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Differential Receptor Binding Affinities of Influenza Hemagglutinins on Glycan Arrays

Hsin-Yu Liao,[†,‡] Che-Hsiung Hsu,[†,§,II] Shih-Chi Wang,[†,‡] Chi-Hui Liang,[†] Hsin-Yung Yen,[†,⊥] Ching-Yao Su,[†,⊥] Chien-Hung Chen,[†] Jia-Tsrong Jan,[†] Chien-Tai Ren,[†] Chung-Hsuan Chen,[†] Ting-Jen R. Cheng,*[,†] Chung-Yi Wu,*[,†] and Chi-Huey Wong*[,†,§]

Genomics Research Center, Academia Sinica, 128 Academia Road, Section 2, Nankang, Taipei 115, Taiwan, Institute of Biochemistry and Molecular Biology, National Yang-Ming University, Taipei 112, Taiwan, Chemical Biology and Molecular Biophysics, Taiwan International Graduate Program, Academia Sinica, Taiwan, Institute of Bioinformatics and Structural Biology, National Tsing-Hua University, Hsin-Chu 300, Taiwan and Institute of Biochemical Sciences, National Taiwan University, Taipei, Taiwan

Received May 28, 2010; E-mail: tingjenc@gate.sinica.edu.tw (T.-J.R.C.); cyiwu@gate.sinica.edu.tw (C.-Y.W.); chwong@gate.sinica.edu.tw (or wong@scripps.edu) (C.-H.W.)

Abstract: A library of 27 sialosides, including seventeen 2,3-linked and ten 2,6-linked glycans, has been prepared to construct a glycan array and used to profile the binding specificity of different influenza hemagglutinins (HA) subtypes, especially from the 2009 swine-originated H1N1 and seasonal influenza viruses. It was found that the HAs from the 2009 H1N1 and the seasonal Brisbane strain share similar binding profiles yet different binding affinities toward various α2,6 sialosides. Analysis of the binding profiles of different HA subtypes indicate that a minimum set of 5 oligosaccharides can be used to differentiate influenza H1, H3, H5, H7, and H9 subtypes. In addition, the glycan array was used to profile the binding pattern of different influenza viruses. It was found that most binding patterns of viruses and HA proteins are similar and that glycosylation at Asn27 is essential for receptor binding.

Introduction

Pandemic influenza outbreaks pose a significant threat to public health as highlighted by the recent emergence of highly pathogenic avian influenza H5N1[1] and the latest 2009 outbreaks of swine-oriented H1N1 viruses (2009 H1N1).[2,3] Influenza virus infection is initiated by virus attachment to cell-surface sialoside receptors via influenza hemagglutinin (HA). The HA binding is then followed by viral neuraminidase cleavage of the terminal sialic acid on receptors, and receptor-mediated endocytosis accompanied by pH-induced conformational changes of HA to allow virus−cell membrane fusion and entry for replication.[4]

Influenza HA is a glycoprotein that forms a trimer on the virus surface.[5] In contrast to protein−protein interactions, the binding of HA to cell-surface receptors is dominated by protein−oligosaccharide interactions mediated by the *N*-acetyl neuraminic acid (Neu5Ac) residue, the most abundant derivative of sialic acid found at the terminal end of glycoproteins or glycolipids on the cell surface.[6] Extensive studies of various influenza virus subtypes and recombinant HAs have established a correlation between sialoside binding preferences and viral species: α2,6 linkage to galactose is preferred by the HA from human isolates, α2,3 linkage to galactose is preferred by the HA from avian isolates, and either α2,6 or α2,3 linkage to galactose or glucose is recognized by the HA from swine viruses.[7−11] It is generally believed that acquirement of the α2,6 sialosides recognition ability of an influenza virus is a prerequisite to its transmissions among humans. Therefore, understanding the receptor binding specificity of influenza viruses would lead to development of sensitive and fast diagnostic tools to detect and differentiate various subtypes of influenza viruses. Glycan arrays have become a powerful tool for use to help identify virus strains by monitoring changes in the receptor

[†] Genomics Research Center, Academia Sinica.
[‡] Institute of Biochemistry and Molecular Biology, National Yang-Ming University.
[§] Taiwan International Graduate Program, Academia Sinica.
[II] Institute of Bioinformatics and Structural Biology, National Tsing-Hua University.
[⊥] Institute of Biochemical Sciences, National Taiwan University.

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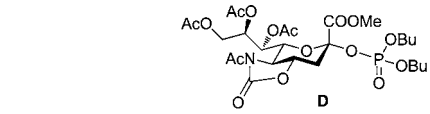
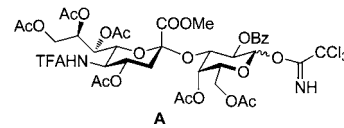
binding profile[12−17] and by dissecting the binding energy contribution via quantitative analysis.[17] This new tool may provide an alternative approach to the current methods based on RT-PCR (which is highly accurate but time-consuming)[18] and ELISA based on antibody−nucleoprotein interaction (which is quick but less accurate).[19] A recent significant discovery by using glycan array is that Tumpey's group examined the glycan binding properties of the 2009 A(H1N1) HA and found that the soluble form of CA/04 HA exhibited specific preference to longer α2,6 glycan (6'SLN-LN) structures. This binding behavior showed a significant difference from seasonal influenza H1N1 viral HA.[15] Moreover, Feizi's group also utilized a glycan array to evaluate the binding specificity of different influenza viruses and found that Cal/09 and Ham/09 H1N1 viruses bound not only to the α2,6-linked sialosides but also to a considerable range of the α2,3-linked sialyl sequences, and Mem/96 bound exclusively to the α2,6-linked sequences.[16]

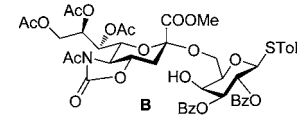
In contrast to the well-known binding preferences of various HA subtypes, the effect of HA glycosylation on receptor binding is still not well understood. The addition and processing of *N*-linked oligosaccharides on a protein has been shown to be important in maintaining protein conformation and stability and in modulating biological activities.[20] In addition, glycosylation may protect proteins from clearance and proteolysis. In the case of virus glycoproteins, glycans may also protect the virus from immune attacks and modulate receptor recognition during infection. In the case of influenza viruses, numerous reports have demonstrated that loss of carbohydrates on HA can affect its biological functions. For example, Deom et al.[21] reported that deletion of a complex oligosaccharide on the tip of the HA would increase virus−cell interaction and make it easier for the mutant viruses to survive in the infected cells. The increased interaction between less-glycosylated HA and sialosides was also demonstrated in our recent studies using sugar microarray:[17] the more outer glycans removed, the higher receptor binding affinity and immunogeneity observed, leading to the development of more potent HA vaccines with broader neutralization activities.[17] To further understand how individual glycans of a full-length HA affect receptor binding, we report here the expression of HA variants with a deleted glycosylation site and study their secondary structure and receptor binding using circular dichroism and a designer synthetic glycan array.

Results and Discussion

Preparation of Sialosides Array for HA Binding Evaluation. In naturally occurring sialosides, Neu5Ac is linked to other sugars through an α-linkage. For example, Neu5Ac is commonly found as a terminal sugar linked to galactosides through the α2,3 or α2,6 linkage in both *N*-linked and *O*-linked glycoproteins and also to *N*-acetyl-galactosamine through the α2,6 linkage in *O*-linked glycoproteins. In addition, Neu5Ac can be linked to another Neu5Ac residue via the α2,8 or α2,9 linkage as a constituent of glycoproteins and glycolipids.[22,23] Though enzymatic sialylation provides α-linked sialosides stereospecifically, the enzymes are not all readily available and are often limited to, though with some exceptions, the synthesis of naturally occurring sialosides due to their substrate specificity.[24,25] This limitation precludes the synthesis of a wide range of sialosdies. We have therefore developed an α-specific sialylation donor (**D**) for the synthesis of various α-sialosides, including the design of "sialylated disaccharide" as building blocks for the one-pot synthesis of sialylated oligosaccharides.[26] We have further extended this strategy to the preparation of a sialoside array. By using disaccharide **A**[27−29] as building block, seventeen α2,3-linked sialosides (**1**−**17**) of disaccharides to hexasaccharide were synthesized, and, using the disaccharide **B** as building block, several α2,6-linked sialosides (**21**−**30**) were prepared (Table S1 and Schemes S2−S13 of the Supporting Information). These glycans were spotted onto the NHS-activated glass slide via amide bond formation[12] to create an array of sialosides for HA binding evaluation.







Establishment of a Platform for Quantitative Analysis of Sialosides Binding (K_D Determination). Recombinant full-length HAs with a C-terminal Strep-tag and a $(His)_6$ tag were prepared from human HEK293 cells for array detection. Strep-tag is an eight-residue minimal peptide sequence with neutral charges and exhibits intrinsic affinity toward streptavidin.[30] The Strep-tag was reported to not to hamper protein folding nor secretion and it usually does not interfere with protein function.[30] Prior to analyze binding specificity of HA toward sialosides, a general

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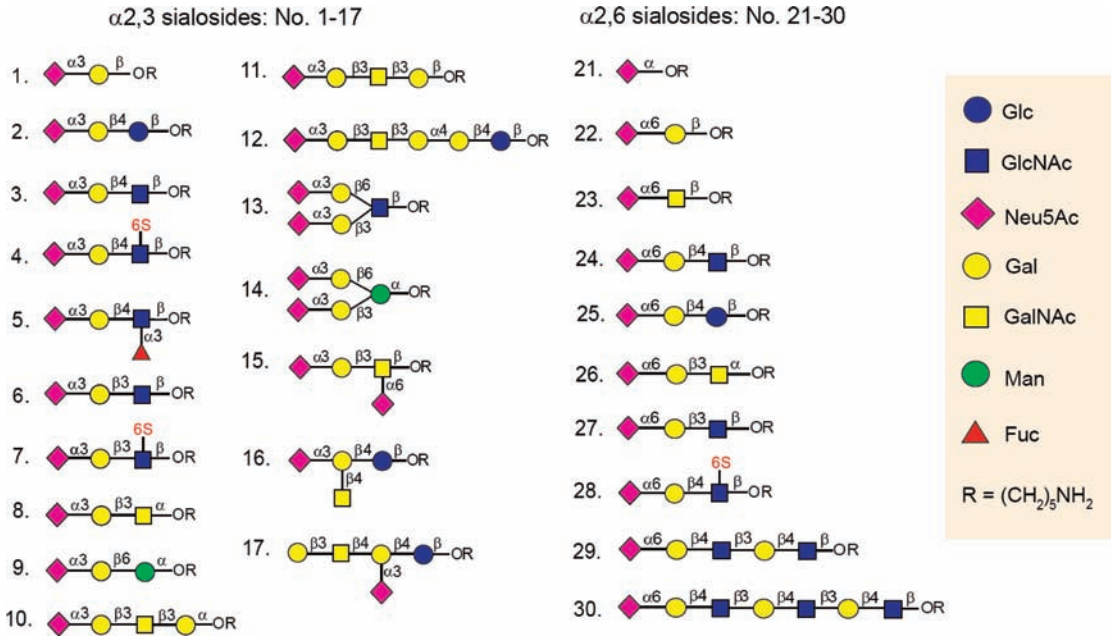

Figure 1. Sialoside structures to be spotted on glass slide for creating an array of sialosides for the HA binding studies.

platform needs to be established. Therefore, detection methods using Cy3-conjugated HA for direct measurement,[17,31] anti-His antibodies followed by Cy3-conjugated secondary antibodies,[13,14] and Cy3-conjugated streptavidin were parallelly compared and evaluated for the feasibility in binding analysis. For the detection using antibodies or streptavidin, a precomplexation strategy that is incubation of HA with the Cy-3 conjugated partners in defined ratios prior to applying the whole complexes onto the slide as used.[13,14] The results showed that: 1) three detection methods resulted in no significant differences in binding patterns nor binding intensities, 2) Cy3-conjugated HA showed binding activities, indicating that HA forms functional trimers and the binding detected using the other two approaches was not due to multimerization caused by the antibodies or streptavidin, and 3) preliminary qualitative binding analysis revealed similar binding affinities for the reported antibody-based approach ($K_{D,surf}$ = 150 for **15** in the case of H5) versus the streptavidin-based strategy ($K_{D,surf}$ = 145 ± 44 for **15** in the case of H5). We therefore used the HA labeled with Cy3-SA for the following binding analysis. An additional advantage of this strategy is that it can provide a clear and consistent binding profile for experiments using low concentrations of HA, which benefits the application of quantitative biochemical analysis.

Differential Receptor Binding of HAs from Seasonal and Pandemic Influenza Viruses. Our ultimate goal is to differentiate influenza virus subtypes by only a specific set of glycans. In this respect, recombinant HAs from the seasonal and pandemic viruses were examined with respect to their receptor binding specificity. The results of binding profiles for HAs from both pandemic H1N1 (California/07/2009) (part A of Figure 2) and seasonal H1N1 Brisbane/59/2007 (Br/59/07) (part B of Figure 2) displayed a similar pattern, with both higher binding activities toward longer α2,6 sialosides. It was noticed that the maximum binding affinity of the 2009 pandemic H1 reached with α2,6 sialoside containing 5 or 7 sugar units. Yet the H1 from Brisbane strains showed the highest binding affinity toward the α2,6 sialoside containing 7 sugar units. The surface dissociation constant values ($K_{D,surf}$) were further determined using glycan microassay based on the Langmuir isotherms.[32] The monovalent HA−sialoside binding is weak, exhibiting solution dissociation constants in the millimolar range ($K_D = 2.5 \times 10^{-3}$ M) if competition-based experiments were conducted.[33] HA, however, is involved in multivalent interactions with sialosides on the host cell surface, which can be seen in the quantitative array profiling.[17,32] By the analysis of $K_{D,surf}$ (Table 1) of both strains, the result revealed stronger binding capability of H1 from Br/59/07 than the H1 from 2009 pandemic strain toward α2,6 sialosides, and this observation was also supported by the phenomena that Br/59/07 H1 showed a high binding affinity even when the protein was used as low as nanomolar concentrations for sugars **29** and **30**, making it difficult for the K_D determination. Compared to the earlier circulating strain H1N1/New Caledonia/1999 (NC/99) (part C of Figure 2), it was shown that recent H1N1 strains showed strong binding affinities toward specific long α2,6 sialosides such as **29** and **30**, implying possibility that broader receptor specificity necessitate efficient transmission of influenza virus. On the other hand, the H3 from Brisbane/10/2007 (Br/10/07) showed a narrower binding profile toward only two α2,6 sialosides, **28** and **30**. It was surprising that binding can only be observed with sialoside **30**, the glycan contains three repeats of LacNAc, but not **29** with two LacNAc repeats (part D of Figure 2).

The same array was also used to profile the binding pattern of avian flu H5 (H5N1/Vietnam/1194/2004) (part E of Figure 2), avian flu H7 (A/H7N7/Netherlands/219/03) (part F of Figure 2), and human flu H9 (A/H9N2/Hong Kong/1073/99) (part G of Figure 2). As expected, the HAs from human and avian viruses showed respective binding profiles. The results also suggested that binding to **28** and **30** is unique to human viruses

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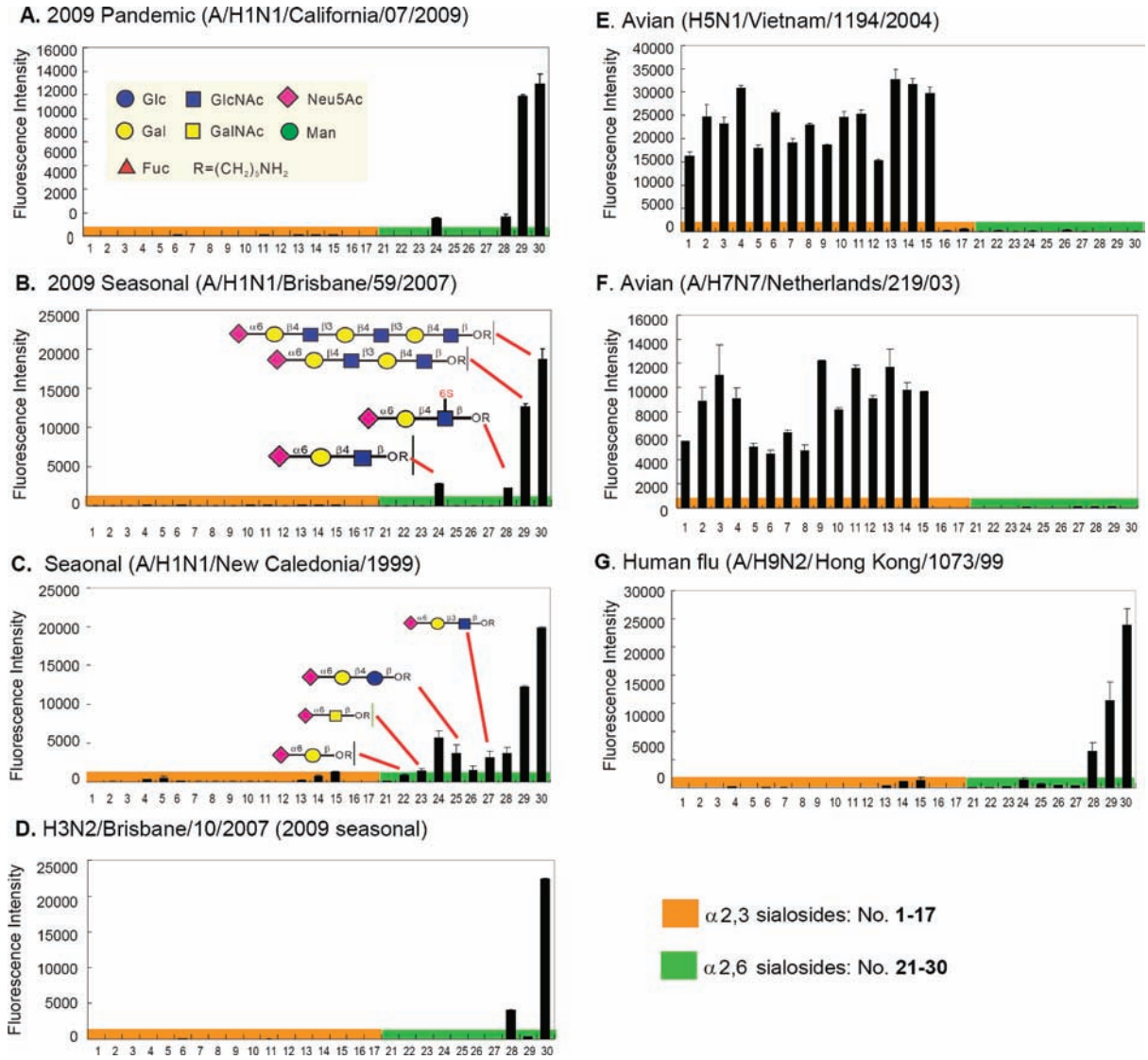

Figure 2. Differential binding patterns of HA from H1N1, H3N2, H5N1, H7N7, and H9N2 viruses.

Table 1. K_D of HA from SOV and Seasonal Flu Towards α2,6-Sialosides

	$K_{D,surf}$ of Hemagglutinin (nM)		
Sialosides[a]	Cal07(H1N1)	Br59 (H1N1)	Br10 (H3N2)
24	376 ± 40	233 ± 23	6350 ± 110
28	1307 ± 533	443 ± 156	2011 ± 746
29	383 ± 9	N.D.[b]	>10[5]
30	686 ± 230	N.D.[b]	836 ± 96

[a] Standard symbols are used. Blue circle, Glc; blue square, GlcNac; red diamond, Neu5Ac; yellow circle, Gal; yellow square, GalNac; green circle, Man; red triangle, Fuc. [b] High binding activities but no concentration-dependence was observed.

but not avian viruses. Furthermore, H1 binds glycan **24** (parts A−C of Figure 2), whereas H3 shows no binding (part D of Figure 2), thereby providing diagnostic potential for the differentiation of H1 from H3. In addition, binding to the disaccharides **22** and **23** may imply strong foothold among human populations. These sugars, together with α2,3-trisaccharides, can be used to differentiate HA subtypes and thus have the potential to provide a method of quick test upon emergence of an influenza outbreak.

Binding Profiles of Real Viruses. To understand the relationship of real viruses and HA proteins toward sialosides binding, receptor-binding profiles of four isolates of the influenza virus by glycan array analysis were compared directly by using the same sialosides array. A clear distinction among the receptor-binding repertoire of the Cal/09 H1N1, Brisbane H1N1, Brisbane H3N1, RG14 H5N1, and B/Lee/40 was observed (Figure 3). The Cal/09 H1N1 (part A of Figure 3) and Brisbane H1N1 viruses (part B of Figure 3) bound not only to the majority of α2,6 linked sialyl sequences but also to a considerable range of α2,3 linked sialyl sequences. In contrast, H5N1 (part D of Figure 3) bound exclusively to α2,3 linked sialyl sequences. Similar to recombinant H3 proteins, the H3N2 influenza viruses showed a preferential binding to α2,6 linked and α2,3 linked sialyl sequences with strongest binding toward **28** and **30** (part C of Figure 3). Interestingly, influenza B showed a similar binding profile to both α2,3 and α2,6 sialosides (part E of Figure

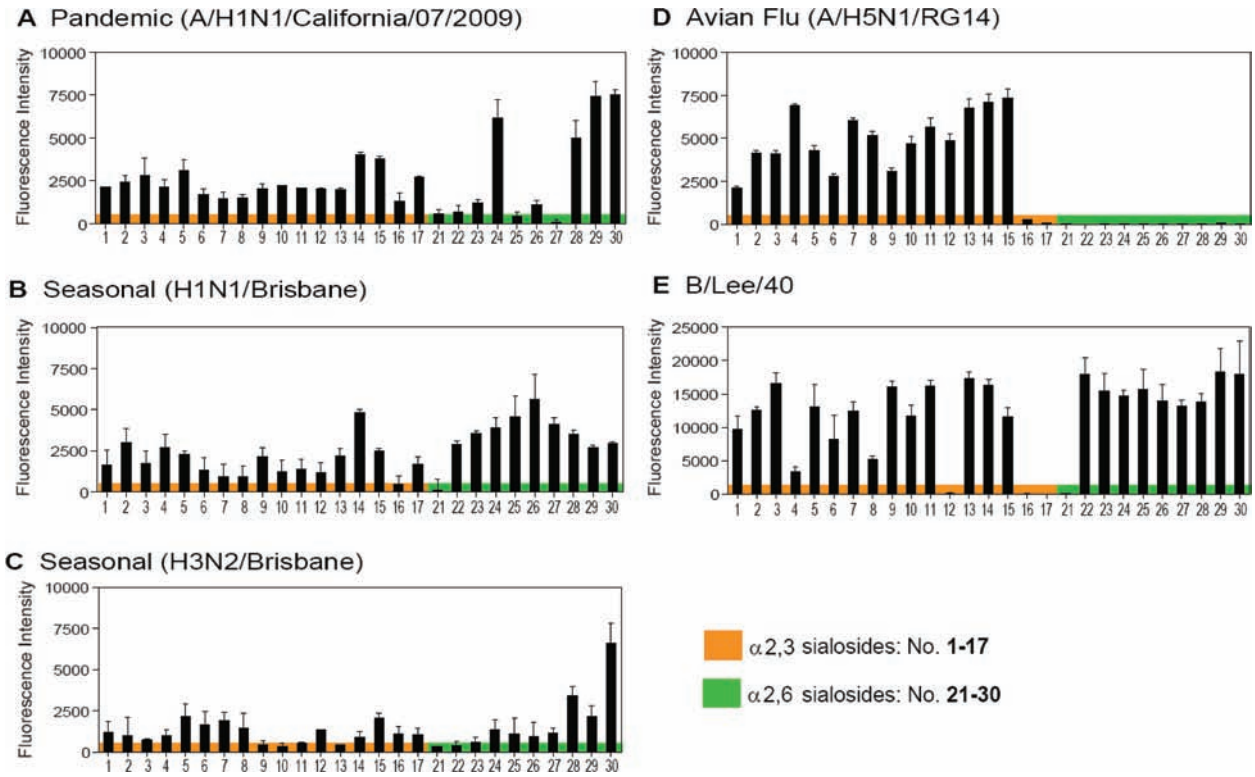

Figure 3. Glycan array analyses of the five viruses investigated. The binding signals are shown as means of duplicate spots at 100 μM per spot. Each experiment was repeated twice. Arrays consisted of twenty seven sialylated oligosaccharide probes, printed on NHS-coated glass slides (NHS: *N*-hydroxy succinimide). The various types of terminal sialic acid linkage are indicated by the colored panels as defined at the bottom of the figure.

3). The broader specificity, namely, the ability to bind to α2,3 in addition to α2,6 linked receptors was also pertinent to the greater virulence of the pandemic virus, and its capacity to cause severe and fatal disease in humans. Binding to α2,3 linked receptors is thought to be associated with the ability of influenza viruses to infect the lower respiratory tract where there is a greater proportion of α2,3 vs α2,6 linked sialyl glycans. Differences in receptor binding among the viruses may therefore formulate a good candidate for classifying the serotype of influenza viruses.

The binding preference of RG14 was the same as that of recombinant H5. In the case of H1N1 virus, the binding profile using the whole virus is slightly different from the profile obtained with recombinant proteins. Like recombinant HAs, viruses showed the strongest binding toward long α2,6 sialosides. However, the viruses also showed significant binding to α2,3 sialosides, which was unusual for recombinant HAs. The intrinsic binding affinity of sialosides for the hemagglutinin is dominated by polyvalent interactions at the cell surface. Therefore, weak monovalent binding may become significant in multivalent interactions, and protein presentation, such as HA orientation and density, on the cell surface may have a major impact in receptor recognition. Furthermore, the tip of the globular region harbors the receptor-binding pocket, which is known to be crucial for the process of virus binding to its receptor. The orientation, quantity, and structure of *N*-glycans neighboring the receptor-binding pocket appear to be important regulators of receptor specificity, which may also cause the differences in binding preferences between recombinant HA and whole virus.

Effects of Site-Specific HA Glycosylation on Sialoside Receptor Binding. It is believed that influenza HA needs to be glycosylated to have proper function, that is to bind sialylated host receptors to mediate virus entry. Glycosylation sites on certain regions of HA are highly conserved.[34] It was also reported that changes in the HA glycosylation pattern may be advantageous or detrimental to HA binding and eventually to the survival of the virus.[35] To elucidate the effects of site-specific glycosylation on the HA binding to sialoside receptors, we produced H5 (consensus HA, CHA5[36]) mutants in which the individual predicted glycosylation sequon Nx(S/T) was changed to NxA to prevent *N*-glycosylation at the specific site (part A of Figure 4). Except for Δ26, Δ170, Δ209, and Δ559, remove of each of the HA glycosylation sites showed an increased rate of gel mobility on SDS-PAGE compared with WT, corresponding loss of glycosylation on the mutant (data not shown). These glycosylation sites were consistent with the results from mass sequencing analysis, where carbohydrates were identified to attach to the sites N26/27, N39, N170, N181, N302, N500, and N559 (Data not shown). The subsequent sugar binding analysis suggested that glycosylation at position 39, 302, 500, or 209 did not significantly change sialoside binding (part B of Figure 4). In contrast, the mutation to block the glycosylation on N27 appeared to be detrimental for receptor binding activities.

The glycosylation on the amino-terminal *N*-glycosylation sites, that is N27, had been implicated in protein transport and folding. The loss of N20/21 of H2 was demonstrated to have a decrease in hemadsorption and cell fusion activities.[37] It was also reported that HA without glycosylation on amino-terminal

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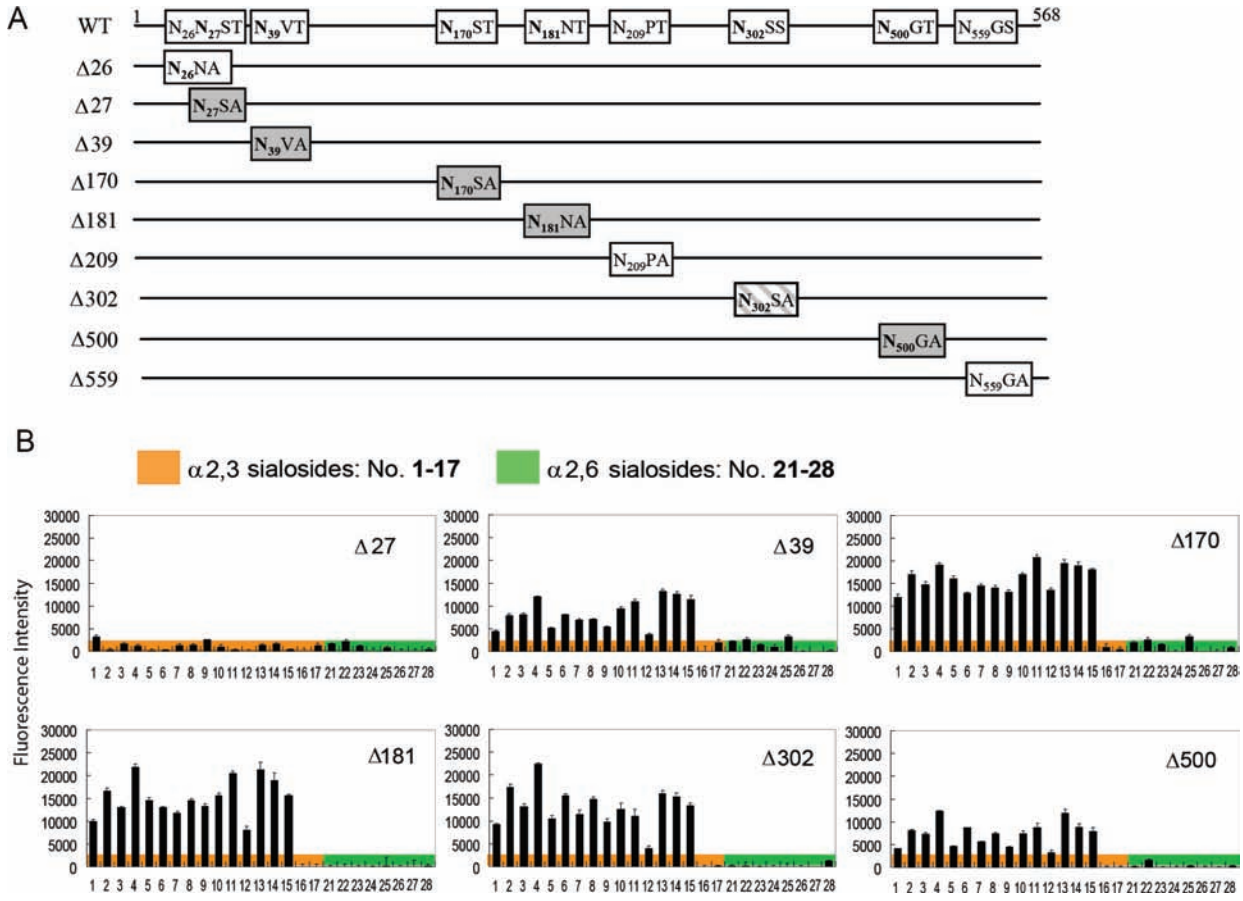

Figure 4. Binding profiles of HA glycosylation mutant. (A) Nine glycosylation sites were predicted using H5 as an example. The predicted glycosylation sites in gray boxes were confirmed to be glycosylated using LCMS. The sites in white boxes were confirmed to be unglycosylated and the one in the hatched box may be partially glycosylated. The glycosylation mutant were created by changing Nx(T/S) to NxA. (B) Sugar binding patterns of individual glycosylation mutants.

sites would be trapped in endoplasmic reticulum and cannot be transported to cell membrane.[38] Our results showed that removal of the N27 glycosylation site almost demolished the sugar binding activity of WT HA. To further find out if the loss of binding affinity is due to improper folding as other reports suggested, circular dichroism (CD) spectroscopy analysis was conducted. The results showed that the secondary structure of Δ27 changed, whereas another glycosylation mutant with similar binding (Δ170) had the same spectrum as the wild-type HA (Figure 5). The results indicated that deletion of glycosylation at position 27 had a major impact on HA structure, implying that *N*-glycosylation on N27 is important for proper HA folding. However, although the glycans found on the receptor binding domain were shown to modulate the antigenic properties, hemadsorption activities, and cell fusion activities,[39] our array studies showed no major change in the binding patterns for glycosylation mutants. The binding affinities, however, decreased for glycosylation mutants (Table 2). Consistent with previous results where soluble HAs were used for array analysis,[13,17] our results showed that high affinity toward sulfated trisaccharides that contains LacNAc can be observed.

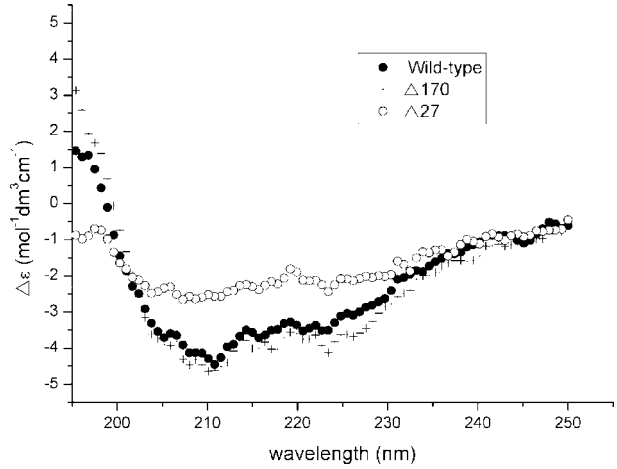

Figure 5. CD spectra showed that Δ170 exhibits similar secondary structure with the wild-type HA, whereas the secondary structure of Δ27 changed.

Conclusions

Using the sialylated disaccharide strategy, 27 sialosides have been synthesized to prepare a sialoside microarray on glass slides which can be used to profile not only the receptor binding specificity of various HA subtypes but also HAs with different glycosylation patterns and real influenza viruses.

The sugar binding results of recombinant HAs (Figure 2) and influenza viruses (Figure 3) suggested that a minimum set of

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Table 2. Sialosides on the Array and the K_D Values (nM) of H5 (CHA5[36]) Glycosylation Mutants for Represented Sialoside

	Sialosides[a]	wt	Δ39	Δ170	Δ181	Δ302
4		207 ± 52	497 ± 81	494 ± 79	795 ± 194	290 ± 118
13		190 ± 87	368 ± 84	424 ± 416	603 ± 381	323 ± 127
14		171 ± 30	440 ± 13	355 ± 162	506 ± 249	286 ± 138
15		145 ± 44	398 ± 42	342 ± 312	990 ± 433	358 ± 53

[a] Standard symbols are used. Blue circle, Glc; blue square, GlcNac; red diamond, Neu5Ac; yellow circle, Gal; yellow square, GalNac; green circle, Man; red triangle, Fuc, $R = (CH_2)_5NH_2$.

oligosaccharides containing **4**, **5**, **24**, **29**, **30** might be useful for influenza subtyping. Our results showed that avian influenza virus and recombinant HAs displayed no difference with their glycan array profiling results, which preferentially bound α2,3 sialosides with relatively no binding was observed for α2,6 sialosides (parts E and F of Figure 2 and part D of 3), providing a unique pattern for differentiating between avian and human viruses. However, broader specificity toward both α2,3 and α2,6 sialosides was shown as a general theme of binding for real human virus, comparing to major α2,6 binding of recombinant human virus HAs. (parts A−C of Figures 2 and parts A−C of 3). Another interesting observation is that use of sialyl mono-, di-, and trilactosamine glycans, which are **24**, **29**, **30** respectively seems to be able to differentiate recombinant human virus HAs such as pandemic H1, seasonal H1, and H3 (parts A−C of Figure 3), wherein subtle binding of H3 to **29** comparing to strong binding of both seasonal and pandemic H1 appeared to be a good hint. Human influenza type A and B also displayed different binding patterns in our real virus glycan array profiling results. Influenza type A virus showed stronger binding to α2,6 sialosides than α2,3 sialosides, whereas type B virus showed similar preference to both categories of sialosides. The strong binding to α2,6 disaccharide **22** implicate high transmission possibilities because seasonal H1 is uniquely binding to it comparing to other H1 HAs.

It has been known that the glycans of influenza hemagglutinins have some important biological functions.[40] For example, the glycans in the head region of hemagglutinins block the antigenic site. Also, the HA receptor binding specificity was affected by the absence of a complex glycan chain near the receptor binding site.[35] To address how glycosylation affects the specificity of HA binding, we adopted the powerful glycan microarray technology to profile HAs produced from different expression systems which generate different *N*-glycosylation patterns on the protein surface. Mutations of certain *N*-glycosylation sequons on HA further suggested that certain glycosylation sequons such as $N_{27}ST$ have detrimental effects on HA function. The studies that use site-specific glycosylation mutants to elucidate the relationship between HA glycosylation and its receptor binding specificity underscore the importance of HA glycosylation.

Experimental Section

Materials. NHS-coated glass slides were obtained from SCHOTT (Nexterion H), monoclonal antibodies to influenza hemagglutinin were kindly provided by the NIH Biodefense and Emerging Infections Research Resources Repository, NIAID, NIH, Cy3-labeled antimouse secondary antibodies, and Cy3-labeled streptavidin were purchased from Jackson ImmunoResearch. Standard chemicals and reagents were purchased from commercial suppliers and used as received.

Construction of Hemagglutinin Expression Plasmids. The full-length genes encoding HA from H1N1 Influenza A Virus including pandemic California/07/2009, Brisbane/59/2007, New Caledonia/20/1999 (ABF21272.1), H3N2 Brisbane/10/2007 (ABW23422.1), H5N1 Vietnam/1194/2004 (ABP51976.1), H7N7 Netherland/219/03 (AAR02640.1), or H9N2 HongKong/1073/99 (CAB95856.1) fused with a C-terminal Strep (Trp-Ser-His-Pro-Gln-Phe-Glu-Lys)[30] and $(His)_6$ tag were cloned into pcDNA (Invitrogen) for expression in human 293T cells. The sequences were confirmed by DNA sequencing and prepared in high quality for expression. The genes were cloned into pcDNA for expression in human 293T cells.

Construction of Hemagglutinin Glycosylation Mutants. The nucleotide sequence of consensus hemagglutinin H5 (CHA5[37]) was cloned into pcDNA with a C-terminal Strep-Tag, and the resulting plasmid was used as the templates for site-directed mutagenesis. The *N*-glycosylation sites of CHA5 were predicted with The NetNGly server provided by Expasy (www.expasy.org). The putative N-X-S/T sequons were then mutated to N-X-A using site-directed mutagenesis with QuikChange II Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA, USA). The mutagenesis was confirmed by DNA sequencing, and the glycosylation knockouts were confirmed by SDS-PAGE analysis.

Expression of Recombinant Full-Length Hemagglutinin from Expressed Cells. For expression in human 293T cells, pcDNA carrying the gene of interest were prepared in high quality and transfected with cation lipids DOTAP/DOPE (1:1) (Avanti Lipids) for transient expression. The expression of hemagglutinin was confirmed with immunoblots using anti$(his)_6$ antibodies (Qiagen) or specific anti-hemagglutinin antibodies and the horseradish peroxidase-conjugated secondary antibodies (PerkinElmer).

Purification of Recombinant Hemagglutinins. The cells containing recombinant full-length HA were lysed in 20 mM Hepes buffer (pH 7.4) using a microfluidizer. The lysates were centrifuged at 12,000 rpm for 10 min and the supernatant were collected for a second centrifugation at 40 000 rpm for 1 h. Next, the pellets were extracted with 20 mM Hepes buffer containing 1% dodecyl maltoside for 2 h followed with a brief centrifugation at 12 000 rpm. The supernatant were then passed through an affinity column packed with Nickel Sepharose Hi Performance (GE Healthcare). After washes with 20 mM Hepes buffer containing 0.5% dodecyl maltoside, the recombinant full-length HA was eluted with 500 mM imidazole in 20 mM Hepes buffer. The purified proteins were concentrated using Amicon (Millipore) and stored at 4 °C. The concentrations were determined using immunoblots with anti-hemagglutinin and horseradish peroxidase (HRP)-conjugated secondary antibodies.

Microarray Analysis of Sugar Binding Activities of Hemagglutinin. Microarrays were prepared by printing (AD3200, BioDot) the glycan with an amide tail to the NHS-activated glass slide (Nexterion H) by robotic pin (SMP2B, TeleChem International Inc.). Nexterion H slides were spotted with solutions of sugar **1**−**17** and **21**−**30** at 100 μM from bottom to top with 12 replicates horizontally in each grid and dried under vacuum. The spotted slides were blocked with ethanolamine in sodium borate for 1 h just before use followed with three washes of 0.05% Tween 20 in PBS buffer (pH 7.4) (PBST). A solution of hemagglutinin at 50 μg/mL in PBST was premixed with Cy3-labled streptavidin in 1:1 molar ratio for 1 h prior to incubation of the preformed complexes with the slides for another hour. After six washes with PBST, one wash with PBS, and three washes with distilled water, the slides were air-dried and scanned with a 532 laser using a microarray fluorescence scanner (GenePix 4000B, Molecular Devices). The PMT gain was set to 600. The resulting images were analyzed with GenePix Pro 6.0

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(Molecular Devices) to locate and quantify the fluorescence intensity of all of the spots on the grid. The median of fluorescence intensity of each spot was taken to calculate the median value of binding activities toward each sugar (12 replicates for each sugar). The medians from at least three independent experiments were averaged for the figures. For K_D determination, the preformed complexes were serially diluted for binding reaction,[31] and the binding intensities were quantified at various concentrations of complexes and fitted to the Langumir isotherms using the Prism (GraphPad, San Diego, CA).[41]

Virus Preparation. Samples of various viruses are collected from the Center for Disease Control and Prevention in Taiwan. All viruses were propagated in 10-day-old embryonated specific-pathogen free chicken eggs. Purified viruses were done by centrifugation at 100 000 g for 30 min and resuspended in PBS. As previously reported,[16] differences in the degree of virus purity did not influence significantly the pattern of receptor binding. Viruses were inactivated by treatment with β-propiolactone (BPL; 0.05% v/v) for 60 min at 33 °C, and resuspended in 0.01 M phosphate buffered saline pH 7.4 (PBS) and stored at −80 °C. Comparison of samples of live and inactivated virus showed that BPL inactivation did not alter receptor binding specificity.

Virus Binding Assay Procedure. Influenza virus A/Vietnam/1194/2004 RG14 (H5N1), A/California/7/2009 (H1N1), A/Brisbane/10/2007 (H1N1), and A/Brisbane/10/2007 (H3N2) were from Taiwan CDC. Influenza virus B/Lee/40 was obtained from ATCC (Manassas, VA, USA) and propagated. Printed slides were analyzed without any further modification. Inactivated whole virus was applied at a concentration around 10^7 virus/mL in PBS buffer containing the neuraminidase inhibitor Oseltamivir carboxylate (10 μM). Suspensions of the inactivated viruses with Oseltamivir carboxylate were overlaid onto the arrays and incubated at room temperature for 1 h. Slides were subsequently washed by successive rinses in PBS-0.05% Tween, PBS, and deionized water three times. Bound viruses were detected using the following antibodies: homemade rabbit anti-H1 antibody both for SOV California/07/2009 and H1N1 Brisbane; anti-H3 antibody for H3N2 Brisbane (NR3118, Biodefense and Emerging Infections Research Resources Repository, National Institute of Allergy and Infectious Diseases, MD, USA); anti-H5 (α-293s) (Sino Biological Inc. Beijing, CH) antibody for H5N1 (RG14), and anti-flu B (Abcam, MA, USA) for B/Lee/40. The slides were gently rocked at room temperature for 60 min. After the repeating washing steps, binding was detected by overlay with labeled secondary antibodies.

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Supporting Information Available: Complete refs 2 and 12 experimental procedures and compound characterization data (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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