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Structure and Receptor Binding Specificity of Hemagglutinin H13 from Avian Influenza A Virus H13N6

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Interspecies transmission (host switching/jumping) of influenza viruses is a key scientific question that must be addressed. In addition to the vigorous research on highly pathogenic avian influenza viruses (HPAIVs), studies of the mechanism of interspecies transmission of low-pathogenic avian influenza viruses (LPAIVs) could also provide insights into host tropism and virulence evolution. Influenza A viruses harboring hemagglutinin (HA) H13 (e.g., H13N6) are LPAIVs. In this study, soluble H13 HA glycoprotein was purified, and its receptor binding activity was characterized. The results revealed that H13 exclusively binds the avian α2-3-linked sialic acid receptor; no binding to the mammalian α2-6-linked sialic acid receptor was detected. Furthermore, the molecular basis of the H13 receptor binding specificity was revealed by comparative analysis of the crystal structures of both receptor-bound H13 and H5 HA s, which might be contributed by the hydrophobic residue V186. Work with an H13N186 mutant confirmed the importance of V186 in the receptor binding specificity of H13 HA, which shows that the mutant protein reduced the binding of an avian receptor analog but increased the binding of a human receptor analog. Detailed structural analysis also demonstrated that the conserved binding sites of the recently well-studied broadly neutralizing human monoclonal antibodies targeting the HA2 domain are found in H13. Our results expand our understanding of virulence evolution, receptor binding preference, and species tropism of the LPAIVs and HPAIVs.

There are three types of influenza virus: A, B, and C. Influenza A viruses account for all known major epidemics and pandemics, though some mild epidemics of influenza B virus have been recorded (1–4). Influenza A viruses are classified into subtypes according to their two surface glycoproteins, hemagglutinin (HA) and neuraminidase (NA) (5, 6). For HA, which functions in sialic acid receptor binding and membrane fusion during virus infection, 16 functional antigenic subtypes (H1 to H16) and one bat HA homolog (H17) have been reported (7–10). For NA, the receptor-destroying enzyme that removes sialic acid from the virus and cellular glycoproteins to release newly made virus from the infected cells, there are 9 functional antigenic subtypes (N1 to N9) and one bat NA homolog (N10) (9–11). Influenza A virus infects a broad spectrum of species, including many animals, humans, and birds (5). Interspecies transmission (host switching/jumping) is a major virulence factor for influenza viruses. In general, human viruses preferentially interact with an N-acetyl sialic acid attached to galactose with an α2-6 linkage, whereas avian viruses mostly bind to N-acetyl sialic acid attached to galactose with an α2-3 linkage (12). This specificity corresponds, to a degree, with the availability and density of such glycans at the principal sites of infection in the host. For example, α2-3-linked glycans predominate in the intestinal tracts of ducks, where viral replication generally occurs during natural infection (13, 14), while in humans, α2-6-linked glycans predominate in the upper respiratory tract (15–17).

The 17 HA subtypes are divided into two groups based on phylogenetic analysis. Group 1 can be further divided into three clades (containing H8, H9, and H12; H1, H2, H5, H6 and H17; and H11, H13, and H16), and group 2 includes two clades (containing H3, H4, and H14 and H7, H10, and H15) (18, 19). Since the first HA structure was solved in 1981 (20), nine of the 17 known HAs (H1, H2, H3, H5, H7, H9, H14, H16, and H17) have been crystallized (19, 21–28), and structural correlates of these subtypes have been made from comparisons of the three-dimensional structures of representative HAs from different clades (9, 19).

To date, only three HA subtypes have adapted to cause pandemics in humans: H1N1 in 1918 and most recently 2009, H2N2 in 1957, and H3N2 in 1968 (18). Other subtypes (e.g., H5N1, H6N1, H7N2, H7N7, and H9N2) have caused epidemics in domestic poultry in certain areas of the world (29). Moreover, some viruses from poultry involving subtypes H5, H7, and H9 have resulted in sporadic infections with high fatality in humans, but their low transmissibility among humans has prevented any new pandemics or epidemics (30–32). However, two recent papers report that only a few mutations in the HA protein give the H5N1 viruses the ability to spread through the air between ferrets (33,
Evidence was also provided that the wild H5N1 virus could potentially evolve to spark a pandemic on its own (33, 34).

Generally speaking, many scientists focus on the highly pathogenic H5N1 virus, and less attention is paid to other avian HA subtypes (e.g., H13 and H16), which are commonly seen in some gulls and shorebirds (35). However, these avian subtypes still have the potential to cross species barriers to infect humans and thus are a major concern for public health. Investigation of the receptor binding properties of these avian subtypes could contribute to the understanding of host range switching during virus transmission.

The H13 subtype virus was first isolated in 1977 from gulls (H13N6) in the United States (36) and has subsequently been detected primarily in gulls and shorebirds as a virus with low pathogenicity (37). To better understand the receptor binding specificity of this subtype, we performed a series of receptor binding experiments, including surface plasmon resonance (SPR) analysis and glycan microarray analysis, and further determined the three-dimensional atomic structure of H13 and that of its complexes with an avian receptor analog (LSTa). We demonstrated here that H13 specifically binds the avian receptor analog but not the human receptor. Comparative analysis of the crystal structures of both receptor-bound H13 and H5 HAs revealed that this specificity might be contributed by the hydrophobic residue V186. We generated the H13N186 mutant and found that this mutant protein reduced the binding of the avian receptor analog but increased the binding of the human receptor analog. These results provide the structural basis for the receptor binding specificity of H13 and important insight into the interaction of H13 with avian hosts.

**MATERIALS AND METHODS**

**H13 cloning, expression, and purification.** Highly stable and pure H13 protein was prepared using previously established methods (38), with slight modifications, using the Bac-to-Bac baculovirus expression system (Invitrogen). The cDNA corresponding to residues 11 to 329 (HA1) and 1 to 176 (HA2) of the ectodomain of HA from the A/gull/Maryland/704/1977 (H13N6) virus was cloned into the baculovirus transfer vector pFastBac1 (Invitrogen), with a GP67 signal peptide at the N terminus, a thrombin cleavage site, a trimerizing sequence, and a His6 tag at the C terminus. The recombinant baculovirus was prepared based on the manufacturer’s protocol (Invitrogen). H13 and H13N186 proteins were obtained from infected Hi5 insect cells using previously reported purification methods (38).

**SPR analysis.** Ion-exchange chromatography-purified H13 and H13N186 proteins were subjected to thrombin digestion (3 U/mg protein overnight at 4°C) and purified by gel filtration chromatography using standard phosphate-buffered saline (PBS) with 0.005% Tween 20 (PBST) as the running buffer. The affinity and kinetics of the binding of soluble HAs to receptor analogs were measured at 25°C on a BIAcore 3000 instrument using streptavidin chips (SA chips; Biacore) by SPR. Two biotinylated receptor analogs, the \( 6'\)- and \( 6'\)-glycans (39). Data collection and processing statistics are summarized in Table 1. The structures were solved by the molecular replacement (MR) method using Phaser (40) from the CCP4 program suite (41), with the structure of H16HA (Protein Data Bank [PDB] identifier 4F23) as the search model. Model building and refinement were performed using the COOT (42) and REFMAC5 (43) programs, respectively. The stereochemical quality of the final models was assessed with the program PROCHECK (44).

**RMSD**

| Protein structure accession numbers.** Crystal structures were deposited in the Protein Data Bank (PDB) under accession numbers 4KPS and 4KPS. |
RESULTS

Preferential avian α2-3-linked receptor binding activity. The sequence encoding the ectodomain of the H13 hemagglutinin from influenza A virus A/gull/Maryland/704/1977 (H13N6) was cloned into the pFastbac1 vector and expressed using a baculovirus expression system as previously described (38). Next, SPR technology and glycan microarrays were used to investigate the receptor binding properties of the H13 protein. SPR experiments revealed that H13 binds exclusively to the canonical avian α2-3-linked receptor and not to the canonical human α2-6-linked receptor (Fig. 1A and B). The large-scale glycan microarray analysis with 611 different glycans, including natural sialosides (α2-3-linkage, α2-6-linkage, α2,8-linkage, and mixed linkage) and other glycans that may be relevant to influenza virus biology, revealed that the H13 protein binds to only a few α2-3-linked glycans, with different relative fluorescence units (RFU) (Fig. 1C; see Table S1 in the supplemental material). The structural formulas of the top representative five glycans are shown in Fig. 1C. Although H13 shares a general preference for sialic acid with an α2-3 linkage to Gal-2, alternative linkages from GlcNAc-3 to Gal-4 and subsequent glycan linkages could affect its binding activity.

Overall H13 structural features. The H13 structure was solved by molecular replacement (MR) at a resolution of 2.5 Å using H16HA (PDB identifier 4F23) (19) as a search model (Table 1), and the sequence identity between H13 and H16 is 80.04%. Although the H13 protein was expressed and purified in HA0 form, the solved H13 structure exhibits as a cleaved HA1/HA2 form. Only one molecule is observed in the asymmetrical unit. The crystal structure displays a classical homotrimer oligomerization as seen in other HA subtypes (Fig. 2). Seven asparagine-linked glycosylation sites are predicted in the H13 HA monomer, but interpretable carbohydrate electron density was observed at only one site, N169. Regarding this site, only one N-acetylglucosamine (GlcNAc) could be interpreted.

The phylogenetic tree in Fig. 2A shows that H13 indeed belongs to group 1 HAs, indicating that the H13 structure should resemble...
the structures of group 1 HAs rather than those of group 2. The superimposition of other HA structures onto the H13 monomer by means of their HA2 domains (root mean square deviations [RMSD] are given in Table 2) demonstrated that H13 is most closely related to the 1957 Singapore H2 subtype (RMSD = 0.634), whereas the avian H14 subtype is the most divergent (RMSD = 1.164). Based on their HA1 domains, H13 is most closely related to the avian H16 subtype (RMSD = 0.480), whereas the 1968 Hong Kong H3 subtype is the most divergent (RMSD = 2.391). Concerning the receptor binding region (R region) and vestigial esterase region (E region), H13 is still most closely related to the avian H16 subtype (RMSD = 0.454 and 0.359, respectively).

Previously solved HA structures demonstrate that there are group-specific features at sites where extensive conformational changes occur for HA activation, including the conformation of the interhelix loop and the rigid-body orientation of the globular domain. Here, we studied only whether the rule of the rigid-body orientation of the globular domain is applicable to recently solved H13 and H16 structures or not. Superimposition with other solved HA structures by means of the long central α-helices of HA2 revealed that the rigid-body orientations of globular domains fall into three clusters: group 1, including H1, H2, H5, and H9; group 2, including H3, H7, and H14; and “cluster 3,” consisting of H16 and H13. Cluster 3 (H13 and H16) is located between the group 1 HAs and group 2 HAs. These differences may result in different mechanisms of HA activation, which should be the subject of a future study.

**Structural basis for receptor binding specificity.** The crystals of H13 were soaked with the pentasaccharides LSTc and LSTA, which represent α2-6- and α2-3-linked glycan analogs of human and avian receptors, respectively. However, only LSTA successfully soaked into the crystal to form a complex, which is consistent with the SPR experiments (i.e., only LSTA binds H13). The structural formula of LSTA is NeuAca2-3Galβ1-3GlcNAcβ1-3Galβ1-4Glc. In the H13/LSTA complex structure, strong electron density for the glycan ligand is observed in the receptor binding sites of two of the three monomers in the asymmetric unit. It is possible that the crystal packing limits the glycan receptor analog binding in the
third monomer or that the soaking time with the receptor analogs was not long enough to get full occupancy. In one site, only Sia-1 and Gal-2 are observed, and in the other site, Sia-1, Gal-2, and GlcNAc-3 are observed. To our surprise, the protein-ligand hydrogen bond interactions converge on Sia-1 (Table 3 and Fig. 3A), with no binding to other sugars (including Gal-2 and GlcNAc-3). Nine hydrogen bonds are formed between Sia-1 and residues (Y98, T135, T136, S137, Q226, and S228) within the receptor binding pocket (Table 3 and Fig. 3A). Notably, Q226 and S228, two key residues for receptor binding specificity and host adaptation, form most (five) of the hydrogen bonds.

Previous studies have revealed that Q226L and G228S substitutions can shift the receptor binding specificity from the α2-3-linked avian receptor to the α2-6-linked human receptor in H2, H3, and H5 (9). Furthermore, the G228S single substitution increases human receptor binding affinity in H5 (24). However, in H13, residue 228 is naturally an S, and the H13 protein still binds exclusively to the α2-3-linked avian receptor. Thus, we compared these two complex structures (H13/LSTa and H5/LSTa) (45) and found that the hydrogen bond interactions are different. First, in the H5/LSTa complex structure, the G228 residue forms a hydrogen bond with Sia-1 (Fig. 3A and B). Second, in the H5/LSTa complex structure, residue E190 forms a hydrogen bond with Sia-1, but in the H13/LSTa complex structure, E190 does not form any hydrogen bond with Sia-1 (Fig. 3A and B). This difference might result from the different residues at position 186. In the H5/LSTa complex structure, N186 forms a hydrogen bond with E190 and stabilizes the side chain of E190 to point toward the Sia-1 (Fig. 3C). However, in the H13/LSTa complex structure, V186 creates a hydrophobic environment and might make the side chain of E190 fold back toward the 190 helix (Fig. 3C). Analysis of the virus isolates in the NCBI Flu database reveals that the residue N186 is highly conserved in the H5 subtype (2,932/2,948 isolates have N) and the residue V186 is highly conserved in the H13 subtype (56/57 isolates have V).

In order to confirm the importance of residue V186 in the receptor binding of H13 protein, we generated the H13N186 mutant protein and measured receptor binding using the SPR method. We found that this mutant protein reduced the binding of the avian receptor analog, but increased the binding of the human receptor analog, compared with wild-type H13 protein (Fig. 4).

Superimposing the receptor binding pockets, the Sia-1 is dragged more toward the 220 loop in H13. This structural feature allows Q226 to form more hydrogen bonds with Sia-1 in H13. It is well established that avian and human HAs distinguish avian and human receptors by atomic contacts at the glycosidic linkage between Sia-1 and Gal-2. The α2-3-linked avian receptor binds avian HA in a trans conformation to form an α2-3 linkage-specific motif, made by the glycosidic oxygen and 4-OH of Gal-2, that is complementary to the hydrogen bonding capacity of Q226, an avian-specific residue. The α2-6-linked human receptor binds human HA in a cis conformation, exposing the glycosidic oxygen to solution and nonpolar atoms of the receptor to L226, a human-specific residue. According to these binding rules, if the α2-6-linked human receptor bound H13, the closer distance would drive the nonpolar atoms of the human receptor into a stronger clash with the hydrophilic residue Q226 (which is in the 220 loop). Therefore, combined with the lack of hydrogen bond interactions mediated by E190, this might explain why the H13 protein binds exclusively to the α2-3-linked avian receptor.

Conserved binding sites for the broadly neutralizing anti-HA2 antibodies. The HA of influenza virus is the major target for vaccine design (46). Recently, several cross-reactive anti-HA2 antibodies have been found to neutralize a wide spectrum of influenza A viruses by binding to highly conserved epitopes in the stem region of HA (47–50). Among these antibodies, F6b binds all HA subtypes (H1 to H16) (48). The crystal structure of the F6b-09H1 complex reveals that the antibody targets a shallow hydrophobic groove on the F subdomain of HA, where the sides of the groove are formed by the residues from the A helix of HA2 (including L38, T41, I45, and I48) and parts of two strands of HA1 (including V40 and T318), as well as the HA2 turn (including W21) encompassing residues 18 to 21 (Fig. 5A). In H13 HA, a similar hydrophobic groove is observed, but it contains two different residues, I40 and K38 (Fig. 5B). Thus, we deduced that the F6b cross-reactive anti-HA2 neutralizing antibody can bind to the H13 protein (Fig. 5).

### TABLE 3 Hydrogen bond interactions between H13 and LSTa

<table>
<thead>
<tr>
<th>Protein, residue</th>
<th>Avg bond distance (Å)</th>
</tr>
</thead>
<tbody>
<tr>
<td>O1A, O, S137</td>
<td>3.41</td>
</tr>
<tr>
<td>NE2, Q226</td>
<td>3.43</td>
</tr>
<tr>
<td>O1B, O, E1, Q226</td>
<td>2.73</td>
</tr>
<tr>
<td>O1B, O, Q1, T136</td>
<td>2.79</td>
</tr>
<tr>
<td>NE2, Q226</td>
<td>2.75</td>
</tr>
<tr>
<td>N5, O, T135</td>
<td>3.38</td>
</tr>
<tr>
<td>O8, O, E1, Q226</td>
<td>2.47</td>
</tr>
<tr>
<td>O8, O, OH, Y98</td>
<td>2.55</td>
</tr>
<tr>
<td>O8, O, OH, S228</td>
<td>3.31</td>
</tr>
</tbody>
</table>

*All protein-ligand hydrogen bond interactions converged on Sia-1.*
DISCUSSION

An increasing number of cases of human infections by the H5N1 highly pathogenic avian influenza virus (HPAIV) underscores the real public health concern about potential pandemics or endemics caused by influenza viruses with an avian origin (51). Recent work (33, 34) on H5N1 virus adaptation to horizontal transmission in ferrets has again alerted the public and the scientific community to the possibility of human infection by avian-origin influenza virus. Studies on the virulence evolution and/or interspecies transmission of avian viruses cannot be suspended under such circumstances. The H13 subtype was originally isolated from seagulls in 1977 (36) and causes illness in ferrets upon direct inoculation (36). Thus, detailed studies of H13 receptor binding and structure are highly relevant to avian-to-mammalian transmission of low-pathogenic avian influenza virus (LPAIV).

In this study, we solved the H13 crystal structure and characterized its receptor binding, revealing that H13 has a typical HA structure and is cleaved into HA1 and HA2 in the crystal structure. Our results demonstrated that the avian H13 displays exclusive binding to the avian receptor (i.e., the α2-3-linked sialic acid receptor), whereas other avian HAS (e.g., H1, H2, H3, and H5) both bind the avian receptor and possess weak binding affinity for the human receptor (i.e., the α2-6-linked sialic acid receptor). Notably, the H13 HA bound well to linear-sequence fragments (structures 2 and 4 in Fig. 1C) and the same sequence on selected O-linked glycans but bound with reduced avidity to glycans containing the same terminal sequences attached to N-linked glycan cores, consistent with previous analysis on a more limited set of glycans (52). Structural analysis revealed that H13 has a typical α2-3-linked sialic acid receptor binding groove, as confirmed by a receptor-H13 complex structure. The hydrophobic interactions between the sialyl glycan receptor and the receptor binding site of H13 converge on the sialic acid (Sia-1), with no binding to the glycans. Furthermore, the residue E190 does not form hydrogen bonds with Sia-1 in the H13/LSTa complex structure. This lack of hydrogen bond interactions results from the fold-back conformation of E190, which might be generated by the nearby hydrophobic residue V186. However, in other avian HAS (H5 and H2 subtypes), the residue at position 186 is usually an asparagine (N) (308/308 virus isolates of H2 in the NCBI Flu database have N). Previous studies show that the residues E190 and N186 contribute to the binding of the human receptor via a water-mediated hydrogen bond network in other HA subtypes (26). Thus, the hydrophobic residue V186 precludes the binding of H13 to the human receptor, which is also confirmed by our work with the H13N186 mutant. In terms of interspecies transmission, a Q226L substitution in the receptor binding site might help H13 to obtain human α2-6-linked sialic acid receptor binding, as the same Q226L substitution helps H5 to obtain...
Therefore, evidence of this mutation should be closely monitored in the future for influenza virus surveillance.

Detailed analysis of the HA structure also revealed that the binding sites of the recently well-studied HA2-targeting neutralizing monoclonal antibodies are highly conserved. Therefore, these human monoclonal antibodies should neutralize viruses encoding H13, but they need to be (minimally) tested in an animal model. Future work should also focus on the structures and receptor binding activities of other LPAIV HAs.

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FIG 4 Receptor binding of the H13N186 mutant protein. (A and B) SPR diagrams of H13 protein binding to the α2-3-linked and α2-6-linked sialic acid receptors with a series of higher concentrations. The H13 protein still displayed exclusive binding to the α2-3-linked receptor. (C and D) SPR diagrams of H13N186 protein binding to the α2-3-linked and α2-6-linked sialic acid receptors. The H13N186 protein reduced the binding to the α2-3-linked receptor but increased the binding to α2-6-linked receptor.

FIG 5 The conserved hydrophobic groove in the H13 protein reveals the structural basis of binding by the broadly neutralizing antibody FI6. Surface representations of the F subdomains of 09H1 HA (A) and H13 HA (B) with selected side chains that contribute to the conserved hydrophobic groove are shown. The approximate boundaries of the hydrophobic grooves are indicated by the black lines. Although the residues contributing to the hydrophobic groove are moderately different between 09H1 and H13, similar hydrophobic grooves guarantee the binding potential by the FI6 antibody.

human receptor binding (34). Therefore, evidence of this mutation should be closely monitored in the future for influenza virus surveillance.

Detailed analysis of the HA structure also revealed that the binding sites of the recently well-studied HA2-targeting neutralizing monoclonal antibodies are highly conserved. Therefore, these human monoclonal antibodies should neutralize viruses encoding H13, but they need to be (minimally) tested in an animal model. Future work should also focus on the structures and receptor binding activities of other LPAIV HAs.

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G.F.G. supervised and oversaw the project; G.F.G., X.L., and Y.S. conceived the research; X.L. performed the protein expression, purification, and crystallization; Y.Z. prepared the insect cells; J.Q. performed the structure determination; D.F.S., and J.H.-M. performed the glycan array experiments; J.C.P. provided advice about receptor binding experiments; G.F.G., Y.S., X.L., H.X., and M.W. performed the data analysis; Y.S., X.L., and G.F.G. wrote the manuscript; all authors reviewed and edited the manuscript.

We declare that we have no conflict of interest.

REFERENCES


