Glycoconjugate Journal (1994) **11**:204–209

Expression of recombinant rabbit UDP-GlcNAc:α3-D-mannoside β-1,2-*N*-acetylglucosaminyltransferase I catalytic domain in Sf9 insect cells

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Received 14 January 1994

UDP-GlcNAc:α3-D-mannoside β-1,2-*N*-acetylglucosaminyltransferase I (GnT I; EC 2.4.1.101) catalyses a key reaction in the conversion of oligomannose to complex and hybrid *N*-glycans. The cytoplasmic tail and transmembrane segment of rabbit GnT I cDNA were replaced with an in-frame cleavable signal sequence and the hybrid construct was inserted into the genome of *Autographa californica* nuclear polyhedrosis virus (AcMNPV) under the control of the polyhedrin promoter. Sf9 insect cells were infected with the recombinant baculovirus and the enzymatically active and soluble catalytic domain of GnT I was purified from the medium (1–5 mg l^{-1}) in two steps to a specific activity of about 2 μmol min^{-1} mg^{-1} protein. Recombinant GnT I has been used for the chemical-enzymatic synthesis of analogues of Manα1-6[GlcNAcβ1-2Manα1-3]Manβ-*O*-octyl.

Keywords: recombinant/*N*-acetylglucosaminyl transferase *I*/*N*-glycans/baculovirus/insect cells

Abbreviations: *Autographa californica* nuclear polyhedrosis virus. AcMNPV; fetal calf serum, FCS; international enzyme unit, 1 μmol min^{-1}; myelin associated glycoprotein, MAG; multiplicity of infection, MOI; plaque forming units, pfu; sodium dodecyl sulfate/polyacrylamide gel electrophoresis, SDS-PAGE; *Spodoptera frugiperda* insect cells, Sf9 cells; UDP-GlcNAc:α3-D-mannoside β1,2-*N*-acetylglucosaminyltransferase I (EC 2.4.1.101), GnT I.

Introduction

UDP-GlcNAc:α3-D-mannoside β-1,2-*N*-acetylglucosaminyltransferase I (GnT I; EC 2.4.1.101) catalyses the conversion of [Manα1-6(Manα1-3)Manα1-6][Manα1-3]Manβ1-4R to [Manα1 - 6(Manα1 - 3)Manα1 - 6][**GlcNAcβ1-2**Manα1-3] Manβ1-4R, where R is GlcNAcβ1-4(+/–Fucα1-6)Glc NAc-Asn-polypeptide [1, 2]. GnT I action is an essential prerequisite for several enzymes in the biosynthetic pathway leading from oligomannose to complex and hybrid *N*-glycans, i.e. *N*-acetylglucosaminyltransferases II, III and IV, α-mannosidase II and core α-1,6-fucosyltransferase; *N*-acetylglucosaminyltransferases V and VI, in turn, require the prior action of *N*-acetylglucosaminyltransferase II. Glycosyltransferases are useful for the chemical-enzymatic synthesis of oligosaccharides [3–13]. More than twenty glycosyltransferase genes have been cloned to date [14–18] suggesting that various recombinant enzymes will shortly become available in amounts sufficient for large-scale chemical-enzymatic syntheses. Recombinant forms of CMP-sialic acid:*N*-acetyllactosaminide α-2,3-sialyltransferase [9] and UDP-Gal:*N*-acetyllactosaminide α-1,3-galactosyltransferase [19, 20] have been expressed in the insect cell/baculovirus system and used for the synthesis of oligosaccharides. We report here the preparation of soluble enzymatically active recombinant GnT I expressed in Sf9 insect cells; preliminary reports of this work have been published [21, 22]. The recombinant enzyme has been used for the synthesis of Manα1-6[GlcNAcβ1-2Manα1-3]Manβ-*O*-octyl analogues [23, 24].

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Materials and methods

Materials

The following materials were purchased from the indicated sources: Grace's insect medium, fetal calf serum (FCS), TC Yeastolate, TC lactalbumin hydrolysate, gentamycin, amphotericin (Fungizone) (Gibco Laboratories, Grand Island, NY); Sep-Pak C_{18} reverse phase cartridges (Waters); AG1-X8, 100–200 mesh, chloride form (Bio-Rad); CM-Sephadex C-50, Protein G-Sepharose, restriction endonucleases, T4 ligase (Pharmacia); bovine serum albumin, Triton X-100, *N*-acetylglucosamine (GlcNAc), goat anti-sheep IgG coupled

to alkaline phosphatase (Sigma). UDP-hexanolamine-Sepharose was synthesized using UDP-hexanolamine (Sigma) and cyanogen bromide-activated Sepharose (Pharmacia) according to the manufacturer's instructions. UDP-[^{14}C]GlcNAc was synthesized as previously described [25] and diluted with non-radioactive UDP-GlcNAc from Sigma. Manα1-6[Manα1-3]Manβ-octyl (M_3-octyl) was kindly provided by Dr Hans Paulsen, University of Hamburg, Hamburg, Germany. Polyclonal antibody raised in sheep against purified recombinant rabbit GnT I fusion protein expressed in *E. coli* [26] was a kind gift from Drs Jo Burke and Paul Gleeson, Melbourne, Australia. Oligonucleotides were synthesized on a Pharmacia DNA synthesizer and purified by the cartridge method (Hospital for Sick Children-Pharmacia Biotechnology Centre, Toronto, Canada). Plasmid pShonex 1.1 and the baculovirus transfer vector pJVP10Z were obtained from Dr R. J. Dunn, Montreal Neurological Institute, Montreal, Quebec, Canada; pShonex 1.1 contains the ATG start site and signal sequence of myelin associated glycoprotein (MAG) cloned into the *Eco* RI site of pUC8. *Spodoptera frugiperda* (Sf9) insect cells and *Autographa californica* nuclear polyhedrosis virus (AcMNPV) were obtained from the laboratory of Dr M. D. Summers (Texas A&M University, College Station, Texas).

General procedures

Sf9 cells were grown at 28 °C in Grace's insect medium supplemented with 10% FCS, TC Yeastolate, lactalbumin hydrolysate, 50 μg ml^{-1} gentamycin sulfate and 2.5 μg ml^{-1} amphotericin (Fungizone) in either Falconware T flasks or in spinner flasks (Bellco Glass Inc., Vineland, NJ) [27–30]. Restriction endonuclease reactions, DNA ligations, miniplasmid preparations and transfections were carried out by standard methods [31]. *E. coli* DH5αF (Gibco Laboratories or BRL) were used for all DNA amplifications. Large-scale plasmid preparations were carried out using CsCl gradient centrifugation [31]. Double-strand DNA sequencing was carried out by the dideoxy method [32] using synthetic oligonucleotide primers and the Sequenase Version 2.0 kit from United States Biochemicals.

Construction of the baculovirus transfer vector pJVP10Z-GnT I

Polymerase chain reaction (PCR) amplification was used to generate a DNA fragment (nucleotides 135–1700) from rabbit GnT I cDNA clone rc2500 [33]. The DNA was inserted into plasmid pShonex 1.1 downstream from and in-frame with the ATG start site and cleavable signal sequence of MAG [34, 35]. The construct lacks the section of DNA encoding the cytoplasmic N-terminus and transmembrane domain of GnT I. The MAG-GnT I hybrid sequence was cloned into a unique *Nhe* I site within the polyhedrin gene of the baculovirus transfer vector pJVP10Z to produce plasmid pJVP10Z-GnTI. This vector also carries the *LacZ* gene under the control of the P10 promoter allowing recombinant selection by staining for β-galactosidase activity.

Recombinant baculovirus

Plasmid pJVP10Z-GnTI was co-transfected with purified wild type AcMNPV viral DNA into Sf9 cells [27–30]. About 3 μg pJVP10Z-GnT I and 1 μg viral DNA were co-transfected using cationic liposomes (Invitrogen) into 1.5×10^5 cells per 60 mm tissue culture dish (about 75% confluent) and the cells were incubated for 4 days at 28 °C to allow homologous recombination and release of virus into the culture medium. Recombinant virus containing β-galactosidase was isolated by repetitive plaque purification [36]. GnTI-encoding recombinant virus (vMAG-GnTI) was detected by infection of Sf9 cells in microtitre plate wells and assay of supernatants for GnT I activity. The most active vMAG-GnTI virus was stored as a stock solution (2×10^8 pfu ml^{-1}) at 4 °C in insect medium.

Time course of GnT I expression during baculovirus infection

Sf9 cells (6×10^6 cells in 250 ml Grace's insect medium) were infected with vMAG-GnTI virus at an MOI of 2 pfu per cell. The cells were cultured in a spinner flask in 250 ml of medium at 28 °C. Aliquots (5 ml) were taken at various times and the cells were sedimented by centrifugation. The cells were resuspended and lysed in 0.5 ml 25 mM MES, 0.1% Triton X-100 and 0.02% sodium azide. Control incubations were carried out either with uninfected Sf9 cells or Sf9 cells infected with wild type AcMNPV virus. Both the cell lysates and the supernatants were assayed for GnT I enzyme activity.

Purification of recombinant rabbit GnT I

Sf9 cells ($2.0–2.8 \times 10^8$ cells/litre) were infected with vMAG-GnTI virus at an MOI of 2 pfu per cell. The cells were cultured in spinner flasks each containing 250 ml of medium at 28 °C. Supernatants were collected at four days post-infection and passed through a CM-Sephadex C-50 column equilibrated with 25 mM MES, pH 6.5, 0.1% Triton X-100 and 0.02% sodium azide (buffer A). The column was washed with 2 l of buffer A containing 0.1 M NaCl. Enzyme was eluted with 0.25 M NaCl in buffer A. Fractions containing enzyme activity were pooled (200 ml) and concentrated to 20 ml by filtration under pressure. The yield from this step was 70%. The concentrated enzyme was diluted 2.5-fold with buffer A to a final NaCl concentration of 0.1 M and loaded on a UDP-hexanolamine-Sepharose column equilibrated with buffer A containing 20% glycerol (buffer B). The column was washed with 0.1 M NaCl in buffer B and enzyme activity was eluted with 0.75 M NaCl in buffer B with a yield of 61%. The over-all yield was 43%. The enzyme was stored at 4 °C at a concentration of 0.4 mg ml^{-1} in 0.75 M NaCl in buffer B.

Electrophoretic analysis and immunoblots of recombinant GnT I

Recombinant GnT I at various stages of purification was analysed by sodium dodecyl sulfate/polyacrylamide gel electrophoresis (SDS-PAGE) by the method of Laemmli [37]; aliquots were mixed with Laemmli buffer containing mercaptoethanol, boiled for 3 min and analysed using 12.5% polyacrylamide. Gels were fixed in methanol:acetic acid:water (50:12:38, by vol) and stained using either the silver staining method [38] or Coomassie blue. For immunoblot analysis, the gels were electrophoretically blotted on to nitrocellulose membranes. Electrophoretic transfer of GnT I protein in the absence of SDS was very inefficient as determined by staining of both gels and membranes after transfer; addition of 0.1% SDS to the transfer buffer resulted in complete transfer of protein to the membrane. The membranes were probed with a sheep polyclonal antibody raised against a recombinant rabbit GnT I fusion protein expressed in *E. coli* [26]. The sheep antiserum was purified by 50% ammonium sulfate precipitation followed by adsorption to a column of Protein G-Sepharose and elution with glycine buffer at pH 2.5. The sheep antibody was detected with goat anti-sheep IgG coupled to alkaline phosphatase.

Amino acid composition and sequence

Protein bands were transferred to Immobilon membrane (Millipore) by electrophoresis, detected on the membrane by staining with Coomassie blue and subjected to analysis for amino acid composition and amino-terminal sequence (Hospital for Sick Children-Pharmacia Biotechnology Centre).

Southern blots

Total DNA was isolated [31] from uninfected Sf9 cells and Sf9 cells infected with wild type AcMNPV and recombinant vMAG-GnTI virus. DNA was digested with *Pst* I and analysed by 1% agarose gel electrophoresis. The DNA was transferred to Hybond-N membrane (Amersham) and hybridized with a 1380 *Pst* I fragment isolated from rabbit GnT I cDNA clone rc2500. The probe was labelled by the random primer method using digoxigenin-11-dUTP and the DIG DNA Labelling Kit (Boehringer-Mannheim) according to the manufacturer's instructions. Prehybridization, hybridization and detection of label by anti-digoxigenin-AP antiserum were carried out according to the instructions in the DIG Luminescent Detection Kit supplied by Boehringer-Mannheim.

GnT I enzyme assays

GnT I was assayed [39] with 0.5 mM M_3-octyl, 1 mM UDP-[^{14}C]GlcNAc (2000 dpm nmole^{-1}). 2.5 mM AMP, 50 mM GlcNAc, 20 mM $MnCl_2$, 1 mg ml^{-1} bovine serum albumin, 0.1% Triton X-100, 0.1 M MES buffer (pH 6.1) and 0.010 ml enzyme in 0.050 ml. After incubation at 37 °C for 30 min, radioactive product was purified either by elution through AG1-X8 or with Sep-Pak C_{18} reverse-phase cartridges [40]. Product formation was proportional to time of incubation and protein concentration. Kinetic parameters were determined [41] by assays at several concentrations of M_3-octyl (0.5, 1.0, 1.5 and 2.0 mM) and UDP-GlcNAc (0.06, 0.08, 0.1 and 0.12 mM).

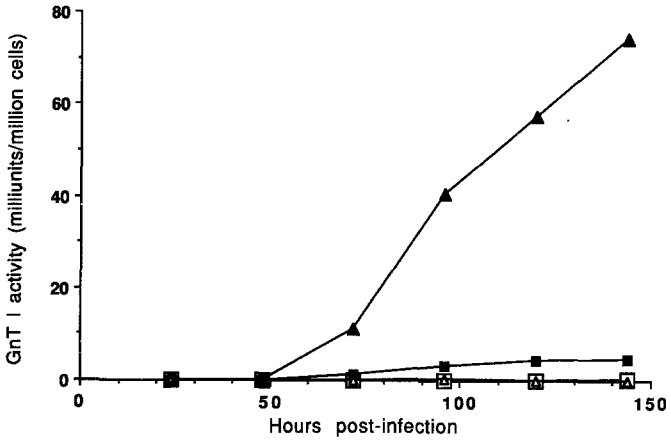

Figure 1. Time course of GnT I expression during baculovirus infection. Sf9 cells infected with vMAG-GnTI virus stock (MOI = 2) at a density of 2.5 × 10^7 cells per l were cultured in a spinner flask in 250 ml of medium at 28 °C. Aliquots (5 ml) were taken at various times and the cells were sedimented and lysed. Control incubations were carried out either with uninfected Sf9 cells or Sf9 cells infected with wild type AcMNPV virus (data not shown). Both the cell lysates and the supernatants were assayed for GnT I enzyme activity (1 mU = 1 nmol product per min). Enzyme activity (mU per 10^6 cells) is plotted against hours after infection with baculovirus. Uninfected pellet (□); uninfected supernatant (△); infected pellet (■); infected supernatant (▲).

Results and discussion

Expression of rabbit GnT I catalytic domain

Recombinant baculovirus vMAG-GnTI encodes a fusion protein in which the N-terminal cytoplasmic domain and non-cleavable signal-anchor transmembrane domain of rabbit GnT I have been replaced with the cleavable MAG signal sequence. This signal sequence effects secretion from Sf9 insect cells of MAG truncated to remove its C-terminal transmembrane domain [35]. Infection of Sf9 cells (2.5 × 10^7 cells per l) with vMAG-GnTI results in the appearance of enzymatically active GnT I in the medium (Fig. 1). Supernatants from uninfected Sf9 cells (Fig. 1) or Sf9 cells infected with wild type AcMNPV virus (data not shown) and insect cell pellets from control and infected cultures (Fig. 1) show little or no GnT I activity. The enzyme concentration in the medium at 6 days post-infection was 74 mU per 10^6 cells (1 mU = 1 nmol min^{-1}) (Fig. 1), equivalent to about 0.2 mg enzyme protein per litre of

medium. Yields of 1–5 mg l^{-1} can be obtained by increasing the Sf9 cell density 10-fold to 2.5×10^8 cells per l (see below). The secreted catalytic domain of CMP-sialic acid:*N*-acetyllactosaminide α:2,3-sialyltransferase [9] expressed in the insect cell/baculovirus system was obtained at a concentration of 20–30 mU per 10^6 cells at 72 h post-infection. The entire UDP-Gal:*N*-acetyllactosaminide α-1,3-galactosyltransferase protein including the transmembrane domain [19] was expressed as an intracellular protein in the insect cell/baculovirus system at a concentration of 50 mU per 10^6 cells at 60 h post-infection. These yields are very similar to those obtained for GnT I in this report.

Attempts to grow virus-infected Sf9 cells in various types of serum-free media led to the appearance and subsequent disappearance of GnT I activity in the medium probably due to proteolysis of GnT I. The presence of fetal calf serum in the medium prevents this loss possibly because the serum has anti-protease activity. Recombinant GnT I in the medium is stable for several months at 4 °C.

The data show that the catalytic domain of rabbit GnT I, lacking the cytoplasmic and transmembrane domains, is enzymatically active; this has also been demonstrated for rabbit [26] and mouse [42] GnT I expressed in *E. coli* and for several other glycosyltransferases [9, 43–46].

Purification of recombinant rabbit GnT I

Sf9 cells ($2.0–2.8 \times 10^8$ cells per l) were infected with vMAG-GnTI virus, medium was collected 4 days after infection (GnT I concentration was about 5 mg l^{-1}) and recombinant GnT I was purified as described above. SDS-PAGE analyses (Fig. 2) showed two major protein bands at about 50 and 42 kDa. Two or three minor bands were usually seen between the two major bands. Immunoblots showed a single band at about 50 kDa (Fig. 3). Amino acid composition analysis and amino-terminal sequence analysis (data not shown) of the separated two bands after transfer to Immobilon membrane suggest that the large band is the catalytic domain of GnT I after cleavage of the MAG signal sequence (predicted to be 48 kDa) and the small band is due to GnT I which had been truncated by removal of the stem region to yield a 42 kDa protein. The data indicate that there has been proteolysis in the stem region during expression and purification of recombinant GnT I. The enzyme is stable for several weeks at 4 °C at a concentration of 0.4 mg ml^{-1} but freezing or excessive dilution leads to enzyme inactivation. It is not known whether the 42 kDa protein is enzymatically active.

We have shown [33, 47, 48] that the catalytic domains of rabbit, human and mouse GnT I are 93% similar while the stem regions show a similarity between species of only 75% or less. Our sheep anti-rabbit GnT I antiserum is probably directed primarily against the stem region thereby explaining why the antiserum reacts with the large band but not with the 42 kDa band.

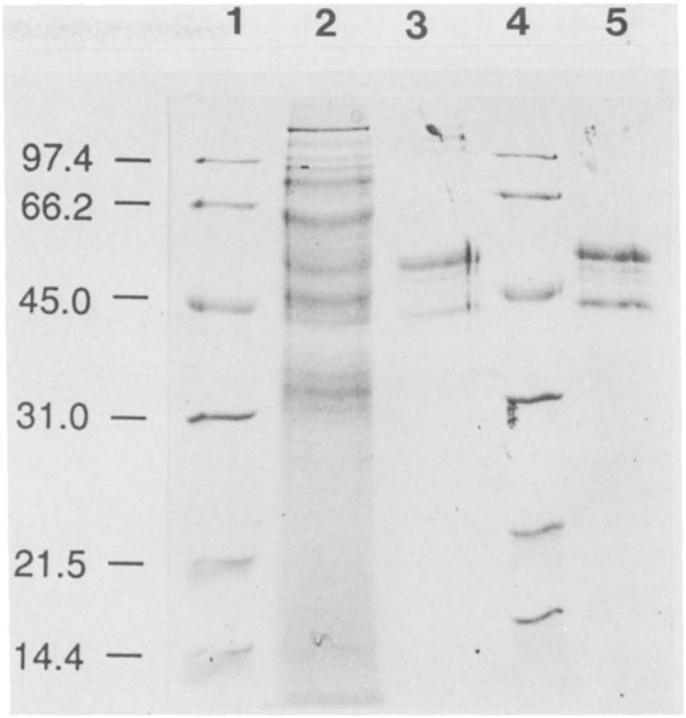

Figure 2. SDS-PAGE of various recombinant GnT I fractions. Recombinant GnT I was analysed by SDS-PAGE by the method of Laemmli [37] in the presence of mercaptoethanol using 12.5% polyacrylamide gels. The gel was stained with Coomassie blue. Lanes 1 and 4: protein standards, 200 and 300 ng per lane per band respectively; lane 2: 0.020 ml of GnT I after the CM-Sephadex step; lanes 3 and 5: 0.005 ml of the two peak fractions from the UDP-hexanolamine-Sepharose column. Molecular weight markers are indicated in kDa.

The specific activity of the recombinant enzyme was about 2 U mg^{-1} (1 U = 1 μmol min^{-1}), somewhat lower than the specific activities of 16.3 and 4.6 U mg^{-1} previously found for GnT I purified from rabbit [41] and rat [39] liver respectively. Recombinant GnT I has also been expressed in *E. coli* but most of the protein was present in an insoluble form which required detergent and sonication for solubilization [26, 42]. The specific activity of crude *E. coli* lysates [42] or of affinity-purified recombinant *E. coli* GnT I [26] was about 0.001–0.02 U mg^{-1} suggesting that solubilization of the *E. coli* enzyme results in denaturation. Recombinant α-1,3-galactosyltransferase [19] was purified to a specific activity of 3 U mg^{-1} and the yield was about 2–3 mg protein per l of culture; this compares well with the results reported in this paper for GnT I.

Southern blots

Southern analysis of *Pst* I DNA digests (data not shown) indicated that only Sf9 cells infected with recombinant vMAG-GnTI baculovirus showed the expected 1380 bp *Pst* I cleavage product. Non-infected cells and cells infected with wild type AcMNPV baculovirus showed no detectable DNA bands.

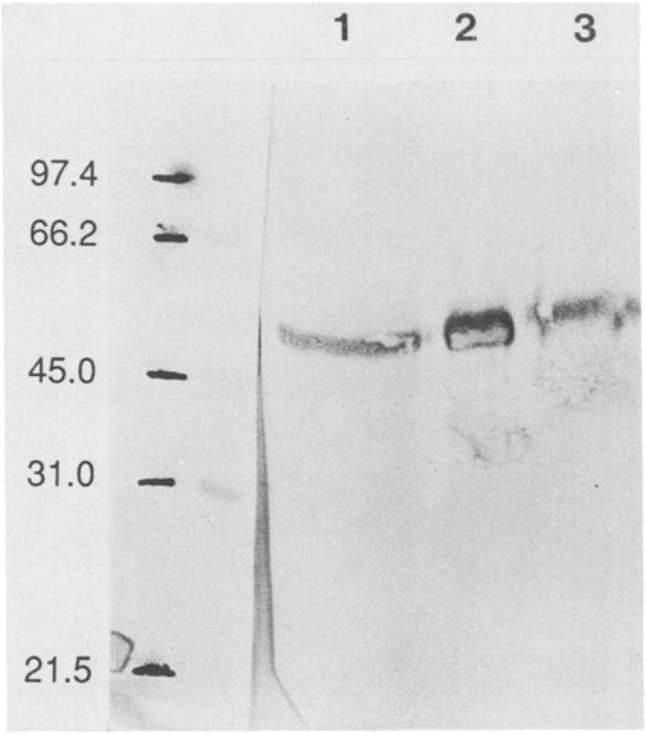

Figure 3. Immunoblots (Western blots) of recombinant rabbit GnT I. GnT I fractions were run on a 12.5% SDS-PAGE gel and blotted on to a nitrocellulose membrane. The membrane was probed with a polyclonal antibody raised in sheep against purified recombinant rabbit GnT I fusion protein expressed in *E. coli*. The antibody was detected with goat anti-sheep IgG coupled to alkaline phosphatase. Only a single band at about 50 kDa was detected. Lane 1: 0.020 ml CM-Sephadex fraction; lanes 2 and 3: 0.005 and 0.010 ml respectively of the UDP-hexanolamine-Sepharose fraction. Molecular weight markers are indicated in kDa.

Characterization of recombinant GnT I

Kinetic analysis of recombinant rabbit GnT I showed K_m values for M_3-octyl and UDP-GlcNAc of 1.0 and 0.1 mM respectively (data not shown). GnT I purified from rabbit and rat [39] livers showed K_m values for M_3-octyl of 0.6 and 1.1 mM respectively. Recombinant glycoproteins expressed in insect cells contain varying amounts of complex *N*-glycans [49–54] and endogenous GnT I activity has been measured in insect cells [55, 56] (L. März, F. Altmann, E. Staudacher and V. Kubelka, personal communication). Recombinant GnT I is not likely to be the endogenous insect enzyme since: (i) enzyme assays on medium from uninfected Sf9 cells and Sf9 cells infected with wild type baculovirus showed no detectable GnT I activity, (ii) Southern analysis of DNA from these control Sf9 cells gave no signal with a rabbit GnT I cDNA probe; (iii) recombinant GnT I binds antiserum against rabbit GnT I expressed in *E. coli*; and (iv) the recombinant and natural rabbit enzymes showed similar kinetic parameters.

The recombinant GnT I enzyme was used to convert several compounds of the type R-Manα1-6[Manα1-3]-Manβ-*O*-octyl to R-Manα1-6[GlcNAcβ1-2Manα1-3]Manβ-*O*-octyl [23, 24]. The products were identified by high resolution proton nuclear magnetic resonance spectroscopy and fast atom bombardment-mass spectrometry. Some of these compounds have been shown to be inhibitors of GlcNAc-transferase II (F. Reck, E. Meinjohanns, M. Springer, R. Wilkens, J. A. L. M. van Dorst, H. Paulsen, G. Möller, I. Brockhausen and H. Schachter, this issue).

Acknowledgements

The author is grateful to Dr R. J. Dunn, Montreal Neurological Institute, Montreal, Quebec, Canada, for plasmids and invaluable help in carrying out these experiments, Mr M. Fabry for technical assistance, Drs J. Burke and P. Gleeson, Melbourne, Australia, for anti-GnT I antibody, Dr H. Paulsen, University of Hamburg, Hamburg, Germany, for GnT I substrate, and Dr H. Schachter, Hospital for Sick Children, Toronto, Canada, for critical review of the manuscript. This research was supported by a grant from the The Protein Engineering Network of Centres of Excellence, Canada (PENCE) to H. Schachter.

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