Identification of Physiologically Relevant Substrates for Cloned Gal: 3-O-Sulfotransferases (Gal3STs)

DISTINCT HIGH AFFINITY OF Gal3ST-2 and LS180 SULFOTRANSFERASE FOR THE GLOBO H BACKBONE, Gal3ST-3 FOR N-GLYCAN MULTITERMINAL Galβ1,4GlcNAcβ UNITS AND 6-SULFOGalβ1,4GlcNAcβ, AND Gal3ST-4 FOR THE MUCIN CORE-2 TRISACCHARIDE*

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Sulfated glycoconjugates regulate biological processes such as cell adhesion and cancer metastasis. We examined the acceptor specificities and kinetic properties of three cloned Gal:3-O-sulfotransferases (Gal3STs) ST-2, ST-3, and ST-4 along with a purified Gal3ST from colon carcinoma LS180 cells. Gal3ST-2 was the dominant Gal3ST in LS180. While the mucin core-2 structure Galβ1,4GlcNAcβ,6(3-O-MeGalβ1,3)GalNAc-O-Bn (where Bn is benzyl) and the disaccharide Galβ1,4GlcNAc served as high affinity acceptors for Gal3ST-2 and Gal3ST-3, 3-O-MeGalβ1,4GlcNAcβ,6(Galβ1,3)GalNAc-O-Bn and Galβ1,3GalNAc-O-Al (where Al is allyl) were efficient acceptors for Gal3ST-4. The activities of Gal3ST-2 and Gal3ST-3 could be distinguished with the Globo H precursor (Galβ1,3Galβ1,4GlcNAcβ,3Gal-O-Me) and fetuin triantennary asialoglycopeptide. Gal3ST-2 acted efficiently on the former, while Gal3ST-3 showed preference for the latter. Gal3ST-4 also acted on the Globo H precursor but not the glycopeptide. In support of the specificity, Gal3ST-2 activity toward the Galβ1,4GlcNAcβ unit on mucin core-2 as well as the Globo H precursor could be inhibited competitively by Galβ1,4GlcNAcβ,6(3-O-sulfoGalβ1,3)GalNAc-O-Bn but not 3-O-sulfoGalβ1,4GlcNAcβ,6(Galβ1,3)GalNAc-O-Bn. Remarkably these sulfotransferases were uniquely specific for sulfated substrates: Gal3ST-3 utilized Galβ1,4(6-O-sulfo)-GlcNAcβ-O-Al as acceptor, Gal3ST-2 acted efficiently on Galβ1,3(6-O-sulfo)GlcNAcβ-O-Al, and Gal3ST-4 acted efficiently on Galβ1,3(6-O-sulfo)GalNAc-O-Al. Mg2+, Mn2+, and Ca2+ stimulated the activities of Gal3ST-2, whereas only Mg2+ augmented Gal3ST-3 activity. Divalent cations did not stimulate Gal3ST-4, although inhibition was noted at high Mn2+ concentrations. The fine substrate specificities of Gal3STs indicate a distinct physiological role for each enzyme.

*Sulfate groups located at various defined positions in glycoconjugates are thought to play crucial roles in biological processes. For example, a sulfated Lewis x determinant has been identified to be a major structural motif in mucins isolated from a nude mouse xenograft tumor produced by the human colon carcinoma LS174T-HM7 cell (1). It is suggested that this sulfated determinant may contribute to the high metastatic potential of this cell. A monoclonal antibody evoked to high molecular weight salivary mucins recognizes the epitope 3-O-sulfoGalβ1,3GlcNAc in mucinous epithelia of salivary glands, colon, and uterine cervix, but this epitope is not detectable in healthy stomach, breast, and small intestine (2). Thus, a tissue-specific distribution of sulfated glycosans is noted. A novel cell substrate recognition phenomenon was demonstrated in the interaction between the lectin domains of chondroitin sulfate proteoglycans and cells expressing sulfated glycolipids (3). It is suggested that such molecular recognition may contribute to cell adhesion and migration.

Carbohydrate sulfation has been shown to be important for the formation of ligands that bind adhesion molecules belonging to the selectin family (4, 5). Studies that examine the ability of sulfated carbohydrates to inhibit selectin-ligand recognition also highlight the importance of sulfation to human health. Polymers displaying the selectin recognition epitopes 3’,6-disulfido Lewis x and 3’,6-sulfo Lewis x as potent inhibitors of L-selectin binding to heparin under static cell-free binding conditions with similar efficacies; however, under the conditions of shear flow, only the polymer displaying 3’,6-disulfido Lewis x inhibited the rolling of L-selectin-transfected cells on the glycoprotein ligand glycosylation-dependent cell adhesion molecule-1 (6). Binding inhibition assays utilizing paucivalent L-selectin also identified 3’,6-sulfo Lewis x and 3’-sulfo Lewis x as potent inhibitors of L-selectin binding (7). In our studies, we have also found that substitution of a sialyl group with a sulfate group in GalNAcβ1,4(Fucα1,3)GlcNAcβ,6(NeuAcα2,3Galβ1,3)-GalNAc-O-Me reduced considerably its inhibitory potential of L- and P-selectin binding (8).

In previous studies (9, 10), we identified two distinct Gal:3-O-sulfotransferases (Gal3STs) in tumor tissues and cancer cells that exhibited distinct acceptor preferences. Specifically, while enzymes from colon cell lines and colon tissue prefer to sulfate the C-3 position of Gal in the Galβ1,4GlcNAcβ-moiety of the mucin core-2 structure (Fig. 1), breast cancer cells prefer the Galβ1,3GalNAcα-moiety. In similar biochemical studies,
Distinct Specificities of Gal3:O-Sulfotransferases

**Gal3:O-Sulfotransferase**

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Gal3:1→4GlcNAcβ1
   6  core-2 structure
Gal3:1→3GalNAcβ1→3Galα→ Globo H backbone
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**Core-2 tetrasaccharide and Globo H precursor.** Enzymes that prefer to act on the Gal residue of the Galβ1→4GlcNAcβ compared with the Gal residue on the mucin core-2 β1,3 branch (11). This enzyme also acted on the cancer antigen Globo H backbone Galβ1,4GlcNAcβ,3Galα-structure in the core-2 tetrasaccharide. The possibility that the Gal3:O-sulfotransferase from colon tissue, similar to the α1,2-l-fucosyltransferase and α2,3-l-fucosyltransferase, also acts on the Globo H antigen remains undetermined, and we tested this here. Further the cloning of three distinct Gal3:O-sulfotransferases recently, GalβST-2 (12), GalβST-3 (13), and GalβST-4 (14), provides us with an opportunity to determine the acceptor specificities and kinetic properties of these proteins with emphasis on their action on core-2-based acceptors. These studies have led us to identify novel, high affinity, and specific acceptors for each of these enzymes, suggesting that each enzyme has a distinct physiological role.

**EXPERIMENTAL PROCEDURES**

**Cell Culture—**Human colon carcinoma LS180 and Chinese hamster ovary CHO-S cells were obtained from American Type Culture Collection (ATCC, Manassas, VA). LS180 was grown in 2-liter roller bottles in Leibovitz’s L-15 medium supplemented with 10% fetal bovine serum (HyClone, Logan, UT) and antibiotics (penicillin, streptomycin, and amphotericin B). Harvested cells were stored frozen at −20 °C prior to GalβST purification. CHO-S was cultured in Dulbecco’s modified Eagle’s medium (Invitrogen) with 10% heat-inactivated fetal bovine serum in tissue culture incubators.

**Expression of Cloned GalβST—**The following plasmids containing genes that encode various Gal3STs were used in the current work: human prostate carcinoma cell LNCaP has α1,2-α1,3GalNAcβ1,4-β1,3GalNAcβ3Galα, a cloned α1,2-sialyltransferase, ST-3GalIV, utilized both the Globo H backbone and the Galβ1,4GlcNAcβ-structure in the core-2 tetrasaccharide. Two sets of primers were designed for PCR amplification. The first set corresponded to the amino acid sequences FLKTHKT, FLKTHKS, DESVLRR, and DESVLRA, which are conserved among all cloned GalβSTs. The sense primers corresponding to these sequences were 5′-TTCTCAAGACTCAAAGCC-3′ and 5′-TGCAAGACATATAACCC-3′, and the antisense primers used were 5′-CCGCAGCAGCTAGGACTCTGC-3′ and 5′-TGCCACGAGAACCCATGACTCATC-3′, respectively.

**RT-PCR to Identify Sulfotransferase Genes in LS180—**Total RNA extracted from LS180 cells using TRIzol reagent (Invitrogen) was subjected to RT-PCR using Superscript one-step RT-PCR with platinum Taq (Invitrogen). Total RNA was reverse-transcribed with oligo(dT) primers at 42 °C for 1 h and then subjected to PCR using Taq DNA polymerase. Two sets of primers were designed for PCR amplification. The first set corresponded to the amino acid sequences FLKTHKT, FLKTHKS, DESVLRR, and DESVLRA, which are conserved among all cloned GalβSTs. The sense primers corresponding to these sequences were 5′-TTCTCAAGACTCAAAGCC-3′ and 5′-TGCAAGACATATAACCC-3′, and the antisense primers used were 5′-CCGCAGCAGCTAGGACTCTGC-3′ and 5′-TGCCACGAGAACCCATGACTCATC-3′, respectively.

**Assay of Sulfotransferase—**The incubation mixture run in duplicate contained 100 ml Tris maleate buffer with 6 ml of Lipopectamine 2000 (Invitrogen)/well along with 6 μg/ml of one of the above plasmids. The cells were passed into T75 flasks 1 day after transfection, cultured for 3 days, then harvested, and kept frozen at −20 °C until use.

**Purification of Gal3:O-Sulfotransferase from LS180 Cells—**5.5 × 10^9 LS180 cells were homogenized in a Dounce all-glass hand-operated grinder with 60 ml of 0.1 M Tris maleate buffer (pH 7.2) containing 10 mM magnesium acetate, 2% Triton X-100, 20% glycerol, 30 μM phenylmethylsulfonyl fluoride, and 0.1% NaCl, and then stirred for 2 h at 4 °C. The supernatant was subjected to chromatography on a 25-ml bed volume of Aleuria aurantia lectin-agarse (Vector Laboratories, Burlingame, CA) column, which had been washed and equilibrated with the above buffer. After entry of the sample into the column bed, the column was washed with 60 ml of the buffer. The bound proteins were eluted sequentially with 100 ml each of 0.5 M sucrose and 2 M NaCl in the same buffer. Both eluates were concentrated separately by Amicon ultrafiltration using PM10 membranes to a small volume and dialyzed against 2 liters of the buffer with five changes in the cold room for 48 h. The concentrated and dialyzed fucose-eluted fraction (10 ml) was then applied to a 10-ml volume affinity gel-DGP (Calbiochem) column equilibrated with the same buffer. The affinity column was washed with 20 ml of the buffer and then eluted with 30 ml of 2 M NaCl in the same buffer. The NaCl eluate was concentrated and dialyzed as above. This preparation (3 ml) was further purified on a Sephacryl S-100 HR column (25.0 × 118.0 cm) at 4 °C, equilibrated, and eluted with 0.1 M Tris maleate, pH 7.2, containing 0.1% Triton X-100 and 0.02% NaCl. Fractions of 2 ml at a flow rate of 6 ml/h were collected, and 10 μl of alternate fractions were assayed for sulfotransferase activity using Galβ1,4GlcNAcβ1,6-O-MeGalβ1,3GalNAcO-Bn as an acceptor. The enzyme activity emerged as a single peak from the column soon after the void volume. The fractions were pooled and concentrated by ultrafiltration and dialyzed against the extraction buffer. This fraction (0.8 ml) was stored frozen at −20 °C and used for enzymology studies.

**Synthetic Acceptors—**The synthesis of several compounds that we have used as acceptors in the present study have been published (8, 15–17). The synthesis of acceptors containing the Globo H precursor structure, namely Galβ1,3GalNAcβ1,3Galα and 5′-TGCCACGAGAACCCATGACTCATC-3′ (antisense), and the other set was 5′-ATAATTCCGGAGACCTGGTCTAGACC-3′ (sense) and 5′-CAGAGACCTGGTCTAGACC-3′ (antisense). PCR was performed in all cases using the denaturation temperature of 94 °C, annealing temperature of 55 °C, and extension temperature of 72 °C. RT-PCR products were sequenced by dideoxy chain termination method to determine the sulfotransferase mRNA in LS180.
Distinct Specificities of Gal:3-O-Sulfotransferases

Table I

| Fraction                  | Volume | Total protein | Enzyme activity<sup>a</sup> | Enzyme specific activity<sup>a</sup> | Purification | Recovery of enzyme activity |
|---------------------------|--------|--------------|-----------------------------|--------------------------------------|--------------|-----------------------------|
| 1. Homogenate             | 65     | 1485         | 3564                        | 0.24                                 | 1.0          | 100.0                       |
| 2. Triton X-100 supernatant | 60     | 810          | 3159                        | 0.39                                 | 1.6          | 88.9                        |
| 3. <i>A. aurantia</i> lectin-agarose chromatography: fucose eluate (concentrated and dialyzed) | 10     | 52           | 169.0                       | 3.25                                 | 13.5         | 47.3                        |
| 4. Chromatography on affinity gel-GDP: NaCl eluate (concentrated and dialyzed) | 3      | 0.8          | 143.0                       | 178.80                                | 745.0        | 40.0                        |
| 5. Chromatography on Sephacryl S-100 HR column (concentrated and dialyzed) | 0.8    | 0.15         | 108.4                       | 722.60                                | 3010         | 30.4                        |

<sup>a</sup> 1 milliunit is defined as the transfer of 1 nmol of sulfate from [35S]PAPS to the acceptor Galβ1,4GlcNAcβ1,6-3-O-MeGalβ1,3GalNAcO-Bn (7.5 mM) catalyzed by Gal3:3-sulfotransferase in 1 h at 37°C.

The acceptor was quantitated by TLC method using CHCl<sub>3</sub>:CH<sub>3</sub>OH:H<sub>2</sub>O (4:1, v/v) as the solvent system (9, 10).

Identification of the [35S]-Sulfated Compound Arising from the Acceptor Galβ1,3GalNAcβ1,3gal-O-Me by the Action of the LS180 Sulfotransferase—A 10-fold standard reaction mixture (200 µl) containing Galβ1,3GalNAcβ1,3gal-O-Me was incubated for 2 h at 37°C with purified enzyme. After dilution with 1.0 ml of water, it was fractionated by the Dowex-1-Cl method. The 0.2 mM NaCl eluate was lyophilized to a small volume (1 ml) and desalted on a Biogel P2 column (1.0 × 116.0 cm) using 0.1 M pyridine acetate, pH 5.4, as the eluting buffer. The radioactive fractions emerging first as a major peak were pooled, lyophilized to dryness, and dissolved in 200 µl of water. A small aliquot was subjected to TLC (silica gel GHLF, 250 µm scored 20 × 20 cm, Analtech) using 1-propanol:NH<sub>3</sub>OH (25%/water (50:10:25, v/v) along with authentic Galβ1,3GalNAcβ1,3Gal-O-Me and 3-0-sulfogalβ1,3GalNAcβ1,3gal-O-Me as reference compounds. The reference compounds were located on TLC plates by spraying with sulfuric acid in ethanol and heating at 100°C. The radioactive compound was located by scraping 0.5-cm-wide segments of the silica and soaking in 2.0 ml of water followed by liquid scintillation counting.

Testing for Competitive Inhibition—For testing the competitive inhibition by the acceptors on Gal:3-O-sulfotransferases, we took advantage of the fact that the radioactive product arising from the monosulfated compounds 3-O-sulfogalβ1,4GlcNAcβ1,6galβ1,3GalNAcO-Bn and Galβ1,4GlcNAcβ1,6-3-O-sulfogalβ1,3GalNAcO-Bn cannot be eluted, whereas the product from the neutral acceptors can be eluted from the Dowex-1-Cl column by 0.2 mM NaCl. For these runs, the concentrated neutral acceptor was left constant (6 mM) and that of the sulfated compound was varied from 0 to 7.5 mM under the standard conditions.

RESULTS

Gal3ST Purified from LS180 Acts Both on Galβ1,4GlcNAcβ in Mucin Core-2 as Well as Terminal Galβ in Globo H Precursor

Gal3ST was purified from LS180 cells using a series of chromatography steps (Table I). The enzyme was purified 750-fold with a recovery of 40% using the three steps of Triton X-100 solubilization, <i>A. aurantia</i> lectin-agarose chromatography, and fractionation on affinity gel-GDP. When this purified preparation was further subjected to chromatography on a Sephacryl S-100 HR column, we obtained a single peak of activity (data not shown) emerging from the column soon after the void volume as measured with blue dextran 2000 but prior to our bovine serum albumin standard (molecular mass, 66,000 Da). The enzyme at this stage was purified 3000-fold with 30% recovery of activity. The enzyme exhibited a relatively low specific activity of ~700 milliunits/mg suggesting that it was only partially purified, and this was confirmed by silver staining of the SDS-polyacrylamide gel of the partially purified fraction (data not shown). GlcNAc:6-O-sulfotransferase activity is not exhibited by sulfotransferases in LS180 cells (10), and this was the case for our fraction as well. Further, as shown below, the partially purified enzyme exhibited activity that resembled only one of the three cloned Gal3STs studied in the current work suggesting that it contains only one Gal3ST.

The activity of the purified LS180 Gal3ST was examined using a series of acceptors based on the core-2 mucin structure (Table II). This enzyme was poorly active with Galβ1,3GalNAcβ-A-O-Al (20.2% activity as compared with its activity toward Galβ1,4GlcNAcβ1,6-3-O-MeGalβ1,3GalNAcO-Bn) and the mucin core-2 analog 3-O-MeGalβ1,4GlcNAcβ1,6Galβ1,3-GalNAcO-Bn (15.9% activity), thus suggesting that it preferred to sulfate the Galβ1,4GlcNAcβ unit on the core-2 mucin. In further support of this, it was noted that Galβ1,4GlcNAcβ1,6-(Galβ1,3),GalNAcO-Bn was a comparable acceptor to Galβ1,4GlcNAcβ1,6-3-O-MeGalβ1,3,GalNAcO-Bn (activities 95.6 and 100.0%, respectively). Galβ1,3,GlcNAcβ1,6,GalNAcO-Bn showed low activity (8.7%), and as anticipated 3-O-MeGalβ1,3,GlcNAcβ1,6,GalNAcO-Bn was an inactive acceptor. Together the above data suggest that the enzyme has very low affinity for the β1,3-linked Gal moiety in mucin core-2, and it is devoid of GlcNAc:sulfotransferase activity. It prefers to act on the β1,4-linked Gal in the mucin core-2 structure.

The acceptors containing the Globo H precursor structure Galβ1,3,GalNAcβ1,3Galo-O-AI and Galβ1,3,GalNAcβ1,3Galo-O-Me served as better acceptors for the enzyme than the LacNAc moiety in mucin core-2. An analog of this structure, Galβ1,3,GlcNAcβ1,3Galo-O-Me, showed low activity (20.6 and 5.0%, respectively). We synthesized a compound by substituting d-Fuc for d-Gal in the acceptor structure Galβ1,3,GalNAcβ1,3Galo-O-Me; this is equivalent to converting the Gal moiety to its 6-deoxy derivative. The newly synthesized compound d-Fucβ1,3,GalNAcβ1,3Galo-O-Me was even better than Galβ1,3,GalNAcβ1,3Galo-O-Me as an acceptor (activities 180.5 and 159.3%, respectively). These results indicate that sulfation does not take place on the C-6 hydroxyl of Gal, and further a free OH on C-6 of Gal is not a requirement for substrate binding of the enzyme.

TLC experiments were conducted to determine that the purified enzyme transferred sulfate to the C-3 hydroxyl of the terminal Gal moiety in Galβ1,3,GalNAcβ1,3Gala (Fig. 2). A monosulfated standard compound, 3-O-sulfogalβ1,3,GalNAcβ1,3Galo-O-Me, was synthesized for this purpose. When the [35S]-sulfated product isolated from the action of LS180 sulfotransferase on Galβ1,3,GalNAcβ1,3Gala was subjected to TLC (Fig. 2) along with the synthetic sulfated standard, it was found that the mobility of the radioactive product coincided with that of the synthetic sulfated compound. Thus, based on the combined results from Table II and Fig. 2, it has been tentatively identified that sulfation by LS180 Gal3ST takes place at the C-3 OH of the terminal Gal moiety in the Globo H precursor.
Distinct Specificities of Gal:3-O-Sulfotransferases

The Globo H structure and its 6-deoxy analog are better acceptors than the mucin core-2 structure for colon carcinoma LS180 Gal3ST Activity of the purified LS180 Gal3-O-sulfotransferase

| Acceptor (7.5 mM) | Activity of the purified LS180 Gal3-O-sulfotransferase |
|------------------|--------------------------------------------------------|
|                  | %                                                      |
| 3-O-MeGalβ1,4GlcNAcβ1,6Galβ1,3GalNAco-O-Bn               | 15.9                                                   |
| Galβ1,4GlcNAcβ1,6Galβ1,3GalNAco-O-Bn                      | 100.0 (59.970)                                        |
| 3-O-MeGalβ1,3GlcNAcβ1,6GalNAco-O-Bn                      | 95.6                                                   |
| Galβ1,3GlcNAcβ1,6GalNAco-O-Al                             | 0                                                      |
| Galβ1,3GlcNAcβ-O-Al                                       | 20.2                                                   |
| Acceptor Specificity of Three Cloned Gal3STs: Comparison with LS180 Gal3ST Activity

We used an array of carbohydrate acceptors to determine the detailed specificity of the cloned Gal3STs (Table III). As seen, both Gal3ST-2 and Gal3ST-3 preferred to act on the C-3 hydroxyl group of Galβ1,4GlcNAc rather than Galβ1,3GalNAco-O-Al. These enzymes efficiently utilized the mucin core-2 acceptor Galβ1,4GlcNAcβ1,6(3-O-MeGalβ1,3)GalNAco-O-Bn and its corresponding non-methylated structure. They also acted to a lesser extent on 3-O-MeGalβ1,4GlcNAcβ1,6- (Galβ1,3)GalNAco-O-Bn (19.4–24.0%) and also on the trisaccharide Galβ1,3(GlcNAcβ1,6)GalNAco-O-Al (45.2–67.9%). The activities of these two enzymes can be distinguished by their action on the Globo H precursor Galβ1,3GlcNAcβ1,3Galo-O-Me in that this was a preferred acceptor for Gal3ST-2 (295.6% active), whereas it was a poor acceptor for Gal3ST-3 (25.8% active). On the contrary, fetuin triantennary asialo-GP was a preferred acceptor for Gal3ST-3 (383.9% active) but a relatively poor acceptor for Gal3ST-2 (81.7% active). LS180 Gal3ST resembled Gal3ST-2 when considering its acceptor utilizing efficiencies.

Unlike Gal3ST-2 and Gal3ST-3, Gal3ST-4 had an absolute specificity toward Galβ1,3GalNAco-O-Al. It utilized 3-O- MeGalβ1,4GlcNAcβ1,6(Galβ1,3)GalNAco-O-Bn but not Galβ1,4- GlcNAcβ1,6(3-O-MeGalβ1,3)GalNAco-O-Al as an acceptor. The trisaccharide acceptor Galβ1,3(GlcNAcβ1,6)GalNAco-O-Al (85.7% active) was 2.5-fold as active as 3-O-MeGalβ1,4- GlcNAcβ1,6(Galβ1,3)GalNAco-O-Bn suggesting that the Gal moiety linked to GlcNAc has an inhibitory effect on the enzyme. It also acted on the Globo H precursor Galβ1,3GlcNAcβ1,3-Galo-O-Me and that it has significant influence on this enzyme activity. As anticipated, Gal3ST-4 showed negligible activity toward the acceptors Galβ1,4GlcNAc, Galβ1,4GlcNAcβ1,6Manα1,6Manβ1,6Man, Galβ1,4GlcNAcβ1,6(3-O-MeGalβ1,3)GalNAco-O-Bn, and fetuin triantennary asialo-GP. It showed significant activity when Galβ1,3GalNAco-O-Al/acrylamide copolymer was used as an acceptor at low concentration of 0.125 mM as compared with the reference acceptor Galβ1,3GalNAco-O-Al, which was used at 7.5 mM.

Selected Structural Modifications of Mucin Core-2 Can Reduce Gal3ST Activity

To determine the structural features of the mucin core-2 that influence Gal3ST activity, studies were undertaken with modified mucin core-2 structures (Table IV). Here, as compared with the acceptor Galβ1,4GlcNAcβ1,6(3-O-MeGalβ1,3)GalNAco-O-Bn, LS180 Gal3ST, Gal3ST-2, and Gal3ST-3 exhibited only 12–16% activity toward Galβ1,4GlcNAcβ1,6(3-F-
3-O-MeGalβ1,4GlcNAcβ1,6Galβ1,3GalNAcβ-O-Bn and 3-F-Galβ1,4GlcNAcβ1,6Galβ1,3GalNAc-O-Bn, respectively. Again, analogous to the situation with Gal3ST-2 and Gal3ST-3 discussed above, the presence of hydrophobic entities attached to the β1,4-linked Gal reduced the activity of the Gal3ST-4 enzyme to the Galβ1,3GalNAcβ moiety.

Sulfated Compounds Can Distinguish Gal3ST-2 from Gal3ST-3

As compared with its activity toward Galβ1,4GlcNAcβ1,6Galβ1,3GalNAc-O-Al and 45–55% activity toward Galβ1,4GlcNAcβ1,6Galβ1,3GalNAc-O-Al, this suggests that hydrophobic entities attached to the Galβ1,3 moiety of the core-2 mucin can influence enzyme activity directed toward the Galβ1,4GlcNAcβ structure.

As compared with 3-O-MeGalβ1,4GlcNAcβ1,6Galβ1,3-GalNAc-O-Bn, Gal3ST-4 had only 60.8 and 23.1% activities toward 4-O-MeGalβ1,4GlcNAcβ1,6Galβ1,3GalNAcO-Bn and 4-F-Galβ1,4GlcNAcβ1,6Galβ1,3GalNAc-O-Bn, respectively. Analogous to the situation with Gal3ST-2 and Gal3ST-3 discussed above, the presence of hydrophobic entities attached to the β1,4-linked Gal reduced the activity of the Gal3ST-4 enzyme to the Galβ1,3GalNAcβ moiety.

**Sulfated Compounds Can Distinguish Gal3ST-2 from Gal3ST-3**

As compared with its activity toward Galβ1,4GlcNAcβ1 and Gal3ST-2 was not able to utilize Galβ1,4GlcNAcβ1,6Galβ1,3GalNAc-O-Al (only 42% active) and Galβ1,4GlcNAcβ1,6Galβ1,3GalNAcβ-O-Al (2.6% active) as acceptors (Table V). On the contrary, Gal3ST-3 acted equally well or even better on these acceptors (102.8 and 131.3% active, respectively). Also Gal3ST-2 could use Galβ1,36-O-sulfoGlcNAcβ-O-Al (78.8% active) as an acceptor, whereas Gal3ST-3 was less active (32.2%) toward this acceptor. Besides providing novel and important specificity data, these experiments also suggest that Gal3ST-2 and Gal3ST-3 can help form disulfated structures. Thus, these enzymes may act in synergy with Galβ1,3GalNAcβ sulfoGalβ1,4GlcNAcβ1,6Galβ1,3GalNAcβ-O-Al moiety increased the acceptor activity (183.9% active), whereas the NeuAca2,3Galβ1,4GlcNAcβ1,6Galβ1,3GalNAcβ-O-Al decreased the activity (22.7% active). Both the β1,6-linked GlcNAc and β1,6-linked GalNAc Lewis x moiety stimulated the acceptor activity (243.8 and 215.7% active, respectively). The data suggest that 3-O-sulfation of both Gal residues in the mucin core-2 is possible.

**A Comparison of LS180 Gal3ST with the Cloned Gal3STs**

Comparison of the enzyme activities in Tables II–IV suggests that the identity of the Gal3ST isolated from LS180 cells is Gal3ST-2. Experiments were conducted to verify this finding. **Effect of Divalent Metal Ions**—The enzymatic transfer of sulfate was measured separately with each Gal3ST using specific acceptors in the presence of varying concentrations of Mg2+, Mn2+, and Ca2+ (Fig. 3). In the case of LS180 sulfotransferase, both Mg2+ and Ca2+ showed a similar profile of activity when Galβ1,4GlcNAcβ1,6Galβ1,3GalNAcβ-O-Bn (Fig. 3A) and Galβ1,3GalNAcβ1,3GalNAcβ-O-Al (Fig. 3B) were used as acceptors. In both cases, Mn2+ stimulated the enzyme activity up to a concentration of 10 mM, and the activity gradually decreased with further increase in Mn2+ concentration. The stimulation of Gal3ST-2 activity by Mg2+, Mn2+, and Ca2+ was similar to that of LS180 Gal3ST (Fig. 3, compare C with A and B).

### Table III

| Acceptor (7.5 mm) | Gal3ST-2 | Gal3ST-3 | Gal3ST-4 | LS180 Gal3ST |
|------------------|---------|---------|---------|-------------|
| Galβ1,3GalNAcβ-O-Al | 15.9 | 0 | 100.0 (5,400) | 12.1 |
| Galβ1,4GlcNAcβ1,6Galβ1,3GalNAcβ-O-Bn | 87.9 | 45.2 | 324.1 | 71.3 |
| Galβ1,3GalNAcβ1,6Galβ1,3GalNAcβ-O-Bn | 24.0 | 19.4 | 335.2 | 12.7 |
| Galβ1,3GalNAcβ1,6Galβ1,3GalNAcβ-O-Al | 89.4 | 100.0 | 1.9 | 74.7 |
| Galβ1,3GalNAcβ1,6Galβ1,3GalNAcβ-O-Bn | 116.9 | 119.4 | 178.0 | |

### Table IV

| Acceptor | Gal3ST-2 | Gal3ST-3 | Gal3ST-4 | LS180 Gal3ST |
|----------|---------|---------|---------|-------------|
| Galβ1,4GlcNAcβ1,6Galβ1,3GalNAcβ-O-Bn | 100.0 (122,655) | 71.1 | 100.0 (26,353) | 58.0 |
| Galβ1,4GlcNAcβ1,6Galβ1,3GalNAcβ-O-Bn | 89.4 | 100.0 | 1.9 | 71.3 |
| Galβ1,4GlcNAcβ1,6Galβ1,3GalNAcβ-O-Bn | 100.0 (122,655) | 71.1 | 100.0 (26,353) | 58.0 |
| Galβ1,3GalNAcβ1,6Galβ1,3GalNAcβ-O-Al | 17.0 | 12.2 | 16.6 | |
| Galβ1,3GalNAcβ1,6Galβ1,3GalNAcβ-O-Al | 17.0 | 12.2 | 16.6 | |

### Table V

| Acceptor | Gal3ST-2 | Gal3ST-3 |
|----------|---------|---------|
| Galβ1,4GlcNAcβ1,6Galβ1,3GalNAcβ-O-Al | 100.0 (83,215) | 100.0 (11,580) |
| Galβ1,4GlcNAcβ1,6Galβ1,3GalNAcβ-O-Al | 100.0 (83,215) | 100.0 (11,580) |

### References

1. See Footnote a in Table II.
2. AA-CP, acrylamide copolymer.

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**Notes:**

- See Footnote a in Table II.
- See Footnote a in Table II.
- See Footnote a in Table II.

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Among the Gal3STs tested, Mg$^{2+}$/H11001 exhibited a stimulating effect only on Gal3ST-3 when fetuin triantennary asialo-GP was used as an acceptor (Fig. 3D). None of the cations, Mg$^{2+}$/H11001, Mn$^{2+}$/H11001, or Ca$^{2+}$/H11001, had a stimulating effect on Gal3ST-4. In fact, a gradual decrease in the enzyme activity of Gal3ST-4 was noticed upon increasing the concentration of Mn$^{2+}$ in the reaction mixture (Fig. 3E).

**Effect of pH on Gal3ST Activities**—The activities of LS180 Gal3ST and Gal3ST-2 were measured over a pH range from 5.2 to 8.4 using the Globo H precursor Galβ1,3GalNAcβ1,6Gal-O-Me as the acceptor. Enzyme activity in both cases had an optimum at pH 6.8 (data not shown). The near identical pH-dependent activity profiles of the two enzymes support the proposition that they have the same identity.

**Acceptor Competition Experiment**—Acceptor competition studies were performed with LS180 Gal3ST and Gal3ST-2 (Fig. 4). Here the enzymatic transfer of sulfate was measured separately for the acceptors Galβ1,4GlcNAcβ1,6Gal-O-Al and Galβ1,3GalNAcβ1,3Gal-O-Al in the presence of varying concentrations of mucin core-2 acceptors containing a sulfo group on C-3 of either Gal, namely 3-O-sulfoGalβ1,4GlcNAcβ1,6Galβ1,3GalNAc-O-Bn and Galβ1,4-GlcNAcβ1,6(3-O-sulfoGalβ1,3)GalNAc-O-Bn. The results of these experiments were identical for LS180 Gal3ST (Fig. 4) and

**TABLE VI**

| Acceptor | Activity |
|----------|----------|
| 3-O-MeGalβ1,4GlcNAcβ1,6(Galβ1,3)GalNAc-O-Bn | 100.0$^a$ (26,353) |
| Galβ1,3(6-O-sulfo)GalNAc-O-Al | 128.6 |
| 3-O-SulfoGalβ1,4GlcNAcβ1,6(Galβ1,3)GalNAc-O-Bn | 183.9 |
| NeuAc2,3Galβ1,4GlcNAcβ1,6(Galβ1,3)GalNAc-O-Me | 28.7 |
| GalNAcβ1,4(Fucα1,3)GlcNAcβ1,6(Galβ1,3)GalNAc-O-Bn | 215.7 |
| Galβ1,3(6-O-Me)GalNAcβ1,6GalNAc-O-Al | 243.8 |

$^a$ See Footnote a in Table II.

**FIG. 3.** Effect of divalent metal ions on Gal3ST activity. Activity of LS180 Gal3ST on acceptor Galβ1,4GlcNAcβ1,6(3-O-Me)Galβ1,3GalNAc-O-Bn (A), LS180 Gal3ST on Galβ1,3GalNAcβ1,6Gal-O-Al (B), Gal3ST-2 on Galβ1,4GlcNAcβ1,6(3-O-Me)Galβ1,3GalNAc-O-Bn (C), Gal3ST-3 on fetuin triantennary asialo-GP (D), and Gal3ST-4 on Galβ1,3GlcNAcβ1,6GalNAc-O-Al (E) was quantified in the presence of varying cation concentrations. The effect of Mg$^{2+}$ is depicted by □, Mn$^{2+}$ is depicted by ◻, and Ca$^{2+}$ is depicted by △. RM, reaction mixture.
Gal3ST-2 (data not shown). In both cases, we found that the enzymatic transfer of sulfate to both acceptors was not inhibited by 3-O-sulfogalβ1,4GlcNAcβ1,6Galβ1,3GalNAcO-Bn. The other sulfated compound, Galβ1,4GlcNAcβ1,6(3-O-sulfogalβ1,3)GalNAcO-Bn, which is a specific acceptor for the Gal3ST acting on the LacNAc moiety of mucin core-2, inhibited the enzymatic transfer of sulfate to both acceptors. The pattern of inhibition by this sulfated compound using both acceptors was quite similar, suggesting that the same enzyme is responsible for the sulfation of terminal β1,4Gal in mucin core-2 as well as terminal β1,3Gal in Globo H precursor. Further, the data support the proposition that the identity of LS180 Gal3ST matches that of Gal3ST-2.

A Comparison of the Kinetic Properties of LS180 Gal3ST with That of Gal3ST-2 and Gal3ST-3—The $K_m$ and $V_{\text{max}}$ values for the acceptors D-Fucβ1,3GalNAcβ1,3Galo-O-Me, Galβ1,3GlcNAcβ1,3Galo-O-Me, Galβ1,4GlcNAcβ1,6Manβ1,6Manβ1,6Man, Galβ1,4GlcNAcβ1,6(3-O-Me)Galβ1,3)GalNAcO-Bn, Galβ1,3GlcNAcβ1,3Galβ-O-Me, and fetuin triantennary asialo-GP were determined for LS180 Gal3ST (Fig. 5A) and Gal3ST-2 (Fig. 5B), and these are tabulated in Table VII. Except for Galβ1,3GlcNAcβ1,3Galβ-O-Me ($K_m$ = 20.0 mm), the $K_m$ value was 4 mm for each acceptor and for both enzymes. These data further reveal the identity of LS180 Gal3ST as Gal3ST-2. The $V_{\text{max}}$ value of Gal3ST-2 for each acceptor was found to be higher than that of LS180 Gal3ST. Both enzymes exhibited higher affinity for Globo H backbone structures. Galβ1,4GlcNAcβ1,6Manβ1,6Manβ1,6Man served as a good acceptor for both enzymes. As anticipated, the N-glycan acceptors, namely fetuin triantennary asialo-GP, were found to be far less active than the Globo H structures. Gal3ST-3 (Fig. 5C) gave the $K_m$ values of 0.43, 0.87, and 0.74 mm for the acceptors fetuin triantennary asialo-GP, Galβ1,4GlcNAcβ1,6-Manβ1,6Manβ1,6Man, and Galβ1,4GlcNAcβ1,6(3-O-Me)Galβ1,3)GalNAcO-Bn, respectively. From the $V_{\text{max}}$ values, it is evident that Gal3ST-3 prefers to act on the terminal Galβ1,4GlcNAc units in N-linked glycans.

RT-PCR—RT-PCR experiments with primers corresponding to the common domain and PAPS domain of previously cloned sulfotransferases (ST-2, ST-3, and ST-4) revealed that LS180 has mRNA corresponding to two Gal:3-O-sulfotransferases, Gal3ST-4, which we obtained using the primers to the common domain, and Gal3ST-2, which was obtained with primers corresponding to the PAPS binding domain. The observations support our suggestion that we have purified Gal3ST-2 from LS180 cells. Gal3ST-4 activity was not detected in our assay.
and we attribute this to possibly low levels of protein expression in the cells or the absence of this enzyme in our purified fraction. We note that the acceptor used to detect sulfotransferase activity during purification (Galβ1,4GlcNAcβ1,6(3-O-Me)Galβ1,3GalNAc-O-Bn, Table I) was not acted upon by Gal3ST-4.

**DISCUSSION**

Studies of cellular glycosyltransferase expression at the mRNA level can be performed using microarrays and Northern blot analysis, and such analysis can also be performed at the protein level using enzymology methods. Both strategies when combined with knowledge of glycan biosynthesis pathways can be useful in predicting oligosaccharide structures on cell glycolipids and glycoproteins. Enzymatic studies using a range of well defined acceptors can quantitatively compare enzyme activities, and they can be used to study the competition of various enzymes for a single substrate. Identification of unique substrates for each enzyme can also allow development of rapid assay strategies for the identification of a particular glycosyltransferase in a complex mixture. We discuss here results of our studies with three cloned Gal:3-O-sulfotransferases: Gal3ST-2, Gal3ST-3, and Gal3ST-4. Emphasis is placed on acceptor studies with the mucin core-2 structure and the Globo H precursor due to the physiological importance of these molecules and because such studies have not been carried out previously.

**Gal3ST in Colon and Breast Cancer Cells and Tissue—** Various Gal:3-O-sulfotransferases have been noted to act on glycolipids and glycoproteins. In one study, it was observed that the human colon cancer cell line SMKT-R3 sulfotransferase utilized both GalCer and LacCer as acceptors but did not act on the terminal β-Gal moiety of oligosaccharides that are not associated with lipids (24). This enzyme was cloned and designated human cerebroside 3’-sulfotransferase (24). Honke et al. (12) further used the cerebroside 3’-sulfotransferase cDNA sequence as a probe and cloned a human β-Gal:3-O-sulfotransferase (GP3ST or Gal3ST-2) that acts on LacNAc types 1 and 2 as well as mucin core 1 structure. The amino acid sequence of Gal3ST-2 indicated 33% identity to the cerebroside 3’-sulfotransferase sequence, and this enzyme acted on the terminal β-Gal moiety of oligosaccharide chains in glycoproteins only (12). Others have noted the existence of a Gal:3-O-sulfotransferase from human respiratory mucosa that acts on terminal LacNAc unit in mucins, but this enzyme did not utilize GalCer (25). The activity of this enzyme does not match any of the Gal3STs tested in the current study. Chance and Mawhinney (26) reported the occurrence of the 3-O-sulfogalβ1,4(6-O-sulfo) GlcNAcβ sequence in tracheobronchial mucin. The present study identifies Galβ1,4(6-O-sulfo)GlcNAcβ-O-Ac as a novel acceptor for Gal3ST-3 and suggests that this enzyme may be responsible for the biosynthesis of this bronchial mucin. The 3-O-sulfoGalβ1,3GalNAcβ1,3Galα1- sequence has also been suggested to be part of sulfated glycolipids in kidney (27, 28).

Our studies suggest that the sulfotransferase involved in this process is either Gal3ST-2 or Gal3ST-4. We have also reported earlier (9, 10) the existence of two distinct Gal:3-O-sulfotransferases showing acceptor preference to either LacNAc type 2 unit (Galβ1,4GlcNAcβ or the T-hapten unit (Galβ1,3GlcNAcα) of the mucin core-2 structure. The enzymes that acted on Galβ1,4GlcNAcβ were observed in colon tumor tissue (9) and colon cancer cell lines (10), while the Gal3STs acting on Galβ1,3GlcNAcα were observed in breast tumor tissues and breast cancer cell lines (9, 10). These two enzymes exhibited significant difference in their kinetic properties such as pH optima and divalent metal ion activation.

In the present study, we further purified the Gal3ST from LS180 cells using a series of chromatography steps. In these studies, we noted that the LS180 Gal3ST can bind to a GDP affinity column. The reason for this binding may be due to the fact that the biological sulfate donor, PAPS, contains adenine (a purine base), and Gal:3-O-sulfotransferase is able to bind to another purine base guanine. Further, even after significant purification, the Gal3ST exhibited the same kind of acceptor specificity and kinetics toward the LacNAc type 2 structure of mucin core-2 as the enzyme from crude lysate. Our enzyme preparation from LS180 was free of GlcNAc:6-O-sulfotransferase as Me-O-Galβ1,3(GlcNAcβ1,6)GalNAc-O-Bn did not act as an acceptor. Upon comparison with the three cloned Gal3STs, the activity of LS180 sulfotransferase most closely resembled Gal3ST-2. Drawing analogies from the current work, based on the acceptor specificity studies, it appears very likely that the predominant Gal3ST in breast cancer cells and tissue is Gal3ST-4.

**Identification of Unique Acceptors for Cloned Gal3STs—** We performed studies on various cloned Gal:3-O-sulfotransferases (Gal3ST-2, Gal3ST-3, and Gal3ST-4) with the objective of determining both unique and overlapping substrates for each of these enzymes. Identification of this acceptor specificity can reveal the glycan structure facilitated by these enzymes and the biological role of these enzymes.

| Table VII | Kinetic data obtained for the purified LS180 Gal3ST, Gal3ST-2, and Gal3ST-3 |
|-----------|---------------------------------------------------------------|
| **Acceptor** | **LS180 Gal3ST** | **Gal3ST-2** | **Gal3ST-3** |
| | $K_m$ (mM) | $V_{max}$ (pmol/h) | $K_m$ (mM) | $V_{max}$ (pmol/h) | $K_m$ (mM) | $V_{max}$ (pmol/h) |
| Galβ1,4GlcNAcβ1,6(3-O-Me)Galβ1,3GalNAc-O-Bn | 4.0 | 5.8 | 4.0 | 11.7 | 0.74 | 1.0 |
| Galβ1,3GlcNAcβ1,3Galα-O-Me | 4.0 | 8.8 | 4.0 | 35.0 | ND | ND |
| Galβ1,3GlcNAcβ1,3Galα-O-Me | 4.0 | 8.8 | 4.0 | 35.0 | ND | ND |
| Galβ1,3GlcNAcβ1,3Galα-O-Me | 9.0 | 7.0 | 20.0 | 11.7 | ND | ND |
| Galβ1,4GlcNAcβ1,6ManO1,6Manβ1,6Man | 4.0 | 11.7 | 4.0 | 11.7 | 0.87 | 2.3 |
| Fetuin triantennary asialo-GP | 4.0 | 5.8 | 4.0 | 11.7 | 0.43 | 2.7 |

*$^*$ ND, not determined.

|$^*$ ND, not determined.
Distinct Specificities of Gal:3-O-Sulfotransferases

a) Galβ1,4GlcNAcβ1,6(Galβ1,3)GlcNAc-O-Bn [Mucin core 2]  
↓ Gal3ST-2 or Gal3ST-3  
3-O-Sulfogalβ1,4GlcNAcβ1,6(Galβ1,3)GlcNAc-O-Bn  
↓ Gal3ST-4  
3-O-Sulfogalβ1,4GlcNAcβ1,6(3-O-Sulfogalβ1,3)GlcNAc-O-Bn

b) Asialo triantennary chain (complex type)  
↓ Gal3ST-3  
Sulfated triantennary chain

c) Galβ1,3GlcNAcβ1,3Galα-O-Me  
↓ Gal3ST-2 or Gal3ST-4  
3-O-Sulfogalβ1,3GlcNAcβ1,3Galα-O-Me

d) Galβ1,3GlcNAcβ1,3Galα-O-AI  
↓ Gal3ST-4  
3-O-Sulfogalβ1,3GlcNAcβ1,3Galα-O-AI

e) Galβ1,4(6-O-Sulfogalβ1,3GlcNAcβ1,3Galα-O-AI  
↓ Gal3ST-3  
3-O-Sulfogalβ1,4(6-O-Sulfogalβ1,3GlcNAcβ1,3Galα-O-AI  
↓ Gal3ST-2  
3-O-Sulfogalβ1,3(6-O-Sulfogalβ1,3GlcNAcβ1,3Galα-O-AI

Dist. 6. Role of Gal3STs examined in this study. 3-O-sulfation of terminal β-galactosyl residue in: a) mucin core-2; b) N-glycan; c) Globo backbone; d) T-hapten; and e) 6-O-SulfogalNAc type 1 and type 2 chains.

disaccharide. These authors also showed that their enzyme preparation was also active toward Galβ1,4GlcNAcβ1,3Galβ1,4Glc-LC4 and Galβ1,3GlcNAcβ1,3Galβ1,4Glc-LC4 (12). On the other hand, Gal3ST-3, which is involved in the biosynthesis of 3'-O-sulfogalα-Le"}, has poor activity toward LC4 but is quite active toward nLC4. (c) We showed that the activities of these two enzymes can be readily distinguished when Globo H precursor Galβ1,3GlcNAcβ1,3Galα-O-Me and fetuin triantennary asialo-GP were used as acceptors. Here we found that the above compounds served, respectively, as high affinity and poor acceptors for Gal3ST-2 (Fig. 6c). The opposite was observed for Gal3ST-3 (Fig. 6b). Fetuin triantennary asialo-lactosylceramide was a better acceptor for Gal3ST-3 (383.9% active) than it was for either Gal3ST-2 (81.7% active) or Gal3ST-4 (14.8% active). (d) Gal3ST-2 and Gal3ST-3 also had distinct activities toward sulfated acceptors (Fig. 6e). While Galβ1,4(6-O-sulfogalβ1,3GlcNAcβ1,3Galα-O-AI was a specific acceptor for Gal3ST-3, Galβ1,3(6-O-sulfogalβ1,3GlcNAcβ1,3Galα-O-AI was specific for Gal3ST-2.

Gal3ST-4 was found to be specific for Galβ1,3GlcNAc (14) (Fig. 6d). Our study further showed the following. (a) As compared with Galβ1,3GlcNAc, as such, the Galβ1,3GlcNAc unit occurring in the mucin core-2 tetrasaccharide structure was a 3-fold better substrate for this enzyme, and the mucin core-2 trisaccharide was 8.5-fold better. These observations suggest that Gal3ST-4 may be an efficient modifier of the core-2 structure. Since the mucin core-2 trisaccharide was 2.5-fold more active than the tetrasaccharide, it appears that the Gal residue linked to GlcNAc has an adverse effect on enzyme activity. (b) It is interesting to note that various modifications of the β1,4-linked Gal moiety in the mucin core-2 tetrasaccharide can either decrease or enhance enzyme activity. While an α2,3-sialyl group on this Gal decreased the Gal3ST-4 acceptor ability (28.7% active compared with core-2 tetrasaccharide), a 3-O-sulfogalNAc at this position enhanced the activity (183.9% active). This enzyme also acted efficiently (130% active) on Galβ1,3(6-O-sulfogalβ1,3GlcNAc-O-AI. Overall the action of Gal3ST-4 can lead to the synthesis of diverse disulfated structures in cells (Fig. 6, a and c). (c) As compared with Galβ1,3GlcNAc-O-AI, Galβ1,3GlcNAcβ1,3Galα-O-Me and d-Fucβ1,3Galβ1,3GlcNAcβ1,3Galα-O-Me were 327.8 and 213.0% active, respectively, indicating that the α-linkage of GlcNAc is not critical for enzyme activity. (d) Since Galβ1,3GlcNAcβ1,3Galα-O-Me was considerably more active than d-Fucβ1,3Galβ1,3GlcNAcβ1,3Galα-O-Me, it appears that C-6 OH group of Gal is necessary for maximum activity.

Mucin Core-2 and Globo H Precursor—We compared the behavior of the cloned sulfotransferases toward the mucin core-2 structure and toward the Globo H precursor. The latter occurs as part of the cancer-associated carbohydrate epitope recognized by antibody MBr1 (29, 30). Previously we have observed that α1,2-fucosyltransferase from prostate cancer cells (LNCaP) that acts on the Galβ1,4GlcNAcβ1 unit of the core-2 branched structure (11) also incorporated fucose at the C-2 position of the terminal galactose in Galβ1,3GlcNAcβ1,3Galα-O-Me. Moreover cloned α2,3-sialyltransferase ST-3GalVI acting on the Galβ1,4GlcNAcβ1 arm in mucin core-2 also efficiently utilized the Globo H trisaccharide. In the current study we observed that Gal3ST-2 acts similarly to α1,2- and α1,3-fucosyltransferase and ST-3GalVI in that it acted both on the Globo trisaccharide and core-2 tetrasaccharide. However, we noted that it is not necessary that enzymes that act on the β1,4-linked Gal in the core-2 tetrasaccharide also act on the Globo backbone. In this regard, while Gal3ST-4 acted upon the Globo H precursor, it did not act on the Galβ1,4GlcNAcβ1 arm of the mucin core-2. Also while Gal3ST-3 acted upon Galβ1,4GlcNAc, it did not act efficiently on the Globo H precursor.

Similarities are observed between the substrate specificities of Gal3ST and ST-3GalIV. ST-3GalIV is a human Galβ1,3GlcNAcβ1,3Galβ1,4Glc-LC4/β-arm in mucin core-2 (11) also incorporated fucose at the C-2 position of the terminal galactose in Galβ1,3GlcNAcβ1,3Galα-O-Me. Moreover cloned α2,3-sialyltransferase ST-3GalVI acting on the Galβ1,4GlcNAcβ1 arm in mucin core-2 also efficiently utilized the Globo H trisaccharide. Therefore, we observed that α1,2-fucosyltransferase from prostate cancer cells (LNCaP) that acts on the Galβ1,4GlcNAcβ1 unit of the core-2 branched structure (11) also incorporated fucose at the C-2 position of the terminal galactose in Galβ1,3GlcNAcβ1,3Galα-O-Me. Moreover cloned α2,3-sialyltransferase ST-3GalVI acting on the Galβ1,4GlcNAcβ1 arm in mucin core-2 also efficiently utilized the Globo H trisaccharide. In the current study we observed that Gal3ST-2 acts similarly to α1,2- and α1,3-fucosyltransferase and ST-3GalVI in that it acted both on the Globo trisaccharide and core-2 tetrasaccharide. However, we noted that it is not necessary that enzymes that act on the β1,4-linked Gal in the core-2 tetrasaccharide also act on the Globo backbone. In this regard, while Gal3ST-4 acted upon the Globo H precursor, it did not act on the Galβ1,4GlcNAcβ1 arm of the mucin core-2. Also while Gal3ST-3 acted upon Galβ1,4GlcNAc, it did not act efficiently on the Globo H precursor.

The current study defines the acceptor specificity of carbohydrate-specific Gal3STs cloned thus far, and it poses new questions for the future. In this regard, the characterization of specific substrates in the current work will likely facilitate assessment of the functional importance of these enzymes in the future. For example, based on the current work, it appears that the generation of monoclonal antibodies against the 3-O-sulfated mucin core-2 tetrasaccharide structure and the 3-O-sulfated Globo H trisaccharide may be useful reagents for future immunohistochemical studies that examine the tissue.
distribution of sulfated glycans. Our enzymatic studies suggest the existence of novel disulfated structures in vivo. They also suggest that it will be important to compare the competitive action of various α2,3-sialyltransferases and GaL3STs on the core-2 mucin structures along with structural analysis of cell surface carbohydrates. Finally we showed here that a 3-fluoro or 4-O-methyl substituent on either Gal moiety of the mucin core-2 tetrasaccharide reduced the function of various GaL3STs. It will be interesting to determine whether such molecules are poor acceptors for these enzymes or whether they act as competitive inhibitors that may find in vivo application as sulfo- and sialyltransferase inhibitors.

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