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†

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ABSTRACT: The sequence in the assembly of the functional unit of selectin ligands containing sulfate, sialic acid, and fucose and also tumor-associated *O*-glycan structures was studied by examining the specificities of α2,3-sialyltransferases (ST). The first enzyme, porcine liver ST, was 57, 37, and 79% active (K_m: 0.105, 0.420, and 0.200 mM), respectively, toward 6-sulfo, 6-sialyl, or 6-*O*-methyl derivatives of the Galβ1,3GalNAcα- unit; C-3 or C-6 substitution on Gal abolished sialylation. An acrylamide copolymer (MW ~ 40 000) containing ~40 T-haptens and asialo Cowper's gland mucin (MW ~ 200 000) containing ~48 T-haptens was -5-fold more active as an acceptor as compared to Galβ1, 3GalNAcα-O-Al on a molecular weight basis. The second enzyme, a cloned α-2,3-ST specific for lactose-based structure, was 70, 102, and 108% active (K_m: 0.500, 0.210, and 0.330 mM), respectively, toward 6-sialyl, 6-sulfo, or 6-*O*-methyl derivatives of the Galβ1,3GlcNAcβ- unit; C-3 and C-6 substitution on Gal abolished sialylation. Galβ1,4GlcNAcβ- and its 6-sulfo derivative were ~20% active; the Lewis a structure, Galβ1,3-(Fucα1,4)GlcNAcβ-, was not an acceptor. The acrylamide copolymers containing ~40 units of Galβ1,-3GlcNAcβ-, Galβ1,3(6-sulfo)GlcNAcβ-, or fetuin triantennary asialo or bovine IgG diantennary glycopeptides were respectively 5.9-, 5.4-, 0.7-, and 0.1-fold as active. A transfer of 7−9 mol of NeuAc per mole of the above copolymers was catalyzed by this ST, the sialyl linkage being susceptible to α2,3-specific sialidase. A partially purified Colo 205 Lewis type (α1, 3/4) fucosyltransferase catalyzed the formation of 3′-sialyl-6-sulfo Lewis a from [9-^{3}H]NeuAcα2, 3Galβ1, 3(6-sulfo)GlcNAcβ-O-Allyl and copolymer containing [9-^{3}H]NeuAcα2, 3Galβ1, 3(6-sulfo)GlcNAcβ- units, using GDP[^{14}C]Fuc as fucosyl donor. The third enzyme, HL-60 ST, was 103% active with Galβ1,3(6-sulfo)GalNAcα- but was only 8% active with 6-sialo compound; it showed 11.6-fold greater activity with the copolymer of T-hapten. Further, we observed the α2,3 sialylation of Galβ1,4GlcNAcβ- but not Galβ1,3GlcNAcβ- by HL60-ST, consistent with the occurrence of 3′-sialyl LacNAc and 3′-sialyl Lewis x units in leukosialin of HL60. The present results indicating that C-6 sulfation or sialylation of Galβ1,3GlcNAcβ- could precede C-3′ sialylation, which is followed by C-4 fucosylation in the biosynthetic pathway, are of importance, considering the occurrence of the 6-sulfoglucosamine moiety as a major constiuent of GLYCAM-I and human immunodeficiency virus envelope glycoproteins. In addition, since C-6′ sulfation in Galβ1,-3GlcNAcβ- or Galβ1,3GalNAcα- abolishes the enzymatic C-3′ sialylation, it is imperative that 3′-sialylation must precede 6′-sulfation in the assembly of the major capping group of GLYCAM-I.

Cell surface sialic acid occurs in a variety of structures, which change in a regulated manner during development, differentiation, and oncogenic transformation (Kimber, 1989; Roos, 1984). Sialyl Lewis x present at the termini of polylactosaminoglycans in granulocytes and monocytes (Spooncer et al., 1984; Fukuda et al., 1984) was found to serve as a ligand for selectins, the adhesive molecules of endothelial cells and platelets (Lowe et al., 1990; Philips et al., 1990; Walz et al., 1990; Larsen et al., 1990). The core structure and sialylation of O-linked oligosaccharides of leukosialin occurring in the myeloid-type HL-60 cells were shown to be disinct from those of the erythroid-type K562 cells (Carlsson et al., 1986; Fukuda et al., 1986; Hanisch et al., 1989); a polylactosamine chain linked β1, 6 to *N*-acetylgalactosamine and bearing sialyl Lewis x structure was found on the cell surface of HL-60 (Maemura & Fukuda, 1992). It is evident from several reports that the levels of sialyl Lewis x and sialyl Lewis a were increased in tumor cells and carcinomas (Fukushima et al., 1984; Fukushi et al., 1984; Magnani et al., 1982). Highly metastatic carcinoma cells expressed more sialyl Lewis x structures at the termini of polylactosaminyl side chains than poorly metastatic carcinoma cells, thus indicating a correlation between increased sialylation and metastatic behavior of carcinoma cells (Saitoh et al., 1992). The level of Galβ1,3GalNAc-R: α2,3-sialyltransferase activity of granulocytes was observed to increase 3-fold in chronic myelogenous leukemia (Baker et al., 1987); this increase probably results in the accelerated termination of β1,3 branches and hence the availability of

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sugar donors and glycosyltransferases solely for the elongation of β1,6 branches containing the terminal sialyl x structure.

For understanding the meaning of the diversity and the regulatory mechanisms for sialylation of glycoconjugates, it is necessary to advance our knowledge on the specificities of these enzymes, and this knowledge would aid in the synthesis of more complex oligosaccharide structures of great therapeutic value. For example, the two endothelial venule associated ligands for L-selectins, GLYCAM-I and CD_{34}, are O-linked glycoproteins containing fucose, sialic acid, and sulfate, the essential ingredients for binding with L-selectins (Imai et al., 1991). Although the sialyltransferases use a common donor substrate, they exhibit specificity for the sequence of their oligosaccharide acceptor substrate. The present paper reports our unique findings on the assembly of sialic acid, fucose, and sulfate moieties in selectin ligands and tumor-associated carbohydrates from a detailed investigation on the specificities of α2,3-sialyltransferases.

EXPERIMENTAL PROCEDURES

Chemical Syntheses of Sulfated, Methylated, or Sialylated Benzyl Glycosides. (i) 3-SulfoGalβ1, 3GalNAcα-O-Bn[1] (**6**). Glycosylation of benzyl 2-acetamido-2-deoxy-4,6-di-*O*-(4-methoxybenzylidene)-α-D-galactopyranoside with 2,3,4,6-tetra-*O*-acetyl-α-D-galactopyranosyl bromide, in the presence of mercuric cyanide, followed by the removal of protecting groups afforded compound **1**, benzyl *O*-(β-D-galactopyranosyl)-(1→3)-2-acetamido-2-deoxy-α-D-galactopyranoside (Flowers & Shapiro, 1965). This was treated with *tert*-butylchlorodiphenylsilane (Hanessian & Lavallee, 1975) in *N,N*-dimethylformamide to give compound **2**, benzyl *O*-(6-*O*-(*tert*-butyldiphenylsilyl)-β-D-galactopyranosyl)-(1→3)-2-acetamido-6-*O*-(*tert*-butyldiphenylsilyl)-2-deoxy-α-D-galactopyranoside in 66% yield: $[\alpha]_D$ +29° (*c* 1.5, $CHCl_3$); ^{1}H-NMR ($CDCl_3$) δ 7.67−7.17 (m, 25 H, arom.), 6.01 (d, *J* ~ 9 Hz, 1 H, NH), 4.88 (d, *J* ~ 3 Hz, 1 H, H-1), 1.87 (s, 3 H, NAc), 0.98 and 0.95 (each s, 18 H, 2 × CMe_3).

Isopropylidenation of compound **2** with 2,2-dimethoxypropane−acetone in the presence of 4-toluenesulfonic acid and followed by the removal of the *tert*-butyldiphenylsilyl group with fluoride ion (Hanessian & Lavallee, 1975) provided compound **3**, the 3′,4′-*O*-isopropylidene derivative, in 93% yield: $[\alpha]_D$ +108° (*c* 0.9, CH_3OH); ^{1}H-NMR (CD_3OD) δ 7.28 (bs, 5 H, arom.), 1.87 (s, 3 H, NAc), 1.40 and 1.26 (each s, 6 H, CMe_2).

Acetylation of this compound with pyridine−acetic anhydride, followed by cleavage of the 3′,4′-*O*-isopropylidene group with chloroform−trifluoroacetic acid−water, furnished compound **4**, benzyl *O*-(2,6-di-*O*-acetyl-β-D-galactopyranosyl)-(1→3)-2-acetamido-4,6-di-*O*-acetyl-2-deoxy-α-D-galactopyranoside in 64% yield: $[\alpha]_D$ +88° (*c* 0.9, $CHCl_3$); ^{1}H-NMR ($CDCl_3$) δ 7.33 (bs, 5 H, arom.), 5.28 (d, *J* ~ 3 Hz, 1 H, H-4), 4.98 (d, *J* ~ 4 Hz, 1 H, H-1), 2.13−1.93 (cluster of s, 15 H, 4 × OAc and NAc).

Compound **4** was converted into its 3′,4′-(ethyl orthoacetate), which was hydrolyzed with 80% aqueous acetic acid to give compound **5**, the key 3′-hydroxy intermediate, in 88% yield: $[\alpha]_D$ +90° (*c* 0.8, $CHCl_3$); ^{1}H-NMR ($CDCl_3$): δ 7.27 (bs, 5 H, arom.), 5.37 (d, J ~ 3 Hz, 1 H, H-4), 5.27 (d, J ~ 3 Hz, 1 H, H-4′), 5.07 (d, J ~ 4 Hz, 1 H, H-1), 2.13−1.97 (cluster of s, 18 H, 5 × OAc and NAc).

Sulfation of compound **5** in N,N-dimethylformamide with sulfurtrioxide-pyridine complex at room temperature followed by de-O-acetylation with methanolic sodium methoxide afforded the title compound **6** in 79% yield; $[\alpha]_D$ +92° (*c* 1.4, H_2O); ^{13}C-NMR (D_2O): δ 107.10 (C-1′), 99.09 (C-1), 82.99 (C-3), 80.39 (C-3′), 63.92 (C-6), 63.68 (C-6′), 51.37 (C-2), 24.72 ($COCH_3$); m/z: 597.9 $[M+Na]^+$, 521.1 $[M-Na]^-$, 574.3 $[M-1]^-$.

(ii) Galβ1, 3(4-O-Me)GlcNacβ-O-Bn (**10**). Glycosylation of benzyl 6-*O*-benzyl-2-deoxy-2-phthalimido-β-D-glucopyranoside with 2,3,4,5-tetra-*O*-acetyl-β-D-galactopyranosyl fluoride under Mukaiyama's conditions (Mukaiyama et al., 1981) ($SnCl_2$-AgOTf) in 5:1 (v/v) dichloromethane−toluene in the presence of 4-Å molecular sieves afforded β-(1→4)-linked disaccharide in 51% yield: $[\alpha]_D$ −16° (*c* 1.8, $CHCl_3$); ^{13}C-NMR ($CDCl_3$) δ 101.53 (C-1′), 97.53 (C-1), 81.99 (C-4), 68.02 (C-6), 61.45 (C-6′), 56.07 (C-2), 20.70, 20.56, 20.49 and 20.34 (4 × OAc). The β-(1→3)-linked disaccharide (compound **7**) was also obtained in 28% yield: $[\alpha]_D$ −27° (*c* 1.6, $CHCl_3$); ^{13}C-NMR ($CDCl_3$) δ 101.12 (C-1′), 97.06 (C-1), 82.15 (C-3), 68.53 (C-6), 61.49 (C-6′), 54.93 (C-2), 20.54, 20.49, 20.34 and 19.74 (4 × OAc).

Methylation of compound **7** with trimethyloxonium tetrafluoroborate−2,6-di-(*tert*-butyl)-4-methylpyridine (Fugedi & Kovacs, 1990) in dichloromethane followed by the treatment with hydrazine hydrate ethanol and pyridine−acetic anhydride gave compound **8**, benzyl *O*-(2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranosyl)-(1→3)-2-acetamido-6-*O*-benzyl-2-deoxy-4-*O*-methyl-β-D-glucopyranoside in 59% yield: $[\alpha]_D$ −26° (*c* 1.1, $CHCl_3$); ^{1}H-NMR ($CDCl_3$) δ 7.43−7.23 (m, 10 H, arom.), 5.76 (d, *J* ~ 8 Hz, 1 H, NH), 5.34 (d, *J* ~ 3 Hz, 1 H, H-4′), 3.43 (s, 3 H, OMe), 2.39−1.96 (cluster of s, 15 H, 4 × OAc and NAc).

Treatment of compound **8** in acetic anhydride−methylene chloride (20:1 v/v) containing 1% (v/v) (trimethylsilyl)-trifluoromethanesulfonate (Angibeaud & Utille, 1991) at ~O° gave compound **9**, benzyl *O*-(2,3,4,5-tetra-*O*-acetyl-β-D-galactopyranosyl)-(1→3)-2-acetamido-6-*O*-acetyl-2-deoxy-4-*O*-methyl-β-D-glucopyranoside in 80% yield: $[\alpha]_D$ −19° (*c* 0.6, $CHCl_3$); ^{1}H-NMR ($CDCl_3$) δ 7.28 (bs, 5 H, arom.), 3.45 (s, 3 H, OMe), 2.12−1.95 (cluster of s, 15 H, 5 × OAc), 1.58 (s, 3 H, NAc). De-*O*-acetylation with methanolic sodium methoxide provided title compound **10** in 86% yield: $[\alpha]_D$ −30° (*c* 0.7, H_2O); ^{1}H-NMR (D_2O) δ 7.64−7.56 (m, 5 H, arom.), 4.98 (d, *J* = 8.2 Hz, 1 H, H-1), 4.60 (d, *J* = 7.5 Hz, 1 H, H-1′), 3.72 (s, 3 H, OMe), 2.11 (s, 3 H, NAc); ^{13}C-NMR (D_2O) δ 102.39 (C-1′), 98.62 (C-1), 78.29 (C-3), 74.09 (C-4), 60.18 (C-6), 59.33 (C-6′), 58.96 (OMe), 54.29 (C-2), 21.25 (NAc); *m/z* 486.5 $[M-H]^-$, 488.2 $[M+H]^+$, 510.0 $[M+Na]^+$.

(iii) Galβ1,3(4,6-di-O-Me)GlcNacβ-O-Bn (**14**). Reaction of benzyl 4,6-*O*-benzylidene-2-deoxy-2-phthalimido-β-D-glucopyranoside with 2,3,4,6-tetra-*O*-acetyl-α-D-galactopyranosyl bromide in the presence of silver triflate and 2,6-di-*O*-(*tert*-butyl)-4-methylpyridine (Nilsson & Norberg, 1988) gave compound **11**, benzyl *O*-(2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranosyl)-(1→3)-4,6-di-*O*-benzylidene-2-deoxy-2-phthalimido-β-D-glucopyranoside (Lemieux et al., 1982) in 79.5% yield, which after removal of the acetal group with

[1] Abbreviations: ST, sialyltransferase; FT, fucosyltransferase; Bn, benzyl; Al, allyl; Me, methyl; AA-CP, acrylamide copolymer; CGM, Cowper's gland mucin; NDV, Newcastle disease virus; TLC, thin-layer chromatography.

80% aqueous acetic acid afforded known diol (compound **12**). Methylation of this diol with trimethyloxonium tetrafluoroborate−2,6-di-(*tert*-butyl)-4-methylpyridine as described for the preparation of compound **10** afforded 4,6-di-*O*-methyl compound **13** in 87.5% yield: $[\alpha]_D$ −30° (*c* 1.3, $CHCl_3$); ^{1}H-NMR ($CDCl_3$) δ 7.79 (bs, 4 H, arom.), 7.03 (bs, 5 H, arom.), 3.53 and 3.47 (each s, 6 H, 2 × OMe), 2.10−1.87 (cluster of s, 12 H, 4 × OAc). Removal of the protecting groups from **13** as described for the synthesis of **10** gave title compound **14** in 82% yield: $[\alpha]_D$ −29° (*c* 1.1, CH_3OH); ^{1}H-NMR (D_2O) δ 7.50−7.40 (m, 5 H, arom), 4.91 (d, *J* ~ 8.1 Hz, 1 H, H-1), 4.45 (d, *J* = 7.8 Hz, 1 H, H-1′), 3.59 and 3.48 (each s, 6 H, 2 × OMe), 1.96 (s, 3 H, NAc); ^{13}C-NMR (D_2O) δ 102.39 (C-1′), 98.68 (C-1), 78.23 (C-3), 77.04 (C-4), 69.85 (C-6), 60.16 (C-6′), 59.03 (OMe), 57.53 (OMe), 54.25 (C-2), 21.24 (NAc); *m/z* 500.4 $[M - H]^-$, 501.9 $[M + H]^+$.

(iv) Galβ1,3(NeuAcα2,6)GalNAcα-O-Bn (**15**). A solution of benzyl *O*-(2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranosyl)-(1→3)-2-acetamido-2-deoxy-α-D-galactopyranoside (1 mmol) and phenyl (methyl 5-acetamido-4,7,8,9-tetra-*O*-acetyl-3,5-dideoxy-2-thio-D-*glycero*-D-*galacto*-2-nonulopyranoside)-onate (Marra & Sinay, 1989) (2 mmol) in propionitrile (20 mL) was stirred for 0.5 h with 4-Å molecular sieves under an argon atmosphere at room temperature. Then, *N*-bromosuccinimide (3 mmol) and tetrabutylammonium triflate (Fukase et al., 1993) (1 mmol) were added to it at −40 °C. The reaction temperature was brought to ~0 °C, and stirring was continued at same temperature for 16 h. The reaction mixture was neutralized with a few drops of *N,N*-diisopropylethylamine. The mixture was filtered through Celite, the solids were thoroughly washed with chloroform, and the filtrate and washings were combined and concentrated in vacuo. The recombined mixture was purified by silica gel filtration, and fractions corresponding to the product were concentrated and de-*O*-acetylated with methanolic sodium methoxide followed by addition of water to the reaction mixture to hydrolyze methyl ester to acid to provide title compound **15** in 30% yield: $[\alpha]_D$ +67° (*c* 0.6, H_2O); ^{1}H-NMR (D_2O) δ 7.52−7.46 (m, 5 H, arom.), 4.98 (d, *J* ~ 3.7 Hz, 1 H, H-1), 4.49 (d, *J* = 7.7 Hz, 1 H, H-1′), 2.79 (dd, $J_{3''e,4''}$ = 4.6 Hz, 1 H, H-3″e) 2.08 (s, 3 H, NAc), 2.00 (s, 3 H, NAc), 1.75 (t, $J_{3''a,4''} = J_{3''e,3''a}$ = 12.2 Hz, 1 H, H-3″a); ^{13}C-NMR (D_2O) δ 103.64 (C-1′), 99.45 (C-2″), 95.08 (C-1), 76.17 (C-3), 67.53 (C-6), 61.61 (C-6′), 59.94 (C-9″), 50.87 (C-2′), 47.57 (C-5″), 39.30 (C-3″), 21.03, 20.92 (NHAc); *m/z* 763.2 $[M - H]^-$, 786.8 $[M + Na]^+$.

(v) NeuAcα2,6Galβ1,3GalNAcα-O-Bn (**20**). Reaction of benzyl 2-acetamido-2-deoxy-α-D-galactopyranoside with trimethylacetyl chloride (pivaloyl chloride) in pyridine afforded a di-*O*-pivaloyl compound and the desired 6-*O*-pivaloyl derivative (compound **16**) in 54% yield: $[\alpha]_D$ +106° (*c* 1.7, $CHCl_3$); ^{1}H-NMR ($CDCl_3$) δ 7.37−7.26 (m, 5 H, arom.), 6.04 (d, *J* = 8.8 Hz, 1 H, NH), 4.90 (d, *J*= 3.8 Hz, 1 H, H-1), 1.96 (s, 3 H, NAc), 1.20 (s, 9 H, CMe_3).

Glycosylation of **16** with 2,3,4,6-tetra-*O*-acetyl-α-D-galactopyranosyl bromide under conditions similar to those described for the preparation of **1** gave **17** in 65% yield: $[\alpha]_D$ +75° (*c* 1.6, $CHCl_3$); ^{1}H-NMR ($CDCl_3$) δ 7.33 (bs, 6 H, arom.), 4.91 (d, *J* = 4 Hz, 1 H, H-1), 2.21−1.91 (cluster of s, 15 H, 4 × OAc and NAc), 1.19 (s, 9 H, CMe_3). De-*O*-acetylation of compound **17** with methanolic sodium methoxide in methanol−methylene chloride (1:1, vv; pH ~ 9) at ~ 0 °C gave **18** in 73% yield: $[\alpha]_D$ +105° (*c* 1.2, CH_3OH); ^{1}H-NMR ($CDCl_3$ + CD_3OD) δ 7.29 (bs, 5 H, arom.), 4.87 (d, *J* = 4 Hz, 1 H, H-1), 1.85 (s, 3 H, NAc), 1.17 (s, 9 H, CMe_3). Reaction of compound **18** with anisaldehyde dimethyl acetal in *N,N*-dimethylformamide in the presence of 4-toluenesulfonic acid and acetylation of 4′,6′-di-*O*-acetal with pyridine−acetic anhydride, followed by hydrolysis with 70% aqueous acetic acid, afforded (along with some acetyl migrated compound) key intermediate benzyl-*O*-(2,3-di-*O*-acetyl-β-D-galactopyranosyl)-(1→3)-2-acetamido-4-*O*-acetyl-2-deoxy-6-*O*-pivaloyl-α-D-galactopyranoside (**19**) in 31% yield: $[\alpha]_D$ +102° (*c* 1.1, $CHCl_3$); ^{1}H-NMR ($CDCl_3$) δ 7.32 (bs, 6 H, arom.), 5.18 (d, *J* = 3 Hz, 1 H, H-4), 4.99 (d, *J* = 4 Hz, 1 H, H-1), 2.15−1.89 (4 s, 12 H, 3 × OAc and NAc), 1.22 (s, 9 H, CMe_3).

Glycosylation of **19** with the same sialic acid donor under reaction conditions similar to those described for the preparation of **15** afforded title compound **20** in 41% yield: $[\alpha]_D$ +55° (*c* 0.8, H_2O); ^{1}H-NMR (D_2O δ 7.45−7.40 (m, 5 H, arom.), 4.94 (d, *J* = 3.6 Hz, 1 H, H-1), 4.43 (d, *J* = 7.7 Hz, 1 H, H-1′), 2.71 (dd, $J_{3''e,4''}$ = 4.5 Hz, 1 H, H-3″e), 2.03 (s, 3 H, NAc), 1.94 (s, 3 H, NAc), 1.64 (t, $J_{3''a,4''} = J_{3''e,3''a}$ = 12.1 Hz, H-3″a); ^{13}C-NMR (D_2O) δ 103.46 (C-1′), 95.19 (C-1), 76.30 (C-3), 67.54 (C-6′), 61.68 (C-6), 60.38 (C-9″), 50.92 (C-2), 47.71 (C-5″), 39.26 (C-3″), 22.14 and 20.96 (2 × NAc); *m/z* 763.4 $[M - H]^-$, 765.1 $[M + H]^+$, 787.0 $[M + Na]^+$.

(vi) Galβ1,3(NeuAcα2,6)GlcNAcβ-O-Bn (**21**). The procedures used for preparing **21** were essentially the same as described for compound **15**, except that benzyl *O*-(2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranosyl)-(1→3)-2-acetamido-2-deoxy-β-D-glucopyranoside was used as starting material, which was prepared using the published procedure. Compound **21**: $[\alpha]_D$ −30° (*c* 1.0, H_2O); ^{1}H-NMR (D_2O) δ 7.51−7.42 (m, 5 H, arom.), 2.81 (dd, $J_{3''e,4}$ = 4.5 Hz, 1 H, H-3″e), 2.09 (s, 3 H, NAc), 1.95 (s, 3 H, NAc), 1.78 (t, $J_{3''a,4''} = J_{3''e,3''a}$ = 12.2 Hz, H-3″a); ^{13}C-NMR (D_2O) δ 102.34 (C-1′), 100.01 (C-2″), 98.77 (C-1), 80.92 (C-3), 67.58 (C-6), 61.65 (C-6′), 59.97 (C-9″), 53.58 (C-2), 50.87 (C-5″), 39.08 (C-3″), 21.17 and 21.02 (2 × NAc); *m/z* 763.3 $[M - H]^-$, 785.3 $[M - 2H + Na]^-$.

(vii) NeuAcα2,6Galβ1,3GlcNAcβ-O-Bn (**24**). A similar sequence of reactions were performed for the synthesis of **24** from benzyl 2-acetamido-2-deoxy-6-*O*-pivaloyl-β-D-glucopyranoside ($[\alpha]_D$ − 66° (*c* 1.6, $CHCl_3$)), as described for the preparation of **20** from **16**. Benzyl *O*-(2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranosyl)-(1→3)-2-acetamido-2-deoxy-6-*O*-pivaloyl-β-D-glucopyranoside (**22**) ($[\alpha]_D$ −11° (*c* 1.7, $CHCl_3$)), after de-*O*-acetylation followed by the formation of 4′,6′-*O*-acetal, acetylation, and the hydrolysis of the acetal ring, provided key intermediate benzyl *O*-(2,3-di-*O*-acetyl-β-D-galactopyranosyl)-(1→3)-2-acetamido-4-*O*-acetyl-2-deoxy-6-*O*-pivaloyl-β-D-glucopyranoside (**23**) ($[\alpha]_D$ −13° (*c* 1.2, $CHCl_3$)), along with some acetyl migrated compound. Compound **23**, after similar glycosylation followed by the removal of protecting groups, provided title compound **24**: $[\alpha]_D$ −40° (*c* 0.7, H_2O); ^{1}H-NMR (D_2O) δ 7.51−7.43 (m, 5 H, arom.), 2.74 (dd, $J_{3''e,4''}$ = 4.8 Hz, 1 H, H-3″e), 2.08 (s, 3 H, NAc), 1.97 (s, 3 H, NAc), 1.74 (t, $J_{3''a,4''} = J_{3''e,3''a}$ = 12.1 Hz); ^{13}C-NMR (D_2O) δ 102.84 (C-1′), 98.68 (C-1), 82.99 (C-3), 67.90 (C-6), 59.93 (C-9″), 53.29 (C-2), 50.81 (C-5″), 39.12 (C-3″), 21.15 and 21.04 (NAc); *m/z* 763.4 $[M - H]^-$, 785.1 $[M - 2H + Na]^-$.

We have already reported the synthesis of Galβ1,4(6-sulfo)GlcNAcβ1,6Manα-*O*-ONP (Liu et al., 1994). The synthesis of allyl glycosides will be reported elsewhere.

Assay of Sialyltransferases. The incubation mixture (20 μL) contained 50 mM Na cacodylate buffer, pH 6.5, 0.57 mM CMP-NeuAc, CMP-[^{14}C]NeuAc (8500 CPM/nmol), Triton X-100 (0.5% W/V), BSA (100 μg), the acceptor (1 or 15 mM or as warranted in kinetic experiments), and sialyltransferase. Incubation was carried out for 2 h at 37 °C.

Porcine liver α2,3-sialyltransferase (Sigma Chemical Co.) was studied by using 16 microunits of the enzyme in each assay (the unit of activity was based on anti-freeze glycoprotein as the substrate). The cloned α2,3-sialyltransferase was made available by Glycomed (Alameda, CA) as an enzyme bound to Sepharose beads; the binding was achieved through protein A (the dimeric segment of the enzyme) to IgG, which had been coupled to Sepharose. As our present studies necessitated a soluble enzyme preparation, 100 μL (packed volume) of the Sepharose beads equivalent to 15 milliunits (activity reported by Glycomed using lactose as substrate) were stripped off for the enzyme, using the conditions suggested by the supplier, except the treatment period was prolonged to 30 min at 4 °C to ensure the release of sufficient enzyme from the beads for completing the studies reported in this paper. The bead slurry (500 μL) was centrifuged for 1 min in a microfuge. After removal of the supernatant, 500 μL of 1 mM Tris-HCl−150mM NaCl, pH 8.0, was added to the beads, mixed gently by finger tapping, and centrifuged. The supernatant was discarded. Then 500 μL of 0.1 M citrate buffer, pH 4.4, was added to the beads, mixed in the cold room for 1/2 h using Speci-Mix (Thermolyne), and then centrifuged for 1 min. The supernatant was mixed with 500 μL of 0.4 M Na cacodylate buffer, pH 6.5, containing 4% Triton X-100 and 10 mg of BSA. The stripped enzyme was stored frozen at −20 °C. No loss of enzyme activity was observed under these conditions for at least 4 months. In each assay, 5 μL of this soluble enzyme was used.

HL60 cells were grown in 250-mL plastic T-flasks in RPMI 1640 supplemented with 10% fetal calf serum (GIBCO), 20 mM HEPES, 2 mM glutamine, penicillin G (50 μg/mL), streptomycin (50 μg/mL), neomycin (100 μg/mL), and fungizone (0.25 μg/mL). Cells were subcultured without trypsin−EDTA treatment. For experimental use, cells were pelleted at 1500 rpm for 5 min, washed twice with PBS, and stored frozen at −20 °C. The cells (2.2×10^8) were homogenized with 1 mL of 15 mM Na cacodylate buffer, pH 6.5, containing 2 mM PMSF, 2 mM EDTA, and 2% Triton X-100 (w/v) and centrifuged at 10000*g* for 1/2 h at 4 °C. The supernatant was stored frozen. A 10-μL aliquot of this preparation was used in each assay run in duplicate, using a reaction volume of 25 μL. Protein was assayed by the BCA method (Pierce Chemical Co.).

Macromolecular and Natural Acceptors. Acrylamide copolymers of Galβ1,3GalNAcα-O-Al, Galβ1,3GlcNAcβ-O-Al, and Galβ1,3(6-sulfo)GlcNAcβ-O-Al were synthesized by following the procedure of Horejsi et al. (1978). These preparations contained ~1.0 μmol of the sugar unit/mg (determination of Gal by anthrone reaction) and were similar in molecular size to dextran of average molecular weight 39 200, as evident from column chromatography on Biogel P60. Asialo CGM, bovine IgG diantennary glycopeptide, and fetuin triantennary asialoglycopeptide were available from earlier studies (Chandrasekaran et al., 1992a, 1994a,b).

Separation and Quantitation of the Radioactive Sialylated Products. The reaction mixtures (either 20 or 25 μL) after incubation for 2 h at 37 °C were subjected to thin-layer chromatography (silica gel GHLF; 250 μm, scored 20 × 20 cm), after a quantitative transfer as 2-cm streaks. The solvent system chloroform−methanol−water (5:4:1, v/v) was used to separate ^{14}C- or ^{3}H-labeled sialylated synthetic allyl or benzyl glycosides from CMP-NeuAc, which did not move much (within 3 cm) from the origin. Radioactivity was measured by scraping the silica gel from 1/2 cm width segments into scintillation vials, soaking in 2 mL of water, and then liquid scintillation counting. A typical separation is illustrated in Figure 1. The reaction mixtures containing acrylamide copolymers, glycopeptides, and glycoproteins were also subjected to silica gel TLC using ethylacetate−pyridine−water−acetic acid (5:5:3:1); the radioactive products remained at the origin, while CMP-NeuAc moved farther away on the plate.

We also established beyond any reasonable doubt that the above TLC methods for the quantitation of radioactive products were quite precise, since we found that the variation in the values we checked for a number of reaction mixtures run in duplicate was within 5%.

The preciseness of the TLC runs was accomplished by adhering to our strict guidelines as follows: (a) Every morning, 1 h before the TLC runs, the TLC tanks were replenished with freshly prepared solvent mixtures. (b) The saturation of the solvent mixture inside the TLC tanks was assured by a rectangular Whatmann 3 MM paper dipping in the solvent of the TLC tank. (c) After each TLC run, the scraping of the silica gel was carried out starting from the origin in order to make sure that everything went fine.

Large-Scale Incubations. (a) Galβ1,3GalNAcα-O-Allyl/AA-CP. One milligram of copolymer was incubated with porcine liver α2,3-sialyltransferase and CMP-[^{14}C]NeuAc in a reaction volume of 200 μL under the same conditions as described above, but extending the incubation period to 21 h. The reaction mixture was subjected to Biogel P60 column (1 × 116.0 cm) chromatography using pyridine acetate, pH 5.4, as the eluent; the radioactive peak emerging first from the column was collected, lyophilized to dryness, dissolved in a small volume of water, and stored frozen.

(b) Asialo Cowper's Gland Mucin (ACGM). Seven milligrams of this glycoconjugate was incubated with porcine liver α2,3-sialyltransferase in a reaction volume of 560 μL under standard incubation conditions as above for a period of 21 h, and the radioactive product was isolated by Biogel P60 chromatography as described above.

(c) Galβ1,3(6-sulfo)GlcNAcβ-O-Allyl. This acceptor (0.6 μmol) was incubated for 16 h at 37 °C with 0.6 μm CMP-NeuAc and 0.5 μCi of CMP-[9-^{3}H]NeuAc in a reaction volume of 90 μL under the standard incubation conditions with 35 μL of the soluble enzyme obtained from the cloned α2,3-sialyltransferase bound to Sepharose beads. After incubation, the reaction mixture was subjected to Biogel P2 column (1 × 116.0 cm) chromatography. The first radioactive peak was lyophilized to dryness, dissolved in 1 mL of water, and stored frozen at −20 °C.

(d) Galβ1,3(6-sulfo)GlcNAcβ-O-Al/AA-CP and Galβ1,3-GlcNAcβ-O-Al/AA-CP. Four milligrams each of these copolymers (0.1 μmol) was incubated separately for 20 h

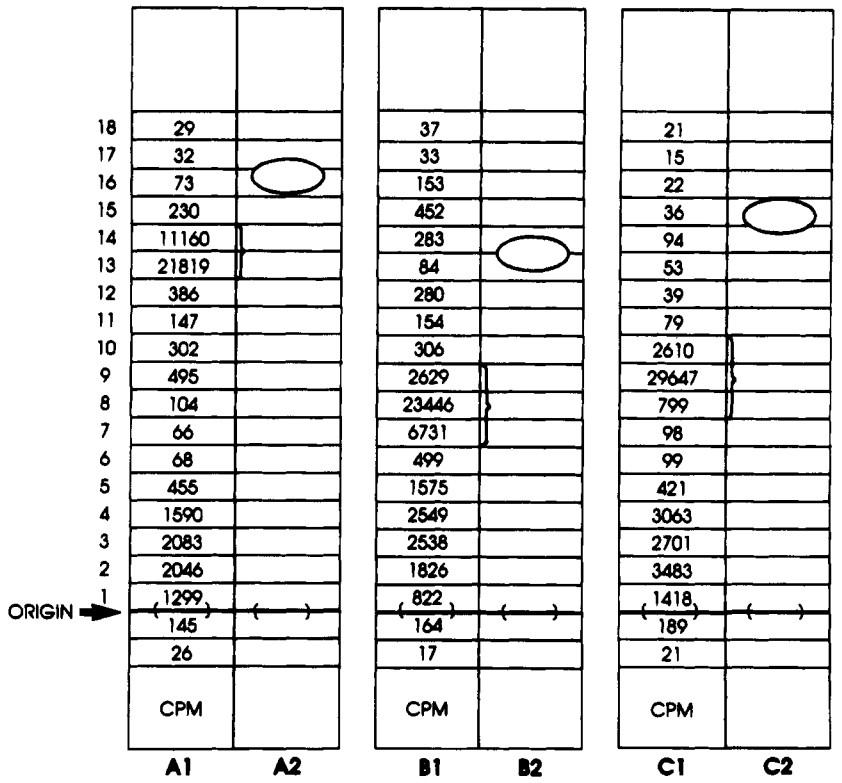

FIGURE 1: Thin-layer chromatographic separation of the radioactive sialyl compounds in the reaction mixture: A_1, reaction mixture containing the acceptor Galβ1,3GalNAcα-O-Al; A_2, Galβ1,3GalNAcα-O-Al; B_1, reaction mixture containing the acceptor Galβ1,3(NeuAcα2,6)GalNAcα-O-Bn; B_2 Galβ1,3(NeuAcα2,6)GalNAcα-O-Bn; C_1, reaction mixture containing the acceptor Galβ1,3(6-sulfo)GalNAcα-O-Al; C_2, Galβ1,3-(6-sulfo)GalNAcα-O-Al.

with 1.2 μmol of CMP-NeuAc and 1.0 μCi of CMP-[9-^{3}H]-NeuAc in a reaction volume of 180 μL under the standard incubation conditions with 70 μL of the soluble cloned α2,3-sialyltransferase. After incubation, the radioactive products were isolated by chromatography on a Biogel P2 column as described above.

Demonstration of α1,4-L-Fucosylation of [9-^{3}H]NeuAc α2,3Galβ1,3(6-sulfo)GlcNAcβ-O-Allyl, [9-^{3}H]NeuAc α2,3Galβ1,3GlcNAcβ-O-Al/AA-CP, and [9-^{3}H]NeuAc α2,3Galβ1,3(6-sulfo)GlcNAcβ-O-Al/AA-CP. Ten-microliter aliquots of ^{3}H-labeled sialyl copolymers isolated as above were incubated with partially purified Colo 205 α1,3/4-L-fucosyltransferase (Chandrasekaran et al., 1994b) in a reaction volume of 25 μL with and without GDP[^{14}C]Fuc for 22 h at 37 °C, and each reaction mixture was subjected to TLC using ethyl acetate−pyridine−water−acetic acid as described above. Both ^{14}C and ^{3}H were measured in each 0.5-cm segment of the silica gel after the gel was scraped as described above.

An 80-μL aliquot from [9-^{3}H]NeuAcα2,3Galβ1,3(6-sulfo)-GlcNAcβ-O-Al, isolated as above, was incubated with partially purified Colo 205 α1,3/4-L-fucosyltransferase (Chandrasekaran et al., 1994b) in a reaction volume of 200 μL for 22 h at 37 °C. The reaction mixture was then subjected to Biogel P2 column chromatography for the separation of the product from GDP-[^{14}C]Fuc and any [^{14}C]Fuc. After lyophilization to dryness, the product was dissolved in 0.50 mL of water. A 20-μL aliquot of this was subjected to TLC using chloroform−methanol−water (5:4:1) as above. The radioactivities were located as described above.

Fractionation on Dowex-1-Cl. 3′-[9-^{3}H]sialyl compounds derived from Galβ1,3(6-sulfo)GlcNAcβ-O-Al, Galβ1,-3GlcNAcβ-O-Al/AA-CP, and Galβ1,3(6-sulfo)GlcNAcβ-O-Al/AA-CP and isolated by Biogel P2 column chromatography were subjected to fractionation separately on Dowex-1-Cl (X-8; 200−400 mesh) columns (1 mL in a Pasteur pipet). The columns were washed with 4 mL of water and then eluted successively with 4 mL each of 0.1, 0.2, 0.3, and 2.0 M NaCl. The fractions from the copolymers were dialyzed exhaustively (48 h at 4 °C) against water and lyophilized to dryness, and each was dissolved in 200 μL of water. The 0.3 and 2.0 M NaCl fractions from 3′-[^{3}H]sialyl Galβ1,3(6-sulfo)GlcNAcβ-O-Al, which contained 57.3% and 37.6% radioactivity, respectively, were lyophilized to dryness, dissolved in 1.0 mL of water, and then desalted separately on a Biogel P2 column as described above. Both fractions emerged from the Biogel P2 column in an identical position, indicating that they are the same, and the higher concentration of NaCl was helpful in the elution of trapped molecules in the Dowex-1-Cl matrix.

α1,4-L-Fucosylation of Dowex-1-Cl Fractionated Materials from 3′-[^{3}H]Sialyl Copolymers. Ten microliters each of the water- and 0.1 M NaCl-eluted materials from 3′-[^{3}H]-sialyl Galβ1,3GlcNAcβ-O-Al/AA-CP and the 2.0 M NaCl-eluted material from 3′-[^{3}H]sialyl Galβ11,3(6-sulfo)GlcNAcβ-O-Al/AA-CP was incubated with partially purified Colo 205 α1,3/4-L-fucosyltransferase (Chandrasekaran et al., 1994b) in a reaction volume of 25 μL. The reaction mixtures were then subjected to TLC as above using ethyl acetate−pyridine−water−acetic acid as the solvent system, and the product remaining at the origin was scraped off and counted for both ^{3}H and ^{14}C.

RESULTS

Activity of Porcine Liver α2,3-Sialyltransferase. The α2,3-sialyltransferase of porcine liver acting on Galβ1,-

Table 1: Specificities of Porcine Liver α2,3-Sialyltransferase Acting on Galβ1,3GalNAcα-OR

	incorporation of [^{14}C]NeuAc into acceptor					
	1 mM acceptor			15 mM acceptor		
acceptor	cpm	NeuAc (nmol)	%	cpm	NeuAc (nmol)	%
Galβ1,3GalNAcα-O-Allyl	15 756	1.9	100	14 568	1.7	100
Galβ1,3(6-sulfo)GalNAcα-O-Allyl	8970	1.1	57	8424	1.0	58
Galβ1,3(NeuAcα2,6)GalNAcα-O-Bn	5831	0.7	37	9834	1.2	68
3-sulfoGalβ1,3GalNAcα-O-Allyl	0	0	0			
3-sulfoGalβ1,3GalNAcα-O-Bn	0	0	0			
3-O-Me-Galβ1,3GalNAcα-O-Bn	0	0	0			
Galβ1,3(6-O-Me)GalNAcα-Bn	12 511	1.5	79	11 491	1.4	79
3-O-Me-Galβ1,3(6-O-Me)GalNAcα-O-Bn	0	0	0	0	0	0
Galβ1,3GalNAcα-O-Bn	13 997	1.7	89	12 759	1.5	88
6-sulfoGalβ1,3GalNAcα-O-Allyl	0	0				
Galβ1,3GalNAcα-O-Allyl/acrylamide copolymer: 100 μg (0.125 mM)	11 373	1.3				
Asialo CGM: 250 μg (0.050 mM)	3565	0.4				

Table 2: Facile C-3′ Sialylation of Galβ1,3GalNAcα- in Multimeric Structures by Porcine Liver α2,3-ST

		donor [^{14}C]NeuAc moiety		product [^{14}C]NeuAc moiety		
acceptor	enzyme (milliunits)	CPM × 10^{-6}	nmol	CPM × 10^{-6}	nmol	utilization of CMP-NeuAc (%)
asialo CGM (28 nmol)	0.45	1.90	224.0	0.47	54.9	24.5
Galβ1,3GalNAcα-O-Al/AA-CP (25 nmol)	0.16	0.68	80.0	0.13	15.0	18.8

3GalNAcα-O-R was measured with sulfated, sialylated, or methylated derivatives of this structure, and the data are presented in Table 1. When the activity was measured at two different concentrations of the acceptor, except in the case of Galβ1,3(NeuAcα2,6)GalNAcα-O-Bn, no appreciable difference in the incorporation of radioactivity was seen, thus indicating that 1 mM acceptor is enough for saturating the enzyme. Galβ1,3GalNAcα-O-Bn showed 89% activity as compared to Galβ1,3GalNAcα-O-Al, indicating that the difference in the aglycon did not much affect the activity of this enzyme. C-6 substitution on GalNAc of Galβ1,-3GalNAcα- with a sulfate, sialic acid, or methyl group resulted in 57, 37, and 79% activity, respectively, as compared to the activity on Galβ1,3GalNAcα-O-Al, thus illustrating that C-6 substitution does not prevent the enzyme activity, except for the reciprocal relationship observed between the activity and the bulkiness of the C-6 substitution group. As anticipated, C-3 substitution (either sulfate or methyl group) on Gal abolished the transfer of sialic acid to these acceptors; this finding thus supports the specificity of the enzyme, i.e., 3′-sialylation. The enzyme activity was also abolished when C-6 of Gal is substituted with sulfate suggesting the enzyme requirement of free hydroxyl group on C-6 of Gal for activity.

When the enzyme was tested for activity with the natural acceptor, asialo CGM (1.0 nmol), and the synthetic copolymer (2.5 nmol) of Galβ1,3GalNAcα-O-Al with acrylamide, 0.4 and 1.3 nmol of sialic acid was transferred, respectively, to these acceptors in 2 h under the standard incubation conditions.

A large-scale incubation of the above two acceptors with the porcine liver sialyltransferase (Table 2) showed that 0.45 milliunit of the enzyme utilized 24.5% of CMP-NeuAc (224 nmol) by transferring 54.9 nmol of NeuAc to asialo CGM, and 0.16 milliunit utilized 18.8% of CMP-NeuAc (80 nmol) by transferring 15 nmol NeuAc to the copolymer.

Specificity of the Cloned α2,3-Sialyltransferase Acting on Galβ1,3/4GlcNAcβ-. This enzyme was highly active on Gal linked β1,3 to GlcNAc (Table 3). When compared to Galβ1,3GlcNAcβ-O-Bn, Galβ1,4GlcNAcβ-O-Bn was only 16% efficient as the acceptor at 1 mM concentration. When the activity was measured at two different concentrations of the acceptor, considerable increase in acceptor efficiency was seen with Galβ1,4GlcNAcβ-O-Bn (67%), Galβ1,3-(NeuAcα2,6)GlcNAcβ-O-Bn (106%) and 6-sulfoLacNAcβ1,-6Manα-O-ONP (54%). Even Galβ1,3GalNAcα-O-Al showed some activity (10%) at 15 mM. The most important finding is that the Lewis a structure, Galβ1,3(Fucα1,4)GlcNAcβ-O-Al, did not show any acceptor activity. As anticipated, C-3 substitution on Gal with sulfate (3-sulfoGalβ1,3ΓlcNAcβ-O-Al and 3-sulfoGalβ1,3GlcNAcβ-O-Bn) abolished the transfer of sialic acid.

C-6 substitution on GlcNAc with a sulfate, sialic acid, or methyl group resulted in 102%, 70%, and 108% activity, thus indicating that this enzyme is less susceptible to this substitution as compared to porcine liver enzyme (see Table 1).

When Fuc was replaced by a methyl group in the Lewis a structure [Galβ1,3(4-O-Me)GlcNAcβ-O-Bn], some activity (29%) was seen, and this would indicate that the bulkiness of a group such as Fuc on C-4 of GlcNAc prevents the enzyme activity. Surprisingly, C-2 substitution on Gal with a methyl group did not abolish the acceptor activity completely (13% activity).

Two copolymers, Galβ1,3GlcNAcβ-O-Al/AA-CP and Galβ1,3(6-sulfo)GlcNAcβ-O-Al/AA-CP, and the natural glycoconjugates fetuin triantennary asialoglycopeptide and bovine IgG diantennary glycopeptide were also tested as acceptors for the cloned enzyme under the standard incubation conditions. The copolymer containing sulfate at C-6 of GlcNAc was nearly as effective as an acceptor as the copolymer containing no substitution at C-6 of GlcNAc. As anticipated, bovine IgG diantennary glycopeptide, which has Galβ1,4GlcNAc as well as a chain lacking a terminal Gal, was a poor acceptor as compared to fetuin triantennary asialoglycopeptide, which has both β1,3- and β1,4-linked terminal Gal resudies.

Table 3: Specificities of the Cloned α2,3-Sialyltransferase Acting on Galβ1,3/4GlcNAc-Containing Structures

acceptor	incorporation of [^{14}C]NeuAc into acceptor					
	1 mM acceptor			15 mM acceptor		
	cpm	NeuAc (nmol)	%	cpm	NeuAc (nmol)	%
Galβ1,3GlcNAcβ-O-Bn	29 458	3.5	100	30 546	3.6	100
Galβ1,4GlcNAcβ-O-Bn	4658	0.6	16	20 554	2.4	67
Galβ1,3(NeuAcα2,6)GlcNAcβ-O-Bn	20 713	2.4	70	32 492	3.8	106
Galβ1,3(6-sulfo)GlcNAcβ-O-Allyl	30 000	3.5	102	35 599	4.2	117
6-sulfoLacNAcβ1,6Manα-O-ONp	5988	0.7	20	16 450	1.9	54
Galβ1,3(Fucα1,4)GlcNAcβ-O-Allyl	0	0	0			
Galβ1,3GalNAcα-O-Allyl				2956	0.4	10
3-sulfoGalβ1,3GlcNAcβ-O-Allyl	0	0	0			
3-sulfoGalβ1,3GlcNAcβ-O-Bn	0	0	0			
6-sulfoGalβ1,3GlcNAcβ-O-Allyl	0	0	0			
Galβ1,3GlcNAcβ-O-Allyl	29 451	3.5	100			
Galβ1,3(4-O-Me)GlcNAcβ-O-Bn	8394	1.0	29			
Galβ1,3(6-O-Me)GlcNAcβ-O-Bn	31 900	3.8	108			
Galβ1,3(4,6-di-O-Me)GlcNAcβ-O-Bn	7766	0.9	26			
2-O-Me-Galβ1,3GlcNAcβ-O-Bn	3923	0.5	13			
Galβ1,3GlcNAcβ-O-Allyl/acrylamide copolymer: 100 μg (0.125 mM)	21 771	2.6				
Galβ1,3(6-sulfo)GlcNAcβ-O-Allyl/acrylamide copolymer: 100 μg (0.125 mM)	19 769	2.3				
fetuin triantennary asialoglycopeptide: 100 μg (1.2 mM)	25 038	3.0				
bovine IgG diantennary glycopeptide: 100 μg (1.4 mM)	4907	0.6				

Table 4: K_m and V_{max} Values Obtained for the Porcine Liver and the Cloned α2,3-Sialyltransferases toward Various Acceptors

acceptor	K_m (mM)	V_{max} (nmol/h)
porcine liver α2,3-ST		
Galβ1,3Ga1NAcα-O-Allyl	0.200	0.59
Galβ1,3GalNAcα-O-Allyl/acrylamide copolymer	0.050	1.18
Galβ1,3(NeuAcα2,6)GalNAcα-O-Benzyl	0.420	0.47
Galβ1,3(6-sulfo)GalNAcα-O-Allyl	0.105	0.98
cloned α2,3-ST		
Galβ1,3GlcNAcβ-O-Allyl	0.330	1.47
Galβ1,3GlcNAcβ-O-Allyl/acrylamide copolymer	0.015	1.76
Galβ1,3(NeuAcα2,6)GlcNAcβ-O-Benzyl	0.500	1.47
Galβ1,3(6-sulfo)GlcNAcβ-O-Allyl	0.210	2.35
6-sulfoLacNAcβ1,6Manα-O-oNP	2.500	1.17

Determination of K_m and V_{max} Values for the Two α2,3-Sialyltransferases. Porcine liver sialyltransferase was incubated with varying concentrations of the acceptors, namely, Galβ1,3GalNAcα-O-Al, Galβ1,3(NeuAcα2,6)GalNAcα-O-Bn, Galβ1,3(6-sulfo)GalNAcα-O-Al, and Galβ1,3GalNAcα-O-Al/AA-CP, under the standard incubation conditions, and the radioactive products were separated by TLC and quantitated as described earlier. Likewise, the cloned enzyme was reacted with varying concentrations of Galβ1,3GlcNAcβ-O-Al, Galβ1,3(NeuAcα2,6)GlcNAcβ-O-Bn, Galβ1,3(6-sulfo)GlcNAcβ-O-Al, Galβ1,GlcNAcβ-O-Al/AA-CP, and 6-sulfoLacNAcβ1,6Manα-O-ONP.

Lineweaver–Burke plots of the above data are presented in Figure 2. K_m and V_{max} were calculated from the intercepts on the x-axis and the y-axis, respectively, and the values are reported in Table 4. For both enzymes, the most effective substrates were the copolymers (K_m: 0.050 mM for the porcine liver enzyme, 0.015 mM for the cloned enzyme) followed by the glycosides of C-6 sulfo disaccharides.

Facile Enzymatic Synthesis of 3′-Sialyl Mono and Multi Units of Galβ1,3GlcNAcβ- and Galβ1,3(6-sulfo)GlcNAcβ-. Figure 3 and Table 5 present the data from a large-scale incubation of the cloned enzyme with Galβ1,3(6-sulfo)-GlcNAcβ-O-Al, Galβ1,3GlcNAcβ-O-Al/AA-CP, and Galβ1,3-(6-sulfo)GlcNAcβ-O-Al/AA-CP. Under the standard incubation conditions, 65.4%, 76.6%, and 57.5% of CMP-NeuAc was used up, respectively, in the formation of the products.

Further 0.4 milliunit of Newcastle disease virus (NDV) sialidase released 100% of the sialic acid (3.9 nmol) present in the product from Galβ1,3(5-sulfo)GlcNAcβ-O-Al; the same amount of sialidase hydrolyzed 49.8% of the sialic acid (7.8 nmol) present in the product from Galβ1,3GlcNAcβ-O-Al/AA-CP and 70.9% of the sialic acid (5.6 nmol) present in the product from Galβ1,3(6-sulfo)GlcNAcβ-O-Al/AA-CP. These results indicate that NDV sialidase is quite efficient in hydrolyzing α2,3-linked sialic acid from the multi units of Galβ1,3GlcNAcβ- and Galβ1,3(6-sulfo)GlcNAcβ-.

Elution Behavior of [^{3}H]Sialyl Compounds from Dowex-1-Cl. The results are presented in Table 6. [^{3}H]NeuAcα2,-3Galβ1,3(6-sulfo)GlcNAcβ-O-Al was eluted from the Dowex-1-Cl column by 0.3 M NaCl (57.3%), and the remaining product, by 2.0 M NaCl. These two materials were subsequently found to be identical (see Experimental Procedures for details); 44.3% and 50.3% of [^{3}H]NeuAcα2,-3Galβ1,3GlcNAcβ-O-Al/AA-CP emerged from the Dowex column in water and 0.1 M NaCl, respectively. A major portion (57.3%) of [^{3}H]NeuAcα2,3Galβ1,3(6-sulfo)GlcNAcβ-O-Al/AA-CP was eluted from the Dowex-1-Cl column by 2.0 M NaCl, whereas water and 0.1, 0.2, and 0.3 M NaCl eluates contained 10.2, 12.8, 8.5, and 11.2% of the radioactivity. We chose [^{3}H]NeuAcα2,3Galβ1,3GlcNAcβ-O-Al/AA-CP (water and 0.1 M NaCl eluates from Dowex-1-Cl, and so less anionic) and [^{3}H]NeuAcα2,3Galβ1,3(6-sulfo)-GlcNAcβ-O-Al/AA-CP (2.0 M NaCl eluate, and so more anionic) as acceptors in order to demonstrate that the enzymatic α1,4-L-fucosylation is really occurring on highly anionic multimeric structures.

Enzymatic α1,4-L-Fucosylation of 3′-Sialyl and 3′-Sialyl-6-sulfoGalβ1,3GlcNAcβ- Units. For this demonstration, [^{3}H]-NeuAcα2,3Galβ1,3GlcNAcβ-O-Al/AA-CP and its Dowex-1-Cl fractions, namely, water- and 0.1 M NaCl-eluted materials, [^{3}H]NeuAcα2,3Galβ1,3(6-sulfo)GlcNAcβ-O-Al/AA-CP and its 2.0 M NaCl-eluted material, and [^{3}H]-NeuAcα2,3Galβ1,3(6-sulfo)GlcNAcβ-O-Al were chosen as the acceptors. The double-radiolabeled products (Figure 4A) were quantitated by TLC (Figure 5; see Experimental Procedures for details). Table 7 shows the incorporation of [^{14}C]Fuc into these acceptors. There was no apparent

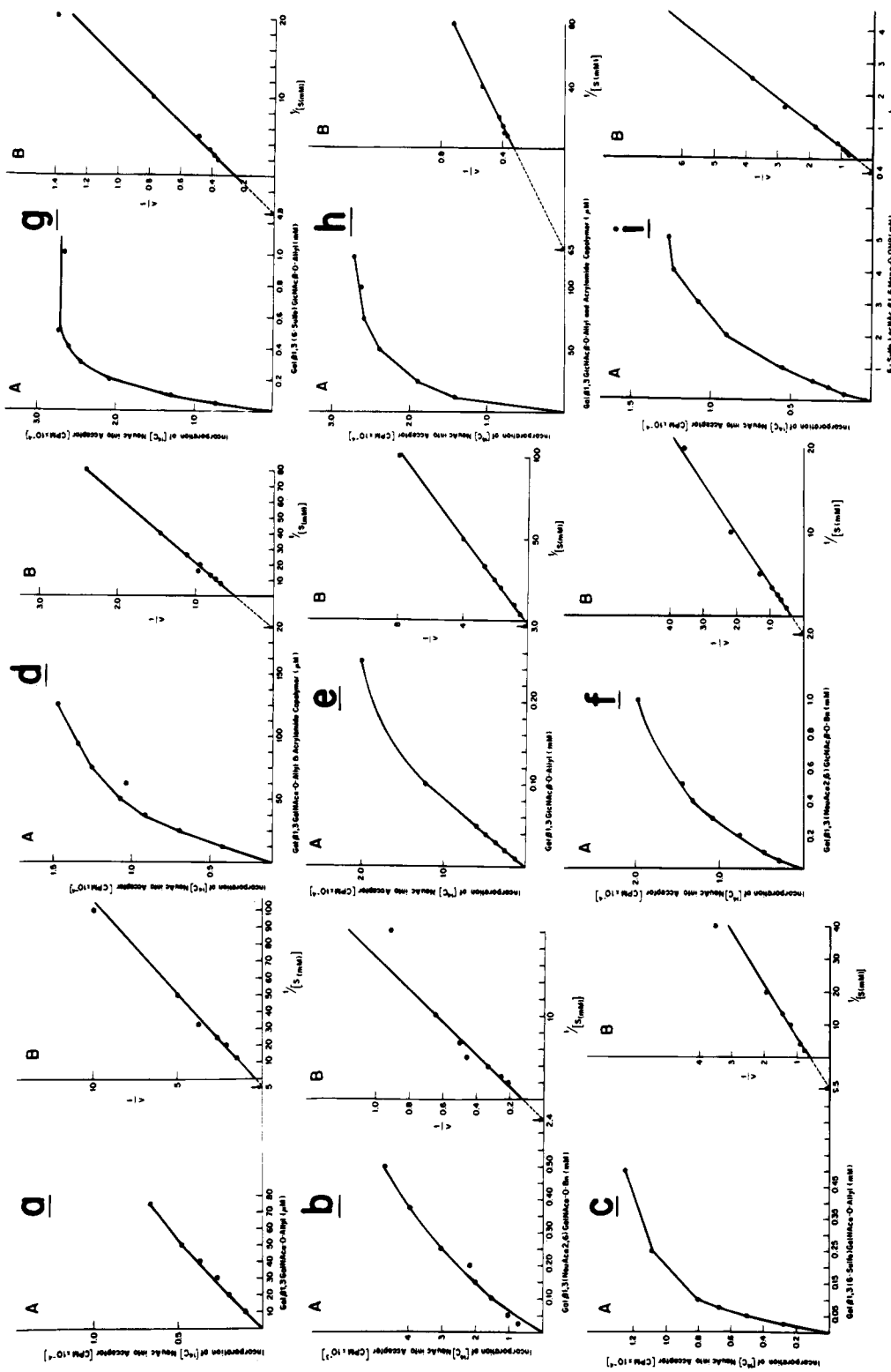

FIGURE 2: Porcine liver α2,3-sialyltransferase activity (a−d) and cloned α2,3-sialyltransferase activity (e−i). In each panel, (A) shows α2,3-sialyltransferase activity at varying concentrations of acceptor, and (B) shows a determination of K_m and V_{max} by Lineweaver−Burke plot. (a) Galβ1,3GalNAcα-O-Al; (b) Galβ1,3(NeuAcα2,6)GalNAcα-O-Bn; (c) Galβ1,3(6-sulfo)GalNAcα-O-Al; (d) Galβ1,3GalNAcα-O-Al/AA-CP; (e) Galβ1,3GlcNAcβ-O-Al; (f) Galβ1,3(NeuAcα2,6)GlcNAcβ-O-Bn; (g) Galβ1,3(6-sulfo)GlcNAcβ-O-Al; (h) Galβ1,3GlcNAcβ-O-Al/AA-CP; (i) 6-sulfoLacNAcβ1,-6Manα-O-ONP.

Table 5: Facile α2,3-Sialylation of Mono as Well as Multi Units of Galβ1,3GlcNAcβ- and Galβ1,3(6-sulfo)GlcNAcβ- by the Cloned Sialyltransferase

acceptor	donor CMP-[^{3}H]NeuAc		product [^{3}H]NeuAc moiety		utilization	susceptibility of products to NDV sialidase[a]		
						amount taken [^{3}H] NeuAc		release of sialic
	CPM × 10^{-5}	μmol	CPM × 10^{-5}	μmol	CMP-NeuAc %	cpm × 10^3	nmol	acid (%)
Galβ1,3(6-sulfo)GlcNAcβ-O-Allyl (0.6 μm)	5.72	0.60	3.74	0.39	65.4	3.7	3.9	100.0
Galβ1,3GlcNAcβ-O-Allyl/AA-CP (4 mg) (0.1 μm)	9.51	1.20	7.27	0.92	76.7	7.8	9.8	49.8
Galβ1,3(6-sulfo)GlcNAcβ-O-Allyl/AA-CP (4 mg) (0.1 μm)	9.66	1.20	5.53	0.69	57.5	5.6	6.9	70.9

[a] 0.4 milliunit of Newcastle disease virus (NDV) sialidase was used in each case.

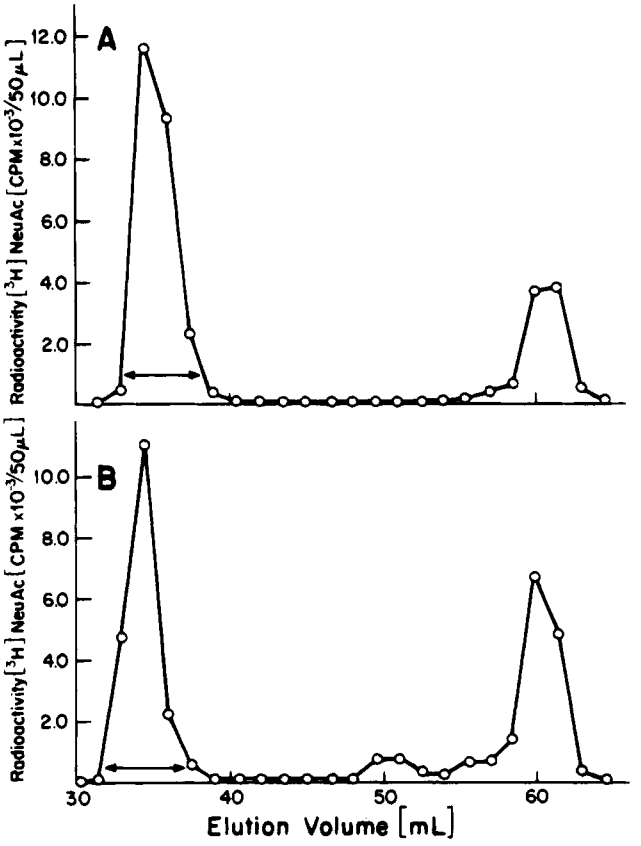

FIGURE 3: (A) Isolation of [9-^{3}H]sialylGalβ1,3GlcNAcβ-O-Al/AA-CP from a large-scale reaction mixture by chromatography on a Biogel P2 column (1.0 × 116.0 cm) using 0.1 M pyridine acetate, pH 5.4, as the eluent. (B) Isolation of [9-^{3}H]sialylGalβ1,3(6-sulfo)-GlcNAcβ-O-Al/AA-CP from a large-scale reaction mixture as described in (A).

quantitative difference in the incorporation of Fuc between the highly anionic copolymer containing both 3′-sialyl and 6-sulfo groups and the other copolymer containing only 3′-sialyl groups. The product from the acceptor [^{3}H]NeuAcα2,-3Galβ1,3(6-sulfo)GlcNAcβ-O-Al was isolated by chromatography on Biogel P2 (Figure 4B) and further separated from the unreacted radioactive acceptor by TLC (see Figure 5, lanes 1 and 2). The purified product contained a similar amount of Fuc.

Activity of HL-60 Sialyltransferase toward Synthetic Acceptors (Table 8). Both Galβ1,3GalNacα-O-Al and its 6-sulfo derivative served as good acceptors. In contrast to the porcine liver ST, the C-6 sialyl derivative served as a poor acceptor for HL-60 ST (only 8.3% activity as compared to Galβ1,3GalNAcα-O-Al). Furthermore, in contrast to the cloned ST, the HL-60 ST was capable of transferring sialic acid to Galβ1,4GlcNAcβ-O-Bn but not to Galβ1,3GlcNAcβ-O-Al, and this activity amounted to about 10% of the activity toward Galβ1,3GalNAcα-O-Al. As seen with the porcine liver enzyme, the copolymer Galβ1,3GalNAcα-O-Al/AA-CP served as a good acceptor for HL-60 ST also.

DISCUSSION

The present study has shown that α2,3-sialylation of Gal by porcine liver ST is affected by the bulkiness of the substituent on C-6 of the penultimate sugar, namely, GalNAcα-; methyl, sulfo, and sialyl derivatives were respectively 79%, 57%, and 37% active as the acceptors for this ST. The C-6 hydroxyl group on Gal appears to be needed for the enzyme activity since C-6 substitution on Gal abolished completely the C-3 sialylation. This enzyme (0.016 milliunit) transferred in a 2-h period 1.34 nmol of NeuAc to Galβ1,3GalNAcα-O-Al/AA-CP, whose concentration was 0.125 mM in the reaction mixture, and 0.42 nmol of NeuAc to asialo CGM, whose concentration was 0.050 mM in the reaction mixture; 0.16 milliunit of the enzyme transferred 15.0 nmol of NeuAc to the copolymer (0.125 mM), and 0.45 milliunit of the enzyme transferred 54.9 nmol of NeuAc to asialo CGM (0.050 mM) when incubation was done for a longer period. The above results would indicate that the above macromolecules serve as very good acceptors for porcine liver ST.

Table 6: Distribution of [^{3}H]Sialyl Compounds in Dowex-1-Cl Fractions

	fractions and radioactivity (%)				
	water	NaCl, 0.1 M	NaCl, 0.2 M	NaCl, 0.3 M	NaCl, 2.0 M
[^{3}H]sialylGalβ1,3(6-sulfo)GlcNAcβ-O-Al[a]	0.6	2.6	1.9	57.3	37.6
[^{3}H]sialylGalβ1,3GlcNAcβ-O-Al/AA-CP[b]	44.3	50.3	4.0	1.3	0.1
[^{3}H]sialylGalβ1,3(6-sulfo)GlcNAcβ-O-Al/AA-CP[b]	10.2	12.8	8.5	11.2	57.3

[a] Fractions after concentration by lyophilization to 1.0 mL were desalted on a Biogel P2 column. [b] Fractions were desalted by dialysis at 4 °C against water. When Galβ1,3(6-sulfo)GlcNAcβ-O-Al/AA-CP as such was subjected to Dowex-1-Cl fractionation, 20.9, 17.1, 11.6, 10.7, and 39.7% of this material (determination of Gal by anthrone reaction) was found in water and 0.1, 0.2, 0.3, and 2.0 M NaCl eluates, respectively. After sialylation of this copolymer, the 2.0 M NaCl elutable material increased from 39.7% to 57.3%.

Table 7: Demonstration of Enzymatic α1,4-L-Fucosylatin of the GlcNAc Moiety in Mono and Multi Galβ1,3GlcNAcβ- Units Containing Either 3′-Sialyl or both 3′-Sialyl and 6-Sulfo Groups

	double-labeled (^{3}H and ^{14}C) radioactive product			
	[9-^{3}H]NeuAc		[^{14}C]Fuc	
acceptor in the reaction mixture	cpm	nmol	cpm	pmol
3-[9-^{3}H]sialylGalβ1,3GlcNAcβ-O-Al/AA-CP[a] (0.29 mM bound and its sialic acid and 0.024 mM CP) and its Dowex-1-Cl fractions[a]	4710	5.9	7349	23.7
water-eluted material	10 160	12.7	18 363	59.2
0.1 M NaCl-eluted material	10 167	12.7	16 403	52.9
3-[9-^{3}H]SialylGalβ1,3(6-sulfo)GlcNAcβ-O-Al/AA-CP[a] (0.22 mM bound sialic acid and 0.018 mM CP) and its Dowex-1-Cl fraction[a]	3994	5.0	10 410	33.6
2.0 M NaCl-eluted material	7047	8.8	17 484	56.4
3-[9-^{3}H]sialylGalβ1,3(6-sulfo)GlcNAcβ-O-Al[b] (0.16 mM)				
before TLC	29 600	31.2	12 012	38.7
after TLC	5690	6.3	7760	25.0

[a] The radioactive product that stayed at the origin when the reaction mixture was subjected to TLC using the solvent system ethyl acetate–pyridine–H_2O–acetic acid (5:5:3:1) was quantitated by liquid scintillation after the silica gel was scraped from the plate. [b] The radioactive product in the reaction mixture was separated from GDP[^{14}C]Fuc and [^{14}C]Fuc on a Biogel P2 column and further separated from the contaminating acceptor by TLC using $CHCl_3$–CH_3OH–H_2O (5:4:1).

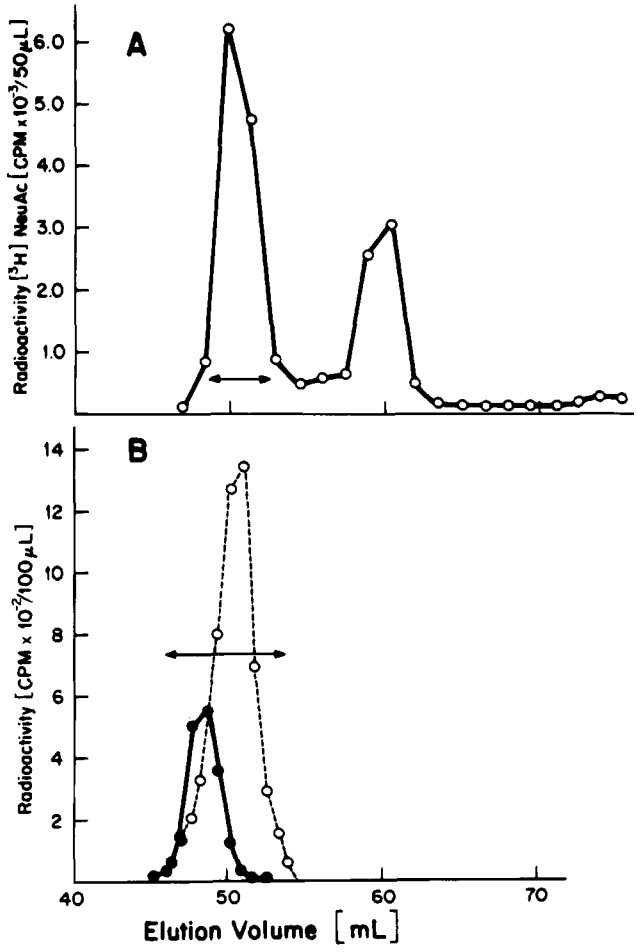

FIGURE 4: (A) Isolation of [9-^{3}H] sialyl Galβ1,3(6-sulfo)GlcNAcβ-O-Al from a large scale reaction mixture as described in Figure 11. (B) Elution profiles of the ^{14}C and ^{3}H radioactivities from the Biogel P2 column when fractionation was done after enzymatic [^{14}C]fucosylation of [9-^{3}H]sialylGalβ1,3(6-sulfo)GlcNAcβ-O-Al. (●) [^{14}C]Fuc; (○) [^{3}H] NeuAc.

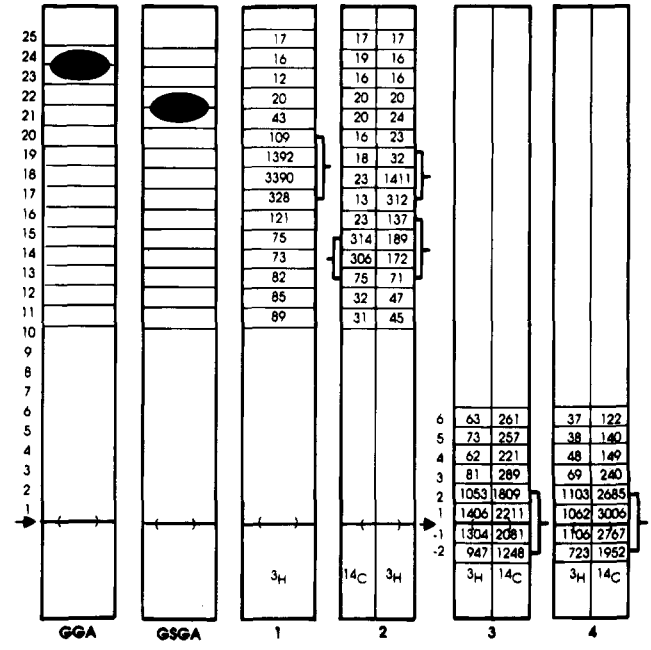

FIGURE 5: Thin-layer chromatography for demonstrating the formation of α1,4-[^{14}C]fucosyl compound from 3′-[^{3}H]sialyl acceptors: GGA, Galβ1,3GlcNAcβ-O-Al;GSGA, Galβ1,3(6-sulfo)-GlcNAcβ-O-Al; 1, [^{3}H]NeuAcα2,3Galβ1,3(6-sulfo)GlcNAcβ-O-Al; 2, separation of the product 3-[^{3}H]-sialylGalβ1,3(4-[^{14}C]fucosyl-6-sulfo)GlcNAcβ-O-Al from the radioactive ^{3}H-labeled substrate; 3, reaction mixture containing the acceptor [^{3}H]NeuAcα2,3Galβ1,-3GlcNAcβ-O-Al/AA-CP; 4, reaction mixture containing the acceptor [^{3}H]NeuAcα2,3Galβ1,3(6-sulfo)GlcNAcβ-O-Al/AA-CP. For details, see Experimental Procedures.

The present investigation identified that Gal linked β1,3 to GlcNAc is the preferred substrate for the cloned ST since the enzymatic 3′-sialylation dropped to 15.8% when Gal was in β1,4 linkage with GlcNAc. Such a preference was also exhibited by the two (H and secretor) types of α1,2-L-fucosyltransferases present in human serum with regard to their ability to transfer Fuc to Gal linked β1,3 or β1,4 to GlcNAc (Sarnesto et al., 1992). In contrast to the porcine liver ST, the cloned enzyme was not affected at all by C-6 substitution on the penultimate sugar, i.e., GlcNAc with either a methyl or a sulfate group; even a bulky group like NeuAc on C-6 of GlcNAc did not much affect the enzymatic transfer of sialic acid to C-3′ (70% activity). The C-4 substitution on GlcNAc by Fuc abolished the 3′-sialylation; even methyl, the least bulky group, on C-4 lowered the acceptor efficiency to 28.5%. Thus, the reduced activity noted above when C-4 hydroxyl is tied up by β1,4-linked Gal and the loss of

Table 8: Levels of Sialyltransferase Activities in HL-60 Cells, as Discerned with Specific Synthetic Acceptors

acceptor (0.8 mM)	incorporation of [9-^{3}H]NeuAc into acceptor			
	cpm	NeuAc (pmol)	%	nmol/h/mg of protein
Galβ1,3GalNAcα-O-Allyl	19 556	65	100	0.41
Galβ1,3(6-sulfo)GalNAcα-O-Allyl	20 068	67	102	0.42
Galβ1,3(NeuAcα2,6)GalNAcα-O-Allyl	1615	5	8	0.03
6-sulfoGalβ1,3GalNAcα-O-Allyl	0	0	0	0
3-sulfoGalβ1,3GalNAcα-O-Allyl	0	0	0	0
3-O-MeGalβ1,3GalNAcα-O-Bn	0	0	0	0
Galβ1,3GlcNAcβ-O-Allyl	0	0	0	0
Galβ1,4GlcNAcβ-O-Bn	2121	7	11	0.04
Galβ1,3GalNAcα-O-Allyl/acrylamide copolymer (100 μg) (0.125 mM)	28 308	95	145	0.59

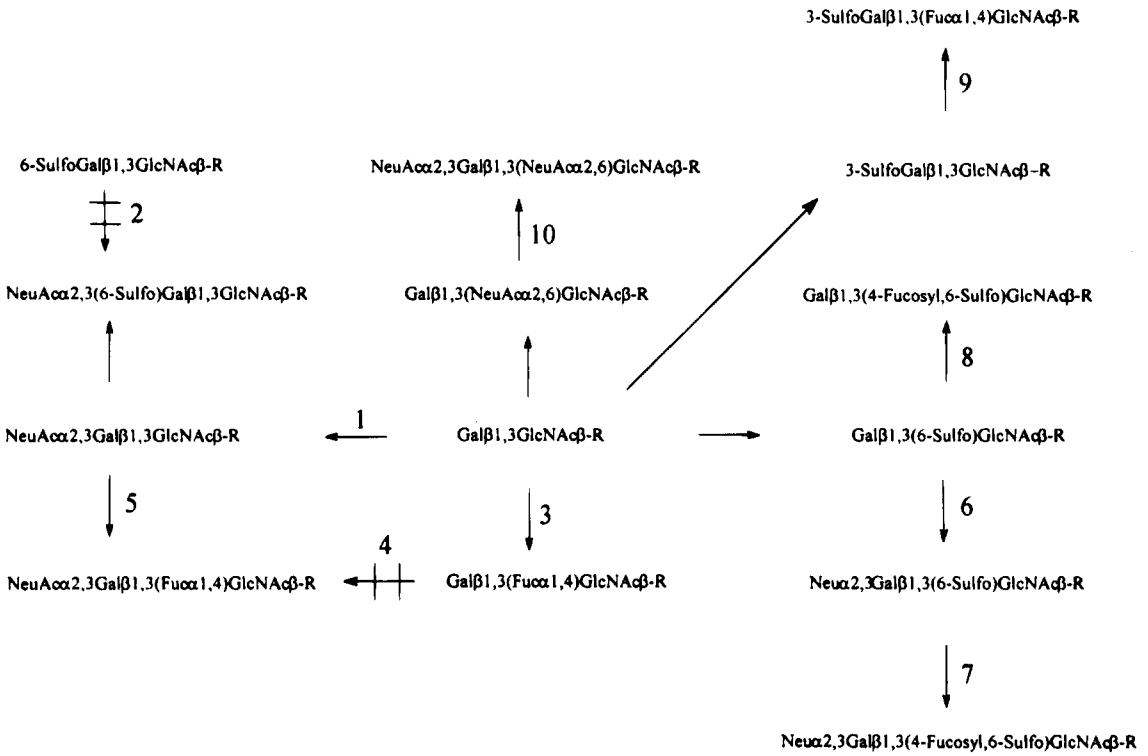

FIGURE 6: Assembly of structures related to selectin ligands as evident from our studies.

acceptor ability when C-4 is substituted with Fuc or a methyl group would suggest that the cloned ST requires the free hydroxyl group at C-4 of GlcNAc for carrying out its catalytic activity.

The α2,3-sialylation of Gal in the Lewis a structure [Galβ1,3(Fucα1,4)GlcNAcβ-] was shown in the present study as not being catalyzed by the cloned ST, thus indicating that sialylation must precede the α1,4-fucosylation in the biosynthetic pathway of this structure. We have gained evidence along these lines, by using the cloned ST for isolating the [9-^{3}H]sialyl compounds from the acceptors Galβ1,3GlcNAcβ-O-Al/AA-CP and its Dowex-1-Cl fractions (water- and 0.1 M NaCl-eluted materials), Galβ1,3(6-sulfo)-GlcNAcβ-O-Al/AA-CP and its Dowex-1-Cl fraction (2.0 M NaCl-eluted material), and also Galβ1,3(6-sulfo)GlcNAcβ-O-Al; the above ^{3}H-labeled compounds were then shown by us to be α1,4-[^{14}C]fucosylated by using a partially purified fucosyltransferase preparation from Colo 205 (Chandrasekaran et al., 1994b).

Very recently Hemmerich and Rosen (1994a) proposed from lectin binding studies the structure of the major capping group of GlyCAM-1, which is a ligand for L-selectin, as NeuAcα2,3(6-sulfo)Galβ1,4(Fucα1,3)GlcNAc. The 3′-sialylation of both Galβ1,3GalNAcα-O-Al by porcine liver ST and Galβ1,3GlcNAcβ-O-Al by the cloned ST was shown in the present study to be completely abolished when a sulfate group was present on C-6 of the Gal moiety. On the other hand, porcine liver ST showed 56.9% activity when the sulfate group was present on C-6 of the GalNAc moiety. Further, when there is a sulfate group on C-6 of GlcNAc in Galβ1,3GlcNAcβ-, the cloned ST is found to be fully capable of 3′-sialylation. Hemmerich et al. (1994b) reported that C-6 sulfation occurs in approximately equal amounts on Gal and GlcNAc residues in GlyCAM-1. The specificities of the lectins used by this group in structural characterization were not tested with saccharides containing sulfate on C-6 of the GlcNAc moiety (Yamashita et al., 1992). Hence, as stated by the above group, it becomes obvious to apply physical techniques and methylation analyses for arriving at confirmation of their above proposed structure for the L-selectin ligand. If their proposed structure turns out to be correct, then it appears to be convincing from our present studies and from the report of Pfeiffer et al. (1992) on the formation of the NeuAcα2,3(6-sulfo)Gal unit present in recombinant human tissue plasminogen activator that C-3 sialylation of Gal in Galβ1,4GlcNAcβ- must precede the C-6 sulfation of

Gal in the biosynthetic pathway. Figure 6 illustrates the biosynthetic pathway leading to selectin ligand related structures and is based on the specificities of the enzymes we have studied so far. Steps 1, 2, 4, 5, 6, 7, and 10 were shown to occur in the present study, and steps 3, 8, and 9 were already reported by us (1992b).

Carlsson, Sasaki, and Fukuda (1986) found O-linked saccharides such as NeuAcα2,3Galβ1,3GalNAcα- and also structures such as NeuAcα2,3Galβ,4GlcNAcβ- in leukosialin of HL-60 cells. Maemura and Fukuda (1992) demonstrated the presence of α2,3-ST in HL-60 cells for the sialylation of Galβ1,3GalNAcα-. We have also shown in the present study the presence of this ST activity in HL-60. Further, we established that C-6 sulfation of GalNAc in Galβ1,-3GalNAcα- did not affect the sialylation by HL-60 cells, but C-6 sialylation almost abolished this activity. Thus, the HL-60 ST seems to differ from the porcine liver ST in its specificities. In addition to the above ST activity, we extended the identification of the structure, NeuAcα2,-3Galβ1,4GlcNAcβ- in leukosialin of HL-60 cells by Fukuda's laboratory, by showing the presence of other α2,3-ST activity in HL-60 acting on Galβ1,4GlcNAc; interestingly, this enzyme appeared to be entirely different from the cloned ST since the former acted only on Galβ1,4GlcNAcβ- but not on Galβ1,3GlcNAcβ-. Maemura and Fukuda (1992) showed the presence of α2,6-ST activity by using the acceptor NeuAcα2,3Galβ1,3GalNAcα-O-PNP. In the present study, we were not able to measure this activity of HL-60 cells by using the acceptors 3-sulfoGalβ1,3GalNAcα-O-Allyl and 3-O-methylGalβ1,3GalNAcα-O-Bn, thus indicating the possible specific requirement of 3′-sialylation for this activity.

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