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Modulation of the basal activity of N-acetylglucosaminyltransferase V by phosphatidylinositol-3-kinase/protein kinase B signaling pathway in human hepatocarcinoma cells

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The modulation of GnT-V activity by signaling molecules in PI-3-K/PKB pathway in human hepatocarcinoma cell line 7721 was studied. GnT-V activity was determined after the transfection of sense or antisense cDNA of PKB into the cells, as well as the addition of activators, specific inhibitors, and the antibodies to the enzyme assay system or culture medium. It was found that the basal activity of GnT-V was up regulated by the sense and down regulated by the antisense cDNA of PKB transfected into 7721 cells. GnT-V was activated by PIP_2, PIP_3 or GTPγ[S] added to the assay system, and the activation of PIP_2 or GTPγ[S] was abolished by LY2940002, a specific inhibitor of PI-3-K, but the activation of PIP_3 was not attenuated by LY2940002. In addition, GnT-V activity in cultured parental or H-ras transfected cells was inhibited by the antibody against PKB or PI-3-K. These findings demonstrated the involvement of PI-3-K/PKB signaling pathway in the regulation of GnT-V. Moreover, ET18-OCH_3, an inhibitor of Raf translocation and PI-PLC enzyme, which produces the activator of PKC, as well as the antibodies against Raf-1 or MEK also inhibited GnT-V activity in the parental and H-ras transfected cells. The inhibitory rates, however, were less in the transfected cells than those in the parental cells. These results reveal that in parental and H-ras transfected 7721 cells, the basal activity of GnT-V is also regulated by the Ras/Raf-1/MEK/MAPK cascade in addition to PI-3-K/PKB signaling pathway. The significance of these two pathways in the regulation of GnT-V and their relations to the activation of PKC previously reported by our laboratory (Ju TZ *et al.*, 1995 *Glyconjugate J* 12, 767–772) was discussed.

Keywords: **signal transduction, N-acetylglucosaminyltransferase V (GnT-V), basal activity, phosphoinositide 3-kinase (PI-3-K), protein kinase B (PKB)**

Abbreviations: **GnT-V, N-acetylglucosaminyltransferase V; GlcNAc, N-acetylglucosamine; Man, mannose; PKB, protein kinase B; PKC, protein kinase C; MAPK, mitogen-activated protein kinase; MEK, MAPK/ERK kinase; Raf, MEK kinase; PI, phosphatidylinositol; PIP_2, phosphatidylinositol-4,5-biphosphate; PIP_3, phosphatidylinositol-3,4,5-triphosphate; PI-3-K, phosphoinositide-3-kinase; GTPγ[S], guanosine 5′-O-(3-thiotriphosphate); LY294002, 2-(4-morpholinyl)-8-phenyl-1(4H)-benzopyran-4-one; PMA, phorbol-12-myristate-13-acetate; ET18-OCH_3, 1-O-octadecyl-2-O-methyl-glycerophosphocholine; cAMP, cyclic-3′,5′-adenosine monophosphate; PVDF, polyvinyline difluoride; ECL, enhanced chemiluminescence; HRP, horse radish peroxidase; TAE, Tris-Acetate-EDTA; PBS, phosphate buffer saline.**

Introduction

UDP-N-acetylglucosamine α-D-mannoside β-1,6-N-acetylglucosaminyltransferase V or N-acetylglycosaminyltransferase V (E.C. 2,1,24,155, GnT-V) is a key enzyme in the processing of asparagine-linked glycans (N-glycans) during the synthesis of glycoproteins. It is located in the Golgi apparatus and catalyses the transfer of a GlcNAc residue from UDP-GlcNAc to the α-1,6 mannose residue in the pentasaccharide core of acceptor glycans to form a β-1,6 branched structure (GlcNAcβ1,6 Manα1,6) in the produced tri- or tetra-antennary N-glycans [1]. GnT-V was considered to be a carcinogenesis-associated

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enzyme [2,3]. We have reported that GnT-V from a human hepatocarcinoma cell line 7721 was regulated by PKC through a direct or indirect phosphorylation mechanism [4], since GnT-V was activated by PMA, the activator of PKC, and inhibited by PKC inhibitors or by treatment with phosphatase. Recently, a new protein kinase, PKB, was discovered which is involved in the signal transduction pathway of insulin and some growth factors, such as nerve growth factors and platelet derived growth factor [5–7]. The signal of insulin or growth factor passes through the receptor with tyrosine kinase activity → PI-3-K → PI-3, 4, 5-P_3 → 3-phophoinositide dependent kinase (PDK or PKB kinase) → PKB. Furthermore, it has been known that GnT-V is regulated by a growth factor signaling pathway containing Ras, Raf, or Src and neu/her-2 [2]. Therefore, it is interesting to study whether GnT-V is regulated by the PI-3-K/PKB pathway other than Ras/Raf pathway and PKC. The present studies dealt with the modulation of GnT-V basal activity in parental and H-ras transfected 7721 cells by PKB and PI-3-K using transfections of the sense and antisense cDNA of PKB, as well as antibodies of PKB and PI-3-K, or specific inhibitors or activators of PI-3-K. The role of Ras/Raf pathway on the regulation of GnT-V was also investigated.

Materials and methods

Materials

RPMI 1640 medium and G418 were purchased from Gibco/BRL. Rabbit polyclonal antibody of PKB and PI-3-K (p110 α kinase domain) were from Biolab and Santa Cruz Company respectively. HRP labeled second antibodies (goat anti-rabbit IgG) was obtained from DAKO Company. Rabbit anti-human Raf-1 sub-domain III antibody, rabbit anti-frog MEK-2 C terminus antibody from Kinetek Biotech Corp, and PIP_3 were gifts from Prof PC Choy in Faculty of Medicine, Manitoba University, ET18-OCH_3 was donated by Prof G Authur in the same university. LY294002 was purchased from Calbiochem Company. UDP-GlcNAc, MEC buffer, PIP_2, GTPγ[S] and the non-immune IgG of rabbit were from Sigma, and DNA restriction endonucleases, T_4 DNA ligase were from Promega. PVDF membrane was purchased from Bio-Rad, ECL assay kit was from Amersham Corp. The fluorescence (2-aminopuridine, AP) labeled acceptor substrate of GnT-V, biantennary N-glycan ($GlcNAc_2Man_3GlcNAc_2$-AP) was prepared by our laboratory [4]. Other reagents were commercially available in China.

7721 human hepatocarcinoma cell line was obtained from the Institute of Cell Biology, Academic Sinica. PKB cDNA containing plasmid (pSGS-PKB^{GAG}) was a kind gift from Prof. P Coffer of University Hospital Utrecht in Netherlands. 7721 cell line transfected with plasmid containing sense cDNA of PKB (pcDNA3/PKB) was established, characterized and provided by our department. 7721 cell line transfected with plasmid pcDNA3/H-ras and the mock-transfected cells were obtained from the Institute of Liver Cancer in our university, pcDNA3 expression vector was from Invitrogen Company, USA.

Cell culture and treatment

7721 cells were cultured at 37°C, 5% CO_2 in RPMI-1640 medium containing 10% fetal calf serum, penicillin and streptomycin as previously described by our laboratory [4]. Inhibitor, antibody or non-immune rabbit IgG was added to the culture medium 30 min before the cells were harvested. When antibody was used, the cells were permeabilized by pre-treatment with 10 μmol/L digitonin for 10 min before the addition of antibody [8].

Construction of anti-sense PKB plasmid, pcDNA3/PKB-AS

The 2.6 kb fragment containing GAG-PKB cDNA was cut out from pSGS-PKB^{GAG} (6.5 kb) with *Eco*RI and *Bgl* II. This fragment was digested with *Nco*I, and the 1.8 kb fragment containing PKB cDNA was separated, purified, and blunt-ended. The fragment was then ligated with *Eco*RI linkers and inserted into the *Eco*RI site of pBluescriptSK. There was a *Pst*I site near the 5'end of the PKB cDNA, and 1.7 kb apart from the down-stream another *Pst*I site (Figure 1). The direction of inserted PKB cDNA can be determined by *Pst*I digestion of the recombinant plasmid. A 1.7 kb fragment can be cut out by *Pst*I (*Pst*I mapping, Figure 2). The plasmid with 5'end of the inserted PKB cDNA closed to the *Hind* III site was selected, and digested with *Hind*III. The linearized cDNA was blunt ended, and treated with *Bam*HI. The blunt-ended *Hind*III-*Bam*HI fragment (1.8 kb) was isolated and ligated with eukaryotic expression plasmid pcDNA3, which was cut with *Eco*R V plus *Bam*H I. The recombinant pcDNA3 inversely inserted with PKB cDNA was verified by sequencing and named pcDNA3/PKB-AS (Figure 1).

Transfection of pcDNA3/PKB-AS

The pcDNA3/PKB-AS plasmid (7.2 kb) or pcDNA3 control (5.4 kb) was transfected into 7721 cells using a standard electroporation method [9]. Briefly, after cells were washed 3 times with PBS and re-suspended in PBS, then 0.8×10^7 cells in 0.8 ml were transfected with 25 μg plasmid or vector by electroporation using Gene Pulser II (Bio-Rad) at 250 V/0.4 cm and 1000 μF. The transfected 7721 cells were diluted to 10.8 ml with PRMI-1640, and transferred to RPMI 1640 medium containing G418 (0.8 mg/ml) after 24 h. Neomycin-resistant cells were obtained after 2 to 3 weeks and re-cloned by serial dilution.

Agarose electrophoresis

Aliquot of $1 \sim 2$ μg DNA was added onto 1% agarose prepared in TAE (pH 7.6) containing 8 μg ethidium bromide, and electrophoresis was carried out in TAE buffer at $60 \sim 80$ V for 1 h. The separated fragments were observed under UV light.

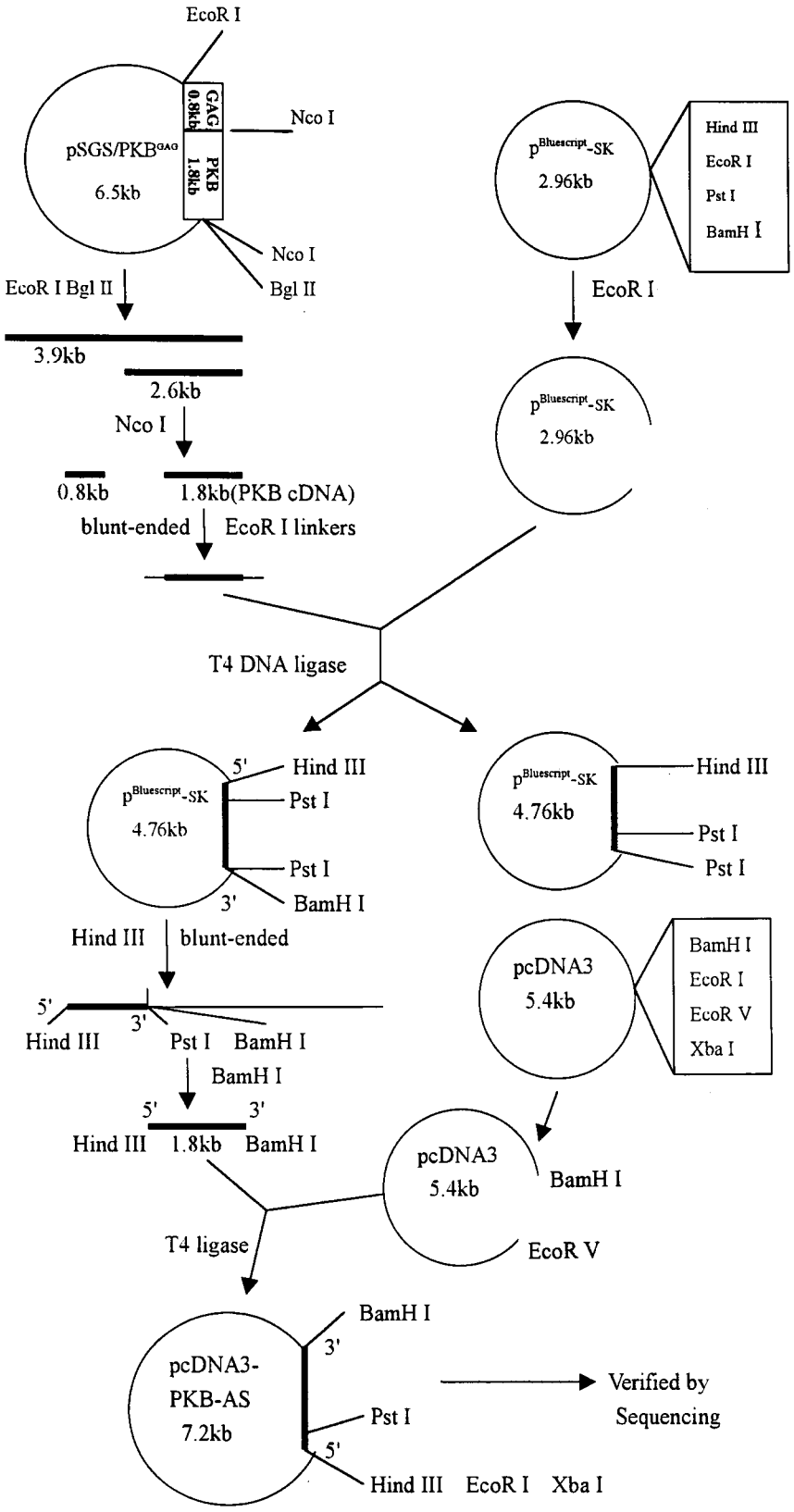

Figure 1. Construction of plasmid containing antisense cDNA of PKB.

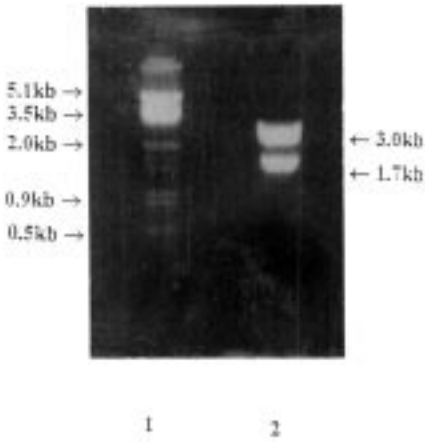

Figure 2. *Pst*I mapping of pBluescriptSK-PKB. (1) Markers λDNA/*Hind*III + *Eco*RI, (2) pBluescriptSK-PKB digested with *Pst*I.

Western blotting

The cells were homogenized in 0.1 M MES buffer, pH 6.5, and centrifuged with $1000 \times g$ at 4°C for 15 min. The protein concentration was determined by the Lowry method [10]. Western blotting was performed according to a modified method of Marone [11]. Aliquots of 30 or 60 μg of protein samples were separated with 10% SDS-polyacrylamide gel electrophoresis and electro-blotted onto a PVDF membrane, which was then blocked with 5% fat-free dry milk in $1 \times$ TBST (0.1 M Tris, 0.15 M NaCl, 0.05% Tween 20, pH 7.4) after blotting. The membrane was treated with a 1:500 dilution of rabbit polyclonal anti-rat PKB antibody in 5% fat-free dry milk in PBS, followed by incubation with 1:200 diluted HRP-labeled second antibody (anti-rabbit IgG), then the staining was performed with the ECL reagent.

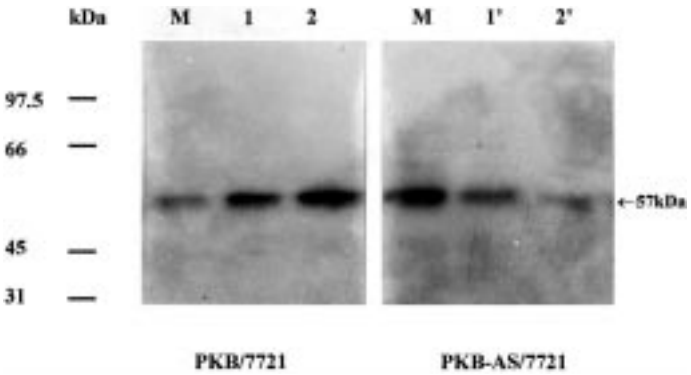

Figure 3. Western blot of PKB protein in pcDNA3/PKB and pcDNA3/PKB-AS transfected 7721 cells. PKB/7721: pcDNA3/PKB transfected 7721 cells (30 μg protein). PKB-AS/7721: pcDNA3/PKB-AS transfected 7721 cells (60 μg protein). M:7721 cells mock-transfected with pcDNA3 vector. 1,2,: Positive pcDNA3/PKB transfectant. 1′,2′,: Positive pcDNA3/PKB-AS transfectants. The experiment was described under "Materials and Methods".

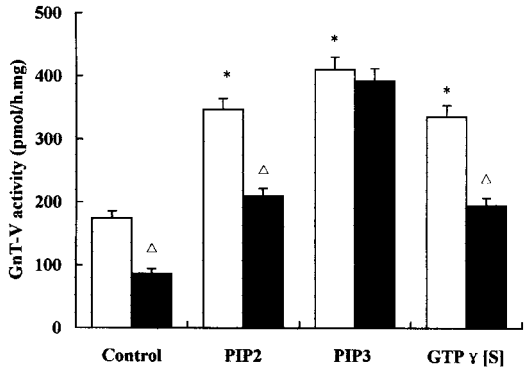

Figure 4. Activation of GnT-V by PIP_2, PIP_3, GTPγ[S] and the abolishment of the activation by LY294002. PIP_2 50 μM or PIP_3 50 μM or GTPγ[S] 10 μM was added to the reaction mixture of GnT-V assay in the absence (open bars) or presence (closed bars) of 15 μM LY294002. Data were expressed as mean value of 3 independent experiments ± S.D.
*$p < 0.01$ compared with control, Δ, $p < 0.05$ compared with the value in the absence of LY294002.

Enzyme preparation and assay of GnT-V

The preparation of crude GnT-V and the assay of GnT-V were carried out according to the methods described previously [4]. A routine reverse-phase HPLC method using an ODS C18 column was used for the separation of the fluorescence labeled substrate and product. The concentration of acceptor substrate was increased to 400 μmol/L. When PIP_2, PIP_3 GTPγ[S] and/or LY294002 were added to the assay system, their concentrations were indicated under Figure 4, and the reaction mixture was incubated for 5 h.

Results

Characterization of PKB protein in PKB/7721 and PKB-AS/7721 cells

PKB is a protein with *M*r. of 57 kDa [12]. The expression of PKB protein in pcDNA3/PKB and pcDNA3/PKB-AS transfected 7721 cells were determined using Western blotting and compared with the control cells transfected with pcDNA3 vector. It was found that the intensity of 57 kDa band was obviously increased in the samples from pcDNA3/PKB transfected 7721 cells (PKB/7721), and markedly decreased in the samples from pcDNA3/PKB-AS transfected 7721 cells (PKB-AS/7721) (Figure 3). These results indicated that the expression of PKB protein was up regulated after pcDNA3/PKB and blocked after pcDNA3/PKB-AS transfection.

GnT-V activity in PKB/7721 and PKB-AS/7721 cells

As shown in Table 1, the specific activities of GnT-V in the PKB/7721 and PKB-AS/7721 cells were increased by 131.9% and decreased by 57.1% respectively from that observed in the cells transfected with pcDNA3 vector ($p < 0.01$).

Table 1. Activity of GnT-V in 7721 cells transfected with pcDNA3/PKB or pcDNA3/PKB-AS.

Cell line	*GnT-V activity (pmol/h.mg)*	*Relative activity (% of control)*
7721	269.9 ± 20.5	
Mock-transfected (control)	253.8 ± 26.9	100.0
PKB/7721	588.6 ± 35.8*	231.9
PKB-AS/7721	108.8 ± 12.1*	42.9

Data were expressed as mean value of 3 independent experiments ± S.D.
PKB/7721 : 7721 cells transfected with pcDNA3/PKB.
PKB-AS/7721 : 7721 cells transfected with pcDNA3/PKB-AS.
*$p < 0.01$ compared with mock-transfected cells.

Activation of GnT-V in 7721 cells by PIP_2, PIP_3, GTPγ[S] and the abolishment of the activation by LY294002

The effects of PIP_2 and PIP_3, the substrate and product of PI-3-K respectively, as well as GTPγ[S], the non-hydrolysable analog of GTP and activator of PI-3-K [13], on the activity of GnT-V were estimated. Among these 3 reagents, PIP_3 was known as a direct activator of PKB [6]. It was found that when PIP_2, PIP_3 and GTPγ[S] were added to the GnT-V assay system, the GnT-V activities were increased to 198.8 %, 235.0 % and 192.0 % of the control value respectively ($p < 0.01$) (Figure 4). Furthermore, the activation of PIP_2 and GTPγ[S] as well as the basal activity of the control were significantly attenuated by LY294002 ($p < 0.05$), a specific inhibitor of PI-3-K [14], while the activation of PIP_3 was not changed by the addition of LY294002.

Effects of inhibitors and antibodies of signal transduction on GnT-V activity in 7721 cells

When the non-permeabilized 7721 cells were treated with LY294002, or ET18-OCH_3, an inhibitor of Raf-1 translocation to membrane [15] and phosphotidylinositol-specific phospholipase, (PI-PLC) [16], the specific activities of GnT-V were markedly decreased ($p < 0.05$ or 0.01). However, no obvious inhibition was found when any of the antibodies against PI-3-K, PKB, Raf-1 and MEK were added to the medium of non-permeabilized cells (Table 2). On the contrary, the specific activities of GnT-V were significantly down regulated after Anti-PI-3-K, Anti-PKB, Anti-Raf-1 or Anti-MEK was added to the culture medium of permeabilized 7721 cell, when compared with the non-immune IgG added control ($p < 0.01$, 0.01, 0.01, 0.05 respectively).

Effects of inhibitors and antibodies of signal transduction on GnT-V activity in H-ras transfected 7721 cells

In order to compare the significance of PI-3-K/PKB signaling pathway in parental and H-ras transfected cells, the H-ras transfected cells were used instead of the parental 7721 cells. As shown in Table 3, after the H-ras transfected cells were

Table 2. Effects of inhibitory agents of signal transduction on the basal activity of GnT-V in permeabilized and non-permeabilized parental 7721 cells.

Addition	*GnT-V activity (pmol/h.mg)*	*% of control*
Non-permeabilized cells		
None (control)	260.5 ± 12.9	100.0
LY294002	148.6 ± 8.7*	57.0
ET18-OCH_3	67.2 ± 2.8**	25.8
Non-immune IgG (control)	252.1 ± 17.3	100.0
Anti-PI-3-K	231.1 ± 16.7	91.7
Anti-PKB	236.0 ± 14.7	93.6
Anti-Raf-1	223.5 ± 13.5	88.7
Anti-MEK	239.9 ± 10.8	95.2
Permeabilized cells		
None	504.2 ± 29.9	102.6
Non-immune IgG (control)	491.5 ± 18.7	100.0
Anti-PI-3-K	205.4 ± 14.2**	41.8
Anti-PKB	186.8 ± 15.7**	38.0
Anti-Raf-1	141.6 ± 10.7**	28.8
Anti-MEK	227.2 ± 15.6*	46.2

Data is expressed as the mean value of 3 independent experiments ± S.D.
In the experiments using permeabilized cells, the cells were treated with 10 μM digitonin as described in Methods. Cells were treated for 30 min with various inhibitor (15 μM) or antibody or non-immune IgG (20 ng/ml).
*$p < 0.05$.
**$p < 0.01$, compared with the "None" or "Non-immune IgG" group.

Table 3. Effects of inhibitory agents of signal transduction on the basal activity of GnT-V in permeabilized and non-permeabilized H-ras-transfected 7721 cells.

Addition	*GnT-V activity (pmol/h.mg)*	*% of control*
Non-permeabilized cells		
None (control)	445.4 ± 20.6	100.0
LY294002	545.2 ± 41.4	122.4
ET18-OCH_3	170.3 ± 9.5**	37.5
Permeabilized cells		
None	501.2 ± 31.5	103.5
Non-immune IgG (control)	484.1 ± 28.4	100.0
Anti-PI-3-K	332.6 ± 32.5*	68.7
Anti-PKB	306.5 ± 25.5*	63.3
Anti-Raf-1	225.1 ± 18.7**	46.5
Anti-MEK	454.7 ± 29.7	93.9

Data were expressed as the mean value of 3 independent experiments ± S.D.
In the experiments using permeabilized cells, the cells were treated with 10 μM digitonin as described in Methods. Cells were treated for 30 min with various inhibitor (15 μM) or antibody or non-immune IgG (20 ng/ml).
*$p < 0.05$ compared with the Non-immune IgG group.
**$p < 0.01$, compared with the "None" or "Non-immune IgG" group.

treated with 15 μM inhibitors or 20 ng/ml antibodies for the same duration as that used in the experiment described in Table 2, the inhibition of GnT-V activity by both of the inhibitors and antibodies was less than those observed in the parental 7721 cells when the data in Table 3 were compared with the corresponding value in Table 2. It was interesting to find that the GnT-V activity was not decreased but slightly increased after the treatment of H-ras transfected cells with LY294002.

Discussion

Wang *et al* reported that the GnT-V activity in 7721 cells was activated by insulin treatment with a ET18-OCH_3 insensitive manner, and suggested that a signal transduction pathway other then the ET18-OCH_3 sensitive Raf pathway existed in 7721 hepatocarcinoma cells [17]. This pathway is important in insulin signaling, known as the PI-3-K/PKB pathway [5–7].

There is a medium expression of PKB in 7721 cells, which can be up or down regulated. Our findings shown in Figure 3 and Table 1 revealed that the increased expression of PKB protein was accompanied by the elevation of GnT-V activity after the transfection of PKB cDNA, and the expression of PKB protein was down regulated with the activity of GnT-V after transfection of PKB-AS cDNA. Furthermore, GnT-V activity was inhibited both in parental and H-ras transfected 7721 cells by the treatment of permeabilized cells with the antibody against PKB (Tables 2, 3). These discoveries demonstrated the regulatory role of PKB on GnT-V. The results in Figure 4, Table, 2 and 3 indicated that besides PKB, PI-3-K might be also involved in the regulation of the basal specific activity of GnT-V in 7721 cells. Figure 4 showed that GnT-V was activated by PIP_2, the substrate of PI-3K, or PIP_3, the product of PI-3-K, when PIP_2 or PIP_3 was added to the assay mixture of GnT-V. Moreover, the activaton of PIP_2 was markedly abolished by the addition of a well-known specific inhibitor of PI-3-K, LY294002 [14], while the activation of PIP_3 was not. The mechanism of the PIP_2 activaton may be caused by the conversion of PIP_2 to PIP_3 by PI-3-K in our enzyme preparation, subsequently, PIP_3 activates PKB [6] and the novel subtypes of PKC [18]. Therefore, the PI-3-K inhibitor could attenuate the activation of PIP_2, but not of PIP_3. Figure 4 also showed the activation of GnT-V by GTPγ[S], and this activation was also partially attenuated by LY294002. It suggests that the activation of GTPγ[S] might in part result from the stimulating effect of GTPγ[S] on PI-3-K via rho A small G protein, as was reported in rat adipocytes [13]. These findings indicate the participation of PI-3K in the activation of GnT-V. The incomplete abolishment of the activation of PIP_2 and GTPγ[S] on GnT-V by LY294002 is probably due to the hydrolysis of PIP_2 by PI-PLC to diacylglycerol, which activates PKC, a documented activator of GnT-V in 7721 cells [4,19], and this process is not sensitive to LY294002. On the other hand, GTPγ[S] also activates G protein Ras and enhances the activity of Ras/Raf signaling pathway, the LY294002 insensitive another positive regulatory pathway of GnT-V activity [2]. Tables 2 and 3 indicated that the GnT-V activity in the parental or H-ras transfected 7721 cell was also inhibited by the addition of LY294002 or antibody against PI-3-K to the culture medium of the cells. These results provided further evidence for the importance of PI-3-K/PKB signaling pathway in the regulation of GnT-V, even in the cells with H-ras over expression. The relation between PI-3K and PKB was documented. The product of PI-3-K, phosphatidyl-3,4,5-triphosphate (PIP_3) and phosphatidyl-3,4-biphosphate (PI-3,4-P_2), producing from their corresponding substrate phosphatidyl-4,5-biphosphate (PI-4,5P_2) and phosphatidyl-4-phosphate respectively, bind to the pleckstrin homology (PH) domain of PKB to localize it to plasma membrane and induces the activation of PKB by PKB kinase (3-phophoinositide dependent kinase-1, PDK-1) via the phosphorylation on Thr308 on PKB [5–7], followed by the phosphorylation on Ser473 by another integrin-linked kinase to fully activate PKB [20].

Ras protein, $p21^{ras}$, which participates in the signaling pathway of Raf/MEK/MAPK cascade, known to be involved in the regulation of GnT-V [2]. It was also evidenced in this study by the comparison of the enzyme activity in control samples from non-permeabilized cells listed in Tables 2 and 3 (260.5 pmol/h mg vs. 445.4 pmol/h mg). Our findings showed that the basal activities of GnT-V in parental and H-ras transfected cells were also down regulated by treatment with antiRaf-1 or anti-MEK antibody, as well as ET18-OCH_3, an inhibitor of Raf translocation to membrane [15] and PI-PLC [16], an enzyme which produces diglyceride for activating PKC. These findings revealed the involvement of Ras/Raf/MEK/MAPK, and possibly PKC, in the regulation of GnT-V, confirming the results of Wang *et al* [17] and our previous reports [4,19]. However, the inhibitory effects of these agents, including antibodies and ET18-OCH_3, on GnT-V activity were less in the H-ras transfected cells than their effects in the parental cells. This was particularly noticeable in the effect of anti-MEK antibody on GnT-V activity, which had little effect in the H-ras transfectant at 20 ng/ml of antibody concentration. This result may be explained by the elevated activities of Ras down-stream signal molecules, such as Raf and MEK in the H-ras transfectant, resulting in decreased sensitivity to the same concentraton of inhibitors or antibodies. It was recently reported that Ras induced the PI-3-K activity in COS-7 cells [21,22]. Therefore, it is reasonable to consider that the PI-3-K/PKB pathway may also be activated in the H-ras-transfected 7721 cells. This appears to be the reason for reduced inhibition of the anti-PI-3-K and anti-PKB antibodies on GnT-V activity in the H-ras transfected cells (31.3 % and 36.7 % compared to 58.2 % and 62.0 % inhibition respectively in parental cells).

It is not clear why the enzyme protein or activity of GnT-V was altered after the transfection of sense or antisense cDNA of PKB, and these are being studied. However, the above-mentioned evidence suggests that GnT-V may be regulated by a post-translational mechanism, such as phosphorylation. Our laboratory has reported that GnT-V activity is activated by

PKC, and inhibited by treatment with phosphatase [4,19]. The activities of GnT-V in the cell cycle of 7721 cells were correlated to the activities of a cell cycle regulating protein kinase, $p34^{cdc2}$, being highest in G2/M phase, but the mRNA and enzyme protein of GnT-V were not altered during the cell cycle [23]. In addition, GnT-V activity was down regulated in 7721 cells transfected with the cDNA of a cell cycle regulating gene, *p16*, without the alteration of the GnT-V mRNA [24]. All of these observations revealed the implication of post-translational phosphorylation mechanism in the regulation of GnT-V activity. However, it remains to be solved that the phosphorylation is direct or indirect on GnT-V. If GnT-V is indirectly phosphorylated by PKB via some regulatory protein(s), the signal molecules between PKB and GnT-V also need to be further studied.

Table 2 also indicated that digitonin treatment itself elevated the activity of GnT-V. It is likely that some macromolecules in the serum contained in culture medium permeated into the cells and stimulate the activity of GnT-V.

In summary, our findings suggest that the signaling molecules in PI-3-K/PKB pathway in addition to the known Ras/Raf/MEK/MAPK cascade and PKC mediate the regulation of GnT-V basal activity. All of these pathways are related to the extra-cellular proliferative signals. Therefore, these results were in consistent with the findings that GnT-V is a cell proliferative or carcinogenesis-associated enzyme [2,3]. However, there appears to be cross-talk among these three pathways, for example, the activation of Ras/Raf pathway may simultaneously stimulate the PI-3-K/PKB pathway via the activation of PI-3-K by Ras as mentioned above [21,22], and the protein kinase domain of PI-3-K can stimulate MAPK [25,26]. The activated PKC can activate the Raf protein in the presence of Ras [27]. In addition, it was reported that PKB was activated by the intracellular elevation of cAMP [28], a signaling pathway not related to carcinogenesis, but associated with benign proliferation of some cell types [29]. Therefore, the biological significance of GnT-V regulated by PI-3-K/PKB pathway remains to be further investigated.

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