Glycoconjugate Journal (1995) **12**: 371–379

Substrate specificity and inhibition of UDP-GlcNAc:GlcNAcβ1-2Manα1-6R β1,6-*N*-acetylglucosaminyl-transferase V using synthetic substrate analogues

INKA BROCKHAUSEN[1,2*], FOLKERT RECK[1], WILLIAM KUHNS[1], SHAHEER KHAN[3‡], KHUSHI L. MATTA[3], ERNST MEINJOHANNS[4], HANS PAULSEN[4], RAJAN N. SHAH[5], MICHAEL A. BAKER[6] and HARRY SCHACHTER[1,2]

[1]*Research Institute, The Hospital for Sick Children, 555 University Avenue, Toronto, Ontario M5G 1X8, Canada*
[2]*Department of Biochemistry, University of Toronto, Toronto, Ontario, Canada*
[3]*Department of Gynecologic Oncology, Roswell Park Memorial Institute, Buffalo, NY, USA*
[4]*Institut für Organische Chemie, Universität Hamburg, Hamburg, Germany*
[5]*Department of Medical Genetics and Biophysics, University of Toronto, Toronto Ontario, Canada*
[6]*Department of Medicine, Toronto Hospital, Toronto, Ontario, Canada*

Received, 4 November, 1994, Revised, 10 January, 1995

UDP-GlcNAc:GlcNAc β1-2Manα1-6R (GlcNAc to Man) β1,6-*N*-acetylglucosaminyltransferase V (GlcNAc-T V) adds a GlcNAcβ1-6 branch to bi- and triantennary *N*-glycans. An increase in this activity has been associated with cellular transformation, metastasis and differentiation. We have used synthetic substrate analogues to study the substrate specificity and inhibition of the partially purified enzyme from hamster kidney and of extracts from hen oviduct membranes and acute myeloid leukaemia leukocytes. All compounds with the minimum structure GlcNAcβ1-2Manα1-6Glc/Manβ-R were good substrates for GlcNAc-T V. The presence of structural elements other than the minimum trisaccharide structure affected GlcNAc-T V activity without being an absolute requirement for activity. Substrates with a biantennary structure were preferred over linear fragments of biantennary structures. Kinetic analysis showed that the 3-hydroxyl of the Manα1-3 residue and the 4-hydroxyl of the Manβ- residue of the Manα1-6(Manα1-3)Manβ-R *N*-glycan core are not essential for catalysis but influence substrate binding. GlcNAcβ1-2(4,6-di-*O*-methyl-)Manα1-6Glcβ-pnp was found to be an inhibitor of GlcNAc-T V from hamster kidney, hen oviduct microsomes and acute and chronic myeloid leukaemia leukocytes.

Keywords: GlcNAc-transferase V, substrate specificity, inhibition, leukaemia, N-linked glycans

Abbreviations: all, allyl; AML, acute myeloid leukaemia; BSA, bovine serum albumin; CML, chronic myelogenous leukaemia; Gal, G, D-galactose; Glc, D-glucose; GlcNAc, Gn, *N*-acetyl-D-glucosamine; HPLC, high performance liquid chromatography; Man, M, D-mannose; mco, 8-methoxycarbonyl-octyl, $(CH_2)_8COOCH_3$; Me, methyl; MES, 2-(*N*-morpholino)ethanesulfonate; oct, octyl; pnp, p-nitrophenyl; T, transferase.

Introduction

Complex *N*-glycans have been implicated in many diverse functions [1], particularly in cell-cell adhesion [2, 3] and in diseases such as metastatic cancer [4, 5]. It has been known for many years that transformed and malignant cells usually present on their surfaces complex N-glycans that are larger than normal due primarily to a combination of increased branching, sialylation and poly-*N*-acetyllactosamines [4–8]. UDP-GlcNAc:GlcNAcβ1-2Manα1-6R (GlcNAc to Man) β1,6-*N*-acetylglucosaminyltransferase V (GlcNAc-T V), which adds a GlcNAcβ1-6 branch to bi-and triantennary N-glycans, plays a major role in this phenomenon.

Comparatively higher amounts of tetraantennary chains, concomitant with higher GlcNAc-T V activity, have been reported after Rous sarcoma virus [9, 10] and Polyoma virus [11] infection of baby hamster kidney cells. The increase in activity was associated with an increase in V_{max} suggesting an induction of GlcNAc-T V [12]. T24H ras transformed rat fibroblasts and metastatic SP1 mammary carcinoma cells also exhibit higher GlcNAc-T V activity [13]. Induction of the *ras* gene with dexamethasone in a stable NIH 3T3 transfectant containing a normal N-*ras* proto-oncogene under the control

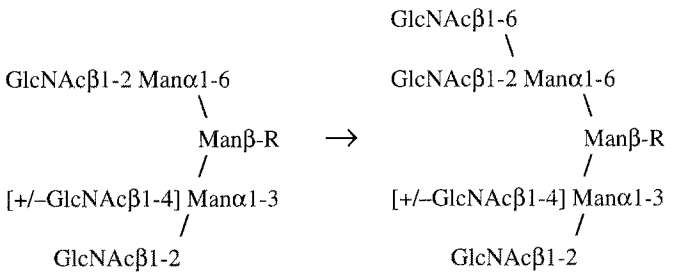
‡ Present address: Perkin-Elmer, Applied Biosystems Division, 850 Lincoln Center Drive, Foster City, CA 94404, USA.
* To whom enquiries should be addressed.

of a glucocorticoid-inducible promoter resulted in increased branching of complex N-glycans and increased GlcNAc-T V activity [14]. An increase in GlcNAc-T V-dependent branching has been reported in malignant human breast tissue compared to benign hyperplastic lesions [15] and in other oncogene-transformed tissues [16, 17]. L-phytohaemagglutinin (L-PHA) reactivity was shown to correlate with GlcNAc-T V activity [15], suggesting that this lectin is useful in detecting N-glycan antennae initiated by β1-6-linked GlcNAc.

The N-glycan GlcNAcβ1-6Manα1-6Manβ- branch, initiated by GlcNAc-T V, favours the addition of poly-*N*-acetyllactosamine chains [18, 19]; highly branched complex N-glycans and poly-*N*-acetyllactosamines carrying cancer-associated antigens have been found in many animal models of tumour progression and acquisition of metastatic potential, and in human melanomas and carcinomas of breast, colon and ovaries [4].

A number of reports suggest the involvement of GlcNAc-T V in cellular activation and differentiation. Upon differentiation of human colonic adenocarcinoma Caco-2 cells, GlcNAc-T V activity increased concomitantly with increased GlcNAc-T II, III, and IV activities and a loss of fucosylated poly-*N*-acetyllactosaminoglycan chains [20]. Mouse F9 teratocarcinoma cells acquired higher GlcNAc-T V activity upon retinoic acid induced differentiation [21]. Interleukin-6 was found to stimulate GlcNAc-T IV and V activities in a human myeloma cell line concomitant with a decrease in GlcNAc-T III [22].

GlcNAc-T V was first described in 1982 [23] and catalyses the conversion of bi- to tri-antennary and of tri- to tetraantennary N-glycans:



The enzyme has been purified from hamster kidney [24], rat kidney [25] and from the culture supernatant of human lung cancer cells [26]. The cDNA encoding the enzyme was isolated from rat and mouse [27] and human fetal liver [28, 29]. The human gene was mapped to chromosome 2q21 [28, 29].

GlcNAc-T V also acts on linear structures representing the Manα1-6 arm of the N-glycan core, i.e. GlcNAcβ1-2Manα1-6Manβ-R where R may be a hydrophobic group or BSA [30–32]. Like other β1,6-GlcNAc-transferases, the enzyme is fully active in the presence of EDTA [25]. The compound GlcNAcβ1-2(6-deoxy)Manα1-6Glcβ-octyl was shown to be a competitive inhibitor of GlcNAc-T V with a K_i of 0.07 mM [12, 24]; this compound lacks the hydroxyl to which the enzyme attaches GlcNAc. The $K_{M(app)}$ of GlcNAc-T V from baby hamster kidney (BHK) cells is 0.18 mM for GlcNAcβ1-2Manα1-6Manβ-mco and 0.25 mM for UDP-GlcNAc [10].

GlcNAc-T V cannot act on substrates which contain a bisecting GlcNAc, or in which either antenna is substituted with a β1-4-linked Gal residue [23, 26, 33, 34]. Removal (deoxy analogue) or substitution of the 4-OH of the terminal GlcNAc of the substrate GlcNAcβ1-2Manα1-6Glcβ-octyl leads to an inactive compound [35]. Although a bisecting GlcNAc attached to the 4-OH of the β-linked Man residue on a biantennary substrate prevents enzyme action, 4-*O*-methyl or 4-deoxy linear substrate analogues are excellent substrates [31]. The GlcNAc-terminal triantennary compound is a better substrate than the biantennary compound [11, 26]. GlcNAcβ1-2(4-deoxy)Manα1-6Glcβ-octyl is a good substrate but the (4-*O*-methyl)Man derivative is an inhibitor but not a substrate [36].

In this study, we investigated a panel of synthetic linear and biantennary compounds as substrates and inhibitors of GlcNAc-T V. GlcNAcβ1-2(4,6-di-*O*-methyl)Manα1-6Glcβ-pnp was found to be an inhibitor of GlcNAc-T V from hamster kidney, hen oviduct microsomes and acute myeloid (AML) and chronic myeloid (CML) leukaemia leukocytes. Kinetic analysis showed that the 3-hydroxyl of the Manα1-3 residue and the 4-hydroxyl of the Manβ- residue of the Manα1-6(Manα1-3)Manβ-R N-glycan core are not required for catalysis but influence substrate binding.

Materials and methods

Materials

AG1-x8 (100–200 mesh, Cl^- form) and Bio-Gel P4 (–400 mesh) were purchased from Bio-Rad. Bovine serum albumin and Triton X-100 were purchased from Sigma. Acetonitrile (190 UV cutoff) was from Fisher Scientific Co. or Caledon Laboratories. UDP-N-[1-^{14}C] acetylglucosamine was synthesized as described previously [37] and diluted with UDP-GlcNAc from Sigma. GlcNAcβ1-2Manα1-6Glcβ-oct and GlcNAcβ1-2Manα1-6Manβ-mco were kindly provided by Dr. O. Hindsgaul, University of Alberta, Edmonton.

The following compounds were chemically synthesized: GlcNAcβ1-2Manα1-6Glcβ-pnp [38]; GlcNAcβ1-2Manα1-6Manβ-Me [39]; GlcNAcβ1-2Manα1-6Glcβ-all, GlcNAcβ1-2Manα1-6Glcβ1-4Glcβ-all [38]; GlcNAcβ1-2Manα1-6Glcβ1-4GlcNAc[40]; GlcNAcβ1-2(4-*O*-Me)Manα1-6Glcβ-pnp [41]; GlcNAcβ1-2(6-*O*-Me)Manα1-6Glcβ-pnp, GlcNAcβ1-2(4,6-di-*O*-Me)Manα1-6Glcβ-pnp [40]; GlcNAcβ1-2Manα-Me, GlcNAcβ1-4(GlcNAcβ1-2)Manα-Me, and GlcNAcβ1-6Manα-Me [39]; GlcNAcβ1-2(6-*O*-Me) Manα-Me and GlcNAcβ1-2(4,6-di-*O*-Me)Manα-Me [42]; GlcNAcβ1-2Manα1-6Manβ-oct, GlcNAcβ1-2Manα1-6(GlcNAcβ1-2Manα1-3)Manβ-oct, GlcNAcβ1-2Manα1-6(GlcNAcβ1-2[3-deoxy] Manα1-3) Manβ-oct, GlcNAcβ1-2Manα1-6(GlcNAcβ1-2[4-deoxy]Manα1-3)Manβ-oct, GlcNAcβ1-2Manα1-6(GlcNAcβ1-2Manα1-3)4-

O-Me-Manβ-oct, GlcNAcβ1-2Manα1-6(GlcNAcβ1-2Manα1-3)4-deoxy-Manβ-oct, GlcNAcβ1-2Manα1-6(GlcNAcβ1-2[4-deoxy]Manα1-3)4-*O*-Me-Manβ-oct [43]; and 3-*O*-pivaloyl-GlcNAcβ1-2Manα1-6Glcβ-pnp (K.L. Matta, unpublished).

High performance liquid chromatography

HPLC separations were carried out with an LKB or a Waters system [34, 39]. Acetonitrile/water mixtures were used as the mobile phase for all columns. Reverse phase C18, amine (NH2) and amine-cyano (PAC) columns were used, depending on the hydrophobicity of the aglycon. Elutions of compounds were monitored by measuring the absorbance at 195 nm and counting the radioactivity of collected fractions [34].

Preparation of enzyme

Homogenates from rat, human and fetal human colon, pig stomach mucosa, leukocytes from patients with acute (AML) and chronic (CML) myelogenous leukaemia and normal human granulocytes, and hen oviduct microsomes were prepared as previously described [34, 37, 44].

GlcNAc-T V was partially purified from hamster kidneys according to Hindsgaul *et al.* [24] as follows. Fifty hamster kidneys (Keystone Biologicals) were homogenized in 50 ml acetone (–20°C) with several short pulses in a Polytron homogenizer. Homogenate was washed with a further 50 ml cold acetone on a Büchner funnel with standard filter paper. Homogenization, washing and filtration of residue were repeated. The acetone powder produced was homogenized briefly with 100 ml buffer A (0.1 M Na-acetate pH 6.0, 10 mM EDTA, 0.2 M NaCl) in a Teflon hand homogenizer, transferred with a further 100 ml buffer A to centrifuge tubes and centrifuged at 15 300 × g for 30 min. The supernatant was discarded and homogenization/centrifugation was repeated with the pellet. Finally, the pellet was homogenized with 180 ml cold water and centrifuged as in the previous step. The pellet was homogenized in 60 ml buffer B (0.1 M Tris/HCl pH 7.6, 0.4 M KCl, 1 mM EDTA and 1% Triton X-100) using two passes with a teflon Dounce homogenizer, stirred 4 h, centrifuged at 23 700 × g for 45 min and supernatant was removed. The pellet was re-homogenized with buffer B (60 ml), stirred overnight, and centrifuged. Both supernatants (crude extracts) were dialysed versus two exchanges of 1 l buffer C (50 mM MES pH 6.5 with 10 mM EDTA and 0.1% Triton X-100). Dialysed crude extracts were loaded onto a UDP-hexanolamine-Sepharose column (10 ml, 10 μmol UDP-hexanolamine per ml gel) equilibrated with buffer C. The column was prepared by coupling UDP-hexanolamine (Sigma) with cyanogen bromide activated Sepharose (Pharmacia) according to the Pharmacia protocol. The column was washed with 30 ml buffer D (buffer C containing 0.25% Triton X-100 and 20% glycerol). Enzyme was eluted with buffer D containing a step gradient of NaCl: 0.1 M, 0.25 M, 0.5 M and 1 M, 15 ml each, in 5 ml fractions. Most of the enzyme was in the 0.25 M NaCl fractions. Enzyme activity (46%) was also found in the flow-through fraction and was applied again to a UDP-hexanolamine-Sepharose column; 50% of enzyme activity was again found in the flow-through fraction. The 0.25 M NaCl fractions from both columns were pooled and dialysed against 50 mM Na-cacodylate buffer pH 6.5 containing 10 mM EDTA, 20% glycerol and 0.1% Triton X-100, and concentrated with polyethyleneglycol (molecular mass 10 000) to 50% volume. Dialysis and concentration were repeated and the enzyme was dialyzed 2 more times to yield a total of 1.27 mU in 12 ml (33% of the activity in the crude homogenate) at a specific activity of 0.06 mU mg^{-1} (1 mU = 1 nmol min^{-1})

Protein determination

Protein was determined by the Bio-Rad method using IgG as the standard.

Assay for β1,6-GlcNAc-transferase V partially purified from hamster kidney

The standard assay mixture for measuring GlcNAc-T V activity contained in a total volume of 30 μl: 1 mM UDP-N-[1-^{14}C]acetylglucosamine (5555 dpm $nmol^{-1}$), 0.1% Triton X-100, 10 mM EDTA, 20% glycerol, 50 mM cacodylate buffer, pH 6.5, acceptor as indicated and 3.13 μU enzyme preparation (3.13 pmol min^{-1}, 30 μl, 53.1 μg protein). Incubations were carried out for 2 h at 37°C and stopped by the addition of 0.4 ml ice cold water. The mixtures were passed through Pasteur pipettes filled with AG1-x8, 100–200 mesh, Cl^- form, equilibrated in water. The columns were washed with 2.6 ml water and the eluates were counted in 17 ml scintillation fluid. The apparent K_m and V_{max} values were determined by double reciprocal Lineweaver-Burk plots.

Assay for β1,6-GlcNAc-transferase V from microsomes and cell homogenates

The standard assay for measuring GlcNAc-T V activity contained in a total volume of 40 μl: 2 mM UDP-*N*-[1-^{14}C] acetylglucosamine (2200 dpm $nmol^{-1}$) or UDP-[6-^{3}H]-*N*-acetylglucosamine (1600–4200 dpm $nmol^{-1}$), 0.25% Triton X-100, 0.125 M GlcNAc, 10 mM AMP, 0.125 M MES buffer, pH 7, acceptor as indicated in the Tables and 10–20 μl microsomes or homogenates (0.12–0.5 mg protein). Incubations were carried out for 2 h at 37°C and stopped by the addition of 0.4 ml 20 mM Na-tetraborate – 1 mM EDTA or 100 μl water. The mixtures were passed through Pasteur pipettes containing AG1-x8, 100–200 mesh, Cl^- form, equilibrated in water. The columns were washed with water and the eluates were lyophilized and stored at –20°C. Aliquots were analysed by HPLC as described in the Tables.

Inhibition of GlcNAc-transferase V

The substrate was incubated at 0.05–0.3 mM concentration under standard assay conditions in the presence of an inhibitor as indicated in the Tables. Potential inhibitors were also preincubated for 10 minutes at room temperature with the enzyme

before the addition of the substrate. Since previous work had shown that irradiation at 350 nm in the presence of nitrophenyl substrate derivatives greatly reduced the activity of core 2 β6-GlcNAc-transferase acting on O-glycans [45], we tested the effect of UV light on inhibition by pnp-containing compounds. Inhibitors were irradiated at 30°C with UV light at 350 nm for 20 min in the presence of the enzyme before incubation, using a Rayonet RPR 100 reactor equipped with 16 RPR 3500 Å lamps.

Results

Substrate Specificity of GlcNAc-T V

Purified hamster kidney GlcNAc-T V was stable for several months at 4°C and acted on a number of compounds with the general structure GlcNAcβ1-2Manα1-6Man/Glcβ-R. The HPLC separation conditions and elution times for various substrates and products are listed in Table 1. The nature of the aglycon group had a strong influence on the effectiveness of the substrate with the octyl compounds giving the highest activities (Tables 2 and 3). Compounds with a biantennary structure (**14–19**) were significantly better substrates than those with only the linear GlcNAcβ1-2Manα1-6 Man/Glc structure (compounds **1**, **5–7**, **10**). The K_m values of the biantennary derivatives ranged from 0.035 to 0.18 mM and enzyme activities were relatively high when compared to the linear substrates (Table 2). Methyl substitution of the 4-hydroxyl of the internal Manβ residue (compound **17**) increased activity but omission of this hydroxyl (compound **18**) increased the K_m 3-fold. Omission of the 4-hydroxyl of the Manα1-3 residue of the biantennary substrate (compound **16**) had little effect on activity whereas the 3-deoxy analogue (compound **15**) was less active (Table 2).

Substitution of the 3-hydroxyl of the GlcNAc residue of GlcNAcβ1-2Manα1-6Glcβ-pnp by a pivaloyl group (compound **9**) prevented catalysis by hamster kidney GlcNAc-T V (Table 2). 3-*O*-pivaloyl-GlcNAcβ1-2Manα1-6Glcβ-pnp (compound **9**) showed 19% and 47% activity with the enzyme from pig stomach and AML cells, respectively, compared to GlcNAcβ1-2Manα1-6Glcβ-pnp (compound **2**). HPLC analysis showed that the pivaloylester was not destroyed by esterases during the incubation and product eluted as one peak before the substrate on reverse phase HPLC (Table 1).

Upon incubation of hen oviduct microsomes with short linear compounds (Table 3), at least two products were usually formed which could be separated by HPLC. Product due to GlcNAc-T VI′ action [39], GlcNAcβ1-4(GlcNAcβ1-2)Manα1-6R, always eluted earlier on HPLC than product due to GlcNAc-T V action, GlcNAcβ1-6(GlcNAcβ1-2)Manα1-6R (Fig. 1). When Mn^{2+} was added to the assay, GlcNAc-T VI′ product was increased about 20-fold and GlcNAc-T V product was not detectable (Fig. 1).

Although there were variations in activity, derivatives with octyl, 8-methoxycarbonyloctyl, p-nitrophenyl, methyl or allyl aglycon groups were active as substrates for GlcNAc-T V from hamster kidney and hen oviduct (Tables 2 and 3). In contrast to GlcNAc-T VI′, GlcNAc-T V acted well on the free reducing tetrasaccharide GlcNAcβ1-2Manα1-6 Glcβ1-4GlcNAc (compound **7**, Tables 2 and 3). Activities towards linear tetrasaccharide substrates were within the same range as for trisaccharides but the disaccharide GlcNAcβ1-2Manα-Me was significantly less active (compound **8**, Table 3).

Table 1. HPLC conditions for separation of substrates and GlcNAc-T V products

Compound No.		*Column*	*Flow rate (ml min^{-1})*	*% Acetonitrile in mobile phase*	*Elution time (min)*	
					Substrate	*Product*
1	GlcNAcβ1-2Manα1-6Glcβ-oct	C18	1	18	36	25
				19	43	32
2	GlcNAcβ1-2Manα1-6Glcβ-pnp	PAC	0.7	82	19	37
3	GlcNAcβ1-2Manα1-6Manβ-mco	PAC	0.7	82	22	51
4	GlcNAcβ1-2Manα1-6Manβ-Me	NH2	0.7	80	41	96
5	GlcNAcβ1-2Manα1-6Glcβ-all	C18	1	15	29	29
		PAC	0.7	82	32	75
6	GlcNAcβ1-2Manα1-6Glcβ1-4Glcβ-all	C18	1	16	34	25
		PAC	0.7	80	29	54
7	GlcNAcβ1-2Manα1-6Glcβ1-4GlcNAc	NH2	1	82	48	112
		PAC	0.7	82	90	113
8	GlcNAcβ1-2Manα-Me	NH2	0.7	82	13	34
9	3-O-pivaloyl-GlcNAcβ1-2Manα1-6-Glcβ-pnp	C18	1	14	34	24

Compounds were separated by HPLC as described in Methods with acetonitrile/water mixtures as the mobile phase on reverse phase (C18), amine (NH2) or amine-cyano (PAC) columns.

Table 2. Specificity of GlcNAc-T V from hamster kidney using linear and biantennary substrates

	Compound	*Activity (pmol mg^{-1} per h)*	*K_m (mM)*
Linear Substrates (0.8 mM)			
10	GlcNAcβ1-2Manα1-6Manβ-oct	2730	0.09
1	GlcNAcβ1-2Manα1-6Glcβ-oct	3180	
7	GlcNAcβ1-2Manα1-6Glcβ1-4GlcNAc	840	
5	GlcNAcβ1-2Manα1-6Glcβ-all	660	
6	GlcNAcβ1-2Manα1-6Glcβ1-4Glcβ-all	< 300	
Biantennary Substrates (0.3 mM)			
14	GlcNAcβ1-2Manα1-6(GlcNAcβ1-2 Manα1-3)Manβ-oct	5880	0.06
15	GlcNAcβ1-2Manα1-6(GlcNAcβ1-2[3-deoxy-]Manα1-3)Manβ-oct	1800	0.13
16	GlcNAcβ1-2Manα1-6(GlcNAcβ1-2[4-deoxy-]Manα1-3)Manβ-oct	5580	0.08
17	GlcNAcβ1-2Manα1-6(GlcNAcβ1-2Manα1-3)4-*O*-Me-Manβ-oct	6300	0.035
18	GlcNAcβ1-2Manα1-6(GlcNAcβ1-2Manα1-3)4-deoxy-Manβ-oct	5340	0.18
19	GlcNAcβ1-2Manα1-6(GlcNAcβ1-2[4-deoxy-]Manα1-3)4-*O*-Me-Manβ-oct	8040	0.06
Substrates (2.7 mM)			
2	GlcNAcβ1-2Manα1-6Glcβ-pnp	1460[a]	
9	3-*O*-pivaloyl-GlcNAcβ1-2Manα1-6Glcβ-pnp	< 300[a]	

Assays were carried out using purified hamster kidney GlcNAc-T V as described in the Methods Section without HPLC separation. Substrates were present in the assay at near saturating concentrations; the.activity values therefore represent the V_{max} values.
[a]Compounds were tested with a less active enzyme preparation.

Table 3. Specificity of GlcNAc-T V from hen oviduct

	Compound	*Activity (pmol/h/mg)*
Conditions A:		
1	1 mM GlcNAcβ1-2Manα1-6Glcβ-oct	485
13	2.5 mM GlcNAcβ1-2(4,6-di-O-Me) Manα1-6Glcβ-pnp + 1 mM GlcNAcβ1-2Manα1-6Glcβ-oct	120
1	2 mM GlcNAcβ1-2Manα1-6Glcβ-oct	664
5	2 mM GlcNAcβ1-2Manα1-6Glcβ-all	359
6	2 mM GlcNAcβ1-2Manα1-6Glcβ4Glcβ-all	313
3	2.5 mM GlcNAcβ1-2Manα1-6Manβ-mco	176
4	1 mM GlcNAcβ1-2Manα1-6Manβ-Me	69
8	2.3 mM GlcNAcβ1-2Manα-Me	60
20	2.3 mM GlcNAcβ1-4(GlcNAcβ1-2)Manα-Me	0
21	1 mM GlcNAcβ1-6Manα-Me	0
Conditions B:		
2	2 mM GlcNAcβ1-2Manα1-6Glcβ-pnp	250
5	2 mM GlcNAcβ1-2Manα1-6Glcβ-all	670
6	2 mM GlcNAcβ1-2Manα1-6Glcβ1-4Glcβ-all	385
7	2 mM GlcNAcβ1-2Manα1-6Glcβ1-4GlcNAc	460

Enzyme assays were carried out as described in Methods by HPLC. Two different experiments (conditions A and B) were carried out using different enzyme preparations.
Conditions A: 1 h incubation, 1 mM UDP-[^{14}C]GlcNAc, 0.5 mg protein per assay.
Conditions B: 2 h incubation, 0.84 mM UDP-[^{14}C]GlcNAc, 0.12 mg protein per assay.

Inhibition studies

Inhibition studies were carried out with hamster kidney GlcNAc-T V using 0.3 mM GlcNAcβ1-2Manα1-6Glcβ-oct as substrate (compound 1, Table 4). The activity was significantly reduced in the presence of inactive substrate analogues with 4- or 6-*O*-methyl substitution of the Manα1-6 residue (compounds **11** and **12**, Table 4). The best inhibitor was GlcNAcβ1-2(4,6-di-*O*-Me)Manα1-6 Glcβ-pnp (compound **13**) which inhibited GlcNAc-T V by 53%. GlcNAc-T V from hen oviduct showed a 75% inhibition by compound **13** (Table 3). Irradiation of the enzyme at 350 nm in the presence of the mono-methylated pnp derivatives resulted in slightly increased inhibition (Table 4).

Several leukaemic leukocyte samples were used as sources of GlcNAc-T V to study the inhibition with compound **13**. The activity was inhibited 37–67% with AML cell extracts and 29–88% with CML granulocyte extracts using a 2.5-fold molar excess of compound **13** over substrate.

GlcNAc-T V in leukaemic leukocytes and other tissues

GlcNAc-T V activity was also found in homogenates from rat colon, adult and fetal human colon, pig stomach mucosa, leukocytes from patients with AML and CML and normal human granulocytes (data not shown). The average GlcNAc-T V activities (measured with 1 mM GlcNAcβ1-2Manα1-6Glcβ-oct as substrate) of extracts from AML (eight samples), CML (four samples) and normal (three samples) leukocytes were 860, 670 and 430 pmol h^{-1} per mg respectively.

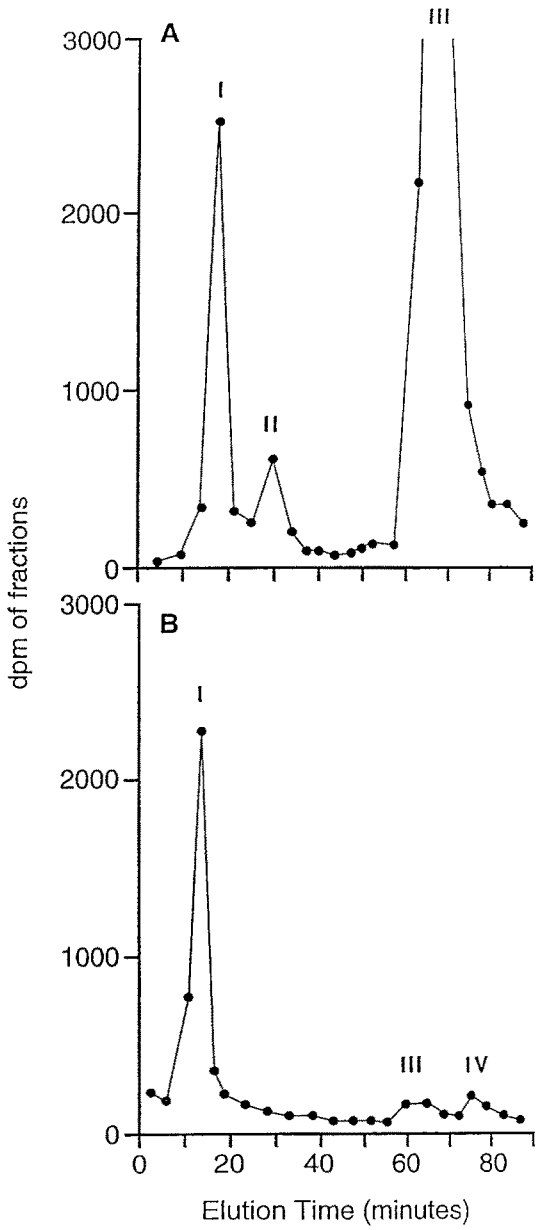


Figure 1. HPLC elution pattern of GlcNAc-T V product using GlcNAcβ1-2Manα1-6Glcβ–allyl as the substrate and hen oviduct microsomes. HPLC was carried out on a PAC column, using acetonitrile:water 82:18 at 0.7 ml min^{-1}. Elution patterns were established with standard compounds GlcNAcβ1-6(GlcNAcβ1-2)Man-R and GlcNAcβ1-4(GlcNAcβ1-2)Man-R [39]; GlcNAcβ1-6(GlcNAcβ1-2)Man-R (GlcNAc-T V product) always elutes after GlcNAcβ1-4(GlcNAcβ1-2)Man-R (GlcNAc-T VI′ product). Peak I, [^{14}C]GlcNAc; peak II, unknown; peak III, GlcNAc-T VI′ product, [^{14}C]GlcNAcβ1-4(GlcNAcβ1-2)Man-R; peak IV, GlcNAc-T V product, [^{14}C]GlcNAcβ1-6(GlcNAcβ1-2)Man-R. (A) In the presence of Mn^{2+} radioactivity due to GlcNAc-T VI′ product was very high and no GlcNAc-T V product was detected. (B) In the absence of Mn^{2+} in the assay, GlcNAc-T V product eluted at 75 min and GlcNAc-T VI′ product eluted earlier at 65 min. The latter product was not seen when mammalian cells were used as the source of GlcNAc-T V.

Discussion

GlcNAc-transferase V adds the GlcNAcβ1-6 branch to the Manα1-6 arm in the biosynthesis of complex N-glycans [23, 34, 46]. It has been suggested that this branch carries most of the long chain poly-*N*-acetyllactosaminoglycans which in turn may carry antigenic determinants and sialic acid residues [18, 19, 21]. Inhibition of GlcNAc-T V would therefore prevent the synthesis of highly branched N-glycan structures and possibly reduce overall sialylation of N-glycans. This inhibition may be beneficial in certain diseases such as cancer or metastasis where increased occurrence of complex structures has been reported [8, 15, 17, 47, 48].

Various cell types exhibit great differences in the expression of GlcNAc-T V activities consistent with regulation of GlcNAc-T V in a tissue-specific fashion and during development and differentiation [4, 11, 20–22]. Typical mucin secreting tissues such as colonic mucosa and pig stomach have comparatively high GlcNAc-T V activity. Our results suggest that GlcNAc-T V is increased in AML and CML leukocytes. This may reflect an altered stage of differentiation of these cells compared to normal granulocytes. We previously reported increased activities in AML and CML cells of another branching GlcNAc-T, core 2 β6-GlcNAc-T [44], which in combination with other changes in the O-glycosylation pathways may be partly responsible for the increased cell surface sialylation in leukaemic cells [49–51]. High GlcNAc-T V activity would be expected to increase the total proportion of tetraantennary chains and thereby contribute to the overall sialylation of leukaemic cells even though sialyltransferase activities acting on N-glycans remain unchanged as previously reported [51]. Inhibition of GlcNAc-T V activity by inhibitors with the ability to penetrate membranes and act on Golgi-localized GlcNAc-T V may therefore be beneficial in reducing the abnormal cell surface sialylation of leukaemic cells. Knowledge of the substrate recognition mechanism used by GlcNAc-T V is an essential prerequisite in the design of effective inhibitors.

We have shown in this study that the size of the substrate influences GlcNAc-T V activity. Three sugars are required for optimal activity [52]. GlcNAc-T VI′ [39] and GlcNAc-T I [53, 54] also require a substrate with a minimum of three sugar residues whereas GlcNAc-T II requires a tetrasaccharide [55–57]. The influence of the peptide sequence of glycoprotein substrates on GlcNAc-T V activity has not yet been investigated. Although there is no absolute requirement for carbohydrate residues other than the GlcNAcβ1-2Manα1-6Manβ structure, the biantennary compounds show higher activities for the hamster kidney enzyme than do the linear compounds. The biantennary compound GlcNAcβ1-2Manα1-6(GlcNAcβ1-2[4-deoxy-]Manα1-3)4-*O*-methyl-Manβ-octyl (compound **19**, Table 2) is of special interest because it shows the highest activity of all the acceptors studied (Table 2) and it is a specific substrate for GlcNAc-T V from mammalian sources (which lack GlcNAc-T VI and VI′) since GlcNAc-T I, II, III and IV cannot act on it.

Table 4. Inhibition of GlcNAc-T V purified from hamster kidney

Compound (0.8 mM)		*Activity*[a] *(pmol mg^{-1} per h)*	*% Inhibition*[b]
In the absence of 0.3 mM GlcNAcβ1-2 Manα1-6 Glcβ-oct:			
11	GlcNAcβ1-2(4-*O*-Me)Manα1-6Glcβ-pnp	ND	
12	GlcNAcβ1-2(6-*O*-Me)Manα1-6Glcβ-pnp	ND	
13	GlcNAcβ1-2(4,6-di-*O*-Me)Manα1-6Glcβ-pnp	ND	
22	GlcNAcβ1-2(6-*O*-Me)Manα-Me	ND	
23	GlcNAcβ1-2(4,6-di-*O*-Me)Manα-Me	ND	
In the presence of 0.3 mM GlcNAcβ1-2 Manα1-6 Glcβ-oct:			
11	GlcNAcβ1-2[4-*O*-Me]Manα1-6Glcβ-pnp		33
		+ UV	48
		- UV	41
12	GlcNAcβ1-2[6-*O*-Me]Manα1-6Glcβ-pnp		28
		+ UV	41
		- UV	28
13	GlcNAcβ1-2[4,6-di-*O*-Me]Manα1-6Glcβ-pnp		53
22	GlcNAcβ1-2(6-*O*-Me)Manα-Me		0

[a] Assays were carried out without HPLC, using purified hamster kidney GlcNAc-T V, as described in Methods with 0.3 mM GlcNAcβ1-2Manα1-6Glcβ-oct as substrate, 1 mM UDP-[^{14}C]GlcNAc, with and without the addition of 0.8 mM of the compounds under test.
ND, activity was not detectable.
+ UV, compounds were preincubated with the enzyme and irradiated at 350 nm.
- UV, compounds were preincubated with the enzyme without irradiation.
[b] Enzyme activity in the absence of inhibitor is 3270 pmol h^{-1} per mg; % inhibition = 100 × (difference in activity of the enzyme in the absence and presence of inhibitor) ÷ (activity in the absence of inhibitor).

Figure 2 displays the structure of a biantennary N-glycan substrate for GlcNAc-T V and the hydroxyls that were found to be important or essential for activity from this study and previous reports [12, 23, 24, 26, 31, 34–36, 40, 42]. Although the Manα1-3 arm is not required, the 3-hydroxyl, but not the 4-hydroxyl, of the Manα1-3 residue appears to be important since the 3-deoxy derivative shows reduced activity and a higher K_m.

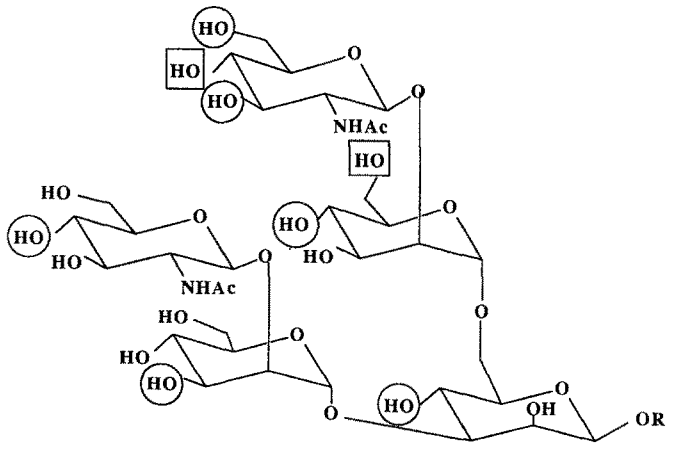


Figure 2. GlcNAc-transferase V specificity towards GlcNAcβ1-2Manα1-6(GlcNAcβ1-2-Manα1-3)Manβ-R substrate. The hydroxyls found to be essential for activity are surrounded by squares. Hydroxyls that have an influence but are not essential for activity are surrounded by circles. The data are from this study and from previous reports [12, 23, 24, 26, 31, 34–36, 40, 42].

The 4-hydroxyl of the internal Manβ residue has been shown to be essential for GlcNAc-T I activity [53, 54, 58]. It is not essential, however, for GlcNAc-T II and V which act on the Manα1-6 residue, although its removal appears to affect substrate binding to GlcNAc-T II [57] and V (Table 2). Methyl substitution of the 4-hydroxyl of Manβ1-4 increases GlcNAc-T V activity. Similar results were reported using the linear trisaccharide [52]. Methyl substitution of the 4-hydroxyl of Manβ1-4 inhibits binding to GlcNAc-T II [57] whereas substitution by the bisecting residue (GlcNAcβ1-4 linked to Manβ1-4) turns off all of the GlcNAc-T acting on N-glycans with the exception of GlcNAc-T VI [26, 34, 54].

The Manβ1-4 residue may be replaced by Glc, indicating that the configuration of the 2-hydroxyl is not important for activity. However, when Glc was replaced by a hexane ring [31], the activity was significantly reduced. Using derivatives of GlcNAcβ1-2Manα1-6Glcβ-R, the crude hamster kidney enzyme was found to be more active when the 4-hydroxyl of Glc was substituted with a methyl group, but the activity was unchanged when this hydroxyl was removed [31].

The 4- and 6-hydroxyls of the GlcNAc residue attached to the Manα1-6 residue are essential for substrate recognition by GlcNAc-T V [35, 59]. Galactosylation, removal or modification of the 4-hydroxyl of the terminal GlcNAc residue results in loss of activity [35]. This is consistent with the finding that GlcNAc-T V from mouse lymphoma cells does

not act on galactosylated or sialylated biantennary substrates [23] and that the enzyme from human lung cancer cells is inactive towards biantennary substrates with Gal-substituted GlcNAc residues on both the Manα1-3 and the Manα1-6 arms [26]. Interestingly, GlcNAc-T I, II, III and IV are also known to be inhibited by galactosylation of GlcNAc [35, 53, 55, 56, 60, 61]. Results using 3-*O*-pivaloyl-GlcNAcβ1-2Manα1-6Glcβ-pnp (compound **9**, Table 2) suggest that the 3-hydroxyl of this GlcNAc residue may also be important [59], or that the pivaloyl group causes steric hindrance or unfavourable electronic interactions which prevent binding.

Both the 4- and the 6-hydroxyls of Manα1-6 are minimally involved in binding to the enzyme substrate binding site. The substrate analogue which is methylated at the 4-hydroxyl of the Manα1-6 residue is a competitive inhibitor and inhibition is probably due to steric hindrance of catalysis by the methyl group since the corresponding 4-deoxy compound is a substrate [36, 62]. The enzyme is competitively inhibited by the substrate analogue lacking the 6-hydroxyl of the Manα1-6 residue, the site of enzyme action [24, 41]. Methylation of the 6-hydroxyl of the Manα1-6 residue of GlcNAcβ1-2 Manα1-6 Glcβ-pnp produced a compound that was not active as a substrate although it inhibited enzyme activity (Table 4). Thus methylation of the Manα1-6 residue does not interfere with binding to the enzyme substrate binding site.

This finding is important for the design of GlcNAc-T V inhibitors. We therefore used the di-methylated derivative GlcNAcβ1-2(4,6-di-*O*-Me)Manα1-6 Glcβ-pnp as an inhibitor for GlcNAc-T V from hamster kidney, hen oviduct and leukaemia cells and found it to be effective *in vitro*. It remains to be shown if compounds with similar structures can be designed to penetrate biological membranes and thereby reach the intracellular site of glycosyltransferase action.

Acknowledgements

This research was supported by grants from the Medical Research Council of Canada to I. Brockhausen, from the Deutsche Forschungsgemeinschaft to H. Paulsen and NIAID No. AI 29326 to K. L. Matta. F. Reck was supported by the Deutsche Studienstiftung and the BASF Aktiengesellschaft.

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