Acta virologica **60**: 121 – 135, 2016 doi:10.4149/av_2016_02_121

REVIEW

The role of fusion activity of influenza A viruses in their biological properties

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Received March 3, 2016; accepted April 29, 2016

Summary. – Influenza A viruses (IAVs) cause acute respiratory infections of humans, which are repeated yearly. Human IAV infections are associated with significant morbidity and mortality and therefore they represent a serious health problem. All human IAV strains are originally derived from avian IAVs, which, after their adaptation to humans, can spread in the human population and cause pandemics with more or less severe course of the disease. Presently, however, the potential of avian IAV to infect humans and to cause the disease cannot be predicted. Many studies are therefore focused on factors influencing the virulence and pathogenicity of IAV viruses in a given host. The virus-host interaction starts by virus attachment via the envelope glycoprotein hemagglutinin (HA) to the receptors on the cell surface. In addition to receptor binding, HA mediates also the fusion of viral and endosomal membranes, which follows the virus endocytosis. The fusion potential of HA trimer, primed by proteolytic cleavage, is activated by low pH in endosomes, resulting in HA refolding into the fusion-active form. The HA conformation change is predetermined by its 3-D structure, is pH-dependent, irreversible and strain-specific. The process of fusion activation of IAV hemagglutinin is crucial for virus entry into the cell and for the ability of the virus to replicate in the host. Here we discuss the known data about the characteristics of fusion activation of HA in relation to IAV virulence and pathogenicity.

Keywords: influenza A viruses; pH optimum of fusion; IAV virulence and pathogenicity; HA conformation change; IAV interspecies transmission; adaptation changes

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Abbreviations: HA = hemagglutinin; HA0 = HA precursor; HA1 = heavy chain of HA; HA2 = light chain of HA; HPAI = highly pathogenic avian influenza; IAV(s) = influenza A virus(es); LPAI = low pathogenic avian influenza; M1 protein = matrix protein; mRNA = messenger RNA; NA = neuraminidase; NEP protein = nuclear export protein; NS1 protein = nonstructural protein 1; PA = polymerase acidic protein; PB1 = polymerase basic protein 1; PB2 = polymerase basic protein 2; RBS = receptor binding site; RNA = ribonucleic acid; RNP = ribonucleoprotein; SA = sialic acid; vRNA = viral RNA

1. Introduction

Influenza infections of humans spreading yearly in the form of epidemics or pandemics represent a health problem for human population all over the world, as they are

accompanied by losses of lives and a negative impact on the economy. The main clinical symptoms of the disease, such as high fever, myalgia, arthralgia (joint pains), headache, dry cough and lethargy, start up to three days after the infection. The course of the disease can differ from mild to severe, depending on the immune status of the host as well as on the infective dose and properties of infecting virus strain. In more complicated cases, however, the disease might result in pneumonia with fatal outcome. IAVs infect broad spectrum of hosts, mainly birds and mammals, including humans, what is, besides other factors, the reason of their high variability.

Influenza A viruses are enveloped spherical particles with 8 segments of negative stranded genomic RNA encoding up to 18 proteins (Yamayoshi *et al.*, 2015). The high mutation rate of influenza A viruses (3.5 amino acids/year or 10^{-3}substitutions per site, per year (Gerhard *et al.*, 2006; Chen and Holmes, 2006) due to the properties of viral RNA polymerase and the ability of reassortment of RNA segments of virus genome enable the viruses to escape the host immune system. Consequently, the selection of variants with growth advantage in the particular host gives rise to mutations allowing the adaptation of IAV to the new host. Therefore, still new IAVs can emerge in human population and some of them could represent a pandemic danger, depending on virulence and pathogenicity of the new strain.

Virulence is genetically determined ability of viruses to penetrate into the cells, to replicate and to spread in the organism from the site of entry to the target organ. It expresses the ability of the virus to cause the special pathological process in the host, as to form lesions in the particular tissue or cause the clinical symptoms of disease in the host (Fislová and Kostolanský, 2005). Pathogenesis of influenza disease is the result of complex interconnected processes that the virus triggers in the organism after the infection. IAV proteins encoded by all 8 segments of viral genome, in cooperation with host cell biosynthetic machinery, participate in the resulting virulence and pathogenicity of the particular viral strain. The most prominent roles are played by viral RNA-polymerase complex and HA (Salomon and Webster, 2009; Fukuyama and Kawaoka, 2011). Here we focused on the HA and its ability to mediate the fusion of viral and cell membranes in relation to the biological properties of IAV.

2. Structure and function of hemagglutinin

Hemagglutinin, the main determinant of IAV pathogenicity, is the best studied molecule of IAV. It is the surface glycoprotein characterized as a transmembrane protein type I. HA is synthesized as a precursor HA0 molecule on the ribosomes of the endoplasmic reticulum (ER) (Daniels *et al.*, 2003; Flint *et al.*, 2009). During the HA synthesis, HA0 monomers are folded by creating the intramolecular disulfide bonds. Consequently, in association with chaperones, they are trimerized and via non-covalent bonds form HA homotrimers with molecular weight around 220 kDa. HA trimers are N-glycosylated, transported through transport vesicles from ER to the Golgi complex (GA) (Flint *et al.*, 2009; Lodish *et al.*, 2013), where the final posttranslation modifications (glycosylation and acylation of the cytoplasmic tail of HA trimer) take place. HA molecules are proteolytically cleaved into HA1 and HA2 intracellularly or extracellularly, depending on the structure of cleavage site on HA. Serine-type proteases (trypsin-like proteases, tryptase Clara), produced by the epithelial cells of the host respiratory tract, recognize the monobasic cleavage site (Q/E-X-R, R arginine, X other aa) of HA (Skehel *et al.*, 1982; Johansson *et al.*, 1989; Skehel and Wiley, 2000). HA0 of some viruses (particularly of H5 and H7 subtypes) is cleaved by subtilizin-like intracellular enzymes present in tissue of various organs (e.g. ubiquitously present furin or PC6). They recognize the polybasic (arginine and lysine) cleavage site comprising R-X-R/K-R sequence. After proteolytic cleavage, HA1 and HA2 remain linked in each HA monomer by a single disulfide bond.

HA trimer is composed of a globular head covering the HA stem, which is anchored in the membrane by the transmembrane domain and an acylated cytoplasmic tail. The heavy chain, HA1 gp, forms the globular head of HA trimer with the receptor binding site (RBS), which is highly conserved among all IAVs. It is composed of aa in positions 98Y, 153W, 183H and 195Y (Cross *et al.*, 2001). These amino acids contribute to the preservation of RBS structure. Three of them (except 195Y) are directly involved in the virus binding to cell surface receptors (Cross *et al.*, 2001). The virus attachment to cell receptors is influenced also by variable amino acids surrounding RBS (Isin *et al.*, 2002). HA globular domain continues in β-sheets of HA1 gp into the HA stem, which is formed predominantly by the light chain of HA, the HA2 gp, mostly of helix conformation. HA2 gp is relatively conserved among different IAV subtypes. The first 11 amino acids of its hydrophobic N-terminus, the fusion peptide, are the most conserved among all HA subtypes. After the proteolytic cleavage of HA precursor, the N-terminus of the fusion peptide is inserted into the pocket (lined by ionizable amino acids) in the cavity near the cleavage site and at neutral pH, it remains buried in the interspace of HA monomers (Skehel and Wiley, 2000; Sriwilaijaroen and Suzuki, 2012). In acidic environment, HA undergoes large structural changes essential for the activation of HA fusion potential (Steinhauer, 1999; Skehel and Wiley, 2000; Sriwilaijaroen and Suzuki, 2012) (Fig. 1).

Hemagglutinin has two significant roles during the replication cycle: the virus attachment to the cell surface receptors and the mediation of viral and endosomal membrane fusion, which makes HA an important player in the IAV virulence.

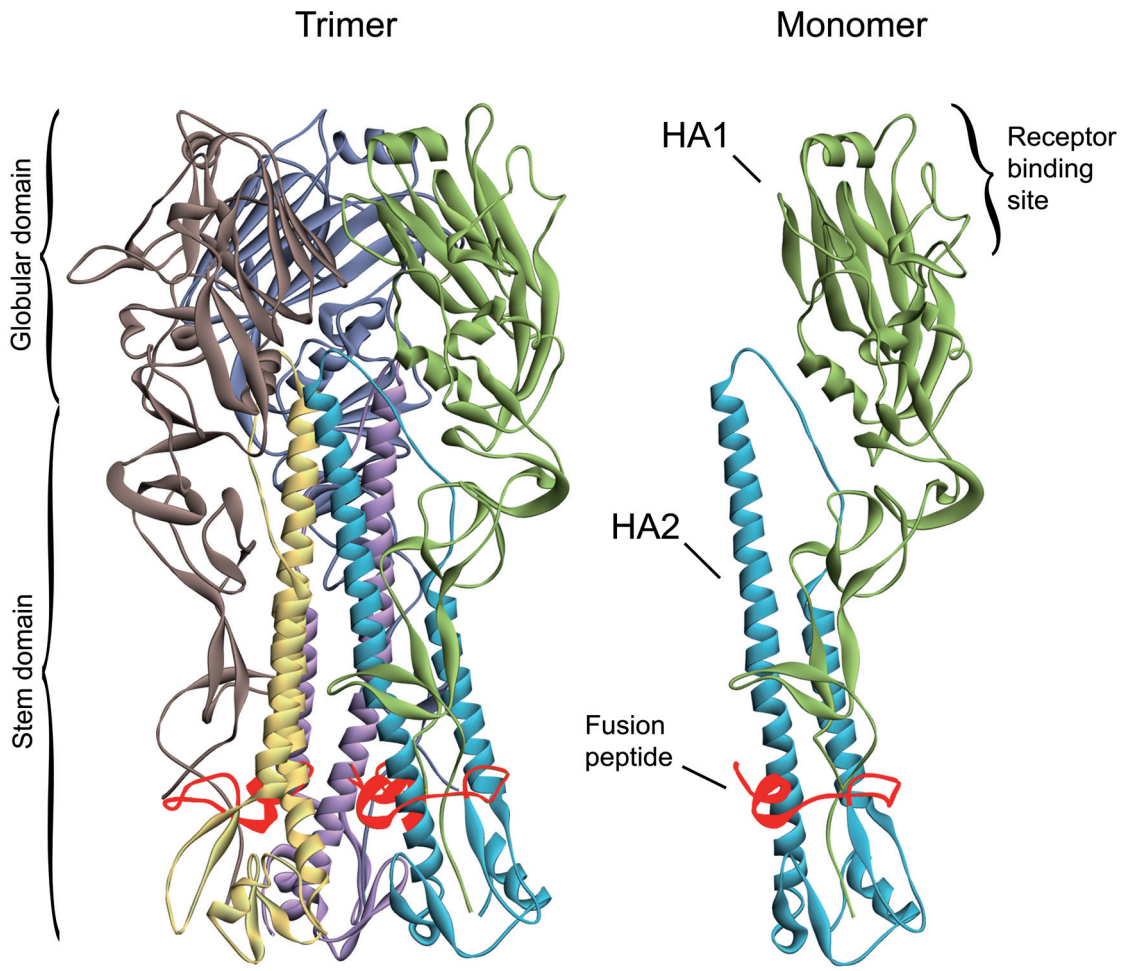

Fig. 1

The structure of hemagglutinin (trimer and monomer) as determined by RTG- analysis

HA1 and HA2 chains of each monomer of HA trimer are differently colored: HA1 gp green, grey and brown, HA2 gp blue, violet and yellow. The first 23 aa of HA2 N-terminus represent fusion peptide (red). The figure was created in Discovery Studio 4.1 visualizer. Source: PBD ID: 1RU7.

2.1 Receptor binding activity and IAV host tropism

The essential step for initiating the virus replication is the recognition and virus binding to the receptors of permissive cells. This property was found already in the forties of the 20th century due to IAV's ability to agglutinate erythrocytes (Hirst, 1941). As the name suggests, hemagglutinin binds to virus receptors present on the surface of erythrocytes and, consequently, is responsible for the agglutination of red blood cells mediated by influenza virus. Later it was shown that hemagglutination mediated by IAV is inhibited in the presence of antiviral serum as well as in the presence of viral or bacterial neuraminidase activity (Gottschalk *et al.*, 1959). Cell-surface receptors for IAV are terminally-linked sialic acids (SA) of glycoproteins and glycolipids present on the host cells (Gottschalk *et al.*, 1959).

The structure of RBS on HA allows the differentiation of the permissive host cells according to the character of linkage between terminal sialic acid and galactose on the cell surface receptors. Sialyloligosaccharides bearing terminal sialic acid linked by Sial(α-2,3)Gal or Sial(α-2,6)Gal linkage have different molecular shapes, therefore, they can be discriminated by IAV according to the aa structure of RBS. In general, human IAV strains preferentially recognize the sialic acid bound to the galactose by α-2,6 glycosidic linkage present on oligosaccharides of non-ciliated cells of the respiratory tract, while the avian isolates prefer the terminal SA bound to the galactose by α-2,3 glycosidic linkage on oligosaccharides of ciliated cells in avian gastrointestinal tract (Skehel and Wiley, 2000). It was confirmed that amino acids in positions 226 and 228 on HA1 gp are responsible for this preference of virus binding. When aa glutamine or glycin are present in these positions,

the IAV binding to Sial(α-2,3)Gal linkage is preferred, while Sial(α-2,6)Gal linkage is preferred when leucin or serin are in these positions (Connor *et al.*, 1994; Cross *et al.*, 2001). In the upper respiratory tract of humans prevail the epithelial cells with α-2,6 terminally-linked SA receptors. Towards the lower respiratory tract, the percentage of α-2,3-linked SA receptors on epithelial cells increases and nears to that of lung alveoli (Wilks *et al.*, 2012). On the other hand, α-2,3 terminally-linked SA receptors prevail at the site of IAV replication in birds, i.e. their gastrointestinal tract, but also in equine respiratory tract. Because of these differences, in general, avian and equine IAVs are usually not transmitted to humans. Chickens and quails, however, represent exceptions. In their intestinal epithelium are present both SA(α-2,3) as well as SA(α-2,6) receptors (Wan and Perez, 2006; Guo *et al.*, 2007). Similarly, in the epithelium of swine respiratory tract are also present both SA(α-2,3) and SA(α-2,6) receptors (Sriwilaijaroen and Suzuki, 2012). These are important facts from the epidemiological point of view. Moreover, it was shown that pigs can be infected by avian as well as by human IAV viruses, even simultaneously. Therefore, pigs represent a potential source of mixed IAVs with a new phenotype, able to infect humans, and could play a special role in biology of IAV viruses. A different cell receptor was found to be recognized by pandemic IAV virus A/California/04/09 (H1N1) as documented by Childs *et al.* (2009). In *in vitro* studies, this virus recognized not only α-2,6, but also α-2,8 glycosidically-linked SA.

It has to be mentioned here that the specificity of binding to the Sial(α-2,6)Gal- or Sial(α-2,3)Gal-linked receptors on the host cells is also influenced by affinity of binding, which is dependent on the temperature at the site of virus attachment and replication. Moreover, the strength of IAV attachment to the cell surface, mediated by HA, is influenced also by other viral proteins, prominently by neuraminidase (NA) with its esterase activity, which closely cooperates with HA during the binding of the virus to permissive cells and during the release of newly assembled (budding) viruses from the cell-surface (will be discussed later).

2.2 Fusion activity and structural rearrangements of HA during the membrane fusion

The second important role of HA during the replication cycle of IAV is to mediate the fusion of viral and endosomal membranes. After the attachment of the virus to the cell receptor and its endocytosis, the low pH causes the refolding of HA trimer into the fusion active form. The fusion peptide is embedded into the endosomal membrane, leading to the formation of the fusion pore required for the release of viral genome into the cytoplasm and its transport to the site of vRNA replication. The cleavage of HA0 to HA1 and HA2 chains is essential for priming of HA fusion activity. It results in the release of a free N-terminus of HA2 gp (fusion peptide), which comprises approximately 20 to 25 aa (Shaw and Palese, 2013; Sriwilaijaroen and Suzuki, 2012). In acidic environment, HA undergoes large structural changes leading to the activation of HA fusion potential and resulting in fusion of membranes or, in the absence of target endosomal membrane, in inactivation of virus infectivity (Sriwilaijaroen and Suzuki, 2012) (Fig. 1). The correct timing of conformational change related to the decrease of pH in the endosome is, therefore, important for the pathogenesis of the infection. Too early release of the genome into the cytoplasm results in vRNA transport to the perinuclear area, while a too long stay of the virus in the endosome could cause virus inactivation (Costello *et al.*, 2014). The optimal pH for the activation of HA fusion potential correlates with complete refolding of HA trimer, which is irreversible.

Spontaneous acquirement of thermodynamically more stable fusion active conformation at neutral pH is prevented by intramolecular bonds among HA monomers. In endosomes, the pH decrease causes weakening of intermolecular bonds in the HA trimer, the distance between globular HA monomers increases and the formely closed globular head is opened. HA2 chain, in native conformation hidden inside the HA trimer, is exposed from the trimer at low pH and its N-terminus is inserted into the target membrane. However, the ability of the virus to bind to the cell surface receptors, as well as disufide bond between HA1 and HA2 in monomers, still remain preserved. This conformational change requires energy. It was shown, however, that approximately 3–7% of HA trimers can undergo such conformational change spontaneously (Kostolanský *et al.*, 1988; Varečková *et al.*, 1993).

HA conformation is influenced by the ionizable amino acid residues at the critical sites of HA trimer, which are the fusion peptide (interacting with the fusion peptide pocket), coiled-coil regions of HA2, and the site of interaction of HA1 and HA2 subunit surfaces (Wang *et al.*, 2015). The globular head formed by HA1 subunits is, at neutral pH, positively charged, while HA2 has a negative charge (Huang *et al.*, 2002; 2003). The intermolecular bonds at the interface between HA1 and HA2 chains contribute to the stability of influenza HA in the acidic environment, which depends on the composition and properties of aa (Wang *et al.*, 2015). Those aa that are preferentially protonated at low pH, play the most important role in the 3-D structure of HA. Such molecule is histidine, which is protonated after the drop of pH under the value of its effective pKa. The positively charged histidines in the molecule of each HA monomer are then strongly repulsed and become distanced. Consequently, HA2 is relocalized to the endosomal membrane (Kampmann *et al.*, 2006; Thoennes *et al.*, 2008). There are several histidine residues in the HA molecule, but the highly conserved His in the position 184 of HA1 was defined as the crucial amino acid contributing to the conformational change of HA. However, the mutations in the amino acids adjacent to this histidine influence its pK_a and, consequently, also the pH optimum of fusion (Mair *et al.*, 2014). More than

90% of human isolates have in the critical positions 205 of HA1 and 72 of HA2 amino acid pair R-H or K-H, while avian viruses have K-N pair of amino acids. The substitutions of these amino acids led to the change of HA stability and pH optimum of the fusion (Mair *et al.*, 2014).

The acidic environment in endosomes causes the complete structural change of HA2 gp (Fig. 2). The N-terminus of HA2 in aa positions 56–75 changes its conformation from the loop to α-helix and in aa position 106–112 α helix is changed to a loop. The consequence is the opposite orientation of HA2 C-terminus

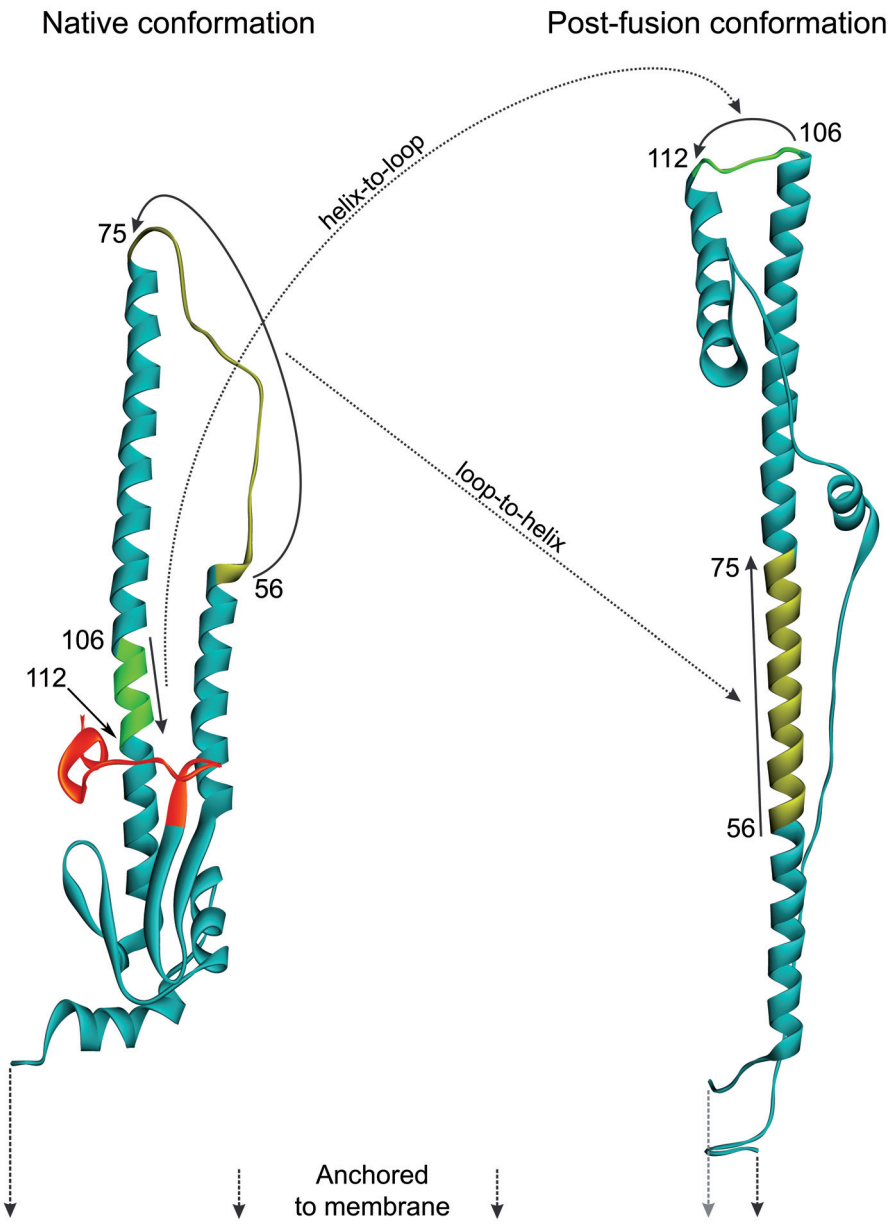

Fig. 2

Conformational changes of HA2 induced by low pH in endosomes

After endocytosis of viral particle, the pH of the virus environment decreases. Consequently, the native (metastable) conformation of HA undergoes structural changes resulting in the fusion of viral and endosomal membranes. The monomers in HA trimer detrimerize, i.e. globular parts of the heavy chain (HA1) are deflected (not shown for simplicity). The light chain (HA2) is released from its trapped position inside the HA trimer and undergoes several conformational transitions towards the stable (irreversible) post-fusion conformation: the region comprising aa 56–75 converts from loop to helix and region in aa position 106–112 converts from helix to loop. The first 23 aa of HA2 N-terminus represent the fusion peptide (red). (The figure was created in Discovery Studio 4.1 visualizer. Source: PBD ID: 1HGF, 1QU1 and modified according to Varečková *et al.*, 2013).

(Skehel and Wiley, 2000; Sriwilaijaroen and Suzuki, 2012; Varečková *et al.*, 2013). The extension of helix structure leads to the spring-out of N-terminus of the HA2 from the cavity near the virus envelope and its anchoring into the target membrane via strong hydrophobic interactions, while the C-terminus of HA2 still remains integrated into the virus membrane (Harrison, 2008). At this step the prolonged intermediate collapses after interconnection of N- and C-membrane HA2 anchors, resulting in hemifusion and fusion pore formation (Hamilton *et al.*, 2012; Sriwilaijaroen and Suzuki, 2012).

The HA conformational change is pH- and temperature-dependent. It can be triggered by lowering the pH at 37°C, or by temperature increase (up to 60°C) at neutral pH (Ruigrok *et al.*, 1986; Shekel and Wiley, 2000). This conformational change is irreversible. The exposure of HA to low pH before virus endocytosis results in loss of HA ability to mediate the membrane fusion and consequently in virus inactivation. Conformation change of HA is therefore an important step preceding the viral and endosomal membrane fusion. While many details of the mechanism of fusion are not yet clear, the principal conditions required for fusion triggering have been described (Fig. 3). The details of the mechanism of membrane fusion are studied by new chemical and physical methods, thus enabling a more precise characterization of this universal process essential for the transport of various molecules, or their engulfment from the environment.

3. The HA cleavability by host proteases and HA fusion activation pH as factors of IAV virulence and pathogenicity

The cleavage activation and membrane fusion are closely associated steps of IAV replication cycle, which can influence the IAV virulence. The pH optimum of HA0 cleavage and consequently of the membrane fusion is limited not only by structure of HA and HA folding, but it is determined also by the route of infection and the site of replication of IAV in the particular host.

3.1 Cleavage activation of HA as a determinant of IAV pathogenicity

Already in the year 1979 it was shown that IAV pathogenicity in chickens directly correlated with the ability of the virus to produce cleaved HA in the infected cells and

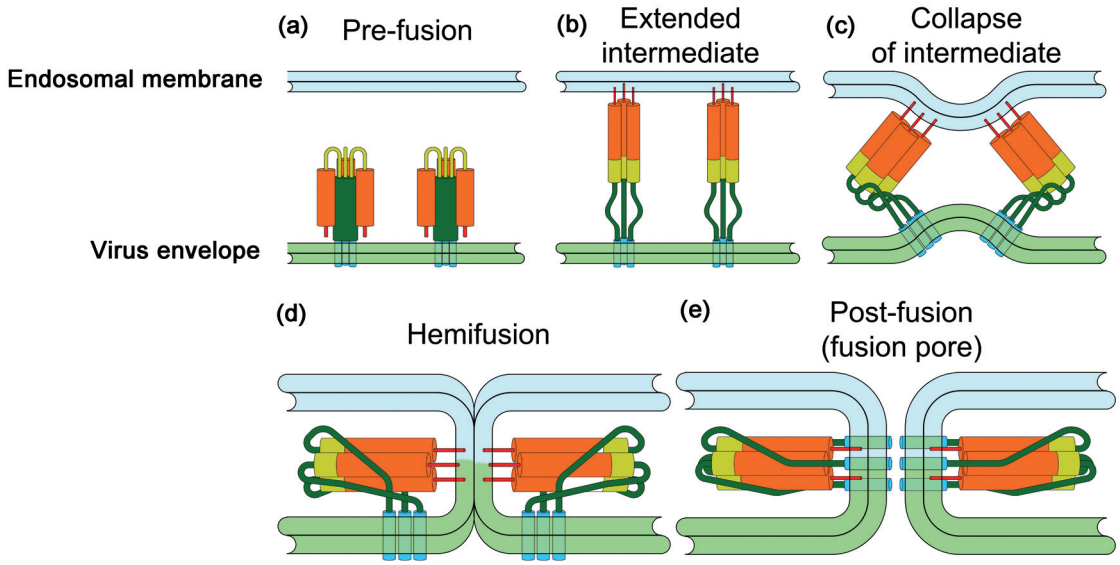

Fig. 3

Schematic illustration of membrane fusion mediated by hemagglutinin

After the virus is endocyted, the HA trimer in pre-fusion conformation (a) undergoes several subsequent structural transitions due to the pH decrease in the endosome. At first, the pre-fusion conformation is changed by acidification of the environment resulting in detrimerization of HA globular domain (not shown). Consequently, the N-terminus of fusion peptide is exposed from HA trimer and targeted towards the endosomal membrane. This exposition is accompanied by complex refolding of the HA2 molecule and an intermediate with extended helix creates link between viral and endosomal membranes (b). Subsequently the intermediate collapses and the most energetically stable conformation is created, which brings both membranes to close proximity (c). The membranes are pulled together to be partially fused (hemifusion) (d). The hemifusion ends up as pore formation (e) and viral genome is released into the cytoplasm. Fusion peptide – red color, C-terminal anchor of HA2 – aquamarine color. Figure adapted according to Harrison (2008).

to form plaques on various cell cultures without addition of trypsin (Bosch *et al.*, 1979). Based on aa sequence at the cleavage site of HA, avian IAVs can be characterized as low pathogenic (LPAI) and highly pathogenic (HPAI) isolates. As HPAI are considered those viral strains of H5 and H7 subtypes, which comprise multibasic aa sequence in HA cleavage site. HA of these viruses is cleaved intracellularly (Skehel and Wiley, 2000). Human viruses belong to LPAI strains, as they contain a monobasic cleavage sequence represented by arginin or lysin. In this case, the HA0 is cleaved at the surface of infected cells or on the released particles (Skehel and Wiley, 2000). Host serine proteases (like trypsin or tryptase Clara) produced by bronchial epithelium are responsible for HA0 cleavage (Skehel *et al.*, 1982). That is the reason why virus replication in humans is restricted to the respiratory tract, the site where such enzymes are present. In contrast, HA of HPAI strains is cleavable by subtilisin-like proteases present in various organs of the host. Therefore, HPAI are able to replicate in organs where such proteases are present and can cause damage to the tissue at the site of replication (Capua and Alexander, 2002; Hilleman, 2002). To the complications of the IAV infections often contribute also bacterial proteases, which are produced during bacterial superinfections by such bacterial strains as are *Staphylococcus aureus* or *Aerococcus viridans*, and which can activate the HA by its proteolytic cleavage with an impact on the efficacy of virus replication (McCullers, 2006; Böttcher-Friebertshäuser *et al.*, 2013).

3.2 Fusion activation pH as a determinant of IAV virulence

The conformation changes of HA resulting in the viral and endosomal membrane fusion are the consequence of the endosomal environment acidification (Wharton *et al.*, 1986). The degree of environment acidification required for the conformation change of HA, activating its fusion potential, is strain-specific and is an important factor influencing the IAV virulence. The pH needed to trigger the conformation change of HA has an impact on the kinetics of replication and correlates with the HA stability at low pH and the preservation of virus infectivity. There were defined more than 70 critical aa positions on HA of different HA subtypes, which alter the pH of HA conformation change at which the membrane fusion is triggered (Russell, 2014).

First studies enabling the attribution of fusion pH optimum elevation to particular amino acid substitutions were described by Daniels *et al.* (1985). They characterized several mutants with pH optimum of conformational change shifted to higher pH values in comparison to the wild type virus (wt). Mutants of influenza A viruses of H3N2 subtypes were obtained by propagation of virus in the cell culture in the presence of amantadine hydrochloride, which is known to increase the pH in endosomes. Nineteen aa substitutions were found in HA of obtained amantadine-resistant mutants. Changes were mostly in the HA2 gp. The only substitution found in HA1 gp was in position 17. In this position the original histidine has been substituted by arginine. The impact of this substitution was the change of the intermolecular ionic interactions in the local area leading to the increase of pH (Δ + pH 0,7) triggering the dissociation of monomers at the site of HA1 and HA2 interaction (Daniels *et al.*, 1985).

Later, more aa substitutions in HA influencing the pH optimum of fusion *in vitro* were reported (Daniels *et al.*, 1985; Steinhauer *et al.*, 1995; Hoffman *et al.*, 1997; Cross *et al.*, 2001; Thoennes *et al.*, 2008; Xu and Wilson, 2011) (Table 1, Fig. 4). In IAV of H3 subtype the substitutions in aa positions H17Y on HA1gp and K51A on HA2 decreased the pH optimum of fusion activation from pH 5.2 to 4.9 (Thoennes *et al.*, 2008). Mutant HAs with these substitutions were more stable at the lower pH than the parental strain. Authors obtained also double mutants with substitutions in HA2 gp ($H106_2A$, $T111_2A$ and $H106_2F$, $T111_2V$), which shifted the pH of the HA conformation change by 0.4 pH units higher in comparison to the wild type virus. However, these viruses with double mutations in HA did not mediate polykaryocyte formation, which indicated that membrane fusion was not completed.

Table 1. Amino acid substitutions in HA resulting in shift of pH optimum of fusion

HA	Position	Aa substitution	pH shift triggering the fusion	HA subtype	References
HA1	17	H → R	↑ pH 0.7	H3	Daniels *et al.*, 1985
HA1 HA2	17 51	H → Y K → A	↓ pH 0.3 (5.2–4.9)	H3	Thoennes *et al.*, 2008
HA2	58	K → I	↓ pH from 0.1 up to 0.7	All subtypes except for H11	Byrd-Leotis *et al.*, 2015
HA2	106	R → H	↓ pH 1.0 (5.2–4.2)	H2	Xu and Wilson, 2011
HA2	106 111	H → A → F T → A → V	↑ pH 0.4	H3	Thoennes *et al.*, 2008
HA2	112	D → G	↑ pH from 0.2 up to 0.6	All subtypes except for virus H5N1 /VietNam	Byrd-Leotis *et al.*, 2015

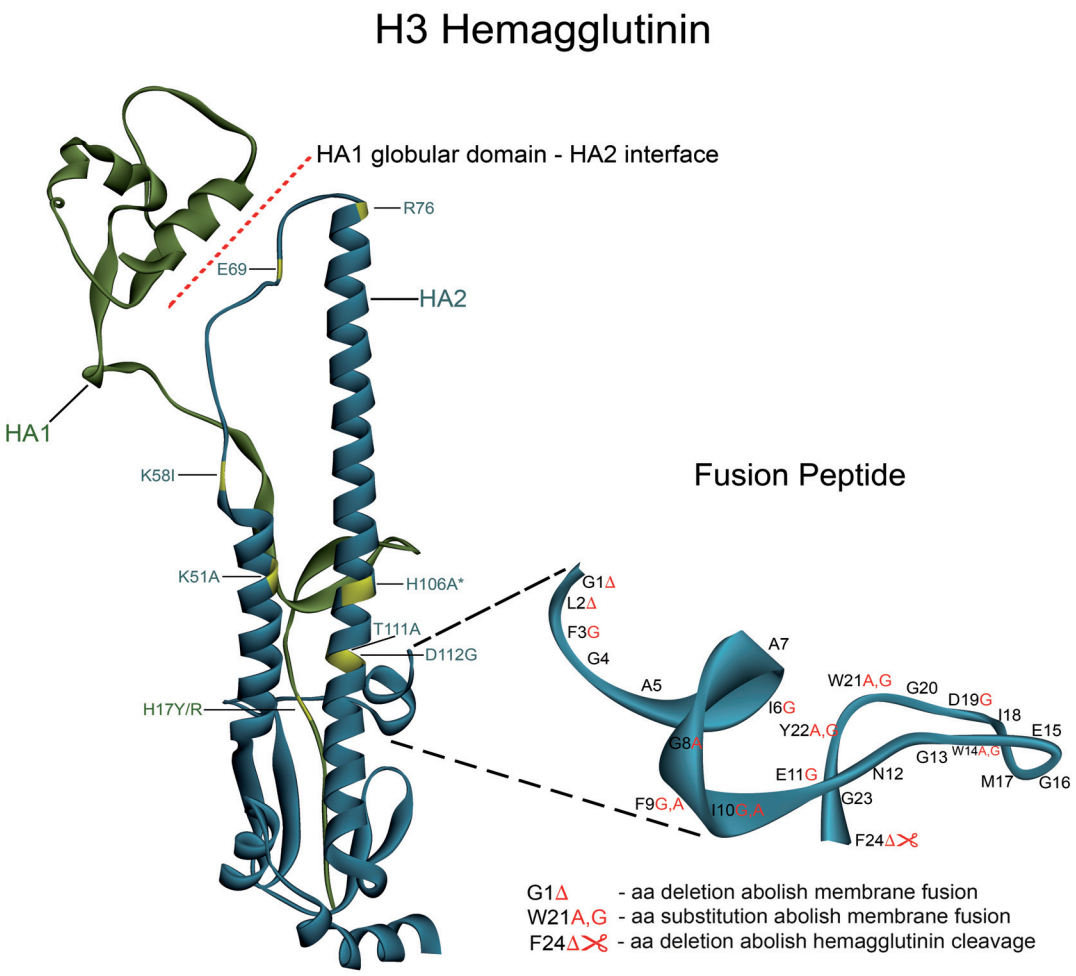

Fig. 4

Mutations in HA altering or preventing the membrane fusion

The mutations affecting the pH optimum of membrane fusion are pointed out on ribbon structure of HA. For simplicity, the heavy chain (green) is only partially shown. Highlighted amino-acid substitutions and important aa regions are described below. The first 24 amino acids of N-terminus of HA2 (fusion peptide) are indicated in details. Amino acid deletions and substitutions influencing the function of fusion peptide are depicted. The substitutions at the interface between HA1 and HA2 (red dashed line) have a potential to influence the pH optimum of HA-mediated membrane fusion. *The position 106 in HA2 is conserved in both phylogenetic groups of HA subtypes (R and K are conserved in the H1 group and H is conserved in the H3 group). (The figure was created in Discovery Studio 4.1 visualizer. Source: PBD ID: 4FNK, the H3 numbering was used).

The substitution of amino acid lysine in position 58 of HA2 gp, highly conserved among all subtypes (except H11, which has arginine in this position), to isoleucine led to a decrease of pH optimum of *in vitro* fusion by about 0.7 units (Byrd-Leotis *et al.*, 2015). This decrease was associated with a better stability of HA in the acidic pH. On the other hand, substitution D to G on HA2 gp ($D112_2G$) increased pH optimum of fusion in all subtypes (except H5N1 A/VietNam/1204/2004 virus) and was the reason of a lower HA stability at the acidic pH (Daniels *et al.*, 1985; Weis *et al.*, 1990; Byrd-Leotis *et al.*, 2015).

The HA2 area restricted by aa 106–111 forms a structure that changes at the low pH from helix to loop. Therefore, aa substitutions in this region could also influence the HA stability (Thoennes *et al.*, 2008). The position 106 of HA2 is conserved within both groups of antigenic HA subtypes. In the first group, comprising subtypes H1, H2, H5, H6, H8, H9, H11, H12, H13 and H16 there is aa arginine or lysin and in the second group (H3, H4, H7, H10, H14 a H15) the aa histidine is conserved in the same position (Ha *et al.*, 2002). The substitution R to H in the position 106 of HA2 of H2 subtype significantly increased the HA stability in the range

Table 2. Amino acid changes in the fusion peptide with a biological impact

Position	Aa substitution	Consequence	HA subtype	References
1	G deletion	Disruption of fusion activity	H3	Steinhauer *et al.*, 1995
2	L deletion		H3	
3	F → G		H3	Cross *et al.*, 2009
6	I → G		H3	Cross *et al.*, 2001
8	G → A		H3	Steinhauer *et al.*, 1995
9	F → G, A		H3	Cross *et al.*, 2001;
10	I → G, A		H3	Lai *et al.*, 2007
11	E → G		H1	Nobusawa *et al.*, 1995
14	W → G, A		H3	Cross *et al.*, 2009
19	D → G		H1	Nobusawa *et al.*, 1995
21	W → G, A		H3	Cross *et al.*, 2009
22	Y → G, A		H3	
24	F deletion	Disruption of cleavage	H3	Langley *et al.*, 2009

of pH from 5.2 to 4.2 (Xu and Wilson, 2011). Another region changing its structure from loop at neutral pH to helix after acidification is between aa 56–75 of HA2. The aa 101–111 of HA1, which interact with HA2 at neutral pH, stabilize the metastable structure of HA and prevent the dissociation of HA1 and HA2 (DuBois *et al.*, 2011). In this region there are also two conserved aa: R76 a E69 on HA2, which create „salt bridges" with aa E107 and K109 of HA1 (DuBois *et al.*, 2011). Therefore, these aa could be crucial for the preservation of fusion activity (Fig. 4).

The fusion peptide, due to its function and its conservation in otherwise variable HA glycoprotein, is the most studied part of HA2. To preserve the function of the fusion peptide, only mutations ensuring its correct structure and activity are allowed (Steinhauer, 2010). Many described aa substitutions or deletions led to the loss of the HA fusion activity (Table 2). Essential for the fusion activity are glycin in position 1 of HA2, and leucin in position 2, the deletion of which abolished the fusion activity (Steinhauer *et al.*, 1995). Later also other authors reported substitutions in the N-terminus of HA2 leading to the loss of fusion activity. There were localized in the positions from 3 to 24 of the fusion peptide: substitution F to G in position 3 (Cross *et al.*, 2009), aa substitution I to G (position 6) (Cross *et al.*, 2001), aa substitution G to A (position 8) (Steinhauer *et al.*, 1995), in the position 9 F to G (Cross *et al.*, 2001) or F to A (Lai and Tamm, 2007), in position 10 I to G (Cross *et al.*, 2001) or I to A (Lai and Tamm, 2007), substitution E to G in position 11 (Nobusawa *et al.*, 1995), W to A or G in position 14 (Cross *et al.*, 2009), D to G in position 19 (Nobusawa *et al.*, 1995), substitution W to A or G (position 21) and Y to A or G (position 22) (Cross *et al.*, 2009). Deletion of F in position 24 of HA2 of H3 subtype caused incorrect HA-folding and consequently the loss of HA cleavability (Langley *et al.*, 2009). The higher is the aa conservation of the fusion peptide among different IAV strains, the less are substitutions or deletions tolerated (Cross *et al.*, 2009).

Table 3. Amino acid changes in HA related to the interspecies IAV transmission

HA	Position	Aa substitution	Impact on the activity	HA, NA subtype	Notes	References
HA1	23	Y → H	↑ pH 5.9–6.3	H5N1 (A/chicken/ VietNam/ C58/04)	Avian to mice	Reed *et al.*, 2009; Zaraket *et al.*, 2013
HA1	24	H → Q	↓ pH 5.9–5.6			
HA2	58	K → I	↓ pH 5.9–5.4			
HA1	158	N → D	Change of receptor specificity	H5N1 H5 (backbone A/H1N1/ pdm09) A/Indonesia/ 5/2005	Avian to ferrets	Imai *et al.*, 2012; Herfst *et al.*, 2012
	224	N → K				
	226	Q → L				
	318	T → I	↓ pH 5.9–5.5			
	138	A → S	Support α-2,6 binding	H7N9 human isolates		Kageyama *et al.*, 2013; Ramos *et al.*, 2013; Xiong *et al.*, 2013; Zhang *et al.*, 2013; Zhou *et al.*, 2013
	186	G → V				
	226	Q → L/I				
	160	T → A	Loss of glycosylation site			

4. The role of fusion activation pH and stability of HA in the adaptation of IAV to the new host

The adaptation to the new host is closely related to the virulence of IAV. It is known that avian IAVs, which are believed to be ancestors of all influenza A viruses, have a potential to overcome the interspecies barrier and infect mammals, including humans. While in humans and mammals as horses, pigs, marine mammals, but also in poultry, influenza infections are associated with more or less expressed clinical symptoms of the disease, IAV infections in wild aquatic birds are generally subclinical. It was documented that avian IAV can cause zoonotic infections in humans. Most of these infections are so-called „dead-end", which means they are not further spread among the individuals of the new host (Mänz *et al.*, 2013). The lack of avian IAV transmission in new host is the result of differences in their interaction with the new host, which require adaptation changes in IAV genome. Therefore, many studies are focused on the characterization of mutations associated with the interspecies transmission. HA is considered to be the most important viral protein responsible for IAV virulence in the new host.

Different HA subtypes display characteristic phenotypes that result from the cleavability of their HA by host proteases, the pH- and temperature- stability of HA, consequently influencing their different kinetics of fusion. To understand these processes, still more data concerning the pH-dependent conformational change of HA in connection with the avian IAV adaptation to mammal/human host are published. The most important observation revealed that the pH optimum of HA conformation change of HPAI viruses is nearly pH 6, while human epidemic viruses are stabile at this pH and require pH around 5 for the HA conformational change, depending on the strain (Zaraket *et al.*, 2013). Viruses, the HA of which undergoes the conformational change at pH higher than 5, can release their genome from endosome faster, posing a growth advantage to them in comparison to the viruses requiring pH 5 for fusion activation. On the other hand, HA of viruses with low pH optimum of fusion activation are more stable in the acidic environment, what can consequently be beneficial.

Waterfowl is considered to be a natural reservoir of IAV, but a complex characterization of avian IAV is still missing. The most studied are H5N1 viruses, because in 1997 a strain of this subtype was isolated from humans and was considered as a dangerous potential pandemic virus (Subbarao and Shaw, 2000). Until the end of the year 2015, already 846 cases of human infections with H5N1 viruses with more than 50% mortality were confirmed (http://www.who.int/influenza/human_animal_interface/). In most cases, the source of human infection was direct contact with infected poultry. Recently, however, several cases of human-to-human transmission of infection caused by a highly pathogenic avian IAV were documented (Beigel *et al.*, 2005; Ungchusak *et al.*, 2005; Eurosurveillance, 2006). Fortunately, such transmissions are rare.

To study the process of IAV adaptation to new hosts, several animal models are used. Results of studies obtained using these models should be evaluated carefully, taking into account the complex characteristics of the particular animal model. As a model organism for the interspecies transmission of IAV and for *in vivo* adaptation studies of avian IAV to mammals are preferentially used mice (Table 3). Mice were infected with wild IAV H5N1 with fusion activation pH 5.9 and then with viruses with confirmed changes of fusion activation pH acquired by introducing mutations in the heavy chain of HA $Y23_1H$ (pH 6.3), $H24_1Q$ (pH 5.6) and in the light chain of HA: $K58_2I$ (pH 5.4) (Reed *et al.*, 2009; Zaraket *et al.*, 2013). These mutations did not have an impact on the protein expression, cleavage, or the virus binding to the cell receptor. The mutation, which lowered pH of HA conformational change from 5.9 to pH 5.4, resulted in higher replication activity of this mutant virus in the nasal cavity of mice in comparison with the parental virus. The decrease of the pH threshold of HA conformational change supports the adaptation of avian IAV H5N1 to the mammalian host. However, it is not the only factor contributing to the host process of IAV adaptation. On the other hand, the mutant virus with pH optimum of fusion 6.3 was not infectious in mice, or in birds (Zaraket *et al.*, 2013). The probable reason was that the environment in the nasal cavity of mice is, similarly as in humans, slightly acidic, ranging from pH 5.5 to 6.3 (Washington *et al.*, 2000). Therefore, virus could be inactivated before its entry into the target cells.

Very useful model for IAV adaptation studies are ferrets. Ferrets were used as animal model for *in vivo* influenza infection since 1933. The disease symptoms in ferrets are very similar to human infection. Controversial data published in the year 2012 (Imai *et al.*, 2012; Herfst *et al.*, 2012) describing the mutations essential for IAV transmission of avian influenza viruses among ferrets split the scientific community into two groups. The first considered them to be the instruction how to construct the new pandemic virus and the second considered these observations to be crucial and important for further scientific work. Imai *et al.* (2012) described mutations in HA1 required not only for adaptation of avian IAV to mammals, but also for spread among them: N158D, N224K, Q226L a T318I. Mutation N158D is responsible for the loss of glycosylation site, N224K a Q226L are needed for the change of receptor specificity and T318I decreases pH of HA fusion activation. When the constructed virus did not have the last T318I aa substitution, pH of HA activation was 5.9, i.e. by 0.2 higher than in wild type with pH 5.7 and virus replication in ferrets was restricted. After introducing the fourth exchange (T318I), pH of fusion has been changed to 5.5, which consequently increased the stability of HA and contributed to the transmissibility of this virus among ferrets (Imai *et al.*, 2012). Very similar substitutions were reported by the Herfst research group (Herfst *et al.*, 2012). After

virus passage in ferrets, they found aa changes in HA never before described in "*in vitro*" or "*in vivo*" experiments. Such results are important from the point of view of designing the human IAV vaccine with protective potential against avian IAV, when the pH of HA activation of vaccine strain should be considered. The recently prepared live attenuated vaccine against HPAI virus of H5N1 subtype met with disappointment, since no protective effect has been achieved (Krenn *et al.*, 2011). The limiting factor of immunogenicity of this vaccine was probably the low pH stability of H5 hemagglutinin of vaccine virus. By introducing $K58_2I$ mutation into HA of the H5 vaccine strain, the pH of fusion activation decreased by 0.5 (Reed *et al.*, 2009). Test of stability of this mutant in the acidic environment showed that virus is inactivated at lower pH than the wild type virus and was successfully used as a vaccine strain. The immunization with this strain, in contrast to the wild parental strain, led to increased immune response of mice (Krenn *et al.*, 2011).

Avian influenza viruses of H7 or rarely H10 subtype cause the human disease, which can also have fatal consequences (Garcia-Sastre and Schmolke, 2014). Avian viruses therefore represent continuing pandemic threat for humans. First infection of humans with IAV of H7N9 subtype was described in March 2013. Three individuals were infected with this virus with a fatal outcome (Gao *et al.*, 2013). From that time until the beginning of the year 2016, 751 cases of H7N9 infections of humans were reported, 294 of which died as a result (http://www.fao.org/ag/againfo/programmes/en/empres/H7N9/situation_update.html). The phylogenetic analysis showed that this virus was a triple reassortant. HA originated from duck virus H7N3, NA from H7N9 virus isolated from wild birds, other segments originated from H9N2 virus (Gao *et al.*, 2013). Sequence analysis of a H7N9 human isolate confirmed mutations in HA1 of this H7 subtype, which were shared also with H5 HA. These mutations were acquired during the adaptation of the avian IAV to the mammalian host. The substitutions A138S, G186V, Q226L/I (H3 numbering) led to the change of receptor specificity and supported the attachment of HA to SA(α 2,6) glycosidically-linked receptors. The substitution T160A resulted in the loss of glycosylation site (Kageyama *et al.*, 2013; Ramos *et al.*, 2013; Xiong *et al.*, 2013; Zhang *et al.*, 2013; Zhou *et al.*, 2013; Zhu and Shu, 2015). However, the correlation between the fusion activity of HA and the pathogenicity of these viruses has not yet been studied. Such characteristics would help to predict the pandemic potential of those mutants of avian IAV viruses, which caused human infections and adapted to humans.

5. Other IAV proteins influencing the virulence

As described above, the cleavability of HA by host proteases, its glycosylation, pH stability of HA trimer, as well as pH optimum of fusion, determined by the structure of HA, are factors influencing the infectivity and virulence of IAV. But many further studies showed that not only HA, but also products of other IAV genes can strongly influence the IAV infectivity and virulence.

Studies using influenza A mutants showed that the second surface glycoprotein, neuraminidase (NA), can also influence the biological properties of IAV (Mayer *et al.*, 1973). Particularly, its activity must be evaluated in relationship to HA properties, as it can influence the effective binding of HA to the receptors on the cell surface as well as the fusion activity of HA. A special case is the NA of A/WSN/33 of H1N1 subtype, which is able to bind to plasminogen and enables the cleavage of HA precursor to HA1 and HA2 gps even in the absence of trypsin protease (Lazarowitz *et al.*, 1973; Goto and Kawaoka, 1998). Due to this activity virus can more easily acquire the infectivity, determining the measure of its pathogenicity (Goto *et al.*, 2001). The virulence of IAV is influenced also by the length of the NA stem protruding from the viral envelope, on which the tetramer „head" of NA with the active center of the enzyme is localized. The optimal length of NA stem is required for virus infectivity and pathogenicity. The NA stem shortened by about 15-20 aa was observed in some H5N1 IAVs isolated since the year 2000 (Wang *et al.*, 2006). Shortening of NA stem might consequently lead to the lower enzymatic activity of NA, influencing *in vitro* replication ability of the virus as well as the host tropism of the virus (Baigent and Cauley, 2003; Li *et al.*, 2011). The compatibility of NA and HA is needed for productive replication of viruses (Hulse *et al.*, 2004), therefore, it is not surprising that the presence of one point mutation in HA can give rise to another mutation in NA and vice versa, to maintain the equilibrium between receptor binding of the virus and its release from the infected cell surface (Maines *et al.*, 2011). Further parameter, which can influence the IAV virulence, is the stability of NA at low pH. Avian and pandemic IAVs isolated in the years 1918, 1957 and 1968 have preserved its NA activity at low pH, while the epidemic viruses are not so stable at low pH (Takashi *et al.*, 2012). Moreover, it was shown that the enzymatic activity of NA influences the *in vitro* fusion activity of HA, as the HA expressed from plasmid DNA mediates membrane fusion more effectively in the presence of NA and that the cleavage of sialic acid residues increases the HA-mediated membrane fusion (Su *et al.*, 2009; Galloway *et al.*, 2013).

An essential role in virulence and the replication ability of IAV plays virus polymerase. The IAV polymerase is composed of three polymerase subunits (PB2, PB1, and PA) and together with the nucleoprotein (NP) forms the ribonucleoprotein (RNP) complex with viral RNA-dependent RNA-polymerase activity. This complex, unlike the surface antigens HA and NA, is highly conserved among all IAVs. However, the host-adaptation mutations were found in all subunits of viral RNA

polymerase (Miotto *et al.*, 2010). In general, they correlate with enhanced replication ability of avian viruses in mammals. Of them, the most important protein for the process of IAV adaptation is PB2, encoded by viral genome segment 1. It has three functional regions: the N-terminus, which interacts with PB1subunit (Ruigrok *et al.*, 2010), the C-terminus, which is responsible for the transport of PB2 into cell nuclei (Tarendeau *et al.*, 2007) and the central part of PB2, which comprises the binding site for host-cell 5'-cap pre-mRNA. The PB1 protein, encoded by viral genome segment 2, interacts with both the PB2 and PA subunits (Shaw and Palese, 2013). Protein PA has endonuclease activity, which is important during "cap snatching" from host mRNA and the start of the IAV replication. NP is the major viral protein in the RNP complex and forms the "scaffold" for the viral RNA (Gabriel and Fodor, 2014). In spite of high conservation of these proteins, host-specific amino acid substitutions were identified during the process of adaptation of avian viruses to the new host and are considered as host markers of IAV viruses. The adaptive changes were identified in all three IAV polymerase subunits (PB2, PB1 and PA) as well as in NP (Mänz *et al.*, 2013). The most frequently described adaptive mutations are E627K and D701N in PB2 protein (Subbarao *et al.*, 1993; Salomon *et al.*, 2006; Hatta *et al.*, 2007; Kim *et al.*, 2010; Gabriel and Fodor, 2014). These substitutions probably influence the replication activity in relation to the temperature. The temperature at the site of IAV replication in humans is 33–35 °C, while the replication in birds takes place in the gastrointestinal tract at 38–40 °C (Hatta *et al.*, 2007). Described substitutions in PB2 were shown to enhance the polymerase activity at lower temperature, but the exact mechanism is not yet clear. In PA and PB1 subunits were also found mutations that increased the avian IAV polymerase activity in mammalian cells (Mänz *et al.*, 2013). Nowadays, the PB1-F2 protein, the product expressed from an alternative open reading frame within the PB1 gene, is considered to be also a viral factor of pathogenicity. Generally, it induces cell death via interaction with mitochondrial membrane proteins, disrupts alveolar macrophages and enhances secondary bacterial infection (Košík *et al.*, 2013). It was demonstrated that mutation N66S contributes to increased virulence in H5N1 and pandemic virus from 1918 (Conenello *et al.*, 2007). NP contributes to the adaptation of avian IAV to mammalian cells by mutation N319K, which consequently results in an increase in virus replication by influencing the interaction of NP with host importins (Gabriel *et al.*, 2008).

The conserved matrix protein M1, underlying the viral envelope and enabling the interaction of internal and surface antigens, also plays an important role in IAV virulence. The infectivity of reassortant viruses comprising HA and NA of H5N1 subtypes was increased in the presence of M1 protein originating from the same HA and NA subtypes (Maines *et al.*, 2006). Membrane protein M2, the product of the same genome segment, has ion channel activity, which can influence the correct folding of HA during its synthesis. Mutations in this protein can, therefore, also influence the IAV virulence.

Two proteins, which may, based on their functions, also influence the pathogenicity, are non-structural protein 1 (NS1) and nuclear export protein (NEP), expressed from the eighth influenza genome segment. NS1 regulates vRNA synthesis and mRNA translation, is important in morphogenesis of viral particles and is known as an antagonist of interferon (Hale *et al.*, 2008). NEP mediates export of vRNP from the nucleus to the cytoplasm required for virus assembly and regulates the transcription of vRNA during virus replication (Paterson and Fodor, 2012). In addition, NEP is important for IAV adaptation to the new host. Known adaptive mutations in NEP of H5N1 human isolates are localized at its N-terminus (M16I, Y41C) as well as at C-terminus (E75G) even in viruses without E627K mutation in PB2. They stimulate the vRNA synthesis mediated by avian IAV polymerase in human cells, enabling to overcome the polymerase restriction in humans (Mänz *et al.*, 2012).

Here should be mentioned, that the characterization of new IAV isolates can uncover further, yet unknown changes of viral genome in association with IAV adaptation to the new host.

6. Conclusion

Though we focused here mainly on the role of HA fusion activity in the virulence and pathogenicity of IAV, it has to be stressed that virulence is a result of many interconnected factors, in spite of the relatively small size of IAV and its genome. Thanks to the replication mechanisms of IAV requiring cooperation with host cell proteosynthetic machinery and interaction with host signaling and metabolic pathways, as well as with the host immune system, the virulence and pathogenicity of newly emerged IAV is currently difficult to predict. Therefore, new approaches, which can bring our knowledge closer to the ability to predict the pandemic potential of newly emerged IAVs and, thus, to prevent the spread of potentially dangerous influenza viruses, are needed.

Acknowledgements. This work was supported by the grants VEGA 2/0146/15, VEGA 2/0100/13, VEGA 2/0153/14, from the Scientific Grant Agency of Ministry of Education of Slovak Republic and Slovak Academy of Sciences and by grants APVV-0250-10 and DO7RP-0025-10 from the Slovak Research and Development Agency.

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