Analysis of the Specificity of Sialyltransferases toward Mucin Core 2, Globo, and Related Structures. Identification of the Sialylation Sequence and the Effects of Sulfate, Fucose, Methyl, and Fluoro Substituents of the Carbohydrate Chain in the Biosynthesis of Selectin and Siglec Ligands, and Novel Sialylation by Cloned α2,3(O)Sialyltransferase†

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ABSTRACT: Sialic acids are key determinants in many carbohydrates involved in biological recognition. We studied the acceptor specificities of three cloned sialyltransferases (STs) [α2,3(N)ST, α2,3(O)ST, and α2,6(N)ST] and another α2,3(O)ST present in prostate cancer cell LNCaP toward mucin core 2 tetrasaccharide [Gal*β*1,4GlcNAc*β*1,6(Gal*β*1,3)GalNAcα-O-Bn] and Globo [Gal*β*1,3GalNAc*β*1,3Galα-O-Me] structures containing sialyl, fucosyl, sulfo, methyl, or fluoro substituents by identifying the products by electrospray ionization tandem mass spectral analysis and other biochemical methods. The Globo precursor was an efficient acceptor for both α2,3(N)ST and α2,3(O)ST, whereas only α2,3(O)ST used its deoxy analogue (D-Fuc*β*1,3GalNAc*β*1,3-Gal-α-O-Me); 2-O-MeGal*β*1,3GlcNAc and 4-OMeGal*β*1,-4GlcNAc were specific acceptors for α2,3(N)ST. Other major findings of this study include: (i) α2,3 sialylation of *β*1,3Gal in mucin core 2 can proceed even after α1,3 fucosylation of *β*1,6-linked LacNAc. (ii) Sialylation of *β*1,3Gal must precede the sialylation of *β*1,4Gal for favorable biosynthesis of mucin core 2 compounds. (iii) α2,3 sialylation of the 6-O-sulfoLacNAc moiety in mucin core 2 (e.g., GlyCAM-1) is facilitated when *β*1,3Gal has already been α2,3 sialylated. (iv) α2,6(N)ST was absolutely specific for the *β*1,4Gal in mucin core 2. Either α1,3 fucosylation or 6-O-sulfation of the GlcNAc moiety reduced the activity. Sialylation of *β*1,3Gal in addition to 6-O-sulfation of GlcNAc moiety abolished the activity. (v) Prior α2,3 sialylation or 3-O-sulfation of *β*1,3Gal would not affect α2,6 sialylation of Gal*β*1,4GlcNAc of mucin core 2. (vi) A 3- or 4-fluoro substituent in *β*1,4Gal resulted in poor acceptors for the cloned α2,6(N)ST and α2,3(N)ST, whereas 4-fluoro- or 4-OMe-Gal*β*1,3GalNAcα was a good acceptor for cloned α2,3(O)ST. (vii) 4-O-Methylation of *β*1,4Gal abolished the acceptor ability toward α2,6(N)ST but increased the acceptor efficiency considerably toward α2,3(N)ST. (viii) Just like LNCaPα1,2-FT and Gal-3-O-sulfotransferase T2, the cloned α2,3(N)ST which modifies terminal Gal in Gal*β*1,4GlcNAc also efficiently utilizes the terminal *β*1,3Gal in the Globo backbone. Utilization of C-3 blocked compounds such as 3-O-sulfo-Gal*β*1,3GalNAc*β*1,3Galα-OMe as acceptors by cloned α2,3(O)ST and analyses of the resulting products by lectin chromatography and mass spectrometry indicate that α2,3(O)ST is capable of attaching NeuAc to another position in C-3-substituted *β*1,3Gal.

Sialyl groups of cell surface and secreted glycoconjugates (*1*, *2*) can act as binding targets for viruses, bacteria, parasites, and toxins (*3*, *4*) and also can recognize mammalian selectin and Siglec lectins (*3*). Sialyltransferases (STs)[1] attach sialic acid to galactose via α2,3 and α2,6 glycosidic bonds and to GalNAc via α2,6 linkages and also form polysialic acid via an α2,8 linkage.

Siglecs in contrast to the majority of immunoglobulin superfamily members, which recognize protein ligands, bind to specific sialylated glycans (*5*−*7*). The carbohydrate recognition motifs of Siglec-2, Siglec-5, and Siglec-7 have been identified as NeuAcα2,6Gal*β*1,4Glc (*8*−*10*). A role for α2,6-linked sialic acid as a modulator of immune cell

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[1] Abbreviations: AA/CP, acrylamide copolymer; Al, allyl; Bn, benzyl; BSA, bovine serum albumin; GlyCAM-1, glycosylation-dependent cell adhesion molecule 1; GP, glycopeptide; LacNAc, Gal*β*1,4GlcNAc; Me, methyl; PNA, peanut agglutinin; PSGL-1, P-selectin glycoprotein ligand 1; RCA, *Ricinus communis* agglutinin; RM, reaction mixture; Siglec, sialic acid-binding lectin of the immunoglobulin superfamily; ST, sialyltransferase; T-hapten, Gal*β*1,-3GalNAcα−; TLC, thin-layer chromatography; WGA, wheat germ agglutinin.

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interaction in B-cells is evident from the finding that B-cell activation with polyclonal anti-IgM and interferon-γ leads to specific loss of α2,6-linked sialic acid, and this creates access to costimulatory molecules for the cell surface (*11*).

Selectins make up another family of sialic acid-recognizing adhesion molecules, containing amino-terminal C-type lectin domains (*12*−*15*). They recognize sialyl Lewis x or sialyl Lewis a motifs (*16*−*18*). Our studies confirmed the importance of α2,3-linked sialic acid and α1,3/4-linked fucose for selectin recognition of ligands, and they demonstrate the role of the mucin core 2 structure in enhancing binding of L- and P-selectin to carbohydrates (*19*). We have also demonstrated the contribution of the NeuAcα2,3Galβ1,3GalNAcα− sequence of the mucin core 2 structure in modulating L- and P-selectin binding function (*20*). Finally, we also showed a unique carbohydrate sequence lacking sialic acid, namely, GalNAcβ1,4(Fucα1,3)GlcNAcβ− (GalNAc Lewis x), that could act as a ligand for E-selectin (*20*). In comparison to NeuAcα2,3Galβ1,4(Fucα1,3) GlcNAcβ-O-Me (sialyl Lewis x), we found GalNAcβ1,4(Fucα1,3)GlcNAcβ1,6(NeuAcα2,-3Galβ1,3) GalNAcα-O-Me to be a 5−6-fold better inhibitor of L- and P-selectin binding.

In a previous study, we used various modified disaccharides as acceptors to study the specificities of a purified porcine liver α2,3ST acting on Galβ1,3GalNAcα− and a cloned α2,3ST utilizing Galβ1,3/4GlcNAcβ− (*21*). In view of the importance of sialic acid residues in various biological recognition phenomena, we decided to study in detail the acceptor substrate specificities of three clonal sialyltransferases and also another sialyltransferase that is strictly specific for the Galβ1,3GalNAcα− sequence. A variety of mucin core 2- and Globo-based synthetic compounds and related simple structures were utilized as acceptors to gain insight into the sialylation sequence in the biosynthesis of complex carbohydrate ligands. The study also reveals a novel action of cloned α2,3(O)sialyltransferase in catalyzing the attachment of sialic acid to another position of galactose in the Galβ1,3GalNAc moiety containing a C-3 substituent.

EXPERIMENTAL PROCEDURES

LNCaP Cells. The prostate carcinoma cell line, LNCaP, was grown in RPMI 1640 supplemented with 10% fetal bovine serum and antibiotics (penicillin, streptomycin, and amphotericin B) under conditions recommended by American Type Culture Collection (Manassas, VA). The cells were homogenized with 0.1 M Tris-maleate (pH 6.3) containing 2% Triton X-100 using a Dounce all glass hand-operated homogenizer. The homogenate was centrifuged at 16000*g* for 1 h at 4 °C. The protein concentration in the supernatant was measured using the BCA assay (Pierce Biotech, Inc., Rockford, IL), with BSA as the standard. The supernatant was adjusted to 5 mg/mL protein by adding the necessary amount of extraction buffer and then stored frozen at −20 °C until use. A 10 μL aliquot of this extract was used in assays run in duplicate.

Cloned Sialyltransferases. Rat recombinant α2,3(O)ST, α2,3(N)ST, and α2,6(N)ST were purchased from Calbiochem and stored at either −20 or −70 °C as recommended by the supplier. Suitable aliquots were diluted with 1.0 mL of 0.1 M sodium cacodylate buffer (pH 6.0) containing 2% Triton CF-54 and 2% BSA and used in the experiments; α2,3(O)ST and α2,6(N)ST as diluted above were found to retain full activity for at least 3 months when stored frozen at −20 °C. α2,3(N)ST was diluted just before it was used in the experiment. Aliquots (10 μL) of the diluted enzymes were used in the assays run in duplicate.

Synthetic Acceptors. The syntheses of several compounds that are used as acceptors in our study have been published (*20*, *22*−*24*). The synthesis of acceptors containing the Globo H precursor structure (namely, Galβ1,3GalNAcβ1,-3Galα-O-Al, Galβ1,3GalNAcβ1,3Galα-O-Me, D-Fucβ,1,-3GalNAcβ1,3Galα-O-Me, 3-O-sulfoGalβ1,3GalNAcβ1,-3Galα-O-Me, and 3-O-sulfo-D-Fucβ1,3GalNAcβ1,3Galα-O-Me) and mucin core 2 tetrasaccharides containing the 4-O-Me group and complex structural units will be published elsewhere. Mass spectrometry analysis of many of these compounds is presented in this work. The synthesis of mucin core 2 tetrasaccharides containing the 3-F group has been reported (*25*).

Macromolecular and Natural Acceptors. Acrylamide copolymer of Galβ1,3GalNAcα-O-Al synthesized by the procedure of Horejsi et al. (*26*) and Fetuin triantennary asialo glycopeptide were available from earlier studies of this laboratory (*21*, *27*−*29*).

Assay of Sialyltransferases. The incubation mixtures run in duplicate contained 0.1 M sodium cacodylate buffer (pH 6.0), the acceptor (typically at 7.5 mM, or as indicated in some experiments), CMP-[9-^{3}H]NeuAc (typically 0.2 μCi; 20 μCi/nmol or as indicated in some experiments), and the enzyme in a total volume of 20 μL. The control incubation mixtures contained everything except the acceptor. Incubation was carried out for 2 h at 37 °C. The enzymatic transfer of [9-^{3}H]NeuAc to a typical acceptor was linear for 2 h, and less than 30% CMP-[9-^{3}H]NeuAc was utilized. Chromatography, using either a Dowex-1-Formate, Sep-Pak C18, or BioGel P2 column, was used to separate the radioactive product from unreacted [9-^{3}H]NeuAc as described below. The values for the duplicate runs did not vary by more than 5%.

The radioactive products from neutral allyl and methyl glycosides as well as non-glycosides were measured by fractionation on Dowex-1-Formate (Bio-Rad, AG-1X8; 200−400 mesh; format form) as follows. The incubation mixture was diluted with 1.0 mL of water and passed through an AG-1-formate column (1.0 mL bed volume in a Pasteur pipet which had been washed with 5 mL of 2 M formic acid followed by 10 mL of water). The column was washed twice with 1.0 mL of water after the entry of the sample and then eluted with 3.0 mL of 0.1 M NaCl. The radioactivity present in water and 0.1 M NaCl eluates was measured separately using 3a70 scintillation cocktail (Research Products International, Mount Prospect, IL) and a Beckman LS6500 scintillation counter. The counts per minute (cpm) values were corrected by subtracting the blank cpm. Any radioactivity present in the water eluate (as noticed in the case of some methyl glycosides) was added to the corresponding cpm value of the 0.1 M NaCl eluate. The radioactive products from sulfated and/or sialylated methyl glycosides were also measured by the procedure described above; only the elution of the AG-1-formate column was continued further with 3.0 mL of 0.2 M NaCl for achieving a complete elution of the radioactive product, a correction being made as before by subtracting the corresponding blank values.

The radioactive products from benzyl glycosides and monosialylated benzyl glycosides were measured by hydrophobic chromatography on a Sep-Pak C18 cartridge (Waters, Milford, MA) and eluting the product with 3.0 mL of methanol (*30*). The radioactivity was determined by liquid scintillation as described above.

Radioactive sialylation products from sulfated benzyl glycosides were quantitated by fractionation of the reaction mixture on a BioGel P2 column, since these products could not be eluted from the AG-1-formate column even by 0.2 M NaCl, and they did not bind to Sep-Pak C18. For such work, a BioGel P2 column (Fine Mesh; 1.0 cm × 116.0 cm) was used with 0.1 M pyridine acetate (pH 5.4) as the eluent at room temperature. The effluent fractions were monitored for radioactivity, and the first peak containing radioactivity was collected, lyophilized to dryness, dissolved in 200 μL of water, and stored frozen at −20 °C for thin-layer chromatography (TLC) and other experimentation.

Thin-Layer Chromatography. TLC was carried out on a silica gel GHLF plates (250 μm, scored 20 cm × 20 cm; Analtech, Newark, DE). 1-Propanol/NH_4OH/H_2O (12/2/5, v/v) and $CHCl_3$/CH_3OH/H_2O (5/4/1, v/v) solvent systems were used. The acceptor compounds were located on the plates by spraying with sulfuric acid in ethanol and heating at 100 °C. The radioactive products were located by scraping 0.5 cm width segments of silica gel and soaking them in 2.0 mL of water in vials followed by liquid scintillation counting. Fluorography of the TLC plates was carried out at −70 °C using Bio-Max MS film (Eastman Kodak) after spraying the TLC plates with Enhance (Dupont).

WGA−, PNA−, and RCA-I−Agarose Affinity Chromatography. A column with a 5 mL bed volume of lectin−agarose (Vector Lab, Burlingame, CA) was employed using 10 mM Hepes (pH 7.5) containing 0.1 mM $CaCl_2$, 0.01 mM $MnCl_2$, and 0.1% NaN_3 as the running buffer. Fractions (1.0 mL) were collected. The bound material was then eluted with 0.5 M GlcNAc after the 10th fraction in the case of WGA−agarose column, or with 0.2 M galactose after the 12th fraction for the RCA-I−agarose and PNA−agarose columns in the same buffer as recommended by the manufacturer. Fractionation was carried out at room temperature.

Mass Spectral Analysis of Enzymatically Sialylated Compounds. Reaction mixtures contained 0.3 μmol of acceptor, 0.2 μmol of CMP-NeuAc, 1.0 μCi of CMP-[9-^{3}H]NeuAc, 30 μg of BSA, 0.1 M sodium cacodylate (pH 6.0), and 20 milliunits of the cloned sialyltransferase in a total volume of 100 μL and were incubated for 18 h at 37 °C. After incubation, the reaction mixture was diluted with 1.0 mL of water and then subjected to column chromatography on a BioGel P2 column (1.0 cm × 116.0 cm) as described above. The first peak containing the radioactive product was collected, lyophilized to dryness, dissolved in 200 μL of water, and stored frozen at −20 °C until mass spectral analysis was carried out.

MS^n experiments (*31*) were carried out with an Esquire-LC, Bruker-HP (Bremen, Germany) ion trap. Samples at 10 pM/μL in MeOH were infused into the electrospray source via a 50 μm inside diameter fused silica capillary using a syringe pump at a flow rate of 5 μL/min. Nitrogen was used as the nebulizing gas (at 5−6 psi) and also as the drying gas (5−7 L/min at 200 °C). The potentials of the spray needle, capillary exit, and skimmer were set to ±4000, 90−150, and 25−50 V, respectively. Helium was the buffer/collision gas. For each spectrum, 100−500 scans were averaged. The fragmentation amplitude was varied between 0.8 and 1.5 depending on the experiment design. The fragmentation amplitude was 1.15 for MS^2 and 1.20 for MS^3. The fragmentation time was 40 ms. Typically, the low-mass cutoff was set at slightly less than one-third of the precursor *m*/*z* value.

All monosialylated or monosulfated oligosaccharides were found to lose protons easily to yield $[M - H]^-$ ion in negative mode. The oligosaccharides bearing both sialyl and sulfate groups were detected as doubly charged $[M - 2H]^{2-}$ ions in negative mode. The monosialylated or monosulfated oligosaccharides yielded singly charged doubly sodiated $[M - H + 2Na]^+$ ions in positive mode. The oligosaccharides bearing both sialyl and sulfate group were detected as doubly charged quadruply sodiated $[M - 2H + 4Na]^{2+}$ ions in positive mode. In contrast to the positive mode, the negative mode could easily prevent the interference of ions from impurity, and this yielded a higher signal intensity of the product ions. However, the sodiated adduct ions detected in the positive mode provided additional information about the molecular weight, and they confirmed the results obtained from negative polarity electrospray mass spectra.

RESULTS

Sialyltransferase Assay. Sialyltransferase activity was measured using methods which we have documented elsewhere (*21*). In addition to these published protocols, in this work, the BioGel P2 column was used to separate 9-^{3}H-labeled benzyl glycosides that were both sulfated and sialylated from unreacted CMP-[9-^{3}H]NeuAc (Figure 1). In Figure 1, sample BioGel P2 elution profiles are shown for three radioactive products along with corresponding autoradiographs of these products following TLC.

Modified Disaccharides and GloboH-Based Acceptors Can Distinguish between the Cloned Enzymes (Table 1, part A). Cloned α2,3(N)ST and α2,6(N)ST displayed a preference for Galβ1,4GlcNAcβ-O-Al rather than Galβ1,3GalNAcα-O-Al, though α2,3(N)ST exhibited 24.1% activity for the latter compound; α2,3(O)ST and LNCaP STs, on the other hand, exhibited higher specificity for Galβ1,3GalNAcα-O-Al than for Galβ1,4GlcNAcβ-O-Al and also acted efficiently on Galβ1,3(GlcNAcβ1,6)GalNAcα-O-Al.

We examined various modifications of the disaccharides described above to determine if unique acceptors could be identified. We observed that α2,6(N)ST utilized Galβ1,-4GlcNAcβ-O-Al and 2-O-MeGalβ1,4GlcNAc as acceptors (100.0 and 90.1% active, respectively), but had very low activity toward 3-O-MeGalβ1,4GlcNAc (8.7% active), Galβ1,-3GlcNAcβ-O-Al (3.0%), 2-O-MeGalβ1,3GlcNAc (3.7%), and 2-O-MeGalβ1,3GlcNAcβ-O-Bn (0%). α2,3(O)ST exhibited either very low or negligible activity with acceptors 2-O-MeGalβ1,3GlcNAc (2.9%), 2-O-MeGalβ1,4GlcNAc (0.2%), and 3-O-MeGalβ1,4GlcNAc (3.6%). α2,3(N)ST was the only enzyme that acted efficiently to sialylate 2-O-MeGalβ1,3GlcNAc (115.3%), suggesting that this may be a unique acceptor for this enzyme. It also acted efficiently on Galβ1,3GlcNAcβ-O-Al (123.1%) and 2-O-Me-Galβ1,-4GlcNAc (60.0%).

The GloboH precursor Galβ1,3GalNAcβ1,3Galα-O-Me and its deoxy analogue D-Fucβ1,3GalNAcβ1,3-Gal-O-Me could be used to distinguish between the enzymes. α2,3-

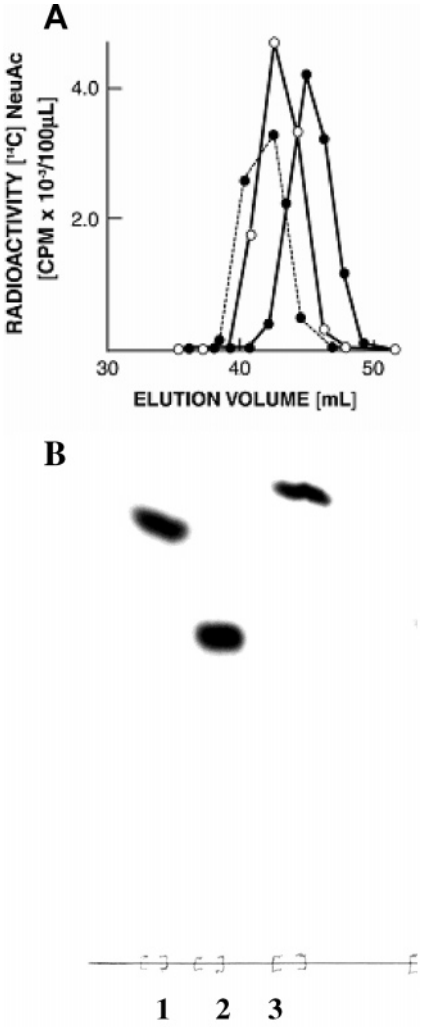

FIGURE 1: (A) Composite picture of BioGel P2 column elution profiles for ^{14}C sialylated products arising from the action of cloned α2,3(O)ST on mucin core 2 acceptors Galβ1,4GlcNAcβ1,6-(Galβ1,3)GalNAcα-O-Bn (○—○), 3-O-sulfoGalβ1,4GlcNAcβ1,6-(Galβ1.3)GalNAcα-O-Bn (●—●), and 3-O-MeGalβ1,4GlcNAcβ1,6-(Galβ1,3)GalNAcα-O-Bn (●---●). CMP-[^{14}C]NeuAc emerged from the BioGel P2 Column at 56−64 mL with a peak at 60 mL. (B) Thin-layer chromatography [12/2/5 1-propanol/NH_4OH/H_2O solvent system (v/v)] followed by autoradiography for [^{14}C]sialyl products isolated from the following acceptors as shown in panel A: 3-O-MeGalβ1,4GlcNAcβ1,6(Galβ1,3)GalNAcα-O-Bn (lane 1), Galβ1,-4GlcNAcβ1,6(Galβ1,3)GalNAcα-O-Bn (lane 2), and 3-O-sulfo-Galβ1,4GlcNAcβ1,6(Galβ1,3)GalNAcα-O-Bn (lane 3).

(O)ST utilized both the GloboH precursor (120.8% active) and its deoxy analogue (115.6%). α2,3(N)ST on the other hand acted efficiently on the GloboH precursor (106.8% active) but not its deoxy analogue (3.1% active), indicating that the C-6 hydroxyl group of the β1,3-linked Gal in the GloboH precursor is absolutely required for this enzyme activity. In contrast, α2,6(N)ST was inactive with both the GloboH-based acceptor (1.1%) and its deoxy analogue (2.5%). The sialyltransferase from LNCaP utilized these two acceptors at lower activities of 34.3 and 9.6%, respectively. Key results regarding the specificity for GloboH precursors were verified using mass spectroscopy (Table 6). Overall, these results demonstrate that 2-O-MeGalβ1,3GlcNAc is a unique acceptor for α2,3(N)ST. All enzymes can also be distinguished by their action on the GloboH precursor and its deoxy analogue.

Specificity of Sialyltransferases for Core 2 Structure. The STs acted on the core 2 tetrasaccharide and 3-*O*-methyl analogues of this molecule in a manner that was largely expected on the basis of our studies with disaccharides (Table 1, part B). α2,3(N)ST and α2,6(N)ST showed specificity toward the Galβ1,4GlcNAcβ side chain in the tetrasaccharide, while the sialyltransferases from LNCaP preferred to act on the Galβ1,3GalNAcα moiety. While α2,3(N)ST was 24.1% active toward Galβ1,3GalNAcα-O-Al, it displayed somewhat lower activity (6.1% activity) toward 3-O-MeGalβ1,4GlcNAcβ1,6(Galβ1,3)GalNAcα-O-Bn. Surprisingly, α2,3(O)ST displayed activity toward both methyl analogues of the tetrasaccharide, suggesting that it can have multiple sites of action including, but not limited to, the 3-position of Gal in Galβ1,3GalNAcα. This finding is elaborated below.

Defining Specific Acceptors To Distinguish between Sialyltransferases from LNCaP and α2,3(O)ST. The sialyltransferase from LNCaP exhibited strict specificity for Galβ1,-3GalNAcα, while α2,3(O)ST displayed broader specificity. This is evident upon comparison of the two methylated tetrasaccharides, 3-O-MeGalβ1,4GlcNAcβ1,6(Galβ1,3)-GalNAcα-O-Bn and Galβ1,4GlcNAcβ1,6(3-O-MeGalβ1,3)-GalNAcα-O-Bn. While the cloned enzyme was 100.0 and 49.6% active, respectively, toward these acceptors, the LNCaP enzyme was 100.9 and 3.0% active, respectively. The strict specificity of the LNCaP enzyme toward Galβ1,-3GalNAcα is also evident from its negligible activity toward Galβ1,3GlcNAcβ1,3Galβ-O-Me (1.4%) as compared to that of the cloned enzyme (37.2%). Further, these two enzymes differ in their action since, while the cloned enzyme utilized both Galβ1,3GalNAcα-O-Al and the T-hapten in the mucin core 2 structure as acceptors to the same extent (112.0 and 100.0%, respectively), the LNCaP enzyme was only one-third efficient toward Galβ1,3GalNAcα-O-Al (34.4% active). The cloned enzyme and the LNCaP enzyme differed in using Galβ1,3GlcNAcβ-O-Al (42.1 and 3.7% active, respectively). Finally, the addition of Gal to the β1,6-linked GlcNAc of the mucin core 2 structure increased the activity of the LNCaP enzyme (from 60.4 to 100.0%) in contrast to the behavior of the cloned enzyme (from 120.5 to 100.0%).

To define a simple specific acceptor for assaying cloned α2,3(O)ST, in the presence of other α2,3(O)STs such as those resembling LNCaP ST, we examined the action of both enzymes on a series of disaccharide analogues, namely, 3-O-MeGalβ1,3GalNAcα-O-Bn, 4-O-MeGalβ1,3GalNAcα-O-Bn, 4-FGalβ1,3GalNAcα-O-Bn, Galβ1,3(6-OMe)GalNAcα-O-Bn, and 3-O-MeGalβ1,3(6-O-Me)GalNAcα-O-Bn along with Galβ1,3GalNAcα-O-Bn (Table 2). Mass spectroscopy analysis was performed with some of the enzymatic products as discussed in Table 6. These studies were conducted at two different acceptor concentrations. We observed that the cloned enzyme exhibited 70.4 and 55.9% activities toward 3-O-MeGalβ1,3GalNAcα-O-Bn and 3-O-MeGalβ1,3(6-O-Me)GalNAcα-O-Bn, respectively, as compared to Galβ1,-3GalNAcα-O-Bn at 0.75 mM, which is the saturation point for maximum activity. LNCaPα2,3(O)ST exhibited negligible activities toward these acceptors (2.6 and 1.1%, respectively). Hence, 3-O-MeGalβ1,3GalNAcα-O-Bn and 3-O-MeGalβ1,3(6-O-Me)GalNAcα-O-Bn could be used as specific acceptors for the cloned α2,3(O)ST enzyme. In fact, the latter compound may be absolutely specific for this

Table 1: Acceptor−Substrate Specificity Analysis of the Cloned Sialyltransferases and LNCaP Cell α2,3(O)ST[a]

acceptor (7.5 mM)	sialyltransferase activity (%) cloned α2,3(N)ST	cloned α2,6(N)ST	cloned α2,3(O)ST	LNCaP α2,3(O)ST
(A)				
Galβ1,4GlcNAcβ-O-Al	100.0[b]	100.0[c]	4.7	0.5
Galβ1,3GalNAcα-O-Al	24.1	0.2	112.0	34.4
Galβ1,4GlcNAcβ1,3Galβ1,4GlcNAcβ-O-Bn			8.7	
Galβ1,3(GlcNAcβ1,6)GalNAcα-O-Al			120.5 (**1**)	60.4
Galβ1,3GlcNAcβ1,3Galβ-O-Me			37.2	1.4
2-O-MeGalβ1,4GlcNAc	60.0	90.1	0.2	
3-O-MeGalβ1,4GlcNAc	1.4	8.7	3.6	
Galβ1,3GlcNAcβ-O-Al	123.1	3.0	42.1	3.7
2-O-MeGalβ1,3GlcNAc	115.3	3.7	2.9	
2-O-MeGalβ1,3GlcNAcβ-O-Bn		0		
Galβ1,3GalNAcβ1,3Galα-O-Me	106.8 (**20**)	1.1	120.8 (**2**)	34.3
D-Fucβ1,3GalNAcβ1,3Galα-O-Me	3.1	2.5	115.6 (**3**)	9.6
fetuin triantennary asialo gp (100 μg)	66.1			
Galβ1,3GalNAcα-O-Al/AA-CP (100 μg)	1.7			
(B)				
Galβ1,4GlcNAcβ1,6(Galβ1,3)GalNAcα-O-Bn	100.7	93.3	94.4 (**4**)	
3-O-MeGalβ1,4GlcNAcβ1,6(Galβ1,3)GalNAcα-O-Bn	6.1	4.4	100.0[d]	100.0[e]
Galβ1,4GlcNAcβ1,6(3-O-MeGalβ1,3)GalNAcα-O-Bn	95.3	97.7	49.6	3.0
Galβ1,4(Fucα1,3)GlcNAcβ1,6(Galβ1,3)GalNAcα-O-Me	19.0	7.1	133.4	
Galβ1,4(Fucα1,3)GlcNAcβ1,6(NeuAcα2,3Galβ1,3)GalNAcα-O-Bn	27.1	31.0		
NeuAcα2,3Galβ1,4GlcNAcβ1,6(Galβ1,3)GalNAcα-O-Me			35.2	5.1
GalNAcβ1,4(Fucα1,3)GlcNAcβ1,6(Galβ1,3)GalNAcα-O-Me			134.9	21.1
6-O-sulfoGlcNAcβ1,6(Galβ1,3)GalNAcα-O-Bn			49.4[f] (**5**)	
Galβ1,4(6-O-sulfo)GlcNAcβ1,6(Galβ1,3)GalNAcα-O-Bn			69.4[f]	
Galβ1,4(6-O-sulfo)GlcNAcβ1,6(Galβ1,3)GalNAcα-O-Me	28.1 (**21**)	30.6 (**17**)		
Galβ1,4(6-O-sulfo)GlcNAcβ1,6(NeuAcα2,3Galβ1,3)GalNAcα-O-Me	50.8 (**22**)	0.9		
Galβ1,4(6-O-sulfo)(Fucα1,3)GlcNAcβ1,6(NeuAcα2,3Galβ1,3)GalNAcα-O-Me	1.7	0		
NeuAcα2,3Galβ1,4(6-O-sulfo)(Fucα1,3)GlcNAcβ1,6(Galβ1,3)GalNAcα-O-Me			7.4	
3-O-sulfoGalβ1,4GlcNAcβ1,6(Galβ1,3)GalNAcα-O-Bn			70.3[f] (**6**)	
Galβ1,4GlcNAcβ1,6(3-O-sulfoGalβ1,3)GalNAcα-O-Bn		127.4[f] (**18**)	33.1[f] (**7**)	

[a] Mass spectroscopy analysis of the reaction products in parentheses was carried out as described in Table 6 and the Supporting Information. [b] Represents 29 520 cpm. [c] Represents 43 500 cpm. [d] Represents 40 560 cpm. [e] Represents 30 930 cpm. [f] This value was obtained by quantitating the ^{14}C sialylated products from fractionation of the reaction mixture on a BioGel P2 column.

Table 2: Unique Sialylation by Cloned α2,3(O)ST Results in Identification of Specific Acceptors that Can Measure α2,3(O)ST Activity in the Presence of Related Enzymes[a]

acceptor	sialyltransferase activity (%) acceptor (0.75 mM) cloned α2,3(O)ST	acceptor (0.75 mM) LNCaP α2,3(O)ST	acceptor (7.5 mM) cloned α2,3(O)ST	acceptor (7.5 mM) LNCaP α2,3(O)ST
Galβ1,3GalNAcα-O-Bn	100.0 (**8**) (42550 cpm)	100.0 (30490 cpm)	100.0 (**8**) (43600 cpm)	100.0 (30600 cpm)
3-O-MeGalβ1,3GalNAcα-O-Bn	70.4	2.6	101.8	15.5
4-O-MeGalβ1,3GalNAcα-O-Bn	104.6 (**9**)	141.5	105.0 (**9**)	188.7
4-F-Galβ1,3GalNAcα-O-Bn	104.6 (**10**)	44.8	102.3 (**10**)	105.1
Galβ1,3(6-O-Me)GalNAcα-O-Bn	107.6 (**11**)	119.2	107.3 (**11**)	189.4
3-O-MeGalβ1,3(6-O-Me)GalNAcα-O-Bn	55.9	1.1	ND[b]	ND[b]

[a] Mass spectroscopy analysis of the reaction products in parentheses was carried out as described in Table 6 and the Supporting Information. [b] Not determined.

enzyme since α2,6-sialyltransferase acting on α-GalNAc cannot act on this acceptor.

Biosynthesis of Mucin Core 2-Based Glycans. Enzyme specificity data from this study (Table 1, part B) indicate the possible sequence in sialylation in the biosynthetic pathways of glycans such as PSGL-1, GalNAc Lewis x, GlyCAM-1, and potential Siglec ligands (Figure 2). However, one must be aware of the possibilities in vivo that (a) the recombinant enzymes may not fully acquire the conformation of the native enzymes, (b) the concentration of the substrates in the cell may dictate the action of enzymes in the competing pathways involved in multiple glycoconjugate synthesis, (c) the conformation of the active site of these enzymes is sensitive to temperature and pH, and (d) alternative, albeit minor, pathways for the same glycan elongation may exist in the cell.

(A) PSGL-1 and GalNAc Lewis x. Singly fucosylated mucin core 2-based glycans with terminal sialyl Lewis x structure and a tyrosine-sulfated peptide display high-affinity binding for P- and L-selectin (*32*). Several investigators have also demonstrated that fucosylation can be the last step in the biosynthesis of glycans borne on PSGL-1 (*33*−*37*). Our enzymatic studies provide additional insight into the biosynthesis of such mucins.

First, our results suggest that α2,3 sialylation of the Gal residue can proceed even after α1,3 fucosylation of the GlcNAc residue. In support of this proposition, we observed that Lewis x and (GalNAc) Lewis x determinants on the core

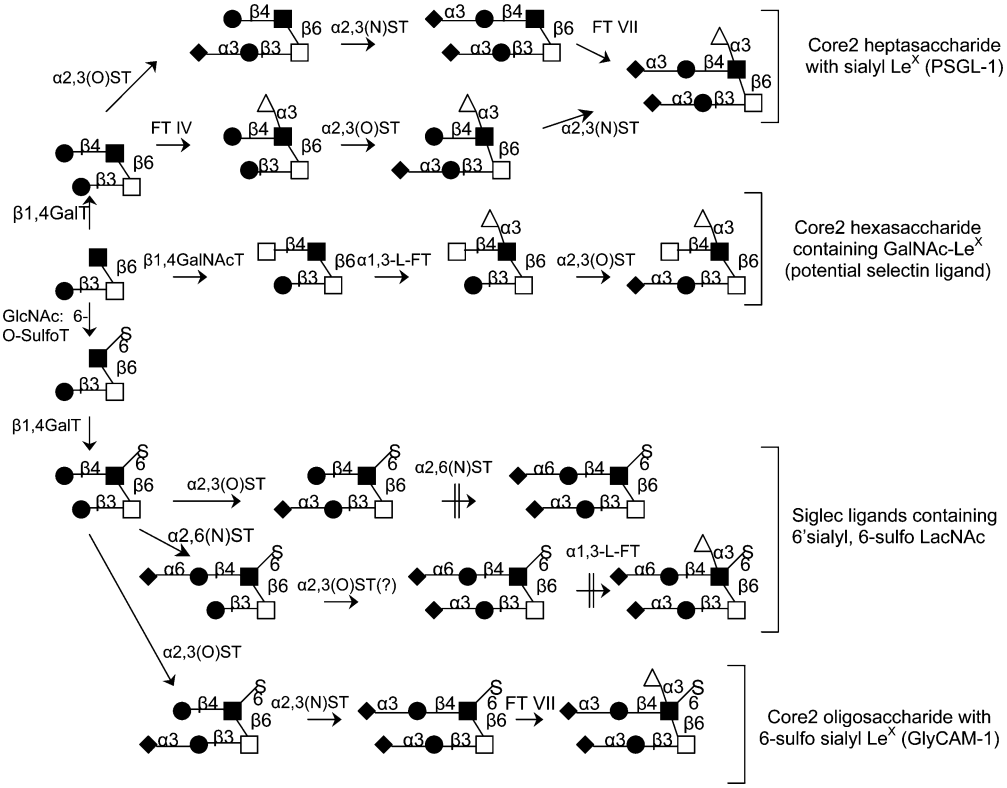

FIGURE 2: Proposed biosynthetic pathways for mucin core 2 heptasaccharide with sialyl Le^x (PSGL-1), core 2 hexasaccharide containing GalNAc Le^x (potential selectin ligand), potential Siglec ligands containing 6′-sialyl, 6-sulfo-LacNAc, and core 2 oligosaccharide with 6-sulfo sialyl Le^x (GlyCAM-1): (□) GalNAc, (■) GlcNAc, (●) Gal, (◆) NeuAc, (△) Fuc, and (S) sulfo group.

2 mucin did not affect the activity of clonal α2,3(O)ST [Galβ1,4(Fucα1,3)GlcNAcβ1,6(Galβ1,3)GalNAcα-O-Me, 133.4%; GalNAcβ1,4(Fucα1,3)GlcNAcβ1,6(Galβ1,3)-GalNAcα-O-Me, 134.9%]. The sialyltransferase from LNCaP was also active toward the GalNAc Lewis x-containing mucin core 2 acceptor, albeit at a lower level (21.1% activity); Galβ1,4(Fucα1,3)GlcNAcβ1,6(Galβ1,3)GalNAcα-O-Me and Galβ1,4(Fucα1,3)GlcNAcβ1,6(NeuAcα2,-3Galβ1,3)GalNAcα-O-Bn served as acceptors for α2,3(N)-ST to a significant, albeit low, level (19.0 and 27.1% active, respectively).

Second, with regard to the sequence of sialylation, our results suggest that sialylation of β1,3-linked Gal must precede the sialylation of β1,4-linked Gal for favorable biosynthesis of mucin core 2 backbone compounds. This proposition is supported by the following lines of evidence. (i) Sialylation of β1,3-linked Gal in mucin core 2 enhanced the α2,3 sialylation of β1,4-linked Gal via α2,3(N)ST, which is evident from a comparison of the activity of this enzyme toward Galβ1,4(Fucα1,3)GlcNAcβ1,6(Galβ1,3)GalNAcα− and Galβ1,4(Fucα1,3)GlcNAcβ1,6(NeuAcα2,3Galβ1,3)-GalNAcα− (19.0 and 27.1%, respectively) and upon comparison of Galβ1,4(6-O-sulfo)GlcNAcβ1,6(Galβ1,3)-GalNAcα− with Galβ1,4(6-O-sulfo)GlcNAcβ1,6(NeuAcα2,-3Galβ1,3)GalNAcα− (28.1 and 50.8%, respectively. (ii) When the LacNAc moiety of mucin core 2 was α2,3-sialylated, which is evident from using the acceptor NeuAcα2,-3Galβ1,4GlcNAcβ1,6(Galβ1,3)GalNAcα-O-Me, the extent of α2,3 sialylation of the Galβ1,3GalNAcα moiety was greatly reduced [35.2% for the cloned α2,3(O)ST enzyme and 5.1% for the LNCaP enzyme].

Taken together, these results suggest that Leukosialin would form from mucin core 2 tetrasaccharide by the sequential action of α2,3(O)ST and α2,3(N)ST. PSGL-1 glycans would form from further action of FTVII. PSGL-1 can also arise from mucin core 2 tetrasaccharide by the sequential action of FTIV (or possibly FTIII, FTV, or FTVI) followed by α2,3(O)ST and α2,3(N)ST (Figure 2). GalNAc Lewis x type structures on the core 2 tetrasaccharide can likewise be formed by the sequential action of β1,4GalNAc transferase, α1,3-fucosyltransferase, and α2,3(O)ST (Figure 2).

(B) GlyCAM-1. From previous studies, it is evident that GlcNAc:6-O-sulfotransferase does not act on the core 2 tetrasaccharide since it requires terminal GlcNAc for its action (*38*−*40*). This suggests that 6-sulfation precedes β1,4-galactosyltransferase activity in core 2 mucins. Building upon this observation, in the current work, we examined the action of sialyltransferases on an array of 6-O-sulfated structures to determine the potential biosynthetic pathway that leads to formation of sulfated core 2 structures. First, we observed that α2,3(N)ST acted more efficiently on the sialylated sulfated mucin core 2 compound Galβ1,4(6-O-sulfo)-GlcNAcβ1,6(NeuAcα2,3Galβ1,3)GalNAcα-O-Me (50.8% active) than on Galβ1,4(6-O-sulfo)GlcNAcβ1,6(Galβ1,3)-GalNAcα-O-Me (28.1% active). Also, this 3′-sialyl-6-O-sulfo Lewis x determinant dramatically decreased the activity of clonal α2,3(O)ST to 7.4%. These observations support the proposition that, like PSGL-1, α2,3 sialylation of the 6-O-sulfoLacNAc moiety in mucin core 2 is facilitated when the β1,3-linked Gal moiety has already been α2,3 sialylated. In other words, α2,3(O)ST action likely precedes α2,3(N)ST action in the case of GlyCAM-1 biosynthesis.

We observed that the human FTVII enzyme, obtained from extracts of COS-7 cells that were transfected with FTVII cDNA, was capable of efficiently transferring [^{14}C]Fuc to

Table 3: Sialyltransferase Activities toward Mucin Core 2-Based Compounds[a]

mucin core 2 compound (7.5 mM)	cloned sialyltransferase activity (%)			
	α2,6(N)ST	α2,3(N)ST	α2,3(O)ST	LNCaP α2,3(O)ST
3-O-MeGalβ1,4GlcNAcβ1,6(Galβ1,3)GalNAcα-O-Bn	6.4	4.5	100.0[d]	100.0[e]
Galβ1,4GlcNAcβ1,6(3-O-MeGalβ1,3)GalNAcα-O-Bn	100.0[b]	100.0[c]	49.6	3.0
4-O-MeGalβ1,4GlcNAcβ1,6(Galβ1,3)GalNAcα-O-Bn	12.6	99.8	103.0	97.1
Galβ1,4GlcNAcβ1,6(4-O-MeGalβ1,3)GalNAcα-O-Bn	101.3	100.6	101.3	112.0
3-FGalβ1,4GlcNAcβ1,6(Galβ1,3)GalNAcα-O-Bn	11.3	7.2	102.9 (**12**)	91.5
Galβ1,4GlcNAcβ1,6(3-FGalβ1,3)GalNAcα-O-Bn	77.7 (**19**)	96.9 (**25**)	77.2	1.7
4-FGalβ1,4GlcNAcβ1,6(Galβ1,3)GalNAcα-O-Bn	2.7	7.9	102.1	ND[f]

[a] Mass spectroscopy analysis of the reaction products in parentheses was carried as described in Table 6 and the Supporting Information. [b] Represents 30 440 cpm. [c] Represents 38 140 cpm. [d] Represents 39 200 cpm. [e] Represents 30 100 cpm. [f] Not determined.

the acceptor NeuAcα2,3Galβ1,4(6-O-sulfo)GlcNAcβ1,-3Galβ1,4(6-O-sulfo)GlcNAcβ-O-Me (E. V. Chandrasekaran, J. Xia, S. Neelamegham, and K. L. Matta, unpublished results). Further, the fucosylated acceptor Galβ1,4(6-O-sulfo)(Fucα1,3)GlcNAcβ1,6(NeuAcα2,3Galβ1,3)GalNAcα-O-Me had very low activity (1.7% active) toward α2,3(N)ST, suggesting that fucosylation must occur after sialylation. On the basis of the observations described above, we suggest that the biosynthesis of GlyCAM-1 starting from GlcNAcβ1,6-(Galβ1,3)GalNAcα− involves the sequential action of GlcNAc:6-O-sulfo-T, β1,4Gal-T, α2,3(O)ST, α2,3(N)ST, and finally FTVII (Figure 2). The sialylation steps in this reaction scheme were validated by analyzing reaction products using mass spectroscopy (Table 6).

While our above data suggest that sulfation must be one of the first steps in the biosynthetic pathway, we also note that 6-O-sulfation of β1,6-linked GlcNAc in mucin core 2 reduces the activity of α2,3(O)STs. In this respect, Galβ1,4-(6-O-sulfo)GlcNAcβ1,6(Galβ1,3)GalNAcα-O-Me was 27.2% active toward clonal α2,3(O)ST and 14.8% active toward sialyltranferase from LNCaP. Similarly, cloned α2,3(O)ST was 49.4 and 69.4% active with acceptors 6-O-sulfoGlc-NAcβ1,6(Galβ1,3)GalNAcα-O-Bn and Galβ1,4(6-O-sulfo)-GlcNAcβ1,6(Galβ1,3)GalNAcα-O-Bn, respectively. Not only 6-O-sulfation but also 3-O-sulfation of the LacNAc moiety also decreased the enzyme activity of α2,3(O)ST in acting on the core 2 mucin since 3-O-sulfoGalβ1,4GlcNAcβ1,6-(Galβ1,3)GalNAcα-O-Bn was only 70.3% active. Our previous study indicated that the sialylated core 2 acceptor, namely, NeuAcα2,3Galβ1,3(GlcNAcβ1,6)GalNAcα-O-Bn, can act as an acceptor for GlcNAc:6-O-sulfotransferase (*40*). Thus, as an alternate scheme (not shown in Figure 2), it may be possible that α2,3(O)ST action may precede 6-O-sulfation of GlcNAc in the GlyCAM-1 biosynthetic pathway.

(C) Siglec Ligands. This study reports the formation of α2,6-sialylated and 6-O-sulfated mucin core 2-based compounds which may serve as potential ligands of Siglecs. In our experiments (Table 1, part B), we observed that α2,6-(N)ST was absolutely specific for the β1,4-linked Gal moiety in mucin core 2. Either α1,3 fucosylation or 6-O-sulfation of the GlcNAc moiety reduced the activity (31.0 and 30.6%, respectively). Sialylation of β1,3-linked Gal in mucin core 2 in addition to 6-O-sulfation of the GlcNAc moiety reduced the activity drastically: Galβ1,4(6-O-sulfo)GlcNAcβ1,6-(NeuAcα2,3Galβ1,3)GalNAcα-O-Me was 0.9% active, and Galβ1,4(6-O-sulfo)(Fucα1,3)GlcNAcβ1,6(NeuAcα2,-3Galβ1,3)GalNAcα-O-Me was 0% active. This suggests that action of α2,3(O)ST cannot be followed by α2,6(N)ST action in the case of 6-O-sulfated core 2 mucin (concept illustrated in Figure 2). Galβ1,4(Fucα1,3)GlcNAcβ1,6(NeuAcα2,-3Galβ1,3)GalNAcα-O-Bn, on the other hand, served as a reasonable acceptor for α2,6(N)ST (31.0%), suggesting that prior α2,3 sialylation of β1,3-linked Gal would not affect α2,6 sialylation of β1,4-linked Gal in nonsulfated mucin core 2 tetrasaccharide. Also, 3-O-sulfation of β1,3-linked Gal in mucin core 2 did not affect the activity of α2,6(N)ST (127.4%). On the basis of these results, the complex Siglec ligand, namely, NeuAcα2,6Galβ1,4(6-O-sulfo)GlcNAcβ1,6-(NeuAcα2,3Galβ1,3)GalNAcα−, could arise from Galβ1,4-(6-O-sulfo)GlcNAcβ1,6(Galβ1,3)GalNAcα− by the sequential action of α2,6(N)ST and α2,3(O)ST. The formation of a more complex ligand, namely, NeuAcα2,6Galβ1,4(Fucα1,3)-(6-O-sulfo)GlcNAcβ1,6(NeuAcα2,3Galβ1,3)GalNAcα−, seems to be impossible since NeuAcα2,6Galβ1,4GlcNAc is inactive as an acceptor for α1,3-L-fucosyltransferases (*41*).

Mucin Core 2-Based Compounds as Acceptors for Sialyltransferases. We examined the action of the sialyltransferases on modified core 2 structures where key hydroxyl groups were replaced with either O-methyl or fluoro substituents (Table 3). We observed that the cloned α2,3(O)ST utilized all acceptors, including Galβ1,4GlcNAcβ1,6(3-FGalβ1,3)GalNAcα-O-Bn, Galβ1,4GlcNAcβ1,6(3-O-MeGalβ1,3)GalNAcα-O-Bn, and Galβ1,4GlcNAcβ1,6(4-O-MeGalβ1,3)GalNAcα-O-Bn exhibiting 77.2, 49.6, and 101.3% activity, respectively. On the other hand, LNCaPα2,3(O)ST acted on only Galβ1,4GlcNAcβ1,6(4-O-MeGalβ1,3)GalNAcα-O-Bn (112.0%), and both 3-*O*-methyl and 3-fluoro substitution of the β1,3-linked Gal abrogated enzyme action. The cloned α2,6(N)ST exhibited very low activity toward 4-O-MeGalβ1,4GlcNAcβ1,6(Galβ1,3)GalNAcα-O-Bn (12.6%), whereas α2,3(N)ST exhibited 99.8% activity toward this acceptor. On the other hand, 3-*O*-methyl, 3-fluoro, and 4-fluoro substitution of Gal in the β1,4-linked Gal resulted in the formation of poor acceptors for both enzymes. The results indicate that besides transferring sialic acid to the 3-position of β1,3-linked Gal, α2,3(O)ST also induces sialylation of the acceptor at another position: either at the C-3 hydroxyl group of β1,4-linked Gal or at another OH group on either β1,4- or β1,3-linked Gal.

We examined in detail the effect of 4-O-methylation of the Gal moiety on the efficiency of mucin core 2 acceptors toward sialyltransferases (Figure 3). When the activity of α2,6(N)ST was measured separately at varying concentrations of Galβ1,4GlcNAcβ1,6(Galβ1,3)GalNAcα-O-Bn and 4-O-MeGalβ1,4GlcNAcβ1,6(Galβ1,3)GalNAcα-O-Bn, it was found (Figure 3A) that the latter compound was almost inactive at the maximum concentration (0.75 mM) that was tested whereas the former compound exhibited considerable

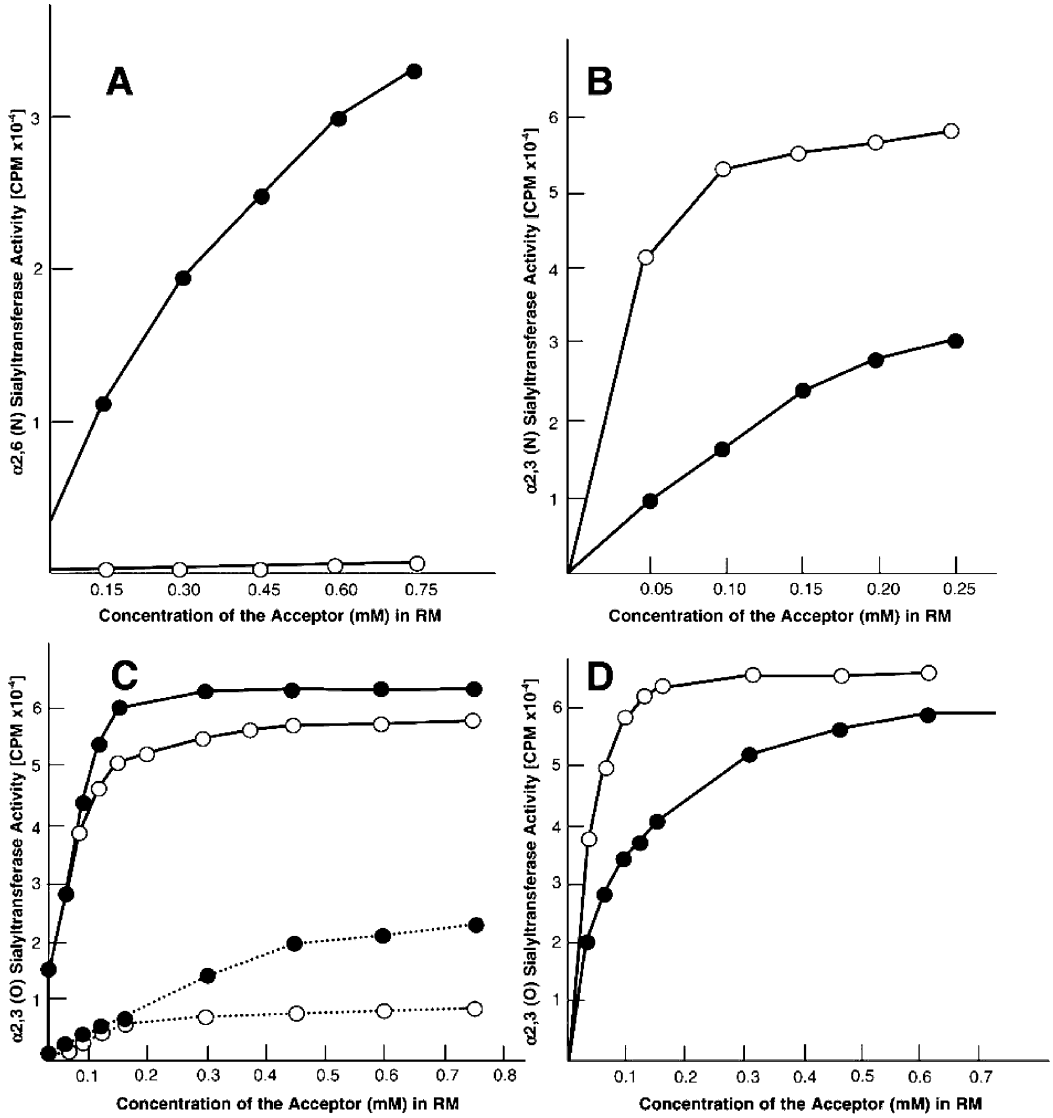

FIGURE 3: Sialyltransferase activity at varying concentrations of mucin core 2-based tetrasaccharide acceptors for cloned α2,6(N)ST (A), α2,3(N)ST (B), α2,3(O)ST (C), and LNCaPα2,3(O)ST (D). The acceptors used in panels A and B are Galβ1,4GlcNAcβ1,6(Galβ1,3)-GalNAcα-O-Bn (●) and 4-O-MeGalβ1,4GlcNAcβ1,6(Galβ1,3)GalNAcα-O-Bn (○). In panels C and D, the acceptors are Galβ1,4GlcNAcβ1,6-(Galβ1,3)GalNAcα-O-Bn (●) and Galβ1,4GlcNAcβ1,6(4-O-MeGalβ1,3)GalNAcα-O-Bn (○). Dotted lines in panel C denote enzymatic reaction data when donor CMP-NeuAc was used at the higher concentration in the reaction mixture.

activity even at the lowest concentration (0.15 mM) that was tested. When the activity of α2,3(N)ST was measured in the same manner with the two acceptors mentioned above (Figure 3B), the latter compound was 4-fold more active at the lowest concentration (0.05 mM) that was tested and 2-fold more active at the maximum concentration (0.25 mM) that was tested as compared to the activities of former compound at these concentrations. Thus, 4-O-methylation of β1,4-linked Gal abolished the acceptor ability toward α2,6(N)ST but increased the acceptor efficiency considerably toward α2,3(N)ST.

When the activity of cloned α2,3(O)ST was measured separately at varying concentrations of Galβ1,4GlcNAcβ1,6-(Galβ1,3)GalNAcα-O-Bn and Galβ1,4GlcNAcβ1,6(4-O-MeGalβ1,3)GalNAcα-O-Bn (Figure 3C), the former compound was found to be a slightly better acceptor under the standard incubation conditions. At a higher concentration of the donor CMP-NeuAc in the reaction mixture, the efficiency difference between these two acceptors was highly pronounced. On the other hand, LNCaPα2,3(O)ST (Figure 3D) utilized the latter compound more efficiently as an acceptor, reaching the maximal activity at 0.15 mM, whereas the former compound exhibited maximal activity at 0.60 mM. Thus, 4-O-methylation of β1,3-linked Gal resulted in an entirely different influence on the activities of cloned α2,3-(O)ST and LNCaPα2,3(O)ST.

Probing the Location of Sialylation by Cloned α2,3(O)-ST on the Acceptor GlcNAcβ1,6(3-O-MeGalβ1,3)GalNAcα-O-Bn. With the objective of determining if sialylation by cloned α2,3(O)ST takes place on the β1,4-linked Gal attached to GlcNAc, we examined the enzymatic products from the acceptors 3-O-MeGalβ1,3(GlcNAcβ1,6)GalNAcα-O-Bn, Fucα1,2Galβ1,3(GlcNAcβ1,6)GalNAcα-O-Bn, and 3-O-sulfoGalβ1,3(GlcNAcβ1,6)GalNAcα-O-Bn. The 9-^{3}H sialylated products from the acceptors 3-O-MeGalβ1,3-(GlcNAcβ1,6)GalNAcα-O-Bn and Fucα1,2Galβ1,3(Glc-NAcβ1,6)GalNAcα-O-Bn were isolated by the Sep-Pak C18 method. The 9-^{3}H sialylated products from the acceptors Galβ1,3(GlcNAcβ1,6)GalNAcα-O-Al and 3-O-sulfoGalβ1,3-(GlcNAcβ1,6)GalNAcα-O-Bn were isolated by BioGel P2

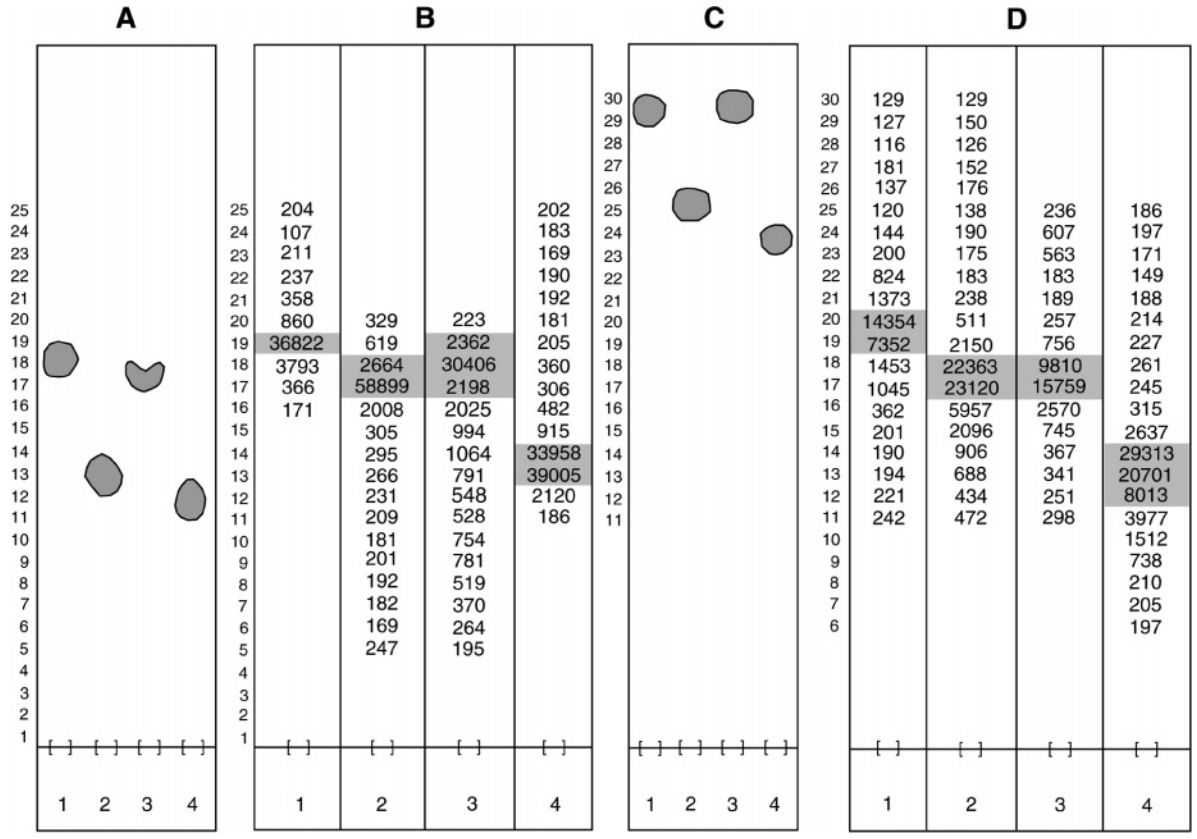

FIGURE 4: Thin-layer chromatography of nonradioactive acceptors: lane 1, 3-O-MeGalβ1,3(GlcNAcβ1,6)GalNAcα-O-Bn; lane 2, Fucα1,-2Galβ1,3(GlcNAcβ1,6)GalNAcα-O-Bn; lane 3, 3-O-sulfoGalβ1,3(GlcNAcβ1,6)GalNAcα-O-Bn; and lane 4, Galβ1,3(GlcNAcβ1,6)GalNAcα-O-Al in a 12/2/5 1-propanol/NH_4OH/H_2O solvent system in panel A (developed once) and a 5/4/1 $CHCl_3/CH_3OH/H_2O$ solvent system in panel C (developed twice). Thin-layer chromatography of [9-^{3}H]sialyl compounds obtained by the action of cloned α2,3(O)ST on the above acceptors (lanes 1−4 in the same sequence). In panel B, the 12/2/5 1-propanol/NH_4OH/H_2O solvent system was used (developed once), and in panel D, the 5/4/1 $CHCl_3/CH_3OH/H_2O$ solvent system was used (developed twice).

Table 4: Unique Sialylation of Mucin Core 2-Based Compounds by Cloned α2,3(O)ST

compound (7.5 mM)	sialyltransferase activity (%)
Galβ1,3(GlcNAcβ1,6)GalNAcα-O-Al[a]	100.0 (29 860 cpm)[c]
3-O-MeGalβ1,3(GlcNAcβ1,6) GalNAcα-O-Bn[b]	24.7
Fucα1,2Galβ1,3(GlcNAcβ1,6)GalNAcα-O-Bn[b]	45.4
3-O-sulfoGalβ1,3(GlcNAcβ1,6)GalNAcα-O-Bn[a]	54.7

[a] The 9-^{3}H sialylated product was quantitated by BioGel P2 column chromatography. [b] Sep-Pak C18 fractionation procedure. [c] Mass spectroscopy analysis of this reaction product was performed as described in Table 6 and the Supporting Information.

column chromatography. These 9-^{3}H sialyl compounds were subjected to TLC using two different solvent systems (Figure 4). The location of the radioactive compounds on TLC plates was determined by scraping $^1/_2$ cm width segments followed by liquid scintillation counting. Such studies indicated that only a single product was being formed. It was found that the enzyme exhibited 24.7, 45.4, and 54.7% activities, respectively, toward these acceptors when its activity toward reference molecule Galβ1,3(GlcNAcβ1,6)GalNAcα-O-Al was set to 100% (Table 4).

The sialylated products from all these acceptors except 3-O-sulfoGalβ1,3(GlcNAcβ1,6)GalNAcα-O-Bn exhibited binding to WGA−agarose via GlcNAc (Figure 5A−D). This indicates that the 3-O-sulfo group on β1,3-linked Gal in mucin core 2 trisaccharide abolishes the binding of WGA to β1,6-linked GlcNAc of mucin core 2. After digestion of these radioactive products with *β-N*-acetylhexosaminidase (Jack bean), the resulting radioactive compounds did not bind to WGA−agarose due to the absence of GlcNAc (Figure 5E−G). Overall, this set of experiments suggests that sialylation by α2,3(O)ST can take place on the β1,3-linked Gal at a position other than the 3-hydroxyl group.

Characterization of the Sialylated Product Arising from Galβ1,4GlcNAcβ1,6(3-O-MeGalβ1,3)GalNAcα-O-Bn by the Action of Cloned α2,3(O)ST. We isolated the product by the Sep-Pak C18 method and then subjected it to RCA-I−agarose chromatography (data not shown). As anticipated, [9-^{3}H]NeuAcα2,3Galβ1,4GlcNAcβ1,6(3-O-MeGalβ1,3)-GalNAcα-O-Bn arising from the action of α2,3(N)ST did not bind to this column. The sialylated compounds formed from Galβ1,4GlcNAcβ1,6(3-O-MeGalβ1,3)GalNAcα-O-Bn and Galβ1,4GlcNAcβ1,6(Galβ1,3)GalNAcα-O-Bn by the action of cloned α2,3(O)ST, however, exhibited weak binding to this column. The results indicated very strongly that sialylation by α2,3(O)ST did not take place on the β1,4-linked Gal moiety, and this is in agreement with the data reported above using WGA−agarose chromatography.

Evidence for Sialylation of Groups Other Than the C-3 or C-6 Hydroxyl Group in the β1,3-Linked Gal Moiety. The action of α2,3(O)ST on a series of compounds over varying acceptor concentrations was examined to better define its specificity. In some of the molecules, Gal was replaced with D-Fuc since both monosaccharides are identical, except that D-Fuc (6-deoxy-Gal) lacks a hydroxyl group at the C-6

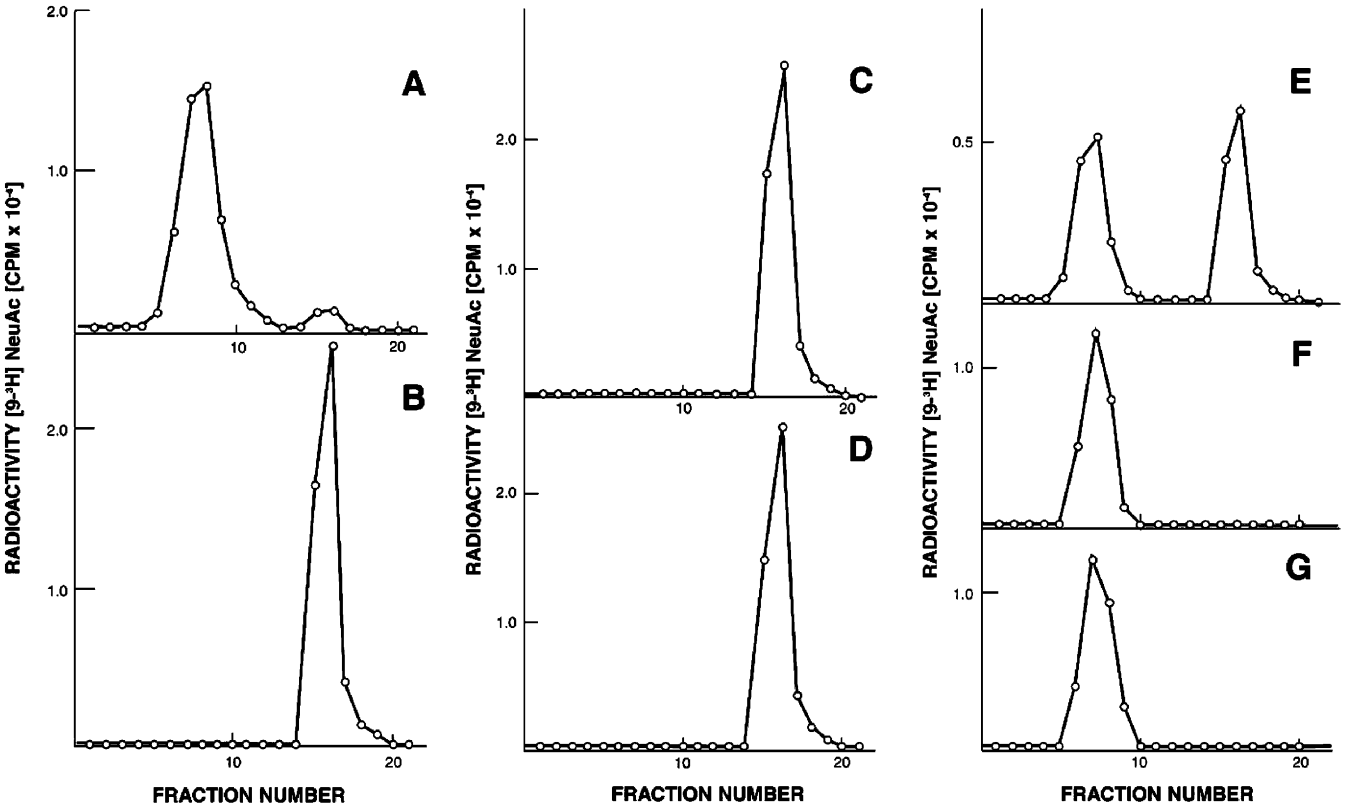

FIGURE 5: WGA−agarose chromatography of 9-^{3}H sialylated product obtained following the action of cloned α21,3(O)ST on (A) 3-O-sulfoGalβ1,3(GlcNAcβ1,6)GalNAcα-O-Bn, (B) 3-O-MeGalβ1,3(GlcNAcβ1,6)GalNAcα-O-Bn, (C) Galβ1,3(GlcNAcβ1,6)GalNAcα-O-Al, (D) Fucα1,2Galβ1,3(GlcNAcβ1,6)GalNAcα-O-Bn, (E) a mixture of the product of the panel B compound incubated with and without β-*N*-acetylhexosaminidase, (F) the product of the panel C compound incubated with β-*N*-acetylhexosaminidase, and (G) the product of the panel D compound incubated with β-*N*-acetylhexosaminidase. In all runs, elution was performed with 0.5 M GlcNAc after the 10th fraction.

position. The compound D-Fucβ1,3GalNAcβ1,3Galα-O-Me as shown above served as a good acceptor for the cloned α2,3(O)ST, indicating that the C-6 OH group of β1,3-linked Gal may not be the target of sialylation. To gain evidence in support of this contention, we also examined D-Fucβ1,-3GalNAcα-O-Bn, D-Fucβ1,3(GlcNAcβ1,6)GalNAcα-O-Bn, D-Fucβ1,3GalNAcβ1,3Galα-O-Me, Galβ1,3GalNAcβ1,3Galα-O-Me, and 3-O-sulfo-D-Fucβ1,3GalNAcβ1,3Galα-O-Me as acceptors for the cloned α2,3(O)ST (Figure 6A,C). Both D-Fucβ1,3GalNAcα-O-Bn and D-Fucβ1,3(GlcNAcβ1,6)-GalNAc-O-Bn at 0.75 mM were found to be 100% efficient as acceptors as compared to 3-O-MeGalβ1,4GlcNAcβ1,6-(Galβ1,3)GalNAcα-O-Bn. Further, the [9-^{3}H]sialyl compound arising from the former did not bind to the WGA−agarose column, whereas that from the latter did bind to this column (data not shown), indicating that sialylation occurs on the D-fucosyl moiety only.

When the activity of the cloned α2,3(O)ST was measured by varying the concentration of GloboH analogues, we found (Figure 6C) that Galβ1,3GalNAcβ1,3Galα-O-Me and D-Fucβ1,3GalNAcβ1,3Galα-O-Me were efficient acceptors, whereas both 3-O-sulfoGalβ1,3GalNAcβ1,3Galα-O-Me and 3-O-sulfo-D-Fucβ1,3GalNAcβ1,3Galα-O-Me also served as acceptors but to a lesser extent. Detailed mass spectroscopy analysis was performed with 3-O-sulfo-D-Fucβ1,3GalNAcβ1,-3Galα-O-Me (compound **S4** in Table 6) and its reaction product (compound **14**) fragmentation analysis using MS^n analysis (Supporting Information). This verified the attachment of sialic acid to this molecule, which is devoid of free hydroxyl at the C-3 and C-6 positions. D-Fucβ1,3GalNAcβ1,-3Galα-O-Me was ∼50 and ∼100% efficient as compared to Galβ1,3GalNAcβ1,3Galα-O-Me at 0.15 and 0.75 mM, respectively, indicating that the C-6 hydroxyl group is required for the maximum enzyme affinity toward the acceptors. Further, almost the same acceptor efficiency of 3-O-sulfoGalβ1,3GalNAcβ1,3Galα-O-Me and 3-O-sulfo-D-Fucβ1,3GalNAcβ13,Galα-O-Me (Figure 6C and Table 5) would suggest that the C-6 hydroxyl group of the Gal moiety is unlikely the site of sialylation in the acceptor 3-O-sulfoGalβ1,3GalNAcβ1,3Galα-O-Me. Sialylation likely takes place at the C-2 or C-4 position at β1,3-attached Gal.

The Unique Catalytic Activities of the Cloned α2,3(O)ST on the Modified Terminal β1,3-Galactosyl Residues. A measurement of the clonal α2,3(O)ST activity at varying concentrations of various acceptors followed by a determination of K_m and V_{max} values by a Lineweaver−Burke plot (see Figure 6 and Table 5) indicated that the Globo backbone compounds Galβ1,3GalNAcβ1,3Galα-O-Me, 3-O-sulfoGalβ1,-3GalNAcβ1,3Galα-O-Me, D-Fucβ1,3GalNAcβ1,3Galα-O-Me; the T-hapten compounds 4-O-MeGalβ1,3GalNAcα-O-Bn, 4-FGalβ1,3GalNAcα-O-Bn, and D-Fucβ1,3GalNAcα-O-Bn; and the mucin core 2 compounds D-Fucβ1,3-(GlcNAcβ1,6)GalNAcα-O-Bn and Fucα1,2Galβ1,3(Glc-NAcβ1,6)GalNAcα-O-Bn exhibited K_m values in the range from 0.12 to 2.00 mM and V_{max} values in the range from 197 to 1873 pmol/h. The results would indicate that the synthesis of these unique sialylated compounds is feasible via utilization of this enzyme.

ESI-MS/MS^n Analysis of the Enzymatically Sialylated Compounds. To confirm our finding that α2,3(O)ST can

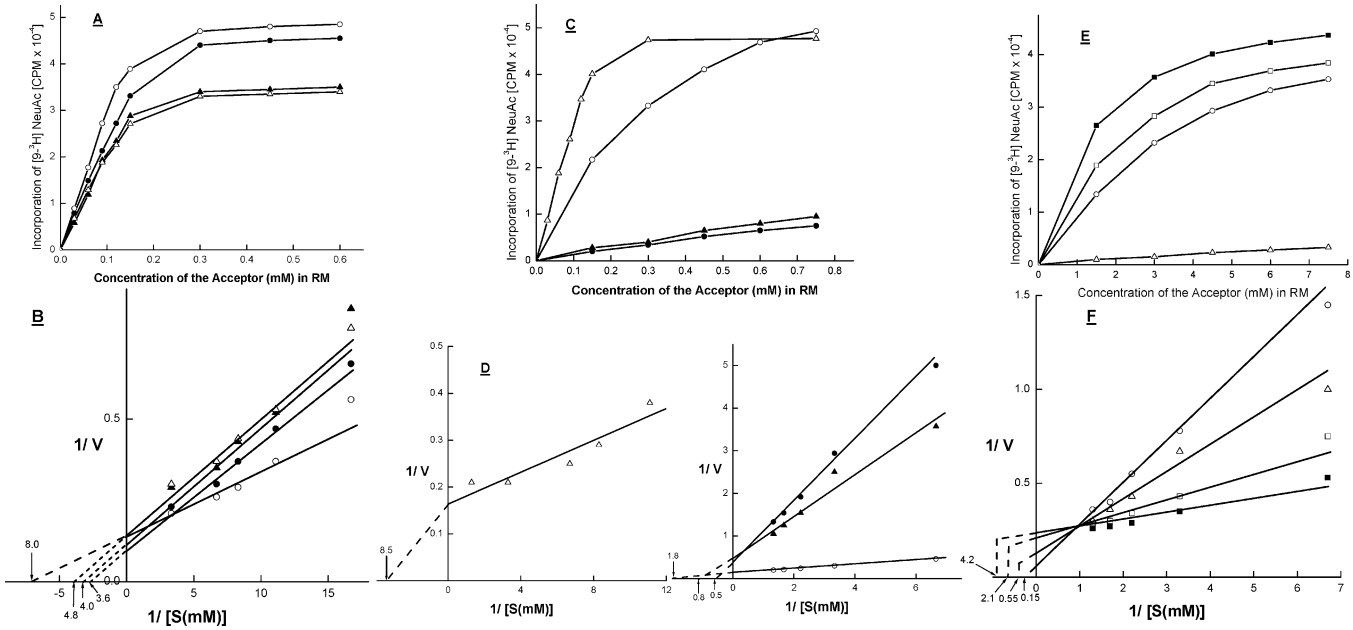

FIGURE 6: Cloned α2,3(O)ST activity at varying concentrations of acceptors containing a modified galactosyl residue linked β1,3 to α-GalNAc. (A) Acceptor concentration vs enzyme activity for D-Fucβ1,3(GlcNAcβ1,6)GalNAcα-O-Bn (●), 4-O-MeGalβ1,3GalNAcα-O-Bn (○), 4-FGalβ1,3GalNAcα-O-Bn (▲), and D-Fucβ1,3GalNAcα-O-Bn (△). (B) Lineweaver−Burke plot for the data in panel A. (C) Acceptor concentration vs enzyme activity for Galβ1,3GalNAcβ1,3Galα-O-Me (△), 3-O-sulfoGalβ1,3GalNAcβ1,3Galα-O-Me (▲), D-Fucβ1,-3GalNAcβ1,3Galα-O-Me (○), and 3-O-sulfo-D-Fucβ1,3GalNAcβ1,3Galα-O-Me (●). (D) Lineweaver−Burke plot for the data in panel C. (E) Acceptor concentration vs enzyme activity for Fucα1,2Galβ1,3(GlcNAcβ1,6)GalNAcα-O-Bn (CMP-NeuAc, 15 μM) (○), Fucα1,-2Galβ1,3(GlcNAcβ1,6)GalNAcα-O-Bn (CMP-NeuAc, 150 μM) (△), 3-O-MeGalβ1,3(GlcNAccβ1,6)GalNAcα-O-Bn (CMP-NeuAc, 0.3 μM) (□), and 3-O-MeGalβ1,3(6-O-Me)GalNAcα-O-Bn (CMP-NeuAc, 0.3 μM) (■). (F) Lineweaver−Burke plot for the data in panel E.

Table 5: Discerning the Unique Catalytic Abilities of the Cloned α2,3(O)ST by Utilizing an Array of Synthetic Compounds Containing Modified Terminal β1,3-Galactosyl Residues as Acceptors[a]

compound	CMP-NeuAc[b] (150 μM) K_m (mM)	V_{max} (pmol/h)
Globo backbone		
Galβ1,3GalNAcβ1,3Galα-O-Me	0.12	1293
3-O-sulfoGalβ1,3GalNAcβ1,3Galα-O-Me	1.25 (**13**)	416
D-Fucβ1,3GalNAcβ1,3Galα-O-Me	0.56	1873
3-O-sulfo-D-Fucβ1,3GalNAcβ1,3Galα-O-Me	2.00 (**14**)	624
T-hapten		
4-O-MeGalβ1,3GalNAcα-O-Bn	0.13	1250
4-FGalβ1,3GalNAcα-O-Bn	0.25	1442
D-Fucβ1,3GalNAcα-O-Bn	0.21 (**15**)	1250
mucin core 2		
D-Fucβ1,3(GlcNAcβ1.6)GalNAcα-O-Bn	0.28 (**16**)	1562
Fucα1,2Galβ1,3(GlcNAcβ1,6)GalNAcα-O-Bn	1.82	197

[a] Mass spectroscopy analysis of the reaction products in parentheses was performed as described in Table 6 and the Supporting Information. **S4** (Table 6) is the synthetic acceptor that yields product **14**. [b] The reaction mixtures (20 μL) contained 0.2 μCi of CMP-[9-^{3}H]NeuAc in 150 μM CMP-NeuAc.

catalyze novel sialylation, we performed MS^n spectral analysis with selected enzymatically synthesized products under collision-induced dissociation (CID). Four isomeric pentasaccharides (compounds **6**, **7**, **18**, and **23**) with different sialyl linkages (siaα2,3, siaα2,2/4, and siaα2,6) and a sulfate residue (at the 3-position of either galactose terminus in mucin core 2 tetrasaccharide) (Figure 7) were detected as doubly charged $[M - 2H]^{2-}$ ion at *m*/*z* 604 in the negative mode and doubly charged quadruply sodiated $[M - 2H + 4Na]^{2+}$ ions at *m*/*z* 650 in the positive mode (Table 6). MS^2 spectra of molecular species at *m*/*z* 604 derived from the four isomers are shown in Figure 8. The fragmentations identified in Figures 8 and 9 were shown in Figure 7 by using established Domon−Costello nomenclature (*42*). The glycosidic cleavage between the sialyl group and branched mucin core 2 yielded B ions (B'_1 ion or B_1 ion) representing sialyl group and Y ions (Y'_2 ion or Y_3 ion) representing the sulfated branched mucin core 2 tetrasaccharides. As seen, the ratios of B ions to Y ions are significantly different among the different linkages (see Figure 8). The same CID patterns are seen in panels A and B of Figure 8 indicating that compounds **6** and **23** shared a common linkage, which is distinct from that of compounds **7** and **18**. On the basis of the enzymatic studies, this is likely an α2,3 linkage. The Y_3 ion from compound **18** is at *m*/*z* 913 which is significantly different from the corresponding ions at *m*/*z* 917 from compounds **6** and **23**. This difference in molecular weight could be attributed to the formation of a lactone bond between two primary hydroxyl groups of Gal and GlcNAc in the Galβ1,4GlcNAc linkage of the branched mucin core 2 tetrasaccharide based on the mechanism reported by Ulrik et al. (*43*). The spectrum of compound **7** (Figure 8C) is distinct from the spectra of compounds **6**, **18**, and **23** indicating a different sialyl linkage and thus likely representing either an α2,2 or α2,4 linkage of sialic acid.

MS^3 spectra of the Y ions representing sulfated branched mucin core 2 structures are shown in Figure 9. The B_2 ion at *m*/*z* 444 shown in Figure 9A confirms that the sulfate group of compound **6** is on the β1,4-linked Gal of the mucin core 2 tetrasaccharide. Similarly, the Y_1 ions at *m*/*z* 552 in panels B and C of Figure 9 indicate that the sulfate group of compounds **23**, **7**, and **18** are on the Gal of Galβ1,3GalNAc in the tetrasaccharide. The formation of a lactone bond was

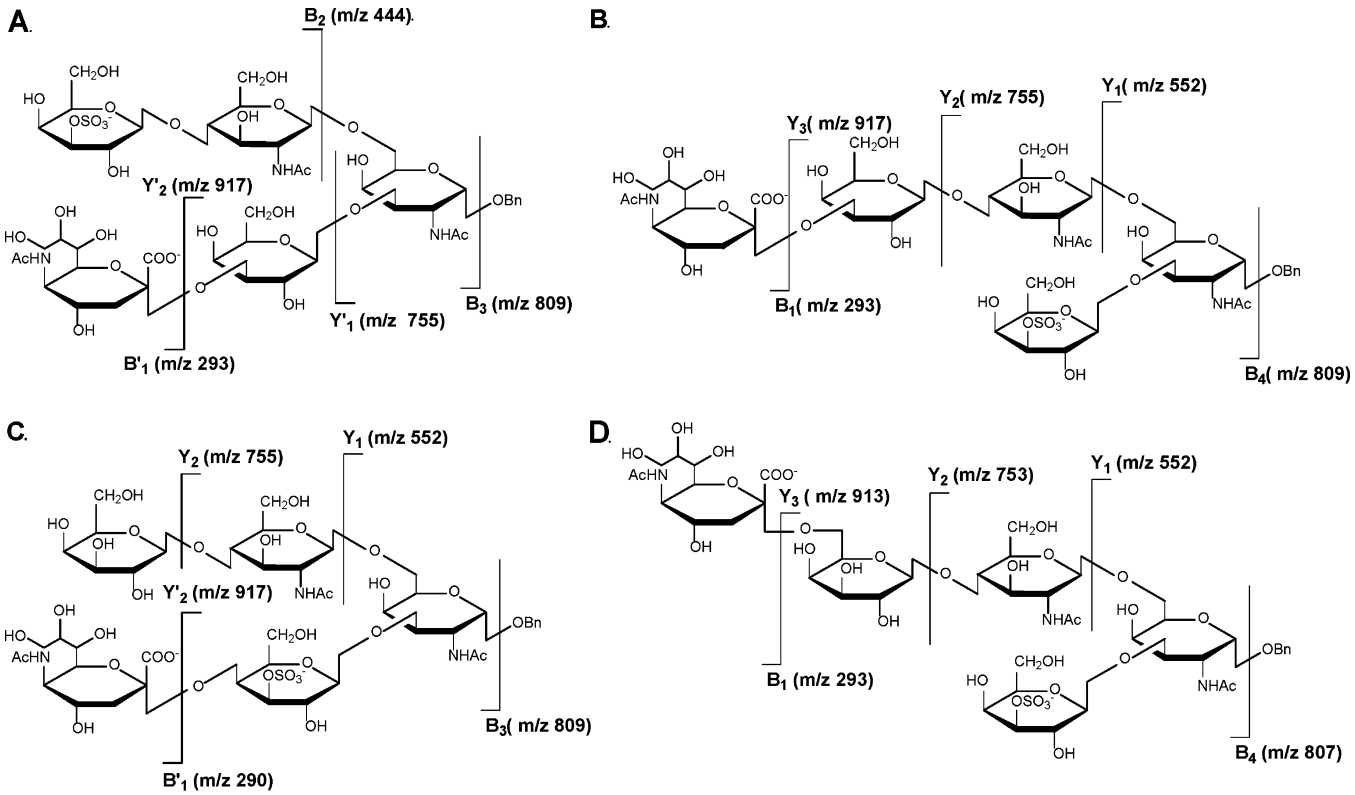

FIGURE 7: Fragmentation of (A) compound **6**, (B) compound **23**, (C) compound **7**, and (D) compound **18** in Table 6 under the same CID conditions of ESI-MS. Fragments are annotated according to the scheme proposed by Domon and Costello (*42*).

also confirmed by the Y_2 ion at *m*/*z* 753 and the B_4 ion at *m*/*z* 807 of compound **18** shown in Figure 9C, which is different from the corresponding ion at *m*/*z* 755 and 809 in panels A and B.

DISCUSSION

This paper examines the acceptor specificity of three cloned rat sialyltransferases and a human sialyltransferase from prostate cancer cell line LNCaP. By defining the specificity of these enzymes for an array of carbohydrate acceptors based on the core 2 and Globo structures, we (i) define unique acceptors that can be used to characterize each of these enzymes in a complex mixture of glycosyltransferases, (ii) describe potential biochemical pathways that can lead to the synthesis of the ligands for selectins and Siglecs, and (iii) propose that the C-2 or C-4 position of β1,3-linked Gal in the core 2 mucin may be a site for the action of cloned α2,3(O)ST, provided the C-3 position has a substituent other than sialic acid.

Some Pertinent Observations on Rat Recombinant and LNCaP Sialyltransferases. Other investigators have performed studies with the sialyltransferases examined in our work. Chemoenzymatic studies of Takano et al. (*44*) identified only one product, namely, Galβ1,4GlcNAcβ1,6(NeuAcα2,3Galβ1,3)GalNAcα-O-R, when the tetrasaccharide acceptor was incubated at 37 °C with the rat recombinant α2,3(O)ST. On the other hand, they identified the above monosialyl compound (8%) and disialyl compound (15%) in addition to the typical product, namely, NeuAcα2,3Galβ1,4GlcNAcβ1,6(Galβ1,3)GalNAcα-O-R (57%), when the tetrasaccharide acceptor was incubated at 37 °C with rat recombinant α2,3(N)ST at pH 7.4 for 24 h. Sengupta et al. (*45*) made a similar observation with the rat recombinant α2,3(N)ST when the tetrasaccharide acceptor was incubated at pH 7.4 and 37 °C for 18 h. To compare our findings with the findings of these investigators, we also extended the reaction times in our experiments from 2 to 20 h under our standard experimental conditions (pH 6.0 at 37 °C) for the acceptor Galβ1,4GlcNAcβ1,6(Galβ1,3)GalNAcα-O-Bn and utilized all three rat recombinant enzymes, α2,3(N)ST, α2,6(N)ST, and α2,3(O)ST. The [9-^{3}H]sialyl products arising from the tetrasaccharide acceptor were isolated using the Sep-Pak C18 cartridge. PNA−agarose affinity chromatography of the radioactive products showed that 92, 93, and 0% of the radioactivity, respectively, was specifically bound to the column, indicating that [9-^{3}H]NeuAcα2,3Galβ1,4GlcNAcβ1,6(Galβ1,3)GalNAcα-O-Bn and [9-^{3}H]NeuAcα2,6Galβ1,4GlcNAcβ1,6(Galβ1,3)GalNAcα-O-Bn were the bound products in case of α2,3(N)ST and α2,6(N)ST, and Galβ1,4GlcNAcβ1,6(9-^{3}HNeuAcα2,3Galβ1,3)GalNAcα-O-Bn was the unbound product in the case of α2,3(O)ST. Triton X-100-solubilized extract of LNCaP cells contained >95.0% α2,3(O)ST activity as evident from the activity of the following acceptors: 3-O-MeGalβ1,4GlcNAcβ1,6(Galβ1,3)GalNAcα-O-Bn (100.0%), Galβ1,4GlcNAcβ1,6(3-O-MeGalβ,13)-GalNAc (3.0%), and Galβ1,4GlcNAcβ-O-Al (0.5%). Overall, our results are in agreement with other data in the literature, and some observed differences may be attributed to variation in incubation conditions between the studies.

Unique Activities of Clonal α2,3(O)ST on a Position Other Than C-3 of β1,3-Linked Gal. The clonal α2,3(O)ST was active with 3-O-MeGalβ1,4GlcNAcβ1,6(Galβ1,3)GalNAcα-O-Bn (100.0%) and Galβ1,4GlcNAcβ1,6(3-O-MeGalβ1,3)-GalNAcα-O-Bn (49.6%) but was almost inactive toward Galβ1,4GlcNAcβ-O-Al (4.7%). When Galβ1,4GlcNAcβ1,6(Galβ1,3)GalNAcα-O-Bn was used as an acceptor, 100%

Table 6: Mass Spectral Analysis of Enzymatically Sialylated Acceptors

Compound[a] No.	Oligosaccharides	Yield[c] (nmol)	Calcd. MW	Found (m/z) Negative mode	Found (m/z) Positive Mode
1	Galβ1,3(GlcNAcβ1,6)GalNAcα-O-Al ↑ **α2,3** **NeuAc**	146	917.3	$[M-H]^-$: 916.0	
2	Galβ1,3GalNAcβ1,3Galα-O-Me ↑ **α2,3** **NeuAc**	138	850.3	$[M-H]^-$: 848.9	$[M-H+2Na]^+$: 894.7
3	*D*-Fucβ1,3GalNAcβ1,3Galα-O-Me ↑ **α2,3** **NeuAc**	160	835.2	$[M-H]^-$: 833.8	$[M-H+2Na]^+$: 879.2
4	Galβ1,4GlcNAc β1,6(Galβ1,3)GalNAcα-O-Bn ↑ **α2,3** **NeuAc**	63	1129.4	$[M-H]^-$: 1127.1	$[M-H+2Na]^+$: 1173.5
5	6-O-SulfoGlcNAc β1,6 (Galβ1,3)GalNAcα-O-Al ↑ **α2,3** **NeuAc**	143	997.9	$[M-2H]^{2-}$: 498.0	$[M-2H+4Na]^{2+}$: 544.0
6	3-O-SulfoGalβ1,4GlcNAc β1,6(Galβ1,3)GalNAcα-O-Bn ↑ **α2,3** **NeuAc**	145	1210.1	$[M-2H]^{2-}$: 604.1	$[M-2H+4Na]^{2+}$: 650.3
7	Galβ1,4GlcNAc β1,6(3-O-SulfoGalβ1,3)GalNAcα-O-Bn ↑ **α2,2/4**[b] **NeuAc**	11	1210.1	$[M-2H]^{2-}$: 604.1	$[M-2H+4Na]^{2+}$: 649.9
8	Galβ1,3GalNAcα-O-Bn ↑ **α2,3** **NeuAc**	146	764.3	$[M-H]^-$: 763.4	
9	4-O-MeGalβ1,3GalNAcα-O-Bn ↑ **α2,3** **NeuAc**	140	778.7	$[M-H]^-$: 777.6	
10	4-F-Galβ1,3GalNAcα-O-Bn ↑ **α2,3** **NeuAc**	124	766.2	$[M-H]^-$: 764.9	$[M-H+2Na]^+$: 813.4
11	Galβ1,3(6-O-Me)GalNAcα-O-Bn ↑ **α2,3** **NeuAc**	117	778.7	$[M-H]^-$: 777.5	$[M-H+2Na]^+$: 823.3
12	3-F-Galβ1,4GlcNAc β1,6(Galβ1,3)GalNAcα-O-Bn ↑ **α2,3** **NeuAc**	94	1131.4	$[M-H]^-$: 1129.4	
13	3-O-SulfoGalβ1,3GalNAcβ1,3Galα-O-Me ↑ **α2,2/4**[b] **NeuAc**	13	931.2	$[M-2H]^{2-}$: 463.9	
14	(3-O-Sulfo)*D*-Fucβ1,3GalNAcβ1,3Galα-O-Me ↑ **α2,2/4**[b] **NeuAc**	17	915.2	$[M-2H]^{2-}$: 455.2	
15	*D*-Fucβ1,3GalNAcα-O-Bn ↑ **α2,3** **NeuAc**	114	748.7	$[M-H]^-$: 747.6	
16	*D*-Fucβ1,3(GlcNAcβ1,6)GalNAcα-O-Bn ↑ **α2,3** **NeuAc**	118	951.3	$[M-H]^-$: 949.7	
17	Galβ1,4(6-O-Sulfo)GlcNAc β1,6(Galβ1,3)GalNAcα-O-Me ↑ **α2,6** **NeuAc**	87	1134.0	$[M-2H]^{2-}$: 566.8	$[M-2H+4Na]^{2+}$: 611.9
18	Galβ1,4GlcNAc β1,6(3-O-SulfoGalβ1,3)GalNAcα-O-Bn ↑ **α2,6** **NeuAc**	121	1210.1	$[M-2H]^{2-}$: 604.3	$[M-2H+4Na]^+$: 649.6
19	Galβ1,4GlcNAc β1,6(3-F-Galβ1,3)GalNAcα-O-Bn ↑ **α2,6** **NeuAc**	69	1131.4	$[M-H]^-$: 1128.9	
20	Galβ1,3GalNAcβ1,3Galα-O-Me ↑ **α2,3** **NeuAc**	175	850.3	$[M-H]^-$: 849.0	$[M-H+2Na]^+$: 895.0
21	Galβ1,4(6-O-Sulfo)GlcNAc β1,6(Galβ1,3)GalNAcα-O-Me ↑ **α2,3** **NeuAc**	129	1134.0	$[M-2H]^{2-}$: 567.2	$[M-2H+4Na]^{2+}$: 611.3
22	Galβ1,4(6-O-Sulfo)GlcNAc β1,6(Galβ1,3)GalNAcα-O-Me ↑ **α2,3** ↑ α2,3 **NeuAc** NeuAc	159	1423.0	$[M-3H]^{3-}$: 472.2	
23	Galβ1,4GlcNAc β1,6(3-O-SulfoGalβ1,3)GalNAcα-O-Bn ↑ **α2,3** **NeuAc**	146	1210.1	$[M-2H]^{2-}$: 603.4	$[M-2H+4Na]^+$: 649.5
24	4OMeGalβ1,4GlcNAcα-O-Bn ↑ **α2,3** **NeuAc**	174	778.7	$[M-H]^-$: 777.3	
25	Galβ1,4GlcNAc β1,6(3-F-Galβ1,3)GalNAcα-O-Bn ↑ **α2,3** **NeuAc**	117	1131.4	$[M-H]^-$: 1130.3	
S1	6-O-SulfoGalβ1,3GalNAcα-O-NP ↑ α2,6 NeuAc		875.8	$[M-2H]^{2-}$: 437.1	
S2	6-O-SulfoGalβ1,4(Fucα1,3)GlcNAcβ-O-Me ↑ α2,3 NeuAc		914.3	$[M-2H]^{2-}$: 456.1	
S3	*D*-Fucβ1,3GalNAcβ1,3Galα-O-Me		543.0	$[M-H]^-$: 542.2	$[M-H+2Na]^+$: 565.8
S4	(3-O-Sulfo)*D*-Fucβ1,3GalNAcβ1,3Galα-O-Me		623.0	$[M-H]^-$: 621.6	$[M-H+2Na]^+$: 667.8

[a] **NeuAc** denotes the sialic acid that was added to specific acceptors by the action of cloned sialyltransferases: α2,3(O)ST for compounds **1**−**16**, α2,6(N)ST for compounds **17**−**19**, and α2,3(N)ST for compounds **20**−**25**. **S1**−**S4** are chemically synthesized compounds. [b] Sialylation may take place at either the C-2 or C-4 position of Gal in the Gal*β*1,3GalNAc residue of the core 2 structure. [c] The yield was calculated from the percentage of radioactivity incorporated into the acceptor from 200 nmol of CMP-[9-^{3}H]NeuAc taken in the reaction mixture.

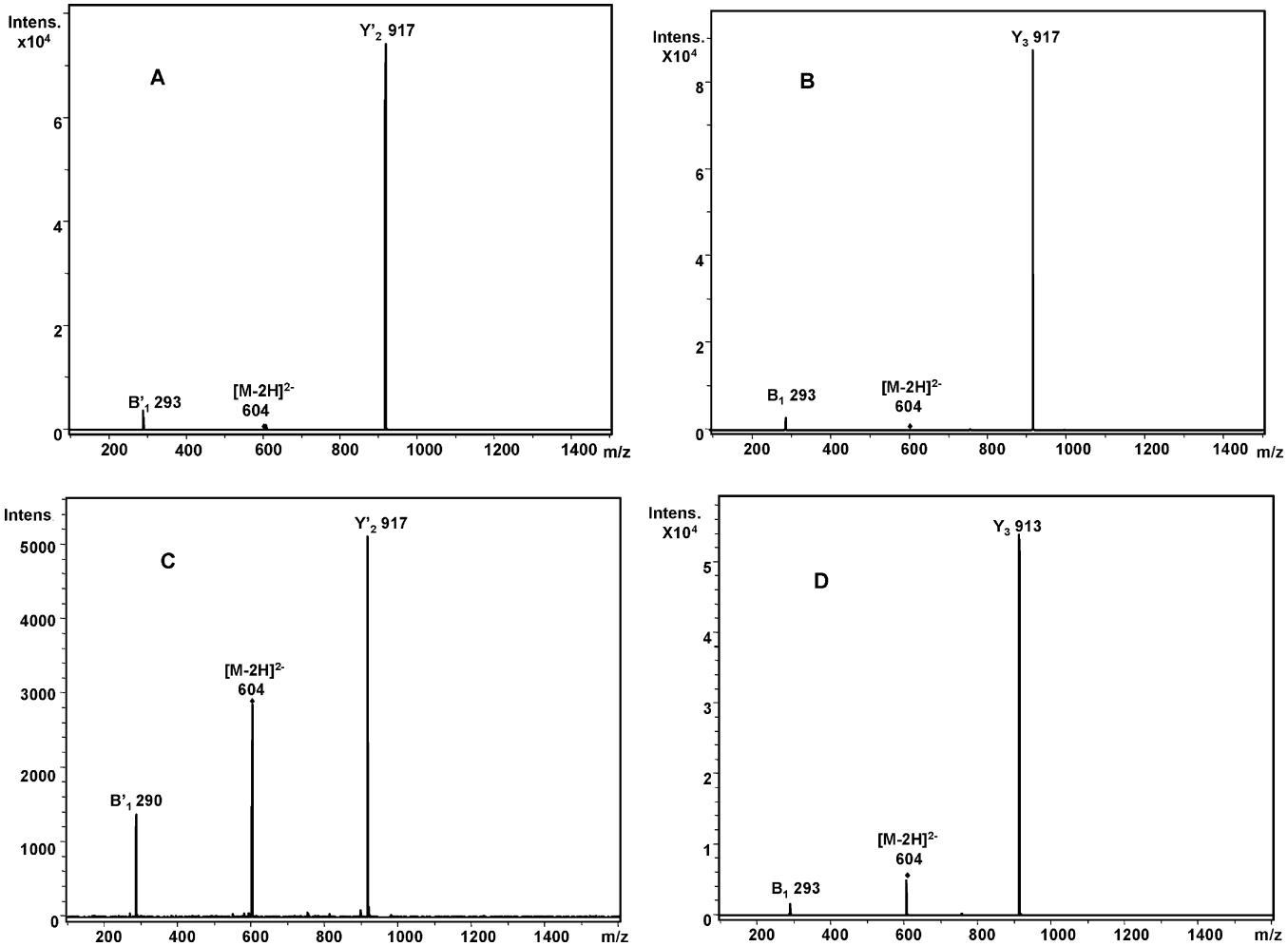

FIGURE 8: MS^2 spectra of doubly charged molecular species $[M - 2H]^{2-}$ at *m/z* 604 derived from (A) compound **6**, (B) compound **23**, (C) compound **7**, and (D) compound **18** in Table 6.

of the isolated [9-^{3}H]sialyl product did not bind to the PNA−agarose column, indicating that sialylation took place only on the β1,3-linked Gal moiety. This suggests two possibilities: (1) that sialylation might occur on β1,4-linked Gal when there is a C-3 block on β1,3-linked Gal moiety and (2) that sialylation may take place on some other position in the β1,3-linked Gal moiety. To explore these possibilities, we used mucin core 2 trisaccharide containing different substituents on the β1,3-linked Gal moiety as the acceptor for the clonal α2,3(O)ST. Using tandem mass spectral analysis, WGA−agarose affinity chromatography on 9-^{3}H sialylated products before and after *β-N*-acetylhexosaminidase treatment, and also RCA−agarose affinity chromatography on [9-^{3}H]sialyl products from mucin core 2 tetrasaccharide acceptors, we demonstrate unequivocally that sialylation took place on a different position of 3-O-substituted β1,3-linked Gal. Since both 3-O-sulfo-D-Fucβ1,3GalNAcβ1,3Galα-O-Me and 3-O-sulfoGalβ1,3GalNAcβ1,3Galα-O-Me served as acceptors with almost the same efficiency for the clonal α2,3(O)ST, the C-6 position in C-3-blocked β1,3-linked Gal is unlikely to be the site for sialylation. It is possible that one of the remaining hydroxyl groups (C-2 or C-4) is the site of action of this enzyme. However, Kobata and his group (*46*) reported the occurrence of NeuAcα2,4Gal terminal N-glycan chains in cold-insoluble globulin isolated from bovine plasma. Further, 3-sulfoGalβ1,3GalNAcβ1,3Galα1,4Gal$\beta\rightarrow$Glcβ ceramide has been reported to occur in rat kidney (*47*). In fact, the 3-sulfo-Galβ1,3GalNAcβ sequence is known to occur in several glycolipids (*48*).

Prediction of Potential Inhibitors of Siglec Binding. Our biochemical investigations suggest new molecules that may act as efficient inhibitors of Siglec binding to its ligand. In this regard, recently, Blixt et al. (*49*) assessed the sialoside specificity of the Siglec family using a novel multivalent platform comprising biotinylated sialosides bound to a streptavidin−alkaline phosphatase conjugate. Human Siglecs-2, -3, and -7−10 exhibited significant binding to the sialoside-SAAP probe H-containing NeuAcα2,6Galβ1,-4GlcNAc. They observed that there was little correlation between the IC_{50} values and the ability of the corresponding SAAP probe to bind to immobilized Siglec chimeras. They also found in the binding assay that the length of the spacer arm can influence the interaction of a sialoside sequence with a given Siglec. Despite these pitfalls, these authors came up with a remarkable finding that NeuAcα2,6(Galβ1,3)GalNAcα-O-Thr, NeuAcα2,3Galβ1,3GalNAcα-O-Thr, and NeuAcα2,-3Galβ1,3(NeuAcα2,6)GalNAcα-O-Thr are 100-, 600-, and 3000-fold more potent than NeuAcα2,6GalNAcα-O-Thr, respectively, in inhibiting Siglec-4 (MAG) binding. On the basis of our studies, it now appears the following mucin core 2-based compounds may also be potential inhibitors for Siglec-4 and perhaps other Siglec binding also: NeuAcα2,-3Galβ1,3(GlcNAcβ1,6)GalNAcα−, NeuAcα2,3Galβ1,3-(NeuAcα2,3Galβ1,4GlcNAcβ1,6)GalNAcα−, NeuAcα2,-

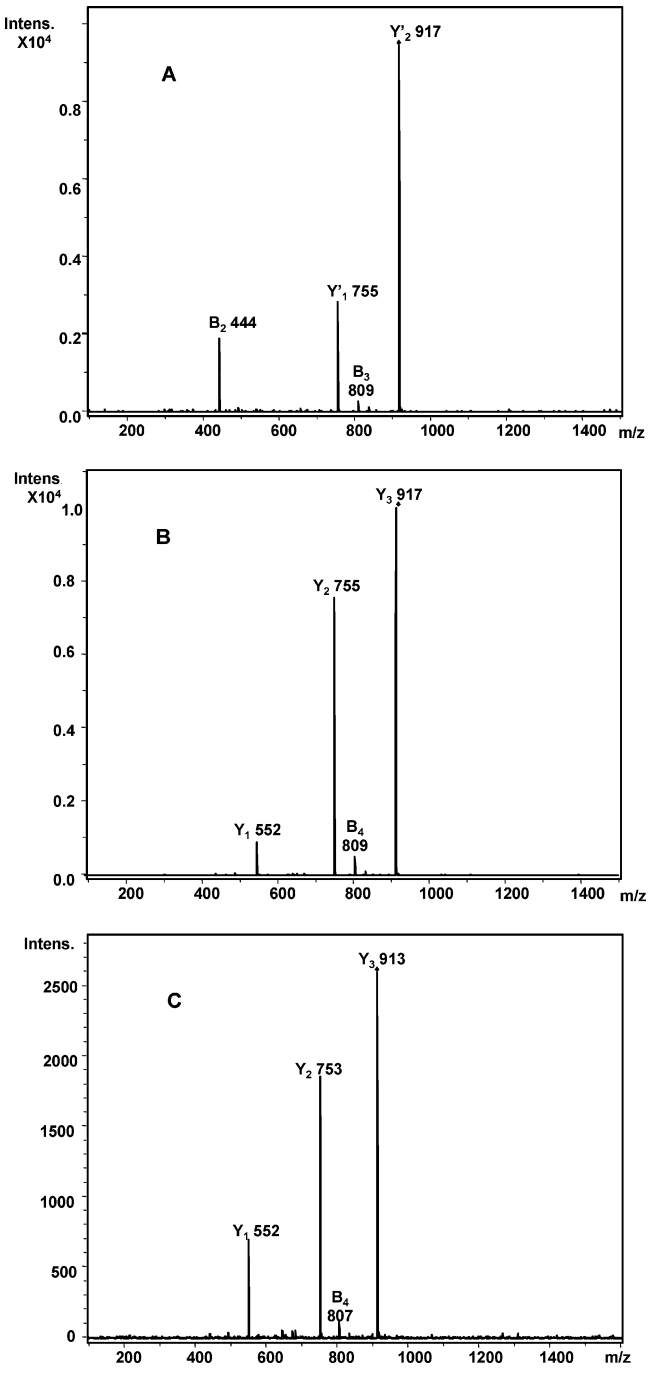

FIGURE 9: MS^3 spectra of (A) the Y'_2 ion at *m/z* 917 derived from compound **6**, (B) the Y_3 or Y'_2 ion at *m/z* 917 derived from compound **23** or compound **7**, and (C) the Y_3 ion at *m/z* 913 derived from compound **18** in Table 6.

3Galβ1,3(NeuAcα2,6Galβ1,4GlcNAcβ1,6)GalNAc$\alpha-$, NeuAcα,α2,3Galβ1,3(6-O-sulfo)GalNAc$\alpha-$, NeuAcα2,-3Galβ1,3(3-O-sulfoGalβ1,4GlcNAcβ1,6)GalNAc$\alpha-$, NeuAcα2,3Galβ1,3[Galβ1,4(6-O-sulfo)GlcNAcβ1,6]-GalNAc$\alpha-$, and NeuAcα2,3Galβ1,3(6-O-sulfoGalβ1,-4GlcNAcβ1,6)GalNAcα. In fact, Blixt et al. (*50*) have recently reported that NeuAcα2,6Galβ1,4(6-O-sulfo)-GlcNAc$\beta-$ is far better than the corresponding nonsulfated structure in binding to human CD22.

Enzymes that Act at the C-3 Position of Gal in LacNAc Also Act Efficiently on the Globo Acceptor. Earlier, we found that prostate cancer cell LNCaP α1,2-L-FT exhibited 4-fold greater activity toward β1,4-linked Gal than with the β1,3-linked Gal in mucin core 2 tetrasaccharide (*51*); the same enzyme also utilized the Globo backbone structures Galβ1,-3GalNAcβ13,Galα-O-Me and D-Fucβ1,3GalNAcβ1,3Galα-O-Me very efficiently. Subsequently, we observed that Gal:3-O-sulfotransferase, Gal3ST-2, which exhibits specificity for LacNAc type 2 structure also acts efficiently on the Globo structures (*52*). Extending these observations, in this study, we show that α2,3(N)ST acted with equal efficiency on LacNAc and the Globo structure Galβ1,3GalNAcβ1,3Galα-O-Me. Thus, we discovered a physiological correlation that different enzymes modifying terminal β1,4-linked Gal also efficiently utilize the terminal β1,3-linked Gal in the Globo backbone.

Rationale for the Difference in the Acceptor Specificity of α2,3(O)ST from Liver and Prostate. All cloned mammalian sialyltransferases contain L-, S-, and VS-sialyl motifs (*53*−*55*). The L-sialyl motif was shown to bind to the donor CMP-NeuAc (*56*), while the S-sialyl motif participated in the binding of both donor and acceptor glycans (*59*). The exact role for the VS-sialyl motif in the catalytic process had not been identified. Kitazume-Kawaguchi et al. (*60*) suggested that His could be a catalytic residue for all sialyltransferases.

Three genes were shown to encode human sialyltransferases ST-4a−c, which catalyze α2,3 sialylation of Galβ1,-3GalNAc$\alpha-$. Each enzyme followed a distinct pattern of tissue specific expression (*59*). The level of ST-4a mRNA was relatively similar in most tissues that were examined except for brain and skeletal muscle which show little and strong expression, respectively (*59*). ST-4b was identified only in liver and, surprisingly, was not detectable in $HepG_2$; ST-4c was most strongly expressed in heart, placenta, testis, and ovary. Prostate tissue also predominantly contains ST-4c (*57*). In the context of our study, it is possible that the human counterpart for rat liver recombinant α2,3(O)ST is ST-4b, and on the basis of the tissue of origin, the sialyltransferases from LNCaP prostate cells may be ST-4c. It remains to be seen whether such a difference in the expression of ST-4 transcripts between liver and prostate can account for the difference in the intricate substrate specificities of enzymes from these sources.

Enzymatic Studies with Synthetic Acceptors Can Complement Conventional Lectin and Antibody Studies. A differential expression of mRNAs for α2,3- and α2,6-sialyltransferases has been noted in rat and human tissues (*60*, *61*). However, the expression of mRNA does not always correlate with actual enzyme levels, which in turn cannot always predict glycosylation. Martin et al. (*62*) examined the terminal glycans in tissues of normal mice and mice deficient in ST6Gal I or ST3Gal I by using plant lectins as histochemical probes. They found cell type specific expression of different linkages of terminal sialic acids in a variety of mouse tissues and identified multiple changes in cell type specific glycan structures in various tissues of deficient mice. Our study was able to provide valuable information about the intricate substrate specificities of sialyltransferases. Such information will be helpful for meaningful interpretation of the histology data obtained using lectin and antibody probes on the tissues of normal and knockout mice. Studies of glycosyltransferase activity using an array of carbohydrate acceptors can also complement genomic and proteomic data that are becoming available from various high-throughput assays.

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SUPPORTING INFORMATION AVAILABLE

Mass spectra of oligosaccharides in Table 6. This material is available free of charge via the Internet at http://pubs.acs.org.

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