

The Hemagglutinin: A Determinant of Pathogenicity

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Abstract The hemagglutinin (HA) is a prime determinant of the pathogenicity of influenza A viruses. It initiates infection by binding to cell surface receptors and by inducing membrane fusion. The fusion capacity of HA depends on cleavage activation by host proteases, and it has long been known that highly pathogenic avian influenza viruses displaying a multibasic cleavage site differ in protease sensitivity from low pathogenic avian and mammalian influenza viruses with a monobasic cleavage site. Evidence is increasing that there are also variations in proteolytic activation among the viruses with a monobasic cleavage site, and several proteases have been identified recently that activate these viruses in a natural setting. Differences in protease sensitivity of HA and in tissue specificity of the enzymes are important determinants for virus tropism in the respiratory tract and for systemic spread of infection. Protease inhibitors that interfere with cleavage activation have the potential to be used for antiviral therapy and attenuated viruses have been generated by mutation of the cleavage site that can be used for the development of inactivated and live vaccines. It has long been known that human and avian influenza viruses differ in their specificity for sialic acid-containing cell receptors, and it is now clear that human tissues contain also receptors for avian viruses. Differences in receptor-binding specificity of seasonal and zoonotic viruses and differential expression of receptors for these viruses in the human respiratory tract account, at least partially, for the severity of disease. Receptor binding and fusion activation are modulated by HA glycosylation, and interaction of the glycans of HA with cellular lectins also affects virus infectivity. Interestingly, some of the mechanisms underlying pathogenicity are determinants of host range and transmissibility, as well.

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Contents

1	Introduction.....	4
2	Proteolytic Cleavage of HA Is a Prerequisite for Membrane Fusion.....	5
2.1	Cleavage Activation of HA: The Prime Pathogenicity Determinant of Avian Influenza Viruses	7
2.2	Proteolytic Activation of Mammalian influenza viruses	9
2.3	Variations in Cleavage Activation of LPAIV and Mammalian Influenza A Viruses	11
2.4	HA Cleavage Is Essential for Pneumotropism and Pathogenicity in Mammalian Hosts	12
2.5	Protease Inhibitors as Drugs for Influenza Treatment	13
2.6	The Use of Protease Activation Mutants for Vaccine Design	14
3	Receptor Specificity of HA	15
3.1	Influenza Viruses Attach to Sia2-3Gal- and Sia2-6Gal-Containing Receptors	15
3.2	Expression of Receptors and Virus Tissue Tropism in the Human Respiratory Tract	17
3.3	Cell Tropism of Viral Infection as Depending on Receptor Specificity.....	19
4	Modulation of HA Functions by Glycosylation.....	21
5	Outlook	23
	References.....	24

1 Introduction

It is generally agreed that most influenza A viruses originate from a large virus pool indigenous to aquatic birds. Since most of these agents cause no or only mild disease they are called low pathogenic avian influenza viruses (LPAIV). Because the host barrier is not an insurmountable obstacle for these viruses, they can occasionally be transmitted to terrestrial birds and mammals (Webster et al. 1992). Most of these transmissions are only transient. On rare occasions, however, the viruses adapt to a new species and give rise to a new lineage. Adaptation is facilitated by the high mutation rate of the viral polymerase and the segmented viral genome that allows reassortment after coinfection with another virus. Transmission and adaptation of LPAIV of subtypes H5 and H7 to domestic poultry may lead to the generation of highly pathogenic avian influenza viruses (HPAIV) (Roehm et al. 1995; Campitelli et al. 2004). Introduction and circulation of avian viruses in pigs may result in mutations allowing efficient growth in man. Moreover, the pig may serve as a mixing vessel in which viruses of different origin reassort to give rise to a new virus. If viruses with new surface antigens generated by these mechanisms are introduced into the human population they may cause a pandemic as has been the case in 1918, 1957, 1968, and 2009. So far, only influenza A viruses of subtypes H1N1, H2N2, and H3N2 have been observed to cause pandemics; influenza B viruses that co-cycle with influenza A viruses are not pandemic.

LPAIV have sporadically infected humans with mostly mild disease symptoms (Peiris 2009; Wei et al. 2013). LPAIV H7N9 that emerged recently (Gao et al. 2013)

is an exception, since most of the human infections (ca. 400 between March 2013 and March 2014) have been severe with a high case-fatality rate. HPAIV have also been transmitted to humans, and the H5N1 infections, in particular, cause severe pulmonary disease with occasional dissemination to other organs (Peiris 2009). Although a few human H7N7 HPAIV infections have been reported before (Kurtz et al. 1996), there has been only one poultry outbreak in 2003 with a larger number of human infections, one of which was fatal (Koopmans et al. 2003; van Kolschooten 2003; Fouchier et al. 2004).

Human influenza is a highly contagious disease that spreads by airborne transmission. Infection is usually confined to the epithelia of the respiratory tract. Severe cases typically develop primary influenza pneumonia or combined viral–bacterial pneumonia. Dissemination into other organs is rare (Kuiken and Taubenberger 2008). Infection of birds by avian influenza viruses is different. LPAIV cause local infections mostly confined to the intestinal tract. Virus is shed in the feces and transmitted through contaminated water. In contrast, HPAIV cause systemic infection. Virus can be isolated from many organs, and widely spread hemorrhages, edema, and cutaneous ischemia are major symptoms of the disease (Pantin-Jackwood and Swayne 2009).

Pathogenesis is the result of the complex interactions that the virus undergoes when it uses the biosynthetic machinery of the cell for replication and when it is exposed to the defence mechanisms of the host (Klenk et al. 2013). All viral proteins are involved in this interplay, but particularly prominent roles are played by the polymerase and the NS1 protein described in other chapters of these volumes and by the hemagglutinin (HA).

HA, the major viral surface glycoprotein, is inserted in the viral envelope as a type I membrane protein. More than 30,000 nucleotide sequences of the 18 HA subtypes identified to date have been determined. The three-dimensional structure of several serotypes has been analyzed by X-ray crystallography: H1 (Russell et al. 2004; Stevens et al. 2004), H2 (Liu et al. 2009), H3 (Wilson et al. 1981; Chen et al. 1998), H5 (Ha et al. 2002; Stevens et al. 2006), H7 (Russell et al. 2004), H9 (Ha et al. 2002), H13 (Lu et al. 2013), H16 (Lu et al. 2012), H17 (Zhu et al. 2013), and H18 (Tong et al. 2013).

HA initiates infection by binding to cell surface receptors and by inducing membrane fusion. Receptor specificity and proteolytic activation of fusion capacity are major determinants of tissue tropism and virus dissemination in the organism. Receptor binding and fusion activation are modulated by HA glycosylation, and evidence is increasing that the carbohydrate side chains may also be directly involved in specific interactions with cell surface-bound and soluble lectins. In the following, the roles of these mechanisms in pathogenesis will be discussed.

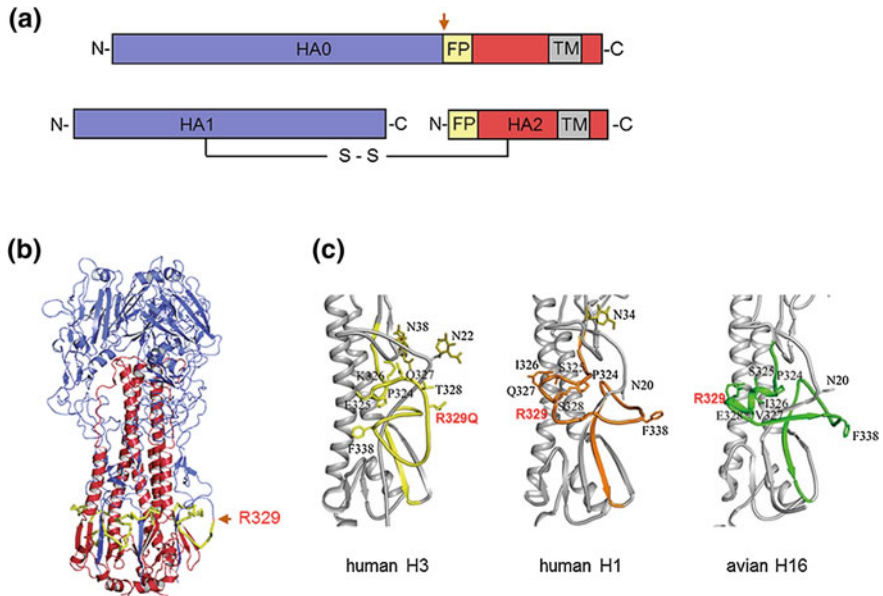


Fig. 1 Cleavage of HA0 into HA1 and HA2 at specific cleavage sites. **a** Schematic illustration of the HA0 precursor and the cleaved form consisting of the disulfide-linked subunits HA1 and HA2. The cleavage site is indicated by an *arrow*. *FP* Fusion peptide. *TM* transmembrane domain. **b** Structure of the trimeric H3 HA0 shown as a ribbon diagram (Galloway et al. 2013). The cleavage site (R329) is located in a surface loop and highlighted by an *arrow*. **c** The structures of noncleaved HA0 have been solved for subtypes H1, H3, and H16. Structural analysis of the H3 precursor (R329Q cleavage site mutant) demonstrated that the HA cleavage site is located in a prominent loop that protrudes from the surface (Chen et al. 1998). The cleavage site loop of HA0 of the 1918 pandemic H1N1 virus is less exposed and abuts the surface of the molecule (Stevens et al. 2004). Interestingly, structural analysis of noncleaved H16 revealed that the cleavage loop has an α -helix structure that hides the arginine residue (Lu et al. 2012). H16 has been shown to be resistant to cleavage by trypsin, but can be activated by TMPRSS2 in vitro (Lu et al. 2012; Galloway et al. 2013)

2 Proteolytic Cleavage of HA Is a Prerequisite for Membrane Fusion

Activation of HA by host proteases has been recognized nearly 40 years ago as a requirement for influenza virus infectivity (Klenk et al. 1975; Lazarowitz and Choppin 1975) and as a prime determinant of avian influenza virus pathogenicity (Bosch et al. 1979). Cleavage of the precursor protein HA0 into the subunits HA1 and HA2 occurs at a conserved arginine-glycine bond (R↓G) and activates fusion capacity (Huang et al. 1981; Maeda et al. 1981). The cleavage site is located in a loop that protrudes from the surface of HA. The amino acid sequence and the conformation of the loop vary with different viruses (Fig. 1, Table 1), and these variations determine cleavage of HA by different proteases (Klenk and Garten

Table 1 Hemagglutinin cleavage site variants of avian influenza A viruses

Virus strain	HA1 aa insertions		HA2	Pathogenicity
A/duck/Alberta/35/1976 (H1N1)	NVP-----SIQ SR	↓	GLF	Low
A/chicken/Potsdam/4705/1984 (H2N2)	NVP-----QIES R	↓	GLF	Low
A/mallard/Alaska/715/2005 (H3N8)	NVP-----EKQ TR	↓	GLF	Low
A/chicken/Germany/N/1949 (H10N7)	NVP-----EVVQ GR	↓	GLF	Low
A/turkey/Wisconsin/1/1966 (H9N2)	NVP-----AVSS R	↓	GLF	Low
A/quail/Shantou/782/2000 (H9N2)	NVP-----ARSS R	↓	GLF	Low ^a
A/quail/Shantou/2061/2000 (H9N2)	NVP-----AR SR R	↓	GLF	Low ^a
A/chicken/Ibaraki/1/2005 (H5N2)	NVP-----QRE TR	↓	GLF	Low
A/ostrich/SA/AI2114/2011 (H5N2)	NVP-----QR RK KR	↓	GLF	Low
A/chicken/HongKong/220/1997 (H5N1)	NTP-----QRE RR R K KR	↓	GLF	High
A/chicken/FPV/Rostock/1934 (H7N1)	NVP-----EPS K K R E K R	↓	GLF	High
A/ostrich/Italy/984/2000 (H7N1)	NVP-----EIP K GS R V R R	↓	GLF	High
A/chicken/Jalisco/CPA1/2012 (H7N3)	NVPENPKDRKSR R R R TR	↓	GLF	High

Amino acid sequences at the HA0 cleavage sites were aligned. The cleavage site between HA1 and HA2 is indicated by a downward arrow. Basic amino acids crucial for HA cleavage by relevant proteases are shown in boldface

^a HA cleavage by matriptase may enhance the virulence of these viruses

1994; Steinhauer 1999). Cleavage of HA exposes a highly conserved fusion peptide consisting of hydrophobic amino acids at the N-terminus of the HA2 subunit. The high structural specificity of the fusion peptide is also indicated by the observation that the protease thermolysin cleaves HA0 C-terminal to the glycine residue (R-G↓L), resulting in fusion-incompetent HA and noninfectious virus (Garten et al. 1981). HA cleavage is a prerequisite for conformational changes at low pH that trigger fusion of the viral and endosomal membranes (Skehel and Wiley 2000) in order to release the viral RNP segments into the cytosol and further in the cell nucleus where transcription and replication of the viral genome takes place (Fig. 2).

2.1 Cleavage Activation of HA: The Prime Pathogenicity Determinant of Avian Influenza Viruses

LPAIV possess a single arginine (R) or rarely a single lysine (K) at the cleavage site and are activated by trypsin in vitro. The available evidence indicates that relevant trypsin-like proteases are restricted to the respiratory and the intestinal

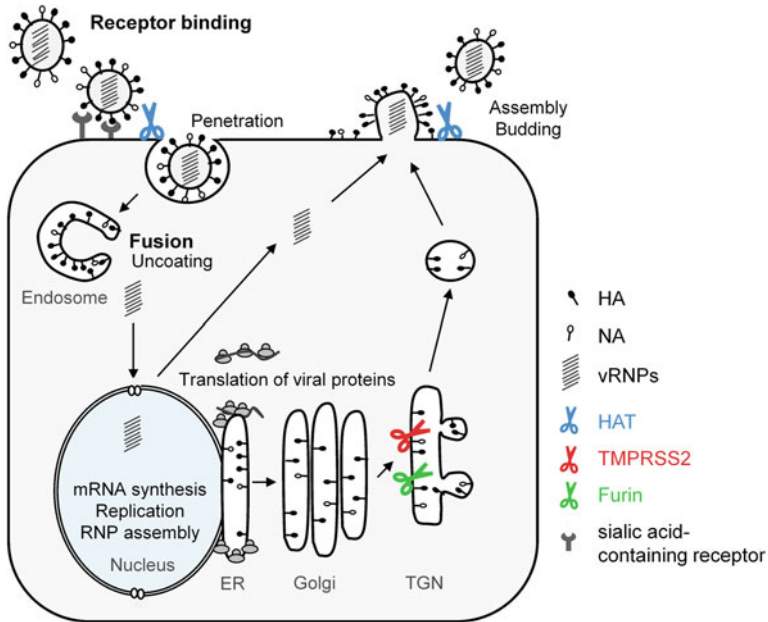


Fig. 2 The role of HA in influenza virus replication and the compartmentalization of the activating proteases. HA mediates binding to sialic acid-containing cell surface receptors and fusion of the viral and endosomal membrane following endocytosis at low pH in order to release the viral ribonucleoprotein complexes (RNPs) into the cell (uncoating). The vRNPs are imported into the nucleus, where transcription and replication occur. Translation of viral mRNAs is performed by the cellular machinery. HA, neuraminidase (NA), and the ion channel protein M2 are transported along the constitutive secretory pathway to the plasma membrane. New vRNPs are assembled in the nucleus and subsequently transported to the plasma membrane, too, where self-assembly of viral proteins leads to budding of new virions. NA cleaves sialic acid from carbohydrate moieties, facilitating release of progeny virus. HA is synthesized as fusion-incompetent precursor that requires posttranslational cleavage by host proteases. HA cleavage occurs by membrane-bound proteases and can take place in different compartments and at different time points during the viral life cycle and is indicated by scissors. HA with multibasic cleavage site is cleaved by furin in the trans Golgi-network (TGN). HA containing a monobasic cleavage site is cleaved by TMPRSS2 in the TGN or by HAT on the plasma membrane, either during assembly and budding of virions or during attachment and entry into the cell

tract of birds. LPAIV infection is therefore confined to these organs (Garten and Klenk 2008). A protease homologous to blood clotting factor Xa was identified activating LPAIV HA in embryonated chicken eggs (Gotoh et al. 1990), but the identity of HA-activating proteases in the intestinal and respiratory tracts of avian species is unknown.

HA of HPAIV is cleaved at the C-terminus of the multibasic consensus motif R-X-R/K-R by the ubiquitous eukaryotic subtilase furin or the closely related proprotein convertase 5/6 (PC5/6) (Stieneke-Gröber et al. 1992; Vey et al. 1992; Horimoto et al. 1994; Feldmann et al. 2000). Furin is a calcium-dependent serine endoprotease that activates pro-proteins and pro-hormones, such as pro-insulin

receptor, pro-albumin and von Willebrand factor, at neutral pH during their transport along the secretory pathway to the plasma membrane (Seidah et al. 2008). Moreover, furin activates also the fusion proteins of a large number of other enveloped viruses (e.g., retroviruses including HIV, paramyxoviruses, flaviviruses), as well as bacterial toxins (e.g., anthrax toxin, botulinum toxin) at multibasic motifs (Klenk and Garten 1994; Thomas 2002). Cleavage of HA of HPAIV by furin takes place in the TGN (Fig. 2). The ubiquitous expression of furin supports proteolytic activation of HA in multiple organs and tissues, causing systemic infection and fatal disease.

It has been demonstrated that HPAIV emerge from LPAIV by insertion of the R-X-R/K-R motif into the cleavage site loop (Table 1). Why acquisition of a multibasic cleavage site susceptible to furin is confined to subtypes H5 and H7 is not known. Different mechanisms underlying the insertion of amino acids at the HA cleavage site have been observed: (1) successive addition of basic amino acids due to polymerase slippage at purine-rich arginine or lysine codons (Horimoto et al. 1995; Garcia et al. 1996; Perdue et al. 1997), (2) recombination of the HA gene with other viral gene segments (Orlich et al. 1994; Suarez et al. 2004; Pasick et al. 2005), or (3) recombination of the HA gene with ribosomal RNAs (Khatchikian et al. 1989; Maurer-Stroh et al. 2013). There are also examples where the insertion mechanism is unknown (Banks et al. 2001).

Introduction of a multibasic cleavage site *in vitro* conferred high cleavability to vector-expressed H3 HA (Ohuchi et al. 1991), but it did not lead to systemic infection and increased pathogenicity of a H3N2 virus in ferrets (Schrauwen et al. 2011). Furthermore, replacement of a monobasic HA cleavage site by a multibasic motif does not automatically confer high pathogenicity to a LPAIV, demonstrating that the emergence of a HPAIV is a multifactorial process (Stech et al. 2009). On the other hand, H5N1 reassortants with modified H2, H4, H8, and H14 HAs containing multibasic cleavage sites caused lethal infections in chickens (Veits et al. 2012). These findings demonstrated that a multibasic HA cleavage site can confer high pathogenicity to subtypes other than H5 and H7 in a suitable genetic background.

2.2 Proteolytic Activation of Mammalian influenza viruses

Like LPAIV, mammalian influenza viruses possess a single arginine at the cleavage site. Relevant host proteases show a restricted tissue distribution, and propagation in cell culture usually requires addition of exogenous trypsin. A number of trypsin-like proteases was isolated from rat and swine lung, including tryptase Clara, mini-plasmin and tryptase TC30, and shown to support proteolytic activation of influenza viruses with a monobasic HA cleavage site *in vitro* (Kido et al. 1992, 2007; Murakami et al. 2001; Sato et al. 2003). Because the genetic identity of these proteases is still unknown, it remains unclear whether they play a role in influenza virus infection *in vivo*. Moreover, plasmin, urokinase, or tissue

kallikreins also have been reported to cleave HA with a monobasic cleavage site *in vitro*, but their role in *in vivo* infection is not clear, either (Lazarowitz and Choppin 1975; Hamilton and Whittaker 2013). Host proteases that activate influenza virus HA with a monobasic cleavage site in the human airways were unknown for a long time.

Cleavage of HA by soluble proteases such as trypsin or by exogenous trypsin in cell culture occurs extracellularly during assembly and budding when HA is present on the plasma membrane or after virus is released from the infected cell. Hence, it was believed that HA with a monobasic cleavage site is activated extracellularly and, therefore, differs from HA of HPAIV, which is cleaved in the TGN by furin. However, in human airway epithelial cells and in human intestinal Caco-2 cells activation of HA with a monobasic cleavage site was shown to take place intracellularly, but the proteases remained unknown in these studies (Zhirnov et al. 2002, 2003).

In 2006, the proteases TMPRSS2 (transmembrane protease serine S1 member 2; also designated as epitheliasin) and HAT (human airway trypsin-like protease; also designated as TMPRSS11D) were identified as proteases present in the human airways that activate influenza virus HA with a monobasic cleavage site *in vitro* (Böttcher et al. 2006). Later on, the TMPRSS2-related protease TMPRSS4 was shown to activate HA with a monobasic cleavage site *in vitro*, too (Chaipan et al. 2009). TMPRSS2- or HAT-homologous proteases in swine and mouse have been identified and were shown to be capable of activating HA at a single arginine, suggesting that homologous proteases are involved in HA cleavage in different host species (Bertram et al. 2012; Peitsch et al. 2014; Tarnow et al. 2014).

TMPRSS2 and HAT belong to the family of type II transmembrane serine proteases (TTSPs) (Szabo and Bugge 2008). HA cleavage by TMPRSS2 and HAT takes place in different cellular compartments and at different time points during the viral life cycle (Fig. 2). HAT localizes to the plasma membrane as an active enzyme that can cleave newly synthesized HA0, probably during assembly and budding of progeny virus, and activates incoming virus at the stage of entry upon attachment to the cell (Böttcher-Friebertshäuser et al. 2010). In contrast, HA cleavage by TMPRSS2 takes place within the cell (Böttcher-Friebertshäuser et al. 2010). TMPRSS2 expressed at the cell surface does not activate incoming virus. Recent studies demonstrated that the protease accumulates in the TGN where it co-localizes with furin, suggesting that HA cleavage by TMPRSS2 and furin occurs in the same cellular compartment (Stieneke-Gröber et al. 1992; Schäfer et al. 1995; Böttcher-Friebertshäuser et al. 2013; Peitsch et al. 2014).

The catalytic domain of TTSPs may also be shed from the cell surface and soluble forms of TMPRSS2 and HAT have been described (Yasuoka et al. 1997; Afar et al. 2001). However, the soluble forms did not support proteolytic activation of influenza viruses, supporting the concept that HA cleavage in the airways occurs by cell-associated proteases (Böttcher-Friebertshäuser et al. 2010). Enhanced shedding of TMPRSS2 and HAT from differentiated human nasal epithelial cells and hence enhanced influenza virus replication has been observed upon exposure to ozone (Kesic et al. 2012). Thus, enhanced protease shedding

under stress might play a role in influenza virus infection, but further investigations are needed to prove this hypothesis.

2.3 Variations in Cleavage Activation of LPAIV and Mammalian Influenza A Viruses

It has long been known that HAs with monobasic cleavage sites differ in protease sensitivity. Thus, A/WSN/33 (H1N1) HA is activated by plasmin (Lazarowitz et al. 1973), whereas A/chicken/Germany/49 (H10N7) HA depends on trypsin (Klenk et al. 1975). Furthermore, comparison of the sensitivity of 16 HA subtypes to TMPRSS2 and HAT as well as pancreatic trypsin in vitro demonstrated also that cleavage efficiency can vary significantly among the different subtypes (Galloway et al. 2013). The mechanisms underlying protease specificity of HA are still unknown and may be related to the structure and exposure of the cleavage site loop (Fig. 1) or steric hindrance by adjacent carbohydrate moieties (Kawaoka et al. 1984).

Although the monobasic HA cleavage site of the mammalian viruses is a major determinant for the confinement of infection to the respiratory tract, there are examples where the protease specificity promotes the spread of such viruses to other organs. A/WSN/33 H1N1 virus contains the unusual H1 HA cleavage site I-Q-Y-R instead of I-Q-S-R (cf. Table 1) that facilitates efficient cleavage by plasmin and has been shown to contribute to the neurotropism of the virus in mice (Goto and Kawaoka 1998; Sun et al. 2010). Another example are H9N2 viruses. Within the last years these viruses have attracted particular attention since they have become highly prevalent in poultry in many countries and are occasionally transmitted to humans and pigs, causing mild disease. Moreover, H9N2 viruses show a high genetic compatibility with other subtypes and have been involved in the emergence of H5N1 and H7N9 viruses (To et al. 2013). When compared to other subtypes, H9N2 viruses vary remarkably in their amino acid sequence at the HA cleavage site, and many isolates from Asia and the Middle East possess uncommon di- or tribasic cleavage site motifs R-S-S-R and R-S-R-R, respectively. In contrast to HPAIV of subtypes H5 and H7, the cleavage sites of H9N2 viruses evolved by substitution and not by insertion of basic amino acids (Table 1). They are not susceptible to cleavage by furin, but are activated by TMPRSS2 and HAT in vitro (Gohrbandt et al. 2011; Soda et al. 2011; Baron et al. 2013). Recent studies suggest that insertion of further amino acids to extend the cleavage loop or removal of a carbohydrate moiety in HA1 can render H9 susceptible to furin (Gohrbandt et al. 2011; Soda et al. 2011; Tse et al. 2014). Interestingly, the type II transmembrane protease matriptase was shown to cleave H9 at R-S-S-R and R-S-R-R motifs, too. Matriptase is expressed in epithelial cells of many tissues (Oberst et al. 2003) and has been suggested to contribute to H9N2 virus replication in the kidney of chickens (Baron et al. 2013). Whether matriptase is also involved in cleavage of other HA subtypes is under discussion (Baron et al. 2013; Beaulieu et al. 2013; Hamilton and Whittaker 2013).

Interestingly, bacterial proteases secreted by some strains of *Staphylococcus aureus* or *Aerococcus viridans* have also been shown to support proteolytic activation of influenza viruses with monobasic cleavage site in vitro. Coinfection of mice resulted in lethal infection with increased virus titers and extended lesions in the lung (Tashiro et al. 1987a, b; Scheiblauber et al. 1992). The susceptibility of HA to bacterial proteases also differed considerably and was specific for both virus and bacteria strains.

2.4 HA Cleavage Is Essential for Pneumotropism and Pathogenicity in Mammalian Hosts

Three recent studies demonstrated that knockout mice that lack expression of the protease TMPRSS2 are protected from pulmonary disease with lethal outcome when infected with influenza viruses of subtypes H7N9 and H1N1 (Hatesuer et al. 2013; Sakai et al. 2014; Tarnow et al. 2014). Intriguingly, H7N9 and H1N1 viruses were apathogenic in TMPRSS2-deficient mice, whereas wild-type mice developed severe infection with 100 and 20 % mortality, respectively. Virus growth kinetics in explants of murine respiratory tissues and in infected mice demonstrated that knockout of TMPRSS2 prevents spread of H7N9 and H1N1 viruses into the lung and consequently development of disease in mice (Tarnow et al. 2014) (Fig. 3a). In contrast, H3N2 virus replication in ex vivo airway models was only marginally affected by knockout of TMPRSS2 and both TMPRSS2-deficient and wild-type mice succumbed to infection. Analysis of the distribution of TMPRSS2 and HAT in the airways demonstrated that both proteases are present in the larynx, trachea and bronchi of mice, but only TMPRSS2 is expressed in the lung (Szabo and Bugge 2008; Sales et al. 2011; Tarnow et al. 2014) (Fig. 3a). Thus, HAT may support virus activation in upper airways and the tracheobronchial epithelium, but expression of TMPRSS2 is crucial for virus spread into the lung. Since, TMPRSS2-knockout mice lack expression of both TMPRSS2 and HAT in the lungs, it appears that multicycle replication of H3N2 virus in murine lung is due to another, not yet identified protease. Taken together, these studies demonstrated for the first time that expression of an appropriate HA-activating protease along the respiratory tract is essential for pneumotropism and pathogenicity of influenza viruses in mammals. Therefore, distribution of both receptors (see below) and virus-activating proteases is crucial for spread of influenza virus in the airways. The protease TMPRSS2 has been identified as a single host cell factor essential for H7N9 and H1N1 influenza virus pathogenicity in mice. It remains to be investigated what contributes to the differences in the protease specificity of H7N9 and H1N1 viruses, on one hand, and of H3N2 virus, on the other hand. Analysis of HAT and TMPRSS2 expression in the respiratory tracts of humans and swine suggests that protease distribution is similar to that in mice, indicating that also in man and pigs TMPRSS2 supports proteolytic activation of HA along the respiratory tract and virus spread into the lung, whereas HA cleavage by HAT seem to be confined to the upper airways,

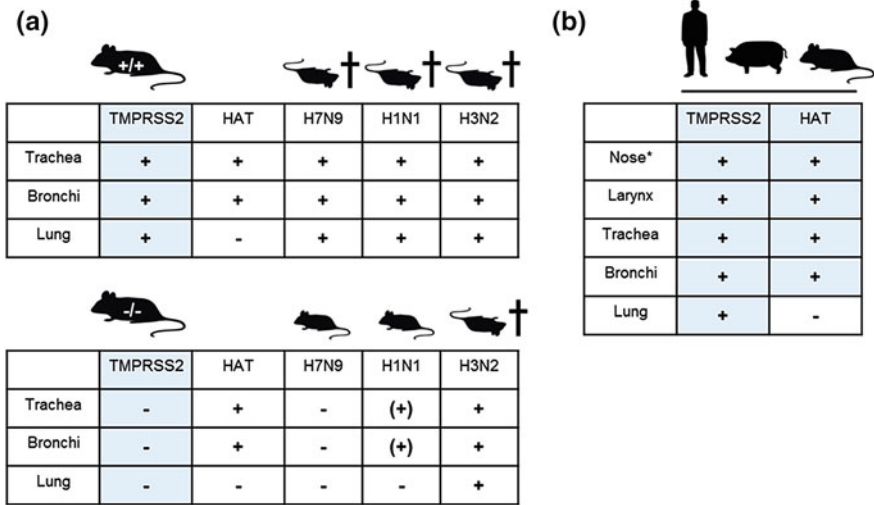


Fig. 3 Role of HA cleavage in pneumotropism and pathogenicity in mice. **a** TMPRSS2 is essential for pneumotropism and pathogenicity of H7N9 and H1N1 virus in mice. Replication and pathogenicity of H7N9, H1N1 and H3N2 influenza viruses, respectively, in TMPRSS2-knockout mice (*TMPRSS2*^{-/-}) and wild-type littermates (*TMPRSS2*^{+/+}) (Tarnow et al. 2014). Knockout of TMPRSS2 inhibits multicycle replication of H7N9 in murine respiratory tissues and prevents spread of H1N1 virus into the lung and thereby protects mice from pulmonary disease with lethal outcome. Low levels of H1N1 replication in trachea and bronchi of *TMPRSS2*^{-/-} mice may be due to HA activation by HAT. In contrast, replication and pathogenicity of H3N2 virus is only marginally affected by knockout of TMPRSS2 and both wild-type and *TMPRSS2*^{-/-} mice succumb to lethal infection. HAT might support multicycle replication of H3N2 virus in trachea and bronchi, but replication of H3N2 virus in the lung depends on a so far unknown protease. Presence (+) and absence (-) of protease expression and multicycle virus replication, respectively. Mice indicate apathogenic infection and lethal outcome of infection, respectively. **b** Expression of TMPRSS2 or HAT (+) and absence of expression (-) in respiratory tissues of human, swine or mouse. *Expression of HAT and TMPRSS2 in nasal epithelial cells of mice has not been determined

trachea and bronchi (Szabo and Bugge 2008; Sales et al. 2011; Bertram et al. 2012; Peitsch et al. 2014). Interestingly, TMPRSS2, but not HAT, has also been detected in human myocytes, indicating that the protease might contribute to influenza-associated myocarditis (Bertram et al. 2012).

2.5 Protease Inhibitors as Drugs for Influenza Treatment

It has long been known that inhibition of HA cleavage by aprotinin, a natural protease inhibitor from bovine lung, suppresses influenza virus replication and spread in mice and reduces symptoms of disease. Inhalation of aerosolized

aprotinin by influenza patients markedly reduced the duration of symptoms without causing side effects (reviewed in Zhirnov et al. 2011). The resistance of TMPRSS2-knockout mice to pulmonary disease upon H7N9 or H1N1 virus infection identified TMPRSS2 as a potential target for drug development. Inhibition of TMPRSS2 activity by peptidomimetics or downregulation of TMPRSS2 expression by anti-sense peptide-conjugated phosphorodiamidate morpholino oligomers (PPMO) have already been shown to efficiently inhibit multicycle replication of human influenza virus in airway epithelial cells (Böttcher-Friebertshäuser et al. 2011, 2012; Meyer et al. 2013). Furthermore, the combination of protease inhibitors and the NA inhibitor oseltamivir carboxylate efficiently blocked influenza virus propagation in a synergistic manner.

Targeting host cell factors instead of viral proteins has the advantage to reduce or even prevent the emergence of drug resistant viruses. A major concern in targeting a host factor is side effects or toxicity due to inhibition of the physiological functions. The physiological roles of TMPRSS2 and HAT are still unknown, but mice deficient in expression of either protease are phenotypically asymptomatic (Kim et al. 2006; Sales et al. 2011), indicating functional redundancy or compensation of the function by other host proteases. Thus, inhibition of TMPRSS2 or HAT during an acute influenza infection seems feasible and the further development of above mentioned inhibitors may lead to novel drugs for influenza chemotherapy. Inhibition of HA cleavage may provide a promising approach also for the treatment of HPAIV infections in humans. Specific inhibitors of furin have been demonstrated to strongly suppress replication of HPAIV of subtype H7N1 in cell culture (Garten et al. 1989; Becker et al. 2010). The first approach for suppression of virus replication by furin inhibitors was performed with peptidomimetics using decanoylated basic tetrapeptide derivatives, such as decRVKR chloromethylketone, that imitate the furin recognition motif (Hallenberger et al. 1992; Stieneke-Gröber et al. 1992; Garten et al. 1994). Recently, furin peptidomimetics with improved stability and efficacy have been designed (Becker et al. 2010, 2012).

2.6 The Use of Protease Activation Mutants for Vaccine Design

Modification of the HA cleavage site has been shown to provide a useful tool for vaccine development. Thus, replacement of the multibasic HA cleavage site of H5N1 and H7N1 viruses by a single arginine led to low pathogenic seed viruses that can be used for the generation of inactivated vaccines under biosafety-level-2 (BSL2) conditions (Webby et al. 2004).

Replacement of the conserved arginine at the HA cleavage site by an elastase motif (V↓G) has been demonstrated to attenuate influenza viruses in mice and to provide a promising strategy for the development of live vaccines (Stech et al. 2005). Such elastase-dependent mutant viruses can be efficiently propagated in the

presence of elastase *in vitro*, but undergo restricted virus replication and spread *in vivo* due to missing HA activation. Generation of elastase-dependent virus variants of both influenza A virus [A/WSN/33 (H1N1)] and influenza B virus (B/Lee/40) has been shown to result in highly attenuated viruses that elicit protective immunity against lethal challenge with the respective wild-type virus after intranasal administration as live vaccine (Stech et al. 2005, 2011). Furthermore, when the multibasic HA cleavage site of the SC35M (H7N7) strain, which is highly pathogenic for mice, was replaced by an elastase motif, an attenuated mutant was obtained that was immunogenic in mice and protected the animals from lethal challenge (Gabriel et al. 2008). Thus, modification of the HA cleavage site may also be a promising strategy for the development of HPAIV live vaccines.

3 Receptor Specificity of HA

As pointed out in the introduction, human influenza viruses typically replicate in the epithelium of the upper respiratory tract (URT), trachea, and bronchi and cause transient uncomplicated rhinitis, pharyngitis, and tracheobronchitis. A major complication, infection of terminal bronchioles and alveoli, results in a severe, occasionally fatal, pneumonia (for review, see Kuiken and Taubenberger 2008; Taubenberger and Morens 2008). Seasonal human influenza viruses rarely cause severe disease, whereas it occurs unusually frequently in the case of zoonotic infections with H5N1 and H7N9 avian viruses (Beigel et al. 2005; Liu et al. 2013). These distinctive patterns of infection may depend, at least partially, on the differences in receptor-binding specificity of seasonal and zoonotic viruses and differential expression of receptors for these viruses in human target tissues.

3.1 *Influenza Viruses Attach to Sia2-3Gal- and Sia2-6Gal-Containing Receptors*

Influenza viruses attach to cells via HA interactions with sialic acids (Sia) expressed at the terminal positions of carbohydrate chains of cell surface glycoproteins and glycolipids. The Sia-binding site is a shallow pocket located in the membrane-distal part of HA (Fig. 4). Individual interactions of HA monomers with the sialyloligosaccharide moieties of the receptors are very weak ($K_{diss} \sim 0.1$ mM), and cooperative binding of multiple HA spikes to multiple copies of the receptor provides for the efficient virus attachment to cells. Attachment therefore depends on (i) HA affinity for the terminal Sia-containing receptor moiety, (ii) HA interactions with the penultimate sugar of the oligosaccharide chain and the

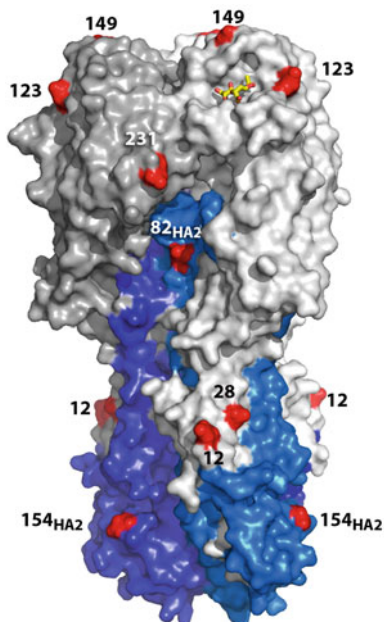


Fig. 4 Attachment sites and functions of the oligosaccharides of the H7 hemagglutinin. The structure of the H7 trimer is shown. HA1 (*gray*) with N-acetylneuraminic acid (*yellow*) attached to the receptor-binding site, HA2 (*blue*), and the asparagine residues at the glycosylation sites (*red*) are indicated. The oligosaccharide at position HA2-82 is of the oligomannosidic type, all other glycosylation sites contain complex or intermediate type glycans. The glycans at position 12, 28, and HA2-154 are conserved and essential for intracellular transport and stability of HA. The glycans at positions 123 and 149 vary with different H7 strains and modulate receptor binding. For references see text

protein/lipid part of the receptor, and (iii) ability of the virus particle to establish multivalent interactions with clusters of receptor molecules expressed on the cell surface. Thus, the receptor-binding properties of influenza viruses can be affected by amino acid substitutions inside the Sia-binding pocket, on the pocket rim, and by more distant mutations resulting in altered glycosylation or electrostatic charge of the HA globular head (for reviews, see Matrosovich et al. 2006, 2008; Gamblin and Skehel 2010; Viswanathan et al. 2010).

Sia species differ from each other by modifications at the amino group (N5) and at four hydroxyl groups (O4, O7, O8, and O9). Influenza A and B viruses typically bind to the most common species, N-acetylneuraminic acid (Neu5Ac), although some viruses can in addition bind to N-glycolylneuraminic acid (Neu5Gc) and 4-O-acetylated Sia (for review, see Matrosovich et al. 2006, 2008). Only viruses of the extinct equine H7N7 lineage bind to Neu5Gc much more strongly than to Neu5Ac (Gambaryan et al. 2012). The structure of natural Sia-containing oligosaccharide chains is highly variable and complex (Glycobiology 2009), and effects of their structural differences on influenza virus binding are still not well

characterized. The best known effect is the ability of influenza viruses from different host species to discriminate between the two major natural sialylgalactosyl moieties, Sia2-3Gal and Sia2-6Gal. Thus, human and swine viruses preferentially bind to Sia2-6Gal-containing oligosaccharides (often called “6-linked” or “human-type” receptors); avian and equine viruses show the opposite specificity and bind to Sia2-3Gal-containing glycans (“3-linked” or “avian-type” receptors).

3.2 Expression of Receptors and Virus Tissue Tropism in the Human Respiratory Tract

The respiratory tract in humans contains a diverse population of cell types which vary along the respiratory tree (Tomashefski and Farver 2008). Two major cell types of the superficial cells of the air-conducting zone (nasal and tracheobronchial epithelium) are ciliated cells and nonciliated secretory cells. Secretory cells, together with the cells of sub-epithelial glands, secrete mucins, and other molecules that become incorporated in the airway surface liquid and mucous blanket. Ciliary beating of ciliated cells moves the blanket toward the pharynx where mucus is swallowed together with trapped particles and microorganisms. The respiratory zone consists of respiratory bronchioles, alveolar ducts, and alveoli. Alveoli, which are responsible for 90 % of the gas exchange, are lined by type I and type II pneumocytes. These flat type I cells cover 90 % of the alveolar surface. They are the main components of the alveolar wall and the barrier between air and blood. The type II cells occupy around 5 % of the alveolar surface. They produce surfactant, resorb fluid from the alveolar lumen, and serve as reserve cells which differentiate into type I cells in response to alveolar damage.

Paulson and colleagues were the first who studied expression of receptors for influenza viruses in the human respiratory tract using fixed paraffin-embedded tissue sections of human trachea and labeled plant lectins and influenza viruses as Sia-binding histochemical probes (Baum and Paulson 1990; Couceiro et al. 1993). They found that Sia2-6Gal-specific lectin and human influenza virus efficiently bound to the apical surface of the epithelium and that binding of Sia2-3Gal-specific lectin and avian virus were restricted to intracellular mucin-containing secretory granules of goblet cells. The authors suggested that the preferential tropism to Sia2-6Gal-containing receptors, which is shared by various human viruses, can be determined by two simultaneous selective pressures, namely abundant expression of 6-linked Sia on airway epithelial cells and abundant expression of 3-linked Sia on decoy receptors of the mucous blanket.

More recently, several groups used the same methodological approach to study expression of receptors in all regions of the human respiratory epithelium from the nasal epithelium to alveoli (Shinya et al. 2006; van Riel et al. 2006, 2007, 2010, 2013; Nicholls et al. 2007a, b; Yao et al. 2008). Table 2 summarizes the main findings. Lectin binding data demonstrated that 6-linked Sia is present in high

Table 2 Expression of 2-3-linked and 2-6-linked sialic acids and virus attachment studied on histological sections of fixed human respiratory tissues

Tissue	Lectin binding ^{1, 2}			Virus attachment ^{3, 4}		
	SNA	MAA-1	MAA-2	Human H1N1, H3N2	H5N1	H7N9
Nasal	++	ND	—	++ ^a	—	+/+ ^a
Nasopharynx	++	++	—	++ ^a	+/-	NT
Trachea	++	ND	—	++ ^a	+/-	+ ^a
Bronchus	++	++	—	++ ^a	+/- ^a	++ ^a
Bronchiole	+	+	++	++ ^a	+ ^c	++ ^{a, c}
Alveolus	+	+	++	+ ^I	+ ^e	++ ^{d, e}
Macrophages	+	+/>++	—	—	+	+

¹ Shinya et al. (2006), Nicholls et al. (2007a), Chan et al. (2013b)

² SNA, *Sambucus nigra* agglutinin, preferentially binds to Sia2-6Gal. MAA-1, *Maackia amurensis* leucoagglutinin, preferentially binds to Sia2-3Gal1-4GlcNAc; MAA-2, *Maackia amurensis* hemagglutinin, preferentially binds to Sia2-3Gal1-3GalNAc and Sia2-3Gal1-3[Sia2-6]GalNAc (Geisler and Jarvis 2011)

³ van Riel et al. (2006, 2007, 2010, 2013)

⁴ Predominant cell type to which the virus attaches: ^a ciliated, ^b nonciliated, ^c Clara, ^d type I pneumocyte, ^e type II pneumocyte

amounts on the surface of epithelial cells of the URT, trachea and bronchi, and in lower but significant amounts on the surface of bronchiolar and alveolar cells. Expression of 3-linked Sia was studied using the lectins MAA-1 and MAA-2 which differ in their fine binding specificity. Sia2-3Gal1-3GalNAc moieties, which are typical for O-linked glycans, were only detected in bronchioles and alveoli. By contrast, Sia2-3Gal1-4GlcNAc-containing glycans were detected throughout the whole respiratory tract (Nicholls et al. 2007a; Walther et al. 2013). Unfortunately, lectin staining is not quantitative, precluding direct comparison of the concentrations in the tissues of different sialic acid types. Van Riel and colleagues studied attachment patterns of labeled human and avian influenza viruses to histological sections (van Riel et al. 2006, 2007, 2010, 2013). In their experiments, pandemic and seasonal human viruses of different subtype attached efficiently to the apical surface of the URT and the tracheobronchial epithelium. H5N1 (and a few other avian viruses tested) attached to these tissues weakly if at all. Both human and avian viruses attached to bronchiolar and alveolar epithelial cells, but displayed a different cell tropism. Human viruses preferentially bound to ciliated cells in the bronchioles and type I pneumocytes, whereas H5N1 virus bound to bronchiolar nonciliated Clara cells, type II pneumocytes and alveolar macrophages.

Avian H7N9 viruses responsible for zoonotic infections of humans since March 2013 were found to bind to both 3-linked and 6-linked Sia (Belser et al. 2013; Watanabe et al. 2013; Xiong et al. 2013; Zhou et al. 2013). In accordance with their dual receptor specificity, these viruses displayed attachment patterns of both human and avian viruses (van Riel et al. 2013). Similarly to human viruses, H7N9 viruses attached, albeit less efficiently, to the ciliated airway epithelium and were able to bind to type I cells in alveoli. As other avian viruses, H7N9 viruses

displayed relatively strong attachment to bronchiolar Clara cells, type II pneumocytes, and alveolar macrophages.

The observed binding patterns (Table 2) correlate with viral receptor specificity and features of infection in humans. Preference of human influenza viruses for 6-linked Sia, abundant expression of 6-linked Sia and efficient attachment of human viruses to the URT, trachea and bronchi are consistent with preferential replication of human viruses in these tissues. This latter effect correlates with the common presentation of human influenza virus infection as tracheobronchitis (Kuiken and Taubenberger 2008) and seems to be essential for airborne transmission of infection via sneezing and coughing (Sorrell et al. 2011; Imai et al. 2013). Thus, mutagenesis of the receptor-binding site of the HA of pandemic H1N1/1918 and H2N2/57 viruses and human seasonal H3N2 virus to switch binding preference from 6-linked Sia to 3-linked Sia did not abolish infection of ferrets, but prevented transmission (Tumpey et al. 2007; Pappas et al. 2010; Roberts et al. 2011). Also, mutations in the HA that switched receptor preference of genetically engineered H5N1 viruses toward 6-linked Sia were critical to ensure their attachment to the epithelium of nasal turbinates in ferrets and render them transmissible in ferrets (Herfst et al. 2012; Imai et al. 2012). It should be noted, that despite a lack of binding of avian viruses to human airway epithelium in histochemical assays (van Riel et al. 2007, 2010, 2013), the presence of 3-linked Sia in the epithelium was confirmed by lectin staining and glycomic analysis, and various avian virus strains were able to infect and replicate in ex vivo explants of human bronchus (Nicholls et al. 2007a, b; Chan et al. 2013a; Walther et al. 2013). Thus, paucity of attachment sites for avian viruses in the human URT, trachea and bronchi does not prevent infection with high virus doses, but limits efficiency of natural infection to an extent incompatible with airborne transmission.

Relatively high concentrations of 3-linked Sia in bronchioles and alveoli (Shinya et al. 2006; Nicholls et al. 2007a; Chan et al. 2013a, b) and the ability of H5N1, H7N7, and H7N9 viruses to attach to cells in the lower respiratory tract suggested that the receptor-mediated pneumotropism of these viruses contributes to their high pathogenicity in humans. It remains to be determined why human viruses, which also can attach to bronchiolar and alveolar epithelial cells (Table 2) and efficiently replicate in human lung explants, only rarely cause severe disease (Walther et al. 2013).

3.3 Cell Tropism of Viral Infection as Depending on Receptor Specificity

Autopsy material obtained from fatal cases of pandemic H1N1, H5N1, and H7N9 influenza has also been analyzed. Viral antigen and/or RNA were typically found in type II pneumocytes, ciliated and nonciliated cells of tracheobronchial epithelium, alveolar macrophages, and several other cell types (Uiprasertkul et al. 2005;

Korteweg and Gu 2010; Zhou et al. 2013). To throw light on cell tropism at the early stages of infection, two useful experimental models were employed in recent years, differentiated cultures of human airway epithelial (HAE) cells and ex vivo cultures of human respiratory tissues removed by biopsy or surgical excision (for review, see Chan et al. 2013b).

Experiments in fully differentiated cultures of human nasal and tracheobronchial epithelial cells showed that early in infection human viruses preferentially infected nonciliated cells, whereas avian viruses mainly infected ciliated cells (Matrosovich et al. 2004). This pattern correlated with the lectin analyses in these cultures, i.e., strong binding of SNA to nonciliated cells and preferential binding of MAA-1 to ciliated cells. The cell tropism was not absolute: for example, human viruses infected all types of cells later in infection, and avian virus infection was not fully restricted to ciliated cells. These findings have been confirmed by other groups (Thompson et al. 2006; Wan and Perez 2007). Interestingly, the H3N2/1968 pandemic virus displayed less prominent tropism to nonciliated cells than seasonal H3N2 and H1N1 human viruses, which correlates with the less strict preference of the 1968 pandemic virus to 6-linked Sia and its weak but significant binding to 3-linked Sia (Matrosovich et al. 2004, 2007; Thompson et al. 2006).

The data on virus infection in human airway epithelial cell cultures suggested for the first time that differences in replication and pathogenicity of human and avian influenza viruses may be partially related to their receptor-mediated differential cell tropism. This concept was also tested in studies on receptor-binding variants of the H1N1/2009 pandemic influenza viruses. These viruses occasionally caused severe and fatal infections, and there was a strong association of the HA mutation D222G with severe disease (Rykkvin et al. 2013). Interestingly, the D222G mutation in the HA was also found in fatal cases from the 1918 influenza pandemic. The D222G mutants of the H1N1/2009 virus were shown to differ from the original human viruses by enhanced binding to 3-linked Sia, enhanced attachment to type II pneumocytes and alveolar macrophages on lung tissue sections and enhanced infection of ciliated cells in HAE cultures (Chutinimitkul et al. 2010; Liu et al. 2010). It is believed therefore that the avian virus-like receptor specificity and cell tropism of the D222G mutants seemed to have contributed to the disease severity.

Based on the differences in the virus attachment patterns in the alveolar tissues (Table 2), it was assumed that human viruses target type I and avian viruses target type II pneumocytes (van Riel et al. 2007, 2010). However, recent studies in human lung explants revealed that all human and avian viruses examined, including avian H5N1 and H7N9, almost exclusively infected type II pneumocytes (Weinheimer et al. 2012; Knepper et al. 2013). These findings agree with the abundance of infected type II pneumocytes in autopsy material of patients that died from severe infection with both H1N1 pandemic and H5N1 avian viruses (Uiprasertkul et al. 2005; Korteweg and Gu 2010) and the rare observation of antigen/RNA-positive type I cells (Liem et al. 2008; Shieh et al. 2010). Therefore, differences in pathogenicity of H5N1 and H7N9 avian viruses and human viruses cannot be explained by replication of human and avian viruses in different types of

pneumocytes. It remains to be determined whether differences in receptor-binding specificity of these viruses could affect the efficiency of their replication in type II pneumocytes.

Antigen-positive alveolar macrophages (AMs) are commonly observed in autopsy material from fatal influenza cases. AMs and peripheral blood monocyte-derived macrophages (PBDMs) were found to express both 3-linked and 6-linked Sia (Yu et al. 2011). In the attachment assay, avian viruses and viruses with dual receptor specificity bound stronger to AMs than human viruses (van Riel et al. 2007; Chutinimitkul et al. 2010). Both AMs and PBDMs were more susceptible to infection with highly pathogenic H5N1 viruses than with human seasonal and pandemic viruses (van Riel et al. 2011; Yu et al. 2011). No infectious virus was released from infected AM suggesting that these cells do not significantly contribute to the spread of infection in human alveolae. As infection of AM likely impairs their protective scavenger function, a high susceptibility of AM to H5N1 viruses may contribute to their unusual high pathogenicity in humans.

In addition to pneumocytes, endothelial cells of alveolar capillaries represent the major type of cells in the lung. Microvascular endothelial cells are separated from pneumocytes by two thin basement membranes. Passive gas exchange in the lung occurs through this epithelio-endothelial barrier. Findings of infected endothelial cells in lung autopsies of patients with severe influenza are rare (Liem et al. 2008; Shieh et al. 2010). Studies of virus infection in cultures of primary human microvascular endothelial cells were recently described. Lectin-based analysis revealed the presence of 3-linked and 6-linked Sia (Chan et al. 2009) with an apparent predominance of the former (Zeng et al. 2012). Highly pathogenic H5N1 viruses infected endothelial cells significantly more efficiently and replicated in these cells to much higher titers than human seasonal and pandemic viruses (Ocana-Macchi et al. 2009; Zeng et al. 2012). Reverse genetics analysis and virus attachment studies demonstrated a major role for the viral HA in the observed tropism of H5N1 to endothelial cells (Ocana-Macchi et al. 2009). After infection, a pronounced release of cytokines and adhesion molecules was observed as well as an elevated rate of cell death. These observations are particularly interesting in view of recent findings suggesting a major role of endothelial cells in the production of cytokines during influenza infection (Tejaro et al. 2011). Thus, enhanced receptor-mediated tropism of H5N1 viruses to endothelial cells in the human lung could contribute to the disease caused by these viruses.

4 Modulation of HA Functions by Glycosylation

The carbohydrate moiety of HA consists of N-glycans synthesized by the cellular glycosylation machinery. The viral carbohydrates therefore show host-dependent variations. The only difference from host glycans is the absence of sialic acid that is removed by NA. There are complex, oligomannosidic, and intermediate type side chains, and there is usually microheterogeneity at a given site (Keil et al.

1985). The glycosylation sites and the functional significance of the carbohydrate side chains of the H7 HA are shown in Fig. 4. The glycosylation sites at Asn-12 and HA2-Asn154 (Asn-478, H7 numbering) are highly conserved, and the glycosylation site at Asn-28 is also present in many strains. The high conservation of these three sites, which are located in the stem region of the HA spike, suggests that they play important structural and functional roles. Indeed, loss of all three oligosaccharides resulted in a temperature-sensitive block of HA transport to the cell surface (Roberts et al. 1993). It was also shown that these carbohydrates stabilize HA in the metastable form susceptible to the conformational change necessary for fusion activation (Ohuchi et al. 1997a). They are therefore essential for the formation of replication-competent virus (Wagner et al. 2002a). In contrast to the oligosaccharides in the stem region, the glycans attached to the globular head of HA vary significantly in number and position with different virus strains (Klenk and Schwarz 1987). It is well known that the carbohydrate side chains modulate HA activities by masking antigenic epitopes and functional domains. On the other hand, evidence is increasing that the glycans may be directly involved in specific interactions with host components. Virus pathogenicity may be altered by both strategies.

Glycosylation of an antigenic epitope prevents antibody binding. It therefore contributes to immune escape and is an important mechanism underlying antigenic drift. This concept is supported by several observations. Thus, intraepidemic variation of HA1 glycosylation has been observed during an H3N2 outbreak in 1974/75, whereby the number of glycans appeared to increase at the end of the outbreak (Seidel et al. 1991). In fact, the H3 HA acquired four additional carbohydrate side chains between 1968 and 1985, and inhibition of glycosylation and site-directed mutagenesis have directly shown that at least one of these glycans interfered with antibody recognition (Skehel et al. 1984; Abe et al. 2004).

Receptor binding may also be affected by glycosylation. When glycans at Asn-123 and Asn-149 in the vicinity of the receptor-binding site of an H7 HA were removed by site-directed mutagenesis, vector-expressed HA showed drastically enhanced hemadsorbing activity, and virions containing HA lacking these glycans were unable to elute from receptors, although they retained NA activity (Ohuchi et al. 1997b). Comparison of human H1 viruses grown in MDCK cells and in eggs revealed that the virus obtained from MDCK cells contained a carbohydrate side chain in close proximity to the receptor-binding site that was not present in the egg-grown virus. The cell and egg-grown viruses differed by receptor-binding properties (Gambaryan et al. 1999). Glycosylation-dependent variations in receptor affinity of HA may have to be balanced by NA variations. Thus it has been shown that acquisition of a carbohydrate close to the receptor-binding site, supposed to interfere with receptor access and to increase virus release, was accompanied by a deletion in the NA stalk that reduced the enzymatic activity and, thus, compensated for the reduced receptor binding (Baigent and McCauley 2001). These observations led to the concept that a functional balance between HA and NA is required for optimal infection (Mitnaul et al. 2000; Wagner et al. 2002b).

It is well known that a carbohydrate can determine pathogenicity by modulating cleavage activation of HA. Apathogenic strains of A/chick/Penn/83(H5N2) virus were found to have a glycan masking the multibasic cleavage site of HA. Loss of the side chain resulted in high cleavability and high pathogenicity (Kawaoka et al. 1984; Deshpande et al. 1987; Kawaoka and Webster 1988). In vitro studies showed that cleavability of human H3 HA with a multibasic cleavage site can also be modulated by a masking oligosaccharide (Ohuchi et al. 1991).

Evidence is increasing that interactions of the glycans of HA and NA with soluble and membrane-bound lectins also play an important role in influenza virus infection. Glycans of the globular head of HA bind to collectins, collagenous Ca⁺⁺- dependent multimeric lectins, including surfactant protein D (SP-D) present in respiratory secretions and mannose-binding lectin (MBL) present in serum. SP-D and MBL neutralize the virus by several mechanisms, such as steric hindrance of the receptor-binding site of HA, inhibition of the enzymatic activity of NA, aggregation of virions, and activation of complement-dependent pathways of the innate immune system (for review see Reading et al. 2007; Hartshorn 2010; Hillaire et al. 2013; Tate et al. 2014). Virus variants with reduced glycosylation obtained by mouse passages or generated by reversed genetics were less sensitive to inhibition by collectins in vitro and more virulent to mice than their fully glycosylated counterparts (Reading et al. 2007, 2009; Tate et al. 2011a, b). Furthermore, the HAs of the influenza viruses that caused the pandemics in 1918, 1957, 1968, and 2009 had only a few glycosylation sites on the globular head, which is a typical feature of LPAIV and LPAIV-derived swine viruses. HA glycosylation of these viruses increased during post-pandemic circulation in humans resulting in more collectin binding sites. Consequently, recombinant influenza viruses with the HAs of pandemic viruses showed lower binding to SP-D and significantly higher pathogenicity for mice than isogenic viruses with the HAs of seasonal human viruses (Vigerust et al. 2007; Qi et al. 2011). These findings indicate that reduced glycosylation and increased resistance to collectins in body fluids might contribute to enhanced pathogenicity of pandemic and zoonotic influenza viruses in humans.

C-type lectin receptors are type II membrane proteins that have also been found to bind to N-glycans of HA and NA. They include MMR (macrophage mannose receptor) and DC-SIGN also recognizing mannose as well as MGL (macrophage galactose-type lectin). All of these lectin receptors have been implicated in promoting infection of macrophages and dendritic cells of mice (Chu and Whittaker 2004; Hillaire et al. 2011; Londrigan et al. 2011). MGL that binds to both galactose and N-acetylgalactosamine has been proposed to function as a secondary receptor mediating endocytosis after virus attachment to the primary sialic acid receptor (Ng et al. 2014). Highly glycosylated virus strains that infected macrophages to high levels were recognized efficiently by MGL, whereas virus containing HA with few oligosaccharides was not, suggesting that differences in MGL-mediated recognition determine the susceptibility of murine macrophages to infection and that this modulates pathogenicity in mice (Upham et al. 2010).

5 Outlook

Future work is needed to focus on the identification of new activating proteases, particularly on enzymes cleaving at monobasic cleavage sites. It will be important to determine the tissue specific expression of the activating enzymes in epithelia, macrophages, and endothelia of the respiratory tract, as well as in nonrespiratory tissues, and to throw light on their role in organ tropism and spread of infection. To better understand the pathogenetic mechanisms involved in coinfection, HA-activating proteases expressed in bacteria and other microorganisms will have to be analyzed. Since evidence is increasing that there is considerable variation in proteolytic activation of mammalian and low pathogenic avian influenza viruses, the specificity of the activating proteases as depending on the structure of the respective cleavage sites will have to be elucidated. It is also desirable to design new inhibitors with a high specificity for these proteases.

It is well known that the linkage between N-acetyl-neuraminic acid and the adjacent galactose is an important determinant for the receptor specificity of the virus. However, little is known about the contribution of the proximal sugar residues and about the roles of the polypeptide and lipid moieties of the receptor molecules. The high variability of the glycan pattern of the respiratory tract is another challenge for structural analysis. Moreover, little is known about the glycans of the respiratory mucus and their roles as decoy receptors that interfere with infection. Thus, there is a need for more glycomics research to fully understand the role of HA in pathogenesis.

Finally, more information is needed about the functional significance of the oligosaccharide side chains of HA. It is fairly well known that they shield cleavage site, receptor-binding site, and antigenic epitopes. However, much has still to be learned about their interactions with lectins and the roles of lectins as secondary receptors and mediators in innate immunity.

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