Effects of epidermal growth factor and insulin on the activity of *N*-acetylglucosaminyltransferase V

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When quiescent rat hepatocellular carcinoma 7919 cells were treated with epidermal growth factor (EGF) or insulin (stimulators of receptor tyrosine kinase activity), the activity of *N*-acetylglucosaminyltransferase V was increased. The effect of EGF reached a maximum after 10 min and remained high for 30 min, while the effect of insulin reached a maximum after 5 min and decreased after 15 min. Preincubation of the cells with 1-*O*-octadecyl-2-*O*-methylglycerophosphocholine (Et₁₈-OH₃), which

blocked the activation of mitogen-activated protein kinase by EGF, also blocked the activation of *N*-acetylglucosaminyltransferase V by this hormone, whereas the activation of *N*-acetylglucosaminyltransferase V by insulin could not be blocked by Et₁₈-OH₃. Our results suggest that *N*-acetylglucosaminyltransferase V may be regulated by different receptor protein tyrosine kinase pathways.

INTRODUCTION

β1,6-branching of asparagine-linked oligosaccharides is directly associated with metastasis [1,2]. *N*-Acetylglucosaminyltransferase V (GnT-V; EC 2.4.1.155) is a key enzyme in the processing of N-linked sugar chains during the synthesis of glycoproteins [3]. It catalyses the transfer of a GlcNAc residue from UDP-GlcNAc to the α1,6-mannoside arm in acceptor *N*-glycans to form a β1,6-branched structure (Scheme 1).

The activity of GnT-V is increased in various malignant cells [4] and tissues [5,6], and the expression of GnT-V was found to be induced in the early stages of hepatocarcinogenesis [7]. Although the cloning of the cDNA of GnT-V and chromosomal mapping were achieved [8,9] and there have been reports about its long-term regulation [10], little is known about its short-term regulation.

Our previous work has shown that GnT-V is regulated by protein kinase C [11]. The activity of GnT-V in human hepatocarcinoma cells was increased after treatment of the cells with phorbol esters. The variation in GnT-V activity was in accordance with the activity of membranous protein kinase C, and could be blocked by protein kinase C inhibitors.

To further the study of the regulation of GnT-V, we have used two different stimulators of receptor tyrosine kinases, epidermal growth factor (EGF) and insulin [12,13], to treat 7919 rat hepatocellular carcinoma cells. Short-term regulation of GnT-V

by these hormones was detected. We also investigated whether these effects could be blocked by 1-*O*-octadecyl-2-*O*-methylglycerophosphocholine (Et₁₈-OH₃), which blocks the activation of mitogen-activated protein kinase (MAP kinase) by EGF (X. Zhou, unpublished work).

EXPERIMENTAL

Materials

RPMT-1640 medium was from Gibco. Bovine calf serum was from the Institute of Medical Radiology, Shanghai Medical University. The standard 2-aminopyridine (PA)-labelled oligosacchar chains GlcNAcβ1-2Manα1-6(GlcNAcβ1-2Manα1-3)Manβ1-4GlcNAcβ1-4GlcNAc-PA and GlcNAcβ1-2(GlcNAcβ1-6)Manα1-6(GlcNAcβ1-2Manα1-3) Manβ1-4GlcNAcβ1-4GlcNAc-PA (confirmed by NMR) were from Dr. N. Taniguchi (Department of Biochemistry, Medical School, Osaka University, Japan). Mannose, UDP-GlcNAc, GlcNAc, Mes, leupeptin and insulin were from Sigma. PMSF was from Merck. EGF was kindly donated by Dr. S. Higashiyama (Department of Biochemistry, Medical School, Osaka University, Japan). The HPLC apparatus LC-9A was a product of Shimadzu.

Cell culture and preparation of crude enzyme

The 7919 rat hepatocellular carcinoma cell line was cultured as described previously [14,15]. Quiescent cells were obtained by incubating 7919 cells for 48 h in 1640 medium supplemented with 0.5 % calf serum. The quiescent cells (preincubated or not with Et₁₈-OH₃) were treated with EGF or insulin for the indicated periods. The cells were then washed with 0.9 % NaCl and homogenized with an ultrasonic disrupter in an adequate volume of buffer A (20 mM Tris/HCl, pH 7.4, containing 0.25 M sucrose, 2 mM EDTA, 10 mM EGTA, 2 mM dithiothreitol, 0.5 mM PMSF and 5 μg/ml leupeptin). Aliquots were taken for assay of GnT-V. Proteins were quantified by Lowry method [16].

Scheme 1 Action of GnT-V

Abbreviations used: EGF, epidermal growth factor; Et₁₈-OH₃, 1-*O*-octadecyl-2-*O*-methylglycerophosphocholine; GnT-V, *N*-acetylglucosaminyltransferase V; MAP kinase, mitogen-activated protein kinase; PA, 2-aminopyridine.

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Figure 1 Product pattern of GnT-V activity

Samples of 100 μ g of protein homogenate were added to a reaction cocktail, as described in the text. The reaction time was 5 h. S, substrate [GlcNAc β 1-2Man α 1-6(GlcNAc β 1-2Man α 1-3)Man β 1-4GlcNAc β 1-4GlcNAc-PA]; P, product [GlcNAc β 1-2(GlcNAc β 1-6)Man α 1-6(GlcNAc β 1-2Man α 1-3)Man β 1-4GlcNAc β 1-4GlcNAc-PA].

Assay of GnT-V

The substrate for GnT-V, the fluorescently labelled biantennary sugar chain GlcNAc β 1-2Man α 1-6(GlcNAc β 1-2Man α 1-3)Man β 1-4GlcNAc β 1-4GlcNAc-PA, was prepared as described [14,17]. A previously described method for the assay of GnT-V activity was adopted [18]. The 50 μ l reaction mixture contained 100 mM Mes (pH 6.25), 100 μ M substrate, 50 mM UDP-GlcNAc, 0.2 M GlcNAc, 1 % Triton X-100 and 100 μ g of crude enzyme protein prepared as described above. After incubation at 37 $^{\circ}$ C for 5 h, the reactions were stopped by heating at 100 $^{\circ}$ C for 3 min and the samples were centrifuged at 10000 *g* in an Eppendorf tube for 15 min. An aliquot (20 μ l of each sample) was applied to HPLC. Elution was performed at 55 $^{\circ}$ C with 10 mM ammonium acetate containing 0.125 % n-butanol for the first 10 min, followed by a gradient of n-butanol (0.125–0.500 %) in 10 mM ammonium acetate for 40 min and then with 0.500 % n-butanol in the same buffer for the final 10 min. The flow rate was 1 ml/min. The elution patterns of the reaction are shown in Figure 1. The product was quantified as: (amount of the standard peak) $\times$ (area under product peak)/(area under standard peak). The specific activity of GnT-V was expressed as pmol of product/h per mg of protein.

RESULTS AND DISCUSSION

Time phase curve for the effect of EGF on GnT-V activity

Quiescent 7919 cells were treated with EGF (2.5 ng/ml) for 5, 10, 15, 20, 25 or 30 min, followed by assay of GnT-V activity. The activity of GnT-V increased gradually after treatment with EGF (Figure 2). After 10 min of treatment, it had reached a maximum that was twice the activity before treatment, and activity remained high for 30 min.

The effect of EGF on GnT-V activity is blocked by Et₁₈-OH₃

Quiescent 7919 cells were preincubated with Et₁₈-OH₃ at a concentration of 10 μ g/ml for 2 h. Then the cells were washed with 0.9 % NaCl three times, and treated with EGF. After 5 and 10 min, the cells were collected for assay of GnT-V activity (Table 1). We found that, after 5 min of treatment with EGF, GnT-V activity in the cells not preincubated with Et₁₈-OH₃ had increased, as shown in Figure 2. However, in the cells preincubated with Et₁₈-OH₃, GnT-V activity could not be detected.

Figure 2 Effect of EGF on GnT-V activity

Quiescent 7919 cells were treated with EGF (2.5 ng/ml) for 5, 10, 15, 20, 25 or 30 min, and samples of 100 μ g of crude enzyme protein were assayed for GnT-V activity. Values are means $\pm$ S.D. (*n* = 3), and are given in units of pmol of product/h per mg of protein.

Table 1 Et₁₈-OH₃ blocks the effect of EGF on GnT-V activity

Quiescent 7919 cells were preincubated or not with Et₁₈-OH₃ (10 μ g/ml), and then treated with EGF (2.5 ng/ml) for 5 or 10 min. Samples of 100 μ g of crude enzyme protein were assayed for GnT-V activity. Values are means $\pm$ S.D. (*n* = 3).

Time (min)	GnT-V activity (pmol of product/h per mg of protein)		
	Control	EGF-treated	
		No Et ₁₈ -OH ₃	+ Et ₁₈ -OH ₃
5	67.2 $\pm$ 4.3	93.7 $\pm$ 5.7	0 (trace amount)
10	59.7 $\pm$ 4.0	139 $\pm$ 7.0	27.2 $\pm$ 2.1

Figure 3 Effect of insulin on GnT-V activity

Quiescent 7919 cells, preincubated or not with Et₁₈-OH₃ (10 μ g/ml), were treated with insulin (2 nM) for 5, 10, 15, 20, 25 or 30 min, and samples of 100 μ g of crude enzyme protein were assayed for GnT-V activity. Values are means $\pm$ S. D. (*n* = 3), and are given in units of pmol of product/h per mg of protein.

After 10 min of treatment with EGF, GnT-V activity had increased greatly in the cells not preincubated with Et₁₈-OH₃, as shown in Figure 2, but the activity in cells preincubated with Et₁₈-OH₃ had only increased slightly and was lower than that in the control cells.

Time phase curve for the effect of insulin on GnT-V activity

Quiescent 7919 cells were treated with insulin (2 nM) for 5, 10, 15, 20, 25 or 30 min, followed by assay of GnT-V activity (Figure 3). The activity of GnT-V increased after treatment, reaching a maximum at 5 min, and then fell gradually, but remained higher than before treatment.

The effect of insulin on GnT-V activity cannot be blocked by Et₁₈-OH₃

Quiescent 7919 cells were preincubated with Et₁₈-OH₃ at a concentration of 10 µg/ml for 2 h. The cells were then washed with 0.9% NaCl three times, and treated with insulin. After 5, 10, 15, 20, 25 and 30 min, the cells were collected for assay of GnT-V activity (Figure 3). Preincubation with Et₁₈-OH₃ had no effect on insulin-stimulated GnT-V activity.

EGF may cause the autophosphorylation of EGF receptors and their binding to SHC [19,20], which in turn binds to and phosphorylates phospholipase C-γ. Phospholipase C-γ catalyses the degradation of PtdIns(4,5)P₂ into diacylglycerol and Ins(1,4,5)P₃. Diacylglycerol activates protein kinase C, which may in turn activate GnT-V, as was shown in our previous work [11]. We propose that this is one of the pathways whereby EGF could activate GnT-V.

Another possible pathway for EGF is that its receptor binds to Grb protein. This protein may be bound to mSos1 and mSos2, in turn activating Ras and Raf proteins, and thus stimulate the MAP kinase pathway. Blocking the activation of Raf may abort the effect of EGF on GnT-V. This suggests that the regulation by EGF of GnT-V is related to the MAP kinase pathway.

The signal transduction pathways of insulin have not been fully elucidated, but they are definitely different from those of EGF. One pathway is that the insulin receptor binds to other signal molecules through insulin receptor substrate-1 [21]. Our research shows that insulin can regulate GnT-V activity but that its effect is not related to the blocking of activation of Raf.

In conclusion, we have shown that: (1) the activation of two different receptor kinases induces the activation of GnT-V; (2) the time phases of activation by EGF and insulin are different; and (3) Et₁₈-OH₃, a new anti-tumour ether lipid that prevents the binding of EGF-activated Raf protein to cell membranes, can

block the effect of EGF, but not that of insulin, on GnT-V activity. This suggests that GnT-V may be regulated via different receptor protein kinase pathways. The detailed mechanisms involved remain to be studied.

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