

Suppression of β 1,3galactosyltransferase β 3Gal-T5 in cancer cells reduces sialyl-Lewis a and enhances poly N-acetyllactosamines and sialyl-Lewis x on O-glycans

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We investigated the role of β 3Gal-T5, a member of the β 1,3galactosyltransferase (β 1,3Gal-T) family, in cancer-associated glycosylation, focusing on the expression of sialyl-Lewis a (sLe^a, the epitope of CA19.9 antigen), poly N-acetyllactosamines, and sialyl-Lewis x (sLe^x) antigen. A clone permanently expressing an antisense fragment of β 3Gal-T5 was obtained from the human pancreas adenocarcinoma cell line BxPC3 and characterized. Both β 1,3Gal-T activity and sLe^a expression are dramatically impaired in the clone. Analysis of the oligosaccharides synthesized in cells metabolically labelled with tritiated galactose shows that a relevant amount of radioactivity is associated to large O-glycans. Endo- β -galactosidase mostly releases NeuAc α 2-3Gal β 1-3[Fuc α 1-4]GlcNAc β 1-3Gal and NeuAc α 2-3Gal β 1-3GlcNAc β 1-3Gal from such O-glycans of BxPC3 membranes, but GlcNAc β 1-3Gal and type 2 chain oligosaccharides, including NeuAc α 2-3Gal β 1-4[Fuc α 1-3]GlcNAc β 1-3Gal, from those of the antisense clone.

Furthermore, BxPC3 cells secrete sLe^a in the culture media but not sLe^x, while antisense clone secretes mostly sLe^x, and accumulation of both antigens is prevented by benzyl- α -GalNAc. These data indicate that β 3Gal-T5 suppression turns synthesis of type 1 chain O-glycans to poly N-acetyllactosamine elongation and termination by sLe^x. In other cell lines and clones, β 3Gal-T5 transcript, β 1,3Gal-T activity, and sLe^a antigen are also correlated, but quantitatively the relative expression ratios are very different from cell type to cell type. We suggest that β 3Gal-T5 plays a relevant role in gastrointestinal and pancreatic tissues counteracting the glycosylation pattern associated to malignancy, and is necessary for the synthesis and secretion of CA19.9 antigen, whose expression still depends on multiple interacting factors.

Keywords: galactosyltransferase; gastrointestinal cancer; Lewis antigen; O-glycan; poly N-acetyllactosamine.

Aberrant glycosylation of glycoproteins and glycolipids is one of many molecular changes that accompany malignant transformation [1]. Perhaps the best known glycosylation change inducing malignancy is enhanced β 1,6GlcNAc branching of N-glycans, leading to poly N-acetyllactosamine sequences frequently terminated by the sialyl-Lewis x (sLe^x) antigenic determinant [2]. GnT-V activity is mostly responsible for this as shown by several pieces of evidence obtained *in vitro* [3,4], and more recently *in vivo* [5].

Moreover, several studies indicated that O-glycan biosynthesis is also abnormal in cancer cells [6]. It has been shown that sLe^x and poly N-acetyllactosamines are associated with increased malignancy of lung and colorectal cancers [7,8], and occur in core 2 and extended core 1 O-glycans in various cells [9,10]. On the other hand, the role of type 1 chain oligosaccharides in cancer-associated glycosylation is unclear. Although type 1 chain structures occur on all glycoconjugate classes, and CA19.9 antigen – that is the sLe^a epitope carried by a mucin backbone [11] – has been used as a tumour marker in clinical practice for several years, little is known about their biosynthesis and differential expression in cancer. β 1,3Gal-T activity was found to be reduced in colon cancer with respect to the normal mucosa [12], and in the CACO-2 cell model of intestinal differentiation β 1,3Gal-T activity [13] and type 1 chain structures [14] were reported to increase with the differentiation process. β 3Gal-T5 is the member of the β 3Gal-T gene family that was proposed to be responsible for β 1,3Gal-T activity and type 1 chain synthesis in epithelial cells of the digestive tract [15]. In a previous paper [16] we reported that β 3Gal-T5 efficiently adds β 1,3Gal residues to GlcNAc β 1-3Gal β 1-4GlcNAc β 1-R branched chains of N-glycans, leading to Le^a and sLe^a synthesis, and preventing poly N-acetyllactosamine extension and sLe^x expression. We also found that the β 3Gal-T5 transcript is downregulated in colon

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Abbreviations: sLe^x, sialyl-Lewis x (NeuAc α 2-3Gal β 1-4[Fuc α 1-3]GlcNAc); sLe^a, sialyl-Lewis a (NeuAc α 2-3Gal β 1-3[Fuc α 1-4]GlcNAc); Le^a, Lewis a (Gal β 1-3[Fuc α 1-4]GlcNAc); Le^b, Lewis b (Fuc α 1-2Gal β 1-3[Fuc α 1-4]GlcNAc); Gal-T, galactosyltransferase; GnT, N-acetylglucosaminyl-transferase; Fuc-TIII, α 1,3/1,4fucosyltransferase; CEA, carcinoembryonic antigen; SNA, *Sambucus nigra* agglutinin; MKN-45-FT, MKN-45 cells permanently expressing Fuc-TIII; HCT-15-T5, HCT-15 cells permanently expressing β 3Gal-T5; T5AS, BxPC3 cells permanently expressing an antisense fragment of β 3Gal-T5.

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adenocarcinomas and is responsible for the differential glycosylation of carcinoembryonic antigen (CEA) in cancer. β 3GalT-5 has a broad acceptor specificity *in vitro* [16,17], but it has not yet been demonstrated *in vivo* if it works on O-glycans that are assumed to be largely expressed in epithelial cells and to be the more relevant carriers of sLe^a epitope in CA19.9 mucin. As no other member of the β 3Gal-T gene family known at present is expressed in epithelial cells and able to synthesize type 1 chain oligosaccharides, the very low levels of β 3Gal-T5 transcript detectable in colon cancer specimens pose the question of whether relevant amounts of type 1 chain O-glycans are formed in cancer cells.

To address these issues, we tried to study the effect of β 3Gal-T5 suppression in the human pancreatic adenocarcinoma cell line BxPC3 that expresses low levels of β 3Gal-T5 transcript but well detectable amounts of β 1,3Gal-T activity and sLe^a, that is presumably carried by O-glycans and even secreted into the culture medium. To this purpose we transfected the cells with a β 3Gal-T5 cDNA fragment placed in the antisense orientation under the control of a strong promoter, and isolated a recombinant clone that stably expresses high levels of the antisense transcript. We then measured the β 1,3Gal-T activity present in the antisense clone, as well as the Lewis antigens expressed on the cell surface or secreted in the culture medium. We also studied the radioactive sugar chains synthesized in parental BxPC3 cells and in the recombinant antisense clone upon metabolic labelling with tritiated Gal, with emphasis on O-glycans and poly N-acetylglucosamines. We also compared the amount of β 3Gal-T5 transcript and β 1,3Gal-T activity with the levels of sLe^a expressed in other cell lines and clones.

Experimental procedures

Cell cultures and treatments

COLO-205, HCT-15, CACO-2, HT-29, SW-1116 (from human colon adenocarcinomas), and MKN-45 (from human gastric cancer) cells were cultured as described previously [16,18]. Human pancreatic adenocarcinoma cells BxPC3 (ATCC CRL-1687) and Panc-1 (ATCC CRL-1469) were cultured in Dulbecco's modified Eagle's medium containing 10% foetal bovine serum, 100 U·mL⁻¹ penicillin, 1.0 mg·mL⁻¹ streptomycin and 2 mM L-Glu. For treating BxPC3 cells and clones with drugs affecting glycosylation, 1×10^5 cells were plated in 12-well plates, incubated for 30 h with regular medium that was replaced with medium containing 1.0 μ g·mL⁻¹ swainsonine (Sigma) or 2 mM benzyl- α -GalNAc (Sigma). After growing for 60 h in the presence of drugs, media were collected again. Media obtained before and after treatment were centrifuged at 3000 g for 10 min and the clean supernatants were used for dot-blots.

Cultured cells were harvested, centrifuged, aliquoted, and freshly processed for flow cytometry as reported [16], or homogenated for RNA extraction or enzyme assay, according to the procedures described [18].

Preparation of pSV2Neo, pcDNA1/Fuc-TIII, and pCDM8/ β 3Gal-T5 was as reported [16]. Antisense plasmid pEFneo/AS β 3Gal-T5 was constructed by cloning a fragment of β 3Gal-T5 cDNA in the antisense orientation in the

vector pEFneo, a generous gift of N. Hiraiwa (Aiki Cancer Center, Nagoya, Japan). Vector relevant features include the strong human elongation factor-1 α promoter [19], the linker sequence containing a 358-bp stuffer between two nonpalindromic *Bst*XI sites, and the simian virus 40 (SV40) polyadenylation signals. cDNA was obtained from COLO-205 total RNA and amplified by PCR with a commercially available 'high fidelity' *Taq* polymerase (LA *Taq*, Takara) as reported [16], using specific primers as follows. Upper strand primer: 5'-GCGCTCTAGACCCAGCGTCTCCA GCTTGCATGGCC-3', having a 4-base filler, an *Xba*I restriction site (underlined), and a 25-base sequence corresponding to nucleotides -192 to -160 from the start ATG codon in the β 3Gal-T5 gene. Lower strand primer: 5'-GCGCAAGCTTGATAATGTCCCCGTGTCGCTG GCTCTC-3', having a 4-base filler, a *Hind*III site (underlined), and a 27-base sequence corresponding to nucleotide 334-360 in the coding region of the gene. PCR reactions were incubated as follows: 94 °C for 3.5 min followed by 25 cycles of 1.5 min at 94 °C (melting) and 3.5 min at 72 °C (annealing plus extension), and a final extension step at 72 °C for 8 min. The amplified DNA was digested with *Xba*I and *Hind*III, for other purposes, or blunt-ended, ligated to *Bst*XI adaptors, and cloned into the corresponding sites of pEFneo, using the procedure described [20]. Direct DNA sequencing of the construct obtained, performed by the dideoxynucleotide chain-termination method using an automated procedure, indicated that the sequence of the construct obtained, pEFneo/AS β 3Gal-T5, was identical to that expected.

Construction of cell clones

HCT-15 expressing β 3Gal-T5, MKN-45 expressing Fuc-TIII, and BxPC3 expressing antisense β 3Gal-T5 construct, were obtained by the calcium phosphate transfection method [21], using a modification of the procedure [16]. The DNA mixture contained 1.5 μ g *Eco*RI-linearized pSV2Neo and 20 μ g *Sca*I-linearized pcDNA1/Fuc-TIII, or 1.5 μ g *Eco*RI-linearized pSV2Neo and 20 μ g *Sca*I-linearized pCDM8/ β 3Gal-T5, or 1.5 μ g *Eco*RI-linearized pSV2Neo and 20 μ g *Tth*1111-linearized pEFneo/AS β 3Gal-T5, respectively. Upon selection with 0.4 mg·mL⁻¹ active G418, colonies were collected using cloning cylinders and grown in 48-well plates. G418-resistant HCT-15 and MKN-45 colonies were stained with anti-sLe^a Ig, analysed by fluorescence microscopy on tissue culture slides, and subcloned [16]. G418-resistant BxPC3 colonies were screened by competitive RT/PCR. Total RNA was extracted from colonies and reverse transcribed, and cDNA submitted to PCR amplification with human β -actin primers, for normalization [16,20], or with primers specific to the antisense construct. Single colonies expressing a constant level of sLe^a, named HCT-15-T5 and MKN-45-T5, or of antisense β 3Gal-T5 construct, named T5AS, were selected and used for further characterization and experiments.

Metabolic labelling and carbohydrate analysis

BxPC3 cells and T5AS clone (4.0×10^6 cells) were plated in 25-mm² flasks containing 0.2 mCi [³H]Gal (Amersham

Pharmacia Biotech) in 4.0 mL culture medium and incubated for 40 h under regular conditions. Labelled cells were harvested, resuspended in phosphate-buffered saline at a density of 4×10^7 cells·mL⁻¹, and processed according to published procedures [9,16,22], with some modifications. Total lysates were obtained by boiling 10 min in phosphate-buffered saline containing 0.5% SDS and 1.0% 2-mercaptoethanol, and spinning at 12 000 r.p.m. for 10 min. The clean supernatants were made 1% for Nonidet P40 and 50 mM for sodium phosphate buffer pH 7.5, and treated with *N*-glycanase (New England Biolabs P0704), 50 000 NEB U·mg⁻¹ cell lysate protein, for 20 h at 37 °C. Lysate protein was 0.8 mg·mL⁻¹. Reaction mixtures were passed through a Sephadex G-50 column (0.7 × 50 cm) equilibrated and eluted with water at a flow rate of 0.11 mL·min⁻¹, 3 min per fraction. Material collected with the inclusion volume of the column was lyophilized and passed through a Bio-Gel P-4 column (0.7 × 50 cm) equilibrated and eluted with water at a flow rate of 0.10 mL·min⁻¹, 5 min per fraction, and the high molecular mass substances, collected with the exclusion volume, lyophilized and referred to as the N-glycans. Material collected with the exclusion volume of the Sephadex G-50 column was lyophilized and submitted to β-elimination, incubating 40 h at 45 °C in 50 mM NaOH containing 0.5 M sodium borohydride. Unreacted NaBH₄ was inactivated with an excess of glacial acetic acid, and the solution neutralized with NaOH and buffered with 0.1 M ammonium bicarbonate. Total reactions were passed through a Bio-Gel P-4 column (1.0 × 50 cm), equilibrated and eluted with water at a flow rate of 0.24 mL·min⁻¹, 5 min per fraction. Radioactive material collected with the inclusion volume of this column was referred to as the small O-glycans, while the material collected in the flow-through of the column was lyophilized and passed through a Sephadex G-50 column (0.7 × 50 cm) equilibrated and eluted as above. Radioactivity collected with the inclusion volume, referred to as the large O-glycans, was lyophilized, resuspended with water at a concentration of 10 000 cpm·μL⁻¹, and submitted to endo-β-galactosidase digestion using the enzyme from *Bacteriodes fragilis* (Sigma E6773), 0.4 mU·μL⁻¹, for 20 h at 37 °C. The reaction mixture was diluted with water and applied to a QAE-Sephadex column to separate neutral and charged sugars, according to a reported procedure [22]. Material collected in the flow-through was referred to as the neutral fraction, while that eluted with NaCl, referred to as the acid fraction, was collected, desalted on a Bio-Gel P-2 column, and treated with α2,3 sialidase (New England Biolabs P0728) according to the manufacturer's recommendations. Neutral and de-sialylated fractions were analysed by a Bio-Gel P-4 column (0.7 × 100 cm), eluted with water at a flow rate of 0.06 mL·min⁻¹, 6.5 min per fraction. The obtained peaks were collected, lyophilized, treated with glycohydrolases, and submitted to Bio-Gel P-2 chromatography for characterization [16]. β1,3-galactosidase (New England Biolabs P0726), α1,3/4-fucosidase (Sigma F-3023), β-N-acetylhexosaminidase (New England Biolabs P0721), and β1,4-galactosidase (Sigma G-0413) digestions were performed on radioactive oligosaccharides, 400–1000 c.p.m.·μL⁻¹, according to the manufacturer's recommendations.

Analytical procedures

For transcript quantification, competitive RT/PCR was performed essentially as reported previously [16,20]. First-strand cDNA was prepared for samples and controls in the presence or absence of the reverse transcriptase, respectively, and reactions incubated under the conditions reported [20]. cDNA was amplified (25 μL reaction volume) in the presence of 10 fg (glycosyltransferases) or 100 fg (antisense construct) of the correct competitor for 35 cycles, or in the presence of 10 pg competitor (β-actin) for 25 cycles, under the conditions reported [16]. No amplification was detected when the control reactions were used as template. Human β-actin and β3Gal-T5 competitors and oligonucleotide primers were those already described [16]. For β3Gal-T5 antisense construct, the competitor was prepared digesting pEFneo/ASβ3Gal-T5 plasmid with *Pma*CI and *Bsp*1407I, blunting the ends, removing the 235-bp fragment, and self re-ligating the truncated plasmid. The following primers were used: upper strand primer, 5'-CCTTCACCATCCTCTCTTCCCCAC-3', corresponding to nucleotides 262–237 of the reverse strand of the β3Gal-T5 coding sequence; lower strand primer, 5'-CAGGTTGAGGGGAGGTGTGGGAG-3', corresponding to nucleotides 31–8 of the reverse strand of the SV40 polyadenylation signal sequence of pEFneo vector.

β1,3Gal-T activity was determined in the reported reaction mixture [16], using 0.6 M GlcNAc as acceptor, in the presence of cell homogenates at protein concentrations of 0.5–4.0 mg·mL⁻¹. Incubations were performed at 37 °C for 60 min. At the end of incubation, reaction products were assayed by Dowex chromatography and characterized according to previously reported protocols [18]. In all cases the reaction product was found to be a disaccharide sensitive to β1,3galactosidase, as expected. In fact, GlcNAc is not used as acceptor by β1,4galactosyltransferases under the reported assay conditions [18,20]. *K_m* calculations were performed as reported [18].

For dot-blot, 50-μL aliquots of the culture media were applied to the blotting membrane by vacuum aspiration. Serial dilution of samples were performed in preliminary experiments to set the amounts needed for detection. Membranes were washed, blocked, stained with primary and peroxidase-labelled secondary antibodies, and visualized by enhanced chemoluminescence as reported for Western blotting [23]. Monoclonal anti-CEA, anti-sLe^a (from hybridoma 1116-NS-19-9), and anti-sLe^x (from hybridoma CSLEX1) Igs were as reported [16,20]. *Sambucus nigra* agglutinin (SNA) staining was preformed as reported [23].

Results

Construction and characterization of a BxPC3 clone expressing an antisense β3Gal-T5 fragment

To study the role of β3Gal-T5, we permanently suppressed the expression in a cell line by an antisense approach. We chose BxPC3 cells for transfection as they express low levels of the transcript (0.2 fg·pg⁻¹ β-actin) but still well detectable amounts of β1,3Gal-T activity (16.0 nmol transferred Gal·mg protein⁻¹·h⁻¹) and sLe^a, but not Le^a,

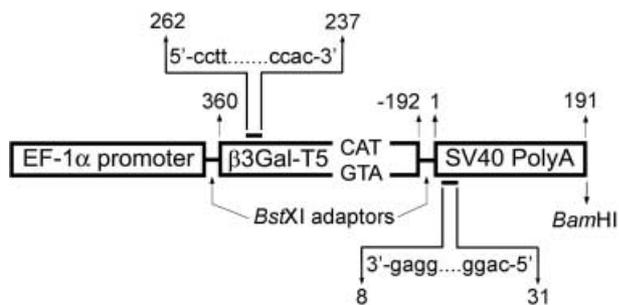


Fig. 1. Schematic representation of β 3Gal-T5 antisense construct. The human elongation factor-1 α promoter and the SV40 polyadenylation signal cassettes present in the pEFneo vector are shown together with the 553-bp fragment amplified from β 3Gal-T5 cDNA, that was cloned in the antisense orientation using adaptors for the *Bst*XI sites available in the vector. Numbers in the β 3Gal-T5 cassette refer to the cDNA sequence starting from the ATG translation initiation codon (indicated). Numbers in the SV40 polyadenylation signal cassette refer to the SV40 sequence in pEFneo vector. The upper strand primer, annealing to the β 3Gal-T5 sequence, and the lower strand primer, annealing to the SV40 sequence, are also indicated. They were used for RT/PCR amplification of the antisense construct expressed in transfected cells, and provided a 515-bp amplification fragment detected as β 3Gal-T5 antisense construct target in Fig. 2A.

Le^b or sLe^x. Moreover, sLe^a expression in these cells is affected by benzyl- α -GalNAc but not by swainsonine. These facts were expected to make the experiment technically feasible, and the high β 1,3Gal-T activity/ β 3Gal-T5 transcript ratio to provide clear-cut results. Cells were transfected with a linearized plasmid containing a 553-bp fragment of β 3Gal-T5 cDNA, that includes the initial 360 bp of the coding sequence and 192 bp of the 5' untranslated region of the gene, placed in the antisense orientation under the control of the elongation factor-1 α promoter, and followed by SV40 polyadenylation signals (Fig. 1). This scheme basically follows the one used successfully by Hiraiwa *et al.* for suppressing fucosyltransferase FucT-VII in lymphoid cells [24]. A cassette for G418 resistance was cotransfected for selection of recombinant clones. To quantify the levels of the antisense construct expressed in G418-resistant clones, we used competitive RT/PCR, taking advantage of primers specific to such a construct (Fig. 1). A clone expressing constant high levels of the antisense construct (60 fg·pg⁻¹ β -actin) was isolated and characterized. The clone, named T5AS, retains a low expression of β 3Gal-T5 transcript as in the parental cell line (Fig. 2A). This indicates that antisense-mediated mechanism of gene suppression does not involve transcript synthesis in this case, as already reported [24]. On the other hand, β 1,3Gal-T activity is dramatically reduced and became faintly detectable in the clone (Fig. 2B). Moreover, the T5AS clone expresses much less sLe^a on the cell surface than BxPC3 cells (Fig. 2C). These data indicate that β 3Gal-T5 is the gene responsible for β 1,3Gal-T activity and sLe^a antigen synthesis in these cells. In addition, T5AS clone became weakly positive to sLe^x, that instead is undetectable in BxPC3 cells, and remains negative to Le^a, faintly positive to Le^x, and moderately positive to SNA, as are the original BxPC3 cells (Fig. 2C). A relevant amount

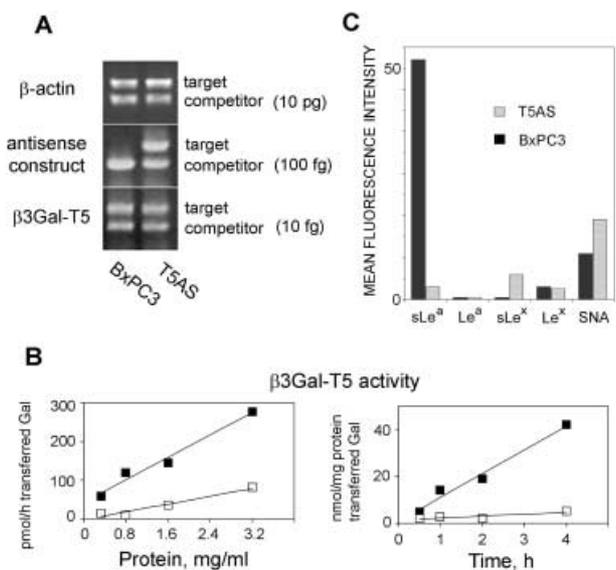


Fig. 2. Characterization of T5AS clone. A cell clone expressing a β 3Gal-T5 antisense construct (T5AS) was obtained from the human pancreatic adenocarcinoma cell line BxPC3. (A) Total RNA was extracted from BxPC3 cells and T5AS clone, reverse transcribed, and the first-strand cDNA obtained was diluted 1 : 20, v/v, with water. PCR amplifications were performed using 0.5- μ L aliquots of the dilutions and primers specific for human β -actin and antisense construct, respectively, or 5.0 μ L of cDNA dilutions and β 3Gal-T5 specific primers, in the presence of the indicated amounts of the respective competitor DNAs. Amplifications were for 25 (β -actin) or 35 cycles (antisense construct and β 3Gal-T5). An aliquot comprising one-fifth of each PCR reaction was analysed by electrophoresis through a 1% agarose gel and visualized by staining with ethidium bromide. (B) β 1,3Gal-T activity in BxPC3 cells (■) or in T5AS clone (□) was determined with GlcNAc as acceptor using different amounts of cell homogenates for a fixed incubation time (1 h), or using a fixed protein concentration (1.6 mg·mL⁻¹) for different incubation times. (C) Cells were stained with monoclonal anti-sLe^a, anti-Le^a (both IgG), anti-sLe^x and anti-Le^x (both IgM) followed by fluorescein-conjugate anti-mouse IgG or IgM, respectively, or with fluorescein-conjugate SNA (*Sambucus nigra* agglutinin) alone, and analysed by flow cytometry.

of sLe^x is also found in the culture medium, where sLe^a, that is secreted by BxPC3 cells, is almost undetectable.

Characterization of sugar chains synthesized in the antisense clone

To understand better the consequences of β 3Gal-T5 suppression on cell glycosylation, we characterized the main oligosaccharide chains synthesized by such cells. To this aim, the antisense clone and parental BxPC3 were metabolically radiolabelled with tritiated Gal, and the distribution of radioactivity studied as outlined in Fig. 3. Table 1 shows that Gal is incorporated into high molecular mass substances attached to the cell membranes, without relevant differences between parental cells and antisense clone. The amount of radioactivity released by *N*-glycanase is moderate in both cases, while the bulk of incorporated

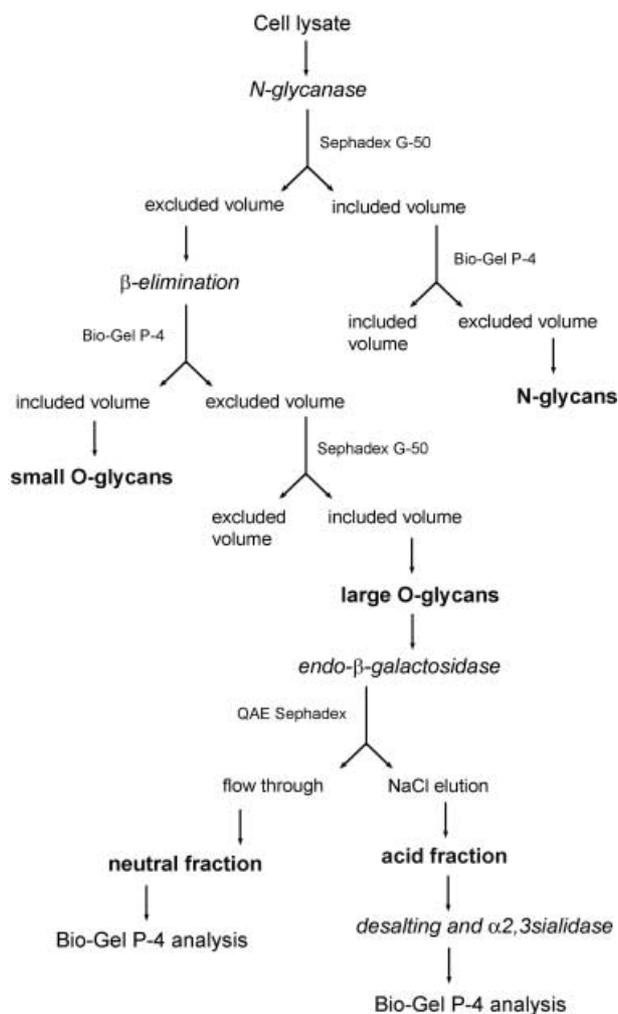


Fig. 3. Scheme of sugar chain purification. The scheme outlines the procedure followed for preparing different sugar fractions from metabolically radiolabelled cells. The main fractions obtained are in boldface, and the more relevant treatments are italicized. The corresponding qualitative results are presented in Fig. 4, and the quantitative data in Table 1.

Table 1. Radioactivity distribution in BxPC3 cells and T5AS clone metabolically radiolabelled with [^3H]Gal. Values are expressed as c.p.m. $\times 10^6 \cdot \text{mg}^{-1}$ cell protein.

	BxPC3 (%)	T5AS (%)
Total cell incorporation	7.40 (100)	7.23 (100)
Glycopeptides	5.92 (80.0)	5.66 (78.2)
N-glycans	0.85 (11.4)	0.74 (10.2)
O-glycans		
Small	2.30 (31.1)	2.41 (33.3)
Large	2.24 (30.4)	2.53 (35.1)
Upon endo- β -galactosidase		
Unbound to QAE-Sephadex	0.38 (5.1)	0.57 (7.8)
Bound to QAE-Sephadex/ eluted with NaCl	1.12 (15.1)	0.91 (12.5)

radioactivity is sensitive to β -elimination providing two fractions: small O-glycans, recovered in the included volume of the Bio-Gel P4 column, and large O-glycans, collected with the excluded volume of the Bio-Gel P4 and the included volume of the Sephadex G-50 column (Fig. 4B and C). Small O-glycans are present in similar amounts in BxPC3 and the T5AS clone (Table 1), and to be mostly constituted by sialylated or neutral disaccharides. They probably represent core 1 O-glycans that are not potential substrates of $\beta 3\text{Gal-T5}$ and were not studied further. Large O-glycans are found in relevant amounts in both cells. Their size was confirmed by Bio-Gel P-4 chromatography performed in 0.1 M acetic acid that shows that they move between N-glycans and small oligosaccharides (Fig. 4D). Large O-glycans are sensitive to endo- β -galactosidase treatment, providing neutral (unbound to QAE-Sephadex) and acid (bound to QAE-Sephadex) oligosaccharides (Table 1). Neutral oligosaccharides released by endo- β -galactosidase from BxPC3 large O-glycans contain a minimal amount of radioactivity and were not analysed further, whereas those released from T5AS clone mostly show a disaccharide peak and a smaller trisaccharide peak (Fig. 4, lower). The disaccharide is sensitive to β -hexosaminidase, giving rise to radioactive Gal, and identified as $\text{GlcNAc}\beta 1-3\text{Gal}$. The trisaccharide was mostly sensitive to $\beta 1,4\text{galactosidase}$, giving rise to a disaccharide and a monosaccharide, and is thus identified as $\text{Gal}\beta 1-4\text{GlcNAc}\beta 1-3\text{Gal}$. The acid fraction of endo- β -galactosidase sensitive large O-glycans from BxPC3 cells, upon specific removal of $\alpha 2,3$ sialyl residues, contains mostly a tetrasaccharide and a trisaccharide, and an oligosaccharide peak close to but separated from the void volume (Fig. 4, lower). The trisaccharide is sensitive to both $\beta 1,3$ - and $\beta 1,4\text{galactosidases}$, giving rise to a disaccharide and a monosaccharide, and is thus identified as a mixture of $\text{Gal}\beta 1-3\text{GlcNAc}\beta 1-3\text{Gal}$ and $\text{Gal}\beta 1-4\text{GlcNAc}\beta 1-3\text{Gal}$. The tetrasaccharide is sensitive to $\alpha 1,3/4$ fucosidase giving rise to a trisaccharide that provides equal amounts of radioactive disaccharide and monosaccharide upon $\beta 1,3\text{galactosidase}$ treatment, and is thus identified as $\text{Gal}\beta 1-3[\text{Fuc}\alpha 1-4]\text{GlcNAc}\beta 1-3\text{Gal}$. The acid fraction of endo- β -galactosidase sensitive O-glycans from the antisense clone, upon removal of $\alpha 2,3$ sialyl residues, contains mostly a trisaccharide, a small shoulder corresponding to a tetrasaccharide, and the oligosaccharides peak separated from the void volume as well. The trisaccharide was mostly sensitive to $\beta 1,4\text{galactosidase}$, giving rise to a disaccharide and a monosaccharide, and is thus identified as $\text{Gal}\beta 1-4\text{GlcNAc}\beta 1-3\text{Gal}$, while the tetrasaccharide was sensitive to $\alpha 1,3/4$ fucosidase, giving rise to a trisaccharide. The latter was sensitive to both $\beta 1,4$ - and $\beta 1,3\text{galactosidases}$, giving rise to a disaccharide and a monosaccharide, and was thus identified as a mixture of $\text{Gal}\beta 1-4[\text{Fuc}\alpha 1-3]\text{GlcNAc}\beta 1-3\text{Gal}$ and $\text{Gal}\beta 1-3[\text{Fuc}\alpha 1-4]\text{GlcNAc}\beta 1-3\text{Gal}$. The calculated amounts of each oligosaccharide are summarized in Table 2. These data indicate that the repression of $\beta 3\text{Gal-T5}$ reduces the synthesis of type 1 chain carbohydrates, including sLe^a , and enhances that of poly N-acetyl-lactosamines and sLe^x on O-glycans. We were unable to characterize the peak separated from the void volume, but we believe that it may represent the reducing end of the sugar chain remaining after endo- β -galactosidase digestion.

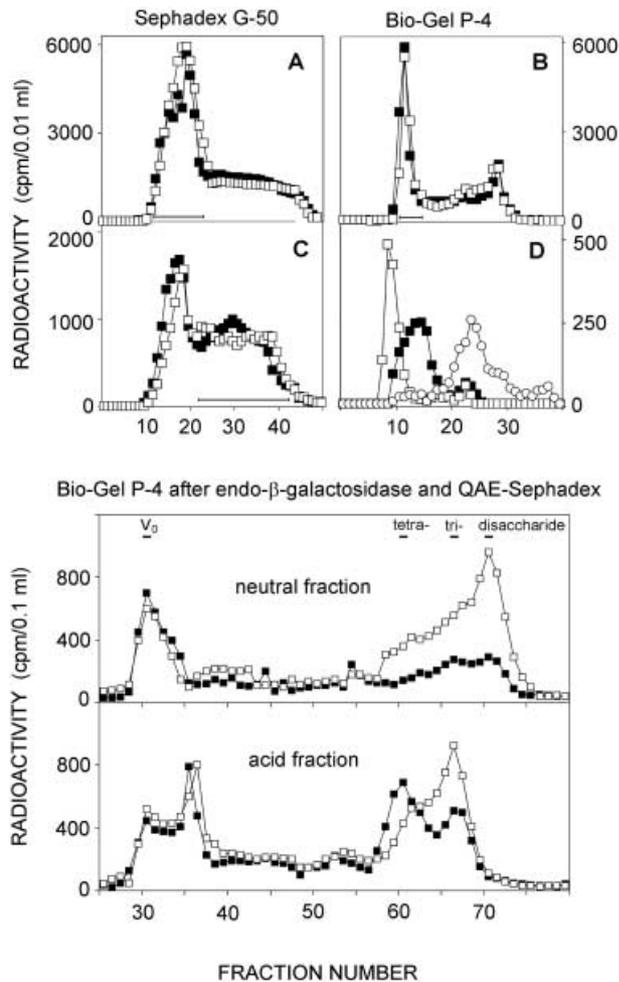


Fig. 4. Characterization of radioactive oligosaccharides formed in metabolically radiolabelled cells. The main radioactive oligosaccharides formed in BxPC3 cells (■ in lower part, and A, B, and C of upper part) and T5AS clone (□ in lower part, and A, B, and C of upper part) metabolically radiolabelled with [3 H]Gal were characterized. Upper part: cell lysates were treated with N-glycanase and passed through a Sephadex G-50 column (A) and the material collected with the flow-through of the column (horizontal bar) was submitted to β -elimination. Upon β -elimination the material was passed through a Bio-Gel P-4 column (B), and the material collected with the excluded volume of the column (horizontal bar) was passed again through a Sephadex G-50 column (C). Material included in this last column (horizontal bar) represents large O-glycans. (D) N-glycans (□), obtained by Bio-Gel P-4 purification of the included volume of the column in (A), large O-glycans (■), obtained as the included volume of the column in (C), and small O-glycans (○), obtained as the included volume of the column in (B), were analysed by a Bio-Gel P-4 column equilibrated and eluted with 0.1 M acetic acid. The profiles obtained with the radioactive fractions prepared from BxPC3 cells are presented, those obtained with fractions from T5AS clone were identical. Lower part: large O-glycans were treated with endo- β -galactosidase and passed through a QAE-Sephadex column. Radioactivity not bound to QAE-Sephadex was lyophilized and applied directly to a long Bio-Gel P4 column (neutral fraction), while radioactivity bound to QAE-Sephadex and eluted with NaCl was desalted, treated with α 2,3 sialidase, and then applied to the column (acid fraction). Column calibration is shown at the top.

If so, it is interesting to note that the O-glycans carrying Lewis antigens in BxPC3 appear to be very complex structures comparable in size to those recently reported in other cells [25].

Secretion of Lewis antigens in the antisense clone

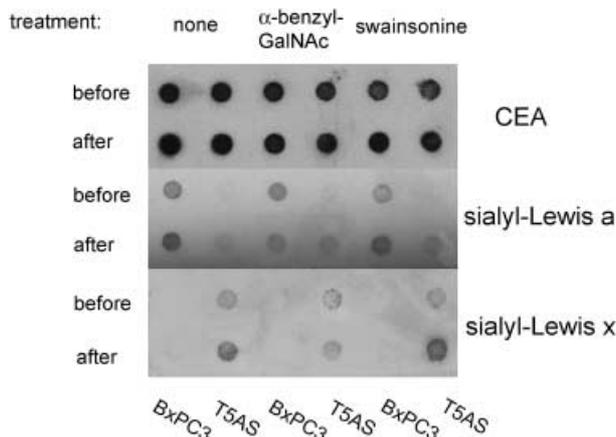
To assess the effect of β 3Gal-T5 repression on the sugar chains of molecules secreted in the culture media, BxPC3 cells and the antisense clone were cultured and the media analysed by dot-blot after adding drugs affecting glycosylation. To obtain comparable data, preliminary experiments were performed in order to normalize the amount of media to be blotted. To this purpose we used CEA as a reference, as it is secreted by the cells, and stained the blots with anti-CEA Ig. Fig. 5 shows the results obtained by staining blots prepared using such amounts of culture media with anti-sLe^a and anti-sLe^x Igs, respectively. BxPC3 cells secrete sLe^a in the media but not sLe^x, while T5AS clone secretes mostly sLe^x. Accumulation of both antigens is prevented by benzyl- α -GalNAc, an inhibitor of O-glycosylation, while it is not affected by swainsonine, an inhibitor of N-glycosylation. These results confirm that β 3Gal-T5 is responsible even for sLe^a secreted by the cells, and that O-glycans carried by secreted molecules are modified upon β 3Gal-T5 repression in a similar manner as those carried by membrane-bound molecules.

β 1,3Gal-T activity, β 3Gal-T5 transcript levels, and sLe^a expression in cancer cell lines and recombinant clones

We also measured the levels of β 3Gal-T5 transcript and β 1,3Gal-T activity in different cancer cell lines and clones, and compared them with the amount of sLe^a antigen expressed on the cell surface. We found that cells expressing high levels of transcript, such as COLO-205, SW-1116 or recombinant HCT-15-T5, express high levels of enzyme activity; cells expressing lower levels of transcript, such as CACO-2, HT-29, or BxPC3, express lower β 1,3Gal-T activity levels; while cells not expressing the transcript at all, such as HCT-15 or Panc-1, have no measurable enzyme activity (Fig. 6). Surprisingly, the range of β 1,3Gal-T activity/ β 3Gal-T5 transcript ratio is very broad. The highest value is found in BxPc3 cells, while it is 16-fold lower in the HCT-15-T5 clone. To verify that the enzyme activities measured are due to β 3Gal-T5 only, we determined the enzyme kinetics from representative cells, and found that the β 1,3Gal-T activities detected are kinetically identical to those of genuine β 3Gal-T5. Altogether these data suggest that β 3Gal-T5 regulation is not exclusively transcriptional in cultured cells, as reported for another glycosyltransferase [26]. Quantitatively, sLe^a expression is also roughly correlated with the levels of β 3Gal-T5 activity, suggesting that many factors control antigen expression besides β 3Gal-T5 expression. In fact, MKN-45 cells express transcript and activity but do not express the antigen at all, while a recombinant clone overexpressing Fuc-TIII, MKN-45-FT, does express a high amount of antigen. In all cell line sLe^a expression is over 90% impaired by benzyl- α -GalNAc treatment, suggesting an involvement of O-glycans in carrying the antigen.

Table 2. Main oligosaccharides released from BxPC3 cells and T5AS clone by endo- β -galactosidase treatment of metabolically labelled O-glycans. Values are expressed as c.p.m. $\times 10^3 \cdot \text{mg}^{-1}$ cell protein.

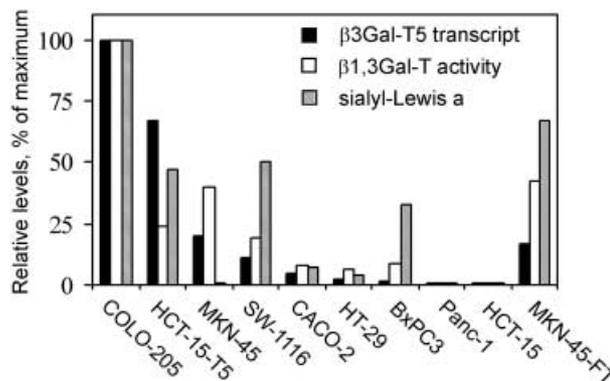
	BxPC3	T5AS
GlcNAc β 1-3Gal	<0.5	8.8
Gal β 1-4GlcNAc β 1-3Gal	<0.5	3.1
NeuAc α 2-3Gal β 1-4GlcNAc β 1-3Gal	2.1	12
NeuAc α 2-3Gal β 1-4[Fuc α 1-3]GlcNAc β 1-3Gal	<0.1	1.4
NeuAc α 2-3Gal β 1-3GlcNAc β 1-3Gal	5.2	1.7
NeuAc α 2-3Gal β 1-3[Fuca1-4]GlcNAc β 1-3Gal	10	0.9

**Fig. 5.** Secretion of Lewis antigens in the culture medium of BxPC3 cells and T5AS clone. Cells were grown under regular conditions for 30 h before treatment, then the tissue culture media were collected and replaced with fresh regular media alone (controls), or containing $1.0 \mu\text{g}\cdot\text{mL}^{-1}$ swainsonine or 2 mM benzyl- α -GalNAc. Media were collected again 60 h after treatment. Aliquots of collected media, normalized with respect to the amount of secreted CEA, were blotted and stained with primary anti-sLe^a or anti-sLe^x Igs followed by peroxidase-labelled secondary antibody.

Discussion

We have found that β 3Gal-T5 is responsible for sLe^a antigen synthesized on O-glycans expressed on or secreted by an epithelial cell line, whereas antisense-mediated suppression of the enzyme turns synthesis of O-glycans to poly N-acetylglucosamine elongation and termination by sLe^x. Taken together with our previous data on β 3Gal-T5 downregulation in colon cancer and N-glycan synthesis [16], the results suggest that β 3Gal-T5 may play a protective role in gastrointestinal and pancreatic cells, counteracting the glycosylation pattern associated to malignancy.

We found in fact that NeuAc α 2-3Gal β 1-3[Fuc α 1-4]GlcNAc β 1-3Gal and NeuAc α 2-3Gal β 1-3GlcNAc β 1-3Gal are the main oligosaccharides released by endo- β -galactosidase treatment of large O-glycans in BxPC3 cells, while in the clone where β 3Gal-T5 is suppressed they are mostly replaced by poly N-acetylglucosamine units differently substituted by sialic acid and fucose. The levels of α 1,3 fucosylation and sLe^x expression were rather low in this case, probably because BxPC3 cells express Fuc-TIII but almost no pure α 1,3fucosyltransferase [27], including Fuc-TVII that is not expressed in any cell line used in the present study [27–29].

**Fig. 6.** Expression of β 3Gal-T5 and sLe^a in different cells. Different cell lines and clones were cultured, harvested, and analysed as follows. β 3Gal-T5 transcript (filled bars) was quantified by competitive RT/PCR starting from RNA extracted from aliquots of the cell pellets, and β 3Gal-T5 activity (empty bars) was determined by *in vitro* assay using homogenates prepared from a second aliquot of the cell pellet. sLe^a antigen expressed on the cell surface (grey bars) was determined by immunostaining and flow cytometry performed on a fresh aliquot of the cell pellet. Results are expressed as relative values, 100% corresponds to $18 \text{ fg}\cdot\text{pg}^{-1}$ β -actin for transcripts, to 190 ng of transferred Gal $\cdot\text{mg}^{-1}$ homogenate protein $\cdot\text{h}^{-1}$ for enzyme activity, and to 50 arbitrary units for fluorescence.

However, moderate amounts of sLe^x were recently proved to be the most efficient in promoting metastatic spread [30]. These data match the finding that CEA synthesized by normal mucosa has abundant N-linked type 1 chains due to β 3Gal-T5 activity, and that are replaced by poly N-acetylglucosamines in cancer where the enzyme is downregulated [16,31]. Altogether they suggest that β 3Gal-T5 synthesizes type 1 chains that do prevent poly N-acetylglucosamine elongation and sLe^x synthesis on both N- and O-glycans. Due to the involvement of such structures in malignancy, β 3Gal-T5 regulation may play an important role in colon cancer, as the residual expression level potentially contributes to prevention of the malignant phenotype.

Synthesis and expression of sLe^a is a relevant issue *per se*, as it is the epitope of the CA19.9 antigen, sometimes found to be elevated in the serum of patients with various abdominal illnesses [32] including cancers of the digestive tract [33–35]. Moreover, it is an E-selectin ligand [36] and may be involved in the metastatic spread of cancer cells, as suggested for other selectin ligands [37]. Previous data indicate that β 3Gal-T5 is the enzyme candidate for synthesis of sLe^a [15–18], but the finding that sLe^a is strongly expressed in normal mucosa

makes this open to question. Here we found evidence that β 3Gal-T5 is actually necessary for sLe^a synthesis on O-glycans in gastrointestinal and pancreatic cells. In fact, in BxPC3 cells antisense suppression of the gene dramatically reduces β 1,3Gal-T activity as well sLe^a antigen expression and secretion. Moreover, only cell lines expressing β 3Gal-T5 express the antigen, and cells not expressing are forced to do by cDNA transfection. On the other hand, sLe^a synthesis and secretion appear to depend on multiple molecular or enzymatic mechanisms. We speculate they may include several interacting factors such as the nature and availability of substrates, including nucleotide sugars [38], the presence of other cooperative or competing enzymes [39], as well their sub-Golgi localization [40]. Our working hypothesis is that the biological role of β 3Gal-T5 includes, but is not restricted to, sLe^a synthesis, that probably requires several concurrent factors *in vivo*. Phylogenetic observations agree with this concept. In fact, while α 1,4 fucosylation and thus sLe^a synthesis are recent evolutionary acquisitions belonging to humans and some primates [41], β 3Gal-T5 is present in other mammals such as mice [42], rats (GenBank accession XM221525), and very probably pigs [43]. While this manuscript was being completed, Isshiki *et al.* reported that β 3Gal-T5 is transcriptionally regulated by homeoproteins specific to the intestinal mucosa [44]. They also found that some of these homeoproteins, as well as β 3Gal-T5, are upregulated during CACO-2 cell differentiation and down-regulated in colon cancer, but that β 3Gal-T5 protein is not correlated with the amount of CA19.9 in cancer tissues. Such results elegantly show that type 1 chain carbohydrates are products of β 3Gal-T5 activity as a part of the specific phenotype of the normal intestinal mucosa. Taken together with our previous [16] and present findings, and with those on CACO-2 differentiation [13,14], they corroborate the hypothesis that β 3Gal-T5 and type 1 chain carbohydrates are 'markers' of normal glycosylation in epithelia of the digestive tract. In this context, the use of CA19.9 antigen as a tumour marker appears paradoxical, since it is a product of β 3Gal-T5 activity on type 1 chain O-glycans. We believe that further studies are needed to elucidate the metabolic origin of CA19.9 circulating in patients and to confirm the actual ability of gastrointestinal and pancreatic cancers to synthesize and secrete large amounts of sLe^a.

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