Molecular Cloning and Characterization of a Novel Human Galactose 3-O-Sulfotransferase That Transfers Sulfate to \( \text{Gal}\beta1\rightarrow3\text{GalNAc} \) Residue in O-Glycans

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We have identified a novel galactose 3-O-sulfotransferase, termed Gal3ST-4, by analysis of an expression sequence tag using the amino acid sequence of human cerebroside 3'-sulfotransferase (Gal3ST-1). The isolated cDNA contains a single open reading frame coding for a protein of 486 amino acids with a type II transmembrane topology. The amino acid sequence of Gal3ST-4 revealed 33%, 39%, and 30% identity to human Gal3ST-1, Gal\( \beta1\rightarrow3/4\text{GlcNAc} \) (Gal3ST-2) and Gal\( \beta1\rightarrow4\text{GlcNAc} \) (Gal3ST-3), respectively. The Gal3ST-4 gene comprised at least four exons and was located on human chromosome 7q22. Expression of Gal3ST-4 in COS-7 cells produced a sulfotransferase activity that catalyzes the transfer of \( ^{35}\text{S} \) sulfate to the C-3' position of Gal\( \beta1\rightarrow3\text{GalNAc} \text{-O-Bn} \). Gal3ST-4 recognizes Gal\( \beta1\rightarrow3\text{GalNAc} \) and Gal\( \beta1\rightarrow3 \text{(GlcNAc}\beta1\rightarrow6)\text{GalNAc} \) as good substrates, but not Gal\( \beta1\rightarrow3\text{GalNAc}\text{-OH} \) or Gal\( \beta1\rightarrow3\text{GlcNAc} \). Asialofetuin is also a good substrate, and the sulfation was found exclusively in O-linked glycans that consist of the Gal\( \beta1\rightarrow3\text{GalNAc} \) moiety, suggesting that the enzyme is specific for O-linked glycans. Northern blot analysis revealed that 2.5-kilobase mRNA for the enzyme is expressed extensively in various tissues. These results suggest that Gal3ST-4 is the fourth member of a Gal\( \beta1\rightarrow3 \)-sulfotransferase family, and that the four members, Gal3ST-1, Gal3ST-2, Gal3ST-3, and Gal3ST-4, are responsible for sulfation of different acceptor substrates.

Sulfation is one of the most extensive modifications for glycan chains in various glycoconjugates. Sulfated glycans are associated with the physiological functions of glycoproteins in the mucosal barrier system, regulation of their half-life, and cell-to-cell interaction. The content of sulfated glycans in mucous glycoproteins is modified in cystic fibrosis and colon cancer (for review, see Refs. 1–3). However, the precise molecular mechanisms in these phenomena and other significances of sulfation in various glycoproteins remain unclear.

The SO\(_3\)\(^{-}\)\(\rightarrow3\)GlcNAc structure in O-linked and N-linked glycans has been found in various glycoproteins including thyroglobulin (4–6), meconium glycoproteins (7), respiratory mucous glycoproteins from patients with cystic fibrosis (8–10) and chronic bronchitis (11), an ovarian cystadenoma glycoprotein (12), LS174T-HM7 colon carcinoma mucin (13), Tamm-Horsfall glycoprotein (14), sulfomucins (15), and oviducal mucins (16). Sulfated residues in these glycoproteins are attached to C-3' of Gal\( \beta1\rightarrow3/4\text{GlcNAc} \), Gal\( \beta1\rightarrow3\text{GalNAc} \), or Gal\( \beta1\rightarrow3\)Gal structure. The occurrence of the SO\(_3\)\(^{-}\) structure has not been fully elucidated to date because of the difficulty of structural studies for sulfated glycans. However, the sulfated structure seems to be distributed extensively because the glycoprotein-specific \( \beta\)-Gal-3'-sulfotransferase (Gal3ST-2) gene is expressed ubiquitously in various human tissues (17).

In relation to the biosynthesis of sulfated glycans, cDNA cloning of more than 20 sulfotransferases has been achieved to date (for review, see Ref. 18). Among these sulfotransferases, the C-3 sulfation of galactose is catalyzed by Gal\( \beta1\rightarrow3 \)-sulfotransferase. Three Gal\( \beta1\rightarrow3 \)-sulfotransferases have so far been cloned. One is glycolipid-specific cerebroside sulfotransferase (Gal3ST-1) (19), which utilizes galactosylceramide (GalCer)\(^1\), lactosylceramide (LacCer), and galactosylsialic glycolipid (GalDG) (20). The second is GP3ST or Gal3ST-2, which utilizes not only Gal\( \beta1\rightarrow3/4\text{GlcNAc} \) but also Gal\( \beta1\rightarrow3\text{GalNAc} \) (17). The third is Gal3ST-3, which utilizes Gal\( \beta1\rightarrow4\text{GlcNAc} \) (21). Three enzymes share about 40% identity of the amino acid sequences, indicating the existence of a family of Gal\( \beta1\rightarrow3 \)-sulfotransferases (17, 21). To search for a novel member of the family, we investigated the expressed sequence tags (EST) data bases using the Gal3ST-1 amino acid sequence and found the fourth member of Gal\( \beta1\rightarrow3 \)-sulfotransferase.

**EXPERIMENTAL PROCEDURES**

**Materials**—\(^{35}\text{S} \) PAPS (96.9 GBq/mmol) and UDP-[4,5-\( ^{3} \text{H} \) N]Gal (1,110 GBq/mmol) were purchased from PerkinElmer Life Science Products (Boston, MA). Gal\( \beta1\rightarrow3\text{GalNAc}1\text{-O-p-nitrophenyl} \) (pNP), N-acetyllactosamine (type 2), Gal\( \beta1\rightarrow3\text{GlcNAc} \), and Gal\( \beta1\rightarrow3/4\text{GlcNAc} \beta1\rightarrow6\text{GalNAc}1\text{-O-pNP} \) (core 2-O-pNP) were purchased from Funakoshi Co., Ltd. (Tokyo, Japan). Lacto-N-biose I (type 1), Gal\( \beta1\rightarrow3\text{GalNAc}1\text{-O-benzyl} \) (Bn), Gal\( \beta1\rightarrow3\text{GalNAc}1\text{-O-Bn} \), Gal\( \beta1\rightarrow3\text{GalNAc}1\text{-O-Bn} \), UDP-Gal, GalCer from bovine brain, LacCer from bovine, and GalDG from whole wheat flour were purchased from Sigma (St. Louis, MO). Streptococcus 6646K \( \beta \) galactosidase was purchased from Seikogaku Co. (Tokyo).

**Ricinus communis agglutinin 1-agarose (4 mg/ml gel)** was purchased from Pharmacia Biochemicals, Uppsala, Sweden.

1 The abbreviations used are: GalCer, galactosylceramide; LacCer, lactosylceramide; GalDG, galactosylsialic glycolipid; EST, expressed sequence tag(s); PAPS, adenosine 3'-phosphate 5'-phosphosulfate; pNP, p-nitrophenyl; type 1, Gal\( \beta1\rightarrow3\text{GlcNAc} \); type 2, Gal\( \beta1\rightarrow4\text{GlcNAc} \); core 2, Gal\( \beta1\rightarrow3/4\text{GlcNAc} \beta1\rightarrow6\text{GalNAc}1\text{-O} \); Bn, benzyl; bp, base pair(s); GalNAc\(_{OH} \), N-acetylgalactosaminol.

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FIG. 1. Nucleotide and deduced amino acid sequence of human Gal3ST-4 cDNA, hydropathy plot of the protein, and genomic organization of the human Gal3ST-4 gene. Panel A, the predicted amino acid sequence for Gal3ST-4 is shown below the nucleotide sequence. The putative membrane spanning domain is underlined, and one potential N-glycosylation site is marked by a dot. The presumptive polyadenylation signal is double-lined. Panel B, the hydropathy plot was calculated by the method of Kyte and Doolittle (32) with a window of 11 amino acids. Panel C, the 5′- and 3′-untranslated regions are shown as open boxes. Shaded boxes represent the coding sequences. The black horizontal lines denote the introns.
from Hohne Oil Co. (Tokyo). Peanut agglutinin-agarose (4.5 mg/ml gel) was purchased from E-Y Laboratories, Inc. (San Mateo, CA).

**Gal3ST Cloning**

**Gal3ST-4**—Based on the amino acid sequence of human Gal3ST-4 (19), we found one colony containing cDNA for Gal3ST-4. The cDNA for Gal3ST-4 contained in pCMV-SPORT, named pCMV-SPORT-Gal3ST-4, was sequenced using Applied Biosystems PRISM® 310 Genetic Analyzer (PE Biosystems).

**Expression of Gal3ST-4 in COS-7 Cells**—The plasmid (1 mg) was transfected into COS-7 cells on 35-mm dishes using Lipofectin Reagent (Life Technologies, Inc.) according to the manufacturer’s instructions. After 48 h, the cells were washed once with phosphate-buffered saline, scraped off from the dishes in 10 mM HEPES-NaOH (pH 7.2), 0.25 mM sucrose, and homogenized. The homogenate was ultracentrifuged at 100,000 × g for 1 h. The precipitated crude membranes were suspended in 20 mM HEPES-NaOH (pH 7.2) containing 0.1 M sodium cacodylate (pH 6.3), 10 mM MnCl₂, 0.1% (v/v) Triton X-100, 0.1 M NaF, 2 mM ATP-Na₂, 6.5 μM [35S]PAPS (2.8 × 10⁶ dpm), 1 mM Galβ1→3GalNac1→O-PnP, and the crude membrane fraction appropriately diluted, was incubated at 37 °C for 1 h. The 35S-labeled products were purified by paper electrophoresis (pyridine/acetic acid/water, 3:1:387, pH 5.4). The solutions were acidified by adding 0.1 M sodium borohydride was added, and the solutions stood for 1 h at room temperature. The solutions were acidified by adding 30 mM NaOH, the mixtures underwent paper electrophoresis.

**Characterization of the 35S-Labeled Product**—The 35S-labeled product was subjected to periodate oxidation (29). The labeled oligosaccharides were dissolved in 20 μl of 75 mM sodium metaperiodate, 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) containing 0.1 M sodium borohydride was added, and the solutions stood for 1 h at room temperature. The solutions were acidified by adding acetic acid and passed through a column (0.5 × 3 cm) of Bio-Rad AG-50W-X8 (H⁺ form). The eluates were evaporated, and residual boric acid was removed by repeated evaporation with methanol. The residues were hydrolyzed in 100 μl of 0.05 N H₂SO₄ at 80 °C for 1 h. After being neutralized with NaOH, the mixtures underwent paper electrophoresis.

**Galβ1→3GalNAc→3’-Sulfotransferase**

| Oligosaccharide | Value of bromphenol blue | Value of 1-O-sulfotransferase | Value of 3',4’'-disulfotransferase |
|-----------------|--------------------------|-------------------------------|-----------------------------------|
| α-Lactosamine   | 1.0                      | 0.69                          | 1.89                              |
| Galβ1→3GalNAc1→O-Pn  | 0.69                      | 1.89                          |                                   |

**Assay of Sulfotransferase Activity**—Twenty μl of reaction mixture consisting of 0.1 mM sodium cacodylate (pH 6.3), 10 mM MnCl₂, 0.1% (v/v) Triton X-100, 0.1 M NaF, 2 mM ATP-Na₂, 105 μM [35S]PAPS (2.8 × 10⁶ dpm), 1 μM Galβ1→3GalNAc1→O-PnP, and the crude membrane fraction appropriately diluted, was incubated at 37 °C for 1 h. The 35S-labeled products were purified by paper electrophoresis (pyridine/acetic acid/water, 3:1:387, pH 5.4). The Rₚ values of 35S-labeled Galβ1→3GalNAc1→O-PnP and PAPS are 0.69 and 1.89, respectively, when the Rₚ value of bromphenol blue is taken as 1.0. After extraction with water, the radioactivities were counted. In the case of glycolipids used as acceptor substrates, the detection of the 35S-labeled products was performed according to the methods reported by Kawano et al. (27).
developed with solvents, pyridine/ethyl acetate/acetic acid/water (5:5:1:3) or 1-butanol/ethanol/water (4:1:1). The radioactivities were monitored by a radiochromatogram scanner.

\[ ^{3}H\text{Gal}1→3\text{GalNAc}1-\text{O-Bn} \] as a positive control for periodate oxidation was prepared as follows. Twenty µl of solution containing 50 mM HEPES-NaOH (pH 7.2), 10 mM MnCl\(_2\), 0.5% (v/v) Triton X-100, 5 mM UDP-\[^{3}H\]Gal, and crude membrane fractions from porcine colonic mucosa was incubated at 37 °C for 1 h. The reaction mixture underwent paper chromatography, which was developed with the solvent pyridine/ethyl acetate/acetic acid/water (5:5:1:3). A radioactive fraction (\[^{3}H\text{Gal}1→3\text{GalNAc}1-\text{O-Bn}\]) with an \( R_F \) value of 0.72 was extracted with water. Linkage position of \[^{3}H\]Gal residue was confirmed by its binding to a peanut agglutinin-agarose column because peanut agglutinin specifically recognizes the \[^{3}H\text{Gal}1→3\text{GalNAc}1-\text{O-Bn}\] structure (29). Synthesis of 6-\[^{35}S\]Sulfate was performed using human GlcNAc:6-sulfotransferase as described previously (30).

\[ ^{35}S \text{Sulfation of Asialofetuin by Gal3ST-4—} \]

\[ ^{35}S\text{SO}_3 \]

**RESULTS**

**Molecular Cloning of a cDNA Homologous to Human Gal3ST-1**—We found small sequences (GenBank AW961058) similar to the sequence of human Gal3ST-1 (19) in the EST data bases. We prepared a sense oligonucleotide, 25 nucleotides in length, the sequence of which is present in the EST, and used it as a probe for GeneTrapper\textsuperscript{®} cDNA positive selection system to screen a human testis cDNA library. One clone was obtained, and the nucleotide sequence was determined (Fig. 1A). The 2,460-bp cDNA had a 5'-translated region of 236 bp, a single open reading frame of 1,458 bp, and a 3'-untranslated region of 766 bp including a poly(A) tail. The translation initiation site conformed to Kozak’s rule (31), and the upstream region contained an in-frame stop codon. The open reading frame predicts a protein of 486 amino acid residues with a molecular mass of 54,173 Da with one potential N-linked glycosylation site. Hydropathy plot analysis of the deduced amino acid sequence revealed one prominent hydrophobic segment, 22 amino acid residues in length in the N-terminal region, predicting that the protein has a type II transmembrane topology (Fig. 1B). The cDNA sequence was compared with the Human Diagnostics using \(^{32}P\text{dCTP}\) (NEN Life Science Products, Inc.) according to the manufacturer’s instructions. The membranes were prehybridized in ExpressHyb Solution (CLONTECH) at 68 °C for 2 h and then hybridized with the \(^{32}P\)-labeled probe in the same solution at 68 °C for 16 h. The Northern blot membranes were washed in 2× SSC, 0.1% SDS at room temperature and then in 0.1× SSC, 0.1% SDS at 50 °C. The radioactivity was detected with FLA-2000 (Fuji Photo Film Co. Ltd., Tokyo).

**Northern Blot Analysis**—Human Multiple Tissue Northern blot membranes (CLONTECH, Palo Alto, CA) were used according to the manufacturer’s instructions. The mRNA content in each lane of the Northern blot membrane is normalized to the mRNA expression level of \( \beta \)-actin. \(^{32}P\)-Labeled probe was prepared from the cDNA fragment (1–1,423; see Fig. 1A) by a Random Primed DNA Labeling Kit (Roche
Genome Project Data Base, and the genomic organization and the chromosomal localization were revealed (Fig. 1C). The gene comprises at least four exons and spans about 10 kilobases in human chromosome 7q22. The intron/exon junctions followed the GT/AG rule (33). The coding region is located in three exons (exons 2, 3, and 4), and two introns were inserted between nucleotides 361 and 362 (at Arg-42) and nucleotide 665 and 666 (between Glu-143 and Val-144) of the cDNA (Fig. 1A).

The amino acid sequence of the protein (named Gal3ST-4) showed 33, 39, and 30% identity with human Gal3ST-1 (19), human Gal3ST-2 (17), and human Gal3ST-3 (21), respectively (Fig. 2). The two highly conserved regions (Fig. 2, underlined and double line) contain putative PAPS binding motifs (34), which are commonly conserved in all sulfotransferases cloned so far (18). There exist other relatively conserved regions in the C-terminal domain (299–314 and 358–374 of Gal3ST-4, in Fig. 2). In searching other sequences homologous to the cDNA for Gal3ST-4, we found one cDNA clone (GenBank accession number AK022178), 2,176 bp in length, which includes the full-length of the open reading frame of cDNA for Gal3ST-4.

Characterization of the Putative Sulfotransferase as Galβ1→3GalNAc→3'-Sulfotransferase—The putative sulfotransferase was expressed in COS-7 cells, and the crude membrane fraction was prepared as an enzyme source. The membrane fraction from the cells transfected with pcMV-SPORT-Gal3ST-4, pcMV-SPORT vector containing the cDNA for Gal3ST-4, had a sulfotransferase activity (5.6 pmol/min/mg of protein) using Galβ1→3GalNAcα1-O-Bn as acceptor. The membrane fractions derived from the transfectant with pcMV-SPORT and wild type had no sulfotransferase activity. The 35S-sulfated product, [35S]SO3–(Galβ1→3GalNAcα1-O-Bn), purified by paper electrophoresis, was resistant to 6646K β-galactosidase digestion and passed through a R. communis agglutinin I-agarose column (data not shown). Because authentic Galβ1→3GalNAcα1-O-Bn was retarded on the R. communis agglutinin I-agarose column, these results suggested that the [35S]sulfate was transferred to the galactose residue. The migrating position of the 35S-sulfated product in paper electrophoresis was close to that of authentic [35S]SO3–GlcNAcβ1→2Man, suggesting that the product is monosulfated disaccharide. To determine the linkage position of [35S]sulfate residue, the 35S-sulfated product was subjected to periodate oxidation. [3H]Galβ1→3GalNAcα1-O-Bn was also subjected to oxidation as a positive control of the reaction; when subjected to TLC developed in the solvent system, 1-butanol/ethanol/water (4: 1:1), [3H]Galβ1→3GalNAcα1-O-Bn and the oxidative product migrated to positions with RF values of 0.45 and 0.56, respectively. The latter compound is theoretically [3H]glycerol because galactose is 3H-labeled on the ring proton at the C-4 and 5. The result indicates that the oxidative reaction went well. The reaction product from [35S]SO3–(Galβ1→3GalNAcα1-O-Bn) underwent paper electrophoresis (Fig. 3A). It migrated to the same position as the untreated 35S-labeled product. If [35S]sulfate is transferred to the C-2, C-4, or C-6 position of galactose residue, or N-acetylglactosamine residue, CH2OH-CH(OSO3–)-CH2OH, CH2OH-CH(OSO3–)-CHOH-CH2OH, CH2OH-CHOH-CH2(OSO3–), or SO3–→
GalNAc1-1-O-Bn should be produced by periodate oxidation, respectively. \[^{35}S\]SO₃⁻→6GalNAcβ1-1-O-Bn and its periodate-oxidized and reduced product, \[^{35}S\]SO₃⁻:OCH₂-CHOH-CH₃OH, migrated faster in paper electrophoresis than \[^{35}S\]SO₃⁻ (Galβ1→3GalNAc1-1-O-Bn) (Fig. 3A). The result suggests that \[^{35}S\]sulfate does not bind to GalNAc residue or the C-2, 4, or 6 position of Gal, but the C-3 position of Gal.

Moreover, the reaction product was subjected to TLC using different solvent systems (Fig. 3, B and C). The reaction product was developed to the same position as the untreated product in both solvent systems. The structure of the reaction product was further confirmed by TLC with authentic \[^{35}S\]SO₃⁻→3GalNAc1-1-O-Bn (Fig. 3D). The reaction product was developed to the same position as the authentic oligosaccharide and the oligosaccharide treated with periodate oxidation. These results suggest that \[^{35}S\]sulfate binds to the C-3 position of Gal residue and that the enzyme cloned here is a Galβ1→3GalNAc:3′-sulfotransferase.

The optimal pH of the enzyme was 6–7, using Galβ1→3GalNAc1-1-O-pNP as acceptor substrate (Fig. 4). In the presence of MnCl₂, the activity increased about 2.2-fold (Table I). EDTA had no effect on the activity, suggesting that MnCl₂ acts on Gal3ST-4 activity when these assays were performed in the presence of MnCl₂.

The concentration of PAPS was 4.2 mM, respectively (Table III).

Galβ1→3GalNAc1-1-O-pNP was used as acceptor substrate (Table III).

### TABLE II

| Substrate | \(K_m\) | \(V_{max}\) |
|-----------|--------|-----------|
| Galβ1→3GalNAc1-1-O-pNP or 6GalNAc1-1-O-pNP | 0.24 | 7.2 |
| Galβ1→3GalNAc1-1-O-pNP | 0.23 | 6.3 |
| PAPS | 0.00029 | 4.2 |

* The concentration of PAPS was 4 μM.
* Acceptor substrate was 3 mM Galβ1→3GalNAc1-1-O-pNP.

### TABLE III

**Kinetic properties of human Gal3ST-4**

The enzyme source was the crude membrane fraction derived from COS-7 cells transfected with pCMV-SPORT-Gal3ST-4.

| Table IV | Action of Gal3ST-4 on asialofetuin |
|----------|-----------------------------------|
| Total radioactivity | Released \[^{35}S\]-oligosaccharides by Alkaline treatment⁻¹ N-Glycanase digestion³ |
| pmol | dpm | dpm | dpm |
|---|---|---|---|
| 14,115 | 12,280 | 0 |

* The enzyme reaction was performed using 40 μg of asialofetuin as described under “Experimental Procedures.”
* \(^{35}S\)-labeled protein was digested with 1 milliunit of N-glycanase after denaturation in 0.5 M Tris-HCl (pH 8.6), 0.5% SDS, and 0.1 M mercaptoethanol at 100 °C for 3 min, and then 1% Nonidet P-40 was added as a stabilizer.

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* The concentration of PAPS was 4 μM.
* Acceptor substrate was 3 mM Galβ1→3GalNAc1-1-O-pNP.

### Incorporation of \[^{35}S\]Sulfate into Asialofetuin by Gal3ST-4

We investigated whether Gal3ST-4 can specifically add sulfate to Galβ1→3GalNAc in asialofetuin, which contains both bi- and tri-antennary N-glycans and O-glycans consisting of Galβ1→3GalNAc (35, 36). After incubating asialofetuin with Gal3ST-4, half of the \[^{35}S\]-sulfated products were subjected to mild alkaline treatment, which specifically releases O-glycans, and the other half of the products were digested with N-glycanase. As shown in Table IV, \[^{35}S\]-sulfated oligosaccharides were released by alkaline treatment, but not by N-glycanase digestion. These results also support that Gal3ST-4 specifically acts on Galβ1→3GalNAc residue in O-glycans.

### Northern Blot Analysis

Among various human tissues, mRNA for Gal3ST-4 with 2.5 kilobases was expressed mainly in placenta, thymus, testis, ovary, spinal cord, trachea, and adrenal

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**Fig. 5. Effect of acceptor concentrations on Gal3ST-4 activity.**

Galβ1→3GalNAc1-1-0-pNP (●) and Galβ1→3GalNAc1-1-0-pNP (○) were used as acceptor substrates.
gland, and moderately in brain, lung, spleen, prostate, small intestine, colon, stomach, thyroid, and lymph node (Fig. 6).

**DISCUSSION**

The present study demonstrates the isolation of a novel cDNA encoding Galβ1→3GalNAc-specific Gal-→3-sulfotransferase by searching an EST homologous to human Gal3ST-1 (19). The amino acid sequence of Gal3ST-4 reveals 33, 39, and 30%, identity with Gal3ST-1, Gal3ST-2, and Gal3ST-3, respectively. This indicates that Gal3ST-4 is the fourth member of a Gal-→3-sulfotransferase family. These enzymes are in common with transferring sulfate to nonreducing terminal Gal but selectively recognize aglycon moieties of β-galactosides. Gal3ST-1 acts on GalCer, LacCer, and GalDG, but not on lactose and Galβ1→4GlcNAcβ1→3Galβ1→4Glcβ1-Cer (20). Gal3ST-2 acts on Galβ1→3/4GlcNAcβ1→R and Galβ1→3GalNAcα1→O-Bn, but not on GalCer and LacCer (17). Gal3ST-3 acts on Galβ1→4GlcNAcβ1→R (21). In contrast, Gal3ST-4 specifically recognizes the Galβ1→3GalNAcα1→structure, and Galβ1→3/4GlcNAc, GalCer, LacCer, and GalDG are not substrates (Table II). The results indicate that the four sulfotransferases are utilized in accordance with distinct acceptor glycoconjugates.

The Gal3ST-4 gene comprises at least four exons (Fig. 1C). The coding region is inserted with two introns at Arg-42 and between Ghu-143 and Val-144. The putative transmembrane domain and two putative PAPS binding domains (5′-PSB and 3′-PB) (34) are localized in exons 2, 3, and 4, respectively. We investigated the intron/exon alignment of Gal3ST-2 gene using the Human Genome Project Data Base and found that the coding region for Gal3ST-2 is also inserted with two introns, at Pro-40 and between Gln-125 and Val-126. The insertion positions of the Gal3ST-2 gene are very close to those of Gal3ST-4 (Fig. 1C). In contrast, the coding region for Gal3ST-4 is inserted with one intron at Thr-44 (37); the position is close to Arg-42 of Gal3ST-4 and Pro-40 of Gal3ST-2. As for the 5′-untranslated region, it has been shown that there exist at least seven exons for the 5′-untranslated region of the human Gal3ST-1 gene and that these exons are alternatively utilized in a cancer-associated manner (37). Whether or not there exist alternative forms of mRNA for Gal3ST-4 remains unclear, but this is an important issue for elucidating transcriptional regulation of the Gal3ST-4 gene.

Gal3ST-4 is highly specific for the Galβ1→3GalNAcα1→structure. Galβ1→3(GlcNAcβ1→6)GalNAcα1→O-pNP is also a good substrate, and the $K_m$ and $V_{max}$ values for the core 2 oligosaccharide are similar to those for Galβ1→3GalNAcα1→O-pNP (Table III), indicating that the substitution of β-GlcNAc at the C-2 of Galβ1 does not affect the substrate recognition of Gal3ST-4. Kuhns et al. (38) showed that Galβ1→3GalNAcβ1→6 N-acetylgalactosaminyltransferases in acute myeloid leukemia cells and rat colon can act on Galβ1→3GalNAcα1→O-Bn, but not on SO3−→3Galβ1→3GalNAcα1→O-Bn and suggested that the substitution of β-GlcNAc at the C-6 of Galβ1→3GalNAc should precede sulfation at the C-3'. Our result that Gal3ST-4 can utilize core 2 oligosaccharide as a good substrate is consistent with their results with regard to biosynthesis of sulfated core 2 glycans.

The enzyme activity of Gal3ST-4 is inhibited by higher concentrations of acceptor substrates (Fig. 5). Similar inhibitory effects have been reported for β1→4-galactosyltransferase I, II, and III (39), β1→4-galactosyltransferase from human colonic mucosa (40), and GlcNAc→6-sulfotransferase (30). These transferase activities are inhibited at concentrations in excess of 2–5 mM, except for β1→4-galactosyltransferase II, the activity of which is inhibited even at $\sim$0.6 mM GlcNAcα1→O-Bn (39). The inhibitory effects of the two substrates for Gal3ST-4 (Fig. 5) appear at similar concentrations as those for the transferases described above, although the molecular mechanism and biological significance remain unclear.

The result in Fig. 6 showed a relatively extensive expression of the mRNA for Gal3ST-4. It is important whether or not the SO3−→3Galβ1→3GalNAcα1→structure is present in the tissues examined in Fig. 6. Although Chance and Mawhinney (10) showed the occurrence of SO3−→3Galβ1→3(R→GlcNAcβ1→6)GalNAcα1→structure in tracheobronchial mucous glycoproteins from a patient with cystic fibrosis, information about the existence of the sulfated glycan has so far been scarce. On the other hand, Gal→3-sulfotransferase activities for the Galβ1→3GalNAcα1→structure have been reported in rat colonic mucosa (38), human breast, colon, and several tumor tissues (41). To assess whether or not the SO3−→3Galβ1→3GalNAcα1→structure is widely distributed, a lectin or antibody that specifically recognizes the sulfated glycan needs to be explored.

Chandrasekaran et al. (41) reported the occurrence of two distinct Gal3-O-sulfotransferases (groups A and B) in human various tumors and normal tissues with a tissue-dependent distribution. Group A sulfotransferase recognizes Galβ1→3GalNAcα-O-allyl and 3-O-MeGalβ1→4GlcNAcβ1→6Galβ1→3GalNAcα-O-Bn as good acceptors, but not Galβ1→4GlcNAcβ1→O-allyl and Galβ1→3GlcNAcβ1→O-allyl, whereas group B sulfotransferase has rather broad substrate specificity (41). The substrate specificity of group A sulfotransferase is similar to...
that of Gal3ST-4. They also showed that group A Gal:-3-sulfotransferase is dominant in breast tumor, some ovarian tumor, and some metastatic ovary, and that the specific activities in breast tumor are higher than those in breast normal tumor, and some metastatic ovary, and that the specific activities in three human breast cancer cell lines. We are in the process of investigating changes in expression level of mRNA for Gal3ST-4 in these cancerous tissues.

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Molecular Cloning and Characterization of a Novel Human Galactose 3-O-Sulfotransferase That Transfers Sulfate to Galβ1→3GalNAc Residue in O-Glycans

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