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Avian and human receptor binding by hemagglutinins of influenza A viruses

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Abstract An understanding of the structural determinants and molecular mechanisms involved in influenza A virus binding to human cell receptors is central to the identification of viruses that pose a pandemic threat. To date, only a limited number of viruses are known to have infected humans even sporadically, and this has recently included the virulent H5 and H7 avian viruses. We compare here the 3-dimensional structures of H5 and H7 hemagglutinins (HA) complexed with avian and human receptor analogues, to highlight regions within the receptor binding domains of these HAs that might prevent strong binding to the human receptor.

Keywords Influenza A virus · Hemagglutinin · H7 · Receptor binding

Introduction

The binding preference of a given influenza virus HA for α2-3- or α2-6-linked sialic acid correlates with the species specificity for infection. HAs of all 16 antigenic subtypes (H1–H16) influenza found in avian influenza viruses bind preferentially to sialic acid in α2-3-linkage [1,2]; swine influenza viruses, are reported to bind sialic acid in either α2-6- or both α2-3- and α2-6-linkages [2,3] and human viruses of the H1, H2, and H3 subtypes that are known to have caused epidemics, recognize sialic acid in α2-6-linkage [4–6]. Since an avian origin is proposed for the HAs of swine and human viruses [7], a change in binding specificity is required for cross species transfer. The mechanism by which HAs of human viruses achieve these changes appears to be different for individual subtypes. For the HAs of the H2 and H3 human viruses a minimum of two changes in binding site amino acids, Gln226Leu and Gly228Ser, correlate with the shift from binding avian to binding human receptors [8,9]. In contrast, HAs of human H1 viruses retain Gln-226 and Gly-228 but can bind to human receptors [3], because of the distinct geometry of the H1 receptor binding site and the consequent novel positioning of these otherwise avian-specific residues [10].

To date, crystal structures of HA in complex with pentasaccharide analogues of avian and human receptors have been elucidated for human H3 [11], avian H3 [12], avian H5 [13], swine H9 [13], swine H1 [10] and human H1 HAs [10]. The avian receptor is bound in a very similar manner by avian H3, avian H5 and human H1 HAs, with the analogue adopting the *trans* conformation about the α2-3-glycosidic linkage. Swine H1 HA however appears not to bind the avian receptor effectively, and human H3 and swine H9 HAs bind it in the *cis* configuration. The human H3, swine H9 and swine H1 HAs show a binding preference for the human receptor which also adopts the *cis* configuration about the glycosidic bond.

To extend these studies we have determined the structures of an avian H7 HA in complex with avian and human receptor analogues and we compare here the H7 complexes with those formed by other HAs especially the H5 HA, which is the other HA subtype associated with the highly pathogenic viruses of recent outbreaks in domesticated poultry.

Methods

Preparation of H7 HA from A/turkey/Italy/02 grown in hens eggs was by bromelain digestion as previously described

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Table 1 Crystallographic statistics. Values in parentheses refer to the highest resolution shell

	H7 HA + LSTa	H7 HA + LSTc
Space group	*R*32	*R*32
Unit cell dimensions (Å)	$a = b = 117.6$, $c = 299.0$	$a = b = 117.1$, $c = 293.8$
Resolution (Å)	2.9 (3.0)	2.9 (3.0)
R_{sym} (%)	10.7 (47.8)	9.2 (51.5)
$I/\sigma I$	9.6 (2.8)	9.4 (2.0)
Completeness (%)	93.9 (94.6)	96.5 (98.6)
Unique reflections	16931	16922
Redundancy	5.7	3.4
R_{work} (%)	21.9	25.6
R_{free} (%)	28.5	31.1
Protein atoms	3771	3771
Solvent atoms	173	–
Rmsd bonds (Å)	0.007	0.008
Rmsd Angles (°)	1.34	1.38

$R_{\text{sym}} = \sum_j \left| < I > - I_j \right| \Big/ \sum < I >$ where I_j is the intensity of the jth reflection and $<I>$ is the average intensity.
$R_{\text{work}} = \sum ||Fo| - |Fc|| \Big/ \sum |Fo|$.
$R_{\text{free}} = \sum_T ||Fo| - |Fc|| \Big/ \sum_T |Fo|$, where T is a test data set of 5% of the total reflections randomly chosen and set aside before refinement.

[13]. Crystals were grown by vapour diffusion in hanging drops consisting of 2 μl of reservoir solution (20% PEG3350 and 0.2 M Sodium citrate dihydrate, pH 8.2) and 2 μl of concentrated protein solution (10 mg/ml in 10 mM Tris-HCl, pH 8.0). Crystals were soaked for ca. 10 min in either 8 mM LSTa or 8 mM LSTc made up in cryo buffer (reservoir solution augmented with 20% glycerol). Data were collected at 100 K on an in-house Rigaku-MSC RU200 rotating anode coupled to a RaxisIIc detector. Diffraction data were integrated using Denzo and scaled with Scalepack [22]. Standard refinement, with CNS [23] and manual model building, with O [24], was performed. Crystallographic statistics are given in Table 1. All figures were created with Pymol [25].

Results

The receptor binding site

The receptor binding sites of HAs are located at the membrane-distal tips of the molecules. Each site is comprised of three secondary structural elements—the 190-Helix (residues 190–198), the 130-Loop (residues 135 to 138) and the 220-Loop (residues 221–228), that together form the rim of the site. A set of conserved residues (Tyr98, Trp153, His183 and Tyr195) form the base of the site and contribute to maintaining its structural integrity and to forming interactions with sialic acid [14]. The conformations of each of these elements are similar in all HAs studied thus far but small differences exist between HAs that account for differences in the mode of binding to receptor analogues.

H7 HA structure

The receptor binding site of H7 HA maintains the essential features of other HAs but a subtype-specific two-residue insertion in the 150-loop at position 158, results in this loop protruding 6 Å into the binding site by comparison with other HAs. Superposition of the unliganded structures of H7, H1 and H5 HAs shows that overall the structures are highly similar (rmsd for the receptor binding domain of 1.2 Å and 1.4 Å, respectively) (Figure 1). However, the precise positioning of Gln226 is of interest since the functional NE2 and OE1 groups are >1 Å higher in the binding sites of H7 and H5 HAs than in the H1 binding site. The lower positioning in H1 HA enables binding of human receptor analogues despite the presence of a glutamine at this position [10].

H7 HA—avian receptor complexes

H7 HA binds the avian receptor in a similar manner to H1 HA and H5 HA, with the α2,3-linkage adopting the *trans* configuration (Figure 2A). All of the interactions previously observed in other HA-avian receptor complexes are conserved, including the hydrogen bond between Gln-226 and the 4-hydroxyl of Gal**2**. An interaction is also observed between the carboxylate group of sialic acid and the side chain oxygen of Ser-137 which is possible due to sialic acid being shifted slightly towards the 220-Loop, sitting lower in the binding site than observed in H5 HA. A potential hydrogen bond also exists between the side chain nitrogen of Gln-226 and the oxygen of the Sia**1**–Gal**2** glycosidic linkage. Overall, H7 HA binds the avian receptor similarly to the H1 HA.

What is evident from the superposition of all the avian receptor complexes is that α2-3-linked Sialic acid adopts a single conformation when bound to HA in the *trans* linkage (Figure 2A–D). In all avian receptor—HA complex structures thus far elucidated, if the α2-3-linkage is in *trans* configuration only the first 3 saccharides of the pentasaccharide analogue are observed suggesting that the fourth and fifth saccharides are highly flexible and do not contribute to binding.

H7 HA—human receptor complex

In a similar manner to that observed with the H5 HA-human receptor complex [13], the electron density for the human receptor analogue bound to H7 HA is too weak to position even

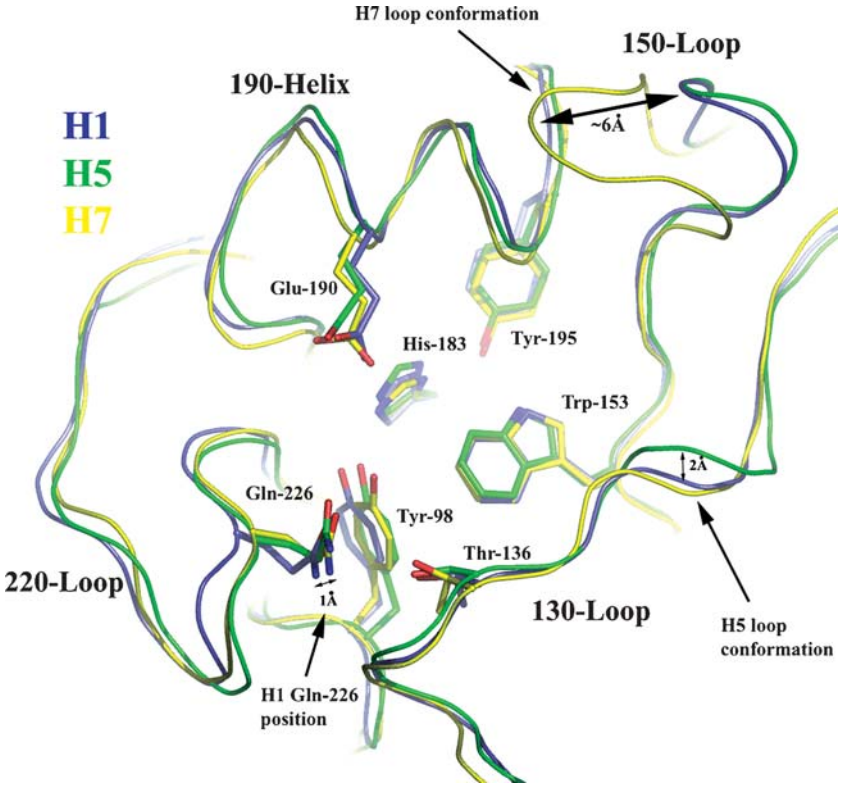

Fig. 1 Superposition of the receptor binding domains of avian H7 HA (yellow), human H1 HA (blue) and avian H5 HA (green). The secondary structure units that make up the site, the 190-Helix, and the 130-, 150- and 220-Loops are indicated. A number of residues important for receptor binding are also shown. The extended 150-Loop of avian H7 HA, the lower position of Gln-226 in human H1 HA and a different conformation of the 130-loop of avian H5 are highlighted

the sialic acid moiety of the receptor, suggesting only very limited binding. In the case of the H5 HA-human receptor complex, the limited binding was attributed to the presence of Gln-226, which would interact unfavourably with the hydrophobic surface presented by Gal-2 C-6 in *cis* configuration about the α2,6-glycosidic linkage. Similar reasoning can be applied to the H7 HA but additional features of the H7 HA receptor-binding site may also play a role.

HA-bound human receptor conformations

The pentasaccharide human receptor analogue has been observed in different conformations in swine H1 HA-, human H3 HA- and swine H9 HA-complexes [10,11,13]. In each conformation, all five rings of the analogue were ordered, but the asialo-portion made specific contacts with different parts of the receptor-binding site (Figure 3). In swine H1 HA, Lys222 forms hydrogen bonds with the 2- and 3- hydroxyls of Gal-2, Asp190 hydrogen bonds with GlcNAc-3 and Ser193 contacts Gal-4; in human H3 HA Lys156 and Ser193 contact Glc-5; in swine H9 HA Ser131, Ser133 and Thr135 contact Glc-5 and Thr155 and Gln156 contact Gal-4.

To investigate potential structural reasons underlying the lack of binding of the human receptor analogue by the H5 and H7 HAs, its structure in the three different conformations observed in swine H1 HA, human H3 HA and swine H9 HA was superimposed onto the H5 and H7 HA receptor binding sites (Figures 4 and 5). Steric clashes between the receptor and the H7 HA occur in all three cases. For both the H1- and H3-bound receptor conformations, steric hindrance by the extended 150-loop of H7 HA occurs that is more pronounced in the case of the H3-bound conformation. In combination with the unfavourable positioning of Gln226, this would make the H7 HA unable to bind the human receptor. Additionally, a number of H7 viruses have a glycosylation site in the extended 150-loop, which could cause an even greater steric block to human receptor binding [15]. The receptor analogue observed in H9 HA complexes is in a fully-folded back conformation and superposition of this conformation onto H7 HA results in a steric clash with the conserved residues, Arg131 and Asn133, in the 130-Loop. The geometry of the H7 HA receptor binding site is such, therefore, that none of the three conformations of the human receptor observed to date can be accommodated.

Similar superpositions to these for the avian H7 HA can be made with avian H5 HA, which also does not bind the human receptor. Superposition of the human receptor analogue in the swine H1-, human H3-, and swine H9- bound conformations onto the receptor binding site of H5 HA, reveals alternative reasons for the inability of this HA to bind any of these conformations (Figure 5). Steric clashes between the subtype-conserved residue Lys193 and GlcNAc**3** of the receptor would occur for both the swine H1 HA- and human H3 HA-bound receptor conformations. Both H1 and H3 HAs have a serine at this position that makes a specific contact with GlcNAc-3 of the human receptor. The fully folded back

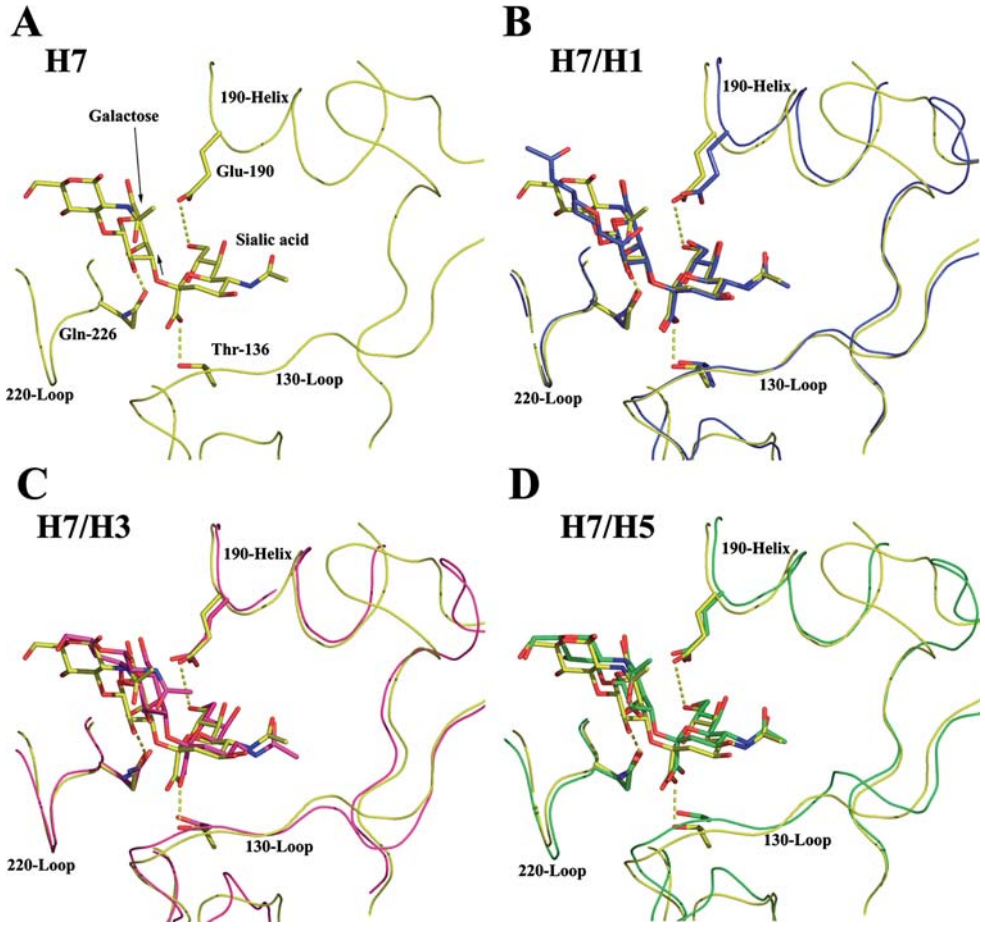

Fig. 2 Binding of the avian receptor to HAs. (A) Avian H7 HA receptor binding site in complex with LSTa. (B) Superposition of avian H7 (yellow) and human H1 (blue) HAs in complex with LSTa. (C) Superposition of avian H7 (yellow) and human H3 (magenta) HAs in complex with LSTa. (D) Superposition of avian H7 (yellow) and avian H5 (green) HAs in complex with LSTa. The *trans* configuration about the glycosidic bond is indicated by an arrow and selected hydrogen bonds shown as dotted lines. The chemical composition of LSTa is: Siaα2-3Galβ1-3GlcNAcβ1-4Glc

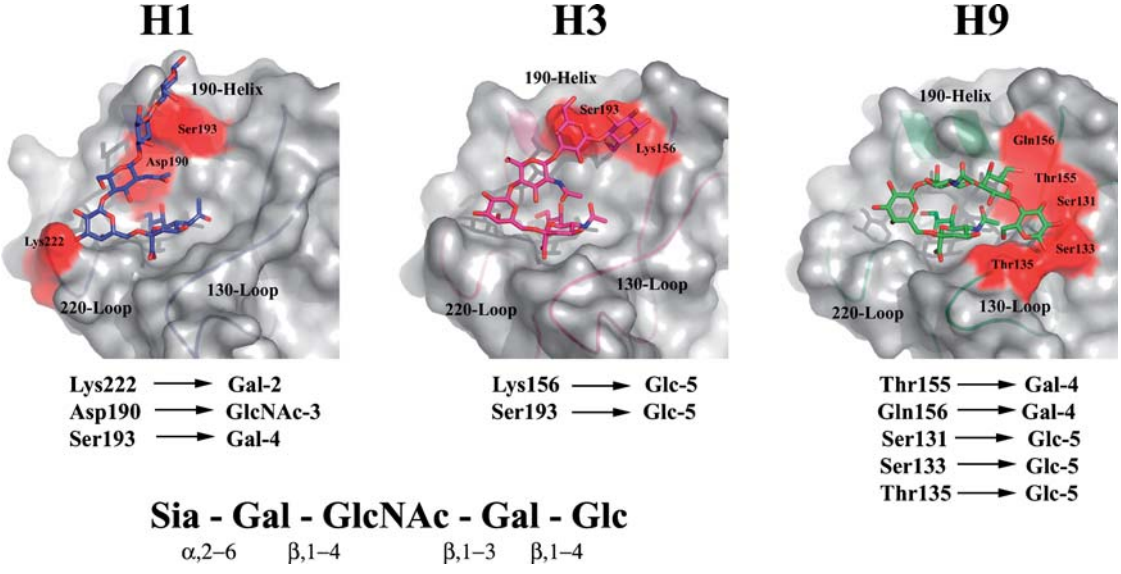

Fig. 3 Interaction surfaces between the pentasaccharide human receptor analogue and swine H1 HA, human H3 HA and swine H9 HA. Residues involved in potential hydrogen bonds between HA and the receptor, excluding those made with sialic acid, are highlighted in red, and the saccharides and the amino acid residues involved are listed below. The chemical composition of the human receptor analogue, LSTc, is given at the bottom

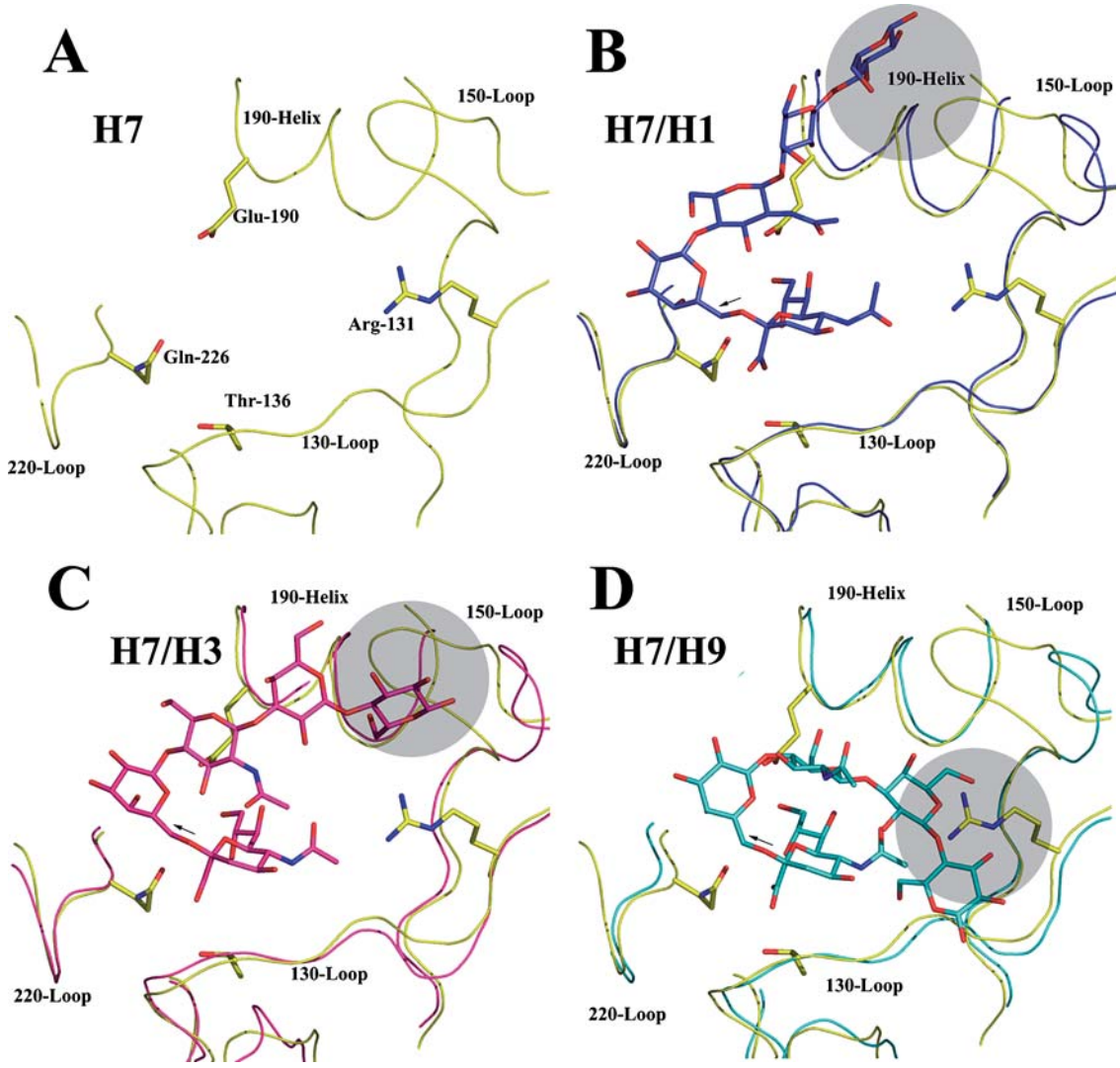

Fig. 4 Comparison of the avian H7 HA receptor binding to swine H1 HA, human H3 HA and swine H9 HA. (A) Avian H7 HA receptor binding site. No binding of the human receptor was observed. (B) Superposition of avian H7 HA (yellow) and swine H1 (blue) HA in complex with LSTc. (C) Superposition of avian H7 (yellow) and human H3 (magenta) HA in complex with LSTc. (D) Superposition of avian H7 HA (yellow) and swine H9 (cyan) HA in complex with LSTc. Regions of potential steric clashes between H7 HA and human receptor are circled. The *cis* configuration about the glycosidic bond is indicated by an arrow and selected residues from the avian H7 HA binding site shown

conformation of the human receptor analogue observed in the H9 HA complex could not be accommodated by avian H5 HA because of a difference in conformation of the protein backbone at the *N*-terminus of the 130-Loop (Figure 5).

Discussion

Unraveling the potential mechanism or mechanisms by which Influenza A viruses change their receptor binding specificities to cross the avian-human species barrier, may provide insights as to which viruses pose a pandemic threat.

The crystal structures compared here, of avian H5 and H7 HAs in complex with both avian and human receptor analogues show that both bind specifically to the avian receptor analogue and fail to interact effectively with the human receptor.

The human receptor analogue binds to HAs of H1, H3 and H9 subtypes in quite different conformations. Despite being bound in the *cis* configuration about the Sia**1**–Gal**2** α2,6-glycosidic bond in all three complexes, the torsion angles are different and result in Gal**2** in swine H1 HA being positioned lower in the site allowing interactions with Lys222. Additionally, the Gal**2** β1-4GlcNAc**3** linkage differs significantly in each conformation, as do the linkages between saccharides **3** and **4** and **4** and **5**. The conformations of the human receptor analogue when bound to H3 and H9 HAs are stabilized by protein-saccharide hydrogen bonds and by intramolecular,

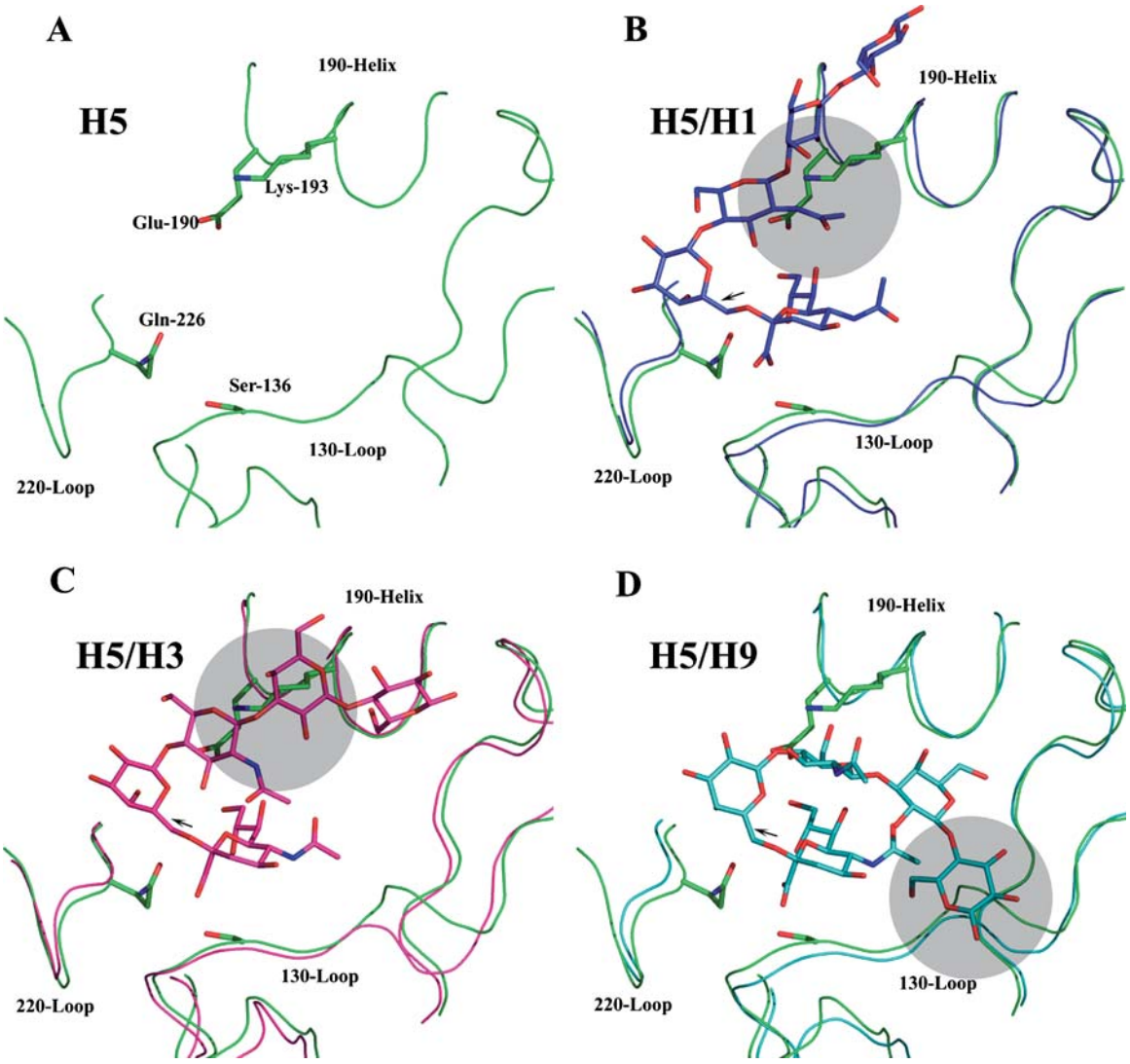

Fig. 5 Comparison of the avian H5 HA receptor binding to swine H1 HA, human H3 HA and swine H9 HA. (A) Avian H5 HA receptor binding site. No binding of the human receptor was observed. (B) Superposition of avian H5 HA (green) and swine H1 (blue) HA in complex with LSTc. (C) Superposition of avian H5 HA (green) and human H3 (magenta) HA in complex with LSTc. (D) Superposition of avian H5 HA (green) and swine H9 (cyan) HA in complex with LSTc. Regions of potential steric clashes between H5 HA and human receptor are circled. The *cis* configuration about the glycosidic bond is indicated by an arrow and selected residues from the avian H5 HA binding site shown

saccharide-saccharide, hydrogen bonds. Fewer intramolecular hydrogen bonds are observed in the H1 complex, where the analogue exits the site in an orientation approximately parallel with the three-fold axis of symmetry, in a more extended conformation.

Only a limited number of crystal structures have been elucidated of proteins complexed with oligosaccharides containing a sialic acid α2-6-galactose linkage, but a considerable variation of the ϕ torsion angle has been observed, ranging from 36° to 294°. By contrast the α2-3-linked avian receptor displays a lower degree of conformational variability ($\phi = 68° \pm 13°$) [16]. Calculations suggest that the global potential energy well of α2-6-linkages is very shallow compared to α2-3-linkages, indicating a larger range of conformations in solution of the former compared to the latter [17].

The variety of HA-bound conformations observed for the human receptor is consistent with the possibility that the analogue has the potential to adopt a conformation, which could complement the shape and size of the H5 and H7 HA receptor binding sites, and be stabilized simply by saccharide-protein interactions. An extended conformation of the human receptor for example may exist that could allow binding in the receptor binding sites of both HAs without steric clashes, in orientations similar to the exit path taken by the avian receptor analogue. A sialic acid α2-6-linkage has in fact been observed to be bound in an extended conformation to Pertussis toxin, with the conformation being

stabilized by specific hydrogen bonds with protein atoms [18]. The avian H7 HA could not bind the human receptor in such a conformation, however, since steric clashes with the 220-loop would probably occur. Interestingly, recent H7 isolates have an 8 residue deletion of this loop (residues 221–228) like influenza C HEF glycoprotein [19] and a mutant H3 HA [20], which both have the same deletion and exhibit no specificity between α2-3- and α2-6-linkages.

Previous crystallographic analysis of the trisaccharide human receptor analogue, α2-6-sialyllactose, bound to human H3 HA revealed an extended conformation rather than the folded conformation of the pentasaccharide human receptor analogue, LSTc [11]. This conformation was presumed not to be representative of natural receptors because of the presence of glucose rather than *N*-acetyl glucosamine at position 3 and the lack of glycosylation beyond this position. The mode of α2-6-sialyllactose binding, however, further demonstrates the plasticity of the HA receptor binding site in accommodating oligosaccharides in different conformations. By contrast, α2-3 sialyllactose binds in a very similar manner to the pentasaccharide, LSTa, again suggesting that there is no role in avian receptor binding for the saccharides beyond GlcNAc**3**. There is therefore the possibility that some HAs are capable of binding sialic acid in α2-6-linkage to galactose, but are not capable of infecting humans because the saccharides beyond GlcNAc**3** sterically clash with the HA. For an avian HA to change its binding specificity from avian to human receptors may therefore involve two sorts of changes, the first, to allow binding of the α2-6-linkage, either via mutation of Gln226 to Leu226 as in H2 and H3 HAs or via a specific positioning of Gln226 as in H1, and the second, to accommodate the additional saccharides linked to galactose**2** in the natural carbohydrate side chain.

Viruses containing H7 HA caused a severe outbreak of avian influenza in 2003 in the Netherlands resulting in one human fatality [21], and have recently emerged in the same highly pathogenic form in North America. H5 avian viruses emerged in 1997 in Hong Kong and are currently widespread across Far East Asia. Thus far also only a low level of human infection has occurred with these viruses and perhaps the special features of the H5 and H7 HA receptor binding sites identified here are, at least in part, responsible for this species restriction.

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