Molecular Cloning and Characterization of a Novel UDP-Gal:GalNAcO Peptide β1,3-Galactosyltransferase (C1Gal-T2), an Enzyme Synthesizing a Core 1 Structure of O-Glycan

Received for publication, June 12, 2002, and in revised form, September 9, 2002. Published, JBC Papers in Press, October 1, 2002, DOI 10.1074/jbc.M205839200.

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Recently, a UDP-Gal:GalNAc peptide β1,3-galactosyltransferase (core 1 synthase; C1Gal-T1) has been purified from rat liver and its complementary DNA cloned from several species. We isolated a second candidate for core 1 synthase from a Colo205 cDNA library and named it C1Gal-T2. The deduced amino acid sequence of C1Gal-T2, having 26% homology to C1Gal-T1, showed a topology typical of a type II membrane protein. Real time PCR analysis revealed that the expression of C1Gal-T2 transcripts was widespread in many tissues and of relatively high level in salivary gland, stomach, small intestine, kidney, testis, thymus, and spleen. LSC cells, having no core 1 synthase activity, were transfected stably with the C1Gal-T2 gene. Their microscopic fraction showed β1,3-galactosyltransferase activity toward GalNAc-o-paranitrophenyl and GalNAc1 peptides resulting in the synthesis of the core 1 structure. The core 1 synthesizing activity of C1Gal-T2 was also determined by flow cytometry and lectin blotting using the LSC cells stably expressing C1Gal-T2. Finally, LSC cells, and Jurkat cells that also lack the core 1 synthase activity, were found to have null alleles of C1Gal-T2. These results indicated that C1Gal-T2 is the second candidate for core 1 synthase that plays an important role in synthesizing O-glycans in digestive organs.

The addition of GalNAc to serine or threonine residues on proteins by UDP-GalNAc:polypeptide N-acetylgalactosaminyltransferases (pp-GalNAc-Ts) initiates the biosynthesis of O-glycans. The GalNAc residue attached to the peptide is usually modified with further glycosylation extended by the action of multiple glycosyltransferases. O-Glycans can be classified into several different groups according to their core structure. There are at least eight such core structures as summarized in our previous paper (1). Core 1, Galβ1–3GalNAc1-serine/threonine, is the major constituent of O-glycan core structures in many cells. The core 1 structure is also called Thomsen-Friedenreich antigen (T antigen) in pathological studies. Core 1 β1,3,galactosyltransferases (C1Gal-T, core 1 synthase, EC 2.4.1.122) transfer a galactose from UDP-Gal to a GalNAc residue on proteins with a β1,3-linkage to synthesize the core 1 structure. It was predicted that there are multiple C1Gal-Ts that differentially recognize the peptide sequences (2, 3). The T antigen is cryptic because it is covalently or structurally masked by more glycosylation and nonimmunoreactive on the surfaces of healthy cells in most tissues. However, it is exposed and becomes immunoreactive on most human cancer cells and T-cell lymphomas (4, 5). Interaction between the T antigen and β-galactoside-binding lectins, such as galectins, has been implicated in tumor cell adhesion and tissue invasion (6, 7). The sialyl Tn (sTn) antigen is synthesized by the action of N-acetylgalactosamine, α2,6-sialyltransferase (ST6GalNAc I), which transfers a sialic acid to GalNAc residues on proteins with an α2,6-linkage (8). These three antigens of the O-glycan core structure, Tn, T, and sTn, have been defined as cancer-associated antigens in many cancer cells (9). It was demonstrated that the expression of these antigens correlated with the clinicopathological parameters and survival probability of cancer patients (9).

IgA nephropathy (IgAN) is a common disease characterized by polymeric IgA1 deposits in the renal glomerular mesangium (10). The hinge portion of human serum IgA1 possesses five O-glycans, all of which are sialylated core 1 structures (11). Many reports confirm that the O-glycans in the IgA1 hinge region of IgAN patient's serum are underglycosylated and have incomplete structures, such as asialo- and/or asialoagalacto-O-glycans (12–14). Allen et al. (15) reported that the activity of UDP-Gal:GalNAcO peptide β1,3-galactosyltransferase initiates the biosynthesis of O-glycans.

* This work was performed as a part of the R&D Project of Industrial Science and Technology Frontier Program (R&D for Establishment and Utilization of a Technical Infrastructure for Japanese Industry) and was supported by the New Energy and Industrial Technology Development Organization. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EMBL Data Bank with accession number(s) AB084170.

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‡‡ The abbreviations used are: GalNAc-T, N-acetylgalactosaminyltransferase; C1Gal-T, core 1 β1,3-galactosyltransferase; EST, expressed sequence tag; ORF, open reading frame; HPTLC, high performance thin layer chromatography; HPLC, high performance liquid chromatography; core 1 (T antigen), Galβ1–3GalNAc1-serine/threonine; GalNAc1-serine/threonine; sTn antigen, NeuAc2–6GalNAc1-serine/threonine; NeuAc, neuraminic acid (sialic acid); pNP, para-nitrophenyl; FITC, fluorescein isothiocyanate; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; UTR, untranslated region; MES, 4-morpholineethanesulfonic acid; RT, reverse transcriptase; mAb, monoclonal antibody; IgAN, IgA nephropathy; PNA, peanut agglutinin.
C1Gal-T1 in homogenates of B cells derived from IgAN patients is down-regulated compared with that of healthy individuals. The idiopathic Tn syndrome, a rare hematological disorder, is characterized by the expression of the Tn antigen on all blood lineage cells (16). The aberrant expression of Tn antigen is caused by an unmasking of T antigen due to down-regulation of C1Gal-T activity. Thus, C1Gal-T(s) are important enzymes that are involved in human disease (17).

Recently, a C1Gal-T has been purified from rat liver membranes (17) and its cDNA cloned for the first time (18). The human, mouse, Drosophila melanogaster, and Caenorhabditis elegans genes orthologous to the rat C1Gal-T gene have also been cloned (18).

We found an EST sequence that encodes a partial cDNA sequence with significant homology to the C1Gal-T1 sequence. In the present study, we successfully cloned a novel gene encoding a second human core 1 β,3-galactosyltransferase and named it C1Gal-T2. LSC cells were chosen as host cells to be transfected with the C1Gal-T2 gene, because they lack C1Gal-T activity endogenously. We analyzed and characterized C1Gal-T2 using microsize fractions of the LSC cells transfected stably with the C1Gal-T2 gene.

**EXPERIMENTAL PROCEDURES**

**In Silico Cloning of cDNA Encoding Human C1Gal-T2**—We performed a BLAST search of the DDBJ database containing human cDNA, EST, and genome sequences, and we identified a cDNA homologous to the amino acid sequence of C1Gal-T1 (18). A number of cDNAs matching part of the sequence of the expected full-length open reading frame (ORF) were found. The partial cDNA sequence (GenBank accession number AT555552), which encodes a putative catalytic region of a novel candidate, was amplified by RT-PCR from Colo205 cDNA, using a forward primer 5′-GAACCATTCTAAGCTTTGTACAAGAAAGCTGGGTCTCAATCATTGTCA-3′ and a reverse primer 5′-GGGGACCACTTTGTACAAGAAAGCTGGGTCTCAATCATTGTCA-3′. The amplified fragment was used as a probe for hybridization to isolate a full-length cDNA clone. We screened the λ ZAPII Colo205 cDNA library constructed in our previous study (8), and we isolated a clone that we named pBS-C1Gal-T2. The 1471-bp insert was subjected to nucleotide sequencing.

**Construction of Vectors for Stable Expression of C1Gal-T1 and C1Gal-T2 in Mammalian Cultured Cells**—The fragment of the ORF encoding C1Gal-T2 was amplified by PCR using pBS-C1Gal-T2 as a template. The PCR was performed with Platinum® Pfx DNA polymerase (Invitrogen). The forward and reverse primer sequences were flanked with attB1 and attB2 sequences, respectively, to create the recombination sites. The forward primer for C1Gal-T2 gene was 5′-GGGACCACTTTGTACAAGCTTTGTACAAGAAAGCTGGGTCTCAATCATTGTCA-3′, and the reverse primer was 5′-GGGGACCACTTTGTACAAGCTTTGTACAAGAAAGCTGGGTCTCAATCATTGTCA-3′. The amplification fragment was used as a template for the amplification and sequencing of cDNAs using primers attP1 and attP2. After digestion of the DNA with BamHI and XhoI, the insert was ligated into the expression vector pDEST12.2 mammalian expression vector using the Gateway system (Invitrogen). The plasmids were named pDEST12.2-C1Gal-T1 and pDEST12.2-C1Gal-T2, respectively.

**Cell Culture and Transfection**—The Jurkat and K562 cell lines were purchased from the ATCC. LSB and LSC cells were kindly provided by Dr. S. Yanai (17). LSB cells, a human colorectal cancer cell line, were transfected with pDEST12.2-C1Gal-T1 or pDEST12.2-C1Gal-T2 expression plasmid DNA using LipofectAMINE 2000 reagent (Invitrogen). The cells were selected in the presence of genetin (0.6 mg/ml) (Invitrogen) in RPMI 1640 medium (Invitrogen) supplemented with 10% heat-inactivated fetal bovine serum. After 3 weeks of exposure to genetin, the cells stably expressing C1Gal-T1 or C1Gal-T2 were subjected to several different assays.

**Flow Cytometric Analysis**—For flow cytometry, the cells were incubated with each monoclonal antibody (mAb) or lectin. The anti-Tn mAb, HB T1 (IgM), and anti-Tn mAb, HB STn1 (IgG1), were both purchased from Dako (Kyoto, Japan), whereas fluorescein isothiocyanate (FITC)-conjugated peanut agglutinin (PNA) lectin was from EY Laboratories, Inc. After incubation with the first antibody, the cells were stained with FITC-conjugated goat anti-mouse IgG or IgM (ICN Pharmaceuticals, Inc.) and then subjected to flow cytometric analysis with a FACSCalibur (BD Biosciences).

**Assay of Galactosyltransferase Activity toward GalNAc Using High Performance Thin Layer Chromatography (HPTLC)**—Galactosyltransferase (Gal-T) activity was assayed in a 20-μl reaction mixture containing 14 mM HEPES buffer (pH 7.4), 12.5 mM MgCl2, 250 μM UDP-Gal (Sigma), 175 nCi of UDP-[3H]Gal (Amer sham Biosciences), 10 mM of GalNAc-α-βpNP or GalNAc-β-βpNP (Calbiochem), and the enzyme source. C1Gal-T enzymes were used as an enzyme source for the HPTLC assay. The cells were solubilized in 20 mM HEPES (pH 7.4), 154 mM NaCl, and 1% Triton X-100 by brief sonication. After incubation at 37 °C for 2 h, the reaction was terminated by adding 200 μl of water. The enzyme product, [14C]Gal-GalNAc-pNP, was separated from free UDP-[3H]Gal using a Sep-Pak Plus C18 cartridge (Water, Milford, MA) and subjected to HPTLC analysis as described in detail previously (1).

**Assay of Galactosyltransferase Activity Using HPLC**—GalNAc-α-βpNP (0.25 mM) was incubated in a 20-μl reaction mixture containing 50 mM MES buffer (pH 6.5), 20 mM MgCl2, 2 mM ATP, 1 mM UDP-Gal, and cell homogenates. After incubation at 37 °C for 2 h, the supernatant of the reaction mixture was filtered with Ultrafree-MC (1.0 μm, Millipore Corp.) and then subjected to HPLC on an ODS-80Ts AQ column (4.6 × 250 mm; Tosoh, Tokyo, Japan). The reaction products were eluted with 12% acetonitrile in water containing 0.1% trifluoroacetic acid at a flow rate of 1.0 ml/min at 40 °C and monitored with an ultraviolet spectrophotometer (absorbance at 210 nm), SPD-10AVP (Shimadzu, Kyoto, Japan).

**Subcellular Fractionation of Cell Lines**—The cells were harvested and suspended in 1 ml of ice-cold buffer containing 0.25% sucrose, 10 mM Tris-HCl (pH 7.4), and 100 μM of protease inhibitor mixture (Sigma). Suspensions containing 1 × 106 cells were homogenized by a Dounce homogenizer. The cell homogenate was centrifuged at 1,000 × g for 10 min to remove cell debris. The supernatant was centrifuged at 100,000 × g for 1 h at 4 °C. Microsome fractions thus obtained were resuspended in 0.25% sucrose and 10 mM Tris-HCl (pH 7.4), and used as an enzyme source. All procedures were carried out at 4 °C.

**Galactosyltransferase Activity Assay on GalNAc Peptides**—An acceptor peptide of the human IgA1 hinge region having a GalNAc residue at the 14th serine (11-GalNAc-HP; 5′TPSTTPPSFS-GalNAcTTPPSFS) was purchased from the Peptide Institute (Osaka, Japan). 11-GalNAc-HP was labeled with C14 by using 14 C-labeled UDP-Gal (Amersham Biosciences). After incubation at 37 °C for 2 h, the supernatant of the reaction mixture was separated from free UDP-[14C]Gal by brief sonication. After incubation at 37 °C for 2 h, the supernatant of the reaction mixture was separated from free UDP-[14C]Gal by brief sonication. After incubation at 37 °C for 2 h, the supernatant of the reaction mixture was separated from free UDP-[14C]Gal by brief sonication.
as follows: the forward primer was 5'-GCTTCTGCTGCCCAAGGCGCTTCC-3', and the reverse primer was 5'-GCCCAAGATTCTCTAATGGTTC-3'. Additional primers for the sequencing of C1Gal-T1 cDNA were 5'-AGAAGCTTGAAGATTTGTTGATG-3' and 5'-ATTACCTTGTGTTATCATGATTCTT-3'. Additional primers for the sequencing of C1Gal-T2 cDNA were 5'-TCAATTCATGTTGAAACC-3' and 5'-TGTGACGCCCCACATCTGTGTCG-3'.

Quantitative Analysis of C1Gal-T1 and C1Gal-T2 Transcripts in Human Tissues Using the Real Time PCR—We employed the real time PCR method for the measurement of C1Gal-T1 and C1Gal-T2 transcripts, as described in detail previously (1). Marathon Ready™ cDNAs derived from various human tissues and cells were purchased from BD Biosciences, Clontech. Standard curves for C1Gal-T1, C1Gal-T2, and an endogenous control, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNAs, were generated by serial dilutions of a pDEST12.2 vector containing the C1Gal-T1 or C1Gal-T2 cDNA and a pCR2.1 vector (Invitrogen) containing the GAPDH cDNA. The primer set and probe for C1Gal-T1 were as follows: the forward primer was 5'-CTGAAGTGGTTTGATGATAGCTAC-3' and the reverse primer was 5'-ACATAGTGAAAAGAAACTGCAAGATCA-3'. The probe set was 5'–CCTCCTGTAGAAGGCTCTGTTGCTGCT-3' with a minor groove binder (20). The primer set and probe for C1Gal-T2 were as follows: the forward primer was 5'-GTTTGGCTGAAATATGCTGGAGTAT-3' and the reverse primer was 5'-AGAAGCTTGAAGATTTGTTGATG-3'. The probe was 5'-CAGAATAAGTGAGATGCTGATGGAAAGATGTA-3' with a minor groove binder.

RESULTS

Isolation of a Novel Human cDNA Clone That Is a Candidate Encoding Core 1 β1,3-Galactosyltransferase (C1Gal-T)—As described under “Experimental Procedures,” we cloned a novel candidate cDNA for C1Gal-T which has a 1,471-bp insert and encodes a full-length ORF. This cDNA clone, named C1Gal-T2, consisted of a 104-bp 5'-untranslated region (UTR), a 957-bp coding region, and a 410-bp 3'-UTR that did not contain a poly(A) tail (Fig. 1A). A hydropathy profile of the putative amino acid sequence based on Kyte and Doolittle hydrophobicity plots indicates the ORF encodes a typical type II membrane protein, which is consistent with the topology of other glycosyltransferases, with a cytoplasmic tail of 10 amino acids, a transmembrane domain of 20 amino acids, and a large catalytic portion of 288 amino acids (Fig. 1A). The cDNA sequence of C1Gal-T2 was compared with a draft genome sequence. The same sequence was found in a clone, GenBank™ accession number AC011890, which is localized on chromosome X at q23. This is the first glycosyltransferase gene to be found on the sex chromosomes. The genomic structure of the C1Gal-T2 gene was determined (Fig. 1B). The C1Gal-T2 gene contains at least two exons, and the ORF was found to be encoded by a single exon. Exon 2 started 5 bp upstream from A and the intron sequences at the intron-exon junctions complied with the acceptor and donor site sequences of the splicing rule, i.e. GT-AG rule.

The amino acid sequence in a putative catalytic region, 288 residues from the C-terminal end of C1Gal-T2, showed only 26% homology to that of human C1Gal-T1; however, all 7 cysteines were conserved in both amino acid sequences (Fig. 2). In a previous study (17, 18), four motifs that were found by a comparison of C1Gal-T1 with other β3Gal-Ts were proposed. However, the four were not highly conserved between C1Gal-T1 and -T2 (Fig. 2). A possible divalent cation-binding site, the DXD motif, was found at two positions in each C1Gal-T, but the position was not aligned in the two enzymes.

Galactosyltransferase Activity of C1Gal-T1 and C1Gal-T2 toward GalNAc-α-pNP and GalNAc-β-pNP and Transcript Levels of Two C1Gal-Ts—LSB and LSC are clonal cell lines, both of which have been derived from LS174T human colon cancer cells. In a previous study (21), it was demonstrated that LSC cells express only the truncated carbohydrate antigen Tn (GalNAc α1-Ser/Thr) and sTn on their mucin molecules because of a lack of C1Gal-T activity, whereas LSB cells, having C1Gal-T activity, express elongated oligosaccharide chains. We chose LSC cells as host cells to be transfected with the C1Gal-T2 gene. LSB cells stably expressing C1Gal-T1 or C1Gal-T2 were established as described under “Experimental Procedures” and named LSC-C1Gal-T1 or LSC-C1Gal-T2 cells, respectively. Concurrently, mock LSC transfected cells were prepared and named LSC-mock cells. The galactose transfer activity of the cell homogenates of seven cell lines, i.e. LSB, LSC, LSC-mock, LSC-C1Gal-T1, LSC-C1Gal-T2, K562, and Jurkat, toward GalNAc-α-pNP and GalNAc-β-pNP was analyzed using the HPTLC assay. As seen in Fig. 3A, the homogenates of LSB, as a positive control, showed a positive band toward GalNAc-α-pNP. K562 gave the same positive band as LSB, but Jurkat, LSC, LSC-mock, and LSC-C1Gal-T1 did not. The homogenates of LSC-C1Gal-T2 cells exhibited significant Gal-T activity toward GalNAc-α-pNP, almost equal to that of LSB cells. However, none of the seven cell homogenates exhibited activity toward GalNAc-β-pNP (data not shown).

The transcript levels for the two enzymes in the cells were determined by real time PCR. In LSB, LSC, and LSC-mock cells, both transcripts, C1Gal-T1 and -T2, were expressed almost at the same level.

The expression of C1Gal-T1 transcript in LSC-C1Gal-T1 cells was increased to ~2–3 times that of LSB and LSC cells. LSC-C1Gal-T2 cells expressed ~1.5 times and twice as much C1Gal-T2 transcript as LSB and LSC cells, respectively. The expression level of C1Gal-T2 in the stable transfec tant, LSC-C1Gal-T2, was in the physiological range, and there was no overexpression. Two leukemia cell lines, K562 and Jurkat cells, expressed both transcripts at relatively higher levels than LSB and LSC cells.

The enzyme reaction was carried out to measure the relative β3Gal-T activity toward GalNAc-α-pNP using equal amounts of homogenates of LSC-C1Gal-T1 and LSC-C1Gal-T2 cells. The reaction products were subjected to HPLC analysis because the HPLC analysis could detect the products more sensitively than HPTLC analysis. A commercially available compound, Galβ1-3GalNAc-α-pNP (core 1-pNP), was used as the standard to estimate the β3-linkage structure (Fig. 4A). The peaks of both enzyme products shifted from the original substrate peak to the position of core 1-pNP (Fig. 4, B and C). Although LSC-C1Gal-T1 showed β3Gal-T activity, its relative activity was only 2% that of LSC-C1Gal-T2. This result demonstrated that C1Gal-T2 expressed in LSC cells exhibits 50 times stronger core 1 synthesizing activity toward GalNAc-α-pNP than C1Gal-T1.

Core 1 Synthesizing Activity of C1Gal-T2 for GalNAc Peptides—GalNAc residues and, in particular, the GalNAc moiety of Muc1a-FITC, were used as acceptor substrates for the assay of core 1 synthesizing activity. The cell homogenates were inappropriate for this assay, because they contained proteases that degraded the acceptor peptides. Therefore, microsome fractions were separated from the cells and used as an enzyme source to avoid degradation of the acceptor peptides.

Representative HPLC profiles of reaction products of C1Gal-T2 are shown in Fig. 5. The retention time of the substrate (peak “S” in Fig. 5), 11-GalNAc-HP-Cy5 and GalNAc-Muc1a′-FITC, were used as acceptor substrates for the assay of core 1 synthesizing activity. The cell homogenates were inappropriate for this assay, because they contained proteases that degraded the acceptor peptides. Therefore, microsome fractions were separated from the cells and used as an enzyme source to avoid degradation of the acceptor peptides.
matrix-assisted laser desorption ionization time-of-flight mass analysis (data not shown). They were confirmed to be mono-galactosylated GalNAc peptides. The microsome fractions of LSB produced a reaction product in the presence of both substrates that had the same retention time as did the reaction product of LSC-C1Gal-T2 (data not shown).

Digestion of the reaction products with a 1,3-linkage-specific galactosidase from B. circulans resulted in the disappearance of P and a shift back to the original position of the substrate. This strongly indicated that C1Gal-T2 transferred Gal to GalNAc with a 1,3-linkage to synthesize the core 1 structure on the peptides (Fig. 5, C and F).

Flow Cytometric Analysis of LSC-C1Gal-T2 Cells—LSC-mock, LSC-C1Gal-T1, and C1Ga-T2 cells were stained with PNA lectin, HB-STn1 (anti-sTn), and HB-T1 (anti-Tn) antibodies and analyzed by flow cytometry (Fig. 6). LSC-mock cells showed positive profiles for both sTn and Tn antigens but were not recognized by PNA lectin. LSC-C1Gal-T2 cells apparently showed positive staining with PNA lectin compared with the negative staining of LSC-mock cells (Fig. 6A). LSC-C1Gal-T2 cells showed significantly less reac-
activity against sTn and Tn antibodies than LSC-mock cells (Fig. 6, B and C). These results strongly indicated the following points. 1) C1Gal-T2 synthesized the core 1 structure which is recognized by PNA lectin. 2) C1Gal-T2 competed with the synthesis of sTn directed by ST6GalNAc(s) to decrease the expression of sTn antigen. 3) Tn antigen was masked by the addition of Gal to GalNAc through the core 1 synthesizing activity of C1Gal-T2.

PNA Lectin Blotting of the Cell Lysates of LSC-C1Gal-T2—PNA lectin blot analysis was performed to identify the molecular sizes of proteins carrying the core 1 structure. As seen in Fig. 7, the homogenates of three cells, LSB, LSC, and LSC-C1Gal-T2, were stained with PNA. LSB cells showed positive staining of smear bands in a range of higher molecular masses, above 100 kDa, in addition to the three discrete bands that were shared by the three cell homogenates. The smear bands of higher molecular weight, probably mucins carrying the core 1 structure, were also detected in LSC-C1Gal-T2 cells but not in LSC cells. Thus, transfection of the C1Gal-T2 gene into LSC cells yielded the PNA-reactive epitope, core 1 epitope, on mucin-like molecules, which are similar to the molecules detected in LSB cells.

LSC and Jurkat Cells Lack C1Gal-T Activity Due to Mutation in the C1Gal-T2 Gene—LSC and Jurkat cells have been reported to lack C1Gal-T activity and to express Tn antigen because of the exposure of GalNAc residues on peptides (21, 22). Neither cell exhibited C1Gal-T activity toward GalNAc-o-\(\rho\)Np, whereas LSB and K562 cells exhibited the activity, as demonstrated in the previous section of this study (Fig. 3). Complementary DNAs encoding C1Gal-T1 or -T2 were amplified by RT-PCR from K562, LSB, LSC, and Jurkat cells and were directly sequenced. As summarized in Fig. 8, the cDNA sequences of C1Gal-T1 from the four cell lines, LSB, LSC, K562, and Jurkat cells, were determined to be identical to the C1Gal-T1 sequence registered by others (18). The cDNA sequence of C1Gal-T2 from LSB and K562 cells was identical to that of Colo205 cells which was demonstrated to encode an active enzyme in this study. Interestingly, the cDNA sequence

![Molecular Cloning of C1Gal-T2](http://www.jbc.org/Downloadedfrom)
of C1Gal-T2 from LSC and Jurkat cells was found to possess mutations in its ORF. The C1Gal-T2 cDNA from LSC cells possessed a single nucleotide insertion of T between T53 and C54 which leads to termination of the C1Gal-T2 translation (Fig. 8, A and B). The C1Gal-T2 cDNA from Jurkat cells had a missense mutation of C428T and a single nucleotide deletion of T568 that also leads to termination of the C1Gal-T2 translation (Fig. 8, A and C). The origin of Jurkat cells is a male patient. Therefore, it is reasonable that we did not detect heterozygosity of the C1Gal-T2 sequence. The origin of LSC cells is unknown. However, we speculate that they were derived from a male patient because the mutant allele (insertion of T at 53) possessed a single nucleotide insertion of T between T53 and C54) found in the C1Gal-T2 gene. We proposed a molecular cloning of C1Gal-T2.

**Tissue Distribution and Quantitative Measurement of C1Gal-T2 Transcripts**—The amount of transcript for the C1Gal-T2 gene was determined in various human tissues and cell lines by real time PCR. The expression level of C1Gal-T2 was shown as relative to that of GAPDH transcript. The amount of transcript for the C1Gal-T2 gene was determined in various human tissues and cell lines by real time PCR. The expression level of C1Gal-T2 was shown as relative to that of GAPDH transcript. The amount of transcript for the C1Gal-T2 gene was determined in various human tissues and cell lines by real time PCR. The expression level of C1Gal-T2 was shown as relative to that of GAPDH transcript.

**DISCUSSION**

Core 1 synthase (C1Gal-T) is an important enzyme in the synthesis of mucin-type O-glycans in most cells. There may be multiple C1Gal-Ts in vertebrates, based on the enzymic activities detected in their tissues (3, 23). C1Gal-T1, the first core 1 synthase to be identified, was originally purified from rat liver though orthologous genes and has since been cloned from various species (17, 18). The present study describes the isolation of a novel human cDNA encoding a core 1 β1,3-galactosidase, named C1Gal-T2, by searching a data base for cDNAs homologous to human C1Gal-T1.

We proposed a β3-glycosyltransferase (β3GT) family in the previous study (1). This family consists of β3Gal-Ts (β3Gal-T1,2,4–6) (24–26), β3GlcNAc-Ts (β3Gn-T2–6) (1, 27, 28), and β3GnAc-T (β3GalNAc-T1/β3Gal-T3; globoside synthase) (29), all of which share amino acid motifs in three regions of the catalytic domain (25). Although each member differs in its specificity for donor and acceptor substrates, all exhibit activity to form a β1,3-linkage. Ju et al. (18) proposed the existence of four motifs, VKXTW, DXDXF, GXXG(Y/V/I)XS, and DLXXG, based on a comparison of C1Gal-T1 with other β3GTs reported previously. Three of the motifs aligned at the same positions as those proposed by us to be conserved in the β3GT family (1, 25). The motifs proposed by Ju et al. (18) were not so well conserved between C1Gal-T1 and -T2, as shown in Fig. 2. More suitable motifs will be determined when additional members of the C1GalT family are identified. Two DXD sequences, possible binding sites of divalent cations, exist in both C1Gal-T1 and -T2; however, their positions were not aligned.

**Fig. 5**. HPLC analysis of the products derived from 11-GalNAc-HP and GalNAc-Muc1a’ peptides. The left column, A–C, shows profiles of the products derived from Cy5-labeled 11-GalNAc-HP (11-GalNAc-HP-Cy5; VPSTPPTSPSPS-GalNAc/TTPTPSPS-Cy5), and the right column, D–F, shows profiles of the products derived from FITC-labeled GalNAc-Muc1a’ peptide (GalNAc-Muc1a’-FITC; FITC-AHGVT(-GalNAc)/H11032). The elution position of 11-GalNAc-HP-Cy5 or GalNAc-Muc1a’-FITC is presented as peak S. The reaction products are indicated with P in B or E. The HPLC profiles after digestion with β1,3-galactosidase are shown in C and F.

**Fig. 6**. Flow cytometric analysis of LSC cells transfected with the C1Gal-T2 gene. The expression of T-related antigens on the surface of mock-transfected LSC cells (LSC-mock) and LSC cells stably expressing C1Gal-T2 (LSC-C1Gal-T2) was analyzed by flow cytometry. LSC-mock or LSC-C1Gal-T2 cells are indicated with a thin or thick line, respectively, in each panel. The cells were stained with PNA lectin (A), anti-sTn (B), and anti-Tn (C).
between the two enzymes. C1Gal-T2 failed to show core 1 synthesizing activity without Mn\(^{2+}\) (data not shown); therefore, one of the DXD sequences of C1Gal-T2 may function in binding divalent cations. C1Gal-T2 shares all 7 Cys residues with C1Gal-T1, indicating that the two enzymes retain a similar tertiary structure, although these Cys residues are not shared by other enzymes of the β3GT family. The ORFs of all members, including C1Gal-T2, were found to be encoded by a single exon except for C1Gal-T1. The cDNA sequence of C1Gal-T2 in this study was still incomplete, lacking a poly(A) tail. We searched for EST sequences to cover the missing 3'-UTR sequence in our clone, and we obtained an additional sequence (GenBank accession number BC011930) which contained a poly(A) tail. However, the 3'-UTR of this sequence was 250 bp shorter than that of our clone. There are at least two isoforms of C1Gal-T2 transcripts that differ in the length of the 3'-UTR.

C1Gal-T2 could not transfer Gal to GalNAc-β-pNp, which is the minimal unit for ganglioside synthesis (GD1b/GM1/GA1). In fact, C1Gal-T2 could not synthesize GD1b, GM1, or GA1 (data not shown). C1Gal-T1 also revealed Gal-T activity against GalNAc-α-pNp (18). Thus, C1Gal-T1 and -T2 can recognize the GalNAc-α-structure at a minimum. In this study, we detected transcripts of both C1Gal-T1 and -T2 in LSB and LSC cells by RT-PCR. LSB cells had active transcripts for C1Gal-T1 and -T2, whereas LSC and Jurkat cells were found to be mutant cells expressing inactive transcripts for C1Gal-T2 but possessing active transcripts for C1Gal-T1, i.e. the same sequence as reported by others (18). This strongly indicated that the activity for core 1 synthesis toward GalNAc-α-pNp in Fig. 3 is directed by C1Gal-T2 and not by C1Gal-T1. As demonstrated in Fig. 4, the level of β3Gal-T activity of C1Gal-T1 for GalNAc-α-pNp was quite low in comparison to that of C1Gal-T2. This is why C1Gal-T1 expressed in LSB and LSC cells did not synthesize the core 1 structure toward GalNAc-α-pNp (Fig. 3).

Flow cytometric analysis was performed on Jurkat cells (data not shown). They were strongly stained with HB-T1 (anti-Tn), faintly stained with HB-STn (anti-sTn), but not with PNA. Jurkat cells expressed a considerable amount of C1Gal-T1 and -T2 transcripts (Fig. 3B); however, the C1Gal-T1

**FIG. 7.** PNA lectin blot analysis. The expression of T antigen in cell lysates of LSB, LSC, and LSC-C1Gal-T2 was analyzed by lectin blotting using horseradish peroxidase-conjugated PNA.

**FIG. 8.** Detection of mutations in cDNAs encoding C1Gal-T2 in LSC and Jurkat cells which lack the core 1 synthesizing activity. A, schematic diagrams of wild-type and mutant C1Gal-T2 alleles in various cell lines. Open columns represent the original sequence encoded by the C1Gal-T2 cDNA derived from Colo205 cells. Hatched columns represent amino acid sequences different from the original due to a frameshift. Closed columns represent untranslated regions after the termination codon. B, insertion of T between the 53rd and 54th nucleotide in the C1Gal-T2 cDNA of LSC cells. C, missense mutation from C428 to T428 (left panel) and deletion of T 468 (right panel) in the C1Gal-T2 cDNA of Jurkat cells.

**FIG. 9.** Quantitative analysis of C1Gal-T2 transcripts in various human tissues by real time PCR. Standard curves for the transcripts of C1Gal-T2 and GAPDH were generated by serial dilution of each plasmid DNA. The expression level of C1Gal-T2 transcripts was normalized to that of the GAPDH transcripts. Both transcripts were measured in the same cDNAs. Data were obtained from triplicate experiments and are indicated as the mean ± S.D.
and -T2 transcripts of Jurkat cells were found to be active and inactive, respectively (Fig. 8). The homogenates of Jurkat cells did not show the Gal-T activity toward GalNAc-α-pNp (Fig. 3). Taken together, the lack of core 1 structure in Jurkat cells is attributed to the inactivation of C1Gal-T2 by the T458 deletion.

The two GalNAc peptides, 11-GalNAc-HP-Cy5 and GalNAc-Muclα-FITC, examined in this study were good acceptors for C1Gal-T2, indicating that it is actually involved in the core 1 synthesis of IgA1 and MUC1 in the cells. We will further examine the substrate specificities of C1Gal-T2 using a variety of GalNAc peptides.

LSC-C1Gal-T2 cells expressed C1Gal-T2 at a physiological level, similar to the level in LSb cells. In a previous study (8), we reported that the active form of ST6GalNAc I, an enzyme responsible for the synthesis of sTn, is expressed to synthesize sTn antigen in LSC cells. The obvious decrease in the expression of sTn antigen in LSC-C1Gal-T2 cells suggested that C1Gal-T2 physiologically competes with ST6GalNAc I for the acceptors in the cells.

The PNA-reactive epitope apparently increased in LSC-C1Gal-T2 cells as detected by both flow cytometry and PNA blotting. The blotting experiment showed that the intensity of sTn in LSC-C1Gal-T2 is almost the same as that of LSb cells. This indicated that many O-glycans in LSC-C1Gal-T2 cells are not terminated at the length of the T antigen but are elongated further by more glycosylation to reach almost the same length as those in LSb cells. In this sense, LSC-C1Gal-T2 cells are the cells that revert to the wild-type LSb cells.

The C1Gal-T2 transcript was highly expressed in digestive organs, such as salivary gland, stomach, and small intestine (Fig. 9). The expression level of C1Gal-T1 transcripts was reported to be low in small intestine (18). Thus, C1Gal-T2 is the most likely candidate for the core 1 synthase on mucin in digestive organs. The C1Gal-T1 transcript was highly expressed in heart, liver, skeletal muscle, and placenta on Northern blot analysis (18). In contrast, the C1Gal-T2 transcripts were faintly expressed in such tissues as determined in this study (Fig. 9). The difference in expression pattern between C1Gal-T1 and C1Gal-T2 transcripts may concern the core 1 synthesis regulated in a tissue-specific or peptide sequence-specific manner.

In a preliminary experiment, IgA+ lymphocytes in peripheral blood expressed a considerable amount of C1Gal-T2, indicating that this enzyme is also responsible for core 1 synthesis on IgA.

It was reported that the core 1 synthase activity decreased in B cells from patients with IgAN (15), and in Tn antigen-positive T lymphocytes of Tn syndrome patients (30). Core 1 synthase(s) might be down-regulated transcriptionally or post-transcriptionally in a cell type-specific manner in such patients. It will be important to determine whether C1Gal-T1, C1Gal-T2, or an unknown core 1 synthase is a key enzyme for the undergalactosylation in IgAN or Tn syndrome. We are currently determining the substrate specificities of each C1Gal-T using glycopeptides or glycoproteins accounting for these diseases.

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Molecular Cloning and Characterization of a Novel UDP-Gal:GalNAcα Peptide β 1,3-Galactosyltransferase (C1Gal-T2), an Enzyme Synthesizing a Core 1 Structure of O-Glycan

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J. Biol. Chem. 2002, 277:47724-47731.
doi: 10.1074/jbc.M205839200 originally published online October 1, 2002

Access the most updated version of this article at doi: 10.1074/jbc.M205839200

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Molecular cloning and characterization of a novel UDP-Gal:GalNAc peptide β1,3-galactosyltransferase (C1Gal-T2), an enzyme synthesizing a core 1 structure of O-glycan.

Takashi Kudo, Toshie Iwai, Tomomi Kubota, Hiroko Iwasaki, Yuko Takayama, Toru Hiruma, Niro Inaba, Yan Zhang, Masanori Gotoh, Akira Togayachi, and Hisashi Narimatsu

We previously reported what we thought was a second core 1 β3-galactosyltransferase or C1Gal-T2 (GenBank™ accession number AF155582) (1) based on the ability of this gene to complement cell lines lacking the activity of the previously identified and characterized core 1 β3-galactosyltransferase or T-synthase (2, 3). However, it was shown by Ju and Cummings (4, 5) that the gene encoding what we thought to be C1Gal-T2 in fact encodes a novel and unexpected molecular chaperone, termed Cosmc, that is required for expression of the T-synthase. The cell lines used in our previous studies, which lack the T-synthase activity, carry mutations in the X-linked gene encoding Cosmc, which we reported as being mutations in the C1Gal-T2 gene.

Although we mistakenly interpreted our experiments as indicating that this gene was a second core 1 β3-galactosyltransferase, the fundamental experimental data remain sound.

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Ligands for the β-glucan receptor, Dectin-1, assigned using “designer” microarrays of oligosaccharide probes (neoglycolipids) generated from glucan polysaccharides.

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The first line of the grant support footnote should be corrected to read as follows: This work was supported by United Kingdom (UK) Medical Research Council Programme Grant G9601454 (to A. M. L. and T. F.) and a UK Glycochips Consortium grant from the UK Research Council Basic Technology Programme (to T. F.)
Dynamic association between the catalytic and lectin domains of human UDP-GalNAc:polypeptide α-N-acetylgalactosaminyltransferase-2.

Timothy A. Fritz, Jayalakshmi Raman, and Lawrence A. Tabak

After the publication of the above referenced paper, during the course of a new study we encountered difficulty in obtaining the peptide sequence for one of the substrates (termed Muc5Ac-13) used in the above referenced paper. We investigated the reasons for this further. Using specific exoglycosidase digestion, we determined that this Muc5Ac-13 peptide contained an N-acetylgalactosamine residue in $\beta$ and not $\alpha$ anomeric linkage with Thr$^{13}$. All remaining substrates were checked and found to be bona fide (i.e. containing N-acetylgalactosamine only in $\alpha$ anomeric linkage). We have now determined kinetic constants with the authentic Muc5Ac-13 peptide, containing N-acetylgalactosamine in $\alpha$ anomeric linkage with Thr$^{13}$.

PAGE 8617:

As a result, the paragraph entitled “Catalytic Domain Activity” (left column) should now read as follows.

“The lack of interaction between the hT2 catalytic and lectin domains observed in the ternary complex suggests that the catalytic domain may not require the lectin domain for activity. Based on the crystal structure, we designed and expressed the hT2 catalytic domain (residues 74–440) lacking the entire lectin domain and compared its activity to the full-length enzyme against peptide and glycopeptide acceptors. As shown in Table 3, both $k_{cat}$ and $K_m$ values (and thus the $k_{cat}/K_m$ ratio) for peptides EA2 and Muc5Ac are similar for full-length hT2 and the hT2 catalytic domain. However, removal of the hT2 lectin domain reduced glycopeptide $k_{cat}/K_m$ ratios of the catalytic domain 4–6-fold compared with full-length hT2 (Table 3). Thus, the absence of the hT2 lectin domain affected the transfer of GalNAc to the glycopeptides but not to the peptide substrates tested. For the Muc5Ac-3 glycopeptide, the smaller $k_{cat}/K_m$ value was dominated by a reduced $k_{cat}$ whereas for the Muc5Ac-13 and Muc5Ac-3/13 glycopeptides, the diminished $k_{cat}/K_m$ value was caused by both a smaller $k_{cat}$ and a larger $K_m$. Because both the $k_{cat}$ and $K_m$ values represent a collection of individual rate constants (and thus are apparent catalytic constants) that have not been determined, the specific step(s) of the catalytic mechanism most affected by the absence of the lectin domain remains unknown. $K_m$ values for UDP-GalNAc were similar for full-length hT2 (11.5 ± 2.4 $\mu$M) and the hT2 catalytic domain (7.9 ± 2.3 $\mu$M).”

These corrections do not in any way alter the conclusions presented in the manuscript.

PAGE 8618:

A corrected version of Table 3 follows.

### TABLE 3

| Peptide | Sequence | Full-length hT2 | hT2 catalytic domain | $k_{cat}/K_m$ (µM$^{-1}$ s$^{-1}$) × 1000 |
|---------|----------|-----------------|----------------------|--------------------------------------|
| EA2     | PTIDSTDAPPTIK | $3.70 ± 0.22$ | $943 ± 120$ | $3.29 ± 0.14$ | $1157 ± 130$ | $3.9 ± 0.5$ | $2.9 ± 0.3$ | $1.3 ± 0.3$ |
| Muc5Ac  | GTPSPVFVTSTTSAP | $0.29 ± 0.02$ | $20.2 ± 4.6$ | $0.22 ± 0.01$ | $17.5 ± 4.3$ | $14.3 ± 3.4$ | $12.5 ± 3.2$ | $1.1 ± 0.4$ |
| Muc5Ac-3 | GTPSPVFVTSTTSAP | $1.56 ± 0.07$ | $28.0 ± 4.8$ | $0.31 ± 0.02$ | $32.9 ± 5.8$ | $55.6 ± 9.8$ | $9.4 ± 1.7$ | $5.9 ± 1.5$ |
| Muc5Ac-13 | GTPSPVFVTSTTSAP | $0.56 ± 0.02$ | $54 ± 6.7$ | $0.25 ± 0.01$ | $89.3 ± 9.8$ | $10.4 ± 1.3$ | $2.8 ± 0.3$ | $3.7 ± 0.6$ |
| Muc5Ac-3,13 | GTPSPVFVTSTTSAP | $0.82 ± 0.06$ | $406 ± 79$ | $0.47 ± 0.04$ | $933 ± 204$ | $2.0 ± 0.4$ | $0.5 ± 0.1$ | $4.0 ± 1.3$ |