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Identification of physiologically relevant substrates for cloned Gal: 3-0-sulfotransferases: Distinct high affinity of Gal 3ST-2 and LS180 sulfotransferase for the Globo H backbone, Gal 3ST-3 for N-Glycan multi-terminal LacNAc units & 6-sulfo LacNAc and Gal 3ST-4 for the mucin core 2 trisaccharide

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Running Title: Distinct Specificities of Gal:3-O-Sulfotransferases

SUMMARY

Sulfated glycoconjugates regulate biological processes such as cell adhesion and cancer metastasis. We examined the acceptor specificities and kinetic properties of three cloned Gal: 3-0-sulfotransferases (Gal 3STs) ST-2, ST-3 and ST-4, along with a purified Gal 3ST from colon carcinoma LS180 cells. Gal 3ST-2 was the dominant Gal 3ST in LS180. While the mucin core 2 structure, Galβ1,4GlcNAcβ1,6(3-O-MeGalβ1,3)GalNAcα-O-Bn, and the disaccharide Galβ1,4GlcNAc served as high affinity acceptors for Gal 3ST-2 and ST-3, 3-O-MeGalβ1,4GlcNAcβ1,6(Galβ1,3)GalNAcα-O-Bn and Galβ1,3GalNAcα-O-Al were efficient acceptors for Gal 3ST-4. The activities of Gal 3ST-2 and ST-3 could be distinguished with the Globo H precursor (Galβ1,3GalNAcβ1,3Galα-O-Me) and Fetuin triantennary asialo glycopeptide. Gal 3ST-2 acted efficiently on the former while ST-3 showed preference for the latter. Gal ST-4 also acted on the Globo H precursor but not the glycopeptide. In support of the specificity, Gal 3ST-2 activity towards the LacNac unit on mucin core-2 as well as the GloboH precursor could be inhibited competitively by Galβ1,4GlcNAcβ1,6(3-O-SulfoGal β1,3)GalNAcα-O-Bn but not 3-O-SulfoGalβ1,4GlcNAcβ1,6(Galβ1,3)GalNAcα-O-Bn. Remarkably, these sulfotransferases were uniquely specific for sulfated substrates: Gal 3ST-3 utilized Galβ1,4(6-0-Sulfo)GlcNAcβ-O-Al as acceptor, Gal 3ST-2 acted efficiently on Galβ1,3(6-0-Sulfo)GlcNAcβ-O-Al and Gal 3ST-4 on Galβ1,3(6-0-Sulfo)GalNAcα-O-Al. Mg^{2+}, Mn^{2+} and Ca^{2+} stimulated the activities of Gal 3ST-2 whereas only Mg^{2+} augmented Gal 3ST-3 activity. Divalent cations did not stimulate Gal 3ST-4, though inhibition was noted at high Mn^{2+} concentrations. The fine substrate specificities of Gal 3STs indicate a distinct physiological role for each enzyme.

INTRODUCTION

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 mice 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 glycans 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-disulfo Lewis x and 3′,6′-disulfo Lewis x inhibited 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-disulfo Lewis x inhibited the rolling of L-selectin-transfected cells on the glycoprotein ligand GlyCAM-1 [6]. Binding inhibition assays utilizing paucivalent L-selectin also identified, 3′,6-Sulfo Lewis x and 3′-Sulfo Lewis a as potent inhibitors of L-selectin binding [7]. In our studies, we have also found that substitution of sialyl group with sulfate group in GalNAcβ1,4 (Fucα1,3) GlcNAcβ1,6 (NeuAcα2,3Galβ1,3) GalNAcα-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 (Gal 3ST)[1] 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, we also observed that prostate carcinoma cell LNCaP has α1,2-L-Fucosyltransferase activity that exhibits four-fold higher activity towards Galβ1,4GlcNAcβ- compared to the Gal residue on the mucin core-2 β1,3 branch [11]. This enzyme also acted on the cancer antigen Globo H backbone Galβ1,3GalNAcβ1,3Galα- very efficiently (Fig. 1). Similar to the activity of the fucosyltransferase enzyme, we have observed that a cloned α2,3 sialyltransferase ST-3GalIV utilized both the Globo H backbone and the Galβ1,4GlcNAcβ- structure in the core-2 tetrasaccharide (Chandrasekaran, *et al.*, unpublished results). The possibility that the Gal:3-O-sulfotransferase from colon tissue, similar to the α1,2-L-Fucosyltransferase and α2,3 sialyltransferase, also acts on the GloboH antigen remains undetermined and we test this here. Further, the cloning of three distinct Gal:3-O-sulfotransferases recently Gal3ST-2 [12], Gal3ST-3 [13] and Gal3ST-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.

[1] Abbreviations: Gal 3ST: Galactose:3-O-sulfotransferase; AA-CP: Acrylamide Copolymer; Al: Allyl; Bn: Benzyl; Me: Methyl; GP: glycopeptide; LacNAc: Galβ1,4GlcNAcβ; PAPS: 3'-phosphoadenosine 5'-phosphosulfate; RM: Reaction mixture; TLC: thin layer chromatography.

EXPERIMENTAL PROCEDURES

Cell Culture: Human colon carcinoma LS180 and Chinese hamster ovarian CHO-S cells were obtained from American Type Culture Collection (ATCC, Manassas, Virginia). 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, amphotericin B). Harvested cells were stored frozen at -20^{o}C prior to Gal:3-O-sulfotransferase (Gal 3ST) purification. CHO-S was cultured in DMEM media (Life Technologies, Grand Island, NY) with 10% heat-inactivated fetal bovine serum in tissue culture incubators.

Expression of cloned Gal 3ST: The following plasmids containing genes that encode various Gal 3ST were used in the current work: pSV-GP3ST for Gal 3ST-2 [12], pCXNGal 3ST for Gal 3ST-3 [13], and pCMV-SPORT for Gal 3ST-4 [14]. These plasmids were kindly provided by Drs. K.Honke (Osaka University Medical School, Japan), K.Uchimura (Nagoya University School of Medicine, Japan) and A. Seko (Sasaki Institute, Japan) respectively. For protein expression, CHO cells in six-well plates at sub-confluency were transiently transfected with 6μl of LipofectAMINE 2000 (Life Technologies, Inc.) per well along with 6μg/mL of one of the above plasmid. The cells were passed into T75 flasks one day after transfection, cultured for 3 days, then harvested and kept frozen at -20^{o} C until use.

Purification of Gal:3-O-sulfotransferase from LS180 cells: 5.5 x 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 Mg Acetate, 2% Triton X-100, 20% glycerol, 30 μM PMSF and 0.1% NaN_3 and then stirred for 2 h at 4^{o}C. The homogenate was centrifuged at 10,000 g for 1 hr at 4^{o}C. The supernatant was subjected to chromatography on a 25 ml bed volume Aleuria aurantia lectin-agarose (Vector Lab, 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 Fucose and 2.0 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 bed volume affinity Gel-GDP (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 (2.5 x 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% NaN_3. 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(3-O-MeGalβ1,3)GalNAcα-O-Bn as the acceptor. The enzyme activity emerged as a single peak from the column soon after the void volume. The fractions under this peak 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.

RT-PCR to identify sulfotransferase genes in LS180: Total RNA extracted from LS180 cells using TRIzol reagent (Invitrogen, CA) was subjected to RT-PCR using Superscript One step RT-PCR with platinum taq (Invitrogen, CA). Total RNA was reverse-transcribed with Oligo dT primers at 42^{o}C for 1 hr, 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, DESLVLLR, and DESLVLLA which are conserved among all cloned Gal 3STs. The sense primers corresponding to these sequences were 5'TTCCTGAAGACTCACAAGACG 3' and 5' TTCCTGAAGACACATAAATCC 3' and the antisense primers used were 5'CCGCAGCAGCACTAGCGACTCGTC 3' and 5' TGCCAGCAGAACCAATGACTCATC 3'. The second set of primers were designed against the PAPS binding domain of the cloned Gal 3STs corresponding to the amino-acid sequence, either HKTASSTV and LRNPVFQLESSFI or HKSGSSSV and RDPAALARSAF. One set of primers for this were 5' CACAAGACGGCCAGCAGCACGGTG 3' (sense) and 5' GTAGATGAAGGAGGACTCCAGCTGGAACACGGGGTTCCT 3' (antisense), and the other were 5' ATAAATCCGGGAGCAGCTCTGTGCTGAGCC 3' (sense) and 5'

CAGAGCGAGCCAGAGCCGCTGGGTCTCG 3' (antisense). PCR was performed in all cases using the denaturation temperature of 94^{o}C, annealing temperature of 58^{o}C and extension temperature of 72^{o}C. RT-PCR products were sequenced by dideoxy chain termination method to determine the sulfotransferase mRNA in LS180.

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 Globo H precursor structure namely: Galβ1,3GalNAcβ1,3Galα-O-Al; Galβ1,3GalNAcβ1,3Galα-O-Me; D-Fucβ1,3GalNAcβ1,3Galα-O-Me; 3-O-SulfoGalβ1,3GalNAcβ1,3Galα-O-Me and mucin core 2 tetrasaccharides containing 4-O-Me group will be published elsewhere. The synthesis of mucin core 2 tetrasaccharides containing 3-F group has been reported [18]. Galβ1,4GlcNAc was purchased from Toronto Research Chemicals (North York, Canada).

Macromolecular and natural acceptors: Acrylamide copolymer of Galβ1,3GalNAcα-O-Al synthesized by the procedure of Horejsi et al. [19] and Fetuin triantennary asialo glycopeptide were available from earlier studies of this laboratory [20-23].

Assay of sulfotransferase: The incubation mixture run in duplicate contained 100 mM Tris-Maleate pH 7.2, 5 mM Mg Acetate, 5 mM ATP, 10 mM NaF, 10 mM BAL, 7.5 mM acceptor, 0.5 μCi [^{35}S] PAPS (2.4 Ci/mmol) and the enzyme in a total volume of 20 μl unless otherwise stated. The control incubation mixtures contained everything except the acceptor. Incubation was carried out for 2h at 37^{o}C. Under our assay conditions, for all acceptors and enzymes tested, less than 25% of the donor [^{35}S] PAPS was utilized. [^{35}S] enzymatic transfer was in the linear range for the first 2 h, and it reached maximum incorporation at 5-6 h. Similar observations were made by us in our earlier studies [9, 10]. Dowex-1-Cl method was used to measure the radioactive product from neutral acceptors as follows: The incubation mixture was diluted with 1.0 ml water and passed through Dowex-1-X8 (200-400 mesh; chloride form) of 1 ml bed volume in a Pasteur pipet. The column was washed twice with 1.0 ml water. A quantitative elution of the radioactive product (the ^{35}S-sulfated compound) was achieved by eluting the column with

3.0 ml of 0.2 M NaCl. The radioactivity present in the NaCl eluate was measured using 3a70 scintillation cocktail (Research Products International, Mount Prospect, IL) and a Beckman LS6500 scintillation counter. The 0.2 M NaCl from the control reaction mixture (containing no acceptor) contained a negligible amount of radioactivity (<50 CPM) in all cases. The values for the duplicate runs did not vary by more than 5%. The [^{35}S] sulfated compound resulting from sialylated or sulfated synthetic acceptor was quantitated by TLC method using $CHCl_3$:CH_3OH:H_2O (5/4/1 v/v) as the solvent system [9,10].

Identification of the [^{35}S] sulfated compound arising from the acceptor Galβ1,3GalNAcβ1,3Galα-O-Me by the action of LS180 sulfotransferase: A ten-fold standard reaction mixture (200 μl) containing Galβ1,3GalNAcβ1,3Galα-O-Me was incubated for 2 h at 37^{o}C with purified enzyme. After dilution with 1.0 ml water, it was fractionated by Dowex-1-Cl method. The 0.2 M NaCl eluate was lyophilized to a small volume (1 ml) and desalted on a Biogel P_2 column (1.0 x 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 picked up in 200 μl water. A small aliquot was subjected to TLC (silica gel GHLF; 250 μm scored 20 x 20 cm; Analtech, NJ) using 1-propanol/NH_4OH (25%)/water (60/10/25 v/v) along with authentic Galβ1,3GalNAcβ1,3Galα-O-Me and 3-O-SulfoGalβ1,3GalNAcβ1,3Galα-O-Me as reference compounds. The reference compounds were located on TLC plates by spraying with sulfuric acid in ethanol and heating at 100^{o}C. The radioactive compound was located by scraping 0.5 cm width segments of the silica , soaking in 2.0 ml water followed by liquid scintillation counting.

Effect of divalent metal ions on Gal:3-O-sulfotransferases: For studying the effect of divalent metal ions, the incubation mixture contained varying concentrations (0-50 mM) of Mg Acetate, Mn Acetate or Ca Acetate under the standard incubation conditions.

Testing for competitive inhibition: For studying 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-SulfoGalβ1,4GlcNAcβ1,6(Galβ1,3)GalNAcα-O-Bn and Galβ1,4GlcNAcβ1,6(3-O-SulfoGalβ1,3)GalNAcα-O-Bn cannot be eluted, whereas, the product from the

neutral acceptors can be eluted from the Dowex-1-Cl column by 0.2 M NaCl. For these runs, the concentration of the 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

Gal 3ST purified from LS180 acts both on Galβ1,4GlcNAcβ in mucin core 2 as well as terminal Galβ in Globo H precursor

Gal 3ST 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, Aleuria aurantia lectin-agarose chromatography and fractionation on Affinity Gel-GDP. When this purified preparation was further subjected to chromatography on 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 BSA standard (MW 66,000 Da). The enzyme at this stage 3000-fold purified 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 SDS-PAGE gel of the partially purified fraction (data not shown). GlcNAc:6-O-sulfotransferase activity is not exhibited by sulfotransferases in LS-180 cell (10) and this was the case for our fraction also. Further, as shown below the partially purified enzyme exhibited activity that resembled only one of the three cloned Gal 3STs studied in the current paper suggesting that it contains only one Gal 3ST.

The activity of the purified LS180 Gal 3ST was examined using a series of acceptors based on the core-2 mucin structure (Table II). This enzyme was poorly active with Galβ1,3GalNAcα-O-Al (20.2% activity as compared to its activity towards Galβ1,4GlcNAcβ1,6(3-O-MeGalβ1,3)GalNAcα-O-Bn) and the mucin core-2 analog 3-0-MeGalβ1,4GlcNAcβ1,6(Galβ1,3)GalNAcα-O-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)GalNAcα-O-Bn is a comparable acceptor to Galβ1,4GlcNAcβ1,6 (3-O-MeGalβ1,3)GalNAcα-O-Bn (Activities 95.6% and 100.0% respectively). Galβ1,3(GlcNAcβ1,6)GalNAcα-O-Bn showed low activity (8.7%), and as anticipated 3-O-MeGalβ1,3(GlcNAcβ1,6)GalNAcα-O-Bn was an inactive acceptor. Together, the above data suggests

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 Galβ1,3GalNAcβ1,3Galα-O-Al and Galβ1,3GalNAcβ1,3Galα-O-Me served as better acceptor for the enzyme than the LacNAc moiety in mucin core-2. An analog of this structure Galβ1,3GlcNAcβ1,3Galβ-O-Me and another acceptor Galβ1,4Glcβ1,6GalNAcα-O-Bn exhibited very low activities (20.6% and 5.0% respectively). We synthesized a compound by substituting D-Fuc for D-Gal in the acceptor structure Galβ1,3GalNAcβ1,3β1,3Galα-O-Me; this is equivalent to converting the Gal moiety to its 6-deoxy derivative. The newly synthesized compound D-Fucβ1,3GalNAcβ1,3Galα-O-Me was even better than Galβ1,3GalNAcβ1,3Galα-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 Galβ1,3GalNAcβ1,3Galα (Fig. 2). A monosulfated standard compound, 3-O-Sulfo-Galβ1,3GalNAcβ1,3Galα-O-Me was synthesized for this purpose. When the [^{35}S] sulfated product isolated from the action of LS180 sulfotransferase on Galβ1,3GalNAcβ1,3Galα-O-Me 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.

Acceptor specificity of three cloned Gal 3 STs: Comparison to LS180 Gal 3ST activity

We used an array of carbohydrate acceptors to determine the detailed specificity of the cloned Gal3ST (Table III). As seen, both Gal 3ST-2 and Gal 3ST-3 prefer to act on the C3 hydroxyl group of Galβ1,4GlcNAc rather than Galβ1,3GalNAcα-O-Al. These enzymes efficiently utilize the mucin core 2 acceptor Galβ1,4GlcNAcβ1,6(3-O-MeGalβ1,3)GalNAcα-O-Bn and its corresponding non-methylated

structure. They also acted to a lesser extent on 3-O-MeGalβ1,4 GlcNAcβ1,6(Galβ1,3)GalNAcα-O-Bn (19.4 to 24.0%) and also on the trisaccharide Galβ1,3(GlcNAcβ1,6)GalNAcα-O-Al (45.2 to 67.9%). The activities of these two enzymes can be distinguished by their action on the Globo H precursor Galβ1,3GalNAcβ1,3Galα-O-Me, in that this was a preferred acceptor for Gal 3ST-2 (295.6% active) whereas it was a poor acceptor for Gal 3ST-3 (25.8% active). On the contrary, Fetuin triantennary asialo GP was a preferred acceptor for Gal 3ST-3 (383.9% active) but a relatively poor acceptor for Gal 3ST-2 (81.7% active). LS180 Gal 3ST resembled Gal 3ST-2 when considering its acceptor utilizing efficiencies.

Unlike Gal3ST-2 and ST-3, Gal3ST-4 had an absolute specificity towards Galβ1,3GalNAcα-O-Al. It utilized 3-O-MeGalβ1,4GlcNAcβ1,6(Galβ1,3)GalNAcα-O-Bn but not Galβ1,4GlcNAcβ1,6(3-O-MeGalβ1,3)GalNAcα-O-Bn as an acceptor. The trisaccharide acceptor Galβ1,3(GlcNAcβ1,6)GalNAcα-O-Al (857.4% active) was 2.5 fold as active as 3-O-MeGalβ1,4GlcNAcβ1,6(Galβ1,3)GalNAcα-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 Galβ1,3GalNAcβ1,3Galα-O-Me and D-Fucβ1,3GalNAcβ1,3Galα-O-Me. Since this enzyme recognizes both the GloboH precursor and the mucin core-2, the results suggest that this enzyme acts on substrates with both α- and β-linked GalNAc. The decreased activity (213.0%) towards D-Fucβ1,3GalNAcβ1,3Galα-O-Me in comparison to Galβ1,3GalNAcβ1,3Galα-O-Me (327.8%) would suggest that the C6 OH group of the Gal moiety has significant influence on this enzyme activity. As anticipated, Gal3ST-4 showed negligible activity towards the acceptors, Galβ1,4GlcNAc; Galβ1,4GlcNAcβ1,6Manα1,6Manβ1,6Man; Galβ1,4GlcNAcβ1,6(3-O-MeGalβ1,3)GalNAcα-O-Bn and Fetuin triantennary asialo GP. But it showed significant activity when Galβ1,3GalNAcα-O-Al/AA-CP was used as an acceptor at low concentration of 0.125mM, as compared to the reference acceptor Galβ1,3GalNAcα-O-Al which was used at 7.5mM.

Selected structural modifications of mucin core 2 can reduce Gal 3ST activity

In order 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 to the acceptor Galβ1,4GlcNAcβ1,6(3-O-MeGalβ1,3)GalNAcα-O-Bn, LS180 Gal 3ST, Gal 3ST-2 and Gal 3ST-3 exhibited only 12-16% activity towards Galβ1,4GlcNAcβ1,6(3-F-Galβ1,3)GalNAcα-O-Bn and 45-55% activity towards Galβ1,4GlcNAcβ1,6(4-O-MeGalβ1,3)GalNAcα-O-Bn. This suggests that hydrophobic entities attached to the Galβ1,3 moiety of the core-2 mucin can influence enzyme activity directed towards the Galβ1,4GlcNAcβ structure.

As compared to 3-O-MeGalβ1,4GlcNAcβ1,6(Galβ1,3)GalNAcα-O-Bn, Gal3ST-4 had only 60.8% and 23.1% activities towards 4-O-MeGalβ1,4GlcNAcβ1,6(Galβ1,3)GalNAcα-O-Bn and 3-F-Galβ1,4GlcNAcβ1,6(Galβ1,3)GalNAcα-O-Bn respectively. Again, analogous to the situation with Gal3ST-2 and ST-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 Galβ1,3GalNAcα moiety.

Sulfated compounds can distinguish Gal3ST-2 from Gal3ST-3

As compared to its activity towards Galβ1,4GlcNAc, Gal3ST-2, was not able to utilize Galβ1,4(6-O-Sulfo)GlcNAc (only 4.2% active) and Galβ1,4(6-O-Sulfo)GlcNAcβ-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 can use Galβ1,3(6-O-Sulfo)GlcNAcβ-O-Al (78.8% active) as an acceptor whereas Gal3ST-3 was less active (32.2%) towards this acceptor. Besides providing novel and important specificity data, these experiments also suggest that Gal3ST-2 and ST-3 can help form di-sulfated structures. Thus, these enzymes may act in synergy with Gal6:O-sulfotransferases to form unique di-sulfated structures in the human physiology.

Effect of the GalNAc moiety substituents on Gal 3ST-4 activity

Studies similar to that in Table IV and V were undertaken for Gal3ST-4 (Table VI). Here, 6-O-Sulfo or 6-O-Me groups did not affect at all the activity (139.2% and 128.6% active respectively) of Gal

3ST-4 whereas 6-O-sialyl group decreased the enzyme activity (30.9% active only). β1,6 linked 3-O-SulfoGalβ1,4GlcNAc moiety increased the acceptor activity (183.9% active) whereas NeuAcα2,3Galβ1,4GlcNAc moiety decreased the activity (28.7% active). Both 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 suggests that 3-O-sulfation of both Gal residues in the mucin core-2 is possible.

A comparison of LS180 Gal 3ST with the cloned Gal 3STs

Comparison of the enzyme activities in Table II-IV suggest that the identity of the Gal 3ST isolated from LS180 cells is Gal 3ST-2. Experiments were conducted to verify this finding:

a) Effect of divalent metal ions: The enzymatic transfer of sulfate was measured separately with each Gal 3ST using specific acceptors in the presence of varying concentrations of Mg^{2+}, Mn^{2+} and Ca^{2+} (Fig. 3). In the case of LS180 sulfotransferase, both Mg^{2+} and Ca^{2+} showed a similar profile of activity when Galβ1,4GlcNAcβ1,6(3-O-MeGalβ1,3)GalNAcα-O-Bn (Fig. 3A) and Galβ1,3GalNAcβ1,3Galα-O-Al (Fig. 3B) were used as acceptors. In both cases, Mn^{2+} stimulated the enzyme activity up to a concentration of 10 mM concentration and the activity gradually decreased with further increase in Mn^{2+} concentration. The stimulation of Gal3ST-2 activity by Mg^{2+}, Mn^{2+} and Ca^{2+} was similar to that of LS180Gal3ST (compare Fig. 3C with 3A and 3B).

Among the Gal 3STs tested, Mg^{2+}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+}, Mn^{2+} or Ca^{2+} had a stimulating effect on Gal3ST-4. In fact, a gradual decreasing in the enzyme activity of Gal 3ST-4 was noticed upon increasing the concentration of Mn^{2+} in the reaction mixture (Fig. 3E).

b) Effect of pH on Gal 3ST activities: The activities of LS180 Gal 3ST and Gal3ST-2 were measured over a pH range from 5.2 to 8.4 using the Globo H precursor Galβ1,3GalNAcβ1,3Galα-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.

c) Acceptor competition experiment: Acceptor competition studies were performed with LS180Gal3ST and Gal3ST-2 (Fig. 4). Here, the enzymatic transfer of sulfate was measured separately for the acceptors Galβ1,4GlcNAcβ1,6(3-O-MeGalβ1,3)GalNAcα-O-Bn and Galβ1,3GalNAcβ1,3Galα-O-Al in presence of varying concentrations of mucin core 2 acceptors containing a sulfo group on C-3 of either Gal, namely 3-O-SulfoGalβ1,4GlcNAcβ1,6(Galβ1,3)GalNAcα-O-Bn and Galβ1,4GlcNAcβ1,6(3-O-SulfoGalβ1,3)GalNAcα-O-Bn. The results of these experiments are identical for LS180Gal3ST (Fig. 4) and 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-SulfoGalβ1,4GlcNAcβ1,6(Galβ1,3)GalNAcα-O-Bn. The other sulfated compound Galβ1,4GlcNAcβ1,6(3-O-SulfoGalβ1,3)GalNAcα-O-Bn, which is a specific acceptor for the Gal 3ST 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 supports the proposition that the identity of LS180 Gal3ST matches that of Gal3ST-2.

d) A comparison of the kinetic properties of LS180Gal3ST with that of Gal3ST-2 and Gal3ST-3 The Km and Vmax values for the acceptors D-Fucβ1,3GalNAcβ1,3Galα-O-Me; Galβ1,3GalNAcβ1,3Galα-O-Me; Galβ1,4GlcNAcβ1,6Manα1,6Manβ1,6Man; Galβ1,4 GlcNAcβ1,6(3-O-MeGalβ1,3)GalNAcα-O-Bn; Galβ1,3GlcNAcβ1,3Galβ-O-Me and Fetuin triantennary asialo GP were determined for LS180Gal3ST (Fig. 4A) and Gal3ST-2 (Fig. 4B), and these are tabulated in Table VII. Except for Galβ1,3GlcNAcβ1,3Galβ-O-Me (Km:20.0mM), the Km value was 4mM for each acceptor and for both enzymes. These data further reveal the identity of LS180Gal3ST as Gal3ST-2. The Vmax 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. Galβ1,4GlcNAcβ1,6Manα1,6Manβ1,6Man served as a good acceptor for both enzymes. But, as anticipated the nature of N-glycan acceptors, namely Fetuin triantennary asialo GP was found to be far less active than the Globo H structures. Gal3ST-3 (Fig. 4C)

gave the Km values 0.43mM, 0.87mM and 0.74mM for the acceptors Fetuin triantennary asialo GP, Galβ1,4GlcNAcβ1,6Manα1,6Manβ1,6Man and Galβ1,4GlcNAcβ1,6(3-O-MeGalβ1,3)GalNAcα-O-Bn respectively. From the Vmax values, it is evident that Gal 3ST-3 prefers to act on the terminal Galβ1,4GlcNAc units in N-linked glycans.

e) 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 LS-180 has mRNA corresponding to two Gal3:0-sulfotransferases, Gal 3ST-4 which we obtained using the primers to the common domain and Gal 3ST-2 which was obtained with primers corresponding to the PAPS binding domain. The observations support our suggestion that we have purified Gal 3ST-2 from LS180 cells. Gal 3ST-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-MeGalβ1,3)GalNAcα-O-Bn, Table I) is not acted upon by Gal3 ST4.

DISCUSSION

Studies of cellular glycosyltransferase expression at the mRNA level can be performed using micro-arrays 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 sulfotransfereases, Gal 3ST-2, Gal 3ST-3 and Gal 3ST-4. Emphasis is placed on acceptor studies with the mucin core-2 structure and the globoH precursor due to the physiological importance of these molecules, and since such studies have not been carried out previously.

Gal 3ST in colon and breast cancer cells and tissue

Various Gal 3:O sulfotransferases have been noted to act on glycoproteins and glycolipids. In one study, it was observed that the human renal 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, CST [24]. Honke *et al.* [12] further used the CST cDNA sequence as a probe and cloned a human βGal:3-O-sulfotransferase (GP3ST or Gal 3ST-2) that acts on LacNAc types 1 and 2, as well as mucin core 1 structure. The amino acid sequence of Gal 3ST-2 indicated 33% identity to the CST 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, which acts on terminal LacNAc unit in mucins, but this did not utilize GalCer [25]. The activity of this enzyme does not match any of the Gal 3STs tested in the current study. Chance and Mawhinney [26] reported the occurrence of 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 Gal 3ST-2 or ST-4. We have also reported earlier, 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 (Galβ1,3GalNAcα) 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 Gal 3ST acting on Galβ1,3GalNAcα 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 Gal 3ST from LS180 cells using a series of chromatography steps. In these studies, we noted that the LS180 Gal 3ST can bind to 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 Gal 3ST exhibited the same kind of acceptor specificity and kinetics towards the LacNAc type 2 structure of mucin core 2 as the enzyme from crude lysate. Our enzyme preparation from LS180 was free of GlcNAc6:O Sulfotransferase as Me-O-Galβ1,3(GlcNAcβ1,6)GalNAcα-O-Bn did not act as an acceptor. Upon comparison with three cloned Gal 3ST, the activity of LS180 sulfotransfersase most closely resembled Gal 3ST-2. Drawing analogies from the current work, based on the acceptor specificity studies, it appears very likely that the predominant Gal 3ST in breast cancer cells and tissue is Gal 3ST-4.

Identification of unique acceptors for cloned Gal 3STs

We performed studies on various cloned Gal 3:O sulfotransfereases (Gal 3ST-2, Gal 3ST-3 and Gal 3ST-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.

Gal 3ST-2 and Gal 3ST-3 have been shown to utilize the substrate Galβ1,4GlcNAc [12, 13]. We further show here that: a) These enzymes act with similar efficiency on the disaccharide when it is presented alone and when this unit is expressed in the context of the core 2 tetrasaccharide (Fig. 6a). We also found that Galβ1,4GlcNAcβ1,6Manα1,6Manβ1,6Man was an efficient acceptor of Gal3ST-2 and Gal3ST-3. b) While Gal 3ST-3 did not act on Galβ1,3GalNAcα-O-Al; Gal 3ST-2 showed low activity (15.9%) towards this acceptor. These enzymes show some activity (20-24%) toward the unoccupied Galβ1,3GalNAc chain in 3-O-MeGalβ1-4GlcNAcβ1,6(Galβ1,3)GalNAcα-O-Bn, and they acted on the trisaccharide Galβ1,3(GlcNAcβ1,6)GalNacα-O-Al at 45-68%. These results are in agreement with observations of Honke *et al.* [12] who show that Gal3ST-2 enzyme has >3-fold activity toward Galβ1,4GlcNAc compared to core 1 Galβ1,3GalNAcα-O-Bn disaccharide. These authors also show that their enzyme preparation was also active toward Galβ1,4GlcNAcβ1,3Galβ1,4Glc(nLC4) and Galβ1,3GlcNAcβ1,3Galβ1,4Glc(LC4). On the other hand, Gal 3ST-3, which is involved in the biosynthesis of 3-O-sulfo-Le^x, has poor activity toward LC4 but is quite active towards nLC4. c) We show that the activities of these two enzymes can be readily distinguished when Globo H precursor Galβ1,3GalNAcβ1,3Galα-O-Me and Fetuin triantennary asialo GP were tested 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 glycopeptide was a better acceptor for Gal3ST-3 (383.9% active) than either Gal3ST-2 (81.7% active) or Gal3ST-4 (14.8% active). d) Gal 3ST-2 and ST-3 also had distinct activities towards sulfated acceptors (Fig. 6e). While Galβ1,4(6-O-Sulfo)GlcNAcβ-O-Al was a specific acceptor for Gal3ST-3, Galβ1,3(6-O-Sulfo)GlcNAcβ-O-Al was specific for Gal 3ST-2.

Gal 3ST-4 was found to be specific for the Galβ1,3GalNAcα [14] (Fig. 6d). We further show in the present study that: a) As compared to Galβ1,3GalNAcα- as such, the Galβ1,3GalNAcα- 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 Gal 3ST-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 decreases the Gal 3ST-4 acceptor ability (28.7% active compared to core-2 tetrasaccharide), a 3-O-sulfo group at this position enhances the activity (183.9% active). This enzyme also acts efficiently (130% active) on Galβ1,3(6-O-Sulfo)GalNAcα-O-Al. Overall, the action of Gal 3ST-4 can lead to the synthesis of diverse di-sulfated structures in cells (Fig. 6a, 6e). c) As compared to Galβ1,3GalNAcα-O-Al, Galβ1,3GalNAcβ1,3Galα-O-Me and D-Fucβ1,3GalNAc β1,3Galα-O-Me were 327.8% and 213.0% active, indicating that the α-linkage of GalNAc is not critical for enzyme activity. d) Since Galβ1,3GalNAcβ1,3Galα-O-Me was considerably more active than D-Fucβ1,3GalNAcβ1,3Galα-O-Me, it appears that C_6-OH group of Gal is necessary for maximum activity.

Mucin core-2 and GloboH precursor

We compared the behavior of the cloned sulfotransferases towards the mucin core-2 structure and towards 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-L-fucosyltransferase from prostate cancer cells, LNCaP, which acts on the Galβ1,4GlcNAcβ unit of the core 2-branched structure [11], also incorporated fucose at the C-2 position of the terminal galactose in Galβ1,3GalNAcβ1,3Galα-OMe. Moreover, cloned α2,3-Sialytransferase ST-3GalVI acting on the Galβ1,4GlcNAcβ- arm in mucin core-2 also efficiently utilized the Globo trisaccharide (unpublished data). In the current study we observed that Gal 3ST-2 acts similarly to α1,2-L-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 must also

act on the GloboH precursor. In this regard, while Gal 3ST-4 acted upon the Globo H precursor, it did not act on the Galβ1,4GlcNAcβ arm of the mucin-core-2. Also, while Gal 3ST-3 acted upon Galβ1,4GlcNAc, it did not act efficiently on the GloboH precursor.

Similarities are observed between the substrate specificities of Gal 3ST and ST-3GalIV. ST-3GalIV is a human Galβ1,3/4GlcNAc:α2,3-sialyltransferase involved in the *in vivo* biosynthesis of sialyl Lewis x. It is also able to utilize glycosphingolipids, the most efficient acceptor being Galβ1,4GlcNAcβ1,3Galβ1,4Glc-Cer (nLC4Cer) [31]. On the other hand, a similar enzyme (STZ) cloned by Kitagawa and Paulson [32] utilized Galβ1,3GlcNAcβ1,3Galβ1,4Glc-Cer 5-fold more efficiently than nLC4Cer. Another cloned α2,3-sialyltransferase was found to be specific for the synthesis of GM3,NeuAcα2,3Galβ1,4Glc-Cer [33]. A cloned human ST-3GalVI exhibited high acceptor specificity towards terminal Galβ1,4GlcNAc sequence in glycoproteins and glycolipids [34]. The α2,3-sialyltransferase obtained from myxoma virus infected rabbit kidney RK13 cells exhibited close homology to mammalian ST-3GalIV but this enzyme unlike other ST-3 enzymes was able to use Lewis x and Lewis a as acceptors [35]. Thus it appears reasonable to assume that just like Gal:α2,3-sialyltransferases, several distinct Gal:3-O-sulfotransferases may exist that play a role in tissue and cell type-specific expression of terminal 3-O-Sulfogalactosyl moieties on glycoproteins and glycolipids.

The current study defines the acceptor specificity of carbohydrate-specific Gal 3STs cloned thus far, and it poses new questions for the future. In this regard, the characterization of specific substrates in 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 GloboH 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 di-sulfated structures *in vivo.* They also suggest that it will be important to compare the competitive action of various α2,3sialyltransferases and Gal 3STs on the core-2 mucin structures, along with structural analysis of cell-surface carbohydrates.

Finally, we showed here that 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 if such molecules are poor acceptors for these enzymes or if they act as competitive inhibitors that may find *in vivo* application as sulfo- and sialyl-transferase inhibitors.

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FIGURE LEGENDS

Fig. 1. Core-2 tetrasaccharide and GloboH precursor. Enzymes that prefer to act on the Gal residue of the Galβ1→4GlcNAcβ moiety on the core-2 structure also typically act on the Gal residue on the GloboH backbone.

Fig. 2. Enzyme specificity verified using TLC. Thin layer chromatography of Galβ1,3GalNAcβ1,3Galα-O-Me (lane A), synthetic 3-O-SulfoGalβ1,3GalNAcβ1,3Galα-O-Me (lane B) and the [^{35}S] sulfated compound arising from the action of the purified LS-180 Gal 3ST enzyme on Galβ1,3GalNAcβ1,3Galα-O-Me (lane C).

Fig. 3. Effect of Divalent metal ions on Gal 3ST activity. Activity of LS180 Gal 3ST on acceptor Galβ1,4GlcNAcβ1,6(3-O- MeGalβ1,3)GalNAcα-O-Bn (panel A), LS180 Gal 3ST on Galβ1,3GalNAcβ1,3Galα-O-Al (panel B), Gal 3ST-2 on Galβ1,4GlcNAcβ1,6(3-O-MeGalβ1,3)GalNAcα-O-Bn (panel C), Gal 3ST-3 on Fetuin triantennary asialo GP (panel D) and Gal 3ST-4 on Galβ1,3(GlcNAcβ1,6)GalNAcα-O-Al (panel E), and was quantified in presence of varying cation concentrations. Effect of Mg^{2+} is depicted by □-□-□, Mn^{2+} by ◇-◇-◇ and $Ca^{2+}$by △-△-△.

Fig. 4. Acceptor competition experiments. Acceptor competition runs were performed for Gal:3-O-Sulfotransferase from LS180 cells, where the action of this purified enzyme on either the modified mucin Core 2 [Galβ1,4GlcNAcβ1,6(3-O-MeGalβ1,3)GalNAcα-O-Bn] or GloboH precursor [Galβ1,3GalNAcβ1,3Galα-O-Al] was quantified in the presence of one of the two sulfated compounds. Data where mucin Core 2 is the acceptor in presence of 3-O-SulfoGalβ1,4 GlcNAcβ1,6(Galβ1,3)GalNAcα-O-Bn (△-△-△) and Galβ1,4GlcNAcβ1,6(3-O-SulfoGalβ1,3)GalNAcα-O-Bn (▲-▲-▲). Data where GloboH precursor is the acceptor in presence of 3-

O-SulfoGalβ1,4GlcNAcβ1,6(Galβ1,3)GalNAcα-O-Bn (○-○-○) and Galβ1,4GlcNAcβ1,6 (3-O-SulfoGalβ1,3)GalNAcα-O-Bn (●-●-●).

Fig. 5. Kinetic studies with cloned and purified enzymes. Linewever-Burke plots for LS180 Gal 3ST (panel A), Gal 3ST-2 (panel B), Gal 3ST-3 (panel C) activities are presented. These plots were obtained by varying the concentration of various acceptors. Acceptors studied in panel A and B were : ●-●-● D-Fucβ1,3GalNAcβ1,3Galα-O-Me; ○-○-○ Galβ1,3GalNAcβ1,3Galα-O-Me; △-△-△ Galβ1,4GlcNAcβ1,6Manα1,6Manβ1,6Man; ▲-▲-▲ Galβ1,4GlcNAcβ1,6(3-O-MeGalβ1,3)GalNAcα-O-Bn; □-□-□ Galβ1,3GlcNAcβ1,3Galβ-O-Me; ■-■-■ Fetuin triant asialo GP. Acceptors in panel C were: ○-○-○ Fetuin triant asialo GP; △-△-△ Galβ1,4GlcNAcβ1,6Manα1,6Manβ1,6Man; ●-●-● Galβ1,4GlcNAcβ1,6(3-O-MeGalβ1,3)GalNAcα-O-Bn.

Fig. 6. Role of Gal3 STs examined in this study.

TABLE I

Purification of Gal 3ST from human colon carcinoma LS180 cells

Fraction	Volume ml	Total Protein mg	Enzyme Activity milliunits	Enzyme Specific Activity milliunits	Fold Purification	Recovery of Enzyme Activity %
1. Homogenate	65	1485	356.4	0.24	1.0	100.0
2. Triton X-100 Supernatant	60	810	315.9	0.39	1.6	88.9
3. Aleuria Aurantia Lectin-agarose Chromatography: Fucose Eluate (concentrated & dialyzed)	10	52	169.0	3.25	13.5	47.3
4. Chromatography on Affinity Gel-GDP: NaCl Eluate (concentrated & dialyzed)	3	0.8	143.0	178.80	745.0	40.0
5. Chromatography on Sephacryl S100 HR column (concentrated & dialyzed):	0.8	0.15	108.4	722.60	3010	30.4

1 milliunit is defined as the transfer of 1 nmol of sulfate from PAP[^{35}S] to the acceptor Galβ1,4GlcNAcβ1,6(3-O-MeGalβ1,3)GalNAcα-O-Bn (7.5 mM) catalyzed by Gal:3-O-sulfotransferase in 1 h at 37^{o}C.

TABLE II

The Globo structure and its 6-deoxy analog are better acceptors than mucin core 2 structure for colon carcinoma LS180 Gal 3ST

Acceptor (7.5 mM)	The activity of the purified LS180 Gal:3-O-sulfotransferase %
Acceptors based on mucin core-2 structure:	
3-O-MeGalβ1,4GlcNAcβ1,6(Galβ1,3)GalNAcα-O-Bn	15.9
Galβ1,4GlcNAcβ1,6(3-O-MeGalβ1,3)GalNAcα-O-Bn	100.0 (59,970)*
Galβ1,4GlcNAcβ1,6(Galβ1,3)GalNAcα-O-Bn	95.6
3-O-MeGalβ1,3(GlcNAcβ1,6)GalNAcα-O-Bn	0
Galβ1,3(GlcNAcβ1,6)GalNAcα-O-Bn	8.7
Galβ1,3GalNAcα-O-Al	20.2
Galβ1,3GlcNAcβ-O-Al	3.0
Acceptors based on GloboH:	
Galβ1,4Glcβ1,6GalNAcα-O-Bn	5.0
Galβ1,3GalNAcβ1,3Galα-O-Al	113.2
Galβ1,3GalNAcβ1,3Galα-O-Me	159.3
Galβ1,3GlcNAcβ1,3Galβ-O-Me	20.6
D-Fucβ1,3GalNAcβ1,3Galα-O-Me	180.5

* Value in parenthesis is the activity in CPM of the reference acceptor (100% activity) against which all other acceptors were compared; Blank samples (without any acceptor) were of less than 50CPM and this corresponds to 0% activity.

TABLE III

A comparison of the acceptor substrate specificities of the three cloned Gal 3ST and the purified Gal 3ST from LS180

Acceptor 7.5mM	Gal 3ST-2	Gal 3ST-3	Gal 3ST-4	LS180 Gal 3ST
	%	%	%	%
Galβ1,3GalNAcα-O-Al	15.9	0	100.0* (5,400)	12.1
Galβ1,4GlcNAc	100.0* (83,215)	100.0* (3,100)	0	100.0* (66,300)
Galβ1,4GlcNAcβ1,6(Galβ1,3)GalNAcα-O-Bn	89.4	100.0	324.1	71.3
Galβ1,4GlcNAcβ1,6(3-0-MeGalβ1,3)GalNAcα-O-Bn	116.9	119.4	1.9	74.7
3-0-MeGalβ1,4GlcNAcβ1,6(Galβ1,3)GalNAcα-O-Bn	24.0	19.4	335.2	12.7
Galβ1,3(GlcNAcβ1,6)GalNAcα-O-Al	67.9	45.2	857.4	33.0
Galβ1,3GalNAcβ1,3Galα-O-Me	295.6	25.8	327.8	178.0
D-Fucβ1,3GalNAcβ1,3Galα-O-Me	296.0		213.0	197.9
Galβ1,4GlcNAcβ1,6Manα1,6Manβ1,6Man	287.7	296.8	1.9	156.6
Fetuin triantennary asialo GP (100μg:2.5mM)	81.7	383.9	14.8	37.7
Galβ1,3GalNAcα-O-Al/AA-CP (100μg:0.125mM)	0.1		61.1	

* See legend to Table II

TABLE IV

A comparison of Gal 3ST activities towards mucin core 2 based acceptors

	Gal 3ST-2	Gal 3ST-3	Gal 3ST-4	LS180 Gal3SulfoT
Galβ1,4GlcNAcβ1,6(3-0-MeGalβ1,3)GalNAcα-O-Bn	100.0* (122,655)	100.0* (12,474)		100.0* (34,369)
Galβ1,4GlcNAcβ1,6(Galβ1,3)GalNAcα-O-Bn	80.0	71.1		76.5
3-0-MeGalβ1,4GlcNAcβ1,6(Galβ1,3)GalNAcα-O-Bn	17.0	16.2	100.0* (26,353)	9.2
Galβ1,4GlcNAcβ1,6(4-0-MeGalβ1,3)GalNAcα-O-Bn	55.0	45.5	7.1	47.2
4-0-MeGalβ1,4GlcNAcβ1,6(Galβ1,3)GalNAcα-O-Bn	12.0	26.9	60.8	7.0
Galβ1,4GlcNAcβ1,6(3-F-Galβ1,3)GalNAcα-O-Bn	16.3	12.2		16.6
3-F-Galβ1,4GlcNAcβ1,6(Galβ1,3)GalNAcα-O-Bn	1.3	1.4	23.1	2.4

* See legend to Table II

TABLE V

Differentiation of Gal 3ST-2 and Gal 3ST-3 by using simple sulfated compounds as acceptors.

Acceptor	Activity (%) Gal 3ST-2	Gal 3ST-3
Galβ1,4GlcNAc	100.0* (83,215)	100.0* (11,580)
Galβ1,4(6-O-Sulfo)GlcNAc	4.2	102.8
Galβ1,4(6-O-Sulfo)GlcNAcβ-O-Al	2.6	131.3
Galβ1,3(6-O-Sulfo)GlcNAcβ-O-Al	78.8	32.2

* See legend to Table II

TABLE VI

Influence of the substituent groups in the GalNAc moiety of Galβ1,3GalNAc-α on Gal 3ST-4 activity

Acceptor	Activity (%)
3-0-MeGalβ1,4GlcNAcβ1,6(Galβ1,3)GalNAcα-O-Bn	100.0* (26,353)
Galβ1,3(NeuAcα2,6)GalNAcα-O-Bn	30.9
Galβ1,3(6-O-Sulfo)GalNAcα-O-Al	139.2
Galβ1,3(6-0-Me)GalNAcα-O-Bn	128.6
3-0-SulfoGalβ1,4GlcNAcβ1,6(Galβ1,3)GalNAcα-O-Bn	183.9
NeuAcα2,3Galβ1,4GlcNAcβ1,6(Galβ1,3)GalNAcα-O-Me	28.7
GalNAcβ1,4(Fucα1,3)GlcNAcβ1,6(Galβ1,3)GalNAcα-O-Bn	215.7
Galβ1,3(GlcNAcβ1,6)GalNAcα-O-Al	243.8

* See legend to Table II

TABLE VII

Kinetic data obtained for the purified LS180 Gal 3ST, Gal 3ST2 and Gal 3ST3

Acceptor	LS180 Gal 3ST		Gal 3ST-2		Gal 3ST-3	
	Km (mM)	Vmax (pmol/h)	Km (mM)	Vmax (pmol/h)	Km (mM)	Vmax (pmol/h)
Galβ1,4GlcNAcβ1,6(3-0-MeGalβ1,3) GalNAcα-O-Bn	4.0	5.8	4.0	11.7	0.74	1.0
Galβ1,3GalNAcβ1,3Galα-O-Me	4.0	8.8	4.0	35.0	ND	
D-Fucβ1,3GalNAcβ1,3Galα-O-Me	4.0	8.8	4.0	35.0	ND	
Galβ1,3GlcNAcβ1,3Galα-O-Me	20.0	7.0	20.0	11.7	ND	
Galβ1,4GlcNAcβ1,6Manα1,6Manβ1,6Man	4.0	11.7	4.0	35.0	0.87	2.3
Fetuin triantennary asialo GP	4.0	5.8	4.0	11.7	0.43	2.7

ND: Not Determined

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Figure 1

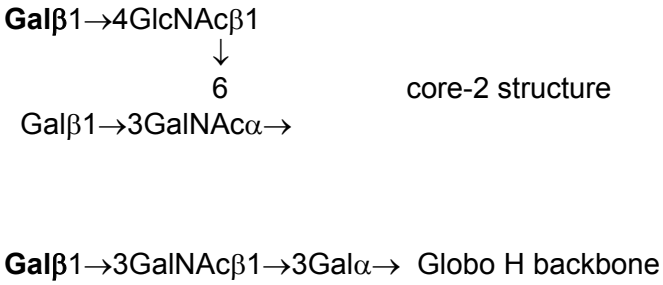

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Figure 2

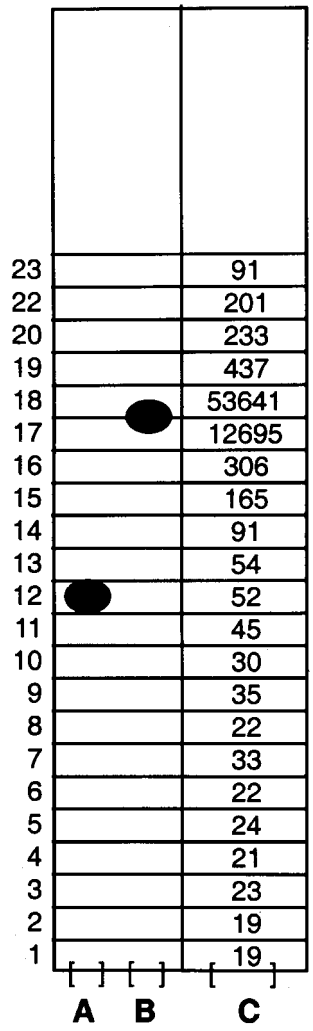

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Figure 3

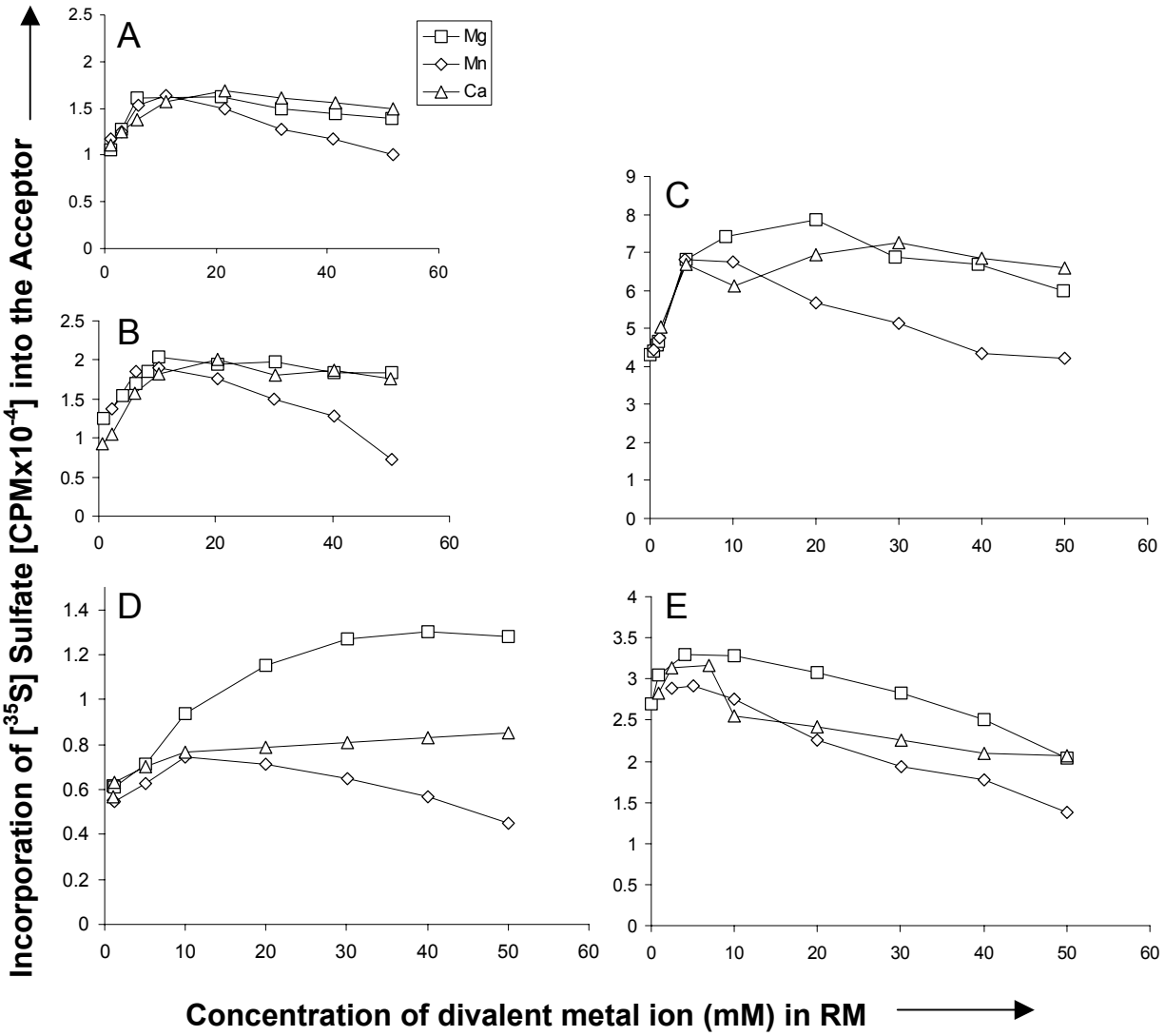

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Figure 4

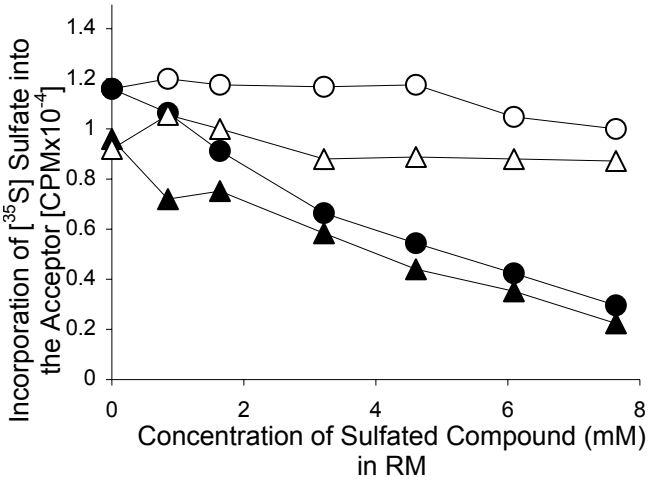

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Figure 5

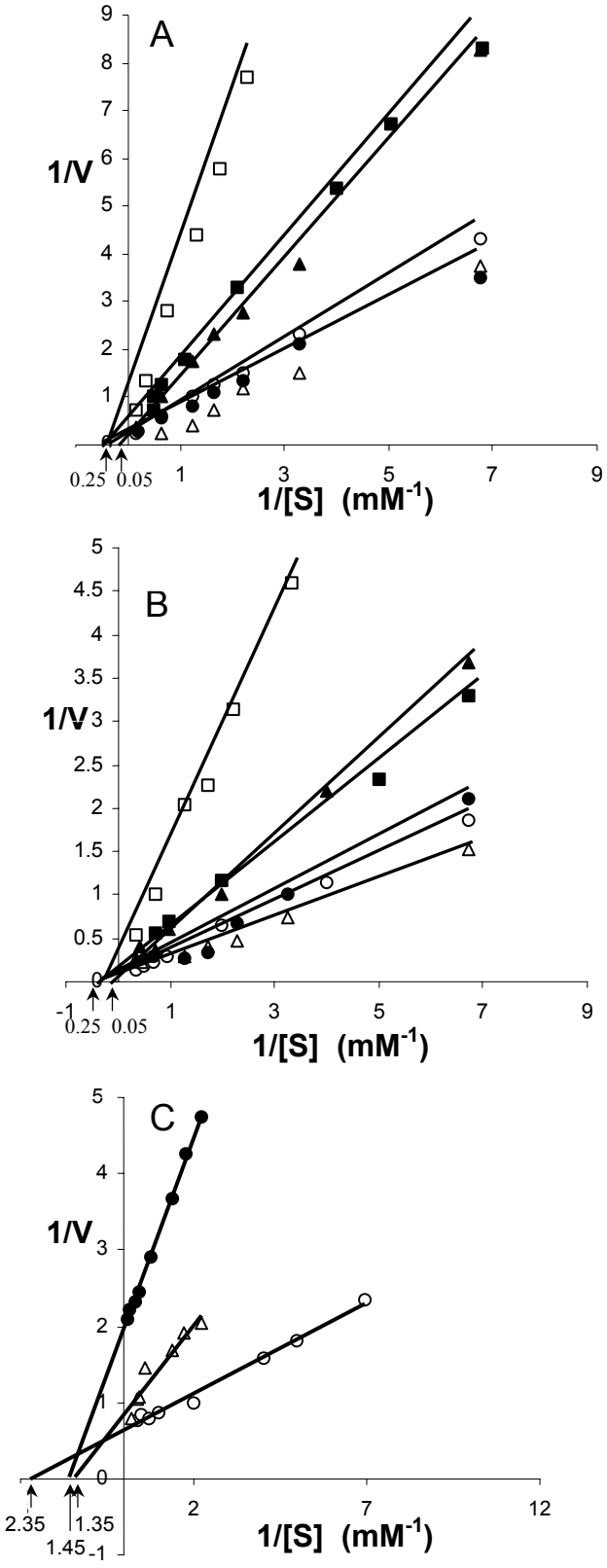

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Figure 6

a) Galβ1,4GlcNAcβ1,6(Galβ1,3)GalNAcα-O-Bn **[Mucin core 2]**

↓ **Gal3ST-2 or Gal3ST-3**

3-O-SulfoGalβ1,4GlcNAcβ1,6(Galβ1,3)GalNAcα-O-Bn

↓ **Gal3ST-4**

3-O-SulfoGalβ1,4GlcNAcβ1,6(3-O-SulfoGalβ1,3)GalNAcα-O-Bn

b) Asialo triantennary chain (complex type) **[N-Glycan]**

↓ **Gal3ST-3**

Sulfated triantennary chain

c) Galβ1,3GalNAcβ1,3Galα-O-Me **[Globo backbone]**

↓ **Gal3ST-2 or Gal3ST-4**

3-O-SulfoGalβ1,3GalNAcβ1,3Galα-O-Me

d) Galβ1,3GalNAcα-O-Al **[T-hapten]**

↓ **Gal3ST-4**

3-O-SulfoGalβ1,3GalNAcα-O-Al

e) Galβ1,4(6-O-Sulfo)GlcNAcβ-O-Al **[Disulfo LacNAc**

↓ **Gal3ST-3** **type 1 and type 2]**

3-O-SulfoGalβ1,4(6-O-Sulfo)GlcNAcβ-O-Al

Galβ1,3(6-O-Sulfo)GlcNAcβ-O-Al

↓ **Gal3ST-2**

3-O-SulfoGalβ1,3(6-O-Sulfo)GlcNAcβ-O-Al