Identification and Characterization of Three Novel β1,3-N-Acetylglucosaminyltransferases Structurally Related to the β1,3-Galactosyltransferase Family*

Norihiko Shiraishi‡‡, Ayumi Natsume‡‡, Akira Togayachi‖, Tetsuo Endo‡, Tomohiro Akashima***, Yoji Yamada‡, Nobuyuki Imai‡, Satoshi Nakagawa‡, Satoshi Koizumi‡, Susumu Sekine‡, Hisashi Narimatsu‡, and Katsutoshi Sasaki‡‡

From the ‡Tokyo Research Laboratories, Kyowa Hakko Kogyo Company, Limited, 3-6-6 Asahi-machi, Machida-shi, Tokyo 194-8533, the Division of Cell Biology, Institute of Life Science, Soka University, 1-236 Tango-cho, Hachioji, Tokyo 192-8577, the Laboratory of Cancer Biology and Molecular Immunology, Graduate School of Pharmaceutical Sciences, University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113, and the **Laboratory of Animal Resources, Faculty of Bioindustry, Tokyo University of Agriculture, 196 Aza-Yasaka, Abashiri-shi, Hokkaido 099-2422, Japan

We have isolated three types of cDNAs encoding novel β1,3-N-acetylglucosaminyltransferases (designated β3Gn-T2, -T3, and -T4) from human gastric mucosa and the neuroblastoma cell line SK-N-MC. These enzymes are predicted to be type 2 transmembrane proteins of 397, 372, and 378 amino acids, respectively. They share motifs conserved among members of the β1,3-galactosyltransferase family and a β1,3-N-acetylglucosaminyltransferase (designated β3Gn-T1), but show no structural similarity to another type of β1,3-N-acetylglucosaminyltransferase (iGnT). Each of the enzymes expressed by insect cells as a secreted protein fused to the FLAG peptide showed β1,3-N-acetylglucosaminyltransferase activity for type 2 oligosaccharides but not β1,3-galactosyltransferase activity. These enzymes exhibited different substrate specificity. Transfection of Na-

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†† These authors contributed equally to this work and should be considered as first authors.

‡‡ To whom correspondence should be addressed. Tel.: 81-427-25-2555; Fax: 81-427-26-8330; E-mail: k SASAKI@KYOWA.co.jp.

The nucleotide sequences reported in this paper have been submitted to the DDBJ/GenBank™/EBI Data Bank with accession numbers AB049584 (β3Gn-T2), AB049585 (β3Gn-T3), and AB049586 (β3Gn-T4).

‡ These authors contributed equally to this work and should be considered as first authors.

The abbreviations used are: β3Gn-Ts, UDP-N-acetylglucosaminyltransferase β1,3-galactosyltransferase; EST, expressed sequence tag; GNA, Galβ1-3GalNAcβ1-4(NeuAcα2-3)Galβ1-4Glc-Cer; GM3, GalNAcβ1-4(NeuAcα2-3)Galβ1-4Glc-Cer; GM1, Galβ1-3GalNAcβ1-4(NeuAcα2-3)Galβ1-4Glc-Cer; PCR, polymerase chain reaction; β3Gn-T, UDP-GlcNAcβ:β-galactose β1,3-N-acetylglucosaminyltransferase; bp, base pairs; kb, kilobase pairs; LEA, L. esculentum agglutinin; PWM, pokeweed mitogen; MOPS, 4-morpholinepropanesulfonic acid; LNT, lacto-N-tetraosyl; LNPH, p-lacto-N-neohexaose; LNT, lacto-N-tetraosyl; LNFP, lacto-N-fucosylpentasose; HPLC, high performance liquid chromatography; RT-PCR, reverse transcription-polymerase chain reaction.
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signifcantly overall similarity to β3Gal-Ts (15–19%) and shares motifs conserved among the β3Gal-Ts, but is structurally distinct from another type of β1,3-N-acetylglucosaminyltransferase (iGnT) that was isolated by expression cloning using an anti-i-antigen antibody (8). β3Gn-T1 exhibits β1,3-N-acetylglucosaminyltransferase activity instead of β3,4-galactosyltransferase activity. This result provides an exception that a glycosyltransferase structurally related to the β3Gal-T family uses distinct donor (GlNAc versus Gal) and acceptor (Gal versus GlcNAc) substrates, maintaining the same linkage specificity (β1,3-linkage).

During the course of study to isolate β3Gal-T1 homologs, we have identified three additional types of putative members of the β3Gal-T family. In this study, we show additional examples that glycosyltransferases structurally related to the β3Gal-T family exhibit β1,3-N-acetylglucosaminyltransferase activity, but not β3,4-galactosyltransferase activity. These results indicate that β1,3-N-acetylglucosaminyltransferases (β3Gn-Ts) form a family having structural similarity to the β3Gal-T family. Alignment of primary sequences of all members of the β3Gn-T and β3Gal-T families revealed that the members are clustered into four subgroups, probably reflecting enzymatic diversity and substrate specificity. Transfection experiments and in vitro enzymatic analysis have demonstrated that β3Gn-T2, -T3, and -T4 are able to catalyze the initiation and elongation of poly-N-acetyllactosamine sugar chains; however, they exhibit different substrate specificity. These results, taken together with the different distributions of these enzymes, indicate that β3Gn-T2, -T3, and -T4 each exert distinct roles in physiological and pathological processes.

**ExPERIMENTAL PROCEDURES**

**Nomenclature of β1,3-Galactosyltransferases and β1,3-N-Acetylglucosaminyltransferases**—To simplify discussion, five members of the cloned human β3Gal-Ts will be called β3Gn-T1, -T2, -T3, -T4, and -T5 according to the designation of Kolbinger et al. (2), Clausen and coworkers (3), and Narimatsu and co-workers (4). Five types of β3Gn-Ts cloned to date will be referred to tentatively as follows: A β3Gn-T and newly isolated β3Gn-Ts in this study, which show structural similarity to the β3Gal-T family, will be called β3Gn-T1, -T2, -T3, and -T4. Another type of β3Gn-T, which was isolated by expression cloning using anti-i-antigen antibody (8) and showed no structural similarity to the β3Gal-T family, will be called iGnT according to Fukuda et al. (8).

**Cell Lines**—Natalwa KJM-1 cells, a subline of the human Burkitt lymphoma cell line Namalwa, was cultivated in serum-free RPMI 1640 medium as described (9, 10). Cell lines SK-N-MC and Colo205 were obtained from the American Type Culture Collection. These cell lines were cultivated in RPMI 1640 medium containing 10% fetal calf serum. SF9 and Sf21 insect cells were cultured at 27 °C in TNM-FH insect medium (Pharmingen) as described previously (11).

**Preparation of cDNA Libraries and Single Strand cDNAs**—cDNA libraries of human gastric mucosa and human placenta were constructed as described previously (12). Single strand cDNAs were synthesized from total RNA prepared from the neuroblastoma cell line SK-N-MC by PCR using primers 5′-CCAGGACACTGACCTCTCGCT-3′ and 5′-AGGATCAATTTGGGATACCCAGATG-3′ and was inserted into the pT7Blue-T vector to make pT7β3Gn-T1, -T2.

**DNA Sequencing**—DNA sequences were determined by the dideoxynucleotide chain termination method using an ABI PRISM™ 377 DNA sequencer (Applied Biosystems, Inc.).

**Construction of Plasmids for Expressing β3Gn-T2, -T3, and -T4 in Animal Cells**—A β3Gn T2 cDNA fragment (prepared from pBS-β3Gn T2 by SfiI and XbaI digestion, followed by linking and addition of the SfiI linker (5′-CTTTAGAGCCA-3′ and 5′-CTCTAAAG-3′)) was inserted between the SfiI sites of pAMo to yield pAMo-β3Gn T2. A β3Gn T3 cDNA fragment prepared from pBS-β3Gn T3 by HindIII and NotI digestion was inserted between the HindIII and NotI sites of pAMo to yield pAMo-β3Gn T3. A β3Gn T4 cDNA fragment (prepared from pT7β3Gn T4 by Smal and HindI digestion, followed by addition of the SfiI linker) was inserted between the SfiI sites of pAMo to yield pAMo-β3Gn T4.

Expression of β3Gn-T2, -T3, and -T4 in Natalwa KJM-1 Cells—Namalwa KJM-1 cells were transfected with pAMo-β3Gn T2, pAMo-β3Gn T3, or pAMo-β3Gn T4 by electroporation as described (9, 10) and grown for 24 h. Stably transfected cells were selected by cultivation for >14 days in the presence of G418 (0.5 mg/ml).

**Flow Cytometric Analysis**—Transfected Namalwa KJM-1 cells (5 × 10⁶ cells) were incubated in 100 μl of phosphate-buffered saline for 60 min at 37 °C in the presence or absence of 20 milliunits of Clostridium perfringens neuraminidase (N2133, Sigma). These cells were stained with human anti-i-antigen serum (Den) (13), followed by fluorescein isothiocyanate-conjugated goat anti-human IgM, and were analyzed on a FACS.calibur apparatus (Becton Dickinson) as described (8). For lectin staining, cells were stained with 10 μg/ml fluorescein isothiocyanate-labeled Lycopersicon esculentum pokeweed mitogen (LEA) or agglutinin (PWM; both from EY Laboratories).

**Construction and Purification of β3Gn-T Proteins** Fused to the FLAG Peptide—The putative catalytic domain of each β3Gn-T2, -T3, and -T4 was expressed as a secreted protein fused to the FLAG peptide in insect cells. A 1.1 kb DNA fragment encoding a COOH-terminal portion of β3Gn-T2 (amino acids 31–397) was amplified by PCR using primers 5′-CCGAGGTCAGGGCGCCAGGAAAGTTGGAAAAAGGGA-3′ and 5′-ATCGGATACGCGGCTTACGTTAATTTGCGACTCCGTGAC-3′, digested with BamHI and NotI, and inserted between the BamHI and NotI sites of pVL1393-F2 to yield pVL1393-F2G2. pVL1393-F2 is an expression vector derived from pVL1393 (Pharmingen) and contains a fragment encoding the signal peptide of human immunoglobulin κ (MHFQVQIFSFLLISASVIMSRG) and the FLAG peptide (DYKD- DDK). Joining in-frame a cDNA fragment with a unique BamHI site of pVL1393-F2 just downstream of the COOH terminus of the FLAG peptide enables the cDNA product to be secreted as a protein fused to the FLAG peptide. A 1.9 kb DNA fragment encoding a COOH-terminal portion of β3Gn-T3 (amino acids 38–372) was amplified by PCR using primers 5′-CCGAGGATCCTCCCCAGGCTTCTGGACAGC-3′ and 5′-GATAGGTGGAGGCGCCGAGAGGCGTACACCGCCTG-3′, digested with BamHI and NotI, and inserted between the BamHI and NotI sites of pVL1393-F2 to yield pVL1393-F2G3. A 0.9 kb DNA fragment encoding a COOH-terminal portion of β3Gn-T4 (amino acids 56–378) was amplified by PCR using primers 5′-ATAAGATCTGGACAGGACCCCA- CGGCCCCAC-3′ and 5′-ATAAGATCTGGACAGGACCCCA- CGGCCCCAC-3′.
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CCAACCCAC-3', digested with BglII and NotI, inserted between the BamHI and NotI sites of pVL1393-F2 to yield pVL1393-F2G4. The PCR-amplified portions of pVL1393-F2G2, pVL1393-F2G3, and pVL1393-F2G4 were sequenced to confirm the absence of possible PCR errors.

Sf9 insect cells were cotransfected with BaculoGold viral DNA (Pharmeningen) according to the manufacturer's instruction and each of plasmids pVL1393-F2G2, pVL1393-F2G3, and pVL1393-F2G4 and were incubated for 3 days at 27 °C to produce individual recombinant viruses. These viruses were amplified three times to reach titers of ~10^9 plaque-forming units/ml. Sf21 insect cells (4 x 10^7 cells; Pharmingen) were infected in a multiplicity of 1.0. TMN-FH insect medium at 27 °C for 72 h to yield conditioned medium including recombinant β3Gn-T proteins fused to the FLAG peptide, which were readily purified by anti-FLAG M1 antibody resin (Sigma) according to the protocol of the manufacturer. Briefly, the culture medium (30 ml) was collected by centrifugation and added to NaCl (150 mM final concentration), Na2SO4 (0.1% final concentration), and M1 antibody resin (30 ml) to absorb the recombinant β3Gn-T proteins on the resin. The resin was recovered by centrifugation and washed three times with buffer (1 ml) consisting of 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, and 1 mM CaCl2. The recombinant β3Gn-T proteins were eluted with buffer (90 μl) consisting of 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, and 2 mM EDTA, followed by the addition of CaCl2 (4 mM final concentration) and stored at 4 °C until use. The amount of the purified protein was not enough for accurate quantification.

Silver Staining and Western Blot Analysis—The enzymes purified above (3 μl) were subjected to SDS-polyacrylamide gel electrophoresis, followed by silver staining or Western blot analysis. Silver staining was performed using a silver staining kit (Wako Bioproducts). Proteins separated on 6% SDS-polyacrylamide gel were transferred to an polyvinylidene difluoride membrane (Immobilon, Millipore Corp.) in a Trans-Blot SD cell (Bio-Rad). The membrane was blocked with phosphate-buffered saline containing 5% skim milk at 4 °C overnight and then incubated with 10 μg/ml M2 antibody (Sigma). The membrane was stained with ECL Western blot detection reagents (Amersham Pharmacia Biotech) according to the manufacturer's instructions.

Glycosyltransferase Assays and Product Characterization—The N-acetylgalosaminyltransferase activities of the purified proteins (15 μl) were assayed in 50 mM MOPS (pH 7.5), 5 mM MgCl2, 5 mM UDP-GlcNAc, and 10 mM unabeled acceptors (a total volume of 40 μl). The following oligosaccharides were used as acceptors: lactose (Galβ1–4Glc), N-acetylactosamine (Galβ1–4GlcNAc), lacto-N-neotetraose (LNT; Galβ1–4GlcNAcβ1–3Galβ1–4Glc), lacto-N-trifucosylactotetraose (LTF; Galβ1–4GlcNAcβ1–3Galβ1–4(Fucα1–3)Glc), lacto-N-fucosyltetraose II (Galβ1–4GlcNAcβ1–3Galβ1–4(Fucα1–3)Glc), and lacto-N-difucoxyhexaose II (Galβ1–4GlcNAcβ1–3Galβ1–4(Fucα1–3)Glc). After incubation at 37 °C for 3 h in the presence or absence of UDP-GlcNAc to identify products and to check hydrolysis of substrate and product. The oligosaccharides were purchased from Oxford Glycosystems and pyridylaminated according to the method of Kondo et al. (15). The amounts of products were determined from their fluorescence intensities using pyridylaminated lactose as a standard.

The reaction product derived from pyridylaminated LNF was identified by comparison of the retention time on HPLC with that of the pyridylaminated standard oligosaccharide Galβ1–3Galβ1–4GlcNAcβ1–3Galβ1–4Glc, which was prepared from pyridylaminated p-LHNH-N by digestion with jack bean β-galactosidase. To further confirm the structure of the reaction product, the reaction product was modified by β1,4-galactosyltransferase to examine whether p-LHNH-N was produced or not. The reaction mixtures (20 μl) was incubated with 200 μl of bovine milk β1,4-galactosyltransferase in the presence or absence of UDP-Gal (20 mM) in a total volume of 30 μl at 37 °C for 15 h according to manufacturer's recommendations. The product further modified by β1,4-galactosyltransferase comigrated with pyridylaminated p-LHNH-N on HPLC. These results indicated that the product was pyridylaminated Galβ1–3Galβ1–4GlcNAcβ1–3Galβ1–4Glc. The galactosyltransferase activities of the purified proteins (15 μl) were assayed using pyridylaminated oligosaccharides (Galβ1–3Galβ1–4Glc and LNT) as substrates as described previously (4).

Preparation and Fractionation of Blood Leukocytes—Human polymorphonuclear leukocytes, monocyte-enriched population, and lymphocyte-enriched population were obtained as described previously (10).

Quantitative Analysis of the Three β3Gn-T Transcripts in Human Tumor Cell Lines and Human Tissues by Competitive RT-PCR—The levels of the β3Gn-T2, -T3, and -T4 transcripts were measured by competitive RT-PCR as described in detail previously (4, 16). Competitive RT-PCR was performed with AmpliTaq Gold™ pre-amplification system for first strand cDNA synthesis (Life Technologies, Inc.) according to manufacturer's instructions. After cDNA synthesis, the reaction mixture was diluted 50-fold with H2O and then stored at −80 °C until use. Competitive RT-PCR was performed with AmpliTaq Gold™ (PerkinElmer Life Sciences). The annealing temperatures and specific primers used are listed in Table II. The amount of each of the β3Gn-T transcripts was determined as the ratio of the amount of β-actin transcripts (4, 16).

Determination of Chromosomal Localization—The chromosomal localizations of the β3Gn-T2, -T3, and -T4 genes were determined by PCR analysis using a series of genomic DNAs from hamster-human somatic hybrids (BIO-SMAPP™ Somatic Cell Hybrid PCRable™ DNAs, BIOS Laboratories)
and specific primers (5'-TTACGGACACTTACGTTGCAAGG-3' and 5'-ATACCCCTCTTCGCTGCGTGGGTGGAG-3'). The predicted fragment of 495 bp was amplified only when genomic DNA from a hybrid containing human chromosome 2 (hybrid 852) was used, indicating that this gene is located on chromosome 2.

RESULTS

Identification and Isolation of β3Gn-T2, -T3, and -T4—A homology search in the EST division of the GenBankTM/EBI Data Bank using the FrameSearch algorithm revealed the existence of six types of cDNAs encoding proteins with low but significant similarity to β3Gal-T1, three of which have been reported recently to be β3Gal-T2, -T3, and -T4 (2, 3). Based on the nucleotide sequence of the ESTs shown in Table I, we prepared specific probes and isolated three types of full-length cDNAs encoding novel proteins (designated β3Gn-T2, -T3, and -T4) of 397, 372, and 378 amino acid residues, respectively, with a significant amount of 11 residues, a transmembrane segment of 19 residues, and a stem region and catalytic domains of 29 and 4 residues, a transmembrane segment of 21 residues, and a stem region and catalytic domain of 340 residues. The predicted coding region of β3Gn-T4 has two potential initiation codons, both of which are in agreement with Kozak's rule (18). Therefore, it is predicted that β3Gn-T4 is composed of two different N-terminal cytoplasmic domains of 29 and 4 residues, a transmembrane segment of 20 residues, and 329 residues containing the stem region and catalytic domain (Fig. 1A).

Fig. 1A shows a multiple alignment of the amino acid sequences of β3Gn-T2, -T3, and -T4 as well as β3Gn-T1 and five members of the β3Gal-T family. β3Gn-T2, -T3, and -T4 show 19–24, 22–26, and 22–25% identities, respectively, to the β3Gal-T family (β3Gal-T1, -T2, -T3, and -T5), whereas they show 15, 18, and 15% identities to β3Gn-T1, β3Gn-T2, -T3, and -T4 show 40–45% identity one another. The sequence similarities are limited to the putative catalytic regions. Several sequence motifs conserved in the β3Gal-T family are also shared by β3Gn-T2, -T3, and -T4 as well as β3Gn-T1. Twenty-five amino acid residues located separately in the putative catalytic regions are identical among all the proteins. Three cysteine residues conserved in all members of the β3Gal-T family are also maintained in β3Gn-T2, -T3, and -T4, whereas two of these are not conserved in β3Gn-T1 (Fig. 1A, white arrows), indicating that β3Gn-T1 is relatively distinct from other members, especially in the context of the three-dimensional structure. There are five potential N-linked glycosylation sites in β3Gn-T2, three in β3Gn-T3, and three in β3Gal-T4. One site in a highly conserved motif is maintained among all the proteins (Fig. 1A, black arrow). The phylogenetic tree of these proteins generated using the amino acid sequences of the putative catalytic domains demonstrates that β3Gn-T2, -T3, and -T4 form a subgroup, indicating that they have similar enzymatic activity (Fig. 1B).

Production of Secreted Recombinant Proteins Fused to the FLAG Peptide—To examine the enzymatic activities of β3Gn-T2, -T3, and -T4, we expressed the putative catalytic domain of each enzyme (amino acids 31–397 of β3Gn-T2, amino acids 38–372 of β3Gn-T3, and amino acids 56–378 of β3Gn-T4) as a secreted protein fused to the FLAG peptide in SF21 insect cells. The FLAG-fused recombinant proteins were partially purified using anti-FLAG M1 antibody resin and analyzed by SDS-polyacrylamide gel electrophoresis, followed by silver staining (Fig. 2A) or Western blotting using anti-FLAG monoclonal antibody (Fig. 2B). Two major bands with apparent molecular masses of 45.5 and 48 kDa, broad bands of 42–45 kDa, and two major bands of 37.6 and 40 kDa were observed specifically for β3Gn-T2, -T3, and -T4, respectively. The FLAG-fused recombinant proteins for β3Gn-T2, -T3, and -T4 have predicted molecular masses of 43,674, 39,507, and 37,608 Da for the respective polypeptides, indicating glycosylation of the recombinant proteins produced by insect cells.

β3Gn-T2, -T3, and -T4 Are β1,3-N-Acetylgalcosaminyltransferases—The glycosyltransferase activities of the partially purified FLAG-fused recombinant proteins were examined. When lactose was used as an acceptor, β3Gn-T2, -T3, and -T4 showed a significant amount of N-acetylgalcosaminyltransferase activity, whereas no activity was detected in a sample prepared from the conditioned medium of insect cells infected with empty vector virus. The structure of the product was estimated to be GlcNAcβ1–3Galβ1–4Glc by comparing the retention time on HPLC with that of the standard oligosaccharide (Fig. 3A). To further confirm the structure of the product, it was digested with endo-β-galactosidase or modified by β1,4-galactosyltransferase. Digestion of the product by E. freundii endo-β-galactosidase yielded two peaks comigrating with the standard oligosaccharides GlcNAcβ1–3Gal and glucose at a 1:1 molar ratio (Fig. 3, compare A and B). Modification of the product by bovine milk β1,4-galactosyltransferase yielded a peak comigrating with LNT (Fig. 3, compare A and C). These results clearly indicated that the product was GlcNAcβ1–3Galβ1–4Glc.

On the other hand, β3Gn-T2, -T3, and -T4 showed no β1,3-galactosyltransferase activity for GlcNAcβ1–3Galβ1–4Glc or LNT. Taken together, β3Gn-T2, -T3, and -T4 were demonstrated to be novel β1,3-N-acetylgalcosaminyltransferases.

Substrate Specificity of β3Gn-T2, -T3, and -T4—Analysis of the substrate specificity of β3Gn-T2, -T3, and -T4 revealed that these enzymes utilized common oligosaccharides as substrates, but the substrate preference was significantly different (Tables III and IV). β3Gn-T2 and -T4 showed more preferential activity for LNT than for LNT, which is consistent with the nature of

| Target gene | Primer sets | Sizes of PCR products | Restriction enzymes for competitor DNA | Annealing temperature |
|-------------|-------------|-----------------------|----------------------------------------|----------------------|
| β3Gn-T2     | Forward: 5'-CTATTACCAAGTGGATCTGACGA-3', Reverse: 5'-GGAAGATGTCGCGTTCATCTGCA-3' | 646 bp | EcoRI, PstI | 55 °C |
| β3Gn-T3     | Forward: 5'-GTGCCATGCGACCTCCTTATGG-3', Reverse: 5'-CCCTGCAGGTAGAAGACCATGTTG-3' | 619 bp | BstEII, PmlI | 65 °C |
| β3Gn-T4     | Forward: 5'-GTCTTCTCTTGCACCATGCTCAG-3', Reverse: 5'-AGTTCGATCTTCCATGATAGCC-3' | 399 bp | Tbm1111 NarI | 65 °C |

**Table II**

Oligonucleotide primers and conditions used for competitive RT-PCR analysis
β3Gn-T1 and iGnT (7, 8). In contrast, β3Gn-T3 utilized LNT as a substrate comparable to LNnT. In common, fucosylation at the penultimate GlcNAc residue in LNnT or LNT yielded poor substrates (LNFP-III, LNFP-II, and lacto-N-difucoylohexaose II). LNFP-V, which is an LNT derivative fucosylated at the reducing terminal glucose residue, was a relatively good substrate for β3Gn-T2 compared with LNT.

β3Gn-T2 transferred GlcNAc efficiently to both lactose and p-LNnH, as well as LNnT, whereas the relative activity N-acetyllactosamine was 21% compared with that of LNnT. β3Gn-T3 preferred lactose (235% relative activity) as a substrate, followed by LNnT (100%) and p-LNnH (45%), whereas it showed no activity for N-acetyllactosamine. β3Gn-T4 showed 33, 9, and 0% relative activities for lactose, N-acetyllac-
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The exo-β-N-acetylβ-d-galactosaminidase activity of the purified β3Gn-T2 protein was assayed using lactose (Galβ1-4Glc) as an acceptor. The reaction mixture was analyzed using high-pH anion-exchange chromatography with pulsed amperometric detection. The peaks for substrate lactose and the generated product are labeled S and P, respectively. Arrow 1 indicates the elution position of the standard oligosaccharide GlcNAcβ1-3Galβ1-4Glc. Based on the elution position, the peak indicated by the asterisk seems to be Galβ1-4N-acetylgalactosamine (Galβ1-4GlcNAc). Arrow 2 indicates the elution position of the standard oligosaccharide LNnT (Galβ1-4GlcNAcβ1-3Galβ1-4Glc). Based on the elution position, the peak indicated by the double asterisk seems to be Galβ1-4GlcNAc, which would be a galactosylation product of GlcNAc indicated by the asterisk in A.

DISCUSSION

In this study, we identified three novel β1,3-N-acetylglucosaminyltransferases (β3Gn-T2, -T3, and -T4) that show structural similarity to β3Gn-T1 as well as the β3Gal-T family, including five members (β3Gal-T1, -T2, -T3, -T4, and T5), demonstrating the existence of a β3Gn-T family now consisting of four members (β3Gn-T1, -T2, -T3, and -T4). The existence of the multiple enzymes showing similar activity is a common feature of glycosyltransferases, which was demonstrated for...
versus N conditions using buffer containing 2 mM EDTA. Since the fused proteins adsorbed to the resin were eluted under mild readily recovered by anti-FLAG M1 antibody resin. The FLAG-enzymes were successfully produced by insect cells and were analysis using anti-FLAG antibody revealed that the secreted galactosyltransferases 1–5 (1–4). Discovery of the activities of \( \beta3 \)N-T2, -T3, and -T4 using pyridylaminated oligosaccharides as substrates Analysis of substrate specificity of \( \beta3 \)N-T2, -T3, and -T4 using unlabeled oligosaccharides as substrates Gal \( \alpha2,3 \)-sialyltransferases I–VI (9, 41–47, 49–52), GalNAc \( \alpha2,6 \)-sialyltransferases I–VI (53–58), NeuAc \( \alpha2,8 \)-sialyltransferases I–V (59–69), \( \alpha1,3 \)-fucosyltransferases III–VII and IX (10, 70–75, 77, 78), core 2 \( \beta1,6 \)-N-acetylglucosaminyltransferases 1–3 (79–82), large I \( \beta1,6 \)-N-acetylglucosaminyltransferases (83), polypeptide N-acetylglucosaminyltransferases (84–91), \( \beta1,4 \)-galactosyltransferases 1–7 (92–100), and \( \beta1,3 \)-galactosyltransferases 1–5 (1–4). Discovery of the \( \beta3 \)N-T family in this study has clearly demonstrated a new feature of glycosyltransferases, that the \( \beta3 \)N-T and -Gal-T families show structural similarity despite of differences in both the transfer sugar (GlcNAc versus Gal) and the acceptor sugar (Gal versus GlcNAc).

We constructed the secreted recombinant proteins for \( \beta3 \)N-T2, -T3, and -T4 fused to the FLAG peptide. Western blot analysis using anti-FLAG antibody revealed that the secreted enzymes were successfully produced by insect cells and were readily recovered by anti-FLAG M1 antibody resin. The FLAG-fused proteins adsorbed to the resin were eluted under mild conditions using buffer containing 2 mM EDTA. Since the eluted proteins showed activity comparative to that of the adsorbed proteins, it was confirmed that EDTA treatment did not damage the enzymes (data not shown). The molecular masses of the recovered proteins were equal to or larger than the predicted ones for their polypeptides, indicating some glycosylation and no significant degradation of the recovered proteins.

All of the recombinant proteins showed Gal \( \beta1,3 \)-N-acetyl-galactosaminyltransferase activity for common oligosaccharides, whereas their substrate preference was significantly different. Since the amount of the recombinant proteins used in this study was not enough for determination of the protein concentration, we could not precisely compare the relative activities of the enzymes. However, the relative activities of \( \beta3 \)N-T2 for LNT, lactose, Gal\( \beta1,4 \)-GlcNAc, and \( p \)-LNnH seemed to be higher than those of other enzymes (Tables III and IV). Considering the variety of acceptor substrates and the different reactivities of the transfected cells to anti-i-antigen antibody or PWM and LEA lectins, the higher activity of \( \beta3 \)Gn-T2 for these oligosaccharides may reflect substrate specificity. To date, \( \beta3 \)Gn-T activities have been detected in several tissues, cells, and sera, some of which were characterized using partially purified enzymes (19–28). Based on the substrate specificity, \( \beta3 \)Gn-T1, but not \( \beta3 \)Gn-T2, -T3, and -T4, may correspond to a \( \beta3 \)Gn-T partially purified from calf serum. \( \beta3 \)Gn-T2 and -T4 showed more preferential activity for LNT than for LNT, which was similar to the nature of the calf serum enzyme as well as \( \beta3 \)Gn-T1 and iGnT (7, 8, 26); however, \( \beta3 \)Gn-T2 and -T4 were distinguished from the calf serum enzyme and \( \beta3 \)Gn-T1 by the activities for lactose (Gal\( \beta1,4 \)-Glc and N-acetyllactosamine (Gal\( \beta1,4 \)-GlcNAc) (Table IV). On the other hand, \( \beta3 \)Gn-T3 is quite unique since it showed activity for LNT comparable to LNT. It has been reported that human colon cancer tissues and the colon cancer cell line Colo205 contain cancer-associated glycosphingolipids with dimeric Le\( ^a \) antigens (Gal\( \beta1,3 \)Fuc\( \alpha1,4 \)-GlcNAc\( \beta1,3 \)Gal\( \beta1,4 \)Fuc\( \alpha1,3 \)Glc) (101). Considering the substrate specificity and expression in colon tissues and Colo205 cells, \( \beta3 \)Gn-T3 is likely to be the most probable candidate involved in the biosynthesis of the backbone structure of dimeric Le\( ^a \) (Gal\( \beta1,3 \)Fuc\( \alpha1,4 \)-GlcNAc\( \beta1,3 \)Gal\( \beta1,4 \)Fuc\( \alpha1,3 \)Glc) (101). Considering the substrate specificity and expression in colon tissues and Colo205 cells, \( \beta3 \)Gn-T3 is likely to be the most probable candidate involved in the biosynthesis of the backbone structure of dimeric Le\( ^a \) (Gal\( \beta1,3 \)Fuc\( \alpha1,4 \)-GlcNAc\( \beta1,3 \)Gal\( \beta1,4 \)Fuc\( \alpha1,3 \)Glc) (101). Considering the substrate specificity and expression in colon tissues and Colo205 cells, \( \beta3 \)Gn-T3 is likely to be the most probable candidate involved in the biosynthesis of the backbone structure of dimeric Le\( ^a \) (101). Considering the substrate specificity and expression in colon tissues and Colo205 cells, \( \beta3 \)Gn-T3 is likely to be the most probable candidate involved in the biosynthesis of the backbone structure of dimeric Le\( ^a \) (101). Considering the substrate specificity and expression in colon tissues and Colo205 cells, \( \beta3 \)Gn-T3 is likely to be the most probable candidate involved in the biosynthesis of the backbone structure of dimeric Le\( ^a \) (101). Considering the substrate specificity and expression in colon tissues and Colo205 cells, \( \beta3 \)Gn-T3 is likely to be the most probable candidate involved in the biosynthesis of the backbone structure of dimeric Le\( ^a \) (101). Considering the substrate specificity and expression in colon tissues and Colo205 cells, \( \beta3 \)Gn-T3 is likely to be the most probable candidate involved in the biosynthesis of the backbone structure of dimeric Le\( ^a \) (101). Considering the substrate specificity and expression in colon tissues and Colo205 cells, \( \beta3 \)Gn-T3 is likely to be the most probable candidate involved in the biosynthesis of the backbone structure of dimeric Le\( ^a \) (101).
transfectants were stained with phosphate-buffered saline (PBS), suggesting that the glycosaminoglycan (GnT) might be isolated by expression cloning using the anti-i-antigen antibody, because it does not have 2 of the 3 cysteine residues conserved in typical Gal glycosyltransferases involved in the biosynthesis of poly-N-acetyllactosamines (e.g. GalNAc1–3Gal, GlcNAc1–3Gal, GlcNAc1–3GalNAc, etc.). Discovery of the multiple βGn-Ts in addition to other multiple glycosyltransferases involved in the biosynthesis of poly-N-acetyllactosamines (e.g. βGal1,4galactosyltransferases) indicates that regulation of poly-N-acetyllactosamine synthesis may be more complex than previously recognized. Definitive determination of the enzymatic activities and expression patterns of the βGn-Ts as well as experiments using knockout mice may provide insight into their functions in physiological and pathological processes.

Recently, Amado et al. (76) have reported the existence of four additional members of the βGal-T family, although their enzymatic activities were not determined. Based on the characteristics of the primary structures and chromosomal localizations, three of them may correspond to βGn-T3, -T3, and -T4. These cells were stained with anti-i-antigen antiserum or poly-N-acetyllactosaminerecognizing lectins (LEA or PWM) and subjected to flow cytometric analysis as described under “Experimental Procedures” (thick lines). As controls, the transfectants were stained with phosphate-buffered saline (thin lines).

In this study, we isolated three types of novel βGn-T genes, which enabled us to discriminate the respective enzymes at the molecular level. Considering the enzymatic activities in vitro and in vivo as well as the expression patterns of the βGn-Ts, the respective enzymes are likely to play different roles. The poly-N-acetyllactosamine or GlcNAcβ1–3Gal structure appears to be a major determinant of physiological and pathological processes.
Additional members of the β3Gn-T and βGal-T families remain to be investigated.

Acknowledgments—We are grateful to Dr. Minoru Fukuda (Burnham Institute) for the generous gift of the anti-i-antigen antiserum. We thank Drs. Shoko Nishihara and Takashi Kudo and Hiroko Iwasaki (Soka University) for great help in and discussion of this study. We also thank Sachiko Kodama for excellent technical assistance, Reiko Kido for DNA sequencing, and Mayumi Ibai and Dr. Atsushi Hasegawa for providing the synthetic oligonucleotides. We thank Kazumi Kuratami-Miura for advice and suggestions throughout this work.

Note Added in Proof—Recently, Zhou et al. (7) have corrected the nucleotide sequence and deduced amino acid sequence of the β1,3-N-acetylgalactosaminyltransferase (β3Gn-T1) cDNA that they previously published (see “Corrections” in Proc. Natl. Acad. Sci. U.S.A. 2000 97, 11673–11675). This cDNA was developed from an unfortuante cDNA clone substitution in their laboratory. The corrected sequence of β3Gn-T1 was identical to that of β3Gn-T2. Consequently, besides iGnT, there are three types of βGn-Ts described to date, not four. The sequence of β3Gn-T1 used in this paper is that of the corrected, substituted cDNA clone. To try to prevent further confusion, we point out this fact but do not change the enzyme names used in this paper.

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Identification and Characterization of Three Novel β1,3-N-Acetylglucosaminyltransferases Structurally Related to the β1,3-Galactosyltransferase Family

Norihiko Shiraishi, Ayumi Natsume, Akira Togayachi, Tetsuo Endo, Tomohiro Akashima, Yoji Yamada, Nobuyuki Imai, Satoshi Nakagawa, Satoshi Koizumi, Susumu Sekine, Hisashi Narimatsu and Katsutoshi Sasaki

J. Biol. Chem. 2001, 276:3498-3507.
doi: 10.1074/jbc.M004800200 originally published online October 19, 2000

Access the most updated version of this article at doi: 10.1074/jbc.M004800200

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