Molecular Cloning and Characterization of UDP-GlcNAc:Lactosylceramide β1,3-N-Acetylgalcosaminyltransferase (β3Gn-T5), an Essential Enzyme for the Expression of HNK-1 and Lewis X Epitopes on Glycolipids*

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A new member of the UDP-N-acetylgalcosamine-β-galactose β1,3-N-acetylgalcosaminyltransferase (β3Gn-T) family having the β3Gn-T motifs was cloned from rat and human cDNA libraries and named β3Gn-T5 based on its position in a phylogenetic tree. We concluded that β3Gn-T5 is the most feasible candidate for lactotriaosylceramide (Le3Cer) synthase, an important enzyme which plays a key role in the synthesis of lacto- or neolacto-series carbohydrate chains on glycolipids. β3Gn-T5 exhibited strong activity to transfer GlcNAc to glycolipid substrates, such as lactosylceramide (LacCer) and neolactotetraosylceramide (nLe4Cer; paragloboside), resulting in the synthesis of Le3Cer and neolactopentaosylceramide (nLe5Cer), respectively. A marked decrease in LacCer and increase in nLeCer was detected in Namalwa cells and 4 were consistent with the Lc3Cer synthase activity in the rat brain development were determined. Points 2, 3, and 4 were consistent with the Le3Cer synthase activity reported previously.

To date, three members of the human β1,3-N-acetylgalcosaminyltransferase (β3Gn-T) family (β3Gn-T2, -T3, and -T4) (1, 2) and five members of the human β1,3-galactosyltransferase (β3Gal-T) family (β3Gal-T1, -T2, -T3, -T4, and -T5) have been identified (3–6). All of them share amino acid motifs (β3Gn-T motifs or β3Gal-T motifs) in three regions of the catalytic domain. The first, β3Gn-T, was cloned by an expression cloning method using an anti-i antibody (7). However, this enzyme is unique in that it does not have the β3Gn-T motifs although it transfers GlcNAc to Gal with an β1,3-linkage, resulting in the synthesis of polylactosamine chains. It was named iGn-T (7). Thereafter, β3Gn-T1 was isolated based on structural similarity with the β3Gal-T family (2). We previously reported three additional β3Gn-Ts, β3Gn-T2, -T3, and -T4, which are also structurally related to the β3Gn-T family (1). However, the cDNA sequence of β3Gn-T1 was recently corrected by Zhou et al. (see Ref. 2). The corrected sequence of β3Gn-T1 was identical to that of β3Gn-T2 which was isolated

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†† The nucleotide sequence(s) reported in this paper has been submitted to the DDBJ/GenBank™/EBI Data Bank with the accession numbers AB045278 for human β3Gn-T5 and AB045279 for rat β3Gn-T5.

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and reported by us (1). So, a total of four β3 Gn-Ts, i.e. iγN-T, β3Gn-T2-T1, -T3, and -T4, have been described to date. To avoid further confusion, we note this fact, but do not change the enzyme names used in this study. By transfection experiments and in vitro enzymatic analysis, these four β3Gn-Ts were found to exhibit β3Gn-T activity that catalyzes the synthesis of polyolactosamine chains, but not β3Gal-T activity.

The monoclonal antibody HNK-1 reacts to a sulfoglucuronyl carbohydrate epitope, SO3-GlcAβ1–3Galβ1–4GlcNAc-R, which is expressed in several glycoproteins involved in neural cell recognition, and in two neolactoglycolipids, named sulfoglucuronylglycolipid (SGGL)-1 and -2 (SGGL-1, SO43-GlcA-1,3-GlcNAcβ1–3Galβ1–4Glcβ1–ceramide; and SGGL-2, SO3-GlcAβ1–3Galβ1–4GlcNAcβ1–3Galβ1–4Glcβ1–ceramide) (8, 9). Neurobiologists have suggested in a series of studies that the HNK-1 epitope functions as a cell-cell interaction molecule during the development of the brain to complete the nervous system (10–14). The expression is developmentally and spatially regulated in the nervous system. In rat cerebellum, SGGLs are expressed in a biphasic manner, with the initial peak at around postnatal days (PD) 1–3, and the second peak at PD 20 (15). In the adult rat cerebellum, the expression of SGGLs is kept constant (16, 17). Immunohistochemical studies localized SGGLs in specific cells during the development of the cerebellum, and suggested some biological functions for neuron guidance (14, 15, 17, 18).

In rat cerebral cortex, the expression of SGGLs peaked at embryonic day (ED) 19 and then decreased until postnatal PD 5, and had almost completely disappeared by PD 20. Carbohydrate chains on SGGLs are extended by stepwise reactions catalyzed by glycosyltransferases. Chou et al. (19–22) reported developmental changes in the activity of each enzyme in correlation with the SGGL expression in rat brain. It was demonstrated that LCβCer synthase, which catalyzes the transfer of GlcNAc to the Gal residue of lactosylceramide (LacCer; Galβ1–4Glcβ1–1Cer) with a β1–3-linkage resulting in the synthesis of LCβCer (GlcNAcβ1–3Galβ1–4Glcβ1–1Cer), is the key enzyme in the expression of SGGLs in the developing rat brain, because only the activity of this synthase was well correlated with the developmental change in SGGL expression.

The expression of the Lewis x (Le x; CD15) carbohydrate structure, α1,3-fucosyl-N-acetyllactosamine, Galβ1–4(Fucα1–3)3Gn-Ts were found in lymphoid cells. The lack of LCβCer synthase in lymphoid cells explains the absence of neolacto-neutral GSLs in the cells, while lymphoid cells exhibited activity for nLCβCer, but not LacCer. The lack of LCβCer synthase activity in lymphoid cells explains the absence of neolacto-neutral GSLs.

LCβCer synthase is the enzyme controlling the expression of neolacto-series GSLs and so plays important roles in many cells. In the present study, we cloned and characterized a fifth member of the β3Gn-T family (β3Gn-T5), and identified β3Gn-T5 as the most likely candidate for LCβCer synthase.

**EXPERIMENTAL PROCEDURES**

**Tumor Cell Lines and Monoclonal Antibodies (MAb)—**The tumor cell lines were cultured in RPMI 1640 medium (Life Technologies, Inc., Rockville, MD) supplemented with 10% fetal bovine serum. The cell lines were donated or purchased from American Type Culture Collection (ATCC, Manassas, VA), Riken Cell Bank (RCB, Tsukuba, Japan), Japanese Cell Research Bank (JCRB, Tokyo, Japan), Immunobiochemical laboratory (IBL, Fujikko, Japan), or Dai-Nippon Pharmaceutical Co., Ltd. (NIPPON SHOKU-IKA, Osaka, Japan).

**Isolation of Rat and Human β3Gn-T5 cDNAs—**We constructed a rat shank cdna library for a random sequencing project. During the sequencing, we found a rat cdna encoding a partial sequence of β3Gn-T5. This novel sequence (738 bp) did not encode the full ORF. But it had the β3Gal-T (β3Gn-T) motifs which are shared by the known β3Gal-Ts and β3Gn-Ts. The cdna library of Colo205 cells constructed in a previous study (6) was screened with the rat partial β3Gn-Ts cdna as a probe to isolate the full-length human β3Gn-T5 cdna which possessed a 3.7-kilobase pair insert cdna. We did not clone a rat full-length β3Gn-T5 in this study.

**Quantitative Analysis of the Four β3Gn-T Transcripts in Human Tissues and Tumor Cell Lines by Competitive RT-PCR—**The principle behind the competitive RT-PCR method for quantification of transcripts was described in detail in our previous studies (6, 38). Total cellular RNAs of various human tissues were purchased from CLONTECH. Those of various tumor cell lines were extracted and purified in our laboratory. As to the measurement of the transcripts for three cloned β3Gn-T genes, β3Gn-T2, -T3, and -T4, the primers and the PCR conditions were also reported previously (1).

Regarding the β3Gn-T5 gene, a standard DNA plasmid was constructed by subcloning a full-length ORF cdna into a pBluescript SK+ vector. A competitor DNA plasmid carrying a small deletion (245 base pairs) within the ORF of the β3Gn-T5 cdna was constructed as follows. The standard DNA plasmid was double digested with appropriate restriction endonucleases, Mscl and BgIII, and was blunt-ended with T4 DNA polymerase. This was followed by self-ligation of the deleted plasmid DNA. As to the measurement of the β3Gn-T5 transcripts by competitive RT-PCR, we used the following primer set: forward primer, 5′-TTTTGAATTCGCTAATGATTTCGCA-3′, and reverse primer, 5′-CTTTAGGATCTGATATTCGATCA-3′.

**Transfection Experiments to Express Each of the Four Human β3Gn-T Genes in Namalwa Cells—**Expression of glycosyltransferase genes subcloned in pMAO was described in detail in a series of previous papers (6, 7, 39–42). The construction of pAMo vector was described in detail in our previous studies (6, 38). Total cellular RNAs of various human tissues were purchased from CLONTECH. Those of various tumor cell lines were extracted and purified in our laboratory. As to the measurement of the transcripts for three cloned β3Gn-T genes, β3Gn-T2, -T3, and -T4, the primers and the PCR conditions were also reported previously (1).

Regarding the β3Gn-T5 gene, a standard DNA plasmid was constructed by subcloning a full-length ORF cdna into a pBluescript SK+ vector. A competitor DNA plasmid carrying a small deletion (245 base pairs) within the ORF of the β3Gn-T5 cdna was constructed as follows. The standard DNA plasmid was double digested with appropriate restriction endonucleases, Mscl and BgIII, and was blunt-ended with T4 DNA polymerase. This was followed by self-ligation of the deleted plasmid DNA. As to the measurement of the β3Gn-T5 transcripts by competitive RT-PCR, we used the following primer set: forward primer, 5′-CTTTATGACCTGCTGATGACAT-3′, and reverse primer, 5′-CTTTAGGATCTGATATTCGATCA-3′.

**Transfection Experiments to Express Each of the Four Human β3Gn-T Genes in Namalwa Cells—**Expression of glycosyltransferase genes subcloned in pMAO was described in detail in a series of previous papers (6, 7, 39–42). The construction of pAMo vector inserted with each of three cloned β3Gn-T genes, β3Gn-T2, -T3, and -T4, was also reported previously (1). Regarding the β3Gn-T5 gene, the β3Gn-T5 ORF fragment was excised from the standard β3Gn-T5 DNA in pBluescript SK+ vector, and then blunt-ended with T4 DNA polymerase. The blunt-ended fragment was flanked with SfiI linker and inserted into the SfiI site of pMAO vector. Each of the four β3Gn-T genes subcloned into a pMAO vector was transfected by electroporation into Namalwa cells. These cells were selected in the presence of genetin (G418) (Life Technologies, Inc.) at a concentration of 0.8 mg/ml. Stable transfected cells were obtained after 25 days exposure to genetin. The levels of the transcripts expressed in the Namalwa transfected cells were measured by means of competitive RT-PCR.
Separation of Glycolipids by Thin Layer Chromatography (TLC) and Immuno-TLC with Anti-ncL_Cer (Anti-paragloboside) MAb—For glycolipid analysis, 1.0 × 10^6 of the Namalwa transfected cells with each β3Gn-T gene were collected, washed twice with phosphate-buffered saline, and then lyophilized. Total glycolipids were extracted from the lyophilized tissue using chloroform/methanol (2:1, v/v), and then with chloroform/methanol/water (30:60:8, v/v/v). Samples dried with the N2 evaporator were dissolved in methanol, then subjected to mild alkaline treatment in 0.1 M ROH/methanol at 40 °C for 2 h, and neutralized with 1 N acetic acid. After the free fatty acids had been removed with n-heptane, the remaining fractions were dried with the N2 evaporator and subjected to Folch’s partition. The lower neutral glycolipid fractions were eluted with 20 mM ammonium acetate buffer (pH 4.0) containing 7% methanol and subjected to immuno-TLC analysis. Neutral glycolipid equivalent to 1.0 × 10^6 cells was applied to each lane of TLC. Neutral glycolipids were separated by TLC (HPTLC, Kieselgel 60, 5841; Merek, Germany) with mixtures of chloroform/methanol/water (60:35:8, v/v/v) and immuno-TLC analysis was performed with the antibody 1B2 as described (36).

Construction and Purification of β3Gn-T Proteins Fused with FLAG Peptide—The putative catalytic domain of each of β3Gn-T2, -T3, -T4 had been expressed as a secreted protein fused with FLAG peptide in insect cells as described in a previous study (1). In the present study, a 1.1-kilobase pair DNA fragment encoding a COOH-terminal portion of β3Gn-T (amino acids 39 to 378) was amplified by PCR. The PCR was performed with Platinum PfX DNA Polymerase (Life Technologies, Inc.), according to the supplier’s manual. The 5’ and 3’ primer sequences were flanked with BamHI and XbaI sequences, respectively, to create the restriction sites. Those sequences were as follows: forward primer, 5’-CCGGATCATTTGAGCTCATATGAAGTCATAT-3’ and reverse primer, 5’-GGTCTAGAAGGCACTGAACATCATACTTACATG-3’. The amplified fragment was first inserted between the BamHI and XbaI sites of the pBluescript SK- vector. Subsequently, the inserted DNA was excised by BamHI and NotI, and was inserted between the BamHI and NotI sites of pVL1393-F2 to yield pVL1393-F2G5. pVL1393-F2 is an expression vector derived from pVL1393 (Pharmin). We prepared recombinant viruses as described previously (1). SF21 insect cells (Pharmining) were infected with each individual recombinant virus at a multiplicity of infection of 10 and incubated at 27 °C for 72 h to yield conditioned media containing recombinant β3Gn-T proteins fused with FLAG peptide. Bacu3GnT proteins were readily purified from the FLAG M1 antibody resin (Sigma) and eluted with 50 mM TBS (50 mM Tris-HCl, pH 7.4, 150 mM NaCl), 4 mM CaCl2 buffer (pH 7.4). The recombinant proteins obtained in this system were named Bacu3GnT2, Bacu3GnT3, Bacu3GnT4, and Bacu3GnT5.

Bacu3GnT proteins separated by 10% SDS-polyacrylamide gel electrophoresis were transferred to an Immobilon PVDF membrane (Millipore, Bedford, MA). The membrane was probed with anti-FLAG monoclonal antibody, and stained with the ECL Western blotting detection reagents (Amer sham Pharmacal Biotech). The intensity of positive bands on Western blotting was measured by densitometer (43) to determine the relative amounts of each Bacu3GnT protein.

Assaying of β3Gn-T Activity—Two types of each recombinant β3Gn-T, the soluble enzyme produced in the baculovirus expression system and the membrane-bound form expressed in the cell homogenates of Namalwa transfected cells and other cultured cell lines, were used to determine the relative activities of each β3Gn-T toward various substrates. Each soluble Bacu3Gn-T purified was adjusted to the same amount as described above and used for the assay. The various cells were solubilized in a 20 mM Hepes buffer (pH 7.2) containing 2% Triton X-100, and 20 μg of total protein in the cell homogenates was used for the enzyme reaction. Various oligosaccharides were fluorescein isothiocyanate labeled with 2-aminobenzamide (2AB) or pyridyaminated (PA) (DYKDDDDK). The human β3Gn-T cDNA contains a full-length ORF encoding a protein of 378 amino acids, as shown in Fig. 1. β3Gn-T is predicted to be a typical type II membrane protein consisting of a NH2-terminal cytoplasmic domain of 12 residues, a transmembrane segment of 20 residues, and a stem region and catalytic domain of 346 residues. β3Gn-T had the three motifs typical of members of the β3Gn-T and β3Gn-T families. On ClustalW analysis (Fig. 1), four cysteine residues were found to be conserved in the four β3Gn-Ts. The human β3Gn-T5 cDNA contains a full-length ORF encoding a protein of 378 amino acids, as shown in Fig. 1. β3Gn-T is predicted to be a typical type II membrane protein consisting of a NH2-terminal cytoplasmic domain of 12 residues, a transmembrane segment of 20 residues, and a stem region and catalytic domain of 346 residues. β3Gn-T had the three motifs typical of members of the β3Gn-T and β3Gn-T families. On ClustalW analysis (Fig. 1), four cysteine residues were found to be conserved in the four β3Gn-Ts which indicates that some of these cysteines are essential for maintenance of the tertiary structure of β3Gn-Ts. In the second β3Gn-T motif, a triplet of aspartic acid residues, DDD, which may be a dicationic binding site as proposed in a crystallization study of other glycosyltransferases (48), was conserved. Four possible N-glycosylation sites were found in the primary sequence of β3Gn-T5. One of them was conserved in all β3Gn-Ts. The β3Gn-T5 gene was found to be localized to a draft genome sequence (GenBank™ accession number AC025833) and the 3.7-kilobase pair cDNA containing the ORF is composed of a single exon.

On a phylogenetic tree (Fig. 2), the four members of the
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β3-N-Acetylgalcosaminyltransferase

β3-Gal-T family apparently formed a cluster which is separated from β3Gal-T members. β3-Gn-T is positioned in the β3-Gal-T family branch, however, it is in an outer branch away from the cluster of the other members. Three enzymes, β3Gn-T2, -T3, and -T4, form a subfamily in the phylogenetic tree and β3Gal-T and iGn-T also form a subfamily. The divergence of β3Gn-T occurred earlier than that of any other β3Gn-T member.

Quantitative Analysis of β3Gn-T5 Transcripts in Human Tissues and Various Cell Lines—As summarized in Fig. 3 and Table I, this gene was expressed in almost all tissues and cell lines with very few exceptions, although the expression level was different depending on the tissue and cell line. The tissues expressing β3Gn-T5 at a considerably high level were lung, colon, placenta, testis, pituitary gland, and cerebellum. Brain, liver (very low), spleen, lymph node, and thymus expressed β3Gn-T at a low level. Colonic adenocarcinoma, Colo205, SW620, lung cancer cell lines, EBC-1, HAL8, LX-1, PC-7, and RERF-LC-MS, and stomach cancer cell lines, KATOIII, MK7N, and HSC43 expressed the β3Gn-T5 transcripts at a high level. All neuroblastoma cells examined, except for NAGAI and GOTO cells, expressed the β3Gn-T5 transcript at a relatively high level. On the other hand, all leukemic cells derived from lymphocytes, except for NALL-1 (lymphoblastic leukemia), expressed β3Gn-T5 almost at an undetectable level. U937 (monocyte-like) and HL-60 (promyelocytic leukemia) cells expressed it at a considerable level. HepG2 cells did not express it at all. The expression levels in the cultured cells reflected those in the original tissues derived therefrom.

Relative Activities of Four Recombinant β3Gn-Ts, Which Were Produced as Truncated Forms of Fusion Protein with the FLAG Peptide in a Baculovirus Expression System, Toward Oligosaccharides—The recombinant enzymes of four β3Gn-Ts, β3Gn-T2, -T3, -T4, and -T5, produced as fusion proteins with a FLAG peptide in the baculovirus expression system were named Bacu3GnT2, -3GnT3, -3GnT4, and -3GnT5, respectively. The amounts of each enzyme were made equal for assaying the β3Gn-T activity.

We observed no galactosyltransferase activity of the four β3Gn-Ts toward LNnT-PA and agalacto-LNnT-PA (data not shown). The β3Gn-T activity of Bacu3GnT2 toward LNnT-PA was strongest among all combinations of the enzyme and the substrate. Thus, the activity of Bacu3GnT2 toward LNnT-PA is presented as 100%, and all other activities are given as relative values.

Bacu3GnT2 exhibited relative activity toward the following pyridylaminated substrates: LNnT-PA, 100%; LNT-PA, 3.3%; LNFP-II-PA, 4.2%; and LNFP-III-PA, 4%. and exhibited no activity toward LNFP-III-PA or LNDFH-II-PA. Bacu3GnT3 exhibited relative activity toward only LNFP-IV-PA, 0.2%, and exhibited no activity toward LNnT-PA, LNT-PA, LNFP-II-PA, LNFP-III-PA, or LNDFH-II-PA. Bacu3GnT3 exhibited relative activity toward pyridylaminated substrates, LNnT-PA, 100%; LNT-PA, 89%; LNFP-II-PA, 89%; and LNFP-III-PA, 90%. It exhibited no activity toward LNFP-IV-PA or LNDFH-II-PA.

The β3Gn-T activity of Bacu3GnT5 toward LNnT-PA was almost one-fifth the activity of Bacu3GnT2. The three Bacu3GnTs, -3GnT2, -3GnT3, and -3GnT5, exhibited weak ac-
activity for LNT-PA and LNFP-V-PA. Bacu3GnT2 and -3GnT5 also exhibited weak activity for LNFP-III-PA. LNFP-II-PA and LNDFH-II-PA could not be utilized as acceptor substrate for any Bacu3GnT examined. Bacu3GnT4 activity was almost undetectable for all substrates except a very faint activity for LNFP-V-PA. In a previous study, we confirmed that Bacu3GnT3 and Bacu3GnT4 apparently exhibited detectable levels of $\beta$3Gn-T activity toward LNnT-PA and LNT-PA because we used an excess of recombinant enzyme for the reaction (1).

The oligosaccharide substrates having the polylactosamine structures, repeats of units of lactosamine (Gal$\beta$1–4GlcNAc; LN), were labeled with 2AB and used as acceptor substrates (44). The LNnT-2AB oligosaccharide was used as a control. 2LN, 3LN, 4LN, and 5LN in Table II indicate that each oligosaccharide has 2-, 3-, 4-, or 5-repeating lactosamine (LN) units, respectively. The $\beta$3Gn-T activity toward LNnT-2AB of Bacu3GnT2 was again the strongest among all combinations of enzyme and substrate, therefore its activity is expressed as 100%, and the activities of the other combinations are expressed relative to this value in Table II. Bacu3GnT2 transferred a GlcNAc with almost the same level of activity to all polylactosamine substrates regardless of the number of LN units. Bacu3GnT3 exhibited low, but apparently positive activity for all lengths of polylactosamine substrate. The activity of Bacu3GnT4 was again hardly detected with the amount of recombinant protein used in the present study. Interestingly, Bacu3GnT5 preferred the shorter substrates, i.e. 2LN-2AB and 3LN-2AB. The activities of Bacu3GnT5 for the longer polylactosamine chains, 4LN-2AB and 5LN-2AB, were almost one-tenth of those for the shorter chains.

Relative Activities of Four $\beta$3Gn-Ts, Which Were Produced in

### Table I

| Cell Lines | Tissue type               | Relative amount of $\beta$3Gn-T transcript ($/\beta$-actin $\times 10^3$) |
|------------|---------------------------|---------------------------------------------------------------------|
| Colo205    | Colon adenocarcinoma      | 3.08                                                                |
| HCT-115    |                           | 23.42                                                               |
| SW620      |                           | 8.03                                                                |
| LS180      |                           | 0.01                                                                |
| A03        | Lung squamous cell carcinoma | 0.24                                                           |
| RBC-1      |                           | 0.54                                                                |
| PC-1       |                           | 0.95                                                                |
| HAL8       |                           | 5.87                                                                |
| HAL24      |                           | 1.23                                                                |
| LX1        | Lung adenocarcinoma       | 6.31                                                                |
| PC-7       |                           | 0.11                                                                |
| PC-9       |                           | 5.21                                                                |
| RERF-LC-MS |                           | 0.44                                                                |
| KATOIII    |                           | 4.17                                                                |
| MKN1       | Stomach cancer            | 9.17                                                                |
| MKN7       |                           | 1.95                                                                |
| MKN45      |                           | 0.39                                                                |
| HSC43      |                           | 1.17                                                                |
| NAGA1      |                           | 1.97                                                                |
| NB-9       |                           | 0.01                                                                |
| SCCH-26    | Neuroblastoma             | 0.01                                                                |
| IMR32      |                           | 0.21                                                                |
| SK-N-SH    |                           | 0.24                                                                |
| GOTO       |                           | 0.23                                                                |
| A172       |                           | 0.71                                                                |
| YKG-1      |                           | 0.42                                                                |
| T98G       | Glioblastoma              | 0.30                                                                |
| U251       |                           | 1.38                                                                |
| U-118-MG   |                           | 0.51                                                                |
| G1–1       |                           | 0.12                                                                |
| Capan-1    | Pancreas cancer           | 1.51                                                                |
| Capan-2    |                           | 1.50                                                                |
| PC-3       | Prostatic cancer          | 0.86                                                                |
| HepG2      | Hepatoblastoma            | 0.86                                                                |
| Namalwa    | B cell leukemia           | 0.04                                                                |
| Daudi      | Burkitt’s lymphoma        | 0.04                                                                |
| Raji       | T cell leukemia           | 0.06                                                                |
| Ramos      | T cell leukemia           | 0.05                                                                |
| Jurkat     | Monocyte-like myeloma     | 0.05                                                                |
| U937       | Lymphoblastic leukemia    | 0.05                                                                |
| U266       |                           | 0.05                                                                |
| NALL-1     | Lymphoblastic leukemia    | 1.06                                                                |
| K562       | Leukemia                  | 1.06                                                                |
| HL-60      | Promyelocytic leukemia    | 0.06                                                                |

*ND, not detected.

**Relative Activities of Four $\beta$3Gn-Ts, Which Were Produced in**

- H1,3-N-Acetylglucosaminyltransferase Synthesizing Lc3Cer

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**Table I**

Quantitative analysis of $\beta$3Gn-T transcripts in various human cell lines by competitive RT-PCR

| Cell Lines | Tissue type | Relative amount of $\beta$3Gn-T transcript (/\(\beta\)-actin $\times 10^3$) |
|------------|-------------|---------------------------------------------------------------------|
| Colo205    | Colon adenocarcinoma      | 3.08                                                                |
| HCT-115    |                           | 23.42                                                               |
| SW620      |                           | 8.03                                                                |
| LS180      |                           | 0.01                                                                |
| A03        | Lung squamous cell carcinoma | 0.24                                                           |
| RBC-1      |                           | 0.54                                                                |
| PC-1       |                           | 0.95                                                                |
| HAL8       |                           | 5.87                                                                |
| HAL24      |                           | 1.23                                                                |
| LX1        | Lung adenocarcinoma       | 6.31                                                                |
| PC-7       |                           | 0.11                                                                |
| PC-9       |                           | 5.21                                                                |
| RERF-LC-MS |                           | 0.44                                                                |
| KATOIII    |                           | 4.17                                                                |
| MKN1       | Stomach cancer            | 9.17                                                                |
| MKN7       |                           | 1.95                                                                |
| MKN45      |                           | 0.39                                                                |
| HSC43      |                           | 1.17                                                                |
| NAGA1      |                           | 1.97                                                                |
| NB-9       |                           | 0.01                                                                |
| SCCH-26    | Neuroblastoma             | 0.01                                                                |
| IMR32      |                           | 0.21                                                                |
| SK-N-SH    |                           | 0.24                                                                |
| GOTO       |                           | 0.23                                                                |
| A172       |                           | 0.71                                                                |
| YKG-1      |                           | 0.42                                                                |
| T98G       | Glioblastoma              | 0.30                                                                |
| U251       |                           | 1.38                                                                |
| U-118-MG   |                           | 0.51                                                                |
| G1–1       |                           | 0.12                                                                |
| Capan-1    | Pancreas cancer           | 1.51                                                                |
| Capan-2    |                           | 1.50                                                                |
| PC-3       | Prostatic cancer          | 0.86                                                                |
| HepG2      | Hepatoblastoma            | 0.86                                                                |
| Namalwa    | B cell leukemia           | 0.04                                                                |
| Daudi      | Burkitt’s lymphoma        | 0.04                                                                |
| Raji       | T cell leukemia           | 0.06                                                                |
| Ramos      | T cell leukemia           | 0.06                                                                |
| Jurkat     | Monocyte-like myeloma     | 0.05                                                                |
| U937       | Lymphoblastic leukemia    | 0.05                                                                |
| U266       |                           | 0.05                                                                |
| NALL-1     | Lymphoblastic leukemia    | 1.06                                                                |
| K562       | Leukemia                  | 1.06                                                                |
| HL-60      | Promyelocytic leukemia    | 0.06                                                                |

*ND, not detected.

**Relative Activities of Four $\beta$3Gn-Ts, Which Were Produced in**

- H1,3-N-Acetylglucosaminyltransferase Synthesizing Lc3Cer
β1,3-N-Acetylglucosaminyltransferase Synthesizing Lc₃Cer

Table II
Specific activity of recombinant β3Gn-Ts expressed in the baculo system toward polylactosamine chains

| Polylactosamine substrate | Bacu3GnT5 | Bacu3GnT2 | Bacu3GnT3 | Bacu3GnT4 |
|---------------------------|-----------|-----------|-----------|-----------|
| LNNT-2AB                  | 38.9      | 100.0     | 2.5       | ND        |
| 2LN-2AB                   | 25.4      | 94.3      | 1.1       | ND        |
| 3LN-2AB                   | 30.8      | 90.4      | 1.8       | ND        |
| 4LN-2AB                   | 3.9       | 91.0      | 1.0       | ND        |
| 5LN-2AB                   | 4.8       | 85.3      | 2.5       | ND        |

* ND, not detected.

Two Different Types of Recombinant Enzymes, Toward Glycolipid Substrates—To elucidate the relative activities toward glycolipid acceptors, two recombinant β3Gn-Ts were compared. First, the homogenates of Namalwa cells stably expressing each β3Gn-T gene were used as a source of recombinant enzyme.

Leading to measurement of β3Gn-T activity in Namalwa transfected cells, we determined the transcript level of each β3Gn-T gene expressed in each Namalwa transfected cell. The wild-type Namalwa cells expressed substantial amounts of β3Gn-T2 endogenously, 3.3 units, but not the other β3Gn-Ts (β3Gn-T3, -T4, and -T5). The amounts of transcript in cells transfected with the β3Gn-T2, β3Gn-T3, β3Gn-T4, or β3Gn-T5 gene, were 21.2, 15.4, 24.4, and 10.0 units, respectively. The amount of each transcript in cells mock-transfected with the pAMo vector alone was just as much as that in Namalwa wild cells. We used HL-60 cells as a control for this experiment. HL-60 expressed substantial amounts of two β3Gn-T transcripts, i.e. β3Gn-T2; 5.3 units and β3Gn-T5; 0.9 units, but possessed no transcript of β3Gn-T3 and β3Gn-T4.

As seen in Fig. 4, the homogenates of Namalwa-3GnT5 cells exhibited strong activity for the synthesis of both Lc3Cer and nLcCer. The homogenates of HL-60 cells also showed positive activities toward both acceptors, LacCer and nLcCer. Relative activities were obtained by measurement of the positive bands in Fig. 4. The activity of Namalwa-3GnT5 toward LacCer is presented as 100%, and all other activities are given as relative values. Namalwa-3GnT5 exhibited strong activity toward LacCer resulting in the synthesis of Lc3Cer (100%), however, the other three β3Gn-Ts showed no activity toward LacCer. The wild-type Namalwa cells and the mock-transfected Namalwa cells showed faint activity, 2.6 and 2.5%, respectively, for the acceptors, LacCer and nLcCer, respectively, in the homogenates of Namalwa-3GnT5 cells.

Immuo-TLC Analysis of Glycosphingolipids (GSLs) Extracted from Namalwa Cells Transfected with Each β3Gn-T Gene—As seen from the orcinol staining results of neutral GSLs (Fig. 5A), among multiple bands of GSLs, the band intensity of LacCer of Namalwa-3GnT5 apparently decreased as compared with that of the other transfectants. By immuno-staining using the 1B2 mAb (Fig. 5B), positive bands were detected in all transfectants including the wild-type and mock transfected cells. However, the nLcCer band of Namalwa-3GnT5 cells showed a very strong intensity as compared with that of the other transfectants. The positive bands faintly detected below that of nLcCer of Namalwa-3GnT5 cells corresponded to nLcCer. It is known that mAb 1B2 reacts with both nLcCer and nLcCer (37). The above results are interpreted as follows. The overexpressed β3Gn-T5 in the cells consumed the substrate, LacCer, and thereafter, the product Lc3Cer was galactosylated by an endogenous β1,4-galactosyltransferase(s) to produce nLcCer, because Namalwa cells are known to endogenously possess an excess amount of β4Gal-T1. Some of the nLcCer produced in the cells was further converted to nLcCer by the overexpressed β3Gn-T5 and again galactosylated to produce nLcCer by endogenous β4Gal-T(s).

Correlation of Lc₃Cer Synthesizing Activity with the Amount of β3Gn-T5 Transcript in Various Cultured Tumor Cells—The Lc₃Cer synthesizing activity was highest in KATOIII cells among various cultured cancer cells examined in this study (Table IV). Thus, the activity of KATOIII was set as 100%, and the values for Lc₃Cer synthesizing activity of the other cells were calculated relative to this (see Table IV). The level of Lc₃Cer synthesizing activity was almost correlated with the amount of β3Gn-T5 transcript (the mathematical correlation coefficient is 0.83), but not with the levels of the other β3Gn-T transcripts (the mathematical correlation coefficients of other β3Gn-Ts are all under 0.45). KATOIII cells possessed the most β3Gn-T5, followed by Colo205, EBC-1, LS180, and A01 cells in decreasing order both for activity and the amount of transcript.
cell. On the other hand, it markedly declined during the monocytic differentiation induced by TPA treatment.

**DISCUSSION**

Le₃Cer synthase plays a key role in the control of carbohydrate synthesis in GSLs during cell differentiation and development. As demonstrated in the present study, β3Gn-T2, -T3, and -T4 are not the Le₃Cer synthase. We did not examine activities of the recombinant enzymes of iGn-T. However, the transcript levels of iGn-T in various tumor cell lines were not correlated with the Le₃Cer and nLe₃Cer synthesizing activities in the respective cell lysates (data not shown). So, we can rule out iGn-T as a candidate for the Le₃Cer synthase. We concluded that β3Gn-T5 is the most feasible candidate for the following reasons. 1) Bacu3GnT5 and the homogenates of Namalwa-3GnT5 cells exhibited strong activity to synthesize Le₃Cer in vitro. 2) LacCer was consumed to be converted to neolacto-GSLs with the respective GSL synthase activity as reported previously. 4) The expression level of the β3Gn-T5 transcript in various cultured cancer cells was well correlated with the Le₃Cer synthesizing activity. 5) The changes in the β3Gn-T5 transcript level during HL-60 differentiation and during rat brain development were consistent with those of the Le₃Cer synthesizing activity as reported by others (15, 20, 35, 46).

The truncated enzyme expressed in the insect cells preferred nLe₃Cer which has a longer carbohydrate chain than LacCer, while the membrane-bound form exhibited almost the same activity toward both substrates. The difference in activity between the two forms may be related to structural difference or the presence of detergent in the reaction mixture. Glycosyltransferases are the Golgi enzymes bound to the transmembrane domain. We assume that the truncated form, which is soluble due to the absence of transmembrane domain, may easily access GSLs with a long carbohydrate chain, which are more hydrophilic than GSLs with shorter carbohydrate chains. The membrane-bound form expresses in Namalwa cells probably has more physiological activity than the truncated form. In the reaction mixture for β3Gn-T assay, the membrane-bound form probably exists as a micellar penetrating the Golgi membrane. It is likely that the membrane-bound enzyme rather than the truncated soluble form interacts with glycolipid substrates.

Le₃Cer synthase has been detected in many tissues, including the developing rat brain (14, 15, 20, 46), hematopoietic cells (35, 36, 47–49), and colorectal tissues (50–52). In all tissues
the respective rat brains at each developmentally stage, fetal (ED19), neonatal (PD3, PD8, and PD14), and adult, and subjected to the measurement of Total RNA was extracted from a mixture of 5–7 rat development.

RT-PCR experiments were performed twice on each sample. The values for up to 4 or 2 days, respectively.

The change of β3Gn-T5 transcript levels during differenti-ation of HL-60 cells by induction with RA or TPA. The expression level of the β3Gn-T5 transcript in HL-60 cells was quantified by competitive RT-PCR. The value was divided by that for the respective β-actin transcript. Non-treated HL-60 cells (○) are indicated at 0 days. HL-60 cells were cultured in the presence of 1 μM RA (■) or 8 nM TPA (●) for up to 4 or 2 days, respectively.

Fig. 6. Change of β3Gn-T5 transcript levels during differenti-ation of HL-60 cells by induction with RA or TPA. The expression level of the β3Gn-T5 transcript in HL-60 cells was quantified by competitive RT-PCR. The value was divided by that for the respective β-actin transcript. Non-treated HL-60 cells (○) are indicated at 0 days. HL-60 cells were cultured in the presence of 1 μM RA (■) or 8 nM TPA (●) for up to 4 or 2 days, respectively.

The homogenates of Namalwa-3GnT5 cells exhibited strong activity for both GSL substrates, LacCer and nLc4Cer, as well as toward LNnT-2AB and the two shorter polyolactosamine chains, 2LN-2AB and 3LN-2AB. This indicated that β3Gn-T5 effectively recognizes a polyolactosamine structure within two units of lactosamine. The marked reduction in the β3Gn-T5 activity for the longer polyolactosamine chain would suggest that GSLs with a long polyolactosamine chain, such as nLc4Cer, are not good substrates for β3Gn-T5. In previous studies (15, 20, 46), the activity of β3Gn-T toward LacCer and nLc4Cer was measured using tissue homogenates of rat brain during development. Both activities showed not only very similar profiles of change during the development, but almost the same level. This may be consistent with the present results showing that the two activities are directed by a single enzyme, β3Gn-T5. The wild-type and mock-transfected Namalwa cells showed weak β3Gn-T activity for nLc4Cer, but no activity for LacCer. We could not identify which enzyme directs this activity in the wild-type Namalwa cells. The activity may be directed by endogenous β3Gn-T2 or there may be some unknown β3Gn-Ts in the Namalwa cells.

The Lc3Cer synthase is a key enzyme in the expression of a series of neolactotryglycolipids, i.e. nLc4Cer and its derivatives. In particular, the expression level of two SGGLs, SGGL-1 and SGGL-2, carrying the HNK-1 epitope is determined by the Lc3Cer synthase (15, 19–22, 46). The change in the level of β3Gn-T5 transcript in developing rat brain almost paralleled that in Lc3Cer synthase activity reported previously (15, 20, 46). This strongly indicated that β3Gn-T5 is the Lc3Cer synthase.

To confirm this, we will examine whether or not β3Gn-T5 is co-localized with HNK-1 on SGGLs and CD15 on neolacto-series GSLs in a future study.

Lc3Cer synthase is also an important enzyme in hematopoietic cell differentiation. The changes in the level of β3Gn-T5 transcript during HL-60 differentiation shown in Fig. 6 are consistent with the results of Nakamura et al. (35). Stults et al. (36) reported that lymphoid cell lines lack Lc3Cer synthase activity, but possess nLc4Cer synthase activity, whereas myeloid cell lines express both activities. Almost all lymphoid cell lines we examined in this study showed very low or undetectable levels of β3Gn-T5 transcript, while HL-60 cells (promyelocytic leukemia) and U937 cells (monocyte-like) expressed substantial amounts of β3Gn-T5. This again supported that β3Gn-T5 is responsible for the synthesis of Lc3Cer in hematopoietic cells.

Holmes (50) reported the β3Gn-T activities for the synthesis of GSL in the cells homogenates of a human colon cancer cell line, SW403. The SW403 cell homogenates showed almost the same level of β3Gn-T activity toward two GSL acceptors, Lac-
Cer and nLc3Cer. This could be interpreted to mean that both activities were directed by a single enzyme, β3Gn-T5, as in the case of rat brain. In the present study, we demonstrated that recombinant β3Gn-T5 effectively catalyzes the biosynthesis of both Lc3Cer and nLc5Cer at almost the same efficiency. There is some controversy (52) over whether a single enzyme, β3Gn-T5, synthesizes both Lc3Cer and nLc5Cer in colon tissue. Based on the Basu study (52), there appear to be unknown β3Gn-Ts in colon tissue, which differentially catalyze the synthesis of Lc3Cer or nLc5Cer. Another controversy with the present study relates to the report by Holmes et al. (51) in which significantly high activity of Lc3Cer synthase was detected in colon adenocarcinoma tissues of patients and cell lines derived therefrom, but in contrast, the activity was undetectable in normal colonic epithelial cells. In the present study, all colonic adenocarcinoma cell lines expressed substantial amounts of β3Gn-T5 transcript. This is consistent with the results of Holmes et al. (51). However, normal colon tissue also expressed substantial amounts of the transcript. We will examine whether or not the β3Gn-T transcripts are markedly up-regulated in the colorectal cancer tissues of patients.

Regarding the other β3Gn-Ts examined in this study, β3Gn-T2 was most active toward polylactosamine acceptors, and it effectively extended the polylactosamine chain even on the longer chain acceptors. Thus, β3Gn-T2 is the most probable candidate for the enzyme which functions to extend the polylactosamine chain. β3Gn-T5 is involved only in the synthesis of short polylactosamine chains or initiation of polylactosamine synthesis. β3Gn-T3 and β3Gn-T4 exhibited very little activity, almost undetectable, toward all substrates examined. They apparently showed positive β3Gn-T activity in a previous study (1), because excess amounts of enzyme were used for the assay. The native acceptor substrates for β3Gn-T3 and -T4 may be different from the Gal residue acceptor, and some unknown native acceptors may exist for β3Gn-T3 and -T4.

The β3Gn-T (βGal-T) family is very large with 10 members, 1Gn-T, β3Gn-T1-5, and β3Gn-T2-5, having been cloned and analyzed to date. The possibility therefore exists that some as yet unidentified β3Gn-T(s) exhibits Lc3Cer synthase activity.

The results of the present study strongly indicated that β3Gn-T5 is the most feasible candidate for Lc3Cer synthase. In the future, we will assess the biological functions of this synthase, the key enzyme determining the expression of biologically functional epitopes on GSLs.

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