Glycoconjugate Journal (1995) **12**: 813–823

Preparation of antisera to recombinant, soluble *N*-acetylglucosaminyltransferase V and its visualization *in situ*

LIN CHEN[1], NING ZHANG[1], BEVERLY ADLER[2], JEFFERY BROWNE[2], NEVIS FREIGEN[3] and MICHAEL PIERCE[1]*

[1]*Department of Biochemistry and Molecular Biology and Complex Carbohydrate Research Center, University of Georgia, Athens, GA 30602, USA*
[2]*Amgen, Inc., Thousand Oaks, CA 91320, USA*
[3]*Department of Cell Biology and Anatomy, University of Miami School of Medicine, P.O. 16960, Miami, FL 33101, USA*

Received 7 April 1995, revised 26 May 1995

N-Acetylglucosaminyltransferase V (GlcNAc-T V) is a glycosyltransferase which transfers *N*-acetylglucosamine in $\beta(1,6)$ linkage to the $\alpha(1,6)$-linked mannose residue of Asn-linked oligosaccharides. This enzyme is characterized by several unusual properties: GlcNAc-T V is the largest lumenal Golgi glycosyltransferase described thus far, and its multiple mRNA transcripts range from 4.5 to about 9.5 kb; GlcNAc-T V mRNA and activity are regulated by the *src* tyrosine kinase signalling pathway; in brain tissue, large levels of GlcNAc-T V mRNA are present, but only relatively low levels of catalytic activity can be detected; a lectin-resistant cell line, Lec4A, expresses active GlcNAc-T V which is mislocalized intracellularly. In addition, the cell surface oligosaccharide products of this enzyme have been hypothesized to regulate intercellular adhesion. In order to devise specific inhibitors of this enzyme it is necessary to understand its physical structure and how structural changes can influence its activity and localization. We have expressed milligram amounts of a soluble form of recombinant rat GlcNAc-T V, purified it from CHO cell-conditioned media, and used it to prepare specific antisera. This antisera binds selectively to GlcNAc-T V and has been used to visualize B-16 mouse melanoma cell GlcNAc-T V on immunoblots after SDS-PAGE. When the antisera was used in immunofluorescence microscopy experiments on permeabilized B-16 and baby hamster kidney cells, intense, specific staining was observed in intracellular structures which appear to correspond to the Golgi apparatus.

Keywords: *N*-acetylglucosaminyltransferase V, antisera, glycosyltransferase, recombinant protein

Abbreviations: GlcNAc-T V, *N*-acetylglucosaminyltransferase V; rsGlcNAc-T V, recombinant, soluble GlcNAc-T V; GlcNAc-T I, *N*-acetylglucosaminyltransferase I; Gal-T, $\beta(1,4)$galactosyltransferase; BHK, baby hamster kidney cells; CHO, Chinese hamster ovary cells; FITC, fluorescein isothiocyanate; BSA, bovine serum albumin; MES, 2-[*N*-morpholino]ethane sulfonic acid; SDS-PAGE, SDS-polyacrylamide gel electrophoresis; KLH, keyhole limpet hemacyanin.

Introduction

Although animal cell glycosyltransferases synthesize a diverse set of oligosaccharide products which have many functions [1], very little is known about their secondary and tertiary structures. The design of reagents to selectively inhibit or modify the catalytic properties of glycosyltransferases would be significantly enhanced by detailed structural information. One goal of this study, therefore, was to develop a system to produce milligram amounts of a particular glycosyltransferase in order to study its structure, catalytic properties, and to gain the ability to regulate its activity. *N*-acetylglucosaminyltransferase V, GlcNAc-T V, is a glycosyltransferase which catalyses the following reaction during Asn-linked oligosaccharide biosynthesis [2]:

*To whom correspondence should be addressed.

$$\text{UDP-}\mathbf{GlcNAc} + \text{GlcNAc}\beta(1,2)\text{Man}\alpha(1,6)\text{Man-R} \xrightarrow[\text{GlcNAc-T V}]{} \begin{matrix}\mathbf{GlcNAc}\\ |\ \beta\mathbf{(1,6)}\\ \text{GlcNAc}\beta(1,2)\text{Man}\alpha(1,6)\text{Man-R}\end{matrix} + \text{UDP}$$

The oligosaccharide products of this enzyme are further modified by other glycosyltransferases and often express polylactosamine [3, 4]. The products of GlcNAc-T V are found on secreted and cell surface glycoproteins which vary depending on the cell type [5, 6]. Changes in the levels of some cell surface GlcNAc-T V products are postulated to function in the regulation of cell adhesion [7, 8], and in some cases, metastatic potential [9, 11]. Recently, the cDNAs encoding human and rat GlcNAc-T V have been isolated and characterized [12, 13].

The investigation of the expression of GlcNAc-T V and its products has uncovered several unusual mechanisms of regulation [14]. First, the mRNA and activity of the enzyme can be increased over basal levels by the pp60*src* tyrosine kinase signalling pathway (Buckhaults P, Margitich I, Freigien N, Pierce M: paper submitted). In adult rat and mouse brain tissue, however, mRNA levels of GlcNAc-T V do not correspond to enzymatic activity. These tissues express the highest levels of GlcNAc-T V mRNA observed for any cell line or tissue, yet the specific activity of the enzyme in rat and mouse brain is relatively very low. This observation suggests that there is a mechanism which inhibits the expression of GlcNAc-T V activity in brain tissue, perhaps by regulating translation of the abundant GlcNAc-T V transcripts or inhibiting the catalytic activity of translated protein. In addition, a CHO lectin-resistant cell line, Lec 4A, isolated by Chaney and Stanley, expresses wild-type CHO GlcNAc-T V activity, but mislocalizes the enzyme [15]. Upon gradient fractionation, Lec 4A GlcNAc-T V activity does not sediment in the Golgi-containing fractions where GlcNAc-T V activity from wild-type CHO cells sediments. This mislocalization causes Lec4A GlcNAc-T V to be ineffective in transferring GlcNAc to Asn-linked cell surface oligosaccharides, ultimately giving rise to the phenotype of resistance to L-phytohaemagglutinin cytotoxicity.

An understanding of these mechanisms of regulating GlcNAc-T V expression would be facilitated if a specific antisera was available. This antisera would allow us to visualize the mislocalized GlcNAc-T V enzyme in the Lec4A cells, as well as allow us to quantify the amount of translated GlcNAc-T V in neuronal tissue. We have been successful in purifying milligram amounts of a recombinant, soluble GlcNAc-T V (rsGlcNAc-T V) expressed by CHO cells. This rsGlcNAc-T V has been used to produce specific antisera which also recognizes native GlcNAc-T V. The antisera has been used to visualize the enzyme after SDS-PAGE of B-16 mouse melanoma cell sonicates and Western blotting. In addition, the antisera has been used to visualize the enzyme *in situ* in B-16 and baby hamster kidney cells using immunofluorescence microscopy.

Materials and methods

Materials

The Hi-trap and CM-fast flow sepharose columns and Protein A sepharose were from Pharmacia. UDP-GlcNAc, alkaline phosphatase-conjugated, peroxidase-conjugated, and FITC-conjugated goat-anti-rabbit IgG were from Sigma. BCA protein assay reagents were from Pierce. SDS-gels, silver stain, and immunoblotting reagents were from Bio-Rad. ECL immunoblotting detection reagents were from Amersham. PCR Magic Prep Kit was from Promega. S&S Elu-Quick DNA Purification Kit was from Schleicher & Schuell. T4 DNA ligase was from Boehringer Mannheim, the Plasmid Midi Kit was obtained from Qiagen, and the Mini Sartocon polysulfone modules were from Sartorius Corp.

GlcNAc-T V assay

Recombinant GlcNAc-T V activity was assayed using radiolabelled UDP-[3H]-GlcNAc and synthetic octyltrisaccharide as described [16]. UDP-[3H]-GlcNAc was from American Radiochemicals, Inc. Assay tubes contained 4.00 mM UDP-GlcNAc, 0.40 mM trisaccharide acceptor, 2.00 mM ADP (as pyrophosphatase inhibitor), and approximately 5×10^5 cpm UDP-[^{3}H]-GlcNAc. This mixture was evaporated to dryness. Five microlitres of the following solution were added prior to assay: 100 mM MES, pH 6.5, 40% glycerol, 1.0% Triton X-100, 1.0 mg ml^{-1} bovine serum albumin, 100 mM NaCl. Five microlitres of the solution to be assayed was then added and the tube incubated for 60 min at 37°. To stop the reaction, 0.5 ml of water was added. Radiolabelled product was isolated using Sep-Pak C18 cartridge chromatography by methanol elution.

SDS-PAGE and immunoblotting

Samples were separated on 10% mini-gels of 1.5 mm thickness and transferred to nitrocellulose by electroblotting. The blot was blocked for at least 2 h in blocking buffer (3% BSA, 100 mM NaCl, 100 mM Tris, pH 7.5, 0.1% Tween-20). Antisera was diluted (1:500) in blocking buffer and incubated with the blot for at least 2 h. Antibody was detected by incubation with peroxidase-conjugated or phosphatase-conjugated-goat anti-rabbit IgG (1:10 000) as described [17]. For immunoblotting of B-16 mouse melanoma cells, cells were homogenized in a protease inhibitor cocktail (50 mM MES, pH 6.5, 150 mM NaCl, 2% Triton X-100, and 25% glycerol plus 1 mg ml^{-1} pefabloc SC, 10 μg ml^{-1} leupeptin, 10 μg ml^{-1} pepstatin, 1 μg ml^{-1} aprotinin and 5 mM EDTA). The ECL detec-

tion system was used according to manufacturer's protocol to visualize antibody binding on X-ray film.

Preparation of soluble recombinant GlcNAc-T V

Soluble, secreted recombinant rat GlcNAc-T V with enzymatic activity was produced by deleting the membrane anchor domain and Golgi apparatus retention signal. These were replaced by sequence information for expressing the cleavable secretion signal of gamma-interferon. PCR was carried out using rat GlcNAc-T V cDNA in pSPORT as template and the following primers:

Primer 553-28 (sense)
CGCGCTCTAGATGCAAAGATGAAATACACCT
CTTACATTTTGGCTTTCCAATTGTGTATTGT
TTTGGGTTCTTTGGGTTGTTACTGTCAGGAT
GGCCCGTATGCCGGTGTC

Primer 516-5 (antisense)
GCGGTCGACCTACTATAGGCAGTCTTTGCAGA
GGG.

Primer 553–28 contains the gamma-interferon signal sequence, one amino acid of the mature gamma-interferon and joins into the rat GlcNAc-T V sequence at amino acid 70. Primer 516-5 contains the rat GlcNAc-T V carboxy-terminal sequence and the TAG stop codon. After 20 cycles, the PCR products were cleaned using a PCR Magic Prep Kit and a portion of the resulting sample was digested with *Xba* I and *Sal* I. The restriction endonuclease digested sample was analysed by agarose gel electrophoresis, and the approximately 2.1 kb PCR product was excised. The DNA was isolated using an S&S Elu-Quick DNA Purification Kit and ligated to an *Xba* I/*Sal* I cut mammalian expression vector overnight at 16° using T4 DNA ligase. A portion of the ligation mixture was electroporated into *E. coli* DH10B cells. Plasmid DNA that contained the correct insert was isolated from eight bacterial colonies using a Plasmid Midi Kit.

COS-7 cells were transfected with a mammalian expression vector containing the secretable rat GlcNAc T V cDNA insert by electroporation [13]. Expression of the GlcNAc T V cDNA was driven by the SV40 early promoter and used a heterologous polyadenylation signal. In addition, the expression vector contained a mouse dihydrofolate reductase (*DHFR*) gene as a selectable marker, a pBR322 backbone with the ampicillin resistance marker gene, and the origin for replication of the plasmid in *E. coli*. Fifteen μl of plasmid DNA was used for each of the eight clones described below. The cells were transferred to T-75 culture flasks containing 10 ml of Dulbecco's Modified Eagle's Medium (DMEM), 10% fetal bovine serum (FBS), and a 1 × solution (GPS) of glutamine, penicillin, and streptomycin; final concentrations in medium: L-glutamine 0.292 mg ml^{-1}; penicillin G, 100 U ml^{-1}; streptomycin sulfate 100 μg ml^{-1}). After a 7 h incubation at 37°, the medium was replaced with 7 ml of DMEM, 1% FBS and 1 × GPS and incubation continued for an additional 3 days. The cell-conditioned medium from each COS-7 plasmid transfection flask was collected and centrifuged to pellet cells and debris. The clear supernatant was frozen at −70° until analysed for GlcNAc-T V activity by the radiochemical assay.

The results obtained from analysis of 10 μl of cell-conditioned supernatant indicated that four of the transfected clones made little or no active, soluble GlcNAc-T V, while four made over 200 pmol mg^{-1} h^{-1}. One clone, which yielded activity of 470 pmol mg^{-1} h^{-1} in 10 μl of supernatant, was analysed further. The PCR-derived insert from the vector used to generate this clone was sequenced. The gamma-interferon signal sequence joined to the truncated rat GlcNAc-T V sequence was found to be identical to the expected sequence. This vector was transfected into CHO dhfr- cells by the calcium phosphate precipitation method [21]. Following selection by growth in media containing 5% dialysed FBS, pools and clones of stably transfected CHO dhfr- cell lines were collected and analysed for GlcNAc-T V activity. The CHO cell line which produced the highest amount of active soluble GlcNAc-T V (709 pmol mg^{-1} h^{-1}) was used to seed a spinner cell culture flask. The cells were propagated in suspension cell culture and then used to seed roller bottles at an initial seeding density of 2.5×10^7 cells in 200 ml of a 50:50 mixture of DMEM and F-12 media supplemented with 5% dialysed FBS, 1 × non-essential amino acids and 2 mM L-glutamine. After 3 days, the roller bottles were shifted to 200 ml of serum-free medium. Harvests were collected at 6 day intervals with new serum-free medium added after each harvest. The conditioned media were pooled and concentrated 25-fold by cross-flow ultrafiltration through Mini Sartocon polysulfone modules, then stored at −80° prior to purification.

Purification of soluble recombinant GlcNAc-T V

Protein assays were performed using the BCA microtitre plate assay method with bovine serum albumin as standard [18]. The concentrated media was thawed and applied to a copper-chelating column. To prepare the copper-chelating column, 100 mM copper sulfate was passed over a 5 ml Hi-trap column, which was then equilibrated in buffer X (50 mM MES, pH 6.5, and 100 mM NaCl). About 20 ml of concentrated CHO medium was loaded directly onto the column, which was then washed with 100 ml of buffer X. The column was eluted with a 0–20 mM linear gradient of imidazole in buffer X. Fractions were assayed for GlcNAc-T V

activity and the active fractions were pooled. EDTA was added to the pool to give a final concentration of 5 mM. GlcNAc-T V typically eluted in fractions containing 20 mM imidazole.

The pooled fractions from the copper-chelating column were diluted at least four-fold in buffer Y (50 mM MES pH 6.5, 5 mM EDTA) and applied to a CM-fast flow sepharose column (1.1 × 15 cm) pre-equilibrated with buffer Y. The column was washed with 300 ml of buffer Y, and GlcNAc-T V was eluted using a 0–500 mM linear gradient of NaCl in buffer Y. GlcNAc-T V typically eluted at a salt concentration of 300 mM. Fractions were then pooled and stored in this buffer.

Production of rabbit antisera to rsGlcNAc-T V

Two New Zealand white female rabbits were injected with purified rsGlcNAc-T V and boosted 2 weeks later. The initial injection and first boost used a total of 250 μg of enzyme. Freund's adjuvant was used in the initial inoculation. Four weeks later, the rabbits were boosted with an additional 250 μg of enzyme. The first bleed came 4 weeks after the primary injection. Antisera used in the experiments described in this study were collected after 8 weeks from the primary injection. The antisera from the two animals were similar in titre and specificity.

Production of rabbit antisera to synthetic peptides

Three peptides derived from the C-terminal region of the rat sequence were synthesized and conjugated to KLH for use as immunogen [17]. The first peptide (A) corresponded to amino acids 546–557 with an additional Cys and Gly at the N-terminus; the second (B) corresponded to amino acids 592–607 with an additional Cys at the N-terminus; the third (C) corresponded to amino acids 577–592 with an additional Cys at the N-terminus. Peptide A was used to inject three rabbits, three times every 2 weeks with Freund's adjuvant using 100 μg of the conjugate for each injection. Antisera was collected every week beginning 11 weeks after the initial injection. Peptide B was used with Hunter's adjuvant to inject three rabbits using 100 μg of the conjugate for each injection. After the first injection, the rabbits were boosted after 1 month and 3 months. Antisera was collected every week beginning 11 weeks after the first injection. Peptide C was used with Freund's adjuvant to inject three rabbits using 200 μg of the conjugate for each injection. Rabbits were injected 1, 3, 5, and 8 weeks after the initial injection. Antisera was collected at 1 month and 2 months after the initial injection. Each antisera was initially screened in an ELISA format to confirm if antibodies were present against the peptide conjugate used as inoculum. Antisera to all three peptide conjugates bound strongly to their corresponding conjugate.

ELISA for antibodies to GlcNAc-T V

Briefly, a 96-well microtitre plate was coated with rsGlcNAc-T V (20 μg ml^{-1}, 50 μl per well) for 2 h at room temperature. The wells were washed twice with TTBS (100 mM Tris, pH 7.5, 100 mM NaCl, 0.1% Tween-20) and blocked overnight with blocking solution (3% bovine serum albumin in TTBS). The wells were then washed three times with TTBS and incubated with various concentrations of antisera at room temperature for 2 h. The wells were washed with TTBS three times and then 50 μl of alkaline phosphatase conjugated goat-anti-rabbit IgG diluted 1:10 000 in blocking solution was added to each well. After a 2 h incubation, the wells were washed with TTBS three times. Finally, 50 μl of substrate solution (0.06 mg ml^{-1} p-nitrophenyl phosphate in 10 mM diethanolamine, pH 9.5, plus 0.5 mM $MgCl_2$) was added to each well, and after 30 min, the absorbances of the wells were read at 405 nm.

Immunoprecipitation with antibody-protein A sepharose

Antisera was diluted in TBS-B buffer (100 mM Tris, pH 7.5, 100 mM NaCl and 3% BSA), and 50 μl of the dilution was added to 50 μl of pre-washed Protein A sepharose beads. After rotation at 4° for at least 2 h, the mixture was pelleted and the beads were washed with 0.2 ml TBS-B three times and twice with 0.2 ml TBS (100 mM Tris, pH 7.5, 100 mM NaCl). Twenty μl of a five-fold dilution of concentrated CHO media was added to the antibody-Protein A sepharose complex. The mixtures were rotated for 1 h at 4°, pelleted, and GlcNAc-T V assays were performed on the supernatant. For assays to determine antisera specificity, 60 μl of protein A beads were incubated with 60 μl of antisera (or buffer alone or preimmune sera) as described above. Beads were washed and incubated with 20 μl of crude CHO-conditioned media or 500 μl of B-16 cell sonicate (0.8 mg ml^{-1}) for 1 h at 4°, after which time the beads were pelleted and the supernatant assayed for GlcNAc-T V, GlcNAc-T I, or β(1,4) galactosyltransferase activity as described [19].

Indirect immunofluorescence of B-16 mouse melanoma cells

Cells were grown on chamber slides under standard culture conditions for 1 day. Cells were fixed with 2% formalin in Hanks solution for 15 min. Slides were washed in PBS and permeabilized in a solution of 0.2% saponin and 3% BSA in PBS for 15 min. Slides were immersed in rsGlcNAc-T V antisera (1:100) for 1 h, washed extensively in PBS, and then incubated in FITC-conjugated goat anti-rabbit antibody (1:1000) for 1 h [20]. After extensive washing, cells were viewed using a Bio-Rad MRC-600 Laserscanning confocal microscope, and the images were recorded on Kodak T-max 100 film.

Results and discussion

Preparation of a secreted form of recombinant rat GlcNAc-T V

Soluble, secreted recombinant rat GlcNAc-T V with enzymatic activity was prepared by deleting the membrane anchor domain and a portion of the stem region, replacing them with a cleavable secretion signal. By PCR techniques described in Materials and methods, the cleavable signal sequence of human gamma-interferon was fused with the rat GlcNAc-T V cDNA at the sequence corresponding to amino acid number 70 [13]. After transfection into cells, expression, and cleavage of the signal sequence, the resulting secreted product had its first 69 amino acids deleted. The new N-terminus of the secreted enzyme included one amino acid from the interferon sequence (Gln) and the remainder of the amino acid sequence corresponded to the rat GlcNAc-T V sequence.

Production of secreted GlcNAc-T V from CHO cells

After demonstration that the construct expressed in COS-7 cells yielded secreted, active enzyme, the construct was sequenced and shown to contain the expected nucleotide sequences. This construct was then transfected into CHO cells, which were then cloned using cloning rings. Several clones were expanded and conditioned media from these were collected and each assayed for GlcNAc-T V activity. The clone producing the media with the highest activity was used to seed roller bottles. After growth for 3 days, the cells were shifted to serum-free media. Harvests were collected, pooled, and concentrated 25-fold by cross-flow ultrafiltration.

Purification of soluble GlcNAc-T V

An aliquot of the CHO conditioned media concentrate was thawed and a purification scheme was developed. The thawed solution was subjected to copper-chelating chromatography as described in Materials and methods, followed by chromatography on a CM-fast flow agarose column. These two purification steps produced a highly purified preparation of active enzyme, summarized in Table 1. The yield was about 67% and the enzyme was purified about 20-fold. Figure 1 depicts a silver-stained gel after SDS-PAGE showing the concentrated CHO-conditioned media, fractions after copper-chelating sepharose chromatography and fractions after CM-sepharose chromatography. It should be noted that the enzyme, even in the crude conditioned media, is efficiently proteolyzed from its 95 kDa form to a 75 kDa form and then finally to a 60 kDa form. Inclusion of EDTA in all buffers could inhibit the majority of the proteolysis, suggesting that at least some of the degradation was caused by a metalloproteinase. The rat kidney enzyme is found to be proteolytically cleaved in a similar manner during purification [18], and GlcNAc-T V released into a lung carcinoma cell line [22] shows similar proteolytic cleavage patterns.

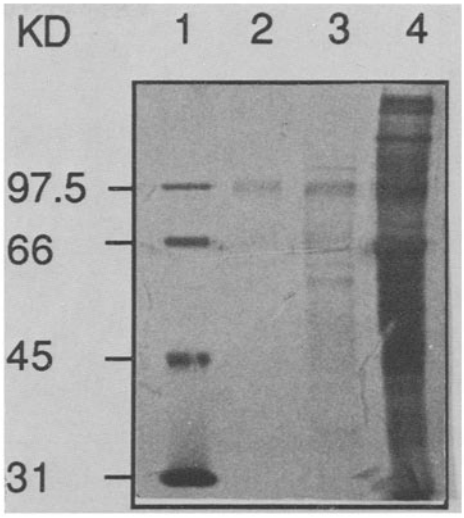


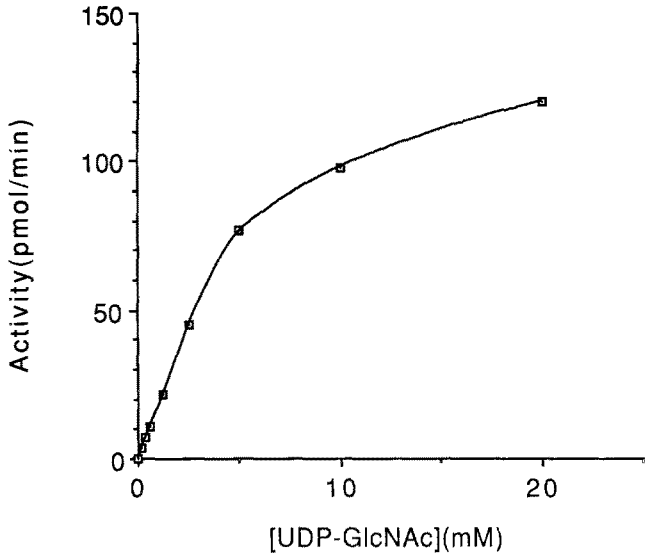
Figure 1. SDS-PAGE of rsGlcNAc-T V during purification. rsGlcNAc-T V after two stages of purification was subjected to SDS-PAGE and visualized with silver staining. Lane 1, molecular weight markers; lane 2, sample after CM-fast flow sepharose chromatography; lane 3, sample after copper-chelating sepharose chromatography; lane 4, concentrated CHO cell-conditioned medium.

Kinetic studies on the purified soluble enzyme

Analysis of the kinetic parameters of rsGlcNAc-T V revealed that its K_m value toward UDP-GlcNAc was 4.9 mM (Fig. 2), while its K_m toward the standard synthetic octyl-trisaccharide acceptor was 1.1 mM (Fig. 3). The UDP-GlcNAc K_m of the soluble enzyme was similar to that measured for the purified rat kidney enzyme and BHK cell crude enzyme [18, 19]. The oligosaccharide K_m for the soluble enzyme, by contrast,

Table 1. Purification of rGlcNAc-T V

Steps	*Protein (mg)*	*Activity (nmol min^{-1})*	*Specific activity (nmol min^{-1} mg^{-1})*	*Yield (%)*	*Fold*
Concentrated CHO-conditioned media	37.92	793.4	20.9	100	1
Copper-chelating column	7.77	723.1	93.1	91	4.5
CM-sepharose column	1.20	531.3	427.8	67	20.5



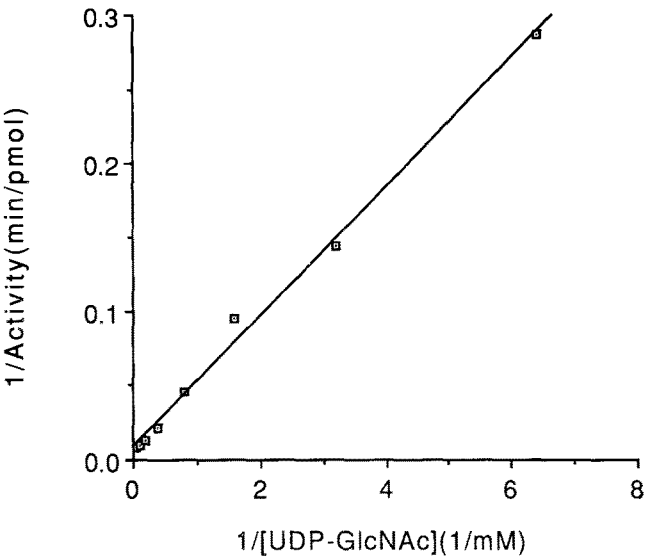
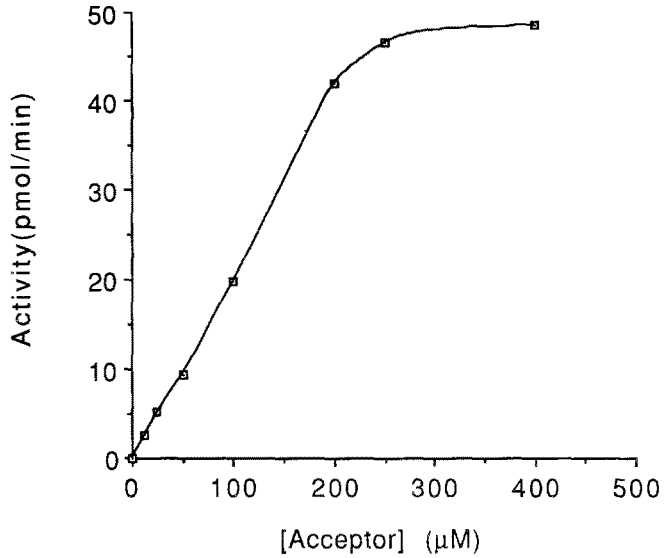


Figure 2. Determination of K_m value of rsGlcNAc-T V toward UDP-GlcNAc. (A) the activity of rsGlcNAc-T V was measured at different concentrations of UDP-GlcNAc using 0.400 mM synthetic trisaccharide acceptor. (B) 1/V vs 1/S plot of the data in Fig. 2A. The K_m was calculated to be 4.9 mM.



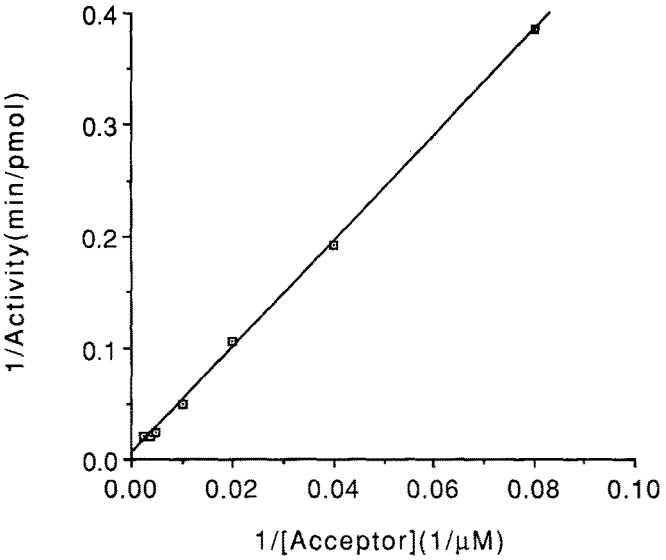


Figure 3. Determination of K_m value of rsGlcNAc-T V toward the synthetic trisaccharide acceptor. (A) the activity of rsGlcNAc-T V was measured at different concentrations of trisaccharide acceptor at 20 mM UDP-GlcNAc. B, 1/V vs 1/S plot of the data in Fig. 3A. The K_m was calculated to be 1.1 mM, but the kinetic behaviour did not conform to the Michaelis-Menten equation.

was about 10-fold higher than those observed for the rat kidney or BHK enzymes. Moreover, the kinetic behaviour of rsGlcNAc-T V toward the acceptor did not follow a classic Michaelis-Menten model, since the acceptor concentration at half V_{max} is not equal to the calculated K_m value (Fig. 3). The V_{max} of the rsGlcNAc-T V was about 420 nmol min^{-1} mg^{-1}, and when its turnover number was calculated, it was about five- to 10-fold less than the purified rat kidney enzyme. It should be noted, however, that the measurement of the protein in the sample of the purified rat kidney enzyme used for the kinetic studies was performed by a different, more indirect method than was used for the rsGlcNAc-T V [18].

Two additional comparisons were made between the rsGlcNAc-T V and the rat kidney enzyme. Both enzymes show similar inhibition by NaCl (Fig. 4) and a similar pH optimum (Fig. 5) [18]. These results demonstrate that the N-terminal truncation that produced the rsGlcNAc-T V did not significantly influence the catalytic properties of the enzyme reflected by pH optima of catalysis or the sensitivity of the enzyme to salt inhibition.

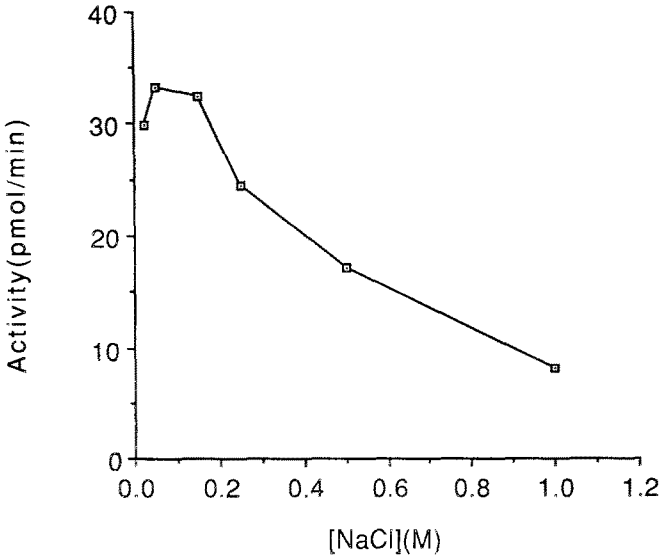


Figure 4. Effect of NaCl on rsGlcNAc-T V activity. The activity of rsGlcNAc-T V was measured at different concentrations of NaCl, as described in Materials and methods.

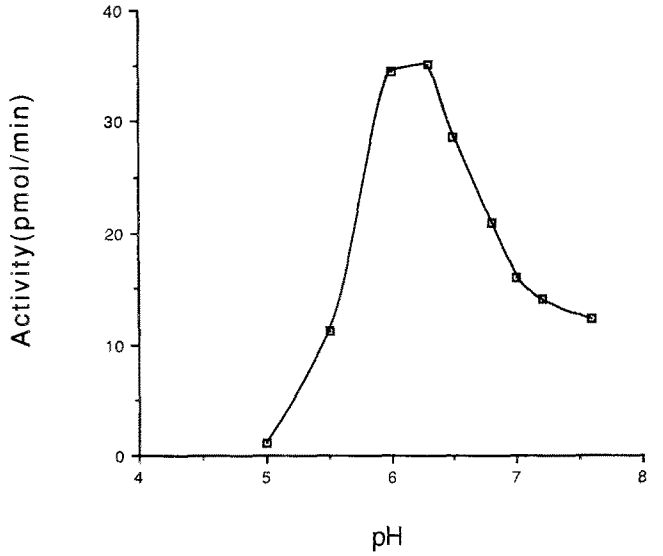


Figure 5. Effect of pH on rsGlcNAc-T V activity. The activity of rsGlcNAc-T V was measured at various pH values, as described in Materials and methods.

The difference in turnover number between the rsGlcNAc-T V and the rat kidney form may be caused by removal of 70 amino acids from the N-terminus in order to construct a soluble secreted form of the enzyme. If the different kinetic properties towards the acceptor trisaccharide were caused by this truncation, it is very interesting, because this truncation did not affect the kinetics toward the sugar nucleotide substrate. Experiments are in progress to produce soluble forms which have only the transmembrane segment removed and compare the kinetic properties of this construct with those of the enzyme truncated at amino acid 70 and the native enzyme.

Production of rabbit antisera against rsGlcNAc-T V protein

Two rabbits were immunized with purified rsGlcNAc-T V, and the antisera generated were assayed against the rsGlcNAc-T V in an ELISA-based assay. After inoculation and two boosts at 4 week intervals, the titre of the antisera of both animals in the ELISA was 1:64000. The specificity of the antisera was tested by both immunoprecipitation and immunoblotting of gels after SDS-PAGE. The results of the immunoprecipitation experiments demonstrate that from the concentrated CHO cell-conditioned media over 90% of the GlcNAc-T V activity could be pelleted after incubation with antisera and protein A-agarose (Fig. 6). By contrast, neither β(1,4)galactosyltransferase in the crude media nor GlcNAc-T I activity in B-16 mouse melanoma cell sonicates was pelleted after incubation with antisera and the immobilized Protein A (Fig. 7). These results show that the antisera bound rsGlcNAc-T V with enough affinity to allow it to pellet with Protein A and did not bind to other glycosyltransferases. In addition, although the antisera bound rsGlcNAc-T V, the binding did not inactivate its enzymatic activity (Fig. 7, incubation with antisera but without Protein A agarose).

An immunoblot of crude media and purified

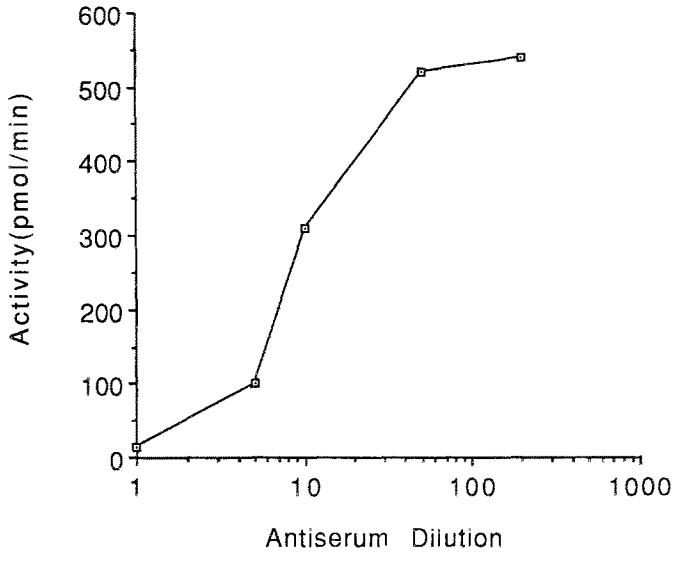


Figure 6. Immunoprecipitation of rsGlcNAc-T V by antibody-Protein A sepharose complexes. Various concentrations of antisera were incubated with Protein A sepharose beads for 2 h, washed, and the complexes incubated with an aliquot of five-fold diluted concentrated CHO-conditioned media. After 2 h of mixing, the beads were pelleted and supernatants assayed for GlcNAc-T V activity.

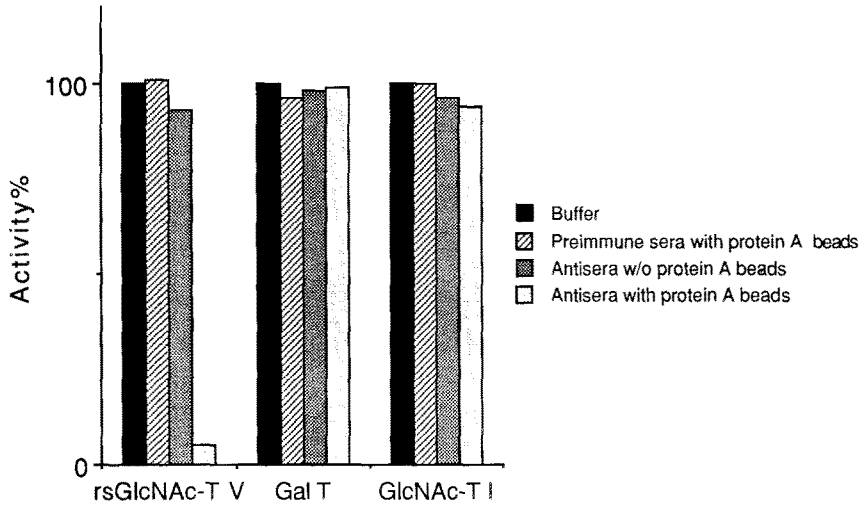


Figure 7. Specificity of rsGlcNAc-T V antisera assayed by immunoprecipitation by antibody-Protein A sepharose complexes. Protein A sepharose-antibody complexes were incubated with crude CHO medium (GlcNAc-T V and Gal T) or B-16 cell sonicates (GlcNAc-T I). After 2 h, beads were pelleted and supernatants assayed for appropriate glycosyltransferase activities.

rsGlcNAc-T V after SDS-PAGE demonstrated that the antisera binds specifically to the band which corresponds to rsGlcNAc-T V (Fig. 8), although reactivity with some lower molecular weight bands is evident (lane 7). To confirm the specificity of the antisera and demonstrate that the bands of lower molecular weights (75 and 60 kDa) represented proteolyzed forms of rsGlcNAc-T V, one rabbit was inoculated and boosted with protein

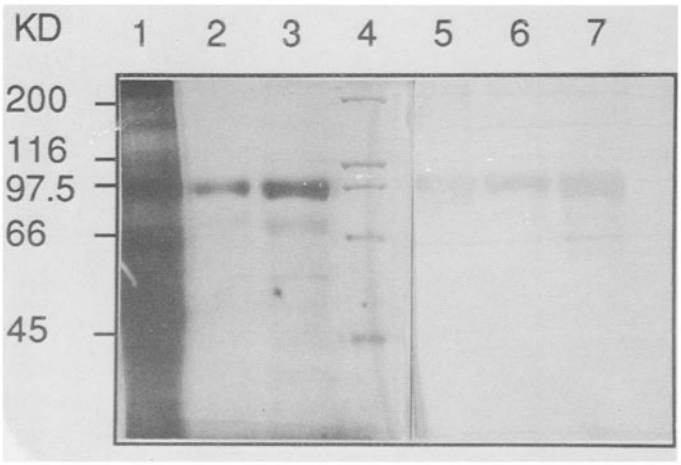


Figure 8. Specificity of rsGlcNAc-T V antisera assayed by Western blotting. Following SDS-PAGE, the gel was divided in half and either stained with silver (lanes 1–4) or subjected to Western blotting using rsGlcNAc-T V antisera (1:500 dilution) and the goat anti-rabbit conjugated alkaline phosphatase detection system (lanes 5–7). Lanes 1 and 5, concentrated CHO-conditioned media; Lanes 2 and 6, purified rsGlcNAc-T V (0.4 μg); lanes 3 and 7, purified rsGlcNAc-T V (1.4 μg); lane 4, molecular weight markers.

electroeluted from the 95 kDa molecular weight area cut from several SDS-gels. The titre of this antisera was significantly lower toward rsGlcNAc-T V; 1:50 dilutions were optimal for immunostaining of Western blots instead of 1:500 dilution for the antisera prepared using the purified preparation. Reactivity of this antisera with the 95 kDa, 75 kDa, and 60 kDa bands, however, demonstrated that the lower molecular weight forms share common epitopes with the 95 kDa band used to produce the antisera (data not shown, see Fig. 10 below).

Production of rabbit antisera to three synthetic GlcNAc-T V peptides

Another protocol was used concurrently to produce antisera specific for GlcNAc-T V. Three peptides from the C-terminus of the rat enzyme [13] were synthesized based on the protein sequence, conjugated to KLH, and used as an immunogen. Polyclonal rabbit antisera against each of these peptides was tested first against its respective peptide in an ELISA assay to ensure that the antisera did indeed recognize the peptide used to produce it. Next, each antisera was then tested for binding to rsGlcNAc-T V using an ELISA assay where the purified enzyme was coated on the wells of a microtitre plate. The results of these experiments demonstrate that antisera to peptide A, but not B and C, was able to recognize and bind GlcNAc-T V (Fig. 9). These results imply that peptide A is more exposed than the other sequences. By ELISA assay, the anti-peptide A antisera has a similar titre toward the rsGlcNAc-T V as the antisera produced against the rsGlcNAc-T V protein described above. Immunoblotting experiments demonstrated that the anti-peptide A antibody (Fig. 10) showed a similar binding pattern to that observed for the antisera produced against the rsGlcNAc-T V protein.

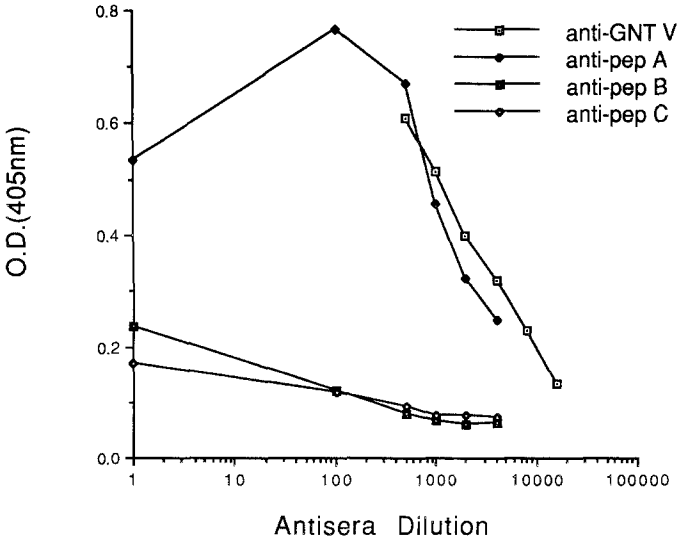


Figure 9. Binding of rsGlcNAc-T V by antisera to three synthetic peptides by ELISA. Wells of a microtitre plate were coated with purified rsGlcNAc-T V, blocked and washed as described in Materials and methods. Various concentrations of the anti-peptide antisera were incubated in the wells, followed by washing and detection of bound antibody by the goat anti-rabbit conjugated alkaline phosphatase detection system. Absorbance at 405 nm was determined after 30 min of colour development. Generation of anti-peptide antibodies are described in Materials and methods.

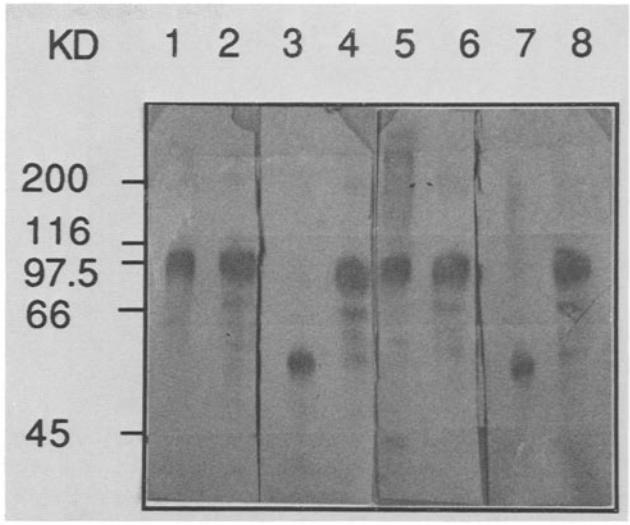


Figure 10. Detection of rsGlcNAc-T V proteolytic degradation by rsGlcNAc-T V antisera and anti-peptide antisera. Following SDS-PAGE, the contents of the gel were blotted to nitrocellulose and the blot divided into halves. The blot of lanes 1–4 was incubated with rsGlcNAc-T V antisera and the blot of lanes 5–8 was incubated with anti-peptide A antiserum. Each blot was then washed and bound antibody detected as described in Fig. 8. Lanes 1 and 5, concentrated CHO-conditioned media; lanes 2 and 6, purified rsGlcNAc-T V; lanes 3 and 7, partially purified GlcNAc-T V after 2 week incubation without EDTA; lanes 4 and 8, a separate preparation of purified rsGlcNAc-T V.

As mentioned earlier, rsGlcNAc-T V is very sensitive to proteolytic cleavage, most likely by a metalloproteinase expressed in the crude CHO media from which rsGlcNAc-T V was purified. Therefore, several preparations of rsGlcNAc-T V contained the 75 kDa and 60 kDa breakdown products of the enzyme. To demonstrate this precursor-proteolytic product relationship, an immunoblot experiment was performed using both rsGlcNAc-T V antisera and antisera produced against synthetic peptide A. Fig. 10 illustrates the results of this experiment using purified rsGlcNAc-T V and a sample of rsGlcNAc-T V which was incubated for 2 weeks without EDTA to accelerate proteolytic degradation. The rsGlcNAc-T V is cleaved finally to a 60 kDa product (lanes 3 and 7) which is recognized by both types of antisera. These results confirm that the small amounts of 75 kDa and 60 kDa bands which can sometimes be detected in the purified rsGlcNAc-T V preparations (Fig. 8) originate from the 95 kDa rsGlcNAc-T V precursor. Indeed, Gu *et al.* (22) purified GlcNAc-T V from the culture medium of a lung carcinoma cell line, QC, and the active enzyme migrated as two species at 73 kDa and 60 kDa on SDS-gels.

Visualization of GlcNAc-T V in cell sonicates

Attempts to visualize GlcNAc-T V on immunoblots after SDS-PAGE were initially unsuccessful due to the very low levels of the enzyme expressed in cells. Using the ECL reagent and the antisera used for the experiments depicted in Fig. 1, however, we were able to detect a 95 kDa band in B-16 mouse melanoma cells (Fig. 11). A band of slightly lower molecular weight, 75 kDa, could also be detected in some experiments; at present it is unknown whether this band originated during sample preparation, or whether this form is found *in situ*. When equivalent amounts of rsGlcNAc-T V activity and B-16 cell sonicate GlcNAc-T V were loaded on a gel, subjected to SDS-PAGE, and visualized by immunoblotting, the intensity of the stain of the bands at 95 kDa were reasonably similar (lanes 2 and 5). These results suggest that the 95 kDa band visualized in the B-16 sonicate corresponds to GlcNAc-T V. The GlcNAc-T V band in the B-16 cell sonicate appears to display a slightly higher apparent molecular mass compared to the rsGlcNAc-T V, which could be due to the differences between the full length sequence of native B-16 GlcNAc-T V and the truncated rsGlcNAc-T V sequence. Alternatively, this difference could be caused by differences in glycosylation. Experiments are in progress to examine the glycosylation in both forms of the enzyme. A faint band at about 75 kDa is visible in lanes 1 and 2, consistent with the observation that GlcNAc-T V is normally cleaved and released from cells as smaller molecular weight forms

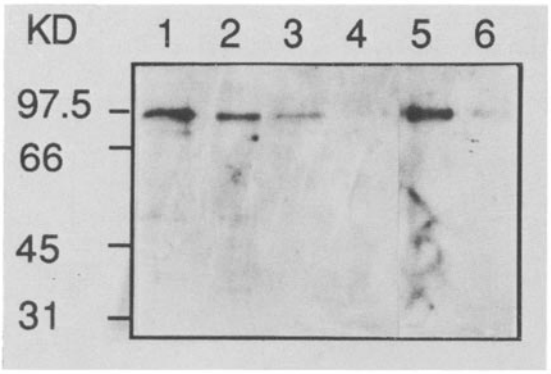


Figure 11. Immunoblot of B-16 cell GlcNAc-T V and rsGlcNAc-T V after SDS-PAGE. B-16 cell lysate (lanes 1–4) or concentrated CHO-conditioned media (lanes 5–6) were subjected to SDS-PAGE and blotting to nitrocellulose. The blot was then incubated with rsGlcNAc-T V antisera and processed for detection of antibody binding using the ECL detection system. Lane 1, 20 μg B-16 cell sonicate protein and 1.88 μU of GlcNAc-T V activity; lane 2, 10 μg protein and 0.94 μU; lane 3, 5 μg protein, 0.47 μU; lane 4, 2 μg and 0.19 μU; lane 5, 1.0 μU GlcNAc-T V activity in concentrated CHO-conditioned media; lane 6, 0.5 μU activity.

[22]. The culture medium of B-16 cells contains large levels of GlcNAc-T V activity; presumably this enzyme is present in the 75 kDa or smaller forms.

GlcNAc-T V visualization in situ

The antisera to rsGlcNAc-T V was used to detect GlcNAc-T V in B-16 melanoma cells and BHK cells by immunofluorescence and visualization by confocal microscopy. These results, depicted in Fig. 12, demonstrate that the antisera stains strongly and selectively the Golgi apparatus of B-16 (Fig. 10B) and Baby hamster kidney cells (Fig. 10C). Pre-immune sera shows no staining of B-16 cells (Fig. 10A), demonstrating the specificity of the staining with GlcNAc-T V antisera. This pattern of staining appears similar to staining patterns of cells by other antisera which recognize Golgi enzymes; for example, αmannosidase II [20].

Future experiments

Immunostaining of sections for electron microscopic analysis should allow us to localize GlcNAc-T V to specific cisternae and compare its intraGgolgi localization to that of other Golgi enzymes. Since the enzyme will not transfer GlcNAc *in vitro* to oligosaccharides which have been acted upon by the β(1,4) galactosyltransferase and contain galactose, it most likely resides in the medial Golgi compartment with GlcNAc-T I. Moreover, a cell line, CHO Lec4A, exists which expresses normal levels of GlcNAc-T V activity but mis-localizes the enzyme

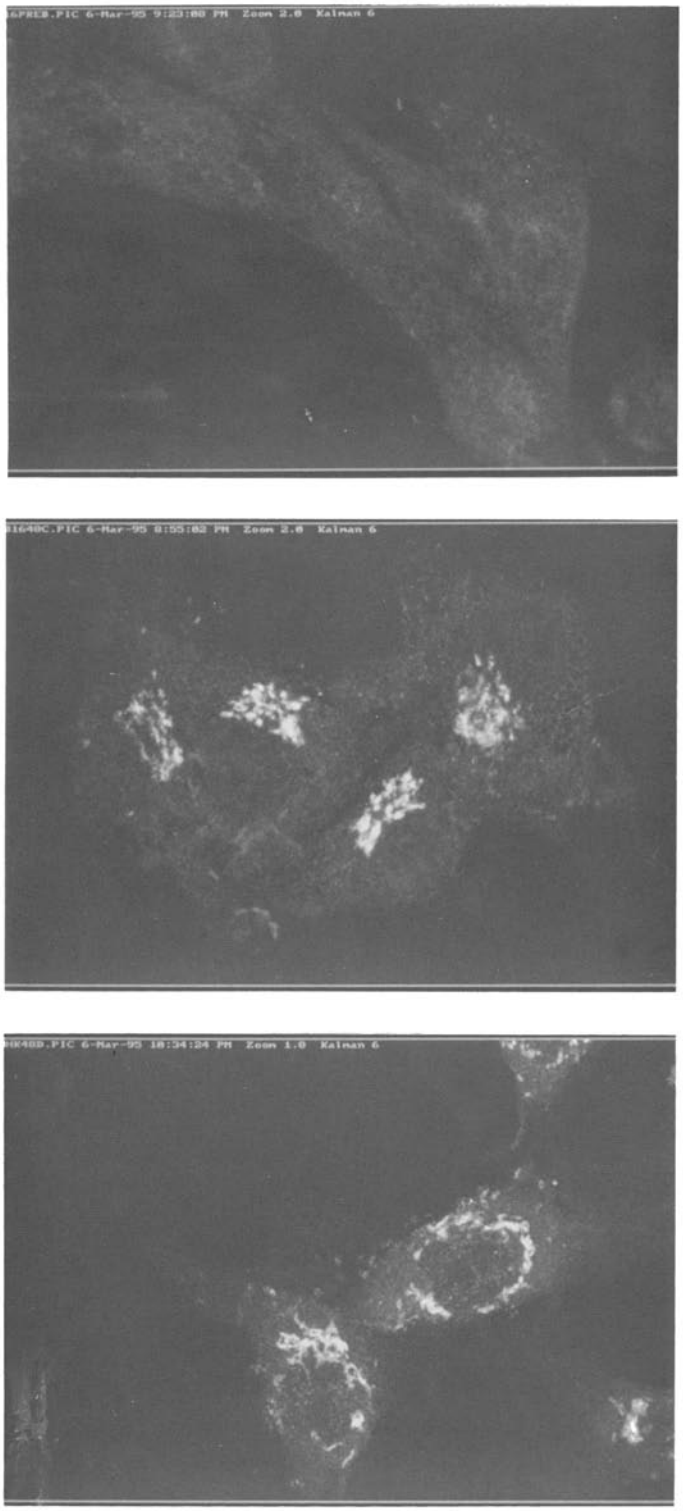


Figure 12. Immunofluorescence detection of GlcNAc-T V in B-16 and BHK cells using rsGlcNAc-T V antisera. Cells were grown on chamber slides and processed as described in Materials and methods. rsGlcNAc-T V antibody binding was detected by FITC-conjugated goat anti-rabbit antibody using a confocal microscope. A, preimmune sera; B, rsGlcNAc-T V antisera binding to B-16 cells; C, rsGlcNAc-T V antisera binding to BHK cells.

[15]. We are now collaborating to visualize the compartment(s) where the mislocalized enzyme is found and to discover by sequence analysis possible mechanisms which cause the mislocalization.

Recent experiments have documented that adult rat and mouse brains express large amounts of GlcNAc-T V mRNA, but, by contrast, express relatively little GlcNAc-T V enzymatic activity [14]. The GlcNAc-T V antisera will be a useful reagent to determine if the low activity in brain is caused by a lack of translation of the GlcNAc-T V mRNA, or whether large levels of GlcNAc-T V protein are translated and the catalytic activity is inhibited by other mechanisms. The availability of milligram quantities of the rsGlcNAc-T V will also allow us to investigate the secondary and tertiary structure of the enzyme, locate its active site using photoactivatable substrates, and attempt X-ray crystallographic studies.

Acknowledgements

We wish to gratefully acknowledge G. Grampp and R. Camacho for generating and concentrating CHO conditioned media for enzyme purification, T. Jones for designing peptide C, M. Rihanek and H. Hockman for conjugating the synthetic peptides and coordinating peptide antibody production, and R. Basu for sequencing the secreted rat GlcNAc-T V construct. We are also very appreciative of the assistance we received from S. Crawley for various enzyme assays.

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