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Identification of amino acid residues of influenza A virus H3 HA contributing to the recognition of molecular species of sialic acid

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ABSTRACT

To identify a determinant of human H3 hemagglutinin (HA) amino acid residues linked to the recognition of molecular species of sialic acid, we generated six mutant viruses possessing either the wild-type HA gene from A/Memphis/1/71 (H3N2) or a genetically single-mutated HA gene at position 137, 144, 155, 158 or 193 from a genetic backbone of A/WSN/33 (H1N1) by reverse genetics. We evaluated the binding ability with four types of synthetic sialylglycolipids. The results indicate that the amino acid substitutions Thr155 to Tyr and Glu158 to Gly in H3 HA facilitate virus binding to *N*-glycolylneuraminic acid.

1. Introduction

Influenza virus hemagglutinin (HA) attaches to sialoglycoconjugates (glycoproteins and glycolipids containing sialic acid) on the host cell surface to initiate infection. Binding specificity of influenza A virus (IAV) for sialyloligosaccharide moieties on the cell surface is a critical factor for acquiring transmission ability to different host species [1,2]. Many derivatives of sialic acid (about 30 species) are known to exist in organisms ranging from bacteria to mammals [3]. Two major molecular species of sialic acid are 5-*N*-acetylneuraminic acid (Neu5Ac) and 5-*N*-glycolylneuraminic acid (Neu5Gc), which are chemically distinguished by a functional group at the C-5 position. Neu5Gc is derived from enzymatic hydroxylation of the *N*-acetyl group of Neu5Ac by CMP-Neu5Ac hydroxylase [4]. Glycoconjugates containing Neu5Gc are expressed in many animal tissues, whereas they cannot be synthesized in normal human tissues owing to a lack of the N-terminus of the CMP-Neu5Ac hydroxylase gene [5]. Determination of the molecular mechanisms of sialic acid recognition by influenza virus will help to clarify the roles of sialic acids in cell tropism and host transmission of IAV, leading to useful information for the development of anti-influenza virus agents.

In our previous study, virus overlay assays of nine human IAV strains (subtype H3N2) against four types of sialylglycolipids that had varied molecular species of a terminal sialic acid and sialyl-linkages showed that amino acid substitution at position 137, 144, 155, 158 or 193 of H3 HA may play a critical role in the recognition of Neu5Ac and Neu5Gc [6]. We therefore generated recombinant viruses from a genetic background of A/WSN/33 (H1N1) that included wild-type HA and five mutant H3 HAs in which one specific amino acid point mutation from A/Memphis/1/71 (H3N2) H3 HA was introduced by using site-specific mutagenesis. We showed that single amino acid substitutions of H3 HA at position 155 from Thr to Tyr and at position 158 from Glu to Gly play a critical role in recognition of two major molecular species of sialic acids.

Abbreviations: HA, hemagglutinin; HAU, hemagglutination unit; IAV, influenza A virus; Neu5Ac, 5-*N*-acetylneuraminic acid; Neu5Gc, 5-*N*-glycolylneuraminic acid; TLC, thin-layer chromatography

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2. Materials and methods

2.1. Sialylglycolipids

The synthetic sialylglycolipids commonly had Galβ1-4Glc-NAcβ1-3Galβ1-4Glc 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. All of the compounds were essentially synthesized by the procedure described by Kameyama et al. [7]. Detailed structures of the four synthetic sialylglycolipids used (IV^3(Neu5Ac)nLc_4B30, IV^6(Neu5Ac)nLc_4B30, IV^3(Neu5Gc)nLc_4B30, and IV^6(Neu5Gc)nLc_4B30) are given in [6].

2.2. Viruses

poll-Mem71HA was generated by insertion of human IAV A/Memphis/1/71 (H3N2) HA gene (accession number: AB298687) into the BsmBI restriction sites of pHH21 plasmid. Six IAVs were generated by a reverse genetics system [8]. Each transfectant virus possessed wild-type HA from A/Memphis/1/71 (H3N2) strain or a single amino acid mutant of the HA at position 137 from Asn to Tyr (N137Y), at position 144 from Gly to Asp (G144D), at position 155 from Thr to Tyr (T155Y), at position 158 from Glu to Gly (E158G), or at position 193 from Ser to Asn (S193N) and all remaining genes from A/WSN/33 (H1N1). HA genes were mutated according to the procedure of the QuickChange II Site-directed Mutagenesis kit (Stratagene, La Jolla, CA) by using respective mutated primer pairs, 5′-CAGAATGGGGGAAGCTATGCTTGCAAAAG-3′ and 5′-CTTTTGCAAGCATAGCTTCCCCCATTCTG-3′ for N137Y mutation, 5′-GCAAAAGGGGACCTGATAGCGGTTTTTTCAG-3′ and 5′-CTG AAAAAACCGCTATCAGGTCCCCTTTTGC-3′ for G144D mutation, 5′-CAGTAGACTGAACTGGTTGTACAAATCAGAAAGC-3′ and 5′-GCTTTC TGATTTGTACAACCAGTTCAGTCTACTG-3′ for T155Y mutation, 5′-GTTGACCAAATCAGGAAGCACATATCCAG-3′ and 5′-CTGGATATGT GCTTCCTGATTTGGTCAAC-3′ for E158G mutation, or 5′-CCAAGAA-CAAACCAACCTGTATGTTCAAGC-3′ and 5′-GCTTGAACATACAGGTT GGTTTGTTCTTGG-3′ for S193N mutation. All viruses were grown in 10-day-old embryonated chicken eggs, concentrated, and purified by sedimentation through 20% glycerol. Viral hemagglutination units (HAU) were determined at 4 °C in microtiter plates as previously described [9]. These mutations of HA genes were

Table 1
Comparison of HA amino acid residues involved in recognition of molecular species of a terminal sialic acid and sialyl-linkages by human H3 IAVs.

Virus (H3N2)	Amino acid residue at position					IAV binding with synthetic sialylglycolipids			
	137	144	155	158	193	Neu5Ac		Neu5Gc	
						α2,3	α2,6	α2,3	α2,6
A/Memphis/1/71	Asn	Gly	Thr	Glu	Ser	•	○	•	•
A/Hong Kong/1/68	Asn	Gly	Thr	Gly	Ser	○	○	○	○
A/Aichi/2/68	Asn	Asp	Thr	Gly	Ser	○	○	○	○
A/Memphis/102/72	Asn	Asp	Tyr	Gly	Ser	•	○	•	○
A/Tokyo/6/73	Asn	Asp	Tyr	Gly	Asn	•	○	•	○
A/Kumamoto/55/76	Ser	Asp	Tyr	Gly	Asp	○	○	○	○
A/Yamanashi/2/77	Ser	Asp	Tyr	Gly	Asp	○	○	○	○
A/Texas/1/77	Tyr	Asp	Tyr	Glu	Asn	•	○	•	○
A/Bangkok/1/79	Tyr	Asp	Tyr	Glu	Asn	•	○	•	○

Binding reactivity of H3 IAV strains with each sialylglycolipid is expressed as positive (open circle) and negative (closed circle) from the result of virus overlay assay in Ref. [6]. Neu5Acα2,3, IV^3(Neu5Ac)nLc_4B30; Neu5Acα2,6, IV^6(Neu5Ac)nLc_4B30; Neu5Gcα2,3, IV^3(Neu5Gc)nLc_4B30; Neu5Gcα2,6, IV^6(Neu5Gc)nLc_4B30.

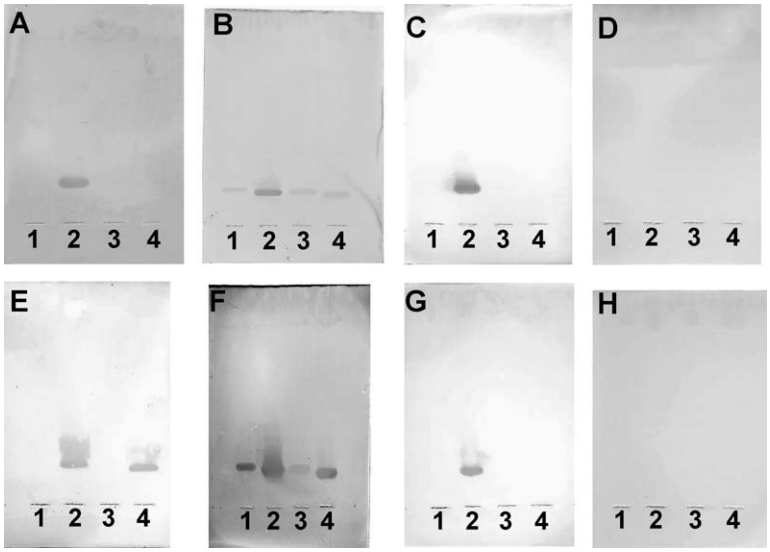

Fig. 1. Binding of reverse genetics IAVs to synthetic sialylglycolipids in virus overlay assays. Binding ability of each recombinant IAV was determined by a virus overlay assay with TLC. (A) A/Memphis/1/71 (H3N2); (B) WT; (C) N137Y; (D) G144D; (E) T155Y; (F) E158G; (G) S193N; and (H) no virus. Lanes: (1) IV^3(Neu5Ac)nLc_4B30; (2) IV^6(Neu5Ac)nLc_4B30; (3) IV^3(Neu5Gc)nLc_4B30; and (4) IV^6(Neu5Gc)nLc_4B30.

confirmed by sequencing with ABI PRISM 310 genetic sequencer (Applied Biosystems Inc., Foster City, CA).

2.3. Antibodies

Rabbit anti-IAV antibodies were prepared as described in [10]. Anti-HA monoclonal antibody (MAb) was generated from mice raised against A/Memphis/1/71 (H3N2) by a previously described [11].

2.4. Virus overlay assay

Synthetic sialylglycolipids (1 nmol) were developed in silica gel thin-layer chromatography (TLC) plates (Polygram Sil G: Nagel, Germany) with chloroform/methanol/12 mM aqueous $MgCl_2$ (65/35/8, v/v/v). The TLC plates were immersed in phosphate-buffered saline (PBS) (pH 7.2, 131 mM NaCl, 14 mM Na_2HPO_4, 1.5 mM KH_2PO_4, and 2.7 mM KCl) containing 1% egg albumin and 1% polyvinylpyrrolidone for 2 h at room temperature and incubated with each IAV suspension (2^{10} HAU) for 12–16 h at 4 °C. The plates were then incubated with rabbit anti-IAV antibodies for 2 h at 4 °C, followed by horseradish peroxidase-conjugated protein A. Finally, the plates were visualized as described in [1].

2.5. Solid-phase binding assay

Synthetic sialylglycolipids were serially diluted in ethanol from a concentration of 12.5 pmol/10 μl. Ten microliters of each diluted sialylglycolipid was placed into a well of a microplate (1 × 8 Strip-well plate, Corning Incorp. Life Sciences, Lowell, MA) until the ethanol had completely dried up. After exposure of the plate to ultraviolet light (254 nm) for 1 min, each well was blocked with 250 μl/well of PBS containing 1% lipid-free BSA overnight at 4 °C. After washing with 0.01% Tween20-PBS, the wells were incubated with 50 μl/well of IAV suspension (2^{10} HAU) at 4 °C for 12 h. After washing, the wells were incubated with mouse anti-HA MAb, followed by horseradish peroxidase-conjugated anti-mouse IgG + M. Finally, the wells were colored as described in [12].

3. Results and discussion

Our previous study showed that nine human IAV strains (H3N2) except A/Memphis/1/71 had the ability to bind to both Neu5Acα2-6Gal and Neu5Gcα2-6Gal linkages as summarized in Table 1 [6]. Comparison of HA amino acid sequences, which are located in and close to the receptor-binding pocket, among the viruses has shown that amino acid substitution at position 137, 144, 155, 158 or 193 of H3 HA may play a critical role in the recognition of Neu5Ac and Neu5Gc. To identify a determinant of HA amino acid residues related to this recognition, we generated six infectious reverse genetics viruses possessing the wild-type HA gene or a genetically single-mutated HA gene of A/Memphis/1/71 from a genetic backbone of A/WSN/33 (H1N1). (Abbreviations of transfectant viruses are given in Section 2.) We examined the ability of the viruses to bind to four types of synthetic sialylglycolipids that had varied molecular species of terminal sialic acids and sialyl-linkages by use of virus overlay assays with TLC plates and solid-phase binding assays with microplates. A/Memphis/1/71 showed preferential binding to the sialylglycolipid IV^6(Neu5Ac)nLc_4B30 possessing Neu5Acα2-6Gal linkage but not to other sialylglycolipids (Figs. 1A and 2A). The transfectant virus possessing wild-type WT HA (WT) preferentially bound to IV^6(Neu5Ac)nLc_4B30 (Figs. 1B and 2B). WT also bound to other sialylglycolipids, but the binding was weak. Parent A/Memphis/1/71 strain did not show binding to the sialylglycolipids except IV^6(Neu5Ac)nLc_4B30 on virus overlay

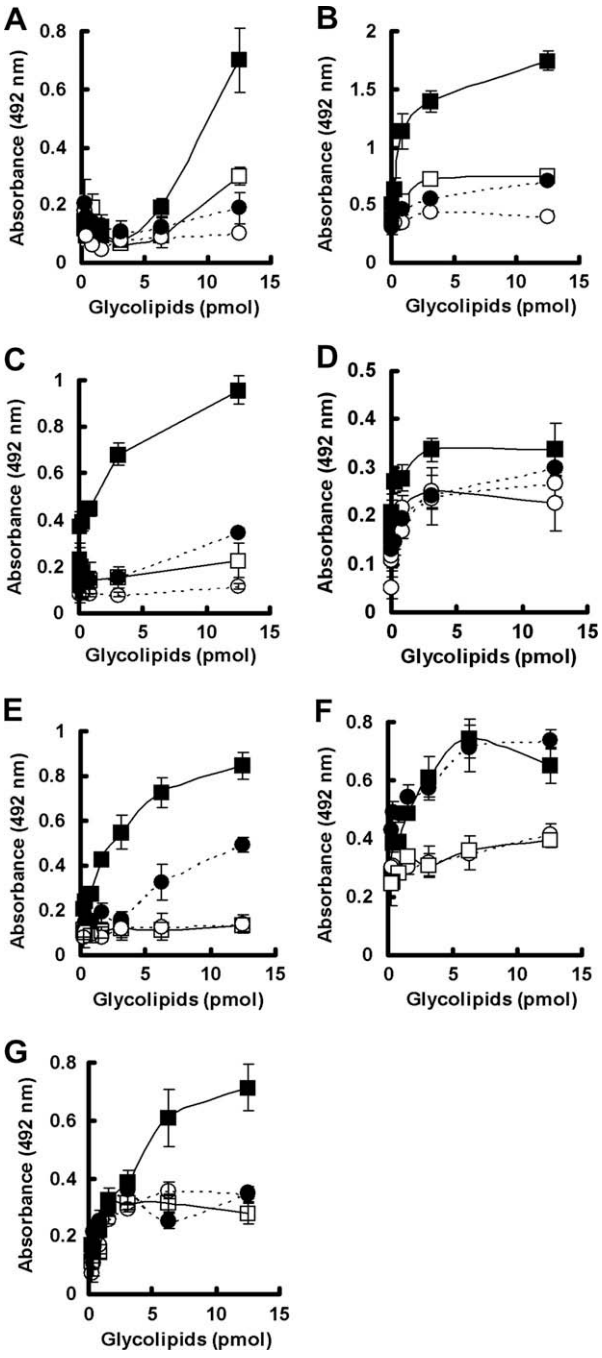

Fig. 2. Binding of reverse genetics IAVs to synthetic sialylglycolipids in solid-phase binding assays. Binding ability of each recombinant IAV was determined by a solid-phase binding assay. (A) A/Memphis/1/71 (H3N2); (B) WT; (C) N137Y; (D) G144D; (E) T155Y; (F) E158G; and (G) S193N. Sialylglycolipids: open square, IV^3(Neu5Ac)nLc_4B30; closed square, IV^6(Neu5Ac)nLc_4B30; open circle, IV^3(Neu5Gc)nLc_4B30; closed circle, IV^6(Neu5Gc)nLc_4B30.

assay. The discrepancy in the binding pattern between WT and A/Memphis/1/71 may be due to matrix protein 1 (M1) of A/WSN/33, which affects binding affinity by interaction with HA at the inner membrane of the virus envelope [13]. N137Y and S193N exhibited the same binding pattern as that of A/Memphis/1/71 strain (Figs. 1C, G and 2C, G). G144D showed no binding activity in the virus overlay assay (Fig. 1D). The binding ability of G144D was therefore evaluated by use of a solid-phase binding assay. G144D preferentially bound to IV^6(Neu5Ac)nLc_4B30; however, the amount of the virus bound to the sialylglycolipids tested was

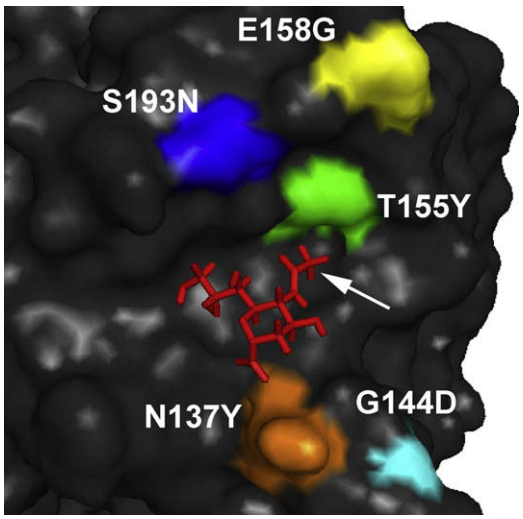

Fig. 3. The receptor-binding pocket with Neu5Gc. Location of altered residues within and neighboring the receptor-binding pocket with Neu5Ac. Orange, N137Y; light blue, G144D; green, T155Y; yellow, E158G; blue, S193N. The arrow indicates the *N*-acetyl group of Neu5Ac. The model was generated by using PyMol Molecular Graphics System 1.1r1 (Delano Scientific LLC).

smaller than the amounts of other transfectants (Fig. 2D). This mutation may lead to significant reduction of binding affinity to sialylglycolipids. T155Y strongly bound to IV^6(Neu5Ac)nLc$_4$B30 and also bound to IV^6(Neu5Gc)nLc$_4$B30 possessing Neu5Gcα2-6Gal linkage but not to sialylglycolipids possessing Neu5Acα2-3Gal or Neu5Gcα2-3Gal linkages (Figs. 1E and 2E). E158G showed preferential binding to both IV^6(Neu5Ac)nLc$_4$B30 and IV^6(Neu5Gc)nLc$_4$B30 regardless of the molecular species of a terminal sialic acid. E158G also bound to both of the sialylglycolipids possessing α2-3 linkage, but the binding ability was weaker than that to α2-6 linkage (Figs. 1F and 2F). The results indicate that the amino acid substitutions Thr155 to Tyr and Glu158 to Gly in H3 HA facilitate virus binding to the sialylglycolipids possessing Neu5Gc. The distribution of molecular species of sialic acid and sialyl-linkages vary among animal species and tissues [3,14]. Avian and equine IAVs preferentially recognize the Neu5Acα2-3Gal linkage, while human IAVs preferentially recognize the Neu5Acα2-6Gal linkage [2]. Genome sequences of H3N2 viruses deposited in the GenBank database indicate that most of the avian viruses possess Gly at position 158 in H3 HA and many swine viruses possess Tyr at position 155 and Gly at position 158. The data suggest that the amino acid substitutions Thr155 to Tyr and Glu158 to Gly are probably responsible for the wide binding pattern recognized by IAVs that potentially infect a variety of animals. Positions of amino acid alterations on the published three-dimensional structure of H3HA [15] are shown in Fig. 3.

Anders et al. suggested that amino acid substitution at position 155 from Thr to Tyr played a critical role in recognition of Neu5Gc by means of hemagglutination of resialylated erythrocytes [16]. The present work is the first study to show, by using a reverse genetics system, that not only residue 155 but also residue 158 in the HA protein contributes to the recognition of molecular species of sialic acid. In the transmission of IAVs between ducks and other animals including pigs, glycoconjugates containing Neu5Gc in the trachea of the pig and crypt cells of the duck colon may be utilized as a common receptor that avian, swine, and equine IAV can recognize [1,6,14,17]. Our finding will be helpful in understanding the significance of molecular species of sialic acid for viral transmission, host range restriction, and pandemics, leading to useful information for the development of anti-influenza virus agents and surveillance of pandemic potential.

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