

Cell Host & Microbe

Structure and Receptor Binding of the Hemagglutinin from a Human H6N1 Influenza Virus

Graphical Abstract

Avian HA	Receptor binding site residues				specificity
	E190	G225	Q226	G228	α 2-3
H6N1 (2013)	V	G	Q	S	α 2-3
H1N1 (1918/2009)	D	D	Q	G	α 2-6
H2N2 (1957)	E	G	L	S	α 2-6
H3N2 (1968)	E	G	L	S	α 2-6
H5N1 (2004/5)	E	G	Q	G	α 2-3
H7N9 (2013)	E	G	Q/L/I	G	α 2-3

H6N1 HA Receptor binding site

190-helix

Val190

Tyr195

His183

Ser228

Gly225

Gln226

Tyr98

220-loop

130-loop

Authors

Netanel Tzarum, Robert P. de Vries, ..., James C. Paulson, Ian A. Wilson

Correspondence

jpaulson@scripps.edu (J.C.P.), wilson@scripps.edu (I.A.W.)

In Brief

To infect humans, avian influenza viruses typically undergo changes in hemagglutinin that permit human receptor binding. Based on structural analyses, Tzarum et al. show that human-isolated H6N1 influenza A virus contains a hemagglutinin receptor binding site that is distinct from other avian and human viruses and retains avian receptor binding.

Highlights

- The human H6N1 HA receptor binding site is distinct from other avian and human HAs
- The HA of a human H6N1 influenza virus retains avian receptor specificity
- The interactions of H6 HA with avian receptor analogs differ from other HAs
- Additional mutations are required to switch H6 HA to human receptor specificity

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Structure and Receptor Binding of the Hemagglutinin from a Human H6N1 Influenza Virus

Netanel Tzarum,¹ Robert P. de Vries,^{2,3,4,6} Xueyong Zhu,¹ Wenli Yu,¹ Ryan McBride,^{2,3,4} James C. Paulson,^{2,3,4,*} and Ian A. Wilson^{1,5,*}

¹Department of Integrative Structural and Computational Biology

²Department of Cell and Molecular Biology

³Department of Chemical Physiology

⁴Department of Immunology and Microbial Science

⁵Skaggs Institute for Chemical Biology

The Scripps Research Institute, 10550 North Torrey Pines Road, La Jolla, CA 92037, USA

⁶Present address: Department of Medicinal Chemistry and Chemical Biology, Utrecht Institute for Pharmaceutical Science, Utrecht University, Universiteitsweg 99, 3584 CG Utrecht, the Netherlands

*Correspondence: jcpaulson@scripps.edu (J.C.P.), wilson@scripps.edu (I.A.W.)

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SUMMARY

Avian influenza viruses that cause infection and are transmissible in humans involve changes in the receptor binding site (RBS) of the viral hemagglutinin (HA) that alter receptor preference from α 2-3-linked (avian-like) to α 2-6-linked (human-like) sialosides. A human case of avian-origin H6N1 influenza virus was recently reported, but the molecular mechanisms contributing to it crossing the species barrier are unknown. We find that, although the H6 HA RBS contains D190V and G228S substitutions that potentially promote human receptor binding, recombinant H6 HA preferentially binds α 2-3-linked sialosides, indicating no adaptation to human receptors. Crystal structures of H6 HA with avian and human receptor analogs reveal that H6 HA preferentially interacts with avian receptor analogs. This binding mechanism differs from other HA subtypes due to a unique combination of RBS residues, highlighting additional variation in HA-receptor interactions and the challenges in predicting which influenza strains and subtypes can infect humans and cause pandemics.

INTRODUCTION

Although a multitude of different avian influenza A viruses have been isolated, only three (H1N1, H2N2, and H3N2) have been able to adapt for transmission in the human population, and these viruses have led to influenza pandemics. To circulate in the human population, an avian influenza virus has to acquire the ability to bind human receptors and lose affinity for avian receptors before being able to transmit from human to human. In the last two decades, an apparent increase has been observed in the reported cases of new avian-origin influenza subtypes infecting humans, including H9N2 (Peiris et al., 1999), H5N1 ("bird flu") (Beigel et al., 2005), H7N9 (Centers for Disease Control and Prevention, 2013), and very recently H6N1 (Shi et al., 2013a; Yuan

et al., 2013) and H10N8 (Chen et al., 2014). This increase in novel zoonotic infections raises concern about the emergence of avian subtypes that could give rise to novel human pandemics.

On June 21, 2013, the first case of a human infection by an avian-origin H6N1 influenza A virus (A/Taiwan/2/2013, Taiwan2) was reported by the Taiwan Centers for Disease Control (<http://www.cdc.gov.tw/english/info.aspx?treeid=bc2d4e89b154059b&nowtreeid=ee0a2987cfba3222&tid=E36A5E9AB3D3A216>). The patient, a 20-year-old female, who had not been exposed to live poultry, was hospitalized after she developed a high fever, cough, headache, and muscle ache. She later fully recovered and no human-to-human transmission of the H6N1 virus was reported (Shi et al., 2013a; Yuan et al., 2013). Phylogenetic analysis of the viral genes from this H6N1 human isolate suggested that an avian virus A/Chicken/Taiwan/A2837/2013 was the possible origin of seven of eight genes of the Taiwan2 (H6N1) virus (for HA sequence, see Figure S1A available online), whereas the source of the eighth gene, coding for PB2, was probably from A/Chicken/Taiwan/0101/2012 (H5N2) (Shi et al., 2013a; Yuan et al., 2013). H6N1 avian viruses are frequently isolated from birds and have been circulating worldwide, including in North America, Europe, and Asia (Cheung et al., 2007; Jonassen and Handeland, 2007; Lee et al., 2006; Senne, 2003; Süß et al., 1994). In Taiwan, the H6N1 virus has been enzootic since the early 1970s (Lee et al., 2006). Although to date only this one case of human infection by an H6N1 virus has been reported, more widespread exposure to H6 viruses likely has already occurred as antibodies against H6 influenza A viruses have been detected in veterinarians in the United States and in workers in live poultry markets in China (Myers et al., 2007; Shortridge, 1992). Furthermore, virus replication studies show that some of the Taiwanese H6N1 viruses can replicate in mice without adaptation (Lee et al., 2006).

Influenza virus is an enveloped virus that contains two surface glycoproteins in its membrane envelope: hemagglutinin (HA) and neuraminidase (NA). The HA is the major glycoprotein and the target of neutralizing antibodies. The HA is a homotrimer where each monomer is synthesized as an inactive single-chain precursor (HA0) that is proteolytically cleaved into two subunits: an N-terminal HA1 and a C-terminal HA2. The mature HA protein is responsible for attachment of the virus to its natural receptors, which are terminal sialic acids (N-acetylneuraminic acid [NeuAc])

on glycoprotein and glycolipids on the host cell, and for fusion of the viral envelope with the host cell in the low pH of endosomal compartments (Skehel and Wiley, 2000). So far 18 HA subtypes (16 avian and 2 bat subtypes) have been identified (Tong et al., 2013), based on serology and antigenic properties, and these can be phylogenetically divided into two groups (Air, 1981; Nobusawa et al., 1991). The H6 HA is within group 1 that also includes H1, H2, H5, and H9 subtypes that have caused human infections (Figure S1A).

The specificity of the HA for glycan receptors is believed to be a key determinant of the viral host range (Matrosovich et al., 2009), where the most important element for host specificity is the linkage between the terminal sialic acid and the penultimate galactose. HAs from avian viruses are characterized by their preference for α 2-3-linked sialosides, whereas HAs from human viruses are specific for α 2-6-linked sialosides. This specificity difference contributes to the inability of most avian influenza viruses to transmit in the human population (Parrish and Kawoka, 2005), and changes in the binding specificity from α 2-3 to α 2-6 sialosides are therefore believed to be essential to cross the species barrier and become human transmissible.

The HA receptor binding site (RBS) is located in the head domain of the HA1 subunit and consists of three conserved secondary elements, the 130 and 220-loops and the 190-helix, and a number of conserved residues in the heart of the binding site (Ha et al., 2001; Skehel and Wiley, 2000). Four key RBS residues have been implicated in the avian-human specificity switch (Matrosovich et al., 1997). In avian viruses, the 220-loop contains Gly225, Gln226, and Gly228, whereas Glu190 is present in the 190-helix (H3 numbering). For H1 subtypes, Glu190Asp and Gly225Asp substitutions are critical for attaining specificity for human receptors, as in the 1918 and 2009 pandemic H1N1 strains (Matrosovich et al., 2000; Stevens et al., 2006b; Tumpey et al., 2007). In contrast, for the 1957 H2N2 and 1968 H3N2 pandemic viruses, Gln226Leu and Gly228Ser substitutions were responsible for the switch between avian-type and human-type receptor specificity (Connor et al., 1994; Matrosovich et al., 2000). These examples have not proven predictive of the amino acid changes required for conversion of receptor specificity in other subtypes. For example, for avian H5N1 virus, Glu190Asp and Gly225Asp mutations abolished receptor binding, while Gln226Leu and Gly228Ser mutations produced partial recognition of α 2-6 linked receptors, but α 2-3 binding was not lost (Stevens et al., 2006b). Further mutations at Asn158 and Asn224, or at Gln196, or combined with the loss of the N-linked glycan at Asn158, were sufficient to switch receptor specificity for H5 viruses to become transmissible in mammals (Chen et al., 2012; de Vries et al., 2014; Herfst et al., 2012; Imai et al., 2012).

RESULTS

Structural Characterization of Taiwan2 H6N1 HA

The H6N1 A/Taiwan/2/2013 virus is one of several emerging influenza viruses over the past 2 years, including H7N9 and H10N8, which have caused human infection but have not yet spread in the human population (Vachieri et al., 2014). The frequency of these infections has elevated concerns that these

viruses will acquire human-type receptor specificity allowing them to transmit between humans.

To analyze the receptor binding of H6N1, we first performed sequence alignment of the Taiwan2 H6N1 HA with human pandemic strains and with avian subtypes from emerging viruses that have caused sporadic outbreaks of human infections over the last few years. Taiwan2 H6 HA contains a unique combination of four key residues in the RBS not seen previously in human influenza viruses or avian viruses that sporadically infect humans (Figure S1B). The H6 190-helix contains an unusual aliphatic substitution of Val at position 190 instead of an acidic residue in all other avian and human subtypes (i.e., Glu or Asp for avian and human subtypes, respectively). In the 220-loop, H6 HA contains the G228S substitution that is associated with the receptor specificity switch of the H2 and H3 human pandemic viruses (Connor et al., 1994; de Graaf and Fouchier, 2014). In addition, two other substitutions could influence receptor binding. At position 222, H6 HA has a small hydrophobic Ala instead of a charged or polar residue (Lys or Gln) in all other subtypes, except for H3, where it is an aromatic residue (Trp). Taiwan/2/2013 H6 HA also contains Leu at position 186 instead of Pro, as in A/Chicken/Taiwan/A2837/2013 (Shi et al., 2013a) (Figure S1B).

To investigate the structural features and properties of this H6 HA, its crystal structure was determined at 2.5 Å resolution (Table S1). Overall, the structure is similar to other known influenza A HAs (Figure 1A), and the disposition of the HA head relative to the stem is most similar to H2N2 HA (PDB ID code 3KU5). Six potential N-linked glycosylation sites per HA protomer are predicted (Wang et al., 2014) (five in HA1), but interpretable electron density consistent with glycosylation is observed only at Asn21 (protomer C), Asn32 (A), and Asn 169 (A and C) from HA1 and Asn154 (C) from HA2, but not at Asn291 and Asn296, presumably due to disorder (i.e., for the trimer, for 18 potential glycosylation sites, only five glycans could be modeled) (Figure 1A). Superposition of the H6 HA on other avian and human HAs confirms that H6 is structurally closest to H1, H2, and H5 (group 1 HAs) (α root-mean-square deviation [rmsd] of 0.8–1.3 Å compared to 2.4–2.5 Å for group 2 H3 and H7). Similar results were also obtained by superposition of the HA1, HA2, and RBS subdomains (Figure 1C; Table S2). The H6 differs from other HAs due to insertion of Asp at position 144a, and a one-residue insertion of Asp at position 157a in the 150-loop compared to H1, H2, H3, and H5 HAs (Figures S1B and 1C). A similar insertion of residues in the 150-loop was found for H7 HA, which contains two additional residues at positions 158a and 158b. The H6 RBS, like all other influenza A HAs, has a conserved floor of Tyr98, Trp153, His183, and Tyr195 (Skehel and Wiley, 2000) for binding the sialic acid moiety of the receptor. The surrounding 130-loop, 150-loop, 220-loop, and 190-helix (Figure 1B) delineate the sides and ends of the RBS.

Receptor Specificity of Taiwan2 H6N1 HA

To determine the receptor specificity, the Taiwan2 H6 HA was expressed in insect cells and analyzed on a custom sialoside glycan array comprised of diverse α 2-3 and α 2-6 sialosides that correspond to N-linked and O-linked glycans, as well as linear fragments of glycans, on mammalian glycoproteins and glycolipids (Figure 2) (Stevens et al., 2006a; Raman et al., 2014). The use of recombinant HA protein eliminates possible interference from the NA on the virus that preferentially cleaves

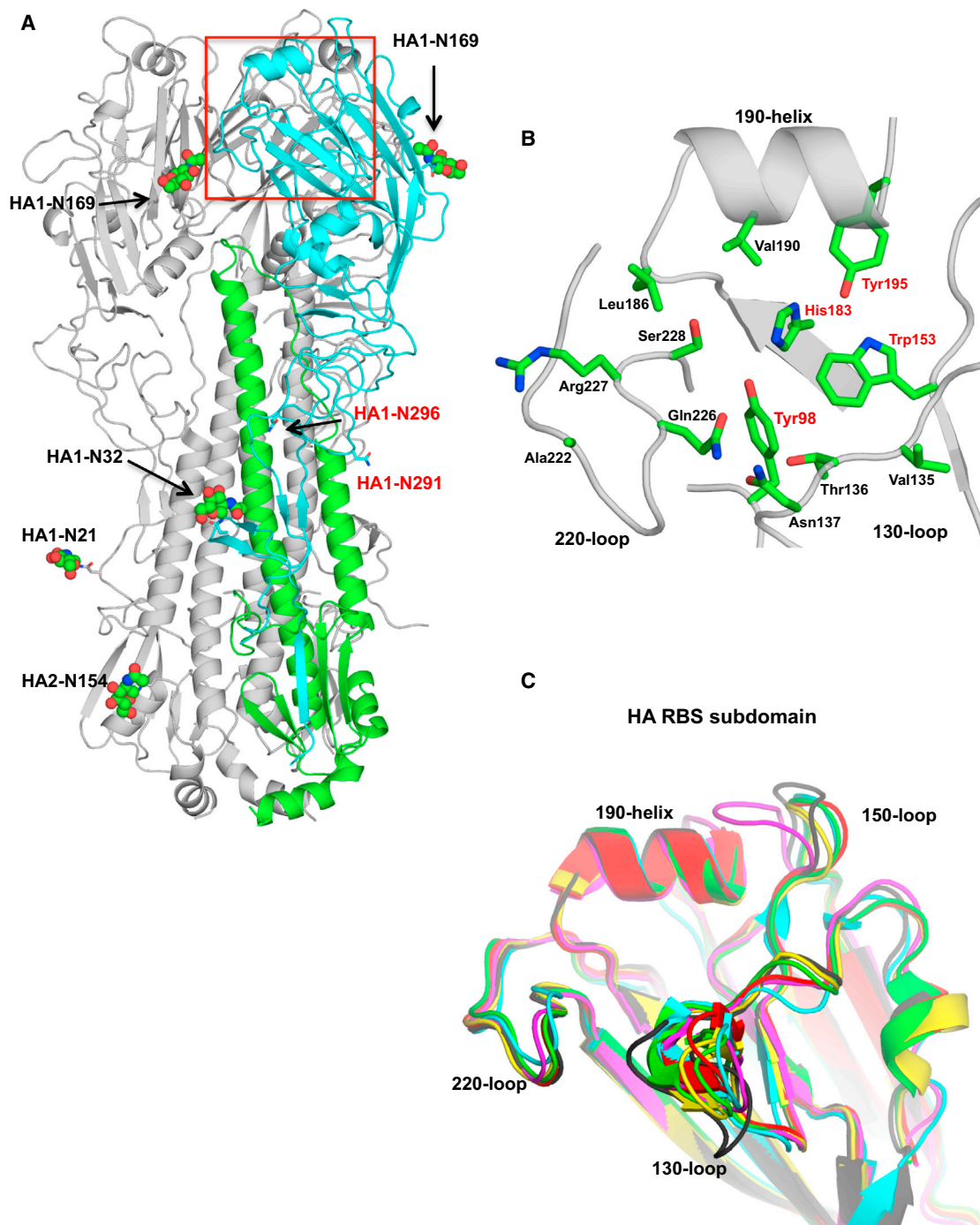


Figure 1. Crystal Structure of Taiwan2 H6N1 HA

(A) Schematic representation of the H6 HA trimer. One protomer is colored in cyan and green for the HA1 and the HA2 subunits, and the RBS is marked with red rectangle. N-linked glycans that could be modeled in the electron density maps are in green spheres, and the corresponding asparagine is shown in sticks and labeled in black. Two other potential glycosylation sites (Asn291 and Asn 296) are labeled in red.

(B) Schematic representation of the H6 HA RBS with sticks representing key residues for receptor binding (colored in green and labeled). The four highly conserved residues in the RBS are labeled in red. The H6 RBS contains Val in position 190 and Ser in position 228.

(C) Superposition of the RBS subdomains of H6 HA (black) and pandemic H1 (yellow, PDB ID code 3AL4), H2 (red, PDB ID code 3KU5), H3 (cyan, PDB ID code 4FNK), A/Vietnam/1203/2004 H5 (green, PDB ID code 2FK0), and A/Shanghai/2/2013 H7 (magenta, PDB ID code 4N5J) HAs (see also Figure S1; Tables S1 and S2).

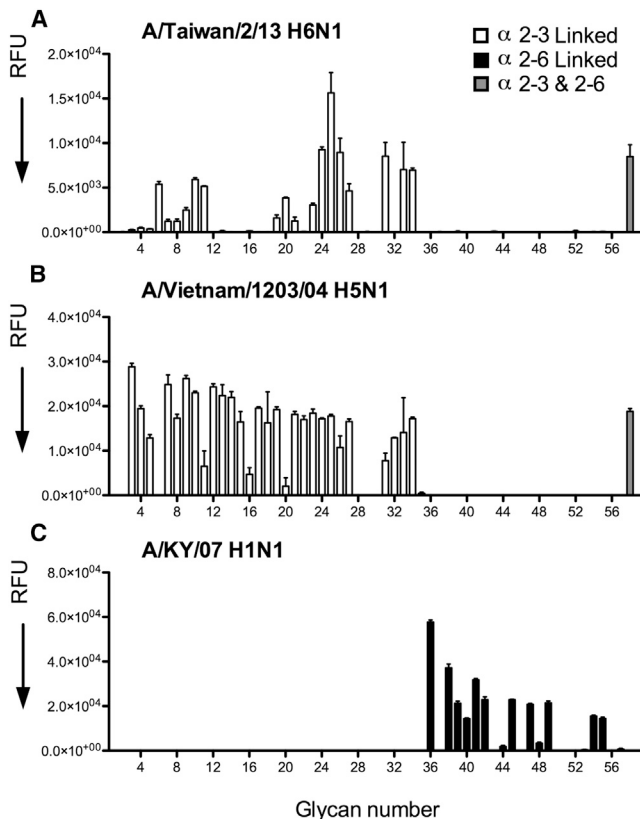


Figure 2. Receptor Binding Specificity of Taiwan2 H6N1 HA

(A) Glycan microarray analysis of recombinant H6 HA protein expressed in insect cells demonstrates specific binding to a subset of α 2-3 sialosides and to a mixed biantennary glycan.

(B and C) The HAs of the A/Vietnam/1203/04 H5N1 (B) and H1N1 seasonal strain KY/07 (C) served as controls for α 2-3 and α 2-6 sialoside binding specificity. The mean signal and standard error were calculated from six independent replicates on the array. α 2-3 linked sialosides in white bars (glycans 3–35 on the x axis), α 2-6 linked sialosides in black (glycans 36–56), and mixed biantennary glycans containing both α 2-3 and α 2-6-linked sialylated glycans in gray bars (glycans 57 and 58). Glycans 1 and 2 are nonsialylated control glycans (see also Figure S2 and Table S3).

α 2-3 linked sialosides. Selective binding was observed to α 2-3 sialosides with no detectable binding to α 2-6 glycans (Figure 2). More specifically, the H6 HA bound preferentially to long branched O-linked and N-linked glycans terminating with α 2-3 linked sialic acids (numbers 24–27) and fucosylated glycan structures (Figure 2A). Recombinant H6 HA expressed in mammalian cells bound with reduced avidity to the same glycans (Figure S2), a property observed previously for the H7N9 HA (Xu et al., 2013) that we attribute to the larger glycans expressed by mammalian cells (Stevens et al., 2006a; de Vries et al., 2010).

Structural Characterization of Taiwan2 H6N1 HA in Complex with Avian Receptor Analogs

Crystal structures of H6 HA in complex with avian receptor analogs 3'-SLN (NeuAc α 2-3Gal β 1-4GlcNAc) and LSTa (NeuAc α 2-3Gal β 1-3GlcNAc β 1-3Gal β 1-4Glc) were determined at 2.3 Å and 2.4 Å, respectively (Figure 3; Table S1). Electron density for 3'-SLN and LSTa was observed in only one RBS of the trimer,

as the two other binding sites are blocked by the HA1 C terminus of a symmetry-related molecule in the crystal. As in other HA subtypes, Sia-1 of 3'-SLN and LSTa forms the usual seven conserved hydrogen bonds with the 220-loop and 130-loop (Figures 3B and 3C). Other conserved interactions include a hydrogen bond between the Tyr98 hydroxyl and the sialic acid (Sia-1) 8-hydroxyl and hydrophobic interactions between Trp153 and Sia-1 C8 and C9 (Figures 3B and 3C). In addition, the H6 RBS contains substitutions at two residues that normally contribute to Sia-1 binding in avian HA subtypes, E190V and G228S. The Gly228Ser substitution, which is also found in human H2 and H3 isolates, eliminates a conserved hydrogen bond between the Sia-1 9-hydroxyl group and the Gly228 main-chain carbonyl. Instead, the Ser228 hydroxyl hydrogen bonds with the Sia-1 9-hydroxyl that results in a small displacement of Sia-1 away from the 220-loop toward the 130-loop. Furthermore, the Glu190Val substitution removes a conserved polar interaction of avian receptor analogs with the 190-helix between Sia-1 and the Glu190 carboxyl.

The second (Gal) and third (GlcNAc) sugars in 3'-SLN and LSTa exit the RBS above the 220-loop (Figures 3B and 3C). For avian HA subtypes, *cis* and *trans* conformations of Sia-Gal bond (C1_{Sia}-C2_{Sia}-O-C3_{Gal}) of avian receptors are found (Figures S4A and S4B). Most analogs bind in a *trans* conformation that is stabilized by hydrogen bonding of Gal-2 to Gln226 and, in some structures, also to Glu190 (Lin et al., 2009; Liu et al., 2009; Xiong et al., 2013a; Xu et al., 2013). For ferret-transmissible H5 (Xiong et al., 2013a; Zhang et al., 2013) and human H7N9 isolates (Shi et al., 2013b; Xiong et al., 2013b; Xu et al., 2013), the *cis* conformation is stabilized by hydrogen bonding of the Gal-2 6-hydroxyl to the Gly225 main chain and by hydrophobic interaction between Gal-2 C3 and Leu226 (except for the A/Shanghai/1/2013 H7N9 HA L226Q mutant in complex with 3'-SLNLN, where the hydrophobic face of Gal-2 makes hydrophobic interactions with other hydrophobic residues of the RBS [PDB ID code 4LKG] [Shi et al., 2013b]). In addition, GlcNAc-3 makes a polar interaction with Lys/Gln222 (for H5 and H7 HAs, respectively) (Figure S4).

In H6 HA, 3'-SLN and LSTa bind in a *cis* conformation, although no hydrophobic residue at position 226 or polar residue at position 222 is present (Gln226 and Ala222 in H6). The *cis* conformation is similar to the conformation in receptor structures, with HAs of ferret-transmissible H5 (Xiong et al., 2013a; Zhang et al., 2013) and human H7N9 isolates (Shi et al., 2013b; Xiong et al., 2013b; Xu et al., 2013) and stabilized by interaction of Gal-2 with Gly225 and by hydrogen bonding of Gal-2 O4 to Asn137 (Figures 3B and 3C). A similar interaction between Gal-2 and the 130-loop has only been reported for an avian analog complex with the transmissible H5 mutant (Zhang et al., 2013). For the 3'-SLN complex, a hydrogen bond is formed between the GlcNAc-3 3-hydroxyl to the Gly225 main chain. In addition, hydrophobic interactions are made from GlcNAc-3 C8 to Arg227 CD in the 3'-SLN and from GlcNAc-3 C6 to Leu186 in the LSTa complex (Figures 3B and 3C).

Structural Characterization of Taiwan2 H6N1 HA in Complex with a Human Receptor Analog

Binding of human receptor analogs to the H6 HA is not detected on the glycan array. Although intrinsic differences in affinity for

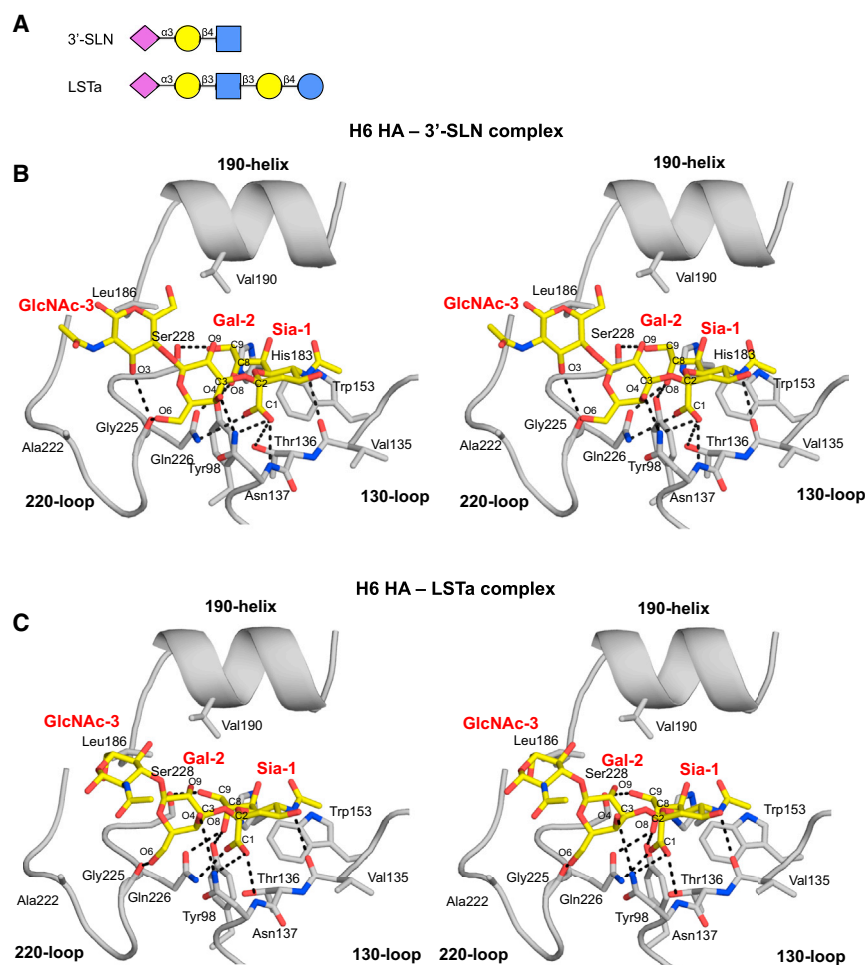


Figure 3. Crystal Structures of the H6 HA in Complex with Avian Receptor Analogs

(A) Cartoon representation of the glycan structures of avian receptor analogs 3'-SLN and LSTa. Sia is the abbreviation for sialic acid, Gal for galactose, GlcNAc for N-acetylglucosamine, and Glc for Glucose.

(B and C) Stereorepresentations of the interactions between the H6 HA RBS and the avian receptor analogs. The conserved secondary elements of the HA RBS (130-loop, 190-helix, and 220-loop) are labeled and shown in cartoon representation. Selected residues and receptor analogs are labeled and shown in sticks. The RBS is colored in gray and the receptor analogs in yellow. Shown are hydrogen bond interactions of Sia-1 and Gal-2 and GlcNAc-3 of 3'-SLN (B) and LSTa (C) with the H6 HA RBS (see also Figures S3 and S4).

more, mutation of Leu226 to Gln results in changes in the phi and psi angles of the Sia-Gal linkage compared to the AH-H7N9 wild-type. Consequently, Gal-2 hydrogen bonds with Gln226 and Gly225 main chain (Figure S4) (Xiong et al., 2013b). This conformation enables hydrogen bonds to be formed between the Gal-2 4-hydroxyl of 6'-SLN with Gln226 and Gly225 main-chain carbonyl (Figure 4B).

DISCUSSION

The first H6 flu virus was isolated in 1965 from turkeys and since then from a

broader range of avian species. Recent biological characterization of 256 H6 viruses from live poultry markets in China revealed that around 34% of H6N2 and H6N6 viruses could bind human-type receptors while maintaining an overall preference for avian receptors (Wang et al., 2014). In addition, most of these viruses can replicate in the respiratory system of mice and guinea pigs without preadaptation (Wang et al., 2014). Although the Gly228Ser mutation was not detected in these isolates, an Ala128Ser substitution, previously associated with human-like receptor binding, was present in a few viruses, but this mutation does not increase binding to human-type receptors (Wang et al., 2014).

Our structural and biological studies indicate that the A/Taiwan/2013 H6N1 virus isolated from an infected human retains the receptor binding properties of an avian virus. Although the H6N1 HA RBS maintains the overall architecture of other HAs, it contains unique substitutions or combinations of residues (Leu186, Val190, Ala222, and Ser228) that influence interaction with receptor analogs. Substitution of the highly conserved acidic residue at position 190 to Val in concert with acquisition of Leu186 increases the hydrophobicity of the 190-helix and precludes formation of hydrogen bonds between receptor analogs and the 190-helix. However, the less frequent *cis* conformation for avian receptors promotes interaction

α 2-3 and α 2-6 linked sialosides are usually small, preferential binding to α 2-3 linked sialosides over α 2-6 linked sialosides is observed as a consequence of the avidity amplification resulting from the HA complex used in the experiment (Sauter et al., 1989), which mimics the multivalent interactions of the HAs on the viral surface. The very weak monovalent binding of α 2-6 linked sialosides can be detected in the crystal soaked at high concentrations of ligand (5 mM; HA:ligand stoichiometry of 1:55), helping to clarify the observed preference in receptor specificity (Xu et al., 2012). In the H6 HA structure with 6'-SLN at 2.2 Å (Figure 4; Table S1), electron density was found for only two sugars, compared to three sugars observed with α 2-3 linked analogs, consistent with the much weaker interaction of α 2-6 sialosides (Figure 2). Hydrogen bonding interactions of Sia-1 with the RBS are largely conserved as in the 3'-SLN and LSTa complexes, except for the polar interaction between the Sia-1 carboxyl and Asn137 due to a side-chain rotamer change (Figure 4A) (Lin et al., 2009, 2012; Liu et al., 2009; Shi et al., 2013b; Xu et al., 2012). The difference in the phi angle may prevent a steric clash between Gln226 and Gal-2 C5 and enable hydrogen bond interactions between Gln226 and Gly225 and Gal-2 of 6'-SLN. Similar interactions with a human receptor analog were observed in an avian AH-H7N9 L226Q mutant with 6'-SLN (PDB ID code 4LKK) (Xiong et al., 2013b). Further-

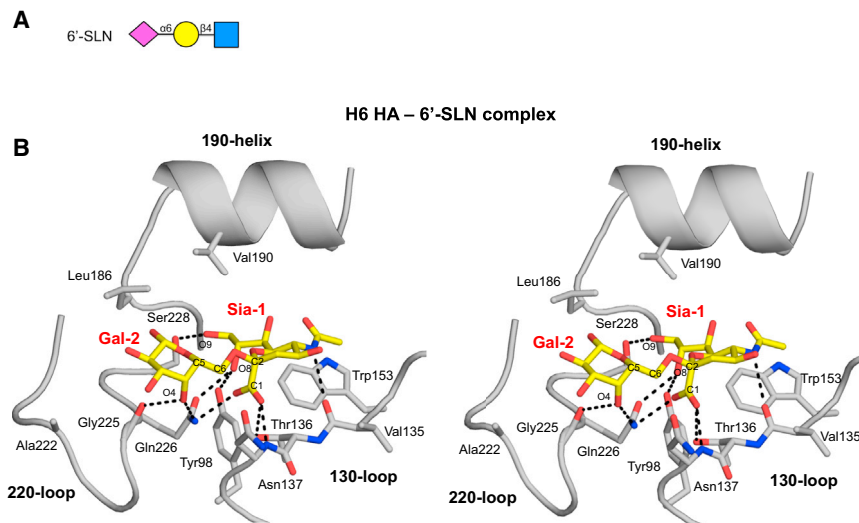


Figure 4. Crystal Structure of the H6 HA in Complex with a Human Receptor Analog

(A) Cartoon representation of the glycan structure of human receptor analog 6'-SLN.

(B) Stereorepresentation of the hydrogen bond interactions of Sia-1 and Gal-2 of 6'-SLN with the H6 HA RBS. The conserved secondary elements of the HA RBS (130-loop, 190-helix, and 220-loop), as well as Tyr 98 and Trp153, are labeled and shown in cartoon representation (see also [Figures S3 and S4](#)).

between Gal-2 of avian analogs and Asn137, thereby enhancing binding with the 130-loop, previously only seen for LSTa in complex with the HA of a transmissible H5 mutant ([Zhang et al., 2013](#)). The H6 crystal structure reveals weaker interaction with 6'-SLN ([Figures 4 and S3](#)), as reflected by electron density for only two sugars and higher B values in the crystal structure for the ligand versus protein (ratio of the B values for receptor homolog/HA protein is 1.23 for 3'-SLN and 1.57 for LSTa compared to 1.72 for 6'-SLN). These structural studies of the H6 HA in complex with tri- and pentasaccharides therefore reveal the structural basis for the preferential specificity for avian versus human receptor analogs. However, other aspects required for transmission could also be impacted by additional characteristics of natural glycan receptors on human lung tissues that are yet to be characterized.

In the last 2 years, an unprecedented number of zoonotic infections of avian-origin viruses have been detected in the human population (H7N9, H10N8, and H6N1) that may be a reflection of new zoonotic events, increased surveillance, or better detection methods and technologies, or a combination of all of these. Notwithstanding, these increasing numbers of zoonotic infections raise concern for the emergence of new pandemic viruses that contain these HA and NA subtypes ([Xu et al., 2013; Vachieri et al., 2014; Zhang et al., 2015](#)). Of primary concern is the potential of these viruses to switch their specificity from avian-type to human-type receptors and, as a result, potentially acquire the ability to transmit from human-to-human and thereby facilitate spread in the human population ([de Graaf and Fouchier, 2014](#)). Human H7N9 and H10N8 isolates have to date largely retained avian-type receptor specificity ([Vachieri et al., 2014; Xu et al., 2013; Zhang et al., 2015](#)), and here we show that the recently isolated H6N1 virus also retains avian-type receptor specificity. RBS mutations that switch specificity from avian-type to human-type receptors in the H1, H2, and H3 subtypes that transmit in humans are well documented ([Connor et al., 1994; de Graaf and Fouchier, 2014; Stevens et al., 2006a](#)). Furthermore, mutations that produce a receptor switch in the H5N1 virus have been demonstrated to increase transmission in ferrets ([de Graaf and Fouchier, 2014; Imai](#)

et al., 2012). Nevertheless, no clear rules have emerged for what it takes to switch receptor specificity for the other 14 HA subtypes (12 avian). Thus, until further insights and better methods for predicting receptor specificity are developed, constant surveillance is required to monitor any changes in avian and other zoonotic viruses that could increase their potential for transmission in the human population.

EXPERIMENTAL PROCEDURES

Expression and Purification of H6 in Insect Cells

The H6 HA cDNA of H6N1 A/Taiwan/2/13 (Global Initiative on Sharing All Influenza Data [GISAID] isolate ID: EPI_ISL_143275) was synthesized by Life Technologies and cloned into a pFastBac vector. Wild-type H6 HA was expressed in Hi5 insect cells with an N-terminal gp67 signal peptide, a C-terminal thrombin cleavage site, a foldon trimerization sequence, and a His₆ tag and expressed as described previously ([Stevens et al., 2006b](#)). The expressed HA0 was purified via a His-tag affinity purification, dialyzed against 20 mM Tris-HCl (pH 8.0), 100 mM NaCl, and then cleaved by trypsin (New England Biolabs, Ipswich, MA) to produce uniformly cleaved (HA1/HA2) and to remove the trimerization domain and His₆ tag. The digested protein was purified further by gel filtration chromatography using a Superdex-200 column (Pharmacia). The HA protein eluted as a trimer and was concentrated to 5 mg/ml. Additional details, as well as expression of H1 and H5 HAs as controls, are given in the [Supplemental Experimental Procedures](#).

Crystallization and Structural Determination of H6 HA

Crystals of H6 HA were obtained using the vapor diffusion sitting-drop method (drop size 4 μ l) at 20°C against a reservoir solution containing 10 mM NiCl₂, 0.1 M Tris (pH 8.5), 20% (w/v) MPEG 2000, and 20% glycerol. Complexes with receptor analogs were obtained by soaking HA crystals in the reservoir solution that contained glycan ligands in a final concentration of 5 mM. Prior to data collection, the crystals were flash cooled in liquid nitrogen. Diffraction data were collected at the Advanced Photon Source (APS) and at the Stanford Synchrotron Radiation Lightsources (SSRL). The H6 apo structure was solved by molecular replacement with an H1 HA structure (PDB ID code 4M4Y) as a search model. The H6 HA apo structure then became the starting model for structure determination of the H6 HA-glycan complex structures.

Glycan Microarray Analysis of HAs Expressed in Insect and Mammalian Cells

Purified, soluble trimeric HA was precomplexed with horseradish peroxidase (HRP)-linked anti-strep-tag mouse antibody and with Alexa 647-linked anti-mouse IgG (4:2:1 molar ratio) prior to incubation for 15 min on ice in 100 μ l PBS-T, and incubated on the array surface in a humidified chamber for 90 min. Slides were subsequently washed and dried by centrifugation and immediately scanned for FITC signal. Fluorescent signal intensity was measured using ImageJ, and mean intensity minus mean background was

calculated. Additional details are given in the [Supplemental Experimental Procedures](#).

ACCESSION NUMBERS

Atomic coordinates and structure factors have been deposited in the Protein Data Bank (PDB) under ID codes 4XKD for Taiwan2 H6 HA in apo form and 4XKE, 4XKF, and 4XKG in complex with 3'-SLN, LSTa, and 6'-SLN.

SUPPLEMENTAL INFORMATION

Supplemental Information includes four figures, three tables, and Supplemental Experimental Procedures and can be found with this article at <http://dx.doi.org/10.1016/j.chom.2015.02.005>.

AUTHOR CONTRIBUTIONS

Project design was by N.T., R.P.d.V., X.Z., J.C.P., and I.A.W.; X-ray structure determination and analysis were by N.T., X.Z., and W.Y.; glycan array studies were by R.P.d.V. and R.M.; and manuscript was written by N.T., R.P.d.V., X.Z., J.C.P., and I.A.W.

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