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An Airborne Transmissible Avian Influenza H5 Hemagglutinin Seen at the Atomic Level

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Recent studies have identified several mutations in the hemagglutinin (HA) protein that allow the highly pathogenic avian H5N1 influenza A virus to transmit between mammals by airborne route. Here, we determined the complex structures of wild type and mutant HAs derived from an Indonesia H5N1 virus bound to either avian or human receptor sialic acid analogs. A *cis*/*trans* conformational change in the glycosidic linkage of the receptor analog was observed, which explains how the H5N1 virus alters its receptor binding preference. Furthermore, the mutant HA possessed low affinities for both avian and human receptors. Our findings provide a structural and biophysical basis for the H5N1 adaptation to acquire human, but maintain avian, receptor binding properties.

In the past 100 years, only three subtypes of influenza virus have adapted to human populations to cause four pandemics: H1N1 in 1918 and 2009, H2N2 in 1957 and H3N2 in 1968 (*1*–*3*). Other subtypes (e.g., H5N1, H6N1, H7N2 and H9N2) have caused epizootics in domestic poultry in certain regions of the world (*4*), with a recent H7N9 human infection in China (*5*). H5N1 virus, especially, has spread through wild and domestic bird populations across Asia and into Europe, the Middle East and Africa (*6*, *7*). H5N1 virus has also caused several hundred sporadic cases of human infections with high fatality (*8*–*10*), but it has not acquired the ability to efficiently transmit among humans.

Two recent studies identified several mutations that allow the H5N1 virus to become transmissible by airborne route in a ferret mammalian model system, raising the question of whether or not these mutations can also confer airborne transmissibility between humans (*11*, *12*). Both reports show that several mutations in hemagglutinin (HA) of the H5N1 virus are sufficient to change the receptor binding specificity from an avian receptor preference (α2,3-linked SA receptor) to a human receptor preference (α2,6-linked SA receptor).

We know how the HAs of the H1, H2 and H3 subtypes bind to α2,3- and α2,6-linked SA receptors (*13*), and that for the HAs of the H2, H3 and H5 subtypes, two amino acid substitutions (Q226L and G228S) in the receptor binding site can switch the avian viruses to human-adapted viruses (*14*–*16*). In H1 subtype, the HA contains Q226 and G228, and the amino acids at position 190 and 225 are important for the binding preference (*17*, *18*). However, in H5 subtype, there is no structural evidence for how the receptor binding preference switches from avian to human receptors.

We generated soluble H5 HA protein (InH5) and its mutant (InH5mut, containing H110Y/T160A/Q226L/G228S, H3 numbering) from the influenza virus A/Indonesia/5/2005 to enable structural and binding studies (*11*). The genes encoding the HA ectodomains were cloned into the pFastbac1 vector (Invitrogen) and expressed using a baculovirus expression system (*3*). The recombinant proteins were purified with good purity and integrity (fig. S1) (*19*).

Surface plasmon resonance (SPR) experiments measured the binding affinities of InH5 and InH5mut to canonical avian and human receptors. The wild type InH5 displayed a strong binding preference for the avian receptor, with an affinity of 60 μM (Fig. 1, A and E), but weak binding to the human receptor (> 1 mM, which is beyond the BIAcore® measurement range) (Fig. 1, B and E). The InH5mut displayed significantly reduced binding affinity to the avian receptor (affinity of 554 μM) (Fig. 1, C and F) and increased binding affinity to the human receptor (affinity of 372 μM) (Fig. 1, D and F), changing the binding preference (Fig. 1, E and F). It is noteworthy that the InH5mut binding affinities for both the avian and human receptors were relatively low.

Using X-ray crystallography, the structures of both InH5 and InH5mut were determined to 2.5 and 2.9 Å, respectively. We solved the structures of both InH5 and InH5mut complexed with the two sialo-pentasaccharides LSTa and LSTc, which are natural sialosides from human milk (*20*). These sialo-pentasaccharides are analogs of the avian and human receptors, respectively, and contain the three terminal saccharides (Sia-Gal-GlcNAc) (*20*). The receptor analogs are in good electron density map (fig. S2).

Conventionally, the receptor binding site (RBS) of H5 is divided into two parts: the base, consisting of conserved residues Y98, W153 and H183; and the side, consisting of three secondary elements, i.e., the 130-loop, 190-helix and 220-loop (*13*). The earlier crystal structures of H5 avian and H9 swine influenza bound to avian and human receptor analogs have identified the α2,3-linkage-specific motif (*trans* conformation) in avian H5 and the α2,6-linkage-specific motif (*cis* conformation) in swine H9 (*21*).

The structure of InH5 with the avian receptor analog LSTa revealed that the ligand binds in a *trans* conformation (Fig. 2A), similar to that seen in a previously reported H5 HA/LSTa complex and nearly all other avian HA/LSTa complexes (*13*, *21*). The 'avian-signature' residue Q226 forms two hydrogen bonds with the ligand: one with the glycosidic oxygen of the α2,3 linkage and the other with the 4-OH of Gal-2. Similarly, the structure of InH5 with the human receptor analog LSTc revealed that the ligand binds in a *trans* conformation (Fig. 2B), by contrast to all human HAs (which are observed in a *cis* conformation). In this structure, the 'avian signature' residue Q226 makes three hydrogen bonds with Sia-1, with no hydrogen bonding to Gal-2 (table S1).

The structure of InH5mut with the avian receptor analog LSTa showed, by contrast to the wild type complexes, that the ligand binds in a *cis* conformation (Fig. 2C). Interestingly, in this structure, the side-chain OH of S137 forms one hydrogen bond with Gal-2, which has not been observed in other HA/receptor complexes. The 'human signature' residue L226 makes extremely weak van der Waals interactions (only two atom-to-atom contacts) with the LSTa (table S1). In this case, most of the interactions are contributed by the 130-loop. Likewise, the structure of

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InH5mut with the human receptor analog LSTc showed that the ligand binds in a *cis* conformation (Fig. 2D). The 'human signature' residue L226 makes stronger van der Waals interactions (eight atom-to-atom contacts) with LSTc than LSTa (table S1).

Structural analysis indicates that a *cis*/*trans* conformational switch can occur when InH5 and InH5mut bind to different receptor analogs as a result of the Q226L substitution. The structures showed that in wild type H5 HA bound to LSTa, the hydrophilic glycosidic oxygen became exposed to the hydrophilic residue Q226 (Fig. 3A) and two hydrogen bonds bridged the Q226 to Sia-1 and Gal-2, and LSTa took up a *trans* conformation. However, when the mutant HA is bound to LSTa, the hydrophobic residue L226 creates an unfavorable environment for the hydrophilic glycosidic oxygen, and the glycosidic oxygen orients away from L226, switching the conformation of LSTa form *trans* to *cis*, and pushing the sialic acid receptor closer to the 130-loop by ~1 Å (Fig. 3A). This conformational switch may reduce the interaction between the InH5mut and LSTa.

By contrast, when wild type HA binds to mammalian receptor analog, LSTc, the hydrophilic residue Q226 creates an unfavorable environment for the non-polar portion of LSTc, and pushes it away from Q226 to take up a *trans* conformation, that tilts the Gal-2 upward (Fig. 3B). However, when the mutant HA binds to LSTc, the hydrophobic residue L226 creates a favorable environment for the non-polar portion of LSTc and the non-polar part orients toward the L226 to adopt a *cis* conformation (Fig. 3B) and makes a tighter interaction between the receptor and ligand. Hence, it appears that the avian and human receptors possess the inherent flexibility to accommodate different amino acid mutations in the RBS of HAs.

Previous studies demonstrate that RBSs of the human and swine influenza virus HAs are larger than those of the avian influenza virus HAs (*21*). Comparison of the wild type H5 HA, the mutant and the 1957 Singapore human H2 (57H2) was made to see if the Q226L and G228S substitutions alone can convert avian type HA into a human-like HA. The distance between the 130-loop and the 220-loop, which form two sides of the receptor binding site, is greater by ~1 Å in the InH5mut structure than in the InH5 structure (Fig. 4A). The distances between the 130-loop and 220-loop are comparable in the InH5mut and 57H2 structures (Fig. 4B). However, the mutant InH5mut does not display as strong a binding affinity for the human receptor, as 57H2 does. Our structural comparisons showed a ~3 Å displacement in the InH5mut/LSTc complex relative to the 57H2/LSTc complex (Fig. 4C), resulting in fewer contacts with the receptor binding site. Further analysis revealed that InH5mut contains an arginine (R) at position 193 in the 190-helix whose long side chain might push the glycan away from the receptor binding site in the InH5mut/LSTc complex. In contrast, 57H2 has a threonine (T) at the same position in its 190-helix whose side chain is much shorter than R193 (Fig. 4C). Similarly, the 1968 Hong Kong human H3 (68H3) contains an S193 in its 190-helix (Fig. 4D).

In summary, our binding studies in vitro showed that the wild type HA of the avian H5N1 influenza virus protein preferentially binds to an analog for the avian receptor. Whereas, binding of the wild type HA to a human sialic acid receptor analog was undetectable (> 1 mM). If the H5 HA was mutated at Q226L, it acquired the ability to bind to both avian and human receptor analogs but with less affinity than that of wild type HA binding to the avian receptor analog. Our structural studies showed that the mutation in HA at Q226L caused a *trans*/*cis* conformational switch in the glycan receptor that affected atomic contacts within the RBS and hence altered binding affinity. Our findings here might be expanded to explain the recent H7N9 virus, whose hemagglutinin has a natural Q226L substitution, with higher human infection rate in China (*5*).

In the HAs of the H2 and H3 subtypes, Q226L and G228S double-substitution mutations switch the affinity of the virus receptor from avian to high avidity for the human receptor (*22*, *23*). However, our SPR results demonstrated that InH5mut still has a low affinity for the human receptor, despite containing Q226L and G228S. The basis for the low affinity appears to be the long side chain of R193, which displaces the glycan from the receptor binding site by ~3 Å in the InH5mut/LSTc. This displacement decreased the atomic contacts between the LSTc and the receptor binding site, resulting in a low binding affinity. Further substitutions may improve the affinity and thus transmissibility between humans.

Two other amino acid substitutions, H110Y and T160A, are also important for the transmissibility of avian H5 virus among mammals (fig. S3A). Temperature-dependent circular dichroism (CD) spectroscopic experiments revealed that InH5mut has a higher thermostability than wild type InH5 proteins (fig. S3B), while structural comparison showed that Y110 in the InH5mut forms a hydrogen bond with the N413 of the adjacent monomer to stabilize the trimeric protein whereas the H110 in the wild type InH5 cannot do so (fig. S3, C and D). The T160A mutation, which results in the loss of a glycosylation site on the head of the HA close to the receptor binding site, enhances H5N1 virus binding to the α2,6-linked human receptor (*11*, *12*). However, in our InH5 structure, we did not observe the glycan in this glycosylation site due to an artifact of baculovirus expression, and this structure will require further research. Moreover, this poor glycosylation in InH5 might be the reason why we get similar affinities of binding of mutant HA to human and avian receptor analogs, which has a discrepancy with respect to the avian receptor binding of the mutant virus in studies by Herfst *et al*. (*11*) and Chutinimikul *et al*. (*24*) using different assays and substrates.

Other amino acid changes elsewhere in the virus may be critical to enable the H5N1 virus to transmit in humans. For example, Herfst *et al*. (*11*) introduced E627K into the PB2 protein (*25*, *26*), together with the two substitutions introduced by reverse genetics and two acquired upon ferret passage in InH5mut, to generate a H5N1 virus that is transmissible among ferrets (*11*).

Our work therefore provides a structural basis to comprehensively evaluate the receptor binding properties of H5N1 virus.

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Supplementary Materials
www.sciencemag.org/cgi/content/full/science.1236787/DC1
Materials and Methods
Figs. S1 to S3
Tables S1 and S2
References (*27*–*36*)

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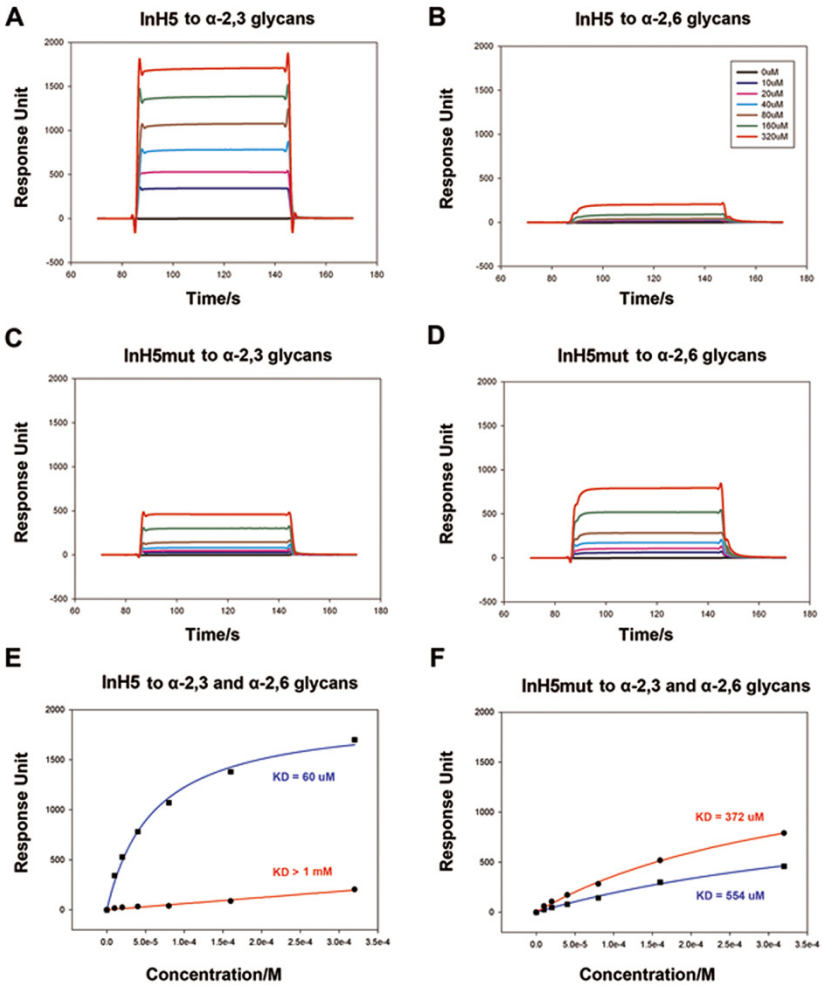

Fig. 1. BIAcore® binding properties of the InH5 and InH5mut HAs to either α2,3-linked or α2,6-linked sialylglycan receptors. (**A** and **B**) BIAcore® diagram of InH5 binding to the two receptors, showing strong binding to the α2,3-linked sialylglycan receptor but little binding to the α2,6-linked sialylglycan receptor. (**C** and **D**) BIAcore® diagram of InH5mut binding to the two receptors, showing reduced binding to the α2,3-linked sialylglycan receptor and increased binding to the α2,6-linked sialylglycan receptor relative to InH5. (**E** and **F**) Response units were plotted against protein concentrations. The K_D values were calculated using a steady affinity state model by the BIAcore® 3000 analysis software (BIAevaluation Version 4.1).

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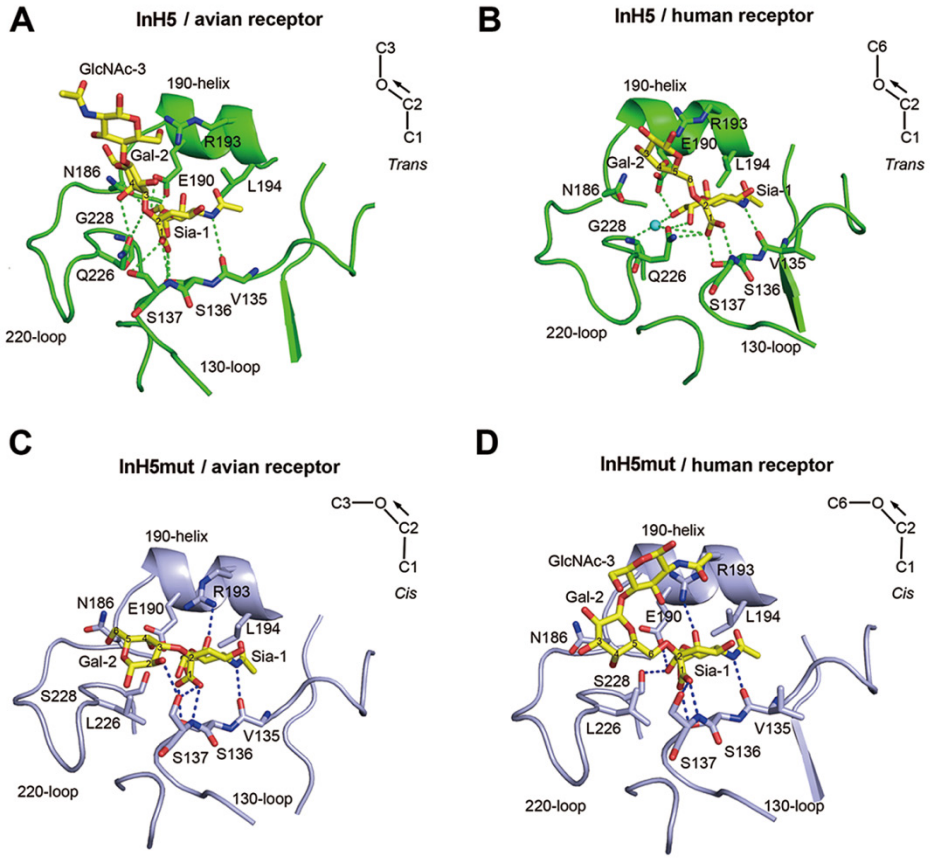

Fig. 2. Interaction of the InH5 and InH5mut HAs with either avian or human receptor analogs. The three secondary structural elements of the binding site (i.e., the 130-loop, 190-helix and 220-loop) are labeled in ribbon representation, together with selected residues in stick representation. The hydrogen bonds are shown as dashed lines. The InH5 HA is colored in green, and the InH5mut HA is colored in light blue. The glycans are colored in yellow. (**A** and **B**) InH5 HA with the avian receptor analog LSTa (α2,3) pentasaccharide (A) or human receptor analog LSTc (α2,6) pentasaccharide (B) bound. Both LSTa and LSTc bind in a *trans* conformation. (**C** and **D**) InH5mut HA with the avian receptor analog LSTa (C) or the human receptor analog LSTc (D) bound. Both LSTa and LSTc bind in a *cis* conformation.

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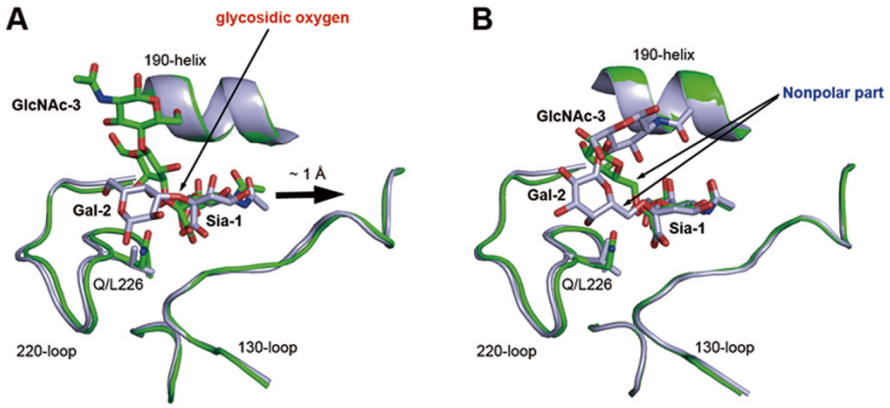

Fig. 3. Molecular mechanism of the *cis*/*trans* conformational switch when InH5 and InH5mut bind either avian or human receptors. (**A**) Comparison of the receptor binding sites between the InH5/LSTa (green) and InH5mut/LSTa (light blue) complexes. The sialic acid moves toward the 130-loop by ~1 Å in the InH5mut/LSTa complex structure relative to the InH5/LSTa complex structure. The hydrophilic glycosidic oxygen of LSTa is exposed to the hydrophilic residue Q226 in the InH5/LSTa complex, while the hydrophilic glycosidic oxygen is exposed away from the hydrophobic residue L226 in the InH5mut/LSTa complex. (**B**) Comparison of the receptor binding sites between the InH5/LSTc (green) and InH5mut/LSTc (light blue) complexes. The sialic acids are similarly located in both complexes. The non-polar portion of LSTc is exposed to the hydrophobic residue L226 in the InH5mut/LSTc complex, while it is exposed away from the hydrophilic residue Q226 in the InH5/LSTc complex.

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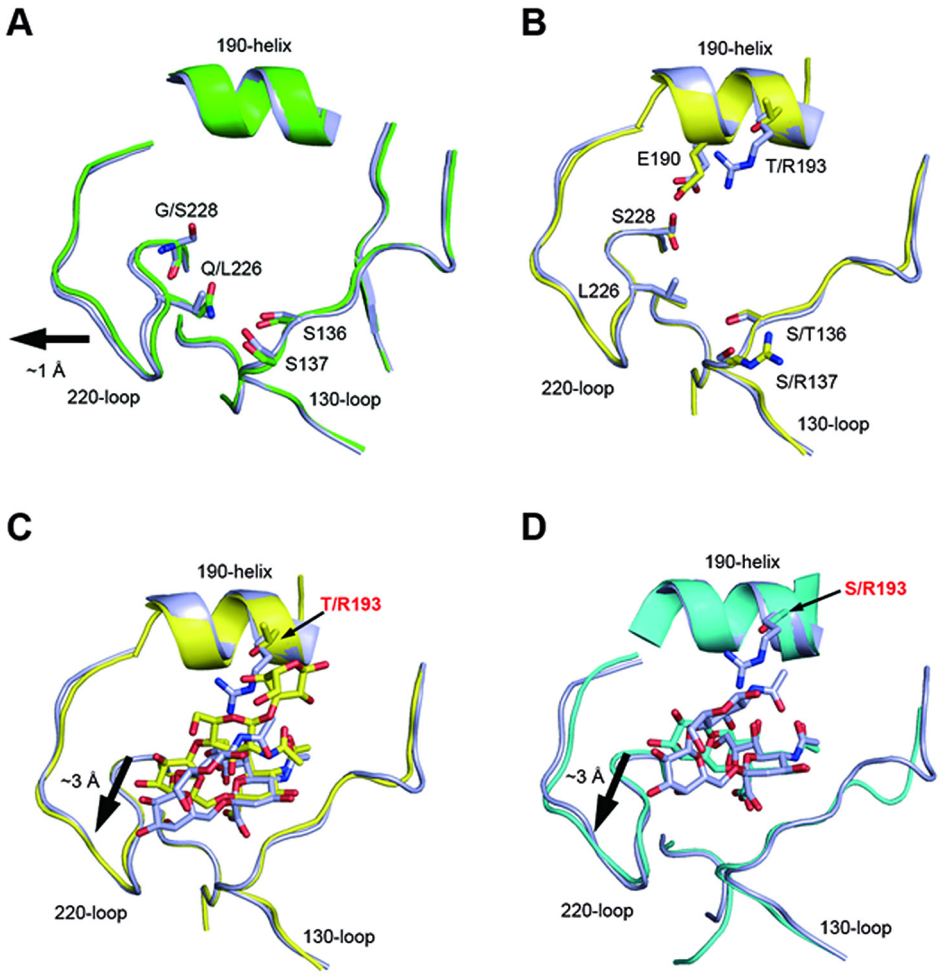

Fig. 4. Comparison of InH5, InH5mut, human H2/H3 and their HA/LSTc complex structures. (**A**) Comparison of receptor binding sites between InH5 (green) and InH5mut (light blue). The receptor binding site of InH5mut is ~1 Å wider than that of InH5 due to the clash between the hydrophobic residue L226 of the 220-loop and the hydrophilic residues S136 and S137 in the 130-loop. (**B**) Comparison of the receptor binding sites between InH5mut (light blue) and 57H2 (yellow). InH5mut has a similar wide receptor binding site compared to 57H2 (PDB code: 2WR7). (**C** and **D**) Comparison of the InH5mut/LSTc (light blue), human H2/LSTc (yellow) and human 68H3/LSTc (cyan) complexes (PDB code: 2YPG). The glycans are displaced away from the receptor binding site by ~3 Å in the InH5mut/LSTc complex relative to those in the 57H2/LSTc and 68H3/LSTc complexes. This displacement may result from the long side chain of R193, while the equivalent residues in human H2 and H3 (T and S) have short side chains.

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