Glycosylation potential of human prostate cancer cell lines

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Abstract Altered glycosylation is a universal feature of
cancer cells and altered glycans can help cancer cells escape
immune surveillance, facilitate tumor invasion, and increase
malignancy. The goal of this study was to identify specific
glycoenzymes, which could distinguish prostate cancer cells
from normal prostatic cells. We investigated enzymatic ac-
tivities and gene expression levels of key glycosyl- and
sulfotransferases responsible for the assembly of O- and
N-glycans in several prostatic cells. These cells included
immortalized RWPE-1 cells derived from normal prostatic
tissues, and prostate cancer cells derived from metastasis in
bone (PC-3), brain (DU145), lymph node (LNCaP), and
vertebra (VCaP). We found that all cells were capable of
synthesizing complex N-glycans and O-glycans with the
core 1 structure, and each cell line had characteristic bio-
synthetic pathways to modify these structures. The *in vitro*
measured activities corresponded well to the mRNA levels
of glycosyltransferases and sulfotransferases. Lectin and
antibody binding to whole cells supported these results,
which form the basis for the development of tumor cell-
specific targeting strategies.

Abbreviations

FUT	fucosyltransferase
Gal3ST	3-O-sulfotransferase
GalT	galactosyltransferase
GalNAcT	GalNAc-transferase
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GnT GlcNAcT	GlcNAc-transferase
GlcNAc6ST	N-acetylglucosaminyl-6-O-sulfotransferase
HPLC	high pressure liquid chromatography
PCR	polymerase chain reaction
ppGalNAcT	polypeptide GalNAc-transferase
PSA	prostate specific antigen
SLe^x	sialyl-Lewis^x
ST3Gal	$\alpha2,3$-sialyltransferase
ST6Gal(NAc)	$\alpha2,6$-sialyltransferase

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Introduction

Prostate cancer (CaP) is the most commonly diagnosed
cancer, and the second leading cause of cancer death in
men in most regions of the world. Altered glycosylation in
cancer cells [1–4] can change antigenicity, facilitate the
escape of cancer cells from the immune surveillance, and
promote invasion and metastasis [5–13]. In particular, sialic
acid is a cell surface carbohydrate involved in multiple
functions such as cell adhesion and immunity, and is often
found in increased amounts in cancer cells [13, 14].

The prostate produces many glycoproteins, including the
N-glycosylated prostate specific antigen (PSA) [15, 16] and

highly O-glycosylated secreted and cell surface-bound mucins [17–19]. PSA glycoforms from the serum of healthy people have mainly bi-antennary complex chains while PSA in the sera of CaP patients has many tri- and tetra-antennary N-glycans, suggesting an increase in branching GlcNAc-transferases (GnT) in CaP [4, 16, 20–22]. Significant changes were observed at the nonreducing ends of the oligosaccharide chains of PSA. The glycosylation of MUC1 mucin is also altered in CaP [2, 3].

The selectin ligand sialyl-Lewis^x, Neu5Acα2-3Galβ1-4 (Fucα1-3)GlcNAcβ-R (SLe^x), and related antigens play a critical role in cancer cell migration [23–26], and are associated with a low survival rate [27]. The O-glycan branching enzyme core 2 β6-GlcNAc-transferase (C2GnT1) synthesizes core 2, GlcNAcβ1-6(Galβ1-3)GalNAc-, which forms a scaffold structure for SLe^x in leukocytes, and may play a role in controlling the adhesion of cancer cells to the endothelium and their invasiveness [22–24, 28]. The expression of C2GnT1 was found to increase with progression of CaP [29]. C2GnT1 also contributes to galectin-1-mediated cell death in LNCaP cells derived from lymph node metastasis [30].

The mechanisms underlying altered glycosylation of cancer glycoproteins involve characteristically abnormal glycosyltransferase activities [7, 31–35]. In the current study, we conducted a comprehensive analysis of the activities and gene expression levels of key enzymes involved in the pathways of N- and O-glycans in four different cultured metastatic CaP cell lines in comparison to cells derived from normal prostate. We correlated transferase activities with mRNA expression levels of transferase genes. We showed that some of the glycosylation pathways are characteristic for a given cancer cell type. In addition, we found that alterations in several enzymes as well as in cell surface carbohydrate structures were common to all of the CaP cell lines. Some of these enzyme patterns resembled those found in colon and breast cancer cells. These studies provide a solid basis for targeting specific transferases and for further work on the biological roles of cancer-associated glycans.

Materials and methods

Materials

Reagents were from Sigma, unless indicated otherwise. Enzyme substrates were also prepared and donated by Hans Paulsen (University Hamburg, Germany) and Khushi Matta (Roswell Park Cancer Institute, Buffalo, NY). The β1,4-Galactosyltransferase (β4GalT) inhibitor **612** was prepared by Walter Szarek (Queen's University, Canada). Lectins were obtained from Vector Labs. Antibodies anti-SLe^x (CSLEX1) was from BD Pharmingen; anti-Le^y, anti-Tn, anti-Le^a and anti-Sialyl-Tn antibodies were from Abcam.

Cell cultures

Human colon cancer Caco-2 cells were obtained from John MacLeod (Queen's University) and grown in Dulbecco's Modified Eagle's medium. The immortalized human prostate cell line RWPE-1 was from ATCC, and cells were grown in Keratinocyte serum free medium. Metastatic CaP cell lines (from ATCC) were kindly donated by Jeremy Squire (Queen's University) and were grown as recommended by ATCC. PC-3 cells, derived from bone metastasis (stage IV), were grown in F-12 K medium from Invitrogen. DU145 cells, derived form brain metastasis, were grown in Eagle's Minimum Essential medium. LNCaP cells, derived from left supraclavicular lymph node metastasis, were grown in RPMI-1640 medium. VCaP cells, derived from vertebral metastasis, were grown in DMEM. All cell lines were grown at 37 °C with 5 % CO_2 and growth media contained 10 % fetal bovine serum (FBS) (except for RWPE-1), as well as 100 U Penicillin/ml and 100 μg Streptomycin/ml.

Preparation of cell homogenates

For glycosyltransferase and sulfotransferase assays, cells were harvested immediately after reaching confluence. Cells were detached with 0.25 % trypsin-EDTA (Invitrogen), washed once in growth medium followed by two washes with phosphate-buffered saline (PBS, Invitrogen). Pellets were stored at −80 °C, then hand homogenized on ice in 0.25 M sucrose (1 ml sucrose/l0^8 cells) and stored in small aliquots at −80 °C. Protein concentrations of homogenates were determined by the Bio-Rad (Bradford) protein assay method using bovine serum albumin as the standard.

Glycosyltransferase assays

Glycosyltransferases were assayed at standard conditions using fresh aliquots of frozen cell homogenates immediately after thawing [36–44]. Enzymes were kept on ice until the time of incubation. Assays were carried out in duplicate with variations between assays of<10 %. Enzyme products were isolated by AG1x8 or Sep-Pak C18, followed by separation with reversed-phase HPLC. Table 1 lists the enzymes and substrates used as well as the HPLC conditions in every assay. The acceptor substrate for β1,2-N-acetylglucosaminyltransferase I (GnT-I) was Manα1-6(Manα1-3)Manβ-octyl (Toronto Research Chemicals). Manα1-6 (GlcNAcβ1-2Manα1-3)Manβ-octyl was enzymatically prepared from Manα1-6(Manα1-3)Manβ-octyl, using recombinant GnT-I (donated by H. Schachter, University of Toronto, Canada), and was purified by reversed-phase HPLC.

Table 1 List of enzymes, reaction substrates and HPLC conditions Assays for the enzymes listed were carried out under conditions described in Materials and methods. The gene names (according to NCBI) indicate specific genes known to be responsible for the activities, and/or genes examined for mRNA expression. Enzyme products were isolated using the acceptor substrates listed, and a C18 column and acetonitrile (AN) - water mixtures as the liquid phase; *% AN* acetonitrile concentration in the liquid phase, *Bn* benzyl, *Gn* GlcNAc, *M* mannose; pnp *p*-nitrophenyl

Enzyme	Short Name	Gene Name	Acceptor Substrate and Assay Concentration (mM)		HPLC (%AN)
Polypeptide-*N*-acetylgalactosaminyltransferase	ppGalNAcT		AQPTPPP	0.5	
Core 1 β1,3-galactosyltransferase	C1GalT	*C1GALT1*	GalNAcα-Bn	0.5	14
	T-synthase				
Core 2 β1,6-*N*- acetylglucosaminyltransferase	C2GnT1	*GCNT1*	Galβ3GalNAcα-Bn	0.5	13
	C2GnT3	*GCNT4*			
Core 3 β1,3-*N*- acetylglucosaminyltransferase	C3GnT		GalNAcα-Bn	1.0	14
	Core 3 synthase	*B3GNT6*			
Core 4 β1,6-*N*-acetylglucosaminyltransferase	C2GnT2	*GCNT3*	Gnβ3GalNAcα-pnp	0.5	12
β1,3-*N*-acetylglucosaminyltransferase	Extension		Galβ4Gnβ-Bn	1.0	13
	β3GlcNAcT	*B3GNT1*			
	iGnT				
β1,3-*N*-acetylglucosaminyltransferase	Elongation		Galβ3(6-deoxy)GalNAcα-Bn	1.0	18
	β3GlcNAcT	*B3GNT3*			
β1,3/4-*N*-acetylgalactosaminyltransferase	β4GalNAcT	*B4GALNT3*	Gnβ3GalNAcα-pnp	0.5	13
	β3GalNAcT		Gnβ-Bn	0.5	12
β1,3/4-Galactosyltransferase	β4GalT	*B4GALT1-6*	Gnβ-Bn	0.5	12
	β3GalT5	*B3GALT5*	Gnβ3GalNAcα-pnp	0.5	13
	β3GalT	*B3GALT1-2*			
β1,2-*N*-acetylglucosaminyltransferase I	GnT-I	*MGAT1*	Mα6(Mα3)Mβ-octyl	0.5	24
β1,4-*N*-acetylglucosaminyltransferase II	GnT-II	*MGAT2*	Mα6(Gnβ2Mα3)Mβ-octyl	0.5	24
N-acetylglucosaminyltransferases III-V	GnT-III-V		Gnβ2Mα6(Gnβ2Mα3)Mβ-octyl	0.5	24
β1,6-*N*-acetylglucosaminyltransferase V	GnT-V	*MGAT5*	Gnβ2Mα6(Gnβ2[4-deoxy]Mα3) 4-*O*-methyl-Mβ-Octyl	1.0	24
α1,2-Fucosyltransferase	α2FUT1	*FUT1*	Galβ-Bn	2.0	11
α1,2-Fucosyltransferase	α2FUT2	*FUT2*	Galβ3GalNAcα-Bn	2.0	13
α1,3-Fucosyltransferase	α3FUT3–9	*FUT3-9*	GalNAcβ4Gnβ-Bn	2.0	13
α1,4-Fucosyltransferase	α3/4FUT3	*FUT3*	2-*O*-Methyl-Galβ3Gnβ-Bn	2.0	13
α2,3-Sialyltransferase	ST3Gal	*ST3GAL1*	Gal(6-deoxy)β3GalNAcα-Bn	1.0	12
α2,3-Sialyltransferase	ST3Gal3-6	*ST3GAL3-6*			
α2,6-Sialyltransferase	ST6Gal	*ST6GAL1*			
α2,6-Sialyltransferase	ST6GalNAc1-4	*ST6GALNAC1-4*			
Galactosyl-3-*O*-sulfotransferase	Gal3ST	*GAL3ST1-4*	Gal(6-deoxy)β3GalNAcα-Bn	2.0	13
N-acetylglucosaminyl-6-*O*-sulfotransferase	GlcNAc6ST	*CHST1-4*	Gnβ-Bn	2.0	12

GalNAc-transferases

Polypeptide α-GalNAc-transferase (ppGalNAcT) [42] and β1,3/4 *N*-acetylgalactosaminyltransferase (β3/4GalNAcT) activities were determined in a total volume of 40 μl containing 0.125 M MES (*N*-morpholino-ethanesulfonic acid) pH 7, 0.125 % Triton X-100, 10 mM AMP, 12.5 mM MnCl$_2$, 0.9 mM UDP-[^{3}H]GalNAc (2,000–4,000 cpm/ nmol), 0.5 mM AQPTPPP (for ppGalNAcT) or GlcNAcβ1-3GalNAcα-*p*-nitrophenyl or GlcNAcβ-Bn (for β3/4GalNAcT), and 10 μl cell homogenate (80 to 150 μg protein). Samples were incubated at 37 °C for 1 h and product determined using AG1x8 and HPLC as described [42].

Glycosyltransferases that synthesize core 1 to 4 glycans

The activities (Table 1) of the T-synthase (C1GalT) and core 3 synthase (C3GnT) that act on GalNAcα-Bn, C2GnT1 that acts on Galβ1-3GalNAcα-Bn or C2GnT2 that acts on both

Galβ1-3GalNAcα-Bn and GlcNAcβ1-3GalNAcα- p-nitrophenyl (Toronto Research Chemicals) to synthesize core 4, GlcNAcβ1-6(GlcNAcβ1-3)GalNAcα-, were determined as described [42].

GlcNAc-transferases-I to V

The assay mixtures for GlcNAc-transferases (Table 1) that synthesize N-glycan branches contained 0.9 mM UDP-[^{3}H] GlcNAc (1,600 cpm/nmol) and 0.5 mM Manα6(Manα3)-Manβ-octyl (for GnT-I, Toronto Research Chemicals), Man α6(GlcNAcβ2Manα3)Manβ-octyl (for GnT-II), GlcNAcβ2-Manα6(GlcNAcβ2Manα3)Manβ-octyl (for GnT-III to V) or 1 mM GlcNAcβ2Manα6(GlcNAcβ2[4-deoxy-]Manα3)[4-O-methyl-]Manβ-octyl in the absence of MnCl$_2$ (for GnT-V) (Table 1). Enzyme parameters for GnT-V were determined using PC-3 cell homogenates. Which exhibited high activity of this enzyme, and the non-linear regression program Origin-Pro 8.1.

Galactosyltransferases

GalT assays were carried out as described [40], using 1 mM UDP-[^{3}H]Gal (1,600 cpm/nmol) and 0.5 mM GlcNAcβ-Bn or GlcNAcβ1-3GalNAcα- p-nitrophenyl as acceptor substrates. Inhibition assays of β4GalT assays were carried out in the presence of 0.5 mM substrates and 0.5 mM GlcNBu-S-2-naphthyl (612). Since this inhibitor was dissolved in methanol, the same amount of methanol (10 %) was present in all assays.

Sialyltransferases

The α2,3-sialyltransferase (ST3Gal) activities (Table 1) were determined in a total volume of 40 μl containing 0.1 M Tris, pH 7, 0.125 % Triton X-100, 12.5 mM MnCl$_2$, 0.8 mM CMP-[^{3}H]sialic acid (2,400 cpm/nmol), 1 mM Galβ1-3(6-deoxy)GalNAcα-Bn as substrate and 10 μl cell homogenates (80 to 150 μg protein). Samples were incubated at 37 °C for 1 h and reaction mixtures applied to Sep-Pak C18 columns. Enzyme product eluted with methanol was analyzed by reversed-phase HPLC.

Sulfotransferases

Sulfotransferase (Gal3ST and GlcNAc6ST, Table 1) activities were determined in a total volume of 40 μl containing 50 mM MES pH 7, 0.1 % Triton X-100, 2 mM ATP, 10 mM NaF, 10 mM 2, 3-mercaptopropanol, 10 mM MnCl$_2$, 0.8 mM 3′-phosphoadenosine 5′-phospho[^{35}S]sulfate (PAP^{35}S) (1,300 cpm/nmol), 2 mM Galβ1-3(6-deoxy)Gal-NAcα-Bn or GlcNAcβ-Bn (for Gal3ST or GlcNAc6ST, respectively) and 10 μl cell homogenate (80 to 150 μg

protein). Samples were incubated at 37 °C for 1 h and the reaction mixtures were eluted through Sep-Pak C18 column, followed by HPLC analysis (C18 column).

Fucosyl-transferases

Assays for α2FUT, α3FUT and α3/4FUT (Table 1) were carried out as described for GalT assays, except that the donor substrate was 1.25 mM GDP-[^{3}H]Fuc (1,000 cpm/nmol) or 1 mM GDP-[^{14}C]Fuc (2,600 cpm/nmol) and the acceptor was either 2 mM Galβ-Bn, Galβ1-3GalNAcα-Bn or GalNAcβ1-4GlcNAcβ-Bn (Table 1). Mixtures were separated by AG1x8 and reversed-phase HPLC.

Real-time PCR analysis of the mRNAs of glycosyl- and sulfotransferase genes

RNA from cultured prostate cells was isolated by TRI-REAGENT according to the manufacturer's instruction. To prepare cDNA, 2 μg RNA were used in a 20 μl reaction mixture using a Verso reverse transcriptase kit (Thermo scientific) as follows: 5 min at r.t., 60 min at 42 °C, and 2 min at 95 °C. Quantitative real-time PCR was performed in 10 μl reaction volume in a 96-well plate using 2 μl of diluted (1:1) cDNA with SYBR® Premix Ex Taq™ (TAKARA BIO INC.) on a Mastercycler Epgradient real-plex (Eppendorf AG, Hamburg, Germany). The PCR conditions included 1 cycle at 95 °C for 2 min followed by 45 cycles at 95 °C for 15 s, 60 °C for 15 s, and 72 °C for 45 s. The data were analyzed using Eppendorf realplex software, version 1.5 (Eppendorf). The amounts of glycosyltransferase transcripts (see Table 1 for gene names) were normalized to the amount of GAPDH transcript in the same cDNA sample. Relative fold differences in transcript expression were determined using the following comparative CT method: CT method: $2^{-[\Delta Ct\ (Target)]} \times 100$ where $\Delta C_t = (Target) = C_t$ (Target)-C_t (GAPDH) as described previously [43]. The results were expressed as the amount (%) relative to that (100 %) of GAPDH and plotted as mean relative amount± SEM. Primer sequences used for expression analysis of all genes including GAPDH are summarized in Supplementary Table 1.

Lectin staining assays of whole cells

Lectins were used to determine cell surface glycosylation. Lectin staining was carried out as described [44] with cells fixed on 96 well microtitre plates. Confluent cells (7.2×10^4 to 1.04×10^5) were incubated with biotinylated lectins followed by alkaline phosphatase-conjugated Avidin and nitrophenyl-phosphate reaction substrate. The color change was measured with a microplate reader at 405 nm. The intensities were normalized to those of 1.0×10^{5}cells. The

differences between means were compared using the Student's *t*-test. In all cases, $p < 0.01$ was considered statistically significant. Each sample was analyzed at least seven times.

Enzyme linked immunosorbent assays (ELISA) of whole cells

Antibodies were also used to assess cell surface glycans. ELISA was carried out as described [45]. Cells were grown to confluency in 96-well plates, and were then fixed. Confluent cells (7.2×10^4 to 1.2×10^5 cells) were incubated with anti-SLe^x (CSLEX1), anti-Le^y, anti-Tn, anti-Le^a or anti-Sialyl-Tn antibodies followed by alkaline phosphatase-linked secondary antibodies and nitrophenyl-phosphate reaction substrate. Samples were analyzed as described above.

Results

The enzymatic activities of several glycosyltransferases and sulfotransferases were compared in homogenates from four different metastatic CaP cell lines and a normal prostatic cell line, as well as the human colon cancer cell line Caco-2 [39] and rat colon mucosal homogenate, which served as positive controls. Enzyme nomenclature and substrates used are listed in Table 1. The activities were correlated to the mRNA expression of isoenzymes and the cell surface expression of lectin and anti-carbohydrate antibody binding sites. The cell surface carbohydrates showed distribution patterns that appeared to be specific for each prostatic cell line.

Synthesis of complex N-glycans

As shown in Table 2, all prostatic cell lines contained GlcNAc-transferase (GnT-I and II) activities involved in the synthesis of bi-antennary N-glycans. The activity of GnT-I which synthesizes the first antenna of N-glycans, *i.e.* GlcNAcβ1-2 linked to the Manα1-3 branch, was reduced in all CaP cells up to 85 % when compared to prostatic RWPE-1 cells. GnT-II, which adds GlcNAc in a β1-2 linkage to the Manα1-6 arm was active in all prostatic

cells. The combined activities of GnT-III, IV and V using a biantennary substrate were low (<0.5 nmol/h/mg) in all cells (data not shown). GnT-V which synthesizes the GlcNAcβ1-6 branch of tetra-antennary N-glycans was also assayed using an acceptor substrate specific for GnT-V (Fig. 1a). The activities of GnT-V were higher in all CaP cells compared to RWPE-1 cells. PC-3 and LNCaP cells exhibited the highest GnT-V activities (0.31 and 0.35 nmol/h/mg, respectively). The reaction rate was linear with respect to protein concentrations up to 0.29 mg/ml and incubation times up to 2 h. Because of the low GnT-V activity in normal prostatic cells it was not possible to obtain an accurate K_M value. For GnT-V in PC-3 cell homogenates, the apparent K_M value for UDP-GlcNAc was 0.93 mM and V_{max} was 0.62 nmol/h/mg (Fig. 1b). For the acceptor substrate, the apparent K_M and V_{max} values were 0.05 mM and 0.38 nmol/h/mg, respectively (Fig. 1c). This K_M value was similar to that previously found for a purified hamster kidney GnT-V (0.06 mM) [41].

Glycosyl- and sulfotransferase activities that assemble O-glycans

Prostatic cancer cells produce highly O-glycosylated mucins that are abnormally expressed and glycosylated [3, 12, 18]. We therefore measured the enzymatic activities of glycosyl- and sulfotransferases that participate in the synthesis of O-glycans. The addition of the first sugar in the O-glycosylation pathways is catalyzed by a family of ppGalNAcT. Using a peptide acceptor with only one O-glycosylation site (AQPTPPP), high activities (3.0 to 12.5 nmol/h/mg) of ppGalNAcT were detected in all prostatic cell homogenates (Table 3). This suggests that glycoproteins and mucins in these prostatic cells are potentially highly O-glycosylated, which corresponds to the expression of the HP lectin epitope (Supplementary Figure 1) and the Tn antigen (Supplementary Figure 2) on cell surfaces. All of these prostatic cells contained the activity of T-synthase, which synthesizes core 1, Galβ1-3GalNAc-, the T antigen recognized by PNA lectin (Supplementary Figure 1). However, the subsequent processing of core 1 appeared to be quite

Table 2 GlcNAc-transferase activities that synthesize N-glycan antennae. Assays were carried out by HPLC as described in Material and Methods. The results were calculated as the average of duplicates that differ by <10 %. Abbreviations are as in Table 1. Caco-2 cells served as positive controls. RWPE-1 cells were derived from normal prostate. PC-3, DU145, LNCaP and VCaP cells are metastatic prostate cancer cells

Enzyme	Activity (nmol/h/mg)					
	RWPE-1	PC-3	DU145	LNCaP	VCaP	Caco-2
GnT-I	7.21	1.65	1.10	3.94	2.60	3.45
GnT-II	3.40	1.19	1.55	5.64	2.79	2.44
GnT-V	0.03	0.31	0.05	0.35	0.23	0.08
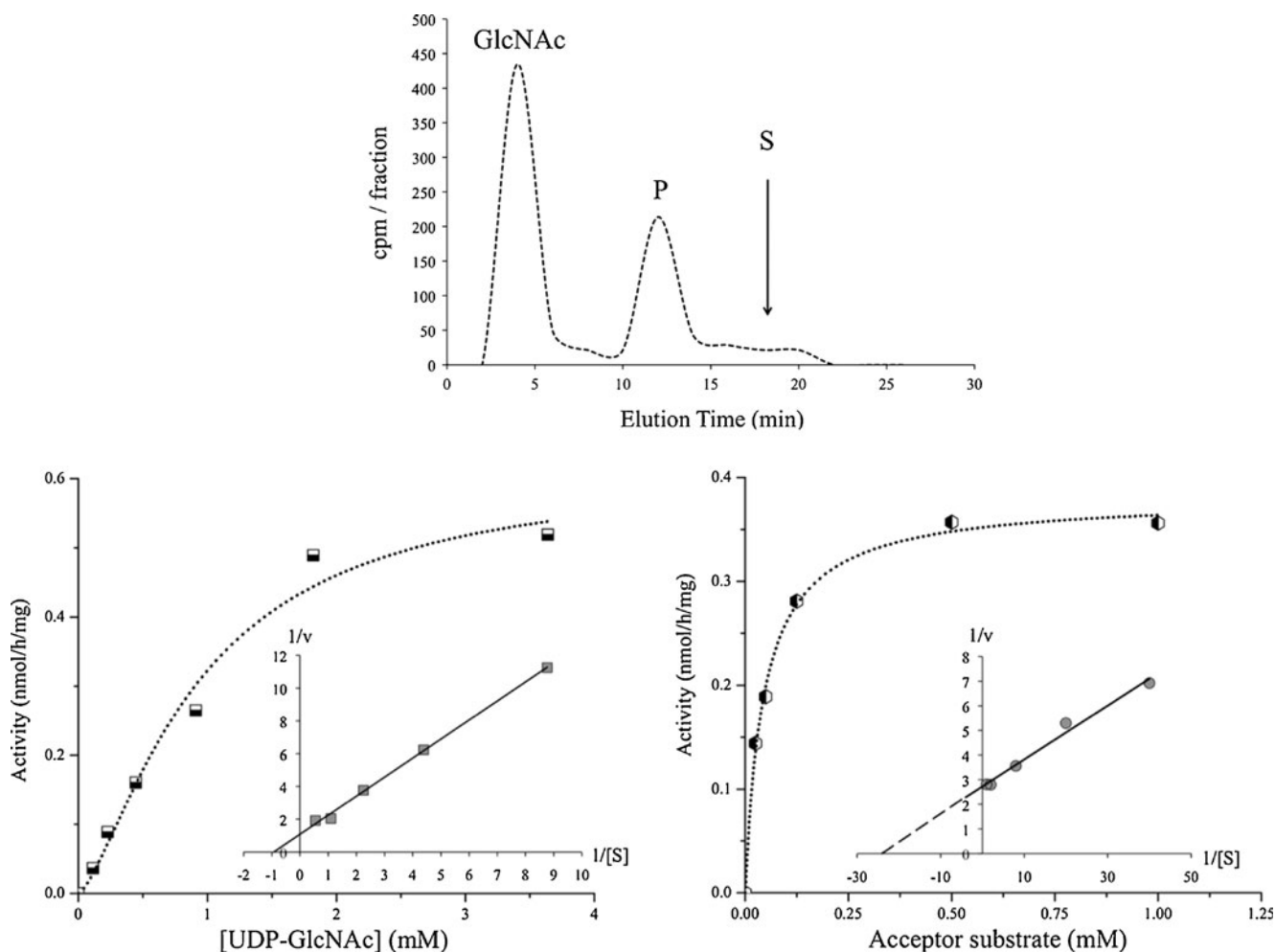
Fig. 1 β1,6-*N*-acetylglucosaminyltransferase V enzymatic activity and kinetics. GnT-V enzymatic activity in homogenates from PC-3 cells (derived from bone metastasis) was measured as described in Materials and Methods. GnT-V enzymatic activity was found to be increased in all prostatic cancer cells compared to normal prostatic cells. **a** Enzyme reaction product was separated by HPLC using 24 % acetonitrile in H_2O as the mobile phase. The radioactivity (cpm) of collected 2 min fractions is shown. The elution of radioactive GlcNAc and specific acceptor substrate (S), GlcNAcβ2Manα6(GlcNAcβ2[4-deoxy-]Manα3)[4-O-methyl-]Manβ-octyl, is indicated by the arrow; this was well separated from the radioactive enzyme product (P). **b** The apparent K_M for UDP-GlcNAc was 0.93 mM and V_{max} was 0.62 nmol/h/mg (with 0.5 mM acceptor substrate). The inset shows the Lineweaver-Burk plot. **c** The apparent K_M for the acceptor substrate was 0.05 mM and the apparent V_{max} was 0.38 nmol/h/mg (with 1 mM UDP-GlcNAc). The inset shows the Lineweaver-Burk plot

different among these cells. The C2GnT activity responsible for the synthesis of branched core 2 O-glycans was not detected in PC-3 and DU145 cells. C2GnT activity was low in normal prostatic RWPE-1 cells (0.3 nmol/h/mg), but was four to ten-fold higher in VCaP and LNCaP cells,

respectively. These cells therefore can synthesize complex branched O-glycan structures.

The activity of core 3 synthase, B3GNT6, that synthesizes core 3, GlcNAcβ1-3GalNAc, was not detected in any of these CaP cells while normal prostatic cells exhibited a

Table 3 Activities of glycosyltransferases that synthesize O-glycan core structures. Assays were carried out by HPLC as described in Material and methods. Abbreviations are as in Tables 1 and 2

[a]Activity in Caco-2 cell homogenates

[b]activity in rat colon mucosal homogenates

Enzyme	Activity (nmol/h/mg)					
	RWPE-1	PC-3	DU145	LNCaP	VCaP	Positive control
ppGalNAcT	11.6	3.0	5.7	11.7	12.5	18.0[a]
C1GalT	3.0	2.3	2.3	2.2	1.0	3.4[a]
C2GnT1	0.3	< 0.1	< 0.1	3.1	1.2	56.1[b]
C3GnT	0.3	< 0.1	< 0.1	< 0.1	< 0.1	1.2[b]
C2GnT2	< 0.1	< 0.1	< 0.1	< 0.1	< 0.1	54.0[b]

low activity (0.3 nmol/h/mg). Therefore, core 3 structure is expected to be produced primarily in RWPE-1 cells. None of the prostatic cells appeared to be capable of synthesizing core 4 from core 3 [7].

Extension and elongation glycosyltransferases

The activities of extension β1,3-*N*-acetylglucosaminyltransferases (β3GlcNAcT, iGnT) and β1,3/4-*N*-acetylgalactosaminyltransferases [32, 46] were low in prostatic cells (Table 4). In contrast, high activities of β3/4GalT were observed in all prostatic cells (Table 4). The β4GalT1 inhibitor **612** (*N*-butyryl-glucosamine-1-thio-2-naphthyl) [40, 47], inhibited GalT activity in prostate cell lines by 65 to 99 % (data not shown). In addition, prostatic cells consistently showed lower GalT activities using O-glycan core 3 substrate, which is a preferred substrate for β3GalT5, as compared to the GlcNAcβ-Bn substrate. These results suggest that the majority of the GalT activity in prostatic cells was due to β4GalT although there was also substantial β3GalT activity.

Terminal modification by glycosyltransferases and sulfotransferases

Fuc-transferase (FUT) activities were compared between normal prostatic and CaP cells (Table 4). The activities of α2FUT1, which synthesizes the Fucα1-2Gal linkage in histo-blood group substances and α2FUT2 activity, which synthesizes the Fucα1-2Gal linkage in secretory blood group substances appeared to be variable and low (Table 4).

The activities of α3FUT and α4FUT3 involved in the synthesis of Lewis blood group structures were generally low, but highest in RWPE-1 cells (Table 4).

All cells exhibited activities of α2,3-sialyltransferases (ST3Gal) (Table 4). A 3-fold higher activity of ST3Gal was seen in VCaP cells, compared to normal prostatic cells, and a dramatic 43- and 19-fold increase was seen in PC-3 and LNCaP cells, respectively. Thus in PC-3 cells, core 1 is expected to be highly α2-3-sialylated and in LNCaP and VCaP cells both cores 1 and 2 are α2-3-sialylated. The high ST3Gal activities are mirrored in the MAAII binding of cell surfaces (Supplementary Figure 1).

The Gal and GlcNAc residues in N- and O-glycans can be sulfated. The sulfotransferase activities (Gal3ST) that synthesize the Gal-3-*O*-sulfate ester of core 1 were reduced in CaP cells to 16 to 50 % of the activity in RWPE-1 cells. The sulfotransferase activities that synthesize the GlcNAc-6-*O*-sulfate ester (GlcNAc6ST) were reduced to 2 and 4 %, in DU145 and PC-3 cells, respectively, relative to the activity in RWPE-1 cells, and were undetectable in LNCaP and VCaP cells (Table 4). Thus, sulfotransferase activities are down regulated in CaP cells, similar to colon cancer or tumorigenic *polyposis coli* cells [7, 37].

Expression profiles of glycosyl- and sulfotransferase genes

To identify specific glycosyltransferase and sulfotransferase isoenzymes responsible for the enzyme activities measured in prostatic cells, and to determine if the activities can be predicted from the mRNA levels of glycosyltransferase and

Table 4 Activities of extension and termination glycosyltransferases and sulfotransferases. Assays were carried out by HPLC as described in Material and methods. Abbreviations are as in Table 1 and 2

Enzyme	Activity (nmol/h/mg)					
	RWPE-1	PC-3	DU145	LNCaP	VCaP	Positive control
Extension β3GlcNAcT	0.1	0.3	0.1	0.3	< 0.1	1.1[b]
Elongation β3GlcNAcT	0.1	0.2	0.1	0.1	0.1	1.4[b]
β3/4GalNAcT (Gnβ3GalNAcα-pnp substrate)	< 0.1	< 0.1	0.2	0.4	0.2	15.3[b]
β3/4GalNAcT (Gnβ-Bn substrate)	< 0.1	< 0.1	< 0.1	0.1	< 0.1	0.2[b]
β3/4GalT (Gnβ-Bn substrate)	25.9	9.3	31.7	39.7	17.2	38.4[a]
β3/4GalT (Gnβ3GalNAcα-pnp substrate)	6.3	3.7	6.9	13.3	8.4	13.1[a]
α2FUT1	0.9	< 0.1	0.2	0.6	0.7	>10[b]
α2FUT2	0.8	0.2	0.8	0.3	0.7	>10[b]
α3FUT	0.6	0.2	0.2	0.2	0.3	3.1[b]
α4FUT3	1.1	< 0.1	< 0.1	< 0.1	0.1	0.8[b]
ST3Gal	0.4	17.3	0.2	7.7	1.3	7.0[c]
	Activity (pmol/h/mg)					
Gal3ST	3.2	0.5	0.7	0.6	1.6	148.7[b]
GlcNAc6ST	5.3	0.2	0.1	< 0.1	< 0.1	0.5[b]

[a] Enzyme activity in Caco-2 cell homogenates

[b] activity in rat colon mucosal homogenates

[c] ST3Gal activity in mouse intestine homogenates from ST3Gal1 overexpressing mice [61]

sulfotransferase genes we carried out real-time PCR (Fig. 2, Supplementary Figure 3). Values for transferase gene expression were related to those of Glyceraldehyde 3-phosphate dehydrogenase (GAPDH), which based on the Ct values varied by <7 % in the different cell lines.

N-glycan transferases

The expression level of the GnT-I gene (*MGAT1*) was highest in RWPE-1 cells and lower in all CaP cells (Fig. 2a), which reflected the GnT-I activities measured in these cells (Table 2). The expression levels of the GnT-II (*MGAT2*) gene were consistent with the enzyme activity profile

(Fig. 2a, Table 2). The GnT-V gene (*MGAT5*) was expressed highest in PC-3 cells, followed by DU145, LNCaP, and then VCaP, and was lowest in RWPE-1 cells (Fig. 2a). Thus, both the enzymatic activities and the mRNA levels of GnT-V were higher in all CaP cells than in RWPE-1 cells.

O-glycan transferases

The *C1GALT1* gene was expressed at similar levels in all five prostatic cells (Fig. 2b), which paralleled the activities (Table 3). The *GCNT1* gene, encoding one of the enzymes that synthesize core 2, was expressed highest in LNCaP cells (Fig. 2c), matching the high C2GnT activity in these
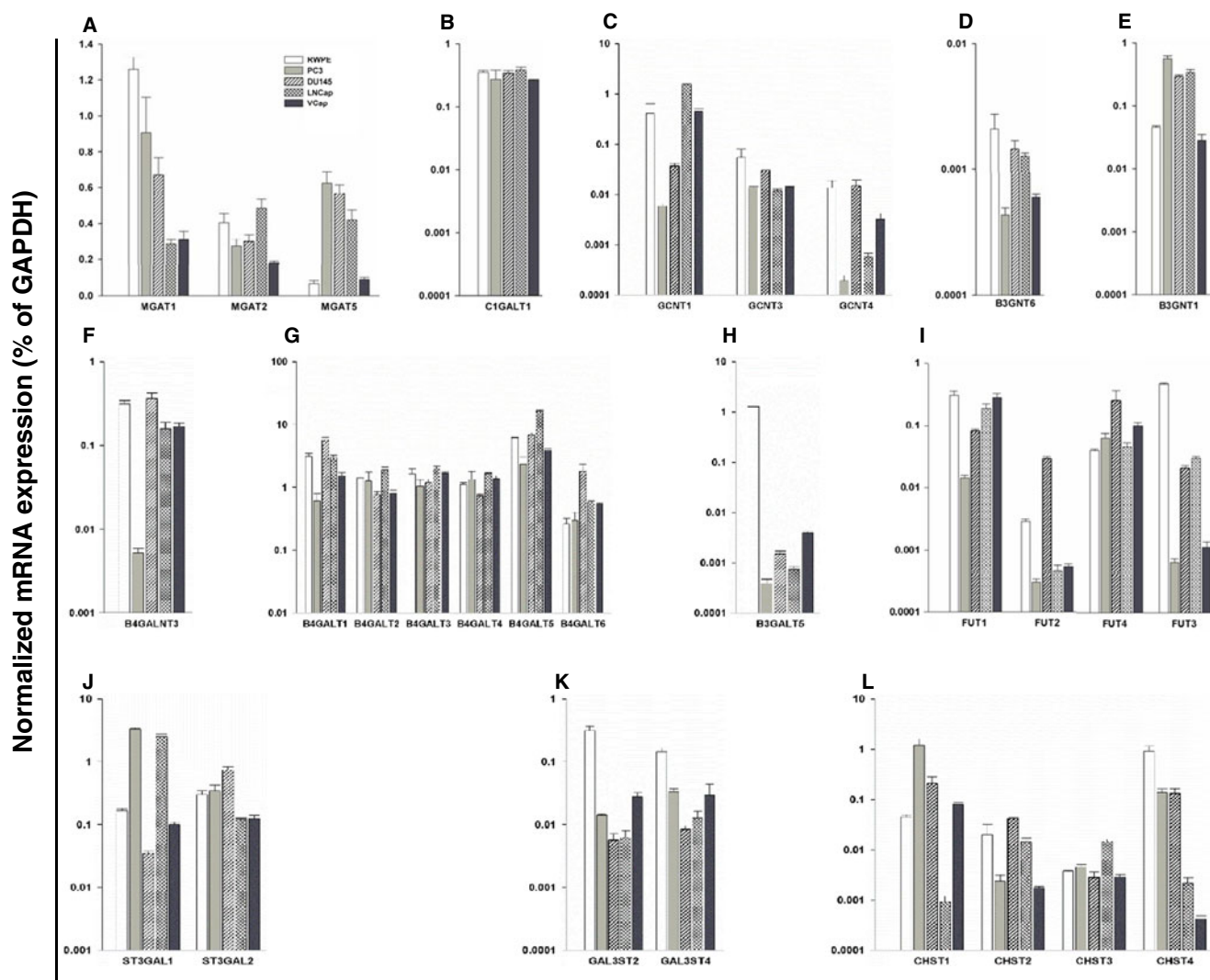
Fig. 2 Quantitative real-time PCR analysis of the mRNAs of glycosyltransferase and sulfotransferase genes in normal and cancerous prostatic cells. The expression levels of glycosyltransferase and sulfotransferase genes are shown, which corresponded to the enzymatic activities measured in this study. The gene expression levels were calculated by the ΔCt method as described in Materials and Methods and expressed as relative amount to that of *GAPDH* (100 %). The enzyme names are listed in Table 1. Results are shown for **a** *MGAT1, 2, 5*; (GnT-I, II, V); **b** *C1GALT1* (C1GalT); **c** *GCNT1,3,4* (C2GnT1-3); **d** *B3GNT6* (Core 3 Synthase); **e** *B3GNT1* (Extension *β3GlcNAcT*); **f** *B4GALNT3* (β4GalNAcT); **g** *B4GALT1-6* (β4GalT); **h** *B3GALT5* (β3GalT5); **i** *FUT1-4*; **j** *ST3GAL1-2*; **k** sulfotransferases *GAL3ST2 & 4*; and **l** sulfotransferases *CHST1-4*. The data were obtained from three independent experiments and expressed as mean ± SEM

cells (Table 3). This was followed by VCaP and RWPE-1 cells. The activity was not detectable in DU145 and PC-3 cells, which corresponded to low expression levels of this gene (Fig. 2c). The expression levels of *GCNT3* involved in core 4 synthesis were also low, explaining why the activity was below the level of detection in our assays.

As could be predicted from the low core 3 synthase activity in RWPE-1 cells and the lack of the activity in CaP cells, the *B3GNT6* gene was expressed at extremely low levels in CaP while RWPE-1 cells were the highest expressors (Fig. 2d). The expression levels of the *B3GNT1* gene (Fig. 2e) correlated well with β3GlcNAcT enzymatic activities (Table 4). Additional members of the β3GlcNAcT gene family (*B3GNT2-5*) were also expressed in prostatic cells (Supplementary Figure 3). The *B4GALNT3* gene involved in the synthesis of GalNAcβ1-4GlcNAc extension was expressed in all prostatic cells (Fig. 2f). This gene plays a role in cell growth and invasion of colon cancer cells [48]. The combined gene expression levels of isoenzymes of the β4GalT family *(B4GALT1-6)*, which synthesize type 2 chains (*N*-acetyllactosamines) were high (Fig. 2g) and paralleled the high activities. In contrast, the transcript level of the *B3GALT5* gene was low in all CaP cells and higher in RWPE-1 cells (Fig. 2h).

Figure 2i shows the gene expression levels of *FUT1* and *FUT2* that matched the patterns of enzymatic activities (Table 4). While *α3FUT4* gene expression was similar in all prostatic cells, the *α3/4FUT3* expression pattern agreed with the highest enzymatic activity detected in normal prostatic cells (Table 4). The expression levels of these enzymes contribute to the synthesis of Le^a and Le^y epitopes and correspond to the high SLe^x expression on normal prostatic cells (Supplementary Figure 2).

Results from the ST3Gal enzyme activities (Table 4) predicted a high expression level of *ST3GAL1*, which synthesizes the sialylα2-3Gal linkages on cores 1 and 2, in PC-3 and LNCaP cells. The mRNA levels of the *ST3GAL1* gene corresponded to the high activities (Fig. 2j). Several other *ST3GAL* genes (2 to 6) were also found to be expressed with minor variations among CaP and normal prostatic cells (Supplementary Figure 3). These sialyltransferases may participate in SLe^x synthesis in all prostatic cells (Supplementary Figure 2).

The α2,6-sialyltransferases that synthesize the sialylα2-6GalNAc linkage on O-glycans to form sialyl-Tn and sialyl-T antigens were variably expressed (Supplementary Figure 3) although the activities were not measured. The expression level of *ST6GALNAC1* responsible for the synthesis of the STn epitope, as well as anti-STn antibody binding was lowest in normal prostatic cells (Supplementary Figure 2). The *ST6GAL1* gene involved in adding sialic acid in α2-6 linkage to terminal Gal residues in N-glycans was variably expressed (Supplementary Figure 3). These enzymes provide the ligands for SNA lectin binding (Supplementary Figure 1).
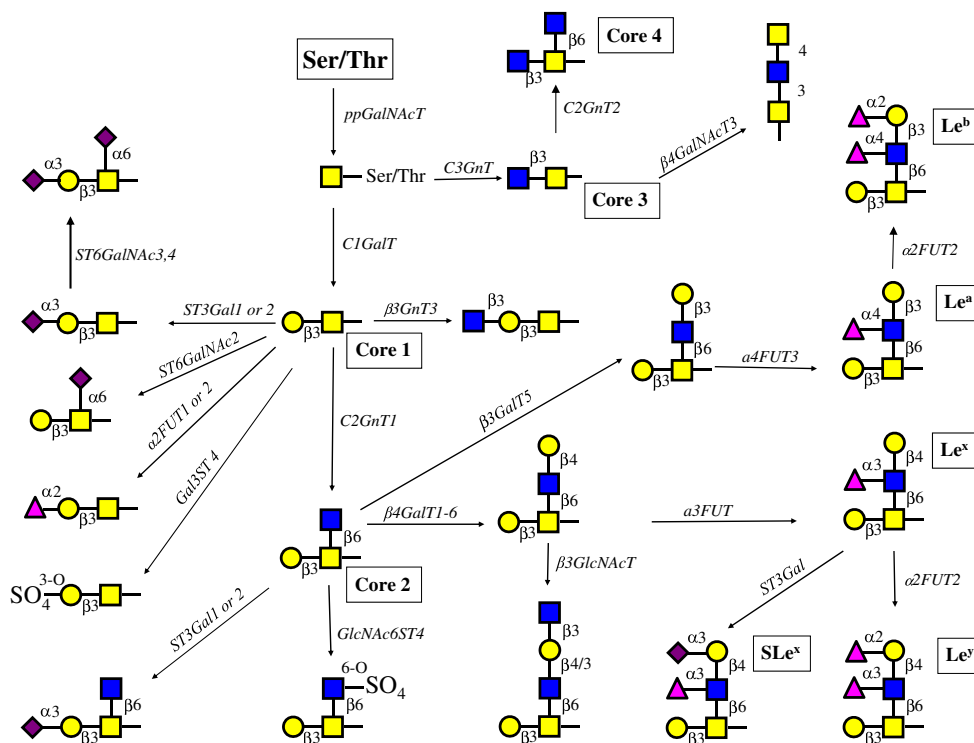
The expression levels of the 3-*O*-sulfotransferase *GAL3ST2&4* genes, responsible for the synthesis of the Gal-3-*O*-sulfate ester linkage in cores 1 and 2, were highest in RWPE-1 cells (Fig. 2k). Among the four *N*-acetylglucosaminyl-6-*O*-sulfotransferase (*CHST1-4*) genes responsible for the synthesis of the sulfate-6-*O*-GlcNAc linkage, *CHST4* was expressed highest in RWPE-1 cells. These expression profiles of these genes matched the pattern of enzyme activities measured in prostatic cells (Table 4).

Discussion

Altered glycosylation of glycoproteins and mucins has been reported in prostate cancer but the enzymes responsible for alterations have not been identified. In this study, we compared the enzymatic activities and the gene expression profiles of glycosyl- and sulfotransferases involved in N- and O-glycan synthesis in normal and cancerous prostatic cells. We showed that the activities corresponded well to the mRNA expression levels for most transferases. We found that each prostatic cell line exhibits characteristic biosynthesis pathways. In addition, we have identified a number of transferases that may in this combination potentially serve as markers for CaP. This includes high GnT-V activity and gene expression, undetectable core 3 synthase activity and low enzymatic activity of sulfotransferases in CaP cells, as well as an upregulation of C2GnT1 and ST3Gal in a selected number of CaP cells. However, this remains to be confirmed with prostate tissue from patients with prostate cancer. The pathways in these cell lines partly correspond to the lectin and antibody binding patterns of prostatic cell surfaces. A lack of correlation may be explained by the selected exposure of glycans on the cell surface, or possibly, by differences in glycosylation of secreted glycoproteins or mucins and cell surface molecules that include glycolipids.

All prostatic cells are capable of producing biantennary chains and show similar binding of ConA. Higher enzymatic activities and mRNA levels of GnT-V in CaP cells compared to those in normal prostatic cells suggest that CaP cells have a greater potential to generate the GlcNAcβ6 (GlcNAcβ2)Manα6 branch of N-glycans, which has been shown to contribute to cancer cell invasion and metastasis [49, 50]. This finding corresponds to the higher branching pattern found in PSA glycans from prostate cancer patients [4, 16, 21, 22].

While N-glycans share a common core (Man)$_3$(GlcNAc)$_2$ structure, O-glycans have a number of different core structures and are more heterogeneous. Figure 3 depicts the biosynthesis pathways of a great variety of the major O-glycan structures in prostatic cells, predicted based on

Fig. 3 Proposed major O-glycosylation pathways in normal and prostatic cancer cells. Based on the major enzymatic activities and the relative gene expression levels of glycosyltransferases and sulfotransferases, the proposed major O-glycosylation pathways in prostate cells were constructed (nomenclature is shown in Table 1). Many other minor pathways are possible. Real-time PCR showed the expression of isoenzymes. RWPE-1 cells can synthesize core 1, 2 and 3 structures. Which can be extended and modified by the addition of sugar residues or sulfate esters and Lewis antigens Le^a, Le^b, Le^x, Le^y and SLe^x Only RPWE-1 cells showed the activity that synthesizes core 3 but not the activity that synthesizes core 4 although the enzyme (C2GnT2) was expressed at low levels. Both PC-3 (from bone metastasis) and DU145 (from brain metastasis) lack C2GnT1 activity and *GCNT1* expression, which results in shorter O-glycan chains with mainly sialylated core 1 structures. DU145 cells exhibit a low ST3Gal enzymatic activity, whereas PC-3 cells exhibit a high enzymatic activity and a high gene expression of *ST3GAL1*. The prostatic cancer cells appear to be unable to synthesize core structures 3 and 4 although significant amounts of mRNA for the respective enzyme genes were found. LNCaP cells (from lymph node metastasis) contain high enzymatic C2GnT activity and gene expression level of GCNT1 and can form complex core 1 and core 2, but not core 3 O-glycans. ST3Gal enzymatic activity and gene expression are high in LNCaP cells. These glycans can be modified to form a variety of complex structures with extension enzymes as well as α1,2-and α1,3FUT4 and Gal3ST, but not α3/4FUT3. Although the activities of α2,6-sialyltransferases were not directly measured, the gene expression levels suggest that sialylα2-6GalNAc- and sialylated core 1 structures can be synthesized. Sulfotransferases using GlcNAc-R substrate are expressed although the activities were not detected. However, sulfotransferases using core 1 substrate were active. VCaP cells differ from LNCaP in that the expression of sulfotransferases Gal3ST1-4 and the enzymatic activity of Gal3ST is also higher in VCaP cells. α3/4FUT3 and α3FUT4 are active in VCaP cells and can synthesize various Lewis antigens

enzymatic activities and mRNA levels of glycogenes obtained from these cells. Many more extended and branched structures may be synthesized in normal and cancerous prostatic cells.

Enzymatic activity assays are often limited by their low sensitivity, and their difficulty to discriminate among different isoenzymes due to the fact that enzymes share substrates. To solve this problem, sensitive real-time PCR analysis of the expression profile of specific isoenzymes was carried out. In our study, most of the enzymatic activities corresponded well with their gene expression profiles, although clearly the complex control of biosynthesis and mechanisms that display carbohydrates on cell surfaces are still not well understood.

Normal prostatic RWPE-1 cells are capable of forming many complex O-glycans with core 1, 2 and 3 structures and

a variety of terminal epitopes that may resemble those found in human colonic mucins [31]. PC-3 and DU145 cells are expected to have only core 1-based O-glycans and appear to be restricted in their ability to form branched chains since the activity of C2GnT1 was undetectable in spite of mRNA expression. In contrast, LNCaP cells are capable of synthesizing a multitude of O-glycan structures with core 1 and 2 structures [51].

With the exception of LNCaP cells, all prostatic cells show a higher Tn-antigen expression compared to normal prostatic cells, which is typically found in cancer cells [31]. The mechanism of this Tn antigen exposure is not clear. The cancer-associated sialyl-Tn antigen, which may play a role in the tumorigenic properties of cancer cells [52] was also exposed in all CaP cells at higher levels compared to normal

prostatic cells. This correlated with higher expression levels of *ST6GALNAC1*. The expression of *ST6GAL* acting on N-glycans has been shown to be increased in colon cancer [53], and in VCaP cells correlated with high SNA binding.

LNCaP cells have a high potential to form the branched core 2 structure and to α2-3-sialylated core 1 and 2 structures. Since C2GnT1 and ST3Gal compete *in vivo* for the common core 1 substrate [54] the high ST3Gal activity can limit the conversion of core 1 to core 2 by C2GnT1.

Although core 2 structures have been shown to be important scaffolds for SLe[x], related to the invasive and metastatic potential of a number of cancer cell types [23, 24, 55], SLe[x] levels expressed in CaP cells did not correlate with C2GnT1 expression, or with the activities or expression levels of the enzymes that directly synthesize SLe[x]. Thus, the regulation of glycan synthesis remains to be further investigated.

Core 3 synthase activity was clearly detected in RWPE-1 cells but not in any of the cancer cells. A decrease in core 3 synthesis has been shown in colon cancer tissues [36] and appears to be a characteristic of colon cancer cell lines [37, 38]. This suggests that the absence of core 3 may be a marker for cancer cells, including CaP cells. PC-3 cells from bone metastasis and LNCaP cells transfected with the *C3GNT6* gene showed reduced ability of migration and invasion through extracellular matrix components, and suppressed tumor formation and metastasis in mice [56, 57]. Most of these findings have been reproduced in a *C3GNT6* gene knockout mouse model [58] suggesting that core 3-associated glycans may have a protective function in the normal prostatic tissue.

Because of the enzymatic activity and gene expression levels of *ST3GAL1*, PC-3 cells are expected to synthesize O-glycans with sialylated core 1 structures, similar to those found on breast cancer cells T47D [59]. In breast cancer, the high ST3Gal enzymatic activity or *ST3GAL1* expression level may be used as a biomarker [33] and appears to protect cancer cells *in vivo* [60–62]. However, high ST3Gal enzymatic activity in CaP is cell-specific and is not a general feature of all metastatic αCaP cells.

The levels of many glycosyltransferase activities and mRNA expression were shown to be cell type-specific in prostatic cells. It remains to be shown if this is due to variations in cancer cell phenotypes or due to the metastatic sites. The results obtained from cultured cells as models for prostate tumors and metastasis need to be validated in primary tumor tissues and cancer tissues from different metastatic sites.

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