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Review

# Receptor binding and pH stability — How influenza A virus hemagglutinin affects host-specific virus infection ☆

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ABSTRACT

Influenza A virus strains adopt different host specificities mainly depending on their hemagglutinin (HA) protein. Via HA, the virus binds sialic acid receptors of the host cell and, upon endocytic uptake, HA triggers fusion between the viral envelope bilayer and the endosomal membrane by a low pH-induced conformational change leading to the release of the viral genome into the host cell cytoplasm. Both functions are crucial for viral infection enabling the genesis of new progeny virus.

Adaptation to different hosts in vitro was shown to require mutations within HA altering the receptor binding and/or fusion behavior of the respective virus strain. Human adapted influenza virus strains (H1N1, H3N2, H2N2) as well as recent avian influenza virus strains (H5, H7 and H9 subtypes) which gained the ability to infect humans mostly contained mutations in the receptor binding site (RBS) of HA enabling increased binding affinity of these viruses to human type (α-2,6 linked sialic acid) receptors. Thus, the receptor binding specificity seems to be the major requirement for successful adaptation to the human host; however, the RBS is not the only determinant of host specificity. Increased binding to a certain cell type does not always correlate with infection efficiency. Furthermore, viruses carrying mutations in the RBS often resulted in reduced viral fitness and were still unable to transmit between mammals. Recently, the pH stability of HA was reported to affect the transmissibility of influenza viruses. This review summarizes recent findings on the adaptation of influenza A viruses to the human host and related amino acid substitutions resulting in altered receptor binding specificity and/or modulated fusion pH of HA. Furthermore, the role of these properties (receptor specificity and pH stability of HA) for adaptation to and transmissibility in the human host is discussed. This article is part of a Special Issue entitled: Viral Membrane Protiens – Channels for Cellular Networking.



**Contents**



*Abbreviations:* BHA, bromelain-cleaved hemagglutinin; E′, vestigial esterase subdomain of the HA1 subunit of hemagglutinin; F′, fusion subdomain of the HA1 subunit of hemagglutinin; F, fusion subdomain of the HA2 subunit of hemagglutinin; HA, hemagglutinin protein; HA0, pre-cursor (uncleaved form) of the hemagglutinin protein; HPAIV, highly pathogenic avian influenza virus; LPAIV, low pathogenic avian influenza virus; NA, neuraminidase of influenza virus; NP, nucleoprotein of influenza virus; R, receptor binding domain of the HA1 subunit of hemagglutinin; RBS, receptor binding site of hemagglutinin; RNPs, ribonucleoproteins (viral RNA and NP); SA, N-acetyl-neuraminic acid generally termed as sialic acid; SAα-2,3Gal, sialic acid linked to different carbohydrates by an α-2,3 glycosidic bond; SAα-2,6Gal, sialic acid linked to different carbohydrates by an α-2,6 glycosidic bond; TBHA2, BHA without HA1 at low pH

☆ This article is part of a Special Issue entitled: Viral Membrane Proteins — Channels for Cellular Networking.

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## 1. Introduction

Influenza A viruses have been a known burden for mankind since the beginning of the 20th century. Infection of a variety of hosts such as birds, horses, dogs and swine causes high morbidity and mortality among these species associated with huge economic losses. Furthermore, seasonal (epidemic) outbreaks of the human flu also have serious consequences, especially for the elderly population. The high variability of different virus strains, their constant evolution in birds and the resulting reassortment of novel, sometimes highly pathogenic influenza viruses in respective hosts (swine) complicates the development of new vaccines which can effectively inhibit or prevent infection with influenza viruses.

The negative stranded RNA virus belongs to the family of *Orthomyxoviridae*. It has a segmented genome encapsulated by the M1 matrix protein. This capsid is surrounded by the host cell-derived lipid membrane. Embedded in the envelope membrane are the proton channel protein M2, essential for proton transport across the viral membrane, and the two glycoproteins hemagglutinin (HA) and neuraminidase (NA) which are important for entry [1] and budding of new viruses from the cell surface [2], respectively. Since they are exposed at the surface of the virus, HA and NA also serve as antigens and thus as major targets for vaccine design. The segmented genome is composed of single-stranded negative sense RNA decorated with the nucleoprotein NP and the trimeric polymerase complex consisting of the proteins PB1, PB2 and PA (Fig. 1).

Based on the antigenic properties of HA and NA, influenza A viruses can be classified into 17 subtypes of HA (H1 to H17) and ten of NA (N1 to N10). Furthermore, the segmented genome allows the virus to reassort with different strains upon co-infection of a host so that novel viruses emerge constantly with a new composition of segments and subtypes (*antigenic shift*) [3]. All of these subtypes circulate in aquatic wild birds, their natural reservoir, mostly without causing any symptoms. Only when transmitted to poultry, lower mammals and humans, they cause respiratory disease. To date only viruses with HA of subtype H1, H2 and H3 and NA of subtype N1 and N2 are known to have successfully adapted to humans among which they keep circulating causing the annual human flu [4]. The avian H5 and H7 subtypes can be further classified in low and highly pathogenic viruses according to their mortality rates in infected chickens. Different to low pathogenic avian influenza viruses (LPAIV) which cause milder respiratory disease, highly pathogenic avian influenza viruses (HPAIV) provoke severe disease resulting in up to 100% mortality among poultry within 48 h [5,6]. In 2004, a very aggressive form of H5N1 HPAIV caused millions of deaths among birds associated with a growing number of spill-over infections from birds to humans or other mammals. Efficient human-to-human transmission has fortunately not been reported due to limited binding to human-type receptors, which is thought to be the major factor in determining the interspecies barrier [7–9]. Still, there is a great risk for the development of a human pathogenic H5N1 strain. In 2001, it was reported that only four mutations in the HA protein would be sufficient to turn the avian pathogenic H5N1 into a human pathogenic strain [6]. Additionally, an increasing number of human infections with avian H7N9 have been reported. From March until May 2013 approximately 130 cases have been detected with a high mortality of 21% [10,11]. Thus, precise surveillance of circulating influenza viruses and a better understanding of human-adaptive mutations would improve the forecast of a potential outbreak of a human pathogenic strain and help to prevent human pandemics.

For successful adaptation to a different host, mutations in several viral genes are required. For example, mutation E627K in the PB2 gene, which is one of the viral polymerase proteins, is known to confer higher replication efficiency at 37 °C [12–14]. Also substitutions in other viral proteins have been detected improving replication efficiency in the respective host [15–17]. These originate from the missing proof reading activity of the viral polymerase resulting in a high error rate during replication and endowing the virus with great flexibility (*antigenic drift*) [3].

Mutations of the influenza virus HA seem to have the most dramatic effect on virus pathogenicity. As interaction partner, fusion initiator and antigen, HA has several important functions in the first, crucial steps of virus infection. It mediates binding of the virus to sialic acid (SA) cell receptors and, after endocytosis of the virus particle, controls the release of the viral genome into the cell by a pH-dependent membrane fusion process (Fig. 2). Only efficient membrane fusion, i.e. formation of a fusion pore and release and transport of the viral RNPs into the nucleus, leads to the replication of new viral RNA, transcription and translation of the viral proteins and subsequent formation of new progeny virus [18,19].

Several studies have shown that mutations within HA can be sufficient for adaptation of an avian HA to a mammalian host [16,20–22]. In most cases these were linked to altered receptor binding or fusion activity. However, the variety of different strains and HA subtypes coupled with difficulty in assessing contributing host factors complicates the confident prediction of a new human pathogenic reassortant based on these studies. The high variability among influenza virus strains and their HAs correlates with a remarkable divergence among hosts, tissues and cells showing different susceptibilities to virus infection. Here, we review the role of HA-mediated cell binding and membrane fusion in influenza A virus infection considering these cell-specific differences. Furthermore, we describe how amino acid changes in different HA subtypes affect receptor binding and fusion behavior of the virus and how these changes contribute to host specific virus infection and successful interspecies transmission.

## 2. Structure and function of the hemagglutinin protein

The first crystal structure of HA from influenza A/Hong Kong/1968 at neutral pH was obtained by bromelain cleavage yielding the water soluble ectodomain of the glycoprotein (BHA) [23]. It is comprised of a triple-stranded coiled-coil of α-helices (stalk region comprising residues of HA1 and HA2) and a globular region of antiparallel β-sheets (receptor binding domain R, HA1) (Fig. 3). The receptor binding site (RBS) and the highly variable antigenic binding loops surrounding the RBS are located on the top of the R domain [1]. They usually carry carbohydrate side chains which are as well crucial for virus infection, especially for evading the host immune response [23–26]. Apart from the receptor binding domain R, the HA1 subunit also consists of the vestigial esterase

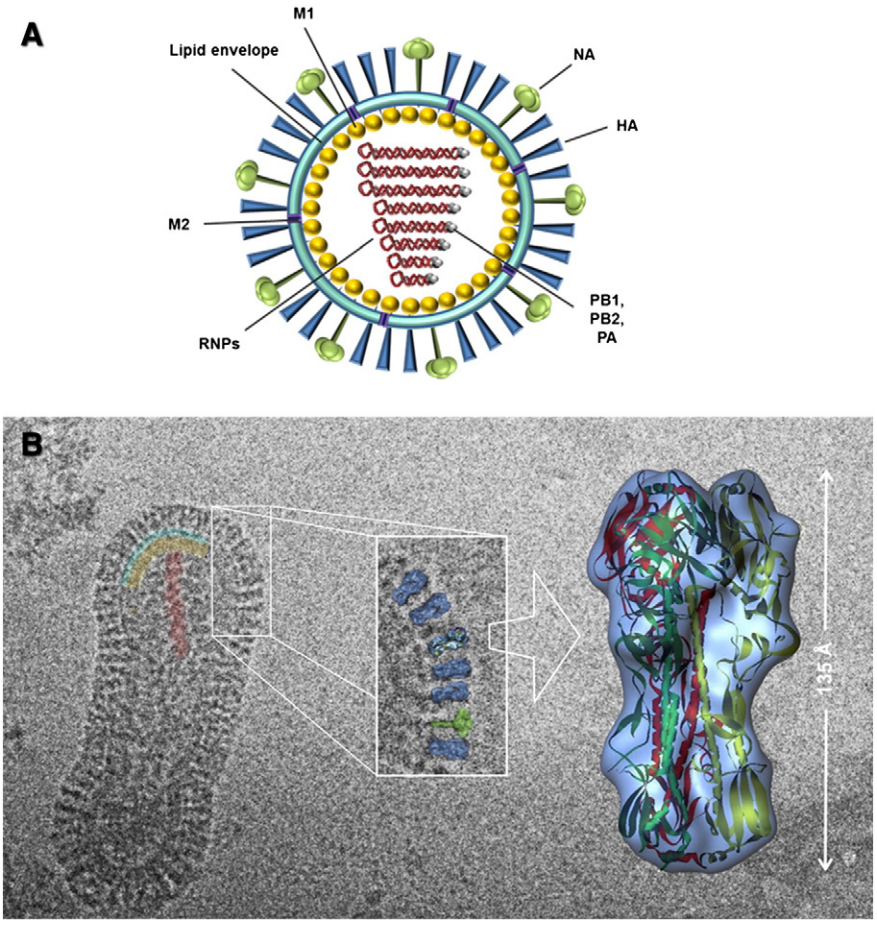


**Fig. 1.** (A) Schematic representation of an influenza A virus particle. The spike proteins hemagglutinin (HA, blue), neuraminidase (NA, green) and the proton channel protein M2 (violet) are embedded in the lipid envelope (turquoise) of the virus. The membrane is lined with the M1 capsid protein at the inside (yellow). The viral genome consists of eight ribonucleoprotein particles (RNPs, red), with each segment formed by viral RNA (vRNA), the nucleoprotein (NP) and the viral polymerase proteins (PB1, PB2 and PA, colored in gray). (B) Transmission electron micrograph of an influenza A/X-31 virion. Regions of the lipid membrane (turquoise) and of the M1 capsid (yellow) as well as one RNP (red) are colorized. In the magnified section of the electron micrograph HA and NA were overlaid with surface representations of the corresponding crystal structures filtered to an EM-comparable resolution (attainable by 3D-TEM-reconstruction techniques) [58]. On the right side the enlarged surface representation of the HA 3D-structure is overlaid with its secondary structure (PDB ID: 2YPG, monomers in green, red, yellow).

(E′) and the fusion (F′) subdomains, which form important contacts with the HA2 subunit of the protein [27].

The coiled-coil structure of HA2 (stalk domain) is crucial for the stabilization of the HA trimer and for anchoring the protein in the membrane via its transmembrane (TM) subdomain. This stabilizing central α-helical rod-like oligomer is also observed in other viral fusion proteins such as the Ebola glycoprotein Gp2 [28] or the HIV-1 gp-41 [29]. Furthermore, the HA2 subunit carries the fusion peptide (20–25 residues) at its N-terminus, which is essential for the membrane fusion activity of the protein [30]. Other important structural elements of the HA2 domain include the B loop connecting the long α-helix (helix A) of the stalk domain with the shorter helix at the outside (helix C) and a short part of the helix A itself (residues 106–112 of HA2). These elements could be determined by comparison of the pre-fusion (BHA) and the post-fusion structure of HA (TBHA2) [31]. The latter was resolved by low pH incubation of BHA with subsequent digestion by trypsin and thermolysin before crystallization [31,32]. It was found that the TBHA2 seems to be the energetically favored state of the protein [33,34]. The receptor binding domain appears to be structurally consistent between the pre-fusion structure [35] and its low-pH dissociated state [36].

### 2.1. Expression and cleavage

In infected cells, the hemagglutinin glycoprotein is produced as the precursor HA0. With the help of chaperones, HA0 monomers assemble into non-covalently linked homotrimers that subsequently travel through the Golgi apparatus to the plasma membrane [37,38]. Each HA0 monomer has to be cleaved by host proteases into the subunits HA1 and HA2 to be functional, i.e. able to induce membrane fusion [23,30,39–41]. Furthermore, cleavage liberates the N-terminal fusion peptide of HA2. Due to the positively charged N-terminal amino group the fusion peptide becomes buried in a negatively charged cavity of HA, the so called fusion peptide pocket [23,42,43]. Cleavage happens either during the course of intracellular transport or extracellularly at the plasma membrane depending on the sequence of the cleavage site.

For most HAs, the cleavage site between HA1 and HA2 is a single arginine residue (*monobasic cleavage site*) and the precursor is cleaved by extracellular enzymes. However, in some avian HAs (H5 and H7), the cleavage site is longer and contains multiple basic residues (arginines or lysines, *polybasic cleavage site*) [44–46]. The presence of a polybasic cleavage site directly correlates with enhanced pathogenicity as it is cleaved by a family of widespread subtilisin-like proteases such as furin and PC6, which act on HA intracellularly after exit from the ER. These subtilisin-like proteases are ubiquitously expressed resulting in a more efficient cleavage and systemic spread of the virus over the course of the infection. In contrast, the monobasic cleavage site of HA of low pathogenic viruses can only be cleaved extracellularly by serine proteases. These trypsin-like enzymes are only secreted in the avian and human lung epithelium (e.g. tryptase Clara, TMPRSS2 and HAT [43,47]) and in the intestinal tract of birds, e.g. factor Xa-like proteases. The restricted range of cells secreting such a protease results in anatomically

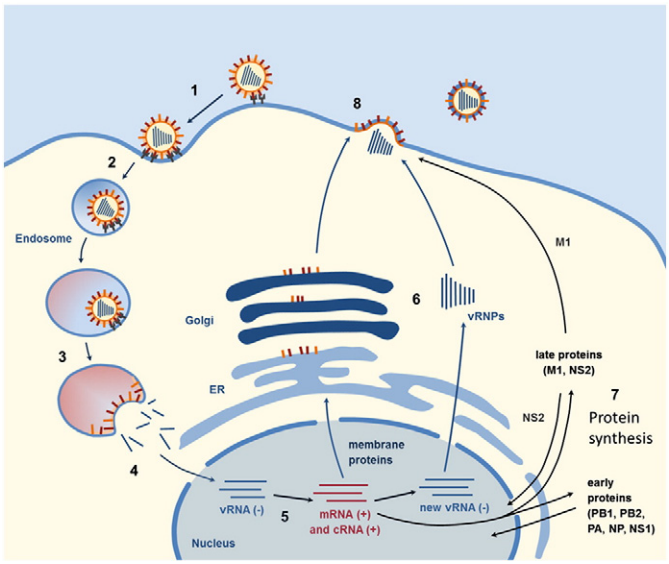


**Fig. 2.** Replication cycle of an influenza A virus. After the virus has bound to sialic acid-containing receptors via hemagglutinin (1) it gets endocytosed (2) and is transported within the endosome along microtubules (3). The declining endosomal pH triggers a conformational change of HA mediating the fusion of the viral and endosomal membrane. As a consequence, the RNPs are released into the cytoplasm and are finally transported into the nucleus (4). There the viral RNA is transcribed into mRNA and new viral RNA (via cRNA intermediate) (5). From the newly synthesized mRNA viral membrane proteins (HA, NA and M2) are produced at the endoplasmic reticulum (ER) and travel through the Golgi apparatus to the plasma membrane of the cell (6). Other early (PB1, PB2, PA, NP and NS1) and late (M1 and NS2) viral proteins are also translated and transported back into the nucleus where new ribonucleoprotein particles (vRNPs) are formed with the nucleoprotein (NP) and the trimeric polymerase complex (PB1, PB2, PA) (7). These new vRNPs, as well as M1, are also transported to the plasma membrane, where assembly of new viral particles takes place which subsequently bud from the plasma membrane (8).

localized and hence milder infections [48]. Therefore, efficiency of proteolytic cleavage does not only depend on the virus strain (LPAIV or HPAIV) but also on the host cell proteolytic enzyme machinery. Secretion of serine proteases activating the HA of LPAIV is limited to a number of tissues and cells leading to reduced viral replication. However, other viral or bacterial lung infections can contribute to the secretion of enzymes such as thrombin and plasmin capable of HA cleavage [41]. The presence of host proteases influencing cleavage activation of HA is only one of several host specific factors affecting the infection potential of an influenza virus which are described later in this review.

### 2.2. Receptor binding

The receptor on the host cell surface is N-acetyl-neuraminic acid, generally called sialic acid (SA), which inserts into the receptor binding pocket at the top of the HA1 subunit. The RBS is formed by three domains, the 130-loop (residues 134–138), the 190-helix (residues 188–195) and the 220-loop (residues 221–228) (Fig. 3). However, only some of the residues directly interact with the receptor sialic acid. For example, in the H3 subtype Y98, W153, E190, Y195 and H183 were identified to interact through hydrogen bonds with the side chains of SA [49,50]. Three of these residues (Y98, W153 and H183) are highly conserved throughout all HA subtypes except from the recently identified H17 subtype of bat derived H17N10 [51]. Other residues of the RBS interact with the adjacent sugars of sialic acid and play an important role for the receptor specificity of HA as described in Section 3.1.

In general, the mechanism of viral sugar binding greatly differs from the binding mechanism of human sugar binding proteins, such as galectins. The latter have an open structure which is much more accessible compared to the hidden cavity of HA and other viral lectins [52]. Despite this discrepancy, the RBS of HA is thought to be derived from the same host galectin as human galectins. Interestingly, H17 has a rather

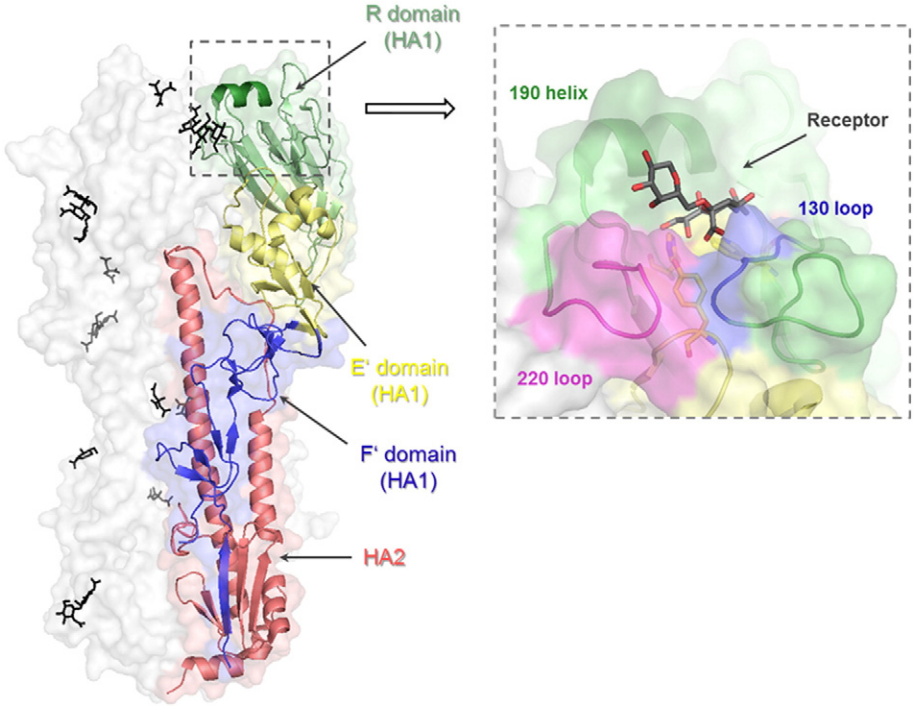


**Fig. 3.** Crystal structure of HA from Influenza A/X-31 virus (H3N2) (PDB ID: 2YPG). One monomer is shown in cartoon representation with subdomains of HA1 labeled in green (receptor binding domain, R), yellow (vestigial esterase domain, E′) and blue (fusion domain, F′) and the HA2 subunit in red. The other monomers are displayed in surface representation with carbohydrate moieties in black. The magnification in the R subdomain of HA1 shows the structural elements of HA1 forming the receptor binding site (130-loop in blue, 190-helix in green, 220-loop in pink) in complex with a human-type receptor analog (dark gray). Conserved residues forming hydrogen bonds with sialic acid are displayed in stick representation (yellow transparent).

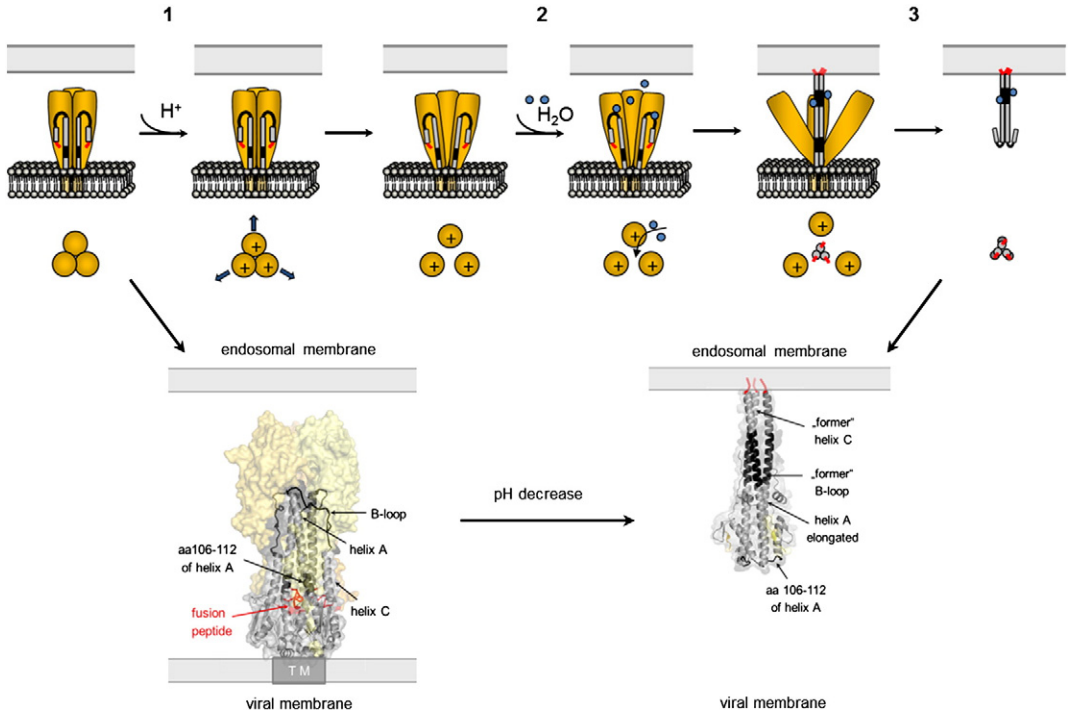


**Fig. 4.** Conformational change of the influenza virus HA at acidic pH (5.0–6.0). In the upper part of the image the steps of conformational change are illustrated (HA1 in gold, HA2 in gray and black, fusion peptide in red). Upon acidification in the endosome protonation of HA1 leads to the dissociation of the HA1 monomers (1). Water can enter triggering the structural transition of the B-loop into a helix and liberating the fusion peptide which inserts into the endosomal membrane yielding the extended intermediate conformation of HA (2). Refolding of amino acid residues (aa) 106–112 of the helix A into a loop finally mediates the apposition of the two membranes triggering fusion (hairpin conformation of HA) (3). The lower part of the image shows the corresponding secondary structures of trimeric HA of A/X-31 (H3N2) at neutral (PDB ID: 2YPG) and low pH (hairpin structure, PDB ID: 1HTM) in surface (HA1, yellow-orange) and cartoon representation (HA2, gray) with structural elements undergoing a conformational change highlighted in black. TM is the transmembrane region of HA.

flat RBS comparable to human sugar binding motifs in contrast to the shallow pocket in all other viral HA proteins. For this HA subtype, binding to sialic acid-containing receptors has not been observed and hence it was speculated that this subtype might have an alternative mechanism of cell attachment [51,52].

### 2.3. Fusion activity

The merger of two membranes is a thermodynamically favored process but has a high kinetic barrier. The energy to overcome this barrier is thought to be provided by the "spring-loaded" mechanism of HA conformational change, also observed for other viral fusion proteins [53,54]. As mentioned above, the fusion peptide of HA2 plays an important role in the membrane fusion process. Upon acidification in the endosome (pH 5.0–6.0) and the conformational change of HA, it gets exposed and inserts into the target membrane inducing the merger of the two membranes [1,42,55–57]. However, the process of conformational change and membrane fusion is not as simple and involves structural rearrangements in both, the HA1 and HA2 domains.

A sequence of three major steps was discussed for the conformational change of HA: (1) Protonation of HA1 leads to the dissociation of intra-trimeric and inter-subunit contacts allowing water to enter the ectodomain having now access to sequences which have originally been shielded from contact with water [55,58]. (2) Interaction with water triggers the B loop of the HA2 N-terminus to undergo a loop-to-helix transition resulting in the extended coiled-coil conformation of the three monomers (extended intermediate) [59]. (3) Due to refolding of helix A into a loop, the extended intermediate collapses and thereby draws the fusion peptide towards the transmembrane region (hairpin conformation) leading to lipid mixing and the formation of a fusion pore [53,59] (Fig. 4). Cryo-EM studies and characterization of HA mutants locking the protein in (mostly) reversible intermediates strongly support this model [58,60,61]. Partial opening of the HA1 monomers (1) was observed by cryo-EM for the trimeric ectodomain (BHA) [58] as well as for HA in intact virus particles [62] upon incubation at low pH. Furthermore, incomplete formation of the extended coiled-coil conformation (2) due to mutations in the B-loop (F63P, F70P) was shown to inhibit membrane fusion emphasizing the importance of a fully extended intermediate state for the formation of a fusion pore [63]. All of these studies report (partly reversible) intermediate states of the HA that are essential for complete membrane fusion and precede the final irreversible hairpin structure of HA2 [58,62,64].

Lee et al. [65] suggested a different model of membrane fusion based on observations of virus–liposome complexes at pH 5.5 by cryo-electron tomography (CET). In contrast to the conventional model of membrane fusion, dissociation of HA1-monomers (1) and insertion of the fusion peptide into the target membrane (2) are proposed to be followed by membrane scission (3) (breakage of the target membrane) while the viral membrane stays intact. The author further proposes that, after the apposition of the two membranes (due to refolding of HA2 into the hairpin structure) leading to lipid mixing (4), dissolution of the matrix layer M1 at pH < 5.0 is required for fusion pore formation (5). Thus, M1 was suggested to play an essential role in membrane fusion controlling the release of the viral RNPs at a lower pH than that of HA conformational change.

## 3. Role of HA for host-specific virus infection and adaptation

The significance of the hemagglutinin glycoprotein for viral infection and host adaptation has been reported several times [1,30,39,66,67]. In the first steps, from viral attachment through membrane fusion, the HA protein plays an important role for antigenicity, host susceptibility and pathogenicity of the virus. Binding and uptake by a certain host cell is mostly determined by the composition of the RBS of the HA1 domain (receptor binding specificity) whereas the fusion potential of HA is correlated to its pH-dependent stability. Hence, for a given virus the

**Table 1**
Residues affecting the receptor binding specificity of different HA subtypes. Residues highlighted were shown to confer transmissibility in the mammalian host.

| Avian HA | Mutation (H3 numbering) | position | receptor specificity | | Delta fusion pH | RD transmission [units] | Literature |
|---|---|---|---|---|---|---|---|
| | | | α–2,3 SA | α–2,6 SA | | | |
| H1 | **E190D, G225D** | R, RBS | – | ++ | n.a. | **Yes** | [79,80,163,164] |
| H2, H3 | **Q226 L, G228S** | R, RBS | – | ++ | n.a. | **Yes** | [68,79,165] |
| | **G218E/W** | R, HA1–HA1 | + | + | +0.4 | No | [108,124,125] |
| | **$T_2$156N** | F, HA1–HA2 | ++ | – | +0.1 | – | [124] |
| | **S223N** | R, RBS | + | + | n.a. | – | [49,166] |
| | **S227P** | RBS | – | + | n.a. | – | [79] |
| H5 | **L133V, A138V** | R, RBS | + | + | n.a. | – | [89,126,167] |
| | **N186K (+ M230I)** | R, RBS | + | + | n.a. | – | [21,90,126] |
| | **Q196R (+ N186K)** | R, RBS | + | + | n.a. | – | [90] |
| | **Q226 L, G228S** | R, RBS | + | + | n.a. | No | [20,21,86,89,120] |
| | **+E190D** | R, RBS | + | + | n.a. | No | [86,87] |
| | **+K193R** | R, far from RBS | + | + | n.a. | No | [86,89] |
| | **+D187G, E190D, K193S** | R, RBS | + | + | n.a. | No | [86,91] |
| | **+Q196R** | R, RBS | – | ++ | n.a. | **Yes** | [91] |
| | **+T160A** | R, close to RBS | – | ++ | + | No | [20,88,89] |
| | **+T160A, H110Y** | E', HA1–HA2 | – | ++ | – | **Yes** | [20,101] |
| | **N224K, Q226 L** | R, RBS | – | + | +0.3 | No | [21] |
| | **+N158D/A** | R, RBS | – | ++ | +0.3 | No | [21] |
| | **+N158D, T318I** | F', HA1–HA2 | – | ++ | -0.3 | **Yes** | [21] |
| | **V152I, Q226 L** | R, close to RBS | + | + | n.a. | No | [21] |
| | **S227N, G228A** | R, RBS | + | + | n.a. | No | [21,77,120] |
| | **S227N, Q196R** | R, RBS | + | + | n.a. | No | [90,91] |
| | **Q226 L, E231G** | R, RBS, close to RBS | + | + | n.a. | No | [21] |
| | **R/K216E, S221P** | R, HA1–HA1, RBS | ++ | – | +0.3 | – | [35,77,87,146] |
| | **K216E** | R, HA1–HA1 | ++ | – | +0.4 | – | [35] |
| | **S221P** | R, RBS | ++ | – | -0.2 | – | [35] |
| H7 | **Q226 L + G186V** | R, RBS | ++ | + | n.a. | – | [127] |
| | **N123 or T125A** | close to RBS | ++ | – | n.a. | – | [93,168] |
| | **G186E/V/A** | R, RBS | + | + | n.a. | – | [70,93] |
| | **K193R** | R, RBS | + | + | n.a. | – | [70] |
| | **220 loop deletion** | R, RBS | – | ++ | n.a. | **Direct contact** | [70,92,93] |
| | **+G186E** | R, RBS | + | + | n.a. | – | [93] |
| | **+R205G, (G186E)** | R, RBS | + | + | n.a. | – | [93] |
| | **S227T** | R, RBS | + | + | n.a. | – | [70] |
| H9 | **226 L** | R, RBS | + | + | n.a. | **Direct contact** | [84] |
| | **+G228S** | R, RBS | + | ++ | n.a. | – | [82] |
| | **+T189A, G$192_2$R** | R, RBS, F, HA2 | + | + | n.a. | **Yes** | [16,121] |

*Exact values of the delta pH of fusion were not provided in the literature.
**Residues 221 to 228

infection potential may also depend on the endosomal acidification characteristic of the host cell. In the following section, we will discuss the importance of receptor binding specificity and HA stability for viral infection and host adaptation in more detail.

### 3.1. Receptor binding specificity

Sialic acid belongs to a diverse family of sugars terminally linked to different carbohydrates (mostly galactose) either by α-2,3 (SAα-2,3Gal) or α-2,6 (SAα-2,6Gal) glycosidic bond. The kind of linkage is of great importance since different HA subtypes have different preferences in binding to one of these SA linkages. Whereas avian influenza HAs preferentially bind to SAα-2,3Gal, human adapted HA subtypes have been shown to favor SAα-2,6Gal [68–71]. The preference of avian viruses to bind SAα-2,3Gal matches the occurrence of this sugar on epithelial cells in the intestinal tract of birds, the replication site of avian influenza viruses. In contrast, the human upper respiratory tract is rich in SA with α-2,6 linked carbohydrates and thus might not be infected by avian influenza viruses [72]. The human lower respiratory tract was shown to contain SAα-2,3Gal, favoring avian influenza virus infection. Furthermore, avian influenza viruses target different cells in the tissue sections of the human lung than human ones [73–75]. Although many studies reported dual receptor binding, meaning the HA protein of some strains binds both, α-2,3 and α-2,6 linked receptors [76,77], there is usually a preference for one of these linkages. Receptor specificity is thus to be understood as preferential binding being an important determinant of host specificity and thus considered as major interspecies barrier [9,78].

#### 3.1.1. Residues affecting HA receptor specificity

Successful host adaptation of different HA subtypes has been shown to require the substitution of certain amino acids in the RBS associated with a switch in receptor binding specificity (from SAα-2,3Gal to SAα-2,6Gal or vice versa). For some subtypes (H1, H2 and H3), amino acid changes in the RBS resulting in a switch to preferential SAα-2,6Gal binding have already been identified (E190D and G225D for H1, G226L and G228S for H2 and H3). These substitutions did not only confer α-2,6 binding and infection of humans but also successful transmission between human hosts resulting in the past four human pandemics (1918: H1N1, 1957: H2N2, 1968: H3N2, 2009: H1N1) [79,80]. In contrast, adaptive substitutions required for the circulation of current avian strains in the human host such as H5N1, H7N9 and H9N2 remain elusive. Only recently, a ferret-transmissible H5N1 strain with mutation Q226L (in combination with N224K and N158D) was found to bind to human-type receptors in the same mode as the HA proteins of previous pandemics [81]. Furthermore, binding to the human receptor of current human infecting H7N9 (which already contain leucine at position 226) was enhanced by mutation G228S (as in human H2 and H3) [82]. It was shown that the substitution of glycine with serine at position 228 results in a network of inter-residue interactions between the RBS of H7 and the human receptor which is comparable to the interaction network of the H3 RBS with its bound receptor. H5, H7 and H9 subtypes are of

specific importance since some of them are highly pathogenic and have been shown to infect humans in a number of cases [11,83,84]. Other mutations that were found to increase SA α-2,6Gal binding or reduce SA α-2,3Gal are listed in Table 1.

Interestingly, the introduction of mutations Q226L and G228S which led to successful adaptation of H2 and H3 subtypes to the human host, also increased the ability of the H5 and the H7 subtypes to bind to human-type receptors [82,85,86]. In contrast, human adaptive mutations of H1 resulted only in a slight increase of SAα-2,6Gal binding in the avian H5 subtype but rather in reduced affinity for SAα-2,3Gal (E190D) or total abolishment of glycan binding (E190D, G225D) [87]. Imai et al. identified by random mutagenesis and glycan array analysis that mutations N224K and Q226L in A/Vietnam/1203/2004 (H5N1) confer binding solely to α-2,6 linked SA. Binding and replication of this mutant virus was further increased by substitution of N158 [21]. A similar study by Herfst et al. using a combination of targeted mutagenesis followed by serial viral passage in ferrets also resulted in an additional mutation of N158 or of T160 [20] in addition to previously inserted Q226L and G228S known to enhance SAα-2,6Gal binding in several HA subtypes. Mutation of asparagine at position 158 or of threonine at position 160 was reported also in other studies to enhance preference for human-type receptors due to the loss of the same glycosylation site on the top of the HA1 globular head [88,89]. These findings support the previous suggestion that glycosylation of HA influences virus infection not only in terms of antigenicity but also at the stage of receptor binding and replication [25]. Other mutations that have been detected in the RBS of avian strains include N186K, K193R, Q196R and S227N either together or in combination with other changes in the avian H5 subtype [21,90,91]. These substitutions have also been associated with reduced α-2,3 and increased α-2,6 sialoside binding. Similar substitutions were found in natural isolates of H7 and H9 subtypes which contribute to increased SAα-2,6Gal recognition [70,84,92]. The most prominent substitution in H7 is the 220 loop deletion which seems to facilitate α-2,6-glycan binding [93], whereas in the H9 subtype leucine at position 226 (such as in human H2 and H3) was shown to enable replication in the human airway epithelium [84].

### 3.1.2. Methods to assess receptor specificity

Rogers et al. in 1983 were among the first to study receptor specificity using sialidase treated (asialo) erythrocytes which were specifically re-sialylated with either α-2,3- or α-2,6-linked SA [68]. Aside from this elegant approach that is still used in current studies [80,94], solid-phase binding assays or glycan arrays represent a convenient way to analyze HA-receptor interactions [95]. These methods allow the analysis of a wide number of SA-linked carbohydrates coupled to a flat surface. This surface can either be probed with intact viruses [21,92] or purified HA [96,97] which are subsequently detected using monoclonal antibodies. Other methods for the evaluation of receptor binding include NMR [98,99], surface plasmon resonance (SPR) and single-virus force spectroscopy (SVFS) [100–102]. Recently, surface bilayer interferometry and microscale thermophoresis have been used for quantitative receptor binding studies [81].

In most of these studies, purified SA receptors were used as binding partners for HA or virus particles providing very accurate binding profiles and affinities. However, results are hardly comparable due to different ligand densities and sample preparation, which most likely affect receptor binding. For example, it was shown that multivalent nanoparticles showed differential virus binding depending on the surface density of sialic acid [103]. For methods such as SPR, dissociation is often underestimated due to the high re-association rates, which can be ascribed to the multivalent character of binding as it is the case for viruses [81,102]. Also using SVFS, unbinding rates must be considered carefully since two dimensional dissociation resulting from immobilization of the ligand to a force transducer leads to reduced ligand entropy. However, SVFS is the only tool that enables virus-cell adhesion measurements in a set-up that closely mimics the natural situation. Using this method it could be shown that the resulting cellular binding specificity of influenza virus may not necessarily resemble the receptor specificity of HA [102]. Hence, the unique presentation of the ligand on the host cell membrane has an important influence on virus binding and should be considered when assessing receptor specificity of different HA subtypes and viruses, i.e. host cell specificity is not only determined by the presence of SAα-2,6Gal or SAα-2,3Gal but also by the organization on the cell surface (see below).

### 3.1.3. Role of receptor binding specificity in viral infection

Despite the many indicated studies stating the importance of specific residues for increased SAα-2,6Gal or SAα-2,3Gal preference, variations in receptor binding do not always correlate with the amino acid composition [76]. For example, H1 from influenza A/Cal/09 (H1N1), an isolate from the recent pandemic (swine flu, 2009), with aspartic acid at positions 190 as well as 225 should preferentially bind human type receptors. In contrast, the virus exhibits pronounced dual binding to both receptors, a property that is opposed to seasonal H1 viruses and might be linked to the pandemic character of A/Cal/09 [76]. H3 from A/X-31, which originates from the pandemic virus A/Aichi/2/68 (H3N2) also shows dual binding behavior [76]. Also, in two independent studies of H5N1 tropism in the respiratory tract of mammals it was found that the viruses replicated well in both the lower and upper respiratory tract of ferrets in vivo [104] and in upper respiratory tract epithelia in humans ex vivo [72] despite the lack of SAα-2,6Gal binding. Hence, it is difficult to make predictions about receptor binding and infection based only on the amino acids present in the RBS and on the kind of SA linkage.

Other critical amino acids have been shown to affect binding [79] leading to a preference for certain types of carbohydrates linked to SA [87,93], i.e. many other factors might play a role in receptor specificity apart from the structure of the receptor binding site such as the carbohydrate modification of SA on the host cell surface. Chandrasekaran et al. [105] and Stevens et al. [97] postulate that human influenza viruses bind to a much more structurally diverse set of SA linked carbohydrates than avian viruses which goes beyond the general preference of α-2,3 or α-2,6 linkage [67]. In another study the glycosylation state of the virus itself (i.e. of its HA) has been shown to affect virus attachment and uptake depending on the specific host cell [96]. Being a transmembrane protein, HA follows the secretory pathway with post-translational modifications taking place in the ER and the Golgi [106]. The glycosylation state of HA thus strictly depends on the cell type and has been shown to alter receptor specificity [97]. But if viral attachment to the cell surface is influenced by the host cell in many ways the question arises of how crucial the initial HA–SA contact is for influenza virus infection. Furthermore, it is not yet known if the presence of sialic acid on the cell surface is strictly required for binding and uptake of the influenza virus particle [107].

Indeed, virus binding does not always correlate with infection per se. For example, MDCK cells have higher infection efficiencies compared to CHO cells [108] although human H3N2 (X-31) displays better binding to CHO than to MDCK cells, suggesting the existence of other post-attachment factors necessary for efficient infection in MDCK cells. This finding is supported by the study of Nunes-Correira et al. [109] who present a kinetic model for virus binding and endocytosis in MDCK cells pointing out two kinds of binding sites: (1) low-affinity receptors responsible for binding and (2) high affinity receptors responsible for uptake. Also other studies have implicated the dependence of viral uptake on other post-attachment factors [110,111].

Stray et al. [107] provided evidence for influenza virus infection in the absence of sialic acid, suggesting other possible attachment factors involved in virus binding. Of these, L-SIGN and DC-SIGN, C-type lectins (LC-SIGN and DC-SIGN), were found to participate in influenza virus attachment independent of SA specificity [112]. Furthermore, blocking of fibronectin was shown to interfere with influenza A virus entry [113]. Interestingly, only α-2,6 specific viruses were affected. All of these

studies suggest a much higher complexity of virus–cell interaction beyond the level of HA–SA binding.

The involvement of multiple entry pathways [114], the de novo induction of clathrin-mediated endocytosis [115] and the use of specific adaptor proteins such as Epsin 1 [116] suggest that more specific signals than the initial HA–SA contact are necessary to trigger internalization and promote infection. Indeed, other cellular signaling factors are activated upon virus infection such as protein kinase C and phosphatidylinositol-3 kinase (PI3K) as reviewed by Grove and Marsh [117]. Clustering of sialylated receptor tyrosine kinases such as the epidermal growth factor (EGF) and the c-Met receptor has been reported to facilitate virus internalization by inducing tyrosine kinase and PI3K signaling [118]. These signals might be the missing link between binding and infection, not only for successful endocytosis, but also in context of the innate immunity, which might also be directly connected to receptor binding. In a recently presented hypothesis, virus binding to either human or avian type receptors activates differential immune signaling pathways [119], a factor that is important considering preferred binding or more dual-binding behavior of influenza viruses.

### *3.1.4. Role of receptor binding specificity for host adaptation and transmissibility*

Many studies aiming at predicting and preventing the emergence of a new pandemic evaluated the impact of receptor binding specificity in the course of viral adaptation to a different host and its transmissibility [67,85]. Despite an increased binding to α-2,6 linked SA and efficient infection of human respiratory tract tissues [72,120], most of the above described substitutions in the avian H5, H7 or H9 strains were not able to confer respiratory droplet (RD) transmission in the ferret model (Table 1) [21,86,93,121]. A possible explanation is that most of these HA mutants retained their capacity to bind SAα-2,3Gal (dual receptor binding) and thus can be inhibited by mucins. These sialic acid-rich substances are secreted from human lung epithelial cells and also carry α-2,3 linked SA specifically inhibiting viruses with SAα-2,3Gal preference [122]. Therefore, it seems that apart from enhanced α-2,6 glycan binding, the virus also pursues a reduction of α-2,3 glycan binding for successful human-to-human transmission. The recently studied receptor preference of a ferret-transmissible H5N1 strain also displayed drastically decreased binding to avian type receptors whereas preference to human type receptors was only slightly increased compared to the non-transmissible wild type, confirming that reduced SAα-2,3Gal binding might be very important for human infection and successful transmission [21,81].

Furthermore, mutations in or near the RBS that conferred enhanced α-2,6-glycan binding were often associated with reduced replication and virulence [21,86,123] suggesting that there are other mutations needed that retain viral fitness during the course of adaptation to a different host. Substitutions in the HA1 domain were often associated with a shift in the pH of membrane fusion, mostly to higher values [21,35,124] and thus it was hypothesized that resulting destabilization has to be compensated by a stabilizing factor. This idea was supported by recent studies on the respiratory droplet (RD) transmission of a recombinant highly pathogenic avian H5 virus in the ferret model. Mutations conferring SAα-2,6Gal binding were shown to increase the pH of fusion and were not able to infect co-housed ferrets by this mode of transmission. Only with an additional stabilizing mutation (T318I) shifting the pH of fusion from 5.9 to 5.7 these recombinant viruses were transmitted through the air and were able to infect other ferrets [20,21].

Also, passage of human H3N2 in mice resulted in much higher virulence, which was not only ascribed to increased α-2,3 glycan binding

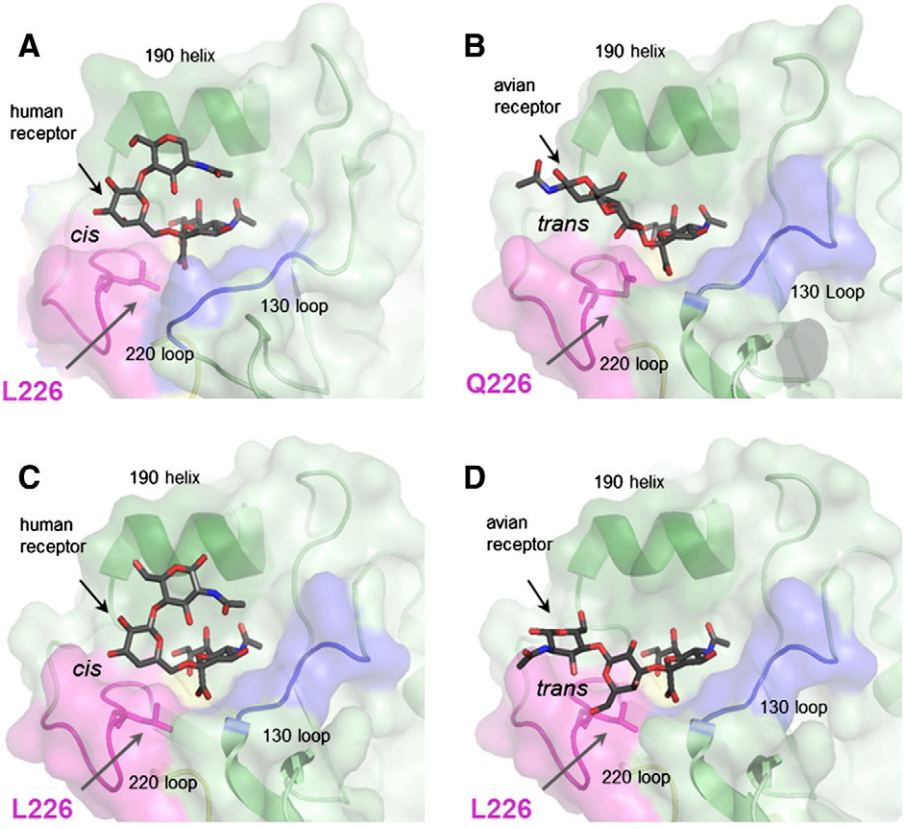


**Fig. 5.** HA receptor binding site from human (A) and avian (B, C, D) influenza viruses complexed with the human (A, C) and/or avian (B, D) cell receptor. HA from human influenza A/X-31 H3N2 (PDB ID: 2YPG) has leucine at position 226 facilitating binding to the human receptor analog with 2-galactose (2-Gal) in cis conformation (A) whereas HA from avian H5N1 (A/VN1194, PDB ID: 4BGY3) has a glutamine at position 226, favoring the trans motif of avian type receptors (B). The ferret-transmissible H5 of A/VN1194 binds the human receptor (PDB ID: 4BH3) in the same mode as human H3, most probably due to the Q226L mutation (C). In contrast, binding of ferret-transmissible H5 to the avian receptor (PDB ID: 4BH4) is substantially decreased due to leucine at position 226 as illustrated in the image (D).

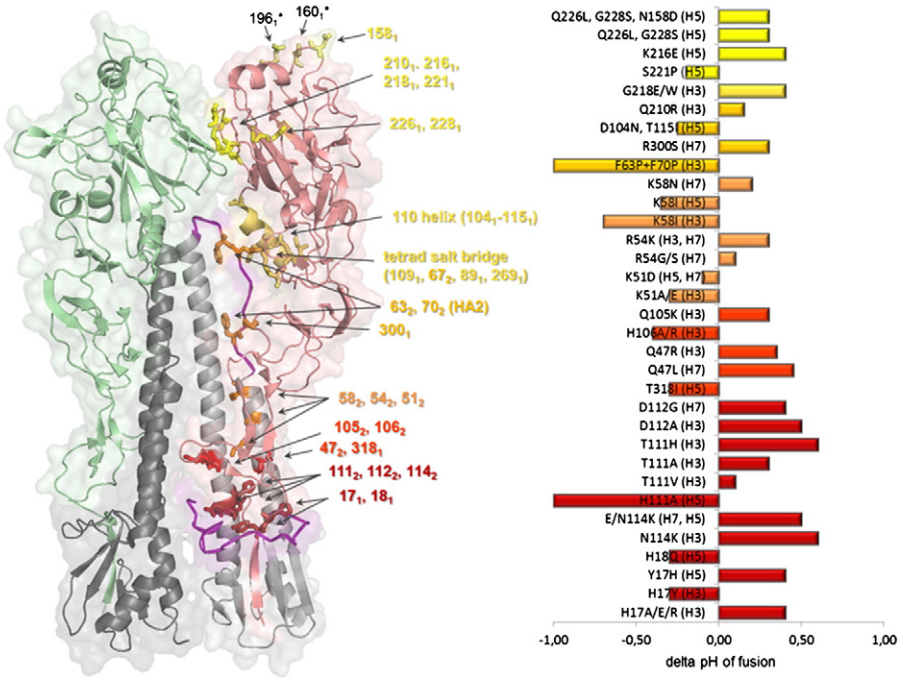


**Fig. 6.** Summary of mutations modulating the pH of fusion of H3, H5 and H7 subtypes. Two monomers of the HA crystal structure of A/VN1194 (PDB ID: 2IBX) are displayed with the HA1 subunits in green and salmon, and the HA2 subunit in gray, respectively. Mutations of residues leading to a pH shift of fusion are shown in stick presentation in red, orange and yellow colors. In the graph these residues are colored correspondingly. The x-axis of the graph shows the delta pH of fusion. Wild-type HA was set to zero and mutations shifting the fusion pH to higher or lower values are listed according to their location in the crystal structure of HA. Remarkably, most of mutations modulating the pH of fusion are located in one of the three major regions of conformational change (HA1–HA1 interface, HA1–HA2 interface including the B-loop and the 110-helix and the fusion peptide region). *Residues 160 and 196 did not affect the pH of fusion; however, both were shown to increase the ability of the H5 subtype to bind to human type receptors as well as to transmit between ferrets. Due to their importance for host adaptation these amino acids were highlighted in the HA structure.

but also to an elevated pH of fusion (from 5.2 to 5.6) due to substitutions at position 218 of HA1 and 156 of HA2 (Table 1). Again, G218W near the RBS resulted in significant destabilization of HA [124,125] whereas $T156_2$[1] did not show a large effect on the pH of fusion. However, $T156_2$ also participates at a potential glycosylation site (position $N154_2$) [124]. These studies indicate that aside from receptor binding specificity also other cell-specific factors contribute to better susceptibility and higher virulence in a different host. Still, over the course of host adaptation of different virus strains, amino acid substitutions mostly occur in or near the RBS, strongly indicating that a "suitable" receptor binding pocket plays a pre-dominant role for host-specific virus infection [82,85]. Recent comparison of the above described "transmissible" H5 subtype with its avian counterpart in complex with avian and human receptors confirms that the substitutions in the RBS (Q226L, N224K) result in an altered sialic acid binding orientation which is more similar to the binding mode of H2 and H3 pandemic viruses [81]. In these subtypes the G226L mutation causes widening of the space between the 130 loop and the 220 loop of the RBS subtypes. This structural change was also previously described as a requirement to bind the α-2,6 *cis* linkage of human type receptors instead of the avian α-2,3 *trans* motif [127] (Fig. 5). Interestingly, a Q226L mutation was shown in many avian viruses to confer SAα-2,6Gal binding and even human infection (Table 1), not only for H5N1 viruses, but also in natural occurring H9N2 [84] and the recent H7N9 strains [127]. For the H5 subtype, leucine at position 226 has not been found in any natural isolate and usually additional substitutions were required for enhanced SAα-2,6Gal binding. These amino acids (G228S, Q196R, N224K) were associated with the binding of other components of the cell or facilitate binding due to the loss of a glycosylation site (T160A, N158D) [20,21,86,88,91]. Recently, the network of inter-residue interactions of H3 and H7 RBS in complex with the human type receptor was analyzed [82]. Residues which interact with the adjacent sugars of sialic acid were shown to include also the 140 and 150 loops of the receptor binding domain possibly influencing the affinity to a certain receptor.

In any case, viral adaptation mostly required several passages to obtain acceptable viral titers for a respective host [20,108] suggesting a multifactorial trait of receptor binding and infection that has to be optimized in a long process of selection.

### 3.2. HA stability

Temperature and acid stability of HA has been shown to differ among HA subtypes [128–130] as well as between viruses of the same subtype [35,131]. The stability of HA is usually measured over a range of different pH values or temperatures. The conformational change is irreversible, which provides inactivation as a marker for stability. In practice, after a pre-incubation step at the respective pH or temperature, the activity of HA is tested, e.g. membrane fusion, to determine the threshold of inactivation [21,132]. The divergence in the inactivation kinetics and thus stability of the HA is ascribed to structural variations in the protein ectodomain that have been identified by comparison of the various crystal structures [35,133–136]. However, the biological significance of HA stability has not yet been fully clarified but it seems to be another hallmark of HA affecting the infection potential of the whole virus.

#### 3.2.1. Residues affecting HA stability

The conformational change of HA, and thus membrane fusion and virus infection, does not depend only on the efficiency of cleavage but also on the stability of the protein itself. The meta-stable HA1–HA2 structure resulting from HA0 cleavage is thought to be stabilized by the HA1 domain acting like a "clamp" on HA2 thereby preventing its

[1] Index 2 indicates that the residue is located at position 156 of the HA2 domain. HA1 residues do not carry an index. All subsequent residues are labeled accordingly. H3 numbering is used throughout.

refolding at neutral pH [34,61]. Several ionic interactions between HA subunits and monomers contribute to the stabilization of this structure. During endosomal acidification, key residues located in these critical interface regions are protonated weakening the interactions and thus triggering the structural rearrangements leading to membrane fusion [125,137–139]. It is well known that the stability, flexibility and function of HA largely depends on electrostatic interactions at interfaces such as hydrogen bonds and salt bridges, and van der Waals interactions [140–142]. These interactions are important for keeping the metastable conformation at neutral pH. Thus, substitution of a number of residues has been shown to modulate HA stability what in turn affects the pH of membrane fusion. For an overview, see Table 2. Analysis of a variety of so called "fusion mutants" has revealed the importance of some key residues and their interactions at several domain and subunit interfaces which are partially conserved throughout HA subtypes [56,125,138] (Fig. 6).

*3.2.1.1. Residues in the fusion peptide pocket.* As mentioned earlier, the most stabilizing part of the protein is the coiled-coil structure of the HA2 stalk domain with its transmembrane region [38] being mostly retained during conformational change; only a small part of the long α-helix (helix A) undergoes a structural transition (Fig. 4). One of the most determinant regions for the HA conformational change includes the fusion peptide and the pocket where it is intercalated. The first ten N-terminal residues of the fusion peptide and some residues in the fusion peptide pocket are highly conserved. For example, the aspartic acids at positions 109 and 112 in the pocket form strong hydrogen bonds with fusion peptide residues 2, 3, 4 and 5 [136] (**Fig. S1**). Accordingly, amino acid deletions or substitutions in the peptide or the cavity surrounding it, disturb these balanced interactions significantly affecting the fusion activity of HA [56,123,125,143–145]. However, not all of the residues lining the fusion peptide pocket are conserved resulting in local structural differences among subtypes. Based on these differences, the known 17 HA subtypes (except bat-derived H17) have been classified into five structural clades which were again divided in two groups according to the characteristics of the fusion peptide region: Whereas residues $K51_2$ (HA2 stalk domain), $D109_2$, and $D112_2$ of the pocket are completely conserved throughout all subtypes, residues H17 (F′ fusion subdomain), $H106_2$ and $H111_2$ of HA2 (F fusion subdomain) are group specific [137]. The H3 group (H3 and H7 subtypes) carries His at positions 17 and 106 and a neutral amino acid (alanine or threonine) at position 111, whereas the H1 group (e.g. H1, H2, H5 subtypes) has a Tyr at position 17, an arginine or lysine at position 106 and a histidine at position 111. Substitution of these amino acids (H17 in H3, Y17 in H5, $H_2106$ in H2 and H3 [61,137] and $H111_2$ in H5 [144]), affected the fusion pH in H3 and H5 subtypes significantly suggesting that important interactions in the HA protein are preserved rather than the amino acid sequence. For example, it was hypothesized that at low pH the protonated $H106_2$ forms repulsive interactions with $K51_2$ of the stalk what might be important for bending of helix A after the formation of the extended intermediate (as described in Section 2.3). These are lost when mutating one of these residues to a neutral amino acid, thus leading to a decrease of the fusion pH [137]. Interestingly, mutating the $H106_2$ to $R106_2$ (which is naturally present in all H1 group HAs) led to a significant stabilization of HA although it is positively charged, even at neutral pH [61]. These results emphasize that it is very difficult to predict important interactions or the exact role of conserved residues for the HA conformational change even with structural information.

*3.2.1.2. Residues at the HA1–HA2 interface.* With the release of the fusion peptide, the inter-helical loop B of HA2 undergoes a loop-to-helix transition upon protonation at low pH. As described earlier, this process requires reorganization of interactions at the HA1–HA2 interface. Thus, involved residues certainly play a role in the regulation of conformational change. The interface is formed between loop B (HA2) and the R, E′ and F′ subdomains of the HA1 subunit [134] with involved residues 85–90, 104–115 and 265–270 of HA1 and 64–72 of the HA2. Some of these residues are conserved and thus also the interactions among them such as the tetrad salt bridge between E89, R109, R269 of HA1 and $E67_2$ of HA2 [138]. Rachakonda et al. [138] demonstrated the

**Table 2**
Residues modulating the pH of membrane fusion of different HA subtypes.

| Avian HA | Residue (H3 numbering) | Position | Delta fusion pH [units] | Literature |
|---|---|---|---|---|
| H1 | **L78P, Q354H** | E', F HA1 | − 0.2 (↕) | [123] |
| H3 | **H17A/E/R** | F', HA1 | +0.4 | [125,137,139] |
| | **H17Y** | F', HA1 | −0.3 | [125,137] |
| | **D132N** | F', HA1 | +0.2 | [169] |
| | **T212E,N216R** | R, HA1–HA1 | No fusion | [138] |
| | **T212C,N216C** | R, HA1–HA1 | No fusion | [147,148,170] |
| | **P162S** | R, HA1 | +0.2 | [124] |
| | **Q210R** | R, close to RBS | +0.15 | [124] |
| | **G218E/W (+$T_2$156N)** | R, HA1–HA1 | +0.4 | [108,124,139] |
| | **$I6_2$M** | F, HA2 | +0.3 | [125] |
| | **$F9_2$L** | F, HA2 | +0.6 | [125] |
| | **$Q47_2$R** | F, HA2 | +0.35 | [125] |
| | **$K51_2$A/E** | F, HA2 | −0.3 | [137] |
| | **$R54_2$K** | F, HA1–HA2 | +0.25 | [125] |
| | **$K58_2$I** | F, HA1–HA2 | −0.6–0.8 | [139,170] |
| | **$F63_2$P, $F70_2$P** | F, HA1–HA2 | No fusion | [63] |
| | **$E81_2$G** | F, HA2 | +0.3 | [125] |
| | **$Q105_2$K** | F, HA2 | +0.3 | [125] |
| | **$H106_2$A/R** | F, HA2 | −0.4 | [61,137] |
| | **$T111_2$H/V/A** | F, HA2 | +0.6,+0.3,+0.1 | [137] |
| | **$D112_2$A/G** | F, HA2 | +0.4, +0.5 | [125,137] |
| | **$N114_2$K** | F, HA2 | +0.6 | [125] |
| | **$T156_2$N** | F, HA1–HA2 | −0.1 | [124] |
| H5 | **Y17H** | F' , HA1 | +0.4 | [144,154,155] |
| | **H18Q** | F' , HA1 | −0.3 | [144,154,155,171] |
| | **D104N, T115I** | E', HA1–HA2 | −0.25 | [35,146] |
| | **K216E** | R, HA1–HA1 | +0.4 | [35] |
| | **S221P** | R, RBS | −0.2 | [35] |
| | **Q226 L, G228S, N158D** | R, RBS | +0.3 | [21] |
| | **+T318I** | F', HA1 | −0.3 | [21] |
| | **Q226 L, G228S, T160A** | R, RBS | +* | [20] |
| | **+H110Y** | E', HA1–HA2 | −* | [20,101] |
| | **$K51_2$D** | F, HA1–HA2 | −0.1 | [144] |
| | **$K58_2$I** | F, HA1–HA2 | −0.3–0.4 | [144,153,155] |
| | **$H111_2$A** | F, HA2 | No fusion | [144] |
| | **$N114_2$K** | F, HA2 | +0.5 | [155] |
| H7 | **R32G** | F', HA1 | +0.2 | [125] |
| | **R91Q/L** | R, HA1 | +0.1, +0.3 | [125] |
| | **R300S** | F', HA1 | +0.3 | [125] |
| | **$F3_2$L** | F, HA2 | +0.4 | [125] |
| | **$G23_2$C** | F, HA2 | +0.1 | [172] |
| | **$Q47_2$L** | F, HA2 | +0.45 | [125] |
| | **$K51_2$D** | F, HA1–HA2 | −0.1 | [144] |
| | **$R54_2$K/G/S** | F, HA1–HA2 | +0.1,+0.3 | [125] |
| | **$K58_2$I/N** | F, HA2 | −0.7 | [170] |
| | **$I81_2$S** | F, HA1–HA2 | +0.1 | [125] |
| | **$D112_2$G** | F, HA2 | +0.4 | [125] |
| | **$E114_2$K** | F, HA2 | +0.5 | [125] |

*Exact values for the delta pH of fusion were not provided in the literature.

importance of this and other salt bridges for the stabilization and thus fusion activity of HA. The deletion of conserved and the introduction of new salt bridges resulted in a pH shift of membrane fusion. Also other authors reported substitutions at the HA1–HA2 interface (positions 17, 18, 104, 110, 115 and 318 of HA1 and 47, 51, 54, 58, 59, 63 and 70) that significantly affect the pH of fusion [20,35,101] (Table 2, Fig. 6).

*3.2.1.3. Residues at the HA1–HA1 interface.* Only a few studies have reported mutations at the HA1–HA1 interface which could potentially influence fusion activity of the HA protein [138,139,146,147]. Thus, there is only little evidence about stabilizing interactions between the globular top domains of HA1 (**Fig. S2**). By the introduction of disulfide bonds [147,148] or salt bridges [138], it was shown that the dissociation of the HA1 monomers is required to allow the whole conformational change of HA. Refolding of HA2 also occurs spontaneously at neutral pH [33,55,59]. It was thus proposed by Huang et al. that protonation of HA1 might be sufficient for HA conformational change and membrane fusion [59]. However, there is no experimental evidence to support this and residues being protonated at low pH are largely unknown. Interestingly, some studies have reported that mutations in or near the RBS may affect the fusion pH of several subtypes suggesting that the HA1 domain is very sensitive to structural variations [21,35,124]. Antigenic drift resulting in the substitution of amino acids in the receptor and antigenic binding sites are often associated with the modulation of inter-monomeric contacts of HA1 and might thus be a strategy of the virus to control several functions at the same time.

To summarize, electrostatic interactions throughout the HA molecule contribute to the stabilization of different HA subtypes and thus play an important role for HA conformational change. The number of HA molecules undergoing a conformational change determines the efficiency of fusion with the endosomal membrane what in turn might affect the infectivity of an influenza virus strain [149,150]. Some of the ionizable residues and their interactions (e.g. H17, R220, R229 of HA1, $D109_2$ and $D112_2$ of HA2) are buried in the cleaved HA without contact to neighboring ions giving them the potential to destabilize the structure upon their protonation [43]. Daniels et al. [125] reported that HA mutants isolated from amantadine resistant strains (X-31 (H3N2) and Weybridge virus (H7N1)) could be divided into two groups depending on the location of amino acid modification: One group had substitutions in proximity to the fusion peptide resulting in the destabilization of interactions between the peptide and residues of the pocket. The other group contained mostly substitutions at inter-subunit contacts leading to structural changes at the HA1–HA2 interface. Also adaptation of egg-grown X-31 (H3N2) to mammalian cells resulted in mutations in these two regions resulting in a shift of the fusion pH [139]. This confirms the central role of these parts for the stability of HA. However, it is still not clear which are the key residues that get protonated upon acidification.

Stevens et al. [87,135] described pH-sensitive histidine patches that might play a role in triggering the dissociation of subunit and domain interactions in the two regions described above. Histidine residues are generally known to act as "pH sensors" in class I and class II fusion proteins due to their pKa~6 (= pH of protonation). Hence, their protonation matches the pH region of membrane fusion in the endosome [151–152]. It was further suggested that sequential protonation of histidines at positions 18 and 38 in HA1 and at positions 106/111 and 142 controls the structural transitions of HA [57,87,151]. The exchange of histidines and of neighboring (mostly charged) residues had a significant effect on the fusion pH (Table 1) proposing that the pKa of histidine can be modulated by the surrounding residues thereby affecting the pH of the conformational change, and in consequence, of membrane fusion [137]. It was also proposed previously that the substitution of charged residues might play a dominant role in the course of virus adaptation [125].

In any case, the described studies highlight the importance of energetically favored interactions between domains and subunits that preserve the HA protein in its native, meta-stable structure. Subtype-specific differences in sequence and structure reflect the versatility of the virus to form these interactions throughout the HA molecule [133–134]. Thereby the precise balance of charged residues might play a key role in the evolution of influenza viruses regulating the electrostatic interactions within the trimer what in turn affects fusion activity in the endosome.

*3.2.2. Role of HA stability for virus infection and host adaptation*

The pathogenicity of avian H5N1 influenza virus strains in chickens has been recently linked to the acid stability of their HA proteins [35,146]. A HPAIV strain had an elevated pH of fusion (pH5.7) compared to a moderately pathogenic virus (pH5.5) due to mutations N104D and T115I which destabilize the 110-helix and its interaction with the neighboring HA2 subunit (Fig. 6). However, for infection of mammals a lower pH of fusion and thus enhanced temperature and acid stability seems to be essential in order to achieve optimal uptake by nasal epithelial cells [153]. It was thus suggested that adaptation to a different host does not only require binding to the cell-surface specific receptors but also needs to acquire an optimal pH of membrane fusion [35,154,155]. Indeed, it could be shown that HA mutant viruses with a higher pH of fusion (pH 5.5–6.0) replicated with better efficiency in ducks than in mice. In contrast, replication of HA mutant viruses characterized by a lower pH of fusion (pH 5.0–5.5) was supported only in mice, but not in ducks. These studies provide evidence for a different requirement of acid stability for avian and mammalian influenza viruses depending on their activation pHs [154,155].

Differences in acid stability of avian and human influenza viruses which have also been reported in other studies [21,130] might have a simple explanation. The endosomal pH determines the time point of fusion and can vary among different cell lines [156]. This has two consequences for the virus: (1) The earlier the viral genome is released from the endosome, the earlier the cellular immune response is stimulated upon recognition of the viral RNA [24]. Therefore, from the viral perspective, membrane fusion in close proximity to the nucleus is favored, so that the time frame between vRNP release and transport into the nucleus is minimized. (2) At the same time, fusion has to occur before the endosome has matured into a lysosome where the virus particle would be otherwise degraded [157]. This explanation is supported by several studies where adaptation to a different host resulted in a pH shift of membrane fusion, no matter if it was adaptation from egg-grown viruses to mammalian cells [139], during passage of a human derived strain (H3N2) in mice [108,124], or as a result of adaptation from MDCK grown viruses to Vero cells [156].

*3.2.3. Role of HA stability for RD transmission*

Another explanation for differential requirements of acid stability can be deduced from the mode of viral transmission which also differs between birds and mammals. Wild birds are the natural reservoir for avian influenza viruses and spreading within these aquatic bird populations occurs through an indirect fecal-oral route involving contaminated water on shared aquatic habitats. It was found that the chemical and physical conditions of the water (pH, salinity, and temperature) can significantly influence the stability of avian influenza viruses [155,158]. Thus, in wild birds a higher acid stability might be favored for efficient spreading of the virus [131]. HPAIV which are associated with rather low stability compared to LPAIV, are known to have developed by adaptation from wild birds to poultry by formation of the polybasic cleavage site [15]. Transmission in poultry might not require high acid stability probably allowing evolution of this highly pathogenic phenotype. However, it remains to be determined if cleavage activation and an elevated pH of fusion evolved in parallel or in an independent manner.

In contrast, for spreading via respiratory droplets, which is generally accepted as the primary transmission mode in humans [159], the virus has to pass the human nasal airway epithelium which is known to be mildly acidic. Thus, in addition to the prerequisite of SAα-2,6Gal binding

a rather stable HA seems to be indispensable to prevent an early conformational change of HA, and thus inactivation of the virus, before cell entry. Indeed, infection efficiency of an avian influenza H5N1 virus in mice was shown to be enhanced by a stabilizing mutation in the HA2 domain ($K58_2I$) [153,154]. This residue is also located at the HA1–HA2 interface where the exchange of a charged residue could lead to a decrease of the fusion pH [137,144]. It was reported that the mutated strain retained infectivity at pH 5.6 whereas infection of the wild type strain was limited to pH 5.8 resulting in reduced infectivity in the intranasal infection of mice, most likely due to the low pH of the mucosal surface [153]. This finding was supported by two recent studies of Zaraket et al. reporting an increased viral growth of the $K58_2I$ mutant in mice as well as in the upper respiratory tract of ferrets due to the lower fusion pH [154,160] whereas in infected mallards no physiological symptoms could be detected [155]. The glands of the nasal epithelia in mice are quite similar to those in humans, however the transmission mode is different. Influenza virus infection of ferrets along with their symptoms is much more comparable to the infection in humans. Hence, ferrets are a widely used model system to study transmissibility. As described above, also in this model it could be shown that, apart from SAα-2,6Gal receptor preference, a stabilizing mutation in the HA protein was required for efficient RD transmission between ferrets [20,21]. Crystal structure analysis of the transmissibility conferring mutation reported by Imai et al. revealed that residue 318 is situated in proximity of the fusion peptide where substitution of threonine to isoleucine contributes to the stabilization of this structure [81]. Herfst et al. reported mutation H110Y to be responsible for better transmissibility between ferrets. The exchange of histidine at position 110 to a tyrosine was shown to stabilize the intra-trimeric contact (HA1–HA2 interface) due to an additional hydrogen bond to the adjacent monomer [20,101].

Accordingly, it could be shown that a stabilizing mutation alone is not sufficient for an avian H5N1 virus to transmit between ferrets, not even via direct contact [160]. Thus, together with increased HA stability, increased SA-2,6Gal binding is indispensable for the transmissibility of an avian influenza virus between mammals [20,21,91,161].

However, studies on the airborne-transmissibility in ferrets were mostly performed with recombinant viruses which contained viral genes of human adapted strains [16,21,91,161] most likely contributing to successful replication and transmission. Thus, the experimental conditions used for assessing ferret transmissibility might not represent the conditions for transmissibility of a natural virus strain. Furthermore, it is difficult to make assumptions for influenza virus transmission in humans based on the results obtained with ferret studies. Still, for human infection and/or human-to human transmission higher acid stability of the viral HA seems to be mandatory. Galloway et al. [130] recently compared cleavage activation and fusion pH of several human and avian influenza virus strains. The last human pandemics were caused by strains of lower pathogenicity and rather high stability. Thus, most HAs derived from human isolates mediated membrane fusion at 0.1–0.5 pH units lower than those of the same subtype derived from an avian species (except from H5) confirming the pre-condition of HA stability for viral spread in the human population. Thus, the pH of triggering the conformational change of the influenza virus hemagglutinin is certainly an important factor to consider in the evaluation of newly emerging strains [130].

## 4. Conclusions

Influenza A virus cell entry is mainly determined by the HA protein which mediates both viral attachment and membrane fusion, eventually leading to the release of the viral genome into the cell cytoplasm.

Both functions have been studied extensively and were also the focus of this review. Some structural features involved in receptor binding and conformational change are conserved throughout evolution and thus small differences in the amino acid composition may significantly affect these functions leading to the diversification of subtypes and clades with different binding and fusion behavior.

In this review, we described the influence of structural variations on the receptor binding specificity and stability of HA and their role in host infection and adaptation. We conclude that infection of a certain host seems to require a more cell-specific adaptation of the HA protein which might not necessarily be related to HA receptor binding. In contrast, for sustained inter-species transmission of influenza viruses, binding to the host-specific SA receptors as well as adaptation of HA stability seem to be required. All strains from the last human pandemics (H1N1 2009, H2N2 1957 and H3N2 1968) as well as more recent ones (H5N1, H9N2, H7N2, H7N9), have acquired substitutions in the RBS that could be related to a switch or at least an increase in human type receptor binding [79,82,84,85,93]. Furthermore, most of the ferret- and also human-transmissible strains contain HA proteins with higher stability [21,130] than their avian counterparts which seems to contribute to the transmissibility of the virus. Therefore, increased human type receptor binding together with an increase of the HA stability is required for the adaptation of an avian influenza virus to the human host [20,85,160].

However, the inefficiency of avian-human and human-to-human transmission for the current H5N1 and H7N9 cannot be explained solely by the inability of these viruses to bind and replicate at these sites. Viral attachment, endocytosis and efficiency of membrane fusion were shown to be influenced also by many cell-specific factors such as plasma membrane composition, existence of multiple entry and signaling pathways, presence of host proteases, cellular immunity and likely many other factors not mentioned here. These additional host cell factors were not subject of the present study and are described elsewhere [119,162].

Another question remains that has not been addressed yet. HA stability and pathogenicity have only been shown to inversely correlate

**Table 3**
Summary of human- and ferret-transmissible influenza virus strains to date.

| Avian HA | Residues in the RBS | Receptor specificity | | Delta fusion pH | RD transmission | Other viral genes | Literature |
|---|---|---|---|---|---|---|---|
| | | α-2,3 SA | α-2,6 SA | | | | |
| H1 | E190D, G225D | – | ++ | n.a. | Yes | All genes of human H1N1 | [80,163,164] |
| H2, H3 | Q226 L, G228S | – | ++ | n.a. | Yes | All genes of human H3N2/H2N2 | [68,79,165] |
| H5 | Q226 L, G228S, Q196R | – | ++ | n.a. | No | HA and NA of avian H5N1, other of H3N2 | [91] |
| | Q226 L, G228S, Q196R | – | ++ | n.a. | No | NA of human H1N1, other genes of avian H5N1 | [91] |
| | Q226 L, G228S, Q196R | – | ++ | n.a. | Yes | mutant NA of human H3N2, other genes of avian H5N1 | [91] |
| | Q226 L, G228S, T160A | – | ++ | + | No | All genes of avian H5N1 | [20,89] |
| | +H110Y | – | ++ | – | Yes | All genes of avian H5N1 | [20] |
| | N224K, G226 L, N158D/A [21,88,89] | – | ++ | +0.3 | No | HA and NA of avian H5N1, | |
| | +T318I | – | ++ | −0.3 | Yes | HA and NA of avian H5N1, other genes of human H1N1 | [21] |
| H7 | Q226 L + G186V | n.a. | n.a. | n.a. | No | All genes of avian H7N9 | [127] |
| | 220 loop deletion | – | ++ | n.a. | No | All genes of avian H7N2 | [70,92,93] |
| H9 | 226 L | + | + | n.a. | No | All genes of avian H9N2 | [84] |
| | +T189A, $G192_2R$ | + | + | n.a. | Yes | NA of avian H9N2, other genes of human H3N3 | [16,121] |

in chicken, but not in mammals. If human-to-human transmission requires high stability of the virus, highly pathogenic avian strains associated with low stability of HA must not necessarily be highly pathogenic in humans as well. In contrast, HPAIV with a high pH of fusion might not replicate very well in the human host. Furthermore, it should not be forgotten that also changes in other viral proteins apart from HA are required for replication in human populations. Human pandemic strains have been in most cases reassortants from viruses of different origins [9], which might have additional adaptations in other genes. In contrast, circulating highly pathogenic avian H5N1 strains in 2004 and 2005 as well as current human infecting H7N9 strains contain all viral genes of avian origin [83,127] which might hinder successful transmission between humans (Table 3).

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.bbamem.2013.10.004.

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