Functional Balance of the Hemagglutinin and Neuraminidase Activities Accompanies the Emergence of the 2009 H1N1 Influenza Pandemic

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The 2009 H1N1 influenza pandemic is the first human pandemic in decades and was of swine origin. Although swine are believed to be an intermediate host in the emergence of new human influenza viruses, there is still little known about the host barriers that keep swine influenza viruses from entering the human population. We surveyed swine progenitors and human viruses from the 2009 pandemic and measured the activities of the hemagglutinin (HA) and neuraminidase (NA), which are the two viral surface proteins that interact with host glycan receptors. A functional balance of these two activities (HA binding and NA cleavage) is found in human viruses but not in the swine progenitors. The human 2009 H1N1 pandemic virus exhibited both low HA avidity for glycan receptors as a result of mutations near the receptor binding site and weak NA enzymatic activity. Thus, a functional match between the hemagglutinin and neuraminidase appears to be necessary for efficient transmission between humans and may be an indicator of the pandemic potential of zoonotic viruses.

Influenza A viruses are negative-sense RNA viruses that circulate in many animal hosts, including birds, pigs, and humans (33). While viruses residing in humans cause seasonal flu virus infections, zoonotic viruses pose imminent risk to human health for their potential of initiating influenza pandemics of devastating effect. In recent years, the threat from zoonotic viruses has been highlighted by concern about the highly pathogenic avian influenza viruses (avian flu) (50) and the emergence of the 2009 H1N1 influenza pandemic virus of swine origin (38).

Wild aquatic birds are the natural hosts of influenza A viruses. Influenza viral subtypes are determined by the serotype combination of the viral surface proteins: hemagglutinin (HA) and neuraminidase (NA). So far, over 100 different subtypes have been isolated from avian species; but only three have sufficiently adapted for circulation in humans, and these were responsible for the flu pandemics of the last 100 years: H1N1 (1918), H2N2 (1957), H3N2 (1968), and H1N1 (2009). A virus must meet at least two criteria in order to attain pandemic potential: substantial antigenic variation from seasonal strains and efficient human-to-human transmission. The former guarantees a large naïve population that is susceptible to the newly emerged virus, whereas the latter initiates new infections and helps the virus spread rapidly among the population.

While other antigenically distinct zoonotic viruses, such as the H5N1 “bird flu” and swine flu cause sporadic infections in humans, they have not resulted in pandemics due to their inability to transmit from human to human (4, 37, 44). Instead, such infections are typically a result of humans coming into close contact with infected animals, and subsequent human-to-human transmission is rare. For avian viruses, one well-documented barrier to transmission arises from different HA binding specificities of avian and human viruses for glycan receptors (29). Human viruses preferentially recognize glycans with terminal α 2-6-linked sialic acids, which are broadly distributed on epithelial cells of the human trachea. In contrast, avian viruses specifically bind α 2-3-linked sialic acids, which are present only deep down in the alveoli

in the lower respiratory tract of humans. To replicate efficiently and transmit in humans, avian viruses must acquire an ability to engage α 2-6-linked sialic acid receptors. The specificity shift from α 2-3 to α 2-6 in H1, H2, or H3 HAs requires as few as two amino acid substitutions near the HA receptor binding site (11, 30), which may explain, in part, why these subtypes adapted to humans. Swine are unique among influenza virus hosts in that their respiratory tracts express both α 2-3- and α 2-6-linked sialic acid receptors (3) and can be infected by viruses with either human or avian-like specificities. It was, therefore, hypothesized that the specificity shift from avian to human likely occurs in an “intermediate host,” like swine (21, 43). This ability to shift receptor specificity in swine is exemplified by the currently circulating Eurasian avian-like H1N1 swine viruses. These viruses were introduced into pigs from birds in the 1970s and have since gained avidity to α 2-6-linked sialic acid receptors (16, 21, 30). However, it is apparent that α 2-6 receptor specificity alone is insufficient for efficient human transmission. Many swine viruses have binding specificity toward α 2-6-linked glycans (16) and have repeatedly entered the human population through close swine-human contacts (37, 44). Yet most caused only isolated human cases, with few human-to-human infections being reported until the emergence of the 2009 H1N1 influenza pandemic.

The 2009 H1N1 influenza pandemic is the first time in recent

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decades that a swine virus has become well established in humans and, thus, provides a valuable opportunity for studying the host barrier between swine and human. The 2009 H1N1 pandemic virus arose from the reassortment of several viruses of swine lineages (17, 47). In particular, the NA and M single-strand RNA gene segments came from Eurasian avian-like swine H1N1, and the other six gene segments came from North American swine H1N2, which itself is a triple reassortant of classical swine H1N1 virus (which provided HA, NP, and NS of the 2009 virus), a North American avian H1N1 virus (which provided PB2 and PA), and a human H3N2 virus (which provided PB1). The complex makeup of the emerged pandemic virus implies that a functional match among the gene segments might be crucial for adaptation to achieve human transmission.

HA and NA both engage glycan receptors on host cells, and their interplay has important implications for viral replication and fitness (49). On viral entry, HA recognizes host glycan receptors and initiates internalization of virus to the endosome, where it mediates membrane fusion at acidic pH (45). During viral budding, nascent viruses remain attached to the cell via HA binding until NA destroys the receptors by removing sialic acids to release the progeny (35). An optimal balance between HA receptor binding and NA receptor destroying is important for viral replication in cultured cells and experimental animals (34, 41, 42). Mismatched pairs of HA and NA can be rescued by adaptive mutations in the HA, NA, or both proteins after several cycles of infection in cell culture (20, 22, 23, 34). However, studies of the functional match of HA/NA in field strains and its role in emerging pandemics have been limited (25, 31, 55).

Here, we show coadaptation of the HA and NA during the most recent entry of a swine H1N1 virus into the human population, suggesting that a functional balance between HA and NA was required to initiate this human pandemic. In particular, the avidity of the HA for its sialylated receptors correlates with its neuraminidase activity for the corresponding receptors. The HA from the earliest human 2009 H1N1 HA exhibited lower avidity for glycan receptors than the swine progenitor HA, while retaining human-type specificity for glycan linkages. This binding profile occurred as a result of amino acid substitutions near the receptor binding site. In a separate event, the virus acquired an NA enzyme of relatively low activity through reassortment. This functional match of low HA avidity and low NA activity coincided with the emergence of the 2009 human pandemic and corresponds to a similar balance of HA/NA activities observed for pandemic viruses of the last century. In contrast to human viruses, the swine progenitors often deviate from such a functional balance, suggesting less stringent selection pressure for transmission in pigs than in humans. Thus, in the surveillance of zoonotic viruses with pandemic potential, appropriately matched activities of HA and NA may be considered indicators of transmission efficiency in humans.

MATERIALS AND METHODS

Viral strains. The HA and NA coding regions of tested viral strains were synthesized without codon optimization (GenScript). Viral strains studied include the following: A/South Carolina/1/18 (H1N1; SC18), A/Japan/305+/1957 (H2N2; Japan57), A/Hong Kong/68 (H3N2; HK68), A/California/04/2009 (H1N1; Cali09), A/New York/06/2009 (H1N1; NY06), A/Netherlands/602/2009 (H1N1; Neth602), A/swine/Indiana/P12439/00 (H1N2; sw/Indiana00), A/swine/Guangxi/13/2006 (H1N2;

sw/Guangxi06), A/Iowa/CEID23/2005 (H1N1; Iowa05), and A/swine/England/WVL7/1992 (H1N1; sw/England92).

HA glycan binding assays. The gene corresponding to the ectodomain of tested hemagglutinin (HA) was expressed as described previously (51). Supernatant from the suspension culture of insect Sf9 cells was batch purified using Ni-nitrilotriacetic acid (Ni-NTA) resin (Qiagen). Fractions containing HA were purified through size exclusion chromatography and concentrated to 1 mg/ml in 20 mM Tris-HCl-100 mM NaCl, pH 8.0. Procedures for an HA glycan microarray binding assay and an enzyme-linked immunosorbent assay (ELISA) using biotinylated glycans were described previously (52).

Crystallization and structural determination of sw/Indiana00 HA. The crystal structure was determined using a stabilizing mutant of sw/Indiana00 HA. The mutations G205C/R220C in sw/Indiana00 HA were introduced by the polymerase incomplete primer extension cloning method (24). The mutant was expressed and purified as described previously (51). Purified protein was concentrated to 7 mg/ml and crystallized by mixing the protein with an equal volume (0.5 μ l) of precipitant solution using the sitting-drop vapor diffusion method at 22.5°C. The precipitant in the reservoir contained 20% polyethylene glycol 1000 (PEG 1000) and 0.1 M Tris, pH 8.9. Crystals were soaked in reservoir solution plus 15% PEG 1000 and flash cooled in liquid nitrogen. Data sets were collected at the Stanford Synchrotron Radiation Lightsource and processed with the HKL-2000 program (39). The structure was solved by molecular replacement by Phaser (32) using the coordinates from the Cali09 HA trimer (Protein Data Bank [PDB] code 3LZG). The structure was then adjusted using COOT (14) and refined with PHENIX (1). Statistics for data collection and structure refinement are presented in Table 1.

Cloning, expression, and purification of NA. The gene corresponding to the ectodomain of neuraminidase (NA) (residues 37 to 469, N2 numbering) was inserted into a baculovirus transfer vector, pFastBacHT-A (Invitrogen), with an N-terminal gp67 signal peptide, a His₆ tag, an N-terminal tetramerization domain, and a thrombin cleavage site between the NA ectodomain and the tetramerization domain, as previously described (53, 56). NA proteins were produced by infecting suspension cultures of Hi5 cells with recombinant baculovirus at a multiplicity of infection (MOI) of 5 to 10. Cells were then incubated at 28°C with shaking at 110 rpm. After 72 h, Hi5 cells were removed by centrifugation, and supernatants containing secreted, soluble NAs were concentrated and buffer exchanged into a solution of 20 mM Tris, pH 8.0, 150 mM NaCl, 2.5 mM CaCl₂, and 10 mM imidazole. The NAs were recovered from the cell supernatants by metal affinity chromatography using Ni-NTA resin (Qiagen). The Ni-NTA-purified NAs with >95% purity were concentrated and exchanged into a buffer consisting of 100 mM imidazole-malate, pH 6.15, 150 mM NaCl, 10 mM CaCl₂, and 0.02% NaN₃ for the NA solution-based activity assay and glycan array activity/specificity assay discussed in the following sections.

NA activity assay with substrate 4-MU-NANA. NA solution-based enzymatic activities were measured in reaction buffer consisting of 100 mM imidazole-malate, pH 6.15, 150 mM NaCl, 10 mM CaCl₂, 0.1 mg/ml bovine serum albumin (BSA), and 0.02% NaN₃ using the fluorescent substrate 2'-(4-methylumbelliferyl)- α -D-N-acetylneuraminic acid (4-MU-NANA; Sigma) (40). The reaction was conducted at room temperature (~22°C) in a total volume of 80 μ l. After the reaction mixture was incubated for 10 min, the reaction was stopped by 80 μ l of 1 M Na₂CO₃. Concentrations of reaction product were measured at excitation and emission wavelengths of 365 nm and 450 nm, respectively. The reactions were all performed in triplicate.

To compare NA cleavage activities at different NA concentrations with a fixed substrate 4-MU-NANA concentration of 0.025 mM, the NA starting solutions were serially diluted 1:2 to achieve 12 different NA test concentrations. From the sigmoidal curve produced for each NA, it is possible to select the concentration that corresponds to the midpoint of the linear section of the curve. At this NA concentration, a linear relationship between NA activity and 4-MU-NANA substrate is found, and this is then

TABLE 1 Data collection and refinement statistics^a

Parameter	Value for sw/Indiana00 HA
Data collection	
Wavelength (Å)	0.97945
Space group	P2 ₁ 2 ₁ 2 ₁
Unit cell parameters	
a, b, c (Å)	93.4, 94.9, 220.5
α, β, γ (°)	90, 90, 90
Resolution (Å)	35–3.20 (3.31–3.20)
No. of observations	230,020
No. of unique reflections	32,867 (2,826)
Completeness (%)	98.2 (85.7)
I/σ(I)	11.0 (2.1)
R _{sym} ^b	0.17 (0.57)
Z _a ^c	1
Refinement statistics	
Resolution (Å)	34.3–3.20
No. of reflections (total)	30,849
No. of reflections (test)	1,569
R _{cryst} (%) ^d	23.7 ^d
R _{free} (%) ^e	29.3 ^e
Avg B value (Å ²)	51.3
Wilson B value (Å ²)	51.4
No. of protein atoms	11,530
No. of carbohydrate atoms	14
No. of waters	0
RMSD from ideal geometry ^g	
Bond length (Å)	0.006
Bond angles (°)	0.76
Ramachandran statistics (%) ^f	
Favored	95.3
Outliers	0.0

^a Data were collected using beamline SSRL 11-1. Numbers in parentheses refer to the highest-resolution shell. Structure data were deposited in the PDB under accession code 4F3Z.

^b $R_{\text{sym}} = \sum_{hkl} | \langle I_i \rangle - \langle I \rangle | / \sum_{hkl} \langle I_i \rangle$, where I_i is the scaled intensity of the i th measurement of reflection (hkl) and $\langle I \rangle$ is the average intensity for that reflection.

^c Z_a is the number of HA trimers per crystallographic asymmetric unit.

^d $R_{\text{cryst}} = \sum_{hkl} | F_o - F_c | / \sum_{hkl} | F_o | \times 100$, where F_o and F_c are the observed and calculated structure factor amplitudes, respectively.

^e R_{free} was calculated as described for R_{cryst} but on a test set comprising 5% of the data excluded from refinement.

^f Calculated using Molprobity (12).

^g RMSD, root mean square deviation.

used for an NA kinetics assay in which the enzyme reaction velocity is measured as a function of substrate concentration. The K_m , V_{max} , and k_{cat} values were obtained by fitting the data to the appropriate Michaelis-Menten equations using nonlinear regression in the GraphPad Prism software program (GraphPad Software, La Jolla, CA).

NA glycan array activity/specificity assay. NA proteins were diluted in a reaction buffer consisting of 100 mM imidazole-malate, pH 6.15, 150 mM NaCl, 10 mM CaCl₂, and 0.02% NaN₃ to a starting concentration based on NA activity against 4-MU-NANA (with a higher concentration used for an NA with lower activity); samples were serially diluted 1:5 to achieve seven different NA concentrations. The seven NA dilutions were applied to seven identical glycan arrays on a glass slide. These are the same arrays used for HA glycan binding (52), and imprinted glycans on the array are listed in Fig. 1. NA incubation was carried out for 1 h, and then each array was washed three times with 1× phosphate-buffered saline (PBS) with 0.05% Tween 20, pH 7.4. Following the third wash, a 100-μl solution of a preformed complex of biotinylated *Erythrina cristagalli* lectin (ECL) (10 μg/ml; VectorLabs) and streptavidin-Alexa Fluor 555 (2 μg/ml; Invitrogen) was applied directly to the array surface and allowed to

incubate for 2 h. Following incubation, the ECL-streptavidin solution was removed, and the array was washed three times with 1× PBS with 0.05% Tween 20, pH 7.4, and subsequently dipped three times in 1× PBS and then three times in double-distilled H₂O (ddH₂O). Washed slides were dried by centrifugation and scanned on a ProScanArray Express HT (PerkinElmer) confocal slide scanner for the Alexa Fluor 555 setting. Image data were stored in the form of a TIFF image, and signal data were collected using Imagen (BioDiscovery) imaging software. Collected data were processed to determine the averaged (mean signal minus mean background) values of four replicate spots on the array for each unique printed glycan.

Protein structure accession number. The atomic coordinate and structure factor of the sw/Indiana00 HA have been deposited in the Protein Data Bank (www.rcsb.org) under accession code 4F3Z.

RESULTS

Receptor binding of the 2009 pandemic virus and its swine progenitor. The HA of the 2009 pandemic virus contains Asp190 and Asp225 in the receptor binding site, implying receptor specificity for α2-6-linked sialic acids (38). The sequence-based prediction was later confirmed by glycan binding of inactivated viruses and recombinant HAs (6, 9, 13, 28, 52, 54). Interestingly, swine viruses that are closely related to human viruses exhibit the same glycan linkage specificity as human viruses that have caused human epidemics and pandemics (6, 9, 13), suggesting that other factors have restricted the transmission of classical swine viruses in the human population.

Here, we studied the HA glycan binding properties of one of the earliest isolated 2009 H1N1 pandemic viruses (A/California/04/2009 [Cali09]) and its closest HA progenitor in swine (A/sw/Indiana/P12439/00 [sw/Indiana00]) (17). Recombinant HAs were purified and tested using a customized glycan microarray (glycans are listed in Fig. 1) and a plate-based ELISA as described previously (52). The quantitative but low-throughput ELISA, coupled with the qualitative analysis of a large group of glycans by the microarray assay, presents a more accurate assessment of HA binding avidities. Cali09 HA binding to the glycan array is very weak, and above-background binding of any significance was observed only for α2-6-linked linear glycans (Fig. 2A) (52). However, sw/Indiana00 HA shows much stronger binding, with broad specificity on the glycan microarray for α2-6-linked glycans with terminal composition of NeuAcα2-6Galβ1-4GlcNAc (where NeuAc is *N*-acetylneuraminic acid) (Fig. 2B). Strong binding is observed for linear glycans (glycans 28 to 31), biantennary glycans (numbers 32 and 34 to 36), as well as a sulfated glycan (number 26). Using a more quantitative ELISA, Cali09 HA binding to the immobilized linear glycan NeuAcα2-6Galβ1-4GlcNAcβ1-3Galβ1-4GlcNAc (6'-SLNLN) was ~15 times weaker than that of sw/Indiana00 HA (Fig. 2C to D and 3A). Linear glycans 6'-SLNLN and 3'-SLNLN were chosen to be representatives of the natural ligands for viral infections because sialosides with terminal SLNLN units are found on the epithelial cells of human and swine respiratory tracts (3, 8) and have been suggested to be critical for viral transmission in humans (8). Moreover, they have been demonstrated to be superior to shorter sialosides for ELISA-based analysis of the avidity of human influenza virus HAs (8).

Comparison of crystal structures (Fig. 3B and C) and protein sequences (Fig. 3D) reveals two amino acid substitutions on the rim of the receptor binding site that differ in the swine progenitor and 2009 pandemic viruses. Ala227 and Arg133_A (H3 HA numbering; insertions relative to H3 are labeled by the preceding res-

FIG 1 List of glycans on the microarray. The glycans include 34 unique natural sialoside epitopes (α 2-3 linkage, numbers 3 to 25; α 2-6 linkage, numbers 26 to 34; mixed linkage, numbers 35 and 36) that are relevant to influenza biology and two neutral glycans (numbers 1 and 2) as controls.

idue with a letter) are highly conserved in classic swine H1N1 viruses but are replaced by Glu227 and Lys133_A in 2009 H1N1 pandemic viruses. The A227E mutation provides a bulky and negatively charged residue on the 220 loop. In the complex structure with human receptor analog LSTc, Glu227 is part of a charge network that involves Lys222 and Asp225, which hydrogen bond with the Gal-2 sugar of the glycan receptor (52). Lys133_A is located at the other end of the binding site and provides a water-mediated hydrogen bond to Sia-1 of the receptor. Ile219 is also highly conserved in human viruses but is Ala219 in classic swine viruses (Fig. 3D). Ile219 does not directly contact glycan receptors but, instead, buttresses Glu227, likely stabilizing it for interaction with Lys222.

Overall, these three amino acid changes, especially the introduction of the bulky Ile219 and Glu227, dramatically change the molecular surface of the HA receptor binding site and glycan binding avidity (see Fig. S1 to S4). The sw/Indiana00 HA has a groove between Lys222 and the 190 helix that is filled by the bulkier Ile219 and Glu227 in Cali09 HA (Fig. 3B and C). These residue changes, in addition to the previously identified A200T mutation (13), appear to be responsible for the shift of receptor binding affinity from swine viruses to early human 2009 H1N1 viruses. The introduction of swine residues Ala227/Arg133_A into Cali09 HA, either individually or combined, improves glycan binding, as measured by glycan microarray analysis

FIG 2 Avidity shift in the HA from swine to human. The avidity shift in the HA is mainly mediated by two changes in the receptor binding site: A227E and R133_AK. The swine virus signature residues Ala227 and Arg133_A, when substituted into Cali09 HA, improve binding for glycan receptors (A and C), whereas human virus residues Glu227 and Lys133_A attenuate receptor binding in sw/Indiana00 HA (B and D). The amino acid substitutions change binding avidity but not binding specificity for glycan linkages as both wild-type strains and mutants maintain favorable binding for α 2-6-linked sialic acids. In glycan microarray binding (A and B), binding signals are shown in filled bars colored by sialic acid linkages: neutral glycans are in black (numbers 1 and 2), α 2-3-linked glycans are in blue (3 to 25), α 2-6-linked glycans in red (26 to 34), and mixed glycans in are cyan (35 and 36). Glycan array results for Cali09 and sw/Indiana00 are shown on different scales. In the plate binding assay (C and D), binding for 6'-SLNLN is shown in red, and that for 3'-SLNLN is in blue. In each panel, wild-type binding of 6'-SLNLN is shown as a dashed line for comparison.

or ELISA (Fig. 2A and C). Meanwhile, A227E/R133_AK mutations attenuate HA binding for sw/Indiana00 (Fig. 2B and D). These mutations have no effect on the specificity for the sialic acid linkage. Neither wild-type nor mutants display noticeable binding for α 2-3-linked glycans. However, the mutations impact the avidity and the breadth of α 2-6-linked glycans recognized by the HA. HAs with the combination of Ala227/Arg133_A show broad bind-

ing for α 2-6-linked glycans on the array, whereas HAs with Glu227/Lys133_A recognize a smaller subset of the α 2-6-linked glycans (Fig. 2A and B).

HA/NA functional match in pandemic viruses. To assess how the changes in HA receptor binding from sw/Indiana00 to Cali09 might be related to virus adaptation from swine to human, we first surveyed other human viruses from previous pandemics, which

FIG 3 Differences in glycan binding avidity and receptor binding site structure between sw/Indiana00 and Cali09. (A) Comparison of receptor binding by ELISA. The sw/Indiana00 is the HA progenitor of pandemic H1N1 virus Cali09. HAs from both strains display specificity for α 2-6-linked sialic acids, but swine HA demonstrates significantly stronger binding toward glycan 6'-SLNLN in a plate-based ELISA. The difference in binding avidities is correlated to structural differences in the HAs (B and C). (B) Crystal structure of the Cali09 HA in complex with the α 2-6 receptor analog LSTc (PDB 3UBE) (52). (C) Crystal structure of the sw/Indiana00 HA. (D) Three residue changes are found in the receptor binding site between swine progenitors and the 2009 H1N1 pandemic strains. Two of these residues are in proximity to bound glycan receptors in Cali09: Glu227 and Lys133_A (highlighted in red circles in panels B and C). The third residue, Ile219, is located at the periphery of the binding pocket (highlighted in a dashed red circle).

revealed a wide range of HA binding profiles in terms of binding avidity and binding specificity for α 2-6-linked glycans (Fig. 4A and C). Human H1N1 viruses from the 1918 (A/South Carolina/1/18 [SC18]) and 2009 (Cali09) H1N1 pandemics show relatively weak and very weak HA binding, respectively, on the glycan microarray, as well as a narrower range of specificity among α 2-6-linked glycans (Fig. 4C). HAs of the 1957 H2N2 pandemic (A/Japan/305+/1957 [Japan57]) and the 1968 H3N2 pandemic (A/Hong Kong/68 [HK68]) exhibit relatively strong HA binding and recognize a wide range of α 2-6-linked sialic acids. These results suggest that the quantity (i.e., high avidity) and extent (i.e., broad breadth) of HA binding to α 2-6-linked glycans of sw/Indiana00 compared to those Cali09 (and even more so with respect to other pandemic viruses) could not explain its lack of fitness for human transmission. We hypothesized then that this highly reduced binding of Cali09 HA to α 2-6-linked glycans was required to achieve a functional balance between the HA and NA in the 2009 H1N1 pandemic viruses (or vice versa) and that such a balance is essential for transmission of influenza viruses in humans.

To then test that notion, we screened for NA activity with the same set of viruses, using an NA activity assay that we developed that utilizes the same glycan microarray as used for HA binding studies, thus enabling a wide range of glycan substrates with α 2-3 or α 2-6 linkages to be investigated. Briefly, we first incubate purified recombinant NA samples on the glycan microarray. NA digestion removes terminal sialic acids from the imprinted glycans and exposes galactose as the terminal sugars. The newly generated

terminal galactose is then specifically detected with fluorescein-labeled *Erythrina cristagalli* lectin (ECL), which is specific for the terminal Gal β 1-4GlcNAc sequence formed as a product of the neuraminidase for the majority of the glycans on the array. The specificity of the ECL excludes certain glycans from analysis, including those that would yield a product with terminal Gal β 1-3GlcNAc (Fig. 1, glycans 13 to 15), Gal β 1-3GalNAc (glycans 16 and 19) Gal β 1-4Glc (glycans 8 and 27), GalNAc β 1-4Gal (glycans 17 and 18), or Gal β 1-4GlcNAc when Gal is sulfated (glycans 5 and 6) or GlcNAc is fucosylated (glycans 4, 6, and 20 to 23). As with the HA microarray binding assay, this NA activity assay allows us to investigate not only the overall enzymatic activity but also the range and relative substrate specificity of each NA for different glycan structures. These results from the microarray assay were complemented by a commonly used quantitative NA activity assay that utilizes the linkage-independent substrate 2'-O-(4-methylumbelliferyl)-N-acetylneuraminic acid (4-MU-NANA) (40). Using both of these assays, it was possible to assess both the intrinsic activity of the NAs and their specificities toward sialosides present on the array.

NAs from the four pandemic viruses displayed nearly 100-fold differences in enzymatic activity toward the artificial 4-MU-NANA substrate (Fig. 4B) and toward the panel of sialosides on the glycan array (Fig. 4D). N2 NAs of Japan57 and HK68 were highly active in both activity assays. SC18 NA showed intermediate activity, and Cali09 NA was the least active among the four pandemic strains. The qualitative ranking of the NA enzymatic

FIG 4 Comparison of HA and NA activities for pandemic viruses. In human pandemic viruses, HA binding to glycan receptors is closely correlated with the efficiency of NA cleavage of these glycan receptors, suggesting that a functional match of these two surface glycoproteins contributes to effective human transmission. Strong HA glycan binding in Japan57 and HK68 is paired up with NAs that exhibit higher activity. Weaker binding in SC18 and Cali09 is complemented by NAs of lower activity. HA binding was measured against biotinylated 6'-SLNIN in a plate assay (A) and printed glycans on a microarray (C) as previously described (52). NA activity was quantified by a solution assay using the fluorescence substrate 4-MU-NANA (B). NA specificity for glycan structures was investigated by the newly developed microarray method (D). All of the NA microarray results shown in the main figures were obtained at 0.8 $\mu\text{g/ml}$. Results with other NA concentrations are presented in Fig S5 to S14 in the supplemental material.

activity parallels HA binding avidity from these pandemic viruses (Fig. 4). Japan57 and HK68 demonstrated both strong HA binding and high NA activity. Cali09 displayed weak HA receptor binding along with weak NA activity. The results demonstrate a remarkable correlation between the relative activities of HA and NA in these human pandemic viruses.

Although the tested pandemic NAs show different levels of activity, they have similar spectra of glycan specificity on the microarray (Fig. 4D). In general, these enzymes exhibit broad specificity for glycan substrates with terminal structures NeuAc α 2-3Gal β 1-4GlcNAc or NeuAc α 2-6Gal β 1-4GlcNAc. Overall, the N1 sialidases exhibited a slightly stronger preference for α 2-3-linked sialosides over α 2-6 sialosides, which was best seen by titration of

the NA on the array (see Fig. S5 to S14 in the supplemental material). This difference was less apparent for the N2 sialidases. The conservation of NA activity for α 2-3-linked glycans is presumed to be important for viral transmission in humans as it helps viruses keep from being trapped by respiratory mucins in the human upper airway that contain α 2-3-linked glycan receptors (26).

HA/NA activities in swine progenitors. The results from human pandemic viruses suggest that the functional balance between HA and NA is a conserved trait among human viruses. Next, we tested several swine viruses to understand if such a trait might then contribute to the species barrier between swine and human. We chose two triple-reassortant H1N2 swine viruses (sw/Indiana00 and A/sw/Guangxi/13/2006 [sw/Guangxi06]) that pos-

FIG 5 HA and NA activities of swine H1 viruses. Swine viruses that are closely related to the 2009 H1N1 pandemic virus show strong HA glycan binding (A and C) but variable NA activities (B and D). HA binding was studied by ELISA (A) and on the glycan microarray (C). NA activity was measured by a solution-based assay using fluorescent substrate 4-MU-NANA (B) and by the NA glycan array activity/specificity assay (D). sw/Indiana00 N2 NA shows very weak NA activity. Iowa05 N1 NA is slightly more efficient and is roughly comparable to the Cali09 N1 NA. sw/Guangxi06 N2 NA is very efficient in catalyzing cleavage of 4-MU-NANA and comparable to Japan57 N2 NA. The mismatched Iowa05 virus was isolated from an infected patient but did not cause further human infections.

sess HA genes closely related to the viruses from the 2009 pandemic (17) and a classic swine H1N1 virus that infected a human (A/Iowa/CEID23/2005 [Iowa05]) in the last decade (18). The HAs of these three strains contain Asp190 and Asp225 that are characteristic for human receptor binding, with the exception of sw/Guangxi06, which has Asn190 (Fig. 3D). They also have Arg133_A and Ala227 that are well conserved in classical swine H1N1 viruses and their reassorted descendants in swine.

The three swine H1 HAs display strong binding for α 2-6-linked sialic acids with binding avidities similar to H2 HA of Japan57 (Fig. 5A and C). Glycan binding is restricted to α 2-6-linked glycans with terminal structures of NeuAc α 2-6Gal β 1-4GlcNAc. sw/Guangxi06 HA also binds sulfated α 2-3-linked glycans (Fig. 1, numbers 4 to 6). However, in the swine viruses, strong HA binding is not always accompanied by equally active NA cleavage activity (Fig. 5B and D). The sw/Indiana00 NA possesses extremely low activity against sialic acid substrates. Iowa05 NA is only slightly more active than sw/Indiana00 and is similar to the NA of Cali09. sw/Guangxi06 also carries a strong binding HA but has a higher-activity NA. Thus, two out of three tested swine viruses show a mismatch in the activities of their surface proteins compared to the strong correlation of these binding and cleavage activities in human viruses. sw/Guangxi06 appears to have a good HA-NA balance, but viruses of this lineage have not entered hu-

man populations, suggesting that a functional match of HA and NA may be necessary, but not sufficient, for viral transmission in humans and that other viral and host factors influence viral fitness.

To better visualize the relationship between the HA and NA activities of the human and swine viruses, we compared the quantitative results from the ELISA-based HA assay and the 4-MU-NANA NA assay. The correlation between HA binding and NA cleavage is clearly illustrated when HA binding avidity is plotted against NA enzymatic activity (Fig. 6 and Table 2). Here, the HA binding constants for glycan 6'-SLNLN were deduced from the HA ELISA binding assay. The K_d values reflect the avidities of multivalent binding between antibody-cross-linked HA molecules and immobilized glycans on the plate surface and are different from the monovalent affinities between HA and glycans. K_m and k_{cat} values of NA were determined by measuring NA activity as a function of substrate 4-MU-NANA concentration (see Fig. S17 in the supplemental material). For comparison of NA enzyme efficiency, we plotted either the commonly used catalytic efficiency k_{cat}/K_m (Fig. 6A) or k_{cat} alone (Fig. 6B). In both plots, it is evident that a functional match between HA and NA is maintained in human pandemic viruses. The swine progenitor strains that were tested mostly deviate from such a functional balance.

Evolution of the 2009 pandemic virus. From the triple-reas-

FIG 6 Functional balance of HA and NA in human pandemic viruses. Human pandemic viruses that readily transmit among humans (red filled circles) exhibit a functional balance of HA receptor binding and NA receptor cleavage activities. The strength of glycan binding in HA is correlated with the efficiency of NA cleavage of glycan receptors. A maladapted human virus (Neth602, red open circles) deviates from such functional balance. Swine progenitors (black filled squares) have strong HA binding but variable levels of NA activity. (A) HA binding avidity plotted against NA activity as expressed in k_{cat}/K_m . (B) HA binding avidity plotted against NA activity as expressed in k_{cat} (the turnover rate under optimum conditions). SC1918, A/South Carolina/1/18.

sortant sw/Indiana00 to the pandemic Cali09, the virus underwent evolution that allowed for efficient transmission in humans. As shown above, significant changes arose in the activities of both surface proteins. Confirming an earlier study (13), the Cali09 HA acquired amino acid substitutions that reduced binding to human receptors. These viruses also obtained an NA gene segment from avian-like swine viruses through reassortment and genetic mutations. These evolutionary changes ended up replacing a mismatched HA/NA pair in sw/Indiana00 with an HA/NA pair of balanced binding and cleavage activities in Cali09. No intermediate strains were isolated during this decade of evolution. It therefore raises the question of whether this adaptation occurred in swine or in humans.

Although these adaptations could have taken place before or after infection of humans, we believe that it is likely that the adaptation events took place in swine. Both parental viruses are of swine origin, and pigs are known as “mixing vessels” that can be coinfecting with multiple stains. Sequence analysis of swine viruses carrying classical H1 HAs suggests that the adaptive mutations A227E/R133_AK likely also arose in pigs. Since 2001, classical swine H1 HAs have gradually accumulated single mutations of A227E or R133_AK. These H1 HAs are on phylogenetic branches distant from the one that led to the pandemic and, thus, are not immediate progenitors of the pandemic virus. Rather, the existence of these mutants suggests a currently unknown evolutionary mechanism in swine that selected for HAs with attenuated glycan binding in the past decade.

TABLE 2 Summary of HA/NA activities in tested viruses

Virus	HA avidity to 6'-SLNLN ($\mu\text{g/ml}$)	NA activity against 4-MU-NANA		
		k_{cat} (sec^{-1})	K_m (μM)	k_{cat}/K_m ($\text{M}^{-1} \text{s}^{-1}$)
Japan57	0.32 ± 0.02	28.8 ± 0.1	78.6 ± 1.7	3.66×10^5
HK68	0.93 ± 0.12	18.2 ± 0.1	19.6 ± 0.1	9.29×10^5
SC18	1.19 ± 0.11	11.5 ± 0.1	80.3 ± 1.1	1.43×10^5
Cali09	6.79 ± 1.25	3.17 ± 0.01	373 ± 8	8.50×10^3
Neth602	1.11 ± 0.08	0.479 ± 0.003	1780 ± 80	2.69×10^2
NY06	1.10 ± 0.06	7.28 ± 0.03	169 ± 4	4.31×10^4
sw/Indiana00	0.34 ± 0.01	0.0257 ± 0.0001	24.8 ± 0.6	1.04×10^3
sw/Guangxi06	0.33 ± 0.03	5.21 ± 0.01	9.57 ± 0.23	5.44×10^5
Iowa05	0.24 ± 0.02	1.06 ± 0.01	78.3 ± 2.8	1.35×10^4
sw/England92	0.47 ± 0.05	9.62 ± 0.07	86.9 ± 3.3	1.11×10^5

The “off-the-shelf” (19) emergence of the pandemic virus was followed by adaptive fine-tuning after its introduction into humans. Cali09 was isolated on 1 April 2009 and was one of the first identified pandemic strains. By the end of April 2009, pandemic viruses had evolved into two clusters, I and II (10, 15), with cluster II viruses becoming dominant by the end of 2009 (10). One of the earliest cluster II virus, A/New York/06/2009 (NY06), accumulated four amino acid substitutions in the HA (P90_AS/T200A/S206T/I323V, H3 numbering) and two mutations in the NA (V106I and N247D, N2 numbering) (Fig. 7A). The NY06 HA shows significantly improved binding for α 2-6-linked sialic acids (Fig. 7B and D). In the ELISA-based plate assay, NY06 HA avidities for 6'-SLNLN approach those of SC18 HA (Fig. 6A and 7B). This binding affinity increase is mediated by the mutation T200A, which is localized in the receptor binding subdomain of H1 HA. Substitution of T200A in Cali09 HA improves glycan binding (13). Notably, mutations in the NY06 NA also resulted in enhanced enzymatic activity relative to the Cali09 NA (Fig. 7C and E). We conclude that these amino acid substitutions occurred in order for NY06 to achieve a new functional balance, with improved HA binding avidity and enhanced NA activity (Fig. 6). The mutations acquired in NY06 are present in most descendants of the pandemic viruses subsequently isolated in humans, suggesting that viruses carrying these mutations are fit for viral circulation in humans.

Viral evolution is driven by natural selection of random mutations, which are generated rapidly due to the poor proofreading mechanism of influenza virus RNA polymerase. Because of the randomness of the process, most mutants have impaired fitness and fail to propagate further, unlike NY06. We looked at another strain isolated at the end of April 2009, A/Netherlands/602/2009 (Neth602), which has two unique amino acid mutations in its NA molecule. The I108V/V411I substitutions occur only once among the 1,147 pandemic NA sequences that are available in the Influenza Virus Sequence Database at the National Center for Biotechnology Information (NCBI) (Fig. 7A). With these mutations, Neth602 NA has greatly diminished activity against sialosides on the microarray, as well as with the 4-MU-NANA substrate in the plate assay (Fig. 7C and E). In contrast, the Neth602 HA shows improved HA binding for α 2-6-linked sialic acids over Cali09 and HA binding avidity similar to that of NY06 (Fig. 7B and D). Thus, in Neth602, the activities of HA and NA diverged from Cali09 HA and NA, suggesting an imbalanced HA/NA pair. The mismatch of

FIG 7 Adaptive fine-tuning of the 2009 pandemic virus. (A) Amino acid substitutions in HA and NA in two strains that were isolated 4 weeks later than the first isolated Cali09 viruses. The incidence of an amino acid occurring at certain position among 2009 pandemic viruses is shown in parentheses. A total of 2,271 H1 HA sequences and 1,147 N1 NA sequences from 2009 pandemic viruses were available in the Influenza Virus Resource at the NCBI in September 2011 (2). (B and D) The amino acid changes in the HA improved binding for glycan receptors. The NA of strain NY06 also shows improved catalytic efficiency (C and E), corresponding to an increase in both HA and NA activities. Descendants of an NY06-like virus became dominant later in the pandemic. On the other hand, Neth602 displays diminished NA activity (C and E) and an HA/NA imbalance, but the NA mutations acquired by Neth602 did not propagate in later pandemic strains.

the viral surface proteins in Neth602 seems to have put the virus in an evolutionary dead end as mutations that impair the HA/NA balance may lower the chance of such mutations being accumulated in later field strains. The I108V/V411I substitutions are not present in any other H1N1 pandemic strains isolated so far (Fig. 7A). It should be noted that Neth602 was shown to transmit among ferrets and guinea pigs through respiratory droplets (36, 48). This virus poses an interesting exception to the balance of HA and NA of other human viruses. It is notable, however, that the Neth602-like virus was reported only once from human infections during the pandemic, suggesting that it might not be fit in humans. Possible reasons for the virulence in animal models include host or environmental differences between humans and housed experimental animals, where other, as yet undefined, fitness factors dominate. Other more trivial explanations are also possible, such as errors in the sequence of the Neth602 virus in the database or compensatory mutations in laboratory viruses used in the animal models that were not appreciated. Clearly, further studies are required to distinguish among these possibilities.

DISCUSSION

Our analysis of recombinant influenza virus surface proteins reveals the relative functional balance between the HA and the NA in

human viruses. Swine viruses do not necessarily share such a property, which may thus present a species barrier for swine viruses to adapt to human transmission. The functional balance between HA and NA, in addition to the previously known linkage specificity of HA, therefore serves as an important host barrier to an emerging virus that has to be overcome before widespread human infections are possible.

The 2009 influenza pandemic, and possibly the 1918 pandemic, directly originated from swine viruses (17, 46, 47). Current circulating swine viruses, including the H2 subtype (27) and the recently emerging triple-reassortant H3N2 viruses (7), raise concerns about the potential of human epidemics and pandemics if a sustained human-to-human transmission is acquired by these swine-origin viruses. Recent studies have observed limited respiratory droplet transmission in ferrets for some triple-reassortant H1N1 swine viruses (5) but not so far for others (55). Although the molecular mechanism of viral transmission remains largely unknown, we conclude that the functional balance of HA/NA is likely a necessary factor for efficient transmission. Iowa05, a human-infecting swine virus, represents a compelling example. This functionally mismatched virus (Fig. 6) did successfully jump into a human host but failed to cause further human infection (18), suggesting an impaired capability for human transmission.

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