Molecular Cloning and Characterization of a Novel Human β1,4-N-Acetylgalactosaminyltransferase, β4GalNAc-T3, Responsible for the Synthesis of N,N'-Diacytllactosediamine, GalNAcβ1–4GlcNAc*

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We found a novel human glycosyltransferase gene carrying a hypothetical β1,4-glycosyltransferase motif during a BLAST search, and we cloned its full-length open reading frame by using the 5′-rapid amplification of cDNA ends method. It encodes a type II transmembrane protein of 999 amino acids with homology to chondroitin sulfate synthase in its C-terminal region (GenBank™ accession number AB089940). Its putative orthologous gene was also found in mouse (accession number AB114826). The truncated form of the human enzyme was expressed in HEK293T cells as a soluble protein. The recombinant enzyme transferred GalNAc to GlcNAc β-benzyl. The product was deduced to be GalNAcβ1–4GlcNAcβ-benzyl based on mass spectrometry and NMR spectroscopy. We renamed the enzyme β1,4-N-acetylglactosaminyltransferase-III (β4GalNAc-T3). β4GalNAc-T3 effectively synthesized N,N'-diacytllactosediamine, GalNAcβ1–4GlcNAc, at non-reducing termini of various acceptors. As some glycohormones contain N,N'-diacytllactosediamine structures in their N-glycans, we examined the ability of β4GalNAc-T3 to synthesize N,N'-diacytllactosediamine structures in N-glycans on a model protein. When fetal calf fetuin treated with neuraminidase and β1,4-galactosidase was utilized as an acceptor protein, β4GalNAc-T3 transferred GalNAc to it. Furthermore, the majority of the signal from GalNAc disappeared on treatment with glycopeptidase F. These results suggest that β4GalNAc-T3 could transfer GalNAc residues, producing N,N'-diacytllactosediamine structures at least in N-glycans and probably in both N- and O-glycans.

To date, seven members of the human β1,4-galactosyltransferase (β4Gal-T) family and six members of the human β1,4-N-acetylglactosaminyltransferase (β4GalNAc-T) family have been identified. β4Gal-T1 to -T6 exhibit activity to transfer Gal to GlcNAc with a β1,4-linkage in N- and O-glycans and glycolipids (1–4). β4Gal-T7 synthesizes the Galβ1–4Xyl structure that occurs in the linkage portion of glycosaminoglycans (5, 6). β4GalNAc-T1 is a key enzyme to catalyze the conversion of GM3, GD3, and lactosylceramide to GM2, GD2, and asialoglycans (7), respectively. Chondroitin synthases, CSS1 and CSS2, have both β1,3-galactosyltransferase and β4GalNAc-T activities and catalyze chondroitin sulfate elongation (10–12). In the case of CSSs, the β4GalNAc-T domain exists in the C-terminal region. Chondroitin sulfate N-acetylglactosaminyltransferase I and II (CSGalNAc-T1 and -T2) both act as β4GalNAc-Ts in the initiation and elongation of chondroitin sulfate synthesis (13–16).

The bovine β4Gal-T1, one of the best understood glycosyltransferases (17, 18), was crystallized, and its structure was analyzed (19–21). An acidic short sequence, GWGGED, which occurs in the linkage portion of glycosaminoglycans (5, 6). To date, seven members of the human β1,4-galactosyltransferase (β4Gal-T) family and six members of the human β1,4-N-acetylglactosaminyltransferase (β4GalNAc-T) family have been identified. β4Gal-T1 to -T6 exhibit activity to transfer Gal to GlcNAc with a β1,4-linkage in N- and O-glycans and glycolipids (1–4). β4Gal-T7 synthesizes the Galβ1–4Xyl structure that occurs in the linkage portion of glycosaminoglycans (5, 6).

The abbreviations used are: (β4) GalNAc-T, (β1,4)galactosyltransferase; (β4 or CS) GalNAc-T, (β1,4- or CS/N-acetylglactosaminyltransferase; CS, chondroitin sulfate; Cer, ceramide; GM3, NeuAcα2–3Galβ1–4Glcβ1–1Cer; GD3, NeuAcα2–3Galβ1–4Glcβ1–1Cer; GM2, GalNAcβ1–4NeuAcα2–3Galβ1–4Glcβ1–1Cer; GD2, GalNAcβ1–4NeuAcα2–3Galβ1–4Glcβ1–1Cer; CSS, chondroitin synthase; LactoNAC, N,N'-diacytllactosediamine (GalNAcβ1–4GlcNAc); LH, lutropin; TSH, thyrotropin; TFPI, tissue factor pathway inhibitor; HEK, human embryonic kidney; HPC, human protein C; β4GT, β4-galactosyltransferase; ORP, open reading frame; RAEC, rapid amplification of cDNA ends; TR, Tris-buffered saline; pNP, para-nitrophenol; Bz, benzyl; HPLC, high performance liquid chromatography; MS, mass spectrometry; MALDI-TOF, matrix assisted laser desorption/ionization-time of flight; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; PEF, fetal calf fetuin; GPF, glycopeptidase F; HRP, horseradish peroxidase; WFA, W. floribunda agglutinin; MES, 4-morpholineethanesulfonic acid; NOE, nuclear Overhauser effect.

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EMBL Data Bank with accession number(s) AB089940 and AB114826.

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human βGalNAc-T (amino acids 57–999) was expressed as a secreted protein fused with a FLAG peptide in HEK293T cells. An ~2.7-kb DNA fragment was amplified by PCR using the Marathon-Ready™ cDNA derived from human stomach as a template and two primers, 5'-GGAATTCGAGGTACGGCAGCTGGAGAGAA-3' and 5'-ACGGCTCAGCGTCAGGGCAGAGAAA-3'. The amplified fragment was digested with the restriction endonucleases EcoRI and SalI and then inserted into pcDNA3.1 (Intronviteg). Following digestion of the vector with restriction endonucleases EcoRI and Pmel, the 2.9-kb fragment was inserted into the EcoRI-EcoRV site of pFLAG-CMV-1 (Sigma) to construct pCMV-βGalNAc-T. The catalytic domain of βGalNAc-T was expressed in HEK293T cells. A 50-ml volume of culture medium was mixed with anti-FLAG M1 antibody resin (Sigma) and incubated with rotating at 4 °C overnight. The resin was washed twice with 50 mM Tris-buffered saline (50 mM Tris-HCl, pH 7.4, and 150 mM NaCl) containing 1 mM CaCl2 and suspended in 100 µl of the assay buffer described below. The mouse βGalNAc-T (mβGalNAc-T) gene encoding its putative catalytic domain (amino acids 57–986) was amplified with two primers, 5'-CCCCAGGTCTGGCCAGGGCGGGAAC-3' and 5'-GGATTCTTCACGGCATCTTTGCTGCGGA-3', by using the cDNA derived from mouse stomach as a template. The amplified 2.7-kb fragment was digested with restriction endonucleases HindIII and EcoRI, and then the digested fragment was inserted into pFLAG-CMV-1.

**Experimental Procedures**

**Isolation of Human βGalNAc-T3 cDNA**—We performed a BLAST search of the GenBank™ data base by using βGalT motifs as query sequences and identified a genomic DNA with accession number AC006205, which was estimated to contain a partial open reading frame (ORF) using GENSCAN software (39), and we showed a high level of homology to the C-terminal region of the βGalT family. To obtain the complete ORF, the four-step 5′-rapid amplification for complementary DNA (cDNA) ends (5′-RACE) method was employed using a Marathon-Ready™ cDNA amplification kit (Clontech, Palo Alto, CA) and eight reverse primers, the nucleotide sequences of which are summarized in Table I. The sequences of the DNA fragments obtained by the 5′-RACE method were determined using a DYEnamic ET Terminator Cycle sequencing kit (Amersham Biosciences). Finally, a cDNA sequence encoding the full-length ORF was obtained by PCR using the Marathon-Ready™ cDNA of human stomach (Clontech) as a template.

**Construction and Purification of Human and Mouse βGalNAc-T3 Proteins Fused with FLAG Peptides**—The putative catalytic domain of
Quantitative Analysis of βGalNAc-T3 Transcripts in Human Tissues by Real Time PCR—For the quantification of human βGalNAc-T3 transcripts, we employed the real time PCR method, as described in detail previously (40, 41). Total RNA of various human tissues was purchased from Clontech. Total RNA of peripheral blood mononuclear cells and various cell lines was extracted with an RNeasy kit (Qiagen, Hilden, Germany). cDNA templates were synthesized from the total RNA with a SuperScript™ II first-strand synthesis system (Invitrogen). Standard curves for the endogenous control, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA, were generated by serial dilution of a pCR2.1 (Invitrogen) DNA containing the GAPDH gene. The primer set and the probe for the βGalNAc-T3 gene were as follows: the forward primer was 5'-CTGGACTTCAGGCTGGCATAG-3' and the reverse primer 5'-GAATGGCATCGATGACTCCAG-3', and the probe was 5'-CTCGTGAAGGACCCGCA-3' with a minor groove binder (42).

Quantitative Analysis of βGalNAc-T3 Transcripts in Human Tissues by Real Time PCR—For the quantification of human βGalNAc-T3 transcripts, we employed the real time PCR method, as described in detail previously (40, 41). Total RNA of various human tissues was purchased from Clontech. Total RNA of peripheral blood mononuclear cells and various cell lines was extracted with an RNeasy kit (Qiagen, Hilden, Germany). cDNA templates were synthesized from the total RNA with a SuperScript™ II first-strand synthesis system (Invitrogen). Standard curves for the endogenous control, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA, were generated by serial dilution of a pCR2.1 (Invitrogen) DNA containing the GAPDH gene. The primer set and the probe for the βGalNAc-T3 gene were as follows: the forward primer was 5'-CTGGACTTCAGGCTGGCATAG-3' and the reverse primer 5'-GAATGGCATCGATGACTCCAG-3', and the probe was 5'-CTCGTGAAGGACCCGCA-3' with a minor groove binder (42). PCR products were continuously measured with an ABI PRISM 7700 Sequence Detection System (Applied Biosystems, Foster City, CA). The relative amount of the transcript was normalized to the amount of...
GAPDH transcript in the same cDNA.

Detection of GalNAc on AsialoAgalacto-FCF—Fetal calf fetuin (FCF), neuraminidase, β1,4-galactosidase, and glycopeptidase F were purchased from Sigma, Nacalai Tesque (Kyoto, Japan), Calbiochem, and Takara, respectively. Asialoagalacto-FCF was prepared from 200 µg of FCF by incubating with 4 microunits of neuraminidase and 12 microunits of β1,4-galactosidase at 37 °C for 16 h. The transfer of GalNAc by β4GalNAc-T3 to glycoprotein was performed in 20 µl of a standard reaction mixture containing 50 µg of asialoagalacto-FCF produced by glycosidase treatment. After incubation at 37 °C for 16 h, 5 µl of the reaction mixture was digested with glycopeptidase F (GPF) according to the manufacturer’s instructions. For detection of transferred GalNAc, horseradish peroxidase (HRP)-conjugated lectin, Wiste-ria floribunda agglutinin (WFA) (EY Laboratories, San Mateo, CA), was used. A 1-µl aliquot of reaction mixture subjected to 12.5% SDS-PAGE was transferred to nitrocellulose membrane (Schleicher & Schuell) and stained with 0.1% HRP-conjugated WFA lectin. The signals were detected using enhanced chemiluminescence (ECL) and Hyperfilm ECL (Amersham Biosciences).

RESULTS

Determination of Nucleotide Sequence of β4GalNAc-T3 cDNA and Its Putative Amino Acid Sequence—We determined a novel full-length cDNA sequence by the 5’-RACE method and registered it in the GenBank™ data base with the accession number AB089940. The nucleotide sequence and putative amino acid sequence are shown in Fig. 1. The gene was located at 12p13.3 and composed of at least 20 exons. This open reading frame consisted of 2,997 bp encoding a predicted 999-amino acid protein with a typical type II topology, which is common in glycosyltransferases. It contained four potential N-glycosylation sites and a DXH sequence, which is conserved in UDP-GalNAc-polypeptide-GalNAc-Ts (pp-GalNAc-T), and is thought to participate in divalent cation binding. In amino acid sequence, β4GalNAc-T3 was more similar to β4GalNAc-Ts than to β4Gal-Ts in the human β4GT family (Fig. 2). β4GalNAc-T3 was included in a GalNAc-T cluster in the phylogenetic tree, not in a Gal-T cluster (Fig. 2). The glycosyltransferase most homologous to β4GalNAc-T3 was chondroitin sulfate synthase 1 (CSS1), which is an enzyme-polymerizing chondroitin, (GalNAcβ1,4GlcUAβ1,3)n. The two showed 29.3% identity in the C-terminal 140-amino acid region. In this region, there was the β4GT motif, GWGEGD, which is highly conserved in the β4GT family except for β4GalNAc-T1 and -T2. Furthermore, a gene possessing a quite similar sequence to human β4GalNAc-T3 was found in mouse (82.2% identity in amino acid sequence) as shown in Fig. 3. This mouse gene is probably orthologous to human β4GalNAc-T3 and was named mβ4GalNAc-T3. It encoded a hypothetical 986-amino acid protein carrying four potential N-glycosylation sites, a DXH sequence, and a β4GT motif, the same as human β4GalNAc-T3.

Substrate Specificity of β4GalNAc-T3—We determined the substrate specificity of the truncated soluble β4GalNAc-T3 expressed in HER293 cells. By utilizing a variety of UDP donors and monosaccharide acceptors with α, βN or βB group, donor and acceptor substrates for β4GalNAc-T3 were screened with HPLC. As shown in Fig. 4, a peak (P) appeared at 28.4 min (Fig. 4B) in addition to the acceptor substrate peak (S) at 23.2 min (Fig. 4A and B) when UDP-GalNAc and GlcNAc-β-O-Bz were used as a donor and an acceptor substrate, respectively. The peak P in Fig. 4B was isolated by reversed phase chromatography and identified with MALDI-TOF MS. It gave three peaks of 515.442, 537.398, and 553.357 m/z as shown in Fig. 4C. These peaks were the same molecular mass as GalNAc-GlcNAc-α-Bz and GalNAc-GlcNAc-β-O-Bz with Na+ and with K+, respectively. On the contrary, no activity was observed with any other donor and monosaccharide acceptor combina-

Fig. 2. A phylogenetic tree of human β4GTs and their structures. The phylogenetic tree of human β4GTs was constructed with GENETYX based on the amino acid (aa) sequences (left). The branch length indicates the evolutionary distance. The structures of these enzymes are illustrated (right). The β4GT domains with and without a WGGED motif are shown as open and hatched boxes, respectively.
tion (Table II, acceptor numbers 1–13). β4GalNAc-T3 showed no activity toward chondroitin-related acceptors containing GlcUA at their non-reducing termini, although its amino acid sequence was similar to that of CSS1 (data not shown).

mβ4GalNAc-T3 exhibited the same substrate specificity as human β4GalNAc-T3 did (data not shown).

**Determination of β4GalNAc-T3 Product with 1H NMR**

- 1H NMR spectroscopy was performed to determine the newly formed glycosidic linkage of the β4GalNAc-T3 product. A one-dimensional 1H NMR spectrum of the β4GalNAc-T3 product is shown in Fig. 5. In the NMR spectra, signal integrals (not shown, 5 phenyl protons of Bz, 2 methylene protons of Bz, 2 anomeric protons, 12 sugar protons except anomeric protons, and 6 methyl protons of two N-acetyl groups) corresponded well with the structure of GalNAc-GlcNAc-O-Bz. As shown in Fig. 5 and in Table III, two anomeric protons revealed resonances at a very close magnetic field with a coupling constant (J1,2) larger than 8 Hz. This indicates that two pyranoses in the samples are in the β-glucopyranose configuration. All 1H signals could be assigned after high resolutional detection in COSY, TOCSY, and NOESY experiments. The anomeric resonance in the lower field showed NOE with two methylene protons of the benzyl group in the sample (not shown); on the other hand, the anomeric resonance in the higher field did not show NOE with...
methylenic protons (not shown). The results mean that the anomic resonance in the lower field is responsible for the anomeric proton of the substrate pyranose (β-GlcNAc, defined as A) and that the anomeric proton in the higher field corresponds to the anomeric proton of the transferred pyranose (β-GalNAc, defined as B). The chemical shifts and coupling constants of the sugar part of the sample are shown in Table III. The chemical shift and signal splitting of B-4 resonance was characteristic in the β-Gal configuration (43), and the order in the chemical shift of A1–A6 protons was characteristic similar to the observed spectrum of β-GlcNAc in LLnT (Galβ1→4GlcNAcβ1→3Galβ1→4Glc). As shown in Fig. 6, a weak NOE cross-peak between B1 and A4 and very weak NOE cross-peaks between B1 and two A6 were observed in addition to strong inner residual NOEs between B1 and B5 and between A1 and A5. These results suggest the existence of a β1→4 linkage between two pyranoses. Results in NMR experiments thus indicated clearly that the product of β4GalNAc-T3 is GalNAcβ1→4GalNAc-β-Bz.

Comparison of Acceptor Substrates—To investigate the specificity for acceptor substrates, N- and O-glycans containing GlcNAc at their non-reducing termini were utilized. As shown in Tables II and IV, a GalNAc residue could be transferred to all acceptor substrates examined, although the transfer efficiency differed. The most efficient acceptor in the O-glycan structures was core 6-pNP (GlcNAcβ1→6GalNAcα-pNP) which accepted GalNAc with a 2.2-fold higher efficiency than GlcNAcβ-Bz. However, core 2-pNP having similar non-reducing terminal structures with core 6-pNP showed less efficiency than the core 3-pNP. On the other hand, the most efficient acceptor substrate in the N-glycans examined was a non-fucosylated bi-antennary one (acceptor substrate number 1 in Table IV). The efficiency decreased as the number of antennae increased (data not shown). However, the presence of Fuc residues had little effect (number 1 and 2, 3 and 4, and 5 and 6) on the activity of β4GalNAc-T3. In contrast, a difference in transfer efficiency was observed between the two antennae, i.e. the GlcNAcβ1→2Manα1→3 antenna was preferable as an acceptor for β4GalNAc-T3 to the GlcNAcβ1→2Manα1→6 antenna (numbers 3 and 5 and 4 and 6).

**Quantitative Analysis of the β4GalNAc-T3 Transcripts in Human Tissues and Cell Lines by Real Time PCR**—We determined the tissue distribution and expression levels of the human β4GalNAc-T3 transcript by the real time PCR method. The expression levels of β4GalNAc-T3 in various tissues were shown relative to the GAPDH transcript (Fig. 7A). The transcript was highly expressed in stomach, colon, and testis, with relatively low levels found in other tissues. In the case of cell lines, the expression levels were comparatively low; however, the transcript was certainly expressed in SW1116 (colon cancer), KATO III (stomach cancer), HEK293, and some other cell lines (Fig. 7B).

**LacdiNAc Synthesizing Activity of β4GalNAc-T3 toward Asialo/Agalacto-FCF—**As demonstrated in Table IV, β4GalNAc-T3 transferred GalNAc to both O- and N-glycan substrates. The LacdiNAc structure has been found in N-glycans of some glycoproteins in humans. Therefore, to determine the activity of β4GalNAc-T3 to transfer GalNAc to a glycoprotein, FCF, which has both N- and O-glycans, was chosen as an acceptor substrate. As shown in Fig. 8, asialo/agalacto-FCF appeared as ~55- and 60-kDa bands (lane 1). β4GalNAc-T3 effectively transferred GalNAc to asialo/agalacto-FCF (lane 5). Furthermore, the band mostly disappeared on GPF treatment, and it was detected at approximately the 45- and 50-kDa positions by Coomassie staining (Fig. 8, lanes 3 and 6).
DISCUSSION

We found a novel human glycosyltransferase carrying the β4GT motif, WGGED, in its C-terminal region. From its similarity in amino acid sequence to CSS1 which transfers GalNAc to GlcA with a 1,4-linkage (10), we expected it to have 4GalNAc-T activity. As expected, it transferred GalNAc to GlcNAc and synthesized the LacdiNAc structure. Its ortholog was also found in mouse. Kawar et al. (23) has reported the cloning of a nematode LacdiNAc synthase, Ceβ4GalNAc-T, which is probably an ortholog of human β4Gal-T1. β4GalNAc-T in the present study is a novel glycosyltransferase, and this is the first report of the molecular cloning and characterization of mammal LacdiNAc synthases, β4GalNAc-T3 and mβ4GalNAc-T3.

By comparing β4GalNAc-T3 with Ceβ4GalNAc-T, the greatest difference is the length, i.e. β4GalNAc-T3 and Ceβ4GalNAc-T consist of 999 and 383 amino acids, respectively (Fig. 1). Furthermore, Ceβ4GalNAc-T has relatively strong homology (35.5% identity) with human β4Gal-T1 (23), and the two are probably orthologous. However, β4GalNAc-T3 was more similar to CSS1 than any other β4GTs (Fig. 2). Although the C-terminal sequence of β4GalNAc-T3 had a conserved β4GT motif, WGGED, the N-terminal sequence was quite unique. As shown in Fig. 3, no protein homologous with the N-terminal sequence of β4GalNAc-T3 was found in any databases by a BLAST search except for mβ4GalNAc-T3. In the middle region, the homology between β4GalNAc-T3 and mβ4GalNAc-T3 was comparatively low, although both enzymes contain many prolines and acidic amino acids, Glu and Asp, in this region (Fig. 3). Functions of the N-terminal region and the middle region, which compose the unique amino acid sequence, remain to be elucidated.

On screening with monosaccharide acceptors and UDP-sugar donors, both β4GalNAc-T3 and mβ4GalNAc-T3 showed GalNAc-T activity toward GlcNAc (Fig. 4). Although the amino acid sequence of β4GalNAc-T3 showed a high level of homology with that of CSS1, and no GalNAc-T activity toward GlcUA was observed. The GalNAc-GlcNAc structure has been found as LacdiNAc in humans, and this enzyme had a motif conserved in the β4GT family; therefore, we expected that it might be a β4GalNAc-T. The results of NMR spectrometry revealed that

| β4GalNAc-T3 product | GlcNAc | GalNAc |
|----------------------|-------|-------|
| 1H chemical shifts (ppm) |       |       |
| δ1  | 4.434 | 4.425 |
| δ2  | 3.647 | 3.831 |
| δ3  | 3.546 | 3.665 |
| δ4  | 3.534 | 3.846 |
| δ5  | 3.411 | 3.628 |
| δ6  | 3.589 | 3.696 |
| δδCH3 | 3.782 | 3.680 |
| Coupling constants (Hz) |       |       |
| Η1,2 | 8.5  | 8.4  |
| Η2,3 | 10.8 | <3.7 |
| Η4,5 | 5.6  | <3.7 |
| Η5,6a | 2.0  | 12.1 |
| Η5,6b | 2.0  | 12.1 |

*a The chemical shifts were set as the higher field signal of the benzyl methylene protons in ppm.
the product was GalNAcβ1–4GlcNAc, LacdiNAc, as expected (Figs. 5 and 6). β4GalNAc-T3 did not show any β3GT activity despite having homology with CSS1, which has both β4 and β3GT activities. However, this is not unexpected because the β3GT motif was not found in β4GalNAc-T3.

Ramakrishnan and Qasba (22) reported that the elimination of a hydrogen bond by mutating the Tyr-289 residue to Leu, Ile, or Asn in bovine β4Gal-T1 enhances the GalNAc-T activity in place of Gal-T activity. In fact, Ceβ4GalNAc-T possesses Ile-257 at the same position as Tyr-289 in bovine β4Gal-T1 (23). The primary sequence of β4GalNAc-T3 is not very homologous with that of bovine β4Gal-T1 or Ceβ4GalNAc-T; however, Asn-927, Leu-931, or Leu-932 might be an important residue for donor binding, judging from the distance between these amino acids and the conserved WGGED sequences.

We analyzed the acceptor preference of β4GalNAc-T3 for oligosaccharides that were derived from N- and O-glycans. The levels of efficiency in Tables II and IV are not comparable,

| Acceptor substrate | Relative activity (%) |
|--------------------|-----------------------|
| 1. GlcNAcβ1-2Manα1-5Manβ1-4GlcNAcβ1-4GlcNAc-PA | 100 |
| 2. GlcNAcβ1-2Manα1-5Manβ1-4GlcNAcβ1-4GlcNAc-PA | 87.1 |
| 3. Galβ1-4GlcNAcβ1-2Manα1-5Manβ1-4GlcNAcβ1-4GlcNAc-PA | 45.0 |
| 4. Galβ1-4GlcNAcβ1-2Manα1-5Manβ1-4GlcNAcβ1-4GlcNAc-PA | 51.7 |
| 5. GlcNAcβ1-2Manα1-5Manβ1-4GlcNAcβ1-4GlcNAc-PA | 21.6 |
| 6. GlcNAcβ1-2Manα1-5Manβ1-4GlcNAcβ1-4GlcNAc-PA | 5.0 |
because the concentrations of acceptor substrates are different. However, it was demonstrated that 4GalNAc-T3 could transfer GalNAc to any of the non-reducing terminal GlcNAc- in vitro. Among O-glycan-derived substrates, core 6-pNp was the most efficient acceptor. The core 6 structures and core 6 synthesizing activity have been found in ovarian cyst fluid, seminal fluid, and meconium (44–46). As shown in Fig. 7, the 4GalNAc-T3 gene was highly expressed in testis; therefore, there is some possibility that carrier proteins of 4GalNAc-T products on the core 6 structure exist in testis. Core 2 and core 3 structures have been found in stomach. 3Gn-T6, which synthesizes core 3, is highly expressed in stomach as reported in our previous paper (40). Although the GalNAcβ1–4core 3 and GalNAcβ1–4core 2 structures have not been found, these O-glycans would be candidates for acceptors of 4GalNAc-T3 in vivo. On the other hand, some human glycoproteins are known to contain the LacdiNAc structure in their N-glycans; therefore, these N-glycans are possible native substrates for 4GalNAc-T3 in vivo. 4GalNAc-T3 could transfer GalNAc residues to N-glycans in asialo/agalacto-FCF (Fig. 8). It is known that FCF is a glycoprotein containing both N- and O-glycans (47). FCF is a convenient acceptor for the screening of N- and O-glycosylation. However, FCF is probably not a physiological acceptor for 4GalNAc-T3, because there is no report that it has the LacdiNAc structure. The major sugar chains in FCF are O-glycans, NeuAc2–3Galβ1–3GalNAc and NeuAc2–3Galβ1–3(NeuAcα2–6)GalNAc, each of which accounts for ~30% of all glycans, with another 10% composed of NeuAc2–3Galβ1–4GlcNAcβ1–6(NeuAcα2–3Galβ1–3)GalNAc (48). FCF also possesses bi- and tri-antennary N-glycans. The results in Fig. 8 demonstrated that 4GalNAc-T3 efficiently transferred GalNAc to the N-glycan of asialo/agalacto-FCF because the strong signal probed with WFA that binds to GalNAc almost disappeared after the GPF treatment. The molecular size of FCF was reduced to ~45 and 50 kDa because of the loss of N-glycans. However, two bands having faint positive signals for

Fig. 7. Quantitative real time PCR analysis of the 4GalNAc-T3 transcript in human tissues and cell lines. Standard curves for 4GalNAc-T3 and GAPDH were generated by serial dilution of each plasmid DNA. The expression level of the 4GalNAc-T3 transcript was normalized to that of the GAPDH transcript, which was measured in the same cDNAs from human tissues (A) and cell lines (B). Data were obtained from triplicate experiments and are indicated as the mean ± S.D. PBMC, peripheral blood mononuclear cells; GOTO and SCCH-26, neuroblastomas; T98G and U251, glioblastomas; PC-7, lung adenocarcinoma; PC-1 and EBC-1, lung squamous cells; A431, esophageus cancer; MKN45, KATO III, and HSC43, stomach cancers; Colo205, HCT15, LSC, LSB, SW480, and SW1116, colorectal cancers; HepG2, hepatocarcinoma; Capan-2, pancreas cancer; SW1736, thyroid cancer; HL-60, promyelocytic leukemia; Namalwa, B cell lymphoma; Daudi, B cell (Burkitt’s) lymphoma; K562, erythroid leukemia; HEK293, embryonic kidney cell; HeLa and HeLa-S3, cervix cancers.
These glycoproteins are synthesized by β4GalNAc-T3 or not. Very recently, we have cloned another gene that has high homology with the gene for β4GalNAc-T3. There is a possibility that both enzymes have similar types of activity, sharing roles in various tissues and cells but differing in tissue distribution.

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