Porcine, mouse and human galactose 3-O-sulphotransferase-2 enzymes have different substrate specificities; the porcine enzyme requires basic compounds for its catalytic activity

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Sulphation of galactose at the C-3 position is one of the major post-translational modifications of colorectal mucin. Thus we partially purified a Gal 3-O-sulphotransferase from porcine colonic mucosa (pGal3ST) and studied its enzymatic characteristics. The enzyme was purified 48 500-fold by sequential chromatographies on hydroxyapatite, Con A (concanavalin A)–Sepharose, porcine colonic mucin–Sepharose, Cu^{2+}-chelating Sepharose and AMP–agarose. Interestingly, the purified pGal3ST required submillimolar concentrations of spermine or basic lipids, such as D-sphingosine and *N*,*N*-dimethylsphingosine, for enzymatic activity. pGal3ST recognized Galβ1 → 3GalNAc (core 1) as an optimal substrate, and had weaker activity for Galβ1 → 3GlcNAc (type 1) and Galβ1 → 4GlcNAc (type 2). Substrate competition experiments proved that a single enzyme catalyses sulphation of all three oligosaccharides. Among the four human Gal3STs cloned to date, the substrate specificity of pGal3ST is most similar to that of human Gal3ST-2, which is also strongly expressed in colonic mucosa, although the kinetics of pGal3ST and human Gal3ST-2 were rather different. To determine whether pGal3ST is the orthologue of human Gal3ST-2, a cDNA encoding porcine Gal3ST-2 was isolated and the enzyme was expressed in COS-7 cells for analysis of substrate specificity. This revealed that porcine Gal3ST-2 has the same specificity as pGal3ST, indicating that pGal3ST is indeed the porcine equivalent of Gal3ST-2. The substrate specificity of mouse Gal3ST-2 was also different from those of human and porcine Gal3ST-2 enzymes. Mouse Gal3ST-2 preferred core 1 and type 2 glycans to type 1, and the K_m values were much higher than those of human Gal3ST-2. These results suggest that porcine Gal3ST-2 requires basic compounds for catalytic activity and that human, mouse and porcine Gal3ST-2 orthologues have diverse substrate specificities.

Key words: colonic mucosa, mucin, spermine, sphingosine, sulphotransferase.

INTRODUCTION

Sulphation of colonic mucosal glycoproteins is believed to be responsible for the viscosity and primary defensive properties of the mucous layer. It has been shown that O-linked and N-linked glycans in colon-derived glycoproteins are sulphated at C-3 and C-6 of Gal and C-6 of GlcNAc residues [1–5]. One of these sulphation events, 3-O-sulphation of Gal, is catalysed by Gal3ST (Gal 3-O-sulphotransferase), which is the focus of the present study.

To date, four human Gal3STs have been cloned. The first, Gal3ST-1 is a cerebroside 3-O-sulphotransferase (CST) [6], which acts on GalCer (galactosylceramide) and GalDG (galactosyldiacylglycerol) [7]. Gal3ST-2 or GP3ST has broad substrate specificity towards Galβ1 → 3GlcNAc (type 1), Galβ1 → 4GlcNAc (type 2) and Galβ1 → 3GalNAc (core 1) structures [8]. Suzuki et al. [9] and El-Fasakhany et al. [10] identified Gal3ST-3, which prefers type 2 structures, whereas Gal3ST-4 [11] is highly specific for core 1 structures. The amino acid sequences of these four Gal3STs share 30–40 % identity, suggesting that the Gal3STs constitute a molecular family.

Recently, we found that Gal3ST-2 expression is down-regulated in human colonic adenocarcinomas [12]. Since the substrate specificity of Gal3ST-2 is very similar to those of Gal3ST in human colonic mucosa and adenocarcinomas, Gal3ST-2 seems to be the major Gal3ST that is expressed in human colonic mucosa and adenocarcinomas. Gal3ST-2 is one of the enzymes that is involved in the synthesis of 91.9H antigen, 3′-sulphoLewis^a [5], and down-regulation of Gal3ST-2 expression in colonic adenocarcinomas seems to be responsible for the lower expression of 91.9H antigen that is observed in colonic adenocarcinomas relative to normal mucosa.

Previously, Kuhns et al. [13] characterized Gal3ST activity in the membrane fraction of rat colonic mucosa. Interestingly, its substrate specificity is slightly different from that of human Gal3ST-2. Specifically, the rat Gal3ST prefers core 1 to type 1 or type 2 glycan structures, whereas human Gal3ST-2 acts efficiently on all three substrates. There are two possible explanations for this phenomenon: (i) rat Gal3ST is encoded by a novel fifth gene, or (ii) rat Gal3ST is the orthologue of human Gal3ST-2, although their enzymatic characteristics differ. To clarify this issue, we partially purified a Gal3ST from porcine mucosa (pGal3ST),

Abbreviations used: biGP, Galβ1 → 4GlcNAcβ1 → 2Manα1 → 3(Galβ1 → 4GlcNAcβ1 → 2Manα1 → 6)Manβ1 → 4GlcNAcβ1 → 4GlcNAc; Bn, benzyl; Con A, concanavalin A; core 1, Galβ1 → 3GalNAcα1 →; core 2, Galβ1 → 3(GlcNAcβ1 → 6)GalNAcα1 →; DTT, dithiothreitol; GalCer, galactosylceramide; GalDG, galactosyldiacylglycerol; Gal3ST, Gal 3-*O*-sulphotransferase; LacCer, lactosylceramide; LNT, Galβ1 → 3GlcNAcβ1 → 3Galβ1 → 4Glc; monoGP, Galβ1 → 4GlcNAcβ1 → 2Manα1 → 3/6Manβ1 → 4GlcNAc; PAPS, adenosine 3′-phosphate 5′-phosphosulphate; pGal3ST, porcine Gal3ST; PNA, peanut agglutinin; pNP, *p*-nitrophenyl; RACE, rapid amplification of cDNA ends; SulT, sulphotransferase; type 1, Galβ1 → 3GlcNAc (lacto-*N*-biose I); type 2, Galβ1 → 4GlcNAc (*N*-acetyllactosamine).

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The nucleotide sequence data reported will appear in DDBJ, EMBL, GenBank® and GSDB Nucleotide Sequence Databases under accession numbers AY216523 for porcine Gal3ST-2 and AY216522 for mouse Gal3ST-2.

which had substrate specificity similar to that of rat Gal3ST. We also isolated a cDNA encoding porcine Gal3ST-2 and showed that porcine Gal3ST-2 has the same substrate specificity as purified pGal3ST. These results suggest that porcine Gal3ST-2 has different substrate specificity to that of human Gal3ST-2. In the process of pGal3ST purification, we also established that purified pGal3ST requires basic compounds for its enzymatic activity.

EXPERIMENTAL

Materials

[^{35}S]PAPS (adenosine 3′-phosphate 5′-phospho[^{35}S]sulphate) (97.0 GBq/mmol) and UDP-[^{3}H]Gal[4,5-^{3}H(N)] (1110 GBq/mmol) were purchased from NEN Life Science Products (Boston, MA, U.S.A.). Type 2, core 1, core 1-*O*-pNP (Galβ1 → 3GalNAcα1-*O*-*p*-nitrophenyl) and core 2-*O*-pNP [Galβ1 → 3-(GlcNAcβ1 → 6)GalNAcα1-*O*-*p*-nitrophenyl] were purchased from Funakoshi (Tokyo, Japan). LNT (Galβ1 → 3GlcNAc-β1 → 3Galβ1 → 4Glc) was isolated from human milk, as described previously [14]. MonoGP (Galβ1 → 4GlcNAcβ1 → 2Manα1 → 3/6Manβ1 → 4GlcNAc) was prepared from the urine of GM_1 gangliosidosis patients [15]. BiGP [Galβ1 → 4GlcNAcβ1 → 2Manα1 → 3(Galβ1 → 4GlcNAcβ1 → 2Manα1 → 6)Manβ1 → 4GlcNAc β1 → 4GlcNAc] was prepared from egg yolk sialoglycopeptide [16] by hydrazinolysis–reacetylation and mild acid hydrolysis. Type 1, GlcNAcβ1-*O*-Bn (where Bn is benzyl), GalNAcα1-*O*-Bn, core1-*O*-Bn, GalCer from bovine brain, LacCer (lactosylceramide) from bovine brain, GalDG from whole wheat flour, L-α-phosphatidic acid from egg yolk lecithin, L-α-phosphatidylcholine from egg yolk, L-α-lysophosphatidylcholine from egg yolk, L-α-phosphatidylethanolamine from egg yolk, L-α-phosphatidylinositol from soybean, L-α-phosphatidyl-L-serine from bovine brain, sphingomyelin from bovine brain, ceramides from bovine brain sphingomyelin, D-sphingosine from bovine brain cerebrosides, *N*,*N*-dimethylsphingosine prepared from D-sphingosine, *N*-acetyl-D-sphingosine prepared from D-sphingosine, *N*-stearoyl-D-sphingosine prepared from D-sphingosine, sphingosine 1-phosphate and stearylamine were all purchased from Sigma–Aldrich. (St. Louis, MO, U.S.A.). *Streptococcus* 6646K β-galactosidase was purchased from Seikagaku (Tokyo, Japan). RCA-I (*Ricinus communis* agglutinin-I)–agarose (4 mg/ml gel) was purchased from Hohnen Oil (Tokyo, Japan). PNA (peanut agglutinin)–agarose (4.5 mg/ml gel) was purchased from E-Y Laboratories (San Mateo, CA, U.S.A.). Con A (concanavalin A)–Sepharose, chelating Sepharose FF and CNBr-activated Sepharose 4B were purchased from Amersham Biosciences (Piscataway, NJ, U.S.A.). Octylamine and UDP-Gal were purchased from Sigma–Aldrich. Hydroxyapatite, hexadecyltrimethylammonium bromide and 1-dodecylpyridinium chloride were purchased from Wako Pure Chemical Industries (Osaka, Japan).

Purification of pGal3ST from porcine colonic mucosa

All procedures were performed at 4 °C. The mucus layer was scraped from 20 kg of porcine colon and homogenized using a mixer in 6 litres of 20 mM Hepes/NaOH (pH 7.2) and 1 mM PMSF. A half volume of 3 % (v/v) Triton X-100, 30 % (v/v) glycerol, 30 mM Hepes/NaOH (pH 7.2), 15 mM $MnCl_2$ and 3 mM DTT (dithiothreitol) was added to the homogenate, and the mixture was stirred at 4 °C for 16 h, followed by centrifugation at 5500 ***g*** for 20 min. To the supernatant (fraction 1), 400 ml of hydroxyapatite was added and stirred gently for 1 h. The supernatant was decanted, and the resin was washed three times in 3 vol. of 10 mM Hepes/NaOH (pH 7.2), 10 % (v/v) glycerol, and 0.05 % (w/v) Triton X-100 (buffer A). The absorbed enzyme was recovered from the resin by extraction three times with 2 vol. of 1.5 M NaCl/buffer A. This batch method using hydroxyapatite was repeated three times for fraction 1. The 1.5 M NaCl/buffer A solution containing the enzyme was dialysed against 10 mM Hepes/NaOH (pH 7.2) and 20 % (v/v) glycerol (buffer B), and applied on a hydroxyapatite column (2.4 cm × 27 cm, equilibrated with buffer A). The enzyme was eluted with 1.5 M NaCl/buffer A, and the eluate was adjusted to a composition of 20 mM Tris/HCl (pH 8.0), 1 M NaCl, 1 mM $MgCl_2$, 1mM $CaCl_2$, 0.05 % (w/v) Triton X-100 and 10 % (v/v) glycerol (buffer C), and applied to a Con A–Sepharose column (1.6 cm × 5.1 cm, equilibrated with buffer C). The enzyme was eluted with buffer C containing 0.4 M α-methylmannoside, and SulT (sulphotransferase)-containing fractions were collected and dialysed against buffer B. The enzyme fractions were subjected to sequential chromatographies on: (i) a hydroxyapatite column [1.6 cm × 2.6 cm, equilibrated with buffer A and eluted with a linear gradient (0–1.5 M) of NaCl in buffer A]; (ii) a porcine colonic mucin-derived glycopeptide–Sepharose column [0.75 cm × 4.4 cm, equilibrated with 10 mM sodium cacodylate (pH 6.6), 10 % (v/v) glycerol and 0.1 % Triton X-100, and eluted with a linear gradient (0–0.1 M) of NaCl in the same buffer]; (iii) a Cu^{2+}-chelating Sepharose column [1.6 cm × 2.6 cm, equilibrated with 50 mM sodium acetate (pH 5.4), 10 % (v/v) glycerol, 0.3 M NaCl and 0.05 % (v/v) Triton X-100, and eluted with a linear gradient (0–0.1M) of glycine in the same buffer]; and (iv) an AMP–agarose column [1.6 cm × 2.6 cm, equilibrated with buffer A and eluted with a linear gradient (0–0.6 M) of NaCl in buffer A].

After each chromatography step, the enzyme fractions were dialysed against buffer B. Finally, fractions obtained (pGal3ST) were collected and used for enzymatic characterization.

Preparation of resins for affinity chromatographies

Porcine colonic mucin-derived glycopeptides were prepared as follows: the mucus layer derived from 250 g of porcine colon was homogenized in 20 mM Hepes/NaOH (pH 7.2) and centrifuged at 2500 ***g*** for 20 min. The precipitate was digested with 80 mg of pronase E in 0.1 M Tris/HCl (pH 8), 10 mM $CaCl_2$ and 0.01 % (w/v) NaN_3 at 37 °C for 24 h. The digest was applied to a Sepharose CL-2B column (1.4 cm × 68 cm, eluted with 0.1 M NaCl), and fractions within Kav = 0.25–0.60 [Kav = (eluate volume − void volume)/(total volume − void volume)] were collected, dialysed against water and freeze-dried (yield, 78.9 mg). After digestion with 0.2 unit of *Arthrobacter ureafaciens* sialidase, the glycopeptides were conjugated with CNBr-activated Sepharose 4B according to the manufacturer's instructions. Sialidase digestion could expose Galβ1 → 3GalNAc and/or Galβ1 → 4GlcNAc moieties at the non-reducing termini of O-linked glycans of glycopeptides. The resin bound 0.56 mg of glycopeptides per ml of gel.

Cu^{2+}-chelating Sepharose was prepared according to the manufacturer's instructions. Binding of periodate-oxidized AMP to Affi-Gel Hz (Bio-Rad Laboratories, Hercules, CA, U.S.A.) was performed essentially as described previously [17] with modifications described by the manufacturer. The resin bound 17 μmol of AMP per ml of gel.

Protein concentrations were estimated using the Bio-Rad Protein dye reagent with BSA as standard.

Assay of SulT activity

Aliquots (20 μl) of the reaction mixture consisting of 0.1 M sodium cacodylate (pH 6.3), 10 mM $MnCl_2$, 0.1 % (w/v) Triton

X-100, 0.1 M NaF, 2 mM Na_2-ATP, 6.5 μM [^{35}S]PAPS (2.8×10^5 d.p.m.), 1 mM Galβ1 → 3GalNAc, 0.01 % (w/v) D-sphingosine and the enzyme solution, diluted appropriately, were incubated at 37 °C for 1 h. The ^{35}S-labelled products were purified by paper electrophoresis [pyridine/ethanoic (acetic) acid/water = 3:1:387, by vol., pH 5.4]. The R_F values of ^{35}S-labelled Galβ1 → 3GalNAc and PAPS are 0.80 and 1.89 respectively, when the R_F value of Bromophenol Blue is taken as 1.0. After extraction with water, incorporated radioactivity was counted.

For assaying SulT activities in the presence of various lipids, required amounts of lipids in chloroform/methanol (1:1, v/v) were dried under vacuum and solubilized by sonication in the enzyme reaction solution described above without the enzyme. Thereafter, an appropriate amount of enzyme was added to the solution.

Characterization of the ^{35}S-labelled product

The ^{35}S-labelled product was subjected to periodate oxidation [18]. The labelled oligosaccharides were dissolved in 20 μl of 75 mM sodium metaperiodate and 75 mM sodium acetate (pH 5.3), and incubated at 4 °C for 24 h in the dark. Excess periodate was destroyed by adding 2 μl of 20 % ethylene glycol. After 1 h at room temperature, 300 μl of 0.1 M sodium borate (pH 9.0) and 0.1 M sodium borohydride were added, and the solutions were maintained at room temperature for 3 h. The solutions were acidified with diluted ethanoic acid and passed through a column (0.5 cm × 3 cm) of Bio-Rad AG50W-X8 (H^+ form). The eluates were evaporated, and residual boric acid was removed by repeated evaporation with methanol. The residues were hydrolysed in 100 μl of 0.025 M H_2SO_4 at 80 °C for 1 h. After neutralization with NaOH, the mixtures were subjected to paper electrophoresis. The ^{35}S-labelled compounds were extracted with water, applied on a thin-layer plate (Kieselgel $60F_{254}$, Merck, Darmstadt, Germany) and developed with a solvent mixture, butan-1-ol/pyridine/water (6:4:3, by vol.) or butan-1-ol/ethanol/water (4:1:1, by vol.). Radioactivity was monitored using a radiochromatogram scanner. Authentic $^{35}SO_3^-$ → 3Galβ1 → 3GalNAcα1-*O*-Bn, $^{35}SO_3^-$ → 3Galβ1 → 3(GlcNAcβ1 → 6)GalNAcα1-*O*-pNP and $^{35}SO_3^-$ → 3Galβ1 → 3GalNAc were prepared by Gal3ST-4, as described previously [11]. [^{3}H]Galβ1 → 3GalNAcα1-*O*-Bn as a positive control for periodate oxidation was prepared as follows: 20 μl of a solution containing 50 mM Hepes/NaOH (pH 7.2), 10 mM $MnCl_2$, 0.5 % (v/v) Triton X-100, 5 mM GalNAcα1-*O*-Bn (Koch-Light Laboratories, Colnbrook, Bucks., U.K.), 2.5 μM UDP-[^{3}H]Gal (3.3×10^6 d.p.m.), 250 μM UDP-Gal and a crude membrane fraction from porcine colonic mucosa was incubated at 37 °C for 1 h. The reaction mixture was subjected to paper electrophoresis, and the neutral fraction was subjected further to paper chromatography, which was developed with the solvent mixture pyridine/ethyl acetate/ethanoic acid/water (5:5:1:3, by vol.). A radioactive fraction ([^{3}H]-Galβ1 → 3GalNAcα1-*O*-Bn) with an R_F value of 0.72 was extracted with water. The linkage position of [^{3}H]Gal residues was confirmed by binding to a PNA-conjugated column, since PNA specifically recognizes Galβ1 → 3GalNAc structures and does not bind to SO_3^- → 3Galβ1 → 3GalNAc [11,19]. Synthesis of 6-[^{35}S]sulpho-GlcNAcβ1-*O*-Bn was performed using human GlcNAc 6-O-sulphotransferase activity, as described previously [20].

Cloning of cDNAs encoding porcine and mouse Gal3ST-2

Based on an EST (expressed sequence tag) and a genome sequence in NCBI BLAST databases (accession numbers BB617404 and NW_021880), a cDNA encoding mouse Gal3ST-2 was amplified by PCR from mouse colonic cDNA prepared with the SuperScript™ Preamplification System (Life Technologies). Oligonucleotide primers used for the PCR were 5′-tttaagcttAATAGGTTACAGCGAATG-3′ (forward primer) and 5′-tttctcgagAGGGTCACTTAGCC-3′ (reverse primer). Bases in lower case contain appropriate restriction sites. Amplified cDNAs were digested with HindIII and XhoI, and cloned between the respective sites of pcDNA3 (Invitrogen). Four independently prepared plasmids were sequenced using Applied Biosystems PRISM 310 Genetic Analyzer (PE Biosystems) to check the accuracy of PCRs.

A cDNA fragment encoding porcine Gal3ST-2 was amplified from porcine colonic cDNA by PCR. Oligonucleotide primers used for the PCR were 5′-tttgtcgac(C/T)TGAAGACICA(C/T)-AA(A/G)ACIG-3′ (forward primer) and 5′-tttatcgatAGIAGI-ACIAGIGACTCGT-3′ (reverse primer). These sequences were deduced from PAPS-binding domains, which are highly conserved among Gal3STs [11]. An approx. 560-bp amplified fragment was digested with SalI and ClaI, and cloned between the respective sites of pBluescript II SK (Stratagene). The plasmids were sequenced and based on the obtained sequence, 5′- and 3′-RACEs (rapid amplification of cDNA ends) were performed with the 5′ RACE System (version 2.0) and 3′ RACE System (Life Technologies), according to the manufacturer's instructions. Oligonucleotide primers used for the RACEs and PCR were 5′-TAGATGAAGGAGGACTCG-3′ (5′-RACE, first primer), 5′-CCGGGACATAGCCCTTG-3′ (5′-RACE, second primer), 5′-TTCC(A/G)GCTGGTGCTCATC-3′ (3′-RACE, first primer), and 5′-tttatcgatCGCCGAGCACTTCGAC-3′ (3′-RACE, second primer). On the basis of the sequences obtained by the RACE procedures, a cDNA encoding porcine Gal3ST-2 was amplified from porcine colonic cDNA by PCR. Oligonucleotide primers used for the PCR were 5′-tttggatccCTGGAAACATGCTGTC-3′ (forward primer) and 5′-tttgaattcTGAGCCTGAGGCAGC-3′ (reverse primer). Amplified cDNA was digested with BamHI and EcoRI, and cloned between the respective sites of pcDNA3. Four independently prepared plasmids were sequenced to check the accuracy of PCRs.

Expression of Gal3STs in COS-7 cells

The plasmids (1 μg) were transfected into COS-7 cells on 35-mm-diameter dishes using Lipofectin Reagent (Life Technologies) according to the manufacturer's instructions. After 48 h, the cells were washed twice with PBS, scraped into 10 mM Hepes/NaOH (pH 7.2) and 0.25 M sucrose, and homogenized. The homogenates were centrifuged at 100000 ***g*** for 1 h. The precipitated crude membranes were suspended in 20 mM Hepes/NaOH (pH 7.2) and stored at − 80 °C until use.

RESULTS

Purification of a SulT from porcine colon

Subsequent to each of the following purification procedures, SulT activities were measured using Galβ1 → 3GalNAc as an acceptor substrate. SulT activity in the mucous layer obtained from a 20 kg sample of porcine colon was solubilized by Triton X-100 treatment, and the extract was applied to an hydroxyapatite column. After a second round of hydroxyapatite chromatography, the enzyme fractions were applied to a Con A–Sepharose column (Figure 1A). A large part of the activity bound to the column and was eluted with 0.4 M α-methylmannoside. The enzyme fractions were rechromatographed on an hydroxyapatite column and then applied to a porcine colonic mucin-derived glycopeptide–Sepharose column (Figure 1B). SulT was eluted from the column

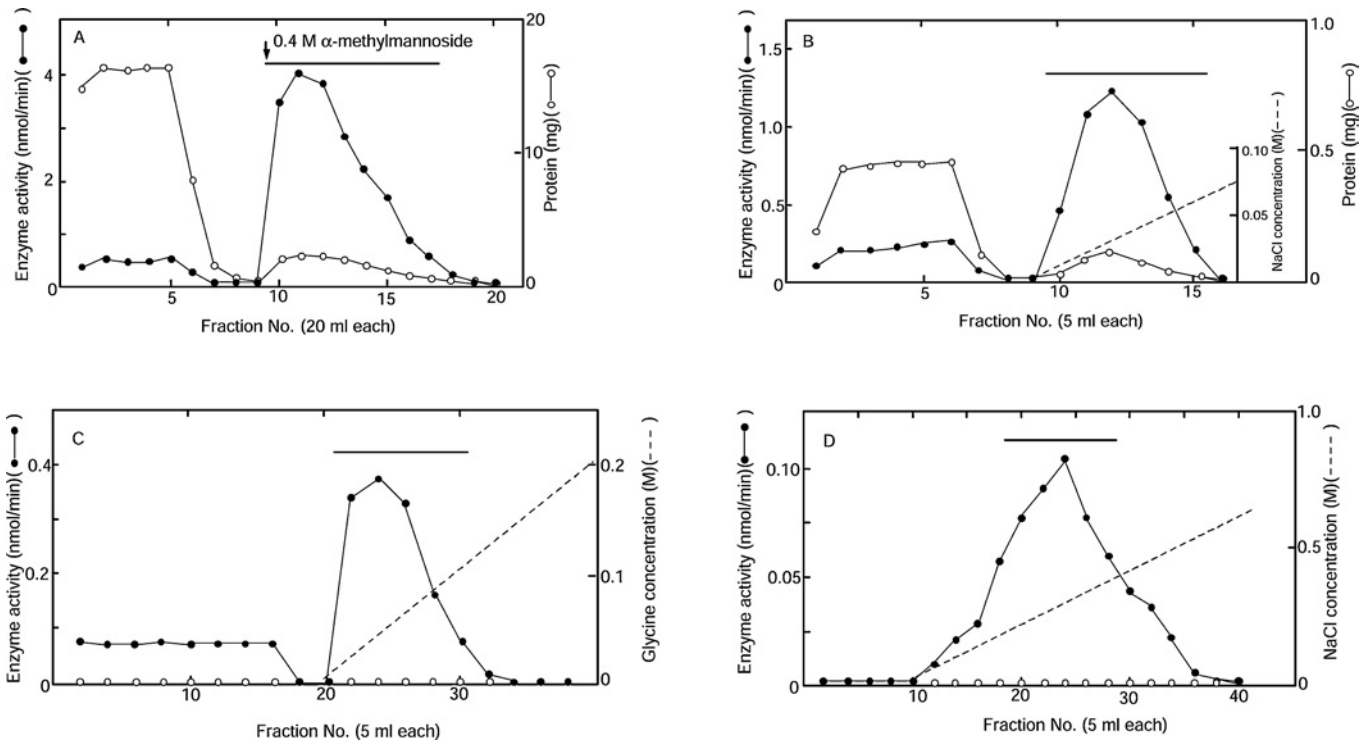

Figure 1 Purification of a Gal3ST from porcine colonic mucosa

(**A**) Con A–Sepharose chromatography of the NaCl-eluted fraction of the second round of hydroxyapatite chromatography. The fractions were monitored for Gal3ST activity (●) and protein level (○). The arrow indicates the starting point of elution with 0.4 M α-methylmannoside in buffer C. Fractions indicated by the horizontal bar were collected. (**B**) Porcine colonic mucin-derived glycopeptide–Sepharose chromatography of NaCl-eluted fractions from the third round of hydroxyapatite chromatography. The fractions were monitored for Gal3ST activity (●) and protein (○). Fractions indicated by the horizontal bar were collected. (**C**) Cu^{2+}-chelating Sepharose chromatography of the enzyme fractions obtained from mucin glycopeptide–Sepharose chromatography. The fractions were monitored for Gal3ST activity in the presence (●) or absence (○) of sphingosine. Fractions indicated by the horizontal bar were collected. (**D**) AMP–agarose chromatography of the enzyme fractions obtained from Cu^{2+}-chelating Sepharose chromatography. The fractions were monitored for Gal3ST activity in the presence (●) or absence (○) of sphingosine. Fractions indicated by the horizontal bar were collected and used for the characterization of pGal3ST.

Table 1 Purification of pGal3ST from porcine colonic mucosa

Gal3ST activities were determined as described in the Experimental section.

Step	Total activity (nmol/min)	Protein (mg)	Specific activity (nmol/min per mg)	Yield (%)	Purification (fold)
Triton X-100 extract	903	3.40×10^4	26.6	100	1
First hydroxyapatite	173	1980	87.4	19	3.3
Second hydroxyapatite	76.0	778	97.7	8.4	3.7
Con A–Sepharose	19.4	11.2	1.73×10^3	2.1	65
Third hydroxyapatite	7.59	3.35	2.27×10^3	0.84	85
Mucin glycopeptide–Sepharose	4.45	0.31	1.44×10^4	0.49	540
Cu^{2+}-chelating Sepharose	2.69	0.030	8.97×10^4	0.30	3370
AMP–agarose	1.29	0.003	1.29×10^6	0.14	48500

at a relatively low concentration of NaCl (0.01–0.06 M). Notably, when the enzyme fractions were applied further to a Cu^{2+}-chelating Sepharose column, no SulT activity was detected (Figure 1C, ○-○). To determine whether this disappearance of activity was caused by loss of membranous lipids during the chromatography step, we added crude lipid fraction derived from porcine colon to the reaction mixture and found that the SulT activity was restored (Figure 1C, ●-●). This is similar to glucuronyltransferase and β1,4-galactosyltransferase, which require specific lipids for their catalytic activities [21,22]. Therefore D-sphingosine was added to the reaction mixture for measuring SulT activity subsequent to the remaining purification procedures. The SulT-active fractions from Cu^{2+}-chelating Sepharose chromatography (Figure 1C, ●-●) were applied to an AMP-agarose column (Figure 1D). The final enzyme fraction had a specific activity of 1.29 mmol/min per mg of protein, representing a purification of 48500-fold (Table 1). Although SDS/PAGE analysis of the final fraction still showed several protein bands, we characterized SulT activity further using this fraction, since both limited protein concentration and protein lability hampered further purification.

Identification of purified SulT as a Gal3ST

We investigated the linkage position of [^{35}S]sulphate in the enzymatic product, $^{35}SO_3^- \rightarrow$ (Galβ1 $\rightarrow$ 3GalNAcα1-*O*-Bn). The

Table 2 Effects of various compounds on pGal3ST activity

The relative activity values were calculated with respect to the value of 'None' which was set as 100. The enzymatic activities represent the means for four independent experiments (S.D. was $<5\%$). NEM, *N*-ethylmaleimide.

Compound	Relative activity (%)
None	100
$MnCl_2$ (10 mM)	172
$CaCl_2$ (10 mM)	116
$MgCl_2$ (10 mM)	114
EDTA (10 mM)	89
NEM (5 mM in 10 mM $MnCl_2$)	101
DTT (5 mM in 10 mM $MnCl_2$)	63

[^{35}S]sulphated product purified by paper electrophoresis was resistant to 6646K β-galactosidase digestion and passed through a PNA–agarose column (results not shown), suggesting that sulphate was transferred to the Gal residue. Next, the [^{35}S]-sulphated product was subjected to periodate oxidation. As a positive control for the oxidation reaction, [^{3}H]Galβ1 $\rightarrow$ 3GalNAcα1-*O*-Bn and $^{35}SO_3^- \rightarrow$ 6GlcNAcβ1-*O*-Bn were also treated with periodate. Importantly, their theoretical oxidation products, [^{3}H]glycerol and $^{35}SO_3^-$-OCH_2-CHOH-CH_2OH respectively migrated to different positions from the original compounds in paper electrophoresis as well as on TLC plates developed in a solvent mixture, butan-1-ol/pyridine/water (6:4:3, by vol.) or butan-1-ol/ethanol/water (4:1:1, by vol.) (results not shown). Periodate oxidation of [^{3}H]Galβ1 $\rightarrow$ 3GalNAcα1-*O*-Bn should generate [^{3}H]glycerol because Gal is ^{3}H-labelled on the ring proton at the C-4 and C-5 residues. Thus the results obtained suggested that the periodate oxidation method worked well. The oxidation product of $^{35}SO_3^- \rightarrow$ (Galβ1 $\rightarrow$ 3GalNAcα1-*O*-Bn) was subjected to paper electrophoresis. If [^{35}S]sulphate is transferred to the C-2, C-4 or C-6 position of a Gal or GalNAc residue, CH_2OH-$CH(OSO_3^-)$-CH_2OH, CH_2OH-$CH(OSO_3^-)$-CHOH-CH_2OH, CH_2OH-CHOH-$CH_2(OSO_3^-)$ or $SO_3^- \rightarrow$ GalNAcα1-*O*-Bn should be produced respectively. The oxidation product migrated to the same position as the untreated ^{35}S-labelled product and authentic $^{35}SO_3^- \rightarrow$ 3Galβ1 $\rightarrow$ 3GalNAcα1-*O*-Bn (R_F value was 0.71, when the position of Bromophenol Blue was taken as 1.0). This suggested that [^{35}S]-sulphate does not bind to GalNAc or to the C-2, C-4 or C-6 position of Gal, but to the C-3 position of Gal. Moreover, the oxidation product was applied to a TLC plate that was then developed with two different solvent systems. In both solvent systems, the oxidation product migrated to the same position as the untreated product and the authentic $^{35}SO_3^- \rightarrow$ 3Galβ1 $\rightarrow$ 3GalNAcα1-*O*-Bn [R_F values were 0.74 and 0.44 using with the solvent systems, butan-1-ol/pyridine/water (6:4:3, by vol.) and butan-1-ol/ethanol/water (4:1:1, by vol.) respectively when the front of solvent was taken as 1.0]. These results suggested that [^{35}S]sulphate binds to the C-3 position of the Gal residue and that the enzyme is a Gal 3-*O*-sulphotransferase. We therefore designated the purified porcine colonic enzyme pGal3ST.

pH-dependency and metal ion requirement of pGal3ST activity

The optimal pH of pGal3ST activity was 6.5–6.7. Hepes buffer had an inhibitory effect on the activity compared with cacodylate buffer. At pH 6.8, the activity in cacodylate buffer was 2.1-fold larger than that in Hepes buffer. In the presence of $MnCl_2$, the activity increased 1.7-fold (Table 2). EDTA slightly inhibited the activity, and NEM (*N*-ethylmaleimide) and DTT also diminished the activity to 59 % and 37 % respectively.

Table 3 Effects of various lipids and amines on pGal3ST activity

Concentrations of lipids and amines were 0.005 % (w/v). The relative activities were calculated with respect to the values of D-sphingosine, which was set as 100. The enzymatic activities represent the means for four independent experiments (S.D. was $<5\%$).

Compound	Relative activity (%)
L-α-Phosphatidic acid	<1
α-Phosphatidylcholine	<1
L-α-Lysophosphatidylcholine	<1
L-α-Phosphatidylethanolamine	<1
L-α-Phosphatidylinositol	<1
L-α-Phosphatidyl-L-serine	<1
Sphingomyelin	<1
Ceramides	<1
Spermine	76
Spermidine	6
Putrescine	2
D-sphingosine	100
N,*N*-Dimethylsphingosine	86
N-Acetyl-D-sphingosine	<1
N-Stearoyl-D-sphingosine	<1
Psychosine	65
Sphingosine 1-phosphate	17
Stearylamine	103
Octylamine	<1
CHAPS	<1
Hexadecyltrimethylammonium bromide	105
1-Dodecylpyridinium chloride	83

Requirement of basic compounds for pGal3ST activity

As shown in Figure 1(C), pGal3ST activity was lost during the purification process. To assess which, if any, compounds restored pGal3ST activity, we assayed the activity in the presence of various compounds. As shown in Table 3, most of phospholipids had no effect, but D-sphingosine and *N*,*N*-dimethylsphingosine effectively restored the activity. Some basic chemosynthetic compounds, stearylamine, hexadecyltrimethylammonium bromide and 1-dodecylpyridinium chloride, also activated pGal3ST activity. With regard to non-lipid compounds, spermine restored the activity much better than spermidine or putrescine. These results suggest that polybasicity is an essential physicochemical property of agents that restore pGal3ST activity. The concentrations of stearylamine, hexadecyltrimethylammonium bromide, D-sphingosine and spermine giving half maximal activity were approx. 0.001 % ($\sim$40 μM), 0.0006 % ($\sim$20 μM), 0.003 % ($\sim$100 μM) and 0.004 % ($\sim$200 μM) respectively.

Substrate specificities of pGal3ST and Gal3ST-2s

As shown in Table 4, pGal3ST recognized core 1 as a good acceptor. Galβ1 $\rightarrow$ 3GalNAc-$_{OH}$ (*N*-acetylgalactosaminitol) was not a substrate, suggesting that the GalNAc residue is required to be in the pyranose form. Substitution of β-GlcNAc at the C-6 of core 1-*O*-pNP decreased enzymatic activity. GalCer, LacCer and GalDG did not function as acceptor substrates. Type 1 and type 2 oligosaccharides including N-linked glycan chains could be utilized, but with lower efficiency than core 1. These results were confirmed by the kinetic analysis (Table 5); the V_{max}/K_m value for core 1 was 3.7- and 8.8-fold greater than those for type 1 and type 2 respectively.

To determine whether pGal3ST activities toward type 1, type 2 and core 1 glycan structures are mediated by a single enzyme, substrate competition experiments were performed (Table 6). When two acceptor substrates compete for one

Table 4 Substrate specificity of pGal3ST

The concentrations of the acceptors were 1 mM. The relative activities were calculated with respect to the value of core 1, which was set as 100. The enzymatic activities represent the means for four independent experiments (S.D. was < 5 %). GalNAc-$_{OH}$, *N*-acetylgalactosaminitol.

Acceptor	Relative activity (%)
Core 1	100
Galβ1 → 3GalNAcα1-*O*-pNP	68
Galβ1 → 3GalNAcα1-*O*-Bn	132
Galβ1 → 3GalNAc-$_{OH}$	< 1
Galβ1 → 3(GlcNAcβ1 → 6)GalNAcα1-*O*-pNP	27
Type 1	34
Type 2	14
Galβ1 → 4Glc	8.5
Galβ1 → 3GlcNAcβ1 → 3Galβ1 → 4Glc	43
Galβ1 → 4GlcNAcβ1 → 2Manα1 → 3/6Manβ1 → 4GlcNAc	17
BiGP	15
GalCer	< 1
LacCer	< 1
GalDG	< 1

Table 5 Kinetic properties of pGal3ST

The concentration of PAPS was 4 μM. The acceptor substrate for PAPS was 5 mM Galβ1 → 3GalNAc.

Substrate	K_m (mM)	V_{max} (nmol/min per mg of protein)	V_{max}/K_m (nmol/min per mg of protein per mM)
Galβ1 → 3GalNAc	3.7	57	15
Galβ1 → 3GalNAcα1-*O*-pNP	5.6	47	8.4
Galβ1 → 3GlcNAc	14	57	4.1
Galβ1 → 4GlcNAc	30	51	1.7
PAPS	0.026	217	

Table 6 Substrate competition experiments with pGal3ST

	Total velocity (nmol/min per mg of protein)			
		Expected		Suggested number of enzymes
Substrates	Observed	One enzyme	Two enzymes	
Type 1 (10 mM)	23.7 ± 1.3			
Type 2 (10 mM)	13.9 ± 0.7			
Core 1 (3 mM)	26.3 ± 0.6			
Type 1 (10 mM) + core 1 (3 mM)	33.5 ± 1.1	34.5	50.0	1
Type 2 (10 mM) + core 1 (3 mM)	30.5 ± 0.5	29.5	40.2	1

enzyme, the total velocity is estimated as $(V_{m1}C_1/K_1 + V_{m2}C_2/K_2)/(1 + C_1/K_1 + C_2/K_2)$, where C_1 and C_2 are the concentrations of the acceptor substrates, V_{m1} and V_{m2} are the respective maximum velocities, and K_1 and K_2 are the respective Michaelis constants. On the other hand, when two acceptor substrates are catalysed by two different enzymes, the total velocity is the simple sum of reaction velocities of the individual enzymes [23]. Here, the observed velocities with a combination of the two acceptor substrates were close to the expected values for a single enzyme (Table 6).

The substrate specificity of pGal3ST was then compared with those of human Gal3ST-1, -2, -3 and -4. The relative activities of pGal3ST, human Gal3ST-2 and human Gal3ST-3 for type 1, type 2 and core 1 substrates were 34:14:100, 55:108:100 and 11:100:14 respectively [12]. Human Gal3ST-1 does not act on the three substrates, and human Gal3ST-4 acts specifically on core 1. We suggested recently that Gal3ST-2 is the major Gal3ST that is expressed in human colon [12]. This led us to speculate that pGal3ST may be the orthologue of human Gal3ST-2, because pGal3ST should be the major Gal3ST in porcine colon. However, the substrate specificities of the two enzymes were different. Type 2 structures were much better substrates for human Gal3ST-2 than for pGal3ST. To clarify this issue, we isolated a cDNA encoding porcine Gal3ST-2. Using two oligonucleotide primers deduced from two highly conserved PAPS-binding domains, we obtained a PCR fragment, the sequence of which is very similar to that of human Gal3ST-2. This fragment was elongated using the 5′- and 3′-RACE methods. Kuhns et al. [13] characterized a rat colon Gal3ST, which, like pGal3ST, prefers core 1 structures as an acceptor to type 2 and type 1 structures. Therefore we also isolated a cDNA encoding mouse Gal3ST-2, which is likely to have close nucleic acid sequence identity with the rat orthologue, which was cloned easily using the BLAST database system. The amino acid sequences of porcine Gal3ST-2 and mouse Gal3ST-2 are shown in comparison with human Gal3ST-2 in Figure 2(A). Porcine Gal3ST-2 and mouse Gal3ST-2 shared 76 % and 67 % amino acid identity with human Gal3ST-2 respectively, while the two Gal3ST-2s shared 30–40 % amino acid identity with human Gal3ST-1, -3 and -4 (Figure 2B). No sequences with greater similarity to human Gal3ST-2 were present in the mouse or porcine databases, suggesting that porcine and mouse Gal3ST-2s are indeed species orthologues of human Gal3ST-2. The cDNAs encoding porcine Gal3ST-2 and mouse Gal3ST-2 were individually inserted into pcDNA3, and each expression vector was transfected into COS-7 cells. The specific activities of transiently expressed porcine Gal3ST-2 and mouse Gal3ST-2 in the membrane fractions were 71 and 34 pmol/min per mg of protein respectively, using core 1 as a substrate. The substrate specificities and kinetic properties of these enzymes are shown in Tables 7 and 8. Porcine Gal3ST-2 had very similar substrate specificity to that of the purified pGal3ST, indicating that pGal3ST corresponds to porcine Gal3ST-2. The V_{max}/K_m value of mouse Gal3ST-2 for type 1 was smaller than for type 2 and core 1, as is the case for human Gal3ST-2. However, the K_m values of mouse Gal3ST-2 were much larger than those of human Gal3ST-2. On the other hand, porcine Gal3ST-2 had a smaller V_{max}/K_m value for type 2 than for type 1 and core 1 substrates, in contrast with human and mouse Gal3ST-2s. These results suggested that the three Gal3ST-2s have different preferences for acceptor substrates.

DISCUSSION

In the present study, we purified pGal3ST 48500-fold from porcine colonic mucosa and examined its enzymatic characteristics. Purified pGal3ST requires basic compounds, such as spermine and sphingosine, for enzymatic activity. We also cloned porcine and mouse Gal3ST-2 enzymes and analysed their substrate specificities. Porcine Gal3ST-2 had very similar substrate specificity to pGal3ST, suggesting that pGal3ST does correspond to porcine Gal3ST-2. Interestingly, porcine Gal3ST-2 and mouse Gal3ST-2 had different substrate specificities from that of human Gal3ST-2.

To date, four human Gal3STs have been cDNA cloned [6,8–11]. Notably, the substrate specificities of these Gal3STs differ significantly. Gal3ST-1 acts specifically on glycolipids, including GalCer, LacCer and GalDG [7]. Gal3ST-2 recognizes type 1, type 2 and core 1 oligosaccharides as good substrates. Gal3ST-3 preferentially acts on type 2 glycan structures and Gal3ST-4 specifically acts on core 1 glycans. In contrast, pGal3ST prefers

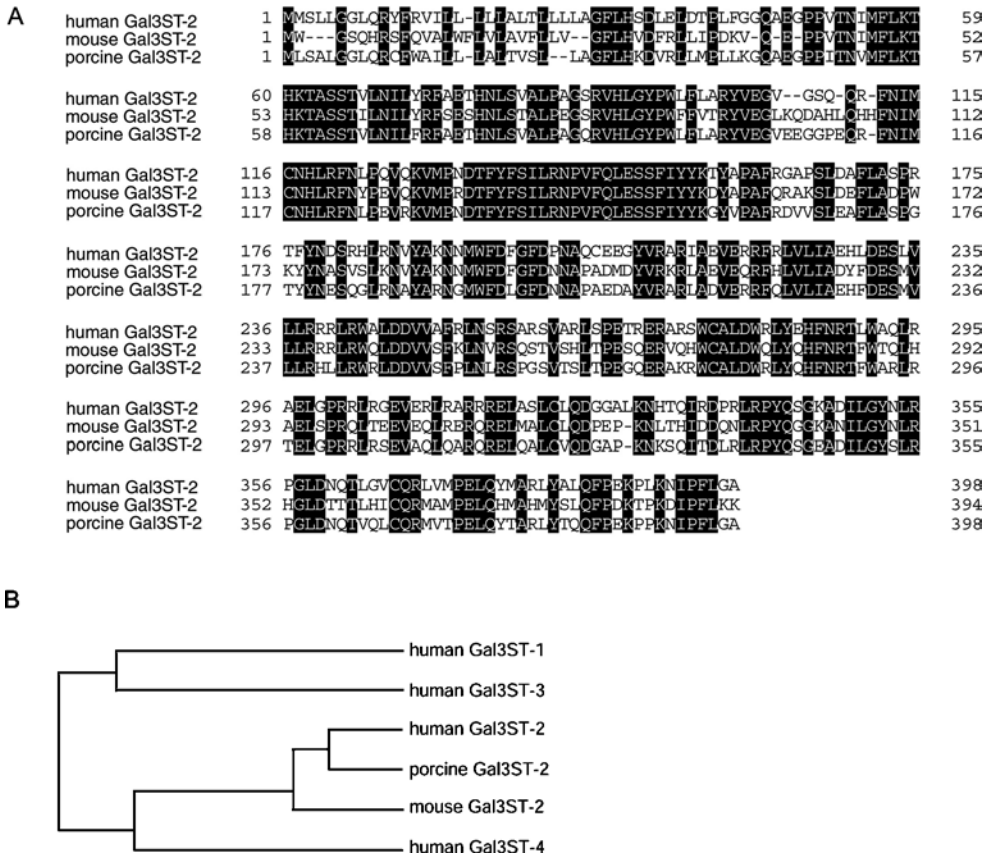

Figure 2 Comparison of porcine and mouse Gal3ST-2s with human Gal3STs

(**A**) Comparison of the amino acid sequences of porcine and mouse Gal3ST-2 enzymes with that of human Gal3ST-2 [8]. The alignment was performed with ClustalW. Highlighted residues indicate the identity among the three Gal3ST-2 enzymes. (**B**) A phylogenetic tree of four human Gal3STs, mouse Gal3ST-2 and porcine Gal3ST-2.

Table 7 Substrate specificities of human, mouse and porcine Gal3ST-2 enzymes

The concentrations of the substrates were 1 mM. The enzymatic activities represent the means for four independent experiments (S.D. was $<5\%$). The relative values were calculated with respect to the value of core 1, which was set as 100.

	Core1	Core1-*O*-pNP	Core1-*O*-Bn	Type 1	Type 2	Core2-*O*-pNP	Lactose	LNT	MonoGP	BiGP
Human Gal3ST-2	100	121	120	55	108	70	114	71	99	80
Mouse Gal3ST-2	100	123	85	15	102	37	60	39	49	52
Porcine Gal3ST-2	100	85	124	39	15	27	11	69	19	17

Table 8 Kinetic properties of human, mouse and porcine Gal3ST-2s

	Human			Mouse			Porcine		
	Type 1	Type 2	Core 1	Type 1	Type 2	Core 1	Type 1	Type 2	Core 1
K_m (mM)	2.9	0.28	0.56	200	10	20	15	29	4.2
V_{max} (pmol/min per mg of protein)	280	240	280	590	500	310	200	170	260
V_{max}/K_m	97	860	500	3.0	50	16	13	5.9	62

core 1 to type 1 and type 2 glycans (Tables 4 and 5). The possibility that pGal3ST represents a mixture of Gal3ST-2 and -4 is unlikely because substrate competition experiments showed that the pGal3ST activity for type 1, type 2 and core 1 is catalysed by a single enzyme (Table 6). Porcine Gal3ST-2 cloned in the present study preferentially acts on core 1 structures relative to type 1 and type 2, and its substrate specificity was identical with that of pGal3ST, suggesting that pGal3ST corresponds to pGal3ST-2. Porcine Gal3ST-2 and mouse Gal3ST-2 exhibited amino acid identity of 76% and 67% with human Gal3ST-2 respectively. Thus it appears that amino acid differences between human, mouse and porcine Gal3ST-2s

Table 9 Glycosyltransferases and sulphotransferases activated (A) or inhibited (I) by sphingosine (Sph) and spermine (Spr)

$(NH_4)_2SO_4$/acetone is ammonium sulphate precipitation and cold-acetone precipitation.

Enzyme	Source	Purification	Effect on the activity	Reference
Gal: → 3SulT	Porcine colonic mucosa	48500-fold	Sph, Spr, A (essential)	Present study
GlcNAc-6S:β1 → 4GalT	Porcine colonic mucosa	24000-fold	Sph, A (essential)	[22]
Chondroitin 4-*O*-SulT	Chick embryo cartilage	270-fold	Spr, A (5.8-fold at 0.6 mM)	[26]
Chondroitin 6-*O*-SulT	Chick embryo cartilage	100-fold	Spr, A (37-fold at 0.6 mM)	[26]
	Foetal calf serum	$(NH_4)_2SO_4$/acetone	Spr, A (1.5-fold at 1 mM)	[27]
Glycoprotein SulT	Rat submandibular Salivary glands	Golgi membranes	Sph, A (1.7-fold at 0.3 mM)	[28]
Heparan sulphate 2-*N*-SulT	Foetal calf serum	$(NH_4)_2SO_4$/acetone	Spr, I (IC_{50}: 2–4 mM)	[27]
Keratan sulphate 6-*O*-SulT	Foetal calf serum	$(NH_4)_2SO_4$/acetone	Spr, A (1.3-fold at 1 mM)	[27]
Tyrosylprotein SulT	Rat submandibular Salivary glands	Golgi membranes	Sph, I (IC_{50}: 0.15 mM)	[29]
GalT	Canine trachea	Microsome fraction	Spr, A (2-fold at 1 mM)	[30]
β1 → 3 GalT	Human trachea	Crude extract	Spr, A (2.3-fold at 2 mM)	[31]
GM2: β1 → 3GalT	Embryonic chicken brain	2000-fold	Sph, A (1.4-fold at 0.1 mM)	[32]
β1 → 4 GalT	Rat mammary	Golgi membranes	Spr, A (8-fold at 2.5 mM)	[33]
α1 → 3FucT	Colo 205 cells	100-fold	Sph, A (1.7-fold at 0.7 mM)	[32]
GalNAcT	Canine trachea	Microsome fraction	Spr, A (1.5-fold at 1 mM)	[30]
GM3: β1 → 4GalNAcT	Guinea-pig bone marrow	600-fold	Sph, A (8-fold at 1.4 mM)	[32]
β1 → 3GlcAT	Embryonic chicken brain	Golgi membranes	Sph, I (IC_{50}: 0.8 mM)	[34]

account for the disparity in their substrate specificities, especially the accessibility to type 2 chains. To our knowledge, this is the first demonstration that orthologous SulTs from different species have distinct substrate specificities. There are examples of two distinct glycosyltransferases having different substrate specificities, despite possessing closely related amino acid sequences. Specifically, blood group A-synthesizing α1,3galactosyltransferase has 98.9% amino acid identity with blood group B-synthesizing α1,3*N*-acetylgalactosaminyltransferase [24]. Akama et al. [25] showed that GlcNAc 6-*O*-sulphotransferase-5 (GlcNAc6ST-5), but not GlcNAc6ST-3, is involved in the elongation of keratan sulphate, although the two enzymes share high amino acid identity (86%). Although these examples are from different genes in one species (human), our observations here suggest that some glycosyltransferase orthologues have very different substrate specificities and thus different functions.

Previously, Kuhns et al. [13] characterized a Gal3ST in rat colon membrane extracts and showed that it prefers the Galβ1 → 3GalNAcα- structure to Galβ1 → 4GlcNAc. This specificity resembles porcine Gal3ST-2, and, since the rat enzyme seems to be the major Gal3ST in colon, it suggests that rat colon Gal3ST may also be categorized as Gal3ST-2.

Purified pGal3ST requires basic compounds for its catalytic activity. Spermine activates pGal3ST much more potently than spermidine or putrescine (Table 3). This suggests that polybasic physiochemistry is effective for activation. In this context, sphingosine–Triton X-100 micelles should be polybasic on their surface. It has been reported that the enzymatic activities of several glycosyltransferases and SulTs are modified in the presence of spermine or sphingosine (Table 9). A few details in this Table deserve mention. (i) The effects of sphingosine or spermine on enzyme activities seem to be related to levels of purification of the enzymes. Highly purified pGal3ST and GlcNAc-6S:β1,4GalT [22] require basic compounds for their activity, while the other transferases listed in Table 9, which were purified to lower levels, exhibit some enzymatic activity even in the absence of the basic compounds. The basic-compound-dependent behaviour of pGal3ST appeared after Cu^{2+}-chelating Sepharose chromatography (Figure 1C). The reason for this behaviour remains unclear, but it may be possible that certain basic cofactor(s) are separated from the enzyme protein during this step. (ii) Despite their large degree of variety, the transferases presented in Table 9 share the common property of residing in the *trans*-Golgi apparatus. Sakamaki et al. [35] reported that treatment of stimulated bovine lymphocytes with biosynthetic inhibitors of polyamine causes both swelling of the Golgi apparatus and a reduction in the specific activity of a GalT. These observations must be interpreted carefully, since polyamine regulates many metabolic processes in living cells [36]. However, it is plausible that polyamine might be involved in the functional regulation of these transferases, which reside in the *trans*-Golgi apparatus. Whether porcine Gal3ST-2 actually binds to sphingosine or spermine *in vivo* should be determined as a next step in the study of these diverse enzymes.

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