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# Regulation of *N*-acetylglucosaminyltransferase V by protein kinases

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When 7721 human hepatocarcinoma cells were treated with 100 nM phorbol-12-myristate-13-acetate (PMA), the activity of *N*-acetylglucosaminyltransferase V(GnT-V) in the cells varied in accordance with the activity of membranous protein kinase C (PKC), but not with that of cytosolic PKC. Quercetin, a non-specific inhibitor of Ser/Thr protein kinase, and D-sphingosine and staurosporine, two specific inhibitors of PKC, blocked the activation of membranous PKC and GnT-V by PMA. Among the three inhibitors, quercetin was least effective. The inhibitory rates of quercetin and staurosporine toward membranous PKC and GnT V were proportional to the concentrations of the two inhibitors. The activities of GnT V and membranous protein kinase A (PKA) were also induced in parallel by dibutyryl cAMP (db-cAMP) and this induction was blocked by a specific PKA inhibitor. When cell free preparations of 7721 cells and human kidney were treated with alkaline phosphatase (ALP) to remove the phosphate groups, the GnT V activities were decreased. These results suggest that GnT V may be activated by membranous PKC or PKA, indirectly or directly, via phosphorylation of Ser/Thr residues.

*Keywords*: N-acetylglucosaminyltransferase V, hepatocarcinoma cells, protein kinase, phosphorylation/dephosphorylation

*Abbreviations*: UDP-, uridine diphospho-; GnT, *N*-acetylglucosaminyltransferase; GlcNAc Gn, *N*-acetylglucosamine; M, mannose; PMA, phorbol-12-myristate-13-acetate; PKC, protein kinase C; PKA, protein kinase A; cAMP, adenosine 3′, 5′-cyclic monophosphate; db-cAMP, dibutyryl cAMP; TPK, tyrosine protein kinase; MES, 2-[*N*-morpholino]ethanesulfonic acid; DTT, dithiothreitol; PMSF, phenylmethylsulfonyl fluoride; EDTA, ethylene diamine tetraacetic acid; EGTA, glycol-bis-($\alpha$-aminoethyl) ether *N*,*N*,*N*′,*N*′-tetraacetic acid; PA, 2-aminopyridine; ALP, alkaline phosphatase; $C_2C_2$, GlcNAc$\beta$1–2Man$\alpha$1–6(GlcNAc$\beta$1–2Man$\alpha$1–3)Man$\beta$R; $C_{2,4}C_2$, GlcNAc$\beta$1–2Man$\alpha$1–6(GlcNAc$\beta$1–4[GlcNAc$\beta$1–2]Man$\alpha$1–3)Man$\beta$R; $C_2C_{2,6}$, GlcNAc$\beta$1–6[GlcNAc$\beta$1–2]Man$\alpha$1–6(GlcNAc$\beta$1–2Man$\alpha$1–3)Man$\beta$R; $C_{2,4}C_{2,6}$, GlcNAc$\beta$1–6[GlcNAc$\beta$1–2]Man$\alpha$1–6(GlcNAc$\beta$1–4[GlcNAc$\beta$1–2]Man$\alpha$1–3)Man$\beta$R where R = 1–4GlcNAc$\beta$1–4GlcNAcAsnX; $Gn_2M_3Gn_2$-PA, $C_2C_2$ where R = 1–4GlcNAc$\beta$1–4GlcNAc-PA; $Gn_3M_3Gn_2$-PA, $C_2C_{2,6}$ where R = 1–4GlcNAc$\beta$1–4GlcNAc-PA.

## Introduction

UDP-*N*-acetylglucosamine: $\alpha$-D-mannoside $\beta$-1,6-*N*-acetylglucosaminyltransferase, or *N*-acetylglucosaminyltransferase-V (GnT-V.EC 2, 4, 1, 155) is a key enzyme in the processing of asparagine-linked sugar chains (N-glycans) during the synthesis of glycoproteins. This enzyme is located in the Golgi apparatus and catalyses the transfer of an N-acetylglucosamine (GlcNAc) residue from UDP-GlcNAc to the $\alpha$-1,6-Mannoside arm in acceptor N-glycans ($C_2C_2$ biantennary or $C_{2,4}C_2$ triantennary complex type) to form a $\beta$-1,6 branched structure (GlcNAc-$\beta$-1,6-Man) in the products ($C_2C_{2,6}$ triantennary or $C_{2,4}C_{2,6}$ tetraantennary N-glycan) [1]. The activity of GnT-V was reported to be increased in some malignant cells, such as azo dye hepatoma, AH66, AH-130, Yoshida Sarcoma [2], *N*-nitrosodiethylamine induced hepatoma [3], breast carcinoma [4], and various cells transformed by tumour viruses [5–7], tumorigenic DNA [8] and oncogenes [4, 7, 9, 10]. Although rat kidney GnT-V was purified and characterized [11], and the cDNA clone for the complete coding sequence for GnT-V was isolated and expressed in COS-7 cells [12], the regulation of this enzyme is not understood. It was found in our laboratory that GnT-V activity was increased in 7721 human hepato-

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carcinoma cells by treatment with phorbol-12-myristate-13-acetate (PMA) [13], an activator of calcium-phospholipid dependent protein kinase (protein kinase C, PKC) [14]. In the present studies, the effects of some inhibitors of protein kinases, including PKC, adenosine 3′5′-cyclic monophosphate (cAMP) dependent protein kinase (protein kinase A, PKA) and tyrosine protein kinase (TPK), on GnT V activity are reported.

## Materials and methods

### *Chemicals*

UDP-GlcNAc, GlcNAc, MES, β-galactosidase (jack bean), Sephadex G-25, 2-aminopyridine, $NaBH_3CN$, Protamine (Grade IV, Salmon), Poly Glu:Tyr (4:1), Leupeptin, DTT, PMA, db-cAMP, quercetin, D-sphingosine, staurosporine, genistein, PKA inhibitor (rabbit sequence, TTYADFIASGRTGRRNAIHD), and human placental ALP were purchased from Sigma. Pronase E and PMSF were purchased from Merck. [$\tau$-$^{32}$P]-ATP (3000 Ci mmol$^{-1}$) was the product of Amersham and other chemicals were commercially available in Shanghai.

### *Cell culture and preparation of crude enzyme*

7721 Human hepatocarcinoma cell line was cultured as previously described by our laboratory [15]. Inhibitors of protein kinase dissolved in DMSO (quercetin, staurosporine, genistein) or 50% ethanol (D-sphingosine) were added to the medium at different concentrations and at different times of culture before cell collection as indicated in the Tables and Figures. One hundred nM PMA was added at 30 min before cell collection except for Fig. 1, in which the time of PMA treatment is shown under the Figure.

The collected cells were homogenized 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 DTT, 0.5 mM PMSF, 5 mg l$^{-1}$ Leupeptin) and centrifuged at 700 × **g** for 20 min. The supernatants were recentrifuged at 100 000 × **g** for 60 min and the second supernatants were used as a source of crude cytosolic protein kinases. The pellets were suspended in Buffer A and aliquots were taken to determine the activities of GnT-V, membranous PKA and TPK. The remaining suspensions were mixed with equal volumes of Buffer A containing 1% Triton X-100 and subjected to ultrasonication for 12 s × six times in an ice bath. After further extraction in an ice bath for 60 min and centrifugation at 100 000 × **g** for 60 min, the supernatants were used as a source of membranous PKC.

### *Preparation of glycan substrate of GnT-V*

Biantennary N-glycans were prepared from human serum transferrin purified by the method of Feng [16], followed by pronase digestion, hydrazinolysis, *N*-acetylation and fluorescence labelling with 2-aminopyridine (PA) at the reducing end of the glycans according to Hase *et al.* [17]. The labelled biantennary glycans were desialylated by acid, treated with β-galactosidase, and the final product ($Gn_2M_3Gn_2$-PA heptasaccharide) was purified by Sephadex G-15 chromatography as described by Gu *et al.* [3].

### *Assay of GnT-V*

A previously described method [2, 3] was used. The reaction mixture (50 μl) contained 100 mM MES (pH 6.25), 100 μM $Gn_2M_3Gn_2$-PA, 50 mM UDP-GlcNAc, 0.2 M GlcNAc, 1% Triton X-100 and enzyme protein 80–100 μg. After incubation at 37 °C for 5 h, the reactions were stopped by heating at 100 °C for 3 min and the samples were centrifuged at 5000 rpm in an Eppendorf centrifuge for 15 min. An aliquot (20 μl) of each sample was applied to a TSK-gel ODS-column (6 × 150 mm) to separate the $C_2C_{2,6}$ $Gn_3M_3Gn_2$-PA product from the acceptor substrate. Elution was performed at 55 °C with 10 mM ammonium acetate (AmAc), containing 0.125% *n*-butanol for the first 10 min, followed by a gradient concentration of *n*-butanol (0.125–0.500%) in 10 mM AmAc for 40 min and 0.500% *n*-butanol in the same buffer for the last 10 min. The flow rate was 1 ml min$^{-1}$. All the samples were assayed in duplicate. The specific activity of GnT-V was expressed as pmol of GlcNAc transferred per h per mg protein.

### *Assay of protein kinases*

PKC was assayed according to the method of Kikkawa *et al.* [18] as described by Yu *et al.* [19], using 500 μg ml$^{-1}$ protamine and 10 μM [$\tau$-$^{32}$P]-ATP (200–300 cpm pmol$^{-1}$) as the substrates.

PKA was determined by a modified method [20] as described by Tang and Chen [21], using 1 mg ml$^{-1}$ Histone 1 and 25 μM [$\tau$-$^{32}$P]-ATP as the substrates. TPK was assayed by a modified method of Kong and Wang [22], using poly Glu:Tyr (4:1) as the $^{32}$P-phosphate group acceptor and 50 μM [$\tau$-$^{32}$P]-ATP [21]. The samples for protein kinase were all assayed in duplicate.

### *Treatment of crude enzyme with alkaline phosphatase*

Cell homogenates of 7721 and human kidney were prepared and centrifuged at 700 × **g** for 30 min and a portion of the supernatant was incubated with 100 nM PMA at 37 °C for 7.5 min. After the supernatants were centrifuged at 100 000 × **g** for 60 min, the pellets were dissolved in 0.1 M Tris-HCl, and the protein contents determined. The pH of the crude enzyme preparations was adjusted to 8.0 after Triton X-100 was added to 1%. Then the enzyme samples were incubated in the absence or presence of 200 mU human placental ALP per mg protein, in the presence of 10 mM $MgCl_2$ at 37 °C for 30 min. The reactions were stopped in an ice bath and

the GnT-V activity was immediately assayed after the pH was checked to be at 6.25.

*Protein determination*

Protein concentration was determined by the Lowry method [23] except for the sample of membranous PKC which was assayed by the Bradford method [24]. Bovine serum albumin was used as standard protein.

## Results

*Activation of PKC and GnT-V by PMA in 7721 cells*

When the 7721 cells were treated with PMA, an activator of PKC, for 60 min and the activities of membranous PKC, cytosolic PKC and GnT-V were assayed simultaneously at different intervals during incubation, the activity of membranous PKC, as shown in Fig. 1, was increased within 10 min, reached a peak at 30 min and then decreased. The activity of cytosolic PKC gradually decreased with prolongation of the incubation time in agreement with previous reports on the regulation of PKC [14, 25]. Interestingly, the activity of GnT-V was elevated in parallel with membranous PKC, and also reached a peak at 30 min, and decreased simultaneously with membranous PKC. This result suggests that GnT-V, like membranous PKC, is activated by PMA.

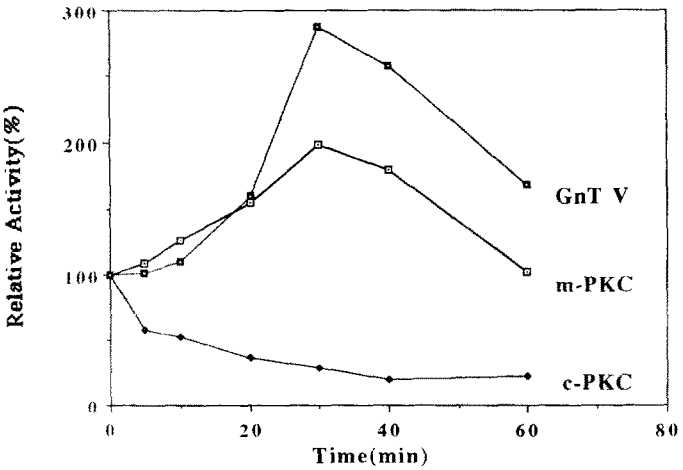


**Figure 1.** Time-phase of the activation of PKC and GnT-V by PMA in 7721 cells. After cells were cultured for 2 days, the 'treated' groups were changed to a medium containing PMA (1 mg PMA dissolved in DMSO, diluted to 0.2 mg ml$^{-1}$ by acetone and added to culture medium to a final concentration of 100 nM) for 5–60 min and control groups were changed to a medium containing the solvent alone. Data are expressed as the mean value of two experiments. m-PKC, membranous PKC; c-PKC, cytosolic PKC.

*Blocking effects of PKC inhibitors on PMA-activated membranous PKC and GnT-V*

After 7721 cells were treated with 100 nM PMA for 30 min, the activities of membranous PKC and GnT-V were increased to 205% and 256% of control values respectively, consistent with the results shown in Fig. 1. When inhibitors of PKC were added at various times before the addition of PMA, both membranous PKC and GnT-V were significantly inhibited. If the value of the sample treated with PMA alone is set at 100%, the GnT-V activities in the presence of quercetin, D-sphingosine and staurosporine were 53.6%, 37.6% and 35.0% respectively (Table 1). The blocking efficiency was more potent for two PKC specific inhibitors, D-sphingosine [26] and staurosporine [27] than that for the non-specific inhibitor of Ser/Thr protein kinase, quercetin [28]. These results indicate that the PKC inhibitors block the activation of GnT-V by PMA pretreatment.

*Dose-effect relationship between PKC inhibitors and GnT-V activities*

7721 cells were pretreated with different concentrations of quercetin and staurosporine for 30 min, followed by the addition of PMA to 100 nM and incubation for another 30 min. GnT-V, membranous and cytosolic PKC were then determined. As indicated in Figs 2 and 3, the activity of GnT-V declined progressively and was almost parallel with membranous and cytosolic PKC as the concentration of quercetin or staurosporine was increased, except that the cytosolic PKC activity was not further decreased, but GnT-V was still decreased when the concentration of staurosporine was more than 30 nM

**Table 1.** Blocking effects of PKC inhibitors on PMA-activated m-PKC and GnT-V.

| *Treatment (concentration)* | | *Membranous PKC* | *GnT-V* | |
|---|---|---|---|---|
| | | *% of control* | *pmol h$^{-1}$ per mg protein* | *%* |
| Control | – | 100.0 | 54.0 | 100.0 |
| PMA | (100 nM) | 205.3 | 138.4 | 256.2 |
| PMA + quercetin | (60 μM) | 36.8 | 75.6 | 140.0 |
| PMA + D-sphingosine | (25 μM) | 33.3 | 52.0 | 96.3 |
| PMA + staurosporine | (60 nM) | 73.1 | 48.4 | 89.6 |

Preincubation time of PMA and PKC inhibitors: PMA 30 min, quercetin 60 min, D-sphingosine 210 min, staurosporine 60 min. The values in the Table are the mean value of two (PMA + PKC inhibitors) or three (PMA treated only) experiments.

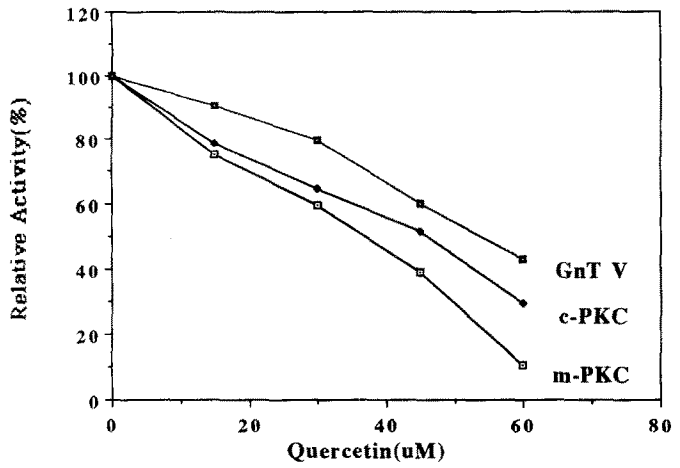


**Figure 2.** Inhibition of PKC and GnT-V by different concentrations of quercetin. 7721 Cells were pretreated with different concentrations of quercetin for 30 min, followed by the addition of 100 nM PMA and incubation for another 30 min. Control cells were treated with 100 nM PMA for 30 min only and the activities of GnT-V and PKC were set at 100%.

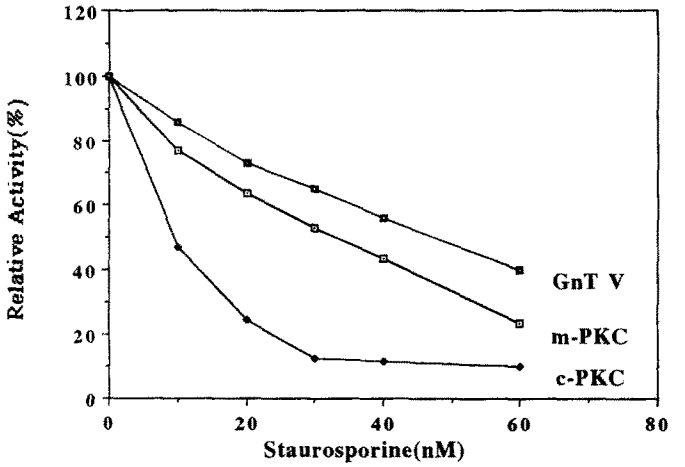


**Figure 3.** Inhibition of PKC and GnT-V by different concentrations of staurosporine. 7721 cells were pretreated with different concentrations of staurosporine for 30 min, followed by the addition of 100 nM PMA and incubation for another 30 min. Control cells were treated with 100 nM PMA for 30 min only and its activities of GnT-V and PKC were set at 100%.

(Fig. 3). It can be concluded that the activity of GnT-V is inversely proportional to the concentration of PKC inhibitors and is in parallel to the activity of membranous PKC.

*Stimulation of membranous-PKA and GnT-V by db-cAMP and the blocking effect of PKA-inhibitor*

As shown in Table 2, when 7721 cells were treated with 0.5 mM db-cAMP for different times, the activities of membranous PKA and GnT-V were simultaneously elevated to a similar degree at 15 min and both reached their maximum at 30 min, being 188.9% and 166.8% of the control value, they then declined to the same degree to a value lower than the control. If the cells were pre-treated with 50 nM of a specific inhibitor of PKA [29] at 60 min prior to db-cAMP, membranous PKA and GnT-V were inhibited to only 88.9% and 111.1% of control levels respectively at 30 min of db-cAMP treatment. These results prove that GnT-V is not only activated by membranous PKC, but also stimulated by membranous PKA, and that the PKA-inhibitor blocks db-cAMP activated GnT-V.

*Inhibition of non stimulated GnT-V by inhibitors of Ser/Thr protein kinases*

In order to study the possibility of regulation of GnT-V by protein kinase in the non-stimulated (basal) condition, the three inhibitors of PKC, quercetin, D-sphingosine and staurosporine, were tested for their effects on GnTV activity without preactivation by PMA. It was found that all the inhibitors also decreased the basal activity of GnT-V. The inhibitory rates were 26.4%, 17.0% and 19.1% for quercetin, D-sphingosine and staurosporine respectively, which were not as significant as the inhibitory rates on PMA activated GnT-V. The specific inhibitor of PKA also inhibited the basal activity of GnT-V and the inhibitory rate was 34.6%, which was similar to the inhibitory rate (34.4%) on db-cAMP activated GnT-V.

*Effects of genistein and ortho-vanadate on the activity of GnT-V*

Genistein was reported as a specific inhibitor of TPK [30], and ortho-vanadate is well known as a specific inhibitor of protein tyrosine phosphatase. The effects of these two compounds at a 100 $\mu$M concentration on GnT-V activity when preincubated with 7721 cells for 120 min and the effect of genistein on TPK activity were investigated. It was discovered that membranous and cytosolic TPK were significantly inhibited by genistein to 34.8% and 41.1% of the control value respectively, but no inhibitory or stimulative effect was found on GnT-V with genistein or ortho-vanadate (106.4% and 98.0% of the control value respectively) which indicates that GnT-V might not be regulated by TPK and tyrosine phosphorylation.

*Effect of ALP treatment on the activities of GnT-V*

As shown in Table 3, PMA also showed an inductive effect on GnT-V (59.5 and 79.9 pmol $h^{-1}$ per mg protein in the absence and presence of PMA respectively) in the 700 × g supernatant of the 7721 cell homogenate, which contained both the membranous and cytosolic fractions. However the effect of PMA was less significant than in the intact cell, suggesting that the membranous PKC may

**Table 2.** Stimulation of m-PKA and GnT-V by db-cAMP and the blocking effect of PKA-inhibitor.

| *Group* | *Treated time (min)* | *Membranous PKA* | | *GnT-V* | |
|---|---|---|---|---|---|
| | | *pmol min$^{-1}$ per mg protein* | *%* | *pmol min$^{-1}$ per mg protein* | *%* |
| Control | 0 | 72.0 | 100.0 | 57.6 | 100.0 |
| db-cAMP | 15 | 84.1 | 116.3 | 68.2 | 118.4 |
| | 30 | 136.0 | 188.9 | 96.1 | 166.8 |
| | 45 | 62.1 | 86.3 | 49.5 | 85.9 |
| PKA-inhibitor | 90 | 64.2 | 88.9 | 64.0 | 111.1 |
| + db-cAMP | 30 | | | | |

Concentrations of db-cAMP and PKA-inhibitor were 0.5 mM and 50 nM respectively.

**Table 3.** Effect of ALP treatment on the activities of GnT-V in crude enzyme preparations.

| *Group* | *GnT-V* | |
|---|---|---|
| | *pmol hr$^{-1}$ per mg protein* | *% of control* |
| Enzyme from 7721 without PMA treatment | | |
| −ALP | 59.5 | 100.0 |
| +ALP | 28.6 | 48.1 |
| Enzyme from 7721 with PMA treatment | | |
| −ALP | 79.9 | 134.3 (100.0) |
| +ALP | 32.0 | 53.8 (40.1[a]) |
| Enzyme from human kidney | | |
| −ALP | 57.0 | 100.0 |
| +ALP | 35.8 | 62.8 |

The experimental procedure is described in 'methods'.
[a]The percentage of ALP treated sample as compared with ALP untreated sample in PMA treated enzyme preparation.
The values in the Table are the mean values of two experiments.

also be activated by PMA in a cell-free system, but to a lesser extent. When the crude enzyme preparations were treated with alkaline phosphatase (ALP) at pH 8.0 for 30 min to remove the covalently bound phosphate groups, the GnT-V activities were significantly decreased both in PMA pre-treated and untreated samples. The same result was obtained when human kidney crude enzyme was used instead of the enzyme from 7721 cells, suggesting that GnT-V is regulated by a phosphorylation/dephosphorylation mechanism.

## Discussion

The regulation of glycosyltransferases by protein kinases has been described previously. Momoi *et al.* [31] and Scheideler and Dawson [32] reported that CMP-*N*-acetylneuraminic acid: lactosylceramide sialyltransferase ($GM_3$ synthase or sialyltransferase I) was activated by PMA via PKC in HL-60 cells, and UDP-*N*-acetylgalactosamine: [$GM_3$] *N*-acetylgalactosaminyltransferase ($GM_2$ synthase) was activated by cAMP. However, these enzymes were both involved in the synthesis of glycolipids. Bruce *et al.* [33] reported that mammalian $\beta$1,4 galactosyltransferase was stimulated three-fold when a $P^{58}$ Ser/Thr protein kinase was expressed *in vivo* and that this enzyme was inhibited by treatment with acid phosphotase or with antibody to the $P^{58}$ protein kinase *in vitro*, but it was not determined whether $\beta$-1,4 galactosyltransferase was regulated by PKC or PKA.

Our results suggest that GnT-V is regulated by Ser/Thr protein kinases, such as PKC and PKA rather than by tyrosine protein kinase. However, the possibility cannot be ruled out that other Ser/Thr kinases, in addition to PKC and PKA, may participate in the regulation of GnT-V *in vitro*, such as $Ca^{2+}$-calmodulin dependent protein kinase. Our unpublished results showed that trifluoparazine, a potent inhibitor of this protein kinase [34] also inhibited GnT-V. It can be speculated that PKC is more important than PKA in the control of GnT-V *in vivo*, because the elevation of GnT-V treated with 100 nM PMA (Fig. 1) is more than that treated with 0.5 mM db-cAMP (Table 2), and the concentration of the latter is far higher than that in physiological conditions. Membranous PKC is possibly more important than the cytosolic enzyme, since the activity change of cytosolic PKC was not in parallel with GnT-V after the cells were treated with PMA at different times (Fig. 1), or treated with different doses of staurosporine (Fig. 3). Moreover,

it was also discovered that trifluoperazine markedly inhibited membranous PKC and GnT-V but did not significantly affect the activity of cytosolic PKC (data not shown). It is reasonable to consider that the Golgi-localized GnT-V is regulated by membranous PKC due to the presence of this PKC and Golgi membranes in our crude GnT-V enzyme preparation.

The induction of GnT-V by PMA and another cAMP derivative (8-Bromo cAMP) was repeated in a rat hepatocarcinoma cell line CBRH-7919; whether this is a common phenomenon in the regulation of GnT-V remains to be investigated.

Using ALP to dephosphorylate the phosphorylated product(s) of the protein kinase(s), it was found that the activities of GnT-V were decreased both in the PMA activated and non-activated cell free systems of 7721 as well as in a human kidney preparation, indicating that the phosphorylated form is more active than the dephosphorylated one. In previous experiments we have proved that in our ALP reaction systems the covalently-bound phosphate groups were almost entirely removed, so the dephosphorylated form may be considered as a low active form rather than an inactive form. This speculation was supported by the similar levels of GnT-V specific activity in the ALP treated samples with or without PMA preincubation (32.0 and 28.6 pmol $h^{-1}$ per mg protein respectively). It is not certain, however, whether GnT-V is regulated by protein kinases directly or indirectly. Protein kinases may phosphorylate GnT-V directly at its Ser/Thr residue(s) within the motif(s) recognized by PKC or PKA [35]. Protein kinases may also first phosphorylate a protein activator or inhibitor, and transform the activator to an active form or the inhibitor to an inactive form; the regulatory protein then stimulates GnT-V by activation or de-inhibition. The above problems as well as the phosphorylated site in GnT-V or regulatory protein must be resolved by further investigation.

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