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Molecular cloning and expression analysis of a mouse UDP-GlcNAc:Gal(β1-4)Glc(NAc)-R β1,3-*N*-acetylglucosaminyltransferase homologous to *Drosophila melanogaster* Brainiac and the β1,3-galactosyltransferase family

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We have isolated a murine cDNA coding for a β1,3-*N*-acetylglucosaminyltransferase enzyme (β3GnT). This enzyme is similar in sequence to *Drosophila melanogaster* Brainiac and to the murine and human β1,3-galactosyltransferase family of proteins. The mouse β3GnT protein is 397 amino acids in length and contains 7 cysteine residues that are conserved in the human orthologue. β3GnT is a type II membrane protein localized to the Golgi apparatus. Enzyme assays with recombinant mouse β3GnT reveal that it has a preference for acceptors with Gal(β1-4)Glc(NAc) at the non-reducing termini. Proton NMR analysis of product showed incorporation of GlcNAc in β1,3 linkage to the terminal Gal of Gal(β1-4)Glc(β1-O-benzyl). Northern blot analysis revealed the presence of a single 3.0 kb transcript in all adult mouse and human organs tested, with highest levels in the kidney, liver, heart and placenta. The β3GnT gene is also expressed in a number of tumor cell lines. The human orthologue of β3GnT is located on chromosome 2pl5.

Keywords: **poly-*N*-acetyllactosamine, glycoprotein, glycosyltransferase, Golgi localization**

Abbreviations: **BAC, Bacterial Artificial Chromosome; β3GalT, β1,3-galactosyltransferase; β3GnT, β1,3-*N*-acetylglucosaminyltransferase; Bn, benzyl; EST, expressed sequence tag; FISH, fluorescence *in situ* hybridization; HRP, horse radish peroxidase; NMR, nuclear magnetic resonance spectroscopy; pNP, para-nitrophenyl; PCR, polymerase chain reaction; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; STS, sequence tagged site; 3′-UTR, 3′-untranslated region; YAC, Yeast Artifical Chromosome**

Introduction

Genetic analysis in *Drosophila* has led to the identification of two developmental regulators, Fringe and Brainiac, with homology to bacterial glycosyltransferase enzymes [1]. Recently, Fringe and its mammalian relatives, Lunatic Fringe and Manic Fringe, have been shown to encode a β1,3-*N*-acetylglucosaminyltransferase that adds *N*-acetylglucosamine (GlcNAc) onto O-linked fucose substrates [2,3]. In contrast, it is not yet clear whether Brainiac is a glycosyltransferase. In the past few years, several groups have defined a large family of mammalian β1,3-galactosyltransferase (β3GalT) enzymes [4–12]. Interestingly, members of this family have striking sequence similarity to *Drosophila melanogaster* Brainiac.

We previously identified the mammalian Fringe gene family through searches in the public expressed sequence tag (EST) database followed by PCR cloning [13]. We therefore used the same approach to identify mammalian Brainiac homologues. Two mouse EST sequences were used to clone a novel mouse cDNA encoding a 397 amino acid protein with high homology to Brainiac and to the mammalian β3GalT enzyme family.

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The protein was found to be a β1,3-*N*-acetylglucosaminyltransferase enzyme (β3GnT) acting on acceptors with Gal(β1-4)Glc(NAc) at the non-reducing termini. The β3GnT is localized to the Golgi apparatus and is expressed as a single transcript in all adult mouse and human organs tested and in a number of tumor cell lines.

Materials and methods

Materials and molecular biology procedures

The following materials were purchased from the indicated sources: Sep-Pak C18 reverse phase cartridges (Waters); Triton X-100, goat anti-mouse IgG (whole molecule) coupled to alkaline phosphatase, Gal(β1-4)Glc(β1-OBn) (Bn = benzyl), lacto-*N*-neotetraose, [Gal(β1-4)GlcNAc (β1-3)Gal(β1-4)Glc], lacto-*N*-tetraose [Gal(β1-3)GlcNAc(β1-3)Gal(β1-4)Glc], Gal (β1-3)GalNAc(α1-OBn), Gal(α1-OpNP) (pNP = para-nitrophenyl), Gal(β1-OpNP), GalNAc(α1-OBn), GalNAc(β1-OpNP), GlcNAc(α1-OBn), GlcNAc(β1-OBn), Fuc(α1-OpNP), Fuc(β1-OpNP) (Sigma); Gal(β1-4)GlcNAc(α1-OpNP), GlcNAc(β1-3)- GalNAc(α1-OpNP), Gal(β1-4)GlcNAc (Toronto Research Chemicals); Gal(β1-4)Glc (BDH Chemicals). The baculovirus transfer vector pVT-Bac-His and methodology for PCR and for expression in the Sf9/baculovirus system have been described previously [14,15]. UDP-GlcNAc-6-[^{3}H] (New England Nuclear, 41.60 Ci/mmol) and UDP-Gal-6-[^{3}H] (American Radiolabeled Chemicals, 60 Ci/mmol) were diluted with non-radioactive UDP-GlcNAc and UDP-Gal (Sigma) to specific activities of 2500 and 10,000 dpm/nmole respectively. Molecular biology procedures were carried out by standard methods [16,17] or as described [13–15]. Northern blot hybridization on Clonetech Northern blots and dot blots was performed as per manufacturer's instructions using a 1.7 kb EcoRl 5′ fragment of the mouse β3GnT cDNA as probe.

Cloning of mouse β3GnT cDNA

Translation products of the public expressed sequence tag (EST) database were screened with *Drosophila* Brainiac (accession number U41449) as query, using the TBLASTN algorithm. Two mouse EST clones were identified (AA119132 and AA204363) which could be translated to generate a peptide sequence with high homology to Brainiac. The EST sequences were used to design PCR primers to clone an 895 bp product from a number of mouse tissues (5′-primer was AGGTATGAGAGATGAGTGTGG and 3′-primer was CTCACTGGGATGTAGTACTTC). The 895 bp clone was sequenced to confirm its identity with the EST clones. This fragment was used to screen a mixed tissue cDNA library [18] from which we isolated a 2676 bp near full length cDNA clone for β3GnT.

Subcellular localization of β3GnT in COS cells

Mouse β3GnT was tagged with the c-myc epitope at its C-terminus by using PCR with 5′-primer GGTGACGTGATCCACAATGCT and 3′-primer CGGCTCGAGTCAAAGGTCCTCTTCGGAGATTAATTTTGTTCTCCGCATTTTAAATTAGGACTTTGC. The 416 nucleotide PCR product containing the myc tag was cloned, sequenced and subcloned together with the 5′-end of the β3GnT gene into *Eco*RI/*Xho*I digested pcDNA3 expression vector to generate plasmid pcDNA3-β3GnT-myc. Mouse Manic Fringe was tagged with a Flag epitope at its C-terminus using PCR with the 5′-primer TCAGCTACGGTGTCTTTGAG and 3′-primer ATTTTGGGCCCTCATTTGTCATCATCATCCTTGTAATCGGGCGCTGCCAGCAGCGGACA. The Flag tagged PCR product was connected to the *N*-terminus of Manic Fringe in pcDNA3 to generate plasmid pcDNA3-Manic Fringe-Flag. Both expression vectors were transfected into COS-7 cells, either alone or in combination (Effectene, Qiagen). After 48 h, the cells were fixed, stained and photographed as described elsewhere [18]. The myc tag was stained with mouse anti-myc antibody (SantaCruz Biotechnology) followed by Texas-Red labeled donkey-anti mouse antibody (Jackson ImmunoResearch Laboratories). The Flag epitope was stained with mouse anti-Flag antibody directly coupled to FITC (Sigma).

Western blot analysis of epitope-tagged proteins

Manic Fringe was tagged with the c-myc epitope at its C-terminus by using PCR with the same 5′-primer as shown in the previous section and 3′-primer (with a myc tag) ATTTTGGGCCCTCAAAGGTCCTCTTCGGAGATTAATTTTTGTTCTCCGGGCGCTGCCAGCAGCGGACA to generate plasmid pcDNA3-Manic Fringe-myc. COS cells were transfected with pcDNA3 and pcDNA3-Manic Fringe-myc or pcDNA3-β3GnT-myc (see above). Culture supernatants and cell lysates were harvested and immunoprecipitated with mouse anti-myc antibody (SantaCruz Biotechnology). Cell were lysed in 50 mM HEPES buffer pH 7.4, 150 mM NaCl, 10% glycerol, 1% Triton x-l00, 1 mM EGTA, 1.5 mM $MgCl_2$, and protease inhibitors. Immunoprecipitates were analyzed on Western blots using rabbit anti-myc antibody (SantaCruz Biotechnology), HRP-labeled donkey anti-rabbit IgG antibody and the ECL detection system essentially as previously described [18].

Expression of β3GnT in the Sf9/baculovirus system

Recombinant β3GnT truncated to remove the cytoplasmic *N*-terminal domain, trans-membrane hydrophobic domain and some of the stem region was expressed in the baculovirus/Sf9 insect cell system [14,15]. β3GnT cDNA was used as a template for PCR amplification with gene-specific primers. The 5′-primer (CACAAGCGGCCGCCCAAGACAAAAATGGAAAG) contained a *Not*1 restriction site (underlined) at the 5′-end followed by a nucleotide encoding a portion of the

β3GnT amino acid sequence commencing at Gln33. The 3′-primer (TATATGGTACCGCTCATGTCTATTTCAGCA) contained a *Kpn*1 restriction site (underlined) at the 5′-end followed by a nucleotide containing the STOP codon. Methods for subcloning of the PCR product into pVT-Bac-His and homologous recombination of the resulting plasmid pVT-Bac-His-GnT with BaculoGold baculovirus linearized DNA (PharMingen) in Sf9 cells have been described [14,15]. Recombinant baculovirus released into the culture medium was amplified twice and used to infect Sf9 cells at a multiplicity of infection of 2 plaque forming units/cell. At 5 days post infection, cells were sedimented, aliquots of the supernatant were concentrated 5-fold and 0.020 ml samples were subjected to Western blot analysis using 12.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) [19]. After transfer by electrophoresis to an ImmobilonPVDF membrane (0.45 microns, Millipore) in the presence of 0.1% SDS, the membranes were probed with 1:5000 diluted mouse monoclonal Anti-Xpress antibody (Invitrogen) followed by treatment with 1:2500 diluted alkaline phosphatase-conjugated goat antimouse IgG and staining with bromochloroindolyl phosphate (BCIP) and nitro blue tetrazolium (NBT) (Promega) [14,15].

Analysis of recombinant β3GnT enzyme activity

Sf9 supernatants were assayed for *N*-acetylglucosaminyltransferase activity in a total volume of 0.055 ml containing various acceptor substrates at concentrations between 0.45–4.5 mM (Table 1), 0.9 mM UDP-[^{3}H]GlcNAc (2500 dpm/nmol), 2.3 mM AMP, 45 mM GlcNAc, 11 mM $MnCl_2$, 45 mM Mes buffer pH 6.5, bovine serum albumin (0.45 mg/ml) and 0.020 ml enzyme. Galactosyltransferase assays were carried out in an incubation mixture (0.050 ml) containing various acceptor substrates (Table 1), 0.32 mM UDP-[^{3}H]Gal (10,000 dpm/nmol), 8 mM $MnCl_2$, 40 mM Mes buffer pH 6.5, bovine serum albumin (0.4 mg/ml) and 0.020 ml enzyme. Time of incubation was 60 min at 37°C. SepPak C18 cartridges were used to obtain radioactive product with substrates containing a para-nitrophenyl or benzyl group [20]. Substrates lacking a hydrophobic aglycone were assayed by passage through AG1-X8 [21]. Endogenous control assays were carried out in the absence of acceptor. Mock control assays were done with supernatants from Sf9 cells infected with a baculovirus construct encoding human UDP-GlcNAc: α-3-D-mannoside β1,2-*N*-acetylglucosaminyltransferase I [14,22]. The β3GnT enzyme was stable at 4°C for at least 2 weeks.

Table 1. Substrate specificity of recombinant mouse β3GnT*. Enzyme activities are expressed as nmoles/0.020 ml concentrated Sf9 supernatant/hour

Donor	*Acceptor*	*mM*	*endogenous*	*GnT I*	β3GnT
UDP-GlcNAc	Gal(β1-4)GlcNAc(α1-OpNP)	4.5	0.02	0.08	6.0
	Gal(β1-4)Glc(β1-OBn)	4.5	0.02	0.06	2.9
	Gal(β1-4)GlcNAc	4.5	0.39	0.07	5.9
	Gal(β1-4)Glc	4.5	0.39	0.01	2.9
	Gal(β1-4)GlcNAc(β1-3)Gal(β1-4)Glc	0.9	0.39	< 0.01	1.4
	Gal(β1-3)GlcNAc(β1-3)Gal(β1-4)Glc	0.9	0.39	< 0.01	0.16
	Gal(β1-3)GalNAc(α1-OBn)	0.45	0.02	ND	< 0.01
	Gal(α1-OpNP)	4.5	0.02	< 0.01	0.01
	Gal(β1-OpNP)	4.5	0.02	< 0.01	0.03
	GalNAc(α1-OBn)	0.9	0.02	< 0.01	< 0.01
	GalNAc(β1-OpNP)	0.45	0.02	< 0.01	0.01
	GlcNAc(α1-OBn)	1.8	0.02	0.02	< 0.01
	GlcNAc(β1-OBn)	0.9	0.02	< 0.01	< 0.01
	GlcNAc(β1-3)GalNAc(α1-OpNP)	1.1	0.02	< 0.01	0.03
	Fuc(α1-OpNP)	1.1	0.03	< 0.01	< 0.01
	Fuc(β1-OpNP)	4.5	0.03	< 0.01	< 0.01
UDP-Gal	Gal(β1-4)Glc(β1-OBn)	5.0	0.01	0.01	< 0.01
	GalNAc(α1-OBn)	1.0	0.01	< 0.01	< 0.01
	GalNAc(β1-OpNP)	0.5	0.01	< 0.01	< 0.01
	GlcNAc(α1-OBn)	2.0	0.01	0.02	0.01
	GlcNAc(β1-OBn)	1.0	0.01	0.05	0.02

*All assays are averages of duplicate assays and were corrected for endogenous assays carried out in the absence of acceptor. GnT I assays were carried out with Sf9 supernatant infected with recombinant baculovirus encoding human UDP-GlcNAc:α-3-D-mannoside β1,2-*N*-acetylglucosaminyltransferase I.

Acceptors without hydrophobic groups were assayed with the AG1-X8 column method and gave high endogenous values. pNP = para-nitrophenyl; Bn = benzyl; ND = not determined.

Identification of the product of β3GnT action

Twenty incubations were carried out as described above for assay of *N*-acetylglucosaminyltransferase activity, containing 4.5 mM Gal(β1-4)Glc(β1-OBn) as acceptor and 13.6 mM non-radioactive UDP-GlcNAc as donor, at 37°C for 72 h with additions of 0.020 ml recombinant β3GnT (Sf 9 supernatant) at 0, 24 and 48 h. Product was purified on SepPak C18 cartridges [20]. Thin layer chromatography [15] showed almost complete conversion of substrate to product. The product was repeatedly exchanged in D_2O (99.9 atom % D) and finally dissolved in 0.8 ml D_2O (99.96 atom % D). Proton NMR spectra were

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GAATTCGGCACGAGGCGGCAACAAGTGCTGGAGCTGAGGCGAGCCGGAGCCGCCCAGACCCCGCCGGGCCGCCCGTCCGCGCATTGCGCATGGAGCGAGA
GCGCGGCGGTCGCGGGGCTGAGCCGCAAGACCGGCTGGGACGTGGATGCGGCCGCGGTCTTCCGCCCCGCCCCGCCGAGCTGGAGGTGTCCCTAGACAAG
GTATGAGAG

ATGAGTGTGGGGCGTCGAAGAGTCAAGTTGCTGGGCATCCTGATGATGGCAAATGTCTTCATTTATTTGATTGTGGAAGTCTCCAAAAACAGTAGCCAAG
M  S  V  G  R  R  R  V  K  L  L  G  I  L  M  M  A  N  V  F  I  Y  L  I  V  E  V  S  K  N  S  S  Q  D
                                                                                                      34

ACAAAAATGGAAAGGGAGGAGTAATAATCCCGAAAGAGAAGTTCTGGAAGCCACCCAGCACTCCCCGGGCATACTGGAACAGGGAACAGGAGAAGCTGAA
  K  N  G  K  G  G  V  I  I  P  K  E  K  F  W  K  P  P  S  T  P  R  A  Y  W  N  R  E  Q  E  K  L  N
                                                                                                      67

CAGGTGGTACAATCCCATCTTGAACAGGGTGGCCAATCAGACAGGGGAGCTAGCCACATCTCCAAACACAAGTCACCTGAGCTATTGTGAACCAGACTCG
 R  W  Y  N  P  I  L  N  R  V  A  N  Q  T  G  E  L  A  T  S  P  N  T  S  H  L  S  Y  C  E  P  D  S
                                                                                                     100

ACGGTCATGACAGCTGTGACAGATTTTAATAATCTGCCGGACAGATTTAAAGACTTTCTCTTGTATTTGAGATGCCGGAATTACTCGCTGCTTATAGATC
T  V  M  T  A  V  T  D  F  N  N  L  P  D  R  F  K  D  F  L  L  Y  L  R  C  R  N  Y  S  L  L  I  D  Q
                                                                                                     134

AACCGAAGAAATGTGCAAAGAAGCCCTTCTTACTATTGGCGATAAAGTCCCTCATTCCACATTTTGCCAGAAGGCAAGCAATTCGGGAGTCTTGGGGCCG
  P  K  K  C  A  K  K  P  F  L  L  L  A  I  K  S  L  I  P  H  F  A  R  R  Q  A  I  R  E  S  W  G  R
                                                                                                     167

AGAAACCAACGTAGGGAACCAGACAGTAGTGAGGGTCTTCCTGTTGGGCAAGACACCCCCAGAGGACAACCACCCTGACCTTTCGGACATGCTTAAGTTT
 E  T  N  V  G  N  Q  T  V  V  R  V  F  L  L  G  K  T  P  P  E  D  N  H  P  D  L  S  D  M  L  K  F
                                                                                                     200

GAGAGTGACAAGCACCAGGACATCCTCATGTGGAACTATAGAGACACATTCTTCAACCTGTCCCTGAAGGAAGTGCTGTTTCTTAGGTGGGTGAGCACTT
E  S  D  K  H  Q  D  I  L  M  W  N  Y  R  D  T  F  F  N  L  S  L  K  E  V  L  F  L  R  W  V  S  T  S
                                                                                                     234

CCTGTCCAGACGCAGAGTTTGTCTTCAAGGGCGATGATGACGTGTTTGTGAACACCCATCACATCCTTAATTACTTGAATAGCTTATCCAAGAGCAAAGC
  C  P  D  A  E  F  V  F  K  G  D  D  D  V  F  V  N  T  H  H  I  L  N  Y  L  N  S  L  S  K  S  K  A
                                                                                                     267

CAAAGACTTGTTCATAGGTGACGTGATCCACAATGCTGGGCCTCACCGGGATAAGAAACTGAAGTACTACATCCCAGAAGTCTTCTACACCGGCGTCTAC
 K  D  L  F  I  G  D  V  I  H  N  A  G  P  H  R  D  K  K  L  K  Y  Y  I  P  E  V  F  Y  T  G  V  Y
                                                                                                     300

CCACCGTATGCCGGGGGTGGTGGATTCCTGTACTCCGGCCCCCTTGCCTTGAGGCTGTACAGTGCGACTAGCCGGGTCCATCTCTACCCTATTGATGATG
P  P  Y  A  G  G  G  G  F  L  Y  S  G  P  L  A  L  R  L  Y  S  A  T  S  R  V  H  L  Y  P  I  D  D  V
                                                                                                     334

TTTATACGGGAATGTGCCTTCAGAAACTGGGCCTTGTTCCAGAGAAGCACAAAGGCTTCAGGACATTTGATATTGAAGAGAAAAATAAGAAAAATATTTG
  Y  T  G  M  C  L  Q  K  L  G  L  V  P  E  K  H  K  G  F  R  T  F  D  I  E  E  K  N  K  K  N  I  C
                                                                                                     367

TTCCTATATAGACCTAATGTTAGTACATAGCAGAAAACCTCAAGAGATGATTGATATCTGGTCTCAGTTGCAAAGTCCTAATTTAAAATGCTGA
 S  Y  I  D  L  M  L  V  H  S  R  K  P  Q  E  M  I  D  I  W  S  Q  L  Q  S  P  N  L  K  C  *
                                                                                                     397

AATAGACATGAGCTGCATTTCACAGAAAGGCCTAGCCTGACTAGTTCCCATGGTGTGCTCTCACAATAGGTGAGTTCTGTGTGAGGCTATTAGCCTTCAT
GAGCAGGTAGCCCCTGGGCTCCCAAGCCCTCAGTCCTTCCCTTGCCTTGTGAAGAGGGAAGGCTGAAGACAGCTCAGCATGGCAGGGTGAGTGGTTATGA
CCCTTCCTCTGGCTGCCGGTCCTCAGTTTCTAATTTGTTTTCTTTCTCCTCCACAATTATGTATGTATGTGTGTATATATGTGTGTGTGTACATACATAC
ATATATATATATGTAGGACACAACCTGGTGGCTTTGTGAAATGGAATTCCTATGTATTTTCATAAGATTTTGAAAGTTGTCTAGAAAGTAGACTGATGTC
AATCTCCCGTCACCCAGCAGTATTGTCCTTGTTACTAGAAACCGTTACTTCCTTTATGCAAGGAAAGCCACGCAGGCGTGTAGTTCATCTTGTCAGGGCT
TATGGCCATGAGGACAGAGGGGATTTTCTTTTTACTTGTGTTTGGTTTCCTGGGTGGCATCATGGTAGTTAACCTATTTTTAGTATTTGAAGATCATGTG
ATTCCCTAATGGCCAACTGGAGACTGAGTAGCCCGACAGCCATGGGTCTGTGAGTGTTCAGAGACTGGGAAGCATTCGCCACTTCTGAGCTTTGGACGTG
ATTAGTCAGTTAAAACCCCAAGATTCTATTCTTGCCATATTATCACGTATTCCTTAGATAAAATTCTGGGTAGTGACACTTCCCTGTCTCAGTGTAGAAG
TGCCTGTGCTTTTATTTATTGTTCAGATCAAACACCAAAACATTTTCTTAAAAAATATTTTGTGTAATATTTTATTTGTATACAGTGTTTGTGAAATATT
TAACTAGAGCATGATATTTTATTTTTTCTCATTTTTAATTCTTTGAGAATTTCATACAATGTGTTTTGATTGTATTCACCTCCCCATCTCCTCCCAGATC
CAGCATGATGTTTTAAATGTTAAGCTGTAACATGTTAGATAAAGTTAACTCTTATTTTTGAATTTTAAAATTTGGATGGGGGGGTATGAACTGCTAGAGA
AAATAAAGTTCTGCCAAAATATTGCATATACTAGTATCTTGTAACATGCTTTCTTGAAATATTTTTGTGCTTTAGAGGGGTCTCACCTGTGCTACAGGGG
ACTGGGAAAAGTGGAATAAAGTGATTGTATTTTTTAATCAAAAAAAAAAAAAAAAAAAAAAAAAAAAA
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Figure 1. Sequence of mouse β3GnT. The 2676 bp mouse β3GnT cDNA and translation product are shown. Amino acid residues are numbered. The hydrophobic transmembrane domain is between amino acid residues Leu10 and Ser28 (underlined). Cysteine residues are shaded and underlined. The sequence has been deposited to GenBank as accession number AY043479.

recorded at 500 MHz at 25°C using a Varian Unity Plus 500 spectrometer (University of Toronto NMR Centre) [23]. One-dimensional and two-dimensional total correlation spectroscopy (^{1}H-^{1}H TOCSY) spectra were recorded. Chemical shifts (δ) were expressed in parts per million relative to an internal acetone standard (δ 2.225).

Chromosomal localization of the human β3GnT orthologue

The human cDNA sequence (not shown) orthologous to mouse β3GnT was obtained by sequencing EST clones identified on the basis of extremely high homology to the 2676 bp mouse β3GnT cDNA sequence. Mouse β3GnT cDNA was radioactively labeled by random priming and used for screening of the human RPCI-11 BAC (Bacterial Artifical Chromosome) library. From the initial screening two positive BACs were identified (H_NH0093M19, H_NH0172O03) which were confirmed positive by blot hybridzation analysis and PCR using primers designed from the 3′-UTR (3′-untranslated region) of the human cDNA sequence. BAC clone H_NH0093M19 was then used as a probe for fluorescence *in situ* hybridization (FISH) mapping experiments on metaphase chromosomes prepared from human lymphocytes. The human β3GnT cDNA sequence was also used to search the human sequence databases. An STS (sequence tagged site, WI-17834, accession number G23485) was identified having 98% identity to the cDNA sequence over 342 bp (1445–1786).

Results

Mouse β3GnT is localized to the Golgi apparatus

Searches of public EST DNA databases with the *Drosophila* Brainiac protein sequence as query allowed us to identify and clone a novel murine cDNA with high homology to both Brainiac (30% identities over 281 amino acid residues) and the

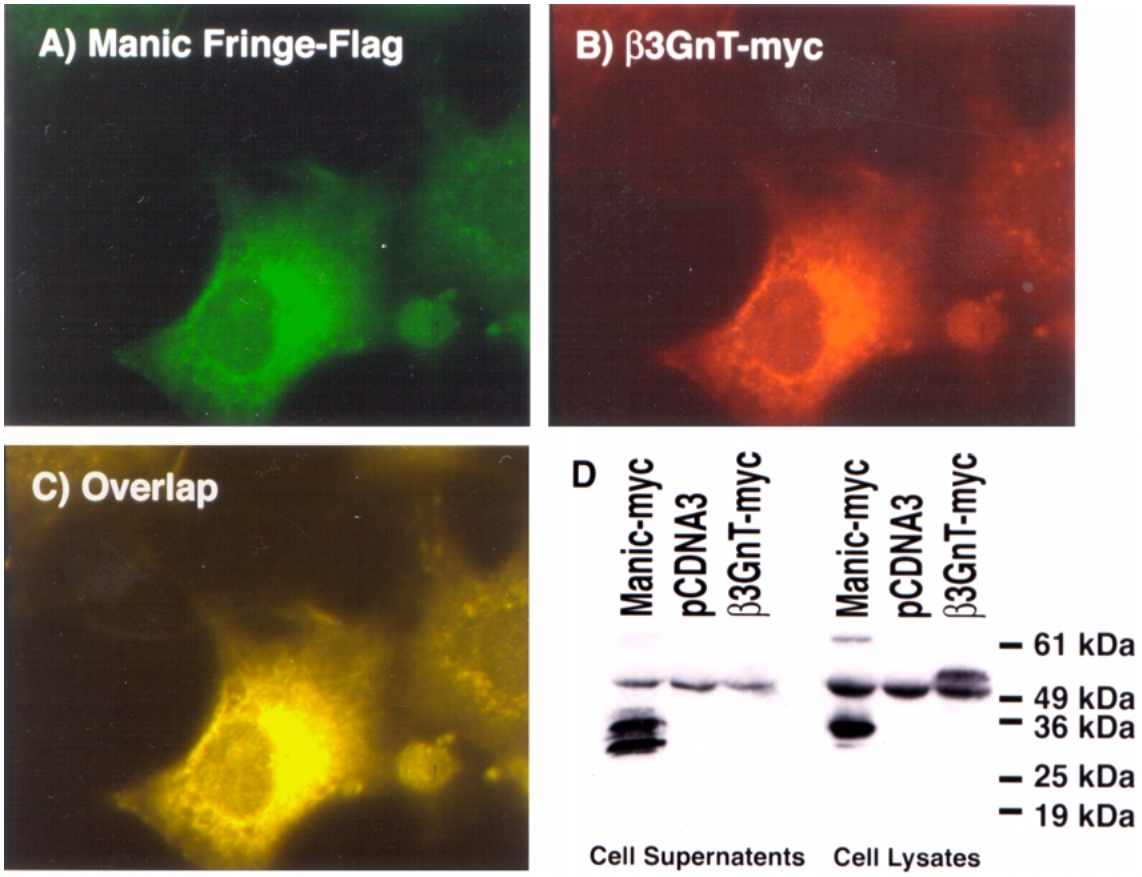

Figure 2. Localization of epitope tagged β3GnT in the Golgi apparatus. (A,B,C) A representative COS-7 cell transfected with both pcDNA3-β3GnT-myc and pcDNA3-Manic Fringe-Flag was stained for tagged Manic Fringe and β3GnT as described in the Methods. (A) Manic Fringe-Flag stained with FITC-labeled anti-Flag. (B) β3GnT-myc stained with anti-myc antibodies and detected with Texas Red. (C) Overlap of images shown in panels A and B reveals co-localization of β3GnT and Manic Fringe in the Golgi compartment. Cells transfected and stained for either Manic Fringe-Flag or β3GnT-myc alone showed similar localization as doubly transfected cells (not shown). (D) COS cells were transfected with pcDNA3, pcDNA3-Manic Fringe-myc or pcDNA3-β3GnT-myc. Culture supernatents and cell lysates were harvested and immunoprecipitated with mouse anti-myc antibody. Precipitates were analyzed on Western blots using rabbit anti-myc antibody (see Methods).

mammalian β1,3-galactosyltransferase (β3GalT) family (30–37% identities over 66–272 residues) [4–12]. This gene encodes a β1,3-*N*-acetylglucosaminyltransferase (β3GnT) (see below). The full length β3GnT cDNA encodes a 397 amino acid protein with an *N*-terminal hydrophobic transmembrane domain (residues 10–28, Figure 1). *N*-terminal hydrophobic sequences can function as leader sequences for secreted proteins or as transmembrane domains of type II transmembrane proteins. To distinguish between these two possibilities we added a c-myc epitope tag at the C-terminus of β3GnT and tested for subcellular localization of the tagged β3GnT-myc protein. Tagged β3GnT co-localizes with an epitope tagged version of Manic Fringe (Figure 2A–2C), a Golgi localized β3GnT that can extend O-linked fucose residues on Notch receptor proteins [2,3]. In addition, whereas Manic Fringe can be secreted from COS cells, overexpressed β3GnT-myc protein (55 kDa) was found exclusively in cell lysates (Figure 2D). β3GnT is therefore a Golgi localized type II transmembrane protein.

Properties of recombinant β3GnT expressed in the Sf9/baculovirus system

In order to study the enzymatic properties of this protein we generated a secreted form of the enzyme by expression in the Sf9/baculovirus system. Western blot analysis of the supernatant obtained 5 days after infection of Sf9 cells with recombinant baculovirus showed a single strong protein band at 47.5 kDa (not shown); the predicted molecular weight of the recombinant protein after cleavage by signal peptidase is 46.96 kDa. The enzyme can transfer GlcNAc to acceptors with Gal(β1-4)Glc(NAc) at the non-reducing termini but is inactive with Gal(β1-3)GlcNAc(β1-3)Gal(β1-4)Glc, Gal(β1-3)GalNAc(αl-OBn), Gal(αl-OpNP), Gal(β1-OpNP) and various acceptors with GalNAc or GlcNAc at the non-reducing termini (Table 1). The data rule out several known GnT enzymes acting on O-glycans [24]. The enzyme cannot transfer GlcNAc to Fuc(αl-OpNP) or Fuc(β1-OpNP) and is therefore different from the β3GnT activity recently attributed to Fringe proteins [2,3]. The enzyme cannot transfer Gal from UDP-Gal to Gal(β1-4)Glc(β1-OBn), GalNAc(α1-OBn), GalNAc(β1-OpNP), GlcNAc(α1-OBn) and GlcNAc(β1-OBn); the data rule out several known galactosyltransferases [5]. The enzyme was inactive in the absence of Mn^{2+} and showed a broad Mn^{2+} concentration requirement between 5 and 20 mM (data not shown).

Proton NMR spectroscopy of the purified β3GnT product using Gal(β1-4)Glc(β1-OBn) as substrate (Table 2) gave data very similar to that reported previously for GlcNAc(β1-3)Gal(β1-4)Glc(β1-OBn) [25]. Comparison of the spectra for substrate and product (Table 2) shows significant shifts for H-1 (0.014 ppm upfield) and H-4 (0.224 ppm downfield) of the galactose residue of the product; similar shifts were found on incorporation of GlcNAc in β1,3 linkage to the terminal Gal residues of Gal(β1-4)Glc(β1-OpNP) [26], lactose [27], Gal(β1-3)GalNAc [28] and Gal(αl-OpNP) [26].

Table 2. Proton NMR data at 25°C for β3GnT substrate Gal(β1-4)Glc(β1-OBn) and product GlcNAc(β1-3)Gal(β1-4)Glc(β1-OBn)[#]

Proton	*Gal*(β1-4)Glc(β1-OBn)	*GlcNAc*(β1-3)-Gal(β1-4)Glc(β1-OBn)
Benzyl		
C_6H_5	7.460	7.459
CH_2	4.936 (11.6)	4.934 (11.6)
Glucose		
H-1	4.552 (8.1)	4.551 (8.1)
H-2	3.341	3.341
H-3	nd	3.61
H-4	nd	3.65
H-5	nd	3.57
H-6*	3.982	3.980
Galactose		
H-1	4.441 (7.8)	4.427 (7.8)
H-2	nd	3.57
H-3	nd	3.69
H-4	3.916 (3.6)	4.140 (3.2)
H-5	nd	3.72
GlcNAc		
H-1		4.677 (8.7)
H-2		3.74
H-3		3.55
H-4		3.463
H-5		3.447
H-6*		3.890
N-acetyl		2.031

[#]Chemical shifts (δ) in parts per million with coupling constants (*J*, Hz) in parenthesis; nd, not determined.
*Signals could be due either to H-6a or H-6b.

It is concluded that the mouse homologue to *Drosophila* Brainiac is a β1,3-*N*-acetylglucosaminyltransferase specific for acceptors with Gal(β1-4)Glc(NAc) at the non-reducing termini.

Northern blot analysis of β3GnT

A multi-tissue RNA dot blot (Clontech) was probed with the β3GnT cDNA to obtain a general expression profile for this gene (not shown). Multiple-tissue Northern blots (Clontech) containing poly(A)$^+$ mRNA from adult mouse tissues (Figure 3A), adult human tissues (Figure 3B), and human tumor cell lines (Figure 3C) were probed with mouse β3GnT cDNA. The data reveal a 3.0 kb transcript that is widely expressed with particularly high levels noted in kidney, liver, placenta, caudal nucleus, putamen, pituitary gland, salivary glands, hematopoietic system, fetal organs and many human cancer cell lines (Figure 3 and data not shown).

Localization of human β3GnT to chromosome 2p15

The STS WI-17834 (see Methods) was localized to chromosome 2 in the reference interval D2S337-D2S147 by mapping

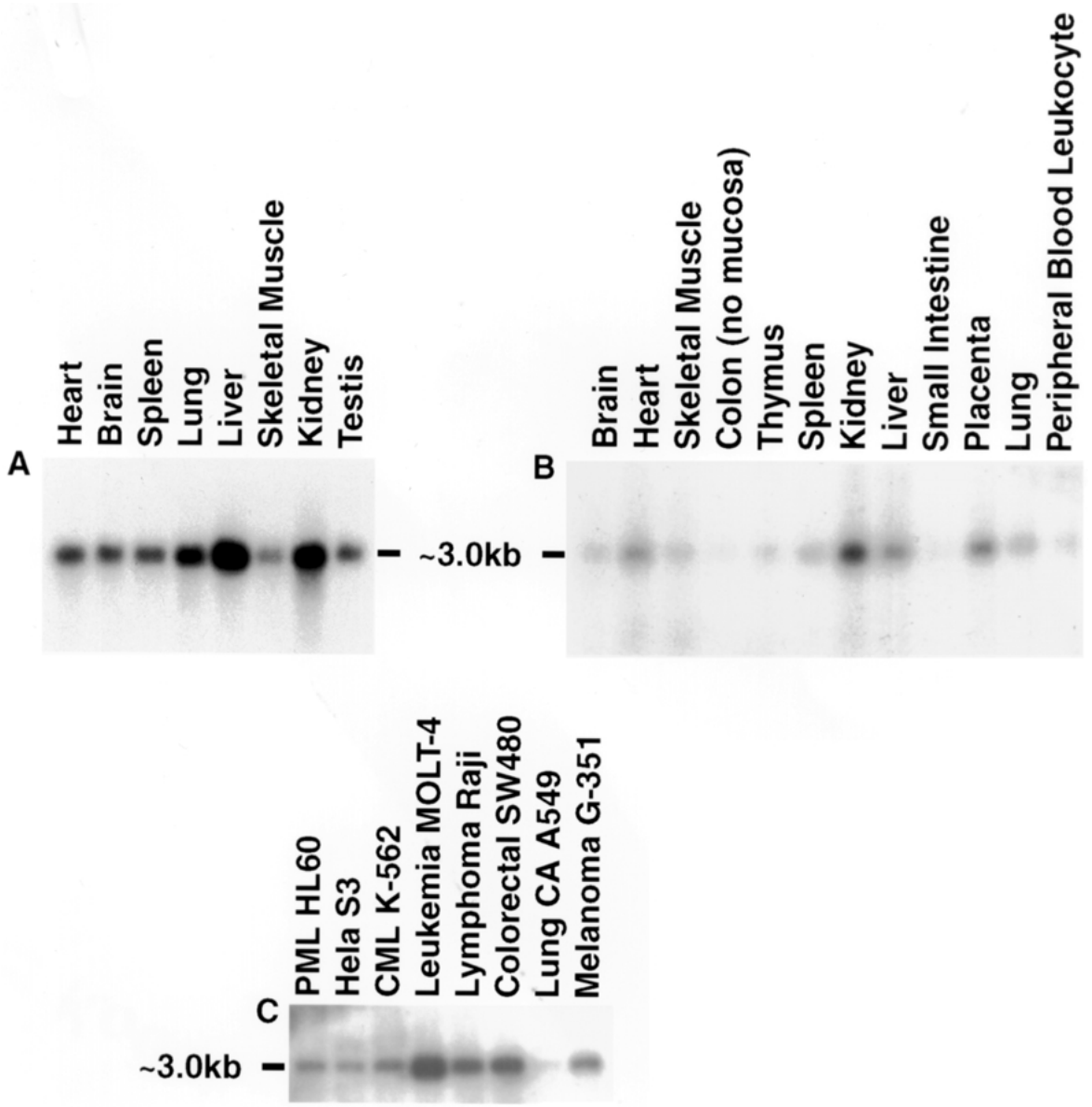

Figure 3. Expression Analysis – Northern blots. Multi-tissue Northern Blots probed with the β3GnT cDNA reveal the presence of an approximately 3.0 kb transcript in all tissues and cell lines analyzed. (A) Mouse adult tissues, (B) Human adult tissues, (C) Tumor cell lines.

with a panel of radiation hybrid cell lines. D2S337 is known to reside in YAC (Yeast Artifical Chromosome) clones localized to chromosome 2p15 by FISH. To confirm the location of this gene, FISH was performed using BAC clone H_NH0093M19 which did indeed map to chromosome 2p15.

Discussion

We have identified a novel widely expressed mouse cDNA that codes for a Golgi localized β3GnT enzyme with striking similarity to *Drosophila* Brainiac and the mammalian β3GalT enzyme family. β3GnT is capable of initiating and elongating poly-*N*-acetyllactosamine chains, i.e., it can transfer GlcNAc in β1,3-linkage to the terminal Gal of both Gal(β1-4)GlcNAc and Gal(β1-4)GlcNAc(β1-3)Gal(β1-4)Glc (Table 1). The enzyme is similar in substrate specificity to β3GnT enzymes previously described in human urine, human and calf serum, rat testis and other tissues [29–35]. A human poly-*N*-acetyllactosamine synthase, a β3GnT, cloned by Sasaki *et al*. in 1997 [36] shows no significant homology to our β3GnT nor to other members of the β3GalT enzyme family. The substrate specificity of our mouse β3GnT is similar to that of a previously cloned human and mouse β3GnT [25], which showed only 26% identity to our enzyme over 245 amino acid residues. A recent correction by the authors of this paper (Proc.Nat.Acad.Sci. **97**, 11673–11675, 2000) showed that their enzyme is identical to our gene product. A recently published paper by Shiraishi *et al*. [37] reported the isolation

of three human β3GnT enzymes designated β3GnT 2, 3 and 4 which all shared a motif conserved among the β1,3-galactosyltransferase and β1,3-*N*-acetylglucosaminyltransferase families. Their human β3GnT 2 is the orthologue of our mouse β3GnT. We have shown in this paper that the human orthologue of our gene is on chromosome 2p15. The physiological roles played by the various β3GnT enzymes in poly-*N*-acetyllactosamine synthesis remain to be determined. It has been shown that poly-*N*-acetyllactosamine chains on the surfaces of tumour cells are related to metastatic potential [38]. It is therefore of great interest that our β3GnT is strongly expressed in several tumour lines (Figure 3C). It is clearly important to identify and study the *in vivo* targets of the mammalian β3GnTs and to determine whether the *Drosophila* Brainiac protein is also a glycosyltransferase.

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References

1 Yuan YP, Schultz J, Mlodzik M, Bork P, Secreted fringe-like signaling molecules may be glycosyltransferases, *Cell* **88**, 9–11 (1997).
2 Moloney DJ, Panin VM, Johnston SH, Chen J, Shao L, Wilson R, Wang Y, Stanley P, Irvine KD, Haltiwanger RS, Vogt TF, Fringe is a glycosyltransferase that modifies Notch, *Nature* **406**, 369–75 (2000).
3 Bruckner K, Perez L, Clausen H, Cohen S, Glycosyltransferase activity of Fringe modulates Notch-Delta interactions, *Nature* **406**, 411–5 (2000).
4 Amado M, Almeida R, Carneiro F, Levery SB, Holmes EH, Nomoto M, Hollingsworth MA, Hassan H, Schwientek T, Nielsen PA, Bennett EP, Clausen H, A family of human beta 3-galactosyltransferases–Characterization of four members of a UDP-galactose:beta-*N*-acetyl-glucosamine/beta-N-acetyl-galactosamine beta-1,3-galactosyltransferase family, *J Biol Chem* **273**, 12 770–8 (1998).
5 Amado M, Almeida R, Schwientek T, Clausen H, Identification and characterization of large galactosyltransferase gene families: galactosyltransferases for all functions, *Biochim Biophys Acta* **1473**, 35–53 (1999).
6 Kolbinger F, Streiff MB, Katopodis AG, Cloning of a human UDP-galactose:2-acetamido-2-deoxy-D-glucose 3 beta-galactosyltransferase catalyzing the formation of type 1 chains, *J Biol Chem* **273**, 433–40 (1998).
7 Isshiki S, Togayachi A, Kudo T, Nishihara S, Watanabe M, Kubota T, Kitajima M, Shiraishi N, Sasaki K, Andoh T, Narimatsu H, Cloning, expression, and characterization of a novel UDP-galactose:beta-N-acetylglucosamine beta 1,3-galactosyltransferase (beta3Gal-T5) responsible for synthesis of type 1 chain in colorectal and pancreatic epithelia and tumor cells derived therefrom, *J Biol Chem* **274**, 12 499–507 (1999).
8 Hennet T, Dinter A, Kuhnert P, Mattu TS, Rudd PM, Berger EG, Genomic cloning and expression of three murine UDP-galactose: beta-N-acetylglucosamine beta 1,3-galactosyltransferase genes, *J Biol Chem* **273**, 58–65 (1998).
9 Zhou D, Berger EG, Hennet T, Molecular cloning of a human UDP-galactose:GlcNAcbeta1,3GalNAc beta1, 3 galactosyltransferase gene encoding an O-linked core3-elongation enzyme, *European Journal Of Biochemistry* **263**, 571–6 (1999).
10 Zhou D, Henion TR, Jungalwala FB, Berger EG, Hennet T, The beta 1,3-galactosyltransferase beta 3GalT-V is a stage-specific embryonic antigen-3 (SSEA-3) synthase, *J Biol Chem* **275**, 22 631–4 (2000).
11 Bardoni A, Valli M, Trinchera M, Differential expression of beta 1,3galactosyltransferases in human colon cells derived from adenocarcinomas or normal mucosa, *Febs Letters* **451**, 75–80 (1999).
12 Miyazaki H, Fukumoto S, Okada M, Hasegawa T, Furukawa K, Furukawa K, Expression cloning of rat cDNA encoding UDP-galactose:G(D2) beta 1,3-galactosyltransferase that determines the expression of G(D1b)/G(M1)/G(A1), *J Biol Chem* **272**, 24 794–99 (1997).
13 Cohen B, Bashirullah A, Dagnino L, Campbell C, Fisher B, Leow CC, Whiting E, Ryan D, Zinyk D, Boulianne G, Hui C-c, Gallie B, Phillips RA, Lipshitz H, Egan SE, *Nature Genetics* **16**, 283–8 (1997).
14 Sarkar M, Pagny S, Unligil U, Joziasse D, Mucha J, Glossl J, Schachter H, Removal of 106 amino acids from the N-terminus of UDP-GlcNAc:alpha-3-D-mannoside beta1,2-N-acetylglucosaminyltransferase I does not inactivate the enzyme, *Glycoconjugate J* **l5**, 193–7 (1998).
15 Sarkar M, Schachter H, Cloning and expression of *Drosophila melanogaster* UDP-GlcNAc:α-3-D-mannoside β1,2-*N*-acetylglucosaminyltransferase I, *Biol Chem* **382**, In press. (2001).
16 Ausubel FM, Brent R, Kingston RE, Moore DD, Seidman JG, Smith JA, Struhl K, *Current Protocols in Molecular Biology*, (John Wiley and Sons, New York, N.Y., 1993).
17 Sambrook J, Fritsch EF, Maniatis T, *Molecular Cloning: A laboratory manual*, (Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y., 1989).
18 Sengar AS, Wang W, Bishay J, Cohen S, Egan SE, *Embo J* **18**, 1159–71 (1999).
19 Laemmli UK, Cleavage of structural proteins during the assembly of the head of bacteriophage T_4, *Nature* **227**, 680–5 (1970).
20 Palcic MM, Heerze LD, Pierce M, Hindsgaul O, The use of hydrophobic synthetic glycosides as acceptors in glycosyltransferase assays, *Glycoconjugate J* **5**, 49–63 (1988).
21 Schachter H, Narasimhan S, Gleeson P, Vella G, Glycosyltransferases involved in elongation of N-glycosidically linked oligosaccharides of the complex or N-acetyllactosamine type, *Methods in Enzymology* **98**, 98–134 (1983).
22 Hull E, Sarkar M, Spruijt MPN, Höppener JWM, Dunn R, Schachter H, Organization and localization to chromosome 5 of the human UDP-N-acetylglucosamine:alpha-3-D-mannoside

beta-1,2-N-acetylglucosaminyltransferase I gene, *Biochem Biophys Res Commun* **176**, 608–15 (1991).
23 Reck F, Meinjohanns E, Tan J, Grey AA, Paulsen H, Schachter H, Synthesis of pentasaccharide analogues of the N-glycan substrates of N-acetylglucosaminyltransferases III, IV and V using tetrasaccharide precursors and recombinant beta-(1→2)-N-acetylglucosaminyltransferase II, *Carbohydr Res* **275**, 221–9 (1995).
24 Schachter H, Brockhausen I, The biosynthesis of serine(threonine)-N-acetylgalactosamine-linked carbohydrate moieties. In *Glycoconjugates. Composition, Structure and Function*, edited by Allen HJ, Kisailus EC (Marcel Dekker, Inc., New York, N.Y., 1992) pp. 263–332.
25 Zhou DP, Dinter A, Gallego RG, Kamerling JP, Vliegenthart JFG, Berger EG, Hennet T, A beta-1,3-N-acetylglucosaminyltransferase with poly-N-acetyllactosamine synthase activity is structurally related to beta-1,3-galactosyltransferases, *Proc Nat Acad Sci USA* **96**, 406–11 (1999).
26 Blixt O, van Die I, Norberg T, van den Eijnden DH, High-level expression of the Neisseria meningitidis lgtA gene in Escherichia coli and characterization of the encoded N-acetylglucosaminyltransferase as a useful catalyst in the synthesis of GlcNAc beta 1 → 3Gal and GalNAc beta 1 → 3Gal linkages, *Glycobiology* **9**, 1061–71 (1999).
27 Brockhausen I, Matta KL, Orr J, Schachter H, Koenderman AHL, van den Eijnden DH, Mucin synthesis. VII. Conversion of R_1-β1-3Gal-R_2 to R_1-β1-3(GlcNAcβ1-6)Gal-R_2 and of R_1-β1-3GalNAc-R_2 to R_1-β1-3(GlcNAcβl-6)GalNAc-R_2 by a β6-N-acetylglucosaminyltransferase in pig gastric mucosa, *Eur J Biochem* **157**, 463–74 (1986).
28 Brockhausen I, Orr J, Schachter H, Mucin synthesis. V. The action of pig gastric mucosal UDP-GlcNAc:Galβ1-3(R_1)GalNAc-R_2 (GlcNAc to Gal) β3-N-acetylglucosaminyltransferase on high molecular weight substrates, *Can J Biochem Cell Biol* **62**, 1081–90 (1984).
29 Takeya A, Hosomi O, Kogure T, The presence of N-acetyllactosamine and lactose: β(1-3)N-acetylglucosaminyltransferase activity in human urine, *Japan J Med Sci Biol* **38**, 1–8 (1985).
30 Piller F, Cartron J-P, UDP-GlcNAc:Galβ1-4Glc(NAc) β1-3N-acetylglucosaminyltransferase. Identification and characterization in human serum, *J Biol Chem* **258**, 12 293–99 (1983).
31 Hosomi O, Takeya A, Kogure T, Separation into two major forms of beta(1-3)N-acetylglucosaminyltransferase from human serum, *Jpn J Med Sci Biol* **42**, 77–82 (1989).
32 Tsuji Y, Urashima T, Matsuzawa T, The characterization of a UDP-N-acetylglucosamine: Gal beta 1-4Glc(NAc) beta 1-3 N-acetylglucosaminyltransferase in fluids from rat rete testis, *Bba-Gen Subjects* **1289**, 115–21 (1996).
33 Kawashima H, Yamamoto K, Osawa T, Irimura T, Purification and Characterization of UDP-GlcNAc-Gal beta 1-4Glc(NAc) beta-1,3-N-acetylglucosaminyltransferase (Poly-N-acetyllactosamine Extension Enzyme) from Calf Serum, *J Biol Chem* **268**, 27 118–126 (1993).
34 Stults C, Macher BA, beta1-3-N-acetylglucosaminyltransferase in human leukocytes: Properties and role in regulating neolacto glycosphingolipid biosynthesis, *Arch Biochem Biophys* **303**, 125–33 (1993).
35 Holmes EH, Characterization of a β1-3N-acetylglucosaminyltransferase associated with synthesis of type 1 and type 2 lacto-series tumor-associated antigens from the human colonic adenocarcinoma cell line SW403, *Arch Biochem Biophys* **260**, 461–8 (1988).
36 Sasaki K, KurataMiura K, Ujita M, Angata K, Nakagawa S, Sekine S, Nishi T, Fukuda M, Expression cloning of cDNA encoding a human beta-1,3-N-acetylglucosaminyltransferase that is essential for poly-N-acetyllactosamine synthesis, *Proc Natl Acad Sci USA* **94**, 14 294–9 (1997).
37 Shiraishi N, Natsume A, Togayachi A, Endo T, Akashima T, Yamada Y, Imai N, Nakagawa S, Koizumi S, Sekine S, Narimatsu H, Sasaki K, Identification and Characterization of Three Novel β1,3-N-Acetylglucosaminyltransferases Structurally Related to the β1,3-Galactosyltransferase Family, *J Biol Chem* **276**, 3498–507 (2001).
38 Dennis JW, Granovsky M, Warren CE, Glycoprotein glycosylation and cancer progression, *Biochim Biophys Acta* **1473**, 21–34 (1999).

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