A Novel Human β1,3-N-Acetylgalactosaminytransferase That Synthesizes a Unique Carbohydrate Structure, GalNAcβ1–3GlcNAc

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We found, using a BLAST search, a novel human gene (GenBank™ accession number BC029564) that possesses β3-glycosyltransferase motifs. The full-length open reading frame consists of 500 amino acids and encodes a typical type II membrane protein. This enzyme has a domain containing β1,3-glycosyltransferase motifs, which are widely conserved in the β1,3-galactosyltransferase and β1,3-N-acetylgalactosaminyltransferase families. The putative catalytic domain was expressed in human embryonic kidney 293T cells as a soluble protein. Its N-acetylgalactosaminyltransferase activity was observed when N-acetylgalcosamine (GlcNAc) β1-O-benzyl was used as an acceptor substrate. The enzyme product was determined to have a β1,3-linkage by NMR spectrosocopic analysis, and was therefore named β1,3-N-acetylgalactosaminyltransferase-II (β3GalNAc-T2). The acceptor substrate specificity of β3GalNAc-T2 was examined using various oligosaccharide substrates. Galβ1–3(GlcNAcβ1–6)GalNAcα1-Para-nitrophenyl (core 2-pNP) was the best acceptor substrate for β3GalNAc-T2, followed by GlcNAcβ1–4GlcNAcβ1–O-benzyl, and GlcNAcβ1–3GalNAcα1-para-nitrophenyl (core 6-pNP), among the tested oligosaccharide substrates. Quantitative real time PCR analysis revealed that the β3GalNAc-T2 transcripts was restricted in its distribution mainly to the testis, adipose tissue, skeletal muscle, and ovary. Its putative orthologous gene, mβ3GalNAc-T2, was also found in a data base of mouse expressed sequence tags. in situ hybridization analysis with mouse testis showed that the transcripts are expressed in germ line cells. β3GalNAc-T2 efficiently transferred GalNAc to N-glycans of fetal calf fetuin, which was treated with neuraminidase and β-galactosidase. However, it showed no activity toward any glycolipid examined. Although the GalNAcβ1–3GlcNAcβ1–R structure has not been reported in humans or other mammals, we have discovered a novel human glycosyltransferase producing this structure on N- and O-glycans.

With the aid of bioinformatics technology, it is now possible to find candidate genes for glycosyltransferases that are distributed in relatively few tissues, are expressed at very low levels, or synthesize unknown structures. The β1,3-glycosyltransferase (β3GT) motifs, originally found in our previous study (1, 2), are conserved in the catalytic domain of β3GT family members. Three β3GT motifs, XIX(S/T)W(G/L/M), (F/Y)XXX(D/E)X, and (E/D)XXGXX, are commonly encoded in β3GTs that combine two sugars with a β1,3-linkage. To date, thirteen members of the β3GT family, i.e. six β1,3-galactosyltransferases (β3Gal-T) (1, 3, 4), six β1,3-N-acetylgalactosaminytransferases (β3Gn-T) (5, 6), and one β1,3-N-acetylgalactosaminytransferase (β3GalNAc-T) (7) have been cloned and characterized. β3Gal-T1, -T2, and -T5 transfer galactose (Gal) to the N-acetylgalcosamine (GlcNAc)-β-R residue from UDP-Gal, resulting in the synthesis of a type 1 chain Galβ1–3GlcNAc. β3Gal-T3 was originally considered to be a β3Gal-T; however, its function was found to be the transfer of N-acetylgalactosamime (GlcNAc) to the Gal-α-R residue of paragloboside with a β1,3-linkage and synthesis of globoside (7). β3Gal-T3 is not a Gal-T, so it was renamed β3GalNAc-T3 as a globoside synthase. β3Gal-T4, the GM1 synthase, efficiently transfers β3GT, β1,3-glycosyltransferase; (β3)GalNAc-T; (β1,3-N-acetylgalactosaminyltransferase); GlcNAc, N-acetylgalosamine; Bz, benzyl; pNP, para-nitrophenyl, oNP, ortho-nitrophenyl); (β3)Gal-T, (β1,3-galactosyltransferase); (β3Gn-T, (β1,3-N-acetylgalactosaminyltransferase); Gal, galactose; GalNAc, N-acetyl-galactosamine; Xy, xylose; MS, mass spectrometry; EST, expressed sequence tag; Lc, Cer, lactotriaosylceramide; HPLC, high performance liquid chromatography; MALDI-TOF, matrix-assisted laser desorption/ ionization time-of-flight; WFA, Wisteria floribunda agglutinin; FCF, fetal calf fetuin; HRP, horseradish peroxidase; NeuAc, neuraminic acid (sialic acid); free-mRNP, free-messenger ribonucleoprotein particles; CBB, Coomassie Brilliant Blue; ORF, open reading frame.

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†‡‡‡ The abbreviations used are: β3GT, β1,3-glycosyltransferase; (β3)GalNAc-T, (β1,3-N-acetylgalactosaminyltransferase); GlcNAc, N-acetylgalosamine; Bz, benzyl; pNP, para-nitrophenyl; oNP, ortho-nitrophenyl); (β3)Gal-T, (β1,3-galactosyltransferase); (β3Gn-T, (β1,3-N-acetylgalactosaminyltransferase); Gal, galactose; GalNAc, N-acetyl-galactosamine; Xy, xylose; MS, mass spectrometry; EST, expressed sequence tag; Lc, Cer, lactotriaosylceramide; HPLC, high performance liquid chromatography; MALDI-TOF, matrix-assisted laser desorption/ ionization time-of-flight; WFA, Wisteria floribunda agglutinin; FCF, fetal calf fetuin; HRP, horseradish peroxidase; NeuAc, neuraminic acid (sialic acid); free-mRNP, free-messenger ribonucleoprotein particles; CBB, Coomassie Brilliant Blue; ORF, open reading frame.

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Fig. 1. Nucleotide and amino acid sequences and genomic structure of β3GalNAc-T2. A, the putative transmembrane domain is underlined. Junctions between exons are shown with triangles. Possible N-glycosylation sites are indicated by open circles. A DXD motif is written in bold. B, genome structure of β3GalNAc-T2.
Gal to the GaINac-β-R residue of GM2 resulting in the synthesis of GM1 (8). β3Gal-T5 exhibits the strongest activity for Gal transfer to GlcNAc among β3Gal-Ts, and is restrictively expressed in colon, intestine, stomach, and pancreas (1). β3Gal-T5 is responsible for the expression of the sialyl Lewis a antigen epitopes, a famous tumor marker, in cancer cells derived from such tissues (9). β3Gal-T6 catalyzes a Galβ1-3Gal linkage and is responsible for the synthesis of GaINap1–3GaINap1–4 xylose (X5y)-(O-Ser/Thr), the core structure of proteoglycans (4).

iGn-T, cloned by the expression cloning method, can express polylactosamine on the cell surface (5). However, iGn-T is not included in the β3GT family because it does not possess the β3GT motif. Four β3Gn-Ts (T2, T3, T4, and T5) have activity for the transfer of GlcNAc to the Gal-β-R residue from UDP-GlcNAc. Transfection experiments with each β3Gn-T showed the expression of a polylactosamine chain on the cell surface (2, 10). β3Gn-T3 was reported to effectively transfer GlcNAc to Gal with a β1,3 linkage on core 1-O-glycan (10). β3Gn-T5 transfers GlcNAc to a Gal residue of lactoelyceramide with a β1,3-linkage and synthesizes lactotriaosylceramide (LeCer) of glycolipid (2). β3Gn-T6 is the core 3-synthesizing enzyme that transfers GlcNAc to GaINac1-O-Ser/Thr with a β1,3-linkage, the core 3 structure of O-glycan (6).

In this study, DNA databases were searched using the amino acid sequences of β3GT family members with particular attention being paid to the existence of a transmembrane domain and the conserved motifs of β3GTs. Thus, a new member of the β3GT family was found and cloned for characterization. The new enzyme was demonstrated to be active in synthesizing a unique carbohydrate structure, GaINacβ1–3GlcNAc, on both N-glycan and O-glycan. Carbohydrate structures determined to date are being accumulated in data bases such as GlycoSuite-edb (11, 12). However, not all carbohydrate structures have been determined. Although the GaINacβ1–3GlcNAc structure has not been found in mammals, there is a possibility that it exists in tissues where this new enzyme is expressed.

**EXPERIMENTAL PROCEDURES**

**Materials—**UDP-[14C]GalNAc was purchased from Amersham Biosciences (Amersham Place, UK) and UDP-[3H]GalNAc from ICN Bio- assay buffers.

**Construction and Purification of Human β3GalNAc-T2 Proteins Fused with FLAG Peptide—**We performed a BLAST search of the data base at NCBI and identified a cDNA (GenBank™ accession number BC029564), homologous in amino acid sequence to the open reading frame (ORF) of BC029564, which was amplified by 5'-AACGCGGATCCGCGCTGTTATCTTGCTTGACATCGA-3' and 5'-CCCAAGCTTGGGCCTGCAGATCAGTTGGCCTTATTCT3'. The putative catalytic domain of β3GalNAc-T2 was expressed as a secreted protein fused with a FLAG peptide in HEK293T cells (a human embryonic renal cancer cell line). A 12-ml volume of culture medium was mixed with anti-FLAG M1 antibody resin (Sigma). The protein-resin mixture was washed twice with 50 mM TBS (50 mM Tris-HCl, pH 7.4, and 150 mM NaCl) containing 1 mM CaCl2 and suspended in 200 μl of each of the assay buffers.

**Screening for Donor and Acceptor Substrates for β3GalNAc-T2—**To determine a donor and acceptor substrate for β3GalNAc-T2, all combinations of nucleotide sugars and monosaccharides were screened by a method described previously (13–15).

**N-Acetylgalactosaminyltransferase Assay—**The basic reaction mixture for assaying GaINAc-T activity contained 14 mM HEPES buffer, pH 7.4, an appropriate concentration of UDP-GalNAc, 10 mM MnCl2, 0.4% Triton CF-54, a suitable amount of acceptor substrate, and the purified enzyme. After incubation at 37 °C for 16 h, the product was analyzed by various techniques as described below.
The acceptor substrates used in this study are listed in Table I. After purchased from Clontech. cDNAs were synthesized using oligo(dT)12 detail previously (16, 17). Total RNA from various human tissues was transcripts, we employed the real time PCR method, as described in

![Fig. 3. Alignment of amino acid sequences of human βGalNAc-T2 and mouse βGalNAc-T2.](http://www.jbc.org/)

**Substrate Specificity of βGalNAc-T2—** A GalNAc-T assay of human βGalNAc-T2 using the synthetic oligosaccharide was performed as follows. 14C]UDP-GalNAc (50 nCi) and the oligosaccharides as the acceptor substrates were added to 10 μL of the basic reaction mixture. The cartridge was activated by washing with 1 ml of 100% methanol (Invitrogen) to construct pCR-TOPO-®-Blunt II-TOPO® (Invitrogen) to construct pCR-TOPO-mβGalNAc-T2. Adult mouse testis fixed in Bouin’s solution was embedded in paraffin and sectioned (7 μm) for in situ hybridization analyses. After linearization of pCR-TOPO-mβGalNAc-T2, sense and antisense digoxigenin-labeled RNA probes were generated using an RNA labeling kit (Roche Applied Science). Hybridization signals were detected with alkaline phosphatase-conjugated anti-digoxigenin antibody and NBT as the chromogen, as described previously (18).

**Detection of βGalNAc-T2 in Mouse Testis by Western blot using Polyclonal Antibody—** Recombinant βGalNAc-T2 enzymes, which were purified from the membrane of HEK293T cells transfected with the pFLAG-CMV3-mβGalNAc-T2 vector, were subjected to Western blotting as controls. Tissue homogenates of mouse testis and spleen were used as experimental samples. Each sample was subjected to 7.5% SDS-PAGE, transferred to a nitrocellulose membrane (Schleicher & Schuell), and treated with the polyclonal antibody against hβGalNAc-T2. The membrane was treated with anti-mouse IgG-HRP (Amersham Biosciences). The signals were detected using enhanced chemiluminescence and Hyperfilm ECL (Amersham Biosciences).

**N-Acetylgalactosaminyltransferase Assay with Glycoproteins**

![Molecular Cloning and Characterization of β-GalNAc-T2](http://www.jbc.org/)

**Alignment of amino acid sequences of human βGalNAc-T2 and mouse βGalNAc-T2.** Identical amino acids are indicated with asterisks. Junctions between exons are indicated with triangles.
TABLE I
Substrate specificity of β3GalNAc-T2
The apparent K values for UDP-GalNAc and GlcNAc-β-Bz for GalNAc-T activity were 5.4 μM (R2 = 0.978) and 11 μM (R2 = 0.959).

| Acceptor substrates | % |
|---------------------|---|
| GlcNAc-α-Bz         | ND* |
| GlcNAc-β-Bz         | 100 |
| Gal-α-2NP           | ND |
| Gal-β-2NP           | ND |
| GalNAc-α-Bz         | ND |
| GalNAc-β-2NP        | ND |
| Glc-α-2NP           | ND |
| Glc-β-2NP           | 2  |
| GlcA-β-2NP          | ND |
| Fuc-α-2NP           | ND |
| Xyl-α-2NP           | ND |
| Man-α-2NP           | ND |
| Lactoside-Bz        | ND |
| Lac-Cer             | ND |
| Gal-Cer             | ND |
| Paragloboside        | ND |
| Globoside           | ND |
| Galβ1-4GalNAc-α-2NP | ND |
| Galβ1-3GlcNAc-β-2NP | ND |
| GlcNAcβ1–4GlcNAc-β-Bz| 20 |
| Galβ1-3GlcNAc(core1)-2NP | ND |
| Galβ1-3GlcNAc(core1)-3NP | 185 |
| GlcNAcβ1–3GlcNAc(core3)-2NP | 8  |
| GlcNAcβ1–3GlcNAc(core6)-2NP | 19 |

*ND, not detected.

TCF was treated with β-galactosidase (Streptococcus 6646K, Seikagaku Corporation, Tokyo, Japan) and neuraminidase (Nakarai Tesque, Tokyo, Japan) in advance of the GalNAc-T assay. A 200-μg amount of each glycoprotein was incubated with β-galactosidase (5 microunits) and neuraminidase (50 microunits) in 50 mM sodium acetate buffer (pH 6.0) at 37 °C for 16 h. The reaction mixture was further incubated at 100 °C for 5 min to inactivate the glycosidases. A 10-μl volume of the glycosidase-treated samples was incubated with β3GalNAc-T2 in the basic reaction mixture containing 2.5 mM UDP-GalNAc (total volume, 20 μl). One microliter of the reaction products was subjected to 12.5% SDS-PAGE, transferred to a nitrocellulose membrane (Schleicher & Schuell, Keene, NH) and stained with 0.1% horseradish peroxidase (HRP)-conjugated Wisteria floribunda agglutinin (WFA) (Ev Laboratorys, San Mateo, CA) for detection of the transferred GalNAc residue. The signals were detected using enhanced chemiluminescence and Hyperfilm ECL (Amersham Biosciences). To remove N-glycans, an aliquot of the reaction product was digested with glycopeptidase F (TaKaRa, Ohtsu, Japan) at 37 °C for 16 h.

RESULTS

Determination of Nucleotide and Amino Acid Sequences of β3GalNAc-T2—We obtained a new sequence for the β3GT family as described under “Experimental Procedures” and named it β3GalNAc-T2. As shown in Fig. 1A, the ORF of β3GalNAc-T2 consisted of 1,500 bp encoding a predicted 500-amino acid protein with a typical type II topology, same as in the other β3GTs. It contained two N-glycosylation sites, a transmembrane segment of 19 residues, and a putative stem region and catalytic domain of 479 residues. The same sequence was found in a clone, GenBankTM accession number AL135928, which is located on chromosome 1. By comparison of the β3GalNAc-T2 cDNA sequence with the genome data base, the genomic structure of the β3GalNAc-T2 gene was determined (Fig. 1B). The β3GalNAc-T2 gene contains at least 12 exons. As shown in a phylogenetic tree (Fig. 2A), β3GalNAc-T2 was most homologous to β3Gal-T6 in the human β3GT family. The three β3GT motifs, one of which contained a DXD motif, were conserved between β3GalNAc-T2 and β3Gal-T6 (Fig. 2B). Partial sequences highly homologous to human β3GalNAc-T2 were found in the mouse EST data base. Based on the partial sequences in EST, we cloned a full-length ORF of this gene. It showed 88.4% identity in amino acid sequence to human β3GalNAc-T2 as shown in Fig. 3. This mouse gene product is probably an ortholog of human β3GalNAc-T2, and was named mβ3GalNAc-T2. The mβ3GalNAc-T2 gene also contained at least 12 exons, and the junctions between exons and introns are at the same positions in hβ3GalNAc-T2 and mβ3GalNAc-T2 (Fig. 3).

Determination of Glycosyltransferase Activity of β3GalNAc-T2—FLAG-tagged recombinant β3GalNAc-T2 was purified from the supernatants of HEK293T cells as described under “Experimental Procedures.” Its calculated molecular mass is 56.6 kDa; however, a major band was observed at around 60 kDa on Western blot analysis with an anti-FLAG antibody (data not shown). This result indicated that the recombinant protein is probably glycosylated in HEK293T cells. Its glycosyltransferase activities, Gal-T, Gn-T, and GalNAc-T activities, were screened using each donor labeled with 14C. No Gal-T or Gn-T activity toward any acceptor substrate was observed (data not shown), whereas GalNAc-T activity was exhibited toward GlcNAc-β-Bz (Table I). The recombinant protein showed faint activity toward Glc-β-2NP, and no activity toward GlcNAc-α-Bz (Table I).

On HPLC analysis as shown in Fig. 4, A and B, we observed a peak of an acceptor substrate (S) at 20.7 min and an additional peak (P) of the reaction product at 19.1 min when UDP-GalNAc and GlcNAc-β-Bz were used as a donor substrate and an acceptor substrate, respectively. The peak P was isolated by reversed-phase chromatography and identified using MALDI-TOF-MS (Fig. 4C). It gave two peaks of 554.154 and 558.194 m/z as shown in Fig. 4C. The two molecular masses, 554.154 and 558.194 m/z, exactly matched those of GalNAc-linked GlcNAc-Bz with Na" and K", respectively.

Determination of the Linkage of the β3GalNAc-T2 Product with 1H NMR—To determine the newly formed glycosidic linkage of the β3GalNAc-T2 product, 1H NMR spectroscopy was employed. Although there were negligible weak signals from contaminants in the 1H NMR spectrum (not shown), signal integrals of five aromatic protons of Bz, two methylene protons of Bz, two anomeric protons, twelve sugar protons except anomeric protons, and six methyl protons of two N-acetyl groups corresponded well with the structure of GalNAc-GlcNAc-β-Bz. All 1H signals could be assigned after high resolutional recordings of COSY, TOCSY, and NOEY spectra. The chemical shifts and coupling constants of the sugar component of the sample are shown in Table II. Two anomeric protons revealed signals with the same coupling constant, (J1,2), 8.4 Hz, as shown in Table II. This indicates that two pyranoses in the samples are in a β-glucopyranose configuration. The anomeric resonance at 4.398 ppm was relatively broad and showed a NOE cross peak with one methylene proton of the Bz group at 4.557 ppm (not shown). On the other hand, the anomeric resonance in the higher field did not show a NOE peak with any methylene protons. This indicates that the anomeric resonance at 4.398 ppm is responsible for the anomeric proton of the substrate pyranose (β-GlcNAc, defined as A), and that the anomeric proton at 4.381 ppm corresponds to the anomeric proton of the transferred pyranose (β-GalNAc, defined as B).

As shown in Fig. 5, there was a weak NOE cross-peak between B1 and A3 in addition to strong inner residual NOEs between B1 and B5 and between A1 and A5. These results suggest the existence of a β1–3 linkage between two pyranoses. Two substrate pyranose signals, A1 and A3, were observed as relatively broadened signals, but signal broadening of A4 was not observed (see 1D spectrum in Fig. 5). The signal broadening...
The product was identified by MALDI-TOF-MS (Fig. 4). HPLC analyses of the standards (upper panel, A) and the reaction mixture of β3GalNAc-T2 with GlcNAc-β-Bz as an acceptor (lower panel, B) were performed using an ODS-80Ts QA column. The acceptor substrate is indicated with S in panels A and B. The reaction product synthesized by the enzymatic reaction is indicated by P in panel B. The product was identified by MALDI-TOF-MS (C).

Table II

| β3GalNAc-T2 product | GlcNAc (A) | GalNAc (B) |
|---------------------|------------|------------|
| δ1                 | 4.398      | 4.381      |
| δ2                 | 3.687      | 3.711      |
| δ3                 | 3.599      | 3.655      |
| δ4                 | 3.435      | 3.811      |
| δ5                 | 3.358      | 3.562      |
| δ6                 | 3.681      | 3.845      |
| δ7                 | 3.844      | 3.698      |
| δ8                 | 1.828      | 1.892      |

The chemical shifts were set as the higher field signal of the benzyl methylene proton is tentatively 4.557 ppm.

The substrate specificity of β3GalNAc-T2 is summarized in Table I. β3GalNAc-T2 showed GalNAc-transfer activity toward all kinds of non-reducing terminal GlcNAcβ1-6GalNAc-O-Bz.

Results in NMR experiments thus indicated clearly two conformations of the molecule by the binding to the Bz group and between two conformations of the molecule by the binding to the mobility of these protons and by the slow equilibrium of the substrate residue was caused by the strong restriction of methylene proton is tentatively 4.557 ppm.

In Situ Hybridization of mβ3GalNAc-T2 in Mouse Testis—In situ hybridization showed germ cell-specific expression of the mβ3GalNAc-T2 gene in adult mouse testis. The mβ3GalNAc-T2 transcript was mainly detected in cells in middle layers of seminiferous tubules at stages XII to II (Fig. 7). As shown in a and c to l in Fig. 7, the metaphase II spermatocytes and the resulting early round spermatids gave strong signals for the mβ3GalNAc-T2 transcript, in contrast, testicular somatic cells such as Sertoli and Leydig cells gave no signal. As shown in b in Fig. 7, no signal was detected in any cell when a sense probe was used.
Detection of β3GalNAc-T2 in Mouse Testis by Western Blotting—As shown in Fig. 8, a band of ~55 kDa was detected in both recombinant hβ3GalNAc-T2 and mβ3GalNAc-T2, but not in the mock-transfectant. This size matched the expected size of the truncated recombinant enzymes. The polyclonal antibody raised against hβ3GalNAc-T2 cross-reacted to mβ3GalNAc-T2. A nonspecific band of ~52 kDa was detected. In mouse testis homogenates, a distinct band was detected at ~60 kDa, which is the molecular size of full-length mβ3GalNAc-T2 that has a transmembrane domain. On the other hand, mouse spleen, which expressed no transcripts of mβ3GalNAc-T2, did not show any positive band.

N-Acetylgalactosaminyltransferase Assay for Glycoproteins—As demonstrated in Table II, β3GalNAc-T2 transferred GalNAc to various kinds of non-reducing terminal GlcNAc. However, the GalNAcβ1–3GlcNAc structure has not been found in humans. To determine the acceptor preference of β3GalNAc-T2 for glycoproteins or glycolipids, FCF, which possesses both N- and O-glycans, and a mixture of glycolipids extracted from mouse testis and commercially available glycolipids were employed as a glycoprotein and glycolipid acceptor, respectively. β3GalNAc-T2 did not show any activity toward glycolipids (data not shown). As shown in Fig. 9, asialo/agalacto-FCF appeared as an ~55 kDa band (lane 3) on Coomassie Brilliant Blue (CBB) staining. β3GalNAc-T2 effectively transferred GalNAc to asialo/agalacto-FCF, as seen from the positive band obtained with WFA (lane 7). The band mostly disappeared on treatment with glycopeptidase F (lane 8), although a faint band obtained with WFA still remained at around 45 kDa (lane 8), which was the size of the glycopeptidase F-treated FCF stained with CBB (lane 4).

Fig. 5. Two-dimensional 1H-1H NMR spectrum of the β3GalNAc-T2 product. 1H-1H NMR spectrum of the same reaction product analyzed in Fig. 4 obtained in the 1H-1H NOESY NMR experiment.
DISCUSSION

With the aid of bioinformatics technology, we performed the cloning of novel human and mouse glycosyltransferases, \( \beta_3 \)GalNAc-T2 and m\( \beta_3 \)GalNAc-T2. All \( \beta_3 \)GTs, i.e. \( \beta_3 \)Gal-T, \( \beta_3 \)Gn-Ts, and \( \beta_3 \)GalNAc-T, share common amino acid motifs in three regions of the putative catalytic domain. Both \( \beta_3 \)GalNAc-T2 (GenBank\textsuperscript{TM} accession number BC029564) and m\( \beta_3 \)GalNAc-T2 (AB116655) are typical members of the \( \beta_3 \)GT family having three \( \beta_3 \)GT motifs, including a DXD motif, and showing the topology of type II membrane proteins. Interestingly, they showed \( \beta_3 \)GalNAc-T activity toward non-reducing terminal GlcNAc residues. Although the GalNAc1–3GlcNAc1 structure has not been reported in humans and other mammals, \( \beta_3 \)GalNAc-T2 was demonstrated to synthesize this unidentified structure on N- and O-glycans.

When we assayed for in vitro activity of \( \beta_3 \)GalNAc-T2 toward various oligosaccharides, we found core 2- \( \beta \)NP to be the best acceptor for \( \beta_3 \)GalNAc-T2, followed by core 6- \( \beta \)NP. FCF is not a physiological acceptor for \( \beta_4 \)GalNAc-T2 because there is no report that it has the GalNAc1–3GlcNAc structure. However, FCF is a convenient acceptor for the screening of N- and O-glycosylation (19–21). \( \beta_3 \)GalNAc-T2 could transfer GalNAc to the GlcNAc termini on both N-glycans and O-glycans in asialo/agalacto-FCF. It was reported that GalNAc\( \beta_1 \)–3GlcNAc\( \beta_1 \)–3Gal\( \beta_1 \)–3GlcNAc\( \beta_1 \)–4GlcNAc\( \beta_1 \)–3Man\( \beta_1 \)–4Glc-\( \beta_1 \)–1 ceramide is present in neutral glycosphingolipids of the green-bottle fly (22). Therefore, we examined the activity of \( \beta_3 \)GalNAc-T2 to transfer GalNAc to glycolipids as acceptors.

FIG. 6. Quantitative real-time PCR analysis of the \( \beta_3 \)GalNAc-T2 transcript in various human tissues. A standard curve for the \( \beta_3 \)GalNAc-T2 transcript was obtained by the serial dilution of plasmid DNA containing \( \beta_3 \)GalNAc-T2 cDNA. Each value represents the mean ± S.D. of triplicate experiments.

FIG. 7. In situ hybridization of mouse \( \beta_3 \)GalNAc-T2 in adult mouse testis. Sections of adult mouse testis were hybridized with the \( \beta_3 \)GalNAc-T2 antisense (a, c–I) and sense (b) probes. Positive signals are colored in brown to dark blue. The higher magnified images (c–I) are shown in the serial panels in order of spermatogenic stage, I to XII; stage I (c), II–III (d), V (e), VI (f), VIII (g), VIII (h), IX (i), X (j), XI (k) and XII (l). Scale bar in b for a and b; 100 \( \mu \)m, bar for magnified images in l; 20 \( \mu \)m.

FIG. 8. Western blotting with polyclonal antibody for h\( \beta_3 \)GalNAc-T2. Recombinant proteins purified with anti-FLAG M1 resin and mouse tissue homogenates were separated by SDS-PAGE. The membrane transblotted with the gel was probed with polyclonal antibody against h\( \beta_3 \)GalNAc-T2.
Molecular Cloning and Characterization of β3-GalNAc-T2

- **Fig. 9.** Assay for the β3GalNAc-T2 activity toward FCF. A slide of agalacto FCF incubated with β3GalNAc-T2 was separated by SDS-PAGE. The gel was stained with CBB in lanes 1, 2, 3, and 4. The membrane transblotted with the gel was probed with WFA lectin in lanes 5, 6, 7, and 8. FCF in lanes 1, 2, 3, 5, and 6 was not digested with neuraminidase and β-galactosidase, whereas FCF in lanes 3, 4, 7, and 8 was digested. The β3GalNAc-T2 products were digested with glycopeptidase F to remove N-glycans (lanes 2, 4, 6, and 8).

Neutral and acidic glycolipids extracted from mouse testis by us and commercially available glycolipids were employed as acceptor substrate(s), which is encoded by the glycan. Male mice lacking the peptidase F to remove N-glycans for the maturation of spermatogenic cells. We speculate that N- and O-glycans might be involved in such cellular interaction. Interestingly, the β3GalNAc-T2 transcripts were most highly expressed in germl cells of mouse testis in a stage-specific manner. The transcripts were strongly expressed in primary and secondary spermatocytes and early round spermatids, but not expressed in spermatogonias, elongating or elongated spermatids, or somatic cells, such as Leydig cells and Sertoli cells. We speculate that β3GalNAc-T2 may be involved in the maturation of sperm. It has been well established that spermatogenic cells show striking differences in patterns of translation from somatic cells (23). As one of these differences, the translation of some mRNA species does not necessarily occur even though the transcripts are expressed in the germ cells. Such mRNAs are mostly transcribed in undifferentiated spermatocytes, such as primary and secondary spermatocytes, and immature round spermatids (23). However, it was confirmed that the β3GalNAc-T2 transcripts are translated in mouse testis by the Western blotting analysis in Fig. 8. The distribution of the enzyme in each tissue will be examined in detail after the establishment of a monoclonal antibody.

Although dramatic changes to carbohydrate structures during the development of testicular cells were suggested by histological analysis using a series of lectins (24), it is very difficult to determine the precise structure of such carbohydrates. In order to detect whether the GalNAcβ1–3GlcNAc structure exists or not, a tool is needed for probing such a structure. We will try to establish an antibody to detect the GalNAcβ1–3GlcNAc structure. In addition, it will be important to find physiological acceptor substrate(s), i.e. glycoprotein(s), for β3GalNAc-T2, which should exist in germ cells of testis.

To date, N-glycans on glycoproteins have been reported to participate in various physiological functions, such as immune reactions, cell adhesion, cell migration and fertilization, etc. (25–28). Recently, Akama et al. (29) reported the importance of N-glycans for the maturation of spermatogenic cells. α-Mannosidase IIX, which is encoded by the MX gene, is an enzyme essential for formation of the intermediate structure of N-glycans. Male mice lacking the MX gene, which is predominantly expressed in male germ cells, exhibited an almost complete loss of fertility because of impairment of the interaction between germ cells and Sertoli cells. Thus, it was demonstrated that incomplete N-glycan synthesis gives rise to a disturbance of cellular interaction during spermatogenic cell maturation. However, the complete N-glycan structure required for cellular interaction has not yet been defined. The GalNAcβ1–3GlcNAc structure at the termini of N- and O-glycans might be involved in such cellular interaction.

In summary, we found two novel members of the β3GT family, β3GalNAc-T2 and mβ3GalNAc-T2, which synthesize an unidentified carbohydrate structure. Cloning technology with the aid of bioinformatics as described in this study has the potential to discover novel glycosyltransferases, although in this report the products were not identified.

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A Novel Human β1,3-N-Acetylgalactosaminyltransferase That Synthesizes a Unique Carbohydrate Structure, GalNAc β1-3GlcNAc
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