Detection, Purification and Characterization of a Human Cancer-Associated Galactosyltransferase Acceptor

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A low-molecular-weight acceptor of galactosyltransferase activity was detected in sera and effusions of patients with extensive malignant disease. This substance was purified to homogeneity from both human serum and effusion by using sequential charcoal/Celite and DEAE-cellulose column chromatography. The purified acceptor was shown to act as substrate for both purified normal and cancer-associated human galactosyltransferase (EC 2.4.1.22) isoenzymes, but had a higher affinity for the cancer-associated isoenzyme ($K_m = 20 \mu M$) than for the normal isoenzyme ($K_m = 500 \mu M$). The substrate was found to be a glycopeptide with mol.wt. approx. 3600 determined by polyacrylamide-gel chromatography. Carbohydrate analysis demonstrated only the presence of glucosamine and mannose. Amino acid analysis revealed that the peptide moiety consisted of eight different amino acids, including two residues of asparagine and one residue of serine, but no threonine. These structural data suggest that the acceptor is a fraction of an asparagine-glucosamine type of glycoprotein.

Several investigators have detected differences between cell-surface-derived glycoproteins from tumours and their normal counterparts (Warren *et al.*, 1972; Codrington *et al.*, 1973; Nicolson & Lacorbiere, 1973; Kim *et al.*, 1974; Rieber & Romano, 1976; VanNest & Grimes, 1977). These studies have not determined the specific functions of these glycoproteins, although several investigators have suggested that glycoproteins may be important in intercellular regulation and as neoplastic determinants (Roseman, 1970; Roth, 1973; Balsamo & Lilien, 1975; Knox *et al.*, 1976; Hausman & Moscona, 1976). Similarly, a number of reports have documented the presence of distinctive glycoproteins in serum or urine of patients with cancer. Although it has been implied that these glycoproteins are derived from the tumour-cell surface, their functions in tumour biology remain unknown (Fuks *et al.*, 1974; Zamcheck, 1974; Bernacki & Kim, 1977; Rudman *et al.*, 1978).

Changes in cell-surface glycosyltransferases have also been observed in transformed and malignant cells, and it has been suggested that these enzymes play a role in the deranged processes of cellular recognition and attachment observed in tumour cells (Roseman, 1970; Roth, 1973; Webb & Roth, 1974; Shur &

Roth, 1975). Various investigators have noted both increased and decreased cell-surface glycosyltransferase activities without any consistent alteration in the different cell systems examined. Workers have also noted increased activity of several serum glycosyltransferases, including both fucosyltransferase (Khilani *et al.*, 1977; Chou *et al.*, 1977) and sialyltransferase (Ip & Dao, 1977, 1978) in patients with cancer. Other glycosyltransferases, notably galactosyltransferase, have shown no quantitative alteration in patients with malignant disease (Kim *et al.*, 1972; Podolsky & Weiser, 1975a; Weiser *et al.*, 1976). However, in previous studies we detected the presence of an electrophoretically distinct form of galactosyltransferase (UDP-galactose-*N*-acetylglucosamine 4- β -galactosyltransferase, EC 2.4.1.22), designated isoenzyme GT-II, in sera and effusions from patients with cancer, which was not detected in control groups despite equivalent activity of total galactosyltransferase in the two populations (Podolsky & Weiser, 1975a; Weiser *et al.*, 1976; Podolsky *et al.*, 1978). In later studies, the normal (GT-I) and cancer-associated (GT-II) isoenzymes were purified to homogeneity from human malignant effusions and found to differ in molecular weights, carbohydrate content and composition, as well as kinetic properties (Podolsky & Weiser, 1978). A similar cancer-associated galactosyltransferase activity (GT-II_H) was found to correlate with tumour growth in an animal model (Podolsky *et al.*, 1977). In these latter studies, hamsters were inoculated with polyoma-transformed BHK (baby-hamster kidney) cells and

Abbreviation used: SGF-fetuin, fetuin lacking terminal sialic acid and penultimate galactose residues.

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an activity similar to serum isoenzyme GT-II_H was solubilized from tumour, but not from other hamster tissues.

A few sera from both humans and hamsters with extensive metastatic cancer exhibited inhibition of galactosyltransferase activity. These previous studies of isoenzyme GT-II used an exogenous glycoprotein derived from fetuin as an acceptor. The inhibition appeared on preliminary experiments to be competitive and suggested to us that the inhibition might reflect an endogenous acceptor. The present paper details the detection, purification and characterization of a glycopeptide galactosyltransferase acceptor from cancer serum and malignant effusion.

Experimental

Materials

UDP-[1-³H]galactose (sp. radioactivity 2.25 Ci/mmol) was obtained from New England Nuclear (Boston, MA, U.S.A.). A stock solution (714 μ M) was prepared to a final specific radioactivity of 0.288 Ci/mmol by using unlabelled UDP-galactose obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Fetuin lacking terminal sialic acid and penultimate galactose residues (SGF-fetuin) was prepared as previously described (Kim *et al.*, 1971).

Preparation of acceptor

Sera were obtained from the Surgical Services of the Massachusetts General Hospital. Samples were prepared by centrifugation at 1000g for 10 min at 4°C and stored at 4°C before use. Acceptor samples were heat-inactivated (20 min, 45°C) and precipitate was removed by centrifugation at 2000g for 5 min at 4°C to eliminate enzyme activity.

Preparation of galactosyltransferase enzyme sources

Pooled malignant effusion containing both isoenzyme GT-I and GT-II activities, as determined by previously described techniques (Podolsky & Weiser, 1975a), was used as enzyme source after partial purification by (NH₄)₂SO₄ precipitation as described (Podolsky & Weiser, 1978). Enzyme from the 30–70%-(w/v)-(NH₄)₂SO₄ fraction was suspended in 0.1 M-sodium cacodylate, pH 7.4, and dialysed against this buffer to give a final activity of 2000 c.p.m./h per 10 μ l with SGF-fetuin as acceptor as described below. Purified isoenzymes GT-I and GT-II were prepared from human malignant effusion as described elsewhere (Podolsky & Weiser, 1978). The Human Studies Committee of the Massachusetts General Hospital approved this study of sera and effusions as consistent with the principles of the Helsinki Declaration.

Assay of galactosyltransferase acceptor activity

Galactosyltransferase activity was measured by

modification of previously described methods (Podolsky & Weiser, 1975a,b). In one assay, acceptor activity was detected by the ability of acceptor samples to inhibit competitively incorporation of labelled galactose into the acid-precipitable acceptor. Assay mixture included 10 μ l of enzyme source, 7.5 μ l of UDP-[1-³H]galactose stock solution, 35 μ l of 0.1 M-sodium cacodylate, pH 7.4, and 0.154 M-NaCl, 7.5 μ l of 0.1 M-MnCl₂ and SGF-fetuin (0.2 μ M-acceptor sites) in a volume of 20 μ l, with 10 μ l of sample being added to test for inhibition. The mixture (85 μ l) was incubated for 60 min at 37°C. The reaction was then terminated by addition of 5% (w/v) phosphotungstic acid in 2 M-HCl, and incorporation of radioactive label into acid-precipitable material was determined as previously described (Podolsky & Weiser, 1975b). Inhibition was calculated as the difference of incorporation between assay mixtures with and without addition of sample.

Direct measurement of acceptor activity was carried out by using an assay mixture identical with that described above except that 20 μ l of the sample being tested replaced SGF-fetuin. At the end of the incubation, the reaction mixture was applied to Whatman 3MM paper, which was then electrophoresed in 1% (w/v) sodium tetraborate buffer, pH 8.0, at 1000 V for 50 min. Strips were evaluated either by a Packard radiochromatogram scanner or, after cutting into 2 cm wide pieces, by counting in a Beckman scintillation counter in a toluene-based scintillation fluid (Podolsky & Weiser, 1975b). The acceptor remained near the origin, whereas UDP-[1-³H]galactose and its breakdown products moved toward the anode.

Identification of product

To determine the identity of incorporated radioactivity, reaction mixtures were scaled up 10-fold and reaction time was increased to 90 min. After termination of the reaction and electrophoresis as above, product was eluted and then hydrolysed in 1.0 ml of 2 M-HCl at 100°C for 6 h. The hydrolysate was subjected to ion-exchange chromatography through a column (1.5 cm $\times$ 10 cm) containing AG 1 (X8; formate form; 200–400 mesh) ion-exchange resin (Podolsky & Weiser, 1975b). The eluates were concentrated to 100 μ l under N₂ and 40 μ l samples applied to Whatman 1MM paper. Descending chromatography was subsequently performed as previously described (Podolsky & Weiser, 1975b) and migration of label was compared with genuine sugar standards.

Purification of cancer-associated galactosyltransferase acceptor

Pooled human malignant effusion (1.4 litres) previously found to contain acceptor activity was

heat-inactivated (65°C, 45 min) and precipitate removed by centrifugation (500g for 30 min). The supernatant was then passed through a filtration membrane with an exclusion size of mol.wt. 10000 (PM-10 membrane; Amicon Co., Lexington, MA, U.S.A.) and the ultrafiltrate obtained. After total hexose (Dubois *et al.*, 1956) and protein (Lowry *et al.*, 1951) determination, the ultrafiltrate was applied to a charcoal/Celite (1:1, w/w) column at a sample loading of 1 g of mixed adsorbent for each 1 mg of hexose in the sample. The column was washed with 10 bed volumes of water, followed by various volumes of aqueous alcohol in increasing concentrations (17%, 20%, 25%, 30%, 50%). The fractions were assayed for hexose, protein and acceptor activity as described above.

Subsequently, the charcoal/Celite fraction containing the acceptor was concentrated by evaporation under vacuum to 2.5 ml and then applied to a column (0.9 cm × 65 cm) of DEAE-cellulose equilibrated in 0.4 M-sodium phosphate buffer, pH 7.6. After washing with the application buffer (100 ml), the column was developed with a linear ascending gradient of 0.5–30 mM-sodium phosphate buffer, pH 7.6, at a rate of 20 ml/h; 5.0 ml fractions were collected. Protein (evaluated by continuous A_{280} monitoring) and hexose were determined for each fraction. Peaks of protein and hexose were individually pooled and acceptor activity was determined. Homogeneity was evaluated in three paper-electrophoretic solvent systems (A, pyridine/acetic acid/water, 25:1:225, by vol., pH 6.4, 400 V for 4 h; B, 0.25 M-acetic acid, pH 3.0, 500 V for 3.5 h; C, 0.05 M- Na_2HPO_4 , pH 8.5, 300 V for 5 h) with Whatman 1 MM paper, and a descending paper-chromatography system (ethyl acetate/pyridine/water/acetone, 10:5:10:1, by vol.) (Johansen *et al.*, 1961). Chromatograms were developed with both ninhydrin reagent and AgNO_3 to detect protein and carbohydrate respectively. Molecular weight was estimated by chromatography on a column (2.5 cm × 100 cm) of polyacrylamide gel (Bio-Gel P-10; Bio-Rad Laboratories, Los Angeles, CA, U.S.A.) developed with water at a rate of 16 ml/h; 5.0 ml fractions were collected. Standards included vitamin B_{12} , ribonuclease, α -lactalbumin and Blue Dextran (Sigma).

Carbohydrate and amino acid composition

Measurement of sugar composition was made by assaying the purified endogenous acceptor after acid hydrolysis (6 M-HCl, 90°C, 120 min). Hexose was determined by the phenol/ H_2SO_4 method (Dubois *et al.*, 1956) and hexosamine by the Elson–Morgan assay (Blix, 1948). Qualitative evaluation of the sugar composition was obtained by application of the hydrolysate to two descending chromatography systems as described (Podolsky & Weiser, 1975b). Sialic acid composition was determined after hydrolysis of

the intact acceptor with 0.05 M- H_2SO_4 at 80°C for 60 min by using the thiobarbituric assay of Warren (1959).

The results of these studies were complemented by parallel evaluation of carbohydrate composition by g.l.c. (Podolsky & Weiser, 1975b). Inositol (25 μg) was added as an internal standard to purified acceptor (125 μg) before drying. The dried material was incubated in 1.0 ml of methanolic 1 M-HCl at 85°C for 60 min, then evaporated again to dryness under N_2 and washed with methanol. A stock solution (0.1 ml) of Syland HRP (Supelco Co., Madison, WI, U.S.A.) was added and the suspension left at room temperature for 60 min. After drying, material was extracted with 60 μl of hexane and 1 μl of this extract applied to a column (152 cm × 0.3 cm) of OV17 (0.1%, g.l.c. 110 support; mesh 120–140; Supelco Co.) by using a Perkin–Elmer 900 g.l.c. instrument programmed over 75–250°C at a rate of 10°C/min.

Amino acid analysis was carried out by modification of the method of Zumwalt *et al.* (1970). After formation of a derivative with trifluoroacetic anhydride in dichloromethane, 1.5 μl was applied to columns of poly(ethylene glycol adipate) or OV-225 by conditions described previously (Podolsky & Weiser, 1975b).

Results

The presence of an acceptor was initially detected by the inhibition of UDP-galactose–SGF-fetuin galactosyltransferase activity. As shown in Fig. 1, serum and effusions from patients with metastatic disease contained a substance that inhibited incorporation into the acid-precipitable SGF-fetuin acceptor. This inhibition of activity was not found in sera from patients with localized disease, normal or disease controls. Inhibition of galactosyltransferase (SGF-fetuin acceptor) activity was found to be linearly related to the amount of inhibiting serum added. That the inhibition of the observed incorporation into an acid-precipitable product was due to the presence of a competitive substrate was suggested by the finding that sera and effusion that contained the inhibitor activity demonstrated competitive inhibition for SGF-fetuin, but not for UDP-galactose as determined by Lineweaver–Burk (1934) plots (Figs. 2 and 3).

The presence of a galactosyltransferase acceptor in sera and effusions was confirmed by observation of direct incorporation of galactose into a product that was separated by paper electrophoresis. Fig. 4 shows the occurrence of a peak radioactivity incorporation near the origin, which was absent from controls or samples lacking capacity to inhibit incorporation into SGF-fetuin. This area was eluted and, after acid hydrolysis, shown to contain radioactively labelled

Fig. 1. Inhibition of galactosyltransferase-SGF-fetuin activity by human sera and effusions

Pooled 30–70%-(w/v)- $(\text{NH}_4)_2\text{SO}_4$ fraction of human malignant tumour effusion was used as enzyme source. Galactosyltransferase activity was assayed by using SGF-fetuin acceptor with or without the heat-inactivated sample ($10\mu\text{l}$) being tested for inhibitor activity as described in the Experimental section and inhibition calculated as the difference between activity in the presence and absence of heat-inactivated sample. Normal controls were 12 healthy laboratory personnel. Disease controls included seven patients with pancreatitis (two acute, five chronic), four patients with cholecystitis and four patients with peptic ulcer disease. Effusions were from patients with cirrhosis (three) or congestive heart failure (two). Patients with localized carcinoma had either carcinoma of colon and rectum (12) without evidence of lymphatic invasion at surgery, or breast carcinoma (three) without evidence of spread at subsequent surgery. Patients with extensive carcinoma (15 colon, six breast, four lung, four pancreas) had evidence of massive hepatic or bone metastases or malignant effusion. Symbols: ●, sera; ○, effusions.

Fig. 2. Effect of increasing amounts of the inhibitor on K_m for UDP-galactose of UDP-galactose-SGF-fetuin galactosyltransferase activity

Determinations of K_m for UDP-galactose were per-

formed by using a complete reaction mixture that included $10\mu\text{l}$ of enzyme diluted with $55\mu\text{l}$ of 0.1M -sodium cacodylate, pH 7.4, $10\mu\text{l}$ of SGF-fetuin containing $0.2\mu\text{M}$ -acceptor sites and $35\mu\text{l}$ of UDP-[$1\text{-}^3\text{H}$]-galactose in increasing concentrations. Samples were incubated at 37°C for 120 min, reaction was stopped and activity determined as c.p.m./mg of protein as described in the Experimental section. Results are plotted by the Lineweaver-Burk (1934) method. Determinations were made in the presence or absence of various amounts of human heat-inactivated inhibitor prepared from pooled sera as described in the Experimental section. Amount of inhibitor present (μl): ●, none; ○, 2.5; ▲, 5; △, 10; △, 15.

galactose by two descending chromatography systems (Fig. 5).

The acceptor activity was found to be diffusible in conventional dialysis tubing, and as Table 1 demonstrates, the inhibitor to galactosyltransferase (SGF-fetuin acceptor) activity was found in the first fraction

Fig. 3. Effect of increasing amounts of inhibitor on the K_m for SGF-fetuin of UDP-galactose-SGF-fetuin galactosyltransferase activity

K_m for SGF-fetuin was determined with a reaction mixture that included $10\mu\text{l}$ of enzyme, $55\mu\text{l}$ of 0.2M -sodium cacodylate, pH 7.4, $10\mu\text{l}$ of 0.15M - MnCl_2 , $15\mu\text{l}$ of UDP-[$1\text{-}^3\text{H}$]-galactose stock prepared as described in the Experimental section and increasing amounts of SGF-fetuin in $60\mu\text{l}$. Assay conditions were as described in the Experimental section and Fig. 2. Results are plotted by the Lineweaver-Burk (1934) method in the presence or absence of increasing amounts of human heat-inactivated inhibitor prepared from pooled sera as described in the Experimental section. Amount of inhibitor present (μl): ●, none; ○, 2.5; ▲, 5; △, 10.

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Fig. 4. Demonstration of galactosyltransferase acceptor activity of malignant serum and purified glycopeptide. Galactosyltransferase activity was assayed as described in the Experimental section by using either heat-inactivated pooled serum or 3 μ g of purified glycopeptide instead of SGF-fetuin as acceptor. Enzyme source was the partially purified preparation from pooled effusions (see the Experimental section). After incubation, mixtures were evaporated to dryness, then resuspended in 15 μ l of water for application to Whatman 3MM paper. Paper electrophoresis was performed in a 1% (w/v) sodium tetraborate buffer, pH8.8, at 1000V for 50min. After electrophoresis, strips were cut into 2cm-wide pieces and radioactivity was determined by counting in toluene-based scintillation fluid. Mobility is compared with UDP-galactose (UDP-Gal) and galactose 1-phosphate (Gal-1-P) standards. (a) Control, no acceptor added. (b) Representative chromatogram with inhibitor-containing serum (see Fig. 1). (c) Purified inhibitor (from peak I, Fig. 6). Chromatograms of sera from normal controls, disease controls and patients with localized carcinoma were similar to those in (a).

Table 1. Charcoal/Celite chromatography of serum filtrates in separation of an inhibitor of UDP-galactose-SGF-fetuin acceptor galactosyltransferase activity. Sera (1.0ml) were heat-inactivated (60°C, 20min) and the ultrafiltrate from an Amicon PM-10 membrane was obtained. The ultrafiltrate was placed on charcoal/Celite columns (1ml/mg of hexose) and eluted in stepwise fashion with increasing concentrations of ethanol. Inhibition of activity was measured as described in the Experimental section, after adjustment of concentration to include 1 μ g of hexose per 10 μ l sample.

Serum	Fraction	Ethanol (%)	Inhibition (%)	Hexose (mg)	Protein (mg)
Cancer	C1	0	47	2.50	0.025
	C2	17	—	1.20	0.160
	C3	20	—	<0.01	0.040
	C4	25	—	<0.01	0.050
	C5	30	—	<0.01	0.020
	C6	50	8	0.03	0.210
Normal	N1	0	4	3.20	0.320
	N2	17	—	0.42	0.060
	N3	20	—	0.40	0.140
	N4	25	—	<0.01	0.030
	N5	30	—	<0.01	0.030
	N6	50	3	<0.01	0.030

Fig. 5. Product identification of UDP-galactose-cancer-associated glycopeptide acceptor galactosyltransferase activity.

Assays were performed as described in the Experimental section and in Fig. 4, except that reaction mixtures were concentrated and applied to Whatman 3MM paper for electrophoresis as described in Fig. 4. The radioactivity incorporated into the new peak located near the origin (Figs. 4b and 4c) was eluted and concentrated to near dryness under N₂. After acid hydrolysis (2M-HCl, 100°C, 6h) the hydrolysate was subjected to ion-exchange chromatography through a column (1.5cm x 19cm) containing AG 1 (X8; formate form; 200–400 mesh) ion-exchange resin. Eluates were concentrated to 100 μ l samples and applied to Whatman 1MM paper. Chromatography was performed in two solvent systems: (a) and (b), butanol/ethanol/water (10:1:2, by vol.), 110h; (c) and (d), ethyl acetate/pyridine/water (12:5:6, by vol.), 15h. Chromatograms were then cut into 2cm strips and radioactivity was determined by counting in a Beckman scintillator counter, in a toluene-based scintillation fluid (Podolsky & Weiser, 1975b). Migration of genuine carbohydrate standards was determined by AgNO₃ stain. (a) and (c), crude effusion as source of acceptor; (b) and (d), purified glycopeptide (from Fig. 6, peak I) as acceptor source. GlcN, glucosamine; Gal, galactose; Glu, glucose; Man, mannose; Fuc, fucose.

Fig. 6. DEAE-cellulose column chromatography of charcoal/Celite column eluate fraction C1 that contained inhibitor activity

Charcoal/Celite column fraction C1 (Table 1) was concentrated under N_2 and applied in a volume of 0.5 ml to a column ($0.9 \text{ cm} \times 60 \text{ cm}$) of DEAE-cellulose equilibrated in 0.0005 M-sodium phosphate buffer, pH 7.5. The column was developed with an increasing concentration gradient ($\cdots$, 0.0005–0.03 M) at a rate of 16 ml/h and 5 ml fractions were collected. Fractions were assayed for neutral hexose by the phenol/ H_2SO_4 method (Dubois *et al.*, 1956; ----, A_{480}) and protein by continuous monitoring at A_{280} (—). Fractions were pooled as indicated and characterized for inhibition of UDP-galactose-SGF-fetuin acceptor galactosyltransferase activity (Table 2) and for the ability to act as a galactosyltransferase acceptor (Fig. 4).

Table 2. Inhibition of UDP-galactose-SGF-fetuin acceptor galactosyltransferase by the three major peaks eluted from DEAE-cellulose

Malignant effusion (10 ml) was heat-inactivated (60°C , 20 min) and the Amicon PM-10 membrane ultrafiltrate obtained. Charcoal/Celite chromatography was performed as described in Table 1. Fraction C1 was concentrated and applied to a column ($0.9 \text{ cm} \times 50 \text{ cm}$) of DEAE-cellulose equilibrated in 0.0005 M-sodium phosphate buffer, pH 7.6. The column was developed with an ascending sodium phosphate buffer gradient as shown in Fig. 5. Peaks were pooled and assayed for hexose (Dubois *et al.*, 1956) and protein (Lowry *et al.*, 1951). The inhibition assay was performed as described in the Experimental section with a $10 \mu\text{l}$ sample adjusted to contain $1 \mu\text{g}$ of hexose.

Peak	Hexose (mg)	Protein (mg)	Inhibition (%)
I	0.43	0.08	86
II	0.05	0.02	6
III	0.26	0.10	3

from a charcoal/Celite column after initial washing with water. No comparable inhibition could be detected in the elution of columns on which controls

Table 3. Carbohydrate composition of cancer-associated galactosyltransferase acceptor

Hexose, hexosamine and sialic acid contents were determined by phenol/ H_2SO_4 (Dubois *et al.*, 1956), Elson-Morgan (Blix, 1948) and Warren (1959) methods respectively after acid hydrolysis (6 M-HCl, 90°C , 120 min). G.l.c. evaluation of carbohydrate composition was carried out as described in the Experimental section, with inositol as an internal standard. Data are expressed as mol/mol of glycopeptide, based on a mol.wt. of 3600 determined by Bio-Gel P-10 polyacrylamide-gel-permeation chromatography.

Method	Carbohydrate	
	Type	Content (mol/mol of glycopeptide)
Assay	Neutral hexose	3.9
	Hexosamine	8.7
	Sialic acid	0.3
G.l.c.	Fucose	0.0
	Galactose	0.1
	N-Acetylglucosamine	9.2
	N-Acetylgalactosamine	0.0
	Mannose	4.2

Table 4. Amino acid composition of cancer-associated galactosyltransferase acceptor

G.l.c. of amino acid derivatives was carried out as described in the Experimental section by using a Perkin-Elmer 900 g.l.c. instrument with attached chromatodigital integrator. Values are expressed as mol of residue/mol of glycopeptide based on a mol.wt. of 3600 determined by Bio-Gel P-10 polyacrylamide-gel chromatography.

Amino acid	Amount (mol/mol of glycopeptide)
Alanine	1.2
Valine	—
Glycine	1.1
Isoleucine	—
Leucine	0.9
Proline	—
Threonine	—
Serine	0.7
Phenylalanine	—
Asparagine/aspartic acid	2.3
Glutamine/glutamic acid	2.7
Lysine	0.8
Tyrosine	1.1
Hydroxylysine	—

(non-malignant effusions and malignant effusion with no detectable inhibition activity) were chromatographed.

Analysis of the inhibiting fraction (C1) (Table 1) showed that it had protein as well as significant

amounts of hexose, with no detectable phosphate, lipid or nucleic acid; this suggested that the inhibitor/acceptor was a glycopeptide. To further purify the inhibitor/acceptor, material in fraction C1 was chromatographed on a column of DEAE-cellulose by using a gradient of increasing concentration of phosphate buffer. Fig. 6 shows that three peaks of material that contained both carbohydrate (neutral hexose by the phenol/H₂SO₄ method) and protein were detected on elution of this column. All inhibitor/acceptor activity was contained within the first peak (fractions 8–12) (Table 2). This material was pooled and found to be homogeneous in three paper-electrophoretic solvent systems and a descending paper-chromatographic system (see the Experimental section). In each system, a single peak that stained for both carbohydrate and protein was detected.

After acid hydrolysis, the purified acceptor was analysed for carbohydrate content by chemical assay and by g.l.c. As Table 3 shows, the purified acceptor contains hexose and hexosamine, as well as some sialic acid, by chemical analysis. Qualitative descending chromatography of the acid hydrolysate revealed

the presence of mannose and glucosamine. These results were confirmed by g.l.c. (Table 3).

Amino acid analysis by g.l.c. revealed the presence of eight amino acids (Table 4). The most notable feature of the amino acid composition is the presence of two residues of asparagine and one residue of serine and no detectable threonine. An approximate mol.wt. of 3600 was determined by polyacrylamide-gel chromatography (results not shown).

The acceptor activity of the purified glycopeptide was measured directly after incubation by separating the glycopeptide by paper electrophoresis as described in the Experimental section. Labelled product was found to co-migrate with purified acceptor (Fig. 4c), and also to co-chromatograph with the purified acceptor in a descending chromatography system (described in the Experimental section). The labelled acceptor was eluted from the chromatograms and the radioactive label identified after acid hydrolysis. All label was found to co-migrate with galactose in two solvent systems (Fig. 5b), confirming the identity of the substance as a cancer-associated galactosyltransferase acceptor.

Finally, the activity of the purified acceptor was assayed by using purified galactosyltransferase isoenzymes (GT-I and GT-II) as enzyme sources. As Fig. 7 shows, the acceptor could be used as a substrate by both normal and cancer-associated isoenzymes. However, determination of Michaelis constant, by assuming a mol.wt. of 3600, indicated that the cancer-associated isoenzyme had a significantly higher affinity ($K_m = 20 \mu M$) for the acceptor than the normal isoenzyme ($K_m = 500 \mu M$).

Discussion

In previous work we described a cancer-associated isoenzyme of galactosyltransferase (isoenzyme II) that was present in sera and cytologically positive effusions of patients with cancer (Podolsky *et al.*, 1978). The present paper describes the presence, isolation and characterization of a competitive inhibitor of galactosyltransferase activity in sera and effusions from patients with extensive malignancy.

Fig. 1 shows that there was significant inhibition of galactosyltransferase activity by sera and effusions obtained from patients with extensive metastatic cancer. This decrease in activity was subsequently shown to be due to competitive substrate inhibition of the exogenous acceptor SGF-fetuin used in the galactosyltransferase assay. Inhibition was then correlated with the presence in these fluids of a galactosyltransferase acceptor substrate that could be detected after separation by paper electrophoresis (Fig. 4). The inhibitor/acceptor was purified (Fig. 5, Tables 1 and 2) and found to be a glycopeptide of approx. 3600 mol.wt. with a composition of almost

Fig. 7. Determination of K_m for cancer-associated galactosyltransferase acceptor for galactosyltransferase activity by using purified normal isoenzyme GT-I (○) or purified cancer-associated isoenzyme GT-II (●).

Isoenzymes GT-I and GT-II were purified from human malignant effusion as described previously (Podolsky & Weiser, 1978). K_m for cancer-associated galactosyltransferase acceptor was determined as described in Fig. 3, except that purified acceptor (peak I, Fig. 6) was substituted for SGF-fetuin, and incorporation was determined as described in the Experimental section and in Fig. 4. Results are plotted by the Lineweaver-Burk (1934) method, assuming a molecular weight for cancer-associated galactosyltransferase acceptor of 3600. Calculated K_m values are for isoenzyme GT-I, $500 \mu M$, and for isoenzyme GT-II, $20 \mu M$.

70% carbohydrate (Table 2). The purified glycopeptide was shown to be a galactose acceptor for galactosyltransferase activity (Figs. 4 and 5), and because of its occurrence in fluids from patients with metastatic cancer, it is referred to as a cancer-associated galactosyltransferase acceptor.

Since this acceptor is a glycopeptide, one would have expected that its presence in fluids would have added to the total galactosyltransferase activity. Its detection as an inhibitor of galactosyltransferase activity utilizing SGF-fetuin as acceptor may be explained by the method of assay, in which the product is detected as an acid precipitate. Glycopeptides of relatively low molecular weight (<10000) are not effectively precipitated by acid, especially if they have a high carbohydrate and low peptide content. In subsequent experiments the purified glycopeptide was found to remain soluble in the phosphotungstic acid/HCl mixture.

The cancer-associated galactosyltransferase acceptor is notable for its limited content of carbohydrates and amino acids. Carbohydrate analysis by g.l.c. or by qualitative descending chromatography after acid hydrolysis demonstrated only glucosamine and mannose in significant quantities (Table 3). The peptide portion when analysed by g.l.c. was composed of only eight different amino acids (Table 4). The cytotoxic polypeptide purified from tumour fluids by Sylvén & Holmberg (1968) also consisted of only a few amino acids, which were similar to those described here. However, that substance was apparently devoid of carbohydrate (Sylvén & Holmberg, 1968). Detection of both asparagine and serine in cancer-associated galactosyltransferase acceptor would permit the possibility of either *N*-glycosidic (asparaginyl) or *O*-glycosidic (seryl) glycopeptide linkage. However, the detection of glucosamine to the exclusion of galactosamine makes an *N*-asparagine linkage most likely. The additional detection of mannose suggests that the glycopeptide may resemble the core region of an asparagine-linked type of glycoprotein (Sharon, 1975).

If the carbohydrate composition does represent the core portion of an asparagine-linked type of glycopeptide, it may indicate that cancer-associated galactosyltransferase acceptor is an incompletely glycosylated glycopeptide (Hughes, 1976). A number of reports have suggested that the surface of transformed and neoplastic cells contains incompletely glycosylated glycoproteins that do not have the variety of carbohydrate composition associated with the normal cell (Kim *et al.*, 1974; Kim & Isaacs, 1975). Alternatively the glycopeptide may represent a partially degraded glycoprotein. Reports have documented an increased carbohydrate content in glycopeptides obtained by proteolytic treatment of transformed cells (Buck *et al.*, 1970, 1971; Ogata *et al.*, 1975). Perhaps these seemingly contradictory findings

can be resolved by the findings of Tabas *et al.* (1978), who determined that during glycoprotein biosynthesis the core region may contain numerous mannose residues that are removed before further glycosylation. Possibly the cancer-associated galactosyltransferase acceptor glycopeptide represents a biosynthetic intermediate form of a core glycoprotein.

Numerous factors have been isolated from tissue fluids in which cancer cells have been growing. A few of these factors have had profound effects on cell growth (Holmberg, 1968; Handler & Magalini, 1966; Rubin, 1966; Helson *et al.*, 1975) and a few have been purified (Sylvén & Holmberg, 1968). Some factors have been attributed to host defences (Carswell *et al.*, 1975) and others have been identified as products of the tumour itself (Wong *et al.*, 1974; Bürk, 1973; Podolsky *et al.*, 1977).

No data are yet available on the source of cancer-associated galactosyltransferase acceptor. It is possible that the glycopeptide is derived from the tumour itself. The observation that cancer-associated galactosyltransferase acceptor seems to appear only after there is metastatic extension of the tumour burden (Fig. 1) supports this suggestion. This implies a relationship of serum cancer-associated galactosyltransferase acceptor to tumour growth, although it is not known yet whether cancer-associated galactosyltransferase acceptor is produced by the tumour or by the host.

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