Cloning of a Human UDP-galactose:2-Acetamido-2-deoxy-p-glucose 3β-Galactosyltransferase Catalyzing the Formation of Type 1 Chains*

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Frank Kolbinger, Markus B. Streiff, and Andreas G. Katopodis‡
From Novartis Pharma AG, Transplantation Preclinical Research, CH 4002 Basel, Switzerland

Biochemical evidence suggests that the galactosyltransferase activity synthesizing type 1 carbohydrate chains is separate from the well characterized enzyme that is responsible for the synthesis of type 2 chains. This was recently confirmed by the cloning, from melanoma cells, of an enzyme capable of synthesizing type 1 chains, which was shown to have no homology to other galactosyltransferases. We report here the molecular cloning and functional expression of a second human β3-galactosyltransferase distinct from the melanoma enzyme. The new β3-galactosyltransferase has homology to the melanoma enzyme in the putative catalytic domain, but has longer cytoplasmic and stem regions and a carboxyl-terminal extension. Northern blots showed that the new gene is present primarily in brain and heart. When transfected into mammalian cells, this gene directs the synthesis of type 1 chains as determined by a monoclonal antibody specific for sialyl Lewisα. A soluble version of the cloned enzyme was expressed in insect cells and purified. The soluble enzyme readily catalyzes the transfer of galactose to GlcNAc to form Gal(β1–3)GlcNAc. It also has a minor but distinct transfer activity toward Gal, LacNAc, and lactose, but is inactive toward GalNAc.

Two types of carbohydrate chains are known to exist in the lacto-series of oligosaccharides, type 1 chains that contain the Gal(β1–3)GlcNAc linkage and type 2 chains containing the topoisomerase Gal(β1–4)GlcNAc. Both types of carbohydrate structures are present in soluble oligosaccharides of human milk (1), are also found on glycoproteins (2) and glycolipids (3), and are important precursors of blood group antigens (4). The differences in function between type 1 and type 2 chains are not well understood. For example, during both embryogenesis (5) and carcinogenesis (6), the ratio of type 1 to type 2 chains produced by the cell changes. Furthermore, both type 1 and type 2 chains can be ligands for the selectin family of leukocyte extravasation receptors (7). The physiological significance of these observations is not yet known (8).

The biosynthesis of type 1 and type 2 structures is catalyzed by specific galactosyltransferases, which transfer galactose to GlcNAc terminating chains. The galactosyltransferase responsible for type 2 chain biosynthesis (β4-Gal-T) has been cloned and well characterized and was shown to be expressed in various tissues and cell types (reviewed in Refs. 9 and 10). This enzyme requires Mn2+ for activity and is regulated by α-lactalbumin to change the kinetics of transfer to glucose, thus favoring the synthesis of lactose. Relatively little information is known about the type 1 elongating enzyme, UDP-galactose:2-acetamido-2-deoxy-p-glucose 3β-galactosyltransferase (β3-Gal-T). This enzyme is clearly different from the β4-Gal-T and is expected to have a more restricted tissue distribution. The type 1 elongating enzyme is thought to be distinct from another β3-Gal-T activity detected in various sources and transferring to lactose or LacNAc (11–13), although this has not been molecularly established.

A β3-Gal-T enzyme catalyzing the synthesis of Gal(β1–3)GlcNAc has been purified from pig trachea and shown to require Mn2+, not to be influenced by lactalbumin, and to have an acceptor specificity consistent with its role of being responsible for elongation of oligosaccharide chains on both mucins and glycolipids (14, 15). Another β3-Gal-T enzyme capable of forming type 1 chains has been detected in colon carcinoma cell lines (16), as well as normal colonic mucosa (17). Moreover, DNA from COLO 205 cells when transfected into mammalian cells produced cell lines de novo synthesizing type 1 chains (18). No molecular information is available for any of these enzymes, and it is therefore difficult to judge their similarity. A β3-Gal-T was recently cloned from the human melanoma WM266–4 cell line using an expression cloning strategy that relied on lectin resistance to identify clones (19). This enzyme, which has no homology to known glycosyltransferases, transfers galactose in vitro to produce Gal(β1–3)GlcNAc(β1–3)Gal(β1–4)Glc and directs the synthesis of sLeα in transfected cells.

We report here the cloning and functional expression of a new β3-Gal-T from human brain tissue distinct from the enzyme present in melanoma cells. The new enzyme is homologous to the melanoma cell enzyme in the putative catalytic domain, and is mainly expressed in brain and heart. When transfected in mammalian cells, the new gene directs the de novo synthesis of type 1 chains. A soluble version of the enzyme expressed in

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† To whom correspondence should be addressed: Novartis Pharma AG, S386.645, CH-4002 Basel, Switzerland. E-mail: andreas.katopodis@pharma.novartis.com.

‡ The abbreviations used are: β4-Gal-T, UDP-galactose:2-acetamido-2-deoxy-p-glucose 4β-galactosyltransferase; β3-Gal-T, UDP-galactose:2-acetamido-2-deoxy-p-glucose 3β-galactosyltransferase; sLeα, Neu5-Acα2–3(Galβ1–3)[Fucα1–4]GlcNAc; sLeβ, Neu5Acα2–3(Galβ1–4)-[Fucα1–3]GlcNAc; EST, expressed sequence tag; FACS, fluorescence-activated cell sorting; UDP-Gal, uridine diphospho-p-galactose; GlcNAc-Lemieux, GlcNAcβ(CH2)4–CO2Me; Gal-Lemieux, Galβ(CHO)2–CO2Me; LacNAc-Lemieux, Galβ1–4GlcNAcβ(CHO)2–CO2Me; GalNAc-benzyl, benzyl 2-acetamido-2-deoxy-a-D-galactopyranoside; BSA, bovine serum albumin; PCR, polymerase chain reaction; HPAGE-PAD, high pH anion exchange chromatography with pulsed amperometric detection; CHO, Chinese hamster ovary; bp, base pairs(s); kb, kilobase pairs(s); PBS, phosphate-buffered saline; FITC, fluorescein isothiocyanate; Fuc-T, fucosyltransferase.
insect cell transfers galactose to GlcNAc to produce Galβ1-3GlcNAc. The recombinant enzyme also transfers galactose to Gal, LacNAc, and lactose, at distinctly lower rates. To distinguish the two β3GalT-enzymes, we propose to name the melanoma enzyme β3GalT1 and the human brain enzyme β3GalT2.

**EXPERIMENTAL PROCEDURES**

**Materials**—All cell culture media, sera, and antibiotics were from Life Technologies, Inc. CHO K1 cells (ATCC CCL-61) were maintained in α-minimal essential medium, 5% fetal bovine serum. S9 insect cells were grown in 5% 900 II SFM medium. GlcNAc, Gal, Gal β1-3GlcNAc, p-nitrophenyl β-D-lactopyranoside, GalNAco-benzyl, and UDP-Gal were purchased from Sigma. Antibody CSLEX was purchased from Becton Dickinson and GSLAI was a gift from Dr. J. Magnani (GlycoTech Corp.). The cDNA for Fuc-T III in the expression vector pcDM7 was a gift from Dr. J. B. Lowe (Howard Hughes Medical Institute, Ann Arbor, MI).

**EST Data Base Searches**—The cDNA sequence of β3GalT2 was cloned by its homology to β3GalT1 (19). Using the complete 322-amino acid protein sequence of β3GalT1, a TBLASTN search was performed (20) on the dbest data base (release: Feb. 4, 1996). The best aligning ESTs (accession numbers R13867, H14861, and R13064) from insect cell libraries identifying the GA4/BAT GAT GGC TCA CAT CCA GGG) were then used to screen for the full-length cDNA of the putative new β3GalT2. Primers, designated galtdia1.pcr (TGA AGC CAG ATC TGC CTC CC) and galtdia2.pcr for the presence of the 307-bp DNA fragment specific for about 68 amino acids to the catalytic domain of β3GalT2. Thirty-eight amino acids deduced from the cDNA sequence of R13867 were then used to search again the dbest data base (release: Feb. 29, 1996) using TBLASTN. An additional human fetal EST clone D81474 was found, further extending the homology within the catalytic domain.

**Screening of agt10 Library**—The sequences of EST clones R13867 and D81474 were artificially combined, and primers were designed, allowing PCR amplification of a 307-bp DNA fragment specific for the 307-bp positive phage plaques could be isolated. Forty-eight single phage lysates were used for further experiments.

**Expression of Full-length β3GalT2 in CHO/Fuc-T III Cells and Fuc-T III Cells**—A mammalian expression vector was prepared containing the complete open reading frame of β3GalT2. Construction was done by PCR amplifying a DNA fragment from plasmid GA4/1.xeo using oligonucleotides NGalTATG.ECO (GCC GAA TAC GCC ACC ATG GTG CAG TGG AGG AGA AGA CAC TGC) and NGalITAG.ECO (GCC GAA TTA ATG TAG TTT ACC AGC GTA CTA GCC GGC GGA ATC CCT GGC). Oligonucleotide NGalITAG.ECO had a 5′ extension containing an EcoRI restriction enzyme sequence (for efficient cloning into the plasmid vector pCR3®-uni) plus suitable cloning sites for the cDNA insertion. The plasmid was prepared by the putative stop codon of the β3GalT2 and an 5′ extension containing an EcoRI restriction endonuclease site. The gel-purified, amplified 1.29-kb PCR fragment was directly ligated into plasmid pCR3®-uni (Invitrogen). Correct orientation of the DNA insert present in plasmid pGA4/ICDS.uni (GenBank accession number AF052010) was verified by restriction analysis.

CHO/Fuc-T III cells seeded overnight in six-well plates were transfected with 4 μg/μl of pCR3®-uni, containing the gene for Fuc-T III (23). Expression vector pcDNA(3)-hyg was constructed by inserting into the single Nhel restriction endonuclease site present in vector pcDNA/Amo (Invitrogen) a Xho1-restricted 1.68-kb DNA fragment encompassing the entire expression unit for hygromycin-B resistance. The fragment was cut with pREPT (Invitrogen) by PCR amplification using primers HygL.Xba (GCT CTA GAG CTT TGT CTG GCG GTG TTC) and HygL.Xba (GCT CTA GAG CTT TGT CTG GCG GTG TCC) (Invitrogen). pcDM7(3)-hyg both, the hygromycin-B resistance gene and inserted gene/cDNA sequences are transcribed in the same direction. The complete cDNA for human Fuc-T III was excised from pcDM7 using restriction endonuclease Xhol and ligated into pcDNA(3)-hyg cut with the same restriction endonuclease to create vector FTIII(3)-hyg. Correct orientation of the Fuc-T III coding sequence was verified by restriction analysis.

CHO/Fuc-T III cells seeded overnight in six-well plates were transfected with 4 μg/well of FTIII(3)-hyg, using LipofectAMINE according to the manufacturer’s instructions. Cells were trypsinized into a T-15 flask, and 0.2 mg/ml hygromycin (Calbiochem) was added to the medium after 24 h. After 3 weeks of selection, surviving cells were sorted by FACS for the surface expression of the sLea epitope by incubation with monoclonal antibody CSLEX-1 and staining with FITC-labeled polyvalent anti-mouse IgM (Jackson ImmunoResearch). The sorted cells were placed in 96-well plates at a density of 0.5 cells/well. Cells from wells containing single colonies were analyzed for surface expression of sLea by FACS with CSLEX, and clones displaying the highest staining were used for further experiments.

**Expression of Full-length β3GalT2 in CHO/Fuc-T III Cells**—A mammalian expression vector was prepared containing the complete open reading frame of β3GalT2. Construction was done by PCR amplifying a DNA fragment from plasmid GA4/1.xeo using oligonucleotides NGalTATG.ECO (GCC GAA TAC GCC ACC ATG GTG CAG TGG AGG AGA AGA CAC TGC) and NGalITAG.ECO (GCC GAA TTA ATG TAG TTT ACC AGC GTA CTA GCC GGC GGA ATC CCT GGC). Oligonucleotide NGalITAG.ECO had a 5′ extension containing an EcoRI restriction enzyme sequence (for efficient cloning into the plasmid vector pCR3®-uni) plus suitable cloning sites for the cDNA insertion. The gel-purified, amplified 1.29-kb PCR fragment was directly ligated into plasmid pCR3®-uni (Invitrogen). Correct orientation of the DNA insert present in plasmid pGA4/ICDS.uni (GenBank accession number AF052010) was verified by restriction analysis.

CHO/Fuc-T III cells seeded overnight in six-well plates were transfected with 4 μg of plasmid pGA4/ICDS.uni using LipofectAMINE. Cells were trypsinized in a T-15 flask, and after 24 h 0.5 mg/ml G418 was added to the culture medium. After 3 weeks of selection, cells were analyzed by FACS for the expression of sLea on their surface. FACS analysis was performed by first incubating the cells with the monoclonal antibody GSLAI and after washing with a FITC-labeled polyvalent anti-mouse IgG (Jackson ImmunoResearch).

Construction of Soluble β3GalT2 Fused with Protein A—A basic mammalian expression vector for the expression of Staphylococcus aureus protein A fusions was prepared as follows. First an expression vector was constructed containing 20 amino acids of the human γ-interferon signal sequence and the first 6 amino acids of mature human γ-interferon (27) plus suitable cloning sites for the cDNA insertion. Oligonucleotides IFNG-NEW1 (GTC GGA AAG CCT TCT AGA GGC GCG CCA CCA CCA GTA AAT CAA GTA GTT TCT GCC TCA ATG TTT ACC AGC GTA CTA GCC GGC GGA ATC CCT GGC) and IFNG-NEW3 (GGA CTA GCT GTA CAA GGA GGT GTT TCT GCC TCA ATG TTT ACC AGC GTA CTA GCC GGC GGA ATC CCT GGC) were then used to amplify the DNA fragment containing the enzyme coding region. The DNA sequences of the amplification products were verified by restriction analysis.
Klenow fragment of DNA polymerase I, and the HindIII/SpeI-restricted DNA fragment was subcloned into vector pcDNAI/neo (Invitrogen) restricted with HindIII and XbaI, resulting in plasmid IFNG-new.neoI. A DNA fragment encoding for the Ig-binding domains of protein A was amplified from plasmid pRIT2T (Pharmacia Biotech Inc.) by PCR using oligonucleotides SPANEW1.SAL (GGT ACG GTC GAC TGG GAT CAA CGC AAT GGT TTT ATC) and SPANEW3.XHO (GGT GCA CTC GAG ATT TGT TAT CTG CAG ATC GAC). This DNA fragment was then cut with restriction endonucleases SalI and XhoI and subcloned into plasmid IFNG-new.neoI restricted with the same enzymes. At the carboxyl-terminal end of protein A were added XhoI and AgeI cloning sites for in-frame insertion of cDNAs. The resulting plasmid, designated sPROTA2.neoI, is capable of directing the expression of secreted protein A or protein A fusion proteins under control of the human cytomegalovirus promoter (data not shown).

A DNA fragment encoding for amino acids 125–422 (Fig. 1) of b3GalT2 was inserted into the XhoI and AgeI sites of vector sPROTA2.neoI. Construction was done by PCR amplification of the corresponding DNA fragment from GA4/1.zeo using oligonucleotides NGALTS1.XHO (GGT GCA CTC GAG AAA GGT ACT GGA CAT CCA AAT TCT TAC) and NGALTEND.AGE (GGT GCA ACC GGT TAC TAA TGT AGT TTA CGG TGG CGA TAC C). The amplified 0.92-kb PCR fragments was restricted with AgeI and XhoI and subcloned into sPROTA2.neoI, cut with the same enzymes. The presence of the b3GalT2 insert in the resulting protein A fusion vector SPA2GATS.neoI was verified by sequencing. The complete expression cassette for the protein A fusion of b3GalT2 of vector SPA2GATS.neoI was then transferred into an insect cell expression system to be able to purify larger quantities of the fusion protein for functional studies. This was achieved by transferring a 1.8-kb XbaI fragment from SPA2GATS.neoI into the unique XbaI site of donor plasmid pFastBac1 (Life Technologies, Inc.). Correct orientation of the DNA insert present in the resulting donor plasmid SPA2GATS.bac1 was verified by restriction analysis.

Expression and Purification of Protein A Fusion of b3GalT2 in Insect Cells—Donor plasmid SPA2GATS.bac1 was used to create in E. coli recombinant SPA2GATS bacmid DNA containing all the genetic elements for the production of recombinant virus particles, by utilizing Tn7-mediated transposition according to protocols given in the instruc-

FIG. 1. Nucleotide and deduced amino acids sequence of human β3- or β3-galactosyltransferase-2. The double underlined amino acids correspond to a putative transmembrane domain. The asterisks indicate potential N-glycosylation sites (Asn-X(Ser/Thr)).
tion manual of the BAC-TO-BAC baculovirus expression system (Life Technologies, Inc.). The recombinant bacmid DNA was then used to transfect SF9 insect cells, and isolated virus was then used to obtain a high titer virus stock.

For large scale production of the β3GalT2/protein A fusion, SF9 cells grown at 28 °C in flasks to a density of 2×10^9 were infected with virus at a multiplicity of infection of 5, and culture supernatants were harvested 72 h post-infection. After centrifugation, filtration and concentration, supernatants were made 25 mM in sodium cacodylate, pH 6.5, and 100 mM in NaCl and loaded onto a SP-Sepharose column (Pharmacia). The column was washed with 25 mM sodium cacodylate, pH 6.5, 200 mM NaCl, and the β3GalT2/protein A fusion protein was eluted with 25 mM sodium cacodylate, pH 6.5, 500 mM NaCl. The eluted fractions were dialyzed against 40 mM sodium cacodylate, pH 6.5, 100 mM NaCl, 20 mM MnCl₂ and loaded onto a UDP-hexanolamine-Sepharose column, equilibrated in the same buffer. The column was washed with loading buffer, followed by washing with loading buffer without MnCl₂. Elution of the enzymatic activity was accomplished with buffer containing 50 mM sodium cacodylate, pH 6.5, 100 mM NaCl, 10 mM EDTA, 1 mg/ml UDP (Fluka). The enzyme was stored at 4 °C.

Expression and Purification of Protein A Fusion of β3GalT1—A protein A fusion chimera of β3GalT1 was cloned and expressed in a manner analogous to that described above for β3GalT2. A portion of β3GalT1 representing the complete stem region and catalytic domains (amino acids 25–326) was amplified by PCR from the genomic DNA of Colo 205 cells (ATCC CCL-222), and recombinant virus were produced in a manner analogous to that described above for β3GalT2. Cell culture and protein purification was performed as indicated above for β3GalT2.

Detection of β3GalT2/Protein A Fusion Protein by Enzyme-linked Immunosorbent Assay—The protein A portion of the β3GalT2/protein A fusion protein was used to semi-quantitatively determine the concentration of the soluble form of β3GalT2. Microtiter plates were coated overnight at 4 °C with 120 μl of human IgG (5 μg/ml; Sigma) in PBS and blocked with 0.5% BSA in PBS for 60 min at room temperature. Samples as well as protein A standard (0.5–50 ng/ml recombinant IgG-binding fragment of protein A; Sigma) diluted in 100 μl of PBS containing 0.5% BSA (PBS/BSA) were added to the microtiter plates and incubated for 60 min at room temperature. Wells were washed with PBS containing 0.05% Tween 20 and incubated successively with 100 μl of biotinylated goat anti-protein A antibody (1:100,000; Sigma) and 100 μl of streptavidin-peroxidase conjugate (1:5000; Boehringer) in PBS/BSA, for 60 min at room temperature. Wells were washed six times with PBS plus 0.05% Tween 20 and developed with TMB substrate solution (Bio-Rad), and absorbance at 450 nm was measured after stopping with 50 μl of 1 M H₂SO₄.

β3GalT2 Assays and Product Characterization—The linkage synthesized by the β3GalT2/protein A fusion protein was analyzed by HPAE/PAD (Dionex) using the following conditions: 70% H₂O, 30% 0.5 M NaOH, at a flow rate of 1 ml/min.

Enzymatic activity of the β3GalT2/protein A fusion protein was determined using a radioactive assay similar to the method of Palcic et al. (28) as follows; the appropriate amounts of enzyme were incubated with 14 μl of assay stock solution (180 mM sodium cacodylate, pH 6.5, 1 mg/ml BSA, 0.26 mM UDP-Gal, 2 μl of [³H]UDP-Gal (Amersham Corp.), 1 μl of MnCl₂ (500 mM)), 25 μl of GlcNAc-Lemieux (37 mg/ml in dimethyl sulfoxide) at 37 °C for 60 min. The reaction was quenched by the addition of 1 ml of water and loaded on a C18 Sep-Pack cartridge (Waters), and the column was washed twice with 5 ml of H₂O and eluted with 5 ml of methanol. All fractions were counted in a BETAmatic β counter (Kontron) after the addition of 10 ml of scintillation fluid. Enzymatic activity was determined by calculating percent UDP-Gal conversion (³H activity in the methanol fraction versus total ³H activity of all fractions).

RESULTS AND DISCUSSION

Cloning and Nucleotide Sequencing of the β3GalT2 cDNA—It is difficult to predict the sequence homology of glycosyltransferases based on their enzymatic activity. Some glycosyltransferases are grouped into families of homologous genes, as for example the α1–3 fucosyltransferases (29), or have characteristic motifs, as is the case for sialyltransferases (30, 31). Many glycosyltransferases, however, have little or no homology even between enzymes that utilize the same activated sugar donor and carbohydrate acceptor. This is the case for galactosyltransferases, which seem to have no common motif except possibly for a hexapeptide (B-D-K-N-A, where A is either E or D and B is either R or K) identified by Joziasse et al. (32). TBLASTN (20) data base searches using all possible permutations of this peptide motif revealed no new homologous sequences.

The cloning of β3GalT1 by an elegant expression cloning method using lectin resistance for phenotype selection (19) provided the opportunity to search for novel galactosyltransferase genes by sequence similarity. Searches performed on the dbest data base using the TBLASTN algorithm revealed several ESTs with homology to portions of the β3GalT1 sequence. From a total of four ESTs identified (all of which were from human brain), a continuous cDNA fragment encoding for about 102 amino acids of a putative new galactosyltransferase gene was assembled. This cDNA fragment allowed the design of primers, which were used to provide a diagnostic PCR signal for the presence of the new putative gene. From the strength of
the PCR signal, the new gene was found to exist mainly in libraries from human brain, heart, and cells of the immune system (data not shown). The human brain library gave the strongest PCR signal and was subsequently used as a starting point for the identification of the new gene. To clone the new gene, the method of D’Esposito et al. (21) was used, which relies on successive levels of splits, tests, and expansions of λ phage pools to obtain successively enriched fractions of the target gene. Starting with approximately 1 million clones, the first split to 192 wells (level 0) provided 17 wells with a positive PCR signal. This translates into an abundance of the cDNA of at least 1 in 84,000 calculated on the basis of 950,000 independent λ phage clones used for the primary infection. Each of the level 0 wells potentially contained a single independent clone from the putative gene. Because the diagnostic PCR amplified signal was from an internal portion of the putative new gene, no information was obtained regarding the length of the various level 0 clones and it was therefore not possible to judge which of the positive wells contained the complete gene. Two level 0 wells were chosen for subsequent expansion solely on the strength and clarity of the PCR signal and were split into 96 separate wells. After testing and splitting again into 96 separate wells, finally single plaques were evaluated for insert size and the largest λgt10 clone GA4/1 was determined to contain an insert of ~3 kb.

DNA sequence analysis of λgt10 clone GA4/1 revealed that it contained a single, long open reading frame encoding for a protein of 422 amino acids with a predicted Mr, of 49,202 (Fig. 1). Hydropathy analysis (Fig. 2) (33), as well as primary amino acid sequence analysis using the Saps program (34), revealed a hydrophobic, putative transmembrane region at the amino-terminal end of the open reading frame (amino acids 27–43), predicting that this protein has a type II transmembrane topology, typical for all mammalian glycosyltransferases cloned to date.

BLAST searches of public data bases using either the deduced amino acids sequence of β3GalT2 or the complete cDNA sequence of clone GA4/1 revealed no significant homology with any protein in the Swissprot (release: June 23, 1997) or with any gene in the GenEMBL (release: June 26, 1997) data base. Furthermore, besides the four human brain EST sequences, which were originally used to design the diagnostic PCR primer pair for the cloning of β3GalT2, no additional matching entries were found in the EST data base (release: June 23, 1997).

Comparison of the new protein sequence with the 326-amino acid protein sequence of β3GalT1 revealed an overall sequence identity of 46%. The highest level of sequence similarity between the two β3-Gal-T sequences was found between positions 47 and 326 for β3GalT1 and between positions 119 and 405 for β3GalT2, respectively. The sequence identity in this region was calculated as 51% (67% similarity), with four 1–3-amino acid insertions found in β3GalT2 as compared with β3GalT1 (Fig. 3).
3B). Due to the high sequence conservation, we assume that these regions most likely represent the catalytic domains of the two enzymes. Both enzymes contain two conserved potential N-glycosylation sites (N-X-S/T) in the putative catalytic domains, with three additional sites present in the stem and putative catalytic domain of β3GalT2 (Fig. 3). The major differences between β3GalT1 and β3GalT2 are a 17-amino acid extension at the carboxyl terminus of β3GalT2 and differences in both the lengths and primary sequences of the putative cytoplasmic, transmembrane, and stem regions (Fig. 3, A and B).

The high sequence homology within the catalytic domain between the two genes suggested that the new enzyme was also a β3-Gal-T. It remained, however, unclear whether the new enzyme transferred galactose to GlcNAc, as would be expected for a type 1 chain extending enzyme, or to other acceptors. Glycosyltransferase stem regions are known to influence enzyme acceptor specificity as has been reported for Fuc-T III and Fuc-T V (35, 36), and cytoplasmic domains are known to influence Golgi localization (37, 38). A distinct possibility therefore existed that β3GalT2 would transfer galactose to GalNAc to make the core 1 structure, or to other acceptors. Such an enzyme would be expected to have both different acceptor specificity and Golgi localization compared with β3GalT1. To test this possibility, we examined the acceptor specificity of β3GalT2 both in cell culture and in enzymatic assays.

Determination of Enzymatic Activity in Cell Culture—The β3GalT2 gene was subcloned in an expression vector and checked for its ability to direct the synthesis of type 1 chains in CHO cells, which do not normally synthesize type 1 chains (39). To readily detect de novo production of type 1 chains, CHO cells were transfected with the gene for Fuc-T III. This enzyme fucosylates both type 1 and type 2 chains so that cells expressing it produce the corresponding fucosylated and also siaylated oligosaccharides (25, 39). CHO/Fuc-T III cells stained brightly with the anti-sLea antibody CSLEX (data not shown) but not with the anti-sLex antibody GSLA1 (Fig. 4, curve A). Transfection of CHO/Fuc-T III cells with a vector containing the newly cloned putative galactosyltransferase produced significant staining of these cells with GSLA1 (Fig. 4, curve B), indicating that the new gene is indeed a type 1 extension enzyme.

Production of Soluble β3GalT2/Protein A Fusion Protein and Enzymatic Assays—The acceptor specificity of β3GalT2 was also directly established in enzymatic assays. To purify sufficient amounts of enzyme for analysis, we constructed and expressed a soluble β3GalT2/protein A fusion protein by removing the putative cytoplasmic, transmembrane, and part of the stem region and replacing them with the IgG binding domain of S. aureus protein A. The β3GalT2/protein A fusion protein was expressed in S9 cells and purified by ion exchange and affinity chromatography. Enzymatic assays with GlcNAc as the acceptor produced significant Golgi localization (37, 38). A distinct possibility therefore existed that β3GalT2 would transfer galactose to GalNAc to make the core 1 structure, or to other acceptors. Such an enzyme would be expected to have both different acceptor specificity and Golgi localization compared with β3GalT1. To test

![Fig. 4. Flow cytometry analysis of β3GalT2 transfected CHO cells.](image)

**Fig. 4. Flow cytometry analysis of β3GalT2 transfected CHO cells.** CHO/Fuc-T III cells transfected with β3GalT2 were analyzed by flow cytometry after staining with sLeα-specific monoclonal antibody GSLA1 and FITC-labeled anti-mouse IgG antibody as indicated under “Experimental Procedures.” Curve A is the histogram obtained from CHO/Fuc-T III control cells, and curve B is that obtained from CHO/Fuc-T III cells transfected with β3GalT2.

![Fig. 5. HPAE/PAD analysis of the β3GalT2 reaction product.](image)

**Fig. 5. HPAE/PAD analysis of the β3GalT2 reaction product.** The enzymatic assay was performed using purified protein A/β3GalT2 fusion protein with GlcNAc as acceptor substrate and analyzed as indicated under “Experimental Procedures.” Curve A is the trace obtained with the authentic standards GlcNAc, LacNAc (Galβ1–4GlcNAc), and Galβ1–3GlcNAc as indicated. Curve B is the trace obtained with the complete assay mix at time 0, and curve C is the trace obtained after incubation for 120 min at 37 °C.

 Surprisingly, β3GalT2 transfers galactose to Gal-terminating acceptors. The results in Table I show Gal to be a relatively good acceptor, with LacNAc and lactose being distinctly worse. The relative efficiency of the substrates in Table I, however, must be interpreted with caution since the hydrophobic groups

2 M. Streiff, unpublished data.
attached on their anomeric sites are different. No transfer was observed to GalNAc-benzyl even when a 10-fold increase of enzyme was used (data not shown). As shown in Table I, 3GalT1 also transfers to Gal, but at a much lower relative rate and with more restricted specificity than 3GalT2. The reactivity pattern of 3GalT2 is partially reminiscent of the human kidney enzyme leaving open the question whether it can of hydrophobic groups at the anomeric site. Unfortunately, no information is yet available about the distribution of 3GalT1. Preliminary PCR experiments using libraries from different tissues and cell types showed clear differences in the distribution of 3Gal-T1 and 3GalT2.3

The 3GalT2 described here represents the second type 1 chain extending enzyme cloned. The homology of the two genes in the catalytic domain suggests that they correspond to an evolutionary related family of 3-gal-galactosyltransferase (29). The presence of additional enzymes would imply a diversity of type 1 bearing structures with perhaps varying roles. The availability of the 3GalT2 sequence will allow addressing these questions by molecular means and possibly uncovering the physiological functions of type 1 chains.

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11. 3GalT2 sequence will allow addressing these questions by molecular means and possibly uncovering the physiological functions of type 1 chains.

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