epithelium.

Because mRNA expression does not always reflect protein levels, we next analyzed matriptase protein expression in human bronchial and alveolar epithelial cells by Western blotting. Using a human matriptase-specific antibody, our data showed a band of an approximate molecular size of 84 kDa, corresponding to the expected range for human matriptase, in Calu-3, NCI-H292, and NHBE bronchial epithelial cells but not in A549 alveolar epithelial cells reflecting mRNA expression (Fig. 1B). In these experiments, loading was normalized using equivalent protein amounts from each extract. Various actin levels reflect the differential expression of actin in each cell type and do not represent unequal protein loading. Furthermore, to assess whether matriptase expression is modulated when cells are polarized, we compared the protein expression of matriptase by Western blotting in cells that were cultured under submerged conditions to cells that were cultured under air-liquid conditions inducing their polarization. Our results showed that matriptase was more strongly expressed under polarizing conditions. These results confirm the presence of matriptase in human bronchial epithelial cells and suggest that protein expression is enhanced when the epithelium is polarized.

Using epifluorescence microscopy, we further characterized the expression of matriptase in human bronchial epithelial cells. Calu-3 cells were cultured on coverslips and stained with anti-matriptase antibody (green) and DAPI (nuclear marker; blue). Results from epifluorescence microscopy showed concentrated green fluorescence, indicating that matriptase was expressed at and near the plasma membrane of Calu-3 cells. Results are representative of at least three independent experiments. GAPDH, glyceraldehyde-3-phosphate dehydrogenase.
trypsin. Nonetheless, these results demonstrate the biochemical capacity of matriptase to cleave specific influenza virus HA consensus cleavage sequences.

Because the octapeptides used to determine the biochemical cleavage capacity of matriptase toward HA consensus cleavage sequences represent only a small portion of the HA protein and because other protein determinants could influence HA cleavage, we next tested whether matriptase (catalytic domain) had the capacity to cleave influenza virus HA of the whole virus at physiological pH. For this purpose, we produced PR8 H1N1 and X-31 H3N2 viruses with noncleaved HA (HA0) by infecting MDCK cells with each virus without the addition of exogenous protease. Viruses were recovered from the supernatant of cells after a single round of replication. We then exposed these viruses to 200 nM or 500 nM matriptase at pH 7.4 and determined HA cleavage by Western blotting. Our data showed, first, that matriptase was able to cleave PR8 H1 within 30 min and that this cleavage was efficient up to pH 7.4 (from 23.1% ± 0.5% at pH 5 to 39.5% ± 1.8% at pH 7.4) with slightly reduced efficiency at pH 9 (31.4% ± 10.4%) (Fig. 4). Together with our previous data, these results suggest that matriptase rapidly cleaves influenza virus H1 hemagglutinin in a broad range of pHs, in particular, at a pH relevant to influenza virus HA activation (cell surface and early to late endosomes).

**Matriptase promotes multicycle replication of H1N1 influenza virus.** We next sought to determine whether the cleavage/activation of influenza virus HA by matriptase led to functional multicycle replication of the virus. To do so, we performed standard viral plaque assays in MDCK cells with the H1N1 PR8 virus or the H3N2 X-31 virus. However, we replaced trypsin by matriptase as the activating exogenous protease to generate viral plaques. Our data convincingly showed that matriptase was able to promote multicycle PR8 virus replication, as determined by viral plaque formation in MDCK cells in the presence of the enzyme (43 nM), compared to the trypsin (43 nM) control (Fig. 5A). No viral plaques were observed with the X-31 virus, thus confirming our previous observations. Next, we assessed, by Western blotting, the state of the PR8 H1 in the supernatant of MDCK cells after 48 h of incubation of PR8 HA0 with increasing concentrations of matriptase. Our data demonstrated a dose-dependent accumulation of virions with cleaved HA, as shown by the increasing intensity of the 55-kDa band with increasing concentrations of matriptase (Fig. 5B). Densitometric analysis showed that viruses had up to 62% cleaved HA within 48 h in the presence of 129 nM matriptase whereas for the trypsin control HA cleavage was 95% (Fig. 5B). We next compared the efficiency of matriptase in promoting the multicycle replication of PR8 viruses bearing either uncleaved (PR8 HA0) or cleaved (PR8 HA+) virus. MDCK cells were
infected with either virus, increasing concentrations of matriptase were added to the culture medium, and viral plaque formation was assessed after 48 h. Our results showed that matriptase was able to support multicycle replication of both viruses in a dose-dependent manner; however, since the HA of the HA1 virus was initially almost exclusively cleaved (~93%) (data not shown), this virus had a significant advantage in the number of internalized and activated viruses capable of forming potential viral plaques from multiple replication cycles. Increasing concentrations of matriptase promoted the generation of the maximum number of viral plaques. In fact, at an equivalent molar concentration (43 nM) the efficacy of matriptase to promote PR8 HA1 viral plaque formation was 62% that of trypsin (Fig. 5C, right panel). On the other hand, in the case of the HA0 virus, only a very minor proportion of HA was initially cleaved (~8%) (Fig. 5B), thereby significantly reducing the number of potential viral plaques following virus internalization. Trypsin allowed the activation of a maximum number of viruses at the cell surface, resulting in the maximal number of viral plaques (Fig. 5C, left panel). Addition of matriptase at an equivalent molar concentration (43 nM) resulted in only 2.5% of the number of viral plaques formed in trypsin-treated cells exposed to PR8 HA0, which is consistent with matriptase’s reduced efficiency at cleaving the H1 consensus cleavage site peptides compared to trypsin (Fig. 2). In all cases, viral plaque sizes were equivalent, indicating that when viral plaque formation was initiated, matriptase efficiently supported viral replication and plaque expansion. Nonetheless, matriptase is capable of...
functional proteolytic cleavage of influenza virus H1 hemagglutinin and promotes multicycle replication of the H1N1 PR8 virus. Taken together, these results suggest that matriptase is capable of, but inefficient at, the initial (first round) functional cleavage activation of influenza virus HA but efficiently promotes the multicycle replication of the virus. To our knowledge, this is the first demonstration of the ability of matriptase to promote multicycle replication of the influenza virus.

**Matriptase is expressed at the apical surface and in endosomes of human bronchial epithelial cells.** To determine the subcellular localization of matriptase, further probing of matriptase’s localization using specific anti-matriptase antibodies and confocal microscopy in polarized Calu-3 cells showed that, in addition to being expressed at the apical surface, matriptase was also expressed in compartmentalized vesicles within the cell cytoplasm (Fig. 6A). Because the influenza virus, after attachment to the cell surface, is internalized into endosomes, we sought to determine whether the compartmentalized matriptase colocalized with these cellular compartments. To do so, first, uninfected Calu-3 cells were stained with anti-matriptase, anti-EAA-1 (early endosome marker), and anti-H1 antibodies, and colocalization of fluorescence in longitudinal cross-sections of cells was analyzed by confocal microscopy. As expected, our data show no anti-H1 staining in these cells; however, matriptase staining colocalized with EEA-1-positive vesicles (34.73%) (Fig. 6B and C). In PR8 H1N1 virus-infected Calu-3 cells (MOI of 0.5), anti-H1 staining colocalized with EEA-1-positive endosomes (11.31%) (Fig. 6B and C). In addition, colocalization of matriptase and HA was observed (17.52%) at the cell surface and in a fraction of EEA-1-positive endosomes (Fig. 6B, bottom left panel). These results suggest that matriptase is particularly well located to cleave influenza virus hemagglutinin either at the apical surface of the cells or within endosomes.

**Matriptase is involved in multicycle replication of influenza virus in human bronchial epithelial cells.** We next investigated whether matriptase had a functional role in H1N1 influenza virus replication in human respiratory tract epithelial cells. We used the Calu-3 human bronchial epithelial cells cultured under air-liquid conditions (tight-junction formation and polarization). First, to assess H1N1 viral replication, we infected polarized Calu-3 cells with the PR8 virus for 1 h to allow viral adsorption before the cells were washed and incubated for periods ranging from 4 h to 96 h postinfection. The apical surface of the cells was sampled by washing with culture medium, and viral load was determined by viral plaque assays. Our data showed that the PR8 virus increasingly replicated to high viral titers from 24 h to 72 h postinfection (>10^7 PFU/ml), at which point replication reached a plateau that was maintained for up to 96 h (Fig. 7A). Similar results were obtained with submerged cells (regular cell culture), but viral titers were lower over the time course (Fig. 7B). To determine whether matriptase was involved in multicycle replication in human bronchial epithelial cells, submerged Calu-3 cells were transfected with an siRNA cocktail specifically targeting matriptase. Matriptase knockdown was assessed by Western blotting. Our data showed that matriptase expression was knocked down by up to 80.8% in siRNA-transfected cells (Fig. 7C). Transfected cells with either an siRNA targeting matriptase or a scrambled siRNA as a control were infected with PR8 at an MOI of 0.0001, and viral production was determined by viral plaque assays in the supernatant at 92 h postinfection. Our results revealed that a significant reduction (up to 1.5 log) in viral titers in Calu-3 cells transfected with matriptase siRNA compared to nontransfected cells (data not shown) or scrambled siRNA-transfected control cells (Fig. 7C). These results demonstrate for the first time that matriptase has a functional role in the proteolytic activation and in multicycle replication of H1N1 influenza virus in human bronchial epithelial cells.

**Pharmacological intervention in the influenza virus replication process.** Current antivirals targeting influenza virus are plagued by increasing viral resistance, and new therapeutic strategies are required. In light of our findings, we designed a novel selective matriptase inhibitor based on the enzyme’s autocatalytic site (P4 to P1 positions) coupled with a unique serine trap capable of covalently and reversibly binding to the enzyme resulting in a slow, tight-binding inhibitor (IN-1) (41). We sought to assess the ability of IN-1 to block influenza virus replication in human bronchial epithelial cells. We tested whether IN-1 was able to block multicycle PR8 replication in Calu-3 human bronchial epithelial cells. Cells were infected, and IN-1 was added at increasing concentrations to the incubation medium. Viral titers were determined in the supernatants of infected cells by viral plaque assays at 48 h postinfection. Our data showed that matriptase inhibitor IN-1 blocked PR8 H1N1 influenza virus replication in a dose-dependent manner with a 50% effective concentration (EC_{50}) of 5.64 μM (Fig. 8A, left panel). In order to compare the efficacy of IN-1, we tested the inhibitory capacity of another published small-molecule inhibitor of matriptase, compound 21 (42), in blocking PR8 multicycle viral replication in Calu-3 cells. Our data revealed that compound 21 was also able to block H1N1 influenza virus replication in Calu-3 cells.
replication in Calu-3 cells; however, it was nine times less effective than IN-1 (EC\textsubscript{50} of 50.21 \textmu M for PR8) (Fig. 8A, right panel). To broaden the scope of our results, we also infected cells with an A/California-like/09 pandemic H1N1 virus (pH1N1/2009; isolated during the pandemic) and assessed the ability of the matriptase inhibitor IN-1 to block its replication. Our data demonstrated that IN-1 is also capable of blocking the replication of the 2009 pandemic H1N1 virus (EC\textsubscript{50} of 6.80 \textmu M), suggesting that matriptase was also involved in the proteolytic activation of this H1N1 virus (Fig. 8B).

**DISCUSSION**

In order to initiate its viral life cycle in humans, the influenza virus must bind to respiratory epithelial cells and fuse with the cell membrane to release its viral ribonucleoproteins that contain its genome. The influenza virus uses its surface hemagglutinin protein (HA\textsubscript{1} subunit) to bind to sialic acids at the cell surface, where \(\alpha2-6\)-linked sialic acids are preferred by human viruses and \(\alpha2-3\)-linked sialic acids are preferred by avian viruses (44). After binding to the respiratory epithelial cell, the virus is rapidly internalized into clathrin-coated pits (45). It then transits through the endosomal pathway, and in late endosomes, the compartment is acidified via endosomal vacuolar proton ATPases (\(\text{v-}[\text{H}^{+}]\text{ATPases}\)) (46), activating the fusion of viral and host membranes. However, in order for this step to occur, the influenza virus hemagglutinin must have undergone proteolytic cleavage either in the extracellular space, at the cell surface, or within endosomes to expose the fusion peptide located at the N terminus of the HA\textsubscript{2} subunit of the protein (post cleavage). Acidification of the endosome induces a permanent conformational change in HA\textsubscript{1}, exposing the hydrophobic fusion peptide and allowing its insertion into the endosomal membrane (47, 48). At this point, the viral genome-containing ribonucleoproteins can be released into the infected cell, and viral replication can ensue. Since the virus does not encode any proteases, a host cell protease, likely a serine protease because of the conserved arginine at the P1 position of the cleavage sequence, must be used. Although progress has been
made in this field, the human host cell protease(s) responsible for this activity remains to be clearly identified.

To date, in vitro studies revealed that several proteases of various species are able to mediate the cleavage/activation of influenza virus HA. Of these proteases, extracellular proteases, such as trypsin Clara and mast cell tryptase, and proteases involved in thrombolysis, such as thrombin, plasmin, kallikrein, and urokinase, all possessing trypsin-like serine protease activities, have been shown to possess HA cleavage capacities in vitro (49–51). More recently, a new family of membrane-bound serine proteases has been implicated in influenza virus HA cleavage/activation, namely, the TTSP family. Among these proteases, mainly HAT and TMPRSS2 but also TMPRSS4, which are known to be expressed in the human respiratory tract, have been shown to activate the influenza virus (19, 52, 53).

In this study, we identify and characterize matriptase in human bronchial epithelial cells as a novel member of the TTSP family expressed in the human respiratory tract capable of functional proteolytic activation of influenza virus HA. First, we showed that matriptase mRNA was expressed in the normal human lung, and we showed that matriptase in its protein form was present in primary human respiratory epithelial cells and in cell lines. Epifluorescence microscopy of Calu-3 bronchial epithelial cells showed that matriptase was expressed at the plasma membrane of these cells. This is consistent with reports showing that matriptase is expressed in the epithelial compartment of most major organ systems and is highly expressed by ciliated and glandular epithelial cells of the nasal cavity and by ciliated columnar epithelial cells of the trachea and bronchioles, where it localizes to the apical surface of the cells (32, 54). Together, this provides an excellent and representative model to study the activation of influenza virus HA by human cellular proteases. Strikingly, to date, only one study has used human bronchial epithelial cells, and all others have used cells from various organs or species origins and transfection systems to study the putative role of TTSPs in the cleavage activation of HA (24).

Matriptase is a trypsin-like serine protease with specific proteolytic activity downstream of an arginine residue at the cleavage site (55), a feature shared with other influenza virus-activating TTSPs identified to date (29). We showed that matriptase has the ability to proteolytically cleave internally quenched fluorescent peptides designed to represent the consensus cleavage sequences of H1 (IQSR GLFG) and H3 (KQTR GLFG) influenza virus hemagglutinins, but not H2 (IESR GLFG). Interestingly, during the

![Fig 6](http://jvi.asm.org/4245)

**Fig 6** Matriptase localizes to the plasma membrane and to endosomes and colocalizes with influenza HA. (A) Calu-3 cells grown on Transwell inserts at an air-liquid interface (ALI) for 10 days. Inserts were then fixed and embedded in paraffin. Samples were cut into 5-μm sections, deparaffinized, and processed for immunostaining with anti-matriptase antibody (red). Nuclei were stained with DAPI (blue). Slides were analyzed using a scanning confocal microscope equipped with an inverted 63× oil immersion objective. (B) Subconfluent submerged Calu-3 cells grown on glass coverslips were infected with A/Puerto Rico/8/34 (PR8) at an MOI of 0.5 and were fixed, blocked, and sequentially stained as described in Materials and Methods. A representative longitudinal cross-section of cells is presented and shows staining of matriptase (Alexa Fluor 647; red), endosomes (EEA-1) (Alexa Fluor 488; green), HA (Alexa Fluor 594; blue), and nuclei (DAPI; gray). A merged image is shown in the middle right panel, and staining colocalization of matriptase, HA, and endosomes (EEA-1) are shown in the lower right panel. (C) The upper left panel shows colocalization of matriptase and EEA-1, the middle left panel shows colocalization of matriptase and HA, and the lower left panel shows colocalization of HA and EEA-1. Right panels show quantitative analyses of colocalization, and numbers in quadrants represent percent colocalization in each panel. Experiments were performed four times.
writing of the manuscript, a study by Hamilton et al. was published ahead of print showing how the catalytic domain of matriptase cleaved H1 and H3 peptides but was very inefficient at cleaving H2 peptides (23). Of note, the authors also showed that matriptase had reduced efficiency in cleaving the A/WSN/33 cleavage sequence that harbors a tyrosine instead of the conserved serine in the P2 position (23). We also compared the cleavage efficiency of influenza virus consensus sequences to the cleavage of a peptide representing matriptase’s reported optimal cleavage sequence (RQRR VVGG) and to the standard trypsin. These data showed that the efficiency of matriptase at cleaving influenza virus consensus sequences in general is reduced compared to that of trypsin and that of an optimal peptide (39). This suggested that even inefficient cleavage of influenza virus consensus sequences might be sufficient to promote viral infection.

In addition to its apical surface expression in respiratory epithelial cells, matriptase has also been found to be expressed at the basolateral side of some epithelial cells, where it is activated and complexes with its inhibitor HAI-1. Following its activation, it then traffics to the apical side of the cell, where it can be shed into the extracellular space (33). Interestingly, cleavage of HAI-1 by a membrane type 1 matrix metalloproteinase (MMP-1) promotes matriptase’s proteolytic activity and shedding of the catalytic domain (56). These features make this protease particularly well suited for the proteolytic activation of the influenza virus hemagglutinin since the enzyme has the potential to activate the virus either in the extracellular space in its shed form or at the cell surface in its transmembrane form at the apical side of respiratory epithelial cells. Since the peptide mimetics used to characterize the cleavage efficiency of the HA consensus cleavage sequences represent only a small portion of the protein, we examined matriptase’s ability to cleave HA on whole virions. Using the soluble form of matriptase (catalytic domain), we showed that the protease was able to cleave the H1-bearing PR8 virus and did so in a dose-dependent manner. In fact, cleavage was observed as quickly as within 30 min, and maximal cleavage was attained after 16 to 24 h, suggesting that the proteolytic activity is well aligned with the time frame of infection. Strikingly, matriptase did not cleave the H3-
bearing X-31 virus though it did cleave the H3 consensus sequence peptide with efficiency similar to that for the H1 consensus sequence. Taken together, these results suggest that other protein determinants, such as tertiary structure and glycosylation, are important for the accessibility of matriptase to the cleavage site (57, 58). Hamilton et al. also reached similar conclusions concerning H1 and H3 cleavage in HEK 293T cells transfected with plasmids containing various H1 and H3 proteins (as expected, H2 was not cleaved in expressing cells) (23). In their paper, the authors also showed that the degree or efficiency of cleavage varied among H1 strains. Together our independent studies confirm the selectivity of matriptase for H1-bearing viruses and the ability of the soluble form of the enzyme to proteolytically activate influenza virus HA.

In contrast to other identified TTSPs seemingly capable of cleaving influenza virus HAs indiscriminately (19–22), this added selectivity in protease usage by H1 strains might represent an evolutionary trait of these viruses and could also constitute a virulence factor. Interestingly, during the revision of the manuscript, a study by Baron et al. described the ability of matriptase to cleave H9-bearing viruses (59). Thus, the repertoire of proteases used by certain viruses might contribute to their virulence in the host.

The experiments presented above clearly show that matriptase is capable of proteolytically processing influenza virus HA; however, they do not show that this proteolysis is functional and can support multicycle replication. We tested whether the soluble form of matriptase could also support the multicycle replication of influenza virus. Using standard viral plaque assays in MDCK cells with either trypsin or matriptase as the activating protease and noncleaved PR8 virus, we showed that matriptase was indeed capable of supporting viral plaque formation and thus multicycle influenza virus replication. Furthermore, we showed that over time, viruses with cleaved HAs accumulated in the supernatant of these cells. Hence, we provide the first formal demonstration that the soluble form of matriptase is capable of supporting multicycle influenza virus replication without any additional proteases in the system. Thus, despite this seemingly inefficient cleavage of the influenza virus HA consensus sequences, matriptase has the ability to support efficient multicycle replication of influenza virus (approximately 60% of the efficiency of trypsin). In relation to our findings, Hamilton et al. showed that the cleavage of HA by the soluble catalytic domain of matriptase at the surface of HA-expressing Vero cells induced cell-cell fusion, indicating that the cleaved HA was able to induce membrane fusion. In addition, they showed nucleoprotein staining in Vero cells exposed to an HA0 virus that had been exposed to matriptase at 6 h postinfection (a time point allowing viral fusion and some viral protein expression but largely preceding new virion generation) but did not show multicycle replication (23). Thus, our results significantly contribute to existing reports showing that overexpression of the TTSP TMPRSS2, TMPRSS4, or HAT in MDCK cells promoted the multicycle replication of influenza virus in these cells (21, 53).

To further characterize the role of matriptase in influenza virus HA activation in humans, we used confocal microscopy in polarized human bronchial epithelial Calu-3 cells infected with the PR8 H1N1 influenza virus and demonstrated that matriptase localizes to the apical surface of the plasma membrane, where it can interact with and proteolytically activate influenza virus HA upon viral

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**FIG 8** Inhibition of matriptase by the slow, tight-binding inhibitor IN-1 significantly blocks influenza virus replication in human bronchial epithelial cells. (A) Confluent submerged Calu-3 cells were infected with A/Puerto Rico/8/34 (PR8) at an MOI of 0.003 with or without various concentrations of the matriptase inhibitor IN-1 or compound 21. Viral titers were evaluated 48 h postinfection. A nonlinear logistic regression curve was fitted to the data, and half-maximal effective concentrations (EC50s) were calculated for each inhibitor using GraphPad Prism, version 5, software. (B) IN-1 inhibition of pH1N1/2009 replication was determined in confluent submerged Calu-3 cells infected at an MOI of 0.003 and treated with increasing concentrations of IN-1. Data represent three independent experiments.
attachment. Since the virus, once attached, is rapidly internalized into endosomes where viral fusion occurs, we further explored the possibility that matriptase could localize to these compartments where viral HA activation might also occur. Indeed, using EEA-1 staining (early endosomal marker), we showed that matriptase colocalizes with these vesicles during influenza virus infection. Viral HA also strongly colocalized with EEA-1-positive vesicles, thus raising the possibility that viral HA could also be proteolytically activated by matriptase within endosomes, promoting conformational change and exposure of the fusion peptide just prior to fusion. It has been shown biochemically that matriptase has a pH optimum of 8 to 9 in cleaving its autocatalytic sequence RQAR VVVG (39). However, upon entry into the respiratory system, the influenza virus is exposed to different degrees of pH ranging from 7.4 in the extracellular space to approximately 6 at the cell surface and to as low as 5 in late endosomes, where HA conformational change and viral fusion take place. Based on these considerations, we tested whether matriptase had the capacity to cleave the H1 hemagglutinin in whole virions at a pH range of 5 to 9. Our data revealed that the protease was indeed capable of efficient cleavage of PR8 HA₀ throughout this pH range, reinforcing the idea that matriptase has the potential to activate the influenza virus at various sites. Thus, taken together, our data provide for the first time a three-pronged model for the activation of influenza virus hemagglutinin by the TTSP matriptase. First, matriptase in its shed form could activate HA of incoming viruses in the extracellular space (step 1). Second, matriptase in its membrane-bound or in its shed form could cleave HA at the apical surface of epithelial cells upon viral attachment or exit (steps 2 and 4). Finally, HA cleavage by the membrane-bound form of matriptase could occur in the endosomes where viral fusion takes place (step 3).

To further demonstrate the role of matriptase in the promotion of influenza virus replication in human bronchial epithelial cells, we conducted infection time course experiments in Calu-3 cells and showed that the H1N1 PR8 virus replicated to high titers in these cells without the presence of any exogenous proteases. Knockdown experiments using siRNA specifically targeting matriptase demonstrated that the protein expression of matriptase could be significantly reduced (up to 80%) and that this was accompanied by a significant (1.5-log) reduction in viral particle production in these cells. These results are the first demonstration of the functional role of matriptase in the proteolytic activation of influenza virus HA in human bronchial epithelial cells. Thus, our study significantly adds to the only other study to date of TTSPs involved in influenza virus activation in human respiratory epi-
thelial cells that showed that TMPRSS2 was an important cellular protease involved in influenza virus activation (24).

Increasing evidence points to specific epithelial proteases as being responsible for influenza virus HA activation, matriptase being a new member of this family. Since these proteases must be considered important candidates for the development of novel therapeutics, we sought to explore the potential of matriptase as a pharmacological target. Bearing in mind that these targets are host and not viral targets that have highly specialized functions in the host, we based our inhibitor (IN-1) on the autocatalytic sequence of matriptase (RQAR) to which was added a ketobenzothiazole moiety acting as a unique serine trap. The ketobenzothiazole serine trap of IN-1 is designed to form a covalent, yet reversible, bond with the catalytic serine residue of the protease, allowing for continued function of the protease after removal of the treatment, which is in sharp contrast to all suicide protease inhibitors that permanently destroy enzyme function. In fact, the binding kinetics of IN-1 follow a tight-binding inhibition pattern, where IN-1 had a $K_i$ of 0.011 nM (41). The $K_i$ was determined using the method of Morrison, which usually gives very low $K_i$s owing to the mode of inhibition (41). IN-1 also displayed an excellent selectivity profile versus other serine proteases (trypsin and thrombin) and TTSPs, including matriptase-2, hepsin, and TMPRSS11D (41). Using the human bronchial epithelial cell line Calu-3 infected with either the PR8 H1N1 or pH1N1/2009 virus, in which the virus replicates to high titers, we showed that IN-1 was capable of significantly blocking influenza virus replication. In fact, the $EC_{50}$ of IN-1 against these viruses was in the low-micromolar range, and up to a 1.5-log viral titer reduction was observed at 50 μM. We confirmed our results using another published matriptase inhibitor from an independent laboratory, compound 21 (42), which also achieved significant inhibition of viral replication, with an $EC_{50}$ of 50.21 μM for PR8. Of interest, serum trap-like inhibitors bocepitre and telaprevir have recently been approved by the FDA against hepatitis C virus protease NS3-A4 (60). Current anti-influenza drugs target viral proteins (neuraminidase and ion channel M2), but the emergence of viral resistance to these drugs underscores the need for novel strategies to fight the disease. Together, our data highlight the possibility of selective and reversible pharmacological intervention targeting the infected host via TTSPs, in this case matriptase, in human bronchial epithelial cells to significantly limit influenza virus production and spread. In addition, the selectivity and reversibility characteristics of IN-1 combined with the limited therapeutic time frame during influenza virus infection might limit the appearance of side effects related to targeting a host enzyme.

In conclusion, our study reveals the new role of a member of the TTSP family, matriptase, in the proteolytic activation of influenza virus hemagglutinin and multicycle viral replication in human respiratory epithelial cells. Our study establishes a three-pronged model of how this activation might occur. Matriptase by its localization could activate incoming viruses either in the extracellular space in its shed form or upon viral attachment (and/or exit) at the apical surface of epithelial cells in its membrane-bound form. It could also activate influenza virus HA within endosomes. We demonstrated that matriptase is active in the proteolytic cleavage of influenza virus HA in a broad pH range that covers all of the above situations. This is the first demonstration of a host protease to function in this manner. Our study also showed that the activation of influenza virus HA by matriptase was specific to the H1 strain, which suggests that the repertoire of host proteases used by influenza viruses might constitute a novel virulence factor. To date, the limited knowledge of host proteases involved in influenza virus HA activation has hindered the development of novel therapeutic strategies that target the host instead of the virus with the potential of limiting viral adaptation. Here, we provide a novel class of selective, slow, tight-binding, reversible serine trap inhibitors capable of targeting a host TTSP and significantly blocking influenza virus production in human bronchial epithelial cells. Further research into the role of TTSPs and other serine proteases in human respiratory epithelial cells will be critical for our understanding of influenza virus HA activation and host-oriented antiviral drug development. However, here, we provide considerable insight into the recent progress in the identification and understanding of human influenza virus-activating proteases.

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