LETTERS

# Haemagglutinin mutations responsible for the binding of H5N1 influenza A viruses to human-type receptors

Shinya Yamada[1,3], Yasuo Suzuki[3,4], Takashi Suzuki[3,5], Mai Q. Le[6], Chairul A. Nidom[7], Yuko Sakai-Tagawa[1,3], Yukiko Muramoto[1,3], Mutsumi Ito[1,3], Maki Kiso[1,3], Taisuke Horimoto[1,3], Kyoko Shinya[8], Toshihiko Sawada[9], Makoto Kiso[9], Taiichi Usui[10], Takeomi Murata[10], Yipu Lin[11], Alan Hay[11], Lesley F. Haire[11], David J. Stevens[11], Rupert J. Russell[11]†, Steven J. Gamblin[11], John J. Skehel[11] & Yoshihiro Kawaoka[1,2,3,12]

**H5N1 influenza A viruses have spread to numerous countries in Asia, Europe and Africa, infecting not only large numbers of poultry, but also an increasing number of humans, often with lethal effects[1,2]. Human and avian influenza A viruses differ in their recognition of host cell receptors: the former preferentially recognize receptors with saccharides terminating in sialic acid-α2,6-galactose (SAα2,6Gal), whereas the latter prefer those ending in SAα2,3Gal (refs 3–6). A conversion from SAα2,3Gal to SAα2,6Gal recognition is thought to be one of the changes that must occur before avian influenza viruses can replicate efficiently in humans and acquire the potential to cause a pandemic. By identifying mutations in the receptor-binding haemagglutinin (HA) molecule that would enable avian H5N1 viruses to recognize human-type host cell receptors, it may be possible to predict (and thus to increase preparedness for) the emergence of pandemic viruses. Here we show that some H5N1 viruses isolated from humans can bind to both human and avian receptors, in contrast to those isolated from chickens and ducks, which recognize the avian receptors exclusively. Mutations at positions 182 and 192 independently convert the HAs of H5N1 viruses known to recognize the avian receptor to ones that recognize the human receptor. Analysis of the crystal structure of the HA from an H5N1 virus used in our genetic experiments shows that the locations of these amino acids in the HA molecule are compatible with an effect on receptor binding. The amino acid changes that we identify might serve as molecular markers for assessing the pandemic potential of H5N1 field isolates.**

We used H5N1 viruses isolated from birds and humans to identify amino acid changes in the HA molecule that could enable the viruses to recognize human-type receptors (Supplementary Table 1 and Fig. 1). A/Vietnam/30262III/04 and A/Vietnam/3028II/04 contained a heterogeneous mixture of HAs on sequence analysis, prompting us to plaque-purify the viruses in Madin–Darby canine kidney (MDCK) cells to obtain viral clones with distinct HA sequences (Supplementary Table 1). We also used plaque-purified clones of A/Vietnam/30408/05 that differed in their HAs[7]. To expand the repertoire of viruses, we synthesized the HAs of eight H5N1 viruses isolated from humans in Thailand, Vietnam and Cambodia, using sequences in the Influenza Sequence Database (https://www.flu.lanl.gov/). Mutant viruses possessing each of these HA genes, the neuraminidase of A/Vietnam/1194/04 (VN1194) and all remaining genes from A/Puerto Rico/8/34 (PR8; H1N1) were then generated by reverse genetics[8].

The receptor specificity of the resulting H5N1 viruses was determined with an assay that measured direct binding to sialylglycopolymers possessing either SAα2,3Gal or SAα2,6Gal. None of the five avian H5N1 isolates bound appreciably to SAα2,6Gal, whereas 3 of the 21 human isolates, A/Vietnam/3028II/04clone3 (VN/3028IIcl3), A/Thailand/1-KAN-1/04RG (Thai/KAN), and A/Vietnam/30408/05clone7 (VN/30408cl7), including subclones, bound to both SAα2,3Gal and SAα2,6Gal; some of the other human H5N1 viruses also recognized SAα2,6Gal but to only a limited extent (Fig. 1 and Supplementary Fig. 2). The specificity of the receptor-binding assay was verified as follows. Sialylglycopolymers possessing either SAα2,3Gal or SAα2,6Gal were treated with *Arthrobacter ureafaciens* sialidase or mock-treated and then incubated with SA–Gal linkage-specific lectins: *Maackia amurensis* lectin II (MALII), specific for SAα2,3Gal; *Sambucus nigra* lectin (SNA), specific for SAα2,6Gal; and *M. amurensis* lectin I (MALI), specific for both Galβ1-4GlcNAc and SAα2,3Galβ1-4GlcNAc. The sialylglycopolymer possessing SAα2,3Gal reacted only with MALII, whereas that possessing SAα2,6Gal reacted only with SNA (Supplementary Fig. 3). The sialylglycopolymers treated with the sialidase no longer bound to the respective SA–Gal linkage-specific lectins, but gained reactivity with MALI, confirming that the sialidase treatment removed only the terminal sialic acid. These lectins did not react with polymers lacking oligosaccharides, as expected. Similarly, viruses that bound to the sialylglycopolymers did not bind to those treated with the sialidase or the polymer backbone without oligosaccharides.

To identify mutations capable of conferring SAα2,6Gal recognition, we focused on the three viruses that bound to the human receptor analogue relatively well: VN/3028IIcl3, Thai/KAN and VN/30408cl7. Comparison of the HA sequences identified two amino acid differences at positions 192 and 223 between VN/3028IIcl3 and VN1194 (a human clade-1 isolate, whose HA is identical to that of avian viruses such as A/duck/Thailand/71.1/2004 and

[1]Division of Virology, Department of Microbiology and Immunology, and [2]International Research Centre for Infectious Diseases, Institute of Medical Science, University of Tokyo, Tokyo 108-8639, Japan. [3]Core Research for Evolutional Science and Technology, Japan Science and Technology Agency, Kawaguchi, Saitama 332-0012, Japan. [4]College of Life and Health Sciences, Chubu University, Kasugai, Aichi 487-8501, Japan. [5]Department of Biochemistry, University of Shizuoka, School of Pharmaceutical Sciences and COE Program in the 21st Century, Yada, Shizuoka 422-8526, Japan. [6]National Institute of Hygiene and Epidemiology, Hanoi, Vietnam. [7]Avian Influenza Laboratory, Tropical Disease Centre, Airlangga University, Surabaya, Indonesia. [8]The Avian Zoonosis Research Centre, Tottori University, Tottori 680-8553, Japan. [9]Department of Applied Bioorganic Chemistry, The United Graduate School of Agricultural Science, Gifu University, Yanagido, Gifu 501-1193, Japan. [10]Department of Applied Biological Chemistry, Shizuoka University, Shizuoka 422-8529, Japan. [11]MRC National Institute for Medical Research, The Ridgeway, Mill Hill, London NW7 1AA, UK. [12]Department of Pathobiological Sciences, University of Wisconsin-Madison, Madison, Wisconsin 53706, USA. †Present address: Centre for Biomolecular Sciences, University of St Andrews, St Andrews KY16 9ST, UK.

A/chicken/Thailand/9.1/2004 and whose HA structure we determined by X-ray crystallography; see below). Introduction of the Gln192→Arg mutation, but not the Ser223→Asn mutation, into the HA of VN1194 appreciably enhanced the capacity of the HA to recognize SAα2,6Gal, and introduction of both mutations increased the binding capacity further. This finding implicates Gln192→Arg as a possible determinant of the shift to recognition of the human receptor by VN/3028IIcl3 (Fig. 2a). The Thai/KAN virus also showed two amino acid changes in HA1 as compared with VN1194: Gly139→Arg and Asn182→Lys. Introduction of either mutation into the VN1194 HA enhanced SAα2,6Gal binding (Fig. 2b), and an additional increase in binding capacity was observed when both mutations were substituted simultaneously. Thus, both Gly139→Arg and Asn182→Lys seem to contribute to recognition of the human-type receptor.

The HA of VN/30408cl7 differed from that of VN1194 by four amino acids at positions 75, 123 and 193 in HA1 and 167 in HA2. When introduced singly into the VN1194 HA, none of these amino acid substitutions enhanced binding to SAα2,6Gal, with the exception of Asn193→Lys, which increased binding capacity slightly (Supplementary Fig. 4a). Different pairs of these amino acid residues substituted at positions 75, 123 and 193 (in HA1) and 167 (in HA2) produced variable increases in SAα2,6Gal recognition (Supplementary Fig. 4b), and the introduction of various combinations of three mutations into the VN1194 HA enhanced SAα2,6Gal binding even further (Supplementary Fig. 4c). These results suggest that two or more of these changes acting in concert are necessary for SAα2,6Gal recognition by the VN/30408cl7 HA.

A group of H5N1 viruses (clade 2) that are antigenically and genetically distinct from previously circulating viruses (clade 1) have become prevalent[1] (Supplementary Fig. 1). As it was unclear whether mutations that conferred SAα2,6Gal recognition to the HA of clade-1 VN1194 virus would act comparably in the HAs of viruses of different clades, we inserted the Gly139→Arg, Asn182→Lys, Gln192→Arg and Asn193→Lys mutations separately into the HA of a clade-2 chicken isolate, A/chicken/Indonesia/N1/05 (CkInd). Either Gln192→Arg or Asn193→Lys, but not Gly139→Arg, enhanced the SAα2,6Gal-binding affinity of CkInd to an extent similar to that observed for the HA of VN1194 (Fig. 3). Introduction of Asn182→Lys into the CkInd HA nearly abolished its binding to both SAα2,3Gal and SAα2,6Gal molecules, indicating the incompatibility of lysine at this position in the CkInd HA.

To address the structural basis for the acquisition of human receptor-binding capacity by the H5 HA, we determined the crystal structure of the VN1194 virus. The structure was solved by molecular replacement using coordinates from the A/duck/Singapore/95 HA (relevant crystallographic statistics are given in Supplementary Table 2). The backbone structure of the VN1194 HA is shown in Fig. 4a, superimposed on the backbone of the HA of A/duck/Singapore/95. The two structures are very similar (root mean-square deviation (r.m.s.d.) on all Cα positions, 0.46 Å), reflecting their 94%

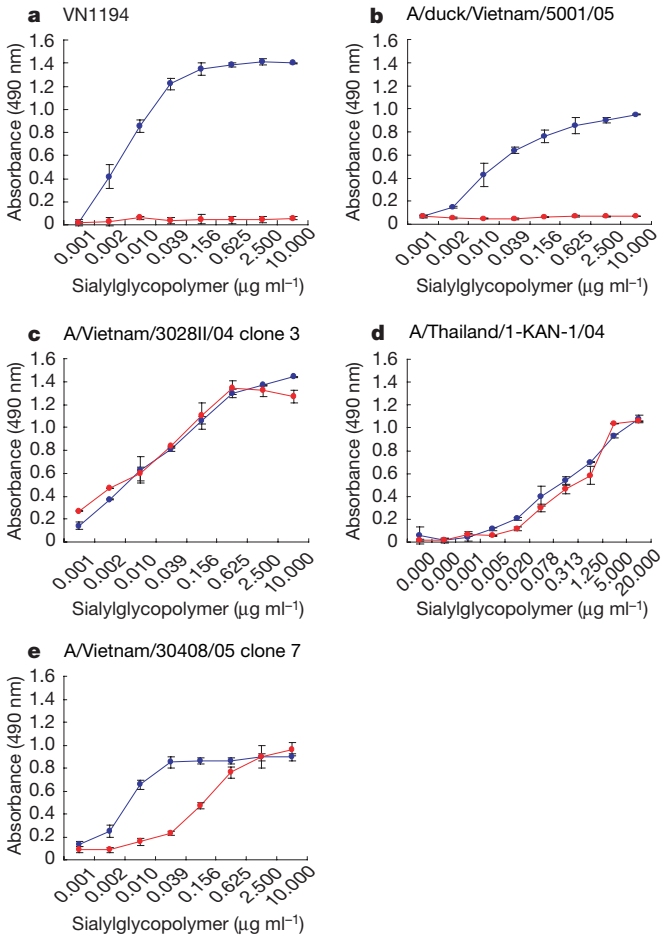


**Figure 1 | Receptor-binding activity of H5N1 viruses.** Direct binding of viruses to sialylglycopolymers containing either α2,3-linked (blue) or α2,6-linked (red) sialic acids was measured. **a**, Human isolate, Vietnam 2004. **b**, Virus isolated from duck. **c**–**e**, Human isolates with capacity to recognize both SAα2,6Gal and SAα2,3Gal. Data are the mean ± s.d. of triplicate experiments.

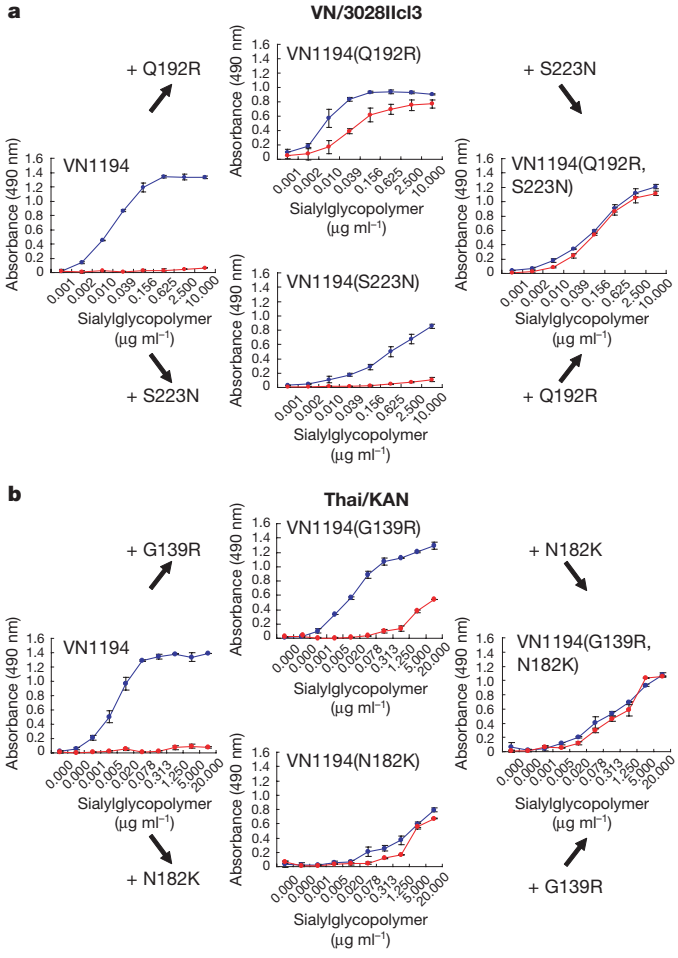


**Figure 2 | Effect of HA mutations on the host cell receptor preference of the VN1194HA.** Shown is the effect of HA mutations found in VN/3028IIcl3 (**a**) and Thai/KAN (**b**) viruses. The mutations (in parentheses) were introduced first singly and then in combination. The HAs of the viruses possessing double mutations, VN1194(Q192R,S223N) in **a** and VN1194(G139R,N182K) in **b**, are identical to those of VN/3028IIcl3 and Thai/KAN, respectively. Data are the mean ± s.d. of triplicate experiments.

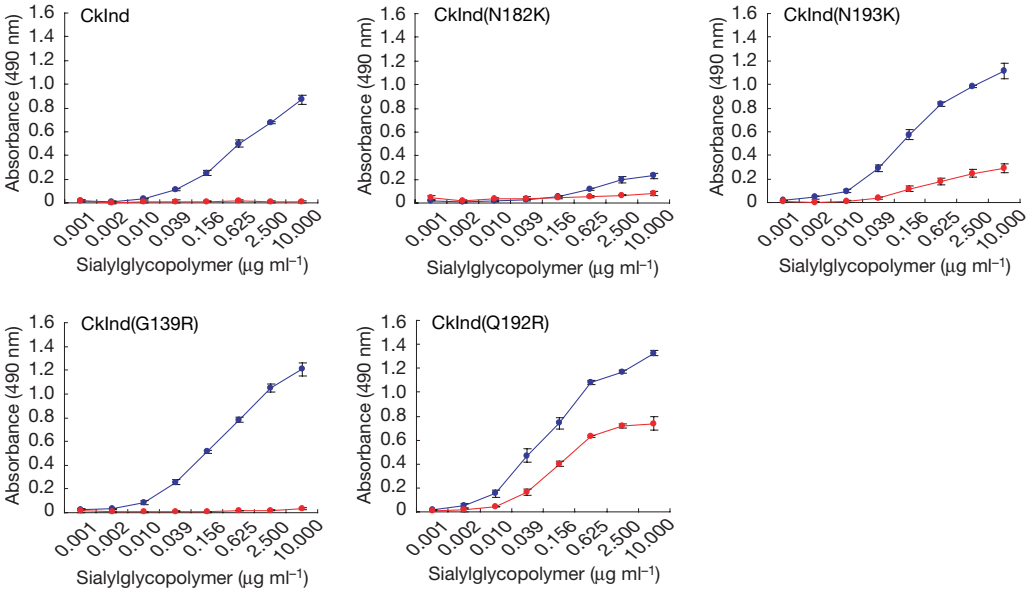


**Figure 3 | Effect of mutations responsible for SAα2,6Gal recognition by clade-1 HAs on a clade-2 HA.** Mutations responsible for SAα2,6Gal recognition by clade-1 HAs (in parentheses) were introduced into the HA of CkInd. The direct binding of each mutant virus to sialylglycopolymers containing either α2,3-linked (blue) or α2,6-linked (red) sialic acids was then measured at different concentrations of sialylglycopolymer. Data are the mean ± s.d. of triplicate experiments.

sequence identity. Figure 4b shows the receptor-binding domain, located on the globular head of the HA (Fig. 4a), of VN1194 superimposed on that of A/duck/Singapore/95 (ref. 9) and A/Vietnam/1203/04 (VN1203; ref. 10), which overlap with an r.m.s.d. of 0.46 Å and 0.5 Å, respectively, on the 175 Cα positions of this domain. The overlap of the structure of the interhelical loop region of the HA2 of VN1194 and A/duck/Singapore/95 is shown in Fig. 4d. Again, the close correspondence of these structures presumably reflects their very high sequence identity. We note that the highly similar overall domain arrangement of the H5 HAs of VN1194 and A/duck/Singapore/95 (Fig. 4a) differs from the arrangement reported for VN1203 (ref. 10). Further studies are needed to understand the basis of these differences.

Residues 75 in HA1 and 167 in HA2 are distant from the receptor-binding site, and the orientation of residues 139 and 193 precludes receptor contact (Fig. 4a). Clearly, further studies will be required to investigate the role of these residues. However, residues 182 and 192 are located at positions in the structure where it is feasible for them to make stabilizing interactions with sugars joined to sialic acid by α2,6 linkages (Fig. 4a, c). Mutations at residue 182 (the equivalent residue in the H3 HA is 186) have been linked to changes in receptor specificity[9,11–13], and mutating Asn 182 *in silico* to a lysine residue leads to

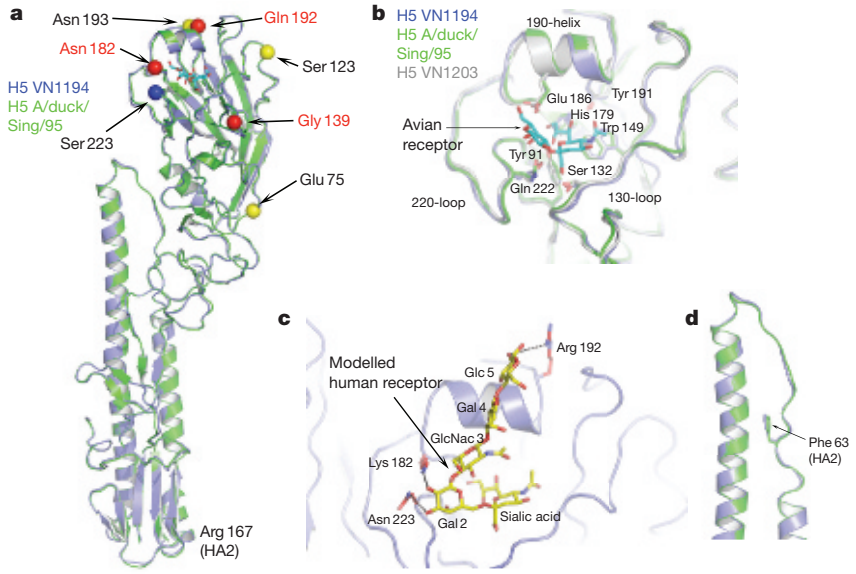


**Figure 4 | Crystal structure of VN1194 H5 HA and the location of mutations conferring SAα2,6Gal-binding capacity. a**, Monomer from the crystal structure of the H5 HA from VN1194 (blue) determined at 2.8 Å, superimposed with a monomer of the H5 HA from A/duck/Singapore/95 (green). The mutations discussed in the text are indicated: residues in red represent positions where single substitutions affect receptor binding; those in yellow increase SAα2,6Gal binding when introduced in combination with other changes. Asn 223 (blue) increases SAα2,6Gal recognition in the presence of Arg 192. **b**, Superposition of the closely related receptor-binding domains of VN1194 (blue), A/duck/Singapore/95 (green) and VN1203 (grey) with the avian receptor analogue taken from the A/duck/Singapore/95 complex structure. The main secondary structure elements of the binding site (130-loop, 220-loop and 190-helix) and key binding site residues are indicated. **c**, More detailed view of the receptor-binding domain of the H5 HA of VN1194 with the human receptor analogue docked into the structure from its complex with the H1 HA. Three of the mutations discussed in the text are modelled in red: Asn182→Lys, Ser223→Asn and Gln192→Arg. Note that the residue numbering differs in the deposited coordinate file; for example, Asn 182 here is numbered 186 in the PDB file. **d**, Close up of the overlap between the interhelical regions of domain F of VN1194 (blue) and A/duck/Singapore/95 (green) and the orientation of the hydrophobic residue Phe 63 (HA2).

a potential hydrogen-bond interaction with the 2-OH of Gal-2 (Fig. 4c). Mutating Gln 192 (196 in the H3 HA) to an arginine residue with selection of a preferred rotamer (from the 'O' database)[14] generates a conformation that places one of the guanidinium nitrogen atoms 4.5 Å from the 2-OH of Glc-5 (Fig. 4c); thus, the mutant HA may be capable of forming a hydrogen bond with human receptor moieties.

Serine 123 (128 in the H3 HA) is located on the turn leading into the 130-loop that forms the front edge of the binding site (Fig. 4b, c). Mutation of this residue could alter the orientation of the 130-loop and thus the attachment angle between the sialic acid and the next residue in the polysaccharide chain, thereby influencing the preference for the α2,3 or α2,6 linkage. Ser 223 is located close to the sialic-acid-binding site and *in silico* substitution of this residue with an asparagine leads to a conformation that places its side-chain nitrogen about 4 Å from the 3-OH of Gal-2 (Fig. 4c). This observation suggests that the mutant HA, with an asparagine at position 223, may be able to form a hydrogen bond with Gal-2 and thus influence SAα2,6Gal recognition, although this amino acid substitution only slightly increases the SAα2,6Gal-binding capacity of the VN1194 HA (Fig. 2a).

The influenza A viruses responsible for the pandemics of 1918, 1957 and 1968 all derived their HAs from avian viruses, which typically recognize SAα2,3Gal (ref. 3). Yet, the HAs of early isolates from humans infected in these pandemics seem to have recognized SAα2,6Gal in preference to SAα2,3Gal (ref. 3), suggesting that conversion of the avian HA to one that can recognize SAα2,6Gal-terminated polysaccharides on host cells is an important step in the generation of pandemic strains. This concept is supported by studies on the distribution of influenza virus receptors in the human airway[15,16]. The critical amino acid substitutions involved in this shift of receptor recognition were residues 226 and 228 in the H2 and H3 HAs[17] (equivalent to residues 222 and 224 in the H5 HA). The introduction of these mutations into the H5 HA permitted its binding to an α2,6 glycan[10], although neither change has been found in the HAs of H5N1 viruses isolated from humans.

In our study, both single and combined amino acid substitutions in the avian H5 HA mediated a shift to SAα2,6Gal recognition. Moreover, two of these changes, lysine at position 182 and arginine at position 192, were present in the HAs of clade-2 H5N1 viruses isolated from two individuals in Azerbaijan and one individual in Iraq, but not in any of the more than 600 avian isolates examined. Although amino acid substitutions in viral proteins other than the HA, including PB2 (refs 18–20), may be needed to confer full pandemic status to an avian virus efficiently replicating in humans, the amino acid residues identified here may be selected during an early phase of human infection involving cells of the upper respiratory tract. Thus, such residues might provide useful molecular markers in assessments of H5N1 field isolates for their capacity to replicate in humans—an essential indicator of pandemic potential.

## METHODS

**Virus preparation.** Viruses were grown in MDCK cells, which were maintained in minimal essential medium supplemented with 5% newborn calf serum (Sigma) and antibiotics at 37 °C in 5% $CO_2$. All experiments with live H5N1 virus were done in a biosafety level-3 containment laboratory.

**Generation of viruses by reverse genetics.** Reassortant viruses were generated with a plasmid-based reverse genetics system[8]. The viral complementary DNAs were cloned into a plasmid under control of the human polymerase I promoter and the mouse RNA polymerase I terminator (referred to as PolI plasmids). All viruses generated by reverse genetics possessed the neuraminidase of VN1194 and the internal genes of PR8. To generate the various HAs of human isolates registered in the Influenza Sequence Database (https://www.flu.lanl.gov/), we introduced mutations into pPolI–VN1194HA. Each construct was sequenced to verify the absence of unwanted mutations.

**Receptor specificity assays.** By using a solid-phase binding assay with the sodium salts of sialylglycopolymers (poly α-L-glutamic acid backbones containing *N*-acetylneuraminic acid linked to galactose through either an α-2,3 (Neu5Acα2,3Galβ1,4GlcNAcβ-pAP) or an α-2,6 (Neu5Acα2,6Galβ1,4GlcNAcβ-pAP) bond) as described[7,21,22], we determined the direct binding capacity of the viruses for the sialylglycopolymers. In brief, polystyrene Universal-Bind microplates (Corning) were incubated with either of the two glycopolymers in PBS at 4 °C for 3 h, and then irradiated under ultraviolet light at 254 nm for 2 min. After removal of the glycopolymer solution, the plates were blocked with 0.1 ml of PBS containing 2% bovine serum albumin (Invitrogen) at room temperature for 1 h. After five washes with PBS, the plates were incubated in a solution containing influenza virus (128 haemagglutination units in PBS) at 4 °C for 12 h. After three washes with PBS, antibody to the virus was added to the plates. After 2 h of incubation at 4 °C, the plates were washed three times with ice-cold PBS and then incubated with horseradish peroxidase (HRP)-conjugated protein A (2000-fold dilution in PBS; Organon Teknika–Cappel) at 4 °C. After four washes with ice-cold PBS, the plates were incubated with *O*-phenylenediamine (Sigma) in PBS containing 0.01% $H_2O_2$ for 10 min at room temperature, and the reaction was stopped with 0.05 ml of 1 M HCl. Absorbance was determined at 490 nm.

To confirm the specificity of the assay, we carried out control experiments as follows. In brief, the glycopolymers were fixed on the plates as described above, 120 μl of *A. ureafaciens* sialidase (80 mU $ml^{-1}$; Nacalai Tesque) was added and the plates were incubated at 37 °C for 16 h. After three washes with PBS, the plates were blocked with 0.1 ml of PBS containing 1% bovine serum albumin at 4 °C for 16 h. Removal of sialic acids from sialylglycopolymers was confirmed by incubating them with 50 μl of biotinylated SNA specific for SAα2,6Gal, biotinylated MALII specific for SAα2,3Gal, or biotinylated MALI specific for both Galβ1-4GlcNAc and SAα2,3Galβ1-4GlcNAc (all Vector Laboratories), at room temperature for 1 h. After three washes with PBS, the plates were incubated with HRP-conjugated streptavidin (Vector Laboratories) at room temperature for 1 h. After four washes with PBS, the plates were incubated with *O*-phenylenediamine in PBS containing 0.01% $H_2O_2$ for 10 min at room temperature, and the reaction was stopped with 0.05 ml of 1 M HCl. Absorbance was determined at 490 nm. Plates containing sialidase-treated glycopolymers and those containing poly α-L-glutamic acid backbones were used to confirm the lack of virus binding to the asialoglycopolymers or poly α-L-glutamic acid backbones.

**Crystal structure determination.** The H5N1 virus NIBRG-14, modified from A/Vietnam/1194/04 by removal of the polybasic cleavage site in HA, was obtained from the National Institute for Biological Standards and Control. HA was prepared by bromelain digestion of purified egg-grown virus for 90 min at 34 °C in 10 mM Tris-HCl (pH 8.0) and 5 mM mercaptoethanol (virus protein/bromelain ratio 10/1 w/w). The HA was purified as described[23]. Crystallization conditions were screened by the sitting-drop vapour diffusion method using Crystal Clear strips (Douglas Instruments). The nanodrops were set up with 0.1 μl of H5 protein solution (10 mg $ml^{-1}$) and 0.1 μl of reservoir solution by using an Oryx1-6 robot (Douglas Instruments). Diffracting crystals were obtained from Index solution 57 (Hampton Research): namely, 30% (v/v) pentaerythritol ethoxylate, 50 mM ammonium sulphate and 50 mM Bis-Tris (pH 6.5). This solution also acted as a cryoprotectant. The H5 diffraction data were recorded on a Raxis4 detector (100-μm scan) mounted on a Rigaku MicroMax 007 HF generator, integrated with Denzo and scaled with Scalepack[24]. The structure was solved by molecular replacement using AmoRe[25] using the PDB file 1JSM (ref. 9) as the initial search model. Standard refinement was carried out with a combination of refmac5 (ref. 25) and CNS[26], together with manual model building with O[12]. Molecular figures were created with Pymol (http://pymol.sourceforge.net/).

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**Supplementary Information** is linked to the online version of the paper at www.nature.com/nature.

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**Author Information** Coordinates for the H5 structure have been deposited in the Protein Data Bank under accession code 2IBX. Reprints and permissions information is available at www.nature.com/reprints. The authors declare no competing financial interests. Correspondence and requests for materials should be addressed to Y.K. (kawaoka@ims.u-tokyo.ac.jp).