ACADEMIC PRESS

Available online at www.sciencedirect.com

SCIENCE DIRECT®

VIROLOGY

Virology 309 (2003) 209–218

www.elsevier.com/locate/yviro

X-ray structure of the hemagglutinin of a potential H3 avian progenitor of the 1968 Hong Kong pandemic influenza virus☆

Ya Ha,[a,b] David J. Stevens,[c] John J. Skehel,[c,*] and Don C. Wiley[b]

[a] *Department of Pharmacology, Yale University School of Medicine, 333 Cedar Street, New Haven, CT 06520, USA*
[b] *Department of Molecular & Cellular Biology, Howard Hughes Medical Institute, Harvard University, 7 Divinity Avenue, Cambridge, MA 02138, USA*
[c] *MRC National Institute for Medical Research, Mill Hill, London, UK NW71 AA*

Received 7 October 2002; returned to author for revision 20 November 2002; accepted 4 December 2002

Abstract

We have determined the structure of the HA of an avian influenza virus, A/duck/Ukraine/63, a member of the same antigenic subtype, H3, as the virus that caused the 1968 Hong Kong influenza pandemic, and a possible progenitor of the pandemic virus. We find that structurally significant differences between the avian and the human HAs are restricted to the receptor-binding site particularly the substitutions Q226L and G228S that cause the site to open and residues within it to rearrange, including the conserved residues Y98, W153, and H183. We have also analyzed complexes formed by the HA with sialopentasaccharides in which the terminal sialic acid is in either α2,3- or α2,6-linkage to galactose. Comparing the structures of complexes in which an α2,3-linked receptor analog is bound to the H3 avian HA or to an H5 avian HA leads to the suggestion that all avian influenza HAs bind to their preferred α2,3-linked receptors similarly, with the analog in a *trans* conformation about the glycosidic linkage. We find that α2,6-linked analogs are bound by both human and avian HAs in a *cis* conformation, and that the incompatibility of an α2,6-linked receptor with the α2,3-linkage-specific H3 avian HA-binding site is partially resolved by a small change in the position and orientation of the sialic acid. We discuss our results in relation to the mechanism of transfer of influenza viruses between species.

Keywords: Influenza; Haemagglutinin; X-ray structure; Receptor specificity

Introduction

An animal or avian virus origin for the hemagglutinin (HA)[1] of the Hong Kong 1968 human influenza virus was deduced at the beginning of the pandemic in 1968, from antigenic analyses of the isolates in comparison with influenza viruses previously recovered from horses and birds. (Coleman et al., 1968; Tumova and Easterday, 1969; Kasel et al., 1969; Zakstelskaya et al., 1969). Subsequent comparisons of the amino acid sequences of HAs from human, avian, and equine viruses (Daniels et al., 1985; Fang et al., 1981; Laver and Webster, 1973; Ward and Dopheide, 1981) supported the proposal of an avian precursor. These studies also revealed that three of the four sequence differences between human and avian HAs that are common to all H3 avian HAs examined (Bean et al., 1992; Kida et al., 1987) are in or near the sialic acid receptor-binding site. Extensive surveys of influenza viruses isolated from a variety of species have shown a correlation of receptor-binding specificity and species of origin, with avian viruses preferring sialic acid in α2,3-linkage and human viruses preferring sialic acid in α2,6-linkage (Connor et al., 1994; Rogers and D'Souza, 1989; Rogers and Paulson, 1983). These preferences may result from the selective pressure of an abundance of α2,6-linked sialosides on airway epithelial cells in humans, where the disease is respiratory, and of α2,3-linked sialosides on the intestinal epithelium of avian species,

☆ The coordinates have been deposited in the Protein Data bank (Accession 1MQL, 1MQM, 1MQN).

* Corresponding author. Fax: +44-0-20-8816-2041.
E-mail address: mbrenna@nimr.mrc.ac.uk (J.J. Skehel).

[1] *Abbreviations used:* Gal, galactose; Glc, glucose; NAG, *N*-acetylglucosamine; HA, haemagglutinin; Sia, sialic acid.

0042-6822/03/$ – see front matter © 2003 Elsevier Science (USA). All rights reserved.
doi:10.1016/S0042-6822(03)00068-0

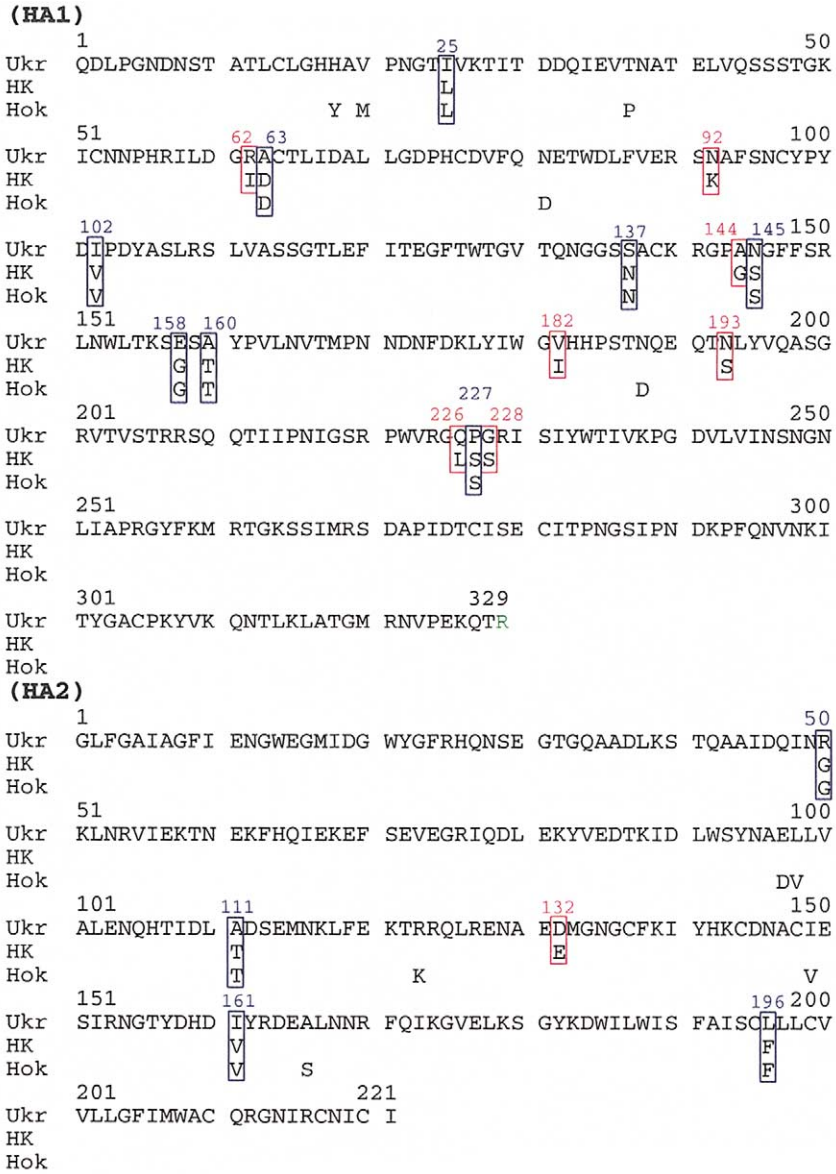
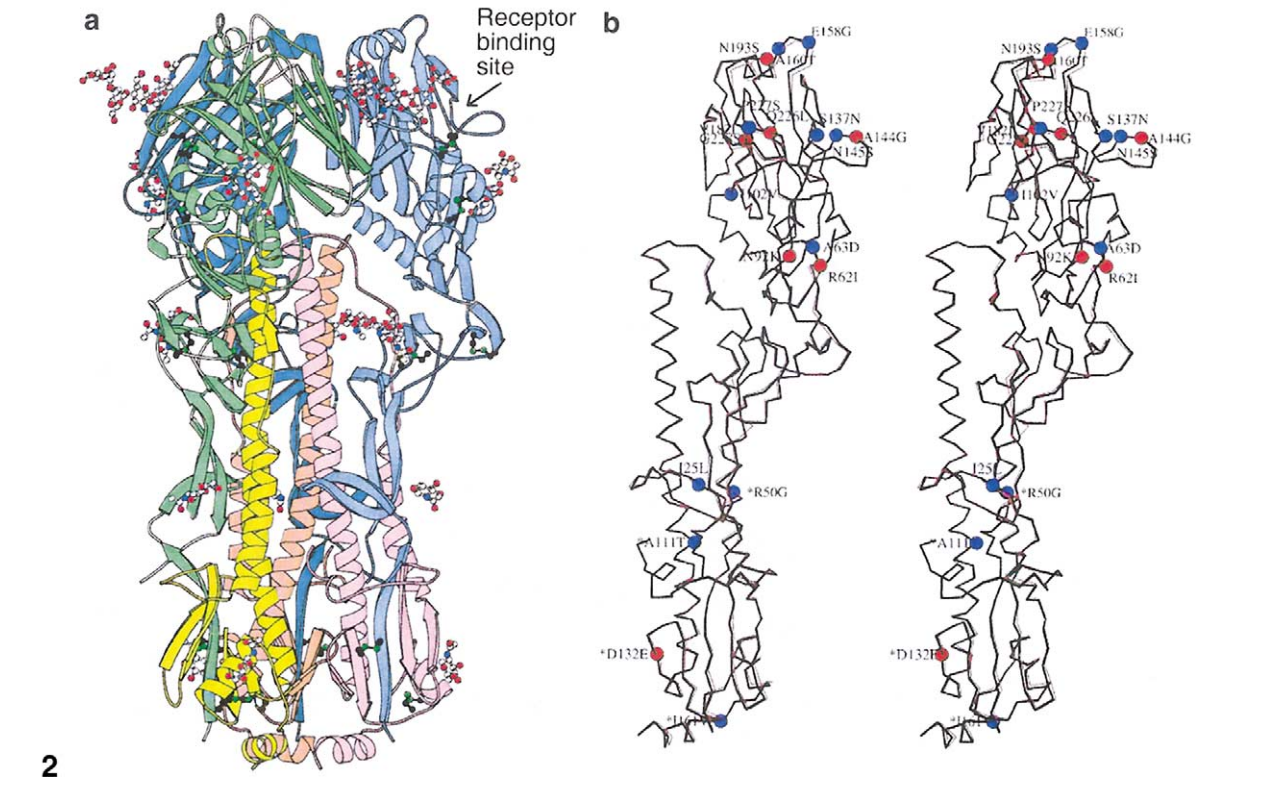


Fig. 1. Amino acid sequence alignment of HAs from A/duck/Ukr/63 (Ukr), X31 (HK), and A/duck/Hokkaido/5/77 (Hok) influenza A viruses. Amino acids (total 8) uniquely different between Ukr and HK are in red boxes. Differences (total 12) observed between both Ukr and HK, and Ukr and Hok are in blue boxes. There are 10 additional amino acid differences between Ukr and Hok. Data from Fang et al. (1981), Verhoeyen et al. (1980), and Bean et al. (1992).

where the disease is enteric (Baum and Paulson 1990; Ito et al., 1998; Skehel and Wiley, 2000). Residue 226 in the receptor-binding site of the HA of the 1968 human virus, A/Aichi/68 (X31) (Wilson et al., 1981) was implicated in distinguishing α2,6- from α2,3-linked sialosides, by analyses of receptor specificity mutants selected by growing virus in nonimmune horse sera, a source of the α2,6-linked sialic acid-rich inhibitory glycoprotein, α2-macroglobulin. Mutants containing Q instead of L at position 226 of the HA had a decreased preference for α2,6-linked sialic acid and an increased preference for α2,3-linked sialic acid, relative to the wild type, which showed preference for α2,6 over α2,3 (Rogers et al., 1983). All HAs of H2 and H3 subtype human viruses have a nonpolar residue, mainly L, at position 226, while HAs of all 15 subtypes from avian viruses have Q at 226 (Nobusawa et al., 1991). By contrast, H1 subtype HAs from either avian or human viruses all have Q at position 226.

A comparison of the structures of the L226Q mutant with wild-type X31 HA showed that the substitution causes a narrowing of the receptor-binding site with the polar amide and carbonyl groups of the Q side chain pointed upward, closely underneath the sialoside linkage (Weis et al., 1988). For the wild type, one methyl group of the L226 side chain contacts conserved Y98 and A138, resulting in a slightly different geometry for adjacent residues and creating a wider site. Recently, structures of an H5 avian HA bound to α2,3- and α2,6-linked sialopentasaccharides were deter-



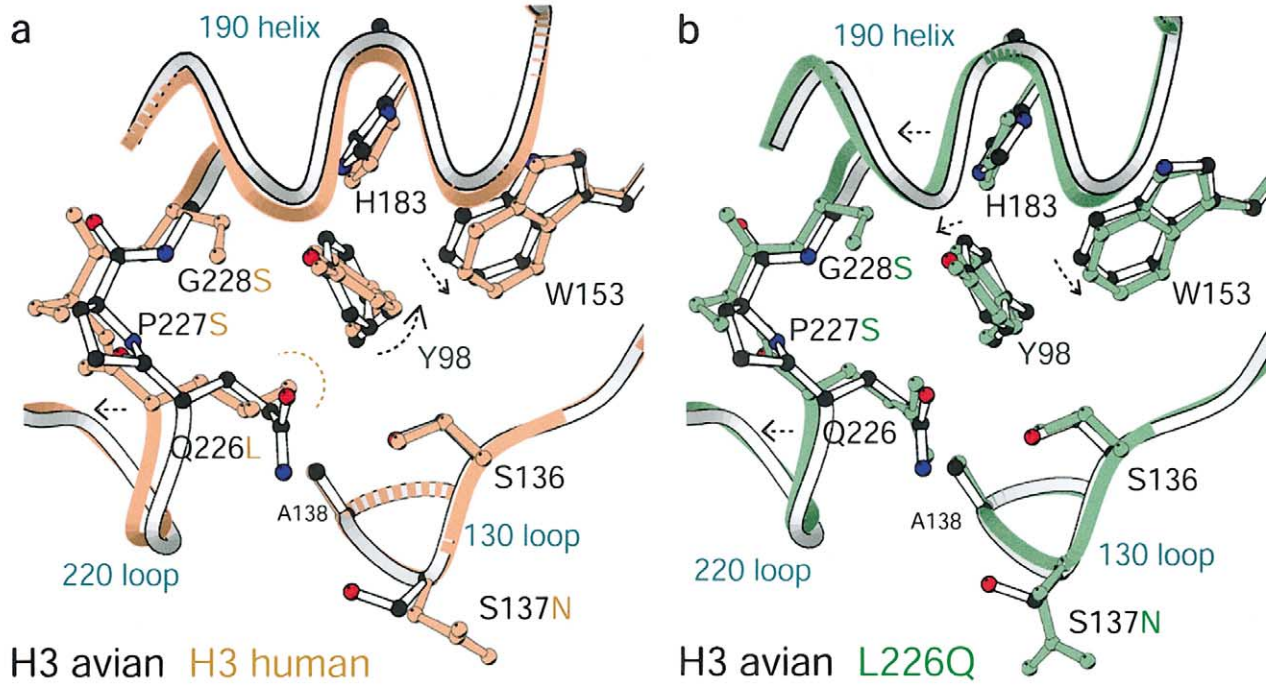


Fig. 2. The three-dimensional structure of the HA ectodomain of the H3 avian HA from A/duck/Ukr/63. (a) A schematic diagram of the trimeric HA showing residues HA1 9–326, HA2 1–172. Modeled carbohydrate side chains are shown in gray, red, and blue. Disulfide bonds are shown in black and green. The six polypeptide chains are shown in light blue (HA1), magenta (HA2), dark blue (HA1′), light red (HA2′), green (HA1″), and yellow (HA2″). (b) Stereo view of the Cα-traces of the H3 avian HA monomer from A/duck/Ukr/63 (black) superimposed on the H3 human HA monomer from X31 (brown). Amino acid differences between the two HAs are circled and colored as in Fig. 1. *HA2 residues.

Fig. 3. Conformational differences in the receptor-binding sites of the H3 avian and H3 human HAs. (a) Comparison between H3 avian-(white) and H3 human (brown)-binding sites. Side-chain and main-chain shifts are indicated by dashed arrows. Of the 20 amino acid differences between avian and human HAs, 3 (S137N, Q226L, and G228S) make contact with receptors. (b) Comparison between H3 avian (white) and L226Q mutant (green) HA-binding sites.

mined which showed that in a narrowed site, an α2,3-linkage is recognized directly by Q226 with the side-chain amide group hydrogen-bonded to the glycosidic oxygen of the linkage and the side-chain carbonyl oxygen hydrogen-bonded to the axial 4-hydroxyl of the penultimate galactose. This arrangement is made possible by the *trans* conformation of the bound linkage atoms in which the glycosidic oxygen is oriented down toward the Q and the galactose ring is projected out of the site (Ha et al., 2001, 2002). By comparison the wider binding site of the H3 human HA with L at 226 has been seen previously in crystal structures to recognize the energetically favored *cis* conformation of the α2,6-linkage in which the glycosidic oxygen of the linkage is projected upward toward solution and the C6 atom of the linkage and the galactose ring are projected downward to make nonpolar contacts with L 226 (Eisen et al., 1997).

We have now determined structures of the HA of A/duck/Ukraine/63 virus bound to both α2,3- and α2,6-linked sialopentasaccharide receptor analogs. The H3 avian HA-binding site is narrow and remarkably similar to the binding sites of the L226Q mutant of the H3 human HA and the H5 avian HA. The α2,3-linked sialoside, interacts with Q226 via hydrogen bonds in the same α2,3 linkage-specific motif exposed by the *trans* geometry of the linkage, as seen in the H5 avian HA. However, the α2,6-linked sialoside is accommodated in the H3 avian HA-binding site, despite the *cis* geometry of the α2,6-linkage which forces nonpolar sialoside atoms down toward the polar Q226 side chain. The bound α2,6-linked sialic acid is shifted slightly upward and twisted within the binding site, affecting the contacts with the binding site surface from one end of the sialic acid to the other. HAs of different subtypes have numerous different amino acids at positions near the sialoside linkage, near the *N*-acetyl substituent across the sialic acid ring from the linkage (Ito et al., 2000), and in regions that may contact the asialo portions of receptor oligosaccharides (Eisen et al., 1997). The small shifts and twists required of the α2,6-linked sialoside to fit into the H3 avian HA-binding site that prefers the α2,3-linkage suggest how the binding sites of different avian HAs, by their slightly different geometries, might differ in their abilities to accommodate α2,6-sialoside receptors, and other receptor modifications such as *N*-acetyl- and *N*-glycolyl substituents (Higa et al., 1985).

Results

The structure of the H3 avian HA from A/duck/Ukraine/63

The three-dimensional structure of the trimeric ectodomain, BHA, from the H3 avian virus, A/duck/Ukraine/63, was determined, with and without complexed receptor analogs. The structure of the H3 avian HA is very similar to the HA of the H3 human virus, A/Aichi/68 (X31) (Wilson et al., 1981) with overall r.m.s.d. (the square root of the mean deviation squared) of the α-carbon positions of 0.5 Å. (Fig. 2b). The positions of all N-linked oligosaccharides and disulfide bonds are conserved in the two HAs, which differ at only 20 positions (Fig. 1; Fig. 2a) (Tumova and Easterday, 1969; Daniels et al., 1985; Fang et al., 1981; Bean et al., 1992). Few of the 20 amino acid differences between the HAs are in positions to affect receptor binding. Only 10, those between residues 137 and 228 (Fig. 1; Fig. 2b), are in the globular membrane distal domain that contains the receptor-binding site and of those only five, 137, 193, 226, 227, 228, are in the site. Only residue 226 is conserved, as Q, in all avian HAs. In H3 human HAs residue 226 is never Q, but always a nonpolar residue, usually L but occasionally V or I (Fig. 1) (Y.P. Lin, and A.J. Hay, personal communication). Residues 137 and 193 may contact some sialosides and residues 227 and 228 may also affect sialoside binding; 227 is S in all H3 human and avian HAs except A/duck/Ukraine/63 where it is P; 228 is G or S in H3 avian HAs but always S in H3 human HAs (Bean et al., 1992; Connor et al., 1994).

When the structures of the H3 avian and human HAs are superimposed, conformational differences are evident in the receptor-binding site (Fig. 3a). The lower left and right edges of the site are formed by two surface loops; residues 225–228, the 220 loop, and 135–138, the 130 loop, respectively. The avian HA-binding site is narrowed by a difference in the position of the main chain of the 220 loop, which is 0.5 Å closer to the 130 loop (Fig. 3a). This difference is apparently a result of the d-methyl group of L226 in the human HA which points downward into the binding site where it contacts A138 and Y98 (Fig. 3a). In the absence of this methyl group, the avian HA site can collapse inward, with the ring of Y98 rotating about 20° and the side chains of W153, H183, and the backbone of the α-helix that forms the top edge of the site, residues 187–194, the 190 helix, also changing slightly.

These structural differences between the H3 avian and human HAs are very similar to the differences observed earlier between the H3 human HA, X31, and the receptor-binding mutant that contains the single amino acid substitution L226Q (Weis et al., 1988; Fig. 3b). The L226Q substitution apparently causes most of the differences in the location of the 220-loop main chain, in the rotation of Y98, and in the shifts of W153, H183, and the backbone of the 190 helix (Fig. 3b) that are observed between the avian and the human H3 HAs (Fig. 3a). The residual differences are probably due to differences at residues 227 and 228, where for the H3 avian HA, 227P and 228G apparently allow the 220 loop closer to Y98 than 227S and 228S in the L226Q mutant (the distance from the Cα of 228 to the phenol OH of Y98 is 3.7 Å in the H3 avian HA but 4.6 Å in the mutant HA).

The structures of the H3 avian HA–receptor analog complexes

A/duck/Ukraine/63 BHA crystals soaked with the receptor analogs, lactoseries tetrasaccharide a, LSTa, and lacto-

series tetrasaccharide c, LSTc, containing α2,3-linked and α2,6-linked sialic acids, respectively (see Ha et al., 2001, for structures), were studied to 2.6 and 3.2 Å resolution. The α2,3-sialopentasaccharide is bound in the *trans* conformation by the H3 avian HA, almost identically to the way in which an H5 avian HA was observed to bind this receptor analog (Fig. 6a; Ha et al., 2001). In this conformation the glycosidic oxygen (* in Fig. 4a) is positioned over the amide group of the Q226 side chain and Gal-2 of the analog is projected upward such that its axial 4-0H group can hydrogen bond to the carbonyl-group of the Q226 side chain (Fig. 4c). This structural complementarity between the Q226 side chain and the analog containing the *trans* α2,3-linkage, forms an α2,3-specific recognition motif (Ha et al., 2001). The electron density for the third saccharide, NAG-3, is incomplete and no electron density was observed for the last two saccharides, Gal-4 and Glc-5, which are apparently disordered. The α2,6-sialopentasaccharide is bound in a *cis* conformation to the H3 avian HA, projecting the C6 methylene group of Gal-2 downward toward the polar atoms of the Q226 side chain. (Figs. 4b and d). The unfavorable juxtaposition of the nonpolar ligand atoms to the polar side chain of Q226 appears to account for the poorer binding of the α2,6-sialoside to the avian HA-binding sites. Electron density was observed for Sia-1 and Gal-2 of the α2,6-linked analog bound to the H3 avian HA but for only Sia-1 of the analog bound to the H5 avian HA (Ha et al., 2001).

Superposition of the structures of the H3 avian HA complexed with either α2,3- or α2,6-linked analogs shows that the α2,3-linked Sia-1 is bound more deeply in the HA binding site (Figs. 5a and b). By comparison the α2,6-linked Sia-1 is raised up, shifted to the front of the site, and slightly rotated counterclockwise, when viewed from above the Sia-1 ring (Fig. 5c). Potential hydrogen bonds between receptor analog and HA atoms are generally longer in the α2,6-complex than in the α2,3-complex (Figs. 5a and b). One interaction, between the side-chain amide of Q226 with the glycosidic oxygen, is eliminated in the α2,6-complex by the presence of the C6 methylene group of Gal-2 and a major effect of the different positions of the Sia-1 ring is to remove the potentially unfavorable contact between the C6 methylene group and Q226.

Discussion

Conversion of the HA of an avian influenza virus into the HA of an influenza virus with the potential to cause an epidemic, whether directly by infection of humans with an avian virus or indirectly by gene reassortment in a mixed infection to produce a new virus containing an avian HA, which then infects humans, requires the avian HA to change its receptor-binding specificity; avian HAs bind preferably to sialic acid in α2,3-linkage; human HAs prefer the α2,6-linkage. The structures reported here of the HA of the H3 avian virus A/duck/Ukraine/63, a potential precursor of the 1968 H3 Hong Kong pandemic virus, in complex with α2,3-linked and α2,6-linked receptor analogs indicate that this avian HA receptor-binding site, while preferring the α2,3-linkage, accommodates the α2,6-linked sialosides preferred by human influenza viruses. Most of the structural adjustments that change the linkage preferences are achieved by one amino acid substitution, Q226L. This is clearly indicated by the close similarity of the structures of the receptor-binding sites of the L226Q mutant of the 1968 human HA and the H3 avian HA both of which prefer the α2,3-linkage, (Fig. 3b). The contributions of amino acid residues other than 226 to this evolutionary process are not known. The small difference in width between the H3 avian HA-binding site and the L226Q mutant binding site (Fig. 3b) appears to be due to differences at residue 227 (P in avian, S in the mutant) and residue 228 (G in avian and S in the mutant). Neither substitution is common to all H3 avian HAs (Bean et al., 1992) but observations of the genetic instability of the selected A/duck/Ukraine/63 Q226L HA mutant by comparison with X31 and its mutant L226Q (Rogers et al., 1985) suggest that residues 227 and 228 influence receptor binding.

When the structure of the H3 avian HA-α2,6-linked receptor analog complex is superimposed on that of the H3 human HA-α2,6-linked analog complex, the sialic acid can be seen to bind slightly up and out of the H3 avian HA-binding site, and, as described above, slightly rotated so that the glycosidic oxygen moves toward the front of the site and the C6 methylene group of Gal-2 is slightly removed from the side chain of Q226 (Fig. 4d). When the backbone atoms of the H3 avian and the H5 avian HAs are superimposed, in complexes prepared at identical ligand concentrations, they are seen to bind the α2,6-linked analog slightly, but significantly differently (Fig. 6b). In the H5 avian HA α2,6-linked sialoside complex Sia-1 is bound in approximately the same position as Sia-1 in all other HA-sialoside complexes examined to date (Weis et al., 1988; Ha et al., 2001; Eisen et al., 1997; Sauter et al., 1992; Watowich et al., 1994), but in the H3 avian α2,6-linked sialoside complex Sia-1 is rotated and shifted toward the 130 loop (Fig. 5; Fig. 6b). As noted above this shift may alleviate unfavorable interactions between the nonpolar C6 methylene group and other nonpolar parts of Gal-2 and the polar atoms of Q226 in the H3 avian HA-binding site. Amino acid sequence differences at positions 133, 155, and 193 may prevent the same positioning of α2,6-linked Sia-1 in the H5 avian HA-binding site (Fig. 6b). The amino acid insertion at position 133 (S133a) and the I at position 155 in the H5 avian HA instead of T in the H3 avian HA may prevent α2,6-linked Sia-1 from shifting away from Q226 and toward residues 155 and 133, as observed here in the H3 avian HA α2,6-linked analog complex (Fig. 6b).

Overall, therefore, α2,6-linked sialic acid is bound by the H3 avian HA differently than by either the H3 human HA or the H5 avian HA, in a way that may be intermediate in the

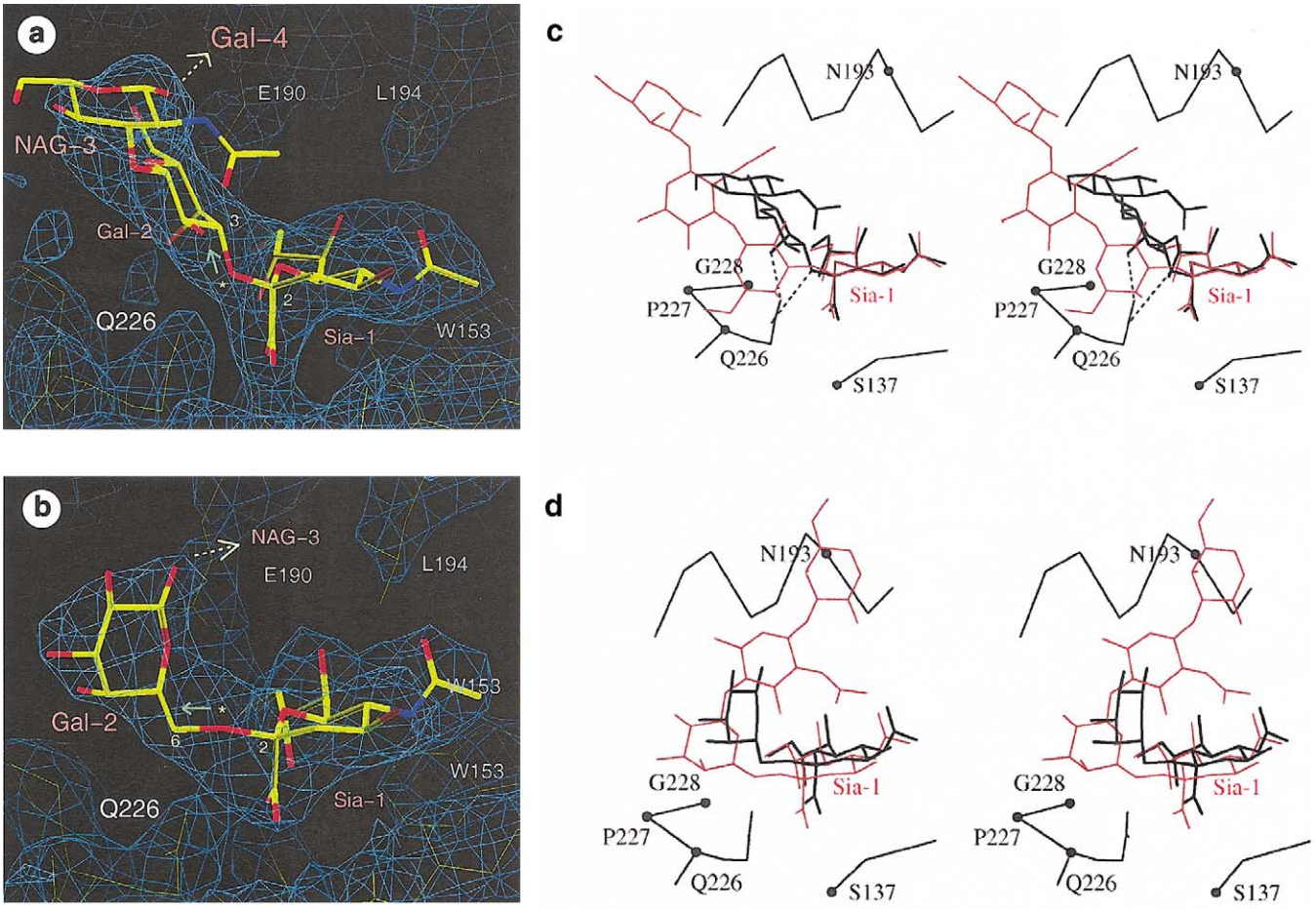


Fig. 4. Receptor binding to H3 avian HA. (a) The electron density map of the H3 avian HA α2,3-linked analog complex with the linkage atoms indicated by light yellow numbers. The density is contoured at 1.2σ level above the mean. The last two sugar residues of the analog (Gal-4 and Glc-5) are not defined in the electron density map. (b) The electron density map of the H3 avian HA α2,6-linked analog complex. Only the Sia-1 and Gal-2 residues of the receptor analog are visible. (c) Comparison of the α2,3-linked analog bound to H3 avian HA (black) and H3 human HA (dark brown) in stereo view. Amino acid differences within 10 Å of the receptor analog are indicated. Sia-1 and the glycosidic oxygen between Sia-1 and Gal-2 superimpose well. For the analog bound to the H3 avian HA (Q226), the glycosidic bond is in *trans* conformation (light green arrow pointing "up" in panel a), allowing hydrogen bonding from Q226 to the glycosidic oxygen and 4-OH of Gal-2 (dashed lines). When the analog is bound to the H3 human HA (L226), the glycosidic bond is in the *cis* conformation. (d) Comparison of the α2,6-linked analog bound to the H3 avian HA (black) and the H3 human HA (dark brown). In both cases, the glycosidic bond between Sia-1 and Gal-2 is in *cis* conformation (light green arrow pointing "down" in panel b). However, in the H3 avian HA analog complex, Sia-1 is shifted away from Q226, and Gal-2 is closer to the 190 helix.

evolution of α2,6-linked receptor-binding specificity from α2,3-linkage specificity.

Numerous studies of mutants selected by growing H3 subtype viruses in cells of different species (Gambaryan et al., 1999) or in the presence of different inhibitors of receptor binding (Ryan-Poirier and Kawaoka, 1991) including antibodies (Daniels et al., 1987) have indicated, directly or indirectly, the importance for receptor binding of residues close to or in the binding site, other than 226. Thus, for example, adaptation of isolates from humans to grow in hens' eggs and to increased binding of α2,3-linked analogs was accompanied by the substitution S186I (Gambaryan et al., 1999); failure of selected antigenic variants to recognize α2,3-linked analogs was correlated with the S193R substitution and the antibody-selected variant G218R also showed decreased affinity for α2,3-linked receptors (Daniels et al., 1987). Additionally the identity of residue 155 appears to be involved in distinguishing receptors containing *N*-acetyl- from those containing N-glycolyneuraminic acid (Masuda et al., 1999), a role that may also be influenced by the small differences in position and orientation of bound sialic acid observed here. In the H5 avian HA studied before (Ha et al., 2001), K at residue 193 instead of N in the H3 avian HA studied here is in the region where Glc-5 of an α2,6-linked sialopentasaccharide interacts with the H3 human HA (Eisen et al., 1997) and may therefore, prevent α2,6-linked receptors in their folded-over conformation (Ha et al., 2001) from fitting well into the H5 avian HA site. Perhaps more importantly from the viewpoint of cross-species transfer of influenza virus HAs, especially from avian viruses as oc-

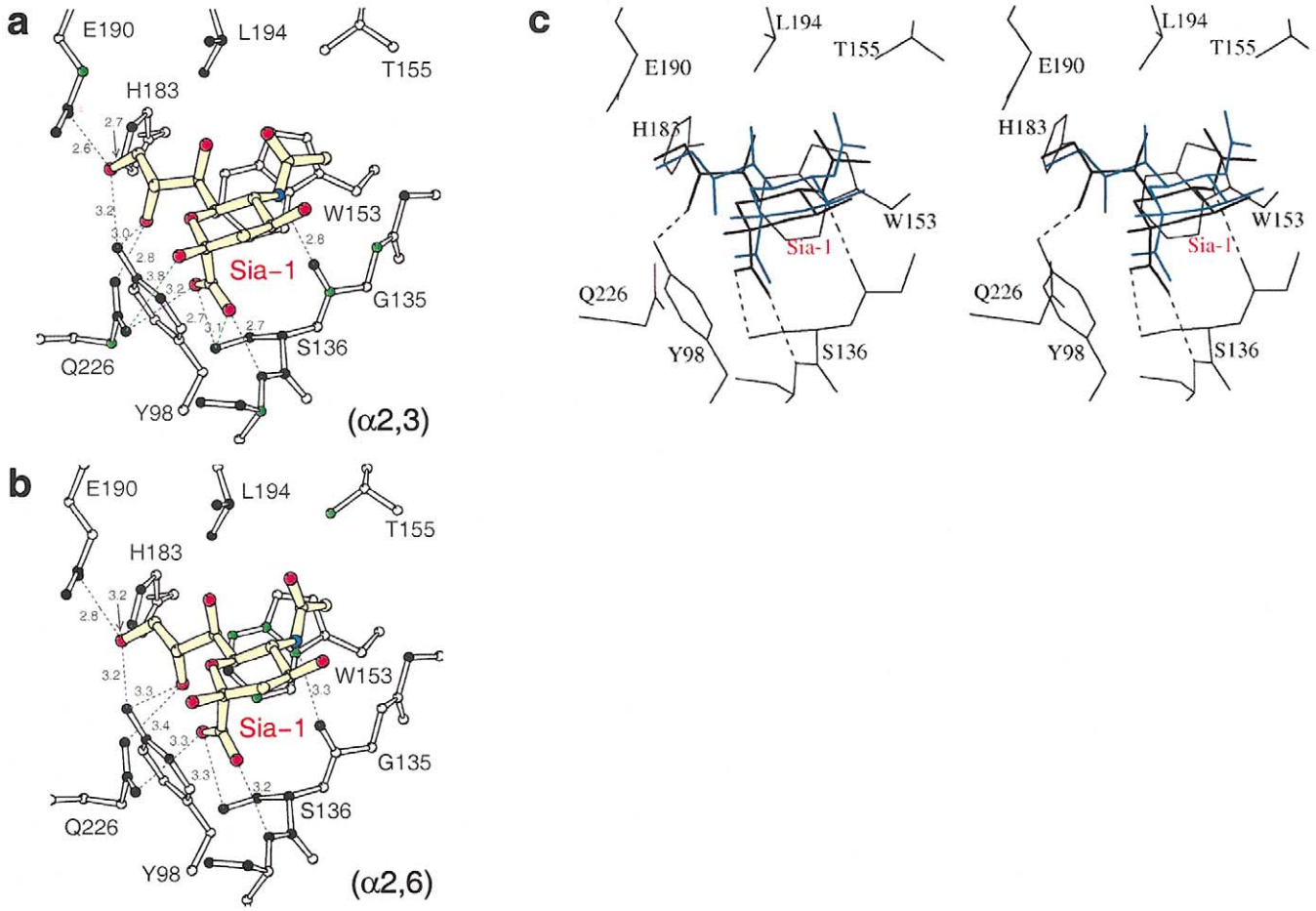


Fig. 5. Differences in the interaction of the H3 avian HA with Sialic acid in α2,3- and α2,6-linked receptor analogs. (a) α2,3-Linked receptor analog bound to H3 avian HA. Only Sia-1 is shown with oxygen as red circles. HA atoms within 4.0 Å of Sia-1 are shown as solid circles. Black circles common in both α2,3- and α2,6-linked analog complexes; green circles unique in the α2,3 complex. Possible hydrogen bonds are represented by dashed lines and bond lengths are indicated. (b) α2,6-Linked receptor analog bound to H3 avian HA. The differences in potential hydrogen bond lengths in the two complexes indicate that Sia-1 in α2,6-linkage is lifted "up" and shifted away from Q226. HA atoms shown as green circles are unique in the α2,6 complex. (c) A stereo view of the superposition of Sia-1 in both types of receptor analog complex: α2,3-linked, black; and α2,6-linked, cyan. The four conserved receptor-binding site hydrogen bonds to Sia-1 are shown as dashed lines.

curred in 1957 and 1968 to cause the Asian and Hong Kong pandemics, differences we observe in α2,6-linked receptor analog binding by the H3 avian and H5 avian HAs suggest that avian HAs of different subtypes may differ in their abilities to accommodate the α2,6-linked receptors abundant in the respiratory tracts of humans, or of an intermediate host such as a pig. As a consequence they may differ in their abilities to gain an initial replication foothold from which with mutation and selection, as noted early in both H2 and H3 pandemics (Matrosovich et al., 2000), mutants clearly preferring sialic acid in α2,6-linkage may derive. Of the 15 subtypes identified in avians, the HAs of the H1, H2, and H3 subtype viruses, which are known to have caused pandemics, may share this ability. Residues other than residue 226, possibly at positions 190 and 225 (Rogers and D'Souza, 1989; Nobusawa et al., 1991; Matrosovich et al., 1997; 2000) would, however, be involved in the accommodation by HAs of the H1 subtype since for these, irrespective of species of origin, residue 226 is always glutamine.

Materials and methods

Bromelain-released hemagglutinin (BHA) was prepared from purified A/duck/Ukraine/63 (H3N8) virus grown in hens' eggs. Digestion was at a virus protein:bromelain ratio of 10:1,in 0.15 M NaCl, 0.025 M 2-mercaptoethanol, 0.01 M Tris, pH 8.0, for 3 h at 37°C. The released BHA was purified by sucrose density gradient centrifugation and anion-exchange chromatography as described before (Ha et al., 2002). Crystals were grown by vapor diffusion in hanging drops. One microliter of 11.0 mg/ml protein solution in 10 mM Hepes buffer at pH 7.5 was mixed with 1 μl of crystal well solution containing 1.4 M sodium citrate, 100

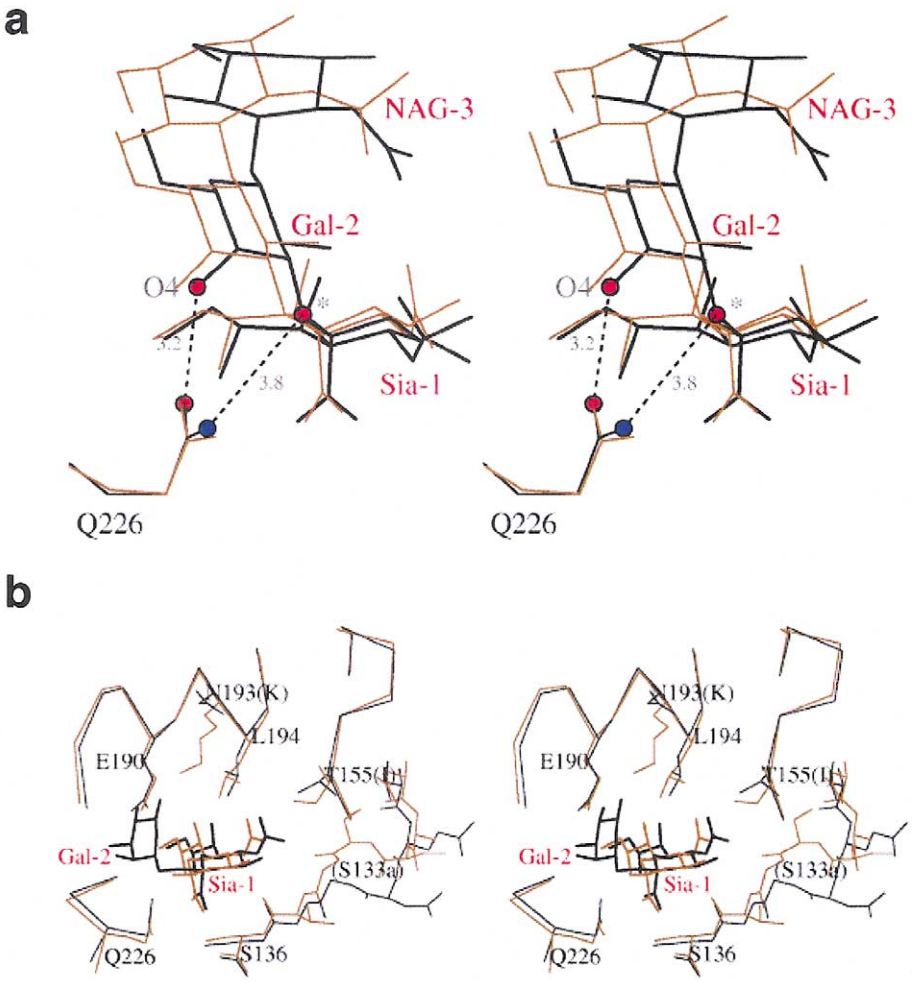


Fig. 6. Comparison of α2,3- and α2,6-linked receptor analogs bound to H3 and H5 avian HAs. (a) A stereo view of the α2,3-linked analog bound to H3 avian HA, black, and H5 avian HA, light brown. Q226, conserved in avian HAs, recognizes an α2,3-linkage-specific motif of glycosidic oxygen (*) and Gal-2 O4 (red circles) with the Sia-1 and Gal-2 linkage in a *trans* conformation. The hydrogen bonds formed by the terminal amide group are shown (red and blue circles). The NAG-3 residues diverge slightly even though the linkage conformations are similar. (b) A stereo view of the α2,6-linked analog bound to H3 avian HA (black) and H5 avian HA (light brown). Different H5 amino acids are in parentheses.

mM Hepes at pH 7.5. Crystals were soaked in the mother liquor containing 16 mM receptor analog LSTa or LSTc (Biocarb) and transferred stepwise to the same solution containing increasing amounts of the cryoprotectant xylitol (final concentration 1.5 M). Each transfer step took 30 min. Crystals were flash-cooled in liquid nitrogen.

Table 1
Crystallographic data statistics[a]

	Ukr	Ukr + LSTa	Ukr + LSTc
Data collection			
Resolution (Å)	40.0–2.9	25.0–2.6	40.0–3.2
Unique reflections	58,957	70,051	44,881
Completeness (%), redundancy, $I/<\sigma>$	98.5 (96.0), 4.3, 8.3	84.2 (84.4), 3.1, 10.6	99.7 (99.9), 5.0, 7.1
R_{merge} (%)	10.0 (54.9)	9.1 (59.5)	15.5 (39.7)
Refinement			
R_{cryst} (%), R_{free} (%)	26.9 (53.0), 30.6 (53.7)	26.5 (48.7), 29.0 (51.3)	23.9 (35.6), 24.3 (40.4)
RMSD bond length (Å), angles (°)	0.008, 1.5	0.010, 1.7	0.011, 1.8
Number of protein, water, and carbohydrate atoms	11,492, 332, 47	11,492, 456, 96	11,492, 397, 100

[a] The H3 A/duck/Ukraine/63 (Ukr) BHA crystallized in space group $C222_1$ (a = 146.9 Å, b = 147.3 Å, c = 250.6 Å). The statistics for the last shell are in parentheses.

Diffraction data were collected at a temperature of 100°K on the 14BMC beamline at APS, Argonne National Laboratory. Data sets were processed with DENZO and SCALEPACK (Otwinowski and Minor, 1997). The A/duck/Ukraine/63 BHA structure was determined by molecular replacement using AMORE in CCP4 and the X31 BHA coordinates (PDB:1HE). LSTa and LSTc were modeled into 2Fo-Fc and Fo-Fc Fourier maps. Modeling of the LSTa Gal-2 was based on density features of the planar pyranose ring, linkage to Sia-1 and NAG-3, and a protrusion normal to the pyranose ring corresponding to the axial 4-0H. The HA-LSTc complex crystal diffracted to a lower resolution and finer features of Gal-2 could not be observed. However, based on the positions and planar structures of Sia-1 and Gal-2, the torsion angles of the bonds connecting them could be defined. Both structures were refined with CNS (Brünger et al., 1998). The statistics are listed in Table 1. The structures were built and compared using 0 (Jones et al., 1991).

Acknowledgments

We thank the beamline staff at 14BMC at APS. This research was supported by NIH Grant AI-13654 and by a supplement to this grant for Expanded International Research on Emerging and Re-Emerging Diseases, by an International Partnership Research Award in Veterinary Epidemiology of the Wellcome Trust, by the Howard Hughes Medical Institute, and by the MRC (UK).

References

Baum, L.G., Paulson, J.C., 1990. Sialyloligosaccharides of the respiratory epithelium in the selection of human influenza virus receptor specificity. Acta. Histochem. Suppl. 40, 35–38.

Bean, W.J., Schell, M., Katz, J., Kawaoka, Y., Naeve, C., Gorman, O., Webster, R.G., 1992. Evolution of the H3 influenza virus hemagglutinin from human and non-human hosts. J. Virol. 66, 1129–1138.

Brünger, A.T., Adams, P.D., Clore, G.M., Gros, P., Grosse-Kuntsleve, R.W., Jiang, J.-S., Kuszerski, J., Nilges, M., Pannu, N.S., Read, R.J., 1998. Crystallography & NMR system: a new software suite for macromolecular structure determination. Acta Cryst. D54, 905–921.

Coleman, M.T., Dowdle, W.R., Pereira, H.G., Schild, G.C., Chang, W.K., 1968. The Hong Kong/68 influenza A2 variant. Lancet 2, 1384–1386.

Connor, R.J., Kawaoka, Y., Webster, R.G., Paulson, J.C., 1994. Receptor specificity in human, avian, and equine H2 and H3 influenza virus isolates. Virology 205, 17–23.

Daniels, R.S., Jeffries, S., Yates, P., Schild, G.C., Rogers, G.N., Paulson, J.C., Wharton, S.A., Douglas, A.R., Skehel, J.J., Wiley, D.C., 1987. The antigenic, receptor binding and fusion properties of variant influenza viruses selected with anti-haemagglutinin monoclonal antibodies. EMBO J. 6, 1459–1465.

Daniels, R.S., Skehel, J.J., Wiley, D.C., 1985. Amino acid sequences of haemagglutinins of influenza viruses of the H3 subtype isolated from horses. J. Gen. Virol. 66, 457–464.

Eisen, M.B., Sabesan, S., Skehel, J.J., Wiley, D.C., 1997. Binding of the influenza A virus to cell-surface receptors: structures of five hemagglutinin sialyloligosaccharide complexes determined by X-ray crystallography. Virology 232, 19–31.

Fang, R., Min Jou, W., Huylebroeck, D., Devos, R., Fiers, W., 1981. Complete structure of A/duck/Ukraine/63 influenza hemagglutinin gene: animal virus as progenitor of human H3 Hong Kong 1968 influenza hemagglutinin. Cell 25, 315–323.

Gambaryan, A.S., Robertson, J.S., Matrosovich, M.N., 1999. Effects of egg-adaptation on the receptor-binding properties of human influenza A and B viruses. Virology 258, 232–339.

Ha, Y., Stevens, D.J., Skehel, J.J., Wiley, D.C., 2001. X-ray structures of H5 avian and H9 swine influenza virus hemagglutinins bound to avian and human receptor analogs. Proc. Natl. Acad. Sci USA 98, 11181–11186.

Ha, Y., Stevens, D.J., Skehel, J.J., Wiley, D.C., 2002. H5 avian and H9 swine influenza virus hemagglutinin structures: possible origin of influenza subtypes. EMBO J. 21, 865–875.

Higa, H.H., Rogers, G.N., Paulson, J.C., 1985. Influenza virus haemagglutinins differentiate between receptor determinants bearing *N*-acetyl-*N*-glycolyl-, and *N,O*-diacetylneuraminic acids. Virology 144, 279–282.

Ito, T., Conceiro, J.N., Kelm, S., Baum, L.G., Krauss, S., Castrucci, M.R., Donatelli, I., Kida, H., Paulson, J.C., Webster, R.G., Kawaoka, Y., 1998. Molecular basis for the generation in pigs of influenza A viruses with pandemic potential. J. Virol. 72, 7367–7373.

Ito, T., Suzuki, Y., Suzuki, T., Takada, A., Horimoto, T., Wells, K., Kida, H., Otsuki, K., Kiso, M., Ishida, H., Kawaoka, Y., 2000. Recognition of N-glycolylneuraminic acid linked to galactose by the alpha 2,3 linkage is associated with intestinal replication of influenza A virus in ducks. J. Virol. 74, 9300–9305.

Jones, T.A., Zou, J.Y., Cowan, S.W., Kjeldgaard, M., 1991. Improved methods for building protein models in electron density maps and the location of errors in these models. Acta Crystallogr. A 47, 110–119.

Kasel, J.A., Fulk, R.V., Couch, R.B., 1969. Antigenic relationship between the equine and the Hong Kong human variant of influenza type A2 virus. J. Immunol. 102, 530–532.

Kida, H., Kawaoka, Y., Naeve, C.W., Webster, R.G., 1987. Antigenic and genetic conservation of H3 influenza virus in Wild ducks. Virology 159, 109–119.

Laver, W.G., Webster, R.G., 1973. Studies on the origin of pandemic influenza. III: Evidence implicating duck and equine influenza viruses as possible progenitors of the Hong Kong strain of human influenza. Virology 51, 383–391.

Masuda, H., Suzuki, T., Sugiyama, Y., Horiike, G., Murakami, K., Miyamoto, D., Jwa Hidari, K.I., Ito, T., Kida, H., Kiso, M., Fukunaga, K., Ohuchi, M., Toyoda, T., Ishihama, A., Kawaoka, Y., Suzuki, Y., 1999. Substitution of amino acid residue in influenza A virus haemagglutinin affects recognition of sialyl-oligosaccharides containing N-glycolylneuraminic acid. FEBS Lett. 464, 71–74.

Matrosovich, M., Tuzikov, A., Bovin, N., Gambaryan, A., Klimov, A., Castrucci, M.R., Donatelli, I., Kawaoka, Y., 2000. Early alterations of the receptor-binding properties of H1, H2 and H3 avian influenza virus hemagglutinins after their introduction into mammals. J. Virol. 74, 8502–8512.

Matrosovich, M.N., Gambaryan, A.S., Teneberg, S., Piskarev, V.E., Yamnikova, S.S., Luov, D.K., Robertson, J.S., Karlsson, K-A., 1997. Avian influenza A viruses differ from human viruses by recognition of sialyloligosaccharides and gangliosides and by a higher conservation of the HA receptor-binding site. Virology 233, 224–234.

Nobusawa, E., Aoyama, T., Kato, H., Suzuki, Y., Tateno, Y., Nakajima, K., 1991. Comparison of complete amino acid sequences and receptor binding properties among 13 serotypes of hemagglutinins of influenza A viruses. Virology 182, 475–485.

Otwinowski, Z., Minor, W., 1997. Processing of X-ray diffraction data collected in oscillation mode. Methods Enzymol. 276, 307–326.

Rogers, G.N., Daniels, R.S., Skehel, J.J., Wiley, D.C., Wang, X.F., Higa, H.H., Paulson, J.C., 1985. Host mediated selection of influenza virus receptor variants. Sialic acid α2,6 Gal-specific clones of A/duck/Ukraine/1/63 revert to sialic acid-α2,3 Gal-specific wild type *in ovo*. J. Biol. Chem. 260, 7362–7367.

Rogers, G.N., D'Souza, B.L., 1989. Receptor binding properties of human and animal H1 influenza virus isolates. Virology 173, 317–322.

Rogers, G.N., Paulson, J.C., 1983. Receptor determinants of human and animal influenza virus isolates: differences in receptor specificity of the H3 haemagglutinin based on species of origin. Virology 127, 361–373.

Rogers, G.N., Paulson, J.C., Daniels, R.S., Skehel, J.J., Wilson, I.A., Wiley, D.C., 1983. Single amino-acid substitutions in influenza haemagglutinin change receptor-binding specificity. Nature 304, 76–78.

Ryan-Poirier, K.A., Kawaoka, Y., 1991. Distinct glycoprotein inhibitors of influenza A virus in different animal sera. J. Virol. 65, 389–395.

Sauter, N.K., Hanson, J.E., Glick, G.D., Brown, J.H., Crowther, R.L., Park, S-J., Skehel, J.J., Wiley, D.C., 1992. Binding of influenza virus hemagglutinin to analogs of its cell-surface receptor, sialic acid: analysis by proton nuclear magnetic resonance spectroscopy and X-ray crystallography. Biochemistry 31, 9609–9621.

Skehel, J.J., Wiley, D.C., 2000. Receptor binding and membrane fusion in virus entry: the Influenza Haemagglutinin. Annu. Rev. Biochem. 69, 531–569.

Tumova, B., Easterday, B.C., 1969. Relationship of envelope antigens of animal influenza viruses to human A2 influenza strains isolated in the years 1957-68. Bull. World Health Org. 41, 429.

Verhoeyen, M., Fang, R., Jou, W.M., Devos, R., Huylebroeck, D., Saman, E., Fiers, W., 1980. Antigenic drift between the haemagglutinin of the Hong Kong influenza strains A/Aichi/1/68 and A/Victoria/3.75. Nature 286, 771–776.

Ward, C.W., Dopheide, T.A., 1981. Evolution of the Hong Kong influenza A subtype. Structural relationship between the hemagglutinin from A/duck/Ukraine/63 (Hav 7) and the Hong Kong (H3) hemagglutinins. Biochem. J. 195, 337–340.

Watowich, S.J., Skehel, J.J., Wiley, D.C., 1994. Crystal structures of influenza virus hemagglutinin in complex with high affinity receptor analogs. Structure 2, 719–731.

Weis, W., Brown, J.H., Cusack, S., Paulson, J.G., Skehel, J.J., Wiley, D.C., 1988. Structure of the influenza virus hemagglutinin complexed with its receptor, sialic acid. Nature 333, 426–431.

Wilson, I.A., Skehel, J.J., Wiley, D.C., 1981. Structure of the haemagglutinin membrane glycoprotein of influenza virus at 3 Å resolution. Nature (London) 289, 366–373.

Zakstelskaya, L.J., Evstigneeva, N.A., Isachenko, V.A., Shenderovitch, S.P., Efimora, V.A., 1969. Influenza in the USSR. New antigenic variant A2/Hong Kong/1/68 and its possible precursors. Am. J. Epidemiol. 90, 400–405.