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Amino Acid 226 in the Hemagglutinin of H4N6 Influenza Virus Determines Binding Affinity for α2,6-Linked Sialic Acid and Infectivity Levels in Primary Swine and Human Respiratory Epithelial Cells▽

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Avian lineage H4N6 influenza viruses previously isolated from pigs differ at hemagglutinin amino acids 226 and 228 from H4 subtype viruses isolated from birds. Using a parental H4N6 swine isolate and hemagglutinin mutant viruses (at residues 226 and/or 228), we determined that viruses which contain L226 had a higher affinity for sialic acid α2,6 galactose (SAα2,6Gal) and a higher infectivity level for primary swine and human respiratory epithelial cells, whereas viruses which contain Q226 had lower SAα2,6Gal affinity and lower infectivity levels for both types of cells. Using specific neuraminidases, we found that irrespective of their relative binding preferences, all of the influenza viruses examined utilized SAα2,6Gal to infect swine and human cells.

Influenza A viruses are important pathogens of human diseases and those of many other species. All hemagglutinin (HA) and neuraminidase (NA) subtypes of influenza A viruses have been isolated from wild waterfowl (32), but only subtypes H1 to H3 have spread widely among humans, and only subtypes H1 and H3 have circulated in swine populations (33, 46). However, in recent years, both humans and pigs have been infected with avian influenza viruses of multiple subtypes, including H5N1 and H9N2 (22, 36, 49).

One factor believed to be important in determining the influenza virus host range is sialic acid binding preference (14, 26). Avian influenza viruses preferentially bind cell surface sialic acid α2,3 galactose (SAα2,3Gal), whereas human and swine influenza viruses preferentially bind SAα2,6Gal (10, 26, 37, 38). Since swine cells express both SAα2,3Gal and SAα2,6Gal (14) and since avian influenza virus replication in pigs can result in a mutation toward a mammalian-like binding preference (25), it has been postulated that pigs serve as intermediate hosts for the generation of human pandemic viruses (reviewed in references 21, 34, 43, and 46).

Our previous full-length sequence analyses of H4N6 influenza viruses isolated from pigs in Canada revealed that all gene segments were of avian origin, indicative of in toto transmission of an avian virus to pigs (15). Compared to the avian H4 sequences in GenBank, these swine isolates differed at HA amino acids 226 and 228 (H3 numbering [31]). These amino acid positions are major determinants of the binding preference of virus subtypes H2, H3, and H9 (7, 27).

To determine if residues 226 and 228 are important in H4 influenza virus binding, we generated the parental H4N6 swine isolate, A/swine/Ontario/01911-1/99 (rgONT/99) (15), and three HA mutant viruses, in which residues 226 and/or 228 were mutated to avian residues (Table 1), using site-directed mutagenesis and reverse genetics (29). Binding affinities were examined by direct virus binding to biotinylated synthetic glycopolymers as described previously (25), with the modification that viruses were equilibrated to 25,000 copies of matrix gene RNA (19) bound per well. Linear regression analysis of Scatchard plots was performed using Prism software (GraphPad, La Jolla, CA). The Neu5Acα2,3Galβ1-4Glcβ (2,3SL) polymer is widely utilized as the avian-like receptor analog, while the Neu5Acα2,6Galβ1-4GlcNAcβ (2,6SLN) polymer is utilized as the human-like analog (11, 24–26, 35). To ensure that the different polymer backbones (lactose versus lactosamine) did not have major effects on binding affinity, we included 2,3SLN and found that viruses bound to 2,3SL and 2,3SLN with similar levels of affinity (Table 1). The 2,6SL polymer was not used because it is believed not to be an appropriate analog for the human influenza virus receptor (8).

Based on calculated approximate constants of dissociation (where lower values represent higher affinity), rgONT/99 and the S228G virus had similar binding profiles, with much higher affinity for SAα2,6Gal (2,6SLN) than for SAα2,3Gal (2,3SL and 2,3SLN) (Table 1). The L226Q virus also had a higher affinity for SAα2,6Gal than for SAα2,3Gal, but compared to that of rgONT/99, the L226Q virus had a lower affinity for SAα2,6Gal. The L226Q/S228G virus had an avian-like virus binding preference, with a higher affinity for SAα2,3Gal than for SAα2,6Gal. Taken together, the results indicate that compared to the affinity of rgONT/99, the virus affinity for SAα2,3Gal increases only when amino acids 226 and 228 are both mutated to avian residues, whereas amino acid 226 is the dominant factor in determining the virus' affinity for

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TABLE 1. Amino acid composition and binding affinity of reverse genetics-generated viruses[a]

Virus	Amino acid composition at:		Polymer binding affinity					
	HA position 226	HA position 228	2,3SL		2,3SLN		2,6SLN	
			$\approx K_d$	R^2	$\approx K_d$	R^2	$\approx K_d$	R^2
rgONT/99	L	S	288	0.88	371	0.90	4	0.99
L226Q	Q	S	311	0.91	314	0.96	90	0.89
S228G	L	G	443	0.88	389	0.90	3	1.00
L226Q/S228G	Q	G	31	0.87	25	0.83	156	0.89

[a] Approximate constant of dissociation ($\approx K_d$) values are expressed in nM^{-1} sialic acid. Lower values indicate higher affinity binding. R^2 is the correlation coefficient. Values shown are from one representative experiment, but highly similar results were obtained in repeated experiments.

SAα2,6Gal. This is consistent with data for the H2, H3, and H9 influenza viruses, where amino acid 226 either plays the predominant role (e.g., H2 and H3 [7]) or is solely responsible (e.g., H9 [27]) for sialic acid binding preference.

To determine if changes at amino acids 226 and 228 influence infectivity, we infected primary swine and human respiratory epithelial cells (SRECs and HRECs, respectively) with each of the viruses. We have previously shown that SRECs are a useful system with which to model influenza virus infection of pigs (3), and primary HRECs have been similarly utilized to investigate infections with rhinovirus, respiratory syncytial virus, parainfluenza virus, and influenza A virus (1, 13, 16, 17). Although medium-submerged cell monolayers lack the differentiation of cells grown at an air-liquid interface (12, 28), when these cells are grown as monolayers, they have the necessary receptors and intracellular factors to support influenza A virus infection and replication (3, 13). SRECs and HRECs were grown (3, 40), infected with three 50% tissue culture infective doses ($TCID_{50}$s)/cell, and stained for the expression of influenza virus nucleoprotein (NP) as described previously (3, 20). By visual inspection of immunocytochemical staining, we determined that the infectivity levels of SRECs and HRECs were similar (Fig. 1). Viruses with L226 infected the majority of the cells, while viruses with Q226 exhibited lower infectivity levels. Detection of NP-positive cells by flow cytometry confirmed this assessment (Fig. 2C and D), demonstrating that amino acid 226 is the major determinant of virus infectivity of SRECs and HRECs.

Although the relative virus infectivity patterns were similar for SRECs and HRECs, the absolute infectivity of each virus was higher for HRECs than for SRECs (Fig. 2C and D). This difference may be due to variations in inner carbohydrate moieties on the cell surface (4, 9), or it could be due to different expression levels of sialic acid species on each cell type. Pigs express both *N*-acetylneuraminic (Neu5Ac) and *N*-glycolylneuraminic acid (Neu5Gc), whereas humans express only Neu5Ac (5, 44). Infectivity may be dependent upon levels of Neu5Ac, in which case the Neu5Gc on SRECs may lead to a lower expression of Neu5Ac, or it may interfere with the availability of Neu5Ac on SRECs.

To verify the association between binding preference and sialic acid utilization to infect cells, prior to the inoculation of cells with viruses, cells were treated for 3 h with 300 U of either an α2,3-specific NA (*Salmonella enterica* serovar Typhimurium; New England Biolabs, Beverly, MA) or an NA that removes both α2,3- and α2,6-linked sialic acids (*Clostridium perfringens*; New England Biolabs). Based on lectin staining (16), we determined that treatment with the α2,3-specific NA decreased the level of α2,3-linked sialic acids on SRECs considerably (with *Maackia amurensis* agglutinin [MAA; Roche Diagnostics, Mannheim, Germany] staining, to less than 32% of untreated SRECs [Fig. 2A]) without depleting α2,6-linked sialic acids (using *Sambucus nigra* agglutinin [SNA; Roche Diagnostics, Mannheim, Germany] staining). Treatment with the α2,3/α2,6 NA decreased the level of α2,3- and α2,6-linked sialic acids (with MAA and SNA staining, to less than 23 and 27% of the untreated SRECs, respectively). Similar results were obtained with HRECs (Fig. 2B). However, treatment with the α2,3-specific NA had no effect on the virus infectivity of SRECs or HRECs (Fig. 2C and D), while treatment with the α2,3/α2,6 NA decreased the infectivity of all viruses for both cell types.

The NA treatments did not cleave all of the sialic acids from the cell surfaces, so it is possible that the viruses utilized the remaining α2,3-linked sialic acid to infect cells. However, treatment with the α2,3-specific NA decreased the α2,3-linked sialic acid but not the α2,6-linked sialic acid and resulted in no change in infectivity levels, while treatment with the α2,3/α2,6 NA decreased both the α2,3- and the α2,6-linked sialic acids, and the infectivity levels of all the viruses decreased significantly. This strongly suggests that these viruses do not generally utilize α2,3-linked sialic acid to infect swine or human cells. Instead, all four viruses, independently of their relative binding preferences, preferentially utilized α2,6-linked sialic acid to infect both SRECs and HRECs.

To determine if the changes at amino acids 226 and 228 might represent swine adaptations that occurred after the introduction of an avian influenza virus into pigs, rgONT/99 and the mutant viruses derived from it were serially passaged in SRECs. Cells were initially inoculated with either 0.5 $TCID_{50}$/cell (with rgONT/99 and the L226Q and S228G viruses) or 2.0 $TCID_{50}$s/cell (with the L226Q/S228G virus) and incubated with 0.5 μg/ml tolylsulfonyl phenylalanyl chloromethyl ketone-treated trypsin at 37°C for 3 days. The supernatant of each well was then collected, diluted 1:3 in fresh medium, and added to confluent SRECs for the next passage. After 10 passages, the amino acid at position 228 remained unaltered in each virus (Table 2). However, the L226 that was carried initially by some viruses was maintained as L226 throughout, while the Q226 carried initially by other viruses changed to L226 within four passages and was maintained as L226 for the remaining passages. This indicates that the Q226L mutation may represent a swine adaptation. Furthermore, beyond being exclusively a swine adaptation, virus infectivity of HRECs indicates that the

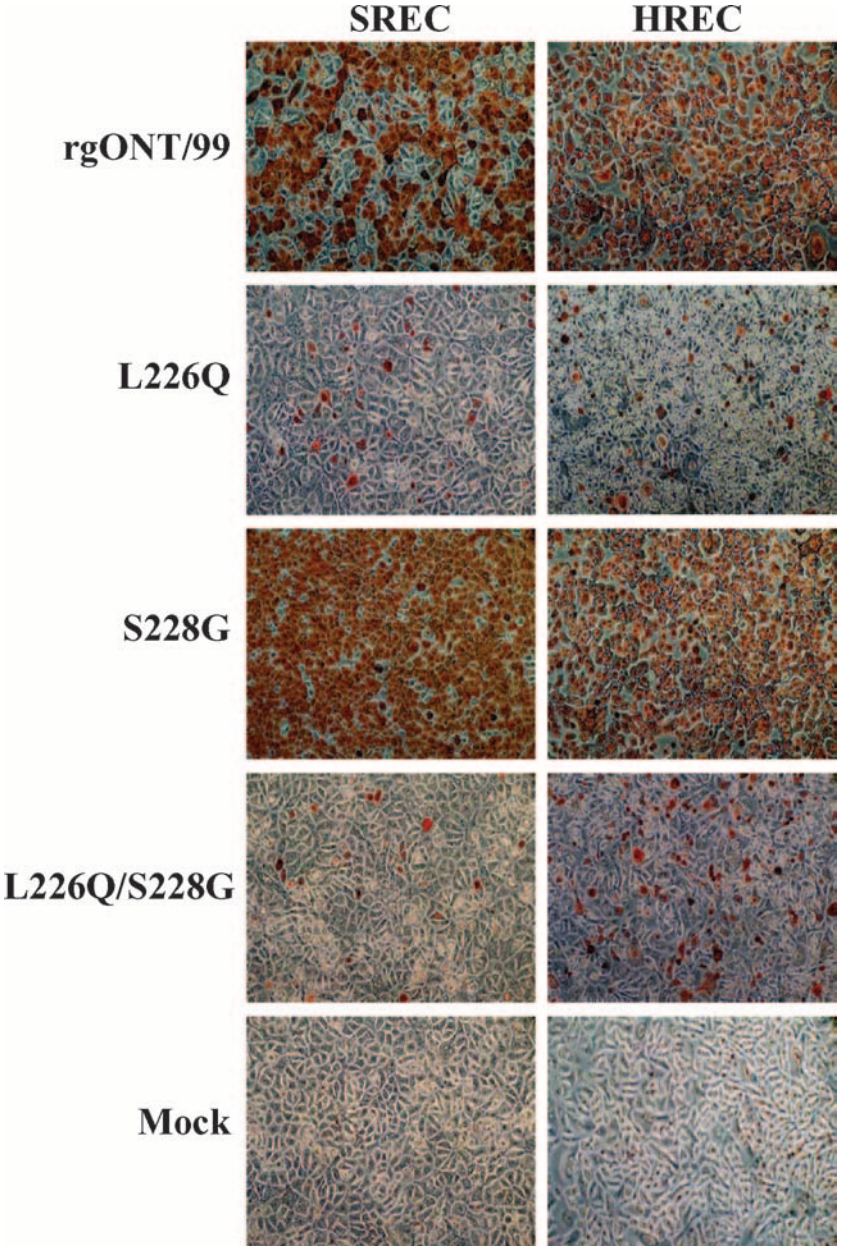

FIG. 1. Infectivity levels of the H4N6 influenza viruses in SRECs and HRECs. Cells were infected with three $TCID_{50}$s/cell for each virus, and infected cells were identified by immunocytochemistry (ICC) using the anti-influenza A NP antibody 68D2 (kindly provided by Y. Kawaoka, University of Wisconsin-Madison). Following ICC staining, the brightness and contrast of HREC micrographs were adjusted with ACDSee Photo Editor (ACD Systems) and PowerPoint (Microsoft) software to match that of the SREC micrographs. Similar patterns were observed in repeated experiments and with cells from different pig and human donors. Magnification, ×60.

Q226L mutation may also be an adaptation used for additional mammalian species.

Reassortant H2N3 influenza viruses with an HA of avian lineage have recently been isolated from pigs (23). These viruses had the Q226L mutation in their HA, suggesting that this is a swine adaptation for the H2 subtype viruses as well. Unlike the H4N6 viruses, the H2N3 viruses did not differ from the avian consensus sequence at amino acid 228. However, based on our passage experiments, the G228S mutation found in the H4N6 swine isolates may not be essential for adaptation to pigs.

Polymer binding studies using assays similar to that presented here have been utilized extensively to investigate influenza virus binding preference (10, 18, 24, 25, 27, 35, 47). However, data presented herein suggest that binding preference in such assays does not correlate uniformly with the receptor determinants utilized for infection. The L226Q/S228G virus had a higher affinity for SAα2,3Gal than for SAα2,6Gal, but based on the infectivity level in NA-treated cells, this virus appears to utilize α2,6-linked sialic acids to infect both SRECs and HRECs. This finding has potential implications for other avian influenza virus subtypes. Our results demonstrate that

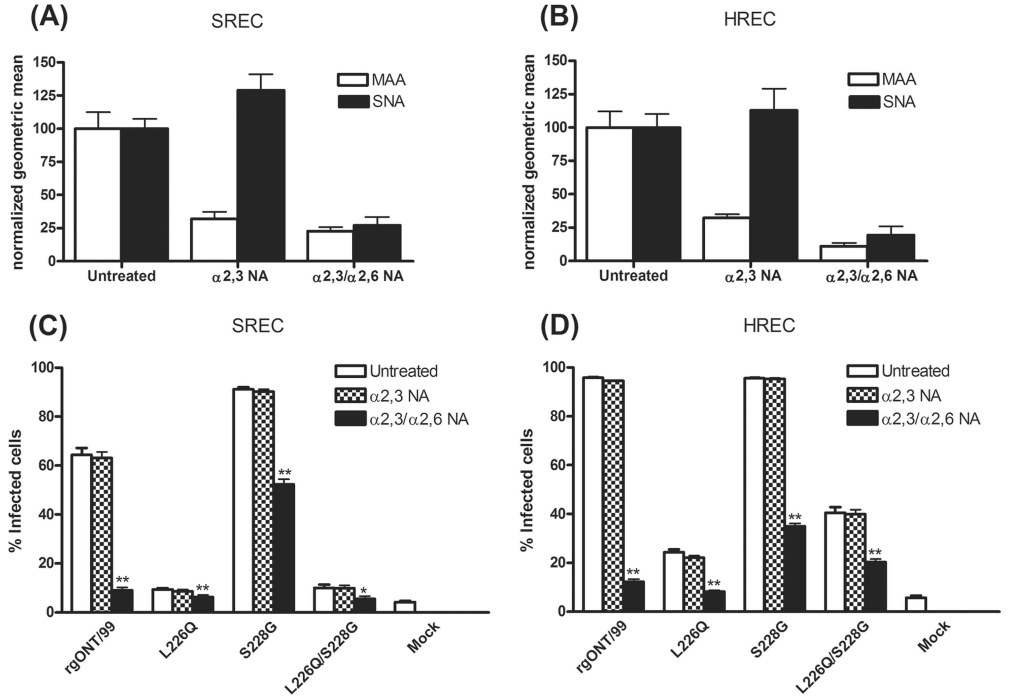

FIG. 2. Lectin staining and H4N6 influenza virus infectivity after NA depletion of cell surface sialic acid are shown. Removal of sialic acids from the surfaces of SRECs (A) and HRECs (B). Cells were treated with either an α2,3-specific NA or an α2,3/α2,6 NA, followed by cell surface lectin staining with MAA (SAα2,3Gal) or SNA (SAα2,6Gal). Values shown are the geometric means of fluorescent intensity (± standard errors of the means [SEM]) normalized such that the fluorescence intensity of untreated cells is 100% and the fluorescence intensity of mock lectin-treated cells is 0%. Data are the results of four separate experiments performed in duplicate. Virus infectivity levels after NA treatment of SRECs (C) and HRECs (D) are shown. Data are the results of four separate experiments with each virus assayed in triplicate for each experiment. Results are the means ± SEM. Statistically significant differences were observed between the infectivity levels of untreated and α2,3/α2,6 NA-treated cells (analysis of variance-protected Student's *t* tests; ∗, $P < 0.05$; ∗∗, $P < 0.01$).

the polymer to which viruses bind with the highest affinity in binding assays may not necessarily be the receptor that the viruses utilize for infection. Therefore, even though α2,3-linked sialic acids are found in the lower human respiratory tract (41, 48) and the H5 influenza viruses can bind to the lower human respiratory tract (45), H5 viruses may still infect human cells via α2,6-linked sialic acids. Indeed, a recent report states that the distribution of SAα2,3Gal in the human respiratory tract is partially inconsistent with the pattern of H5N1-infected cells (48), and Nicholls et al. (30) suggest that binding sites other than SAα2,3Gal may mediate the H5N1 virus infection of human epithelium. In addition to α2,3- and α2,6-linked sialic acids, alternate or coreceptors may also be involved in influenza virus infection, because influenza viruses have been shown to infect desialylated cells (42). The particular proteins or lipids to which sialic acid-containing oligosaccharides are attached are also likely to be important, as cell surface N-linked glycoproteins are necessary for influenza virus infection (6).

TABLE 2. Virus passaging in SRECs[a]

Passage	aa mutations at HA positions for the indicated virus							
	rgONT/99		L226Q		S228G		L226Q/S228G	
	aa 226	aa 228	aa 226	aa 228	aa 226	aa 228	aa 226	aa 228
0	L	S	Q	S	L	G	Q	G
1	L	S	Q	S	L	G	Q	G
2	L	S	Q/L	S	L	G	Q	G
3	L	S	L	S	L	G	Q/L	G
4	L	S	L	S	L	G	L	G
10	L	S	L	S	L	G	L	G

[a] Amino acid (aa) residues at HA positions 226 and 228 are shown for each virus at each passage. No other mutations in the HA were found upon passaging.

Amino acid 226 in the HA of rgONT/99 and related mutant viruses predominantly controls virus affinity for SAα2,6Gal and infectivity of SRECs and HRECs. Independent of binding preference, these viruses appear to utilize α2,6-linked sialic acids to infect both cell types. Thus, in order to establish an efficient infection in pigs, it appears that viruses either must already bind or must rapidly adapt to bind α2,6-linked sialic acids. This finding does not negate the "mixing vessel" hypothesis (39), but it does indicate that a main rationale for this theory (i.e., that pigs express both SAα2,3Gal and SAα2,6Gal) may be less important than previously hypothesized. Pigs still hold considerable potential to serve as mixing vessels, as they are susceptible to both avian and human viruses (2, 34, 46), and viral replication in pigs can lead to a mammalian-like virus binding preference (25). Furthermore, the avian-to-mammalian Q226L mutation that occurred upon virus passage in SRECs suggests that this change is a swine adaptation mutation that may also be an adaptation used for infection of additional species, providing more support for the hypothesis that pigs are

an intermediate adaptation host for the generation of pandemic human viruses.

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