Substitution of amino acid residue in influenza A virus hemagglutinin affects recognition of sialyl-oligosaccharides containing *N*-glycolylneuraminic acid

Hiroyuki Masuda[a], Takashi Suzuki[a], Yoshikuni Sugiyama[a], Goh Horiike[a], Kohji Murakami[a], Daisei Miyamoto[a], Kazuya I.-P. Jwa Hidari[a], Toshihiro Ito[b], Hiroshi Kida[c], Makoto Kiso[d], Kyoko Fukunaga[d], Masanobu Ohuchi[e], Tetsuya Toyoda[f], Akira Ishihama[g], Yoshihiro Kawaoka[h], Yasuo Suzuki[a,*]

[a]*Department of Biochemistry, University of Shizuoka, School of Pharmaceutical Sciences, Shizuoka 422-8526, Japan*
[b]*Department of Veterinary Public Health, Faculty of Agriculture, Tottori University, Tottori 680-8553, Japan*
[c]*Department of Disease Control, Graduate School of Veterinary Medicine, Hokkaido University, Sapporo 060-0818, Japan*
[d]*Department of Applied Bioorganic Chemistry, Gifu University, Gifu 501-1193, Japan*
[e]*Department of Microbiology, Kawasaki Medical School, Kurashiki 701-0192, Japan*
[f]*Department of Virology, Kurume University, School of Medicine, Kurume 830-0011, Japan*
[g]*Department of Molecular Genetics, National Institute of Genetics, Mishima 411-8540, Japan*
[h]*Department of Pathobiological Science, School of Veterinary Medicine, University of Wisconsin-Madison, Madison, WI 53706, USA*

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Abstract Sialic acids are essential components of cell surface receptors used by influenza viruses. To determine the molecular mechanisms of viral recognition of two major species of sialic acids, *N*-acetylneuraminic acid (Neu5Ac) and *N*-glycolylneuraminic acid (Neu5Gc), we tested the binding reactivity of nine human H3 influenza A viruses to sialylglycolipids containing type II sugar chain and different molecular species of terminal sialic acids. All human H3 viruses tested except A/Memphis/1/71 bound both Neu5Ac and Neu5Gc. Nucleotide sequence analysis suggests that amino acids at 143, 155, and 158 are linked to the viral recognition of Neu5Gc.

Key words: Influenza virus; Hemagglutinin; Sialic acid; Sialyl-oligosaccharide; *N*-Glycolylneuraminic acid

1. Introduction

Influenza virus infection is initiated by attachment of virus to host cell receptors that are thought to be sialic acid containing glycoproteins and glycolipids. Sialyl-oligosaccharide moieties of cell surface glycoproteins and glycolipids exhibit significant structural diversity, and numerous studies indicate that the ability of sialic acid to serve as a receptor determinant influenza virus may be influenced by the carbohydrate structure and sialic acid moiety of sialylglycoproteins [1–5], gangliosides [6–9], or sialyl-oligosaccharides [10–13] to which it is attached. There are about 30 derivatives of sialic acid in nature [14]. Two major sialic acids are 5-*N*-acetylneuraminic acid (Nue5Ac) and 5-*N*-glycolylneuraminic acid (Neu5Gc) which are chemically distinguished by a functional group at the C-5 position [15]. Neu5Gc is derived from Neu5Ac by enzymatic hydroxylation of the *N*-acetyl group of CMP-Neu5Ac [16–18]. Glycoconjugates containing Neu5Gc are widely found in many animals [14], however they have not yet been detected in normal human tissues. Understanding the molecular mechanisms of sialic acid recognition will help to clarify the roles of sialic acids on cell tropism and transmission of viruses. Moreover, the observation would provide useful information for development of anti-influenza virus agents.

It was reported that the amino acid change at position 155 of hemagglutinin (HA) 1 from Thr to Tyr played a critical role in the recognition of Neu5Gcα2-6Gal [19]. Over the last decade, we have examined the binding reactivity of influenza A viruses isolated from various animal species by several virus binding assays [2,6–9]. Recently, we found that equine and swine influenza A viruses recognized glycolipids containing Neu5Gcα2-3Gal sequences [20,21]. The nucleotide sequences of HA available in GenBank showed that amino acid Thr at 155 of HA1 was conserved in all equine strains tested, and that the amino acid of swine influenza A viruses was Thr or Tyr. In this report, we investigated the binding reactivity of human H3 influenza A viruses to four types of sialylglycolipids which had varied molecular species of terminal sialic acids and sialyl linkage, and found that amino acid substitutions at 143 and 158 are also linked to the viral recognition of Neu5Gc.

2. Materials and method

2.1. Sialylglycolipids

The synthetic sialylglycolipids commonly had Galβ1-4GlcNAcβ1-3Galβ1-4Glc as a core structure, a sialic acid residue (Neu5Ac or Neu5Gc) linked to a non-reducing galactose terminal, and the ceramide portion substituted by a branched hydrocarbon chain containing 30 carbons (Table 1). All of the compounds were synthesized [22] essentially by the procedure described by Kameyama et al. [23], namely, glycosylation of the GlcNAcβ1-3Galβ1-4Glc acceptor [23] with the Neu5Acα2-3Gal [23], Neu5Acα2-6Gal [24], Neu5Gcα2-3Gal [25], and Neu5Gcα2-6Gal donors, and subsequent introduction of the lipid tail to the reducing end [26].

*Corresponding author.
E-mail: suzukiy@ys7.u-shizuoka-ken.ac.jp

Abbreviations: HA, hemagglutinin; HAU, hemagglutination unit; Neu5Ac, 5-*N*-acetylneuraminic acid; Neu5Gc, 5-*N*-glycolylneuraminic acid; TLC, thin-layer chromatography; RBS, receptor binding site

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2.2. Viruses

Seeds of all influenza A viruses were gifts from the Department of Disease Control, Graduate School of Veterinary Medicine, Hokkaido University and the Department of Virology and Molecular Biology, St. Jude Children's Research Hospital. Viruses were grown in 10-day-old embryonated chicken eggs and were purified by sucrose density gradient centrifugation. Viral hemagglutination units (HAU) were determined at 4°C in microtiter plates as previously described [6].

2.3. Binding assay

The binding reactivity of influenza A viruses to sialic acid-containing glycolipids was examined by thin-layer chromatography (TLC)/virus binding assay. Synthetic sialylglycolipids (500 pmol) were developed in silica gel TLC plates by chloroform/methanol/12 mM aqueous $MgCl_2$ (5/4/1, v/v). TLC plates were immersed in phosphate buffered saline containing 1% egg albumin and 1% polyvinylpyrrolidone. TLC plates were then overlaid with influenza virus suspension (2^8 HAU) for 12–16 h at 4°C. TLC plates were incubated with rabbit anti-influenza virus antiserum, followed by horseradish peroxidase-conjugated protein A. Finally, the plates were visualized as described in [20].

2.4. Sequence determination

To determine the nucleotide sequences, RT-PCR was performed. HA genes of influenza viruses were amplified using the primers 5′H3 (5′-AGCAAAAGCAGGGGATAA-3′, sense) and 3′H3 (5′-TGTTGCACCTAATGTTGC03′, antisense). PCR products from viruses were sequenced with an automatic sequencer (Applied Biosystems Inc.).

3. Results and discussion

We investigated the binding reactivity of nine human H3 influenza A viruses to four types of synthetic sialylglycolipids which had varied molecular species of terminal sialic acids and sialyl linkages, such as Neu5Acα2-3Gal, Neu5Acα2-6Gal, Neu5Gcα2-3Gal and Neu5Gcα2-6Gal (Table 1). All human H3 influenza A virus strains recognized the Neu5Acα2-6Gal linkage more strongly than Neu5Acα2-3Gal as described previously [3,4,27–29]. Eight strains bound glycolipids containing both types of sialic acid residues, Neu5Acα2-6Gal and Neu5Gcα2-6Gal. On the other hand, A/Memphis/1/71 (H3N2) bound only glycolipids containing Neu5Ac, but not Neu5Gc (Fig. 1). In further experiments, we carried out TLC/virus binding assays with various concentrations of synthetic glycolipids (500–1500 pmol) and a high concentration of the virus (2^{12} HAU). In both cases, A/Memphis/1/71 showed no binding to glycolipids containing Neu5Gc (data not shown). To investigate the amino acid residues of HA involved in recognition of Neu5Gc, we determined the nucleotide sequences of all tested influenza viruses including A/Tokyo/6/73, A/Kumamoto/55/76 and A/Yamanashi/2/77 which were not available in the GenBank data base. Fig. 2 shows the deduced amino acid sequences of HA1. We noticed that the amino acids of HA were similar between A/Aichi/2/68 and A/Memphis/1/71, but binding reactivities to Neu5Gc were different. We compared the amino acid sequence of HA1 of A/Memphis/1/71 with that of A/Aichi/2/68. The sequences were almost identical except for seven changes at positions 2, 31, 78, 143, 144, 158, and 319. However, both viruses had conserved Thr-155. We assume that amino acids which vary from A/Memphis/1/71 to A/Aichi/2/68 will affect the ability to bind Neu5Gc.

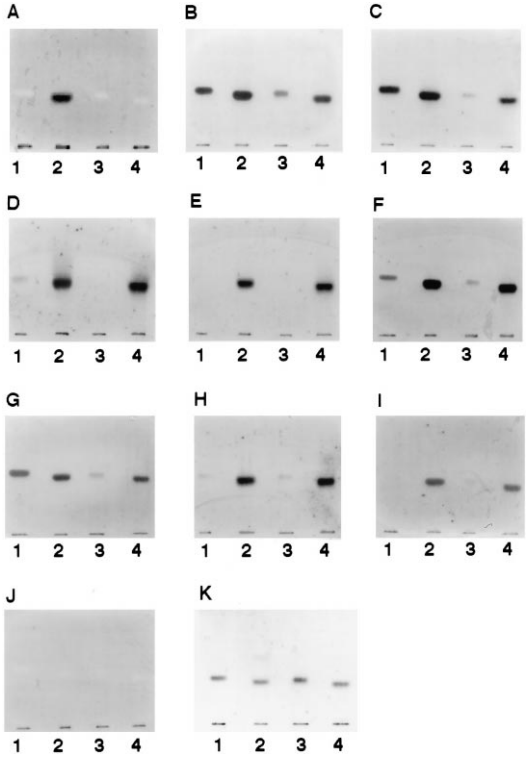


Fig. 1. Binding reactivity of influenza A viruses to sialylglycolipids was determined as described in Section 2 by the TLC/virus binding assay. A: A/Memphis/1/71 (H3N2); B: A/Aichi/2/68 (H3N2); C: A/Hongkong/1/68 (H3N2); D: A/Memphis/102/72 (H3N2); E: A/Tokyo/6/73 (H3N2); F: A/Kumamoto/55/76 (H3N2); G: A/Yamanashi/2/77 (H3N2); H: A/Texas/1/77(H3N2); I: A/Bangkok/1/79 (H3N2); J: no virus; K: chemical staining sprayed orcinol. Lanes: 1, IV^3(Neu5Ac)nLc_4B30; 2, IV^6(Neu5Ac)nLc_4B30; 3, IV^3(Neu5Gc)-nLc_4B30; 4, IV^6(Neu5Gc)nLc_4B30.

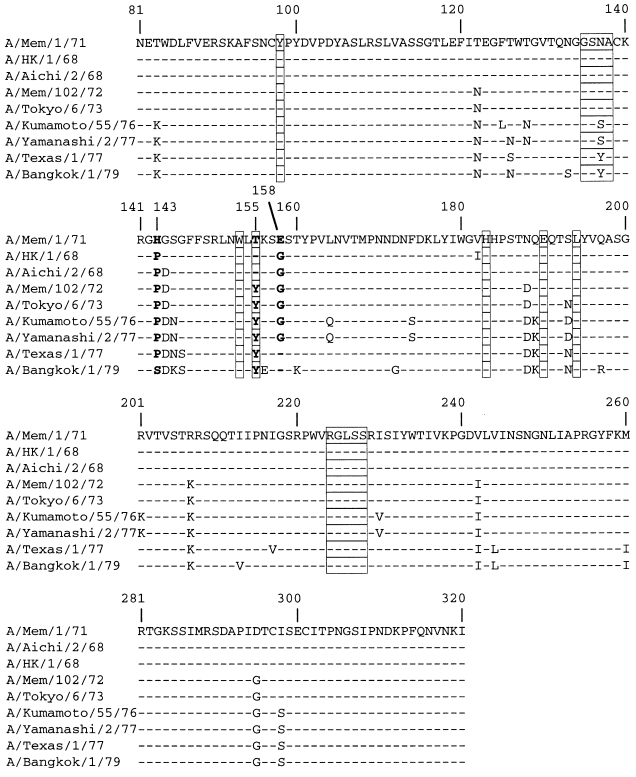


Fig. 2. The deduced amino acid sequences of HA1 regions were determined. The single letter codes for amino acids are used. Boxed indicates receptor binding site. The amino acids at positions 155, 158 and 143 are shown in bold.

The tertiary structure of the influenza A virus HA (X-31) complexed with sialic acid has already been elucidated [12,30,31]. The receptor binding site (RBS) was a depression, the bottom of which is formed by the phenolic hydroxy group of Tyr-98 and aromatic rings of Trp-153. Gln-190 and Leu-194 project down from a short helix to define the rear of the site with His-183 and Thr-155. Amino acid residues 134–138 form the right side and residues 224–228 form the left side of the binding site (Fig. 3).

The amino acid residues at 2, 31, 78, and 319 of HA are distant from the RBS. Thus, we did not think that these amino acid changes affect the recognition of Neu5Gc. The amino acids at 143, 144 and 158 do not take part in RBS, but are located near the RBS. To understand the influence of these amino acids in recognition, we compared the amino acid sequence of A/Memphis/1/71 with that of the other H3 viruses tested. In other viruses, His-143 was replaced by Pro or Ser. Gly-144 was substituted by Asp but was preserved in A/Hongkong/1/68 which could recognize both types of sialic acid residues. These data suggest that the amino acid at 143 in HA affects the recognition of Neu5Gc. As to the amino acid at position 158, Glu-158 was preserved in A/Bangkok/1/79 and A/Texas/1/77, but was changed in other virus strains. However, the binding reactivity of A/Memphis/1/71 differed from the binding reactivity of A/Bangkok/1/79 and A/Texas/1/71. A previous study showed that amino acid substitution at position 155 from Thr to Tyr played a critical role in recognition of Neu5Gc [19]. Viruses isolated after 1972 showed the ability to bind Neu5Gc, because these strains have Tyr-155 in HA. A/Aichi/2/68 and A/Hongkong/1/68 bound Neu5Gc-containing glycolipids, although they were isolated before 1972. In these viruses, Gly-158 played a critical role in recognition of Neu5Gc. We suppose that the amino acid at position 158 influences the structural features around this position of RBS. These data indicate that not only the amino acid at

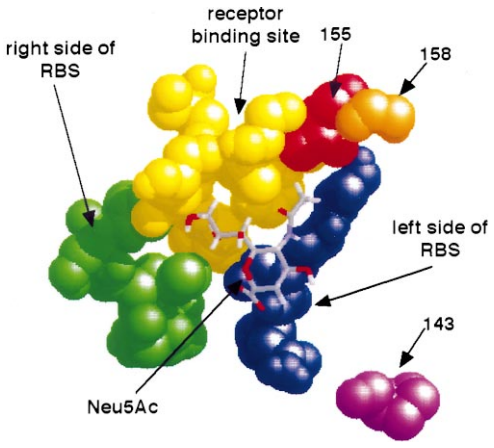
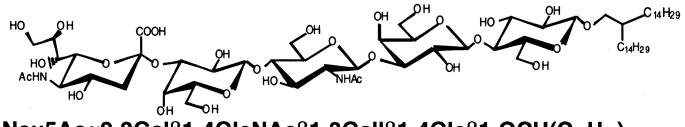
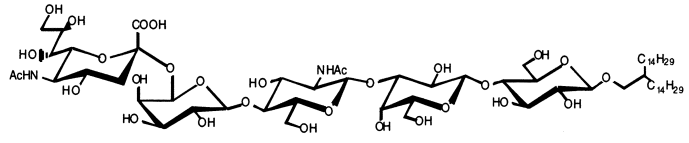
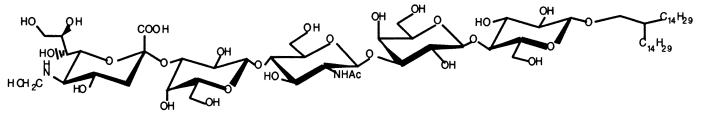
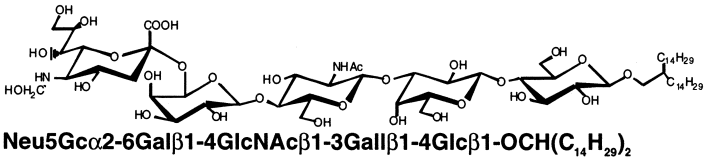


Fig. 3. Amino acids in the RBS of influenza H3 virus are shown on the model of the X-31 HA complexed with Neu5Ac. Yellow atoms are the receptor binding site (98, 153, 155, 190, 194); blue atoms are the left side of the receptor binding site (134–138); green atoms are the right site of the receptor binding site (224–228); the amino acid at position 155 is red; the amino acid at 158 is orange; the amino acid at 143 is purple. The amino acids at 158 and 143 are located close to the RBS, and influenced the recognition of Neu5Gc. This figure is based on the H3 HA structure of X-31 (H3N2) complexed with sialic acid (PDB ID: 1HGG structure) as determined by Sauter et al. [30].

Table 1
Carbohydrate structures of synthetic glycolipids

Name	Chemical Structures of Glycolipids
$IV^3(Neu5Ac)nLc_4B30$	 Neu5Acα2-3Galβ1-4GlcNAcβ1-3Galβ1-4Glcβ1-OCH($C_{14}H_{29}$)$_2$
$IV^6(Neu5Ac)nLc_4B30$	 Neu5Acα2-6Galβ1-4GlcNAcβ1-3Galβ1-4Glcβ1-OCH($C_{14}H_{29}$)$_2$
$IV^3(Neu5Gc)nLc_4B30$	 Neu5Gcα2-3Galβ1-4GlcNAcβ1-3Galβ1-4Glcβ1-OCH($C_{14}H_{29}$)$_2$
$IV^6(Neu5Gc)nLc_4B30$	 Neu5Gcα2-6Galβ1-4GlcNAcβ1-3Galβ1-4Glcβ1-OCH($C_{14}H_{29}$)$_2$

155 but also those at 143 and 158 are linked to the viral recognition of Neu5Gc.

Our findings disagree with a previous study. Anders et al. reported that human H3 influenza A viruses isolated before 1972 did not bind Neu5Gcα2-6Gal-containing glycoprotein [19]. On the other hand, our findings indicated that A/Aichi/2/68 and A/Hongkong/1/68, which were isolated before 1972, bound Neu5Gcα2-6Gal-carrying glycolipids.

We excluded the possibility that either another strain of influenza virus had contaminated our preparation of A/Aichi/2/68, or point-mutated viruses generated during the propagation of A/Aichi/2/68 had overcome the original strain. The pattern of binding reactivity to each sialic acid-containing oligosaccharide was identical between cloned viruses and the originals. Comparing the nucleotide sequences of the cloned viruses, the HA1 amino acid sequences of all viruses were identical to the sequence obtained from the GenBank data base (accession number J02090) except for the amino acids at positions 144 and 182. Our result clearly showed that A/Aichi/2/68 exhibited the same binding reactivity of Neu5Gc as other virus strains that were never contaminated. Anders et al. [19] examined the binding reactivity of influenza viruses to Neu5Gc by a hemagglutination assay using erythrocytes whose oligosaccharides were modified by sialyltransferases. Conversely, we used a TLC/binding assay with defined sialylglycolipids which contained different molecular species of sialic acid residues and different sialyl linkages. A previous study indicated that the structure and length of core oligosaccharides or diversity of oligosaccharides affected the binding activity of the viruses [32]. The sialylglycolipids we used have a lacto-series type II chain, Galβ1-4GlcNAcβ1-3Galβ1-4Glc. The structure of core oligosaccharides on the surface of erythrocytes modified with sialyltransferases was unknown. These structural differences may affect viral recognition. Recently, M protein was correlated with the binding reactivity of HA of influenza A virus (H1N1) [33]. In the case of H3 viruses, during propagation in eggs, mutations in M protein as well as HA may affect the recognition of receptor oligosaccharides. To further investigate the involvement of other proteins, it will be necessary to employ different approaches such as assays using recombinant particles expressing HA.

In this study, we demonstrated that not only the amino acid at position 155 but also the amino acids at 143 and 158 of HA1 of influenza viruses in the vicinity of RBS affect binding reactivity to the molecular species of sialic acid (Neu5Ac, Neu5Gc). This finding will be helpful in understanding the significance of molecular species of sialic acid for viral transmission and host range restriction.

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