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ORIGINAL ARTICLE

Amino acid residues at positions 222 and 227 of the hemagglutinin together with the neuraminidase determine binding of H5 avian influenza viruses to sialyl Lewis X

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Abstract Influenza viruses isolated from ducks are rarely able to infect chickens; it is therefore postulated that these viruses need to adapt in some way to be able to be transmitted to chickens in nature. Previous studies revealed that sialyl Lewis X (3′SLeX), which is fucosylated α2,3 sialoside, was predominantly detected on the epithelial cells of the chicken trachea, whereas this glycan structure is not found in the duck intestinal tract. To clarify the mechanisms of the interspecies transmission of influenza viruses between ducks and chickens, we compared the receptor specificity of low-pathogenic avian influenza viruses isolated from these two species. Glycan-binding analysis of the recombinant hemagglutinin (HA) of a chicken influenza virus, A/chicken/Ibaraki/1/2005 (H5N2), revealed a binding preference to α1,3 fucosylated sialosides. On the other hand, the HA of a duck influenza virus, A/duck/Mongolia/54/2001 (H5N2) (Dk/MNG), particularly bound to non-fucosylated α2,3 sialosides such as 3′-sialyllactosamine (3′SLacNAc). Computational analysis along with binding analysis of the mutant HAs revealed that this glycan-binding specificity of the HA was determined by amino acid residues at positions 222 and 227. Inconsistent with the glycan-binding specificity of the recombinant HA protein, virions of Dk/MNG bound to both 3′SLacNAc and 3′SLeX. Glycan-binding analysis in the presence of a neuraminidase (NA) inhibitor revealed that the NA conferred binding to 3′SLeX to virions of Dk/MNG. The present results reveal the molecular basis of the interaction between fucosylated α2,3 sialosides and influenza viruses.

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Introduction

Influenza A viruses are zoonotic pathogens that are widely distributed among mammalian hosts such as humans, pigs and horses, as well as avian species such as chickens, ducks, many other poultry and wild birds [1]. The mechanism of the interspecies transmission of influenza viruses has been intensively analyzed for the control of human and animal influenza [2–6]. Influenza A virus is an enveloped virus that contains two envelope glycoproteins: hemagglutinin (HA) and neuraminidase (NA), both of which recognize sialoglycans [7–9]. HA is a viral lectin that specifically binds to terminal sialic acid (*N*-acetylneuraminic acid or *N*-glycolylneuraminic acid: Sia) linked to penultimate galactose (Gal) by an α2,3 or 2,6 linkage, and

it is responsible for the attachment of virions to host cell surface receptors. The glycan-binding specificity of HA varies depending on the host species from which the virus is isolated; avian influenza viruses preferentially bind α2,3 sialosides, whereas human influenza viruses prefer α2,6 sialosides [10, 11]. This specificity is consistent with the receptor distribution in host tissues; α2,3 sialosides are predominantly detected on epithelial cells of the duck intestine [12, 13], whereas α2,6 sialosides are predominantly detected on epithelial cells of the human trachea [14].

Influenza A viruses of each of the known subtypes (H1 to H16 and N1 to N9) have been isolated from water birds, particularly migratory ducks [15–17]. Therefore, migratory ducks are the natural hosts for influenza A viruses. Chickens are rarely infected directly with viruses isolated from ducks, although most influenza viruses isolated from chickens prefer α2,3 sialosides, similar to the viruses of duck origin [10, 18, 19]. It was previously reported that influenza viruses isolated from terrestrial poultry prefer fucosylated α2,3 sialosides such as sialyl Lewis X antigen (3′SLeX) or 6-O-sulfo-3′SLeX for the receptor [20]. In our previous study, we revealed that 3′SLeX was predominantly detected on epithelial cells of the upper respiratory tract of chickens, which is the primary replication site of chicken-adapted influenza viruses [21]. On the contrary, 3′SLeX was not detected on epithelial cells of duck colon, suggesting that α1,3 fucosylation of antepenultimate *N*-acetylglucosamine (GlcNAc) in α2,3 sialosides is a species barrier between ducks and chickens. Modifications on the antepenultimate GlcNAc of α2,3 sialosides is considered a key factor explaining differential susceptibility of ducks and chickens to influenza virus infection. A previous study on the structural basis of recognition of this particular glycan motif suggested that the fucose moiety of 3′SLeX is positioned close to the 220-loop of the HA [22]. The role of the 220-loop, particularly an amino acid residue at position 222, in recognition of α1,3 fucosylated sialosides has been discussed previously for H3, H5, and H7 HA [23–25]. However, the effects of this particular amino acid motif in the 220-loop on the glycan-binding properties of H5 HA are not known; thus, the detailed molecular basis of this interaction between H5 HA and α1,3 fucosylated sialosides should be analyzed.

The other envelope glycoprotein of influenza A virus, NA, cleaves terminal Sia from penultimate Gal, which is essential for virus release from host cells and also facilitates cell attachment by destroying "decoy" receptors [26, 27]. Previous studies suggested that the functional balance between HA (receptor binding) and NA (receptor destroying) contributes to the pathogenicity and host range of influenza A viruses [28]. Nevertheless, the mechanism by which NA contributes to virus–glycan interaction has not been extensively studied because information about the interaction of NA with modified α2,3 sialosides is still limited [29].

Here, we identified two amino acid residues, located at positions 222 and 227 in the HA of H5 influenza A viruses, that have a crucial role in recognition of 3′SLeX. In addition, the present results reveal a contribution of NA to the binding of virions of an influenza A virus to 3′SLeX.

Materials and methods

Viruses and cells

A/chicken/Ibaraki/1/2005 (H5N2) (Ck/IBR) [30] was kindly provided by the National Institute of Animal Health, National Agriculture and Food Research Organization, Tsukuba, Ibaraki, Japan. A/duck/Mongolia/54/2001 (H5N2) (Dk/MNG) was isolated from fecal samples of migratory ducks in Mongolia in 2001 [31]. The viruses were propagated in 10-day-old embryonated chicken eggs at 35 °C for 48 h, and the infectious allantoic fluids were used as virus stocks. Madin-Darby canine kidney (MDCK) cells were maintained in Minimum essential medium (MEM; Nissui Pharmaceutical, Tokyo, Japan) supplemented with 0.3 mg of L-glutamine, 100 U of penicillin G, 0.1 mg of streptomycin, and 8 µg of gentamicin per ml and 10 % calf serum. Human embryonic kidney (HEK) 293T cells were maintained in Dulbecco's modified Eagle's medium (D-MEM; Life Technologies, Carlsbad, CA, USA) supplemented with 0.3 mg of L-glutamine, 100 U of penicillin G, 0.1 mg of streptomycin, and 8 µg of gentamicin per ml and 10 % fetal calf serum. HEK 293S GnTI−/− cells were maintained in pyruvate-free D-MEM (Life Technologies) supplemented with 0.3 mg of L-glutamine, 10 U of penicillin G, and 0.01 mg of streptomycin per ml and 10 % fetal calf serum.

Reverse genetics

Eight genes from Ck/IBR and Dk/MNG each were cloned to produce viruses by reverse genetics as described previously [21, 32, 33]. Amino acid substitutions R222K and R227S in the HA of Ck/IBR, as well as K222R and S227R in the HA of Dk/MNG, were generated by site-directed mutagenesis using a QuikChange II site-directed mutagenesis kit (Agilent Technologies, Santa Clara, CA, USA) according to manufacturer's instructions (primer sequences are available upon request). The mutant viruses were rescued by reverse genetics. All eight segments of the genome were sequenced to confirm the existence of the introduced mutations and the absence of undesired mutations.

Expression of the recombinant HAs (rHAs)

The cDNAs of the HA genes of Ck/IBR, Dk/MNG and their mutants were cloned into the pCD5 expression vector as described previously [34]. The rHA proteins were expressed in HEK293S GnTI−/− cells [35] and purified from the cell culture supernatants as described previously [36].

Glycan microarray

The glycan microarray was carried out with 53 glycans, focused on α2,3 sialosides, which are the preferred receptors of avian influenza viruses. On the array slide, 10 non-sialylated glycans (glycans #1–10), 36 non-fucosylated α2,3 sialylated glycans (glycans #11–46) and 7 fucosylated and α2,3 sialylated glycans (glycans #47–53) were printed. Most of the glycans used were reported previously [34, 37], except for a few with extended glycan chains that were synthesized using methods reported previously (#31-35, 39-42, 51) [38, 39]. The rHA was used at 50 μg/ml pre-complexed with horseradish peroxidase (HRP)-linked anti-Strep-tag mouse antibody and with Alexa 488–linked anti-mouse IgG (4:2:1 molar ratio) prior to incubation for 30 min on ice in 100 μl of PBS containing 0.05 % Tween 20 (PBST) and incubated on the array surface in a humidified chamber for 90 min. Slides were subsequently washed by successive rinses with PBST, PBS and deionized H_2O. Washed arrays were dried by centrifugation and immediately scanned for fluorescence signals on a Perkin-Elmer ProScanArray Express confocal microarray scanner (Waltham, MA, USA). Fluorescence signal intensity was measured using Imagene (Biodiscovery, Hawthorne, CA, USA), and the mean intensity minus mean background was calculated and graphed using MS Excel. For each glycan, the mean signal intensity was calculated from six replicates. The highest and lowest signals of the six replicates were removed, and the remaining four replicates were used to calculate the mean signal and standard error (SE).

In silico prediction of the binding of the HA of Ck/IBR and 3′SLeX

The three-dimensional (3D) structure of the H5 HA of Ck/IBR was constructed on the basis of the HA crystal structure of A/Vietnam/1194/2004 (H5N1) (PDB code 2IBX) [40] as described previously [41]. A predicted 3D structure of 3′SLeX was downloaded from GLYCAM-Web (http://glycam.org/). To obtain the structure of HA of Ck/IBR bound to 3′SLeX, each structure was first superimposed on the crystal structure of the H2 HA in complex with 3′-sialyllactosamine (3′SLacNAc) (PDB code 2WR3) [42], and coordinates of 2WR3 were then removed using Discovery Studio version 4.1 (Dassault Systemes Biovia, San Diego, CA, USA). This model structure was refined by energy minimization followed by a 5 ns of molecular dynamics with the AMBER 14 software (Conflex USA, San Diego, CA, USA). The ff99SB force field and GLYCAM06 force field were used for the HA and 3′SLeX, respectively. The energy minimization and molecular dynamics calculations were conducted in a similar way, as described previously [43]. The definition of hydrogen bonds was as follows: distance between the donor and acceptor is less than 3.5 Å, and the angle between the donor, hydrogen and acceptor is greater than 120°.

Solid-phase direct binding assay

The receptor-binding specificity of viruses was assessed using a solid-phase direct binding assay with sialylglycopolymers 3′SLacNAc-PAA and 3′SLeX-PAA (Cosmo Bio Co., Ltd., Tokyo, Japan) as described previously [6, 21]. Briefly, each sialylglycopolymer was serially diluted and added to each well of a Universal-BIND™ 96-well polystyrene strip well microplate (Corning, Corning, NY, USA). Each well was blocked with 1 % bovine serum albumin (BSA) at room temperature for 1 h. After washing with PBST, an influenza virus suspension (16 HAU in PBS) was added to each well, and the plates were incubated at 4 °C for 12 h. After washing, mouse anti-HA monoclonal antibodies were added to each well, and the plates were incubated at 4 °C for 2 h. The wells were then washed and incubated with goat anti-mouse IgG-HRP conjugate (Bio-Rad, Hercules, CA, USA) at 4 °C for 2 h. After washing, 100 μl of the substrate solution including 0.5 mM 3,3′-tetramethylbenzidine (TMB) and 0.04 % H_2O_2, was added to each well. After incubation at room temperature for 10 min, the reactions were stopped using 50 μl of 2N H_2SO_4, and absorbance at 450/630 nm was measured using a MULTISCAN JX (Thermo Fisher Scientific, Waltham, MA, USA). The solid-phase direct binding assay was also performed in the presence of the NA inhibitor peramivir, which was a kind gift from Dr. Masanori Kobayashi of Shionogi & Co. Ltd. In this case, viruses were pre-incubated with either 2.5, 10 or 40 nM peramivir in PBS for 1 h on ice. The solution was used as a virus-containing solution.

A solid-phase direct binding assay was also conducted using rHA. Each sialylglycopolymer was serially diluted and added to each well of a Nunc Immobilizer Amino C8 96-well strip well microplate (Thermo Fisher Scientific), and the plates were incubated at 37 °C for 1 h. Each well was then blocked with PBST containing 2 % BSA at room temperature for 3 h. The rHA was used at 5 μg/ml, pre-complexed with HRP-linked anti-Strep-tag mouse antibody (1:2000 dilution) and with goat anti-mouse IgG-HRP

conjugate (1:1000 dilution) prior to incubation for 30 min on ice in PBST containing 0.5 % BSA with or without 40 nM peramivir. After washing the plates, the complexes were added and incubated at room temperature for 1 h. After washing, 100 µl of TMB substrate solution was added to each well. The reactions were stopped using 50 µl of 2N H_2SO_4, and absorbance at 450/630 nm was measured using a MULTISCAN JX.

Amino acid sequence comparison of the H5 HA

A total of 2901 amino acid sequences of H5 HA were obtained from GenBank. Sequence data were aligned with mafft version 7.215 (http://mafft.cbrc.jp/alignment/software/). Amino acid sequence data for highly pathogenic avian influenza viruses were identified based on the sequence of the HA cleavage site and removed from the dataset. The remaining 631 sequences were further divided into viruses isolated from Anseriformes, non-chicken Galliformes or chickens according to the original host of each virus given in the strain name.

Results

Computational analysis of the binding of the HA of a chicken influenza A virus to 3′SLeX

The binding model of an HA derived from a chicken influenza A virus, Ck/IBR, to 3′SLeX was predicted using *in silico* analysis (Fig. 1A and B). The fucose moiety of 3′SLeX is positioned close to two arginine (R) residues at positions 222 and 227 (H3 numbering is used throughout) of the HA. The R residues at positions 222 and 227 are located within 3.5 Å of the C-2 and C-3 hydroxyl groups of the fucose, indicating potential hydrogen bonding. By comparing the amino acid sequence of Ck/IBR with that of a duck influenza virus, Dk/MNG, we observe a lysine (K) residue at position 222 and a serine (S) residue at position 227 instead of arginines. We hypothesized that these amino acid substitutions contribute to differences in receptor-binding specificity.

Glycan-binding specificity of the HA of Ck/IBR, Dk/MNG and their mutants

Soluble trimeric recombinant HAs (rHA) of Ck/IBR and Dk/MNG were generated and subjected to glycan microarray analysis to evaluate their glycan-binding specificity (Fig. 2A and B). The rHA of Ck/IBR interacted with glycans #51 (3′SLeXTriLN-Core4) and #52 [Siaα2,3Galβ1,4(Fucα1,3)(6O-sulfo)GlcNAcβ-propyl-NH2] with high relative avidity. Although the rHA of Ck/IBR slightly interacted with non-fucosylated α2,3 sialosides, these signals were much weaker than those of glycans #51 and #52. No interaction was observed between the rHA of Dk/MNG and fucosylated α2,3 sialosides. Interestingly, most of the glycans, which were bound by the rHA of Dk/MNG, are biantennary glycans with multiple lactosamine (LacNAc) repeats (e.g., #30-32 and #35). The results indicate that the rHA of Ck/IBR selectively binds α1,3 fucosylated sialosides, whereas that of Dk/MNG selectively binds non-fucosylated α2,3 sialosides. To evaluate the contribution of amino acid residues at positions 222 and 227 of the HA to this binding specificity of rHAs, we generated mutant rHAs of Ck/IBR and Dk/MNG in which amino acid residues at positions 222 and 227 were altered (IBR-R222K,R227S and MNG-K222R,S227R, respectively) and subjected these rHAs to glycan microarray analysis (Fig. 2C and D). The rHA of IBR-R222K,R227S interacted with non-fucosylated α2,3 sialosides, whereas no binding was observed with fucosylated

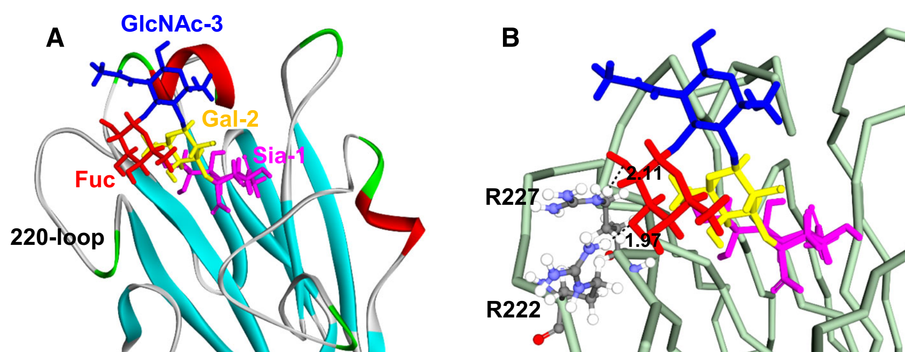

Fig. 1 Structure model of sialyl Lewis X (3′SLeX) bound to the hemagglutinin (HA) of Ck/IBR, which was taken from a snapshot at 5 ns. The receptor-binding site of the HA of Ck/IBR is shown in either a ribbon (A) or line (B) cartoon representation. In the glycan structure, *N*-acetylneuraminic acid (Sia) is shown in purple, galactose (Gal) is shown in yellow, *N*-acetylglucosamine (GlcNAc) is shown in blue, and fucose (Fuc) is shown in red. In panel B, dashed lines indicate hydrogen bonds, and the numbers indicate the predicted length of each hydrogen bond (color figure online)

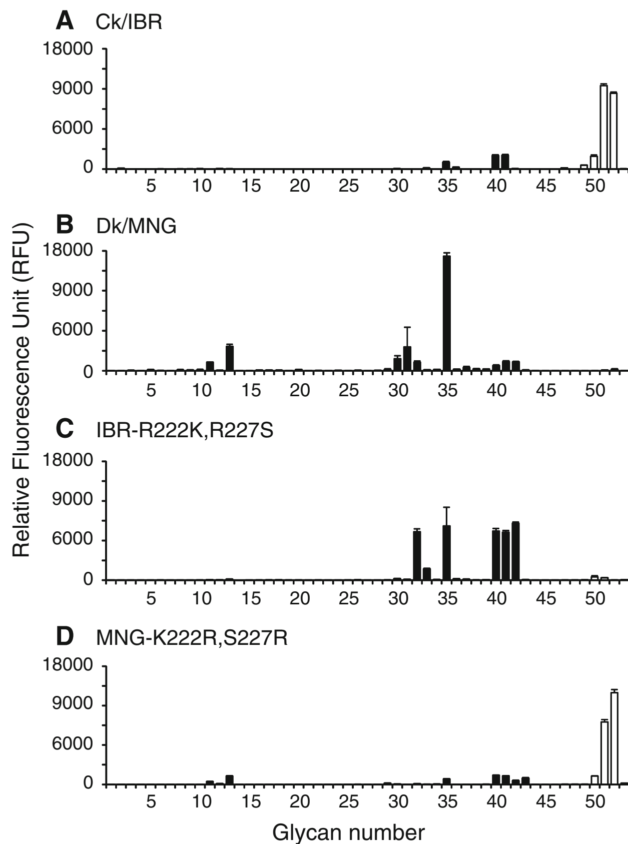

Fig. 2 Glycan-binding specificity of the soluble trimeric recombinant hemagglutinins (rHAs). The glycan-binding specificity of rHA of Ck/IBR (A), Dk/MNG (B), IBR-R222K,R227S (C) and MNG-K222R,S227R (D) was analyzed by glycan microarray. Non-sialylated controls are shown as grey bars (#1–10), non-fucosylated α2,3 sialylated glycans are presented as black bars (#11–46), and fucosylated and α2,3 sialylated glycans are shown in white bars (#47–53). Each bar represents the mean signal minus background for each glycan sample, and error bars represent the SE value. Glycans imprinted on the array are listed in Table S1

α2,3 sialosides. The rHA of MNG-K222R,S227R interacted with glycans #51 and #52 with high specificity, whereas almost no binding was observed with non-fucosylated α2,3 sialosides. These results indicate that the glycan-binding specificity of rHA for fucosylated and non-fucosylated α2,3 sialosides is determined by the amino acid motifs at positions 222 and 227 of the HA.

Glycan-binding specificity of the virions of Ck/IBR, Dk/MNG and their mutants

In our previous study, virions of Ck/IBR specifically bound to α1,3 fucosylated sialosides and 3′SLeX in a solid-phase direct binding assay, whereas those of Dk/MNG bound to both non-fucosylated α2,3 sialosides, 3′-sialyllactosamine (3′SLacNAc) and 3′SLeX [21]. To investigate the importance of the amino acid motifs at positions 222 and 227 of HA, mutant viruses, namely rgIBR/HA-R222K,R227S and rgMNG/HA-K222R,S227R, were generated, and their receptor-binding preferences were characterized using a solid-phase direct binding assay (Fig. 3). Consistent with our previous study, virions of Ck/IBR specifically bound to 3′SLeX, whereas those of Dk/MNG bound to both 3′SLacNAc and 3′SLeX. rgIBR/HA-222K,227S bound to both 3′SLacNAc and 3′SLeX, indicating that the R222K and R227S mutations facilitate binding of non-fucosylated glycans. rgMNG/HA-222R,227R also bound to both 3′SLacNAc and 3′SLeX; however, the binding avidity to 3′SLacNAc was significantly lower than that to 3′SLeX.

Dk/MNG virions bind to fucosylated α2,3 sialosides through the neuraminidase

The rHA of Dk/MNG specifically bound to non-fucosylated α2,3 sialosides, whereas virions of Dk/MNG bound to both fucosylated and non-fucosylated sialosides (Fig. 2B and 3B). To investigate the contribution of NA, which is the other Sia-interacting viral protein of influenza A viruses, to glycan binding by influenza virus virions, a solid-phase direct binding assay was conducted in the presence of the NA inhibitor peramivir (Fig. 4). Binding of Dk/MNG to 3′SLeX was diminished in the presence of 2.5 nM peramivir. Although binding to 3′SLacNAc was slightly affected in the presence of a higher concentration of peramivir, almost no binding to 3′SLeX was observed in the presence of 10 or 40 nM peramivir. To confirm that peramivir specifically inhibits glycan binding by NA and has no effect on the glycan-binding mediated by HA in the assay, a solid-phase direct binding assay was conducted with the rHA of Dk/MNG in the presence of peramivir (Fig. 5). Consistent with the results from the glycan microarray (Fig. 2B), the rHA of Dk/MNG preferentially bound 3′SLacNAc in the absence of peramivir (Fig. 5A). The binding of the rHA to 3′SLacNAc and 3′SLeX was similar in the presence of 40 nM peramivir, and no inhibition was observed (Fig. 5B). These results confirmed that peramivir has no effect on the interaction between sialosides and the rHA of Dk/MNG. Accordingly, the present result indicates that the NA of Dk/MNG binds to fucosylated α2,3 sialosides.

Amino acid sequence comparison of HA of H5 avian influenza viruses

The amino acid motifs at positions 222 and 227 in HA of H5 avian influenza viruses isolated from members of the avian orders Galliformes and Anseriformes were analyzed (Table 1). The majority of low-pathogenic avian influenza viruses (LPAIVs) isolated from chickens have a glutamine (Q) residue at position 222 and an arginine (R) residue at

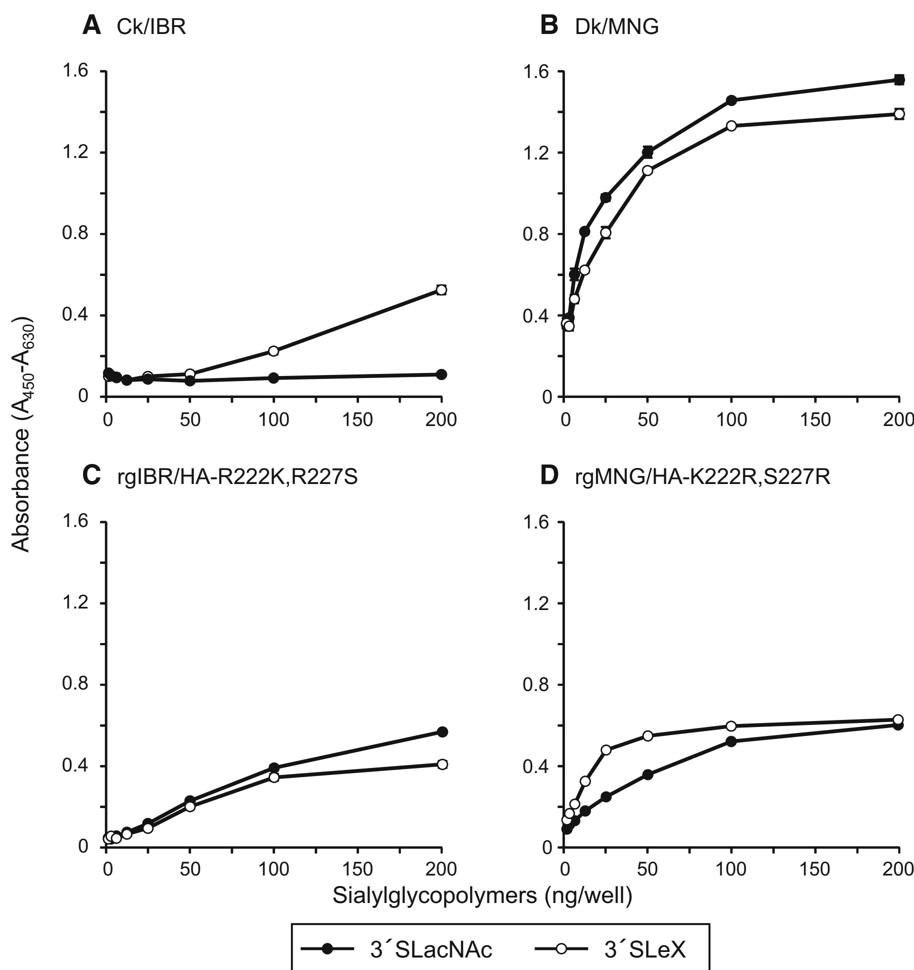

Fig. 3 Glycan-binding specificity of virions. The glycan-binding specificity of Ck/IBR (A), Dk/MNG (B), rgIBR/HA-R222K,R227S (C), and rgMNG/HA-K222R,S227R (D) for sialylglycopolymers containing 3′sialyllactosamine (3′SLacNAc, black circles) and sialyl Lewis X (3′SLeX, white circles) was investigated using a solid-phase direct binding assay. The data are presented as the mean ± SE of triplicate experiments

position 227. On the other hand, the majority of viruses isolated from members of the Anseriformes have a lysine (K) residue at position 222 and a serine (S) residue at position 227. Interestingly, most of the viruses isolated from non-chicken Galliformes have a K residue at position 222 and an S residue at position 227, which is the same amino acid sequence present in isolates from Anseriformes. An R residue at position 222 as observed in Ck/IBR is a relatively rare motif in H5 avian influenza viruses. We also generated mutant viruses possessing the amino acid residues that are present in the consensus motif of chicken LPAIVs at positions 222 and 227 of the HA, namely rgIBR/HA-R222Q and rgMNG/HA-K222Q,S227R. Glycan-binding analysis by solid-phase direct binding assay revealed that rgIBR/HA-R222Q specifically bound to 3′SLeX (Fig. 6A). Also, the glycan-binding preference of rgMNG/HA-K222Q,S227R was similar to that of rgMNG/HA-222R,227R (Fig. 6B). These results indicate that Q and R residues at position 222 of the HA have similar effects on the glycan-binding properties of HA in combination with the R residue at position 227.

Discussion

We previously reported that α2,3 sialosides expressed on epithelial cells of the chicken trachea are fucosylated [21]. Although modification of α2,3 sialosides has a critical impact on the glycan-binding specificity of influenza viruses [20, 21, 23], the interaction of these modified α2,3 sialosides with HA is not fully understood. In the present study, we analyzed the binding specificity of two H5 LPAIVs, Ck/IBR and Dk/MNG, to fucosylated and non-fucosylated α2,3 sialosides and found that R222 and R227 in the HA of Ck/IBR are located close to the α1,3 fucose linked to antepenultimate GlcNAc (Fig. 1). A previous structural analysis indicated that this fucose moiety is positioned close to K222 in the HA of a highly pathogenic avian influenza virus and that it destabilized the interaction of the HA with glycan [22]. Glycan microarray analysis of rHAs revealed that the K222R substitution in combination with S227R in the HA of Dk/MNG altered its glycan-binding specificity from non-fucosylated to fucosylated α2,3 sialosides (Fig. 2B and D). This suggests that these

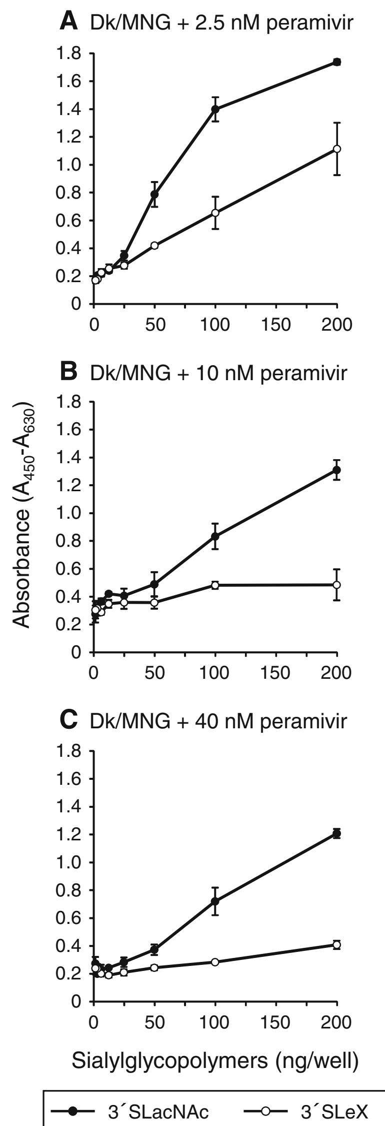

Fig. 4 Glycan-binding specificity of virions of Dk/MNG in the presence of peramivir. The glycan-binding specificity of virions of Dk/MNG to sialylglycopolymers containing 3′sialyllactosamine (3′SLacNAc, black circles) and sialyl Lewis X (3′SLeX, white circles) was investigated in the presence of either 2.5 (A), 10 (B) or 40 (C) nM neuraminidase inhibitor peramivir. The data are presented as the mean ± SE of triplicate experiments

substitutions in HA alter the interaction with fucose and stabilize the binding.

Sequence comparison of HA of H5 influenza A viruses revealed that the amino acid motif K222 and S227 were highly conserved among LPAIVs circulating among migratory ducks (Table 1). Receptor-binding analysis indicated that viruses with K222 and S227 specifically bound to non-fucosylated α2,3 sialosides, whereas viruses with R222 and R227 as well as Q222 and R227 specifically bound fucosylated α2,3 sialosides (Figs. 3 and 6). This suggests that H5 LPAIVs isolated from chickens prefer fucosylated α2,3 sialosides and that those isolated from ducks prefer non-fucosylated α2,3 sialosides. Interestingly, viruses isolated from non-chicken Galliformes species, such as quails or turkeys have the same amino acid motif as LPAIVs isolated from Anseriformes. Influenza viruses circulating among migratory ducks are transmitted to chickens via terrestrial poultry such as quails or turkeys [1, 44]. This suggests that the acquisition of "chicken-type receptor specificity" may not occur during adaptation in ducks or later in non-chicken terrestrial poultry but occurs during the multiple replication and transmission events in chickens.

Glycan-binding analysis of virions and rHAs revealed different glycan-binding preferences (Fig. 2 and 3). Interestingly, virions of Dk/MNG bound to both fucosylated α2,3 sialosides, 3′SLeX and non-fucosylated α2,3 sialosides, 3′SLacNAc, whereas the rHA of Dk/MNG bound only non-fucosylated α2,3 sialosides. A solid-phase direct binding assay in the presence of peramivir suggests that the NA contributes to the receptor-binding specificity of Dk/MNG virions to 3′SLeX (Fig. 4). A solid-phase direct binding assay using rHA confirmed that the rHA of Dk/MNG preferentially bound non-fucosylated α2,3 sialosides (Fig. 5A). The presence of peramivir had no effect on the glycan-binding of the rHA of Dk/MNG (Fig. 5B), indicating that peramivir specifically inhibited glycan-binding of the NA in the solid-phase direct binding assay using virions of Dk/MNG (Fig. 4). Accordingly, we concluded that the glycan-binding specificity of virions of Dk/MNG results from additive effects of glycan-binding to 3′SLacNAc mediated by the HA and to 3′SLeX mediated by the NA, and thus, the binding to 3′SLeX was diminished by the NA inhibitor. NA of influenza viruses has sialidase activity, which means that NA also binds sialylated glycans [7, 9]. In the case of H7N9 influenza viruses recently isolated in China, it was proposed that the preferential cleavage of α2,3 sialosides by the NA along with intrinsic weak binding to α2,6 sialosides by the HA exaggerated the receptor-binding preference of these viruses for α2,6 sialosides [34]. Thus, the NA modulates the receptor-binding preference of influenza virus by eliminating certain receptor interactions through its sialidase activity. The NA of Dk/MNG modulates receptor specificity via a mechanism that is distinct from the previous model, as the NA obviously promotes binding to 3′SLeX (Fig. 2B and 3B). However, we could not determine whether glycan binding by the NA is functional, i.e., that it would lead to infection. Thus, determining the receptor-binding preferences of influenza viruses requires complementary assays using rHA and complete virions to elucidate the fine receptor binding preferences of influenza viruses.

It should also be pointed out that the rHA of Dk/MNG did not exhibit binding to glycan #15 (Fig. 2B), which has

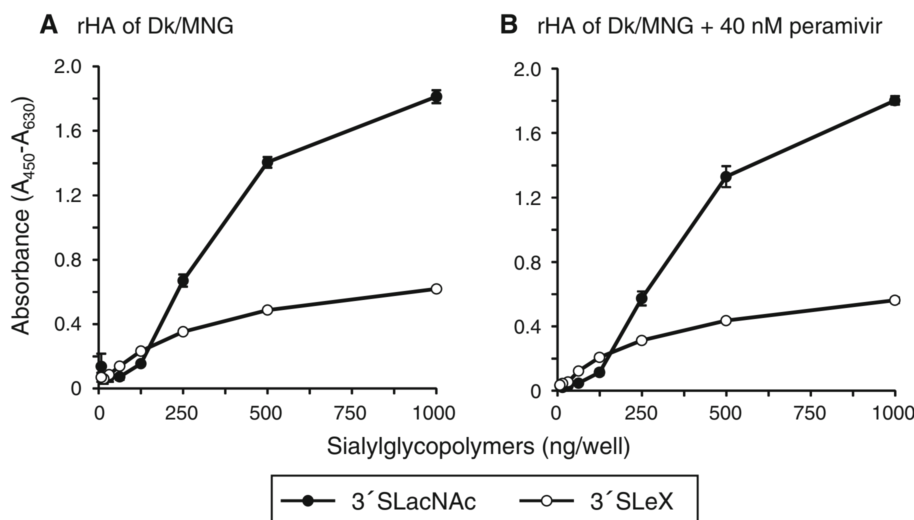

Fig. 5 Glycan-binding specificity of the rHA of Dk/MNG in the presence of peramivir. The glycan-binding specificity of the rHA of Dk/MNG to sialylglycopolymers containing 3′sialyllactosamine (3′SLacNAc, black circles) and sialyl Lewis X (3′SLeX, white circles) was investigated in the absence (A) or presence (B) of a neuraminidase inhibitor peramivir. The data are presented as the mean ± SE of triplicate experiments

Table 1 Amino acid sequence comparison of 222/227 motifs in HA of H5 LPAIVs

Host	Amino acid residue		Number of strains
	222	227	
Chicken (98 strains)	Q	R	70
	K	S	26
	R	S	1
	R	**R**	1
Anseriformes (506 strains)	**K**	**S**	471
	R	S	18
	Q	S	13
	E	R	2
	N	S	1
	R	R	1
Non-chicken Galliformes (27 strains)	K	S	25
	R	S	1
	Q	R	1

Amino acid sequences of H5 HA were obtained from GenBank

Amino acid motifs of Ck/IBR and Dk/MNG are shown in bold

exactly the same glycan structure with the non-fucosylated α2,3 sialoside as that used in the solid-phase direct binding assay. Virions of Dk/MNG strongly bound this glycan structure in the solid-phase direct binding assay (Fig. 3B). In the case of the solid-phase direct binding assay, glycans are polymerized with polyacrylamide. In addition, we used whole virus particles for the solid-phase direct binding assay. Therefore, the interactions of HA and glycans are multivalent in this assay. On the other hand, monomeric glycans were printed in the case of the glycan microarray. These difference in the settings of each assay may have led to the observed differences in the results of these two assays.

The present results reveal the molecular basis of the interaction between α1,3 fucosylated sialosides and HA of influenza viruses. α2,3 sialosides are structurally divergent; the penultimate Gal and the antepenultimate GlcNAc can be sulfated, the linkage between Gal and GlcNAc can be a β1,3 or a β1,4 linkage, and the core structure of glycans can be N-linked, O-linked or glycolipid. It has been reported that the presentation and internal complexity of the glycan structure influence their interaction with HA (See ref. 39 and references therein). Indeed, we found that the internal structure of glycans affected binding specificity of HA; the rHA of Dk/MNG preferred non-fucosylated α2,3 sialosides with multiple LacNAc repeats (Fig. 2B). Similarly, rHA of Ck/IBR bound to glycan #51 with high avidity, whereas binding to glycan #47, 49 and 50, which contain the same glycan sequence with glycan #51 on the non-redacting end, was much weaker (Fig. 2A). The importance of the internal glycan structure for influenza

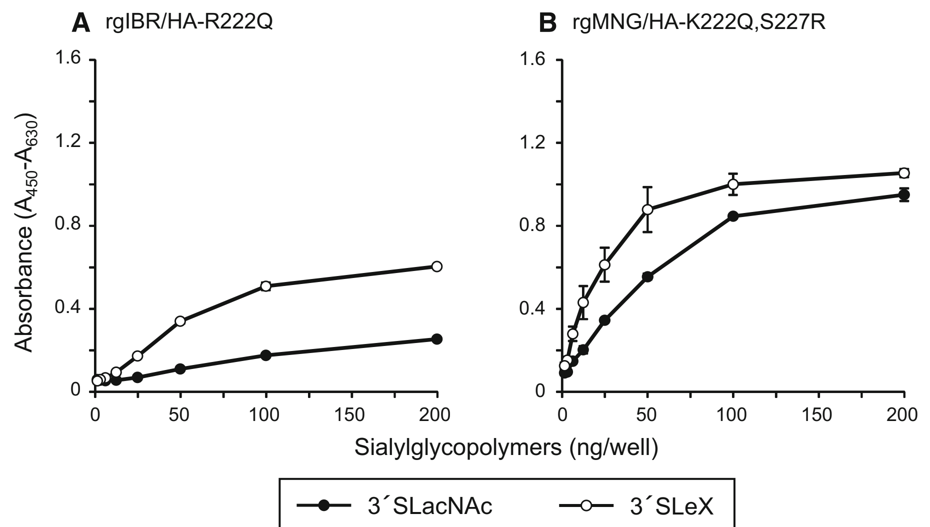

Fig. 6 Glycan-binding specificity of rgIBR/HA-R222Q and rgMNG/HA-K222Q,S227R. Glycan-binding specificity of virions of rgIBR/HA-R222Q (A) and rgMNG/HA-K222Q,S227R (B) to sialylglycopolymers containing 3′sialyllactosamine (3′SLacNAc, black circles) and sialyl Lewis X (3′SLeX, white circles) was investigated using a solid-phase direct binding assays. The data are presented as the mean ± SE of triplicate experiments

virus infection is not at all understood, even though glycan microarray data on the receptor-binding specificity of influenza viruses are widely available. The present results demonstrate the significance of the structural diversity of sialosides on the interaction of influenza A viruses with host cell surface receptors.

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