Reversible Sialylation: Synthesis of Cytidine 5′-Monophospho-*N*-acetylneuraminic Acid from Cytidine 5′-Monophosphate with α2,3-Sialyl O-Glycan-, Glycolipid-, and Macromolecule-Based Donors Yields Diverse Sialylated Products†

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ABSTRACT: Sialyltransferases transfer sialic acid from cytidine 5′-monophospho-*N*-acetylneuraminic acid (CMP-NeuAc) to an acceptor molecule. Trans-sialidases of parasites transfer α2,3-linked sialic acid from one molecule to another without the involvement of CMP-NeuAc. Here we report another type of sialylation, termed reverse sialylation, catalyzed by mammalian sialyltransferase ST3Gal-II. This enzyme synthesizes CMP-NeuAc by transferring NeuAc from the NeuAcα2,3Gal*β*1,3GalNAcα unit of O-glycans, 3-sialyl globo unit of glycolipids, and sialylated macromolecules to 5′-CMP. CMP-NeuAc produced in situ is utilized by the same enzyme to sialylate other O-glycans and by other sialyltransferases such as ST6Gal-I and ST6GalNAc-I, forming α2,6-sialylated compounds. ST3Gal-II also catalyzed the conversion of 5′-uridine monophosphate (UMP) to UMP-NeuAc, which was found to be an inactive sialyl donor. Reverse sialylation proceeded without the need for free sialic acid, divalent metal ions, or energy. Direct sialylation with CMP-NeuAc as well as the formation of CMP-NeuAc from 5′-CMP had a wide optimum range (pH 5.2−7.2 and 4.8−6.4, respectively), whereas the entire reaction comprising in situ production of CMP-NeuAc and sialylation of acceptor had a sharp optimum at pH 5.6 (activity level 50% at pH 5.2 and 6.8, 25% at pH 4.8 and 7.2). Several properties distinguish forward/conventional versus reverse sialylation: (i) sodium citrate inhibited forward sialylation but not reverse sialylation; (ii) 5′-CDP, a potent forward sialyltransferase inhibitor, did not inhibit the conversion of 5′-CMP to CMP-NeuAc; and (iii) the mucin core 2 compound 3-*O*-sulfoGal*β*1,4GlcNAc*β*1,6(Gal*β*1,3)GalNAcα-*O*-benzyl, an efficient acceptor for ST3Gal-II, inhibited the conversion of 5′-CMP to CMP-NeuAc. A significant level of reverse sialylation activity is noted in human prostate cancer cell lines LNCaP and PC3. Overall, the study demonstrates that the sialyltransferase reaction is readily reversible in the case of ST3Gal-II and can be exploited for the enzymatic synthesis of diverse sialyl products.

The sialylation of carbohydrates is catalyzed by sialyltransferases (sialylTs)[1] and trans-sialidases. During the biosynthesis of glycoconjugates, enzymes belonging to the glycosyltransferase family (including sialylT) catalyze the transfer of a monosaccharide unit from an activated glycosyl donor to an appropriate acceptor molecule. In the case of sialic acid (NeuAc), the activated donor consists of NeuAc that is *β*-glycosidically linked to the aglycan cytidine 5′-monophosphate (CMP) to form CMP-NeuAc (*1*). In contrast to other activated nucleotide sugars, CMP-NeuAc contains a monophosphate. This activated sugar is also unique since it exclusively forms α-glycosidically linked sialic acid residues in nature (*2*).

Besides sialylTs, trans-sialidases (TS) exist in parasites and these acquire sialic acids from the host for incorporation into the parasite's surface (*3*). For example, *Trypanosoma cruzi*, an agent of Chagas' disease, is unable to synthesize sialic acid de novo (*4*). This parasite employs TS to transfer terminal α2,3-linked sialic acid residues to terminal *β*-linked Gal in glycoproteins and glycolipids (*5*, *6*). In the absence of a suitable acceptor, TS transfers sialic acid to water molecules, thus functioning as a sialidase similar to viral, mammalian, and bacterial sialidases (*7*). Some members of the TS family lack enzyme activity but still retain their Gal-binding properties. Such molecules function as lectins during parasite−host interaction (*8*). Recently, a multifunctional sialyltransferase has also been identified from *Pasteurella multocida* that exhibits multiple functions including α2,3- and α2,6-sialylation and trans-sialidase activity (*9*).

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[1] Abbreviations: sialylT, sialyltransferases; TS, trans-sialidases; AA-CP, acrylamide copolymer; Bn, benzyl; Me, methyl; BSM, bovine submaxillary mucin; CDP, cytidine diphosphate; CGM, porcine Cowper's gland mucin; CMP, cytidine monophosphate; FOG, fetuin *O*-glycosidic glycopeptide; FTG, fetuin triantennary glycopeptide; Fuc, fucose; Gal, galactose; GalNAc, *N*-acetylgalactosamine; GlcNAc, *N*-acetylglucosamine; LC/MS/MS, liquid chromatography coupled with tandem mass spectrometry; NeuAc, sialic acid; PNA, peanut agglutinin; SNA, sambucus nigra agglutinin; Sulfo, sulfate ester; T-hapten, Gal*β*1,-3GalNAcα; UDP, uridine diphosphate; UMP, uridine monophosphate; WGA, wheat germ agglutinin.

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Besides sialylT and TS activity, in this paper we report yet another action of sialylTs. This activity, which we term "reversible sialylation", involves the enzymatic transfer of NeuAc by mammalian sialylT ST3Gal-II, from linear carbohydrate, branched core-2 mucin and fetuin glycoprotein-based sialylated donors to acceptor CMP. This results in the formation of CMP-NeuAc. This newly synthesized CMP-NeuAc is then available for transfer to another acceptor by the same sialylT (ST3Gal-II). It is also available to other sialylTs like ST6Gal-I and ST6GalNAc-I for sialylation of other macromolecules. Thus, via reversible sialylation, diverse chemical entities can be readily synthesized from an array of sialyl donors without the need for CMP-NeuAc.

Indeed, reversible biochemical reactions are known to occur in metabolic pathways. For example, the hydrolysis of maltose by maltase is reversible provided high glucose concentrations (40% w/v) are present in the reaction mixture (*10*). Plant sucrose synthase can also reversibly catalyze the synthesis of UDP-Glc from sucrose and UDP (*11*, *12*). Recently, Zhang et al. (*13*) also showed that four different glycosyltransferases that participate in natural product biosynthetic pathways related to calicheamicin and vancomycin readily catalyze reversible reactions allowing sugars and aglycons to be exchanged with ease. Our studies now show that sialylation is also a reversible process. Of the three enzymes studied (ST3Gal-II, ST3Gal-III, and ST6Gal-I), reversible sialylation was observed to occur readily in the case of ST3Gal-II. Forward and reverse sialylation could also be independently regulated by citrate ions, 5′-CDP, and selected synthetic molecules, and this suggests that two distinct catalytic activities of the enzyme may regulate the forward and reverse sialylation reactions. Overall, our data support the concept that reverse sialylT activity can be exploited for the chemical synthesis of diverse sialylated glycoconjugates. The physiological significance of this novel enzymatic activity remains to be established.

MATERIALS AND METHODS

Materials. Rat recombinant ST3Gal-II [α2,3(O)ST], ST3Gal-III [α2,3(N)ST], and ST6Gal-I [α2,6(N)ST] were purchased from Calbiochem (*14*). Several different lots of ST3Gal-II have been used for this study and all yielded similar findings. Cloned ST6GalNAc-I (chicken) was kindly provided by Dr. James C. Paulson (Scripps Research Institute, La Jolla, CA). All nucleoside phosphates and CMP-NeuAc were from Sigma. The synthesis strategy of most acceptors/compounds used in this study is described elsewhere (*15*, *16*). Acrylamide copolymer of Galβ1,3GalNAcα-*O*-allyl, asialo-Cowper's gland mucin (CGM), antifreeze glycoproteins, fetuin-*O*-glycosidic asialoglycopeptide (asialo-FOG), and fetuin triantennary asialoglycopeptide (asialo-FTG) were available from earlier studies (*17*−*19*). The culturing of human cancer cell lines and the preparation of cell extracts is reported elsewhere (*20*). All cell extracts were frozen at −20 °C prior to use. Asialo bovine submaxillary mucin (asialo-BSM) was made by heating BSM (Sigma) (5 mg/mL) at 80 °C in 0.1 N HCl for 1 h, neutralizing with 1.0 N NaOH, dialyzing against distilled deionized water in the cold room for 24 h with four changes of water, and then lyophilizing the product.

Enzymology Studies. All enzymatic sialylation reactions were typically carried out in 100 mM sodium cacodylate buffer, pH 6.0, in the presence of enzyme, synthetic acceptor (at 7.5 mM or as indicated in each experiment) and 0.2 μCi of CMP-[9-^{3}H]NeuAc (NEN−Dupont, 29 mCi/μmol). The concentration of total CMP-NeuAc was adjusted in individual reactions by supplementing with additional cold CMP-NeuAc. Reaction volume was 20 μL. Products formed were separated by four different chromatographic procedures (below). In all cases, the radioactive content of isolated products was determined by using 3a70 scintillation cocktail (Research Products International, Mount Prospect, IL), and a Beckman LS6500 scintillation counter.

(a) Bio-Gel P2 Chromatography. A Bio-Gel P2 column (Fine Mesh; 1.0 × 116.0 cm) was used with 0.1 M pyridine acetate (pH 5.4) as the eluent at room temperature. In cases where radiolabeled donor compounds were prepared by use of this column, the peak fraction containing radioactivity was collected, lyophilized to dryness, dissolved in a small volume of water, and stored frozen at −20 °C for further experimentation.

(b) Lectin−Agarose Affinity Chromatography. A column of 5 mL bed volume of WGA−agarose or PNA−agarose (Vector Lab, Burlingame, CA) was employed with 10 mM *N*-(2-hydroxyethyl)piperazine-*N*′-ethanesulfonic acid (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 of 1.0 mL were collected. The bound material was then eluted with 0.5M GlcNAc or 0.2M Gal in the same buffer. SNA−agarose (Vector Lab) affinity chromatography was also carried out as above except that fractions of 2.0 mL were collected and the bound material was eluted with 0.5 M lactose.

(c) Hydrophobic Chromatography. This was done with a Sep-Pak C18 cartridge (Waters, Milford, MA) and the product was eluted with 3.0 mL of methanol.

(d) Dowex-1−Formate Column. Radioactive products from neutral allyl and methyl glycosides were measured by fractionation on Dowex-1−formate (Bio-Rad:AG-1 × 8; 200−400 mesh; formate form) as described previously (*14*).

Calculation of Equilibrium Constant K_{eq}. Equilibrium constant was calculated for selected reversible bimolecular reactions that are denoted by

$$[9\text{-}^3\text{H}]\text{D}^* + \text{A} \overset{K_{eq}}{\Leftrightarrow} \text{D}' + [9\text{-}^3\text{H}]\text{A}^* \qquad (1)$$

Here [9-^{3}H]D* denotes the radiolabeled donor and D′ is the donor after removal of sialic acid. Similarly, A and [9-^{3}H]-A* denote the acceptor before and after incorporation of NeuAc. In each run, unreacted [9-^{3}H]D* and product [9-^{3}H]-A* are subjected to radioactivity measurements. The equilibrium constant ($K_{eq} = k_{reverse}/k_{forward}$, dimensionless units) are then determined from the following equation, where terms in square brackets denote concentrations:

$$K_{eq} = \frac{[\text{D}^*][\text{A}]}{[\text{D}'][\text{A}^*]} = \frac{[\text{D}^*]}{([\text{D}_0] - [\text{D}^*])} \frac{([\text{A}_0] - [\text{A}^*])}{[\text{A}^*]} \qquad (2)$$

$[D_0]$ and $[A_0]$ are initial donor and acceptor concentrations. A plot of $[D^*]/([D_0] - [D^*])$ versus $[A^*]/([A_0] - [A^*])$ yields K_{eq} from slope data. $K_{eq} < 1$ implies that the forward reaction is favored and the reaction proceeds to the right side to form [9-^{3}H]A* efficiently.

Liquid Chromatography Coupled with Tandem Mass Spectrometry. The LC separation was performed on a C18

reverse-phase column at a flow rate of 220 μL/min. Two buffers were used, 0.1% formic acid in acetonitrile and 0.1% formic acid in water, with a linear gradient of 8%/min increase of the organic buffer starting from 20%. The sample injection volume was 20 μL. Negative-ion electrospray ionization (ESI) was used for the detection of sialic acid derivatives due to its greater sensitivity than positive-ion ESI (*14*). The identification was accomplished in precursor ion scan mode at unit resolution [full width at half-maximum (fwhm) 0.6−0.8 amu] by selectively detecting the parent ions in the third quadrupole (Q3) of a triple quadrupole instrument that give rise to the diagnostic fragment ion (sialic acid ion $[M - H]^-$ at *m*/*z* 290) created by collisions in Q2.

RESULTS

ST3Gal-II Reverse-Sialylates 5′-CMP. The cloned and purified rat sialyltransferase α2,3(O)ST (ST3Gal-II) (*14*) is used in many experiments presented in this paper. This enzyme has been shown to mediate α2,3-sialylation of terminal Gal residues in the O-glycan core-2 trisaccharide unit Galβ1,3(GlcNAcβ1,6)GalNAcα (*14*, *21*). We examined whether this sialylation reaction is reversible (Figure 1). For this, [9-^{3}H]NeuAcα2,3Galβ,13(GlcNAcβ1,6)GalNAcα-O−allyl, [9-^{3}H]-**1**, was prepared by use of ST3Gal-II in the presence of CMP-[9-^{3}H]NeuAc and the trisaccharide acceptor Galβ1,3(GlcNAcβ1,6)GalNAcα-*O*-allyl as previously described (*14*), and the radiolabeled product was isolated by Bio-Gel P2 chromatography. During this isolation, radiolabeled [9-^{3}H]-**1** appeared prior to unreacted trisaccharide (data not shown). Two different reaction mixtures were then prepared: (i) [9-^{3}H]-**1** and ST3Gal-II but without 5′-CMP and (ii) [9-^{3}H]-**1** and 5′-CMP along with ST3Gal-II. When the products formed were subjected to WGA−agarose affinity chromatography, a majority of the radioactive component from mixture i but not mixture ii bound the column (Figure 1). The results of the first run indicate that the enzyme ST3Gal-II does not exhibit any sialidase activity. Furthermore, the efficient (>90%) transfer of radioactive [9-^{3}H]NeuAc from 150 μM [9-^{3}H]-**1** to 1.0 mM 5′-CMP in the presence of ST3Gal-II in the second panel suggests that the reverse reaction in Scheme I proceeds at an appreciable rate.

Similar results were observed with increasing concentrations of 5′-CMP when [9-^{3}H]NeuAcα2,3Galβ1,3(4-F-GlcNAcβ1,6)GalNAcα-*O*-Bn **[**9-^{3}H]-**2** was donor (Figure 2a,b). The concentration of 5′-CMP that mediated half-maximal transfer of [9-^{3}H]NeuAc was 80 μM. The bimolecular equilibrium constant (K_{eq}) for this reaction was 0.35 (dimensionless units, Table 1). Indeed, the K_{eq} varied depending on the donor and it was 98.9 when the donor was based on the globo glycolipid [9-^{3}H]NeuAcα2,3Galβ1,-3GalNAcβ1,3Galα-*O*-Me ([9-^{3}H]-**3)** (Figure 2c,d). When [9-^{3}H]-**2** and [9-^{3}H]-**3** are compared, it is apparent that reverse sialylation proceeds more effectively in the case of [9-^{3}H]-**2**. To further confirm the above estimates of K_{eq} for core-2-based structures, we also calculated this parameter for our previously published data [Figure 3C (*14*)] where [9-^{3}H]CMP-NeuAc was the donor and mucin core-2 tetrasaccharide Galβ1,4GlcNAcβ1,6(Galβ1,3)GalNAcα-*O*-Bn was the acceptor. In this case, K_{eq} measured with respect to

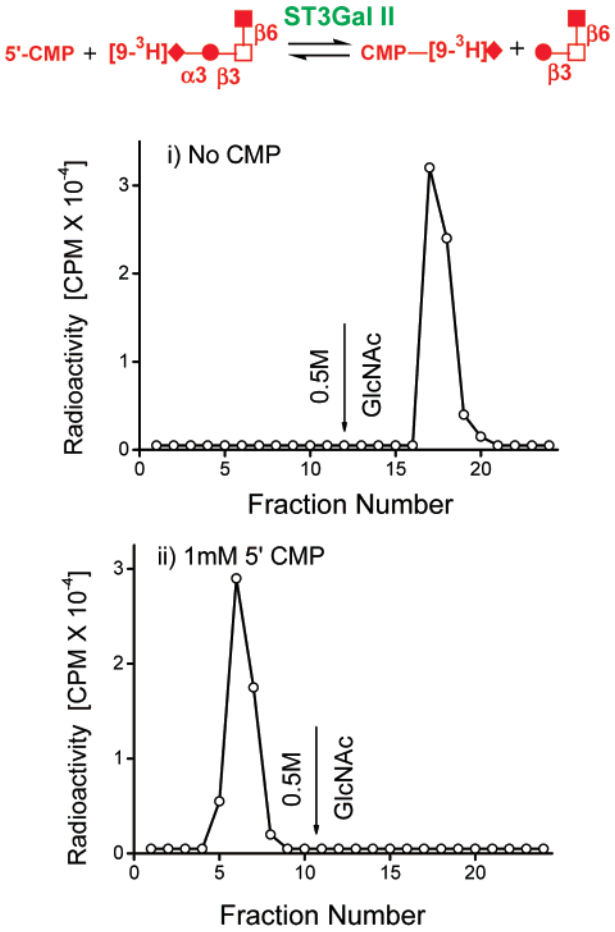


FIGURE 1: Nucleotide phosphates as acceptors of radioactivity from [9-^{3}H]NeuAcα2,3Galβ1,3(GlcNAcβ1,6)GalNAcα-*O*-allyl ([9-^{3}H]-**1)** in the presence of ST3Gal-II (Scheme I). Reaction mixtures (RM) with the following composition were incubated at 37 °C for 21 h in 100 mM sodium cacodylate buffer, pH 6.0: (i) RM containing 150 μM (0.4 μCi) [9-^{3}H]-**1** (donor) along with 0.8 milliunit of ST3Gal-II but no 5′-CMP; (ii) RM containing [9-^{3}H]-**1** and 1.0 mM 5′-CMP, along with ST3Gal-II. Products formed were fractionated on a WGA−agarose column. Bound products were released by 0.5 M GlcNAc at fraction 12. The radioactive product formed in reaction ii does not bind WGA. Thus, the forward reaction of Scheme I occurs at an appreciable rate. The feasibility of the reverse reaction is well established in literature. (◆) Sialic acid; (●) Gal; (□) GalNAc; (■) GlcNAc.

the rate of mucin core-2 sialylation was 5.55 (data not shown), which translates to a reverse sialylation K_{eq} of 0.18. Table 1 provides a summary of K_{eq} values for reverse sialylation.

When other nucleotide phosphates were substituted for 5′-CMP, their effectiveness varied. 5′-UMP, 5′-CDP, and 2′-deoxy-5′-CMP were 55.9%, 28.4% and 26.3% effective in comparison with 5′-CMP. Dosage studies further confirm the formation of UMP-NeuAc via the reverse sialylation mechanism (Supporting Information Figure 1). In these studies where [9-^{3}H]-**3** was donor and 5′-CMP or 5′-UMP was acceptor, CMP-[9-^{3}H]NeuAc was observed to form more efficiently than UMP-[9-^{3}H]NeuAc. Other nucleotide phosphates (3′-CMP, 5′-CTP, 5′-GMP, 5′-AMP, 5′-TMP, and 5′-IMP) had lower activity (<3.0%). 5′-CMP in the presence of cold sialic acid did not show any decrease in accepting [9-^{3}H]NeuAc from donor (100.3%), indicating that reverse sialylation did not involve the formation of free sialic acid as intermediate. Such free sialic acid could be formed

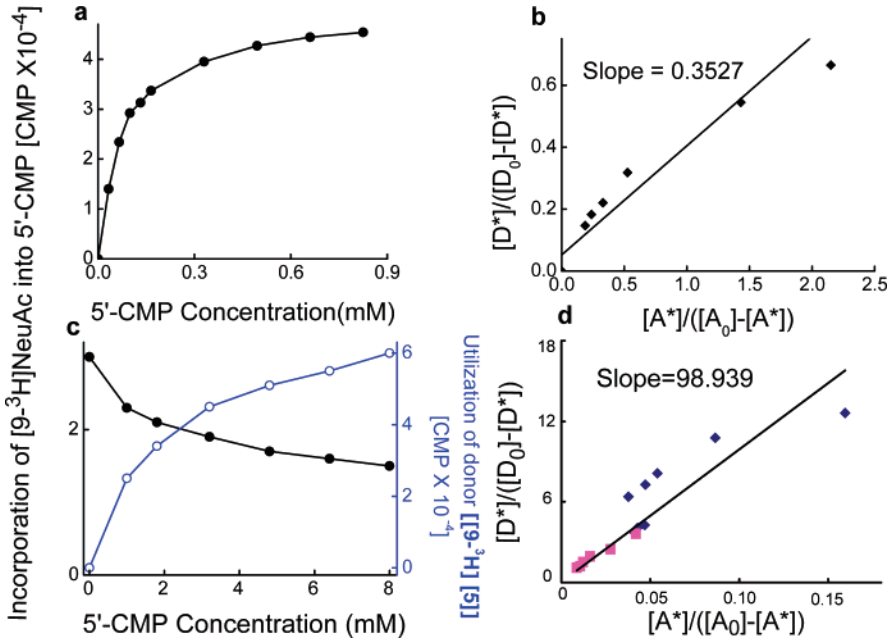


FIGURE 2: Equilibrium constant. (a) Reversible sialyltransferase activity at increasing concentrations of 5′-CMP. Donor [9-^{3}H]NeuAcα2,-3Galβ1,3(4-F-GlcNAcβ1,6)GalNAcα-*O*-Bn ([9-^{3}H]-**2**, 0.15 mM) was incubated with varying concentrations of 5′-CMP and 0.15 milliunit of ST3Gal-II under conditions described for Figure 1 for 2 h. Consumption of the [9-^{3}H]NeuAc benzyl glycoside donor was measured by subjecting the incubation mixture to Sep-Pak C18 fractionation, which binds the donor and not CMP-[9-^{3}H]NeuAc. The reaction proceeded to half the maximum extent at 80 μM 5′-CMP. (b) Equilibrium constant K_{eq} (= 0.35) calculated for data in panel a. (c) Varying concentrations of 5′-CMP were sialylated with ST3Gal-II (2.0 milliunits) by use of either 0.15 mM (panel c) or 1.5 mM (not shown) sialyl donor [9-^{3}H]-NeuAcα2,3Galβ1,3GalNAcβ1,3Galα-*O*-Me ([9-^{3}H]-**3**) for 4 h. The product CMP-[9-^{3}H]NeuAc and the unused [9-^{3}H]-**3** were separated and quantitated by the Dowex-1−formate method. (d) Equilibrium constant K_{eq} = 98.9. (◆) Data from 1.5 mM runs; (■) data from 0.15 mM runs.

Table 1: Reverse Sialylation Equilibrium Constant

donor	acceptor	K_{eq} (dimensionless units)
[9-^{3}H]NeuAcα2,3Galβ1,3(4-F-GlcNAcβ1,6)GalNAcα-*O*-Bn**,** [9-^{3}H]-**2**	5′-CMP	0.35
[9-^{3}H]NeuAcα2,3Galβ1,3GalNAcβ1,3Galα-*O*-Me, [9-^{3}H]-**3**	5′-CMP	98.9
[9-^{3}H]NeuAcα2,3Galβ1,3(Galβ1,4GlcNAcβ1,6)GalNAcα-*O*-Bn	5′-CMP	0.18

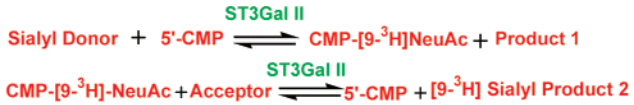
following hydrolysis of donor. Overall, the data support the reverse sialylation mechanism shown in Scheme I.

ST3Gal-II Utilizes CMP-NeuAc Formed in the Reverse Reaction To Sialylate O-Glycans. We tested the possibility that CMP-NeuAc formed above may be available for transfer to other acceptors via ST3Gal-II (Scheme II, Figure 3). For this, three radiolabeled donors were prepared by methods described above: two were based on the mucin core-2 structure ([9-^{3}H]-**1** and [9-^{3}H]NeuAcα2,3Galβ1,3(6-*O*-sulfoGlcNAcβ1,6)GalNAcα-*O*-allyl ([9-^{3}H]-**4**), while the third was based on the globo glycolipid ([9-^{3}H]-**3**). The transfer of [9-^{3}H]NeuAc from these sialylated donors to various T-hapten (Galβ1,3GalNAcα) and mucin core-2-based glycoside acceptors in the presence of 5′-CMP and ST3Gal-II was assessed (Table 2). All three donors of ST3Gal-II ([9-^{3}H]-sialyl-**1**, **-3,** and **-4**) allowed the formation of CMP-[9-^{3}H]NeuAc and a diverse array of products. In contrast to this, two other molecules ([9-^{3}H]NeuAcα2,3Galβ1,4GlcNAcβ-*O*-allyl and [9-^{3}H]NeuAcα2,6Galβ1,4GlcNAcβ-*O*-allyl), which were formed by sialylation of a poor acceptor of ST3Gal-II (Galβ1,4GlcNAcβ-*O*-allyl) by enzymes ST3Gal-III and ST6Gal-I, did not act as donors in the reverse sialylation reaction. Thus, while the reversible function of ST3Gal-II is not unique to a given donor−acceptor pair, the α2,3-sialic acid linkages on different donors have vastly different K_{eq} values (see Table 1).

The transfer of [9-^{3}H]NeuAc from [9-^{3}H]-**1** to D-Fucβ1,-3GalNAcα-*O*-Bn (**5**) in the presence of 5′-CMP and ST3Gal-II increased linearly in the first 2 h and reached saturation at 4 h (Supporting Information Figure 2), and thus the data in Table 3 (4 h time point) represent equilibrium conditions. In Table 3, **5** and D-Fucβ1,3(GlcNAcβ1,6)GalNAcα-*O*-Bn (**6**) were observed to serve as good acceptors followed by 4-F-Galβ1,3GalNAcα-*O*-Bn (**7**) and Galβ1,3(6-*O*-Me)GalNAcα-*O*-Bn (**8**), while 4-O-methylation of β1,3-linked Gal (**11**) reduced acceptor efficiency. As anticipated, Galβ1,-4GlcNAcβ-*O*-Bn (**12**) was an inactive acceptor since ST3Gal-II does not act on it. Since CMP-NeuAc is formed via the same first reaction of Scheme II for a given donor, the data in Table 3 suggest that acceptor specificity for ST3Gal-II may govern the extent of reaction. Further, diverse sialylated products can be formed by use of the same synthetic sialylated donor.

In order to compare the efficiency of individual reactions of Scheme II, independent runs were performed where either 0.15 mM [9-^{3}H]CMP-NeuAc, 0.15 mM [9-^{3}H]-**2,** or 0.15 mM [9-^{3}H]-**4** was the donor and **5** was the acceptor (Figure 3). 5′-CMP (2 mM) was added in runs with core-2 donors **2** and **4**, and thus reverse sialylation of these molecules was feasible. K_{eq} for transfer of sialic acid from CMP-NeuAc to **5** was 5.55 (Figure 3a). As seen in Figure 3b, 10-fold lower amounts of acceptor (0.6 mM **5**) was required for comparable

Scheme II



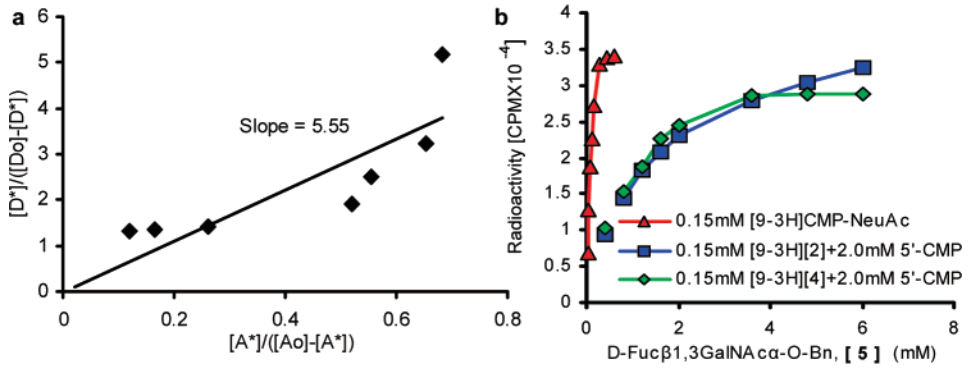


FIGURE 3: Serial transfer of sialic acid according to Scheme II was followed at increasing concentrations of acceptor D-Fucβ1,3GalNAcα-*O*-Bn (**5**). The donor used was either (i) 0.15 mM [9-^{3}H]CMP-NeuAc (▲), (ii) 0.15 mM [9-^{3}H]NeuAcα2,3Galβ1,3(4-F-GlcNAcβ1,6)-GalNAcα-*O*-Bn ([9-^{3}H]-**2**) in the presence of 2.0 mM 5′-CMP (■), or (iii) [9-^{3}H]NeuAcα2,3Galβ1,3(6-*O*-sulfoGlcNAcβ1,6)GalNAcα-*O*-allyl ([9-^{3}H]-**4)** in the presence of 2.0 mM 5′-CMP (◆). In runs where [9-^{3}H]-**2** was donor since both **5** and **2** bind C18 cartridges, control runs were performed in the absence of D-Fucβ1,3GalNAcα-*O*-Bn to estimate the amount of [9-^{3}H]CMP-NeuAc formed under the experimental conditions. In these runs, 80−85% of the radioactivity was transferred to CMP-NeuAc. The amount of CMP-NeuAc remaining in runs with **5** was then subtracted from CMP-NeuAc radioactivity in the control run to determine the amount of sialylated **5**. To complement this run, studies were also performed with [9-^{3}H]-**4**, a molecule with allyl at the anomeric position, which does not bind C18. In these runs, the amount of sialylated **5** was determined by measuring radioactivity retained in the C18-cartridge. (a) Equilibrium constant was 5.55 when CMP-[9-^{3}H]NeuAc was the donor. (b) An ∼10-fold lower acceptor concentration was required for similar extents of reaction when CMP-[9-^{3}H]NeuAc was the donor, compared to core-2-based donors **[2]** and **[4]**.

Table 2: Incorporation of [9-^{3}H]NeuAc from Various Donors[a]

acceptor (2.5 mM)	incorporation from donor,[b] % cpm		
	[9-^{3}H]-**1**[c]	[9-^{3}H]-**4**[d]	[9-^{3}H]-**3**[e]
D-Fucβ1,3GalNAcα-*O*-Bn, **5**	100.0	100.0	100.0
D-Fucβ1,3(GlcNAcβ1,6)GalNAcα-*O*-Bn, **6**	nd	96.9	106.5
4-F-Galβ1,3GalNAcα-*O*-Bn, **7**	60.8	33.3	4.1
Galβ1,3(6-*O*-Me)GalNAcα-*O*-Bn, **8**	43.0	21.5	3.3
3-*O*-MeGalβ1,4GlcNAcβ1,6(Galβ1,3)GalNAcα-*O*-Bn, **9**	22.4	10.0	nd
Galβ1,4GlcNAcβ1,6(Galβ1,3)GalNAcα-*O*-Bn, **10**	20.5	8.4	nd
4-*O*-MeGalβ1,3GalNAcα-*O*-Bn, **11**	8.9	2.7	0.2
Galβ1,4GlcNAcβ-*O*-Bn, **12**	nd	1.9	0.5
Galβ1,4GlcNAcβ1,6(4-*O*-MeGalβ1,3)GalNAcα-*O*-Bn, **13**	1.3	0	nd

[a] [9-^{3}H]-labeled donor (150 μM) was incubated with 1 mM 5′-CMP and 2.5 mM either T-hapten or mucin core-2-based acceptor in the presence of 0.2 milliunit of ST3Gal-II for 4 h under reaction conditions identical to those in Figure 1. Acceptors were separated from donor on a C-18 cartridge due to hydrophobicity of the benzyl (Bn) group. Radioactivity of the eluate was quantified. The blank containing no acceptor had cpm < 100. [b] Incorporation of [9-^{3}H]NeuAc is expressed as percent of cpm incorporated into **5**. nd, not determined. [c] [9-^{3}H]NeuAcα2,3Galβ1,3(GlcNAcβ1,6)GalNAcα-*O*-allyl; 100% = 34 569 cpm incorporated into **5**. [d] [9-^{3}H]NeuAcα2,3Galβ1,3(6-*O*-sulfoGlcNAcβ1,6)GalNAc-*O*-allyl; 100% = 97 675 cpm incorporated into **5**. [e] [9-^{3}H]NeuAcα2,3Galβ1,3GalNAcβ1,3Galα-*O*-M; 100% = 6224 cpm incorporated into **5**. The value for this donor is low because 10-fold cold CMP-NeuAc (i.e., lower specific radioactivity of CMP-[9-^{3}H]NeuAc) was used to synthesize it as compared to other two donors.

transfer when CMP-NeuAc was donor versus the case where the donor was either [9-^{3}H]-**2** (∼6 mM) or [9-^{3}H]-**4** (∼6 mM). Thus, while the two-step mechanism results in lower conversion than CMP-NeuAc alone, the transfer of NeuAc still takes place at an appreciable rate.

Reverse Sialylation Occurs More Readily with ST3Gal-II Compared to ST3Gal-III and ST6Gal-I. We determined whether reverse sialylation was more pronounced for ST3Gal-II in comparison to other sialyltrasferases. Thus two other cloned rat sialyltransferases, ST3Gal-III [or α2,3(N)ST] and ST6Gal-I [or α2,6(N)ST] (*14*), were examined. For these studies we generated two molecules: (i) [9-^{3}H]NeuAcα2,-6Galβ1,4GlcNAcβ-*O*-allyl **(14)** was made by reaction of Galβ1,4GlcNAcβ-*O*-allyl and CMP-[9-^{3}H]NeuAc in the presence of α2,6-sialyltransferase, ST6Gal-I. The radioactive product was separated on a Bio-Gel P2 column by a protocol similar to that described above. (ii) [9-^{3}H]NeuAcα2,3Galβ1,-4GlcNAcβ-*O*-allyl **[15]** was similarly produced by reacting Galβ1,4GlcNAcβ-*O*-allyl and CMP-[9-^{3}H]NeuAc in the presence of α2,3-sialyltransferase ST3Gal-III. We note that the acceptor used in the above runs, Galβ1,4GlcNAcβ-*O*-allyl, does not undergo significant sialylation in the presence of CMP-NeuAc and the sialyltransferase that is the focus of our paper, ST3Gal-II (*14*). However, it is efficiently sialylated by both ST3Gal-III and ST6Gal-I (*14*). The ability of these two radiolabeled molecules (**14** and **15**) to act as [9-^{3}H]-NeuAc donors was assayed by using reaction mixtures where these compounds were mixed with excess 5′-CMP, enzyme, and acceptors with benzyl aglycan group. C18 cartridges were then used to measure the extend of [9-^{3}H]NeuAc transferred to acceptor. The formation of CMP-NeuAc was negligible when either [9-^{3}H]NeuAcα2,6Galβ1,4GlcNAcβ-

Table 3: CMP-NeuAc but Not UMP-NeuAc Formed by Reverse Sialylation Is an Efficient Sialyl Donor for Forward Sialyltransferase Reaction[a]

activity assayed	acceptor	incorporation of [9-^{3}H]NeuAc into acceptor, cpm	
		5′-CMP → CMP-[9-^{3}H]NeuAc	5′-UMP → UMP-[9-^{3}H]NeuAc
(a) ST3Gal-II activity	D-Fucβ1,3GalNAcα-*O*-Bn	134 122	1243
(b) ST3Gal-III activity	4-*O*-MeGalβ1,4GlcNAcβ-*O*-Bn	59 531	3804
(c) ST6Gal-I activity	Galβ1,4GlcNAcβ-*O*-Bn	15 066	182

[a] Incubation mixtures run in duplicate contained the sialyl donor [9-^{3}H]-**3** (0.0625 mM), 8 mM 5′-CMP or 5′-UMP as indicated, and benzyl disaccharide glycoside acceptor (2.5 mM). Blank samples did not contain 5′-CMP or 5′-UMP. Additional contents: (a) 3.0 milliunits of ST3Gal-II; (b) 3.0 milliunits of ST3Gal-II and 3.0 milliunits of ST3Gal-III; (c) 3.0 milliunits of ST3Gal-II and 1.0 milliunit of ST6Gal-I. The reaction mixtures were incubated for 4 h at 37 °C and then processed by the Sep-Pak C18 method, which binds benzyl glycosides. The amount of [9-^{3}H]NeuAc transferred from donor to individual acceptors was obtained after subtraction of blank values (<400 cpm). The variation of duplicate values was less than 5% in all cases.

O-allyl or [9-^{3}H]NeuAcα2,3Galβ1,4GlcNAcβ-*O*-allyl was the donor in the presence of a series of acceptors and sialyltransferases ST6Gal-I and ST3Gal-III, respectively. Thus, reverse sialylation takes place more readily with ST3Gal-II. When these two radiolabeled donors were used in the presence of ST3Gal-II, also, we observed the formation of negligible amounts of product, suggesting that CMP-NeuAc was not formed via reverse sialylation from these two donors. While the possibility that ST3Gal-III and ST6Gal-I can mediate reverse sialylation under different conditions cannot be ruled out, the data do suggest that ST3Gal-II may have unique structural properties that confer the reverse sialylation activity.

pH Dependence of Reverse Sialylation. We examined whether the pH range in which ST3Gal-II catalyzes the formation of CMP-NeuAc from sialylated donor and the total reaction, that is, the incorporation of NeuAc from CMP-NeuAc produced in situ into acceptor, are distinct. First, in studies that measured the transfer of [9^{3}H]NeuAc from donor [9-^{3}H]-**2** to 5′-CMP, we observed optimum transfer in the pH range 4.8−6.4 (Supporting Information Figure 3a). In contrast, the transfer of [9-^{3}H]NeuAc from CMP-[9-^{3}H]-NeuAc to Galβ1,3GalNAcα-*O*-Bn (**16**) was maximal at pH 5.2−7.2 (Supporting Information Figure 3b). Even though the pH curves for direct sialylation with CMP-[9-^{3}H]NeuAc and for the formation of CMP-[9-^{3}H]NeuAc by reverse sialylation have a wide range (almost 2 pH units) and appear very much overlapping, it is really significant to note that when the entire reaction was measured by monitoring the transfer of [9-^{3}H]NeuAc to **5** from the donor [9-^{3}H]-**3** via 5′-CMP, a sharp optimum at pH 5.6 (Supporting Information Figure 3c) was observed. The possibility that reversible sialylation may have additional unique properties was explored as described below.

Distinct Effects of Citrate on Forward and Reverse Sialyltransferase Activities. In addition to p,H other distinctions were also observed between forward and reverse sialylation. In this regard, citrate ions tended to inhibit forward sialylation but not reverse sialylation activity. For these studies, the effect of citrate on the forward sialylation activity of ST3Gal-I, ST3Gal-II, ST3Gal-III, and ST6Gal-I was examined. Reverse sialylation activity of ST3Gal-II was also measured in terms of (i) the formation of CMP-NeuAc from 5′-CMP and (ii) the transfer of NeuAc from this newly synthesized CMP-NeuAc to another O-glycan. Citrate inhibited 48−98% of the direct sialylation activity of all the enzymes mentioned above. However, synthesis of CMP-[9-^{3}H]NeuAc from 5′-CMP, as well as the synthesis of [9-^{3}H]NeuAcα2,3-D-Fucβ1,3 GalNAcα-*O*-Bn from the newly formed CMP-[9-^{3}H]NeuAc through reverse sialylation by ST3Gal-II, was not inhibited at all by citrate. The effect of citrate at high concentration (40 mM) may be due to complexing with cytidine, but it is interesting to note that citrate was present in excess (twice the concentration) of 5′-CMP in the reverse sialylation incubation mixtures.

Effect of 5′-Nucleotides on Reverse Sialylation. The effect of 5′-nucleotides on forward sialylation (Figure 4a) and reverse sialylation (Figure 4b) were examined. As seen in Figure 4a, both 5′-CDP and 5′-CMP inhibited the forward sialylation activities of ST3Gal-II, ST3Gal-III, and ST6Gal-I with the sialyl donor CMP-[9-^{3}H]NeuAc and specific acceptors for the respective enzymes. 5′-CDP was a more potent inhibitor in all cases. In studies of reverse sialylation (Figure 4b), 5′-CDP did not inhibit the first step in the reverse sialylation reaction of ST3Gal-II, namely, the formation of CMP-[9-^{3}H]NeuAc from 5′-CMP. The overall formation of [9-^{3}H]NeuAcα2,3-D-Fucβ1,3GalNAcα-*O*-Bn by the two-step reverse sialylation process, however, was inhibited by 5′-CDP, and this is consistent with the finding that 5′-CDP inhibits forward sialylation in the second step. 5′-UMP and 2-deoxy-5′-CMP served as controls in these experiments since they did not alter the extent of product formation (Figure 4b). Overall, our studies show that 5′-CDP, an analogue of 5′-CMP, inhibits the forward sialylation reaction without altering the reverse reaction.

Effect of O-Glycans with Sialyl or Sulfo Substituents on Reverse Sialyltransferase activity. Another distinction between forward and reverse sialylation was observed in studies where the effect of three compounds, NeuAcα2,3Galβ1,4 GlcNAcβ1,6(Galβ1,3)GalNAcα-*O*-Bn (Figure 5a), 3-*O*-sulfoGalβ1,4GlcNAcβ1,6(Galβ1,3)GalNAcα-*O*-Bn (Figure 5b), and Galβ1,4GlcNAcβ1,6(3-*O*-sulfoGalβ1,3)GalNAcα-*O*-Bn (Figure 5c), on the sialylation reaction was measured. For these studies, [9-^{3}H]sialyl fetuin was prepared by the action of ST3Gal-II on fetuin in the presence of CMP-[9-^{3}H]NeuAc. Radioactivity incorporation into the O-glycan chain of the glycoprotein was confirmed by treating the labeled molecule with alkaline borohydride (1 M $NaBH_4$ in 0.1 M NaOH at 45 °C for 24 h) and detecting the released radioactivity. During these studies, [9-^{3}H]sialyl fetuin was incubated with 5′-CMP, one of the above-mentioned compounds, and sialyltransferase for 20 h, and the product formed was separated on a Bio-Gel P2 column. As seen in Figure 5, the radioactivity associated with fetuin can be resolved from that associated with CMP-NeuAc and the synthetic

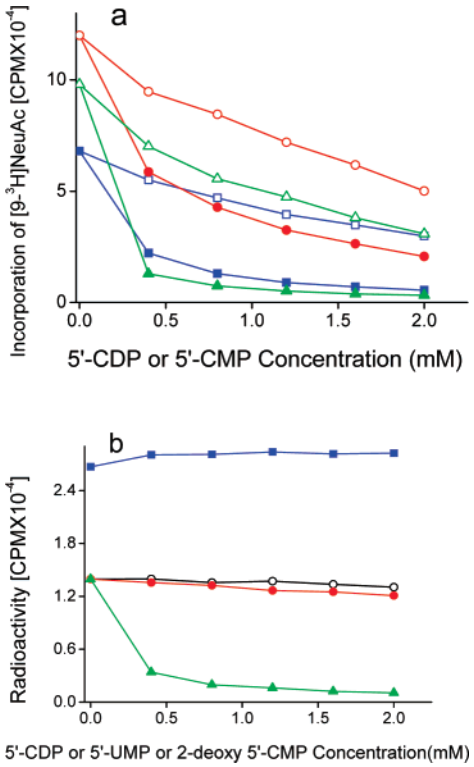


FIGURE 4: Effect of 5′-nucleotides on (a) direct sialyltransferase activity and (b) reverse sialylation. (a) Varying concentrations of 5′-CDP or 5′-CMP were added to 100 mM acceptor (specified below), 0.15 mM CMP-[9-^{3}H]NeuAc, and either ST3Gal-II (0.2 milliunit), ST3Gal-III (0.5 milliunit), or ST6Gal-I (0.2 milliunit) for 4 h at 37 °C. Products were separated by the Sep-Pak C18 method. ST3Gal-III activity was measured with acceptor 4-*O*-MeGalβ1,4GlcNAcβ-*O*-Bn in the presence of 5′-CDP (●) or 5′-CMP (○). ST6Gal-I activity was measured with Galβ1,4GlcNAcβ-*O*-Bn in the presence of 5′-CDP (▲) or 5′-CMP (△). ST3Gal-II was measured with D-Fucβ1,3GalNAcα-*O*-Bn (**5**) in the presence of 5′-CDP (■) or 5′-CMP (□). (b) Reverse sialylation by ST3Gal-II was measured in reaction mixtures containing 0.2 mM [9-^{3}H]-**3**, 7 mM 5′-CMP, ST3Gal-II (2 milliunits), D-Fucβ1,-3GalNAcα-*O*-Bn (3.0 mM), and varying doses of either 5′-CDP (▲), 5′-UMP (○), or 2-deoxy-5′-CMP (●) for 4 h at 37 °C. Reaction product ([9-^{3}H]NeuAcα2,3-D-Fucβ1,3GalNAcα-*O*-Bn) was isolated by the Sep-Pak C18 method. In some runs, where **5** was absent, the amount of CMP-NeuAc produced was quantified by the Dowex-1−formate method in the presence of varying doses of 5′-CDP (■).

acceptor. The radioactive fractions under the peaks corresponding to the product (the sialylated compound) and CMP-NeuAc were pooled separately, concentrated to dryness by lyophilization, and then identified by mass spectrometry as shown in Figure 5. Here, it is observed that 3-*O*-sulfoGalβ1,-4GlcNAcβ1,6(Galβ1,3)GalNAcα-*O*-Bn (Figure 5b) inhibits formation of the intermediate CMP-NeuAc. Consequuently no radioactivity was associated with the acceptor. NeuAcα2,-3Galβ1,4GlcNAcβ1,6(Galβ1,3)GalNAcα-*O*-Bn (Figure 5a), on the other hand, prevents product formation without inhibiting CMP-NeuAc formation. Thus the sulfated compound 3-*O*-sulfoGalβ1,4GlcNAcβ1,6(Galβ1,3)GalNAcα-*O*-Bn, in contrast to its sialylated analogue NeuAcα2,3Galβ1,-4GlcNAcβ1,6(Galβ1,3)GalNAcα-*O*-Bn, inhibits the formation of CMP-NeuAc from 5′-CMP. Finally, Galβ1,4GlcNAcβ1,6-(3-*O*-sulfoGalβ1,3)GalNAcα-*O*-Bn permits both the formation of CMP-NeuAc from 5′-CMP and the transfer of [9-^{3}H]NeuAc from the newly synthesized CMP-[9-^{3}H]NeuAc

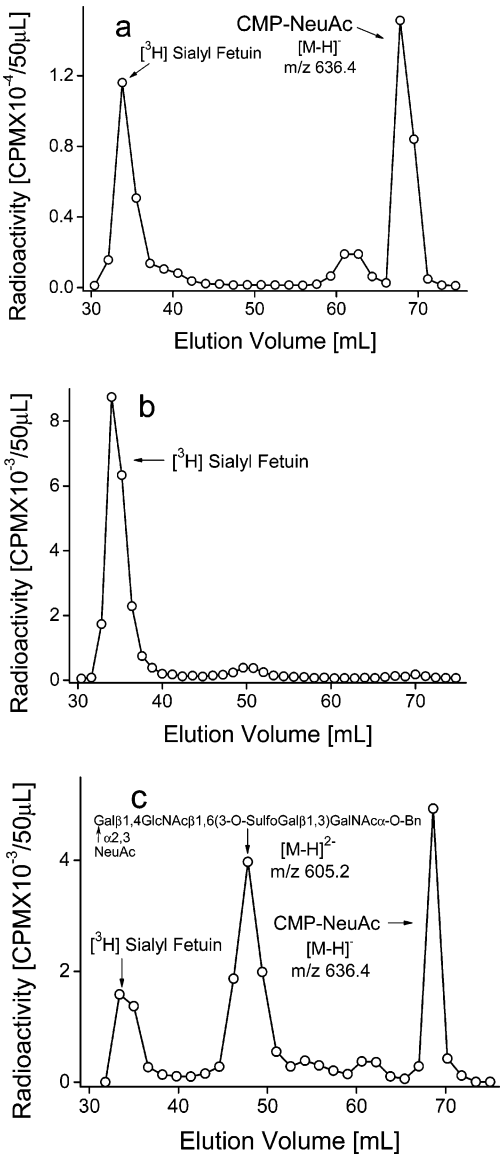


FIGURE 5: Effect of sialyl or sulfo substituents in the O-glycan chain on reverse sialylation by ST3Gal-II. Incubation mixtures (600 μL) contained [9-^{3}H]sialyl fetuin (5 mg), 200 mM sodium cacodylate, pH 6.0, 20 mM 5′-CMP, 50 mM ST3Gal-II, and the following: (a) 6.0 mM NeuAcα2,3Galβ1,4GlcNAcβ1,6(Galβ1,3)-GalNAcα-*O*-Bn, (b) 6.0 mM 3-*O*-sulfoGalβ1,4GlcNAcβ1,6-(Galβ1,3)GalNAcα-*O*-Bn, or (c) 6.0 mM Galβ1,4GlcNAcβ1,6(3-*O*-sulfoGalβ1,3)GalNAcα-*O*-Bn along with 50 milliunits of ST3Gal-III. Products were fractionated on a Bio-Gel P2 column after 20 h at 37 °C. Unused [9-^{3}H]sialyl fetuin (peak 1) appears prior to [9-^{3}H]-sialyl product from acceptor (peak 2) and CMP-[9-^{3}H]NeuAc (peak 3). Product identities were verified by LC-MS as indicated by molecular weights noted in the panels.

into Galβ1,4GlcNAcβ1,6(3-*O*-sulfoGalβ1,3)GalNAcα-*O*-Bn in the presence of ST3Gal-III (Figure 5c). The use of distinct compounds to differentially alter reverse and forward sialylation functions further supports the concept that ST3Gal-II may have more than one catalytic function.

Reverse Sialylation Activity of ST3Gal-II Allows Formation of Sialylated Globo Backbone Structure from Sialyl Fetuin. As shown in Figure 6a, [9-^{3}H]sialyl fetuin served as a good donor that allowed the formation of CMP-[9-^{3}H]-NeuAc by ST3Gal-II through the reverse sialylation mechanism. Figure 6b further shows that ST3Gal-II can synthesize NeuAcα2,3Galβ1,3GalNAcβ1,3Galα-*O*-Me from [9-^{3}H]sia-

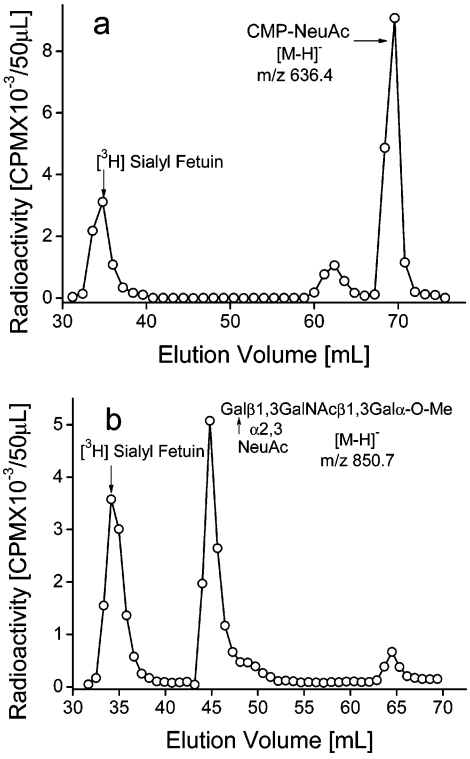


FIGURE 6: Sialylation of globo backbone structures via reverse sialylation by ST3Gal-II. Incubation mixtures (500 μL) contained [9-^{3}H]sialyl fetuin (5 mg), 200 mM sodium cacodylate, pH 6.0, 20 mM 5′-CMP, and the following: (a) 50 milliunits of ST3Gal-II or (b) 6.0 mM Galβ1,3GalNAcβ1,3Galα-*O*-Me and 50 milliunits of ST3Gal-II. Reaction products were isolated on a Bio-Gel P2 column after 20 h at 37 °C, and product identity was verified by mass spectrometry as indicated by the molecular weights in the panel. Peak I, unused [9-^{3}H]sialyl fetuin; peak II, [9-^{3}H]sialyl product from acceptor; peak III, CMP-[9-^{3}H]NeuAc from 5′-CMP.

lyl fetuin via the reaction in Scheme II. Thus sialyl fetuin may be a useful compound for the inexpensive, enzymatic synthesis of an array of α2,3-sialylated compounds including globo backbone-based analogues.

CMP-NeuAc Formed by Reverse Sialylation Serves as a Sialyl Donor for Macromolecules in the Presence of ST3Gal-II, ST6Gal-I, and ST6GalNAc-I. The ability of ST3Gal-II (see Figure 3, Scheme II) to mediate the formation of sialylated macromolecules was studied by use of CGM (porcine Cowper's gland mucin, Supporting Information Figure 4a), BSM (bovine submaxillary mucin, Supporting Information Figure 4b), antifreeze glycoprotein FTG (fetuin triantennary glycopeptide, Supporting Information Figure 4c), and FOG (fetuin O-glycosidic glycopeptides, Supporting Information Figure 6). In the case of CGM (Supporting Information Figure 4a), the acrylamide copolymer Galβ1,-3GalNAcα-*O*−allyl/AA-CP (a synthetic macromolecular acceptor) and asialo-CGM were incubated separately with donor ([9-^{3}H]-**1**) and ST3Gal-II in the presence of either 5′-CMP or 5′-UMP for 21 h at 37 °C, and then subjected to Bio-Gel P2 chromatography to separate [9-^{3}H]sialyl macromolecule from [9-^{3}H]sialyl donor and CMP- or UMP-[9-^{3}H]NeuAc. Both the acrylamide copolymer and asialo-CGM served as good acceptors in the presence of 5′-CMP, whereas lower amounts of sialylated macromolecules were formed in the presence of 5′-UMP (Supporting Information Figure 4a). UMP-sialic acid was a poor donor of sialic acid not only for ST3Gal-II but also for ST3Gal-III and ST6Gal-I (Table 3).

[9-^{3}H]Sialylated CGM could also act as donor to form CMP-[9-^{3}H]NeuAc in presence of 5′-CMP and ST3Gal-II (Supporting Information Figure 5). In this study, [9-^{3}H]-sialylated CGM was first synthesized from CGM and CMP-[9-^{3}H]NeuAc in the presence of ST3Gal-II. Subsequently, it was observed that the radiolabeled CGM could donate [9-^{3}H]NeuAc to 5′-CMP in the presence of ST3Gal-II to form new CMP-[9-^{3}H]NeuAc.

Similar to the case of CGM, BSM, asialo-BSM and antifreeze glycoprotein also gave rise to efficient formation of radiolabeled sialylated macromolecules by use of ST3Gal-II, [9-^{3}H]-**1**, and 5′-CMP.

α2,6[9-^{3}H]Sialylated FTG could be formed from 5′-CMP and asialo-FTG upon incubation with donor [9-^{3}H]-**2** and two enzymes, ST3Gal-II and ST6Gal-I simultaneously (Supporting Information Figure 4c). Here, reverse sialylation by use of ST3Gal-II and 5′-CMP resulted in the formation of CMP-NeuAc. The newly formed CMP-NeuAc was then utilized by ST6Gal-I to form α2,6-sialylated FTG as shown by its binding to SNA−agarose column.

Similar results were observed with FOG by PNA−agarose chromatography. In these studies, the formation of either [9-^{3}H]NeuAcα2,6(Galβ1,3)GalNAcα-*O*-Ser/Thr or [9-^{3}H]-NeuAcα2,3Galβ1,3GalNAcα-*O*-Ser/Thr units was detected upon incubation of asialo-FOG and CMP-[9-^{3}H]NeuAc with ST6GalNAc-I (Supporting Information Figure 6a) or ST3Gal-II (Supporting Information Figure 6b), respectively. Here, the α2,6-sialylated compound formed bound PNA−agarose. The incubation of asialo-FOG with 5′-CMP, the donor [9-^{3}H]-**2**, ST3Gal-II, and ST6GalNAc-I also gave rise to product that bound PNA−agarose (Supporting Information Figure 6c). This observation is consistent with the notion that CMP-NeuAc can be formed from 5′-CMP by reverse sialylation in the presence of ST3Gal-II. This new CMP-NeuAc can then be utilized by ST6GalNAc-I to form [9-^{3}H]-NeuAcα2,6(Galβ1,3)GalNAcα-*O*-Ser/Thr units. Finally it was observed that sialylated FOG itself could participate in reverse sialylation (Supporting Information Figure 6d). Sialylated FOG for these runs was first enzymatically prepared by reacting asialo-FOG with CMP-[9-^{3}H]NeuAc in the presence of ST3Gal-II. Subsequently, it was observed that this sialylated FOG could act as a sialic acid donor for D-Fucβ1,3GalNAcα-*O*-Bn in the presence of 5′-CMP and ST3Gal-II. Formation of NeuAcα2,3-D-Fucβ1,3GalNAcα-*O*-Bn in the above experiment was verified by mass spectrometry.

Reversible Sialyltransferase Activity in Human Cells. While the above experiments were performed with cloned rat enzymes, we examined whether human cells also exhibited this novel enzyme activity (Table 4). Thus, [9-^{3}H]-**4** was used as donor and D-Fucβ1,3GalNAcα-*O*-Bn (**5**) as acceptor in the presence of 5′-CMP and solubilized cell extracts. Of the cells tested, human prostate cancer cell lines LNCaP and PC-3 contained significant reversible sialyltransferase activity. These result shows that reverse sialylation occurs in human cells also.

DISCUSSION

This paper reports a novel enzymatic reaction mechanism, which we term reverse sialylation. This enzyme activity is

Table 4: Reversible Sialyltransferase Activity in Human Cancer Cell Lines[a]

cancer	cell lines	transfer of [9-^{3}H]NeuAc from [9-^{3}H]-**4** to **5**, cpm × 10^{-3}/mg of protein
breast	T47D	0.52
breast	ZR75-1	1.32
breast	MDA-MB231	0.09
breast	MDA-MB-435S	0.69
breast	MCF-7	0.57
colon	LS180	0.01
prostate	LNCaP	19.89
prostate	PC3	4.72
leukemia	HL60	0.76

[a] [9-^{3}H]NeuAcα2,3Galβ1,3(6-*O*-sulfoGlcNAcβ1,6)GalNAc-*O*-allyl ([9-^{3}H]-**4**, 0.15 mM) was added to 3.0 mM D-Fucβ1,3GalNAcα-*O*-Bn (**5**) in the presence of 1.0 mM 5′-CMP and 100 μL of Triton-X-solubilized cell extract for 16 h at 37 °C at pH 6.0. Total reaction volume was 180 μL. The product [9-^{3}H]NeuAcα2,3-D-Fucβ1,3GalNAcα-*O*-Bn was measured by Sep-Pak C18 separation followed by liquid scintillation counting. (When 5′-CMP was omitted in the incubation mixtures of LNCaP and PC3, there was no incorporation of radioactivity in D-Fucβ1,3/GalNAcα-*O*-Bn thus indicating the absence of trans-sialidase reaction.)

exhibited by rat ST3Gal-II and lysates of human prostate cancer cell lines LNCaP and PC3. This reaction catalyzes the formation of CMP-NeuAc according to Scheme I, in the presence of 5′-CMP and a range of donors including those based on mucin core-2, core-1, glycolipid, and macromolecule structures. The reaction mechanism is novel because the reversibility of sialyltransferase activity has not been reported in the literature. While previous studies have demonstrated that biochemical reactions can be reversible, this paper shows that in the case of ST3Gal-II the reaction rate is much more reversible than previously appreciated. The K_{eq} value is less than 1 when some donors are used, and this suggests that at least in some cases the formation of CMP-NeuAc in the case of this enzyme occurs more readily than the conventional, forward sialylation reaction.

Besides transfer of NeuAc to 5′-CMP from an array of donors, this enzyme activity also transfers sialic acid quite efficiently to 5′-UMP. The newly synthesized UMP-NeuAc, however, was a poor sialyl donor, and thus formation of UMP-NeuAc may be an efficient mechanism for depleting sialyl donors.

CMP-NeuAc formed by reverse sialylation above was available for transfer to a range of substrates by use of sialylTs that catalyzed both α2,3- and α2,6-linkage formation. The enzymes that could transfer NeuAc from CMP-NeuAc formed by reverse sialylation to other acceptors include ST3Gal-II, ST6Gal-I, and ST6GlcNAc-I. Together these observations suggest that the reversible sialylation function of ST3Gal-II can be exploited for the synthesis of sialylated glycoconjugates.

The requirements for formation of CMP-NeuAc by reverse sialylation activity were unlike that of conventional CMP-NeuAc synthetase function (*22*−*26*). CMP-NeuAc synthetase utilizes CTP and NeuAc as substrates in the presence of Mg^{2+} to produce CMP-NeuAc and pyrophosphate (*22*, *23*). In contrast, direct transfer of NeuAc to 5′-CMP proceeded without the need for free sialic acid, divalent metal ions, or energy from the breakdown of CTP to CMP and pyrophosphate.

Some of the studies performed in this paper address the possibilities (a) that the mechanism of reverse sialylation simply involves a reversal of forward sialylation and (b) that reverse and forward sialylation occur via different reaction coordinates. In this regard, we note that reverse and forward sialylation are optimal at different, albeit overlapping, pH ranges. While direct/forward sialylation by ST3Gal-II was optimal over a wide pH range from 5.2 to 7.2, the formation of CMP-NeuAc from 5′-CMP by reverse sialylation occurred between pH 4.8 and 6.4. The entire reversible sialylation reaction of ST3Gal-II utilizing CMP-NeuAc produced in situ for sialylation exhibited a sharp peak at pH 5.6. These data suggest that direct/forward sialylation and reverse sialylation by ST3Gal-II may be governed by two distinct catalytic mechanisms. In support of this proposition, we also found the following: (i) While strong inhibition of sialyltransferase activity occurs upon addition of sodium citrate, reverse sialylation (namely, the formation of CMP-NeuAc from 5′-CMP) was not inhibited by citrate. (ii) We also observed that while 5′-CDP was a potent inhibitor of direct/forward sialyltransferase activities, it did not affect the synthesis of CMP-NeuAc from 5′-CMP in the reverse sialylation reaction mediated by ST3Gal-II. (iii) While the mucin core-2 compound 3-*O*-sulfoGalβ1,4GlcNAcβ1,6(Galβ1,3)GalNAcα-*O*-Bn inhibited the conversion of 5′-CMP to CMP-NeuAc via the reverse sialylation mechanism, the corresponding α2,3-sialyl substituent NeuAcα2,3Galβ1,4GlcNAcβ1,6-(Galβ1,3)GalNAcα-*O*-Bn did not inhibit reverse sialylation. In previous studies (*14*), we showed that NeuAcα2,3Galβ1,-4GlcNAcβ1,6(Galβ1,3)GalNAcα-*O*-Bn was a poor acceptor for ST3Gal-II compared to 3-*O*-sulfoGalβ1,4GlcNAcβ1,6-(Galβ1,3)GalNAcα-*O*-Bn. Thus the latter acceptor can be sialylated by ST3Gal-II, but it inhibits the formation of CMP-NeuAc from 5′-CMP by ST3Gal-II. Taken together, the data suggest that forward and reverse sialylation may follow different reaction coordinates and that the enzyme ST3Gal-II may have more than one catalytic activity. Indeed, there is precedence in literature for sialyltransferases that exhibit multiple functions (*9*).

The primary amino acid sequence and protein tertiary structure that contribute to reverse sialylation remain to be determined. All mammalian sialyltransferases characterized to date have type II transmembrane topology and contain highly conserved motifs called sialyl motifs L (long), S (short), and VS (very short) (*26*−*28*). Sialyl motif L is characterized by a 45−60 amino acid region in the center of the protein and has been shown to be involved in the binding of a donor substrate, CMP-NeuAc (*29*). Sialyl motif S is located in the COOH-terminal region and consists of a 20−30 amino acid stretch. It has been shown to be involved in the binding of both the donor and acceptor substrates (*30*). Sialyl motif VS is also located in the COOH-terminal region and is thought to be involved in the catalytic process (*28*, *31*). From the fact that both sialyl motifs L and S participate in the binding of the donor substrate CMP-NeuAc, it seems that the two cysteine residues, which are important for the enzyme activity, bring the two sialyl motifs closer together by forming an intramolecular disulfide linkage and, thus, form the conformation required for binding of the donor substrate (*30*). A comparison of V and VS motifs between

sialyltransferases (*30*, *31*) reveals a substantial difference in the amino acid sequences of the corresponding motifs. This difference could be attributed to a unique conformational difference between ST3Gal-I and ST3Gal-II in the carboxy-terminal portion and thus may explain the unique catalytic ability of the cloned rat liver α2,3(O)ST (ST3Gal-II). It is suggested that subtle differences in the amino acid sequences in the active sites of sialyl motifs may regulate the unique substrate specificities of this family of enzymes, and they may regulate enzyme function in the presence of cofactors, products, and transition-state analogues (*30*−*32*). This difference may also account for the unique ability of ST3Gal-II, but not ST3Gal-III or ST6Gal-I, to mediate the reversible sialylation reaction. If it is proven that normal/forward and reverse sialylation are distinct processes, these structural features may also contribute to important differences in these activities.

Reverse sialylation provides opportunities for the development of a novel glycoconjugate synthetic scheme. For example, since ST3Gal-II readily exchanges sialic acid residues between CMP-NeuAc and the α2,3-sialyl T-hapten unit, such a reaction mechanism may provide a rapid scheme for the synthesis of NeuAc analogues. In such reactions, addition of CMP-NeuAc analogues to mucin glycoproteins containing α2,3-sialyl T-hapten units in the presence of ST3Gal-II can catalyze the formation of modified T-hapten units via the reverse sialylation mechanism. Indeed, a recent report has suggested that reverse glycosyltransferase activity can be exploited for the rapid synthesis of natural products (*13*). Further, it is noteworthy that if one considers the high cost of CMP-sialic acid and the commercially cheap and easily available fetuin (a glycoprotein containing sialylated O-glycan chains), the synthesis of CMP-sialic acid from the cheap precursor CMP and ST3Gal-II with the sialyl donor fetuin appears to be an alternative approach in the in situ production of CMP-sialic acid for an economical synthesis of sialyl oligosaccharides as demonstrated in this study.

The physiological relevance and biochemical features that contribute to reverse sialylation will be the subject of future investigations. It remains to be determined whether reverse sialylation is unique to ST3Gal-II and whether this feature is species-specific. Since sialyltransferases contain multiple domains for binding acceptors and donors and for catalytic activity (*26*−*31*), the exact primary amino acid sequence (*30*, *31*) and protein tertiary structure (*32*) that contribute to reverse sialylation need to be determined. Further, numerous studies have documented the existence of soluble glycosyltransferases in body fluids (*33*−*38*) and in the growth media of normal and transformed cells (*39*, *40*). Further, in light of the observation that both 5′-UMP and 5′-CMP are efficient acceptors of sialic acid but only CMP-NeuAc is a facile donor for subsequent sialylation, the possible existence of a control mechanism for the level of intra- and extracellular sialylation based on the concentration of 5′-UMP and 5′-CMP in the cellular Golgi and extracellular milieu needs to be explored.

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SUPPORTING INFORMATION AVAILABLE

Figures 1−6 as described in the text. This material is available free of charge via the Internet at http://pubs.acs.org.

REFERENCES

1. Haverkamp, J., Spoormaker, T., Dorland, L., Vliegenthart, J. F. G., and Schauer, R (1979) Determination of the β-anomeric configuration of cytidine 5′-monophospho-*N*-acetylneuraminic acid by carbon-13 NMR spectroscopy, *J. Am. Chem. Soc. 101*, 4851−4853.
2. Kolter, T. and Sandhoff, K. (1997) Sialic acids: why always α-linked, *Glycobiology 7*, vii−ix.
3. Engstler, M., Wirtz, E., and Cross, G. A. M. (1997) Generation of constitutive and inducible trans-sialylation dominant-negative phenotypes in *Trypanosoma brucei* and *Trypanosoma cruzi*, *Glycobiology 7*, 955−964.
4. Schauer, R, Reuter, G., Muhlpfordt, H., Andrade, A. F., and Pereira, M. E. (1983) The occurrence of *N*-acetyl- and *N*-glycolylneuraminic acid in *Trypanosoma cruzi*, *Hoppe-Seyler's Z. Physiol. Chem. 364*, 1053−1057.
5. Previato, J., Andrade, A., Pessolani, M., and Priviato, L. M. (1985) Incorporation of sialic acid into *Trypanosoma cruzi* Macromolecules: A proposal for a new metabolic route, *Mol. Biochem. Parasitol. 16*, 85−96.
6. Zingales, B., Carniol, C., de Lederkremer, R. M., and Colli, W. (1987) Direct sialic acid transfer from a protein donor to glycolipids of trypomastigote forms of *Trypanosoma cruzi*, *Mol. Biochem. Parasitol. 26*, 135−144.
7. Scudder, P., Doom, J. P., Chuenkova, M., Manger, I. D., and Pereira, M. E. A. (1993) Enzymatic characterization of beta-D-galactoside alpha 2,3-trans-sialidase from *Trypanosoma cruzi*, *J. Biol. Chem. 268*, 9886−9891.
8. Cremona, M. L., Campetella, O., Sanchez, D. O., and Frasch, A. C. C. (1999) Enzymically inactive members of the trans-sialidase family from *Trypanosoma cruzi* display beta-galactose binding activity, *Glycobiology 9*, 581−587.
9. Yu, H., Chokhawala, H., Karpel, R., Yu, H., Wu, B., Zhang, J., Zhang, Y., Jia, Q., and Chen, S. A. (2005) Multifunctional *Pasteurella multocida* Sialyltransferase: A Powerful Tool for the Synthesis of Sialoside Libraries, *J. Am. Chem. Soc. 127*, 17618−17619.
10. Hill, A. C. (1898) Reversible zymohydrolysis, *J. Chem. Soc. Trans. 73*, 634−658.
11. Ellings, L., Grothus, M., and Kula, M. (1993) Investigation of sucrose synthase from rice for the synthesis of various nucleotide sugars and saccharides, *Glycobiology 3*, 349−355.
12. Romer, U., Schrader, H., Gunther, N., Nettelstroth, N., Frommer, W. B., and Elling, L. (2004) Expression, purification and characterization of recombinant sucrose synthase 1 from *Solanum tuberosum* L. for carbohydrate engineering, *J. Biotechnol. 107*, 135−149.
13. Zhang, C., Griffith, B. R, Fu, Q., Albermann, C., Fu, X., Lee, I. K, Li, L., and Thorson, J. S. (2006) Exploiting the Reversibility of natural products: Glycosyltransferase-catalyzed Reactions, *Science 313*, 1291−4.
14. Chandrasekaran, E. V., Xue, J., Xia, J., Chawda, R., Piskorz C., Locke, R. D., Neelamegham, S., and Matta, K. L. (2005) Analysis of the Specificity of Sialytransferases toward Mucin Core 2, Globo, and Related Structures. Identification of the Sialylation Sequence and the Effects of Sulfate, Fucose, Methyl, and Fluoro Substiuents of the Carbohydrate Chain in the Biosynthesis of Selectin and Siglec Ligands, and Novel Sialylation by Cloned 2,3-(O)Sialyltransferase, *Biochemistry 44*, 15619−35.
15. Jain, R. K, Piskorz, C. F., Chandrasekaran, E. V., and Matta, K. L. (1998) Synthesis of Gal-β1-(1→4)-GlcNAc-β-(1→6)-[Gal-β-(1→3)]-GalNAc-α-Obn Oligosaccharides Bearing O-methyl or O-sulfo Groups at C-3 of the Gal Residue: Specific Acceptors for Gal:3-O-sulfotransferases, *Glycoconjugate J. 15*, 951−959.
16. Jain, R. K., Piskorz, C. F., Huang, B. G., Locke, R. D., Han, H. L., Koenig, A., Varki, A., and Matta, K. L. (1998) Inhibition of L- and P-selectin by a Rationally Synthesized Novel Core 2-like Branched Structure Containing GalNAc-Lewis^x and Neu5Acα2−3Galβ1−3GalNAc Sequences, *Glycobiology 8*, 707−717.

17. Chandrasekaran, E. V., Jain, K. R., Larsen, R. D., Wlasichuk, K., and Matta, K. L. (1995) Selectin ligands and tumor-associated carbohydrate structures: Specificities of α2,3-sialyltransferases in the assembly of 3′-sialyl-6-sulfo/sialyl Lewis a and x, 3′-sialyl-6′-sulfo Lewis x, and 3′-sialyl-6-sialyl/sulfo blood group T-hapten, *Biochemistry 34*, 2925−2936.
18. Chandrasekaran, E. V., Jain, R. K., Vig, R., and Matta, K. L. (1997) The Enzymatic Sulfation of Glycoprotein Carbohydrate Units: Blood Group T-hapten Specific and Two Other Distinct Gal:3-O-sulfotransferases as Evident from Specificities and Kinetics and the Influence of Sulfate and Fucose Residues Occurring in the Carbohydrate Chain and on C-3 Sulfation of Terminal Gal, *Glycobiology 7*, 753−768.
19. Chandrasekaran, E. V., Chawda, R., Locke, R. D., Piskorz, C. F., and Matta, K. L. (2002) Biosynthesis of the carbohydrate antigenic determinants, Globo H, blood group H, and Lewis b: a role for prostate cancer cell alpha1,2-L-fucosyltransferase, *Glycobiology 12*, 153−162.
20. Chandrasekaran, E. V., Chawda, R., Rhodes, J. M., Locke, R. D., Piskorz, C. F., and Matta, K. L. (2003) The binding characteristics and utilization of *Aleuria aurantia, Lens culinaris* and few other lectins in the elucidation of fucosyltransferase activities resembling cloned FT VI and apparently unique to colon cancer cells, *Carbohydr Res 338*, 887−901.
21. Tsuji, S., Datta, A. K., and Paulson, J. C. (1996) Letters to the Glyco-Forum: Systematic nomenclature for sialyltransferases, *Glycobiology 6*, v−vii.
22. Roseman, S. (1962) Enzymatic Synthesis of Cytidine 5′-monophospho-sialic Acids, *Proc. Natl. Acad. Sci. U.S.A. 48*, 437−441.
23. Warren, L., and Blacklow, R. S. (1962) The Biosynthesis of Cytidine 5′-Monophospho-*N*-acetylneuraminic Acid by an Enzyme from *Neisseria meningitidis*, *J. Biol. Chem. 237*, 3527−3534.
24. Kapitonov, D., and Yu, R. K. (1999) Conserved domains of glycosyltransferase, *Glycobiology 9*, 961−978.
25. Mosimann, S. C., Gilbert, M., Dombrowski, D., To, R., Wakarchuk, W., and Strynadka, N. C. (2001) Structure of a Sialic Acid-activating Synthetase, CMP-acylneuraminate Synthetase in the Presence and Absence of CDP, *J. Biol. Chem. 276*, 8190−8196.
26. Drickamer, K. (1993) Letters to the Glyco-Forum: A conserved disulphide bond in sialyltransferases, *Glycobiology 3*, 2−3.
27. Livingston, B. D., and Paulson, J. C. (1993) Polymerase chain reaction cloning of a developmentally regulated member of the sialyltransferase gene family, *J. Biol. Chem. 268*, 11504−11507.
28. Geremia, R. A., Harduin-Lepers, A., and Delannoy, P. (1997) Letters to the Glyco-Forum: Identification of two novel conserved amino acid residues in eukaryotic sialyltransferases: implications for their mechanism of action, *Glycobiology 7*, v−xi.
29. Datta, A. K., and Paulson, J. C. (1995) The Sialyltransferase "Sialylmotif" Participates in Binding the Donor Substrate CMP-NeuAc, *J. Biol. Chem. 270*, 1497−1500.
30. Datta, A. K., Sinha, A., and Paulson, J. C. (1998) Mutation of the Sialyltransferase S-sialylmotif Alters the Kinetics of the Donor and Acceptor Substrates, *J. Biol. Chem. 273*, 9608−9614.
31. Kitazume-Kawaguchi, S., Kabata, S., and Arita, M. (2001) Differential Biosynthesis of Polysialic or Disialic Acid Structure by ST8Sia II and ST8Sia IV, *J. Biol. Chem. 276*, 15696−15703.
32. Erlandsen, H., Abola, E. E., and Stevens, R. C. (2000) Combining structural genomics and enzymology: completing the picture in metabolic pathways and enzyme active sites, *Curr. Opin. Struct. Biol. 10*, 719−730.
33. *33.* DeboseBoyd, R. A., Nyame, A. K., Smith, D. F., and Cummings, R. D. (1996) α1,4-Fucosyltransferase Activity in Human Serum and Saliva, *Arch. Biochem. Biophys. 335*, 109−117.
34. Fujita-Yamaguchi, Y., and Yoshida, A. (1981) Purification and characterization of human serum galactosyltransferase (lactose synthetase A protein, *J. Biol. Chem. 256*, 2701−2706.
35. Lammers, G., and Jamieson, J. C. (1988) The role of a cathepsin D-like activity in the release of Gal beta 1−4GlcNAc alpha 2−6-sialyltransferase from rat liver Golgi, *Biochem. J. 256*, 623−631.
36. Paulson, J. C., Beranek, W. E., and Hill, R. L. (1997) Purification of a sialyltransferase from bovine colostrum by affinity chromatography on CDP-agarose, *J. Biol. Chem. 252*, 2356−2362.
37. Piller, F., and Cartron, J. P. (1983) UDP-GlcNAc:Gal beta 1−4Glc-(NAc) beta 1−3N- acetylglucosaminyltransferase. Identification and characterization in human serum, *J. Biol. Chem. 258*, 12293−12299.
38. Serafinicessi, F., Malagolini, N., and Dallolio, F. (1988) Characterization and partial purification of *β*-N-acetylgalactosaminyltransferase from urine of Sd(a+) individuals, *Arch. Biochem. Biophys. 266*, 573−582.
39. Klohs, W. D., Mastrangelo, R., and Weiser, M. M. (1981) Release of Glycosyltransferase and Glycosidase Activities from Normal and Transformed Cell Lines, *Cancer Res. 41*, 2611−2615.
40. Serafinicessi, F., Malagolini, N., Guerrine, S., and Turrini, I. (1995) A soluble form of Sd^a-*β*1,4 −N-acetylgalatosaminyl-transferase is released by differentiated humanecolon carcinoma CaCo-2 cells, *Glycoconjugate J. 12*, 773−779.

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