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E190V substitution of H6 hemagglutinin is one of key factors for binding to sulfated sialylated glycan receptor and infection to chickens

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Abstract

Avian influenza viruses (AIVs) recognize sialic acid linked α2,3 to galactose (SAα2,3Gal) glycans as receptors. In this study, the interactions between hemagglutinins (HAs) of AIVs and sulfated SAα2,3Gal glycans were analyzed to clarify the molecular basis of interspecies transmission of AIVs from ducks to chickens. It was revealed that E190V and N192D substitutions of the HA increased the recovery of viruses derived from an H6 duck virus isolate, A/duck/Hong Kong/960/1980 (H6N2), in chickens. Recombinant HAs from an H6 chicken virus, A/chicken/Tainan/V156/1999 (H6N1), bound to sulfated SAα2,3Gal glycans, whereas the HAs from an H6 duck virus did not. Binding preference of mutant HAs revealed that an E190V substitution is critical for the recognition of sulfated SAα2,3Gal glycans. These results suggest that the binding of the HA from H6 AIVs to sulfated SAα2,3Gal glycans explains a part of mechanisms of interspecies transmission of AIVs from ducks to chickens.

KEYWORDS
avian influenza virus, hemagglutinin, interspecies transmission, sialic acid receptor, sulfated glycans

Abbreviations: AIV, avian influenza virus; Ck/Tainan, A/chicken/Tainan/V156/1999 (H6N1); Dk/HK, A/duck/Hong Kong/960/1980 (H6N2); DMEM, Dulbecco's modified Eagle's medium; d.p.i., days postinoculation; HA, hemagglutinin; HEK, human embryonic kidney; Le^x, Lewis X; MEM, minimum essential medium; RFU, relative fluorescence unit; rHA, recombinant HA; SAα2,3Gal, sialic acid linked α2,3 to galactose; SAα2,6Gal, sialic acid linked α2,6 to galactose; SE, standard error.

Yuto Kikutani and Masatoshi Okamatsu contributed equally to this work.

1 | INTRODUCTION

Migratory ducks are the natural reservoir of avian influenza viruses (AIVs).[1] AIVs can infect most avian species and be transmitted to mammalian species, including humans. Chickens, however, are rarely infected directly with AIVs circulating in ducks and are thought to be infected by them following their adaptation to other galliform species, including turkeys and quails.[2] Therefore, there must be some interspecies barrier between ducks and chickens for AIVs. H6 low-pathogenic AIVs have circulated in domestic poultry in Asian countries[3,4] and infected humans.[5] In general, a single infection of H6 AIVs does not induce clinical signs in poultry; however, the co-infection of bacteria aggravates the clinical signs.[6] The adaptation of H6 duck influenza viruses to chickens increases the risk of economic damages.

Influenza viruses recognize sialylated glycans on host epithelial cells as their receptor. The binding specificity of hemagglutinin (HA) of influenza viruses to sialylated glycans depends on the host species from which the virus was isolated: HAs of human influenza viruses preferentially bind to glycans terminating with sialic acid linked α2,6 to galactose (SAα2,6Gal), whereas the HAs of AIVs preferentially bind to glycans with sialic acid linked α2,3 to galactose (SAα2,3Gal).[7,8] It has been reported that AIVs isolated from terrestrial birds, including chickens, prefer 6-sulfo sialyl Lewis X (Le^x) (Siaα2-3Galβ1-4[Fucα1-3][6-Sulfo]GlcNAc) as receptors, to which AIVs isolated from ducks do not bind.[9] The result suggests that the binding of AIVs to fucosylated and/or sulfated SAα2,3Gal glycans is one of the determinants for a successful infection in chickens. Previous studies have revealed that H5Nx chicken influenza viruses preferentially bind to fucosylated SAα2,3Gal glycans.[10,11] However, the role of sulfation in SAα2,3Gal glycans which are not fucosylated in infections by chicken influenza viruses remains unknown.

Several amino acids in the 190 helix and 220 loop in HA are known to alter the receptor-binding specificity of influenza viruses; amino acid residues at the 226 and 228 positions of H3 HA (H3 numbering is used throughout) determine the binding specificity of influenza viruses to SAα2,6Gal glycans or SAα2,3Gal glycans.[12,13] In several studies, a relationship has been reported between fucosylated and/or sulfated SAα2,3-Gal glycans and the 190 helix and 220 loop on the HA of influenza viruses; the fucose moiety of the host fucosylated SAα2,3Gal glycans is positioned close to the 220 loop on the HA of the influenza virus.[14] In particular, amino acid residues at positions 222 and 227 on the 220 loop of HA are involved in binding H5 chicken influenza viruses to sialyl Le^x.[11,15] The sulfo moiety of 6-sulfo sialyl Le^x is thought to be positioned close to the 190 helix on the HA of chicken influenza viruses[9]; however, reports on the mechanisms of the recognition of the sulfo moiety are limited. Previously, the cocrystallization of HA from an H7N9 human isolate and sulfated SAα2,3Gal glycan revealed the mechanism of the recognition of the sulfo moiety: amino acid residues at positions 190 and 227 of H7 HA determine the binding of HA to sulfated SAα2,3Gal glycans,[16] whereas the situation for other subtypes remains unreported.

The distribution of sialylated glycans is highly variable in each species; SAα2,6Gal glycans are predominantly detected in human respiratory epithelial cells,[17] whereas SAα2,3Gal glycans are dominant on duck cloacal epithelial cells.[18] The tissue tropism of influenza viruses, which circulate in each host, is consistent with the distribution of their preferred glycans in hosts. In a previous study, we detected fucosylated SAα2,3Gal glycans in chicken tracheal epithelial cells, where chicken influenza viruses primarily replicate, whereas this glycan structure was not detected in duck colon.[10] By contrast, there are no reports on the detection of sulfated SAα2,3Gal glycans on chicken tracheal epithelial cells to date.

In the present study, we aimed to clarify the mechanism of interspecies transmission between ducks and chickens. The growth capacity in chickens and the binding specificity to sulfated glycans of AIVs isolated from chicken and duck were comparatively assessed. The results indicated that E190V and N192D substitutions of the HA increased the virus recovery in chickens and the binding to sulfated SAα2,3Gal glycans is one of the determinants for the increasing infectivity of duck-derived AIVs in chickens.

2 | MATERIALS AND METHODS

2.1 | Viruses and cells

Influenza virus A/chicken/Tainan/V156/1999 (H6N1; Ck/Tainan) was provided by the Animal Health Research Institute, Council of Agriculture, Taiwan. Influenza virus A/duck/Hong Kong/960/1980 (H6N2; Dk/HK) was kindly provided by Professor Ken F. Shortridge, The University of Hong Kong, Hong Kong SAR. These viruses were propagated in 10 day old embryonated chicken eggs at 35°C for 48 hr, and the infectious allantoic fluids were used as virus stocks. MDCK cells were maintained in minimum essential medium (MEM; Nissui Pharmaceutical, Tokyo,

Japan) supplemented with 0.3 mg/ml L-glutamine, 100 U/ml penicillin G, 0.1 mg/ml streptomycin, 8 μg/ml gentamicin, and 10% FCS (Sigma Aldrich, St. Louis, MO, USA). Human embryonic kidney (HEK) 293T cells were maintained in Dulbecco's modified Eagle's medium (DMEM; Thermo Fisher Scientific, Waltham, MA, USA) supplemented with the same concentration of L-glutamine and antibiotics with MEM and 10% FCS (Cambrex, East Rutherford, NJ, USA). HEK 293S GnT (–) cells, which lack *N*-acetyl-glucosaminyltransferase I activity, were maintained in pyruvate-free DMEM (Thermo Fisher Scientific) with the same supplements as DMEM.

2.2 | Reverse genetics

Eight gene segments from Dk/HK were cloned into pHW2000 vector according to previously described methods.[19–21] Amino acid substitutions of E190V and N192D in the HA of Dk/HK were generated by site-directed mutagenesis using KOD -Plus- Neo (Toyobo, Tokyo, Japan) with specific primers (Table S1). All eight plasmids were transfected into a mixed culture of MDCK and HEK 293T cells and mutant viruses were rescued (Supplemental Figure). These viruses were propagated in 10 day old embryonated chicken eggs at 35°C for 48 hr, and the infectious allantoic fluids were used as virus stocks. Virus stocks were kept at −80°C until use. All eight gene segments of rescued viruses were sequenced to confirm the existence of the introduced mutations and the absence of undesired mutations.

2.3 | Virus titration

Plaque assays were performed to titrate the viruses. Tenfold dilutions of viruses were inoculated onto confluent monolayers of MDCK cells and incubated at 35°C for 1 hr. Unbound viruses were removed, and the cells were washed with PBS. The cells were then overlaid with FCS-free MEM containing 0.7% Bacto Agar (Thermo Fisher Scientific) and 5 μg/ml Trypsin Acetylated (Sigma Aldrich). After incubation for 48 hr at 35°C, cells were overlaid with FCS-free MEM containing 0.7% Bacto Agar and 0.005% neutral red. After 24 hr, visible plaques were counted and expressed as plaque forming unit/milliliter.

2.4 | Experimental infection of chicken with viruses

Four week old conventional chickens (*Gallus gallus*, Julia), which were free of anti-influenza virus antibodies, were obtained from HOKKAI STAR CHICK (Hokkaido, Japan). Of the 18 chickens purchased, three chickens was intranasally inoculated with 10^5 pfu of either Ck/Tainan or Dk/HK viruses. On 3 and 5 days postinoculation (d.p.i.), oral and cloacal swabs were collected. Further, other three chickens were inoculated with 10^5 pfu of mutant viruses derived from Dk/HK. Tracheal and cloacal swabs and tissue samples were collected at 3 d.p.i. The tissue samples were homogenized using a Multi-Beads Shocker (Yasui Kikai, Osaka, Japan) to prepare 10% suspensions with MEM. The infectivity titers of the swabs were calculated by a plaque assay. All infected chickens were kept in self-contained isolator units (Tokiwa Kagaku, Tokyo, Japan). Although the experiments were possibly conducted in ABSL2 condition, all the animal experiments were conducted in the higher biosafety level ABSL3 facility at the Faculty of Veterinary Medicine, Hokkaido University, Japan due to the limitation of access to the animal facility.

2.5 | Expression of the recombinant HAs

The complementary DNAs of the HA genes of Ck/Tainan, Dk/HK, and their mutants were cloned into a pCD5 expression vector.[22] In this vector, HA genes without transmembrane regions were fused with a GCN4IL motif and Strep-tag II (WSHPQFEK; IBA, Göttingen, Germany). The soluble trimeric recombinant HA (rHA) proteins were expressed in HEK293S GnT (–) cells and purified from the cell culture supernatants according to a previously described method.[22] In brief, rHA proteins were purified using Strep-Tactin Sepharose beads (IBA). The rHAs bound to Strep-Tactin beads were treated by D-desthiobiotin buffer (IBA) for 15 min at 4°C to elute the rHAs from the beads. Mutant HAs were prepared by the single-site mutagenesis method using specific primers (Table S1).

2.6 | Glycan microarray

Purified rHAs were analyzed according to a previously described method.[17] The glycan microarray contained two nonsialylated glycans (glycans 1 and 2) and 56 α2,3 and α2,6 sialylated glycans (glycans 3–58). Of the 56 sialylated glycans, five were sulfated α2,3 sialylated glycans (glycans 3–7), 28 were nonsulfated α2,3 sialylated glycans (glycans 8–35), and 23 α2,6 sialylated glycans (glycans 36–58) were printed on the array side (Table S2). For each glycan, the mean signal intensity

was calculated from six replicates. The highest and lowest signals of the six were removed, and the remaining four replicates were used to calculate the mean signal shown as relative fluorescence unit (RFU) and standard error (SE).

2.7 | Solid-phase direct binding assay

The receptor-binding specificity of rHAs was assessed using a solid-phase direct binding assay with Neu5Acα2-3Galβ1-4GlcNAc-β-PEG-biotin (SAα2,3Gal-bio) and Neu5Ac α2-3Galβ1-4(6-Sulfo)GlcNAc-β-PEG-biotin (Su-SAα2,3Gal-bio; Tokyo Chemical Industry Co., Ltd., Tokyo, Japan). Each sialylated glycan was serially diluted from 0.039 to 5 μM per well and added to each well of a Nunc Immobilizer streptavidin 12 × 8 strips microplate (Thermo Fisher Scientific). Each well was blocked with 2% BSA at room temperature for 1 hr. After washing with PBST, the rHAs (5 μg in PBST containing 1% BSA) were added to each well, and plates were incubated at 4°C for 12 hr. After washing, anti-Strep-tag mouse antibody (IBA; 1000-fold dilution in PBST containing 1% BSA) was added to each well, and the plates were incubated at 4°C for 2 hr. The wells were then washed and incubated with goat antimouse IgG-HRP conjugate (Bio-Rad, Hercules, CA) at 4°C for 2 hr. After washing, 100 μl of the substrate solution, including 0.5 mM 3,3′-tetramethylbenzidine and 0.04% H_2O_2, was added to each well. After incubation at room temperature for 30 min, the reactions were stopped using 50 μl of 2 N H_2SO_4, and absorbance at 450/630 nm was measured using a Multiskan JX microplate photometer (Thermo Fisher Scientific). The data were presented with the mean values of three technical replicates with SE.

2.8 | Amino acid sequence comparison of the H6 HA

A total of 638 amino acid sequences of H6 chicken and duck influenza viruses were obtained from GenBank. Sequence data were aligned with GENETYX version 12.01 (GENETYX Co., Tokyo, Japan).

2.9 | Ethics statement

All *in vivo* experiments were authorized by the Institutional Animal Care and Use Committee of the Faculty of Veterinary Medicine, Hokkaido University (approval number: 18-0037; 18-0040) and performed according to the guidelines of this committee.

3 | RESULTS

3.1 | Infectivity of chicken and duck influenza viruses in chickens

To confirm the growth of chicken virus, Ck/Tainan, and duck virus, Dk/HK, in chickens, the viral growth was compared *in vivo*. Each virus containing 10^5 pfu was intranasally inoculated into three 4 week old chickens. All chickens inoculated with each virus did not show any clinical signs. Ck/Tainan viruses were recovered from the respiratory tracts of all three infected chickens at 3 and 5 d.p.i., whereas Dk/HK was not recovered from any of the inoculated chickens (Table 1).

3.2 | Preparation of E190V and N192D mutant viruses derived from Dk/HK and their viral growth in chickens

A previous study reported that 190 helix of HA is thought to be positioned close to the sulfo moiety of sulfated SAα2,3Gal glycans.[9] Therefore, the amino acid sequences of 190 helix on HA of these two viruses were compared. The amino acid properties of positions 190 and 192 were found to be different between them. The majority of H6 AIVs isolated from ducks have amino acid residues E190 and N192 based on information from a public database (GenBank/DDBJ/EMBL). Almost one-third of H6 AIVs isolated from chickens, including Ck/Tainan, have amino acid residues V190 and D192 (Table 2). To clarify the role of amino acid residues at positions 190 and 192 of HA in the viral growth in chickens, Dk/HK viruses containing various amino acid substitutions at these positions (rgDk/HK, rgDk/HK-HA190V, rgDk/HK-HA192D, and rgDk/HK-HA190V/192D) were prepared using a reverse genetics method. To compare the virus growth in chickens, 10^5 pfu of each recombinant virus was intranasally

TABLE 1 Virus recovery from chickens intranasally inoculated with chicken and duck H6 influenza viruses

	Swabs (log pfu/ml)			
	Oral		Cloacal	
Viruses	3 d.p.i.	5 d.p.i.	3 d.p.i.	5 d.p.i.
Ck/Tainan	5.3	2.3	–[†]	–
	2.7	2.8	–	–
	3.4	–	–	3.2
Dk/HK	–	–	–	–
	–	–	–	–
	–	–	–	–

[†]Dash (–) indicates <1.0.

TABLE 2 Comparison of amino acid residue 190/192 motifs in HA of H6 chicken and duck influenza viruses

Host	Amino acid residue 190	Amino acid residue 192	Number of strains
Chicken (101 strains)	E	N	42
	V	**D**	32
	A	N	8
	V	N	4
	E	E	4
	E	D	3
	G	N	2
	E	A	2
	L	E	2
	L	N	1
	R	N	1
Duck (537 strains)	**E**	**N**	454
	E	E	51
	E	A	20
	A	N	5
	E	D	4
	V	N	1
	V	D	1
	L	D	1

Note: Amino acid sequence of H6 HA was obtained from GenBank. Amino acid motifs of Ck/Tainan and Dk/HK are shown in bold.

inoculated into three 4 week old chickens. Viruses were recovered from tracheal swabs at 3 d.p.i from the chickens infected with rgDk/HK-190V, rgDk/HK-HA192D, and rgDk/HK-HA190V192D (Table 3). Viruses were also recovered from respiratory organs of one of three rgDk/HK-HA190V-infected chickens and of all three rgDk/HK-HA190V192D-infected chickens. These results suggest that both E190V and N192D substitutions are involved in the replication of mutants derived from Dk/HK in chickens.

3.3 | Glycan-binding specificity of mutant viruses derived from Dk/HK

To clarify the molecular basis of different viral growth in chickens, the glycan-binding specificities of HAs from these two viruses were compared in a glycan microarray (Figure 1, Table S3). The rHAs of Ck/Tainan bound to sulfated SAα2,3Gal glycans (glycans 3, 4, and 7, Figure 2). The rHAs of Dk/HK bound to linear and nonsulfated SAα2,3Gal glycans (glycans 14 and 24–26, Figure 2) with high RFU. The rHAs of Dk/HK also bound to sulfated SAα2,3Gal glycans with lower RFU than those of the rHAs of Ck/Tainan (glycans 3, 4, and 7), indicating that the glycan-binding profile of rHA of Dk/HK is different from that of rHA of Ck/Tainan. These results indicated that the rHA of the chicken isolate, Ck/Tainan, selectively bound to sulfated SAα2,3Gal glycans, whereas that of the duck isolate, Dk/HK, preferentially bound to linear and nonsulfated SAα2,3Gal glycans.

3.4 | Key amino acid residues for recognition of sulfated SAα2,3Gal glycans by HA

To evaluate the contribution of amino acid residues at positions 190 and 192 of the HA to the glycan-binding specificity of these viruses, mutant rHAs of Ck/Tainan and Dk/HK, in which amino acid residues at positions 190 and/or 192 were substituted (Ck/Tainan-HA190E, Ck/Tainan-HA192N, Ck/Tainan-HA190E/192N, Dk/HK-HA190V, DK/HK-HA192D, and Dk/HK-HA190V/192D), were prepared and these rHAs were subjected to solid-phase direct binding assays (Figures 3,4). The rHAs of Ck/Tainan preferentially

TABLE 3 Virus recovery from chickens intranasally inoculated with wild-type and mutant viruses derived from A/duck/Hong Kong/960/1980 (H6N2)

Viruses	Swabs (log pfu/ml)		Tissues (log pfu/g)		
	Tracheal	Cloacal	Trachea	Lung	Colon
rgDk/HK	–[†]	–	–	–	–
	–	–	–	–	–
	–	–	–	–	–
rgDk/HK-HA190V	–	–	–	–	–
	1.4	–	–	1.7	–
	1.7	–	–	–	–
rgDk/HK-HA192D	1.7	–	–	–	–
	1.5	–	–	–	–
	–	–	–	–	–
rgDk/HK-HA190V/192D	2.4	–	2.6	–	–
	2.3	–	2.5	1.8	–
	4.3	–	4.4	3.3	–

[†]Dash (–) indicates <1.0. rgDk/HK, A/duck/Hong Kong/960/1980 (H6N2) generated by reverse genetics. Samples were collected at 3 d.p.i.

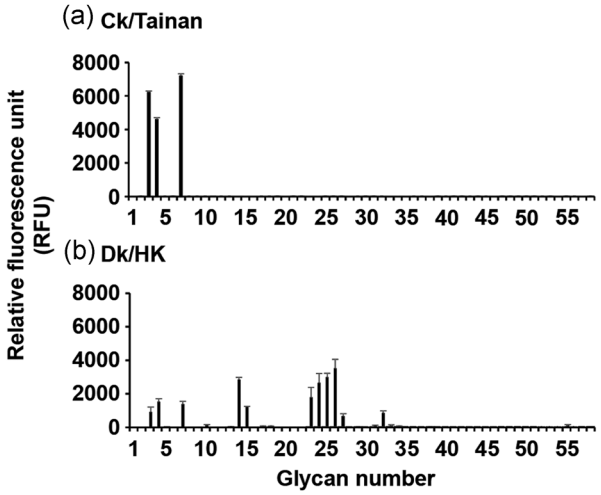

FIGURE 1 Glycan-binding specificity of the soluble trimeric rHAs. The glycan-binding specificities of rHAs from (a) Ck/Tainan and (b) Dk/HK were analyzed using a glycan microarray. Nonsialylated controls (glycans 1 and 2), sulfated α2,3 sialylated glycans (glycans 3–7), nonsulfated α2,3 sialylated glycans (glycans 8–35), and α2,6 sialylated glycans (glycans 36–58) were printed on the array. The data are presented as the mean ± SE of triplicate experiments. The structures of glycans that were preferentially bound (glycans 3, 4, 7, 14, and 24–26) are shown in Figure 2

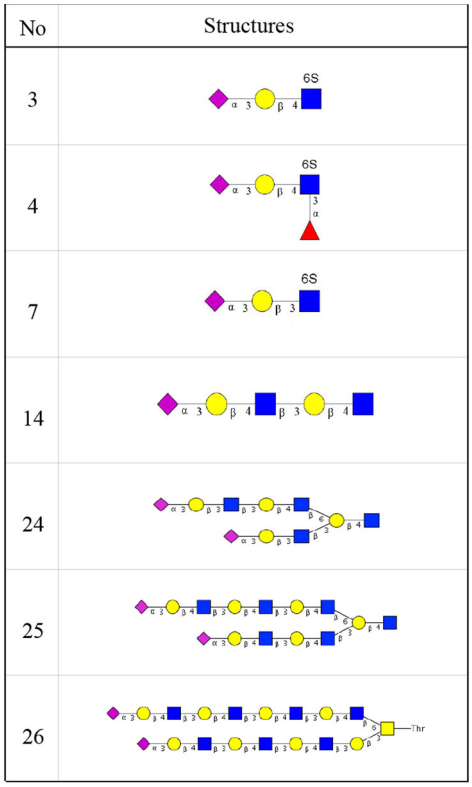

FIGURE 2 Structures of glycans that were bound by rHAs on the array. Each symbol indicates sialic acid (purple diamonds), galactose (yellow circles), *N*-acetylglucosamine (blue squares), *N*-acetylgalactosamine (yellow square), fucose (red triangle), and sulfo (S). The schematic structures of the glycans that yielded significant signals in the glycan microarray analysis with rHA from Ck/Tainan and Dk/HK (Figure 1) are shown in this figure [Color figure can be viewed at wileyonlinelibrary.com]

bound to Su-SAα2,3Gal-bio (Figure 3a) and Ck/Tainan-HA190E HAs preferentially bound to SAα2,3Gal-bio (Figure 3b). The introduction of the 192 N mutation to Ck/Tainan-HA and its mutant resulted in the diminished binding to both SAα2,3Gal-bio and Su-SAα2,3Gal-bio; yet the binding preference to Su-SAα2,3Gal-bio was slightly observed in 190 V HA (Ck/Tainan-HA192N) and to SAα2,3Gal-bio in 190E HA (Ck/Tainan-HA190E/192N; Figure 3c,d). The rHAs of Dk/HK bound to SAα2,3Gal-bio (Figure 4a), whereas Dk/HK-HA190V acquired binding to Su-SAα2,3Gal-bio (Figure 4b). The introduction of the 192D in Dk/HK-HA resulted in weaker binding to glycans without changing binding preference to SAα2,3Gal-bio (Figure 4c). The introduction of the same mutation in Dk/HK-HA190V (Dk/HK-HA190V/192D) also resulted in diminished binding to the glycans, although the binding to Su-SAα2,3Gal-bio was more affected when compared with that to SAα2,3Gal-bio (Figure 4d). These results indicate that E190V substitution of the HA determines the binding of H6 AIVs to sulfated SAα2,3Gal glycans. By contrast, the discrepancy of the 192D/N and the backbone of the HA resulted in the diminished binding to the glycans.

4 | DISCUSSION

In the present study, the virus growth in chickens and the receptor-binding specificities of viruses were analyzed to understand the mechanisms of interspecies transmission of AIVs from ducks to chickens. From the growth capacity of mutant viruses, E190V and N192D substitutions of the HA increased the recovery of the viruses derived from the H6 duck virus, Dk/HK, in experimentally infected chickens. The H6 AIV isolated from a chicken, Ck/Tainan, bound specifically to sulfated SAα2,3Gal glycans, whereas Dk/HK preferred to bound to nonsulfated SAα2,3Gal glycans. The difference of the binding specificity was thought to be related to the host range of H6 AIVs. Solid-phase direct binding assay of rHAs revealed that only an E190V substitution in the HA of Dk/HK altered its glycan-binding specificity from linear and nonsulfated SAα2,3-Gal glycans to sulfated SAα2,3Gal glycans. These results indicate that the amino acid residue at position 190 of H6 HA is a key component for the recognition of sulfated SAα2,3Gal glycans. Furthermore, position 190 of HA might be the key for the interspecies transmission of AIVs from ducks to chickens.

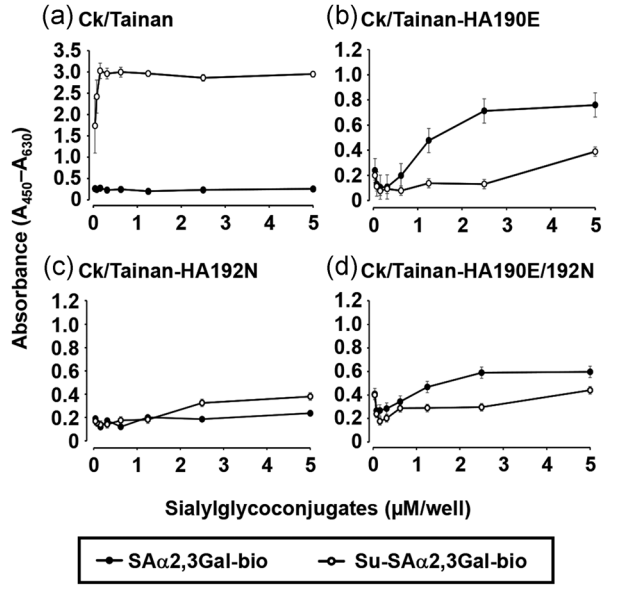

FIGURE 3 Glycan-binding specificity of wild-type and mutant rHAs of Ck/Tainan. The glycan-binding specificities of rHAs of (a) Ck/Tainan, (b) Ck/Tainan-HA190E, (c) Ck/Tainan-HA192N, and (d) Ck/Tainan-HA190E/192N to sialylglycoconjugates containing SAα2,3Gal-bio (closed circles) and Su-SAα2,3Gal-bio (open circles) were investigated using solid-phase direct binding assays. The data are presented as the mean ± SE of triplicate experiments

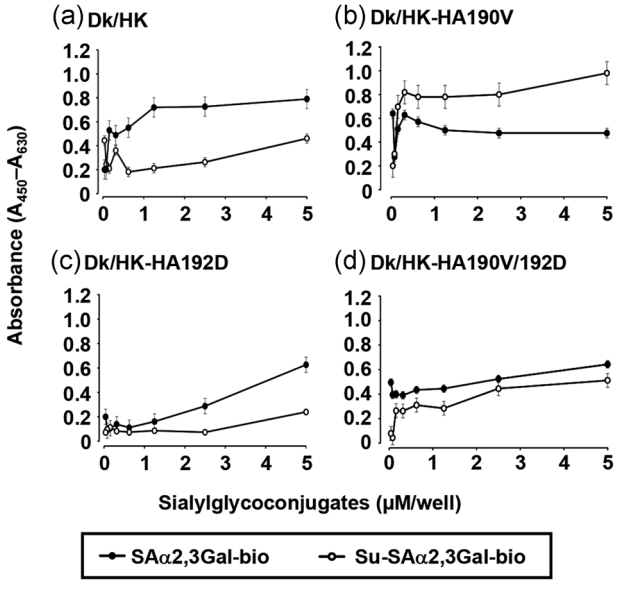

FIGURE 4 Glycan-binding specificity of wild-type and mutant rHAs of Dk/HK. The glycan-binding specificities of rHAs of (a) Dk/HK, (b) Dk/HK-HA190V, (c) Dk/HK-HA192D, and (d) Dk/HK-HA190V/192D to sialylglycoconjugates containing SAα2,3Gal-bio (closed circles) and Su-SAα2,3Gal-bio (open circles) were investigated using solid-phase direct binding assays. The data are presented as the mean ± SE of triplicate experiments

By contrast, both V190 and D192 were important for the virus growth in chickens (Table 3). Interestingly, viruses with V190/D192 were selected in chickens in the field (32/101 strains) rather than those with V190/N192 (4/101 strains; Table 2). These facts suggested that these two mutations, E190V and N192D, synergistically act in the adaptation process of duck influenza viruses to chickens. Moreover, the mutation at position 192 of the HA modulated receptor binding avidity (Figures 3,4). A previous report on the structural analysis demonstrated that the amino acid residue at position 192 was close to the penultimate Gal moiety of the sialylated glycan.[23] Perhaps, the mutation at position 192 might be related to the recognition of modified Gal moiety (e.g., 6-sulfo-Gal), which was not analyzed in the present study.

Nevertheless, approximately 40% of chicken H6 viruses still have the E residue at position 190 of the HA (Table 2). By contrast, viruses possessing the V residue at the same position were exceptional among duck H6 viruses; only two of 537 strains possess V at the position. Interestingly, similar phenomena were observed in the combination of H5 viruses and fucosylated SAα2,3Gal.[15] These facts suggest that viruses recognizing linear and nonsulfated SAα2,3Gal were selected among the duck population. In addition, a previous study demonstrated that co-infection of *Mycoplasma gallisepticum* exaggerated infection of duck influenza viruses in experimentally infected chickens.[24] This suggested that duck influenza viruses, which originally recognize linear and nonsulfated SAα2,3Gal are able to establish infections in chickens in the field without altering their receptor-binding specificity and viruses with "chicken-type receptor specificity" were selected during multiple replications and transmission events in chicken flocks, rather than in the adaptation among ducks or nonchicken terrestrial poultry.

Although rgDk/HK-HA190V, rgDk/HK-HA192D, and rgDk/HK-HA190V/192D were recovered from infected chickens, the virus recovery titers from swabs at 3 d.p.i were slightly lower compared with those from the chickens inoculated with Ck/Tainan. These viruses contain E190V and/or N192D substitution in their HA, indicating that both these mutations enhance viral infection and replication in chickens; however, the backbone of the HA as well as other viral proteins remains the same as wild-type Dk/HK, which could not replicate well in chickens. Therefore, some other interspecies barrier between ducks and chickens for AIVs may explain these lower titers of mutant viruses in chickens. Influenza viruses have eight segmented genes, and each gene encodes for different proteins. Of these proteins, HA is responsible for the attachment and fusion of viral and cellular membranes. Other proteins

have roles for each step of viral replication. It has been reported that PB2 and NS proteins are important for the interspecies transmission of influenza viruses.[25,26] In a previous study, a duck influenza virus adapted to chickens through mutations in its PB2 and NP proteins,[27] although the functional details of these mutations have not been reported.

The mechanisms of receptor recognition by HA of influenza viruses were revealed through the cocrystallization of HA with sialylated glycans.[9] However, reports on the detailed molecular interactions between HAs with modified SAα2,3Gal glycan receptors, such as fucosylated and/or sulfated receptors, are limited. One possible explanation for the lack of receptor binding of HAs from duck influenza viruses to sulfated SAα2,3Gal glycans is the negative charge of the sulfo moiety.[9] Position 190 in the HA of Dk/HK is glutamic acid, E, whereas in Ck/Tainan, it is valine, V. The negatively charged glutamic acid is perhaps not compatible with the similarly negatively charged sulfo moiety of the glycans. Therefore, the amino acid substitution to the uncharged amino acid, E190V, in HA must be involved in binding to sulfated glycans, thereby allowing H6 viruses to infect and replicate in chickens. In conclusion, it is speculated that sulfated SAα2,3Gal glycans are expressed on the chicken trachea and binding to sulfated SAα2,3Gal glycans contributes to the interspecies transmission of H6 AIVs from ducks to chickens. There is limited information on the distribution of sialylated glycans, which can be detected by lectins and antiglycan antibodies on the surface of host cells. To clarify the role of sulfated SAα2,3Gal glycans in the interspecies transmission, it is necessary to analyze the receptor distribution on the host cells. Furthermore, to fully understand the molecular mechanisms of interspecies transmission of AIVs, it is necessary to clarify the structural and quantitative details of sialylated glycans used as receptors by these viruses.

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DISCLOSURE

The authors declare that there are no conflict of interests.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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