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Adaptation of avian influenza A (H6N1) virus from avian to human receptor-binding preference

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Abstract

The receptor-binding specificity of influenza A viruses is a major determinant for the host tropism of the virus, which enables interspecies transmission. In 2013, the first human case of infection with avian influenza A (H6N1) virus was reported in Taiwan. To gather evidence concerning the epidemic potential of H6 subtype viruses, we performed comprehensive analysis of receptor-binding properties of Taiwan-isolated H6 HAs from 1972 to 2013. We propose that the receptor-binding properties of Taiwan-isolated H6 HAs have undergone three major stages: initially avian receptor-binding preference, secondarily obtaining human receptor-binding capacity, and recently human receptor-binding preference, which has been confirmed by receptor-binding assessment of three representative virus isolates. Mutagenesis work revealed that E190V and G228S substitutions are important to acquire the human receptor-binding capacity, and the P186L substitution could reduce the binding to avian receptor. Further structural analysis revealed how the P186L substitution in the receptor-binding site of HA determines the receptor-binding preference change. We conclude that the human-infecting H6N1 evolved into a human receptor preference.

Keywords crystal structure; H6N1; hemagglutinin; receptor binding
Subject Categories Microbiology, Virology & Host Pathogen Interaction
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Introduction

Influenza A virus is an enveloped, negative-strand RNA virus with a segmented genome containing eight gene segments and that can stably adapt to humans, leading to sustained human-to-human transmission (Taubenberger & Kash, 2010; Shi *et al*, 2014) and cause mild or severe diseases (Taubenberger & Morens, 2008). Two major proteins are embedded on the envelope membrane of the influenza virus, hemagglutinin (HA) and neuraminidase (NA) (Gamblin & Skehel, 2010). HA binds to sialic acid receptors on target cells to initiate the virus infection, and NA cleaves the sialic acid receptor to allow virus release (Palese *et al*, 1974; Sauter *et al*, 1989; Liu *et al*, 1995; Gambaryan *et al*, 1997). Influenza A viruses are classified into distinct subtypes based on the antigenicity of their HA and NA proteins. To date, 16 functional HA subtypes (H1-H16) and nine functional NA subtypes (N1-N9) have been identified, not including recently identified HA and NA homologues (H17, H18, N10, and N11) from bat-derived influenza-like virus genomes (Li *et al*, 2012; Tong *et al*, 2012, 2013; Zhu *et al*, 2012, 2013; Sun *et al*, 2013; Wu *et al*, 2014). The functional balance between HA and NA activities is important for viral pathogenicity, replication efficiency, and transmissibility (Baum & Paulson, 1991; Ohuchi *et al*, 1997; Wagner *et al*, 2000; de Wit *et al*, 2010).

In the past century, there have been four severe influenza pandemics in 1918 (Spanish flu), 1957 (Asian flu), 1968 (Hong Kong flu), and 2009 (09-pH1N1), which were caused by H1N1, H2N2, H3N2, and again H1N1, respectively. The slightly mild 1977 Russian flu was also caused by H1N1 (Gregg *et al*, 1978; Shenderovich *et al*, 1979). To date, only the H1, H2, and H3 subtypes of influenza A viruses have adapted to humans, causing annual seasonal flu worldwide, in addition to the recorded pandemics mentioned above (Taubenberger & Kash, 2010). Furthermore, the H5, H7, H9, and H10 subtypes have been reported to cause sporadic infections in humans (Claas *et al*, 1998; Yuen *et al*, 1998; Guo *et al*, 1999; Koopmans *et al*, 2004; Gao *et al*, 2013; Chen *et al*, 2014). The seasonal flu virus preferentially binds to α2,6-linked sialic acid receptors, whereas the avian influenza virus preferentially binds to α2,3-linked sialic acid receptors (Imai & Kawaoka, 2012; Shi *et al*, 2014). The receptor-binding properties of HA are a major determinant for the interspecies transmission of influenza A virus (de Graaf & Fouchier, 2014; Shi *et al*, 2014). A relatively clear molecular understanding of the receptor-binding properties of HA has been established in limited HA subtypes, including H1, H2, H3, H5, H7, H9, H10, H13, and H16 (Eisen *et al*, 1997; Ha *et al*, 2001; Gamblin *et al*, 2004; Liu

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et al, 2009; Lin *et al*, 2012; Lu *et al*, 2012, 2013a,b; Xu *et al*, 2012, 2013; Shi *et al*, 2013b, 2014; Xiong *et al*, 2013a,b; Yang *et al*, 2013; Zhang *et al*, 2013a,b; Vachieri *et al*, 2014), but is unknown in other HA subtypes, which represents a potential threat to human health.

The H6N1 virus has been isolated from migratory birds and domestic poultry in many countries, is considered to have low pathogenicity, and causes outbreaks in poultry with low mortality (Choi *et al*, 2005; Lee *et al*, 2006; Siembieda *et al*, 2010; Corrand *et al*, 2012; Muzyka *et al*, 2012). Recently, a human case of infection with the H6N1 virus was reported for the first time in Taiwan in June 2013 (Wei *et al*, 2013). Sequence analyses reveal that the human isolate (A/Taiwan/2/2013, human-H6N1) from the patient is highly homologous to the chicken H6N1 virus isolates in Taiwan (Shi *et al*, 2013a; Wei *et al*, 2013; Yuan *et al*, 2013). Indeed, since 1972, infection with the H6N1 virus has been prevalent in domestic chickens in Taiwan (Lee *et al*, 2006), and the virus circulating in Taiwan poultry has developed into a genetically unique lineage, different from the H6 subtype viruses circulating in southern China (Lee *et al*, 2006). Recently, Wang *et al* (2014) have assessed the receptor-binding preference, replication, and transmissibility in mammals of a series of H6 viruses isolated from live poultry markets in southern China from 2008 to 2011, pointing that H6 influenza viruses pose a potential threat to human health. However, mechanistic clues of receptor-binding determinant of H6 subtype viruses are unclear yet. An increased understanding of the molecular mechanism involved in receptor-binding properties of H6 subtype viruses could help us to predict the pandemic or epidemic potential.

Here, we performed comprehensive analysis of key residues in the receptor-binding site of Taiwan-isolated H6 HAs from 1972 to 2013. We propose that the evolution of receptor-binding properties of Taiwan-isolated H6 HAs has undergone three major processes: initially avian receptor-binding preference, secondarily obtaining human receptor-binding capacity, and recently human receptor-binding preference. This hypothesis has been confirmed by receptor-binding assessment of three representative virus isolates from these three stages, including the avian isolate (A/duck/Taiwan/0526/72, duck-H6N1) in 1972, the human-H6N1, and a homologous avian isolate (A/chicken/Taiwan/A2837/2013, chicken-H6N1). The duck-H6N1 HA preferentially binds the avian receptor, and both the chicken-H6N1 and human-H6N1 HAs bind avian and human receptor analogs, but the human-H6N1 displayed dramatically reduced binding to the avian receptor relative to the human receptor, a prerequisite for a human-adapting virus (de Graaf & Fouchier, 2014). Mutagenesis experiments have revealed that the E190V and G228S substitutions are important to obtain the human receptor-binding capacity, and further, P186L substitution is responsible for the receptor-binding preference change. Moreover, crystal structures of the human and avian HAs in complex with the receptor analogs elucidated the structural basis for the receptor-binding change.

Results

Comprehensive analysis of receptor-binding-related key residues in Taiwan-isolated H6 HAs

To obtain the mechanistic clues of receptor-binding properties of the Taiwan-isolated H6 subtype viruses, we analyzed the receptor-binding-related key residues of HAs from virus isolates between 1972 and 2013. There are totally 60 H6 HA sequences from Taiwan in the GISAID (Global Initiative on Sharing All Influenza Data) database. Previous studies have been showed that amino acid substitutions at site 190, 186, 226, and 228 are important for receptor-binding change in several HA subtypes including H1, H2, H3, H5, and H7 (Shi *et al*, 2014). Thus, we presented the residue compositions in these four sites for the 60 Taiwan-isolated H6 HA sequences (Table 1). We found that, from 1972 to 1998, the residue combination is P186/E190/Q226/G228. While from 1999 to 2004, amino acid substitutions at site 190 and 228 occurred, that the residue at site 190 could be E, L, or V and the residue at site 228 could be G or S. In 2004, a P186T substitution was also observed, but only one case in those 60 HA sequences. From 2005 to 2013, only the residue combination P186/V190/Q226/S228 was observed in the isolated H6 subtype viruses. Most recently in 2013, a P186L substitution occurred in the HA sequence of human-H6N1, compared with the highly homologous chicken-H6N1. The change of four-residue combinations can be divided into different stages, an initial stage with residue combination of P186/E190/Q226/G228, a second mutated stage with amino acid substitutions at site 190 and 228, a third stable stage with residue combination of P186/V190/Q226/S228, and a case of human infection with residue combination of L186/V190/Q226/S228.

Table 1. Comprehensive analysis of key residues in the receptor binding site for Taiwan-isolated H6 subtype viruses from 1972 to 2013.

Year	No.	186	190	226	228
1972	1	P	E	Q	G
1987	2	P	E	Q	G
1997	1	P	E	Q	G
1998	1	P	V	Q	G
1999	8	P	E(2/8)/L(1/8)/V(5/8)	Q	G
2000	5	P	L(2/5)/V(3/5)	Q	G(2/5)/S(3/5)
2001	4	P	V	Q	S
2002	9	P	L(2/9)/V(7/9)	Q	G(2/9)/S(7/9)
2003	4	P	E(1/4)/L(1/4)/V(2/4)	Q	G(2/4)/S(2/4)
2004	5	P(4/5)/T(1/5)	E(2/5)/L(1/5)/V(2/5)	Q	G(3/5)/S(2/5)
2005	2	P	V	Q	S
2009	6	P	V	Q	S
2010	3	P	V	Q	S
2012	6	P	V	Q	S
2013	3	P(2/3)/L(1/3)	V	Q	S
Total	60				

Note: The numbers in the parentheses represent the frequency.

Receptor-binding properties of duck-, chicken-, and human-derived H6N1

To evaluate the evolution process of receptor binding for the Taiwan-isolated H6 subtype viruses, we chose three representative virus

isolates with different residue combinations in the four sites (186/190/226/228) of HA, including the avian isolate (A/duck/Taiwan/0526/72, duck-H6N1) in 1972, the human-H6N1, and a homologous avian isolate (A/chicken/Taiwan/A2837/2013, chicken-H6N1). To assess the binding affinities of duck-H6N1, chicken-H6N1, and human-H6N1 to canonical avian-like and human-like receptor analogs (Jagger *et al*, 2012), we prepared soluble HA proteins and showed by surface plasmon resonance (SPR) that the duck-H6N1 HA preferentially binds to avian receptor (with an affinity of 1.17 μM), with an undetectable binding to human receptor (Fig 1A–C). While the chicken-H6N1 and human-H6N1 HAs bind to both avian and human receptors. The chicken-H6N1 HA still displays a binding preference for the avian receptor, with an affinity of 5.17 μM, but low binding affinity for the human receptor (203 μM) (Fig 1D–F). By contrast, the human-H6N1 HA displays significantly reduced binding affinity to the avian receptor (298 μM) and a comparable binding affinity to the human receptor (268 μM), and the binding preference for the human receptor was still evident (Fig 1G–I).

To characterize the receptor-binding properties of the avian-(duck-H6N1 and chicken-H6N1) and human-derived H6N1 (human-H6N1) at the virus level, we rescued the viruses with reverse genetics technology (Sun *et al*, 2011) and named them rDuck-H6N1,

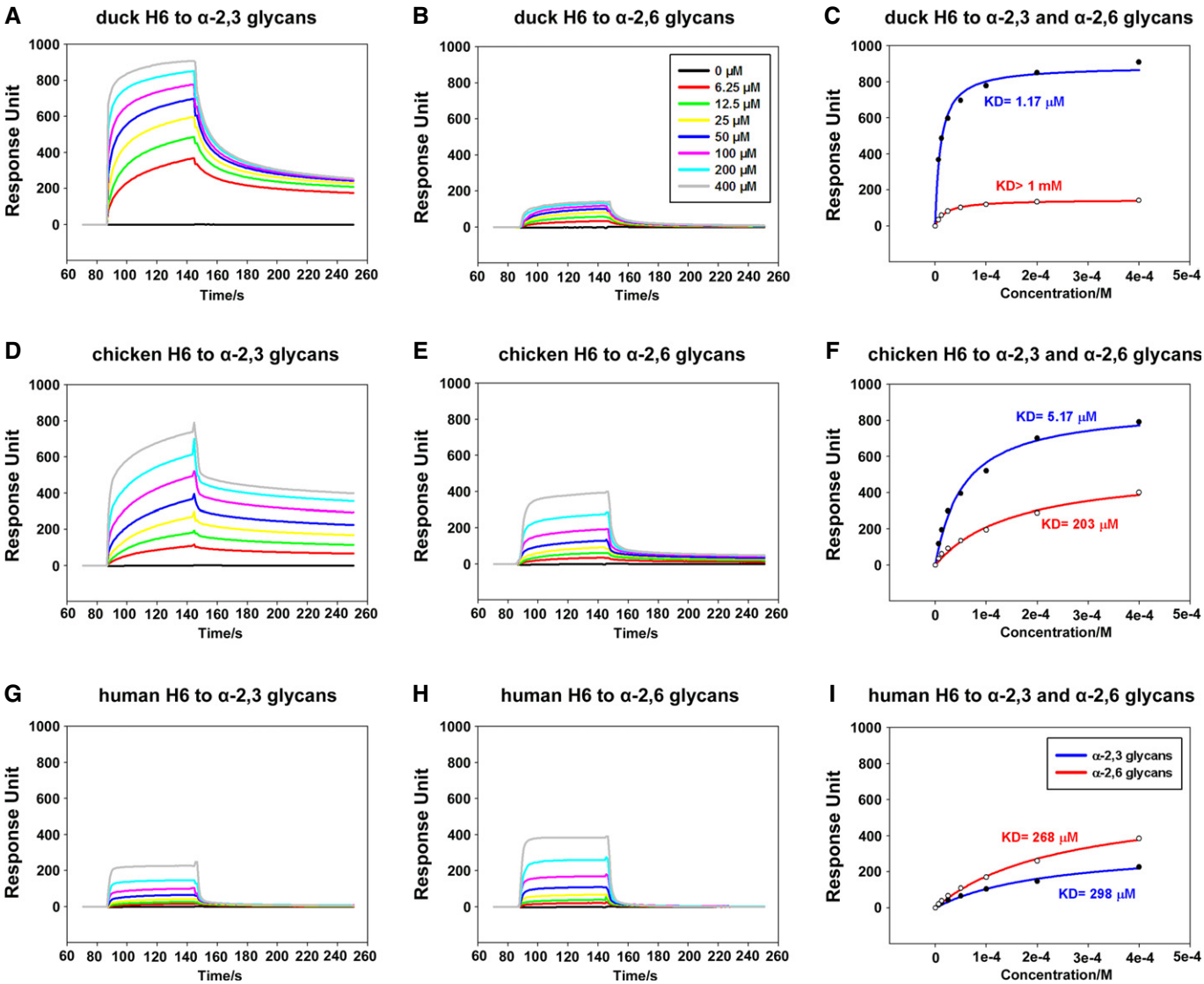

Figure 1. BIAcore® binding properties of duck-H6N1, chicken-H6N1, and human-H6N1 HA proteins to either α2,3-linked or α2,6-linked sialylglycan receptors.

A–I BIAcore® diagram of duck-H6N1 HA (A, B), chicken-H6N1 HA (D, E), and human-H6N1 HA (G, H) binding to the two receptors. Duck-H6N1 HA shows strong binding to the α2,3-linked sialylglycan receptor but undetectable binding to the α2,6-linked sialylglycan receptor. Chicken-H6N1 HA shows strong binding to the α2,3-linked sialylglycan receptor but weak binding to the α2,6-linked sialylglycan receptor. Human-H6N1 HA shows significantly reduced binding to the α2,3-linked sialylglycan receptor and comparable weak binding to the α2,6-linked sialylglycan receptor relative to chicken-H6N1 HA. Response units were plotted against protein concentrations (C, F and I). The K_D values were calculated by the BIAcore® 3000 analysis software (BIAevaluation version 4.1). The curve of binding to α2,3-linked sialylglycan receptor is shown in blue, and the curve of binding to α2,6-linked sialylglycan receptor in red.

rChicken-H6N1, and rHuman-H6N1, respectively (for details, see Materials and Methods). Subsequently, we analyzed their receptor-binding properties through solid-phase binding assays using the 2009 pandemic influenza virus isolate (A/California/04/2009 (H1N1), CA04-H1N1) and avian H5N1 influenza virus isolate (A/Anhui/1/2005 (H5N1), AH05-H5N1) as control viruses that have typical human or avian receptor-binding specificity, respectively. rDuck-H6N1 preferentially bound the avian receptor, with negligible binding to human receptor (Fig 2A). rChicken-H6N1 bound both the human and avian receptors, as did rHuman-H6N1, though with reduced binding to the avian receptor (Fig 2B and C). As a control, CA04-H1N1 specifically bound the human receptor (Fig 2D) and AH05-H5N1 specifically bound the avian receptor (Fig 2E).

Mutagenesis work on chicken-H6N1 HA

To confirm the residues at four positions 186–190–226–228 are receptor-binding determinants of H6 subtype HAs, we investigated the effect of amino acid substitutions at these four positions in the same chicken-H6N1 HA background. We found that the chicken-H6-190E-228G mutant mimics the binding mode of duck-H6N1 HA, which preferentially binds to avian receptor (Fig 3A–C), and both the mutant and duck-H6N1 HA share the residue combination P186/E190/Q226/G228. Then, the chicken-H6-186L mutant mimics the binding mode of human-H6N1 HA, which displays a dramatically reduced binding to avian receptor and preferential binding to human receptor (Fig 3D–F), and both of the mutant and human-H6N1 HA share the residue combination L186/V190/Q226/S228. In a word, the mutagenesis work on chicken-H6N1 HA reveals that the E190V and G228S substitutions are determinants to confer the H6 HA with human receptor-binding capacity, and the P186L substitution can dramatically reduce the binding to avian receptor.

Structures of chicken- and human-H6N1 HAs in complex with receptor analogs

To elucidate the structural basis of receptor binding for chicken-H6N1 HA (cH6) and human-H6N1 HA (hH6), we used X-ray crystallography to determine the structures of cH6 and hH6, both in free form and in complex with two sialo-pentasaccharides: 3′SLNLN and 6′SLNLN. These sialo-pentasaccharides are analogs of the avian and human receptors, respectively, containing the three terminal saccharides (Sia-Gal-GlcNAc). Both cH6 and hH6 display a typical homotrimer oligomerization as seen in other HA subtypes and exhibit a cleaved HA1/HA2 form.

The structure of cH6 with the avian receptor analog 3′SLNLN reveals that the ligand binds in a *cis* conformation (Fig 4A), unlike what is seen in all other previously reported avian HA/avian

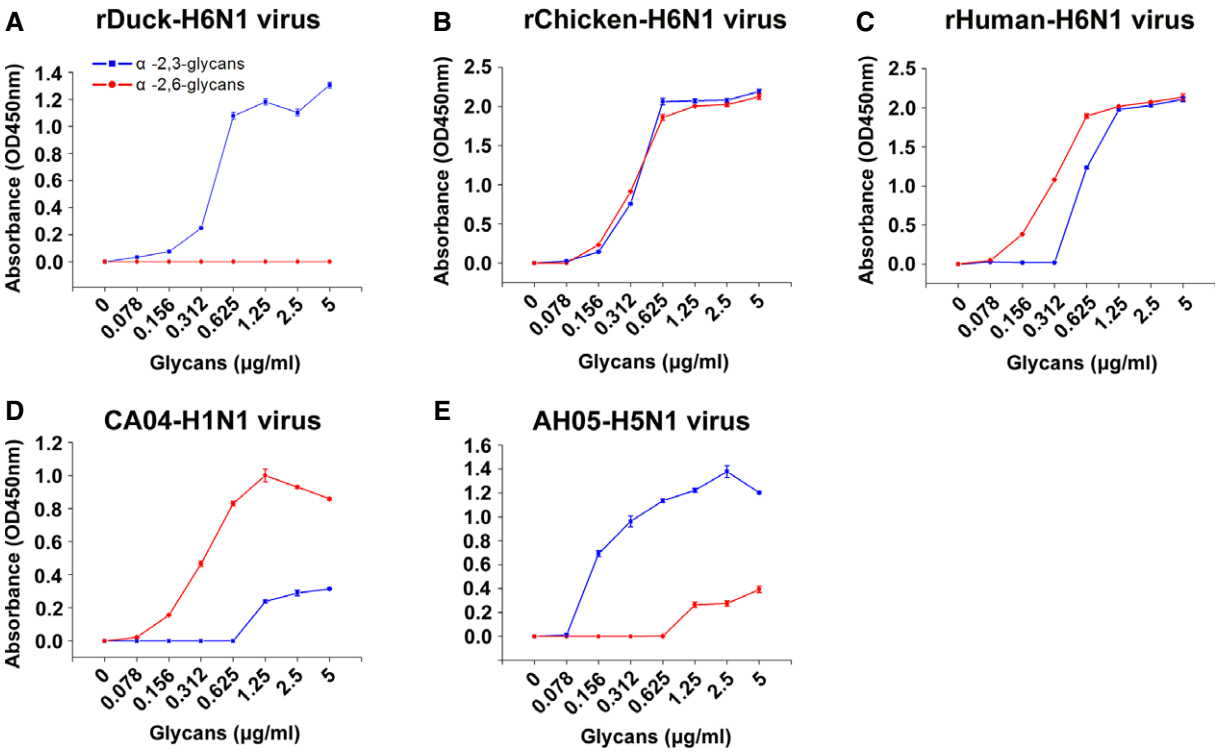

Figure 2. Receptor-binding properties at the virus level.

A–E Solid-phase binding assay for (A) rDuck-H6N1 (reverse genetics rescued A/duck/Taiwan/0526/72), (B) rChicken-H6N1 (reverse genetics rescued A/chicken/Taiwan/A2837/2013), (C) rHuman-H6N1 (reverse genetics rescued A/Taiwan/2/2013), (D) CA04-H1N1 (A/California/04/2009), and (E) AH05-H5N1 (A/Anhui/1/2005) viruses to both α2,3-linked (3′SLNLN) and α2,6-linked sialylglycan receptors (6′SLNLN). Binding to 3′SLNLN is colored in blue and 6′SLNLN in red. rDuck-H6N1 preferentially bound to 3′SLNLN, with no detectable binding to 6′SLNLN. rChicken-H6N1 bound both 3′SLNLN and 6′SLNLN, while rHuman-H6N1 preferentially bound to 6′SLNLN. As controls, CA04-H1N1 specifically bound to 6′SLNLN and AH05-H5N1 specifically bound to 3′SLNLN were used. Error bars represent SD of the mean, which is calculated from three independent repeats.

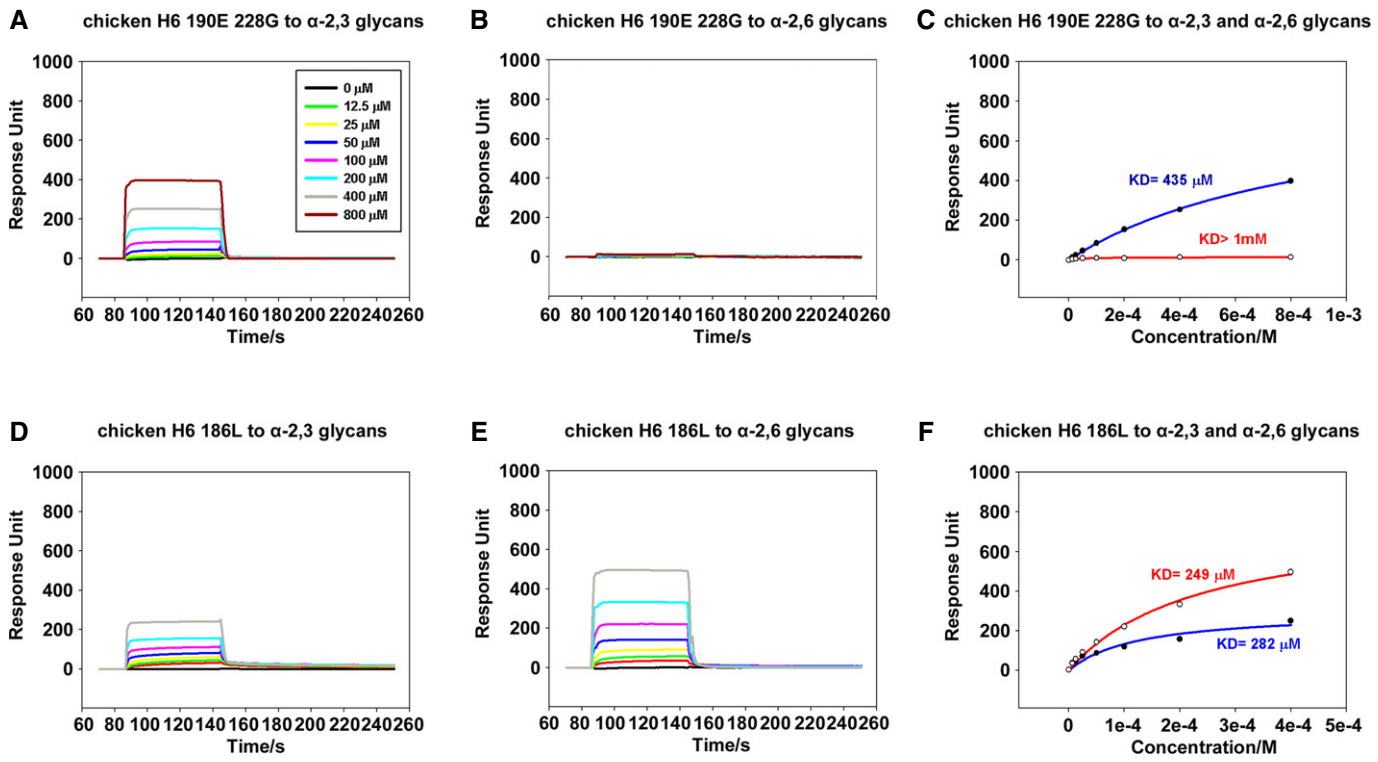

Figure 3. Mutagenesis experiments on chicken-H6N1 HA.

A–F BIAcore® diagram of chicken-H6N1-190E-228G HA mutant (A, B) and chicken-H6N1-186L HA mutant (D, E) binding to the two receptors. The chicken-H6N1-190E-228G HA mutant shows preferential binding to the α2,3-linked sialylglycan receptor but undetectable binding to the α2,6-linked sialylglycan receptor, which mimics the duck-H6N1 HA. The chicken-H6N1-186L HA mutant shows significantly reduced binding to the α2,3-linked sialylglycan receptor and weak binding to the α2,6-linked sialylglycan receptor, which mimics human-H6N1 HA. Response units were plotted against protein concentrations (C, F). The K_D values were calculated by the BIAcore® 3000 analysis software (BIAevaluation version 4.1). The curve of binding to α2,3-linked sialylglycan receptor is shown in blue, and the curve of binding to α2,6-linked sialylglycan receptor in red.

receptor analog complexes. The 'avian-signature' residue Q226 forms two hydrogen bonds with the Sia-1, and the residue S228 forms one hydrogen bond with the Sia-1. Interestingly, N137 forms two hydrogen bonds with the Gal-2, which has not been observed in other naturally occurring HA/receptor complexes. Usually, the residues of the 130-loop only form hydrogen bonds with the Sia-1, aside from one example, that is, that of the airborne-transmissible H5 mutant bound to the avian receptor (Zhang *et al*, 2013a). By contrast, the structure of cH6 with the human receptor analog 6′SLNLN reveals that the ligand binds in a *trans* conformation (Fig 4B). Similarly, the 'avian-signature' residue Q226 forms two hydrogen bonds with the Sia-1, and S228 forms one hydrogen bond with the Sia-1. However, N137 forms only one hydrogen bond with the Gal-2.

The structure of hH6 with the avian receptor analog 3′SLNLN reveals that the ligand binds in a *cis* conformation (Fig 4C), similar to that seen in the cH6/avian receptor complex described above. The 'avian-signature' residue Q226 forms two hydrogen bonds with the Sia-1, and S228 forms one hydrogen bond with the Sia-1. In this case, N137 does not form any hydrogen bonds with the Gal-2 but forms one hydrogen bond with the Sia-1. The structure of hH6 with the human receptor analog 6′SLNLN shows that the ligand binds in a *trans* conformation (Fig 4D), similar to that seen in the cH6/human

Figure 4. Molecular interactions of cH6 and hH6 with either avian or human receptor analogs.

The three secondary structural elements of the binding site (i.e., the 130-loop, 190-helix, and 220-loop) are labeled in ribbon representation, together with selected residues in stick representation. The hydrogen bonds are shown as dashed lines. The Sia-1 moiety of the receptor analogs is colored in red, the Gal-2 moiety is colored in blue, and the GlcNAc-3 moiety is colored in yellow.

A, B cH6 with the avian receptor analog 3′SLNLN (α2,3) pentasaccharide (A) or human receptor analog 6′SLNLN (α2,6) pentasaccharide (B) bound. The 3′SLNLN binds in a *cis* conformation, and the 6′SLNLN binds in a *trans* conformation.

C, D hH6 with the avian receptor analog 3′SLNLN (C) or the human receptor analog 6′SLNLN (D) bound. The 3′SLNLN binds in a *cis* conformation, and the 6′SLNLN binds in a *trans* conformation.

E The detailed differences in the interaction with the avian receptor analog are shown via comparisons between cH6/3′SLNLN and hH6/3′SLNLN complexes. The residues at position 186 exhibit different effects on the overall conformations of the receptor analog, due to different lengths of the side chains of the residues. In the cH6/3′SLNLN complex, N137 forms two hydrogen bonds with the avian receptor analog, ensuring strong binding. In the hH6/3′SLNLN complex, the hydrogen bonds between N137 and receptor analog are destroyed, resulting in reduced binding.

F In the cH6/6′SLNLN complex, N137 forms a hydrogen bond with the Gal-2. In the hH6/6′SLNLN complex, Q226 forms a hydrogen bond with the Gal-2. The Gal-2 rotates by ~60°, which might result from the P186L substitution.

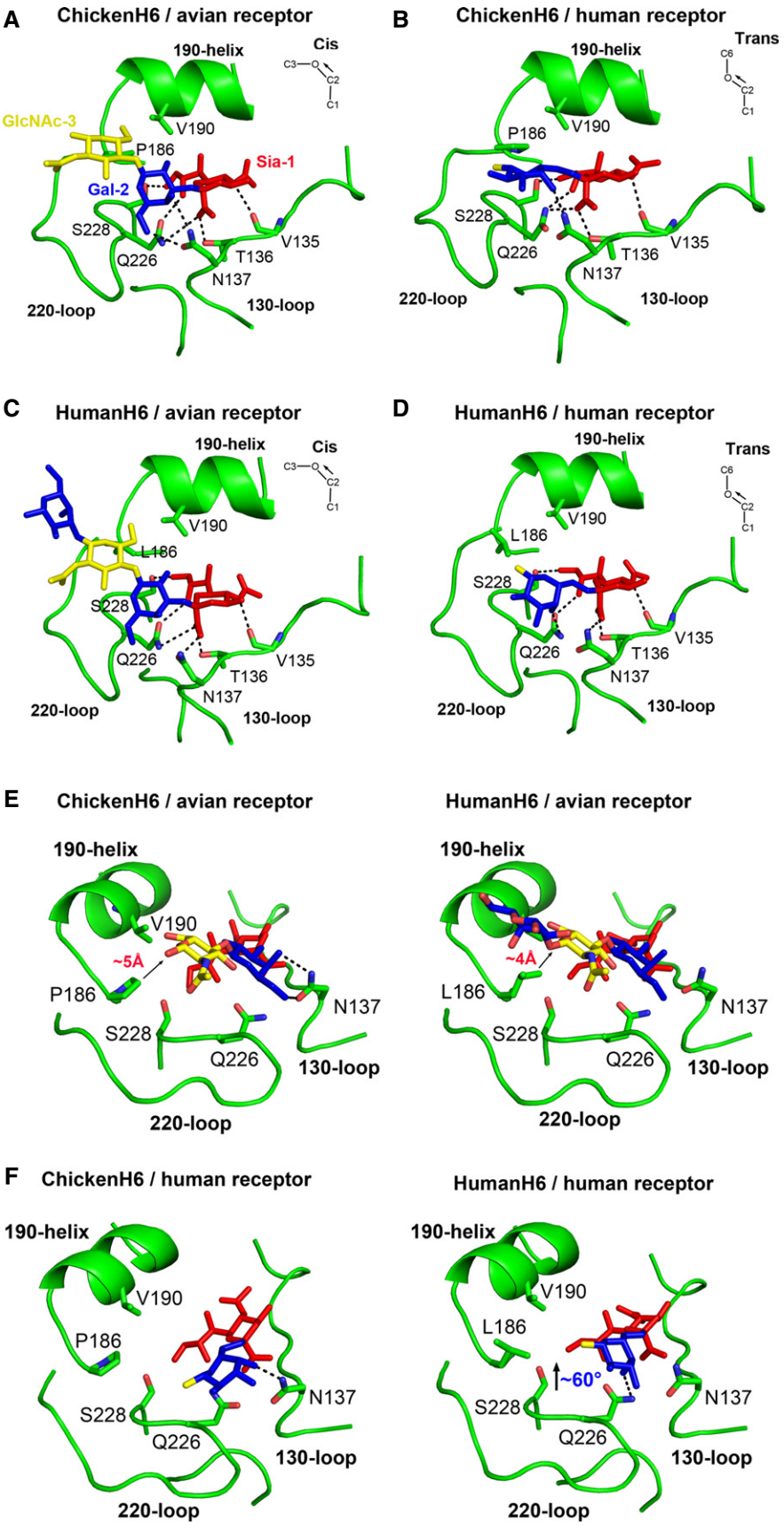

Figure 4.

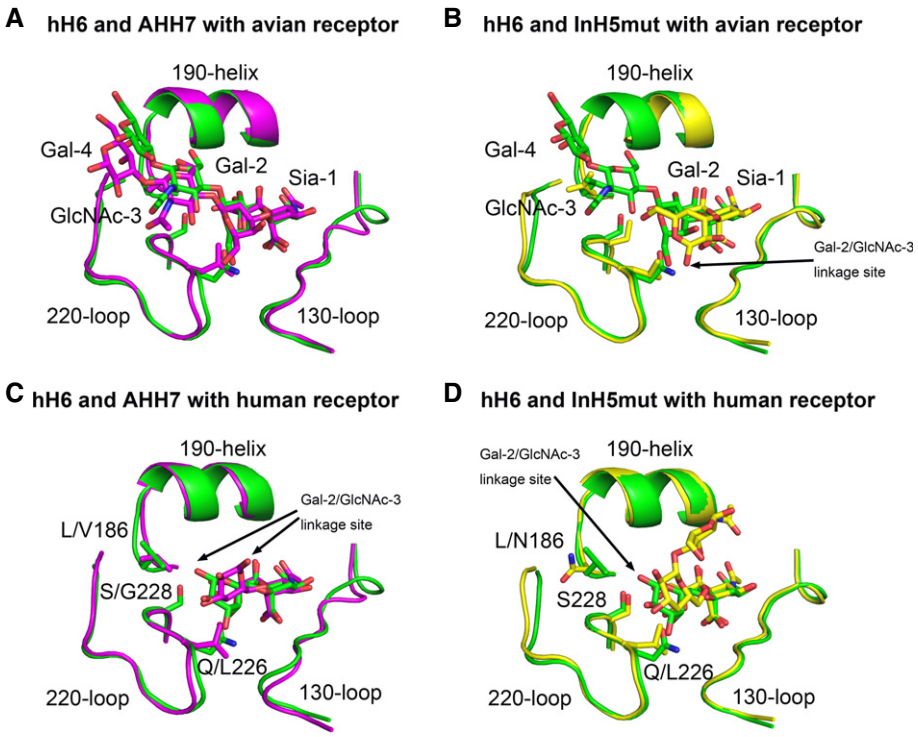

Figure 5. Comparison of the complex structures of hH6, human-infecting H7, and transmissible H5 bound to the avian or human receptor analog.
The HAs from the human-infecting H7N9 isolate (Anhui/1/2013, AHH7) and transmissible H5N1 isolate (mutant form of A/Indonesia/5/2005, InH5mut) were chosen to compare.

A, B Comparison of hH6, AHH7, and InH5mut complexes with avian receptor analogs. The avian receptor analogs bind in a similar *cis* conformation in both the hH6 and AHH7 complexes. Despite the avian receptor analog adopting a similar *cis* conformation in the InH5mut complex, the Gal-2 moiety rotates by ~180°, pointing the Gal-2/GlcNAc-3 linkage site away from the receptor-binding site.

C, D Comparison of hH6, AHH7, and InH5mut complexes with human receptor analogs. The human receptor analog binds in a *trans* conformation in the hH6 complex, and the Gal-2/GlcNAc-3 linkage site points toward the space between the 220-loop and 190-helix. In contrast, the human receptor analogs bind in a similar *cis* conformation in the AHH7 and InH5mut complexes, but the directions of the Gal-2/GlcNAc-3 linkage site are different. The Gal-2/GlcNAc-3 moieties fold back and go through the 190-helix in the InH5mut complex, like that in the pandemic H1, H2, and H3 complexes, while the Gal-2/GlcNAc-3 linkage points out from the receptor-binding site in the AHH7 complex.

receptor complex described above. The 'avian-signature' residue Q226 forms one hydrogen bond with the Sia-1 and one hydrogen bond with the Gal-2, and S228 forms one hydrogen bond with the Sia-1. Similarly, N137 forms one hydrogen bond with the Sia-1.

Structural basis of the receptor-binding preference change

Further structural analysis indicated that a subtle conformation adjustment occurs when cH6 and hH6 bind to different receptor analogs, as a result of the P186L substitution. The structures show that in cH6 bound to 3′SLNLN, the distance between P186 and the GlcNAc-3 is ~5 Å, without any van der Waals (vdw) interactions (Fig 4E). However, when hH6 is bound to 3′SLNLN, the distance between L186 and the GlcNAc-3 is ~4 Å due to the long side chain of L186 (Fig 4E). L186 creates a more hydrophobic environment for the GlcNAc-3 and affects the overall conformation of the ligand through vdw interactions. Thus, the hydrogen bonds between N137 and the Gal-2 are missing in structure of hH6 bound to 3′SLNLN compared to that in cH6 bound to the ligand. This lack of hydrogen bonds may result in the slightly reduced binding of the avian receptor analog by hH6.

In contrast, when both cH6 and hH6 bind to the human receptor analog 6′SLNLN, the ligands adopt a similar *trans* conformation. However, there is a ~60° difference in rotation around the Gal-2 C5-C6 bond between the cH6 and hH6 complexes (Fig 4F). As a result of this rotation, the Gal-2 forms a hydrogen bond with the N137 in the cH6 complex (Fig 4F), while in the hH6 complex, the Gal-2 forms a hydrogen bond with Q226 (Fig 4F). This rotation may result from the P186L substitution, as we cannot observe the remaining moieties of the ligand in the cH6 and hH6 complexes due to possible flexible binding.

Comparison with other HA subtypes

To explore the pandemic potential of the human-derived avian influenza A H6N1 virus from the basis of structural features, we compared the hH6 complex structures with those from the recent human-infecting avian influenza A H7N9 virus and a transmissible H5N1 virus. For comparison, we chose the HA complex structures from the H7N9 virus isolate (A/Anhui/2013/1, AHH7) and the transmissible H5N1 mutant virus isolate (mutant form of A/Indonesia/5/2005, InH5mut).

When bound to the avian receptor, hH6 binds the ligand in a *cis* conformation similar to that of AHH7, but the glycan moieties sit lower in the AHH7 complex structure than in the hH6 complex structure (Fig 5A). In contrast, despite the fact that the avian ligand adopts a *cis* conformation in the InH5mut complex, the Gal-2 moiety is rotated by ~180°, pointing the Gal-2/GlcNAc-3 linkage site out from the receptor-binding site (Fig 5B). This discrepancy may result from the different residues at position 186. Hydrophobic residues (L186 and V186) are observed for hH6 and AHH7, but a hydrophilic residue (N186) is found in InH5mut.

When bound to the human receptor, hH6 binds the ligand in a *trans* conformation, and the Gal-2/GlcNAc-3 linkage site points toward the space between the 220-loop and 190-helix (Fig 5C). In contrast, AHH7 and InH5mut bind the ligand in a similar *cis* conformation, but the directions of the Gal-2/GlcNAc-3 linkage site are different. The Gal-2/GlcNAc-3 moieties fold back and go through the 190-helix in the InH5mut complex, as in the pandemic H1, H2, and H3 complexes, while the Gal-2/GlcNAc-3 linkage points out from the receptor-binding site in the AHH7 complex (Fig 5C and D).

Discussion

During the adaption of influenza A virus to humans, the receptor-binding properties of the HA protein play a major role in interspecies transmission (Shi *et al*, 2014). To date, only the H1, H2, and H3 subtypes of influenza virus have naturally adapted to humans, causing seasonal flu or occasional pandemics (Taubenberger & Kash, 2010). Other HA subtypes are yet circulating in avian species, and some of them can cause sporadic human infections, such as H5, H7, H9, H10, and recently H6 (Claas *et al*, 1998; Yuen *et al*, 1998; Guo *et al*, 1999; Koopmans *et al*, 2004; Gao *et al*, 2013; Wei *et al*, 2013; Chen *et al*, 2014). Prior to the 'host jump', the receptor-binding properties must change from an avian receptor preference to human receptor preference via amino acid substitutions in the receptor-binding site of HA. Understanding of the molecular mechanisms of receptor-binding shifts is important to pre-warn against and control influenza virus infections, including possible future pandemics or epidemics.

For different HA subtypes, the molecular mechanisms of receptor-binding shifts are distinct (Shi *et al*, 2014). For H1 subtype, it is known that the shift in receptor-binding specificity is achieved by two amino acid substitutions (E190D and G225D, amino acids in H3 numbering) in the receptor-binding site (Gamblin *et al*, 2004; Xu *et al*, 2012; Zhang *et al*, 2013b), while for the H2 and H3 subtypes, Q226L and G228S substitutions occur in the receptor-binding site (Connor *et al*, 1994; Eisen *et al*, 1997; Liu *et al*, 2009). For the H5 subtype, the Q226L substitution and loss of glycosylation in the 150-loop of the receptor-binding site are enough to change the receptor-binding preference (Herfst *et al*, 2012; Imai *et al*, 2012; Lu *et al*, 2013b; Xiong *et al*, 2013a; Zhang *et al*, 2013a). In contrast, for H7 subtype, the human receptor-binding capacity can be achieved by several substitutions from hydrophilic residues to hydrophobic residues, including S138A, G186V, T221P, and Q226L (Shi *et al*, 2013b; Xiong *et al*, 2013b; Xu *et al*, 2013; Yang *et al*, 2013). To date, the H7 subtype retains strong avian receptor-binding capacity, which is a restraining factor for efficient human-to-human transmission.

In the case of the H6 subtype reported here, the E190V and G228S substitutions are important for the human receptor-binding capacity, and the P186L substitution is important for the receptor-binding preference shift by reducing avian receptor binding. It is noted that the chicken-H6N1-190E-228G HA mutant does not have strong binding affinity as the duck-H6N1 HA does (Fig 3), indicating other amino acid substitutions in the receptor-binding site could alter the binding affinity to avian receptor. The structural analysis revealed that the more bulky Leu residue creates a more hydrophobic environment for the GlcNAc-3 of the avian receptor analog and affects the overall conformation of the ligand via van der Waals interactions. Thus, this conformational adjustment destroys the hydrogen bonds between N137 and the Gal-2, resulting in weaker avian receptor binding. In addition to H7 subtype, previous studies also reveal the importance of the residue at position 186 for the receptor binding in other HA subtypes (Liu *et al*, 2009; Lu *et al*, 2013a). In avian H2, N186 makes hydrogen bonds with the Gal-2 of the receptor analog through a water molecule, acquiring effective human receptor-binding capacity (Liu *et al*, 2009). In the H13 subtype, V186 is important for its exclusive avian receptor-binding capacity, and an artificial V186N substitution reduces the binding to the avian receptor analog while increasing the binding to the human receptor analog(Lu *et al*, 2013a). In the revision course of this manuscript, Tzarum *et al* (2015) reported the receptor-binding properties of human H6N1 HA (hH6), showing this hH6 still maintains avian receptor-binding preference, which are contradictory to our results. The discrepancy could be potentially explained by different synthetic glycans and other experimental variables. In our case, we performed additional analyses and also did mutagenesis work to confirm the key amino acid substitutions responsible for the receptor-binding change.

Previously, before the appearance of the P186L substitution, the H6N1 viruses persistently circulating in poultry in Taiwan have acquired E190V and G228S substitutions (Wei *et al*, 2013), which are also helpful for human receptor binding. Currently, the affinity for the human receptor is yet low for most of the H6 subtype viruses, and high affinity for the avian receptor has been maintained, which may hamper the efficient infection and transmission in humans. Moreover, it should be aware that, during the viral entry of the host tissue, the receptor-binding HA is not acting in isolation but in a direct balance with the receptor-cleaving NA (Wagner *et al*, 2000). Besides receptor binding of HA, HA–NA balance should also be taken into consideration when we evaluate the epidemic or pandemic potential of avian influenza viruses.

Otherwise, the Taiwan hH6N1 virus has not acquired the mammalian-adapted E627K substitution in its PB2, which is also important for efficient infection and transmission in humans (Manz *et al*, 2013; Wei *et al*, 2013). However, if the H6N1 virus gains its internal genes from H9N2 (as did the human-infecting H7N9), the E627K mutation may occur and the ability to infect humans might also increase. Therefore, extensive surveillance is crucial to pre-warn against and control potential H6N1 human infections.

Materials and Methods

Solid-phase binding assay

The experiments were performed in approved biosafety level 3 laboratories. The chicken- and human-derived H6N1 viruses were generated by plasmid-based reverse genetics technology (Sun *et al*, 2011)

to avoid any impurities of the initial isolated viruses (PR8 virus was used as a backbone for the six internal genes, the H6 and N1 genes were derived from A/duck/Taiwan/0526/72, A/chicken/Taiwan/A2837/2013, and A/Taiwan/2/2013, respectively). The rescued viruses were named rDuck-H6N1, rChicken-H6N1, and rHuman-H6N1, respectively, to distinguish them from the natural isolates. All rescued viruses were sequenced to exclude any unwanted mutations. Virus stocks were propagated in specific pathogen-free chicken eggs. Virus concentrations were determined using hemagglutination assays with 1% (v/v) chicken red blood cells. Receptor-binding specificity was analyzed by a solid-phase direct binding assays as described previously (Watanabe *et al*, 2011). Briefly, serial dilutions (0.078125, 0.15625, 0.3125, 0.625, 1.25, 2.5, and 5 μg/ml) of the biotinylated glycans 3′SLNLN and 6′SLNLN were prepared in PBS, and 100 μl was added to the wells of 96-well microtiter plates (Polystyrene Universal-Bind Microplate; Corning) and allowed to attach overnight at 4°C. The plates were then irradiated with 254 nm ultraviolet light for 2 min. After removal of the glycopolymer solution, the plates were blocked with 0.1 ml PBS containing 2% bovine serum albumin (BSA) at room temperature for 1 h. After washing with ice-cold PBS containing 0.1% Tween-20 (PBST), the plates were incubated in a solution containing influenza virus (64 HA units in PBST) at 4°C for 12 h. After washing with PBST, chicken antisera against the A/duck/Taiwan/0526/72 (Duck-H6N1), A/chicken/Taiwan/A2837/2013 (Chicken-H6N1), A/Taiwan/2/2013 (Human-H6N1), A/California/04/2009 (CA04-H1N1), and A/Anhui/01/2005 (AH05-H5N1) viruses was added to each well, and the plates were incubated at 4°C for 2 h. The wells were then washed with ice-cold PBST and incubated with HRP-linked goat anti-chicken antibody (Sigma-Aldrich, www.sigmaaldrich.com) for 2 h at 4°C. After washing with ice-cold PBST, the plates were incubated with O-phenylenediamine in PBS containing 0.01% H_2O_2 for 10 min at

Table 2. Statistics for crystallographic data collection and structure refinement for Chicken H6.

	Chicken H6	Chicken H6-2,3	Chicken H6-2,6
Data collection			
Space group	P21	P3	P3
Cell dimensions			
a, *b*, *c* (Å)	67.61, 106.26, 125.28	96.77, 96.77, 132.51	97.06, 97.06, 131.68
α, β, γ (°)	90, 102.75, 90	90, 90, 120	90, 90, 120
Resolution (Å)	50.00–2.60 (2.69–2.60)	50.00–3.00 (3.11–3.00)	50.00–3.10 (3.21–3.10)
R_{merge}	0.115 (0.872)	0.100 (0.834)	0.118 (0.812)
$I/\sigma I$	12.5 (1.8)	13.9 (2.2)	8.5 (1.5)
Completeness (%)	99.5 (99.9)	99.0 (100.0)	92.6 (93.9)
Redundancy	3.9 (3.9)	4.2 (4.2)	3.5 (3.4)
Refinement			
Resolution (Å)	41.37–2.60	45.45–3.00	45.54–3.10
Number of reflections	53,268	27,681	23,294
R_{work}/R_{free}	0.2200/0.2714	0.1986/0.2260	0.2095/0.2542
Number of atoms			
Protein	11,626	7,880	7,880
Water	69	0	0
Ligand	0	92	64
B-factors			
Protein	60.9	93.7	112.3
Water	76.9		
Ligand		107.7	117.7
R.m.s. deviations			
Bond lengths (Å)	0.008	0.007	0.005
Bond angles (°)	1.489	1.001	0.966
Ramachandran plot (%)			
Most favoured regions	86.1	85.5	83.6
Additional allowed regions	13.1	13.8	15.2
Generously allowed regions	0.8	0.7	1.2
Disallowed regions	0.1	0	0

Values in parentheses are for the highest resolution shell.

room temperature. The reaction was stopped with 0.05 ml 1 M H_2SO_4, and the absorbance was determined at 450 nm.

Gene cloning, protein expression, and purification

The sequences encoding the ectodomain of the HAs from A/duck/Taiwan/0526/72, A/Taiwan/2/2013(hH6), A/Chicken/Taiwan/A2837/2013(cH6), and its mutants were cloned into the baculovirus transfer vector pFastBac1 (Invitrogen) as previously described (Stevens *et al*, 2004; Zhang *et al*, 2010; Shi *et al*, 2013b), in-frame with an N-terminal gp67 signal peptide for secretion, a C-terminal thrombin cleavage site, a trimerization foldon sequence, and a His_6 tag at the extreme C-terminus for purification. Transfection and virus amplification were performed according to the Bac-to-Bac baculovirus expression system manual (Invitrogen).

HA proteins were produced by infecting suspension cultures of Hi5™ cells (Invitrogen) for 2 days. Soluble HA was recovered from cell supernatants by metal affinity chromatography using a 5-ml HisTrap HP column (GE Healthcare) and then purified by ion-exchange chromatography (IEX) using a Mono-Q4.6/100PE column (GE Healthcare). The purified proteins were subjected to thrombin digestion (BD Biosciences, 3 U/mg HA; overnight at 4°C) to remove the C-terminal trimerization foldon sequence and His_6 tag. For crystallization, the proteins were further purified by gel filtration chromatography using a Superdex®-200 16/60 GL column (GE Healthcare) with a running buffer (pH 8.0) of 20 mM Tris–HCl and 50 mM NaCl. The collected protein fractions were concentrated to 8 mg/ml.

Crystallization, data collection, and structure determination

hH6 and cH6 were both crystallized via the sitting-drop vapor diffusion method at 18°C. The Human-H6N1 crystals grew in a reservoir solution of 0.2 M ammonium acetate, 0.1 M sodium

Table 3. Statistics for crystallographic data collection and structure refinement for Human H6.

	Human H6	Human H6-2,3	Human H6-2,6
Data collection			
Space group	P63	P63	P63
Cell dimensions			
a, *b*, *c* (Å)	113.90, 113.90, 163.84	114.19, 114.19, 164.96	114.39, 114.39, 166.05
α, β, γ (°)	90, 90, 120	90, 90, 120	90, 90, 120
Resolution (Å)	50.00–2.60 (2.69–2.60)	50.00–2.60 (2.69–2.60)	50.00–2.60 (2.69–2.60)
R_{merge}	0.109 (0.554)	0.126 (0.494)	0.137 (0.846)
$I/\sigma I$	19.4 (5.3)	22.0 (6.8)	21.2 (3.8)
Completeness (%)	99.9 (99.9)	99.9 (100.0)	99.9 (99.9)
Redundancy	7.3 (7.6)	13.1 (13.2)	12.2 (12.6)
Refinement			
Resolution (Å)	47.23–2.60	46.95–2.60	39.77–2.60
Number of reflections	36,901	37,568	37,317
R_{work}/R_{free}	0.1914/0.2247	0.1821/0.2082	0.1924/0.2265
Number of atoms			
Protein	3,938	4,016	3,938
Water	188	136	91
Ligand	0	57	32
B-factors			
Protein	47.7	45.8	59.3
Water	48.9	44.5	52.8
Ligand		100.7	120.4
R.m.s. deviations			
Bond lengths (Å)	0.007	0.006	0.008
Bond angles (°)	1.047	1.037	1.111
Ramachandran plot (%)			
Most favoured regions	87.8	88.3	87.8
Additional allowed regions	11.2	10.8	11.2
Generously allowed regions	1	0.9	1
Disallowed regions	0	0	0

Values in parentheses are for the highest resolution shell.

acetate pH 4.0, and 15% w/v PEG 4000. The Avian-H6N1 crystals grew in a reservoir solution of 0.2 M sodium thiocyanate and 20% w/v polyethylene glycol 3350. For receptor analog complexes, crystals were soaked in a reservoir solution containing 10 mM LSTa (NeuAcα2-3Galβ1-4GlcNAcβ1-3Galβ1-4Glc) or LSTc (NeuAcα2-6Galβ1-4GlcNAcβ1-3Galβ1-4Glc) for 4 h. All crystals were flash-cooled in liquid nitrogen after a brief soaking in reservoir solution with the addition of 17% (v/v) glycerol. The X-ray diffraction data were collected at the Shanghai Synchrotron Radiation Facility (SSRF) beamline 17U. All data were processed with HKL2000 software.

The hH6 and cH6 structures were solved by molecular replacement using Phaser from the CCP4 program suite, with the structure of the H2 HA (PDB code: 2WRD) as a model. The HA receptor analog complexes were subsequently determined using the refined HA structure as the input model. The receptor analogs were manually built using COOT based on the simulated annealing omit Fo–Fc maps and were further refined by PHENIX. Final statistics for data collection and structure refinement are represented in Tables 2 and 3. The stereochemical quality of the final model was assessed with the program PROCHECK.

SPR experiments

The affinity and binding kinetics of Duck-H6N1 HA, Human-H6N1 HA, Chicken-H6N1 HA, and its mutants for receptor analogs were both analyzed at 25°C on a BIAcore® 3000 machine with streptavidin chips (SA chips, GE Healthcare). PBST was used as the kinetics analysis buffer. Two biotinylated receptor analogs, the α-2,6 glycan (6′SLNLN: NeuAcα2-6Galβ1-4GlcNAcβ1-3Galβ1-4GlcNAcβ1-SpNH-LC-LC-Biotin), and the α-2,3 glycan (3′SLNLN: Neu Acα2-3Galβ1-4GlcNAcβ1-3Galβ1-4GlcNAcβ1-SpNH-LC-LC-Biotin) were kindly provided by the Consortium for Functional Glycomics (Scripps Research Institute, Department of Molecular Biology, La Jolla, CA). Approximately 400 response units of biotinylated glycans were immobilized on the chip, and a blank channel was used as the negative control. Thrombin-digested HAs were purified by gel filtration using PBST buffer as running buffer and serially diluted to concentrations ranging from 12.5 to 800 μM. The HA protein preparations were then flowed through the chip, and the response units were measured. The sensor surface was regenerated with 10 mM NaOH at the end of each cycle. Sensorgrams were locally fitted with BIAcore® 3000 analysis software (BIAevaluation® version 4.1) using a 1:1 Langmuir binding mode and HA monomer as the identity to assume a 1:1 binding. The affinity values of duck- and chicken-H6N1 HAs to receptors were calculated with a Kinetics simultaneous ka/kd affinity model, and the affinity values of human-H6N1 HA and chicken-H6N1 HA mutants to receptor were calculated with a steady state affinity model.

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Author contributions

GFG designed and coordinated the study. FW, WZ, and MinW conducted the experiments. JQ collected the datasets and solved the structures. YS and GFG wrote the manuscript, and BZ, MingW, JL, and JY participated in the manuscript editing and discussion.

Conflict of interest

The authors declare that they have no conflict of interest.

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