Effect of all-*trans*-retinoic acid and phorbol 12-myristate 13-acetate on the activity of human hepatocellular carcinoma cell-surface β -1,4-galactosyltransferase

Dapeng ZHOU, Songmin JIANG, Zhonghou SHEN and Jianxin GU*

Gene Research Center and Department of Biochemistry, Shanghai Medical University, Shanghai 200032, People's Republic of China, and Key Laboratory of Glycoconjugate, Ministry of Public Health, People's Republic of China

A human hepatocellular carcinoma cell line, SMMC-7721, was treated with all-*trans*-retinoic acid (RA) and phorbol 12-myristate 13-acetate (PMA) to induce its differentiation and proliferation respectively. A biantennary sugar chain fluorescently labelled with 2-aminopyridine (PA), GlcNAc β 1-2Man α 1-6(GlcNAc β 1-2Man α 1-3)Man β 1-4GlcNAc β 1-4GlcNAc-PA, was used to detect

the activity of β -1,4-galactosyltransferase on the cell surface by HPLC. The results show that the activity of β -1,4-galactosyltransferase on the cell surface increases when the cell is treated with RA, but decreases when it is treated with PMA, whereas the activities of the whole cell remain stable during the treatments.

INTRODUCTION

β -1,4-Galactosyltransferase (GalTase; EC 2.4.1.38) is the first glycosyltransferase to have been cloned (reviewed in [1]). It catalyses the transfer of galactose from UDP-galactose to terminal *N*-acetylglucosamine residues on elongating oligosaccharide chains. Two distinct forms of GalTase have been found in various cells. One is located within the Golgi compartment; the other is on the cell membrane. GalTase on the cell membrane is thought to work as a recognition molecule through binding to specific ligands on the adjacent cell surface and the extracellular matrix, and its distribution on the cell surface is related to fertilization [2], cell adhesion [3] and cell migration [4]. However, little is known about its relationship to the degree of malignancy of tumour cells [5].

In this study a human hepatocellular carcinoma cell line, SMMC-7721 [6], was treated with all-*trans*-retinoic acid (RA), a differentiation inducer [7], and phorbol 12-myristate 13-acetate (PMA), which stimulates the proliferation of human hepatocellular carcinoma cells as revealed in our previous research [8]. The activity of GalTase on the cell surface is determined with reverse-phase HPLC.

EXPERIMENTAL

Materials

The SMMC-7721 cell line was established by the Second Military Medical College of China. RPMI-1640 medium was from Gibco. Bovine calf serum was from the Institute of Medical Radiology, Shanghai Medical University. RA and PMA were from Sigma. DEAE-cellulose (DE-52) and Sephadex G-25 were from Whatman. Pronase E, β -galactosidase, UGP-galactose and 2-aminopyridine (PA) were from Merck. HPLC (LC-9A) was a product of Shimadzu. Standard PA-labelled oligosugar chains GlcNAc β 1-2Man α 1-6(GlcNAc β 1-2Man α 1-3)Man β 1-4GlcNAc β 1-4GlcNAc-PA, GlcNAc β 1-2Man α 1-6(Gal β 1-4GlcNAc β 1-2Man α 1-3)Man β 1-4GlcNAc β 1-4GlcNAc-PA, Gal β 1-4GlcNAc β 1-2Man α 1-6(GlcNAc β 1-2Man α 1-3)Man β 1-4GlcNAc β 1-4GlcNAc-PA and Gal β 1-4GlcNAc β 1-2Man α 1-

6(Gal β 1-4GlcNAc β 1-2Man α 1-3)Man β 1-4GlcNAc β 1-4GlcNAc-PA (confirmed by NMR) were kindly donated by Dr. Taniguchi, Osaka University, Japan.

Culture of SMMC-7721 cells

The cells were cultured in RPMI-1640 medium containing 10 % (v/v) bovine calf serum. Cells were induced to differentiate by the addition of RA from a 3 mg/ml solution in ethanol to a final concentration of 300 ng/ml. Cells were allowed to differentiate for 1, 3 or 5 days before being used for GalTase assays. To induce cell proliferation, PMA was added from a 200 mg/ml solution in acetone to a final concentration of 60 μ g/ml. The time for proliferation was also from 1 to 5 days. Control cells for both experiments were cultured in a medium containing the same quantity of ethanol or acetone respectively.

The cells were dissociated with 2 mM EDTA in ice-cold 0.9 % NaCl solution containing 1 μ M PMSF and blown gently into single cells. After being sampled for cell counting, they were washed three times with 0.9 % NaCl containing PMSF. Cells (10⁶; 95 % viable, confirmed by staining with Trypan Blue) were precipitated in a 0.5 ml Eppendorf tube for the assay of cell-surface GalTase. Then the same cells were washed with 0.9 % NaCl containing PMSF, dissolved in 100 μ l of water and homogenized with a Toyoden ultrasonic disrupter for the detection of total cell GalTase.

Preparation of PA-labelled substrate

The method used was a modification of that of Hase [9]. Briefly, human IgG was purified on a DEAE-cellulose column and was identified by SDS/PAGE. The glycan was released from the IgG after hydrolysis with Pronase E and elution through Sephadex G-25. The sugar contents were quantified by the phenol/sulphuric acid method and peaks rich in saccharides were collected. Then the glycans were hydrolysed in 0.1 M HCl to remove the salic acid and fucose, followed by hydrazination, N-acetylation,

pyridylation and digestion with β -galactosidase. Subsequently the pyridylated sugar chains were purified by HPLC at 55 °C with a 0.01 M ammonium acetate buffer, pH 4.0, with a linear concentration gradient of n-butanol established between 0.1 and 0.5 % over 60 min. The flow rate was 1 ml/min. The substrate was collected and its concentration was measured against a standard sugar chain.

Assay of GalTase activity

The method used was that of Shur [10]. The standard incubation mixture for the GalTase assay contained the following components in a final volume of 50 μ l: 0.9 % NaCl, 25 mM sodium dimethyl arsine, 25 mM MnCl₂, 0.2 M galactose, 0.25 mM UDP-Gal, 1 μ M PMSF, 1 μ g/ml leupeptin, 40 nmol of acceptor substrate and 10⁶ whole cells. The mixture was incubated at 37 °C for 2 h, after which the reaction was stopped by being placed in boiling water for 3 min. The samples were filtered and one aliquot of 20 μ l was applied to HPLC as described. The products were quantified by comparison with standard sugar chains. The specific activity of the enzyme is expressed as nmol/h per 10⁶ cells.

In each test an experiment was adopted to exclude the possibility that the cell-surface GalTase reaction products might result from soluble GalTase because of cell lysis. The same numbers of whole cells were incubated for 2 h in the absence of UDP-galactose in the cell-surface reaction cocktail. After that, the supernatants were collected and UDP-galactose was added to check soluble GalTase activity. Cells treated with RA and PMA were also checked accordingly.

The GalTase activity of the whole cell was also assayed. The total volume of the reaction mixture was also 50 μ l and contained 25 mM sodium dimethyl arsine, 25 mM MgCl₂, 0.2 M galactose, 0.25 mM UDP-Gal, 40 nmol of substrate, 0.5 % Triton X-100 and 20 μ l of cell homogenate prepared as described. The incubation time was 1 h.

RESULTS AND DISCUSSION

Product patterns of the GalTase reaction

For both the cell-surface enzyme and the whole-cell activity, three products, 1, 2 and 3, were eluted. The retention time for the substrate and the three products were 17.71, 19.58, 20.17 and 22.15 min respectively (see Figure 1). The structures of the products were identified by comparison with the standard sugar chains confirmed by NMR. They were: product 1, GlcNAc β 1-2Man α 1-6(Gal β 1-4GlcNAc β 1-2Man α 1-3)Man β 1-4GlcNAc β 1-4GlcNAc-PA; product 2, Gal β 1-4GlcNAc β 1-2Man α 1-6(GlcNAc β 1-2Man α 1-3)Man β 1-4GlcNAc β 1-4GlcNAc-PA; and product 3, Gal β 1-4GlcNAc β 1-2Man α 1-6(Gal β 1-4GlcNAc β 1-2Man α 1-3)Man β 1-4GlcNAc β 1-4GlcNAc-PA.

It is not clear why three products appeared. We propose that the enzyme catalyses the addition of Gal to both of the terminal *N*-acetylglucosamine moieties of the biantennary substrate sugar chain, but prefers to form product 2. The steps in the reactions are shown in Scheme 1. In this study, the quantities of the products are determined by: amount of the substrate $\times$ (area under peak 1 + area under peak 2 + twice the area under peak 3)/total peak area.

Activity of GalTase

In SMMC-7721 cells, the average activity of cell-surface GalTase was approx. 2.1 nmol of product/h per 10⁶ cells, and the total activity approx. 42 nmol of product/h per 10⁶ cells. While

Figure 1 Product patterns of GalTase

(a) Assay of cell-surface GalTase. Cells (10⁶) were incubated for 2 h in the cocktail described in the Experimental section. (b) Assay of total GalTase. A homogenate of 2 $\times$ 10⁶ cells was incubated for 1 h in the cocktail described in the Experimental section. (c) Assay of soluble GalTase while detecting cell-surface GalTase. Cells (10⁶) were incubated for 2 h in a cell-surface reaction cocktail except that no UDP-galactose was added. Then the supernatant was collected, UDP-galactose was added and the mixture was incubated for 2 h. In all three panels the peaks of substrate and three products are: peak s (substrate), GlcNAc β 1-2Man α 1-6(GlcNAc β 1-2Man α 1-3)Man β 1-4GlcNAc β 1-4GlcNAc-PA; peak 1 (product 1), GlcNAc β 1-2Man α 1-6(Gal β 1-4GlcNAc β 1-2Man α 1-3)Man β 1-4GlcNAc β 1-4GlcNAc-PA; peak 2 (product 2), Gal β 1-4GlcNAc β 1-2Man α 1-6(GlcNAc β 1-2Man α 1-3)Man β 1-4GlcNAc β 1-4GlcNAc-PA; and peak 3 (product 3), Gal β 1-4GlcNAc β 1-2Man α 1-6(Gal β 1-4GlcNAc β 1-2Man α 1-3)Man β 1-4GlcNAc β 1-4GlcNAc-PA.

Table 1 Effect of RA on the activity of GalTase of SMMC-7721 cells

Living whole cells were used as sources of cell-surface GalTase activity; cell homogenates were used for the assay of total GalTase activity. The concentration of RA was 300 ng/ml. One unit of enzyme activity is defined as nmoles of product/h per 10⁶ cells. Results are means $\pm$ S.D. (*n* = 3). The statistical significance of the effect of RA on cell-surface GalTase was *P* < 0.05 compared with the controls (Student's *t* test).

Treatment time with RA (h)	Distribution	Enzyme activity (units)		RA-treated/control (%)
		Control	RA-treated	
0	Cell surface	2.03 $\pm$ 0.31	1.98 $\pm$ 0.35	97.5
	Total cell	45.6 $\pm$ 4.38	44.8 $\pm$ 3.54	98.2
12	Cell surface	1.87 $\pm$ 0.35	2.23 $\pm$ 0.11	119
	Total cell	41.5 $\pm$ 1.70	43.7 $\pm$ 2.55	105
24	Cell surface	2.01 $\pm$ 0.37	3.64 $\pm$ 0.20	181
	Total cell	37.4 $\pm$ 1.13	39.6 $\pm$ 5.09	105
72	Cell surface	2.14 $\pm$ 0.14	4.03 $\pm$ 0.40	188
	Total cell	43.8 $\pm$ 4.10	40.4 $\pm$ 4.67	92.2
120	Cell surface	1.94 $\pm$ 0.38	3.76 $\pm$ 0.30	193
	Total cell	40.3 $\pm$ 4.38	42.2 $\pm$ 1.27	104

Scheme 1

Table 2 Effect of PMA on the activity of GalTase of SMMC-7721 cells

Living whole cells were used as sources of cell-surface GalTase activity; cell homogenates were used for the assay of total GalTase activity. The concentration of PMA was 60 µg/ml. One unit of enzyme activity is defined as nmoles of product/h per 10⁶ cells. Results are means ± S.D. (*n* = 3). The statistical significance of the effect of PMA on cell-surface GalTase was *P* < 0.05 compared with the controls (Student's *t* test).

Treatment time with RA (h)	Distribution	Enzyme activity (units)		PMA-treated/control (%)
		Control	PMA-treated	
0	Cell surface	2.11 ± 0.01	2.08 ± 0.13	98.5
	Total cell	42.6 ± 2.76	42.3 ± 2.31	99.2
6	Cell surface	2.01 ± 0.40	1.08 ± 0.18	53.7
	Total cell	41.4 ± 1.56	40.6 ± 1.56	98.1
12	Cell surface	2.02 ± 0.18	0.96 ± 0.04	47.5
	Total cell	45.8 ± 1.48	45.4 ± 5.94	99.1
24	Cell surface	2.24 ± 0.13	1.09 ± 0.23	48.6
	Total cell	45.4 ± 6.86	44.8 ± 0.64	98.6
72	Cell surface	2.12 ± 0.17	1.02 ± 0.26	48.1
	Total cell	40.9 ± 0.92	43.1 ± 3.32	105
120	Cell surface	2.12 ± 0.37	1.09 ± 0.18	51.4
	Total cell	42.9 ± 3.75	42.5 ± 3.39	99.1

assaying the cell surface GalTase, only a trace amount of soluble GalTase, which might have resulted from cell lysis, was detected (shown in Figure 1c).

Changes in cell-surface GalTase activity after the addition of RA or PMA are shown in Tables 1 and 2. Treatment with RA caused the GalTase activity on the cell surface to double, whereas PMA caused it to halve. The effects of RA and PMA began at 12 and 6 h respectively after addition. At the same time, neither RA nor PMA altered the GalTase activity of the whole cell, which mainly resulted from the Golgi complex. While assaying the cell surface GalTase in drug-treated cells, the possibility of cell lysis

was also checked as described; only a trace amount of soluble enzyme was detected.

It has been widely reported that in murine cells such as F9 cells and Swiss 3T3 cells, the cell-surface form and the Golgi form of Golgi pools are independently regulated (reviewed in [1]). Our results indicate for the first time that the two forms are also regulated independently in human cells. The mechanism for the regulation is currently the focus of our research.

It has long been known that GalTase is a cell-surface receptor during fertilization. More recently it was found to bind specifically to the E8 domain laminin and to function as a laminin receptor [11]. Their interaction influences various cell events such as cell spreading [12], cell migration on laminin [4] and neurite outgrowth [13]. Blocking or removing GalTase-binding sites within laminin inhibits these events, and such events might be inhibited or stimulated by reagents that selectively inhibit or stimulate cell-surface GalTase [14,15]. We are also studying whether the changes in cell-surface GalTase are related to cell behaviour in human hepatocellular carcinoma cells.

REFERENCES

- Shur, B. D. (1991) *Glycobiology* **1**, 563–575
- Shur, B. D. and Hall, N. G. (1982) *J. Cell Biol.* **95**, 574–579
- Hathaway, H. L., Romagonano, L. C. and Barbiarz, B. S. (1989) *Dev. Biol.* **134**, 351–361
- Appeddu, P. A. and Shur, B. D. (1994) *Proc. Natl. Acad. Sci. U.S.A.* **91**, 2095–2099
- Lopez, L. C., Maillet, C. M., Oleszkowicz, K. and Shur, B. D. (1989) *Mol. Cell. Biol.* **9**, 2370–2377
- Dong, R. (1980) *J. Second Military Medical College* **1**, 5–9
- Al, Z., Zha, X., Liu, Y. and Chen, H. (1990) *Acta Biochim. Biophys. Sin.* **22**, 135–140
- Chai, X. and Chen, H. (1993) *Chinese J. Oncol.* **15**, 182–184
- Hase, S., Ibuki, T. and Ikenaka, T. (1984) *J. Biochem. (Tokyo)* **95**, 197–203
- Shur, B. D. (1982) *Dev. Biol.* **91**, 149–162
- Begovac, P. C., Hall, D. E. and Shur, B. D. (1991) *J. Cell Biol.* **113**, 637–644
- Runyan, R. B., Versalovic, J. and Shur, B. D. (1988) *J. Cell Biol.* **107**, 1863–1871
- Begovac, P. C. and Shur, B. D. (1990) *J. Cell Biol.* **110**, 461–470
- Eckstein, D. J. and Shur, B. D. (1989) *J. Cell Biol.* **108**, 2507–2517
- Evans, S. C., Lopez, L. C. and Shur, B. D. (1993) *J. Cell Biol.* **120**, 1045–1057