# Proteolytic Activation of human Influenza Viruses

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**Index**

## 1. Abstract

The influenza virus hemagglutinin (HA) mediates viral entry into target cells. Newly synthesized HA is inactive and requires cleavage by host cell proteases to transit into an active form. Activation is indispensable for viral infectivity and the responsible proteases are targets for antiviral intervention. However, the identity of the HA-activating proteases is incompletely defined. Highly pathogenic avian influenza viruses are activated by ubiquitously expressed subtilisin-like proteases. In contrast, the proteases responsible for activation of human influenza viruses and low pathogenic avian influenza viruses are largely unknown, and type II transmembrane serine proteases (TTSPs) have recently been suggested as candidates. Interestingly, the highly pathogenic 1918 influenza virus, the causative agent of the Spanish influenza, and the closely related virus A/WSN/33 seems to have evolved special mechanisms to ensure HA activation: Both viruses employ their neuraminidase (NA) protein to ensure HA cleavage. The A/WSN/33 NA accomplishes HA cleavage by recruiting the preprotease plasminogen, while the mechanism underlying 1918 NA-driven cleavage of 1918 HA is unknown. The goal of the present study was to examine if 1918 NA, like A/WSN/33 NA facilitates HA cleavage by recruiting plasminogen, and to analyze the role of TTSPs in the activation of human influenza viruses.

Binding studies revealed that A/WSN/33 NA but not 1918 NA recruited plasminogen and analysis of viral infectivity showed that A/WSN/33 NA was unable to functionally replace 1918 NA. Thus, 1918 NA and A/WSN/33 NA evolved different mechanisms to facilitate HA activation. In addition, evidence was obtained that 1918 NA-dependent activation of 1918 HA is a cell line-dependent phenomenon, casting doubts on the relevance of this process for viral spread in the host. The analysis of the NA-independent activation of 1918 HA showed that TMPRSS2, a TTSP previously found to activate human influenza viruses, also activated the 1918 HA and the related protein TMPRSS4 was newly identified as an HA-activating protease. The activation of HA by TTSPs was observed in transfected cells, raising the question whether endogenously expressed TTSPs also activate HA. Expression of TMPRSS2 and TMPRSS4 was detected in the Caco-2 cell line and siRNA knock-down revealed that these proteases facilitated viral spread in Caco-2 cells in the absence of an exogenously added HA-activating protease. Finally, TMPRSS2 and α-2,6-linked sialic acid, the major receptor determinant for human influenza viruses, were found to be coexpressed on type II pneumocytes, major viral target cells. These results indicate that TMPRSS2 could support viral spread in the infected host and constitutes an attractive target for antiviral intervention.

Keywords:

Influenza virus, hemagglutinin, typ II transmembrane serine proteases

## 2. Zusammenfassung

Das Hämagglutinin (HA) von Influenza Viren vermittelt den viralen Eintritt in Zielzellen. Es wird als inaktive Form synthetisiert und durch Wirtszellproteasen in die aktive Form überführt. Die proteolytische Aktivierung von HA ist für die Infektiosität unverzichtbar, jedoch sind die HA-aktivierenden Proteasen nur teilweise bekannt. Hoch pathogene aviäre Influenza Viren werden durch Subtilisin-ähnliche Proteasen gespalten. Welche Proteasen humane und gering pathogene aviäre Influenza Viren aktivieren ist dagegen weitgehend unklar. Als mögliche Kandidaten-Proteasen für die Aktivierung dieser Viren wurden Typ II Transmembran-Serinproteasen (TTSP) vorgeschlagen und die Rolle dieser Proteasen in der Influenza Virus-Aktivierung sollte im Rahmen dieser Arbeit untersucht werden. Der Erreger der Spanischen Grippe, das hoch pathogene 1918 Influenza Virus, und das verwandte Virus A/WSN/33 scheinen einen speziellen Mechanismus zur Spaltung des HA entwickelt zu haben. Die Neuraminidase- (NA) Proteine beider Viren vermitteln die Spaltung von HA. Die NA des A/WSN/33 Virus vermittelt die Spaltung des HA durch die Bindung der Präprotease Plasminogen. Die Rolle von Plasminogen in der NA-abhängigen Spaltung des 1918 HA ist dagegen unbekannt und sollte im Rahmen dieser Arbeit geklärt werden.

Bindungsstudien zeigten, dass die A/WSN/33 NA jedoch nicht die 1918 NA Plasminogen bindet. Eine Analyse der Infektiosität demonstrierte, dass die A/WSN/33 NA nicht die Funktion der 1918 NA ersetzen kann. Es ist daher wahrscheinlich, dass das 1918 Influenza Virus und A/WSN/33 Virus unterschiedliche Mechanismen zur NA-abhängigen HA-Aktivierung entwickelt haben. Zusätzlich wurden Hinweise erbracht, dass die 1918 NA-abhängige Aktivierung des 1918 HA Zelllinien-abhängig ist. Die Relevanz dieses Prozesses für die Virusvermehrung im Wirt ist daher unklar. Die Analyse der NA-unabhängigen Aktivierung des 1918 HA zeigte, dass die Serinprotease TMPRSS2 und TMPRSS4 das 1918 HA in transfizierten Zellen aktivieren. Die Analyse der mRNA- und Protein-Expression von TMPRSS2 und TMPRSS4 zeigte, dass beide Proteasen in der Zelllinie Caco-2 exprimiert werden und siRNA *knock-down* Experimente demonstrierten, dass beide Proteasen die Virusvermehrung in diesen Zellen in der Abwesenheit einer exogen zugegebenen HA-aktivierenden Protease ermöglichen. Schließlich konnte die Koexpression von TMPRSS2 und α-2,6-verknüpfter Sialinsäuren, einer wichtigen Rezeptordeterminante humaner Influenza Viren, in Typ II Pneumozyten, wichtigen viralen Zielzellen, nachgewiesen werden. Diese Ergebnisse zeigen, dass TMPRSS2 die Virusvermehrung im infizierten Wirt fördern könnte und somit ein attraktives Ziel für die antivirale Intervention darstellt.

Schlagwörter:

Influenza Viren, Hämagglutinin, Type II Transmembran-Serinproteasen

## 3. Introduction

Influenza is an infectious disease that affects birds and mammals such as humans, swine, horses and dogs. In humans, the influenza virus infections usually affect the lungs and airways whereas viral infections in birds can also occur in the gastrointestinal tract. The most common symptoms of the disease are chills, fever, muscle pains, severe headache, coughing, weakness and general discomfort [Modrow *et al.*, 2003; Behrens *et al.*, 2006]. The human influenza viruses are readily transmitted from person-to-person by inhaling droplets from the nose and throat of an infected person who is coughing and sneezing. Transmission may also occur through direct skin-to-skin contact or indirect contact with respiratory secretions (touching contaminated surfaces then touching the eyes, nose or mouth). Infected adults begin to shed influenza virus from up to two days before the onset of symptoms, and are infectious for three to five days after the initial signs of diesease. Young children can spread the virus for up to six days before, and for ten days after they become ill [Modrow *et al.*, 2003; Behrens *et al.*, 2006]. Influenza exhibits a low mortality rate and infections with human influenza viruses are rarely fatal in healthy individuals [Reid and Taubenberger, 2003; Taubenberger and Morens, 2008]. Infections are more severe in the elderly, young children, people with respiratory or cardiac disease, and those who are immunosuppressed [Behrens *et al.*, 2006; Taubenberger and Morens, 2008]. Death is most commonly associated with development of pneumonia, which can be viral, bacterial or both. In viral pneumonia, the influenza virus spreads into the lower parts of the lung. In bacterial pneumonia, a secondary infection with bacteria (such as *Streptococcus pneumoniae* and *Staphylococcus aureus*) attacks the person's weakened defences [Modrow *et al.*, 2003; Behrens *et al.*, 2006].

Influenza emerges as epidemic (seasonal) outbreaks in annual cycles, usually in the winter months in temperate climates, and as pandemic outbreaks caused by a new strain of influenza virus [Taubenberger and Morens, 2008; Taubenberger and Kash, 2010]. Up to 50% of the population can be infected in a single pandemic year and the number of deaths caused by influenza can dramatically exceed up to millions as seen for the Spanish influenza [Simonsen, 1999; Taubenberger and Morens, 2008]. In the last 100 years four influenza pandemics occured: Spanish influenza in 1918, Asian influenza in 1957, Hong Kong influenza in 1968 and Swine-origin influenza in 2009. Despite intensive research, it is difficult to predict the next outbreak: Where will it takes place? What will be the source of the virus? How virulent the virus will be? These are just some of the questions that arise concerning the future of influenza.

## 3.1 Epidemiology of influenza viruses

Influenza poses a significant public health problem worldwide. Seasonal influenza epidemics occur in temperate regions every autumn and winter. These epidemics emerge from an accumulation of subtle mutations, mainly amino acid changes, in the viral surface glycoprotein hemagglutinin (HA), a process termed antigenic drift [Webster *et al.*, 1992; Steinhauer, 1999; Fauci, 2006]. The annual epidemics result in about three to five million cases of severe illness and about 250.000 to 500.000 deaths worldwide [WHO, 2010a].

In addition to seasonal epidemics, influenza pandemics unfold every 10 to 50 years and arise by the emergence of a new virus in an immunologically naïve human population. The antigenically new virus can result from the reorganization of genome segments (see also section 3.3.3 Genome structure) from two different influenza A viruses (reassortment) which co-infected one cell (antigenic shift). The influenza pandemics are usually associated with much higher mortality rates than seasonal epidemics and, thus, can generate catastrophic public health crises as exemplified by the pandemic of the year 1918 (see below Spanish influenza).

The recognition of the first influenza pandemic goes back to the year 1510 A.D. and 14 more pandemics can be documented until now [Morens and Taubenberger, 2009; Morens *et al*., 2010]. In the last century, three pandemics of influenza occurred: The “Spanish influenza” (1918), the “Asian influenza” (1957) and the “Hong Kong influenza” (1968). The 21$^{st}$ century has seen its first influenza pandemic with the “Swine-origin influenza” (2009).

**Spanish influenza (1918-1919)**

The Spanish influenza pandemic represents the most fatal event in human history, which killed an estimated 50 million people or more worldwide in 1918 and 1919 [Johnson and Mueller, 2002; Morens and Fauci, 2007; Morens and Taubenberger, 2009]. Usually, influenza associated morbidity and mortality is highest among young children, the elderly, and immunosuppressed individuals, but the majority of victims of the Spanish influenza were healthy young adults aged between 20 and 40 years, resulting in a W-shape of the age-specific mortality curve (Figure 1) [Glezen, 1996; Reid *et al.*, 2001; Palese, 2004; Morens and Fauci, 2007]. The explanation for this unexpected high influenza mortality in persons 20-40 years of age in 1918 is still an unsolved fact. Several suggestions were made to explain the high death rates: People of this age group did not have enough antibody protection against this H1 subtype virus in 1918 because they had only contact with an H3 influenza virus circulating

around 20 years before the Spanish influenza pandemic [Morens and Fauci, 2007]. A vigorous immune response directed against the virus in healthy young people could have also caused severe disease in 1918. Additionally, it needs to be mentioned that in 1918 many severe cases of influenza disease featured both severe bronchopulmonary tissue damage and severe secondary bacterial infection. On the other hand, people older than 55 years showed lower mortality rates compared to other influenza pandemics because they may have had partial protection through contact to a related virus that was circulating before 1889 [Reid *et al.*, 2001; Palese, 2004].

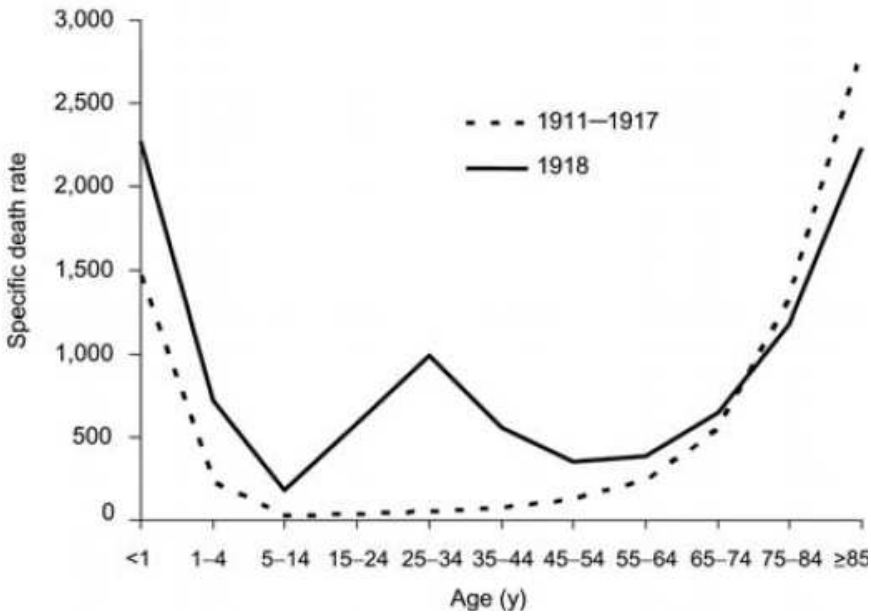


**Figure 1:** U- and W-shaped curve of combined influenza and pneumonia mortality. The graph shows the age at death with 100000 persons in each age group in the United States from 1911 to 1918. Influenza- and pneumonia-specific death rates are plotted for the interpandemic years 1911-1917 (dashed line) and for the pandemic year 1918 (solid line) [Figure was taken from Taubenberger and Morens, 2006].

In contrast to other pandemic influenza viruses, the Spanish influenza virus did not result from a reassortment event. Sequence analysis by Taubenberger and colleagues revealed that all genome segments of the 1918 influenza virus are more closely related to avian influenza viruses than to influenza viruses from other species [Taubenberger *et al.*, 1997]. The HA gene segment of the virus in particular may be derived from an avian source which differs from those currently circulating [Reid *et al.*, 1999]. It was also postulated that the virus, or some of its gene segments may have evolved in a so-far unidentified intermediate host before its introduction into the human population [Reid and Taubenberger, 2003; Taubenberger, 2006]. Taken together, the Spanish influenza was caused by a particular virulent virus, the origin of which has not been fully elucidated.

**Asian influenza (1957-1958)**

The virus responsible for the Asian influenza emerged in Southeast Asia and, subsequently, spread all over the world. More than two million deaths were attributed to this pandemic outbreak [WHO, 2005]. As postulated for pandemic viruses, the virus responsible for the Asian influenza resulted from the reassortment of a human and an avian virus. Specifically, the gene segments encoding for the two surface glycoproteins, HA and neuraminidase (NA), of the human virus were replaced by the corresponding gene segments present in avian influenza virus strains. The gene segment encoding for the polymerase protein PB1 was also replaced by an avian-like gene segment [Scholtissek *et al.*, 1978; Kawaoka *et al.*, 1989].

**Hong Kong influenza (1968-1969)**

The virus responsible for the Hong Kong influenza also emerged in South Asia. The pandemic outbreak of the Hong Kong influenza claimed around one million lives and was thus associated with lower mortality rate compared to the pandemic viruses of the years 1918 and 1957 [WHO, 2005]. Like the Asian influenza virus, the Hong Kong influenza virus also resulted from a reassortment event between avian and human influenza viruses. The gene segments encoding for the HA and the PB1 polymerase of the human virus were exchanged with avian-like segments while the remaining six segments (see also section 3.3.3 Genome structure) were retained from the 1957 influenza virus [Scholtissek *et al.*, 1978; Kawaoka *et al.*, 1989]. It has been suggested that the modest mortality rate of the 1968 virus resulted from conservation of the NA gene and some protection of the population which had previously been exposed to the 1957 influenza virus [Kilbourne, 1997; Lipatov *et al.*, 2004].

**Swine-origin influenza (2009-2010)**

The virus responsible for the 2009 pandemic emerged in Mexico and California in early April 2009 and then spread quickly around the world by human-to-human transmission [Dawood *et al.*, 2009; Fraser *et al.*, 2009; Scalera and Mossad, 2009]. As of May 16 2010, 214 countries had reported 18.097 deaths, out of approximately hundred millions of infections worldwide [WHO, 2010b]. The Robert-Koch Institute in Germany noted more then 220000 cases, including 250 deaths, of pandemic influenza until March 2010 [RKI, 2010]. A disproportionately high infection rate of children and young adults compared to older age groups was noted for the 2009 influenza virus. This age distribution could be explained by the older population being partially immune, since antibodies recognizing both the 1918 influenza

virus and the 2009 influenza virus were found only in the elderly [Garten *et al.*, 2009; Rothberg and Haessler, 2010].

The 2009 pandemic resulted from the reassortment of recent North American swine influenza viruses (triple reassortant of avian/human/swine viruses) with European avian-like swine viruses [Garten *et al.*, 2009; Dunham *et al.*, 2009]. These viruses possess the polymerase PB2 and the polymerase acidic protein gene segments from the North American avian virus origin, a polymerase PB1 gene segment of human virus origin, HA, nucleoprotein, and non-structural protein gene segments of classical swine virus origin, and NA and matrix gene segments from the Eurasian avian-like swine virus origin [Smith *et al.*, 2009; Dawood *et al.*, 2009].

### 3.2 Treatment and prevention of influenza

For the treatment of influenza virus infections, two classes of antiviral drugs are available. The drugs target the viral entry into cells and release of progeny particles from infected cells.
Amantadine and rimantadine block the matrix 2 (M2) ion channel and, thus, inhibit uncoating of the incoming virion in target cells [Hay *et al.*, 1985; Merck, 2009]. These drugs are only effective against influenza A viruses because influenza B viruses lack the M2 ion channel. A further drawback of using amantadine and rimantadine is the rapid emergence of viral resistance against these agents [Monto and Arden, 1992; Merck, 2009].
In addition, a second class of antivirals exists, comprising oseltamivir and zanamivir, which inhibit the NA protein of the influenza virus. These agents interfere with the release of the influenza virus from infected cells and represent important tools for treating influenza A and B virus infection [Mendel *et al.*, 1998; Colman, 1999; Merck, 2009].
A study by Tumpey and colleagues showed that both classes of antivirals were effective against viruses carrying the reconstructed genes of the 1918 influenza virus [Tumpey *et al.*, 2002]. In contrast, the Swine-origin influenza virus of 2009 exhibits resistance to the M2 protein inhibitors amantadine and rimantadine but is susceptible to the NA inhibitors oseltamivir and zanamivir [CDC, 2009].
Besides these agents, several experimental antiviral drugs that target the NA or polymerase proteins are now in different stages of development. The NA inhibitor peramivir is active in *in vitro* tests against viruses of all nine NA subtypes (see section 3.3.1 Aetiology and classification of influenza viruses) and resides now in phase II of clinical investigations [Babu *et al.*, 2000; Boltz *et al.*, 2008]. A pro-drug of the new NA inhibitor R-125489, named CS-8958, is a long-acting neuraminidase inhibitor including oseltamivir-resistant viruses and was found to be effective in phase II clinical trials against seasonal influenza [Yamashita *et al.*, 2009; Kiso *et al.*, 2010]. T-705 acts as a nucleoside analogue that interferes with polymerase activity of influenza A, B and C viruses, but also other RNA viruses [Furuta *et al.*, 2002]. The phase II clinical trials against seasonal influenza have been completed, and phase III clinical trials are scheduled.
Furthermore, monoclonal antibodies binding HA are being developed for the treatment of influenza virus infections. Some antibodies demonstrated prophylactic and therapeutic efficacy against a lethal challenge with the H5N1 virus in mice, suggesting monoclonal antibody treatment as an alternative strategy to treat influenza virus infections [Sui *et al.*, 2009].

Vaccination can provide protection against seasonal influenza. However, in order to produce highly effective vaccines they must be adapted to the virus strains currently circulating for every influenza season. The annual vaccine contains two influenza A viruses and one influenza B virus of the most prevalent strains [Merck, 2009].

Two conventional types of vaccines are being used: the trivalent inactivated influenza vaccine (TIV) and the live-attenuated influenza vaccine (LAIV). The TIV represents the most recently licensed influenza vaccines with three major formulations available. All formulations of TIV primarily work through the generation of antibodies against the HA. The first preparations of TIV contained whole particles while more recent formulations comprised of split virions derived from whole viral particles disrupted by detergents. The third preparation contain a subunit form with enriched HA and NA content on the surface of viral particles, which are disrupted by detergent treatment [Ellebedy and Webby, 2009; Merck, 2009]. Problems associated with inactivated vaccines are the labour intensive production system, embryonated chicken eggs, and the long time interval between the selection of vaccine strains and the availability of the first vaccine doses [Gerdil, 2003; Fedson, 2005].

In comparison to TIV, LAIV is produced by the introduction of the HA and the NA of the target strain into the backbone of an attenuated, cold-adapted virus [Maassab *et al.*, 1990; Wareing and Tannock, 2001]. One advantage of live-attenuated vaccines is the fact that theoretically both a humoral and a cell-mediated immune response can be generated. However, the generation time of LAIV does not substantially differ from inactivated vaccines at present.

These findings suggest that further work needs to be invested to develop new vaccines with shorter production times and higher efficacy against new emerging viruses. To increase the speed of vaccine preparation and delivery, the usage of reverse genetics can enable to design faster seed strains which represent the background for TIV and LAIV vaccines [Neumann *et al.*, 1999; Hoffman *et al.*, 2000]. The usage of adjuvants can also increase the protective response to influenza vaccines and, additionally, are suggested to expand the breadth of an immune response [Ellebedy and Webby, 2009].

## 3.3 Biology of influenza viruses

### 3.3.1 Aetiology and classification of influenza viruses

Influenza viruses belong to the family of *Orthomyxoviridae* and carry a segmented single-stranded RNA genome with negative polarity, which is embedded into a viral envelope derived from the host cell membrane [Palese and Shaw, 2007]. The family consists of five different genera: Influenza A virus, influenza B virus, influenza C virus, thogotovirus and isavirus [ICTV, 2009; Modrow *et al.*, 2003].

**Influenza A virus**

Influenza A virus is the most severe representative of influenza viruses. It mutates much faster than influenza B viruses and is responsible for the pandemics. This influenza virus subgroup includes avian, swine, equine, and canine viruses, as well as the human influenza A viruses. Influenza A viruses are classified into subtypes based on their HA and NA proteins. A total number of 16 HA types (H1-H16) and nine NA types (N1-N9) can be distinguished. The subtypes that are found in humans are H1N1, H1N2 and H3N2. In addition to the division into subtypes, influenza A viruses are classified into strains that are named by their type (A, B or C), host, place of first isolation, number of strain (if available), year of subtype isolation, and antigenic subtype [Hay *et al.*, 2001].

*Avian influenza viruses*

Avian influenza viruses are a genetically and antigenically diverse group of influenza A viruses and are found in a wide variety of domesticated and wild birds [Taubenberger and Morens, 2008; CDC, 2010]. The natural reservoirs of avian influenza viruses are aquatic birds of the orders *Anseriformes* (ducks, geese, swans, *etc.*) and *Charadriiformes* (gulls, terns, etc.). The virus replicates in the gastrointestinal or respiratory tracts of aquatic birds and domestic poultry, usually causing no or only mild disease. The virus causing this generally mild infection is termed low pathogenic avian influenza virus (LPAIV). In contrast, a mutation in the surface protein HA, making the virus more susceptible for activating proteases (see section Proteolytic activation by cellular proteases), renders the virus highly pathogenic (high pathogenic avian influenza virus (HPAIV)) that causes severe disease in the infected animal.

**Influenza B Virus**

Influenza B virus causes less severe diseases than influenza A virus. It usually leads to epidemics but not to pandemics like influenza A virus. This genus almost exclusively infects humans but seals and ferrets are also susceptible to influenza B virus infection [Hay *et al.*, 2001; Osterhaus *et al.*, 2000; Jakeman *et al.*, 1994]. So far, just one subtype of influenza B virus has been described corresponding to a lower mutation rate and less diverse glycoproteins relative to influenza A virus. Usually, one influenza B strain is included in the annual vaccine formulation.

**Influenza C virus**

Influenza C virus is the rarest and most stable (slowest evolving) of the three species of influenza viruses. It usually causes mild illness and was until now never associated with large scale epidemics. However, a nationwide epidemic of influenza C virus was reported in Japan in 2004 [Matsuzaki *et al.*, 2007]. Influenza C viruses mainly infect humans but have also been found in animals, like dogs and pigs [Youzbashi *et al.*, 1996; Kimura *et al.*, 1997]. In contrast to influenza A and B viruses, this genus carries seven genomic segments and harbours one surface glycoprotein, which combines the functions of HA and NA [Palese and Shaw, 2007].

### 3.3.2 Viral particle

The influenza viruses particle is approximately 120 nm in diameter and usually roughly spherically shaped, although filamentous virions have also been observed [Modrow *et al.*, 2003; Palese and Shaw, 2007; Bouvier and Palese, 2008]. Despite differences in morphology, the viral particles of all influenza viruses are similar in their composition. The virion is enveloped with a host cell-derived lipid membrane wrapping around a central core (Figure 2).

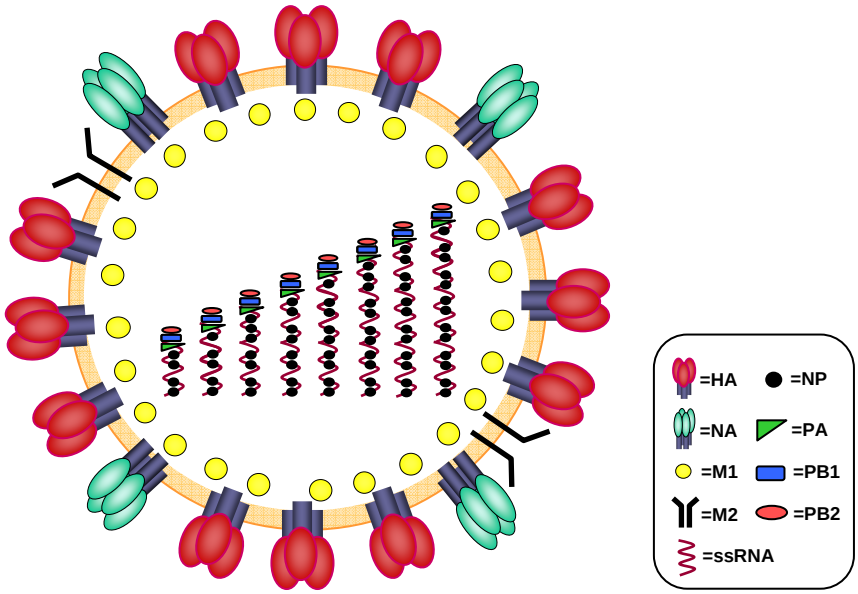


**Figure 2:** Influenza virus particle. The two major surface glycoproteins, homotrimers of HA and homotetramers of NA, and small amounts of the matrix 2 protein (M2) ion channel protein are embedded in the viral envelope. The matrix 1 protein (M1) protein underlies the envelope and interacts with surface proteins as well as with the viral ribonucleoproteins (vRNPs). The RNPs consist of eight negative-stranded RNA segments, nucleoproteins (NP) and the polymerase complex heterotrimer, consistent of the polymerase basic protein 1 (PB1), the polymerase basic protein 2 (PB2), and the polymerase acidic protein (PA).

Three proteins are embedded in the viral envelope: the homotrimers of HA, the homotetramers of NA, and monomers of the ion channel protein M2. Influenza C viruses bear only one major glycoprotein on their surface, called hemagglutinin-esterase-fusion (HEF), and one minor envelope protein (CM2), a putative ion channel protein [Hongo *et al.*, 2004; Palese and Shaw, 2007]. Beneath the membrane, the matrix 1 protein (M1) covers the inner leaflet of the lipid bilayer and interacts with the cytoplasmic domains of the surface glycoproteins HA and NA, as well as with the viral ribonucleoprotein (vRNP) complexes. Moreover, the nuclear export protein (NEP; also called non-structural protein 2, NS2) is associated with the matrix formed by M1 [Ye *et al.*, 1999; Shimizu *et al.*, 2011]. The virion core is located in the center of the viral particle and exhibits the RNP complexes. The RNPs consist of the eight (for influenza C viruses seven) negative-stranded RNA segments, which are covered by viral nucleoproteins (NP). Additionally, each genomic segment is associated with the three polymerase proteins: the polymerase basic protein 1 (PB1), the polymerase basic protein 2 (PB2), and the polymerase acidic protein (PA).

### 3.3.3 Genome structure

The size of the genome of influenza viruses ranges from 12.9 kb (for influenza C viruses) to 14.6 kb (for influenza B viruses) and consists of eight (or seven for influenza C viruses) negative-sense, single-stranded viral RNA (vRNA) segments, which encode at least nine (for influenza C viruses) or eleven (for influenza A and B viruses) open reading frames (ORFs). In influenza B and C viruses, the first five segments encode for one protein where as the remaining three or two segments code for two proteins. In contrast, the second and the two last segments of influenza A viruses encode for two proteins and the remaining five segments code for only one protein [Modrow *et al.*, 2003; Taubenberger and Kash, 2010].
The 3'- and 5'-ends of each vRNA segment form helical hairpins and possess non-coding regions, which function as promoter for vRNA replication and transcription by the viral polymerase complex. These non-coding regions also include the mRNA polyadenylation signal and are part of the packaging signals for virus assembly. Furthermore, the helical hairpin structure of the 3'-end is bound by the heterotrimeric RNA polymerase complex and the remaining single-stranded vRNA is associated with arginine-rich NP [Compans *et al.*, 1972; Murti *et al.*, 1988; Modrow *et al.*, 2003].

### 3.3.4 Replication cycle

The first step of the influenza virus life cycle constitutes the HA-mediated (or HEF-mediated for influenza C viruses) binding of the viral particle to its receptor, sialic acid (SA) on glycoproteins or glycolipids, expressed on the host cell surface (Figure 4) [Modrow *et al.*, 2003; Bouvier and Palese, 2008; Taubenberger and Kash, 2010]. After receptor binding, the viral particle is internalized by endocytosis [Lakadamyali *et al.*, 2004]. The acidic environment of the endosomal compartment triggers a conformational change in the HA, during which a fusion peptide is inserted into the endosomal membrane and both viral and endosomal membrane are pulled into close contact and ultimately fused. As a result, the vRNPs are released into the host cell cytoplasm (Figure 4) [Stegmann, 2000; Sieczkarski and Whittaker, 2005]. Accordingly, hydrogen ions from the endosome are pumped into the virus particle via the M2 ion channel. The internal acidification of the viral particle then disrupts the protein-protein interactions between NP- and M1-proteins, allowing vRNPs to be released from the viral matrix into the cellular cytoplasm [Martin and Helenius, 1991].

After the vRNPs enter the cytoplasm, they are directly transported to the host cell nucleus due to nuclear localization signal of the M1 proteins [Ye *et al.*, 1995; Cros and Palese, 2003]. Inside the nucleus, the viral RNA-dependent RNA polymerase (PB1) uses the negative-sense vRNA as a template to synthesize two positive-sense RNA species: mRNA templates for viral protein synthesis and complementary RNA (cRNA) intermediates from which the RNA polymerase subsequently transcribes more copies of negative-sense, genomic vRNA. For capping of the vRNA, PB1 and PB2 proteins exploit the 5'-capped primers from host pre-mRNA transcripts and connect them to the 3'-end of the viral RNA segment thereby allowing the initiation of viral mRNA synthesis to occur (Figure 4). This cap-steeling mechanism is called "cap snatching" [Krug, 1981; Modrow *et al.*, 2003]. The mRNAs of viral origin are exported and translated like host mRNAs, whereas the export of vRNAs is mediated by the viral proteins M1 and NEP/NS2 [Cros and Palese, 2003].

The translation of the envelope proteins HA (or HEF), NA and M2 occurs on membrane-bound ribosomes accompanied by co-translational translocation into the endoplasmic reticulum, where the proteins are folded, glycosylated (only HA and NA) and trafficked to the Golgi apparatus for post-translational modification (Figure 4). Golgi vesicles released from the Golgi apparatus direct the envelope proteins to the cell membrane for virion assembly [Modrow *et al.*, 2003].

All remaining viral proteins (PB1, PB2, PA, NP, NS1, NS2/NEP and M1) are transported back to the host cell nucleus where the NP, PB1, PB2 and PA proteins associate with newly synthesized vRNAs producing new vRNPs (Figure 4). Afterwards, the nucleocapsids are covered with the M1 proteins, exported into the cytoplasm through the function of NS2/NEP proteins and transported to subdomains of the plasma membrane containing increased amounts of envelope proteins (HA or HEF, NA and M2).

Virus budding is initiated at the cell membrane by an accumulation of M1 proteins at the cytoplasmic side of the lipid bilayer. During this initial budding structure, the vRNPs are associated with the M1 proteins and bind to the cytoplasmic domain of the surface proteins. Subsequently, the cellular membrane protrudes and covers the nucleocapsids, which are released as progeny virions through budding (Figure 4). The interaction of HA with its receptor, sialid acid, is abrogated by the activity of the NA protein and viral particles can be released from the host cell.

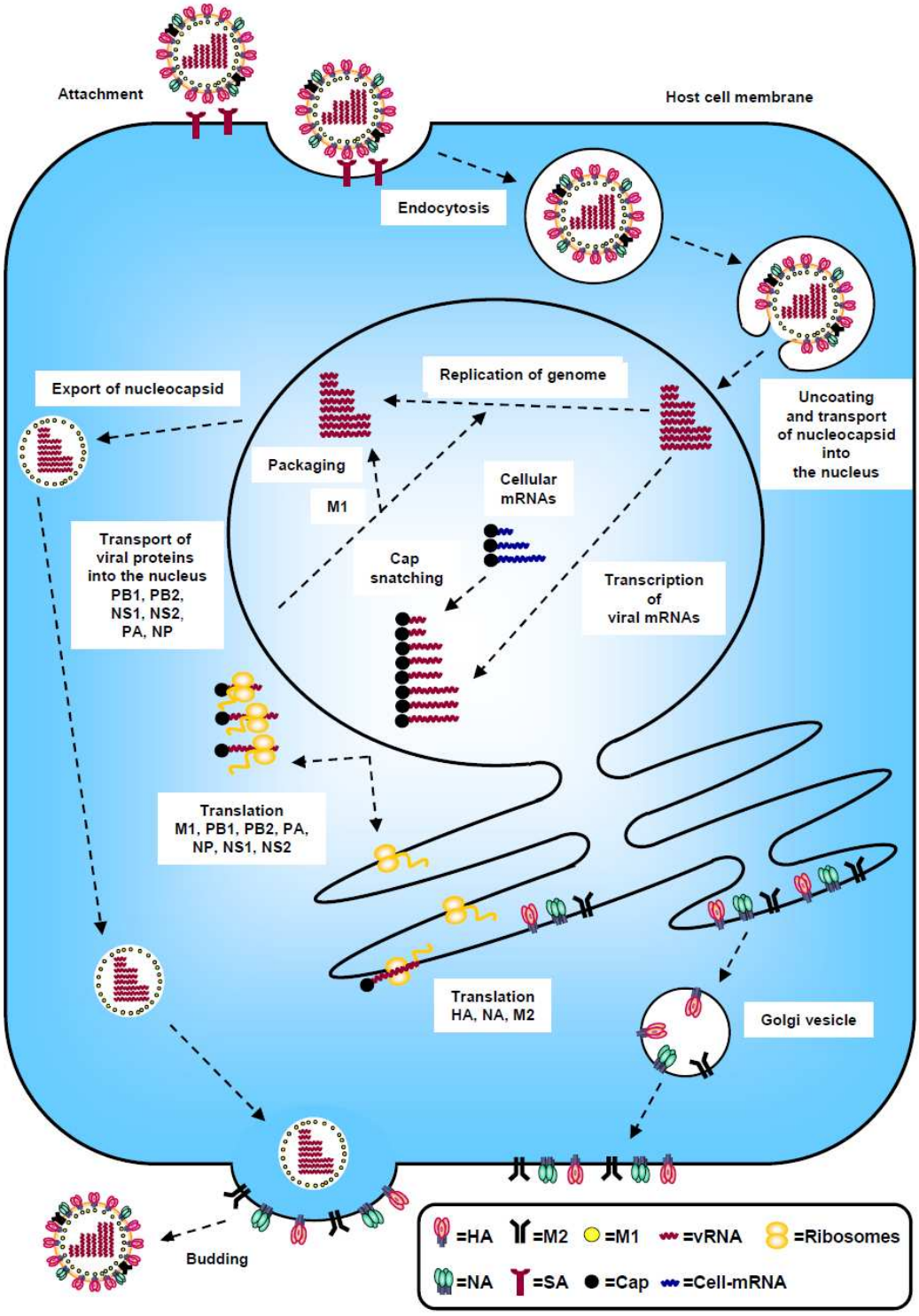


**Figure 3:** Replication cycle of influenza viruses. The first step of the influenza virus life cycle constitutes the HA-mediated binding of the viral particle to SA expressed on the host cell surface. After receptor binding, the viral particle is internalized by endocytosis. The acidic environment of the endosomal compartment triggers a conformational change in the HA, which induces the fusion between the viral and the endosomal membrane. As a result, the vRNPs are released into the host cell cytoplasm and were directly transported to the host cell nucleus. Inside the nucleus two positive-sense RNA species are synthesized: mRNA templates for viral protein synthesis and complementary RNA (cRNA) intermediates from which more copies of negative-sense, genomic vRNA are transcribed. For capping of the vRNA, the 5'-capped primers from host pre-mRNA transcripts are connected to the 3'-end of the viral RNA segment thereby allowing the initiation of viral mRNA synthesis to occur (cap

snatching). The mRNAs of viral origin are exported and translated like host mRNAs. The translation of the envelope proteins occur on membrane-bound ribosomes of the endoplasmatic reticulum, where the proteins are folded, glycosylated and trafficked to the Golgi apparatus for post-translational modification. Golgi vesicles released from the Golgi apparatus direct the envelope proteins to the cell membrane for virion assembly. All remaining viral proteins are transported back to the host cell nucleus and associate with newly synthesized vRNAs. Afterwards, the nucleocapsids are covered with the M1 proteins, exported into the cytoplasm, and transported to the plasma membrane containing increased amounts of envelope proteins. During budding, the vRNPs are surrounded by the cellular membrane and progeny virions are released [Figure was adapted from Modrow *et al.*, 2003].

### 3.3.5 Viral entry

The viral HA binds to sialic acids (SA) on glycoproteins or glycolipids, which triggers endosomal uptake of the viral particle [Lakadamyali *et al.*, 2004]. The acidic environment of the endosomal compartment prompts a conformational change in the HA which encompasses the insertion of a fusion peptide into the endosomal membrane and ultimately results in the fusion of the viral envelope with the endosomal membrane. As a result of membrane fusion, the vRNPs are released into the host cell cytoplasm [Modrow *et al.*, 2003; Bouvier and Palese, 2008; Taubenberger and Kash, 2010].

**Hemagglutinin**

The HA is a type I transmembrane fusion protein, which contains an N-terminal signal sequence (removed post-translationally), a surface unit, HA1, which binds the receptor, a transmembrane unit, HA2, which mediates fusion with the target cell membrane, and a short cytoplasmic tail (Figure 4) [Steinhauer, 1999; Bouvier and Palese, 2008; Harrison, 2008]. The HA also belongs to the group of class I fusion proteins, which exhibit an N-terminal or N-proximal fusion peptide and heptad repeat (HR) regions. In contrast, class II fusion proteins (e.g., the alphavirus E1 and the flavivirus E fusion proteins) lack HR regions and have an internal fusion peptide [Bosch *et al.*, 2003; Kielian and Rey, 2006]. Like influenza virus HA, several other viral glycoproteins (GP) are members of the class I fusion proteins: the HIV-1 gp160 protein, the Ebola virus GP protein, the SARS-CoV spike protein, and the Simian virus 5 F protein [Kielian and Rey, 2006].

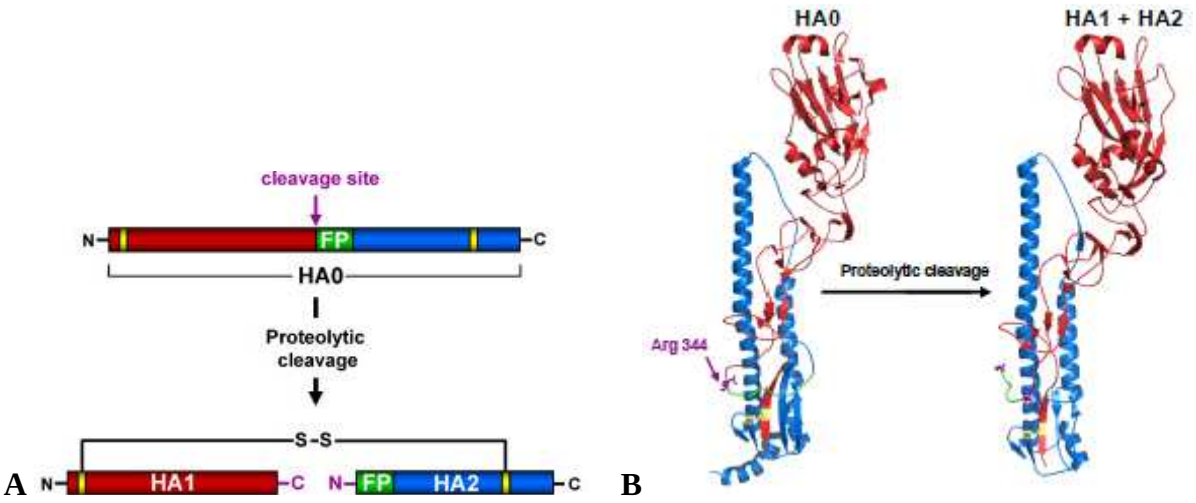


**Figure 4:** Structural rearrangements of influenza virus HA associated with the cleavage by host cell proteases. The HA of influenza viruses is produced as an precursor protein, HA0, in the infected cell and to obtain its complete fusion potential the HA0 needs to be cleaved by host cell proteases. Influenza viruses differ in their cleavage site sequence: HPAIVs harbour a polybasic cleavage site of multiple arginines and/or lysines whereas LPAIVs and human influenza viruses exhibit a monobasic cleavage of a single arginine (Arg 344 in purple in case of the 1918 influenza virus). The activating proteases recognize a cleavage site in the HA1-HA2 connecting sequence and expose the fusion peptide (green). The two subunits HA1 (red) and HA2 (blue) are still covalently linked by a single disulfide bond (yellow). (A) Schematic scheme of the HA cleavage. (B) Structural changes associated with the cleavage of the 1918 influenza virus HA (A/South Carolina/1/18 (H1N1); Accession No: ADD17229, HA0 (1RD8.pdb), HA1+HA2 (1RUZ.pdb)) [Figure was taken from Bertram *et al.*, 2010].

The HA is synthesized as an inactive precursor protein (HA0) that associates with chaperones to homotrimers in the endoplasmic reticulum and is transferred through the Golgi apparatus [Skehel and Wiley, 2000]. Furthermore, during the transport through the secretory pathway, the envelope protein is modified by the addition of glycans and the linkage of fatty acids (palmitoylation) [Modrow *et al.*, 2003]. After post-translational modification in the Golgi apparatus, the HA is transported to the host cell membrane. Membrane regions with high amounts of envelope proteins (including HA and NA) serve as budding areas for newly produced nucleocapsids. Interactions of M1 with M1, M1 with vRNP, and M1 with HA and NA facilitate concentration of viral components and exclusion of host proteins from the budding site. The M1 protein interacts with the cytoplasmic tail and transmembrane domain of HA and NA and thereby functions as a bridge between the viral envelope and vRNP. To complete the budding process, the receptor-destroying activity of NA is needed which allows the release of virus particles from SA residues on the cell surface [Ali *et al.*, 2000; Nayak *et al.*, 2004; 2009].

For maturation of HA, it needs to be post-translationally cleaved by host cell proteases where the cleavage site sequence determines the pathogenicity of avian influenza viruses (see

section 3.3.6 Proteolytic activation of the hemagglutinin) [Klenk and Garten, 1994; Steinhauer, 1999; Choi *et al.*, 2009; Bertram *et al.*, 2010]. Cleavage of HA0 generates mature HA1 and HA2, which are covalently linked by a disulfide bond (Figure 4). After proteolytic cleavage, the remaining amino acid residue (arginine or lysine) on the C-terminus of the progeny HA1 is removed. The cleavage of HA is indispensable for viral infectivity and also liberates the fusion peptide (FP), which is present at the N-terminus of HA2 and is necessary for membrane fusion.

**Receptor**

The influenza virus HA binds SA (N-acetylneuraminic acid) on the host cell surface. Sialic acids are monosaccharides with a nine-carbon backbone, usually found on terminal sugar residues (galactose) of surface glycoproteins and glycolipids. Expression of SA is found widely distributed in different animal tissues and to a lesser extent in other species ranging from plants and fungi to yeast and bacteria [Varki and Schauer, 2009].

Sialic acids and galactose can be linked by α-2,3- or a α-2,6-glycosydic bond [Skehel and Wiley, 2000; Harrison, 2008; Nicholls *et al.*, 2008]. Human influenza viruses bind to α-2,6-SA while avian influenza viruses recognize the α-2,3-SA [Rogers and D'Souza, 1989; Connor *et al.*, 1994; Matrosovich *et al.*, 1997; Ito *et al.*, 1998; Taubenberger, 2006]. The expression of α-2,3- and α-2,6-linked SA correlates with the viral cell tropism: In humans, α-2,6-linked SA is highly expressed in the lung epithelium and influenza is a respiratory infection [Baum and Paulson, 1990; Skehel and Wiley, 2000], while high levels of α-2,3-linked SA are found in the intestinal epithelium of waterfowl [Ito *et al.*, 1998; Causey and Edwards, 2008], the natural reservoir of influenza viruses [Webster *et al.*, 1992], and in these animals influenza is an enteric infection [Skehel and Wiley, 2000]. In contrast to water birds, poultry exhibit a higher concentration of α-2,3-linked SA in the lungs and only low concentrations of α-2,3-linked SA in the colon [Kim *et al.*, 2005].

Based on these receptor expressions being restricted to a specific host, one could assume that the viral replication only occurs in the appropriate host (avian viruses replicate in birds and human viruses replicate in humans). But the past showed that the correlation between specificity for α-2,3- or α-2,6-SA and influenza virus species tropism is not absolute: Avian influenza viruses can infect humans, although with reduced efficiency compared to human viruses [Beare and Webster, 1991]. The ability of avian viruses to replicate in humans is in agreement with the finding that SA in α-2,3-linkage is also expressed in human lung [Matrosovich *et al.*, 2004], but to a much lesser extent than SA in α-2,6-linkage and

predominately in the lower respiratory tract. Since the lower respiratory tract is not as accessible to airborne viruses as the upper respiratory tract, human-to-human transmission of avian viruses is usually inefficient or absent. However, once avian viruses have reached the lower respiratory tract, viral spread is efficient and can result in severe and rapidly progressive pneumonia [Gambotto *et al.*, 2008]. In 2003, a human-to-human transmission of an H7N7 avian influenza virus was detected during a large outbreak in commercially poultry farms in the Netherlands [Koopmans *et al.*, 2004].

**Membrane fusion**

Upon transport of influenza virus into host cell endosomes, the low endosomal pH triggers the membrane fusion activity of HA2. Lipid mixing of the viral and the endosomal membrane allows the formation of a fusion pore through which the vRNPs are released into the host cell cytoplasm. The FP in HA2 plays an essential role in membrane fusion: The FP is located at the N-terminus of HA2, which is liberated from HA1 upon HA cleavage by host cell proteases. Cleavage of the HA induces minor local rearrangements, in the course of which the newly generated N-terminus of HA2, and thus the FP, inserts into a pocket [Skehel and Wiley, 2000; Floyd *et al.*, 2008; Harrison, 2008]. Upon exposure of cleaved HA to endosomal pH (pH 5 to 6), HA undergoes large-scale conformational rearrangements: The FP is released from its pocket and inserts into the endosomal membrane and an extended intermediate is formed (Figure 5). At this stage, HA2 is inserted into both the endosomal membrane (via the FP) and the viral membrane (via the transmembrane domain) whereas HA1 remains flexibly tethered to the corresponding HA2 by a disulfide-bond (Figure 5).

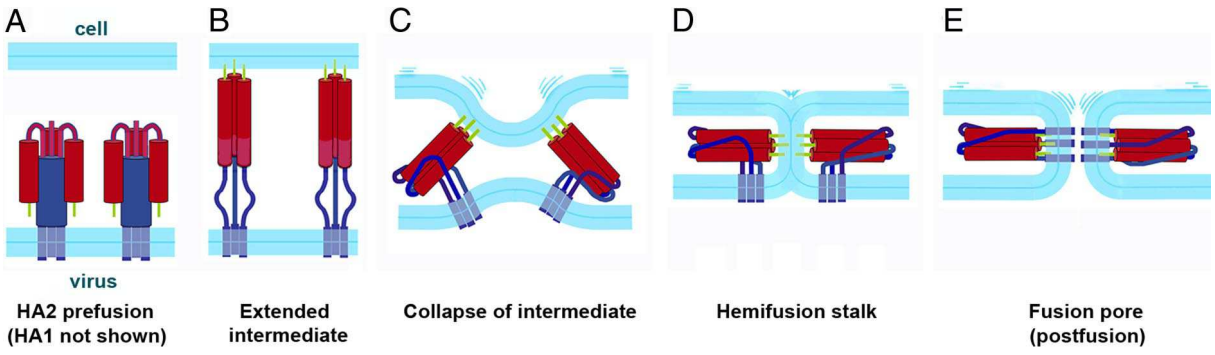


**Figure 5:** Schematic course of membrane fusion mediated by influenza virus HA. (A) In the prefusion state, the HA protein, anchored in the viral membrane by a C-terminal transmembrane domain, folds so that the fusion peptide (green) is sequestered. (B) The proton-binding induces a conformational change in which the fusion peptide projects toward the target membrane forming an extended intermediate that bridges the two membranes. (C) The intermediate collapses by zipping up of the C-terminal part of the ectodomain (dark blue) alongside the

trimer-clustered N-terminal part (red). (D) The collapse pulls the two membranes together leading to formation of a hemifusion stalk. (E) A fusion pore opens up and snapping into the place of the membrane-proximal and transmembrane segments of the HA completes the conformational transition and stabilizes the fusion pore [Figure was taken from Floyd *et al.*, 2008; Harisson, 2008].

Subsequently, two domains in HA2, the FP and the transmembrane anchor, fold back onto each other and the extended intermediate collapses. As a consequence of the back-folding reaction, the viral and the host cell membrane are pulled into close contact and the HA2 transits into an intermediate conformation, termed hemifusion stalk, in which lipid mixing but no content mixing occurs (Figure 5). Finally, the HA2 acquires a stable post-fusion conformation, in which the N- and C-terminal segments of the ectodomain of HA2 are tightly packed onto each other and which is associated with opening and/or stabilization of the fusion pore.

### 3.3.6 Proteolytic activation of the hemagglutinin

The HA of influenza viruses mediates vial attachment and fusion with the host cells. To acquire its membrane fusion potential, the precursor protein HA0 needs to be cleaved by host cell proteases [Klenk and Garten, 1994; Garten and Klenk, 1999; Steinhauer, 1999]. The proteolytic cleavage of HA0 separates the two subunits HA1 and HA2, which remains covalently connected by a disulfide bond and exposes the FP in HA2, which is essential for membrane fusion (Figure 5). The cleavage site sequences determine which type of cellular protease can activate HA and, in turn, the tissue distribution of these proteases determines influenza virus pathogenicity, as discussed below.

**Role of hemagglutinin cleavage in pathogenicity**

Influenza virus pathogenicity is multigenic including different viral genes and the determinants of pathogenicity may differ between hosts. Several viral proteins were identified which impact influenza virus pathogenicity, including the polymerase subunits, NA and HA [Almond, 1977; Webster and Bean, 1978; Oxford *et al.*, 1978; Scholtissek *et al.*, 1985; Snyder *et al.*, 1987; Treanor *et al.*, 1989; Wasilenko *et al.*, 2009; Ping *et al.*, 2010; Imai *et al.*, 2010]. The major determinant of viral pathogenicity in HA is the cleavage site and in the following we will discuss on how HA activation by host cell proteases determines the ability of influenza viruses to spread and cause disease [Horimoto and Kawaoka, 1994].

For avian influenza viruses, a strict correlation between cleavage site and pathogenicity has been established: Low pathogenic avian influenza viruses (LPAIV) contain a monobasic cleavage site consisting of one single arginine or lysine. Monobasic cleavage sites are believed to be recognized by certain trypsin-like proteases exclusively expressed in the gastrointestinal tract of waterfowl and in the respiratory and gastrointestinal tract of poultry [Garten and Klenk, 1999; Klenk and Garten, 1994]. Therefore, viral replication is restricted to these compartments and is associated with only mild symptoms. In contrast, highly pathogenic avian influenza viruses (HPAIV) possess a polybasic cleavage site which harbours several arginine or lysine residues with R-X-R/K-R as a consensus motif [Bosch *et al.*, 1981; Perdue *et al.*, 1997; Webster and Rott, 1987]. Some HPAIV do not contain a polybasic cleavage site, but instead lack a carbohydrate side chain or several amino acids close to the cleavage site and these alterations increase cleavability [Kawaoka *et al.*, 1984; Deshpande *et al.*, 1987; Ohuchi *et al.*, 1989; 1991; Skehel and Wiley, 2000]. The polybasic cleavage site of HPAIV is recognized by eukaryotic subtilisin-like proteases, particularly furin, which are ubiquitously expressed and thus allow systemic viral spread which is associated with severe disease in the avian host [Stieneke-Gröber *et al.*, 1992; Thomas, 2002].

Given the correlation between cleavability and pathogenicity of avian influenza viruses, one would expect that the same holds true for human influenza viruses. However, none of the pandemic viruses contained a polybasic cleavage site including the highly pathogenic 1918 influenza virus and it is unclear which proteases activate human influenza viruses. Therefore, the potential implications of the cleavage site for pathogenicitiy of human viruses are unclear and the identification of the proteases responsible for activation of human viruses should shed light onto this matter.

**Different host cell proteases are involved in the cleavage of influenza viruses**

Different host cell proteases can activate the HA of influenza viruses in different cellular compartments and at different stages of the viral life cycle (Figure 7) [Klenk and Garten, 1994; Steinhauer, 1999; Bertram *et al.*, 2010]. Cleavage can be mediated by soluble and by transmembrane proteases. Some of these enzymes are also required for activation of other viral and non-viral pathogens, as discussed below.

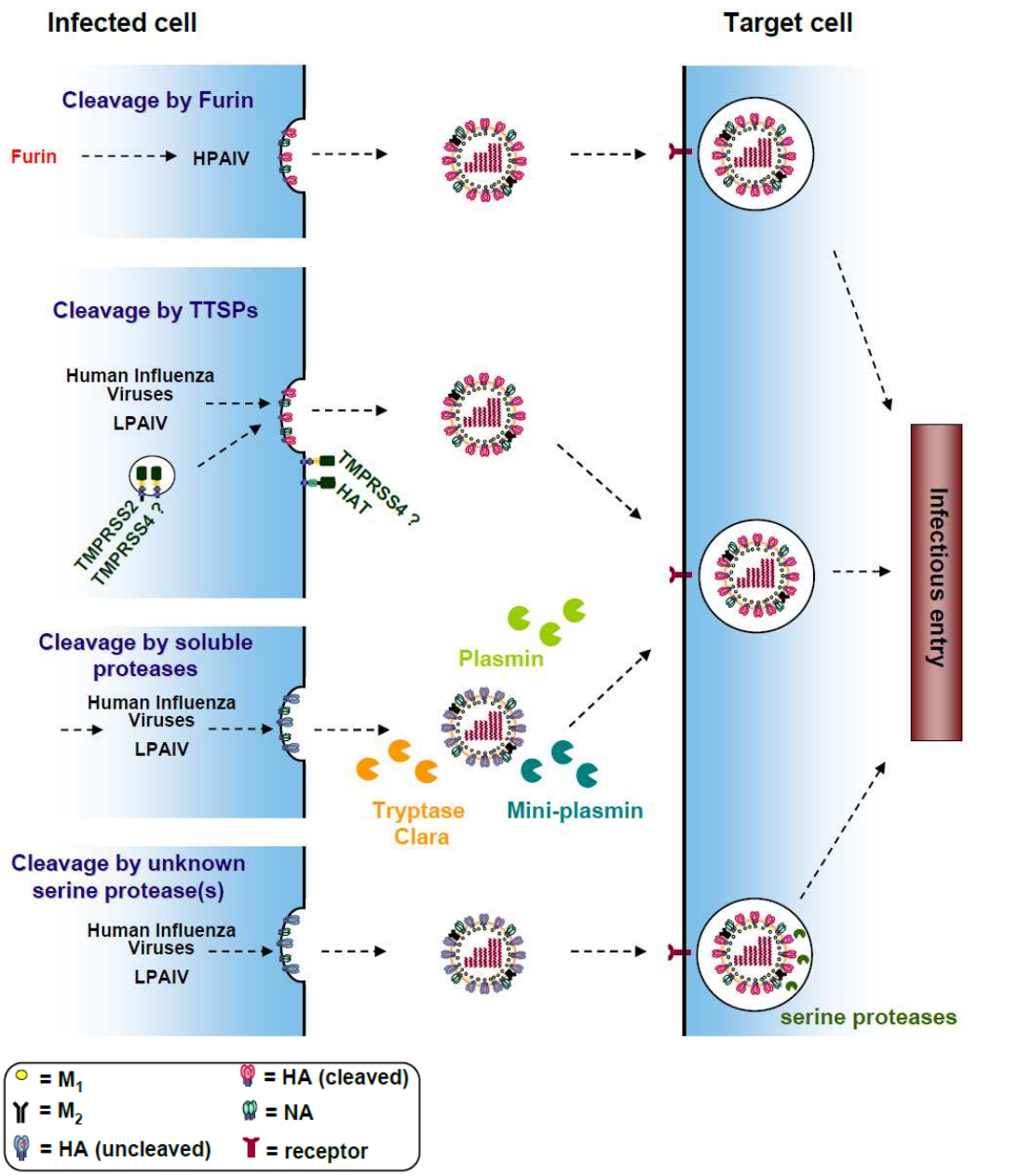


**Figure 7:** Activation of the influenza virus HA by different proteases. The influenza virus HA binds to α-2,3-linked (avian viruses) or α-2,6-linked (human viruses) sialic acids presented by glycoproteins or -lipids on the host cell surface. During endocytosis of the viral particle, protons are pumped in the virions to trigger the membrane fusion between the viral and the endosomal membrane. After fusion, the viral ribonucleoproteins are released in the cytoplasm and transported to the nucleus where the genomic and viral RNAs are synthesized. The viral membrane proteins are synthesized in the secretory pathway of infected cells. The HA of HPAIVs are cleaved in the Golgi apparatus by subtilisin-like proteases, as furin or PC5/6, and the cleaved HA is incorporated into the progeny virions. The HA of human influenza viruses or LPAIVs can be incorporated in a uncleaved form into progeny particles and the cleavage could occur by, at the moment unknown, serine proteases in the endosomal compartment of the newly infected cell. The unprocessed HA of human influenza viruses and LPAIVs could be cleaved by soluble proteases in the extracellular compartment by tryptase clara, plasmin and mini-plasmin. Alternatively, the HA could be also processed by the TTSPs TMPRSS2, TMPRSS4 and HAT in the route to the cell membrane (for TMPRSS2) or at the cell surface during insertion into the cellular membrane

(for HAT). The cellular locations for HA cleavage by TMPRSS4 is at present unclear [Figure was taken from Bertram *et al.*, 2010].

***Subtilisin-like proteases***

Highly pathogenic avian influenza viruses are activated by subtilisin-like proteases in the trans-Golgi network (TGN) of productively infected cells. These proteases are ubiquitously expressed and thus allow systemic infection resulting in severe disease. Two members of the protein family, named furin and PC5/6 (pro-protein convertase 5/6), were identified to activate the influenza virus HA by cleavage [Remacle *et al.*, 2008].

The endoprotease furin is the product of the *fur* gene, which is located upstream of the c-*fes/fps* proto-oncogene [Roebroek *et al.*, 1986]. It is responsible for the processing of several cellular pro-proteins (like proalbumin and insulin pro-receptor) in the secretory pathway [Nakayama, 1997] and plays a key role in embryonic development [Thomas, 2002]. In addition, furin activates several viral fusion proteins, including the spike protein of several mouse hepatitis coronavirus (MHV) strains and of human immunodeficiency virus [Hallenberger *et al.*, 1992; de Haan *et al.*, 2004]. Furin is mainly expressed in the Golgi apparatus, but is also found at the cell surfaces [Nakayama, 1997; Thomas, 2002]. The furin consensus sequence is R-X-K/R-R. The HA cleavage site of HPAIV comprises this sequence [Molloy *et al.*, 1992] and Stieneke-Gröber and colleagues identified furin as the cellular factor responsible for activation of HPAIV (Figure 7) [Stieneke-Gröber *et al.*, 1992]. Peptides spanning the consensus sequence and non-peptidic furin inhibitors were shown to suppress spread of HIV and influenza virus [Stieneke-Gröber *et al.*, 1992; Hallenberger *et al.*, 1992]. Furin also activates several bacterial toxins, including anthrax toxin and diphtheria toxin [Klimpel *et al.*, 1992; Molloy *et al.*, 1992; Tsuneoka *et al.*, 1993]. Another member of the subtilisin-like protein family, PC5/6, can also activate the HAs of HPAIV and, as shown for furin, activation occurs in the TGN [Horimoto *et al.*, 1994]. In summary, furin and to a lesser degree PC5/6 constitutes a therapeutic target in clinically important viral and bacterial diseases.

***Extracellular and endosomal trypsin-like proteases***

Low pathogenic avian influenza viruses and human influenza viruses bear a monobasic cleavage site and are believed to be cleaved extracellularly by various trypsin-like proteases (Figure 6). Consequently, uncleaved HA is incorporated into progeny viral particles [Lazarowitz *et al.*, 1971; Compans, 1973], at least in some cellular systems, and addition of trypsin, plasmin or related proteases to the culture medium strongly increases the infectivity

of these viruses [Appleyard and Maber, 1974; Lazarowitz and Choppin, 1975; Klenk *et al.*, 1975; 1977].

For instance, an HA-activating protease was isolated from chicken embryos, which are used as experimentally hosts for influenza virus amplification in the laboratory [Gotoh *et al.*, 1990]. The protease was found to be similar to the blood clotting factor X, a member of the pro-thrombin family, and localized to tissues in direct contact with allantoic and amniotic fluids, in which virus growth was detected [Ogasawara *et al.*, 1992]. Also Sendai virus and non-pathogenic Newcastle disease viruses as well as paramyxoviruses were activated by the isolated factor X-like protease [Gotoh *et al.*, 1990; Ogasawara *et al.*, 1992]. However, the contribution of the factor X-like protease to influenza virus spread in chicken embryos is unknown.

Proteases potentially responsible for cleavage activation of mammalian influenza viruses were isolated from various animal tissues, including rat lungs. Examples for these enzymes are tryptase Clara, which is produced by bronchial epithelial Clara cells, mini-plasmin, which is generated in epithelial cells of the upward divisions of bronchioles and ectopic anionic trypsin I, which is located in the stromal cells in peri-bronchiolar regions [Kido *et al.*, 1992; Murakami *et al.*, 2001; Towatari *et al.*, 2002]. The distinct expression patterns of theses proteases suggest that different enzymes might activate influenza viruses in different sections of the respiratory tract [Kido *et al.*, 1992; Ogasawara *et al.*, 1992; Murakami *et al.*, 2001; Towatari *et al.*, 2002], although evidence for a role of these proteases in influenza virus spread *in vivo* remains to be established. Finally, the efficiency of HA activation by soluble proteases might be modulated by naturally occurring inhibitors of these enzymes, like secretory leukoprotease inhibitor and pulmonary surfactant, which were both shown to inhibit viral replication *in vitro* and *in vivo* [Kido *et al.*, 2007].

Development of severe disease upon influenza virus infection is often due to a bacterial superinfection [Scheiblauer *et al.*, 1992; Kuiken and Taubenberger, 2008]. Interestingly, certain bacteria, including *Staphylococcus aureus* and *Aerococcus viridans*, were shown to secrete influenza virus activating proteases and to exacerbate disease in influenza virus infected mice [Tashiro *et al.*, 1987a; 1987b]. Whether a similar mechanism – augmentation of viral spread due to expression of HA-activating proteases by bacterial pathogens – is operational in humans is at present unknown. In summary, several soluble trypsin-like proteases can activate influenza viruses. However, for most of these enzymes (with the exception of bacterial proteases) an important role in influenza virus spread in the host remains to be established.

***Proteolytic activation of HA mediated by NA***

Two influenza viruses rely on their NA protein to accomplish efficient proteolytic activation of HA: The laboratory strain A/WSN/33 (H1N1) and the 1918 influenza virus (H1N1). The A/WSN/33 was obtained by extensive passaging of the parental virus, WS/33, in different animals and exhibits a broadened tropism, in particular neurotropism, relative to the parental virus. In contrast to other human influenza viruses, A/WSN/33 replicates in cultured cells in the absence of exogenous trypsin [Choppin, 1969; Castrucci and Kawaoka, 1993] and Lazarowitz and colleagues found that serum plasminogen was essential for trypsin-independent replication [Lazarowitz *et al.*, 1973]. Genetic analyses by Schulman and Palese indicated that the A/WSN/33 NA was critical for HA cleavage-activation [Schulman and Palese, 1977] with adequate NA glycosylation being important for neurovirulence [Li *et al.*, 1993]. Goto and Kawaoka connected the above discussed observations by demonstrating that A/WSN/33 NA binds plasminogen, which upon conversion to plasmin, activates HA (Figure 6) [Goto and Kawaoka, 1998]. Amino acids N146 (N2 numbering), which should miss a oligosaccharide side chain [Goto and Kawaoka, 1998] and residue L453, which is conserved among the NAs of the N1 subtype [Goto *et al.*, 2001], were found to be essential for plasminogen recruitment by NA.

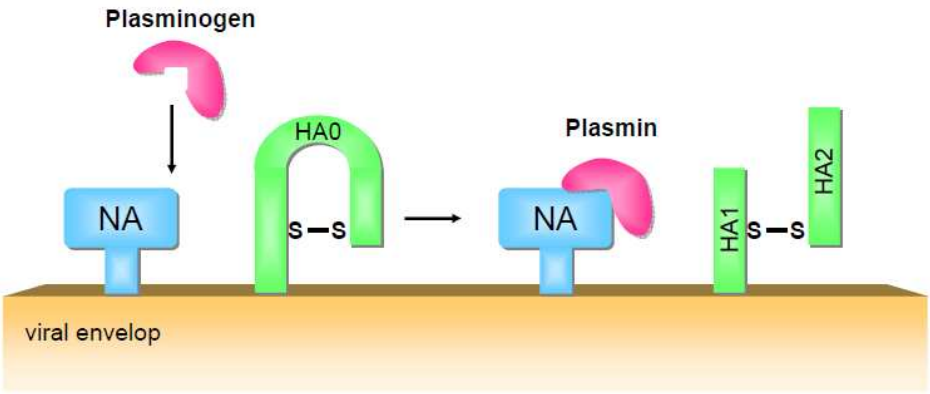


**Figure 6:** Schematic representation of plasminogen-mediated HA cleavage. Plasminogen binds to a lysine at the C-terminus of A/WSN/33 NA. Bound plasminogen is activated to plasmin by plasminogen activator (most likely of cellular origin). The enzymatically active plasmin then cleaves HA0 into HA1 and HA2 subunits [Figure adapted from Goto and Kawaoka, 1998].

The 1918 influenza virus replicated in the dog kidney cell line (MDCK) with high efficiency in the absence of trypsin and trypsin-independent spread was dependent on the presence of the 1918 NA [Tumpey *et al.*, 2005]. Analysis of 1918 NA revealed the presence of the L453 crucial for plasminogen binding, but also showed the presence of the glycosylation signal

incompatible with plasminogen recruitment by A/WSN/33 [Reid *et al.*, 2000]. These observations indicate that the 1918 NA, like the A/WSN/33 NA, might facilitates HA cleavage by recruiting a protease, which might not be identical to plasminogen. Alternatively, the 1918 NA might bind a factor which promotes plasminogen conversion into plasmin, like annexin II, which was shown to be incorporated into influenza virus particles and to support viral replication by activating plasminogen in an NA-independent manner [LeBouder *et al.*, 2008; 2010].

***Type II transmembrane serine proteases (TTSPs)***

A study conducted with primary human adenoid epithelial cells (HAECs), which model the upper respiratory tract of humans, demonstrated that activation of human influenza viruses was cell-associated and mediated by a serine protease [Zhirnov *et al.*, 2002]. These results suggest that proteolytic activation of human influenza viruses and potentially also LPAIV might be accomplished by unknown serine proteases.

A seminal study by Böttcher and colleagues revealed that type II transmembrane serine proteases (TTSPs) might be the elusive proteases responsible for activation of human influenza viruses and LPAIV (Figure 7). They could show that TMPRSS2 (transmembrane serine protease 2) and HAT (human airway trypsin-like protease) cleavage-activate the HA of all influenza subtypes (H1, H2, H3) which were previously pandemic in humans [Böttcher *et al.*, 2006]. These findings were confirmed by Wang and colleagues [Wang *et al.*, 2008] and a recent study identified two further TTSPs, MSPL (mosaic serine protease large form) and its splice variant TMPRSS13 as activating enzymes for HPAIV with a low efficiency recognition site for furin [Okumura *et al.*, 2010]. Notably, other enveloped viruses are also activated by TTSPs. Thus, the human metapneumovirus (HMPV), a paramyxovirus, is activated by TMPRSS2 to allow multiplicative replication in target cells constitutively expressing the serine protease [Shirogane *et al.*, 2008] and TMPRSS2 also activates the SARS-CoV spike protein for virus-cell and cell-cell fusion [Matsuyama *et al.*, 2010; Shulla *et al.*, 2011]. Finally, TMPRSS11a, another TTSP, enhances SARS-CoV entry into human bronchial epithelial cells [Kam *et al.*, 2009].

The TTSPs are an emerging family of proteases with important functions in embryonic development and tissue homeostasis. Furthermore, dysregulated expression of these proteases is involved in several diseases, including cancer. The TTSPs exhibit a characteristic domain organization: a short intracellular N-terminus, a transmembrane domain and a large extracellular C-terminus (Figure 8). The C-terminus consists of several protein domains and

their arrangement allows the division of TTSPs into four subfamilies: the matriptase subfamily, the corin subfamily, the hepsin/TMPRSS subfamily comprising e.g. TMPRSS2 and MSPL, and the HAT/DESC subfamily comprising e.g. HAT and TMPRSS11a [Szabo *et al.*, 2003; Szabo and Bugge, 2008; Choi *et al.*, 2009; Bertram *et al.*, 2010].

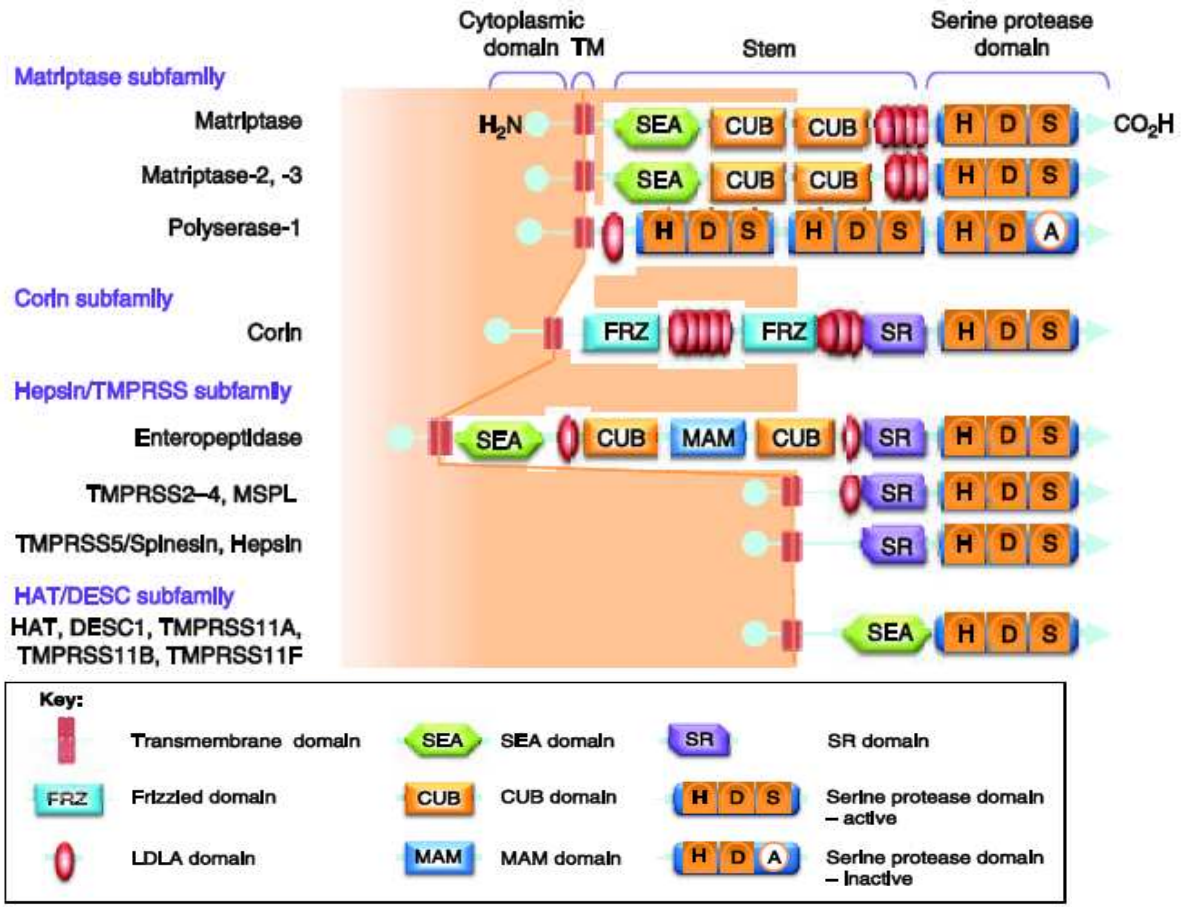


**Figure 8:** Domain organization of human TTSPs. The human TTSPs are grouped into four subfamilies based on the similarity in domain structure and phylogenetic analysis of the serine protease domains: the matriptase, the corin, the hespin/TMPRSS and the HAT/DESC subfamilies. Each diagram was drawn using the web-based SMART software (http://smart.embl-heidelberg.de) with the TTSP amino acid sequences obtained from GenBank. Letters H, D and S in the serine protease domain (active) indicate the position of the three catalytic residues histidine, aspartate and serine, respectively. Letter A in the serine protease domain (inactive) indicates a serine to alanine exchange. CUB (Cls/Clr, urchin embryonic growth factor and bone morphogenic protein-1 domain); DESC-1 (differentially expressed squamous cell carcinoma gene 1); FRZ (frizzled domain); HAT (human airway trypsin-like protease); LDLA (low-density lipoprotein receptor domain class A); MAM (a meprin, A5 antigen and receptor protein phosphatase m domain); MSPL (mosaic serine protease long-form); Polyserase-1 (polyserine protease-1); SEA (a single sea urchin sperm protein, enteropeptidase, agrin domain); SR (scavenger receptor cysteine-rich domain); TM (transmembrane domain) [Figure was taken from Choi *et al.*, 2009].

*TMPRSS2*

The serine protease TMPRSS2 (also known as epitheliasin) is highly expressed in prostate epithelial cells and lower levels of TMPRSS2 mRNA have also been detected in epithelia of the gastrointestinal, urogenital and respiratory tracts [Donaldson *et al.*, 2002; Szabo *et al.*, 2003; Szabo and Bugge, 2008]. The extracellular domain of TMPRSS2 is composed of low-density lipoprotein receptor domain class A (LDLA), scavenger receptor cysteine-rich domain (SR) and serine protease domain [Szabo *et al.*, 2003; Szabo and Bugge, 2008; Choi *et al.*, 2009; Bertram *et al.*, 2010].

In the lung, TMPRSS2 was shown to affect the regulation of the epithelial sodium channel, which controls the airway surface liquid volume and thus the efficiency of mucociliary clearance [Donaldson *et al.*, 2002]. Furthermore, the *tmprss2* gene was shown to be involved in prostate cancer development mainly due to recombination with genes encoding for ETS (E-twenty six) family transcription factors [Szabo *et al.*, 2003; Szabo and Bugge, 2008; Choi *et al.*, 2009; Bertram *et al.*, 2010].

Like other TTSPs, TMPRSS2 is generated as a zymogen and needs to be proteolytically activated, which may result in the release of an enzymatically active protease in the extracellular space [Afar *et al.*, 2001; Szabo and Bugge, 2008]. Whether the released enzyme can activate influenza virus is controversial [Garten *et al.*, 2004; Böttcher-Friebertshäuser *et al.*, 2010]. A study by Garten and colleagues demonstrate influenza replication in culture mediated by recombinant soluble TMPRSS2, which might be able to activate influenza viruses in the extracellular space of the human lung [Garten *et al.*, 2004]. In contrast, a work by Böttcher-Friebertshäuser and colleagues showed only marginal enzymatic activity of soluble forms which was not sufficient to support the cleavage of HA [Böttcher-Friebertshäuser *et al.*, 2010]. These results demonstrate that the membrane-bound and not the soluble form of TMPRSS2 could be responsible for activation of influenza.

*TMPRSS4*

Messanger RNA for TMPRSS4 was detected in the gastrointestinal tract, kidney, eye, skin, and lung [Szabo *et al.*, 2003; Szabo and Bugge, 2008; Chaipan *et al.*, 2009]. Until now, comprehensive data on TMPRSS4 protein expression in normal tissues are not available. Similar to TMPRSS2, TMPRSS4 belongs to the hepsin/TMPRSS subfamily and its extracellular domain consists of LDLA, SR and a serine protease domain [Szabo *et al.*, 2003; Szabo and Bugge, 2008; Choi *et al.*, 2009; Bertram *et al.*, 2010]. The physiological functions of TMPRSS4 are incompletely understood. Like several TTSP proteins, TMPRSS4 can

modulate ion channel activity [Garcia-Caballero *et al.*, 2008; Szabo and Bugge, 2008] where dysregulated expression was found to be associated with several cancers, including gastric, liver, lung, ovarian, pancreatic, and thyroid cancer [Choi *et al.*, 2008].

*HAT*

The HAT mRNA, like TMPRSS2 mRNA, was detected in many tissues including the respiratory and gastrointestinal tract [Szabo and Bugge, 2008]. Protein localization studies in human airway demonstrate HAT expression on bronchial ciliated epithelial cells and their cilia [Takahashi *et al.*, 2001]. The extracellular domain of HAT, like those of all members of the HAT/DESC subfamily, exhibits the simplest modular structure of all TTSPs and consists of a single SEA domain and a serine protease domain.

Under inflammatory conditions in chronic airway diseases (like asthma), high amounts of HAT are released in the airway fluids [Yasuoka *et al.*, 1997; Yamaoka *et al.*, 1998; Szabo and Bugge, 2008] and might activate influenza viruses in the extracellular compartment. However, a study by Böttcher-Friebertshäuser and colleagues showed, like for TMPRSS2, low proteolytic activity of soluble recombinant HAT which was not sufficient to activate influenza HA [Böttcher-Friebertshäuser *et al.*, 2010]. Human airway trypsin-like protease is involved in mucus production, deposition of fibrin in the airway lumen, activation of the protease-activated receptor 2 (PAR-2), and proteolytic inactivation of urokinase receptor (uPA) [Yoshinaga, *et al.*, 1998; Miki *at al.*, 2003; Chokki *et al.*, 2004; Chokki *et al.*, 2005; Beaufort *et al.*, 2007].

*Summary*

Type II transmembrane serine proteases were shown to activate human influenza viruses in transfected cells and it was proposed that these proteins, and potentially related ones, might be responsible for influenza virus activation in the infected host. However, it is unclear if endogenously expressed TTSPs activate influenza viruses. The highly pathogenic 1918 influenza virus and the related virus A/WSN/33 both employ their NAs to promote HA activation. The A/WSN/33 facilitates HA activation by recruiting plasminogen, but the role of this preprotease in 1918 NA-dependent activation of 1918 HA is unclear.

## 4. Aim of the study

The hemagglutinin (HA) of influenza viruses mediates viral entry into target cells. Host cell proteases activate HA by cleavage and activation is essential for viral infectivity. Highly pathogenic avian influenza viruses are cleaved by subtilisin-like proteases, but little is known about the proteases facilitating the activation of low pathogenic avian influenza viruses and human influenza viruses. A landmark study by Böttcher and colleagues suggested that type II transmembrane serine proteases (TTSP) can activate human influenza viruses, at least upon expression in transfected cells. However, it is currently unclear if these proteases are expressed in viral target cells and if endogenous expression supports viral spread. The highly pathogenic 1918 influenza virus and the laboratory adapted virus A/WSN/33 employ their neuraminidase (NA) proteins to facilitate HA activation. The NA of A/WSN/33 facilitates activation of HA by recruiting the preprotease plasminogen, but the molecular mechanism underlying HA activation by the 1918 NA is unknown.

The first aim of this study was to determine if 1918 NA, like A/WSN/33 NA, binds to plasminogen and thereby facilitates HA activation. For this, plasminogen binding to 1918 NA was to be examined in fluorescence-activated cell sorting (FACS)-based assays and HA cleavage was to be assessed by Western blot. In addition, lentiviral pseudotyping was to be employed to analyze if A/WSN/33 NA can functionally replace 1918 NA. Finally, in collaboration with colleagues at BSL4 facilities, it was to be assessed if NA-dependent activation of the 1918 influenza virus is a universal phenomenon or if this process is restricted to certain target cell lines.

The second aim of the study was to determine if HA-activating TTSPs are endogenously expressed in viral target cells and facilitate viral spread. To this end, TTSP expression in cell lines was to be analyzed on the mRNA and protein level and was to be correlated with the ability of influenza virus to spread in the absence of an exogenously added HA-activating protease. Finally, knock-down of protease expression by siRNA was to be used to determine the relevance of specific proteases for influenza virus activation, and immunohistochemistry was to be employed to determine if the respective proteases are expressed in viral target cells in the human lung.

## 5. Manuscripts

Stage of publication

Title

**Proteolytic Activation of the 1918 Influenza Virus Hemagglutinin**

Journal

Journal of Virology, Apr. 2010; 83(7):3200-3211.

Title

**TMPRSS2 and TMPRSS4 Facilitate Trypsin-Independent Spread of Influenza Virus in Caco-2 Cells**

Journal

Journal of Virology, Oct. 2010; 84(19):10016-10025

First manuscript

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# Proteolytic Activation of the 1918 Influenza Virus Hemagglutinin▽

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**Proteolytic activation of the hemagglutinin (HA) protein is indispensable for influenza virus infectivity, and the tissue expression of the responsible cellular proteases impacts viral tropism and pathogenicity. The HA protein critically contributes to the exceptionally high pathogenicity of the 1918 influenza virus, but the mechanisms underlying cleavage activation of the 1918 HA have not been characterized. The neuraminidase (NA) protein of the 1918 influenza virus allows trypsin-independent growth in canine kidney cells (MDCK). However, it is at present unknown if the 1918 NA, like the NA of the closely related strain A/WSN/33, facilitates HA cleavage activation by recruiting the proprotease plasminogen. Moreover, it is not known which pulmonary proteases activate the 1918 HA. We provide evidence that NA-dependent, trypsin-independent cleavage activation of the 1918 HA is cell line dependent and most likely plasminogen independent since the 1918 NA failed to recruit plasminogen and neither exogenous plasminogen nor the presence of the A/WSN/33 NA promoted efficient cleavage of the 1918 HA. The transmembrane serine protease TMPRSS4 was found to be expressed in lung tissue and was shown to cleave the 1918 HA. Accordingly, coexpression of the 1918 HA with TMPRSS4 or the previously identified HA-processing protease TMPRSS2 allowed trypsin-independent infection by pseudotypes bearing the 1918 HA, indicating that these proteases might support 1918 influenza virus spread in the lung. In summary, we show that the previously reported 1918 NA-dependent spread of the 1918 influenza virus is a cell line-dependent phenomenon and is not due to plasminogen recruitment by the 1918 NA. Moreover, we provide evidence that TMPRSS2 and TMPRSS4 activate the 1918 HA by cleavage and therefore may promote viral spread in lung tissue.**

Influenza A viruses exhibit high genetic variability. The accumulation of relatively subtle changes in the surface proteins hemagglutinin (HA) and neuraminidase (NA) of currently circulating viruses, termed antigenic drift, is responsible for the annual influenza virus epidemics. However, the reassortment of genomic material between human and animal influenza A viruses can occasionally lead to emergence of viral variants with radically different antigenic properties, a phenomenon termed antigenic shift (9, 32). Due to the lack of preexisting immunity in the human population, these viruses can cause pandemics. Three influenza pandemics were recorded in the last century. The so-called Asian influenza in 1957 and the Hong Kong influenza in 1968 caused approximately >2 million and 1 million deaths (World Health Organization, Geneva, Switzerland; www.who.int/csr/disease/influenza), respectively, and the etiologic agents were reassortants between human and avian influenza A viruses (2). The third influenza pandemic, which occurred in 1918 and is commonly termed Spanish influenza, differed in several aspects from the previously mentioned pandemics (1, 30). First, the mortality associated with the 1918 pandemic was extraordinarily high, and it is estimated that about 20 to 50 million people died from the disease. Second, instead of infants and the elderly, who are usually the main populations affected in influenza virus epidemics, adults between the ages of 18 and 30 had to bear the brunt of the 1918 pandemic (1, 30). Third, evidence is accumulating that the 1918 virus has similarities with avian influenza viruses and was not the product of a reassortment between human and animal viruses (43, 44).

Reconstitution of the 1918 influenza virus by reverse genetics (45) showed that HA, NA, and PB1 critically contribute to high virulence (22, 31, 47). The HA protein mediates binding to the cellular receptor, alpha 2,6 sialylated glycans and, upon exposure to endosomal low pH, drives fusion of the virus and a cellular membrane, a prerequisite to infectious entry (39). Cleavage of the precursor protein $HA_0$ into the covalently linked subunits $HA_1$ and $HA_2$ by a cellular protease is required for viral infectivity (20, 23) and is an important determinant of viral tropism (40). Usually a single arginine residue is present

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at the border between $HA_1$ and $HA_2$ and is part of the motif recognized by cellular proteases such as serine family proteases (17, 18), like the recently identified HA-processing proteases TMPRSS2 (transmembrane protease, serine 2) and HAT (human airway trypsin-like protease) (5). Since expression of these proteases is limited to the respiratory tract in mammalian hosts, virus replication is confined to this target site. However, an optimized, multibasic cleavage site is present in all highly pathogenic avian influenza viruses. The HA protein of these viruses is cleaved by ubiquitously expressed subtilisin-like proteases, and consequently the respective viruses can spread systemically in susceptible domestic poultry (17, 18). The requirement for addition of trypsin to support efficient virus replication in cell culture is determined by the nature of the cleavage site in the HA protein. Viruses harboring a multibasic cleavage site can spread in the absence of trypsin, while trypsin activation is essential for the replication of viruses that do not encode an HA protein with a multibasic cleavage site.

The 1918 influenza virus HA does not harbor a multibasic cleavage site, yet the virus replicates to high titers in MDCK cells irrespective of trypsin activation (45). Notably, NA is essential for trypsin-independent spread of the 1918 virus in MDCK cells (45). The NA of a related virus, A/WSN/33 (H1N1), also facilitates efficient viral replication in the absence of trypsin and is required for an expanded viral tropism (14, 15). The A/WSN/33 NA abrogates the need for trypsin by sequestering plasminogen which, upon conversion to plasmin, facilitates HA cleavage (14, 15). A comparable mechanism can be envisioned for the 1918 influenza virus NA. However, plasminogen binding by the A/WSN/33 NA critically depends on the absence of a N-linked glycosylation motif in NA that is otherwise conserved in all N1 subtype NA proteins of naturally occurring viruses, including the 1918 virus (34). The molecular mechanism behind NA-dependent, trypsin-independent replication of the 1918 influenza virus is therefore at present unclear.

We established a pseudotyping system to analyze the proteolytic activation of the 1918 HA. We report that the previously noted 1918 NA-dependent, trypsin-independent activation of the 1918 HA in MDCK cells does not occur in Huh-7 and 293T cells and is not due to plasminogen recruitment by the 1918 NA. In addition, we show that the transmembrane proteases TMPRSS2 and TMPRSS4 activate the 1918 HA by cleavage, suggesting that these proteases might facilitate replication of the 1918 influenza virus in lung cells.

## MATERIALS AND METHODS

**Cell culture and plasmids.** 293T, Huh-7, and MDCK cells were maintained in Dulbecco's modified Eagle's medium (PAA) supplemented with 10% fetal bovine serum (FCS; Cambrex) and the antibiotics penicillin and streptomycin. Plasmids encoding the 1918 HA (South Carolina), NA, and M2 (Brevig Mission) proteins as well as the Zaire Ebola virus glycoproteins (ZEBOV-GP), A/WSN/33 HA, A/WSN/33 NA, vesicular stomatitis virus G protein (VSV-G), TMPRSS2, TMPRSS4, and mouse matriptase-3 have been described previously (11, 13, 19, 33, 34, 36, 38, 41, 46).

**Production of virus-like particles (VLPs) and Western blot analysis.** 293T cells were cotransfected with the human immunodeficiency virus type 1 (HIV-1) Gag (p55) encoding plasmid p96ZM651gag-opt (12) and the indicated combinations of the 1918 influenza virus HA, NA, and M2 expression plasmids or empty vector. The culture medium was changed 12 h after transfection, and VLPs in the supernatant were harvested 36 h later. VLPs were passed through 0.45-μm pore-size filters and concentrated by centrifugation through a 20% sucrose cushion for 2 h at 4°C and 14,000 rpm or by using Vivaspin centrifugal concentrators. VLPs were resuspended in medium and, if applicable, treated with trypsin and subsequently soybean trypsin inhibitor (Sigma, Germany). Sodium dodecyl sulfate (SDS) buffer was added, and the samples were incubated at 95°C for 20 min and loaded onto a 10% SDS-polyacrylamide gel electrophoresis gel. HA was detected using a mouse monoclonal antibody (13) at a 1:500 dilution.

**Reporter viruses and infection assays.** For generating HIV-1 NL4-3-based luciferase reporter viruses, 293T cells were transiently cotransfected with pNL4-3 $E^-R^-$ Luc (8) and combinations of 1918 influenza virus HA, NA, and M2 expression plasmids; A/WSN/33 HA and NA expression plasmids; or ZEBOV-GP and VSV-G expression plasmids (38) as controls. At 48 h posttransfection, cell culture supernatants were harvested, passed through 0.45-μm pore-size filters, aliquoted, and stored at −80°C. Subsequently, the p24 content of virus stocks was determined by enzyme-linked immunosorbent assay (Murex, Germany), or infectivity was assessed by inoculation of target cells with different dilutions of virus stocks, followed by quantification of luciferase activities in cellular lysates. For infection experiments, target cells were seeded in 96-well plates at a density of $1 \times 10^4$ cells/well 24 h prior to infection. The cellular monolayer was inoculated with 50 μl of virus stocks normalized for p24 content or infectivity. Medium was replaced 12 h after virus infection, and luciferase activities in cell lysates were determined 72 h postinfection employing a commercially available kit (Promega).

**Influence of trypsin pretreatment and spinoculation on infectivity of HA pseudotypes.** Viruses pseudotyped with 1918 influenza virus proteins or A/WSN/33 HA and NA were pretreated with L-1-tosylamido-2-phenylethyl chloromethyl ketone (TPCK)-treated trypsin (100 μg/ml final concentration) (Sigma, Germany) for 10 min at room temperature. Subsequently, trypsin was inactivated by adding soybean trypsin inhibitor (Sigma, Germany) to a final concentration of 100 μg/ml. Viruses were either added to the cells and the cells were directly placed in the incubator, or cells were infected by centrifugation (spinoculated) at 1,200 rpm and 25°C for 2 h. Medium was replaced 12 h after transduction, and luciferase activities in cell lysates were determined 72 h postinfection using a commercially available kit (Promega).

**Protease inhibitor experiments.** Cells were pretreated with the indicated concentrations of E64d (Sigma, Germany) for 30 min at 37°C and infected with infectivity-normalized pseudotypes. In the case of HA NA pseudotypes, viruses were pretreated with trypsin as described above or treated with phosphate-buffered saline (PBS) and spinoculated onto cells. Medium was replaced 12 h postinfection, and luciferase activities in cell lysates were measured after 3 days using a commercially available kit (Promega).

**Activation of pseudotype infectivity by cellular lysates.** MDCK and 293T cells were grown in 10-cm dishes, scraped off the culture dishes, and counted, and 5 million MDCK or 293T cells were resuspended in 1 ml of PBS containing 10 mM $CaCl_2$ and 2% 1-deoxymannojirimycin hydrochloride (Sigma, Germany), as described previously (6). Cells were then sonicated on ice for 3 s. Pseudotyped viruses were pretreated with either the indicated amounts of cellular lysates for 1 h at 37°C or 100 μg/ml TPCK-treated trypsin for 10 min at room temperature. Virus particles were concentrated by centrifugation through a 20% sucrose cushion at 14,000 rpm for 2 h at 4°C. Virus pellets were resuspended in medium and used for infection of Huh-7 and 293T cells. Medium was replaced 12 h postinfection, and luciferase activities in cell lysates were determined 60 h later using a commercially available kit (Promega).

**Plasminogen binding to HA and NA.** 293T cells were seeded in T-25 flasks and transiently transfected with plasmids encoding the 1918 NA, 1918 HA, A/WSN/33 HA, A/WSN/33 NA, and ZEBOV-GP or were control transfected with empty vector. Culture medium was replaced at 12 h posttransfection, and at 2 h before analysis of plasminogen binding, the cells were washed with FCS-free medium and cultured in the absence of FCS. For assessment of plasminogen binding, cells were incubated with PBS or purified plasminogen (Sigma, Germany) at a final concentration of 10 μg/ml for 30 min at 4°C. Subsequently, unbound plasminogen was removed by washing, and bound plasminogen was detected employing a plasminogen-specific goat serum (Immunology Consultants Laboratory) and an Cy5-labeled anti-goat secondary antibody (Jackson ImmunoResearch). Cell staining was analyzed by fluorescence-activated cell sorting (FACS) using a Beckman Coulter Cytomics FC 500.

**Replication of the 1918 influenza virus in MDCK and Huh-7 cells with or without trypsin activation.** Based on the published sequences of the 1918 influenza virus genes, the complete viral and complementary sequences were synthesized as 40-mer oligonucleotides, with a 20-nucleotide overlap between each forward and reverse primer. The ligase chain reaction was used for initial assembly of subgenomic fragments from the pooled primers for each gene, and the full-length genes were assembled by amplification from the pool of gene fragments by using PCR according to the protocol described by Rouillard and

colleagues (35). Each gene was further amplified by PCR using the gene-specific universal primer pairs described by Hoffmann and colleagues (16) and subcloned into the pPolI vector for reverse genetics (16, 27). The 1918 influenza virus was rescued as previously described (22) and titers were determined by standard plaque assay on MDCK cells in the presence of TPCK-treated trypsin. MDCK or Huh-7 cells were seeded in six-well tissue culture plates and infected at a multiplicity of infection (MOI) of 0.001, and then culture medium was added with or without 1 μg/ml of TPCK-treated trypsin. Supernatants of the virus-infected MDCK cultures were collected at 24, 48, and 72 h and at 48 h postinfection (p.i.) from infected Huh-7 cultures and stored at −80°C. Virus titers were determined by the 50% tissue culture infective dose on MDCK cells seeded in 96-well plates. Samples from cultures maintained without trypsin were treated with trypsin (at a final concentration of 1 μg/ml) prior to titration. Procedures for the production and propagation of the 1918 virus and all subsequent experiments involving infectious 1918 influenza virus were performed in a biosafety level 4 facility of the National Microbiology Laboratory of the Public Health Agency of Canada.

**Detection of TMPRSS4 mRNA in lung cells.** mRNA from cells in bronchoalveolar lavage was extracted, DNase treated, and reverse transcribed employing commercially available kits (Qiagen, Germany, and Invitrogen, Germany). Subsequently, GAPDH (glyceraldehyde-3-phosphate dehydrogenase) and TMPRSS4 sequences were amplified by nested PCR. The following sets of primers were used for amplification of TMPRSS4: the pair 5′-GAGAGCTG GACTGTCCCTTG-3′ (p5 TMPRSS4 out) and 5′-TCGTACTGGATGCTGA CCTG-3′ (p3 TMPRSS4 out) and the pair 5′-GACGAGGAGCACTGTGTCAA-3′ (p5 TMPRSS4 in) and 5′-CTTCCCACAGGCAAGACAGT-3′ (p3 TMPRSS4 in).

## RESULTS

**Trypsin treatment of 1918 influenza virus HA- NA-bearing pseudotypes is required for infectivity.** It has been shown previously that the genetically reconstituted 1918 H1N1 influenza virus replicates in MDCK cells to high titers irrespective of the presence of trypsin (45). The expression of the 1918 virus NA was essential for trypsin-independent growth (45), and it is possible that NA might recruit a cellular protease, which facilitates HA cleavage—a prerequisite to HA-dependent infectious cellular entry (39). In order to further characterize the role of NA in HA activation, we sought to establish a pseudotyping system, which allows convenient analysis of HA and NA in the absence of infectious 1918 influenza virus. Since the influenza virus M2 protein is present in the viral envelope and can prevent premature triggering of HA (3), we also included the 1918 influenza virus M2 protein in our analysis. We first investigated if Env-defective HIV-1 NL4-3-based reporter viruses (8) pseudotyped with the 1918 influenza virus HA, NA, and M2 or combinations thereof are infectious for 293T cells. To this end, virus preparations were normalized for equal content of p24 capsid protein, treated with TPCK-trypsin or PBS, and, after the addition of a trypsin inhibitor, used to infect 293T cells. No luciferase activities above background levels were observed in lysates of cells infected with Env-negative "bald" pseudotypes, while VSV-G-bearing pseudotypes allowed efficient virus entry independent of the presence of trypsin (Fig. 1A). Robust infection of 293T cells with pseudotypes bearing the membrane proteins of the 1918 influenza virus was observed but only when both HA and NA were expressed in the cells used for pseudotype production and when pseudotypes were treated with trypsin before addition to target cells (Fig. 1A). In contrast, pseudotypes produced in the absence of NA were not infectious. This defect was most likely due to a lack of virion incorporation of HA. Thus, lentiviruses are inefficiently released from influenza virus HA-transfected cells in the absence of NA (Fig. 1B, lane 3) (4), and the particles that are released under these conditions do not harbor detectable amounts of HA (4), consistent with the established key role of NA in facilitating virus release due to its receptor-destroying function (40). Finally, we observed that coexpression of HA and NA together with the M2 protein reduced HA incorporation into pseudotypes and thus diminished pseudotype infectivity (Fig. 1B, lane 5).

We next sought to confirm whether trypsin treatment indeed resulted in cleavage of HA. Western blot analysis of V5 epitope-tagged versions of HA, NA, and M2 showed robust and comparable expression in 293T cell lysates (Fig. 1B and data not shown), and efficient incorporation of HA, NA, and M2 into HIV-1 Gag p55-based VLPs could be detected with V5-specific (for NA and M2) or HA-specific monoclonal antibodies (Fig. 1B and data not shown). Importantly, trypsin treatment of VLPs did not impact gel migration of NA or M2 (data not shown), while trypsin treatment reduced the size of HA from approximately 75 kDa to 50 kDa, consistent with cleavage at the border between $HA_1$ and $HA_2$ (Fig. 1B).

**Augmentation of cellular attachment allows inefficient infectious entry of the 1918 HA NA pseudotypes in the absence of trypsin activation.** We next investigated if the infectivity of the 1918 influenza virus HA and NA pseudotypes for different cell lines is invariably trypsin dependent. Moreover, we asked if the requirement for trypsin can be overcome when virus attachment and, thus, infection efficiency are increased. For this, 293T, Huh-7, and MDCK cells were infected with pseudotypes harboring the 1918 influenza virus HA, NA, and M2 proteins or VSV-G in their envelopes. All pseudotypes were pretreated with trypsin or PBS and used for infection of two plates in parallel. One plate was instantly incubated at 37°C; the other was used for spinoculation (2 h at 25°C and 1,200 rpm) (28) and then kept at 37°C. All cell lines were readily susceptible to infection with VSV-G (independent of trypsin treatment) and trypsin-activated HA NA pseudotypes, while none of the cell lines was susceptible to HA NA pseudotypes in the absence of trypsin treatment and spinoculation (Fig. 2A). Spinoculation substantially augmented entry of HA- NA-bearing viruses in the absence of trypsin treatment (Fig. 2A), and analysis of p24-normalized, non-trypsin-treated virus preparations confirmed that the presence of HA and NA was required for appreciable infectious entry under these conditions (data not shown). However, even upon spinoculation, infectious entry of non-trypsin-treated virus was less efficient than entry of trypsin-activated virus (Fig. 2A). Finally, in agreement with our previous observations (25), spinoculation had little effect on infection by pseudotypes exhibiting high infectivity in the absence of experimentally optimized attachment, i.e., reporter viruses bearing trypsin-activated HA, NA, or VSV-G proteins (Fig. 2A). Thus, increased attachment efficiency allows some infectious entry of HA- NA-bearing viruses in the absence of trypsin but does not abrogate the requirement for trypsin activation for high infectivity.

The observation that HA NA pseudotypes can infect cell lines with low but detectable efficiency without prior trypsin activation (Fig. 2A) suggests that uncleaved HA might also be activated by proteases in target cells. The pH-dependent endosomal cysteine proteases cathepsin B and L activate the severe acute respiratory syndrome (SARS)-coronavirus and EBOV-GP (7, 37) and are required for infectious entry. In order to analyze if the activity of cysteine proteases also con-

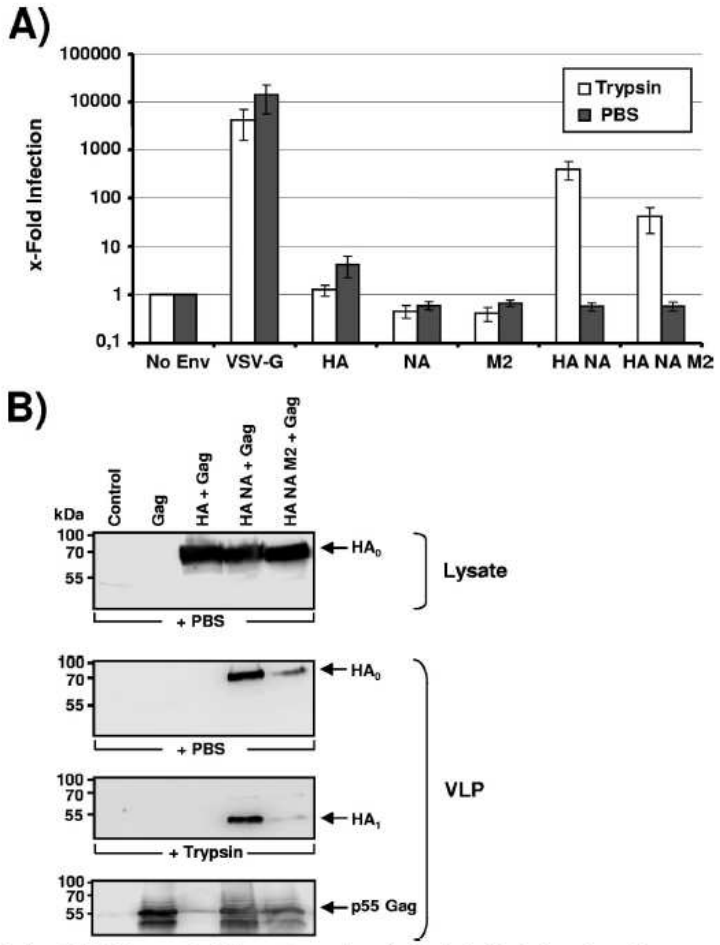


FIG. 1. Trypsin activation of 1918 influenza virus HA pseudotypes is required for infectivity independent of the presence of NA. (A) 293T cells were infected with *env*-defective, p24-normalized HIV-1 NL4-3 reporter virus pseudotyped with the indicated glycoproteins. Prior to infection, the virions were treated with trypsin or PBS, and after 10 min soybean trypsin inhibitor was added. Three days after infection, luciferase activities in cellular lysates were determined. Infection observed with glycoprotein harboring pseudotypes is shown relative to infection detected with control particles bearing no glycoprotein. The average of four independent experiments with different virus stocks is shown. Error bars indicate standard errors of the means. (B) HIV-1-based VLPs with the indicated combinations of surface proteins were generated in 293T cells and concentrated by centrifugation through a 20% sucrose gradient. The VLP preparations were normalized for comparable content of HIV-1 Gag (p55), pretreated with trypsin or PBS, separated by SDS-polyacrylamide gel electrophoresis, and analyzed by Western blotting. The 1918 influenza virus HA was detected with a mouse monoclonal antibody. Similar results were obtained in two independent experiments with different VLP preparations.

tributes to 1918 influenza virus HA- NA-mediated entry, Huh-7 cells were incubated with the cysteine protease inhibitor E64d prior to infection with pseudotypes in the absence of trypsin activation. Pseudotypes bearing ZEBOV-GP or VSV-G, as well as trypsin-activated HA NA pseudotypes served as controls. Huh-7 cells were chosen as targets because these cells were more susceptible to spinoculation with non-trypsin-activated HA NA pseudotypes than MDCK and 293T cells (Fig. 2A). As expected from previous reports, ZEBOV-GP-mediated entry was efficiently and dose-dependently reduced by E64d, while VSV-G-driven entry was unaffected (Fig. 2B). E64d had no appreciable impact on HA- NA-driven infection irrespective of trypsin treatment, indicating that activation by cysteine proteases in target cells is not required for trypsin-independent infectious entry of HA NA pseudotypes.

**The A/WSN/33 NA but not 1918 NA sequesters plasminogen but fails to facilitate cleavage-activation of the 1918 HA.** The A/WSN/33 virus is related to the 1918 virus, and both viruses

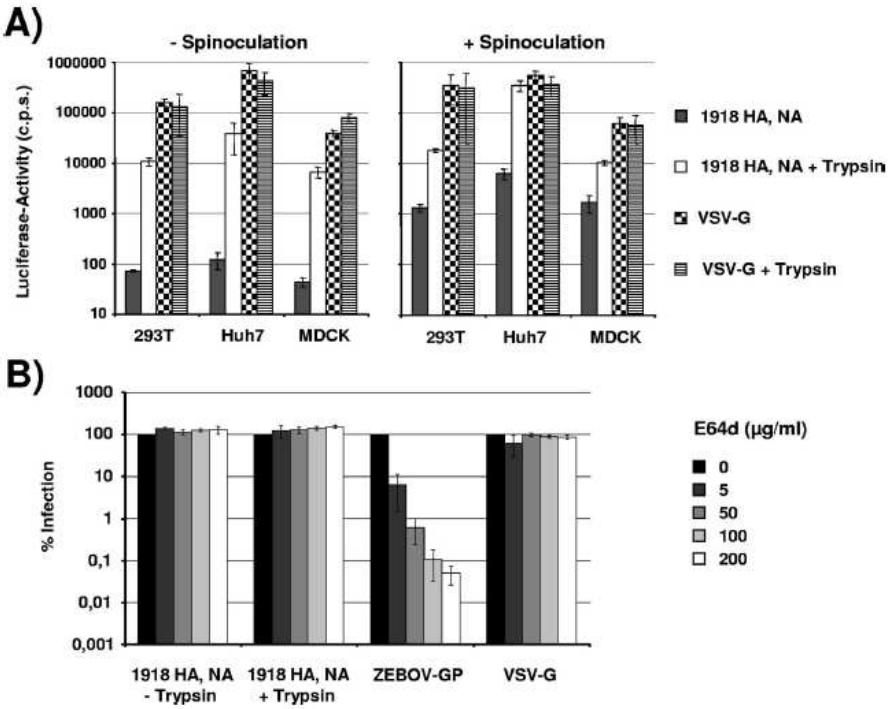


FIG. 2. Impact of spinoculation and protease inhibitors on 1918 influenza virus HA-driven viral entry. (A) 293T, Huh-7, and MDCK cells were seeded in 96-well plates and infected with p24-normalized pseudotypes bearing 1918 influenza virus HA, NA, and M2 or VSV-G. The virions were pretreated with trypsin or PBS and, subsequently, a trypsin inhibitor, before addition to target cells. Two plates were infected in parallel: one plate was incubated at 37°C immediately after addition of virus; the other plate was centrifuged for 2 h at 25°C and 1,200 rpm after infection and then incubated at 37°C. Three days after infection, luciferase activities in cellular lysates were determined. A representative experiment performed in triplicates is shown. Error bars indicate standard deviations. Similar results were obtained in two independent experiments. (B) Huh-7 cells were incubated with the indicated concentrations of the cysteine protease inhibitor E64d prior to infection with infectivity-normalized pseudotypes carrying the 1918 influenza virus HA, NA, M2, ZEBOV-GP, or VSV-G. Pseudotypes bearing influenza virus proteins were pretreated with PBS or trypsin for 10 min at room temperature, and soybean trypsin inhibitor was added. Three days after infection, luciferase activities in cellular lysates were determined. Infection of Huh-7 cells in the absence of protease inhibitors was set as 100%. The average of three independent experiments performed in triplicate is shown. Error bars indicate standard errors of the means. cps, counts per second.

replicate in a trypsin-independent, NA-dependent fashion in cell culture. The NA of A/WSN/33 mediates trypsin-independent replication by capturing plasminogen, which is present in high concentrations in FCS, and, upon conversion to plasmin, facilitates HA cleavage activation. In order to assess a potential role of plasminogen in proteolytic activation of the 1918 HA, we compared trypsin dependence of pseudotypes bearing the 1918 HA and NA, A/WSN/33 HA and NA, or viruses in which the NA proteins were exchanged. In accordance with published data (14, 15) and the results described above (Fig. 1), we found that the A/WSN/33 HA and NA but not the 1918 HA and NA pseudotypes readily infected Huh-7 cells without prior trypsin activation (Fig. 3A). However, viruses bearing the 1918 HA and A/WSN/33 NA or A/WSN/33 HA and the 1918 NA were completely dependent on trypsin treatment for infectivity. In agreement with these observations, Western blot analysis of VLPs revealed that trypsin but not purified human plasminogen promoted 1918 HA cleavage (Fig. 3B), and incubation of 1918 HA-bearing pseudotypes with purified human plasminogen failed to render these viruses infectious (Fig. 3C), further indicating that plasminogen is unlikely to be involved in proteolytic activation of the 1918 HA. Finally, Western blot analysis of HA-transfected cells showed that plasminogen was able to cleave the A/WSN/33 HA but not the 1918 HA (data not shown), confirming that the plasminogen preparation employed for the studies described above was indeed active.

To further characterize the contribution of plasminogen to 1918 HA cleavage, we performed FACS analysis to assess binding of purified plasminogen to cells transiently expressing HA or NA proteins. Plasminogen binding to control-transfected cells was low but detectable by FACS analysis (Fig. 3D), indicating that unmodified 293T cells express an inefficient plasminogen capture activity. Expression of the A/WSN/33 NA profoundly augmented plasminogen binding, as expected, with signals in the absence of purified protein being most likely due to incomplete removal of plasminogen contained in FCS. In

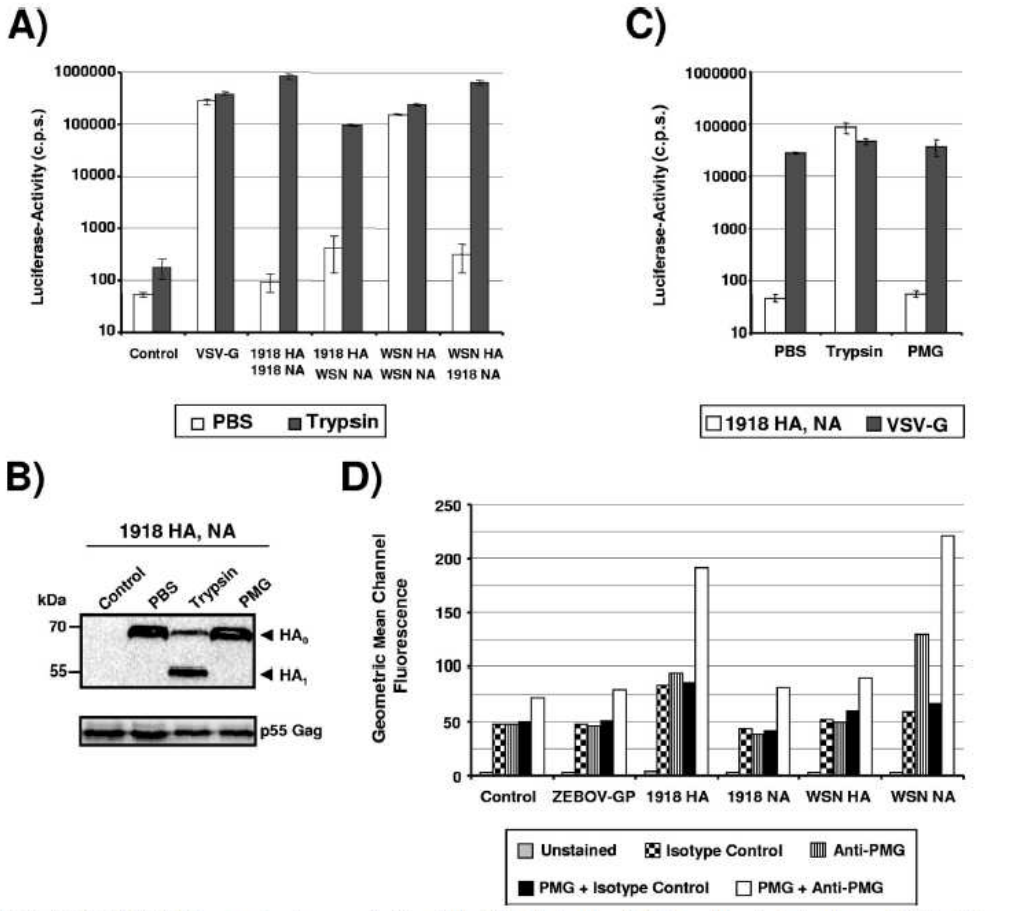


FIG. 3. A/WSN/33 NA fails to promote cleavage activation of the 1918 HA despite efficient recruitment of plasminogen. (A) Pseudotypes bearing the indicated glycoproteins were PBS or trypsin treated and used for infection of Huh-7 cells. Three days after infection, luciferase activities in cellular lysates were determined. The results of a representative experiment are shown and were confirmed in two independent experiments. Error bars indicate standard deviations. (B) VLPs bearing the 1918 HA and NA were incubated with PBS, trypsin, or plasminogen, and the 1918 HA was visualized by Western blotting. (C) The experiment was carried out as described for panel A. However, pseudotypes were treated with PBS, trypsin, or plasminogen (100 μg/ml). The results of a representative experiment are presented and were confirmed within a separate experiment. (D) The indicated proteins were transiently expressed in 293T cells, the transfected cells were incubated with PBS or purified plasminogen (10 μg/ml), and plasminogen binding was detected by FACS. A representative experiment is shown. Similar results were obtained in two independent experiments. PMG, plasminogen; cps, counts per second.

contrast, plasminogen binding to 1918 NA-expressing cells was in the background range. To our surprise, expression of the 1918 HA also facilitated robust plasminogen sequestration (Fig. 3D), suggesting that plasminogen capture is not an exclusive function of NA expression. Finally, control cells expressing ZEBOV-GP did not exhibit specific binding to plasminogen, indicating that expression of an irrelevant viral glycoprotein does not alter endogenous plasminogen capture activity of 293T cells.

In summary, our analyses of pseudotype infectivity (Fig. 3A and C), proteolytic processing of HA (Fig. 3B), and plasminogen binding (Fig. 3D) suggest that under the conditions tested the A/WSN/33 NA but not the 1918 NA recruited plasminogen for HA cleavage and that A/WSN/33 NA-dependent plasminogen sequestration was insufficient to confer infectivity to pseudotypes bearing the 1918 HA and NA.

**Lysates of MDCK but not 293T cells activate infectivity of pseudotypes bearing the 1918 HA and NA.** MDCK cells were found to allow for 1918 influenza virus replication in the absence of trypsin (45) and should thus express a protease, which

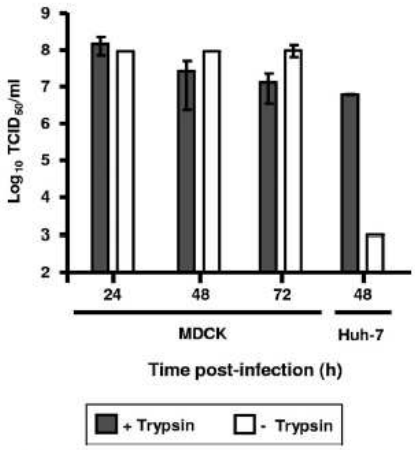


FIG. 5. The 1918 influenza virus replicates efficiently in MDCK but not Huh-7 cells in the absence of trypsin. MDCK and Huh-7 cells were infected at an MOI of 0.001 and then cultivated in the absence or presence of trypsin. Supernatants were collected at 24, 48, and 72 h p.i. from MDCK cultures and at 48 h p.i. from Huh-7 cultures, and the viral titers were determined by a 50% tissue culture infective dose ($TCID_{50}$) assay on fresh MDCK cells. A representative experiment performed in duplicate is shown; error bars indicate standard deviations.

titers of virus were recovered, supporting the observation that efficient trypsin-independent growth of the 1918 virus is cell line dependent.

**Proteolytic activation of the 1918 HA by TMPRSS2 and TMPRSS4.** Infection of MDCK, Huh-7, and 293T cells with the 1918 influenza virus or pseudotypes serves as a model for virus spread outside the lung. In order to investigate proteolytic activation of the 1918 HA in lung cells, we asked if transmembrane serine proteases expressed in lung tissue could activate 1918 HA by cleavage upon coexpression in 293T cells. We selected TMPRSS2 and TMPRSS4 for these studies since TMPRSS2 has already been shown to cleave and activate the HA of A/Hong Kong/1/68 (5) by cleavage, and TMPRSS4 shares 31% amino acid sequence identity with TMPRSS2. Murine matriptase-3, which is not expressed in the lung, was also tested. Transient transfection of the 1918 HA and NA alone did not result in HA cleavage, as expected, while cleavage was readily observed upon treatment of transfected cells with trypsin (Fig. 6A). Similarly, expression of matriptase-3 or the subtilisin-like protease furin did not promote proteolytic processing of the 1918 HA (Fig. 6B). However, coexpression of the 1918 HA with TMPRSS2 and TMPRSS4 resulted in efficient processing of HA independent of the presence of NA (Fig. 6A and B). In this context, it needs to be noted that total HA signals were generally reduced upon coexpression of TMPRSS4 and were often reduced upon coexpression of TMPRSS2, albeit to a lesser extent. The degree of signal reduction was dependent on the ratio of HA and protease plasmids used for transfection (3:1 for the experiment shown in Fig. 6A and 1:1 for the experiment shown in Fig. 6B) and might reflect "overdigestion" of HA under conditions of protease overexpression. In agreement with the specific cleavage of the 1918 HA by TMPRSS2 and TMPRSS4, the 1918 HA NA pseudotypes produced in the presence of TMPRSS2 or TMPRSS4 but not matriptase-3 were fully infectious for Huh-7 cells without prior trypsin activation (Fig. 6C). Finally, reverse transcription-PCR analysis of cells contained in bronchoalveolar lavage fluid (Fig. 6D) and PCR analysis of commercially available lung cDNA (Clontech) (data not shown) confirmed that TMPRSS4 is expressed in human lung tissue (Fig. 6D), suggesting that TMPRSS2 and TMPRSS4 might be able to support spread of the 1918 influenza virus in the human lung.

## DISCUSSION

The virus responsible for the devastating 1918 influenza virus pandemic has been "resurrected" by employing a reverse genetics approach (45). Exchange of specific genomic segments of the 1918 influenza virus against those of well-characterized influenza viruses revealed that HA and NA critically contribute to virus pathogenicity (22, 31, 47). Interestingly, the NA protein facilitates virus spread in MDCK cells in the absence of trypsin activation (45), most likely by recruiting a cellular protease which cleaves HA. Employing a lentiviral pseudotyping system, we show that the respective HA-activating protease is expressed in a cell-type-specific manner and is unlikely to be plasminogen. Moreover, we demonstrate that the transmembrane proteases TMPRSS2 and TMPRSS4, which are expressed in lung tissue, activate the 1918 HA by cleavage in an NA-independent fashion.

Analysis of the 1918 influenza virus will continue to provide important insights into the determinants of influenza virus transmission and pathogenicity but is hampered by the requirement for a high level of biocontainment. Taking advantage of the well-known ability of retroviruses to incorporate heterologous proteins in their envelopes during release from infected cells, we generated HIV-1-derived particles bearing the HA, NA, and M2 proteins of the 1918 influenza virus. The particles were infectious but replication defective and thus constitute a safe and convenient tool for analyzing cellular entry of the 1918 virus and its inhibition by, e.g., neutralizing antibodies. Small amounts of the M2 protein are incorporated into influenza virus particles, and the ion channel function of M2 plays an important role in uncoating and, for some viruses, in maturation (3). Increased infectivity of pseudotypes bearing the fowl plague virus HA has been described upon coexpression of M2 in virus-producing cells (26). We observed efficient M2 expression and incorporation into VLPs (data not shown). Nevertheless, the presence of M2 decreased incorporation but not cellular expression of HA in our system (Fig. 1B), resulting in reduced particle infectivity. The reasons for the discrepancy between the published observations (26) and the data presented here are at present unclear and might be due to differences in the influenza virus proteins and pseudotyping systems used or might be explained by differences in M2 expression levels.

Infectivity of pseudotypes bearing the surface proteins of the 1918 virus was dependent on the presence of both HA and NA. However, the requirement for NA was not due to NA facilitating HA cleavage, at least under the conditions tested. Instead, NA was necessary for efficient particle release (Fig. 1B),

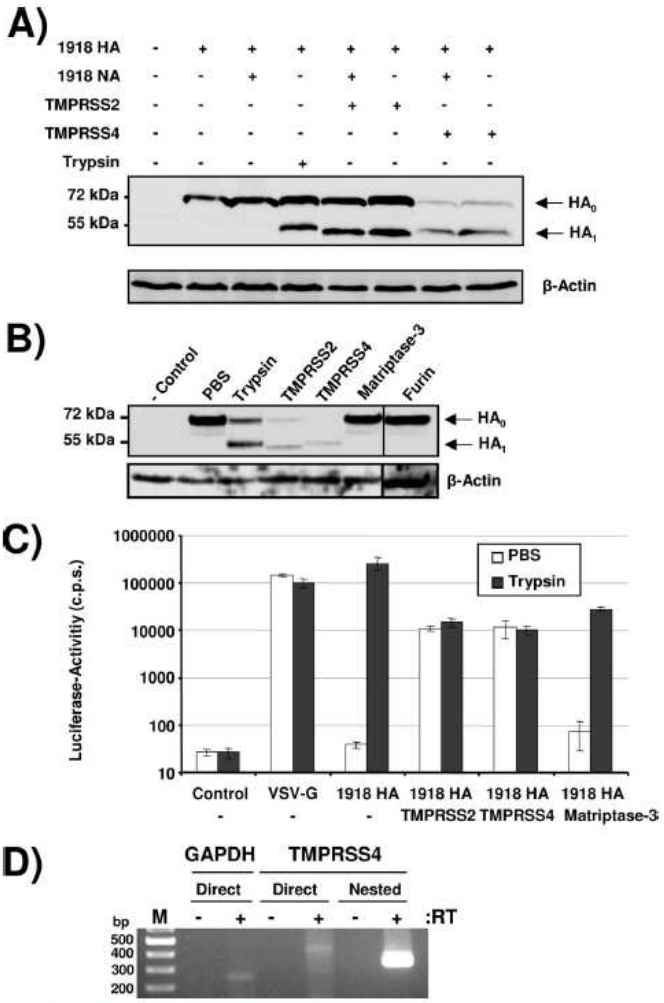


FIG. 6. TMPRSS2 and TMPRSS4 activate 1918 HA by cleavage. (A) 293T cells were transiently cotransfected with the 1918 HA (jointly with pcDNA3 or the 1918 NA) and TMPRSS2, TMPRSS4, or mouse matriptase-3 (HA and protease expression plasmids were transfected at a 3:1 ratio). Subsequently, the transfected cells were treated with trypsin or PBS, and proteolytic processing of HA was analyzed by Western blotting. (B) 293T cells were transfected with the 1918 HA alone or in combination with the indicated proteases (HA and protease expression plasmids were transfected at a 1:1 ratio); the cells were treated with PBS or trypsin, and proteolytic processing of HA was analyzed by Western blotting. (C) Pseudotypes were generated in 293T cells expressing the empty vector or the indicated proteases (HA and protease expression plasmids were transfected at a 3:1 ratio), normalized to p24 (150 pg/well), and employed for infection of Huh-7 cells. Three days after infection, luciferase activities in cellular lysates were determined. A representative experiment is shown, and similar results were obtained in two independent experiments. Error bars indicate standard deviations. (D) RNA was obtained from cells present in bronchoalveolar lavage fluids and reverse transcribed within reaction mixtures containing reverse transcriptase (RT) enzyme or PBS, and GAPDH and TMPRSS4 were amplified by PCR. M, molecular weight marker. cps, counts per second.

consistent with the well-established receptor-destroying function of this protein, and in the absence of NA most likely particles devoid of HA were released. Particle-associated HA exhibited a size of approximately 75 kDa, which is expected for uncleaved HA (10). Trypsin activation reduced the HA size to approximately 50 kDa and was indispensable for viral infectivity (Fig. 1), demonstrating that in the 293T cell-based system examined here particle-associated HA was uncleaved, most likely due to lack of expression of adequate proteases in this cell type (see below). Nevertheless, when viruses were concen-

trated onto target cells by centrifugation, a procedure termed spinoculation (28), some infectivity of HA NA pseudotypes was observed in the absence of trypsin activation. Thus, proteases in target cells might be able to process HA, albeit cleavage efficiency might be low. The endosomal/lysosomal cysteine proteases cathepsin B and cathepsin L activate the glycoproteins of EBOV (7), SARS-coronavirus (37), and the fusion protein of Hendra virus (29) by cleavage, and cathepsin B/L activity has been shown to be essential for virus infectivity. However, inhibitors of cysteine proteases, which were previously shown to block EBOV and SARS-coronavirus entry (7, 37), had no effect on infectious entry of HA NA pseudotypes, suggesting that a different class of proteases might be involved in HA cleavage in target cells (Fig. 2B).

In the absence of artificially enhanced entry, infectivity of the 1918 HA NA pseudotypes was strictly dependent on trypsin activation (Fig. 1A and 2). Thus, proteases that efficiently activate HA were not present in the FCS and were not secreted from the cell lines tested, at least not in sufficient concentrations to activate HA. Particularly the former finding is noteworthy since it has been shown previously that NA of A/WSN/33, a close relative of the 1918 influenza virus, recruits plasminogen present in FCS and facilitates generation of plasmin, which in turn activates HA (14, 15). This process is required for trypsin-independent growth and neurotropism of A/WSN/33 (14, 15). Our analysis confirmed efficient plasminogen binding by cells expressing the A/WSN/33 NA (Fig. 3D). In contrast, we failed to detect plasminogen capture by 1918 NA-positive cells, which is in agreement with the notion that the 1918 NA harbors a glycosylation site incompatible with plasminogen recruitment (42). Moreover, the A/WSN/33 NA failed to confer infectivity to 1918 HA-containing pseudotypes in the absence of trypsin treatment (Fig. 3A). In conjunction with the inability of purified human plasminogen or plasminogen-containing FCS to appreciably activate 1918 HA by cleavage and to render the 1918 HA NA pseudotypes infectious (Fig. 3B and C), these observations indicate that plasminogen is likely not involved in 1918 HA cleavage-activation. Nevertheless, 1918 HA-expressing cells displayed robust plasminogen binding activity (Fig. 3D). The significance of this finding for 1918 influenza virus biology is at present unclear. One can speculate, however, that influenza viruses might have evolved several independent strategies to recruit plasminogen either to ensure cleavage of HA or to facilitate a so far undiscovered proteolytic cleavage event involved in influenza virus replication. In fact, a recent study revealed that plasminogen enhances the replication of several influenza A viruses and that incorporation of annexin II into influenza virus particles promotes conversion of plasminogen to its proteolytically active form, plasmin (24). Collectively, our data suggest that the 1918 NA might allow trypsin-independent replication of the 1918 virus by recruiting a cellular protease other than plasminogen or, maybe less likely, that NA facilitates HA activation by an entirely different mechanism.

Lysates prepared from MDCK but not 293T cells activated pseudotype infectivity, albeit with low efficiency, suggesting that HA-activating proteases are expressed in the former but not the latter cell line. The observation that pseudotype infectivity for 293T and MDCK cells was not detectable in the absence of spinoculation and was comparable upon spinoculation (Fig. 2) indicates that the HA-activating protease detected in MDCK lysates (Fig. 4) might not be present (or active) in endocytic vesicles (and might thus be unable to activate HA on incoming viruses) but might be recruited by NA during transport in the secretory pathway or at the cell surface. Unfortunately, production of pseudotypes in MDCK cells was inefficient (data not shown), preventing us from investigating this hypothesis. The finding that MDCK lysates were clearly less efficient in activating pseudotype infectivity than trypsin suggests that insertion into an intact lipid membrane or the integrity of a specific subcellular compartment might be indispensable for efficient proteolytic processing of HA. In any case, infectious 1918 influenza virus replicated in MDCK cells with similar kinetics and, as reported previously (45), peak titers in the presence and absence of trypsin (Fig. 5), indicating efficient function of the activating protease in the context of intact MDCK cells. Furthermore, efficient replication of the 1918 virus in Huh-7 hepatoma cells in the presence but not in the absence of trypsin confirmed that the trypsin-independent growth of the virus in non-lung cells is a cell line-dependent phenomenon. This observation may have implications for understanding the tissue tropism of the virus which is primarily restricted to the respiratory tract in experimentally infected animals (21, 45). Further investigation as to the nature of the protease activity in MDCK cells is required, as is the proof that this protease activity is present and can facilitate virus spread in human cells.

It is believed that several proteases expressed in lung tissue can facilitate influenza virus spread. However, the nature of these HA-activating pulmonary proteases is incompletely understood. Böttcher and colleagues presented evidence that the transmembrane serine proteases TMPRSS2 and HAT activate the influenza viruses A/Memphis/14/96 (H1N1), A/Mallard/Alberta/205/98 (H2N9), and A/Texas/6/96 (H3N2). Coexpression of TMPRSS2 also facilitated cleavage of the 1918 HA independent of the presence of NA (Fig. 6A and B), and similar results were obtained with TMPRSS4, for which mRNA expression could be detected in human lung cells (Fig. 6A and B), suggesting that these proteases might support the spread of influenza viruses in human lung. In contrast, 293T, Huh-7, and MDCK cells were negative for TMPRSS4 transcripts when analyzed by direct reverse transcription-PCR (signals were observed for Huh-7 and MDCK cells upon nested PCR [data not shown]), indicating that these cell lines might express no TMPRSS4 protein or very small amounts thereof. These results are in agreement with the findings that infectivity of viruses produced in 293T and Huh-7 cells is trypsin dependent (Fig. 1 and 5) and that trypsin-independent virus spread in MDCK cells requires NA-dependent recruitment of a so far unidentified cellular protease (45) (note that TMPRSS2/TMPRSS4 cleavage of the 1918 HA is NA independent) (Fig. 6A). Notably, cleavage of the 1918 HA by TMPRSS2 and TMPRSS4 proteases resulted in $HA_1$ products of slightly different sizes (Fig. 6A and B), a finding that needs further investigation. One possible explanation might be that TMPRSS2 cleaves the 1918 HA at the border between $HA_1$ and $HA_2$ and, in addition, in $HA_1$, thereby producing an $HA_1$ fragment exhibiting slightly faster gel migration than the respective fragments produced by TMPRSS4 or trypsin cleavage. In any event, this study identifies TMPRSS4 as a novel influ-

enza virus HA-processing protease expressed in lung tissue (Fig. 6C), and the impact of TMPRSS4, HAT, and TMPRSS2 on spread of the 1918 influenza virus and other influenza viruses in vivo deserves assessment.

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# TMPRSS2 and TMPRSS4 Facilitate Trypsin-Independent Spread of Influenza Virus in Caco-2 Cells▽

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**Proteolysis of influenza virus hemagglutinin by host cell proteases is essential for viral infectivity, but the proteases responsible are not well defined. Recently, we showed that engineered expression of the type II transmembrane serine proteases (TTSPs) TMPRSS2 and TMPRSS4 allows hemagglutinin (HA) cleavage. Here we analyzed whether TMPRSS2 and TMPRSS4 are expressed in influenza virus target cells and support viral spread in the absence of exogenously added protease (trypsin). We found that transient expression of TMPRSS2 and TMPRSS4 resulted in HA cleavage and trypsin-independent viral spread. Endogenous expression of TMPRSS2 and TMPRSS4 in cell lines correlated with the ability to support the spread of influenza virus in the absence of trypsin, indicating that these proteases might activate influenza virus in naturally permissive cells. Indeed, RNA interference (RNAi)-mediated knockdown of both TMPRSS2 and TMPRSS4 in Caco-2 cells, which released fully infectious virus without trypsin treatment, markedly reduced the spread of influenza virus, demonstrating that these proteases were responsible for efficient proteolytic activation of HA in this cell line. Finally, TMPRSS2 was found to be coexpressed with the major receptor determinant of human influenza viruses, 2,6-linked sialic acids, in human alveolar epithelium, indicating that viral target cells in the human respiratory tract express TMPRSS2. Collectively, our results point toward an important role for TMPRSS2 and possibly TMPRSS4 in influenza virus replication and highlight the former protease as a potential therapeutic target.**

Infection with influenza viruses—negative-stranded, segmented RNA viruses of the *Orthomyxovirus* family—is responsible for substantial morbidity and mortality, particularly among the young and the elderly (10). A hallmark of influenza A viruses is their ability to adapt to immune pressure, which allows constant circulation of these viruses in the human population (seasonal influenza) (8, 31). In addition, antigenically novel viruses, arising from the large pool of animal influenza viruses, are occasionally transmitted to humans and may spread in a pandemic manner (pandemic influenza) (8, 31). The high mutation rate of influenza viruses has major consequences for antiviral prevention and therapy. First, the vaccine against seasonal influenza needs to be reformulated almost annually and will not be effective against a new pandemic virus. Second, antiviral therapy, which targets viral proteins essential for uncoating and release, is plagued by the rapid development of viral resistance (32). Consequently, new targets for antiviral intervention are under investigation, and therapeutic inhibition of invariant host cell factors essential for influenza virus spread is an attractive strategy, since it may allow the suppression of resistance development.

The viral envelope protein hemagglutinin (HA) mediates the first essential steps in the viral life cycle: attachment to target cells and virus-cell fusion (14, 38). Attachment of virions to target cells is mediated by the surface unit of HA, HA1, while the fusion of the viral envelope with a target cell membrane is driven by the transmembrane unit, HA2 (14, 38). In infected cells, HA is initially synthesized as an inactive precursor protein, HA0, in which HA1 und HA2 are connected by a protease-sensitive linker sequence. Cleavage of the linker by host cell proteases generates mature HA1 and HA2 and is crucial for viral infectivity (23, 24, 26, 27), making the responsible proteases attractive targets for therapeutic intervention (2, 11). However, their exact identity has not been determined previously.

Highly pathogenic avian influenza viruses (HPAIV) harbor a cleavage site composed of several arginines and lysines (multibasic cleavage site), which is recognized by furin or related subtilisin proteases (40). Since these proteases are ubiquitously expressed, HPAIV can spread systemically and cause severe disease (1, 17, 21, 22, 28). In contrast, the cleavage site of low-pathogenic avian influenza viruses (LPAIV) consists of a single arginine or lysine residue (monobasic cleavage site) and is believed to be exclusively recognized by as yet uncharacterized proteases expressed in the respiratory and enteric tracts

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(1, 17, 21, 22, 28, 39). As with LPAIV, human influenza viruses contain a monobasic cleavage site, and the identity of the protease(s) activating these viruses is unclear.

It has been suggested that soluble proteases might activate human influenza viruses in the infected host (18, 19, 30, 43). However, analysis of cultured human respiratory epithelial cells revealed efficient HA cleavage during HA biogenesis and upon uptake of virions into target cells, suggesting that HA cleavage in the human host is a cell-associated process (47). Böttcher and colleagues amplified the type II transmembrane serine proteases (TTSPs) TMPRSS2 (transmembrane protease, serine 2) and HAT (human airway trypsin-like protease) from the human lung and showed that these proteases, upon engineered expression, support the spread of human influenza viruses (2–4). Employing lentiviral vectors to analyze HA activation, we have demonstrated previously that TMPRSS2 and another TTSP, TMPRSS4, activate the HA of the highly pathogenic 1918 influenza virus (5). These results suggest that TTSPs might contribute to the activation of influenza virus in the human host. However, it remained to be determined whether HA-activating TTSPs are endogenously expressed in viral target cells and whether they support viral spread in the absence of an exogenously added protease (trypsin).

We addressed the relevance of TMPRSS2 and TMPRSS4 for the activation of influenza virus in permissive cells. A combination of expression and knockdown analyses revealed that endogenous TMPRSS2 and TMPRSS4 activate influenza virus in cell lines and, in the case of TMPRSS2, most likely also in human alveolar epithelium, suggesting a major role for TMPRSS2 in viral replication in human hosts.

## MATERIALS AND METHODS

**Plasmids.** Expression plasmids for the 1918 influenza virus HA, the 1918 influenza virus neuraminidase (NA), TMPRSS2, TMPRSS3, and TMPRSS4 have been described previously (5, 15, 34, 36). cDNAs encoding full-length human hepsin (hMU004062), TMPRSS3 (KU004606), and TMPRSS6 (hMU004435) were provided by the Korean UniGene Information Center (http://kugi.kribb.re.kr/), Daejeon, South Korea. The cDNA encoding each gene was amplified by PCR using primer pairs 5′-GAGGCTAGCCACCATGGCGCAGAAGGAGGGTG G-3′ (forward) and 5′-GAGGCGGCCGCTCAGAGCTGGGTCACCATGC-3′ (reverse) for hepsin, 5′-GAGGCTAGCCACCATGGGGGAAAATGATCCGC CTGCT-3′ (forward) and 5′-GAGGCGGCCGCTCAGGTTTTTAGGTCTCTC T-3′ (reverse) for TMPRSS3, and 5′-GAGGCTAGCCACCATGTTGTTACTC TTCCACTCCAA-3′ (forward) and 5′-GAGGCGGCCGCTCAGGTCACCAC TTGCTGGA-3′ (reverse) for TMPRSS6. The PCR products were then cloned into plasmid pcDNA3.1 (Invitrogen) by NheI and NotI digestion, and the integrity of the cloned cDNAs was verified by sequencing.

**Cell culture.** The following media were used for cell culture: Dulbecco's modified Eagle's medium (DMEM; Invitrogen) (for culture of Vero E6, Huh-7, and 293T cells), Glutamax DMEM (DMEM enriched with glutamate; Invitrogen) (for culture of Caco-2 cells), and minimum essential medium (MEM; Invitrogen) (for culture of MDCK II cells). All media were supplemented with 10% fetal calf serum (FCS), penicillin, and streptomycin, and all cell cultures were maintained at 37°C under a 5% $CO_2$ atmosphere.

**VLPs.** For the production of virus-like particles (VLPs), 293T cells were transiently cotransfected with the HIV-1 Gag (p55)-encoding plasmid p96ZM651gag-opt, 1918 HA and NA expression plasmids, and expression plasmids for TTSPs or empty vector. At 48 h posttransfection, the supernatants were harvested and concentrated by ultrafiltration employing Vivaspin columns (Sartorius). As a control for HA cleavage, VLPs were treated either with phosphate-buffered saline (PBS) or with tosylsulfonyl phenylalanyl chloromethyl ketone (TPCK)-trypsin (Sigma), followed by incubation with soybean trypsin inhibitor (Sigma).

**Generation of replication-competent influenza virus.** Ten-day-old pathogen-free embryonated hens' eggs were inoculated via the allantoic sac with 0.2 ml of virus dilutions. Eggs were incubated for 48 h in an incubator at 37°C with passive humidity. Thereafter, the allantoic fluids were collected, tested for hemagglutinating activity, pooled, aliquoted, and stored at −70°C. The titer of the virus was determined by the focus formation assay.

**Infection experiments with replication-competent influenza viruses.** For infection experiments with replication-competent viruses, 293T cells, transiently transfected with plasmids encoding proteases or empty vector, were seeded in 6-well plates at a density of $1.2 \times 10^5$ cells/well, washed, and inoculated with PR8 (H1N1) or SC35M (H7N7) virus (contained in Dulbecco's PBS [DPBS] supplemented with 0.2% bovine serum albumin [BSA]) at a multiplicity of infection (MOI) of 0.01. Alternatively, Caco-2 cells were either mock treated; transfected with a nonsense small interfering RNA (siRNA), a TMPRSS2 siRNA, or a TMPRSS4 siRNA (Santa Cruz); or cotransfected with both TMPRSS2 and TMPRSS4 siRNAs. Then the cells were washed and infected as described above for 293T cells. Viruses were allowed to adsorb to target cells for 1 h. Subsequently, the infection medium was removed; MEM supplemented with 0.2% BSA and either 1 μg/ml TPCK-trypsin or PBS was added; and the cells were incubated for 24 h. Thereafter, the cell culture supernatants were collected and stored at −80°C for subsequent quantification of infectious virus particles.

**Generation of HA-bearing pseudotypes.** Pseudotypes were generated essentially as described previously (5, 37). Briefly, 293T cells were cotransfected, by use of $CaPO_4$, with the HIV-1-derived vector pNL4-3 E-R-Luc (7) and expression plasmids for 1918 HA, 1918 NA, or vesicular stomatitis virus glycoprotein (VSV-G). For analysis of HA activation by TTSPs, TMPRSS2, TMPRSS3, TMPRSS4, TMPRSS6, or hepsin was coexpressed with viral components during the production of pseudoparticles. At 16 h posttransfection, the culture medium was replaced by fresh medium, and the cultures were maintained at 37°C under 5% $CO_2$. At 48 h posttransfection, the supernatants were harvested, passed through 0.45-μm-pore-size filters, and stored at −80°C. The concentration of HIV-1 capsid protein (p24) in virus stocks was determined by a p24 enzyme-linked immunosorbent assay (ELISA) (AIDS Research and Reference Reagent Program). For the production of pseudotypes in Caco-2 cells, cells were cotransfected with pNL4-3 E-R-Luc and an expression plasmid for 1918 HA, 1918 NA, or VSV-G by use of Lipofectamine 2000 (Invitrogen) and were processed as described above for 293T cells.

**Infection experiments with HA-bearing pseudotypes.** For infection experiments with pseudotyped viruses, Huh-7, Caco-2, and 293T cells were seeded in 96-well plates at a density of $0.8 \times 10^5$ cells/well, washed, and incubated overnight with p24- or luciferase-normalized virus stocks pretreated with either PBS or TPCK-trypsin. After 72 h, the cells were lysed, and the luciferase activities in cell lysates were determined by employing a commercially available kit (Promega). Alternatively, 293T target cells were first transfected with protease-encoding plasmids and then infected as described above.

**Detection of HA in cell lysates and virions by Western blotting.** For the detection of HA in cell lysates, 293T cells were cotransfected with 1918 HA and TTSP expression plasmids or empty vector. The medium was replaced with fresh culture medium at 16 h posttransfection. At 48 h posttransfection, the cells were harvested, treated with PBS or TPCK-trypsin, and lysed in 2× sodium dodecyl sulfate (SDS) loading buffer. For analysis of HA cleavage by endogenous proteases, Caco-2 cells were Lipofectamine transfected with 1918 HA and lysed at 48 h posttransfection in SDS loading buffer. For analysis of the incorporation of HA into VLPs and pseudotypes, the particle preparations were pelleted by centrifugation through a 20% sucrose cushion at 42,000 × *g* for 2 h at 4°C, and the pellets were lysed in SDS loading buffer. For immunoblotting, the lysates were separated by SDS-gel electrophoresis, and HA was detected by staining with a mouse anti-HA antibody (12) at a dilution of 1:500, followed by incubation with a horseradish peroxidase (HRP)-coupled anti-mouse secondary antibody. As a loading control, an anti-β-actin antibody (Sigma) or an anti-p24 hybridoma supernatant (183-H12-5C) was used.

**Analysis of HA glycosylation.** For enzymatic deglycosylation of 1918 HA/NA-bearing VLPs produced in the presence of TMPRSS2 and hepsin, a commercially available kit (New England Biolabs) was used. For this purpose, the VLPs were concentrated via Vivaspin columns and were additionally ultracentrifuged through a 20% sucrose cushion at 42,000 × *g* for 2 h at 4°C. The resulting pellets were harvested with TNE buffer (Tris-HCl [pH 7.4], 0.15 M NaCl, and 10 mM EDTA) and digested by peptide:*N*-glycosidase F (PNGase F). The samples were analyzed by immunoblotting as described above.

**Focus formation assay.** MDCK II cells ($6 \times 10^4$/well) were seeded in 96-well culture plates and were incubated at 37°C under 5% $CO_2$ for 24 h. On the next day, serial 10-fold dilutions of supernatants (collected from the infected cells) in DMEM containing 0.1% BSA and 2.5 μg/ml *N*-acetylated trypsin (NAT; Sigma) were prepared in a separate 96-well plate, and then 50 μl of each dilution was transferred to the confluent monolayers of MDCK II cells in 96-well culture plates. The plates were incubated at 37°C under 5% $CO_2$ for 1 h with shaking at

20-min intervals. The inoculates were aspirated and replaced with 100 μl of a 1% Avicel overlay containing 0.1% BSA and 2.5 μg/ml NAT (Sigma). The infected cells were incubated at 37°C under 5% $CO_2$ for 24 h. Subsequently, cells were washed twice with PBS and were then fixed with 4% formalin in PBS (100 μl/well) for 10 min at room temperature. The formalin was removed; the cells were first washed as described above and then incubated for 10 min with 100 μl/well Quencher (0.5% Triton X-100, 20 mM glycine in PBS). After 10 min, the cells were washed with wash buffer (WB) (0.5% Tween 20 in PBS) and were then blocked with 50 μl of blocking buffer (BB) (0.5% Tween, 20% BSA in PBS) at 37°C under 5% $CO_2$ for 30 min. The primary antibody (a polyclonal goat antibody against the influenza virus nucleocapsid [NP]; Virostat) and the secondary antibody (an HRP-conjugated anti-goat antibody; Kirkegaard & Perry Laboratories, Gaithersburg, MD) were diluted 1:1,000 in BB. A 50-μl volume of the primary antibody was added to each well and was incubated at room temperature for 1 h. After 1 h of incubation, the cells were washed three times with WB, incubated with 50 μl of the secondary antibody for 45 min, washed again, and incubated with 50 μl of the substrate (True Blue; Kirkegaard & Perry Laboratories, Gaithersburg, MD) until blue spots appeared. Foci were counted, and viral titers were calculated as focus-forming units (FFU) per milliliter.

**Immunohistochemistry.** Tissue samples, obtained with full ethical approval from the National Research and Ethics Service (Oxfordshire Research and Ethics Committee A; reference 04/Q1604/21), were stained with hematoxylin and eosin using standard techniques and were either immunostained for TMPRSS2 (mouse monoclonal antibody P5H9 [29]) or stained with *Sambucus nigra* lectin (Vector Laboratories, Peterborough, United Kingdom). Immunostaining was performed using a Bond Max immunostaining machine (Leica Microsystems Newcastle Ltd., Newcastle, United Kingdom) with the manufacturer's standard protocols and reagents for mouse primary antibodies. The biotinylated *Sambucus nigra* lectin was detected directly by means of the Bond Intense R kit (Leica Microsystems Newcastle Ltd., Newcastle, United Kingdom). Negative controls were as follows: standard immunostaining with a mouse monoclonal antibody against Melan-A (clone A103; Leica Microsystems Newcastle Ltd., Newcastle, United Kingdom) for the anti-TMPRSS2 antibody and the Bond Intense R kit alone for the biotinylated *Sambucus nigra* lectin. Stained sections were photographed with a Nikon DS-FI1 camera with a Nikon DS-L2 control unit (Nikon UK Limited, Kingston-upon-Thames, United Kingdom) and an Olympus BX40 microscope (Olympus UK Limited, Watford, United Kingdom).

**Reverse transcription-PCR (RT-PCR) analysis of TTSP mRNA expression in cell lines.** mRNAs were extracted from different cell lines (293T, Caco-2, Huh-7, Vero E6), treated with DNase, and reverse transcribed employing commercially available kits (Qiagen and Invitrogen, Germany). Subsequently, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), TMPRSS2, TMPRSS4, and hepsin sequences were amplified by nested PCR. The following set of primers was used for amplification of TTSPs: TMPRSS2 outer primers 5′-TACCTGCATCAACCCC TCTAACTG-3′ (p5 TMPRSS2 out) and 5′-CTTCTGAGGTCTTCCCTTTCTC CT-3′ (p3 TMPRSS2 out), TMPRSS2 inner primers 5′-GCCTTTACGGACCA AACTTCATCC-3′ (p5 TMPRSS2 in) and 5′-CGCAAATGCCGTCCAATGCC ATGG-3′ (p3 TMPRSS2 in), TMPRSS4 outer primers 5′-GAGAGCTGGACT GTCCCTTG-3′ (p5 TMPRSS4 out) and 5′-TCGTACTGGATGCTGACCT G-3′ (p3 TMPRSS4 out), TMPRSS4 inner primers 5′-GACGAGGAGCACTG TGTCAA-3′ (p5 TMPRSS4 in) and 5′-CTTCCCACAGGCAAGACAGT-3′ (p3 TMPRSS4 in), hepsin outer primers 5′-CCCTGCTACTTCTGACAGCCAT C-3′ (p5 hepsin out) and 5′-TCGTTGCTGTTCTCCTCGCTGTT-3′ (p3 hepsin out), and hepsin inner primers 5′-GCACGTCGGGCTTCTTCTGTGTGG-3′ (p5 hepsin in) and 5′-CCACGGCACCGGCAAACACTCGC-3′ (p3 hepsin in).

**Quantitative RT-PCR analysis of TMPRSS2 and TMPRSS4 mRNA expression in cell lines.** Total RNA (500 ng) was reverse transcribed using 50 U of BioScript RNase H Low reverse transcriptase (BIO-27036; Bioline) in 20-μl reaction mixtures. The enzyme was then inactivated for 10 min at 70°C. Aliquots (1 μl) of the cDNA samples generated (25 ng total RNA equivalents) were used for real-time PCR in 10-μl reaction mixtures with the ABI 7500 Fast real-time PCR system (Applied Biosystems). Specific amplification was ensured with TaqMan gene expression assays (catalog no. 4331182; Applied Biosystems) according to the manufacturer's recommendations. The following specific assays were used: Hs00237175_m1 (TMPRSS2), Hs00212669_m1 (TMPRSS4), and Hs99999908_m1 (β-glucuronidase [GUSB]). The average cycle threshold ($C_T$) for each individual assay was calculated from triplicate measurements by means of the instrument's software in "auto Ct" mode (ABI 7500 Fast system software, version 1.3.0). Average $C_T$ values calculated for TMPRSS2 and TMPRSS4 were normalized by subtraction from the $C_T$ values obtained for GUSB (housekeeping reference). Template-free cDNA reaction mixtures were analyzed in parallel, using both TaqMan assays; no specific signal was detected in any of these experiments.

## RESULTS

**TMPRSS2, TMPRSS4, and hepsin cleave influenza virus hemagglutinin.** We have previously demonstrated that influenza virus HA is proteolytically processed by TMPRSS2 and TMPRSS4 (5). We now asked whether other TTSPs known to be expressed in lung tissue (41) also cleave HA. For this purpose, we coexpressed HA (from the 1918 influenza virus) with TMPRSS3, TMPRSS6, or hepsin in 293T cells and analyzed HA cleavage by Western blotting (Fig. 1A). Trypsin treatment and coexpression of TMPRSS2 and TMPRSS4 served as positive controls for HA cleavage; cells transfected with empty vector (pcDNA) or nontransfected cells (Mock) were employed as negative controls. As expected, trypsin treatment or coexpression of TMPRSS2 or TMPRSS4 facilitated HA cleavage, while HA was not cleaved when empty vector was cotransfected (Fig. 1A). No evidence for cleavage was observed upon coexpression of TMPRSS3 and TMPRSS6. In contrast, a band corresponding to HA1 appeared upon expression of hepsin, indicating that hepsin cleaves HA between HA1 and HA2.

We then asked whether cleaved HA was incorporated into viral particles. For this purpose, we produced virus-like particles by using an HIV-1 Gag expression plasmid, and we analyzed the incorporation of HA and Gag into particles by Western blotting (Fig. 1B). Cleaved HA was present in particles produced in the presence of TMPRSS2 or TMPRSS4, in agreement with our previous results (5). Similarly, cleaved HA was found in particles produced in hepsin-expressing cells (Fig. 1B), indicating that cleavage by hepsin is compatible with the incorporation of HA into virions.

Closer inspection of the HA1 bands obtained upon HA processing by TMPRSS2, TMPRSS4, hepsin, or trypsin reproducibly revealed size differences: HA digestion by trypsin or TMPRSS4 yielded HA1 bands of identical sizes, as did HA digestion by TMPRSS2 or hepsin, and the latter bands migrated slightly faster than the former (Fig. 1A and B). We hypothesized that the size differences might be due to altered glycosylation of HA in hepsin- or TMPRSS2-transfected cells. To address this hypothesis, we digested virus-like particles (VLPs) from TMPRSS2-, TMPRSS4-, or hepsin-transfected cells, as well as PBS- or trypsin-treated control VLPs, with PNGase F, which removes all N-linked glycans. Indeed, the differences in HA1 size observed in mock-treated cells were not detected after treatment with PNGase F (Fig. 1C), indicating that engineered expression of TMPRSS2 and hepsin modulates HA glycosylation. In summary, our results add hepsin to the list of TTSPs that cleave influenza virus HA and indicate that the expression of particular TTSPs modulates HA glycosylation by a currently unknown mechanism.

**Cleavage by TMPRSS2 and TMPRSS4, but not cleavage by hepsin, activates hemagglutinin.** Since hepsin, like TMPRSS2 and TMPRSS4, was able to cleave HA, we next asked whether cleavage by hepsin allows HA to transit into an activated state. A previous study by others (45) and our own published results (5) demonstrate that *trans*-complementation (pseudotyping) of *env*-defective retroviral vectors with influenza virus HA is an adequate system for the analysis of HA activation. We employed this approach to analyze HA activation by hepsin. For this purpose, we produced HA-bearing lentiviral pseudotypes in cells cotransfected with TTSP expression plasmids or empty

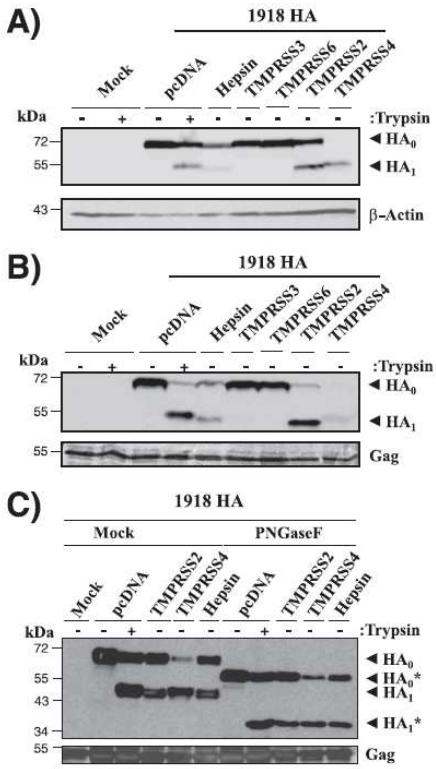


FIG. 1. Cleavage of influenza virus hemagglutinin by TMPRSS2, TMPRSS4, or hepsin. (A) Cleavage of hemagglutinin (HA) in 293T cells expressing TMPRSS2, TMPRSS4, or hepsin. The HA of the 1918 influenza virus and the protease indicated were transiently coexpressed in 293T cells. The cells were then treated with trypsin or PBS, and HA cleavage was detected by Western blot analysis of cell lysates. Detection of β-actin served as a loading control. Mock, cells transfected with empty vector alone; pcDNA, cells cotransfected with an HA expression plasmid and empty vector. (B) Incorporation of cleaved HA into virions. HA was expressed as described for panel A; however, cells were additionally cotransfected with plasmids encoding the HIV-1 Gag protein and the 1918 influenza virus NA. VLPs were concentrated from culture supernatants by centrifugation, and HA cleavage and Gag contents were analyzed by Western blotting. Mock, VLPs produced in cells transfected with a Gag expression plasmid and empty vector; pcDNA, VLPs produced in cells cotransfected with plasmids encoding Gag, HA, and NA and empty vector. (C) Expression of TMPRSS2 or hepsin, but not TMPRSS4, alters the glycosylation of HA1. 293T cells, transfected either with expression vectors for the indicated proteases or with empty vector, were used for the production of HA-bearing VLPs as described for panel B, and concentrated particles were subsequently treated with trypsin or PBS. Thereafter, the VLPs were either digested with PNGase F or mock treated, and HA cleavage was analyzed by Western blotting.

vector, and we then analyzed whether the pseudotypes were infectious or whether they required trypsin treatment in order to acquire infectivity. In agreement with our previous results (5), viruses produced in the presence of TMPRSS2 or TMPRSS4 were fully infectious in the absence of trypsin treatment, while viruses generated in cells transfected with empty vector (pcDNA) required trypsin treatment in order to transit into an infectious form (Fig. 2A, left). Unexpectedly, coexpression of hepsin did not render viruses infectious (Fig. 2A, left), despite the incorporation of cleaved HA into virions (Fig. 2A, right), and infectivity was not acquired upon exposure to trypsin.

We then determined whether the activation of HA pseudotypes reflects the activation of replication-competent influenza viruses. To this end, we analyzed whether transient expression of TMPRSS2, TMPRSS4, or hepsin facilitated the spread of PR8 (H1N1), a virus that has a monobasic cleavage site and requires exogenous trypsin for its activation (in most cell lines). 293T cells were chosen for these experiments, because this cell line is readily transfectable, and it is well documented that 293T cells do not express an endogenous HA-activating protease (5, 9). As a control, infection of 293T cells with SC35M (H7N7) (33) was analyzed. This influenza virus harbors a multibasic cleavage site and does not depend on trypsin treatment to acquire infectivity. TMPRSS2 or TMPRSS4 expression or trypsin treatment increased the spread (measured as the release of infectious virus particles into the supernatant) of PR8 (H1N1) about 10-fold compared to cells transfected with empty vector (Fig. 2B, left), indicating that these proteases were able to support the spread of influenza virus in the absence of an exogenous protease. In contrast, expression of hepsin did not augment viral spread, in agreement with the results obtained with pseudotypes (Fig. 2A, left). It should be noted in this context that PR8 (H1N1) was prepared in hens' eggs and thus contained activated HA. Therefore, the virus was able to undergo a single round of replication without proteolytic activation, resulting in the release of a readily quantifiable number (upon viral activation by trypsin) of infectious units in the supernatant of control-transfected 293T cells. Finally, as expected, the spread of SC35M (H7N7) was not modulated by the expression of TTSPs (Fig. 2B, right), with the exception of hepsin expression, which reduced viral spread about 10-fold. Collectively, these results demonstrate that engineered expression of TMPRSS2 or TMPRSS4 facilitates HA activation and allows the trypsin-independent spread of influenza virus.

**Expression of TMPRSS2 and TMPRSS4 correlates with the trypsin-independent spread of influenza virus in cell lines.** The activation of influenza virus HA by transfected TMPRSS2 and TMPRSS4 raised the question of whether these proteases are endogenously expressed by cell lines that allow trypsin-independent viral spread. To address this question, we investigated TMPRSS2 and TMPRSS4 expression in 293T, Huh-7, and Vero cells, which allow influenza virus to spread only in the presence of exogenous trypsin (5, 9, 16), and in Caco-2 cells, which support trypsin-independent viral spread (48). Direct RT-PCR analyses of TTSP mRNA expression revealed the presence of TMPRSS2 transcripts in Caco-2 cells, and a nested approach detected TMPRSS2 message in Huh-7 cells also, but not in the other cell lines analyzed (Fig. 3A). No TMPRSS4 mRNA could be detected by direct RT-PCR in any of the cell lines examined (Fig. 3A), and similar results were obtained for hepsin (data not shown). Nevertheless, nested PCR demonstrated the presence of TMPRSS4 message in

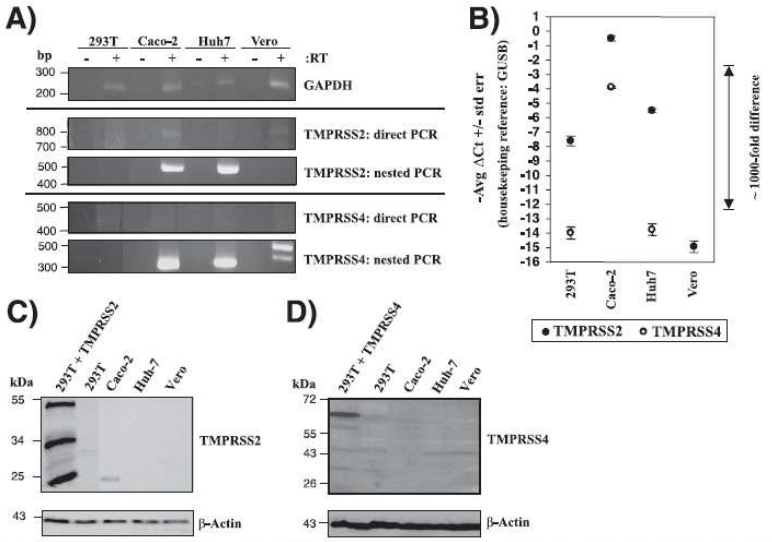


FIG. 3. TMPRSS2 and TMPRSS4 mRNAs and TMPRSS2 protein are expressed in Caco-2 cells. (A) Analysis of TMPRSS2 and TMPRSS4 mRNA expression by RT-PCR. Total RNA was prepared from the indicated cell lines, treated with DNase, reverse transcribed, and used for the amplification of TMPRSS2, TMPRSS4, and GAPDH mRNAs by nested PCR. The results of single gels from which irrelevant lanes were removed are shown. (B) Analysis of TMPRSS2 and TMPRSS4 mRNA expression by quantitative RT-PCR. RNA was prepared as described for panel A, and TMPRSS2, TMPRSS4, and GUSB (housekeeping control) were amplified by TaqMan gene expression assays. Similar results were obtained in two independent experiments. No TMPRSS4 signal was detected in Vero E6 cells. (C and D) Expression of TMPRSS2 (C) or TMPRSS4 (D) in cell lines as determined by Western blotting. The indicated cell lines were lysed, and the expression of TMPRSS2 or TMPRSS4 was analyzed by Western blotting. Detection of β-actin served as a loading control. The results of a single gel from which irrelevant lanes were removed are shown in panel C. 293T + TMPRSS2, 293T cells transiently expressing TMPRSS2; 293T + TMPRSS4, 293T cells transiently expressing TMPRSS4.

target cells. Efficient infection of Caco-2 cells by HA-bearing pseudotypes was dependent on previous activation of these viruses by trypsin (Fig. 4D), indicating that the HA-activating enzyme either is not present or does not recognize HA in virion-containing endocytic vesicles in Caco-2 cells. Similar results were obtained upon overexpression of TMPRSS2 and TMPRSS4 in 293T cells (Fig. 4E), suggesting that HA activation in Caco-2 cells and in TMPRSS2-expressing 293T cells most likely occurs in the secretory pathway of productively infected cells.

**TMPRSS2 and TMPRSS4 are required for the efficient trypsin-independent spread of influenza virus in Caco-2 cells.** Our results obtained so far suggested that endogenous expression of TMPRSS2 and/or TMPRSS4 might contribute to the trypsin-independent spread of influenza virus in Caco-2 cells. To directly test this hypothesis, we examined the consequences of TMPRSS2 and TMPRSS4 knockdown for the spread of influenza virus in Caco-2 cells. For this purpose, Caco-2 cells either were transiently transfected with a TMPRSS2 or a TMPRSS4 siRNA or were cotransfected with both siRNAs, and expression of TMPRSS2 protein was analyzed by Western blotting (Fig. 5A). Transfection of a nonsense siRNA served as a negative control. Transfection of cells with a TMPRSS2-specific siRNA or with a 1:1 mixture of TMPRSS2- and TMPRSS4-specific siRNAs resulted in a decrease in TMPRSS2 expression, which was not observed with the nonsense siRNA or the TMPRSS4-specific siRNA alone (Fig. 5A), indicating that our protocol allowed efficient and specific reduction of TMPRSS2 expression. The lack of detectable TMPRSS4 expression in Caco-2 cells (Fig. 3) precluded evaluation of the efficiency of siRNA-mediated TMPRSS4 knockdown. However, analysis of 293T cells transiently cotransfected with a TMPRSS4 expression plasmid and siRNAs demonstrated that the TMPRSS4-specific siRNA was active (data not shown). When PR8 (H1N1) infection of siRNA-treated Caco-2 cells was analyzed, a substantial reduction in the level of viral spread was observed upon transfection of either TMPRSS2- or TMPRSS4-specific siRNAs, and this effect was enhanced upon cotransfection of both TMPRSS2- and TMPRSS4-specific siRNAs. Notably, the blockade of viral spread by siRNAs could be fully rescued by trypsin treatment (Fig. 5B, left), demonstrating that a lack of proteolytic activation of influenza virus was the underlying inhibitory mechanism. In stark contrast, TMPRSS2- and TMPRSS4-specific siRNAs had no significant effect on SC35M (H7N7) spread (Fig. 5B, right). These observations suggest that TMPRSS2 and TMPRSS4 expression in Caco-2 cells is responsible, at least in part, for the ability of these cells to support the trypsin-independent spread of influenza viruses.

**TMPRSS2 is coexpressed with 2,6-linked sialic acids on type II pneumocytes and alveolar macrophages.** The important contribution of TMPRSS2 expression to the trypsin-independent spread of influenza virus in Caco-2 cells raised the ques-

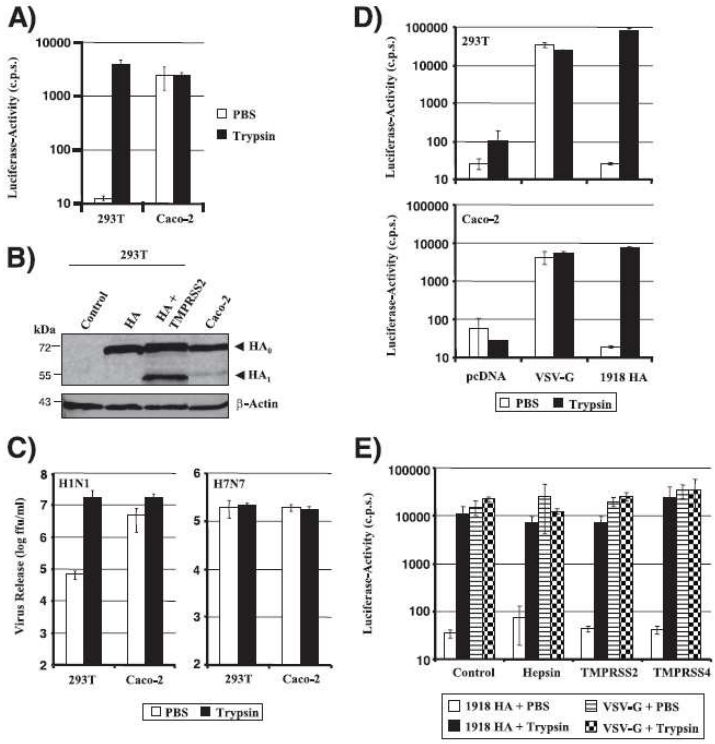


FIG. 4. Influenza virus HA is activated in Caco-2 cells in the absence of trypsin. (A) Activation of HA pseudotypes in Caco-2 cells. Caco-2 and 293T cells were transiently cotransfected with plasmids encoding a lentiviral vector and 1918 HA and NA. The supernatants were treated with trypsin or PBS and were then used to infect Huh7 target cells. Luciferase activity in cell lysates was determined at 72 h postinfection. The results ± standard deviations of a representative experiment performed in triplicate are shown and were confirmed in three separate experiments. (B) Analysis of HA cleavage in Caco-2 and 293T cells. 293T and Caco-2 cells were transfected with 1918 HA, or 293T cells were cotransfected with 1918 HA and TMPRSS2 expression plasmids, and HA cleavage in cell lysates was detected by Western blotting. The results of a single gel from which irrelevant lanes were removed are shown. (C) Activation of influenza viruses in Caco-2 cells. Caco-2 and 293T cells were first infected with PR8 (H1N1) or SC35M (H7N7) at an MOI of 0.01 and then treated with PBS or trypsin. The release of infectious particles in the supernatant was determined by a focus formation assay. Results of a representative experiment performed in triplicate are shown, and activation of PR8 (H1N1) in Caco-2 but not 293T cells was confirmed in two separate experiments. (D) Infection of Caco-2 and 293T cells with pseudotypes bearing nonactivated or trypsin-activated HA. Pseudotypes bearing the 1918 HA, the 1918 NA, or the G protein of VSV were first treated either with PBS or with trypsin and then used to infect 293T and Caco-2 cells. Luciferase activities in cell lysates were determined at 72 h postinfection. Similar results were obtained in two independent experiments. (E) Infection of TTSP-expressing 293T cells with pseudotypes bearing nonactivated or trypsin-activated HA. The indicated TTSPs were transiently expressed in 293T cells, and the cells were infected with HA-bearing pseudotypes as described for panel D.

tion of whether this protease is expressed in influenza virus target cells in the human lung. To address this question, we immunostained human lung tissue with labeled *Sambucus nigra* lectin, which recognizes 2,6-linked sialic acids, and a monoclonal antibody specific for TMPRSS2 (29). Microscopic examination of stained tissues revealed coexpression of TMPRSS2 and 2,6-linked sialic acids in type II pneumocytes and alveolar macrophages (Fig. 6). The former have been shown to bind to human influenza viruses, albeit less frequently than type I pneumocytes (44), and to constitute viral target cells (35), indicating that TMPRSS2 might also support influenza virus spread in this cell type.

## DISCUSSION

We investigated the contributions of TMPRSS2 and TMPRSS4 to the proteolytic activation of influenza viruses in cell lines and in primary target cells. Transient expression of these proteases facilitated HA cleavage and allowed influenza virus to spread in 293T cells in the absence of exogenously added trypsin. The related protein hepsin was also able to cleave HA but failed to render HA-bearing viruses infectious, for reasons that remain unclear. Caco-2 cells, which allowed trypsin-independent viral spread, were found to express endogenous TMPRSS2 (mRNA and protein) and TMPRSS4 (mRNA), and

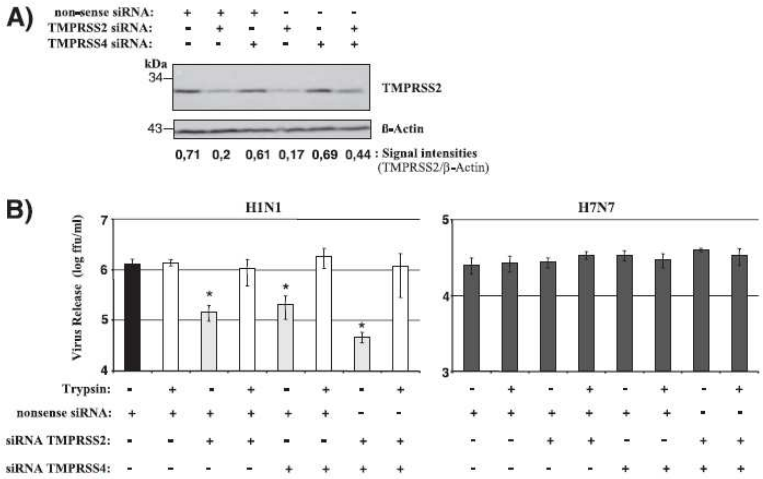


FIG. 5. Knockdown of TMPRSS2 and TMPRSS4 inhibits the trypsin-independent spread of influenza virus in Caco-2 cells. (A) Analysis of TMPRSS2 knockdown. Caco-2 cells either were transfected, by use of Lipofectamine, with a nonsense control siRNA or a TMPRSS2- or TMPRSS4-specific siRNA in the presence or absence of the control siRNA or were cotransfected with a 1:1 mixture of TMPRSS2- and TMPRSS4-specific siRNAs. TMPRSS2 expression in transfected cells was analyzed by Western blotting. Detection of β-actin served as a loading control. All siRNA transfection mixtures contained 200 pmol siRNA. Thus, if the transfection mixture contained a single siRNA species, 200 pmol of the siRNA was added. If two different siRNA species were cotransfected, 100 pmol of each was added. (B) Caco-2 cells were first transfected with siRNAs as described for panel A and then infected with PR8 (H1N1) or SC35M (H7N7) at an MOI of 0.01 in the presence or absence of trypsin. The release of infectious particles into the supernatant was determined by a focus formation assay. The results of a representative experiment ± standard deviations are shown and were confirmed by three separate experiments for PR8 (H1N1) and by one separate experiment for SC35M (H7N7). The statistical significance (asterisks) of the inhibitory effects of TMPRSS2- and TMPRSS4-specific siRNAs relative to that of the nonsense control siRNA was calculated by using the two-tailed Student *t* test for correlated samples.

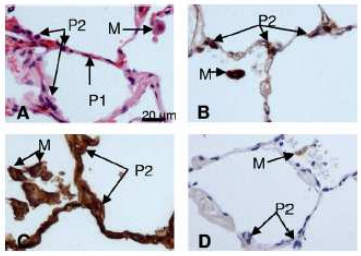


FIG. 6. Expression of TMPRSS2 in human alveolar epithelium. (A) Hematoxylin-and-eosin-stained section of a normal lung showing several alveolar spaces, in which alveolar macrophages (M), type I pneumocytes (P1), and type II pneumocytes (P2) are labeled. Bar, 20 μm. (B) A serial section of panel A immunostained for TMPRSS2 using the peroxidase technique (brown) shows strong positive staining in type II pneumocytes and alveolar macrophages. (C) A serial section of panel B stained with *Sambucus nigra* lectin shows strong positive membrane staining of all cell types, including type II pneumocytes and alveolar macrophages. (D) A serial section of panel C immunostained with an irrelevant mouse primary antibody (Melan-A), as a negative control for panel B, shows no immunostaining. Alveolar macrophages show a faint brown tint, due to the presence of carbon, but not the strong brown staining of macrophages seen in panel B. A serial section of panel D immunostained using a goat polyclonal serum as a primary antibody, as a negative control for panel C, appeared very similar to panel D (data not shown).

expression of these proteases was shown to be required for efficient influenza virus activation. Finally, analysis of human lung tissue revealed that type II pneumocytes and alveolar macrophages coexpressed TMPRSS2 and 2,6-linked sialic acids, indicating that TMPRSS2 could support viral spread in the infected host.

Proteolytic activation of influenza virus HA primes the protein for low-pH-induced membrane fusion and is indispensable for viral infectivity (23, 24, 26, 27). Our previous study showed that TMPRSS2 and TMPRSS4 can activate influenza virus HA (5, 6), in agreement with other published work (2, 3, 45), and raised the possibility that other members of the TTSP family might also cleave and activate HA. Indeed, the TTSP hepsin mediated HA cleavage (Fig. 1A and B), and this cleavage resulted in the production of HA1 and HA2 fragments of the expected sizes (Fig. 1 and data not shown), indicating that it occurred at the junction between HA1 and HA2. However, hepsin-dependent HA cleavage did not confer appreciable infectivity on influenza virus PR8 (H1N1) or on lentiviral pseudotypes bearing HA, despite detectable incorporation of cleaved HA into virions (Fig. 2). The reasons for this apparent contradiction are at present unclear and might involve quantitative differences between HA cleavage by hepsin and that by TMPRSS2 and TMPRSS4. In this context, it is noteworthy that hepsin and TMPRSS2 interfered with HA1 glycosylation; the underlying mechanism and its potential relevance for HA function remain to be clarified.

Transient expression of TMPRSS2 and TMPRSS4 increased

influenza virus spread at least 10-fold above background in 293T cells (the readily measurable background was due to the infection of cells with activated viruses generated in hens' eggs) (Fig. 2B). This observation prompted us to investigate whether endogenous expression of these proteases also facilitates the spread of influenza virus in cell lines in the absence of exogenously added proteases. Many cell lines, including 293T, Huh-7, and Vero E6, support the spread of human influenza virus in the presence of exogenous trypsin (5, 9, 13, 26). However, only the Caco-2 cell line, which was derived from intestinal epithelium, has been shown to allow the spread of different influenza viruses in the absence of trypsin (48). Our findings that Caco-2 cells released fully infectious HA-bearing pseudotypes and allowed the replication of PR8 (H1N1) virus in the absence of trypsin confirmed these results (Fig. 4A to C), although we did not obtain evidence that HA was activated upon viral uptake, as previously suggested (48). This discrepancy might be due to differences in the experimental systems used; we examined HA pseudotypes, while Zhirnov and colleagues employed replication-competent influenza viruses (48). Alternatively, different cell culture conditions might account for the conflicting results, since Zhirnov and coworkers observed HA activation upon viral uptake only in proliferating Caco-2 cells (48). Nevertheless, both studies demonstrated efficient HA activation in Caco-2 cells in the absence of trypsin and raised questions concerning the nature of the protease(s) responsible.

We detected robust expression of TMPRSS2 (mRNA and protein) and TMPRSS4 (mRNA) in Caco-2 cells (trypsin independent) but not in Huh-7, Vero E6, or 293T cells (trypsin dependent) (Fig. 3), suggesting a correlation between TMPRSS2 and TMPRSS4 expression and trypsin-independent replication of influenza virus. Furthermore, siRNA knockdown demonstrated that expression of both TMPRSS2 and TMPRSS4 was essential for the efficient trypsin-independent spread of influenza virus in Caco-2 cells (Fig. 5). Why TMPRSS2 knockdown was not fully rescued by the presence of TMPRSS4 and vice versa is at present unclear, and a definite answer might have to await the availability of efficient TMPRSS4 detection reagents. However, the enhanced inhibitory effect of a cocktail of TMPRSS2- and TMPRSS4-specific siRNAs relative to each siRNA alone clearly indicates that both proteins facilitate HA activation and can, at least in part, functionally complement each other. Collectively, these results provide a molecular mechanism to explain the ability of Caco-2 cells to support the spread of influenza virus in the absence of exogenous HA-activating protease, and they demonstrate for the first time that endogenous TTSPs can cleave and activate influenza viruses.

A contribution of TMPRSS2 and TMPRSS4 to the spread of influenza virus in the host requires that these proteases be expressed on permissive cells. A major determinant for permissiveness to infection by human influenza viruses is the expression of surface structures modified with 2,6-linked sialic acids, which are bound by HA (38). Seasonal and, in particular, pandemic influenza viruses, including the 2009 H1N1 virus (46), can spread to the alveolar epithelium, and infection of these target cells parallels the development of primary viral pneumonia (25, 42). Immunohistochemistry of human alveoli revealed that type II pneumocytes and alveolar macrophages coexpress TMPRSS2 and 2,6-linked sialic acids (Fig. 6), suggesting that TMPRSS2 might promote the spread of influenza virus in these cells. Indeed, attachment of human influenza viruses to type II pneumocytes and, rarely, to alveolar macrophages has been demonstrated previously (44), and recent evidence indicates that type II pneumocytes and, occasionally, macrophages are viral targets (35). Notably, type I pneumocytes have been reported to bind influenza virus more efficiently than type II pneumocytes (44), and the protease responsible for viral activation in these target cells remains to be defined—with TMPRSS4 being an attractive candidate. In contrast, it remains unknown whether TMPRSS2 (and TMPRSS4) is expressed and promotes viral spread in target cells in the upper airways. In addition, it is noteworthy that TMPRSS2 expression in the colon, stomach, and kidney has been demonstrated previously (29), and it is possible that this protease might contribute to the rare extrapulmonary spread (25) of influenza viruses in humans.

In summary, we provide evidence that TMPRSS2 and TMPRSS4 activate human influenza viruses in cell culture and potentially promote the spread of influenza viruses in and between humans. The finding that knockdown of a single protease, either TMPRSS2 or TMPRSS4, was sufficient to reduce viral spread markedly in Caco-2 cells lends further weight to the concept of targeting host cell proteases for influenza therapy—an approach that has already been shown to be feasible and effective in animals (50) and humans (49). Partial inhibition of viral spread by the blockade of a single protease might well be sufficient to produce a clinical benefit. However, our findings that two proteases can activate influenza virus in Caco-2 cells also indicate that high antiviral activity might be achieved only by targeting several proteases simultaneously. In any case, the characterization of the exact roles of TMPRSS2 and TMPRSS4 in influenza virus spread and pathogenesis is an important task, and *Tmprss2* knockout mice, which do not show an obvious phenotype in the absence of infection (20), might be valuable tools for these endeavors.

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# 6. Discussion

## 6.1 First manuscript: Proteolytic activation of the 1918 influenza virus hemagglutinin

The 1918 influenza virus is responsible for the most devastating pandemic in human history. Since the sequence of the complete viral genome is available, reconstruction of the virus by reverse genetics allows the analysis of the factors contributing to the extraordinary high virulence [Tumpey *et al.*, 2005]. Studies using reassortant viruses that contain gene segments of the 1918 influenza virus and well characterized seasonal influenza viruses showed that HA and NA represent determinants of virulence [Kobasa *et al.*, 2004; Pappas *et al.*, 2008; Tumpey *et al.*, 2004]. Consequently, the analysis of the interaction of the 1918 influenza virus HA and NA with host cell factors is of particular interest. Notably, the virus spreads in cultured MDCK cells in the absence of exogenous trypsin in an NA-dependent manner [Tumpey *et al.*, 2005], suggesting a role of NA in HA activation. A similar observation has been previously reported for A/WSN/33, a close relative of the 1918 influenza virus, and has been linked to the ability of A/WSN/33 NA to recruit plasminogen, which, upon conversion to plasmin, can activate HA [Goto and Kawaoka, 1998; Goto *et al.*, 2001]. The goal of the present study was to elucidate if the trypsin-independent spread of the 1918 influenza virus is also due to plasminogen recruitment by NA and, in case of a negative answer, to investigate which proteases can activate the 1918 HA.

The investigation of the biological properties of the 1918 influenza virus needs to be conducted in a laboratory providing a high level of biocontainment. In order to be able to analyse 1918 HA activation without the risk of accidental infection with the 1918 influenza virus, a lentiviral vector system was used. For this, an env-defective human immunodeficiency virus-derived vector was transiently co-expressed with 1918 HA, NA and M2, resulting in the release of HIV particles pseudotyped with HA, NA and M2 (pseudotypes), as expected from the well documented property of retroviruses to incorporate heterologous proteins in their envelope [Sandrin *et al.*, 2003]. The generation of infectious pseudotypes, bearing influenza virus surface proteins, requires the presence of both HA and NA. Although NA is usually not required to facilitate HA cleavage, the receptor-destroying activity of the protein is necessary for release of infectious particles from the virus-producing cell [Modrow *et al.*, 2003; Bouvier and Palese, 2008; Taubenberger and Kash, 2010] and in the absence of NA mainly HA-free particles are released [Bosch *et al.*, 2001]. Hemagglutinin is essential for binding of the viral particle to the target cell and for fusion of the viral and the

endosomal membranes [Modrow *et al.*, 2003; Bouvier and Palese, 2008; Taubenberger and Kash, 2010]. In agreement with these findings the presence of 1918 NA or 1918 HA alone in virus producing cells was not sufficient to confer infectivity to the particles released. Pseudotypes bearing both 1918 HA and 1918 NA were infectious, but only upon treatment with trypsin, indicating that in 293T cells the presence of 1918 NA was not sufficient to mediate 1918 HA activation. Indeed, Western blot analysis of 1918 HA- and 1918 NA-bearing virions revealed an HA size of approximately 75 kDa, which is expected for uncleaved HA [Elliot *et al.*, 2006], while trypsin treatment reduced the HA size to approximately 50 kDa, the size expected for the HA1 subunit [Elliot *et al.*, 2006]. Finally, the additional incorporation of 1918 M2 in pseudotypes bearing 1918 HA and 1918 NA reduced viral infectivity due to decreased particle incorporation of HA in the presence of M2. In natural infection, small amounts of the M2 protein are incorporated into influenza viral particles and the ion channel function of M2 plays an important role in uncoating and, for some viruses, in maturation [Betakova, 2007]. For lentiviruses pseudotyped with the fowl plaque virus HA, M2 expression was found to increase viral infectivity [McKay *et al.*, 2006]. The contradictory observations reported in this study and our analysis could be explained by the usage of a different influenza virus M2 proteins and pseudotyping systems, or by differences in M2 expression levels. In sum, these observations indicate that 1918 NA-dependent activation of 1918 HA is not a general phenomenon, but might depend on the viral producer cell type (293T cells in our system and MDCK cells in the published study [Tumpey *et al.*, 2005]).

Next, it was addressed if 1918 HA, NA bearing pseudotypes might exhibit residual but potentially biologically relevant infectivity (in the absence of trypsin treatment) upon optimization of infection conditions. The concentration of virions onto target cells by centrifugation, a procedure termed spinoculation [O'Doherty *et al.*, 2000], can substantially increase viral infectivity. Spinoculation allowed infectious entry 1918 HA, NA bearing pseudotypes into 293T and MDCK cells in the absence of trypsin treatment, although infection efficiency was clearly reduced compared to trypsin-treated viruses. These observations suggested that 293T and MDCK cells express proteases, which can activate 1918 HA on incoming virions, but activation is inefficient. The role of the endosomal/lysosomal cysteine proteases cathepsin B and L in the activation of the 1918 HA was assessed, since these proteins activate the glycoproteins of Ebola virus, SARS-CoV, and the fusion protein of Hendra virus [Chandran *et al.*, 2005; Pager and Dulch, 2005; Simmons *et al.*, 2005]. However, a cysteine protease inhibitor, which was shown to block Ebola virus and SARS-CoV entry

[Chandran *et al.*, 2005; Simmons *et al.*, 2005], could not inhibit the infectivity of 1918 influenza virus HA, NA bearing pseudotypes. These data suggest the involvement of another class of proteases in the low level cleavage-activation of pseudotypes bearing the 1918 HA and NA.

The NA protein of A/WSN/33, which is closely related to the 1918 influenza virus, recruits plasminogen from serum and thereby facilitates cleavage-activation of NA [Goto and Kawaoka, 1998; Goto *et al.*, 2001]. In agreement with these findings, binding of plasminogen to A/WSN/33 NA was detected and it could be demonstrated that pseudotypes bearing A/WSN/33 HA and NA are infectious in the absence of trypsin treatment [Taubenberger, 1998]. In contrast, the 1918 NA was unable to bind plasminogen and incorporation of A/WSN/33 NA into pseudotypes bearing 1918 HA did not render the respective viruses infectious. These observations indicate that plasminogen capture by 1918 NA is not the mechanism allowing the previously reported trypsin independent spread of the 1918 virus in MDCK cells [Tumpey *et al.*, 2005]. Interestingly, binding of plasminogen to 1918 HA expressing cells was detected, but the significance of this finding for influenza virus biology is at present unclear. However, it is tempting to speculate that plasminogen recruitment by 1918 HA contributes to viral spread either by promoting HA activation or by facilitating an as yet unappreciated cleavage event involved in viral replication. Of note, plasminogen is bound by the cellular protein annexin II, which facilitates the conversion of plasminogen into plasmin [LeBouder *et al.*, 2008]. Annexin II has been detected in influenza virus particles [LeBouder *et al.*, 2008] and it has been suggested that virion associated annexin II promotes viral spread by mediating HA cleavage [LeBouder *et al.*, 2010]. It is therefore conceivable that 1918 NA recruits annexin II or another plasminogen-activating factor and thereby promotes activation of 1918 HA. Alternatively, 1918 NA might promote 1918 HA cleavage by a completely different, so far unappreciated mechanism.

Based on our findings with 1918 HA, NA bearing pseudotypes generated in 293T cells (see above), the possibility was explored that the trypsin-independent spread of the 1918 influenza virus, which was observed in the MDCK cell line [Tumpey *et al.*, 2005], might be a cell type specific phenomenon. To further test this hypothesis, it was assessed whether lysates prepared from different cell lines were able to activate 1918 HA, NA bearing viruses. The lysates of MDCK cells, but not those from 293T cells increased the infectivity of 1918 HA, NA bearing pseudoparticles. However, the infectivity rate of 1918 HA, NA bearing pseudoparticles upon spinoculation of MDCK and 293T cells resided in the same range, suggesting that differential recruitment of endosomal proteases might not account for the differential activation of NA

protein by cell lysates. Instead, NA might recruit a protease during transport through the secretory pathway or at the cell surface of the virus-producing MDCK but not 293T cells. In any case, it needs to be noted that the cleavage-activation of 1918 HA, NA bearing pseudotypes by MDCK, but not 293T cell lysates, was inefficient. This may be due to the HA-activating protease unfolding its full activity only upon incorporation into an intact lipid membrane or a specific subcellular compartment. To further explore the potential cell type-dependence of activation of 1918 HA by 1918 NA, replication of the authentic 1918 influenza virus in MDCK cells and the human hepatoma cell line Huh-7 in the presence and absence of trypsin was compared. In agreement with published results [Tumpey *et al.*, 2005], it was found that the 1918 influenza virus replicated in MDCK cells in the presence or absence of trypsin with the same kinetics and viral peak titers. In contrast, replication in Huh-7 cells was only robust in the presence of trypsin, indicating that these cells do not express a 1918 HA activating protease. Collectively, activation of 1918 HA by 1918 NA is cell type dependent and the responsible cellular protease and the underlying molecular mechanism remain to be elucidated.

Influenza virus infection in humans is largely restricted to the respiratory tract [Skehel and Wiley, 2000], which expresses the receptors for viral entry and is also believed to express HA-activating proteases. A study by Böttcher and colleagues identified the enzymes TMPRSS2 and HAT, two members of the class of type II transmembrane serine proteases (TTSPs), as candidate proteases for activation of different influenza A virus subtypes (H1, H2, H3) in the human respiratory tract [Böttcher *et al.*, 2006; 2009]. Therefore, these serine proteases as well as other members of the TTSPs could also be involved in the activation of the 1918 influenza virus. The co-expression of TMPRSS2 or TMPRSS4 with 1918 HA resulted in 1918 HA cleavage in a 1918 NA-independent manner and allowed generation of 1918 HA, NA bearing pseudotypes, which were infectious in the absence of trypsin treatment. The detection of TMPRSS4 mRNA in human lung tissue, which was previously found to express TMPRSS2 mRNA as well [Donaldson *et al.*, 2002], indicates that these proteases might facilitate spread of the 1918 influenza virus in human lung [Szabo *et al.*, 2003; Szabo and Bugge, 2008]. In contrast, TMPRSS4 mRNA was not detected in 293T, Huh7 and MDCK cells, in agreement with the finding that the former two cell lines do not allow trypsin-independent 1918 HA activation and that trypsin-independent viral spread in MDCK cells requires the recruitment of a still unknown host cell protease by 1918 NA. Finally, it is noteworthy that cleavage of 1918 HA by TMPRSS2 and TMPRSS4 leads to the production of HA1 subunits of slightly different sizes and these size differences might be due to cleavage of

HA at different sites or, more likely, differential glycosylation. Taken together, TMPRSS2 and TMPRSS4 can activate 1918 HA in cell culture and might promote viral spread in infected individuals. While TMPRSS2 had previously been implicated in influenza virus activation [Böttcher *et al.*, 2006; 2010], the protease TMPRSS4 was identified as a new TTSP, capable of activating the 1918 influenza virus HA.

## 6.2 Second manuscript: TMPRSS2 and TMPRSS4 facilitate trypsin-independent spread of influenza virus in Caco-2 cells

The cleavage activation of influenza virus HA is essential for viral infectivity. Polybasic cleavage sites of highly pathogenic avian influenza viruses (HPAIVs) are recognized by ubiquitously expressed subtilisin-like proteases, allowing systemic viral spread and development of severe disease. In contrast, the monobasic cleavage sites of low pathogenic avian influenza viruses (LPAIVs) and human influenza viruses are recognized by trypsin-like proteases, the expression of which is believed to be restricted to the respiratory tract or gastrointestinal tract in waterfowl/and gastrointestinal tract in poultry [Steinhauer, 1999]. Consequently, viral spread is limited to the aforementioned organs and usually results in only mild symptoms [Steinhauer, 1999]. The identity of proteases activating viruses with a monobasic cleavage site is at present not well defined. Recently, a new class of trysin-like serine proteases, named TTSPs, were proposed to activate viruses with a monobasic cleavage site [Böttcher *et al.*, 2006; 2009; Böttcher-Friebertshäuser *et al.*, 2010]. However, these studies had exclusively been conducted with cell lines transfected to express the proteases in question. It was therefore assessed if endogenous protease expression in cell lines could activate influenza HA and correlate with the ability to support trypsin-independent influenza virus spread.

We and others had previously shown that the TTSPs TMPRSS2 and TMPRSS4 can activate influenza HA [Böttcher *et al.*, 2006; 2009; Chaipan *et al.*, 2009; Böttcher-Friebertshäuser *et al.*, 2010]. Based on these findings, it was first tested whether other members of the TTSP family can activate influenza viruses. The serine protease hepsin, which is expressed in high levels in the liver, but is also detected in the thymus, lung, pancreas, prostate and kidney [Tsuji *et al.*, 1991; Szabo and Bugge, 2008] was able to cleave the influenza virus HA into its subunits HA1 and HA2. The HA1 subunits generated by TMPRSS2 on the one side and hepsin/TMPRSS4 on the other side were of slightly different sizes and differential HA

glycosylation was shown to be responsible for these size differences. How TTSP expression impacts HA glycosylation and if this effect occurs in infected cells remains to be determined. Interestingly, hepsin expression was unable to confer infectivity to HA bearing pseudotypes or to PR8 (H1N1) influenza virus, despite the incorporation of cleaved HA into virions. Why hepsin failed to activate influenza virus HA is at present unclear, but might be explained by differences in quantitative cleavage compared to TMPRSS2 and TMPRSS4.

Next, it was determined if TMPRSS2 and TMPRSS4 were not only able to activate HA incorporated into lentiviral pseudotypes, but could confer infectivity to replication competent influenza viruses. Spread of the PR8 (H1N1) influenza virus, which bears a monobasic cleavage site, in 293T cells was about 10-fold augmented by expression of TMPRSS2 and TMPRSS4 relative to control cells and a similar effect was seen upon trypsin treatment. In contrast, expression of TMPRSS2 and TMPRSS4 had no effect on spread of an influenza virus with a polybasic cleavage site, as expected. The relatively high background measured in these experiments resulted from the usage of already activated viruses, generated in embryonated hen eggs, which express HA-activating proteases. Thus, TMPRSS2 and TMPRSS4 can activate replication-competent influenza viruses when exogenously expressed in cell lines.

Based on these observations, it was then investigated if cell lines susceptible to influenza virus infection express TMPRSS2 and TMPRSS4 and if expression allows viral spread in the absence of trypsin treatment. Viral spread in 293T, Huh7 and Vero E6 cells was examined, which are known to support influenza virus replication only upon the addition of an exogenous protease [Lazarowitz and Choppin, 1975; Govorkova *et al.*, 1995; de Wit *et al.*, 2004; Chaipan *et al.*, 2009]. In contrast, the Caco-2 cell line, which was derived from intestinal epithelium [Pinto *et al.*, 1983; Jumarie and Malo, 1991], was reported to allow influenza virus replication in the absence of an exogenous protease [Zhirnov and Klenk, 2003], suggesting that these cells might express an HA-activating protease, potentially TMPRSS2 and/or TMPRSS4. Indeed, TMPRSS2 and TMPRSS4 mRNA was detected by quantitative reverse transcription PCR in this cell line and TMPRSS2 protein was detected by Western blot. Evidence for expression of TMPRSS4 protein was not obtained, due to the lack of an appropriate antibody. Next, it was examined if HA was cleaved in Caco-2 cells. Transient expression of HA in Caco-2 but not 293T cells resulted in the generation of an HA fragment corresponding to HA1. The cleavage of HA in Caco-2 cells resulted in HA-activation, since pseudotypes generated in Caco-2 but not 293T cells were infectious in the absence of trypsin treatment and PR8 (H1N1) replicated in Caco-2 cells with similar

efficiency in the presence and absence of trypsin. The ability of Caco-2 cells to generate infectious HA-bearing pseudotypes indicates that the HA is activated during the passage of the constitutive secretory pathway. In contrast, activation did not occur during viral entry into Caco-2 cells, since pseudotypes bearing non-activated HA were unable to infect Caco-2 cells. This observation is compatible with a recent publication, which demonstrates that TMPRSS2 cleaves newly synthesized HA within the cell and is not able to activate HA on incoming viruses [Böttcher-Friebertshäuser *et al.*, 2010]. However, the results contrast those reported by Zhirnov and Klenk [Zhirnov and Klenk, 2003], who suggested that proteolytic activation of HA can occur during viral uptake in Caco-2 cells. This discrepancy might be explained by the different experimental procedures used in both studies: Zhirnov and colleagues used replication-competent influenza viruses instead of pseudoparticles and might have employed different cell culture conditions [Zhirnov and Klenk, 2003]. Regardless, Zhirnov and Klenk as well as the present study both observed efficient activation of influenza virus in Caco-2 cells in the absence of trypsin, raising the question whether TMPRSS2 and TMPRSS4 are responsible for HA-activation in Caco-2 cells.

In order to assess the role of TMPRSS2 and TMPRSS4 in HA-activation in Caco-2 cells, siRNA-mediated knockdown was employed. Knockdown of either TMPRSS2 or TMPRSS4 markedly reduced PR8 (H1N1) spread in the absence but not in the presence of trypsin while the spread of a virus with a polybasic cleavage site was not affected. Thus, both TMPRSS2 and TMPRSS4 can support viral spread in Caco-2 cells. Why the knockdown of TMPRSS2 could not be rescued by TMPRSS4 and *vice versa* is at present unclear. It can be speculated, however, that expression of both proteases is linked and that knockdown of one protease affected expression of the other – an effect that would have remained unnoticed due to the absence of reagents for detection of TMPRSS4 protein.

To allow influenza virus replication in humans, TMPRSS2 and TMPRSS4 need to be expressed in viral target cells. Human influenza viruses infect cells which express glycoproteins or glycolipids modified with α-2,6-linked sialic acid (SA) [Skehel and Wiley, 2000; Modrow *et al.*, 2003; Bouvier and Palese, 2008; Taubenberger and Kash, 2010]. Pandemic influenza viruses, including the Swine-origin influenza virus 2009, are able to spread in the alveolar epithelium and infection of this tissue is associated with the development of viral pneumonia [Kuiken and Taubenberger, 2008; Taubenberger and Morens, 2008; Yeh *et al.*, 2010]. The analysis of human lung tissues by immunohistochemistry revealed co-expression of TMPRSS2 and α-2,6-linked SA in type II pneumocytes and alveolar macrophages, suggesting that TMPRSS2 might support viral replication in these cells.

A study by van Riel and colleagues demonstrated that human influenza viruses preferentially bind to type I pneumocytes and to a lesser degree to type II pneumocytes and occasionally to macrophages [van Riel *et al.*, 2007]. In contrast, a recent study showed that the 2009 H1N1 virus mainly infects type II pneumocytes [Shieh *et al.*, 2010] and it is conceivable that TMPRSS2 supports viral spread in these cells. Expression of TMPRSS2 was also detected in the colon, stomach, and kidney [Paoloni-Giacobino *et al.*, 1997; Jacquinet *et al.*, 2001; Szabo and Bugge, 2008], and might be responsible for the rare extrapulmonary viral spread in humans [Kuiken and Taubenberger, 2008; Lucas *et al.*, 2008]. The expression of TMPRSS4 protein in human lung remains to be determined.

In summary, the present study showed for the first time that endogenously expressed TMPRSS2 and TMPRSS4 can facilitate the spread of a human influenza virus in cell culture and potentially constitute targets for therapeutic intervention. Previous work unambiguously established that inhibition of proteolytic activation is a suitable strategy to suppress influenza virus spread in cell culture, animals, and in infected humans [Zhirnov *et al.*, 1982; 1984; Garten *et al.*, 1989; Stieneke-Gröber *et al.*, 1992; Böttcher *et al.*, 2009; Böttcher-Friebertshäuser *et al.*, 2010]. Our results suggest that at least two proteases, TMPRSS2 and TMPRSS4, must be targeted to efficiently suppress viral spread. However, inhibition of only one of these proteases reduced viral replication in Caco-2 cells and might be sufficient to obtain a therapeutic benefit. One way to address the relative importance of TMPRSS2 and TMPRSS4 for influenza virus spread are knockout mice. *Tmprss2* knockout mice are available and do not display an obvious phenotype in the absence of expression [Kim *et al.*, 2006] and are thus ideally suited for this endeavour.

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# 8. Appendix

## List of abbreviations

| | |
|---|---|
| A | Alanine |
| A.D. | Lateinisch: *a dato* (since this day) |
| CDC | Centers of Disease Control and Prevention |
| CM2 | Influenza C virus minor envelope protein |
| cRNA | Complementary ribonucleic acid |
| CUB | Cls/Clr, urchin embryonic growth factor and bone morphogenic protein-1 domain |
| D | Aspartate |
| DESC-1 | Differentially expressed squamous cell carcinoma gene 1 |
| ETS | E-twenty six |
| FP | Fusion peptide |
| FRZ | Frizzled domain HAT (human airway trypsin-like protease |
| GP | Glycoprotein |
| H | Histidine |
| HA | Hemagglutinin |
| HAEC | Human adenoid epithelial cell |
| HAT | Human airway trypsin-like protease |
| HEF | Hemagglutinin-esterase-fusion protein |
| HMPV | Human metapneumovirus |
| HPAIV | High pathogenic avian influenza virus |
| ICTV | International Committee on Taxonomy of Viruses |
| kb | Kilo bases |
| kDA | Kilo Dalton |
| LAIV | Live-attenuated influenza vaccine |
| LDLA | Low-density lipoprotein receptor domain class A |
| LPAIV | Low pathogenic avian influenza virus |
| M1 | Matrix 1 protein |
| M2 | Matrix 2 protein |
| MAM | Meprin, A5 antigen and receptor protein phosphatase m domain |
| MDCK | Mardin-Darby Canine Kidney cell line |
| MHV | Mouse hepatitis coronavirus |

| | |
|---|---|
| mRNA | Messanger ribonucleic acid |
| MSPL | Mosaic serine protease large form |
| NA | Neuraminidase |
| NEP | Nuclear export protein |
| NP | Nucleoproteins |
| NS1 | Non-structural protein 1 |
| NS2 | Non-structural protein 2 |
| ORF | Open reading frame |
| PA | Polymerase acidic protein |
| PAR-2 | Protease-activated receptor 2 |
| PB1 | Polymerase basic protein 1 |
| PB2 | Polymerase basic protein 2 |
| PC5/6 | Pro-protein convertase 5/6 |
| R | Arginine |
| RNA | Ribonucleic acid |
| RKI | Robert Koch-Institute |
| S | Serine |
| SA | Sialic acid |
| SARS-CoV | Severe Acute Respiratory Syndrome-Coronavirus |
| SEA | Single sea urchin sperm protein, enteropeptidase, agrin domain |
| SR | Scavenger receptor cysteine-rich domain |
| SsRNA | Single-stranded ribonucleic acid |
| TGN | Trans-Golgi network |
| TIV | Trivalent inactivated influenza vaccine |
| TM | Transmembrane domain |
| TTSP | Type II transmembrane serine protease |
| uPA | Urokinase receptor |
| vRNA | Viral ribonucleic acid |
| vRNP | Viral ribonucleoprotein |
| WHO | World Health Organization |

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**Academical education**

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**Congresses**

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| 09/26-29/2009 | 5th European Congress of Viral Zoonosis (St. Raphael, France)<br>Oral presentation: **"Role of type II transmembrane serine proteases in activiation of influenza A virus"** |
| 03/18-21/2009 | 19th Annual Meeting of the Society for Virology (Leipzig, Germany)<br>Poster: **"Proteolytic activation of the 1918 influenza virus hemagglutinin"** |
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| 09/01-05/2007 | Third European Congress of Virology (Nürnberg, Germany) |

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## List of Publications

### First author

Simmons G.,***** **Bertram S**.,***** Glowacka I.,***** Steffen I., Chaipan C., Agudelo J., Lu K., Rennekamp A.J., Hofmann H., Bates P. and Pöhlmann S., *Different host cell proteases activate the SARS-coronavirus spike-protein for cell-cell and virus-cell fusion*. Virology. **2011**; 413(2):265-274. *****contributed equally

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**Review/book chapter**

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Choi S.Y., **Bertram S.**, Glowacka I., Park Y.W. and Pöhlmann S., *Type II transmembrane serine proteases in cancer and viral infections.* Trends Mol Med. **2009**; 15(7): 303-312.

**Declaration (*Erklärung*)**

gemäß §6(1) der Promotionsordnung der Naturwissenschaftlichen Fakultät der Gottfried Wilhelm Leibnitz Universität Hannover

Hierdurch erkläre ich, dass ich meine Dissertation mit dem Titel

**Proteolytic Activation of human Influenza Viruses**

selbstständig verfasst und die benutzten Hilfsmittel und Quellen sowie gegebenenfalls die zu Hilfeleistungen herangezogenen Institutionen vollständig angegeben habe.

Die Dissertation wurde nicht schon als Masterarbeit, Diplomarbeit oder andere Prüfungsarbeit verwendet.

Hannover, den

Stephanie Bertram