The Galβ1→4(SO3−→6)GlcNAc moiety is present in various N-linked and O-linked glycans including keratan sulfate and 6-sulfosialyl-Lewis X, an L-selectin ligand. We previously found β1,4-galactosyltransferase (β4GalT) activity in human colonic mucosa, which pre-
fers GlcNAc 6-O-sulfate (6SGN) as an acceptor to non-
substituted GlcNAc (Seko, A., Hara-Kuge, S., Nagata, K.,
Yonezawa, S., and Yamashita, K. (1998) FEBS Lett. 440,
307–310). To identify the gene for this enzyme, we puri-
ﬁed the enzyme from porcine colonic mucosa. The puri-
fication and characterization of the enzyme revealed that the puriﬁed
β4GalT has a similar sequence to human β4GalT-IV. To conﬁrm this result, we prepared cDNA for each of the seven β4GalTs cloned to date and examined substrate
speciﬁcities using the membrane fractions derived from
β4GalT-transferred COS-7 cells. When several N-
linked and O-linked glycans with or without 6SGN res-
dues as acceptor substrates, only β4GalT-IV efﬁciently
recognized 6SGN, keratan sulfate-related oligosaccha-
drides, and Galβ1→3(SO3−→6GlcNAcβ1−6) GalNAcα1-O-
pNP, a precursor for 6-sulfosialyl-Lewis X. These results
suggested that β4GalT-IV is a 6SGN-speciﬁc β4GalT and
may be involved in the biosynthesis of various glycopro-
teins carrying a 6-O-sulfated N-acetyllactosamine moiety.

The Galβ1→4(SO3−→6)GlcNAc structure has been found in
N-linked and O-linked glycans of glycoproteins and is involved
in various biological events (1, 2). The sulfated disaccharide
unit has been believed to be biosynthesized by 6-O-sulfation of
non-reducing terminal GlcNAc and, following β1,4-galactosyla-
tion, on the basis of the substrate speciﬁcities of GlcNAc 6-O-
sulfotransferases (GlcNAc6STs)1 so far reported (2–11). Al-
though the galactosylation step had long been unclear, we
partially puriﬁed a GlcNAc 6-O-sulfate (6SGN)-speciﬁc β1,4-
galactosyltransferase (β4GalT) from human colonic mucosa
(12). The enzyme preferred 6SGN and 6SGN-containing oligo-
saccharides to non-sulfated GlcNAc residues as acceptor sub-
strates, whereas bovine milk β4GalT-I showed the opposite
speciﬁcity. The Neu5Acα2→6GlcNAcβ1−4 sequence was a poor
substrate for the 6SGN-speciﬁc β4GalT, suggesting that a sul-
fated residue at the C-6 of GlcNAc is essential for the acceptor
recognition. This preference for 6SGN residues suggests that
the enzyme is involved in the biosynthesis of keratan sulfate
(KS), (SO3−→6)Galβ1→4(SO3−→6GlcNAcβ1−3) (ref. in Ref.
13), and 6-sulfosialyl-Lewis X, known as an L-selectin li-
gand (14–17). However, molecular cloning of the enzyme has
yet not been performed.

It has been clariﬁed so far (18–29) that seven β4GalT genes
exist. All the β4GalTs are synthesized as type II membrane-
bound proteins and reside in the Golgi apparatus (30). These
seven enzymes have been characterized in terms of substrate
speciﬁcity. β4GalT-I is abundant in bovine and human milk in
a soluble form and is the ﬁrst galactosyltransferase for which
the corresponding cDNA has been isolated (18–21). β4GalT-I
acts on non-reducing terminal GlcNAc as an acceptor, and
whereas in the presence of α-lactalbumin, the enzyme prefers
Glc as a lactose synthase to GlcNAc (31, 32). This enzyme is
involved in the elongation of poly-N-acetyllactosamine repeats
(33). β4GalT-II and -III act on GlcNAc residues in several
glycoproteins and speciﬁc glycolipids, and β4GalT-II is aﬀected
by α-lactalbumin in a similar manner to β4GalT-I (22).
β4GalT-IV acts on Le3 (GlcNAcβ1→3Galβ1→4Glcβ1→3Glcβ1−
Cer) (26) and Galβ1→3(GlcNAcβ1−6GlcNAcα1−2) (core 2); the latter

galactosylation at the C-4 of GlcNAc leads to the formation of
the sialy-Lewis X structure (34). β4GalT-V has been shown to
have strong activity for core2 and core6(GlcNAcβ1→6GlnAnac)
(35). The enzyme also recognized GlcNAcβ1→2-(GlcNAcβ1−
6Manα1−6 moieties in N-linked tetra-antennary

3(GlcNAcβ1→2-Manα1−1)−6Manα1−1−GlcNAcβ1→4GlcNAcβ1→4Glc
Core 2, Galβ1→3(GlcNAcβ1−6GlcNAcα1−2)−GL, GlcNAcβ1−3Galβ1−4Glc
GlcCer, glucosylceramide; L2L2, Galβ1→4(SO3−→6GlcNAcβ1−3Galβ1→4(SO3−
→6GlcNAcβ1−3Galβ1→4(SO3−→6GlcNAcβ1−3Galβ1→4(SO3−→6GlcNAcβ1−3Galβ1→4(SO3−
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→6GlcNAcβ1−3Galβ1→4(SO3−→6GlcNAcβ1−3Galβ1→4(SO3−→6GlcNAcβ1−3Galβ1→4(SO3−
→6GlcNAcβ1−3Galβ1--
glycans (23, 36) and was suggested to be involved in the biosynthesis of tumor-associated N-glycans in concert with β9252- N-acetylglucosaminyltransferase V (37). Lactosylceramide synthase has been purified from rat brain, and its molecular cloning was performed based on partial amino acid sequences (25). The enzyme is an orthologue of human β9252-4GalT-V (24, 27). β9252-4GalT-VII is a β-Xyl:1,4GalT, equal to galactosyltransferase-I which is involved in the synthesis of the proximal sequence in various glycosaminoglycans, Gal

In this study, we first purified 6SGN-specific β4GalT from porcine colonic mucosa and partially determined its amino acid sequence. We obtained a sequence similar to human β4GalT-IV. Moreover, we isolated cDNA for each of the seven β4GalT cloned to date, and we introduced expression vectors containing these cDNAs individually into COS-7 cells and prepared membrane fractions to study their substrate specificities. As a result, we found that among the seven β4GalTs, β4GalT-IV is the only enzyme to efficiently act on KS-related oligosaccharides and GlcNAc 6-O-sulfated core2, a precursor of 6-sulfosialyl-Lewis X.

**EXPERIMENTAL PROCEDURES**

**Materials**—UDP-[3H]Galactose (1400 GBq/mmol) was purchased from PerkinElmer Life Sciences. PAPS, UDP-Gal, p-nitrophenyl(pNP)-β-d-xylene (Xyl-O-pNP), and glucosylceramide (GlcCer) were purchased from Sigma. Galβ1→3GlcNAcβ1→4GalNAca1-O-pNP (core2-O-pNP) was obtained from Funakoshi Co., Ltd. (Tokyo, Japan). GlcNAcβ1→
TABLE II

**Effects of various lipids on purified 6SGN-specific β4GalIT activity**

| Lipids* | Relative activity |
|---------|------------------|
| 1→6-Phosphatidic acid | 0% |
| 1→2-Phosphatidylcholine | 0% |
| 1→4-Lyosphosphatidylcholine | 0% |
| 1→2-Phosphatidylethanolamine | 0% |
| 1→4-Phosphatidylinositol | 0% |
| 1→2-Phosphatidylserine | 0% |
| d-Sphingosine | 100% |
| N,N-Dimethylphosphoglyceride | 155% |
| N-Acetylphosphoglyceride | 0% |
| N-Stearoylphosphoglyceride | 0% |
| Ceramides | 0% |
| Sphingosine 1-phosphate | 27% |
| Sphingomyelin | 69% |
| Stearoylamine | 0% |
| Octylamine | 0% |

*The concentrations of lipids in the enzyme reaction mixture were 0.001%.

**Fig. 2.** Effects of d-sphingosine (●), N,N-dimethylphosphoglyceride (○) and stearoylamine (▲) on the purified 6SGN-specific β4GalIT. Indicated concentrations of lipids were added to the enzyme reaction mixture. The concentration of Triton X-100 was 0.05% (w/v) in these assays.

**Fig. 3.** SDS-PAGE analysis of the enzyme fractions obtained by asialo-agalacto-ovomucin-Sepharose column chromatography. The enzyme fractions (10 μg as protein) were subjected to SDS-PAGE (10% gel) under reducing conditions and visualized with Coomassie Brilliant Blue.

β4GalIT-IV Is Specific for GlcNAc 6-O-Sulfate

2Manα1→3GlcNAcβ1→2Manα1→3-galactosidase (41) from lacto-N-tetraose (41) and bovine cornea) was purchased from Seikagaku Corp., Tokyo, Japan. Keratan sulfate (sodium salt) was purchased from Hohnen Oil Co. (Tokyo, Japan). Bovine brain cerebrosides; L-lysophosphatidylcholine, and L-α-phosphatidylcholine were from egg yolk lecithin; and L-α-phosphatidylethanolamine were purchased from Sigma. Octylamine was purchased from Aldrich.

Purification of 6SGN-specific β4GalIT from Porcine Colonic Mucosa—

The following procedures were performed at 4°C. Six kg of porcine colon was purchased from Tokyo Shibaura Zokki Co. Ltd. (Tokyo, Japan). The mucus layer was scraped off the colon; 3 liters of PBS was added, and the mixture was homogenized with a Potter-Elvehjem type homogenizer and then centrifuged at 1,000 × g for 30 min. The supernatant was removed, and the two supernatant fractions were mixed and ultracentrifuged at 100,000 × g for 1 h. The precipitated microsomes were washed once with 0.5 M KCl and extracted twice with 1 liter of 20 mM HEPES-NaOH (pH 7.2), 1 M NaCl, 10 mM MnCl2, 1% (w/v) Triton X-100, 1 mM dithiobisreitol, 1 mM phenylmethanesulfonyl fluoride, and 10% (v/v) glycerol followed by ultracentrifugation. The extract was dialyzed against 10 mM HEPES-NaOH (pH 7.2), 5 mM MnCl2, 1 mM dithiothreitol, and 20% glycerol (buffer A), and applied on a UDP-hexanolamine-Sepharose column (1.4 × 6.5 cm) equilibrated with 20 mM HEPES-NaOH (pH 7.2), 10 mM MnCl2, 0.1% (w/v) Triton X-100, 1 mM dithiothreitol, and 10% glycerol (buffer B) (12). After washing with buffer B containing 0.15 M NaCl, the enzyme fractions were eluted with buffer B containing 1 M NaCl and dialyzed against buffer A. Next, the dialyzed fraction was reapplied on a UDP-hexanolamine-Sepharose column (1.4 × 6.5 cm) equilibrated with buffer B and eluted with buffer B containing 1 mM UDP. Each fraction was dialyzed against buffer A and assayed for β4GalIT activity. The enzyme fractions were applied on an asialo-agalacto-ovomucin-Sepharose column (6.7 μg protein/ml of gel; 1 × 7.5 cm; equilibrated with buffer B) (12). The enzyme was eluted with a linear gradient of NaCl (0–0.2 M) in buffer B and then dialyzed. The enzyme fractions were concentrated and used for biochemical analyses.

Cloning of the cDNAs Encoding β4GalITs—cDNAs encoding β4GalITs II, III, IV, V, and VI were amplified from SuperScript™ human testis cDNA library (Invitrogen) by PCR, and cDNAs encoding β4GalIT-I and VI were amplified by PCR from QUICK-Clone™ cDNA for human
colonic adenocarcinoma and mouse brain (Clontech, Palo Alto, CA), respectively. Oligonucleotide primers used for the PCRs were forward primer for μGalT-I, 5'-tttgatcatCGATTGAGGCTTGACCT-3' (reverse primer for μGalT-I), 5'-tttgatcatGCCCATGGAGGCTTGACCT-3' (reverse primer for μGalT-II), 5'-tttgatcatGGCTGCAGCATGC-3' (forward primer for μGalT-III), 5'-tttgatcatATCAGGCTTGACCT-3' (forward primer for μGalT-IV), 5'-tttgatcatATCCAGGGTCATGCACACTG-3' (forward primer for μGalT-V), 5'-tttgatcatTGCTGCAGCATGCACCTG-3' (forward primer for μGalT-VI), 5'-tttgatcatCCAGGCTTGACCT-3' (forward primer for μGalT-VII), and 5'-tttgatcatTGCTGAGGCTTGACCT-3' (reverse primer for μGalT-VII). Sequences in lowercase letters contain appropriate restriction sites. Amplified cDNAs were transfected into COS-7 cells on 35-mm dishes using Lipofectin Reagent (Gibco BRL). The 6-azaglycosylated acceptor substrate, and the membrane fraction approximately diluted with 107 ml of reaction mixture consisting of 50 mM HEPES-NaOH (pH 7.2), 10 mM MnCl$_2$, 0.5% (w/v) Triton X-100, 250 μM UDP-Gal, 0.3 μM UDP-[3H]Gal (4.9 × 10$^8$ dpm), 0.5 mM 2-oxoglutarate, and the membrane fraction was incubated at 37 °C for 1 h. In the case of purified porcine 6S-βGalT, specific activity was 8 nmol/h when using 1 mM UDP-Gal, 4.9 × 10$^8$ dpm, 0.5 mM 2-oxoglutarate, and the membrane fraction was incubated at 37 °C for 1 h. In the case of purified porcine 6S-βGalT, specific activity was 8 nmol/h when using 1 mM UDP-Gal, 4.9 × 10$^8$ dpm, 0.5 mM 2-oxoglutarate, and the membrane fraction was incubated at 37 °C for 1 h. In the case of purified porcine 6S-βGalT, specific activity was 8 nmol/h when using 1 mM UDP-Gal, 4.9 × 10$^8$ dpm, 0.5 mM 2-oxoglutarate, and the membrane fraction was incubated at 37 °C for 1 h. In the case of purified porcine 6S-βGalT, specific activity was 8 nmol/h when using 1 mM UDP-Gal, 4.9 × 10$^8$ dpm, 0.5 mM 2-oxoglutarate, and the membrane fraction was incubated at 37 °C for 1 h.
Experimental Procedures.

When solvents A and B were 0.09% (v/v) aqueous trifluoroacetic acid and 0.075% (v/v) trifluoroacetic acid in 80%(v/v) acetonitrile, respectively, selected peptides were subjected to Edman degradation using a model 477A automated protein sequencer (Applied Biosystems, Inc.) connected on-line to a model 120A PTH analyzer (PerkinElmer Life Sciences) and to a Reflex matrix-assisted laser desorption ionization time of flight mass spectrometer (Bruker-Franzen Analytik, Bremen, Germany) in linear mode using 2-mercaptobenzothiazole (44) as a matrix.

**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 was normalized to the mRNA expression level of β-actin. 32P-Labeled probe was prepared from the cDNA fragment (excised from pcDNA3-4GalT-IV by BamHI and EcoRV digestion) with a Random Primed DNA Labeling Kit (Roche Diagnostics) using [α-32P]dCTP (PerkinElmer Life Sciences) according to the manufacturer's instructions. The membranes were pre-hybridized in ExpressHyb Solution (Clontech) at 68 °C for 2 h and then hybridized with the 32P-labeled probe in the same solution at 68 °C for 16 h. The Northern blot membranes were washed in 2× SSC, 0.05% SDS at room temperature and then in 0.1× SSC, 0.1% SDS at 50 °C. The radioactivity was detected with FUA-2000 (Fuji Photo Film Co. Ltd., Tokyo).

**RESULTS AND DISCUSSION**

**Purification of 6SGN-specific 4GalT from Porcine Colonic Mucosa and Its Basic Lipid Requirement for Activity**—We showed previously (12) that a 6SGN-specific 4GalT exists in human colonic mucosa. To purify this enzyme and to isolate a corresponding cDNA based on partial amino acid sequences, we used porcine colonic mucosa as an enzyme source. Porcine colonic mucosa also contained 6SGN-specific 4GalT activity. When the enzyme was purified 2,300-fold by a second UDP-hexanolamine-Sepharose column chromatography (Table I), the enzymatic activity disappeared (Fig. 1A). Because such a loss of enzymatic activity has been reported for a glucuronyltransferase, which requires sphingomyelin for activity in a highly purified state (45), we added various lipid compounds to the enzyme reaction mixtures. As shown in Table II, most of the phospholipids and ceramides had no ability to restore the enzymatic activity, whereas the activity appeared in the presence of d-sphingosine and N,N-dimethy1sphingosine. Stearylamine could also restore the activity, whereas octylamine could not, suggesting that basic, lipidous compounds with long hydrophobic chains are essential at least for the \textit{in vitro} enzymatic activity. The minimum concentrations of d-sphingosine, N,N-dimethylphosphoginosine, and stearylamine giving the maximal activity were ~10, 20, and 10 μM, respectively (Fig. 2).

After asialo-agalacto-ovomucin-Sepharose chromatography, 6SGN-specific 4GalT was purified 24,000-fold (Fig 1B and Table I). We tried further purification by various chromatographic methods but were unsuccessful. The final enzyme fraction was extracted from the gel and separated on columns of DEAE-5PW (2 × 20 mm; Tosoh, Tokyo) and Mighty GP-BP-18 (2 × 50 mm; Kanto Chemical, Tokyo) connected in series with a model 1100 (Hewlett-Packard) liquid chromatography system. Peptides were eluted at a flow rate of 0.1 ml/min using a linear gradient of 0–60% solvent B, where solvents A and B were 0.09% (v/v) aqueous trifluoroacetic acid and 0.075% (v/v) trifluoroacetic acid in 80%(v/v) acetonitrile, respectively.

**TABLE V**

Relative values of the enzymatic activities of 4GalT-IV for various acceptor substrates

| Substrate | Structure | 0.1 mM | 0.5 mM |
|-----------|-----------|--------|--------|
| 6SGN      | SO₃⁻—6GlcNAc | 45     | 100    |
| 6S-core2-O-pNP | SO₃⁻—6GlcNAcb1—6Galb1—3GalNAca1-O-pNP | 92   | 40     |
| agL2L2    | SO₃⁻—6GlcNAcb1—3Galb1—4SO₃⁻—6GlcNAc | 112  | 144    |
| agL2L4    | SO₃⁻—6GlcNAcb1—3SO₃⁻—6Galb1—4SO₃⁻—6GlcNAc | 72   | 168    |
| 6S-haGP   | SO₃⁻—6GlcNAcb1—2Manb1—4Fucb1—6Galb1—4SO₃⁻—6GlcNAc | 108  | 172    |
| agLST-b   | Neu5Acb2—6GlcNAcb1—3Galb1—4Glc | 7     | 23     |

**TABLE VI**

Kinetic analysis of 4GalT-IV for several oligosaccharides containing 6-O-sulfated or non-substituted GlcNAc

| Substrate | 6SGN | GlcNAc | 6S-haGP | haGP | 6S-core2-O-pNP | core2-O-pNP | agL2L2 | agL2L4 |
|-----------|------|--------|---------|------|---------------|------------|--------|--------|
| \(K_m\) (mm) | 0.43 | 330    | 0.11    | 7.7  | 0.091         | 0.50       | 0.38   | 0.63   |
| \(V_{max}/K_m\) (nmol/min/mg protein) | 3.6  | 1.5    | 3.2     | 0.42 | 4.8           | 1.2        | 7.8    | 7.8    |
| \(V_{max}\) (nmol/min/mg protein) | 8.4  | 0.0045 | 29      | 0.055| 53            | 2.4        | 21     | 12     |
sections contained two major (45kDa and 42kDa) and two minor (59kDa and 52kDa) proteins as determined by SDS-PAGE analysis (Fig. 3). Each protein band was digested with a lysine-specific protease, and the peptide fragments were separated by reversed phase high pressure liquid chromatography, and their amino acid sequence was analyzed. One or two peptide fragments from each band were sequenced (Table III), and the sequences were compared with known protein sequences using the BLAST search system. The band a contained one sequence that is equal to that of human polypeptide: N-acetylgalactosaminyltransferase-2 (46). The band b contained two sequences that are very similar to that of human N-acetylgalacosaminyltransferase-I (47, 48). Because the two enzymes utilize UDP-sugars as donor substrates, they may be co-purified with 6SGN-specific β4GalT by UDP-hexanolamine-Sepharose column chromatographies. On the other hand, the bands c and d contained a common peptide sequence, which is similar to that of human β4GalT-IV (24, 26). This result suggested that the purified 6SGN-specific β4GalT corresponds to β4GalT-IV.

Expression of Seven β4GalTs in COS-7 Cells and Their Substrate Specificities—To confirm that β4GalT-IV is 6SGN-specific, and the only 6SGN-specific β4GalT among the seven β4GalTs so far identified, we prepared expression vectors containing each β4GalT cDNA and analyzed substrate specificities using the membrane fractions derived from vector-transfected COS-7 cells. Seven β4GalT genes have been identified within the data bases provided by the human genome project to date (18–29). The membrane fraction derived from pcDNA3-transfected COS-7 cells (C-MF) was used as a control for intrinsic β4GalT activities. C-MF had weak β4GalT activity; the specific activities using biGP, 6SGN, agl2L2, Xyl-O-pNP, GL, core2-O-pNP, GlcCer, and 6S-core2-O-pNP as acceptor substrates were 0.20, 0.34, 0.43, 0.20, 0.12, 0.47, 0, and 0.16 nmol/min/mg of protein, respectively. To calculate the amount of exogenous β4GalT activities, values derived from C-MF were subtracted from those of the apparent enzymatic activities obtained under the same conditions.

The specific activities of β4GalT-I, -II, -III, -IV, -V, -VI, and -VII were 6.6 (using biGP as an acceptor), 3.4 (core2-O-pNP), 2.6 (core2-O-pNP), 1.5 (6SGN), 1.5 (GlcCer), 0.32 (GlcCer), and 3.7 nmol/min/mg of protein (Xyl-O-pNP), respectively. The relative activities of the seven β4GalTs for eight acceptor substrates are summarized in Table IV. Among the seven β4GalTs, only β4GalT-IV recognized 6SGN as a good acceptor in comparison with biGP and GL, and this profile of β4GalT-IV was equal to the substrate specificity of human colonic 6SGN-specific β4GalT previously reported by us (12). The linkage position of [3H]Gal in [3H]Galβ1→agl2L2 and ([3H]Galβ1→6S-core2-O-pNP synthesized by β4GalT-IV was confirmed to be the C-4 of GlcNAc by RCA-I-agarose affinity chromatography (Fig. 4), which binds to Galβ1→4GlcNAc (49). [3H]Galβ1→agl2L2 (Fig. 4B, solid line) and ([3H]Galβ1→6S-core2-O-pNP (Fig. 4C, solid line) bound to the column weaker than ([3H]Galβ1→core2-O-pNP (Fig. 4A). To examine whether the weak binding is caused by the presence of 6-O-sulfate, the sulfated products were treated with mild methanolysis (0.05 N HCl/MeOH, 25 °C, 4 h) (50) and applied onto the lectin column. The digests bound to the column and were eluted with 10 mM

**Fig. 5.** Enzymatic activities at various concentrations of acceptor substrates. The activities were measured as under “Experimental Procedures.” Acceptor substrates used were 6SGN (●), core2-O-pNP (○), 6S-core2-O-pNP (▲), and agl2L2 (△).
lactose (Fig. 4, B and C, dotted lines), but the digests flowed through the column by digestion with Galβ1-4GlcNAc-specific diplococcal β-galactosidase (data not shown), showing that [3H]Gal is attached to the C-4 of GlcNAc in the sulfated products. 6SGN was a poor substrate at best for the other 4GalTs (Table IV), even at the lower concentrations, 0.1 or 0.2 mM (data not shown), excluding the possibility that the absence or low level of activity was due to the inhibitory effect of a high concentration of 6SGN; such as has been found in several 4GalTs (34, 51). These results indicated that 4GalT-IV is the only 6SGN-specific enzyme among the seven 4GalTs, consistent with the amino acid sequence of the purified porcine 6SGN-specific 4GalT as described above.

4GalT-III prefers O-linked type core2-O-pNP to N-linked type biGP, whereas 4GalT-I and -II recognize both biGP and core2-O-pNP as good acceptors. It has been shown that 4GalT-V acts on core2 (35) and a specific branching structure of N-linked glycans (36). As shown in Table IV, 4GalT-V could act on GlcCer, similar to 4GalT-VI. Recently, Lee et al. (52) reported that 4GalT-VI-deficient CHO cells have an ability to synthesize lactosylceramides, and they suggested the existence of a lactosylceramide synthase other than 4GalT-VI. 4GalT-V may be the second lactosylceramide synthase.

4GalT-IV recognizes several 6SGN-containing oligosaccharides as good acceptors (Table V). KS-related oligosaccharides, agL2L2 and agL2L4, were good substrates for 4GalT-IV. AgL2L2 was a poor substrate at best for the other 4GalTs (Table IV). KS is also a substrate for 4GalT-IV with a specific activity of 0.045 nmol/min/mg of protein at 1 mM KS. To expose GlcNAc residues at the non-reducing termini of KS chains, we treated KS by mild acid hydrolysis to remove sialic acid and Streptococcus 6646K β-galactosidase digestion. The enzymatic activity for the asialo-agalacto-KS was 3.3-fold higher than that for intact KS. This result suggests that 4GalT-IV can also act on KS long chains. Because agLST-b was a poor substrate for 4GalT-IV, sialic acid cannot replace sulfate at the C-6 of GlcNAc. This substrate selectivity is the same as for human colonic 6SGN-specific 4GalT (12).

4GalT-IV also efficiently acts on 6S-biGP and 6S-core2-O-pNP; the 6SGN moiety is present in N-linked and O-linked glycans in various glycoproteins (1). The 6-sulfosialyl-Lewis X on core2 glycans, which is synthesized from 6S-core2 by 1,3-sialylation, and 1,3-fucosylation, functions as an L-selectin ligand moiety in the early step of lymphocyte homing in lymph nodes (14–17). 4GalT-IV is the only enzyme recognizing 6S-core2-O-pNP as a good substrate among the seven 4GalTs (Table IV), suggesting that 4GalT-IV is involved in the synthesis of 6-sulfosialyl-Lewis X.

Results of kinetic analysis of 4GalT-IV for several glycans with or without 6-O-sulfation at GlcNAc are summarized in Table VI. AgL2L4 and 6S-core2-O-pNP had an inhibitory effect on 4GalT-IV activities at high concentrations (Fig. 5), so that kinetic constants were calculated using only the data obtained with lower concentrations of these substrates. The $K_m$ value for 6SGN was lower than that for GlcNAc, and the $V_{max}/K_m$ value for 6SGN was 1900-fold higher than that for GlcNAc. Similarly,
the $V_{\text{max}}$/$K_m$ values for 6S-biGP and 6S-core2-O-pNP were much higher than those for biGP and core2-O-pNP, respectively. These results indicate that 6-O-sulfation of GlcNAc residues is important for efficient catalytic activity of β4GalT-IV.

Expression of β4GalT-IV in Various Human Tissues—Lo et al. (24) and Schwientek et al. (26) reported the expression profile of β4GalT-IV in human adult and fetal tissues, but they had not examined its expression in colon or lymph node, where 6SNG-specific β4GalT should be expressed. To clarify the expression pattern more extensively, we analyzed it using commercial Northern blot membranes. As shown in Fig. 6, a 2.4-kb transcript of β4GalT-IV was expressed ubiquitously. Relatively high expression levels of the enzyme were observed in kidney, placenta, lymph node, prostate, stomach, thyroid, tongue, and trachea. Recently, it has been shown that the 6-sulfosialyl-Lewis X determinant is present in colonic mucosa (53); in colon, β4GalT-IV was also moderately expressed (Fig. 7).

General Discussion—in this study, we showed the following.

(i) Porcine 6SGN-specific β4GalT was purified and identified as β4GalT-IV. (ii) The substrate specificities of the seven β4GalTs so far cloned were investigated, and β4GalT-IV is the only 6SNG-specific β4GalT among them and is involved in the synthesis of several 6-O-sulfated N-acetyllactosamine glycans. (iii) Porcine β4GalT-IV requires basic lipidic compounds for the catalytic activity at least at a highly purified state. It has been reported that sphingosine increases several times the enzymatic activities of α1-3-fucosyltransferase, GM2:β1→3GalT, GM3:β1→4N-acetylgalactosaminyltransferase (54), and glycoprotein sulfotransferase (55) and decreases that of β1→3-glucuronyltransferase (56). However, to our knowledge, this is the first demonstration that basic lipidic compounds are essential for the catalytic activity of glycosyltransferases. Sphingosine is an intermediate of the metabolism of sphingolipids and a precursor for sphingosine 1-phosphate, a second messenger for sphingosine 1-phosphate, a second messenger molecule derived from several culture cells (66), thyroglobulin (67), zona pellucida glycoproteins (68, 69), gp120 of human immunodeficiency virus type 1 (70), respiratory mucins (71, 72), carinoembryonic antigen (73), hyosohorin (74), and oviducal mucins (75). 6SNG-specific β4GalT-IV identified in this study may be useful to elucidate the biological roles of these sulfated moieties.

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