

An Avian Influenza H5N1 Virus That Binds to a Human-Type Receptor[∇]

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Avian influenza viruses preferentially recognize sialosugar chains terminating in sialic acid- α 2,3-galactose (SA α 2,3Gal), whereas human influenza viruses preferentially recognize SA α 2,6Gal. A conversion to SA α 2,6Gal specificity is believed to be one of the changes required for the introduction of new hemagglutinin (HA) subtypes to the human population, which can lead to pandemics. Avian influenza H5N1 virus is a major threat for the emergence of a pandemic virus. As of 12 June 2007, the virus has been reported in 45 countries, and 312 human cases with 190 deaths have been confirmed. We describe here substitutions at position 129 and 134 identified in a virus isolated from a fatal human case that could change the receptor-binding preference of HA of H5N1 virus from SA α 2,3Gal to both SA α 2,3Gal and SA α 2,6Gal. Molecular modeling demonstrated that the mutation may stabilize SA α 2,6Gal in its optimal *cis* conformation in the binding pocket. The mutation was found in approximately half of the viral sequences directly amplified from a respiratory specimen of the patient. Our data confirm the presence of H5N1 virus with the ability to bind to a human-type receptor in this patient and suggest the selection and expansion of the mutant with human-type receptor specificity in the human host environment.

In contrast to most avian influenza viruses, which do not readily infect humans, highly pathogenic avian influenza H5N1 virus strains can transmit directly from avian species to humans and cause severe disease. Despite the ability to infect and cause severe disease in humans, most H5N1 viruses do not bind the sialic acid- α 2,6-galactose (SA α 2,6Gal) receptor with high affinity (17). It is believed that this receptor binding property is the major factor preventing the H5N1 virus from efficiently transmitting from person to person and causing a pandemic (17). The receptor binding preference of H5N1 viruses can be altered by only a few amino acid substitutions in the hemagglutinin (HA) protein. Mutations that change the receptor binding preference from the avian to the human type could potentially enable the virus to transmit efficiently in the human population and cause a catastrophic pandemic. Monitoring of the viral changes is therefore extremely important in the current situation where H5N1 viruses are spreading progressively.

A previous study showed that mutations at positions 226 and 228 (H3 numbering) (Q226L and G228S), which are the adaptive mutations for H2 and H3 (5, 19), could reduce the binding affinity to sialic acid- α 2,3-galactose (SA α 2,3Gal) of an H5N1 virus isolated in 1997 (5, 19). Human H5N1 isolates from Hong Kong that were isolated in 2003, which contain a mutation at position 227 (S227N) (H3 numbering), were shown to have a reduced binding affinity toward SA α 2,3Gal and an increased affinity toward SA α 2,6Gal (1). Another report demonstrated

previously that neither these mutations nor the mutations that could adapt H1 viruses to the human receptor (E190D and G225D) (H3 numbering) could completely convert a Vietnam H5N1 virus isolated in 2004 to the 2,6-type receptor specificity (16). Except for S227N, these mutations have not been found in H5N1 viruses isolated from humans or animals. Recently, N182K and Q192R mutations were shown to enhance the binding of a Vietnam H5N1 HA isolated in 2004 to the SA α 2,6Gal receptor (20). Although it was described previously that the N182K mutation was found in Kan-1 HA (20), the original Kan-1 virus as well as the HA sequence of Kan-1 in the GenBank Database do not contain this mutation. It is not clear from where this mutation was derived. The Q192R mutation was found in a clone that was present as a minor population in a viral isolate and was identified after plaque purification. In contrast, here, we show a naturally occurring mutant with an ability to bind SA α 2,6Gal that was directly identified in a human nasopharyngeal specimen.

MATERIALS AND METHODS

Cloning and generation of mutants. A fragment of the HA gene covering the receptor binding site (nucleotides 413 to 905) was amplified from RNA extracted from the nasopharyngeal specimen using the high-fidelity enzyme *Pfu* and primers HHAf2 (GGTCCAGTCATGAAGCCTCA) and HA-H5r12 (TTTATCGCC CCCATTGGAGT). The PCR product was cloned into pGEM-T Easy. One hundred clones were picked up and sequenced. Two clones were pooled for each sequencing reaction. A clone with L129V and A134V mutations was used in a spliced overlapping extension reaction, which joined two PCR fragments with overlapping sequences. The reaction swapped the sequence with the mutations into the HA gene of Kan-1 virus in the reverse genetic plasmid pHw2000, in which the cleavage site had been modified to a low-pathogenic sequence. Wild-type or the mutant pHw2000 HA was transfected into Vero cells together with the other seven genomic segments of A/Puerto Rico/8/34 (H1N1) to generate the viruses.

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Hemagglutination assay. A 10% suspension of goose (*Anser cygnoides*) red blood cells was prepared in phosphate-buffered saline (PBS), and a 50- μ l aliquot was treated with 1.25 U of α 2,3-sialidase cloned from *Salmonella enterica* serovar Typhimurium LT2 (7) (Takara, Japan) for 1 h at 37°C. Untreated and treated red blood cells were used in the hemagglutination assay.

HA-receptor binding assay. The receptor binding preference was analyzed by a solid-phase direct binding assay as previously described (20) by using the sialylglycopolymer, which contains *N*-acetylneuraminic acid linked to galactose through either an α 2,3 or an α 2,6 bond (Neu5Ac α 2, 3LacNAc β -pAP, and Neu5Ac α 2,6LacNAc β -pAP) (18). Serial dilutions of each sialylglycopolymer were prepared in PBS, and 100 μ l was added to the wells of 96-well microtiter plates (Polystyrene Universal-Bind Microplate; Corning) and allowed to attach overnight at 4°C. The plate containing sialylglycopolymer was irradiated under UV light at 254 nm for 10 min and then washed five times with 200 μ l PBS. The plate was blocked for 8 h at 4°C with PBS containing 2% skim milk powder. After washing five times with 200 μ l PBS containing 0.1% Tween 20, virus culture supernatant containing 128 HA units was allowed to attach onto the plate on ice overnight. After the incubation, the plate was washed five times with ice-cold PBS–0.1% Tween 20, and 50 μ l of anti-HA goat hyperimmune serum at a dilution of 1:2,000 was added to each well and was allowed to incubate on ice for 2 h. The plate was then washed again with ice-cold PBS–0.1% Tween 20, and 50 μ l of the polyclonal rabbit anti-goat immunoglobulin-horseradish peroxidase conjugate (DakoCytomation) at a dilution of 1:2,000 was added to each well. After incubation on ice for 2 h, the wells were extensively washed with PBS–0.1% Tween 20, and 100 μ l per well of premixed tetramethylbenzidine–H₂O₂ substrate was added. After incubation at room temperature for 15 min, the reaction was stopped with 50 μ l of 1 M H₂SO₄, and the absorbance at 450/630 nm was read.

Molecular dynamic simulation. The crystal structures of HA from A/Duck/Singapore/3/97 (H5N1) (4) were used as templates for the simulations of HA binding to SA α 2,3Gal (Protein Data Bank [PDB] accession number 1JSN) and SA α 2,6Gal (PDB accession number 1JSO). In the structural template under PDB accession number 1JSO, the sialic residue has no galactose unit connected; therefore, we added it with the torsion angle of 55°. Both glycosides in the two structures were terminated with a methoxy group and used as the input for molecular dynamic simulations. In homology modeling, the wild-type and mutant HA (L129V/A134V) were three-dimensionally aligned via the SWISS-MODEL server (12) using PDB accession numbers 1JSN and 1JSO and used as the initial input structures for molecular dynamic simulation. In order to provide a control of classical SA α 2,6Gal-tropic HA, we ran a human influenza virus H1 HA structure (PDB accession number 1RVZ) (2) in a similar simulation.

All structures were solvated using the TIP5P water model (10), and we performed energy minimization to relieve bad contacts caused by unreasonable distances in the initial structures and then equilibrated for 100 ps before a 3-ns productive run at 300K using the SANDER module in the AMBER9 program (University of California, San Francisco) with a Glycam04 parameter (<http://glycam.crc.uga.edu>). Xmgrace (<http://plasma-gate.weizmann.ac.il/Grace/>), VMD (8), and AMBER tools running on UNIX were exploited to visualize and manipulate all figures.

RESULTS

We screened for H5N1 isolates with altered receptor-binding preferences by a hemagglutination assay using goose red blood cells that were treated with a SA α 2,3Gal-specific sialidase (7). Theoretically, the sialidase digestion should abolish hemagglutination by SA α 2,3Gal-specific viruses, whereas viruses that can bind to SA α 2,6Gal should maintain hemagglutination activity with the treated red blood cells. The sialidase treatment did not affect the hemagglutination titer of human influenza viruses. An example of H1N1 virus (A/Thailand/Siriraj-12/06), the hemagglutination titer of which was not affected by the treatment, is shown in Fig. 1a. We have tested four human H5N1 isolates [A/Thailand/1(KAN-1)/2004, A/Thailand/3(SP-83)/2004, A/Thailand/5(KK-494)/2004, and A/Thailand/676/2005]. The sialidase treatment completely abolished the hemagglutination activity of all the viruses with more than a 256-fold reduction in the hemagglutination titer except for A/Thailand/676/2005 (Th676), which partially maintained

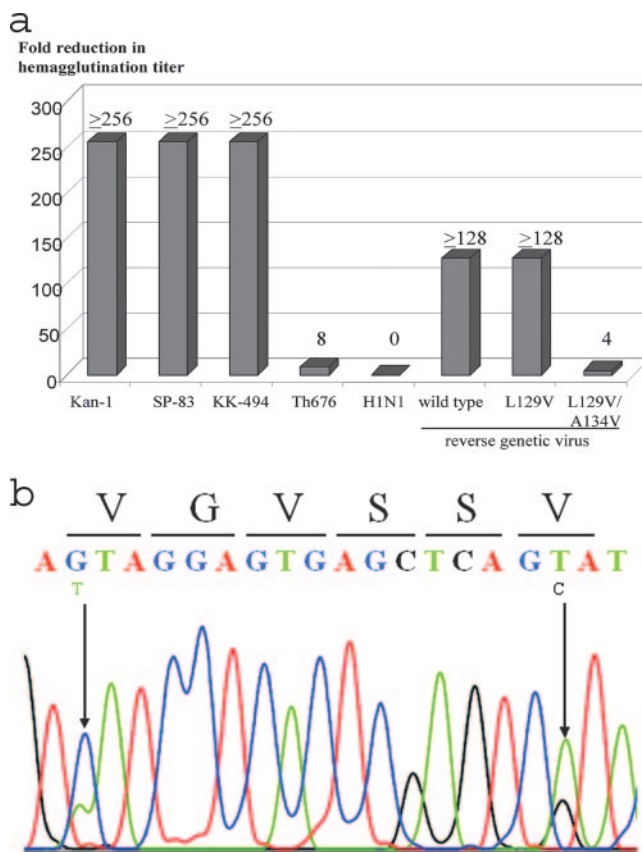


FIG. 1. (a) Abolition of hemagglutination titers in SA α 2,3Gal-specific sialidase-treated red blood cells compared to those in untreated cells indicated SA α 2,3Gal monospecificity for H5N1 viruses except for Th676 and the L129V/A134V reverse genetic virus, the titers of which were only modestly affected, suggesting an enhanced binding to SA α 2,6Gal. (b) Electropherogram of sequencing reaction of the Th676 virus shows a T-to-G mutation changing the amino acid from leucine to valine at position 129 and a C-to-T mutation changing the amino acid from alanine to valine at position 134 (arrowheads).

its hemagglutination activity in sialidase-treated red blood cells with some reduction in the hemagglutination titer (Fig. 1a). This suggested an enhanced binding of this virus to SA α 2,6Gal. The virus was isolated from a 5-year-old boy in Thailand. The patient had a progressive viral pneumonia that led to respiratory failure and death by 12 days after onset of illness.

Sequence of the HA gene of Th676 (GenBank accession number DQ360835) revealed two substitutions at position 129 (leucine to valine [L129V]) and position 134 (alanine to valine [A134V]). These two substitutions were particularly interesting because they are located close to the 130 loop of the receptor binding domain (14). Direct sequencing of viral RNA from culture showed double peaks at both positions, indicating a mixture of wild-type and mutant viruses. The mutant peaks at both positions were two- to threefold higher than the wild-type peaks (Fig. 1b). We have also amplified the viral sequence directly from a nasopharyngeal specimen. Cloning and sequencing of the amplification products showed the substitution L129V in 54 out of 92 clones and the substitution A134V in 52 out of 92 clones. Among these mutant clones, 42 had both substitutions (45.6% of total clones). In order to test whether

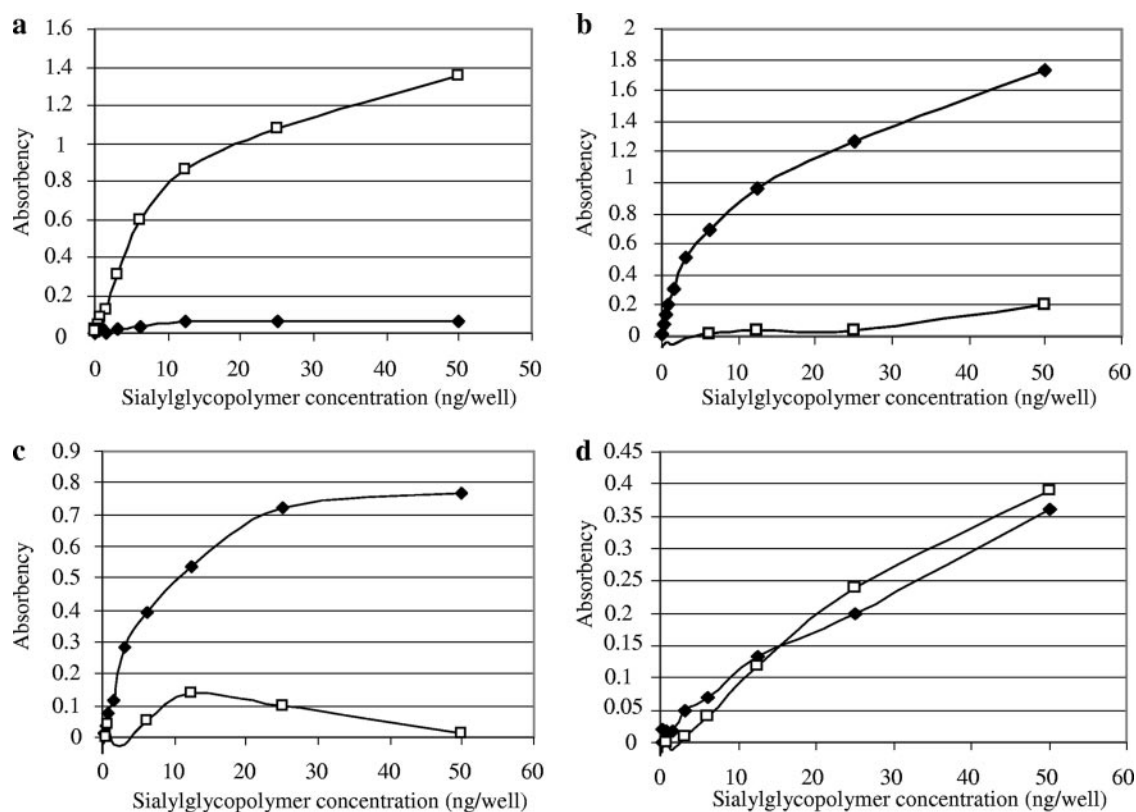


FIG. 2. Direct binding assay using sialylglycopolymers. Human H1N1 virus (A/Thailand/Siriraj-12/06) showed receptor preference for SA α 2,6Gal (a), whereas the reverse genetic virus with Kan-1 wild-type HA showed receptor preference for SA α 2,3Gal (b). The reverse genetic virus carrying HA with the L129V mutation showed a receptor preference similar to that of the wild-type virus (c), whereas the virus carrying HA with L129V/A134V mutations showed receptor preference for both SA α 2,3Gal and SA α 2,6Gal (d). Diamonds represent absorbencies of SA α 2,3Gal binding, and squares represent absorbencies of SA α 2,6Gal binding.

these two substitutions were responsible for the alteration of receptor binding preference, we generated reverse genetic viruses with wild-type HA or mutated HA carrying these two substitutions individually or simultaneously by the reverse genetics method (6). However, the mutated HA carrying A134V alone did not yield viable virus, probably because the mutation was not compatible with the genetic background of the reverse genetic virus. The wild-type HA clone was derived from A/Thailand/1(KAN-1)/2004 (H5N1) (Kan-1). Other genomic segments of the reverse genetic viruses were obtained from A/PR/8/34 (H1N1). In contrast to the Kan-1 HA described recently by Yamada et al. to bind SA α 2,6Gal (20), our Kan-1 HA clone (GenBank accession number EF107522) as well as the Kan-1 viral isolate (GenBank accession number AY555150) do not contain the N182K mutation, which was described to enhance binding to SA α 2,6Gal (20). The hemagglutination pattern of the L129V/A134V reverse genetic virus indicated an enhanced SA α 2,6Gal binding, whereas that of the L129V virus did not (Fig. 1a). The lesser degree of reduction of the hemagglutination titer of the L129V/A134V reverse genetic virus (fourfold) compared to that of Th676 (eightfold) might reflect the fact that Th676 was a mixture of wild-type and mutant viruses. We also used a recently described direct binding assay using sialylglycopolymers (3) and confirmed the effect of the L129V/A134V mutation on the receptor binding preference. The reverse genetic virus carrying wild-type Kan-1 HA showed

SA α 2,3Gal specificity and did not bind significantly to SA α 2,6Gal, whereas a human H1N1 virus, A/Thailand/Siriraj-12/06, bound only to SA α 2,6Gal (Fig. 2a and b). Also, while the L129V mutation alone did not change the receptor binding preference, the HA containing both L129V and A134V mutations bound equally well to both SA α 2,3Gal and SA α 2,6Gal (Fig. 2c and d).

In the HA binding pocket, the SA α 2,3Gal and SA α 2,6Gal receptors were shown to have a specific conformation, either *cis* or *trans* (4). Torsion angle (Φ) is the angle between two planes containing O6, C1 of the SA unit, and O3 (or O6), C3 of Gal unit (Fig. 3a). The angle indicates whether the glycoside is in the *cis* ($\Phi = 56^\circ$) or *trans* ($\Phi = -55^\circ$) conformation (Fig. 3a). Within its bound state to H5 in an X-ray cocrystal structure (4), SA α 2,3Gal was found in the *trans* conformation. The *trans* conformation of SA α 2,3Gal allows 4-OH (O4) and the glycosidic oxygen (O3) of the Gal unit to interact optimally with Gln222 of H5 (4). In contrast, if bound in the *trans* conformation, the 4-OH in SA α 2,6Gal would be too far away from Gln222, and if bound in the *cis* conformation, the glycosidic oxygen (O6) in SA α 2,6Gal would be too far away from Gln222. The lack of interactions between 4-OH and Gln222 in the *trans* conformation and between O6 and Gln222 in the *cis* conformation makes the binding of H5 to SA α 2,6Gal unstable in both conformations. The conformation of SA α 2,6Gal in solution was shown to have a *cis*-to-*trans* ratio of 9:1 (11),

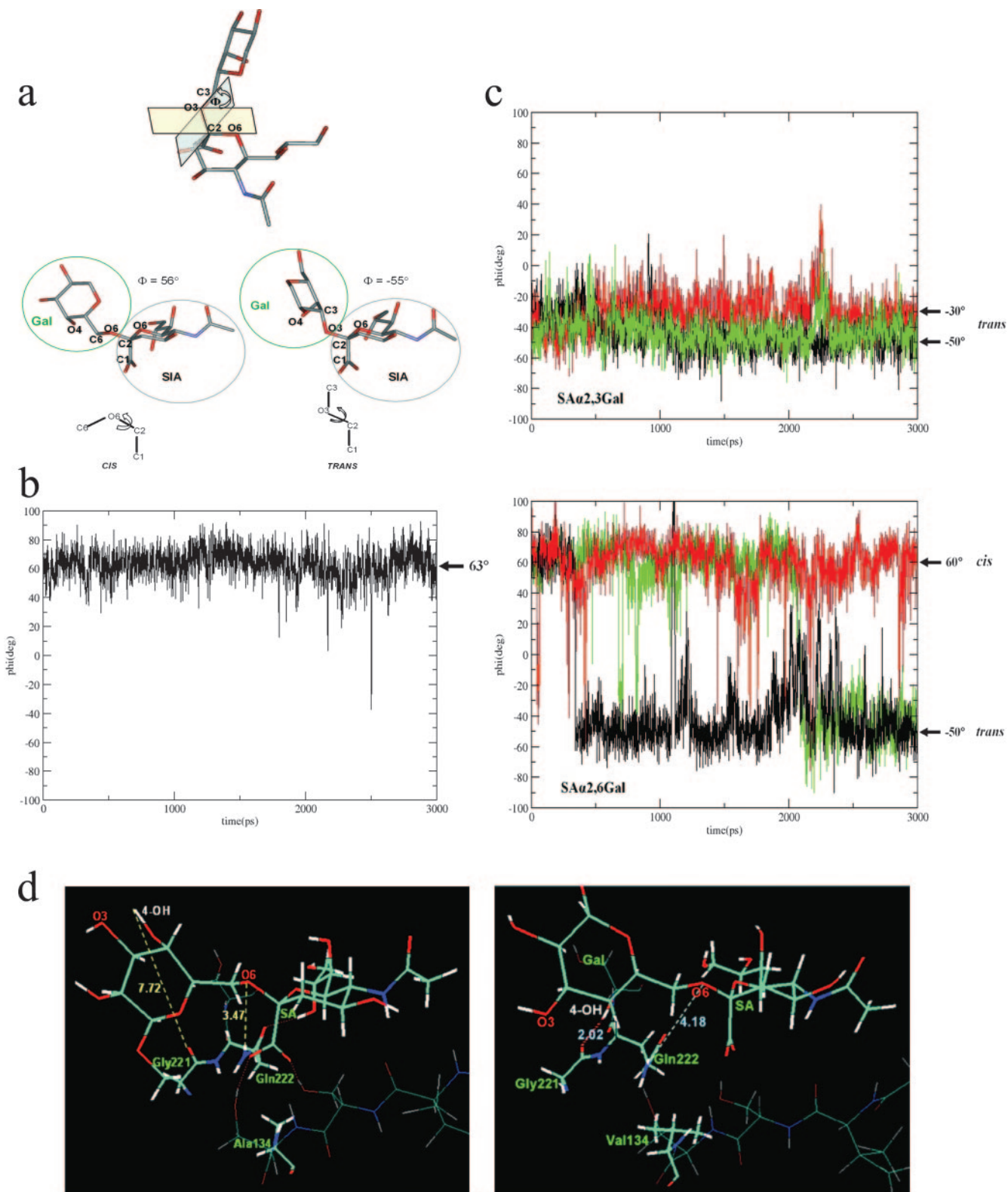


FIG. 3. The two glycoside backbone conformations, SA α 2,6Gal and SA α 2,3Gal, cut from the X-ray cocrystals under PDB accession numbers 1J5I and 1J5IN (4) with different values for torsion (Φ) angles that were used as the *cis* or *trans* conformation characteristics (a). For a human H1 virus (PDB accession number 1RVZ), SA α 2,6Gal showed an average Φ angle of 63° , indicating a *cis* conformation in the molecular dynamic simulation (b). The amount of time that each receptor analog spent having a particular Φ angle for SA α 2,3Gal (top) and SA α 2,6Gal (bottom) in the binding sites of the template H5 (A/Duck/Singapore/3/97) (black), wild-type Kan-1 (green), and the L129V/A134V mutant (red) as the results of molecular dynamics simulations indicated an altered binding conformation in the L129V/A134V mutant (c). The modeling showed that SA α 2,6Gal in a *cis* conformation in the wild-type Kan-1 HA pockets had long distances between O6 and Gln222 (3.47 Å) and between 4-OH and Gly221 (7.72 Å) (left). However, in the L129V/A134V mutant, the glycoside was stabilized with the Gly221 and Gal interactions as observed by the shorter distance between 4-OH and Gln221 (2.02 Å) (right) (d).

which suggests that the optimal binding conformation for SA α 2,6Gal is *cis* because no additional energy would be required for the conformational change. Furthermore, recent molecular modeling has predicted SA α 2,6Gal in H5 binding to be in the *cis* conformation (9). In our simulation, human H1 (PDB accession number 1RVZ) showed an average SA α 2,6Gal Φ angle of 63°, which indicated a *cis* conformation (Fig. 3b). This provides a positive control for SA α 2,6Gal binding in our simulation and indicates that the Φ angle can be used as an indicator of the receptor preference. In order to have structural insight into the altered receptor specificity of the mutant HA, we performed molecular dynamic simulations using two sialic acid-H5 cocrystals as reference structures for the two types of glycosidic linkages, SA α 2,3Gal (PDB accession number 1JSN) and SA α 2,6Gal (PDB accession number 1JSO) (4). In our modeling, both the wild-type and the mutant L129V/A134V HAs shared similar binding patterns within the sialic binding pocket as previously reported for both SA α 2,3Gal and SA α 2,6Gal binding (14, 19). However, it was observed that over the period of simulations, hydrophobicity and spatial constraint changes at residues 129 and 134 due to the different alkyl side chains, especially the A134V mutation, which happened near the glycosidic linkage, caused some changes in glycoside binding patterns. The Φ angle indicated that SA α 2,6Gal in the L129V/A134V HA spent most of the time in the low-energy *cis* conformation, whereas those in the wild-type Kan-1 HA and the template H5 were forced to change from their *cis* conformation in the initial input structures to the *trans* conformation (Fig. 3c). Although SA α 2,6Gal in both wild-type Kan-1 HA and the template HA was in the *trans* conformation at the end of the simulation period, SA α 2,6Gal in Kan-1 HA stayed longer in the *cis* conformation than that in the template HA. This suggested that wild-type Kan-1 HA might have a slightly increased affinity for SA α 2,6Gal compared to the template HA of A/Duck/Singapore/3/97. The increased stability in the *cis* conformation of the binding to L129V/A134V HA was due to an alternative interaction between Gly221 and 4-OH or 3-OH (O3) of Gal caused by a displacement of Gly221 by the A134V mutation (Fig. 3d). In the simulations of SA α 2,3Gal binding to L129V/A134V HA, the Φ angle significantly moved away from the preferred angle (from -55° to -30°) (Fig. 3c). Although SA α 2,3Gal was still in the *trans* conformation, the A134V mutation pushed Gln222 slightly away from the optimal binding condition. The widening of the gap between Gln222 and the glycosidic oxygen (O3) caused by larger hydrophobic side chain of Val134 might reduce the binding affinity of the mutant HA for SA α 2,3Gal.

DISCUSSION

In humans, the SA α 2,6Gal receptor is expressed mainly in the upper airway, while the SA α 2,3Gal receptor is expressed in alveoli and the terminal bronchiole (13). A virus with good affinity to both SA α 2,3Gal and SA α 2,6Gal receptors may be a very dangerous one, which could both infect efficiently via its binding to SA α 2,6Gal in the upper airway and cause severe infection in the lung via its binding to SA α 2,3Gal. This hypothesis is supported by the fact that one of the two well-characterized HA genes from the H1N1 1918 pandemic virus binds efficiently to both SA α 2,3Gal and SA α 2,6Gal (3, 15).

Although receptor binding preference is a major factor determining host species tropism, we do not know whether the alteration in receptor binding properties is sufficient to enable the virus to transmit from person to person efficiently. Nevertheless, our finding indicated that an adaptation of H5N1 virus to the human host by receptor binding site modification could and did indeed happen. Since the identification of this patient, there have been three confirmed human cases in the country, but none of the viral isolates from these cases contained the L129V and A134V substitutions. It is likely that this particular mutant has been eliminated by infection control measures.

Our report demonstrates that avian influenza H5N1 virus could be naturally adapted to the human-type receptor. We need to intensify our effort to detect such viruses as early as possible. Our data also provide a genetic marker that can be used to screen for an H5N1 virus with pandemic potential. H5N1 viruses may have several ways to adapt their receptor binding properties, but mutations that were found directly in patients, such as L129V/A134V, are more likely than those artificially generated and tested mutations to be the mutation that could cause a pandemic.

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