Hemagglutinin-mediated Influenza Viral Entry: Identification of Molecular Determinants on Host Tropism

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THESIS

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LIST OF ABBREVIATIONS

293T	Human embryonic kidney cells
A549	Human lung epithelia cells
ACIP	Advisory Committee on Immunization Practices
BSA	Bovine serum albumin
CO-HA	Codon-optimized H5 hemagglutinin
DNA	Deoxyribonucleic Acid
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-Linked Immunosorbent Assay
ER	Endoplasmic reticulum
Flu	Influenza
GFP	Green fluorescence protein
GnT I	N-acetylglucosaminyltransferase I
HA	Hemagglutinin
HA assay	Hemagglutination assay
HIV	Human Immunodeficiency Virus
HPAI	High pathogenic avian influenza
HPIV	Highly pathogenic influenza viruses
HRP	Horseradish peroxidase
LAIV	Live attenuated influenza vaccine
LPIV	Low pathogenic influenza viruses
MDCK	Madin Darby canine kidney

LIST OF ABBREVIATIONS (continued)

NA	Neuraminidase
NP	Nucleoprotein
NS1	Nonstructural protein 1
NS2	Nuclear export protein/nonstructural protein 2
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PEI	Polyethylenimine
PKC	Protein Kinase C
RBS	Receptor binding site
RFP	Red fluorescence protein
RNA	Ribonucleic Acid
SA	Sialic acid
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
TIV	Trivalent inactivated vaccine
VSV-G	Vesicular stomatitis virus glycoprotein
WHO	World Health Organization

SUMMARY

Influenza A virus causes seasonal flu in humans and occasionally pandemics in humans and poultry, resulting in high mortality diseases and huge economic loss. One major concern comes from the highly pathogenic avian influenza which may break the species barrier and become the source of a potential flu pandemic. The first step of influenza infection is viral entry, which is mediated by viral glycoprotein hemagglutinin (HA) binding to host cell surface sialic acid (SA)-terminated gangliosides. Species specific SA plays an important role in determining influenza host specificity. The SA binding pocket of the HA has been well studied and the important residues for receptor binding have been previously identified. It is thought that avian influenza viruses preferentially bind to NeuAcα2,3 gangliosides, while human influenza viruses exhibit a preference for NeuAcα2,6 containing gangliosides.

The objectives of my thesis work were: 1) to elucidate the potential role(s) of the surface located, highly conserved residues of HA1 subunits (outside of the receptor binding pocket) in influenza A viral entry and host tropism determination; 2) to elucidate the specificity of H5 HA1 subunits in H5N1 viral entry; and 3) to gain more insight into the structural determinants of HA in limiting influenza host range and to help in the development of new inhibitors or drugs in influenza viral entry.

First, I evaluated the potential roles of seven highly conserved residues of HA surface-located amino acids in receptor binding and viral entry. Among them, mutant Y161A showed cell type dependent viral entry without any obvious defect in HA protein expression or viral

incorporation. This mutant also displayed dramatically different ability in agglutinating different animal erythrocytes. Oligosaccharide binding analysis showed that substituting alanine at Y161 changed the HA SA binding preference from NeuAc to NeuGc. Rescued mutant Y161A viruses demonstrated a mild growth defect, but were robust in viral replication and plaque forming ability. We concluded that Y161 is a critical residue for the recognition of different SA species and it may play a role in determining influenza virus host tropism. Second, to further examine the specificity of H5 HA in viral entry, I investigated the potential roles of 36 charged, highly conserved and surface located residues of H5N1 HA in viral entry and the role in influenza host preference. The results revealed that positive charge is favored at reside 117 and at reside 241 the negative charge is critical for NeuAcα2,3 linked SA receptor recognition and viral entry.

In summary, two major categories of residues, which are critical in SA recognition and viral entry, have been identified. One residue is Y161 which plays a critical role in influenza host tropism by affecting viral recognition of different species of SAs from different hosts. The other category includes H117 and D241, which are H5 HA1 specific conserved residues, whose charge at a specific position plays an essential role in avian receptor SA binding, therefore affecting viral entry, however, without affecting SA species specificity. My work contributes to a better understanding of the role of different domains of HA in influenza viral entry. These new insights will help elucidate the mechanism of influenza A viral entry and provide insights for development of HA inhibitors.

1 INTRODUCTION

1.1 Influenza Virus

Influenza, commonly referred to as the flu, is an infectious disease caused by segmented, negative-stranded RNA viruses of the family Orthomyxoviridae. Three genera of influenza virus A, B, and C are different from each other in their nucleoprotein and matrix protein. Influenza B virus almost exclusively infects humans [1] and is less common than influenza A. Influenza C virus infects humans, dogs and pigs, however, it is less common than the other types and usually only causes mild disease in children [2-3]. Influenza A virus has the widest host range and it has been isolated from hosts including humans, swine, poultry, horses, canines, wild waterfowl and other migrating birds [1]. Among these species, wild waterfowl is the natural reservoir of the virus. Influenza A virus can cause human influenza pandemics and avian influenza pandemics.[4].

Influenza A, B and C are similar in viral structure (Fig. 1). The viral particle is usually spherical with the diameter of about 100 nanometers, although elongated filaments can occur [5]. Influenza viruses possesses a lipid envelope derived from the host cell. Hemagglutinin (HA), neuraminidase (NA) and ion channel protein M_2 are the three major viral surface proteins. The matrix protein M_1 lies underneath the envelope. The core of the virus is comprised of 8 RNA segments associated with nucleoprotein (NP) and polymerase. These 8 RNAs encode at least 10 proteins which play different roles in viral life cycle: HA, NA, M_2, M_1, NP, polymerase protein PB_1, PB_2 and PA, nonstructural protein 1 (NS_1) and nuclear export protein/nonstructural protein 2 (NS_2) [6]. HA and NA form distinctive spikes on the viral particle surface which play important roles in viral entry and exit, respectively, with an approximate ratio of 4:1 (HA/NA). The nomenclature of influenza is based on different subtypes of HA and NA. To date, 16

subtypes of HA (H1 to H16) and 9 subtypes of NA (NA1 to NA9) have been identified for influenza A virus [7-8].

Flu replication is initiated by the attachment of a virus to the host receptor sialic acid (SA) terminated glycans or glycoproteins which is mediated by HA [9-10]. After endocytosis, influenza virus travels from the early endosome to the late endosome where the fusion process driven by HA takes place. M_2 ion channel is activated by low pH in the endosome and is responsible for the influx of protons into the viral particle, triggering the uncoating and nuclear trafficking of RNP. In the nucleus, virus genetic RNA (-strand RNA) produces messenger RNA which directs viral protein synthesis. The -strand RNA also copies itself to make +strand RNA for more genetic RNA synthesis. Influenza virus is unique because it replicates in the nucleus of its host cell. Protein production starts in the endoplasmic reticulum (ER) and processes through the Golgi apparatus. Surface glycoproteins HA and NA are synthesized and transported to the cell membrane surface for new viral particle incorporation. Some newly synthesized proteins go back to the nucleus for viral genome packing and nucleocapsid formation. Although several models have been proposed, either by a specific packing system or random packing, the mechanism of precise packing of a full complement of 8 RNA genome segments and viral assembly is still controversial [11]. New viral particles are assembled under the cell membrane when all the components are in place. NA plays a major role in the viral budding process by releasing SA binding progeny virions from the host cell [12-13]. It is believed that influenza virus has to build an optimal balance between HA and NA during entry and exit processes since both glycoproteins recognize the same target molecule SA but exert opposing effects (receptor binding vs. receptor cutting).

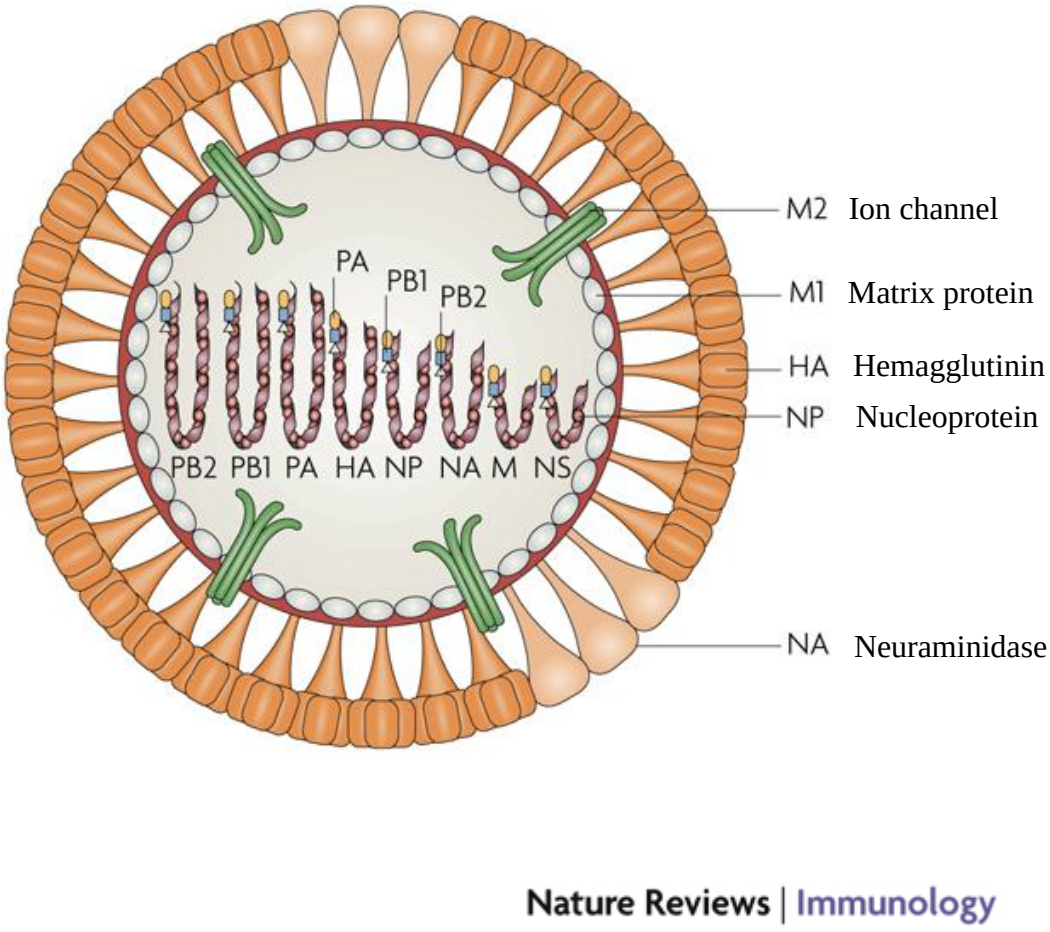

Figure 1. Schematic of an influenza A viral particle. The proteins coded by the eight RNA genes are indicated next to the vRNPs (Subbarao, et al. 2007, modified).

1.2 Influenza epidemiology and pandemics

Influenza A and B viruses isolated in 1933 and 1940, respectively, are associated with significant morbidity and mortality [14-15]. Influenza A virus is responsible for most seasonal illness and pandemics. All the known HA and NA subtypes of influenza A virus have been isolated from avian hosts, but only H1N1, H2N2, and H3N2 subtypes have been associated with epidemics in humans. The incidence of seasonal influenza usually peaks during the winter months. In the Northern Hemisphere, influenza activity occurs between November and March, whereas in the Southern Hemisphere, influenza occurs between April and September. In tropical countries, influenza activity is on throughout the year [16-17]. The mechanism of influenza transmission is still poorly understood, but it is generally accepted that viruses are primarily spread by virus-containing aerosols. Quantification of influenza mortality is complicated because deaths are usually caused by both influenza and other chronic diseases. The average influenza mortality in developed countries is approximately 12 in 100,000 persons [17]. Immunity is the major defense in fighting influenza. The severity and morbidity of seasonal flu are dependent on both host factors and virus virulence. Three major influenza pandemics have attacked humans and caused about 50-100 million deaths worldwide in the 20th century [18].

1918 H1N1 Spanish Influenza

Although the first cases were registered in U.S. long before the spread to Spain, the 1918 pandemic has the nickname "Spanish flu". The geographic origin of the 1918 Spanish flu is still controversial. The source strain of the virus is also unclear. Earlier speculation was that the influenza virus was transmitted directly from birds to humans, but more recent data suggested that the 1918 Spanish flu may have originated from a non-human mammalian species [19]. This

virus had caused unprecedented severe illness and death, especially among young adults, leading to death of 3% to 6% of the global population. The reason for the high mortality remains unclear, but recent studies using recombinant flu viruses suggest that the virus itself was more virulent because of its specific virulence factors PB_1, NA and HA, and was able to cause pneumonia and a cytokine storm [20].

1957 Asian Influenza A H2N2 and 1968 Hong Kong Influenza A H3N2

1957 H2N2 virus was originated in Guizhou, China and spread rapidly worldwide. The highest attack rates were in children aged 5 to 19 years [21-22]. 1968 H3N2 was first isolated in Hong Kong and the mortality was reduced compared to previous pandemics. Both of the pandemic viruses were thought to be resulting from the reassortment of avian and human influenza viruses by possessing completely new HA and/or NA different from the previously circulating flu viruses [16].

In April 2009, a pandemic influenza infection was caused by an H1N1 strain, which was a triple reassortment of bird, swine and human flu viruses [23]. After the first outbreak in Mexico, 2009 H1N1 virus outbreaks were shortly reported all over the world. Although it has the misnomer ‘swine flu’, the virus definitely has gained stronger ability of transmission in humans. Confirmed case rates were higher in children, but the mortality rates (over 1%) were the highest among adults between 30 to 59 years , which is similar to the wave of 1918 Spanish flu pandemic [24].

Two characteristics of influenza virus have been the major reasons for flu pandemics. One such feature is called antigenic drift, which means continual, gradual point mutations in viral surface antigen HA or NA, producing new strains that antibodies to previous infection cannot recognize and neutralize. This is due to the lack of proofreading function from its own RNA polymerase, and thus influenza is constantly undergoing mutations within its genome. Certainly some of these substitutions are silent mutations which are likely not to be consequential, but others may be critical for viral virulence and altering species tropism. For example, critical mutations in HA may render the avian viruses the ability to infect human hosts and mutations in the polymerase may allow successful replication of avian viruses in human hosts.

Antigenic shift is another unique feature of influenza virus. As mentioned above, influenza A virus carries eight independent RNA segments. If a host cell is infected with two or more different influenza viruses, new strains of influenza virus can be generated by RNA segment reassortment. Most of the pandemics in history have been attributed to antigenic shift, resulting in a new pandemic strain from reassortment of an avian and a human strain. However, antigenic drift is thought to be more common since all influenza viruses experience some form of antigenic drift during each replication cycle.

The question is not if, but when the next influenza pandemic will come? Will it be another virus resulting from antigenic shift or a direct jump of highly pathogenic avian strain (e.g. H5N1) into humans? Future studies are necessary to answer these questions.

1.3 Hemagglutinin in viral entry

The hemagglutinin (HA) was originally named for its ability to agglutinate erythrocytes [25-26]. HA mediates viral entry, the first step in influenza virus life cycle. HA is encoded by RNA segment 4, and it is synthesized in the ER as a single peptide HA0 of approximately 550 amino acids. On its way to the cell membrane for virus assembly, HA is highly glycosylated in the Golgi complex. Cleavage of HA0 into two subunits HA1 and HA2 is required for the virus to be infectious, and thus is critical for viral pathogenicity (Fig. 2B) [27].

The proteolytic site in the external loop which links HA1 and HA2 subunits determines HA sensitivity to host proteases. The loop may contain either a monobasic cleavage site (a single Arg or Lys residue) or a multibasic cleavage site (several Arg or Lys residues with an R-X-K/R-R motif). HA with monobasic cleavage site is activated by secreted trypsin-like proteases, which is generally thought to occur only extracellularly, while HA with multibasic cleavage site can be cleaved by a subtilisin-like serine type endoproteases which is nearly ubiquitously expressed in many cell types, allowing systemic infection in hosts [28-29]. Strain subtypes H5 and H7 possessing such multibasic cleavage site are the highly pathogenic influenza viruses (HPIV), whereas viruses with a monobasic HA site are the low pathogenic influenza viruses (LPIV).

The HA trimer extends from the membrane like a spike and is composed of two regions: (a) the globular head derived from HA1 residues consists mostly antiparallel β-sheet; (b) a long fibrous stem mostly derived from HA2 residues consists α-helices (Fig. 2A). HA glycoprotein is a homotrimer of noncovalently linked monomers. HA1 and HA2 are covalently linked by a disulfide bond. The cleavage of HA0 by host proteases liberates N-terminus of HA2, which

contains a highly conserved hydrophobic fusion peptide involved in the fusion process in the entry step.

1.3.1 **Receptor binding site**

The receptor binding site (RBS) is a pocket located on the distal end of each monomer and is composed of three secondary structure elements: the 130 loop (residues 135-138); the 190 helix (residues 190-198); and the 220 loop (residues 221-228), with the base made up of the conserved residues Tyr^{98}, Trp^{153}, His^{183}, and Tyr^{195}, based on H3 numbering (Fig. 2A&2C) [30]. SAs that terminate glycoproteins or glycolipids are the cellular receptors for influenza virus [9]. The receptor binding specificity differs between different hosts. The human HA has a higher affinity to N-5acetylneuraminic acid α2,6-glactose (Neu5Acα2,6Gal), while the avian HA prefers N-5acetylneuraminic acid α2,3-glactose (Neu5Acα2,3Gal), and HA from swine virus appears to recognize both (Fig. 3) [31-33]. These differences are important in limiting influenza A virus species transmission. Amino acids involved in the interaction of SA are highly conserved among 16 subtypes of HA. However, critical mutations among these residues have been detected and linked to the receptor specificity during the adaptation of influenza [34].

In particular, two residues at position 226 and 228 are critical in receptor recognition or host switch for several influenza subtypes [35-36]. Avian HA tends to have glutamine (Q) and glycine (G), while human HA carries leucine (L) and serine (S) at the respective site. Mutations of L226Q and S228G of the HA of the H3 subtype are required for human virus replication in duck intestine where human influenza virus growth is usually restricted [37]. However, influenza A virus of H1 subtype (1918 H1N1 virus) having Q226 and G228 based on sequence study,

which should render the virus preferential binding to avian α2,3Gal receptor, demonstrated binding to human α2,6Gal receptor. Further analysis of 1918 HA revealed that residues at 190 and 225 are important for 1918 H1N1 recognition for human receptor and a single amino acid substitution D190E or double mutation D190E and D225G can change the virus binding specificity from human receptor to avian receptor [38]. Residues including those at position 186, 218, and 193 have also been linked with differences in HA receptor specificity, other than 190, 226 and 228 [39-40]. This illustrates the complex nature of influenza virus receptor binding.

A second ligand binding site has been reported to be located in an interface of two HA1 subunits and an HA2 domain [41]. Ligand binding study suggested that this second binding concave pocket does not fit another important flu receptor molecule α2,6-linked SA and the binding affinity of the α2,3-linked SA to this newly identified site is at least four times weaker than the primary binding site, plus there is no evidence of the physiological importance of the second binding site, thus the biological relevance of this newly revealed ligand binding pocket has not been demonstrated.

1.3.2 **Membrane fusion**

The viral particle goes through the endocytic pathway after entering the target cell and at the endosomes where pH is between 5 and 6, an irreversible conformational change occurs in the HA (Fig. 2D). The exposure of HA2 N-terminal fusion peptide, which is the most highly conserved region in the HA, initiates the fusion of the viral and endosomal membranes [42-44]. At neutral pH, the fusion peptide is buried in a pocket formed by amino acids adjacent to the HA0 cleavage site. Incubation at low pH leads to the conformational change of HA, releasing the

buried fusion peptide and inserting in the membrane. This membrane fusion activity and HA conformational change can also be induced by heat. Therefore, it is thought that native HA is at a metastable state, and at the fusion pH, the HA refolds to a more stable form. [45]. This conformational change does not change the association of the HA1 and HA2 subunits covalently linked by a disulfide bond, and the structure of the HA1 receptor binding site remains preserved [46-47].

1.3.3 Antibody binding site

HA is the primary target for neutralizing antibodies and the most critical component of flu vaccines. HA1 subunit contains almost all the antigenic sites of HA: Sa, Sb, Ca, and Cb, predominantly surrounding the receptor-binding site. Therefore, anti-HA antibodies can neutralize virus infectivity by blocking virus attachment to the receptors. As a consequence, changes in HA structure that prevent antibodies binding are required for the new viruses to avoid immune response. Under rapid antigenic drift, the isolates of Hong Kong pandemic between 1968 and 1999 have accumulated amino acid substitutions in HA1 at a rate of ~3.5 residues per year [48]. Analysis of 2248 HA1 sequences collected from 1968 to 2005 revealed that 95 substitutions occurred very rapidly in 63 sites, of which 57 are antigenic sites [49]. Interestingly, the 2009 H1N1 pandemic virus displays distinct antigenic properties from the seasonal H1 viruses, but exhibits a largely conserved antigenic surface compared with 1918 H1N1 Spanish flu, which may explain the similar age-related immunity to these two pandemics [50]. In 2009, a new antibody recognition site was identified as a highly conserved helical region in the membrane-proximal stem of HA1/HA2. Antibody that recognizes this site can neutralize HAs of

H1, H2, H5, H6, H8, and H9 subtypes, which provides a new lead in designing the new influenza vaccines [51].

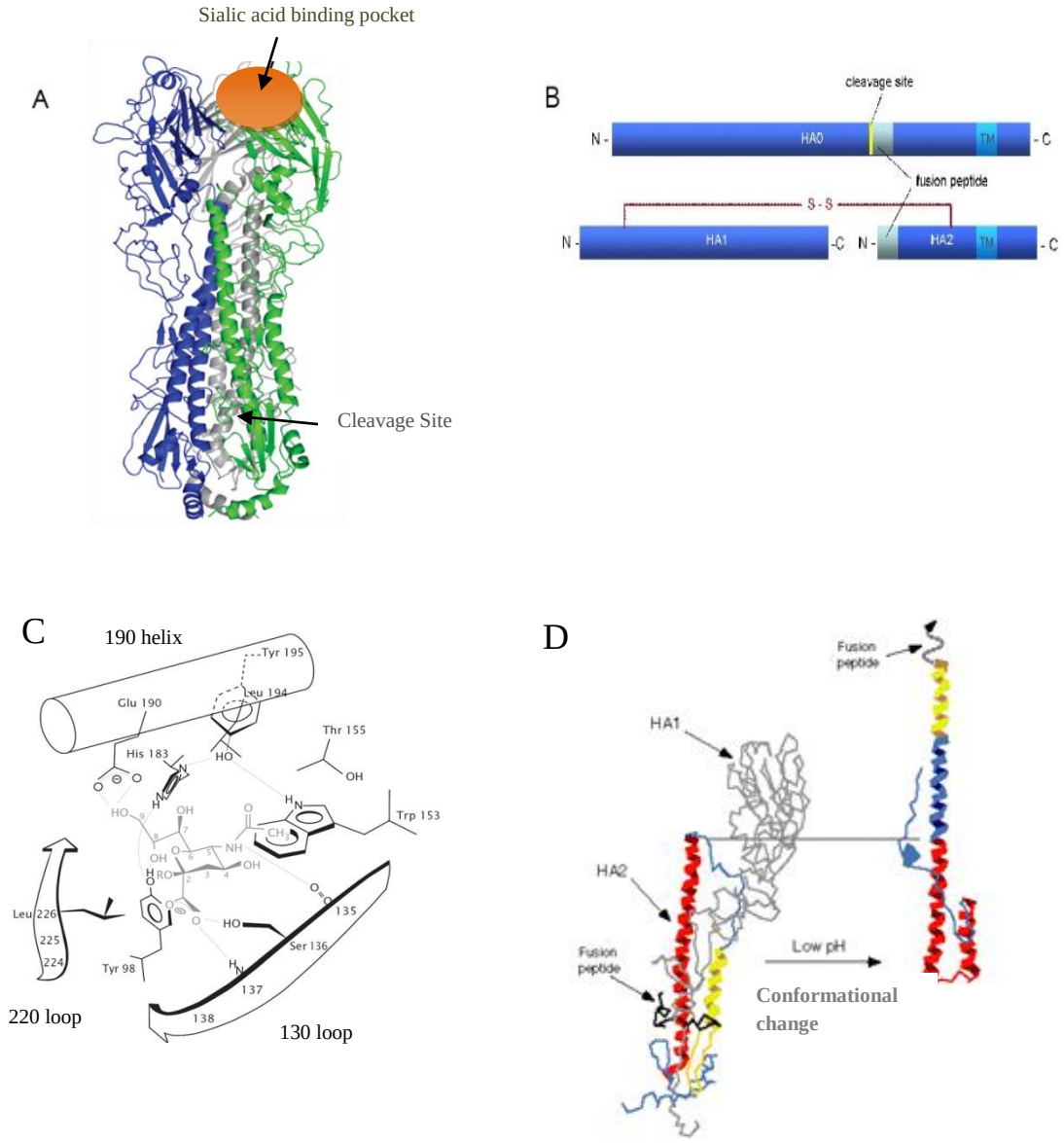

Figure 2. Schematic of influenza hemagglutinin. A) Ribbon representation of the uncleaved precursor HA0. Orange shade: receptor binding site. Arrow marks the cleavage site. B) Cleavage of HA0 into the disulfide linked subunits HA1 and HA2 at a specific cleavage site. TM: transmembrane domain (Wolfgang Garten, Proteases of the respiratory tract activating influenza viruses: Identification, characterization and cellular compartmentalization, modified). C) Sialic acid binding to hemagglutinin. Sialic acid bound in the pocket appears in gray (Immunology and Evolution of Infectious Disease, Chapter 13, 2002, modified). D) Ribbon diagram of the neutral (left) and low pH (right) forms of the influenza virus HA (Wilson et al., 1981, modified).

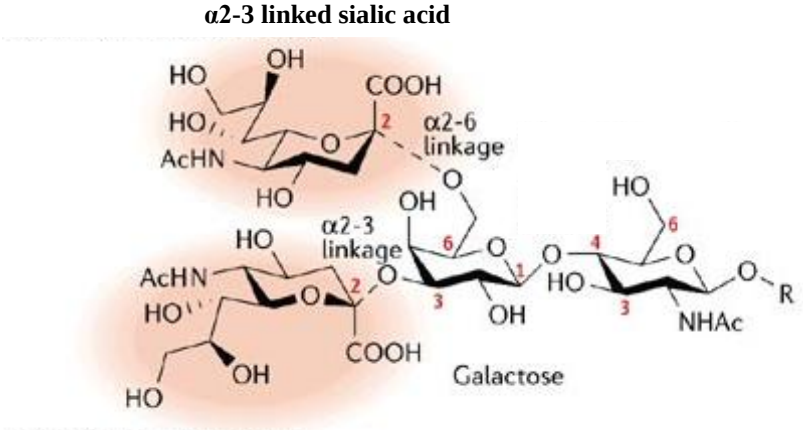

Figure 3. The two possible positions of the sialic-acid linkage to a vicinal galactose (α2-3 and α2-6), which are recognized by the hemagglutinin of avian and human viruses respectively (Stevens et al. 2006, modified).

1.4 Sialic acids in viral entry

Sialic acids (SAs) were discovered by Gunnar Blix, Ernst Klenk, and other investigators as major products released by brain glycolipids or salivary mucins [52]. SAs are monosaccharides with nine-carbon backbones, typically found to be terminal components of N-glycans, O-glycans, and gangliosides. The structures of SAs, comprising a family of over 80 types, have been elucidated, however, challenges in SA research remain due to its significant diversity. The first level of diversity comes from the different α linkage between the C-2 of SAs and the underlying sugars. The most common linkages are to the C-3 or C-6 positions of galactose residues or to the C-6 position of N-acetylgalactosamine residues, resulting in α2-3 linkage and α2-6 linkage. SAs can also be the internal link within glycans in the form of a single SA unit or repeating SA units. The second level of diversity arises from various modifications of SA. The C-5 position can have an N-acetyl group (giving Neu5Ac), or a hydroxyl group (giving Kdn), or hydroxylated 5-N-acetyl group (giving Neu5Gc), or an amino group (giving Neu), composing the four core SA molecules, with Neu5Ac being the most common member of the large family. Neu5Ac and Kdn appear to be the precursors for all known animal SAs. Different sialy-transferases are involved in the metabolism of SAs to generate various forms of SAs. Sialidase or neuraminidase can release SAs which are then either recycled or degraded.

SAs can be found in most animal tissues, bacterial, plants, fungi, and yeasts. The wide distribution, tissue-specific expression of linkages and modifications, and the high expression of SAs on membranes and on secreted glycol-proteins suggest that they have multiple functions in cell stabilization and modulation. One of the prominent roles is as the cellular receptor for influenza virus.

Binding to SA is the first step in flu viral entry. SA is a critical host tropism determinant for influenza viruses as the receptor specificity governs virus entry into target cells. As mentioned above, avian influenza A viruses preferentially bind to SAs linked to the penultimate glactose by α2,3 linkage (Neu5Acα2,3Gal), while human influenza A viruses prefer α2,6 linked SAs (Neu5Acα2,6Gal). SA binding lectins have been used to study the distribution of different forms of SAs in different tissues. Early work showed that the avian virus receptor α-2,3 linked SA is abundant in avian gastrointestinal tract, correlating with the ability of the virus to enter and replicate there. In the respiratory epithelium of the human airway, the binding target of avian influenza viruses is ciliated respiratory epithelium, in contrast, human viruses infect nonciliated cells, which reflects the distribution that nonciliated cells possess a higher proportion of α-2,6 linked sialic acid, while ciliated cells possess both sialic acid linkages [53]. Avian receptor α-2,3 linked SAs are also found to be prevalent in the lower respiratory tract of humans, while human virus receptor α-2,6 linked SAs are abundant in the upper respiratory tract. Avian influenza viruses must surmount obstacles in the upper air way to build infection in human epithelia cells, for example, soluble mucins displaying high level of α-2,3 linked SAs can function as false receptors to impede virus delivery to the target cells. The array of sialyloligosaccharides presented on the human airway epithelium has exerted a positive selection in the evolution of HA receptor specificity in human from other species.

Although a lot of methods (linkage-specific lectins, sialidases, monoclonal antibodies, mass spectrometric, and nuclear magnetic resonance) have been used to define many aspects of the SAs on a given glycans or tissues, the presentation of SAs on different cell types and the

accessibility of SA to the receptor binding site in influenza viral infection are still not well known.

1.5 Other factors in viral entry

SA plays a significant role in viral attachment to initiate entry process by binding to one of the surface glycoproteins HA, and it is the only known cellular molecule so far as a receptor for influenza virus. However, new evidence has indicated that some other factors, in addition to SA, may also be involved in influenza virus entry. It is reported that mutant Lec1 cells that lack surface N-linked glycoprotein are restricted for influenza viral entry at the step of virus internalization, although these cells still posses surface α2,3- and α2,6-SAs. The mutation in the N-acetylglucosaminyltransferase I (GnT I) of the Lec 1 cell is responsible for the defect of cell surface terminal N-linked glycosylation [54]. These data suggest that SA alone is not sufficient for influenza entry and N-linked glycoprotein is required for virus entry and infection. Another group showed that Protein Kinase C (PKC) is crucial for influenza virus entry as the entry step was inhibited by a highly specific PKC inhibitor [55]. Consistent with the work of Chu et al., our lab has also reported that the presence of α2,3- and α2,6-SAs on the target cells does not always correlate with efficient pseudotyped H5N1 influenza virus transduction. Further, we demonstrated that substitutions of the residues that are important for human and avian switching did not dramatically affect virus transduction, suggesting that other host factors are likely involved in influenza viral entry [56].

Several other lines of evidence suggest that SA is not necessary for influenza viral entry. Javier Martin et al found that Y98F mutation in the SA binding site of HA did not affect virus

replication in MDCK (Madin Darby canine kidney) cells and embryonated hens' eggs, although Y98F mutant virus was unable to bind to SAs on erythrocytes [57]. In addition, recent data showed influenza virus infection in desialylated cells, supporting two possible models for influenza virus entry. One is through SA-independent way, the other one is multistep pathway in which SA binding initiates subsequent binding to an unknown receptor(s) [58]. It has been shown that H5N1 virus can infect human upper respiratory epithelia cells where α2-3-linked SAs are absent, implying that other binding sites on the epithelium may mediate viral entry [59].

1.6 Prevention and treatment of influenza virus

Influenza is an acute infection that spread easily from person to person. Worldwide, seasonal influenza kills more than 250,000 people each year and influenza viruses continue to be the threat of a potential pandemic. Effective control requires optimal use of vaccine, antiviral drugs, and a global surveillance network.

1.6.1 Vaccination and infection control

Vaccination is an effective strategy to prevent and control influenza infection. The term "vaccine" is derived from the Latin word "vacca" which means cow because the injection of the material from cowpox can protect people against the attack of smallpox [60]. Currently the primary influenza vaccine is a trivalent inactivated vaccine (TIV) including two influenza A subtypes, H1N1 and H3N2, and influenza B. TIV is prepared from virus grown in fertilized hen eggs and is standardized to contain 15 ug of the hemagglutinin protein from each virus [61]. In 2003, a live attenuated influenza vaccine (LAIV) with the same antigenic characteristics as the inactivated-virus vaccine was formulated for intranasal application, which is easier for vaccine

administration compared to TIV [62]. In the United States, CDC's Advisory Committee on Immunization Practices (ACIP) provides annual recommendations for the prevention and control of influenza. Influenza vaccine is recommended to all persons aged 6 months and older, especially people who are at higher risk, including people of aged 6 months to 4 years or over the age of 50, pregnant women, patients with immunosupression and, health-care personnel [63]. LAIV is only approved to use for persons aged 2-49 years. Due to the fast antigenic change of influenza virus, new vaccine is developed annually according to the flu epidemiology of previous year. The 2010-11 trivalent vaccines containing A/California/7/2009 (H1N1)-like antigens which are derived from a 2009 H1N1 pandemic influenza A virus seem effective to protect against the pandemic. For persons known to have anaphylactic hypersensitivity to eggs or to other components of the influenza vaccine, prophylactic use of antiviral agents is an option to control influenza infection. There is no effective vaccine for H5N1 virus due to the lack of overall production capacity and that the pandemic H5N1 might be lethal to chickens. Cell-based vaccine technology can be applied to influenza vaccines as they are used to produce most other vaccines.

Another key feature to prevent and control influenza infection is building international surveillance networks and collaboration. A broader geographical surveillance of the virus would be helpful in detecting the new antigenic variants of influenza, therefore providing information about the next pandemic and optimizing the efficacy of influenza vaccines [64]. Other operative ways to reduce the transmission of influenza include good personal health and hygiene habits, using protective equipment, and preventing or minimizing the spread of pandemic influenza within certain areas.

1.6.2 Influenza treatments and antiviral drugs

Current treatments for influenza are symptomatic therapy and antiviral drugs. Symptomatic treatment includes home rest, adequate liquid supplement, no smoking or alcohol drinking, and over-the-counter medications for flu symptom relief, until the patient's own immune system works to recover from the infection.

In the United States there are two categories of licensed antiviral drugs. One is the M_2-inhibitors Amantadine and Rimantadine, the other one is the NA inhibitors Zanamivir and Oseltamivir. Both Amantadine and Rimantadine act by blocking the ion channel formed by the flu M_2 protein, which is a proton-selective ion channel for hydrogen ions to enter the viral particle in the low pH environment to facilitate viral uncoating and RNPs releasing [65-66]. NA inhibitors act as analogues of SA, which is the substrate of NA, so the viral releasing process is blocked. NA is necessary for viral budding by hydrolyzing the glycosidic bond of SA to release the membrane tethered virions by HA-SA interaction [12-13].

M_2 ion channel inhibitors are only effective for the prevention of influenza A infection and illness, since influenza B viruses have a different ion channel, the NB protein [67]. NA inhibitors are effective against both influenza A and B viruses. However, the optimal rate of protection of these four drugs can only be achieved when the administration of the drug is started during the beginning of the infection, usually the first two days. At present, the most concerning scenario would be the pandemic resulting from the highly pathogenic H5N1 viruses. However, only anecdotal information is available on the treatment of H5N1 infection with Oseltamivir, and it has not yet reduced the overall H5N1 case-fatality below 50% [68-70]. Rapid emergence of

drug resistance, especially with the Adamantine, made influenza treatment even more challenging. During 2005-2006, the frequency of viral resistance among seasonal flu H3N2 to the M_2 inhibitors has reached >90% in Asia and the United States [70]. NA inhibitors are the best drugs available on the market. They were first effective for treatment of 2009 H1N1 pandemic virus. However, NA drug resistant flu strains have formed and are spreading worldwide, including some of 2009 H1N1 strains which rapidly gained resistance after Zanamivir and Oseltamivir treatment. The seasonal flu in USA in 2009 is more than 95% Oseltamivir -resistant. Mutations within the NA active site, for example mutations at positions of H274 and I222, are responsible for the 2009 H1N1 flu virus gaining resistance to the NA inhibitors [71-72].

Vaccines combined with NA inhibitors treatment is recommended in the cases of severe influenza infection. New potential agents are under active exploration for new antiviral treatments, including parenteral NA inhibitors, long-acting NA inhibitors, polymerase inhibitors, and attachment inhibitors.

1.7 Objectives of this study

The objectives of this study were: 1) to explore the new function of the surface located, highly conserved residues within all 16 subtypes of HA1 subunits (in addition to those in the receptor binding pocket) in influenza A viral entry and host tropism determination; 2) to elucidate the specificity of H5 HA1 subunits in H5N1 viral entry; 3) to gain more insight into the structural information of HA in limiting influenza host range and to help in the development of new inhibitors or drugs in influenza viral entry.

2. MATERIAL AND METHODS

2.1 Cell lines, antibodies and plasmids

Human embryonic kidney 293T cells and human lung epithelia A549 cells were maintained in Dulbecco's modified Eagle's Medium supplemented with 10% fetal bovine serum, 100 μg/ml streptomycin and 100 units of penicillin.

Goat polyclonal anti-influenza virus H5 HA, A/HongKong/213/03 (H5N1), NR163 was obtained from BEI Resources. The mouse anti-β-actin monoclonal antibody was purchased from Sigma (St Louis). The mouse anti-HIV p24 monoclonal antibody was obtained from the National Institute of Health AIDS Research and Reference Reagent Program.

Mutant HAs were generated by QuickChange Site-Directed Mutagenesis Kit from Stratagene following the supplier's protocol with custom designed primers. All HA mutants were confirmed by DNA sequencing of the full-length HA gene.

2.2 Production of pseudovirions

Influenza pseudotyped viruses were produced by transiently co-transfect 293T producing cells with plasmids encoding hemagglutinin (HA), neuraminidase (NA), vesicular stomatitis virus glycoprotein (VSV-G) and Envelope-deficient HIV vector (pNL4.3.Luc-R-E- or pNL4.3.GFP-R-E-) using a polyethylenimine (PEI) based transfection protocol. CO-HA which is codon-optimized H5 HA was used as a parental HA. In those experiments, 6 ug of PEI were added to 250 μl Opti-MEM I media (GIBCO) and the mixture was incubated at room temperature for 20 min. 500 ng of HA or mutant HA, 250 ng of NA and 3 μg of HIV vector were then added to the mixture and incubated at room temperature for 20 min. For pseudotyped VSV-

G virus, 250 ng of VSV-G were mixed with 3 μg of HIV vector. For HIV-NA, only 250 ng of NA was mixed with HIV vector. Seven hours after transfection, cells were washed by phosphate-buffered saline (PBS), 6 ml of fresh medium was added to each plate. Forty-eight hours post transfection, the supernatants were collected and filtered through 0.45 μm pore size filter (Nalgene) and were used directly for infection. The remaining pseudovirions were stored at -80℃ until further use.

2.3 Infection assay

The pseudovirions prepared above were incubated (500 μl/well) with 293T and A549 target cells which were seeded in 24-well plates at a density of 5×10^4/well 24 h prior to infection. The target cells were lysed in 150 μl lysis reagent (Promega) 48 h after infection. The luciferase activity was measured with a luciferase assay kit (Promega) and an FB12 luminometer (Berthold detection system). Each experiment was done in triplicate and repeated at least three times.

2.4 Western Blot

To examine the expression of HA, the 293T producer cells were lysed in 500 μl Triton X-100 lysis buffer containing 50 mM Tris-HCL (pH7.5), 150 mM NaCl, 5 mM EDTA (Ethylenediaminetetraacetic acid), 1% Triton X-100 and protease inhibitors cocktail consisting of 10 μg of leupeptin per ml, 5 μg of aprotinin per ml and 2mM phenylmethylsulfonyl fluoride at 48h after transfection. To examine the incorporation of HA protein into pseudovirions, 4 ml of collected supernatants were layered onto a 1-ml cushion of 20% (wt/vol) sucrose in PBS and centrifuged at 55,000 rpm for 1 h in a Beckman SW55 rotor at 4℃. The pseudovirus pellets were lysed in 50 μl of Triton X-100 lysis buffer. The samples were then subjected to sodium dodecyl

sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a polyvinylidene difluoride membrane. After blocking in PBS with 1.5% BSA for 1 h, the membrane was first incubated with NR163 antibody (1:5000 dilutions) for 2 h and then probed with peroxidase-conjugated rabbit anti-goat antiserum (Pierce) for 1 h. The bands were visualized by the chemiluminescence method according to the protocol of the supplier (Pierce). In these experiments, mouse anti-β-actin (1:10,000) and anti-HIV p24 (1:10,000) monoclonal antibody were used as indicators for cell lysate loading control and the relative amount of the pseudovirions.

2.5 Surface immunofluorescence staining

Seed cells onto cover slips the night before transfection of 500μg CO-HA and other mutant HAs by PEI method. The cover slips were washed twice with PBS 48h after transfection. Cells were then fixed with 0.5ml 4% paraformaldehyde in PBS for 30min at RT. After blocking for 30 min, add primary antibody NR163 (1:200 dilutions) then probe with peroxidase-conjugated rabbit anti-goat antiserum (Pierce). Following with PBS wash, cover slips were further incubated with FITC-conjugated goat anti-rabbit IgG (Bio Rad) at a 1:200 dilution at room temperature for 1 hour. Cover slips were mounted in a medium containing DAPI (4′,6-diamidino-2-phenylindole) (Vector Labs) and examined by a fluorescent microscope.

2.6 Hemagglutination assay

Supernatants from producer 293T cells were harvested 48 h post-transfection. 4 ml of filter-sterilized pseudoparticles were concentrated over a 30% Sucrose-NTE cushion consisting of 100 mM NaCl, 10 mM Tris (pH 7.4), and 1 mM EDTA. The samples were spun at 55,000 rpm

for 1 h in a Beckman SW55 rotor at 4ºC. Pseudovirion pellets were resuspended in 200 µl of Tris-buffer containing 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, and 5 mM EDTA. Two-fold serial dilutions were mixed with an equal volume of 0.5% animal erythrocyte suspension (CRBCs; Lampire Biological Laboratories) in a U-bottomed 96-well plate. HA titers were recorded after 2h incubation at 4℃. HA assay experiments were repeated at least three times.

2.7 Oligosaccharide binding assay

Streptavidin-coated high binding capacity 384-well plates (Pierce) were incubated overnight at 4℃ with 50µl of 3µM biotinylated saccharides in Table 1. Saccharides were provided by the Consortium of Functional Glycomic (http://www.functionalglycomics.org). Pseudotyped viruses were concentrated as above. 50 µl viruses diluted in PBS with 1% (w/v) BSA (PBS-BSA) were added to saccharide-coated wells and were incubated with oligosaccharides overnight at 4℃. Wells were then washed 3 times with PBST (PBS, 0.1% Tween-20), 3 times with PBS and then blocked with PBS-BSA for 2h at 4℃, before incubating with anti-HA antibody NR163 diluted in PBS-BSA for 4h at 4℃. After washing as above, wells were incubated with rabbit anti-goat IgG HRP conjugated antibody in PBS-BSA. After washing as above, 50 µl of 1-stepTM ultra TMB-ELISA substrate (Thermo) was added to each well and the plate was incubated at room temperature for 20 min. Binding of 2nd antibody was detected by measuring the absorbance of each well at 450 nm after adding 50 µl stop solution of 2M sulfuric acid to each well. Appropriate negative controls were included. Assays were performed in duplicates and were repeated at least three times.

2.8 Generation of recombinant viruses and viral growth curve

Mutations of Y161A and K307A/K310A in the HA protein of Influenza HAlo virus generated from influenza A/Vietnam/1203/04 (H5N1) were produced by PCR mutagenesis, and the resulting DNAs were cloned into pPolI transcription plasmids. Generation of recombinant virus by reverse genetics was described as before [73]. Briefly, a coculture of MDCK and 293T cells was transfected with four expression plasmids coding for the PB1, PB2, and PA proteins, and nucleoprotein (NP) of HAlo and seven pPolI, each coding for one of the HAlo virus vRNA segments, omitting the HA plasmids. A total of 0.5 to 1 μg of each plasmid was transfected by using Lipofectamine 2000 (Invitrogen, Carlsbad, Calif.). At 12 h posttransfection the medium was replaced by DMEM containing 0.3% bovine serum albumin, 10 mM HEPES, and 1 μg of TPCK-treated trypsin/ml. At 3 days posttransfection, virus within the cell supernatants was plaque purified by titration on MDCK cells. All the segments of the recovered mutant viruses were analyzed by RT-PCR and confirmed by sequencing.

MDCK cells were infected at an MOI of 0.01 with recombinant influenza A viruses expressing either HA Y161A, HA K307A&K310A or parental HAlo. At 0, 12, 24, 36, 48, and 60 h postinfection, cell supernatants were harvested and titrated by plaque assay on fresh MDCK cells.

3. RESIDUE Y161 OF INFLUENZA HEMAGGLUTININ IS INVOLVED IN SIALIC ACID RECOGNITION FROM DIFFERENT HOSTS

3.1 Introduction

Influenza A virus causes seasonal flu and occasionally pandemics. Influenza A viruses have been isolated from a wide range of animals including humans, swine, poultry, horses, canines, wild waterfowl and other migrating birds. Among these animals, wild waterfowl is thought be to the natural reservoir of influenza A virus. Occasionally viruses can break the host barrier by antigenic shift and antigenic drift, transmitted from the natural host to humans and domestic poultry, causing human influenza pandemics and economic loss [4]. It is believed that the recent 2009 H1N1 flu pandemic was the result of a triple reassortment of bird, swine, and human influenza viruses [74].

The hemagglutinin (HA) of influenza A virus is one of the viral surface glycoproteins and is responsible for binding of the virus to host cells and subsequent membrane fusion within the late endosome [75]. HA also plays an important role in host immune responses by harboring the major antigenic sites in producing neutralizing antibodies. The mature HA is a spike-like homotrimer of about 220 kD. Each monomer is synthesized as a precursor HA0 which is then cleaved into HA1 and HA2 subunits by the host proteases and modified by multiple glycosylations. Most of the HA1 subunit forms the head region of HA, while the HA2 subunit is the primary feature of the stem region [76-78]. The receptor binding site (RBS), which has been well characterized, is located on the head region of each HA monomer [75]. Amino acids involved in the interaction of SA are highly conserved among 16 subtypes of HA. However, critical mutations among these residues have been detected and linked to the receptor specificity during the adaptation of influenza [34]. In particular, two residues at position 226 and 228 are critical in receptor recognition shift or host switch for several influenza subtypes [35-36].

One of the determinants of the influenza A virus host range is the receptor recognition. Glycans terminated with sialic acid (SA) play a crucial role as the receptor in influenza A viral entry. Multiple lines of evidence have demonstrated that SA binding preference switches the host tropism of human and avian influenza A viruses: the former virus preferentially binds to N-5acetylneuraminic acid α2,6-glactose (Neu5Acα2,6Gal), while the latter prefers N-5acetylneuraminic acid α2,3-glactose (Neu5Acα2,3Gal) [31-33]. Although Neu5Ac is recognized as an essential determinant of the cell surface receptor, influenza A viruses also differentially bind to receptors which are modified by N-glycollylneuraminic acid (NeuGc), another common SA found in mammalians and that potentially serves as an entry and host determinant [79-81].

In this study, we have targeted and characterized the function of 7 highly conserved, surface-located amino acids which have not been investigated previously in viral entry. Through alanine scanning strategy, we have identified two interesting targets. The double mutant K307A/K310A abolished virus erythrocyte binding but had reasonably good viral entry and replication, suggesting that the stem region is also important for influenza receptor binding and viral entry. In particular, we have discovered that residue Y161 is critical in viral receptor recognition of NeuAc or NeuGc SAs, which is important in understanding the structure information of HA in limiting influenza host range. These findings may help in the development of effective inhibitors or drugs in influenza viral entry.

3.2 Results

3.2.1 Design of the amino acid substitutions within influenza HA1

HA1 mediates the attachment of viral particle to the target cell surface, which is the first step in viral entry, while HA2 subunit mediates the fusion process after the virus enters the endosome [75]. To further explore the potential roles of other critical amino acids besides those which are already known to be involved in the interaction with SA, we compared the HA1 sequences from all 16 HA subtypes. Overall, 89 highly conserved residues were identified, and seven residues, which are located on the surface of the HA crystal structure (Fig. 4), were chosen for further analysis. In addition, we also selected residues K144 and K307, which are located on the HA surface and on the interior surface of the stem region of HA, respectively, for this study. Together, 11 alanine substitution mutants of HA were generated by site-directed mutagenesis and their phenotypes were characterized.

3.2.2 Effects of alanine substitutions on pseudotyped viral entry

To study the entry mechanism of the highly pathogenic influenza A virus with alleviated safety concerns, we have developed an HIV-based system to produce pseudotyped viruses. HIV-particles pseudotyped with CO-HA or mutant HAs were used to challenge the target cells (293T and A549). The luciferase activities of the transduced cells were used as a measure for viral entry (Fig. 5). In these experiments, virions pseudotyped with VSV-G were used as a positive control, as VSV-G can be incorporated into pesudotyped particles with a very high efficiency and the pseudovirions have a broad host range [82-83]. The effect of alanine substitution mutants of HA on viral entry can be classified into two groups: 1) mutants that behaved like the parental HA (W127A, K144A, F147A, and F148A); 2) mutants with impaired viral entry (F147A/F148A,

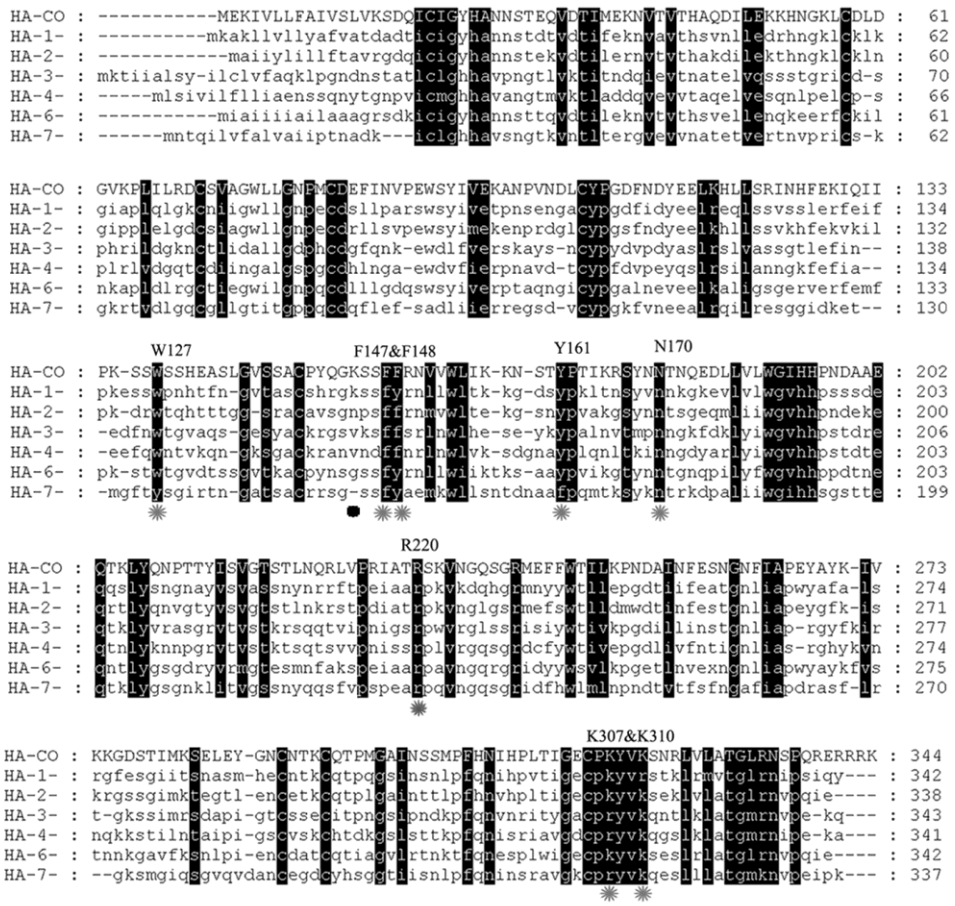

Figure 4. Sequence alignment of HA1 from 16 different HA subtypes. For the space restriction, only the alignment of H1-H7 is shown. CO-HA is a representative of H5 HA. Residues shaded in black are the highly conserved ones in all 16 subtypes. Residues targeted for mutation are indicated by asterisks. The black dot represents control residue K144. The number of each residue is named according to H3 HA number.

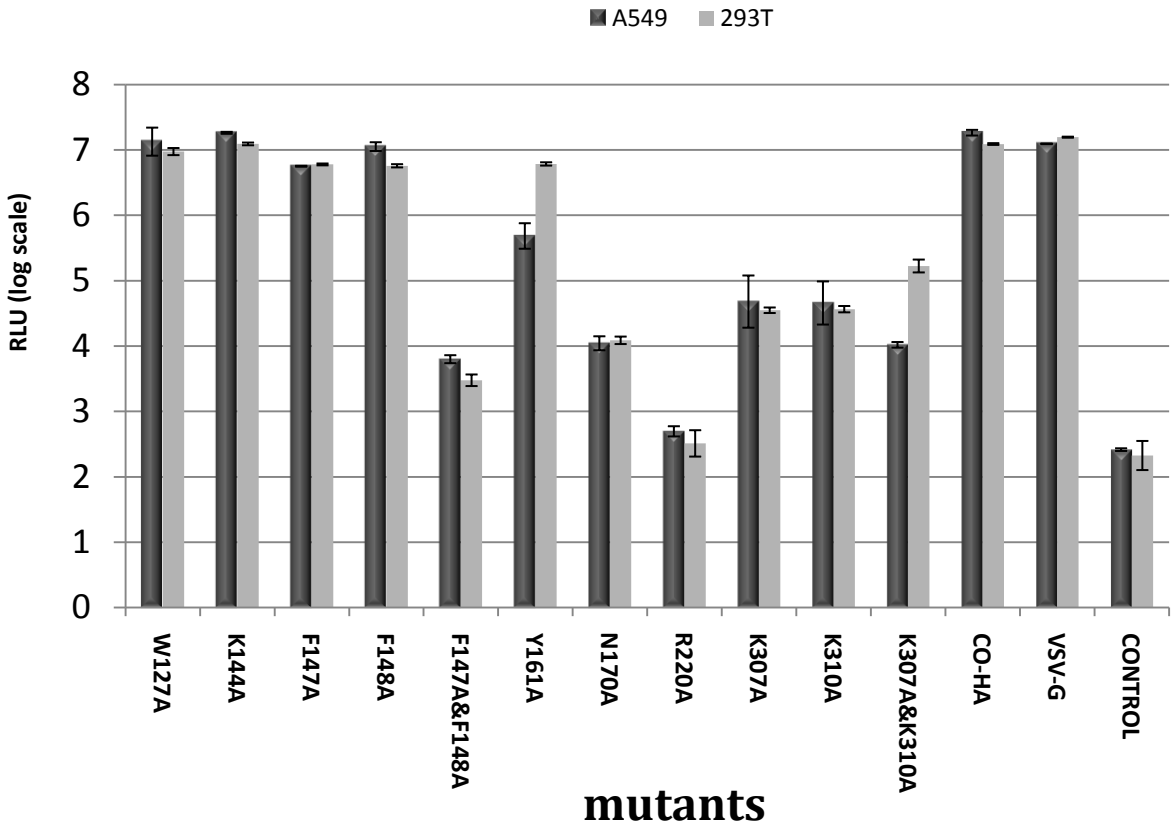

Figure 5. Mutational effects on viral entry as measured by luciferase activity. (A) Relative luciferase units (RLU) from 293T target cells. (B) RLU from A549 target cells. Data represent at least 3 repeated experiments with similar trends. The error bars indicate the S.D. of three independent experiments.

Y161A, N170A, R220A, K307A, K310A, and K307A/K310A). Among them, Y161A, K307A/K310A appeared to display cell type dependent viral entry since there was roughly one log difference in the luciferase activity between 293T and A549 target cells (Fig. 5).

3.2.3 Effects of alanine substitutions on HA expression and incorporation

We next examined the possible effects of alanine substitutions on HA expression, processing or incorporation. First, cell lysates derived from the cells transiently transfected with HIV-luc vector and different mutant HA plasmids were subjected to SDS-PAGE and Western blot analysis, as shown in Fig. 6A. Three bands, which corresponded to HA0, HA1 and HA2, with the size of about 75kDa, 50kDa and 25kDa, respectively, were detected for CO-HA, the parental HA (lane 12), indicating that CO-HA was expressed and processed in 293T cells as predicted. Similarly, eight of the eleven HA substitution mutants had the same pattern as CO-HA (lanes 1, 2, 3, 4, 6, 9, 10, 11), suggesting that these substitutions did not greatly alter the expression or processing of HA in producer 293T cells. In contrast, F147A/F148A, N170A, and R220A (lanes 5, 7, 8) displayed no or little cleavage products compared to CO-HA, suggesting that these alanine substitution mutations adversely affected the processing and/or proper folding of HA.

To test if alanine substitutions affected HA viral incorporation, the viral particles were pelleted by ultracentrifugation in 20% sucrose cushion and subjected to SDS-PAGE and Western blot analysis (Fig. 6B). The pseudovirions carrying CO-HA showed the presence of correct size of HA0, HA1 and HA2 (lane 12), indicating proper incorporation of HA into pseudoparticles. Mutants W127A, K144A, F147A and F148A were detected on the viral particles (lanes 1, 2, 3,

4). In contrast, there were no detectable HA0 and the cleaved HA products from F147A/F148A or N170A on the pseudotyped viruses (lanes 5, 7). Intriguingly, however, a band corresponding to the HA2 was detectable from mutant F147A/F148A. Similarly, for mutant R220A, even though no HA2 was detected from the cell lysate (Fig 6A, lane 8), there were still a detectable but low amount of HA1 and more HA2 in the viral particles (Fig. 6B, lane 8). Mutants Y161A, K307A, K310A, and K307A/K310A were also detected on the viral particles (lanes 6, 9, 10, 11).

In addition, immunostaining was used to examine mutational effects on HA surface transportation and expression. There was no significant difference in HA surface expression between each mutant and the parental HA, except for F147A/F148A and N170A, with slightly weaker surface immunofluorescence intensity (data not shown).

Together, our results suggest that alanine substitutions at position 147/148, 170, and 220 affected HA protein expression and/or proper incorporation into viral particles, which explains why these HA mutants were greatly impaired in mediating viral entry. Alanine substitutions at position 127, 144, 147, 148, 161, 307, and 310 did not dramatically influence HA protein processing or incorporation into viral particles.

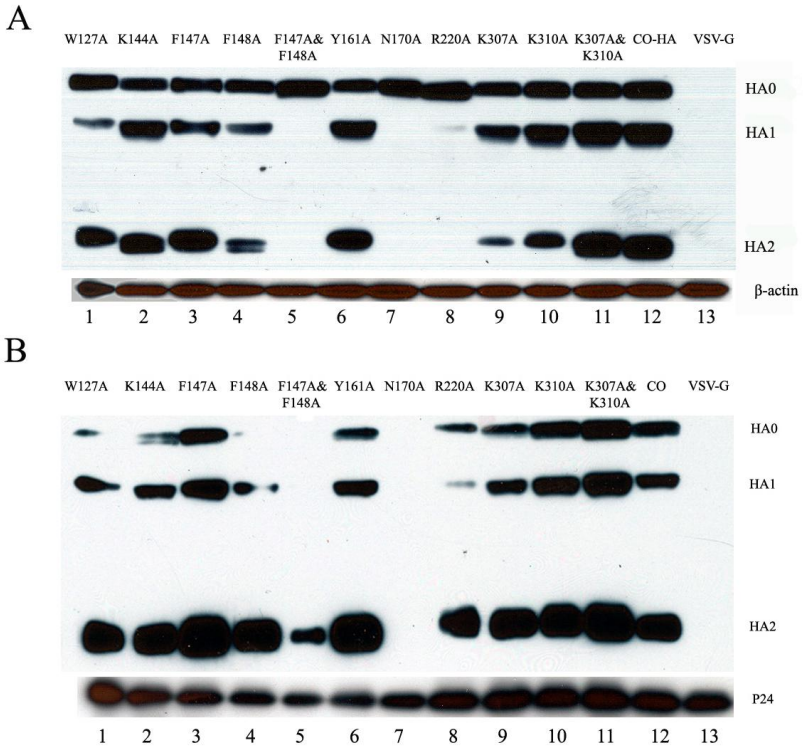

Figure 6. Western blot analysis of HA protein expression (A) and viral incorporation (B). The HA precursor (HA0) and two proteolytic products HA1 and HA2 are shown.

3.2.4 Effect of alanine substitutions on sialic acid recognition

The binding of influenza virus to erythrocytes is mediated by the interaction of HA with cell surface receptors containing SAs. As the influenza viruses attach to multiple RBCs, a lattice-structure forms [84], therefore we used hemagglutination assay (HA assay) to further determine whether alanine substitutions changed SA binding preference and thus altered viral entry. Chicken, horse and swine erythrocytes were chosen based on the previous studies that erythrocytes from these hosts have different surface SA structures and that influenza A viruses receptor specificity correlates with the agglutinating pattern from different animal erythrocytes [85].

Briefly, two-fold serial dilutions of purified pseudotyed viral particles were mixed with chicken or horse erythrocytes. The hemagglutination results of parental HA (CO-HA) and the selected HA mutants (F147A/F148A, Y161A, R220A, K307A, K310A, K307A/K310A) which displayed reduced entry (see Fig. 5), are shown in Fig 7. The HA titer of parental virus is 64 for both erythrocytes. HA assay of mutants from the first group (W127A, K144A, F147A, and F148A) showed similar pattern with parental HA (data not shown). For mutants F147A/F148A, N170A, R220A, and K307A/K310A, the alanine substitutions totally abolished SA binding as their HA titers were zero on both erythrocytes (Fig 7A and 7B). K307A and K310A displayed lower HA titer than CO-HA on both animal erythrocytes (Fig 7A and 7B). Interestingly, Y161A pseudotyped viruses showed dramatically different pattern of agglutination on chicken and horse erythrocytes: no agglutination was observed when mixed with chicken erythrocytes, while one unit higher (128) titer than CO-HA (64) pseudoparticles was revealed in HA assay with agglutinating horse erythrocytes.

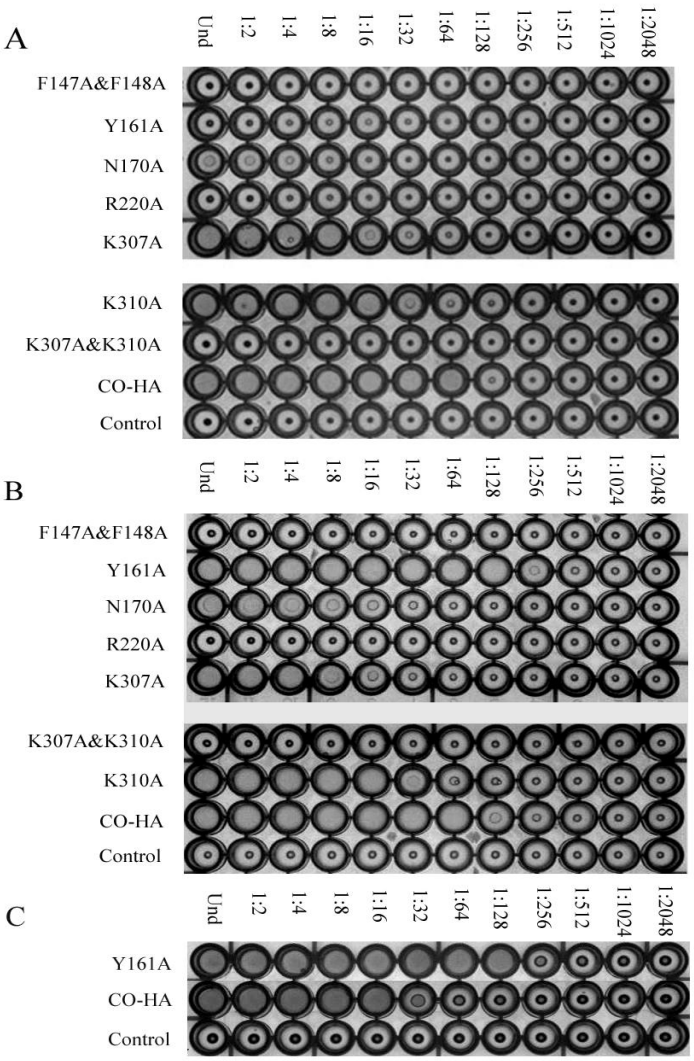

Figure 7. Hemagglutination assay of titrated pseudovirions mixed with chicken erythrocytes (A), horse erythrocytes (B) and swine erythrocytes (C). HA assay of mutants didn't show reduced viral infectivity are not shown here.

Considering the SA structure and species difference on the surface of these two erythrocytes: chicken erythrocytes contain 95-100% N-acetylneuraminic acid (NeuAc) SA of both α2,3-Gal and α2,6-Gal linkages, with slightly more abundant α2,6-Gal SAs; horse erythrocytes contain 95-100% of N-glycolylneuraminic acid (NeuGc) SAs, with more abundant α2,3-Gal SAs compared to α2,6-Gal SAs [85-87], therefore we hypothesized that alanine substitution at Y161 changed the viral receptor preference from NeuAc to NeuGc SA. We thus further tested the ability of Y161A mutant virus in agglutinating swine erythrocytes which had been shown to contain about 66% of NeuGc and 34% of NeuAc of both α2,3-Gal and α2,6-Gal SAs, with more of α2,6-Gal SAs [87]. The results from swine HA assay (Fig. 7C) showed the similar pattern with that from horse agglutination, supporting our hypothesis.

To further confirm our hypothesis, we used Enzyme-Linked Immunosorbent Assay (ELISA) to investigate the direct binding activity of mutant Y161A and parental CO-HA pseudoparticles to five biotinylated saccharides baring varied species of terminal SA and sialyl linkages (Table 1 and Fig. 8). Controls including CO-HA virus alone (without incubation with any biotinylated oligosaccharides), Y161A virus alone, HIV-NA virus pseudotyped with only NA glycoprotein, oligosaccharides alone and 2nd antibody alone were performed. Only the first 3 controls are shown in Fig. 8 since all the controls mentioned above displayed similar levels of absorbance at 450 nm. CO-HA pseudotyped viruses bound to Neu5Ac α2,3 linked SA (3'SLN) in a dose-dependent manner (Fig. 8A), but not Neu5Ac α2,6 linked SA (6'SLN) (Fig. 8C), which is consistent with the notion that avian influenza viruses preferentially bind to NeuAcα2,3 linked SAs [33]. Y161A mutant showed the background binding level to 3'SLN (Fig. 8A). Interestingly,

TABLE 1
NAME AND STRUCTURE OF BIOTINYLATED SYNTHETIC OLIGOSACCHARIDES

Compound No.	Saccharide Name and Type of Synthetic Spacer	Abbreviation
B85	Neu5Acα2-3Galβ1-3GlcNAcβ-Sp*NH-LC-LC*-Biotin	3'SLe[c]
B84	Neu5Acα2-3Galβ1-4GlcNAcβ-Sp*NH-LC-LC*-Biotin	3’SLN
B87	Neu5Acα2-6Galβ1-4GlcNAcβ-Sp*NH-LC-LC*-Biotin	6’SLN
B90	Neu5Gcα2-3Galβ1-4GlcNAcβ-Sp*NH-LC-LC*-Biotin	3’S(Gc)LN
B93	Neu5Gcα2-6Galβ1-4GlcNAcβ-Sp*NH-LC-LC*-Biotin	6’S(Gc)LN

* Synthetic spacer

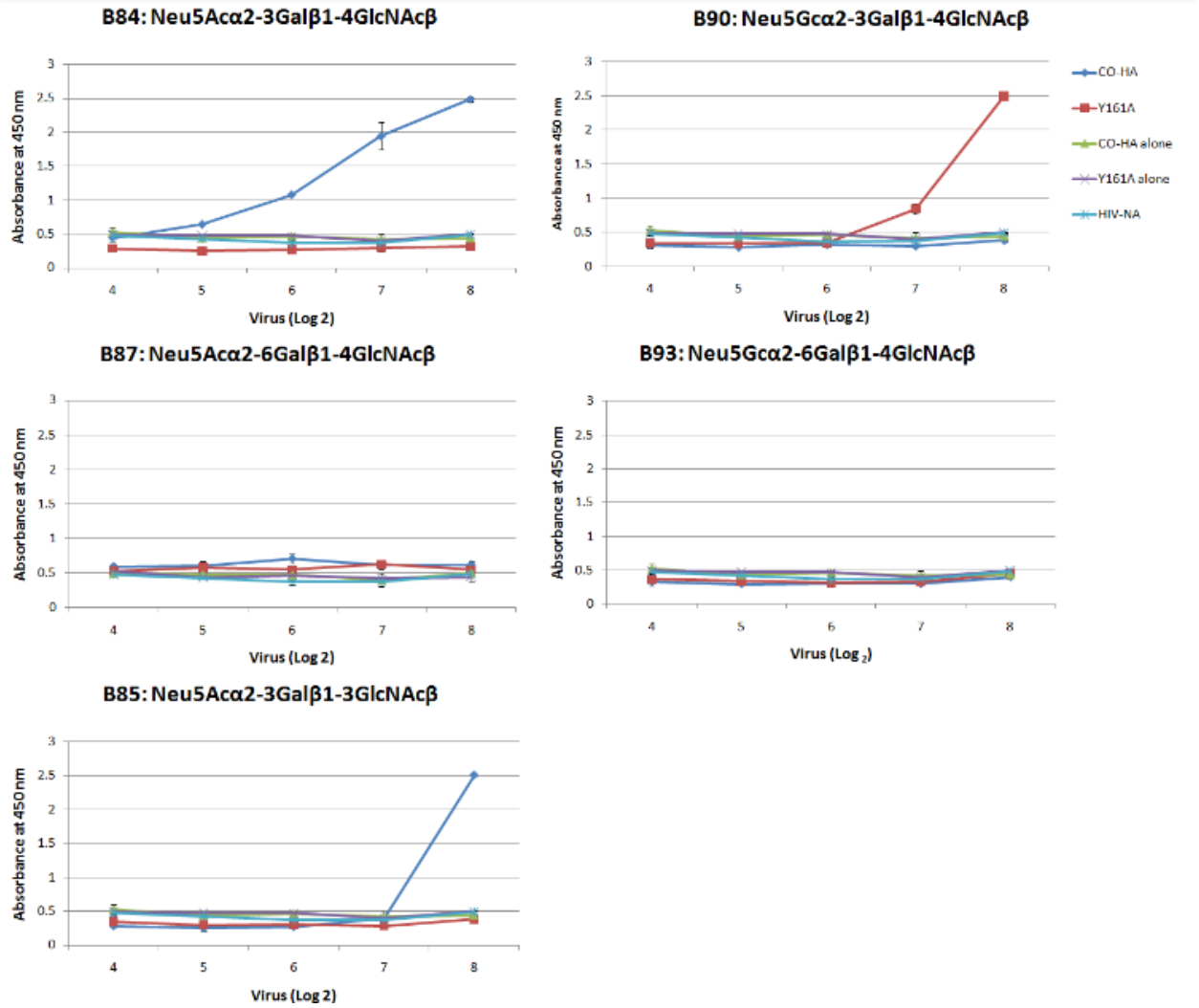

Figure 8. Y161A virus switched HA binding preference. Biotinylated oligosaccharides indicated were used to measure saccharid binding of parental CO-HA and mutant Y161A pseudotyped viruses. Binding of graded amounts of virus (from 16HAU to 256 HAU) to immobilized oligosaccharides was measured by the binding of an anti-HA antibody as described in the Methods. Wells with Y161A or CO-HA viruses alone without adding oligosaccharides served as negative controls. HIV-NA pseudotyped viruses binding to different saccharides were used as another negative control. All other controls including oligosaccharides alone and 2nd antibody alone were performed and displayed similar levels as negative controls here (data not shown).

when Neu5Gc α2,3 linked SA (3'S(Gc)LN) was incubated with these two viruses, the binding pattern was totally divergent. CO-HA viruses showed background binding level, while Y161A mutant viruses displayed a dose-dependent binding to 3'S(Gc)LN (Fig. 8B), which strongly supported our hypothesis that alanine substitution at Y161 switched virus SA receptor preference from NeuAc to NeuGc. Neither of the two viruses (Y161A and CO-HA) showed binding to Neu5Gc α2,6 linked saccharide (6'S(Gc)LN) (Fig. 8D) at current concentrations assayed, which further confirmed that 2,6 linkage is not preferred by avian influenza virus.

It has been reported that influenza virus adaptation was determined not only by the receptor terminal head which has the linkage of α2,3 and α2,6 motif, but also by the inner fragments of the carbohydrate chain [88-89]. Another oligosaccharide, 3'SLec, which differs from 3'SLN by penultimate saccharide linkage, was used for the ELISA binding assay (Fig. 8E). Not surprisingly, Y161A mutant did not show binding to 3'SLec since this oligosaccharide ends with Neu5Ac. CO-HA parental viruses also exhibited weaker binding to 3'SLec than 3'SLN at lower concentration, suggesting that H5 avian influenza has higher relative binding affinity to saccharides with β1-4 linkage rather than β1-3 linkage between the 2nd and 3rd sugars.

3.2.5 Growth property of Y161A and K307A/K310A mutants

The in vitro characteristics of the mutants Y161A and K307A/K310A were studied by generating recombinant viruses through plasmid transfection. We used HAlo as parental HA. HAlo is a modified HA from H5 avian strain influenza A/Vietnam/1203/04(H5N1) in which the HA polybasic cleavage peptide has been removed [73]. The relative plaque sizes produced by both mutants were morphologically similar to those produced by parental HAlo, except Y161A

showed about 50% reduction in the plaque size (Fig. 9A), suggesting impaired cell-cell transmission of Y161A on MDCK cells. The growth curve was evaluated on MDCK cells and the results are shown in Fig. 9B. Both Y161A and K307A/K310A mutant viruses replicated with a mild defect after 24 h (less than 10 fold) but with similar kinetics to HAlo viruses.

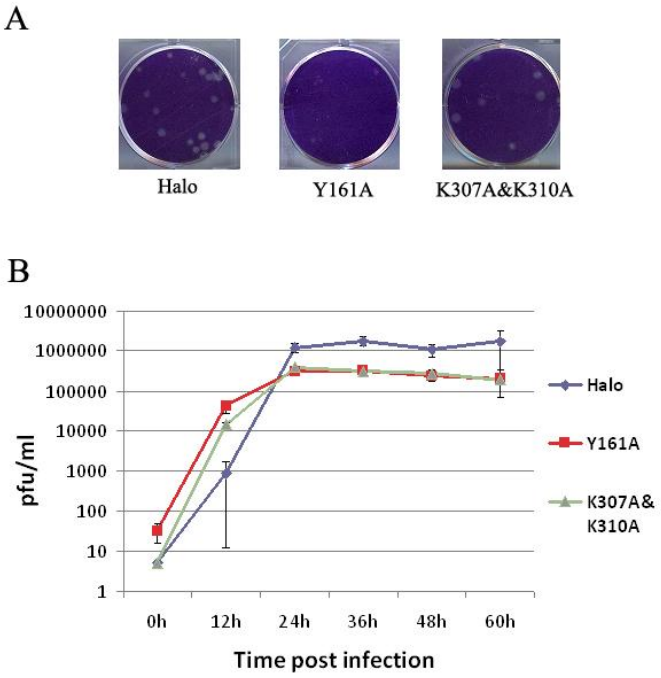

Figure 9. Growth curves of recombinant influenza HAlo viruses expressing parental HA and mutant Y161A or K307A&K310A proteins. MDCK cells were infected at an MOI of 0.01 with the different recombinant viruses. Viruses released to the supernatant were titrated at 0, 12, 24, 48, and 60 h postinfection by plaque assay on fresh MDCK cells.

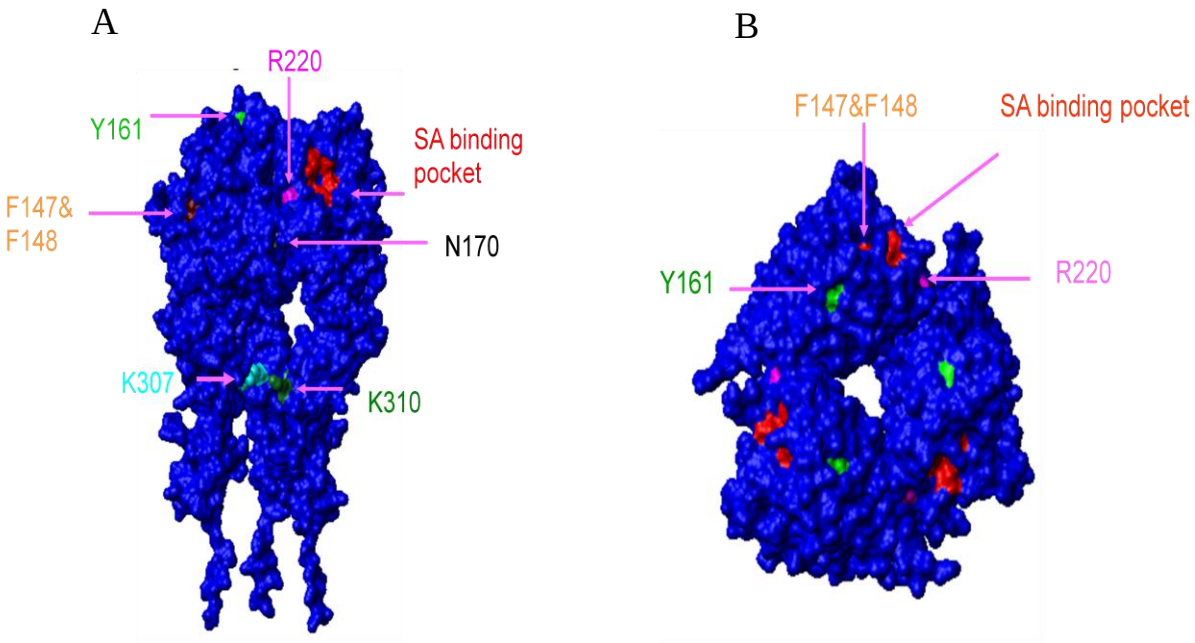

Figure 10. Target residues and sialic acid (SA) binding pocket are mapped onto a surface representation of the H5 HA1 trimer. A) Side view of the HA1 trimer. B) Top view of HA1 trimer. Only visible residues from the top view are marked on HA1 monomer.

3.3 Discussion

Receptor binding site of HA has been well studied, with the following amino acids identified as critical for receptor binding and viral entry: 98, 134 to 138, 153, 155, 183, 190, 194 and 224-228 [75, 90]. Most of these resides are highly conserved among all 16 subtypes of HA. However, little is known regarding the function of other conserved amino acids which are not involved in SA binding in viral entry. In this report, we have targeted 7 highly conserved residues of HA1 by alanine substitution and characterized their potential roles in HA-mediated viral entry. We have identified several key residues (F147/F148, Y161, N170, R220, K307, K310, and K307/K310) which are important for HA cellular processing and receptor recognition. Of particular interest, we have identified residue Y161 that is critical for different SA species recognition. These findings have important implications for understanding the function of the structure of HA in influenza entry during pathogenesis and host tropism determination.

Here, 7 selected residues and the receptor binding site (RBS) of HA are highlighted on H5N1 HA crystal structure (Figure 10A). F147, F148 and R220 are in the vicinity to RBS (Fig. 10B), and alanine substitutions at F147/F148 and R220 affected HA cleavage (see Fig. 6 lanes 5, 8) with most of the HA proteins retained in their precursor state in the producing cells. It is possible that alanine substitutions at these positions may destroy the recognition of HA to SAs on the surface of chicken and horse erythrocytes, resulting in the defective phenotype in receptor binding and viral entry.

N170 is a potential glycosylation site based on the Asn-Xaa-Thr/Ser sequence. Deshpande et al. [91] have reported that glycosylation affected cleavage of an H5N2 influenza

virus hemagglutinin and its virulence. It was thus possible that alanine substitution at N170 could affect HA cleavage and viral entry. To test this, N170A and CO-HA transfected cell lysates were digested with N-glycosidase F (Roche) and were analyzed on 7% polyacrylamide SDS gel (data not shown). Comparison of the shift between digested and undigested CO-HA and Y170A lysates suggested that N170 is likely not a glycosylation site, and further studies are needed in the future to resolve this issue.

The stem region of HA has been recently shown to be a potentially good target for therapeutic treatment for influenza. Ekiert et al. have identified a antibody CR6261 that binds to a highly conserved influenza virus epitope in the membrane-proximal stem region of HA1 and HA2 [92]. Interestingly, K310 is on the surface of the stem region of HA and near this conserved epitope, and alanine substitutions at K307 and K310 decreased the pseudovirus binding to animal erythrocytes and reduced viral entry more than 99%. Also interestingly, although the double mutant K307A/K310A totally abolished pseudotyped virus binding to both chicken and horse erythrocytes, and displayed zero HA titer, just like mutant R220A (see Fig 7), viral entry of K307A/K310A was 10-100 fold higher than R220A (see Fig 5). Viral growth curve of mutant K307A/K310A showed active viral replication in the first 20 h and was only about 10 fold lower than parental virus after 24 h. These results suggest that mutant K307A/K310A is mechanistically different from R220A in receptor recognition.

One important finding of the current work is that Y161A showed a clean switch of receptor recognition from NeuAc to NeuGc α2,3 linked SA. NeuAc and NeuGc SAs are the most prevalent SAs found in mammalian cells. The only difference between these two SAs is the

additional hydroxyl group in the N-glycolyl group of Neu5Gc [93]. It is known that humans are deficient of NeuGc SAs which is explained by an genetic mutation in CMP-N-acetylneuraminic acid hydroxylase, the rate-limiting enzyme in generating NeuGc from precursor NeuAc in cells of other mammals [94]. Hiroyuki M et al. have reported that amino acids at 143, 155 and 158 in HA of human H3 influenza virus are linked to the viral recognition of NeuGc [81]. In our study, we identified a highly conserved residue Y161 located at the top of HA head region outside of the known RBS (Fig. 10) which plays a critical role in virus recognition of NeuGc and NeuAc SAs. Alanine substitution at this position abolished the HA-mediated viral entry on 293T target cells (only 2% of the parental HA), while the reduction on A549 cells was about 50%. This cell type-dependent reduction is probably due to the different species SAs distribution on the two cell lines. Investigation of the SAs on these two cell lines is needed to explain the cell type specificity in viral entry. Rescued mutant Y161A viruses produced 50% reduction in plaque size, but were robust in viral replication, suggesting that alanine substitution at Y161 is important for viral transmission and infectivity. To further explore the mechanism of SA binding switching caused by Y161A, four surface-located amino acids T160, P162, Y195 and N248 surrounding Y161 were mutated to alanine. Also, Y161F mutagenesis was performed since F161 was used by some other influenza A strains. Only T160A showed weak ability in NeuAc to NeuGc switching, while the other mutants did not have detectable effect in different SA species recognition (data not shown), suggesting only Y161 is critical in changing receptor recognition.

In summary, this study has revealed new roles of the highly conserved, surface-located residues of HA in viral entry. We demonstrated that stem region (K307/K310) is also important in SA recognition and viral entry. Specially, single amino acid change at Y161 of HA1 of

influenza virus affected binding activity to different species of SA (NeuAc, NeuGc). This finding provides important insight in understanding the significance of SA species as a determinant in viral transmission and host range restriction.

4. IDENTIFICATION OF SPECIFIC DETERMINANTS IN H5N1 HA CRITICAL FOR VIRAL ENTRY

4.1 Introduction

Avian influenza virus subtype A (H5 and H7 subtype), a high pathogenic avian influenza (HPAI) can cause fatal infectious disease in animals and humans. The first outbreak of HAPI in poultry was due to virus of H5 subtype in 1959 [95]. Since then HPAI has attracted extensive attention not only because it has resulted in huge economic loss in the poultry markets, but also due to its increased capability of sustained bird-to-human and human-to-human transmission, posing the threat of an avian influenza A pandemic. The 1997 Hong Kong avian influenza infection is the most remarkable case with 6 deaths out of 18 total infections [96]. In addition to the primary infection in the lung, the virus has been isolated from other organs, including intestines, liver, spleen, brain and the fetus by penetrating the placental barrier [97], which means this HPAI H5N1 not only infects pulmonary epithelial cells and causes alveolar damages in the lungs, but can result in systemic infection in human. The pathogenic mechanism of H5N1 has not been fully elucidated, however, like 1918 Spanish flu pandemic the cytokine storm and dysregulation of chemokine may be the key mechanism of the severity of HPAI H5N1 virus [98-99].

In general, the transmission of avian influenza viruses from birds to humans is a rare event, which depends on multiple factors of host and virus, including the means of exposure, virus dose and virus strain. However, since error-prone RNA synthesis of influenza virus generates mutations constantly during replication due to the lack of proofreading of its own RNA polymerase, the new strains can break the species barrier and build infection in a new host by escaping the old immune system [100].

As one of the surface glycoproteins, hemagglutinin (HA) is a critical virulence and host tropism determinant for its function in mediating viral entry, which is the first step in the viral life cycle. HA binds to the cellular receptor sialic acid (SA) to initiate the attachment of the virus to the target cell. SA is a 9-carbon sugar located at the end of cell surface glycans and glycoproteins. Avian influenza virus preferentially binds to SA connected to the underlying sugar by α2,3 linkage, while human influenza virus prefers binding to SA of α2,6 linkage [30, 101]. Swine influenza virus binds to both SAs, explaining the high susceptibility of pigs to both avian and human influenza viruses, reassortment of which in pigs can create new strains of influenza viruses. The receptor binding site is at the distal end of each HA monomer, composed of three major parts 130 loop, 190 helix and 220 loop [102]. Residues at position 226 and 228 have been shown to be important in host tropism. Avian influenza HA contains Gln226 and Gly228, which forms a narrow receptor binding pocket in favor of α2,3SA, while human influenza HA contains Leu226 and Ser228, which forms a broader pocket that fits better for α2,6SA [30, 103]. The HA protein of 1918 Spanish flu possesses avian-like Gln226 and Gly228, however, residues at 190 and 225 are critical for 1918 virus binding to human receptor and virus transmission [38, 104-105]. These findings demonstrate the essential role of influenza HA for the virus infection and transmission.

In the previous study, we have demonstrated the role of a single amino acid Y161 of HA in determining influenza host tropism. To further examine the specificity of H5 HA in viral entry, we investigated the potential roles of the highly conserved and surface located residues of H5N1 HA in viral entry and the role in influenza host preference. Our study revealed that amino acid H117 and D241 play critical roles in HA-mediated influenza viral entry.

4.2 Results

4.2.1 Generation of the amino acid substitution mutants of influenza H5N1 HA1 subunit

The receptor binding domain within the HA1 subunit of HA has been well characterized and several critical residues have been identified which are responsible for mediating the binding of the virus to its receptor and are highly conserved among all the influenza strains. To further dissect and evaluate the critical residues of H5N1 HA in viral entry, an alignment of several H5 strain sequences obtained from NCBI gene bank was made and the conserved amino acids in HA1 subunit are highlighted in black in Fig. 11. Among these conserved amino acids, we selected 88 surface located residues by MolMol, which is a molecular graphics program for analyzing the three-dimensional structure of biological macromolecules. In this report, 36 charged, surface located, and highly conserved residues in H5 among the selected 88 residues were first targeted and analyzed by site-directed mutagenesis (Fig. 11). We have chosen codon-optimized HA (CO-HA, H5 subtype) as the parental HA. The substitution numbers are based on residue numbers within H3 HA.

4.2.2 Effects of the substitution mutants of HA on pseudotyped viral entry

Alanine scanning was first used to examine the role of the selected 36 target resides in viral entry. For studying the entry mechanism of the highly pathogenic avian influenza A virus, we have developed an HIV-based system to produce pseudotyped viruses. HIV-particles pseudotyped with CO-HA or mutant HAs were used to challenge 293T and A549 target cells. The luciferase activities from the target cells were used as an indicator for the viral entry level (Fig. 12A). In these experiments, virions pseudotyped with VSV-G were used as a positive

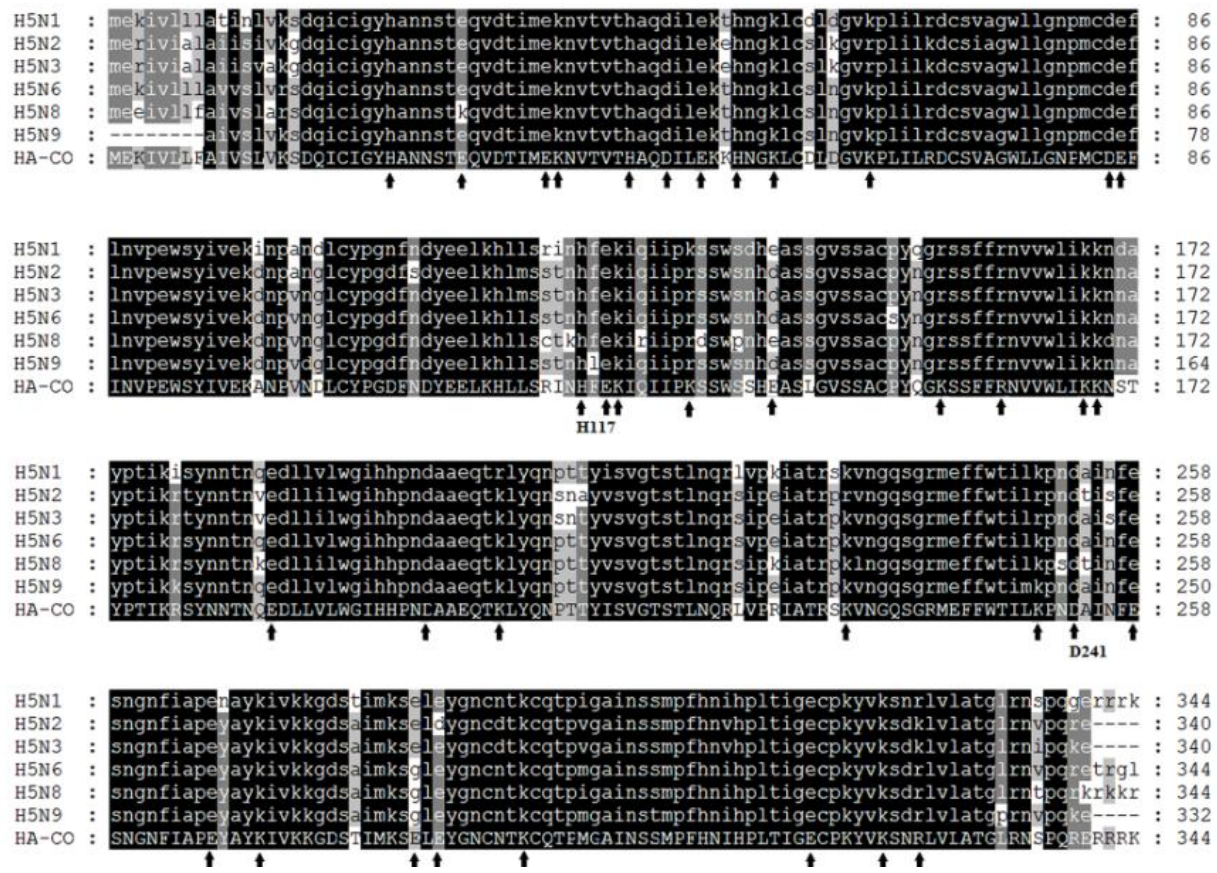

Figure 11. Sequence alignment of H5 HA1 sequences. CO-HA is the parental H5 HA. Residues shaded in black are the highly conserved ones in all H5 HA1. Charged residues located on the HA surface are marked by arrows. The number of each residue is named according to H3 HA number.

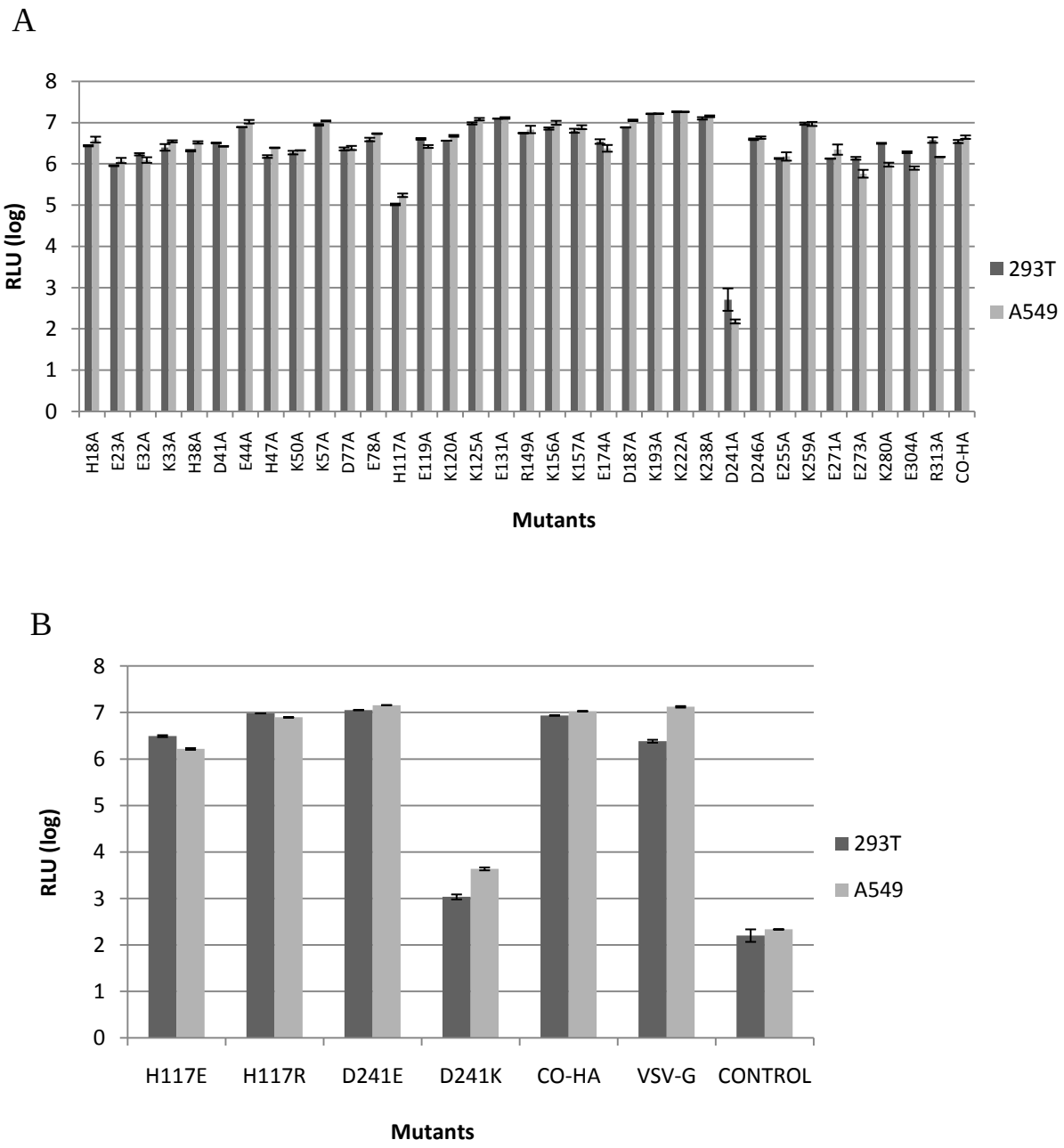

Figure 12. Mutational effects on viral entry as measured by luciferase activity. Relative luciferase units (RLU) of 36 target residues (A) and charged amino acid substitutions (B) were measured from 293T and A549 target cells (see results of substitutions K144A and K310A in Chapter 3). Data represent repeated experiments with similar trends. The error bars are the S.D. of three independent experiments.

control, as VSV-G has a broad spectrum of susceptible cell types [82-83]. Among the 36 alanine substitutions, H117A, D241A and K310A showed more than 90% of reduction in viral entry on both cell types, while the rest of the mutants behaved like parental HA-CO (Fig. 12A). Analysis of K310A has been described in Chapter 3 and will not be further discussed here. Since H117 and D241 are charged residues, we further analyzed whether the charges play a critical role in viral entry and host tropism by substituting with amino acids possessing different charges H117E and H117R; D241E and D241K. The luciferase reporter assay (Fig. 12B) showed that at position 117 when the original positively charged residue His (H) was replaced by the negatively charged amino acid Glu (E), viral entry was reduced by 81% and 71% in target cells A549 and 293T, respectively. In contrast, when the original positively charged residue (H) was replaced by another positively charged residue Arg (R), the mutant HA behaved like the parental HA-CO, suggesting that at position 117, a surface positive charge is preferred for HA-mediated viral entry. For position 241, replacement of the original negatively charged residue Asp (D) with a positively charged amino acid Lys (R) abolished the HA-mediated viral entry, while a negatively charged residue Glu (E) rescued viral entry, suggesting a critical role of the negative charge at position 241 in HA mediated viral entry.

4.2.3 Mutational effects on HA expression and viral incorporation

Western blot analysis was used to examine whether the reduced viral entry was due to a defect in HA protein expression, processing or incorporation as a result of amino acid substitution. Cell lysates from 293T producing cells transiently transfected with HIV-luc vector and different mutant HA plasmids were used to detect mutational effects on HA expression and processing, as shown in Fig. 13A and Fig. 14A. Mutants E119A and E174A were chosen as

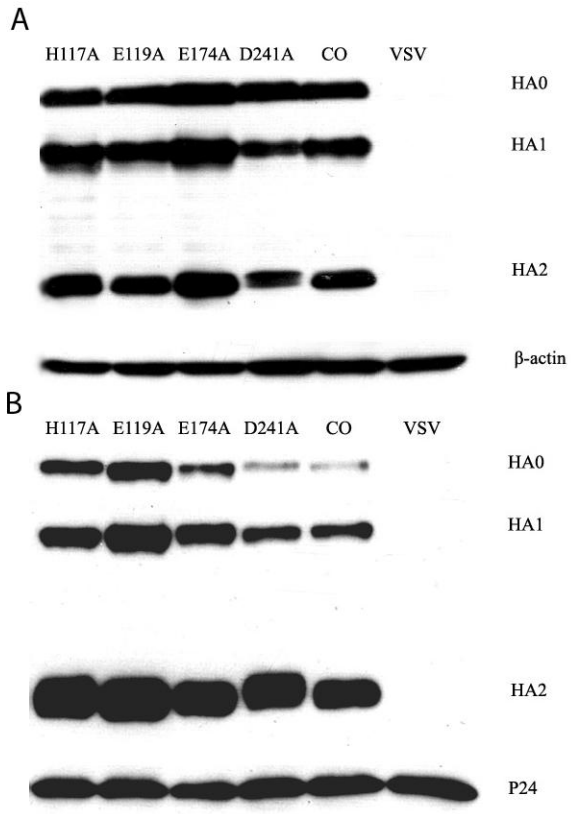

Figure 13. Western blot analysis of HA protein expression (A) and viral incorporation (B) after alanine substitutions. The HA precursor (HA0) and two proteolytic products HA1 and HA2 are shown.

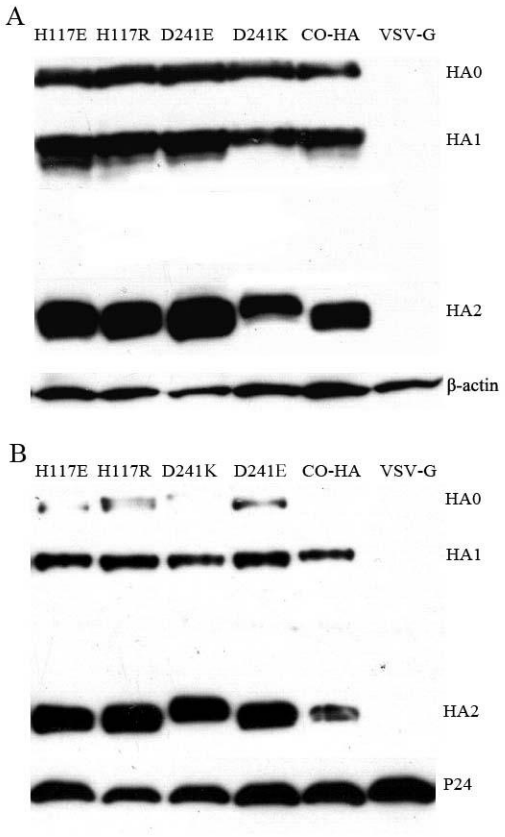

Figure 14. Western blot analysis of HA protein expression in 293T producer cells (A) and viral incorporation (B) after charged amino acid substitutions. The HA precursor (HA0) and two proteolytic products HA1 and HA2 are shown.

positive controls since alanine substitutions at these two positions did not affect pseudotyped viral entry. Western blot of parental HA showed three bands which correspond to HA0, HA1 and HA2, with the size of about 75kDa, 50kDa and 25kDa respectively (Fig. 13A, lane 5 and Fig. 14A, lane 5). This indicates that CO-HA was expressed and processed properly by host proteases in 293T cells. There was no obvious defect for all the mutants (H117A, E119A, E174A, D241A, H117E, H117R, D241E and D241K) in HA expression and cleavage (Fig. 13A and Fig. 14A).

To examine whether amino acid substitutions affected HA viral incorporation, parental HA-CO and individual HA mutants were cotransfected with the HIV vector to 293T cells, and the pelleted viral particles were subjected to SDS-PAGE and Western blot analysis (Fig.13B and Fig. 14B). The pseudovirions carrying CO-HA showed the presence of correct size of HA0, HA1 and HA2, indicating proper incorporation of HA into pseudoparticles (Fig. 13B, lane 5 and Fig. 14B, lane 5). Again, there was no obvious defect in HA incorporation into viral particles for any of the HA mutants.

4.2.4 Mutational effects on sialic acid recognition

Influenza viruses have been found to be able to agglutinate erthrocytes by binding to cell surface SAs [106]. The hemagglutination assay (HA assay) has been widely applied in studying influenza virus receptor recognition and influenza species transmission. We have used HA assay to further determine whether amino acid substitutions changed SA binding preference and thus altered viral entry. Chicken and horse erythrocytes were chosen based on the previous studies that influenza A virus receptor specificity correlates with the agglutinating pattern from different animal erythrocytes [85].

Two-fold serial dilutions of purified pseudotyed viral particles were mixed with chicken or horse erythrocytes. The hemagglutination results of parental HA (CO-HA) and HA mutants with chicken erythrocytes are shown in Fig. 15 (the HA assay result with the horse erythrocytes was similar to that of chicken's, data not shown). H117A (Fig. 15A) and H117E(Fig. 15B) both showed lower HA titer than CO-HA, while H117R (Fig. 15B) had the same HA titer with CO-HA, suggesting that H117R recognized SAs as well as CO-HA and that H117E and H117A weakened HA-SA interaction, with H117A displaying more severe reduction in SA binding. This is consistent with the luciferase based viral entry assay, suggesting that positive charge in 117 is preferred for sufficient SA recognition and viral entry. D241A (Fig. 15A) and D241K (Fig. 15B) showed zero HA titer while D241E (Fig. 15B) displayed the same HA titer with CO-HA, suggesting that a negatively charged residue at position 241 is important for HA binding to SA and viral entry.

To further confirm our results, we used Enzyme-Linked Immunosorbent Assay (**ELISA**) to investigate the direct binding activity of mutant and parental CO-HA pseudoparticles to biotinylated saccharide Neu5Acα2-3Galβ1-4GlcNAcβ- bearing α2,3 linked terminal SA which is preferred by avian influenza virus. Controls including CO-HA virus alone (without incubation with any biotinylated oligosaccharides), mutant viruses alone, HIV-NA virus pseudotyped with only NA glycoprotein, oligosaccharides alone and 2^{nd} antibody alone were performed and only the HIV-NA control is shown in Fig. 16 since all the controls displayed similar levels of absorbance at 450 nm. CO-HA pseudotyped viruses bound to Neu5Ac α2,3 linked SA in a dose-dependent manner (Fig. 16A and B). H117E showed less yet reasonably well binding to SA compared with CO-HA. H117R even exhibited better recognition of SA than parental CO-HA,

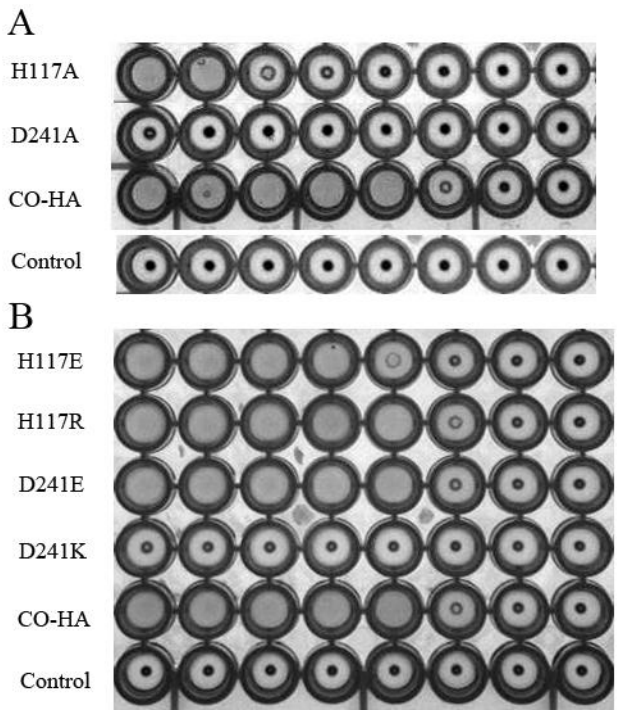

Figure 15. Hemagglutination assay of titrated pseudovirions mixed with chicken erythrocytes. A) Alanine substituted mutants HA assay. B) Charged amino acid substituted mutants HA assay. HA assay from horse erythrocytes showed the same pattern with chicken erythrocytes (data not shown).

suggesting that the amino acid Arg at position 117 may be more favored by Neu5Ac α2,3 linked SA than the original amino acid His (Fig. 16A). D241K which showed background level of viral entry also displayed background level of receptor binding. On the contrary, D241E rescued virus binding of SA to the level of parental virus (Fig. 16B). These data are consistent with the luciferase viral entry and HA assay, further confirming that specific charges at positions 117 and 241 are important for receptor recognition and therefore are essential for viral entry.

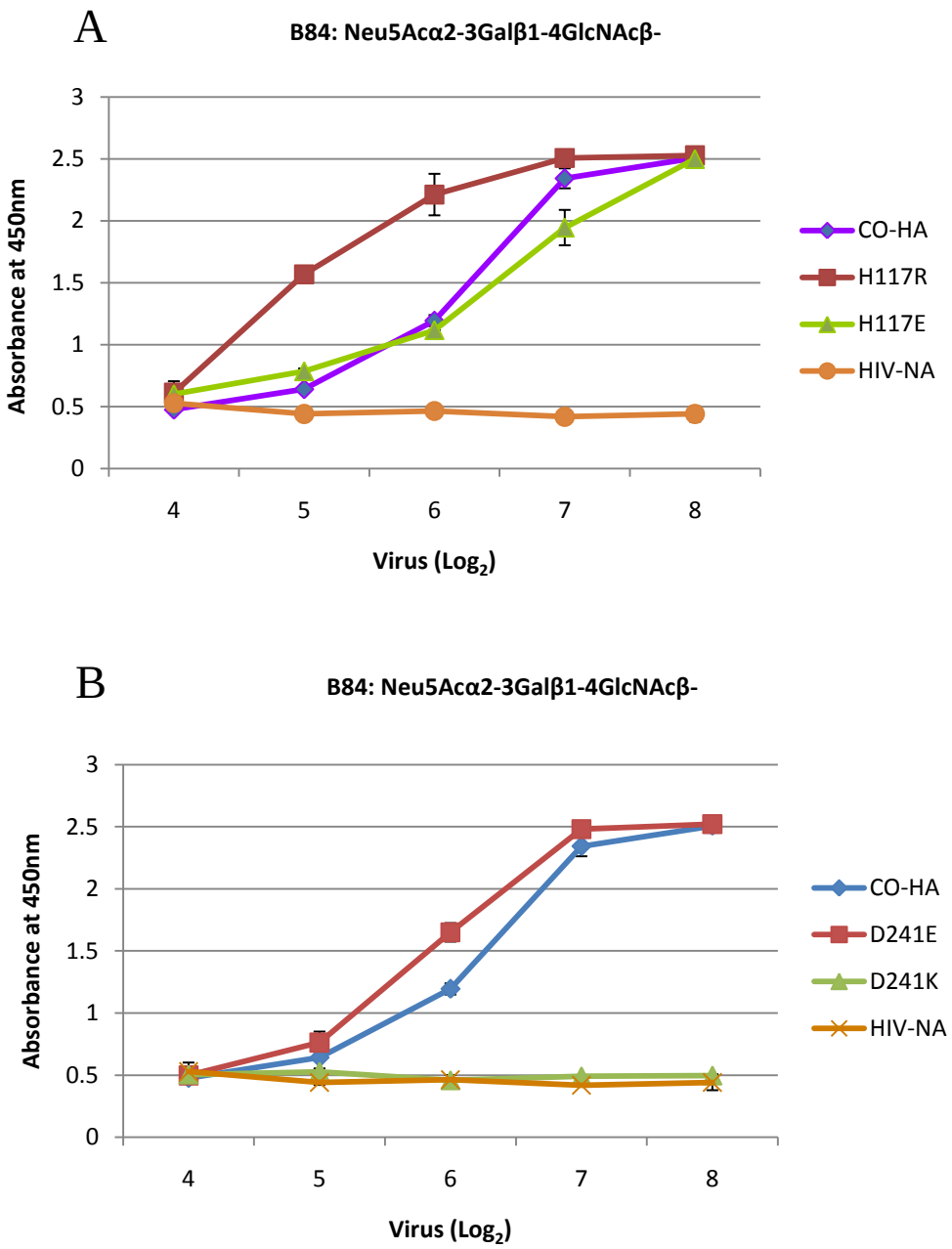

Figure 16. Sialic acid binding preference. Biotinylated oligosaccharides indicated were used to measure saccharid binding of parental CO-HA and mutant pseudotyped viruses. Binding of graded amounts of virus (from 16HAU to 256 HAU) to immobilized oligosaccharides was measured by the binding of an anti-HA antibody as described in the Methods. HIV-NA pseudotyped viruses binding to different saccharides were used as a negative control. All other controls including viruses alone without adding oligosaccharides, oligosaccharides alone and 2nd antibody alone were performed and displayed similar levels as negative control HIV-NA here (data not shown).

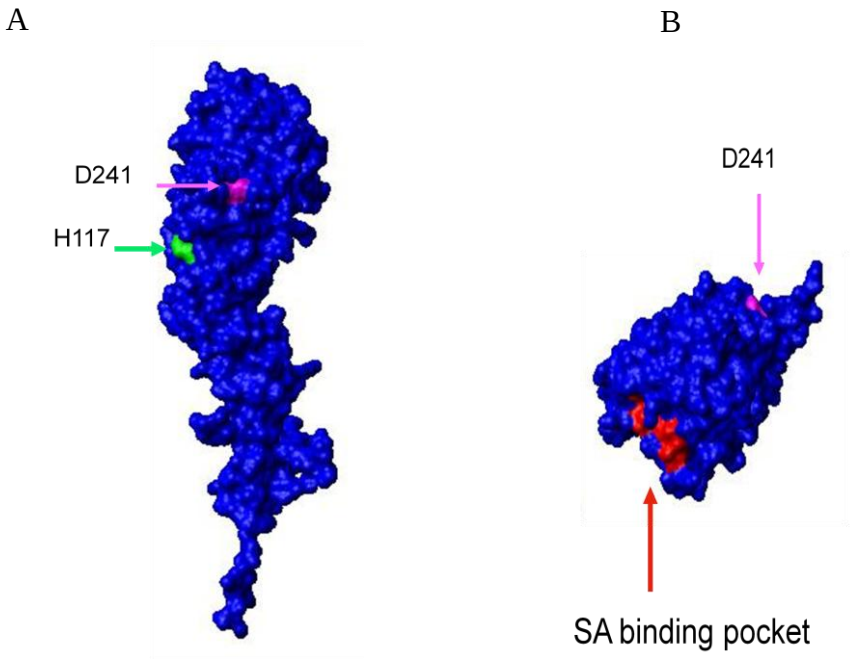

Figure 17. Target residues and sialic acid (SA) binding pocket are mapped onto a surface representation of the H5 HA1 monomer. A) Side view of the HA1 monomer. b) Top view of HA1 monomer. H117 is not visible from the top view.

4.3 Discussion

In this study we investigated the possible roles of a selected group of residues of H5N1 HA1, which are surface located, highly conserved, and charged amino acids, in viral entry. By sequence alignment and MolMol computer modeling, we had a list of 88 highly conserved and surface located residues, which were further narrowed down to 36 charged residues as the targets. Analysis of alanine substitution mutants of these residues showed that three substitution mutants were impaired in viral entry: H117A, D241A and K310A (K310A is discussed in previous chapter). Further, we demonstrated that a positively charged residue is favored at position 117 and a negatively charged residue at position 241is critical for HA-mediated viral entry.

Analysis of HA expression and incorporation suggested that there were no obvious defects for almost all of the HA mutants tested, including the entry defective mutants except mutant D241K (see Fig. 15). Although H117 and D241 are not in the vicinity of SA binding pocket (Fig. 17), HA assay and glycan binding assay (see Fig. 15 and Fig. 16) showed a good correlation between SA binding and viral entry level: the lower the HA titer, the less the HA binding to SA, the lower the viral entry. The mutants which showed no detectable HA titer also displayed background level of viral entry (for example, mutants D241A and D241K, see Fig. 12 and Fig. 15). HA assay carried out using either chicken erythrocytes or horse erythrocytes showed the same pattern, suggesting that those mutants did not show difference in recognition of different species of SAs, since on chicken erythrocytes surface the majority SA is NeuAc, while on horse erythrocytes surface the majority SA is NeuGc [85-87].

Overall, the data suggested that positive charge of the residue is favored at 117 and at 241 the negative charge is critical for NeuAcα2,3 linked SA receptor recognition and viral entry. Charged residues H117 and D241 may be important for HA interaction with SA or other host factors, or they are involved in intramolecular or intermolecular interactions of HA via ionic interactions. More studies are needed to elucidate the mechanism of these two resides in affecting viral entry.

5. CONCLUSIONS AND DISCUSSION

Influenza A and B viruses are the major causative agents for seasonal influenza. Influenza A virus has caused four major flu pandemics in recent history resulting in over 50 million deaths worldwide. HPAI (H5N1) virus continues to cause outbreaks and deadly disease in poultry and migratory birds, and occasional high fatality human infections, leading to the concern that this strain might be the source of the next pandemic once it gains the ability to break the species barrier and infect humans. Influenza A virus is a segmented, negative-stranded RNA virus. RNA segment 4 encodes an important viral surface glycoprotein HA which is essential in the initial stages of infection. Sixteen subtypes of HA have been identified and segregated into two groups [107-108] based on their sequence homology. HA is synthesized as a precursor HA0, then cleaved by enzymes into two subunits HA1 and HA2, mediating viral attachment to the receptor and fusion with cellular membranes respectively. The role of HA in viral entry has been extensively studied for many years, however, questions still remain to be answered with regard to HA determination in host tropism and viral entry in addition to mediating SA binding and fusion process .

One objective of my thesis was to examine the role of the residues which are surface located, highly conserved among all 16 HA1 subunits in viral entry and host tropism determination. The second objective was to study the specificity of H5 HA in viral entry. Two studies revealed three major interesting residues Y161, H117, and D241, which play critical roles in influenza viral SA receptor recognition and viral infection by different mechanisms. Y161A leads a dramatic switch of the virus recognition of different species of SA from NeuAc to NeuGc. While for H117 and D241, the charge of specific residue appears to affect flu viral entry by affecting SA receptor binding without changing SA species preference.

Alanine substitution at Y161 did not affect HA processing and incorporation into viral particle. The rescued virus by reverse genetics showed 50% reduction in plaque formation and 5-10 fold reduction in viral growth after 24 h, but still with robust viral replication on MDCK cells, which is consistent with 50% reduction of luciferase activity of Y161A pseudovirus on A549 cells. However pseudovirus Y161A displayed 98% reduction in viral entry on 293T target cell. Since 293T and A549 cell surface have different amounts of α2,3 linked and α2,6 linked SAs [56], I first speculated that Y161 might be important for recognition of SA with different linkages, therefore contributing to influenza host species preference. However, the data from the HA assay (see Fig. 7) did not support this notion. The distribution of different levels of α2,3 linked and α2,6 linked SAs did not correlate with HA titer of Y161A pseudovirus in HA assay. On the contrary, I observed a good correlation between Y161A HA titer and different species of SA (NeuGc and NeuAc) which are only specifically expressed on certain animal species. Thus I hypothesized that Y161 is a critical residue in HA for recognition of different species of SA. Indeed, the glycan binding ELISA assay demonstrated that alanine substitution at Y161 switched the receptor recognition from NeuAc SA to NeuGc SA (see Fig. 8), suggesting that this highly conserved residue Y161 is essential in determining influenza virus binding to different species of SAs and host tropism. However, Y161 is not in the vicinity of the known RBS on the surface (see Fig. 10), but it is still proximate to the RBS in the three-dimensional structure, close to residues 194, 195 which are known to be involved in SA binding. The difference between NeuAc SA and NeuGc SA is that NeuGc SA has an extra hydroxyl group at the position 5 of the pyranose ring (Fig. 18) Therefore, I speculate that Y161A changed influenza virus receptor recognition from NeuAc SA to NeuGc SA and host tropism by altering the RBS shape through residues 194, 195 to allow the extra room for hydroxal group of NeuGc.

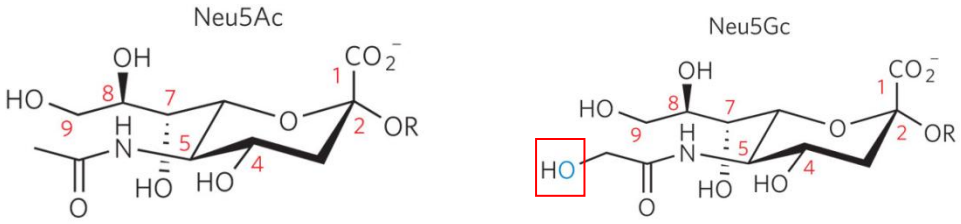

Figure 18. Structure of N-acetyl (Neu5Ac) and N-glycolyl (Neu5Gc) neuraminic acids. Neu5Ac is the precursor of Neu5Gc, which is generated by enzymatic hydroxylation of Neu5Ac at position 5 of the pyranose ring (Essentials of Glycobiology. 2nd edition, Chapter 44, modified).

Alanine substitution at H117 or D241 reduced H5 HA mediated viral entry by more than 95%. HA assay of mutant viruses H117A and D241A showed a good correlation between the reduced SA binding ability and the reduced viral entry level. Considering these two residues are surface located and highly conserved only in H5 HA1, I speculated that the charge of each residue may be important for receptor recognition. To test this hypothesis, I generated four more mutants: H117E, H117R; D241E, D241R. Mutation of H 117 to a negatively charged amino acid Glu (E) increased SA binding compared to mutant H117A, but still with lower SA binding level compared to parental CO-HA, as measured by HA assay and glycan binding ELISA (see Fig. 15 and Fig. 16). The entry level of mutant H117E was about 50% of the parental CO-HA, while H117A only showed background level of viral entry (less than 1%) (See Fig. 12). Interestingly, when H117 was mutated to Arg (R), both SA binding and viral entry were enhanced compared to CO-HA. These results strongly suggest that a positively charged amino acid at position 117 is preferred for SA binding and viral entry. On the contrary, for position 241 the negative charge is essential for SA recognition and viral entry, as substitution of D241 with either Lys (K) or Ala (A) totally abolished SA binding and viral entry, while substitution of D241 with Glu (E) restored the mutant receptor binding and entry level of that of parental CO-HA. Since neither of these two residues is in the vicinity of SA binding pocket on HA (see Fig. 17), it suggests that not only the direct binding of the HA residues to SA is critical for viral entry, but other parts of HA may also affect SA binding. Further, my results showed that residue H117 and D241 did not affect viral entry by changing different species of SA recognition as HA assay of all the mutations at 117 and 241 displayed the same pattern from chicken and horse erythrocytes. Together, these results suggest that a specific charge at position 117 and 241 is important for H5 HA mediated influenza viral entry by affecting SA receptor binding, or affecting binding to other

factors via ionic interactions. The mechanism of these two residues in H5 influenza viral entry needs to be further addressed.

In addition, I also identified other important resides which are important for influenza viral entry. In the first study (see Chapter 3), double alanine substitutions at position 307 and 310 (located at the stem region of HA) affected SA binding and viral entry, without disrupting HA expression and incorporation into pseudovirus. Interestingly, although the double mutant K307A/K310A showed zero HA titer on both chicken and horse erythrocytes by HA assays (see Fig.7), its ability to mediate viral entry was not totally impaired. Rescued recombinant virus with K307A/K310A by reverse genetics also showed robust viral growth and similar plaque size compared to parental HAlo, suggesting the critical role of stem region of HA in viral entry.

In addition to the results I described above, the HA substitution mutants generated in these studies have been used to characterize the binding pockets/sites of HA inhibitors. Our collaborators at Microbiotix Inc. (Wocester, MA, USA) have utilized the HA/HIV pseudotyping system developed by our lab to screen and identify anti-HA inhibitors. Among roughly 100,000 small compounds screened, three potent anti-HA inhibitors have been discovered. Further, these anti-HA inhibitors were shown to be highly effective against pandemic and oseltamivir-resistant H1N1 strains as well as avian influenza H5N1 strains.

The 16 subtypes of HA are categorized into 2 groups different from each other by a specific hydrophobic pocket at the interface of the HA1 monomers near the HA2 fusion domain [109-111]. This region is recognized by a neutralizing antibody which binds to the conserved

domain at the junction of HA1 and HA2 [51, 112]. Primary data suggested that the 3 inhibitors acted in a specific manner, inhibiting only influenza viruses with group 1 HA (H1 and H5 subtype) and not influenza viruses with group 2 HA (H3 and H7 subtype). Therefore, it is hypothesized that the 3 inhibitors bind to the specific conserved pocket of group 1 HA. Binding in this conserved picket is expected to inhibit the conformational changes of HA to its fusogenic form. In an attempt to predict putative binding sites for small molecule influenza inhibitors on HA, SiteMap calculations of computational modeling were used. They are designated as S1 through S3 (Fig. 19). Of the three pockets, S1 is calculated to be hydrophobic in nature and is at the junction of HA1 and HA2, a region which is conserved among the HAs and is near the interaction of the HA monomers.

To map the inhibitor binding site(s) on HA, the HA mutants generated by us were used to generate HA/HIV pseudovirions and each of the mutant pseudoviruses were incubated with the target cells in the presence or absence of the HA inhibitors. It was found that viruses carrying mutants L51A, D54A, K57A, R114E, and H117E could escape the inhibition by the inhibitors. More interestingly, escape mutants L51A, D54A, K57A, R114E, and H117E fall into the predicted S1 pocket. Therefore, S1 is predicted to be the potential inhibitor binding site for the 3 HA inhibitors.

Although more studies will be needed to elucidate the mechanism of these inhibitors for the development of an influenza specific inhibitor targeting HA, to be used alone or in combination with anti-influenza compounds, as a prophylactic and/or therapeutic drug during influenza infection, these results illustrate the power of an integral approach of molecular,

structural, and modeling techniques in developing potential anti-influenza therapeutics. These escape mutants may cause extra attention in the future influenza surveillance since they may occur in nature and produce newly developed drug resistant strains.

In conclusion, I have studied several crucial residues that have not been discussed previously and that are important for influenza viral entry by different mechanisms. Although they are highly conserved in the evolution of influenza virus, due to the unique features of influenza antigenic drift and antigenic shift, these mutations may occur in nature. For example, residue Y161 is highly conserved among all the 16 HA1 subunits, mutation at Y161 to alanine can be tolerated by influenza virus and can change the viral tropism toward hosts which have the NeuGc SA on the cell surface. Therefore, evolution of influenza at this particular position may be related to the future epidemiology of influenza in all the species which bearing NeuGc SAs. The studies described in this thesis may provide important insight in understanding the structural information in studying influenza virus entry and the evolution.

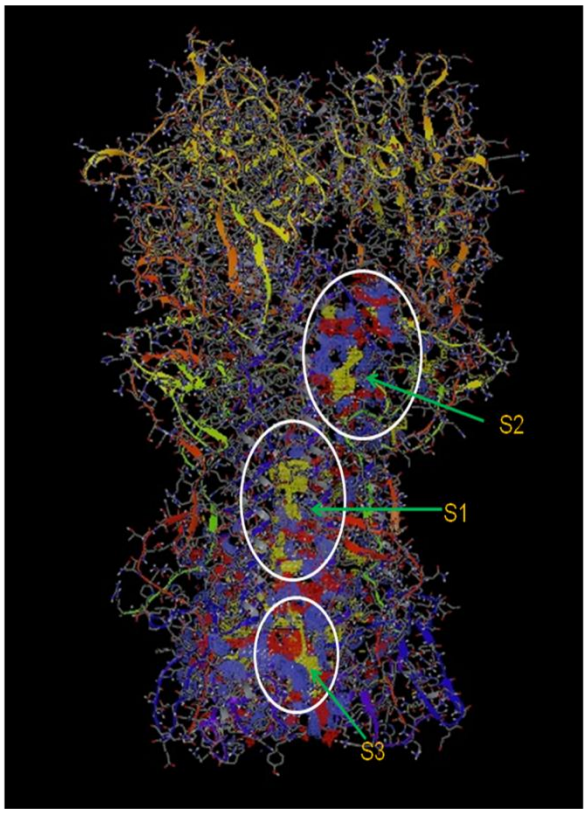

Figure 19. Computational studies by Schrödinger Sitemap locate three hydrophobic pockets (S1-3) in the HA (H1) pre-fusion crystal structure (Microbiotix, Inc.).

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PUBLICATIONS

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