A Sialylated Glycan Microarray Reveals Novel Interactions of Modified Sialic Acids with Proteins and Viruses*

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Many glycan-binding proteins in animals and pathogens recognize sialic acid or its modified forms, but their molecular recognition is poorly understood. Here we describe studies on sialic acid recognition using a novel sialylated glycan microarray containing modified sialic acids presented on different glycan backbones. Glycans terminating in β-linked galactose at the non-reducing end and with an alkylamine-containing fluorophore at the reducing end were sialylated by a one-pot three-enzyme system to generate α2–3- and α2–6-linked sialyl glycans with 16 modified sialic acids. The resulting 77 sialyl glycans were purified and quantified, characterized by mass spectrometry, covalently printed on activated slides, and interrogated with a number of key sialic acid-binding proteins and viruses. Sialic acid recognition by the sialic acid-binding lectins Sambucus nigra agglutinin and Maackia amurensis lectin-I, which are routinely used for detecting α2–6- and α2–3-linked sialic acids, are affected by sialic acid modifications, and both lectins bind glycans terminating with 2-keto-3-deoxy-D-glycero-D-galactonononic acid (Kdn) and Kdn derivatives stronger than the derivatives of more common N-acetylaceulaminuronic acid (Neu5Ac) and N-glycolylneuraminic acid (Neu5Gc). Three human parainfluenza viruses bind to glycans terminating with Neu5Ac or Neu5Gc and some of their derivatives but not to Kdn and its derivatives. Influenza A virus also does not bind glycans terminating in Kdn or Kdn derivatives. An especially novel aspect of human influenza A virus binding is its ability to equivalently recognize glycans terminated with either α2–6-linked Neu5Ac9Lt or α2–6-linked Neu5Ac. Our results demonstrate the utility of this sialylated glycan microarray to investigate the biological importance of modified sialic acids in protein-glycan interactions.

Protein-glycan interactions play important roles in cellular adhesion, signal transduction, and host-pathogen interactions and are mediated through specific glycan recognition by glycan-binding proteins (1–3). Sialic acids (Sia),6 which are targets for glycan-binding protein recognition because they commonly occur at the non-reducing ends of glycans, are uniquely diverse among monosaccharides in that many different modified sialic acids have been identified (4). Sialylation of cell surface glycoconjugates is highly regulated by sialyltransferases and/or sialidases (5) where the most common enzymes add or cleave sialic acid in α2–3, α2–6, or α2–8 linkages to underlying glycan structures.

Sialylated glycans play important roles in the pathogenesis of many microorganisms. The species specificity of influenza A virus is associated with differential binding of the virus to sialic acids linked either α2–3 or α2–6 to galactose on cell surface glycoconjugates (6). However, modification of sialic acids, such as hydroxylation, that converts N-acetylaceulaminuronic acid (Neu5Ac) to N-glycolylneuraminic acid (Neu5Gc), also plays an important role in sialic acid ligand recognition by pathogens and is a component of the surface receptor for certain bacterial toxins (7). Humans, unlike most other mammals including other primates, are unable to naturally synthesize Neu5Gc (8, 9), although they can incorporate it from dietary sources (10). Overall, the most commonly studied sialic acids are Neu5Ac, Neu5Gc, and their 9-O-acetylated derivatives (Neu5,9Ac2 and Neu5Gc9Ac) (11–14). The investigation of sialosides containing more diverse sialic acids was limited until recent successes

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5 The abbreviations used are: Sia, sialic acid(s); AEAB, 2-amino-ethyl-benzamide; GAEAB, glycan-AEAB conjugate; LNT, Galβ1–4GlcNAcβ1–3Galβ1–4Glc; LNT, Galβ1–3GlcNAcβ1–3Galβ1–4Glc; NA2, Galβ1–4GlcNAcβ1–2Manβ1–6Galβ1–4GlcNAcβ1–2Manβ1–3Manβ1–4GlcNAcβ1–4GlcNAc; NHS, N-Hydroxysuccinimide; RFU, relative fluorescence unit(s); RCA-I, R. communis agglutinin I; SNA, S. nigra agglutinin; MAL-I, M. amurensis lectin-I; Neu5Ac, N-acetylaceulaminuronic acid; Neu5Gc, N-glycolylneuraminic acid; PGC, porous graphitized carbon; hPIV, human parainfluenza virus; Kdn, 2-keto-3-deoxy-D-glycero-D-galactonononic acid, SGM, sialylated glycan microarray; CFG, Consortium of Functional Glycomics.
in chemoenzymatic synthesis of sialosides containing natural and non-natural sialic acid residues using multiple promiscuous sialoside biosynthetic enzymes (5, 15–20).

We hypothesized that the existence of multiple modified sialic acid structures has biological importance related to their interaction with proteins or microorganisms. To further explore the recognition and function of modified sialic acid residues, we exploited the development of new chemoenzymatic methods to synthesize modified sialic acids (5), along with recent developments of glyc an microarray technology, which provide an innovative approach to studying glycan recognition and function by glycan-binding proteins. We adapted our recent development of natural glycan microarrays (21–24) to the production of a diverse array of sialylated glycans using fluorescent derivatives of glycans that terminate with β-linked galactose, which are sialyltransferase acceptors, in a one-pot three-enzyme approach for the microscale chemoenzymatic synthesis of sialosidoses (19, 20, 25). The resultant microarray consists of 77 sialylated glycans incorporating 16 modified sialic acids in α2–3 and α2–6 linkages to different underlying structures. This novel sialylated glycan microarray is useful for rapidly screening glycan-binding proteins and viruses for their interactions with a wide variety of modified sialic acids and represents a new glycomic technology to explore glycan function and recognition.

**EXPERIMENTAL PROCEDURES**

Free reducing glycans lactose, lacto-N-tetraose (Galβ1-3GlcNAcβ1–3Galβ1–4Glc; LNT), and lacto-N-neotetraose (Galβ1–4GlcNAcβ1–3Galβ1–4Glc; LNNt) and all chemicals were purchased from Sigma-Aldrich and used without further purification. Asialo, biantennary-N-glycan (Galβ1–4GlcNAcβ1–2Manβ1–6Galβ1–4GlcNAcβ1–2Manβ1–3 Manβ1–4GlcNAcβ1–4GlcNAc; NA2) was prepared from a chicken egg yolk glycopeptide (26) by mild acid hydrolysis and peptidase-N-glycosidase F digestion. The bifunctional linker, 2-amino(Ν-aminomethyl) benzamide (AEAB), was prepared as described previously (27). HPLC solvents were purchased from Fisher. An Ultraflex-II TOF/TOF system from Bruker Daltonics was used for MALDI-TOF mass spectrometry analysis of glycan conjugates. Biotinylated ConA, *Ricinus communis* agglutinin I (RCA-I), *Sambucus nigra* agglutinin (SNA), and *Maackia amurensis* lectin (MAL-I) were from Vector Laboratories; Cy5- and Alexa488-labeled streptavidin were from Invitrogen; and *Arthrobacter ureafaciens* sialidase was from Sigma.

Glycan-AEAB Conjugation, Sialylation, and Purification—Free reducing glycans were conjugated with AEAB as described previously (27). Briefly, 1–10 mg of glycan was mixed with 50–250 μl of AEAB hydrochloride salt solution freshly prepared at 0.35 mM in DMSO/ACOH (7:3, v/v) followed by an equal volume of sodium cyanoborohydride solution freshly prepared in the same solvent. The mixture was vortexed briefly and incubated at 65 °C for 2 h. The mixture was chilled, and the products were precipitated upon the addition of 10 volumes of acetonitrile. After bringing the suspension to −20 °C and maintaining that temperature for 30 min, the products were separated by centrifugation at 10,000 × g for 3 min. The pellet was redisolved in 200–500 μl of water and purified by HPLC. AEAB derivatives of LNNt, LNT, and the biantennary-N-glycan, NA2, were prepared as precursors for presentation of modified sialic acids.

Sialyl glycans containing various natural modifications of sialic acid linked α2–3 or α2–6 to the three different fluorescent glycans described above were synthesized using a highly efficient one-pot three-enzyme system similar to that reported previously (19, 20, 25, 28). The fluorescent glycan-AEAB conjugates (GAEABs) of LNT, LNNt, or NA2 (130–200 μg) were incubated with three enzymes, including *Escherichia coli* sialic acid aldolase (28) (10–15 μg), *Neisseria meningitidis* CMP-sialic acid synthetase (28) (10–15 μg), and *Photobacterium damsela* ae2–2–sialyltransferase (25) (10–15 μg; used for the synthesis of α2–2–linked sialosidoses) or *Pasteurella multocida* ae2–3–sialyltransferase PmST1 (19) (3–6 μg; used for the synthesis of α2–3–linked sialosidoses) in a reaction mixture of 25 μl containing 100 mM Tris-HCl buffer, pH 8.5. The reaction mixtures were incubated at 37 °C for 2 h (for preparing α2–3–sialylated glycans using PmST1) or 24 h (for preparing α2–2–6–sialylated glycans using *P. damsela* ae2–2–sialyltransferase). A Tris-HCl buffer, pH 7.5, was used for substrates containing a base sensitive functional group (e.g. acetyl or lactyl group). The following were added to this mixture: (a) sialic acid precursors (20, 25, 28, 29) (e.g. mannos, N-acetylmannosamine, or their derivatives) at 2.0 or 4.0 eq relative to LNT or LNNt and NA2 (biantennary-AEAB conjugate), respectively; (b) sodium pyruvate (5 eq for LNT or LNNt, 10 equiv. for NA2); (c) CTP (2.0 eq for LNT or LNNt, 4.0 eq for NA2); and (d) MgCl2 (20 mM). The reaction mixtures were incubated at 37 °C. Product formation was monitored by TLC in EtOAc/MeOH/H2O/HOAc (4:2:1:1) and visualized by a UV lamp to follow modification of the fluorescent sialic acid acceptor. When the product formation reached a maximum, the reaction mixtures were frozen. The reaction mixtures were then mixed with 2 volumes of ethanol to precipitate enzymes and cooled at −20 °C for 30 min. After centrifugation at 10,000 × g for 3 min, the supernatant containing the products and excess reactants was purified by porous graphitized carbon (PGC)-HPLC. The collected peaks were dried in a SpeedVac, reconstituted in 100 μl of water, and purified again by PGC-HPLC when necessary. The purified major product of each reaction was repurified using ion exchange HPLC to confirm homogeneity and charge state and to quantify each glycan based on fluorescence and UV. They were also characterized by MALDI-TOF to confirm the generation of the anticipated product of the sialyltransferase from starting N-acetylhexosamine, hexosamine, hexose, or appropriately modified derivative, pyruvate, and CTP.

High Performance Liquid Chromatography—A Shimadzu HPLC CBM-20A system, coupled with a UV detector SPD-20A and a fluorescence detector RF-10Axl, was used for HPLC analysis and separation of GAEABs. The column effluent was monitored by UV absorption at 330 nm and/or fluorescence at 330 nm excitation and 420 nm emission. Both UV absorption and fluorescence intensity were used for the quantification of the AEAB derivatives using lacto-N-fucopentaose (LNFPIII)-AEAB as a standard. For reverse phase HPLC with a PGC column (150 × 4.6 mm), the mobile phase was acetonitrile and water with 0.1% trifluoroacetic acid (TFA). For the glycan anal-
A Novel Sialylated Glycan Microarray

FIGURE 1. The design and preparation of an SGM. Fluorescent glycans (GAEABs) terminating in β-linked galactose were acceptors in a one-pot three-enzyme synthetic scheme with pyruvate, CTP and mannose, N-acetylmannosamine, or various derivatives to generate fluorescent sialyl glycans containing α2–3- and α2–6-linked sialic acid forms. The purified fluorescent sialyl glycans were structurally confirmed and printed as an SGM.

RESULTS

Preparation of Sialylated GAEABs—We chose a synthetic strategy that combined two earlier developments, the bifunctional fluorescent tag AEAB (27) and the one-pot three-enzyme sialylation reaction (19, 20, 25) (Fig. 1). GAEABs are fluorescent and enable us to isolate and process glycans on a microscale. In addition, they have a primary amino group for immobilization onto activated glass surfaces to generate glycan microarrays. Using GAEABs with terminal galactose residues as acceptors for sialyltransferases, we explored a one-pot three-enzyme sialylation system to synthesize terminally sialylated glycans with various sialic acid modifications. The GAEAB acceptors included lactose, LNnT, LNT, and NA2, which were prepared from natural glycans as described previously (27) and purified prior to the multienzyme sialylation. Aliquots (50 μg to 1 mg) of the GAEABs were subjected to one-pot three-enzyme sialylation in a combinatorial fashion (16). The application of N-acetylmannosamine, N-glycolylmannosamine, Man, or their derivatives in the enzymatic reactions containing a sialic acid aldolase, a CMP-sialic acid synthetase, and a CMP-SiaI transferase allows formation of corresponding sialosides or their derivatives in the enzymatic reactions containing a CMP-SiaI transferase.
The enzymatic sialylation efficiency varied dramatically among different reactions, the products were purified by HPLC. Profiles of four of the 77 reaction mixtures are shown as examples in Fig. 2, and the structures and homogeneity of each purified sialylated glycan were confirmed by MALDI-TOF and HPLC analysis shown for each sialyl glycan in supplemental Fig. 1. Because the sialylated glycan products are fluorescent, even small amounts of the isolated products (~1 nmol) are accurately quantified for printing on NHS-activated glass slides. As shown in Table 1 and supplemental Table 2, we generated 77 different terminal sialic acids (Fig. 3a) on four different underlying glycan structures (Fig. 3b) that were tagged with a functional fluorescent linker (Fig. 3c). Each glycan was quantified based on its fluorescence and printed in replicates of n = 4 on NHS-activated glass slides as described previously (27) to provide a sialylated glycan microarray (SGM). The selection of this set of sialic acid structures is based on structures of known importance, natural occurrence, and higher abundance, along with current chemical accessibility. Of the 16 sialic acid structures selected for synthesis, four (Neu5Ac, Neu5,9Ac2, Neu5Ac9Lt, and Kdn) are known to occur in humans, and only three (Neu5Ac9Me, Kdn9Me, and Kdn7Me) are not known to occur or are yet to be discovered in nature. The large diversity of sialic acid structures on common acceptors printed as a glycan microarray facilitates defining the specific interactions of different binding proteins and microorganisms.

**Lectin Binding Confirms Efficient Printing of Sialylated Glycans**—To test whether the sialylated glycans were efficiently printed on the SGM and to evaluate its utility, we interrogated it with plant lectins of known specificities (Figs. 4a–d). ConA, a lectin that binds high mannose-, hybrid-, and complex-type bi-antennary N-glycans (32), showed strong binding to all of the sialylated bi-antennary N-glycans (NA2) possessing the trimannosyl core structure (18–30) and 58–72 (glycan numbers are shown in boldface type), the acceptor bi-antennary N-glycan galactoside used for the sialylation reaction (79), and the Man3GlcNAc2, N-glycan (80).

RCA-1, which binds to terminal Galβ1–4GlcNAc (32), showed stronger binding, as expected, to bivalent NA2 (79) compared with monovalent LNnT (78). Consistent with its known specificity, RCA-1 bound glycans with α2–6-linked sialic acid on Galβ1–4GlcNAc (18–30) but did not bind glycans terminating in α2–3–linked sialic acid, except for the monosialylated, bi-antennary NA2 structures (59–61, 66, 68, and 70–72). The presence of α2–6-linked sialic acid on Gal residues in Galβ1–4GlcNAc interferes with but does not prevent binding of RCA-1, as seen by comparing binding to NA2 (79) and its disialylated (α2–6) derivative (18). The α2–6 monosialylated derivatives of NA2 (28–30) show binding that was essentially equivalent to unsubstituted NA2 (79). Glycan 20, which is an α2–6 monosialylated derivative of NA2, bound RCA-1 before and after neuraminidase, although binding was not enhanced after neuraminidase treatment. RCA-1 bound weakly to α2–6-sialylated lactose and LNnT (1–17), unlike the corresponding α2–3-sialylated lactose and LNnT (41–57), which were not bound. Although most sialic acid derivatives moderately decreased RCA-1 binding to NA2, α2–6-sialylation with Neu5Ac8Me (19) and Neu5Gc9Ac (25) greatly inhibit RCA-1 binding.

After treatment of the slides with mild acid to remove the terminal sialic acid from the sialylated glycans on the array, RCA-1 binding was generally increased over control levels in sialylated type 2 glycans substituted with sialic acids. The acid treatment slightly reduced binding to the control glycans (LNnT and NA2, 78 and 79) compared with the untreated slide probably as a result of slight damage to the derivatized surface. Binding of RCA-1 to LNnT (3–17 and 31–40) was low but detectable on the mild acid-treated slide compared with the untreated slide. When the SGM was treated with A. ureafaciens sialidase, we observed increased binding of RCA-1 for those glycans cleavable by the enzyme as discussed below. These data demonstrate that the sialylated glycans were efficiently printed on the slides. The Neu5Ac8Me derivatives of NA2 (19 and 59) and all Kdn derivatives (67–72) showed no significant increase in binding by RCA-1 after sialidase compared with the untreated and mild acid-treated slides. Although Kdn is generally more resistant than Neu5Ac and Neu5Gc derivatives, 9-O-acetylation of Neu5Ac (6, 7, and 22) and Neu5Gc (10 and 25) also increase their resistance to sialidase. This finding is important and indicates that caution is needed in studies using plant lectins and sialidase treatments of biological samples to explore expression of sialylated glycoconjugates.

**Binding of Sialic Acid-binding Plant Lectins to the SGM**—We analyzed binding of the sialic acid-binding lectins SNA and MAL-1 to the SGM. SNA bound to the Sia2-6Galβ1–4GlcNAc-terminated glycans (LNnT and NA2 backbones) (Figs. 4 lane 5 and 6a), which is consistent with its known specificity for this sialic acid linkage (33, 34). However, it differentiates among the sialyl derivatives and does not bind either LNnT or NA2 derivatized with α2–6-linked Neu5Ac8Me on 4 and 19, respectively, suggesting a requirement of a free 8-OH in sialic acid for SNA binding. Interestingly, SNA binds poorly to Sia2–6 derivatives of LNT, which contains penultimate Galβ1–3GlcNAc, but there is significant binding to the Kdnα2–6 derivatives of LNT. This is consistent
with the strong binding of SNA to Kdnα2–6 derivatives of NA2 (27–30) compared with the corresponding Neu5Ac and Neu5Gc derivatives of NA2 (18, 20, and 23–26). MAL-I, which is specific for Siaα2–3Galβ1–4GlcNAc-R-terminated glycans (35), bound only sialylated glycans terminating with that sequence (Figs. 4 (lane 8) and 6d). MAL-I

### TABLE 1

The chart ID and glycan structures of the SGM

| Chart ID | Structure |
|----------|-----------|
| 1        | Neu5Ac6Galβ4GlcNAcβ3Galβ4Glcitol-AEAB |
| 2        | Neu5Gc6Galβ4GlcNAcβ3Galβ4Glcitol-AEAB |
| 3        | Neu5Ac9Me6Galβ4GlcNAcβ3Galβ4Glcitol-AEAB |
| 4        | Neu5Ac9Me6Galβ4GlcNAcβ3Galβ4Glcitol-AEAB |
| 5        | Neu5Ac9Me6Galβ4GlcNAcβ3Galβ4Glcitol-AEAB |
| 6        | Neu5Ac9Me6Galβ4GlcNAcβ3Galβ4Glcitol-AEAB |
| 7        | Neu5Ac9Me6Galβ4GlcNAcβ3Galβ4Glcitol-AEAB |
| 8        | Neu5Ac6Galβ4GlcNAcβ3Galβ4Glcitol-AEAB |
| 9        | Neu5Ac6Galβ4GlcNAcβ3Galβ4Glcitol-AEAB |
| 10       | Neu5Ac6Galβ4GlcNAcβ3Galβ4Glcitol-AEAB |
| 11       | Neu5Ac6Galβ4GlcNAcβ3Galβ4Glcitol-AEAB |
| 12       | Neu5Ac6Galβ4GlcNAcβ3Galβ4Glcitol-AEAB |
| 13       | Neu5Ac6Galβ4GlcNAcβ3Galβ4Glcitol-AEAB |
| 14       | Neu5Ac6Galβ4GlcNAcβ3Galβ4Glcitol-AEAB |
| 15       | Neu5Ac6Galβ4GlcNAcβ3Galβ4Glcitol-AEAB |
| 16       | Neu5Ac6Galβ4GlcNAcβ3Galβ4Glcitol-AEAB |
| 17       | Neu5Ac6Galβ4GlcNAcβ3Galβ4Glcitol-AEAB |
| 18       | Neu5Ac6Galβ4GlcNAcβ3Galβ4Glcitol-AEAB |
| 19       | Neu5Ac6Galβ4GlcNAcβ3Galβ4Glcitol-AEAB |
| 20       | Neu5Ac6Galβ4GlcNAcβ3Galβ4Glcitol-AEAB |
| 21       | Neu5Ac6Galβ4GlcNAcβ3Galβ4Glcitol-AEAB |
| 22       | Neu5Ac6Galβ4GlcNAcβ3Galβ4Glcitol-AEAB |
| 23       | Neu5Ac6Galβ4GlcNAcβ3Galβ4Glcitol-AEAB |
| 24       | Neu5Ac6Galβ4GlcNAcβ3Galβ4Glcitol-AEAB |
| 25       | Neu5Ac6Galβ4GlcNAcβ3Galβ4Glcitol-AEAB |
| 26       | Neu5Ac6Galβ4GlcNAcβ3Galβ4Glcitol-AEAB |
| 27       | Neu5Ac6Galβ4GlcNAcβ3Galβ4Glcitol-AEAB |
| 28       | Neu5Ac6Galβ4GlcNAcβ3Galβ4Glcitol-AEAB |
| 29       | Neu5Ac6Galβ4GlcNAcβ3Galβ4Glcitol-AEAB |
| 30       | Neu5Ac6Galβ4GlcNAcβ3Galβ4Glcitol-AEAB |
| 31       | Neu5Ac6Galβ4GlcNAcβ3Galβ4Glcitol-AEAB |
| 32       | Neu5Ac6Galβ4GlcNAcβ3Galβ4Glcitol-AEAB |
| 33       | Neu5Ac6Galβ4GlcNAcβ3Galβ4Glcitol-AEAB |
| 34       | Neu5Ac6Galβ4GlcNAcβ3Galβ4Glcitol-AEAB |
| 35       | Neu5Ac6Galβ4GlcNAcβ3Galβ4Glcitol-AEAB |
| 36       | Neu5Ac6Galβ4GlcNAcβ3Galβ4Glcitol-AEAB |
| 37       | Neu5Ac6Galβ4GlcNAcβ3Galβ4Glcitol-AEAB |
| 38       | Neu5Ac6Galβ4GlcNAcβ3Galβ4Glcitol-AEAB |
| 39       | Neu5Ac6Galβ4GlcNAcβ3Galβ4Glcitol-AEAB |
| 40       | Neu5Ac6Galβ4GlcNAcβ3Galβ4Glcitol-AEAB |
| 41       | Neu5Ac6Galβ4GlcNAcβ3Galβ4Glcitol-AEAB |
| 42       | Neu5Ac6Galβ4GlcNAcβ3Galβ4Glcitol-AEAB |
| 43       | Neu5Ac6Galβ4GlcNAcβ3Galβ4Glcitol-AEAB |
| 44       | Neu5Ac6Galβ4GlcNAcβ3Galβ4Glcitol-AEAB |
| 45       | Neu5Ac6Galβ4GlcNAcβ3Galβ4Glcitol-AEAB |
| 46       | Neu5Ac6Galβ4GlcNAcβ3Galβ4Glcitol-AEAB |
| 47       | Neu5Ac6Galβ4GlcNAcβ3Galβ4Glcitol-AEAB |
| 48       | Neu5Ac6Galβ4GlcNAcβ3Galβ4Glcitol-AEAB |
| 49       | Neu5Ac6Galβ4GlcNAcβ3Galβ4Glcitol-AEAB |
| 50       | Neu5Ac6Galβ4GlcNAcβ3Galβ4Glcitol-AEAB |
| 51       | Neu5Ac6Galβ4GlcNAcβ3Galβ4Glcitol-AEAB |
| 52       | Neu5Ac6Galβ4GlcNAcβ3Galβ4Glcitol-AEAB |
| 53       | Neu5Ac6Galβ4GlcNAcβ3Galβ4Glcitol-AEAB |
| 54       | Neu5Ac6Galβ4GlcNAcβ3Galβ4Glcitol-AEAB |
| 55       | Neu5Ac6Galβ4GlcNAcβ3Galβ4Glcitol-AEAB |
| 56       | Neu5Ac6Galβ4GlcNAcβ3Galβ4Glcitol-AEAB |
| 57       | Neu5Ac6Galβ4GlcNAcβ3Galβ4Glcitol-AEAB |
| 58       | Neu5Ac6Galβ4GlcNAcβ3Galβ4Glcitol-AEAB |
| 59       | Neu5Ac6Galβ4GlcNAcβ3Galβ4Glcitol-AEAB |
| 60       | Neu5Ac6Galβ4GlcNAcβ3Galβ4Glcitol-AEAB |
| 61       | Neu5Ac6Galβ4GlcNAcβ3Galβ4Glcitol-AEAB |
| 62       | Neu5Ac6Galβ4GlcNAcβ3Galβ4Glcitol-AEAB |
| 63       | Neu5Ac6Galβ4GlcNAcβ3Galβ4Glcitol-AEAB |
| 64       | Neu5Ac6Galβ4GlcNAcβ3Galβ4Glcitol-AEAB |
| 65       | Neu5Ac6Galβ4GlcNAcβ3Galβ4Glcitol-AEAB |
| 66       | Neu5Ac6Galβ4GlcNAcβ3Galβ4Glcitol-AEAB |
| 67       | Neu5Ac6Galβ4GlcNAcβ3Galβ4Glcitol-AEAB |
| 68       | Neu5Ac6Galβ4GlcNAcβ3Galβ4Glcitol-AEAB |
| 69       | Neu5Ac6Galβ4GlcNAcβ3Galβ4Glcitol-AEAB |
| 70       | Neu5Ac6Galβ4GlcNAcβ3Galβ4Glcitol-AEAB |
| 71       | Neu5Ac6Galβ4GlcNAcβ3Galβ4Glcitol-AEAB |
| 72       | Neu5Ac6Galβ4GlcNAcβ3Galβ4Glcitol-AEAB |
| 73       | Neu5Ac6Galβ4GlcNAcβ3Galβ4Glcitol-AEAB |
| 74       | Neu5Ac6Galβ4GlcNAcβ3Galβ4Glcitol-AEAB |
| 75       | Neu5Ac6Galβ4GlcNAcβ3Galβ4Glcitol-AEAB |
| 76       | Neu5Ac6Galβ4GlcNAcβ3Galβ4Glcitol-AEAB |
| 77       | Neu5Ac6Galβ4GlcNAcβ3Galβ4Glcitol-AEAB |
| 78       | Neu5Ac6Galβ4GlcNAcβ3Galβ4Glcitol-AEAB |
| 79       | Neu5Ac6Galβ4GlcNAcβ3Galβ4Glcitol-AEAB |
| 80       | Neu5Ac6Galβ4GlcNAcβ3Galβ4Glcitol-AEAB |
differenates among the sialyl derivatives; it does not bind α2–3-sialylated LNnT or NA2 with Kdn5Me on 56 and 71, respectively. This observation and the relatively high binding of MAL-I for glycans α2–3-sialylated with C5-substituted Kdn derivatives (Kdn5Ac (54 and 69) and Kdn5,9Ac2 (55)) indicate that an acetyl group on the 5-OH of Kdn is needed for MAL-I binding. Like SNA, MAL-I bound better to O-acetylated derivatives of Kdn than to corresponding glycans terminated in the common sialic acids, Neu5Ac and Neu5Gc. The observation that MAL-I binds poorly to α2–3-sialylated LNT, which contains the subterminal sequence Galβ1–3GlcNAc, is consistent with the specificity of MAL-I for the type 2 sequence Galβ1–4GlcNAc. Removal of sialic acids from the glycan array by mild acid hydrolysis eliminated binding by SNA and MAL-I (Figs. 4 (lanes 7 and 10) and 6 (c and f)), and the residual binding after A. ureafaciens sialidase digestion (Figs. 4 (lanes 6 and 9) and 6 (b and e)) is consistent with the earlier observation of the sialidase resistance of the Kdn derivatives.

To better evaluate the specificity of these sialic acid-binding lectins for this diverse group of sialic acid derivatives, we analyzed the binding of lectins in a dose-dependent manner on the SGM as described previously for SNA on a mammalian cell glycan array (34). Briefly, the binding of each lectin at different concentrations to each glycan was normalized to percentages of the highest RFU value for each analysis, and the percentage of maximum binding for each glycan at the different lectin concentrations was averaged to obtain an average ranking. The detailed ranked analyses for SNA and MAL-I are shown in supplemental Tables 3 and 4. Assuming that the rankings represent strong binding at high values with rankings below 10% considered weak binders or non-binders, the influence of sialic acid modification on the SNA and MAL-I binding can be evaluated. This is, however, a complex analysis due to the number of sialic acid modifications and the fact that significant differences in ranking are observed between monovalent and divalent interactions created by the precursor glycans (LNnT and NA2). To simplify the comparisons of the effects of sialic acid modification on SNA and MAL-I binding, Tables 2 and 3 only show the ranking of the potential monovalent glycan ligands for SNA (Sia2,6 derivatives of the tetrasaccharides) and MAL-I (Sia2,3 derivatives of LNnT), respectively.

Consistent with the known specificity of SNA for glycans terminating in Sia2–6Galβ1–4GlcNAc, ranking analysis (Table 2) showed that the highest binding glycans on the array were those possessing sialic acids linked α2–6 to type 2 precursors. Interestingly, the LNnT terminating with the most common sialic acids, Neu5Acα2–6- and Neu5Gcα2–6- (3 and 8, respectively) were among the weakest of the sialylated glycans bound by SNA at a ranking of 8 compared with glycans terminating in modified Kdns (15, 16, 6, 14, 13, 17, and 38) at rankings of ~30–100, representing a 10-fold higher level of binding for this lectin in certain cases. If Neu5Ac is modified with a methyl group at the C8-position (4), even this weak interaction is lost; the 8-O-methyl derivative of Neu5Gc is not avail-
A Novel Sialylated Glycan Microarray

| Sialyl glycans # | Structure | Lectins | Viruses |
|------------------|-----------|---------|---------|
| 1                | NeuSAcα2,3Lac |         |         |
| 2                | NeuSacα2,6LacNT |         |         |
| 3                | NeuSacα2,8Man5NT |         |         |
| 4                | NeuSacα2,8Man5NT |         |         |
| 5                | NeuSacα2,8Man5NT |         |         |
| 6                | NeuSacα2,8Man5NT |         |         |
| 7                | NeuSacα2,8Man5NT |         |         |
| 8                | NeuSacα2,8Man5NT |         |         |
| 9                | NeuSacα2,8Man5NT |         |         |
| 10               | NeuSacα2,8Man5NT |         |         |
| 11               | NeuSacα2,8Man5NT |         |         |
| 12               | Kdn9Acα2,3LacNT |         |         |
| 13               | Kdn9Acα2,6LacNT |         |         |
| 14               | Kdn9Acα2,6LacNT |         |         |
| 15               | Kdn9Acα2,6LacNT |         |         |
| 16               | Kdn9Acα2,6LacNT |         |         |
| 17               | Kdn9Acα2,6LacNT |         |         |
| 18               | NeuSacα2,3LacNA2 |         |         |
| 19               | NeuSacα2,3LacNA2 |         |         |
| 20               | NeuSacα2,3LacNA2 |         |         |
| 21               | NeuSacα2,3LacNA2 |         |         |
| 22               | NeuSacα2,3LacNA2 |         |         |
| 23               | NeuSacα2,3LacNA2 |         |         |
| 24               | NeuSacα2,3LacNA2 |         |         |
| 25               | NeuSacα2,3LacNA2 |         |         |
| 26               | NeuSacα2,3LacNA2 |         |         |
| 27               | NeuSacα2,3LacNA2 |         |         |
| 28               | NeuSacα2,3LacNA2 |         |         |
| 29               | NeuSacα2,3LacNA2 |         |         |
| 30               | NeuSacα2,3LacNA2 |         |         |
| 31               | NeuSacα2,3LacNT |         |         |
| 32               | NeuSacα2,3LacNT |         |         |
| 33               | NeuSacα2,3LacNT |         |         |
| 34               | NeuSacα2,3LacNT |         |         |
| 35               | NeuSacα2,3LacNT |         |         |
| 36               | NeuSacα2,3LacNT |         |         |
| 37               | NeuSacα2,3LacNT |         |         |
| 38               | NeuSacα2,3LacNT |         |         |
| 39               | NeuSacα2,3LacNT |         |         |
| 40               | NeuSacα2,3LacNT |         |         |
| 41               | NeuSacα2,3LacNT |         |         |
| 42               | NeuSacα2,3LacNT |         |         |
| 43               | NeuSacα2,3LacNT |         |         |
| 44               | NeuSacα2,3LacNT |         |         |
| 45               | NeuSacα2,3LacNT |         |         |
| 46               | NeuSacα2,3LacNT |         |         |
| 47               | NeuSacα2,3LacNT |         |         |
| 48               | NeuSacα2,3LacNT |         |         |
| 49               | NeuSacα2,3LacNT |         |         |
| 50               | NeuSacα2,3LacNT |         |         |
| 51               | NeuSacα2,3LacNT |         |         |
| 52               | NeuSacα2,3LacNT |         |         |
| 53               | NeuSacα2,3LacNT |         |         |
| 54               | NeuSacα2,3LacNT |         |         |
| 55               | NeuSacα2,3LacNT |         |         |
| 56               | NeuSacα2,3LacNT |         |         |
| 57               | NeuSacα2,3LacNT |         |         |
| 58               | NeuSacα2,3LacNT |         |         |
| 59               | NeuSacα2,3LacNT |         |         |
| 60               | NeuSacα2,3LacNT |         |         |
| 61               | NeuSacα2,3LacNT |         |         |
| 62               | NeuSacα2,3LacNT |         |         |
| 63               | NeuSacα2,3LacNT |         |         |
| 64               | NeuSacα2,3LacNT |         |         |
| 65               | NeuSacα2,3LacNT |         |         |
| 66               | NeuSacα2,3LacNT |         |         |
| 67               | NeuSacα2,3LacNT |         |         |
| 68               | NeuSacα2,3LacNT |         |         |
| 69               | NeuSacα2,3LacNT |         |         |
| 70               | NeuSacα2,3LacNT |         |         |
| 71               | NeuSacα2,3LacNT |         |         |
| 72               | NeuSacα2,3LacNT |         |         |
| 73               | NeuSacα2,3LacNT |         |         |
| 74               | NeuSacα2,3LacNT |         |         |
| 75               | NeuSacα2,3LacNT |         |         |
| 76               | NeuSacα2,3LacNT |         |         |
| 77               | NeuSacα2,3LacNT |         |         |
| 78               | NeuSacα2,3LacNT |         |         |
| 79               | NeuSacα2,3LacNT |         |         |
| 80               | NeuSacα2,3LacNT |         |         |

Legend:
- **High**:
- **Low**:

**Notes:**
- Con A, RCA I, RCA II, SNA, SNA + Neuraminidase, MAL-I, MAL-I + Mild acid, MAL-I + Neuraminidase, HPV1, HPV2, HPV3, AOK444/08 H1N1, AOK468/08 H3N2.
able (36). On the other hand, if Neu5Ac is derivatized with an \(\text{O}\)-acetyl group at the 9-position (6), SNA-binding rank increases to 50. Modification of Neu5Ac with a methyl group at the C9-position (5) had little effect on binding to SNA, whereas modification at the C9-position with a larger lactyl group (7) increased binding 3-fold to a ranking of 24. Methylation or acetylation of the \(\text{N}\)-glycolyl group at C5 in Neu5Gc (9 and 11, respectively) resulted in a slight increase in SNA binding, whereas 9-\(\text{O}\)-acetylation of Neu5Gc (10), like 9-\(\text{O}\)-acylation of Neu5Ac, resulted in a significant increase in SNA binding. The \(\alpha\)2–6Kdn derivative of LNnT (12), which is the simplest of the sialic acid derivatives, was bound by SNA almost 3-fold stronger than the corresponding Neu5Ac and Neu5Gc-terminated LNnT. This binding increased an additional 4-fold when the Kdn was modified by \(\text{O}\)-acylation at the 5- and 9-positions (15); interestingly, this Kdn5,9Ac \(\alpha\) derivative (15) was still 2-fold more strongly bound than the corresponding Neu5,9Ac \(\alpha\) derivative of LNnT (6). Other \(\text{O}\)-acylated and \(\text{O}\)-methylated derivatives of Kdn (13, 14, 16, and 17) showed increased binding over glycans containing Kdn alone.

Consistent with the known specificity of MAL-I for glycans terminating in Neu5Ac-2–3Gal-\(\beta\)1–4GlcNAc, ranking analysis of this lectin (Table 3) showed that the strongest binding glycans on the array were those possessing sialic acids linked to type 2 precursors. Similar to what we observed for SNA, LNnT terminating with the most common sialic acids, Neu5Ac, and Neu5Gc-terminated LNnT. This binding increased an additional 4-fold when the Kdn was modified by \(\text{O}\)-acylation at the 5- and 9-positions (15); interestingly, this Kdn5,9Ac \(\alpha\) derivative (15) was still 2-fold more strongly bound than the corresponding Neu5,9Ac \(\alpha\) derivative of LNnT (6). Other \(\text{O}\)-acylated and \(\text{O}\)-methylated derivatives of Kdn (13, 14, 16, and 17) showed increased binding over glycans containing Kdn alone.

Consistent with the known specificity of MAL-I for glycans terminating in Neu5Gc-2–3Gal-\(\beta\)1–4GlcNAc, ranking analysis of this lectin (Table 3) showed that the strongest binding glycans on the array were those possessing sialic acids linked to type 2 precursors. Similar to what we observed for SNA, LNnT terminating with the most common sialic acids, Neu5Ac, and Neu5Gc-terminated LNnT.
case of MAL-I binding, modification of Neu5Ac with a methyl group at the 8-position (44) resulted in increased binding. Other modifications of Neu5Ac, including 9-O-acetylation (46) and 9-O-methylation (45), increased binding by MAL-I, whereas the addition of an O-lactyl group at position 9 (47) had little effect. Although the α2,3-linked Kdn derivative of LNnT (52) shared similar apparent binding levels with the corresponding Neu5Ac and Neu5Gc derivatives (43 and 48), MAL-I binding was very sensitive to modifications of Kdn with significant increases associated with Kdn5,9Ac2 (55), Kdn7Me (57), and Kdn5Ac (54), whereas the binding to the 9-O-acetyl derivative of Kdn (53) was unchanged, and the binding to the 5-O-methyl derivative of LNnT (56) was severely reduced. These results demonstrate that sialic acid modifications affect recognition by SNA and MAL-I in novel ways and that the SGM provides a new platform for studying the specific
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TABLE 2
The relative ranking of the sialylated glycans that bind SNA

| Average Rank | Structure | Chart ID |
|--------------|-----------|----------|
| 100          | KDn5,9Ac6Glcb6Galβ4GlcNAcβ3Galβ4Glcitol-AEAB 15 |
| 50           | Kdn9Me6GlcNAcβ3Galβ4Glcitol-AEAB 16 |
| 50           | Neu5Ac9Me6GlcNAcβ3Galβ4Glcitol-AEAB 6 |
| 50           | Kdn9Ac6Galβ3GlcNAcβ3Galβ4Glcitol-AEAB 14 |
| 32           | Kdn9Ac6Galβ3GlcNAcβ3Galβ4Glcitol-AEAB 13 |
| 27           | Kdn9Ac6Galβ3GlcNAcβ3Galβ4Glcitol-AEAB 17 |
| 25           | Neu5Gc9Ac6Galβ3GlcNAcβ3Galβ4Glcitol-AEAB 10 |
| 24           | Neu5Ac9Pn6GlcNAcβ3Galβ4Glcitol-AEAB 7 |
| 23           | Kdn6GlcNAcβ3Galβ4Glcitol-AEAB 12 |
| 22           | Kdn5Ac6Galβ3GlcNAcβ3Galβ4Glcitol-AEAB 14 |
| 20           | Neu5GcAc6Galβ3GlcNAcβ3Galβ4Glcitol-AEAB 11 |
| 18           | Neu5Gc9Me6GlcNAcβ3Galβ4Glcitol-AEAB 9 |
| 12           | Kdn5Me6GlcNAcβ3Galβ4Glcitol-AEAB 40 |
| 8            | Neu5Gc6GlcNAcβ3Galβ4Glcitol-AEAB 8 |
| 8            | Neu5Ac6GlcNAcβ3Galβ4Glcitol-AEAB 3 |
| 7            | Neu5Ac9Me6GlcNAcβ3Galβ4Glcitol-AEAB 5 |
| 6            | Kdn6GlcNAcβ3Galβ4Glcitol-AEAB 37 |
| 5            | Neu5Ac9GlcNAcβ3Galβ4Glcitol-AEAB 39 |
| 1            | Manα1(Mans3)Manα1(Mans3)Manβ4GlcNAcβ3Galβ4Glcitol-AEAB 80 |

TABLE 3
The relative ranking of the sialylated glycans that bind MAL-I

| Average Rank | Structure | Chart ID |
|--------------|-----------|----------|
| 100          | KDn5,9Ac6Galβ4GlcNAcβ3Galβ4Glcitol-AEAB 55 |
| 42           | Kdn9Me6GlcNAcβ3Galβ4Glcitol-AEAB 57 |
| 37           | Kdn5Ac6Galβ3GlcNAcβ3Galβ4Glcitol-AEAB 54 |
| 32           | Neu5Ac9Me6GlcNAcβ3Galβ4Glcitol-AEAB 45 |
| 27           | Neu5Ac8Me6GlcNAcβ3Galβ4Glcitol-AEAB 44 |
| 23           | Neu5GcAc6Galβ3GlcNAcβ3Galβ4Glcitol-AEAB 51 |
| 18           | Neu5Gc9Me6GlcNAcβ3Galβ4Glcitol-AEAB 50 |
| 15           | Neu5Ac9GlcNAcβ3Galβ4Glcitol-AEAB 48 |
| 14           | Kdn9Ac6Galβ3GlcNAcβ3Galβ4Glcitol-AEAB 53 |
| 13           | Neu5Gc6GlcNAcβ3Galβ4Glcitol-AEAB 49 |
| 10           | Neu5Ac9GlcNAcβ3Galβ4Glcitol-AEAB 43 |
| 10           | Neu5Ac9Ac6Galβ3GlcNAcβ3Galβ4Glcitol-AEAB 46 |
| 9            | Kdn6GlcNAcβ3Galβ4Glcitol-AEAB 52 |
| 8            | Neu5Ac9GlcNAcβ3Galβ4Glcitol-AEAB 47 |
| 1            | Kdn5Me6GlcNAcβ3Galβ4Glcitol-AEAB 56 |
| 3            | Manα1(Mans3)Manα1(Mans3)Manβ4GlcNAcβ3Galβ4Glcitol-AEAB 80 |

The Binding of Viruses to the SGM—Influenza viruses bind sialic acid on cell surfaces via hemagglutinin, and their specificities toward α6-linked sialic acid and α3-linked sialic acid are associated with their ability to infect humans or birds, respectively (6). Their specificities for the various modifications of sialic acid, however, have not been systematically studied due to the lack of available glycans. We screened several human viruses, including influenza A/Oklahoma/447/08 H1N1, influenza A/Oklahoma/483/08 H3N2, and hPIVs hPIV1 (strain C-35), hPIV2, and hPIV3 on the SGM (Fig. 4, lanes 11–15).

Although binding of the three strains of hPIV was restricted to sialic acids in α2–3-linkages to type 2 glycan chains (Galβ1–4GlcNAc) of LNnT and NA2 (Figs. 4, lanes 11–13 and 7 (a–c)), each demonstrated significant differences in binding to the diverse sialic acids on the SGM. These compounds were not available on the glycan microarray of the Consortium of Functional Glycomics (CFG), on which hPIV1 and hPIV3 were analyzed and reported earlier (37). Although hPIV1 and hPIV2 bound glycans with similar efficiency based on the RFU values, they demonstrated very different specificities for different sialic acid derivatives. The strictest specificity was observed with hPIV1, which bound to the monovalent Neu5Ac derivative of LNnT (43) and had stronger interaction with the corresponding Neu5GcMe derivative (49), whereas no binding was observed with the Neu5Gc derivative (48). This observation suggests that the virus hemagglutinin prefers the hydrophobic center provided by the CH3 group associated with the N-acetyl group of the sialic acid at the 5-position of Neu5Ac and that the methoxy group of the glycolyl function on Neu5Gc may even be more preferred. This is consistent with the observation that the bulker Neu5GcAc is not bound. Presentation of these sialyl derivatives as divalent structures on NA2 (58, 64, and 63) did not enhance binding by hPIV1. This is in contrast with commonly observed stronger binding of proteins to ligands presented at higher valence and may be due to interference between the two branches on an N-glycan core.

hPIV2 had a much broader specificity and bound to the monovalent Neu5Ac (43), Neu5Ac9Lt (47), and Neu5GcMe (49) derivatives of LNnT that were bound by hPIV1 as well as the corresponding Neu5Gc (48) and Neu5GcAc (51) and the corresponding divalent (NA2) derivatives (58, 62, and 64). No binding of hPIV2 was observed toward either the monovalent or divalent derivatives of Neu5Ac8Me (44 and 59), Neu5Ac9Me (45 and 60), and Neu5,9Ac6 (46 and 61), suggesting that binding requires a free 9-OH on the sialic acid. In light of this observation, it is not obvious why this virus binds the 9-O-lactyl derivative of Neu5Ac linked α2–3 to LNnT (47) or α2–3 to NA2 (62) to approximately the same degree (relative RFU) as the corresponding α2–3-linked Neu5Ac derivatives (43 and 58). The HPIV2 tolerated the hydrophilic HO- on the glycolyl of Neu5Gc on Neu5Gcα2–3LNnT (48), but the corresponding divalent structure on NA2 was not bound. Unlike hPIV1 specificity, the bulker Neu5GcAc derivative of LNnT (51) was bound by hPIV2, but the divalent Neu5GcAc derivative of NA2 was not available for comparison (66 is a monovalent Neu5GcAc derivative of NA2). By contrast, the binding of hPIV3 to the SGM was approximately an order of magnitude lower in RFU than the other hPIVs (Fig. 7, a–c). The lower RFU values are presumably due to variations in the preparation of the viruses and/or differences in the fluorescent labeling efficiency of virus; however, this should not affect the relative binding of virus to different glycans. Although somewhat similar to hPIV2, the pattern of binding exhibited broader specificity, showing binding to the Neu5Ac and Neu5Gc derivatives α2–3–linked to type 1 underlying structures (LNT) (73 and 75). None of the hPIVs showed binding to Kdn derivatives. Clearly, additional studies are needed.
using structural approaches to define the interactions of such unusual sialic derivatives with hPIV hemagglutinins. Binding of hPIV2 to Neu5Gc was seen on the CFG array, but the other modified derivatives are not present on the CFG array.

Influenza viruses A/Oklahoma/447/08 H1N1 and A/Oklahoma/483/08 H3N2 were analyzed for binding to the SGM, and both bound only glycans with α2–6-linked sialic acid. These viruses demonstrated a preference for only two sialic acid derivatives, namely the common α2–6-linked Neu5Ac (1, 3, 18, and 31) and interestingly the α2–6-linked Neu5Ac9Lt (7, 22, and 33), an equally strong binder (Figs. 4 (lanes 14 and 15) and 7 (d and e)). The H1N1 had a broader specificity for the underlying glycan, binding the α2–6-sialylated NA2 structures (18 and 22) that were not bound by the H3N2. The broader specificity of the H1N1 compared with H3N2 was observed on the defined glycan array from the CFG (38), but binding to Neu5Ac9Lt, which is not present on the CFG defined array, has not been observed previously. In general, the hydrophobicity at 5-position and hydrophilicity at 9-position substitution groups are usually important in determining virus binding (Fig. 8).

**DISCUSSION**

Our results demonstrate the development of a novel sialylated glycan microarray that permits studies on the roles of sialic acid modifications on molecular recognition by proteins and viruses. Analyses of specific expression and functional rec-
ognition of modified sialic acids has been difficult due to their unavailability. Previous development of sialoside arrays focused on sialosides containing four common natural sialic acid forms (Neu5Ac, Neu5Gc, Neu5Ac9Ac, and Neu5Gc9Ac) presented on diverse underlying glycans (17). An earlier chemoenzymatically synthesized biotinylated sialoside array with diverse sialic acid forms was presented on microplates without purification, which introduced some complexity to protein-binding studies (16). We report here the development of a comprehensive and well defined sialylated glycan microarray printed covalently on glass slides. The diversity of the SGM includes four type 1 and type 2 underlying structures, α2–3/6-sialyl linkages, and 16 different modified sialic acids, 13 of which are known to exist in nature (39), and the natural occurrence of Neu5Ac9Me, Kdn9Me, and Kdn7Me is yet to be explored. Although modified sialic acid-containing glycans are challenging to synthesize, we adapted the bifunctional fluorescent labeling of natural glycans and the one-pot multienzyme sialylation in a combinatorial fashion to synthesize this otherwise difficult-to-achieve large microscale library of sialylated glycans. The fluorescent labeled precursor enabled the parallel microscale enzymatic synthesis and facilitated the purification of products by HPLC. The well characterized one-pot multienzyme sialylation technology enabled the generation of terminal sialic acid diversity. The final products can be directly immobilized onto NHS- or epoxy-activated glass slides without further modification. Although the purification of the final products from the reaction mixtures was relatively time-consuming and yields varied, the final product library represents a durable resource for systematic study of sialic acid-binding proteins and organisms in the format of a microarray.

Much of our understanding about the occurrence of sialic acids in cells and tissues has come from chemical analysis or histochemical staining of samples with the sialic acid-specific lectins SNA and MAL-I (40, 41). Detailed specificity studies on SNA using the glycan array from the CFG, which has a limited number of different sialic acid derivatives, indicated that SNA has high binding activity toward Siaα2–6Galβ1,4GlcNAc-containing glycans (34). However, our results show that sialic acid recognition by SNA and MAL-I are affected by sialic acid modifications and raise new questions as to the use of these lectins to generally define sialic acid expression in tissues.

An unexpected observation was that both lectins generally bound well to Kdn, in comparison with the more common Neu5Ac and Neu5Gc derivatives. Kdn is a deaminated sialic acid that occurs widely in all vertebrates and in bacteria (42) and has been observed in human erythrocytes (11, 43). Importantly, certain modifications of sialic acid residues interfered with the binding of lectins. For example, SNA bound poorly to Neu5Ac8Meα2–6, and MAL-I bound poorly to Kdn5Meα2–3. Furthermore, inspection of the binding patterns of these sialic acid-specific lectins after treatment of sialylated glycans by either mild acid hydrolysis or sialidase indicated that some modifications of sialic acid also result in resistance to sialidase. Whereas glycans with Kdn were more resistant to A. arenicola sialidase than those with either Neu5Ac or Neu5Gc, several modifications of Neu5Ac and Neu5Gc, including 8-methylation and 9-acylation, inhibited their desialylation by this sialidase. This sialidase was studied because it is routinely used in sialylation-related studies because it is the most general sialidase known and is capable of releasing sialic acids in α2–3, α2–6, and α2–8 linkages (44). Our studies indicate that histological staining using lectins and specific sialidase treatment should include additional methods, such as specific antibodies to modified sialylated glycan determinants (45), in order to explore sialic acid expression on cells and in tissues. In addition, the information provided by this and other glycan microarrays composed of modified sialic acids will greatly facilitate our understanding of sialidase specificities and perhaps contribute to the design of new sialidase inhibitors and identification of new types of sialidases.

An important utility of the SGM will be its use as a discovery platform to detect novel binding specificities of proteins, antibodies, bacteria, and viruses. Here we have described screening assays using several samples of viruses that are known or suspected to bind to sialylated glycans. During the initiation of infection, many viruses, such as influenza A, bind to cell surface sialic acids (6, 8). Although significant data have accumulated that correlate virus binding specificities directed at α2–3 or α2–6-linked sialic acid with infection of avian and human populations, respectively, most studies have focused attention on differences in the underlying glycans. However, very little information is available on the relative binding of such infectious agents to glycans presenting modified sialic acid residues. The SGM, which includes the most comprehensive group of naturally occurring sialic acid structures on a single slide, provides an ideal discovery platform to explore the impact of modified sialic acids on molecular recognition.

We found that three hPIVs showed very different binding patterns on the SGM. Interestingly, these viruses bound only to modified Neu5Ac or Neu5Gc found in mammals but not to Kdn and its derivatives, which are expressed more commonly in prokaryotes and invertebrates. Although most hydroxy groups and the amide at position 5 of Neu5Ac and Neu5Gc are necessary for hPIV binding (Kdn lacks the 5-amide), the subtle changes at the 5- and 9-positions can result in different binding affinities. In most cases, the hydrophobicity at the 5-position and the hydrophilicity at the 9-position need to be retained for binding by hPIVs (Fig. 8). We observed a similar phenomenon for influenza viruses H1N1 and H3N2, for which α2–6-linked Neu5Ac and Neu5Ac9Lt are the best ligands. It is interesting that for several of the viruses interrogated on the SGM, the best recognized ligands were actually not directed toward Neu5Ac, the most abundant form of sialic acid. For example, the highest bound ligand for hPIV1 and -2 contained Neu5GcMe3, and for both H3N2 and H1N1 influenza A virus, the highest bound ligand was Neu5Ac9Lt. This is an exciting finding because this modified sialic acid occurs in significant amounts in humans (i.e. 20% of the sialic acid in normal human serum is Neu5Ac9Lt) (46). It is noteworthy that in previous studies, the motifs recognized by hPIV1 and -3 also include fucosylation of GlcNAc and sulfation of Gal in the Neu5Aca2-3Galβ1–3/4GlcNAc motif (37). Furthermore, the difference in N-glycolyl and N-acetyl neuraminic acid has been a source of enigma for antibodies recognizing sialyl Lewis X and 6-sulfosialyl Lewis X (47, 48). Although our current modified sialic acid array lacks fucosylation and sulfation, future expansion of
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this array using fucosylated and sulfated acceptors will facilitate further investigation into the context of relevant modifications to glycan backbones.

The general occurrence and localization of these modified sialic acid structures in human tissues are poorly understood. Our results indicate the need to reexamine modified sialic acid expression in human and animal tissues in regard to virus interactions beyond analyses of the common Neu5Ac and Neu5Gc glycan derivatives. The resulting binding information and specificity analysis could also further the development of pharmacotherapy for virus infections (49). The SGM could serve as a general platform to promote the functional studies of modified sialic acids toward more glycan binding proteins and viruses in the future.

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REFERENCES

1. Avci, F. Y., and Kasper, D. L. (2010) Annu. Rev. Immunol. 28, 107–130
2. Stowell, S. R., Arthur, C. M., Dias-Baruffi, M., Rodrigues, L. C., Gourdine, J. P., Heimburg-Molinaro, J., Ju, T., Molinari, R. J., Rivera-Marrero, C., Xia, B., Smith, D. F., and Cummings, R. D. (2010) Nat. Med. 16, 295–301
3. Taylor, M. E., and Drickamer, K. (2007) Currr. Opin. Cell Biol. 19, 572–577
4. Varki, A., Cummings, R. D., Esko, D. J., Freeze, H., Stanley, P., Bertozzi, C. R., Hart, G. W., and Etzler, M. E. (2009) Essentials of Glycobiology, 2nd Ed., pp. 199–217, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY
5. Chen, X., and Varki, A. (2010) ACS Chem. Biol. 5, 163–176
6. Nicholls, I. M., Chan, R. W., Russell, R. J., Air, G. M., and Peiris, J. S. (2008) Trends Microbiol. 16, 149–157
7. Byres, E., Paton, A. W., Paton, J. C., Löfling, J. C., Smith, D. F., Wilce, M. C., Talbot, U. M., Chong, D. C., Yu, H., Huang, S., Chen, X., Varki, N. M., Rossjohn, J., and Beddoe, T. (2008) Nature 456, 648–652
8. Schauer, R. (2009) Currr. Opin. Struct. Biol. 19, 507–514
9. Gagneux, P., and Varki, A. (2001) Mol. Phylgenet. Evol. 18, 2–13
10. Talbot-Uomantakul, P., Gagneux, P., Diaz, S., Bardor, M., Varki, N., Varki, A., and Muchmore, E. (2003) Proc. Natl. Acad. Sci. USA. 100, 12045–12050
11. Bratosin, D., Palii, C., Moiocean, A. D., Zaneta, J. P., and Montreuil, J. (2007) Biochimie 89, 355–359
12. Bulai, T., Bratosin, D., Pons, A., Montreuil, J., and Zaneta, J. P. (2003) FEBS Lett. 534, 185–189
13. Schultze, B., Gross, H. J., Klenk, H. D., Brossmer, R., and Herrler, G. (1990) Adv. Exp. Med. Biol. 276, 115–119
14. Varki, A. (2009) Glycoconj. J. 26, 231–245
15. Cao, H., Li, Y., Lau, K., Muthana, S., Yu, H., Cheng, J., Chokhawala, H. A., Sugarto, G., Zhang, L., and Chen, X. (2009) Org. Biomol. Chem. 7, 5137–5145
16. Chokhawala, H. A., Huang, S., Lau, K., Yu, H., Cheng, J., Thon, V., Hurtado-Ziola, N., Guerrero, J. A., Varki, A., and Chen, X. (2008) ACS Chem. Biol. 3, 567–576
17. Padler-Karavani, V., Hurtado-Ziola, N., Pu, M., Yu, H., Huang, S., Muthana, S., Chokhawala, H. A., Cao, H., Secrest, P., Friedmann-Morvinski, D., Singer, O., Ghaderi, D., Verma, I. M., Liu, Y. T., Messer, K., Chen, X., Varki, A., and Schwab, R. (2011) Cancer Res. 71, 3322–3326
18. Yu, H., Cheng, J., Ding, L., Khedri, Z., Chen, Y., Chin, S., Lau, K., Tiwari, V. K., and Chen, X. (2009) J. Am. Chem. Soc. 131, 18467–18477
19. Yu, H., Chokhawala, H., Karpel, R., Yu, H., Wu, B., Zhang, J., Zhang, Y., Jia, Q., and Chen, X. (2005) J. Am. Chem. Soc. 127, 17618–17619