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Transient Expression of a p58 Protein Kinase cDNA Enhances Mammalian Glycosyltransferase Activity

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The effect of expression of a p58 protein kinase on mammalian β-1,4 galactosyltransferase enzyme activity was examined in vitro and in vivo. We found that p58 protein kinase expression enhanced galactosyltransferase enzyme activity approximately three-fold in vivo when compared to reporter gene activity. Galactosyltransferase enzyme activity was also substantially reduced in vitro when dephosphorylated, or when p58 specific antibodies were used to inhibit kinase activity. These results suggest that galactosyltransferase activity is influenced by phosphorylation, and that the p58 protein kinase may mediate this effect.

A new protein kinase, p58^{GTA}, was previously isolated using a non-specific β1-4 galactosyltransferase (GalTase) polyclonal antibody (1, 2). Molecular characterization of the p58^{GTA} cDNA indicated that it encoded a protein of 434 amino acids, and that 299 amino acids of this sequence was 68% homologous to both yeast and human p34^{cdc2} kinases (3). This protein also contains a unique amino-terminal domain with putative calmodulin binding, nuclear localization and PEST sequences (3). A specific antibody was made to a portion of this amino-terminal domain and then used to demonstrate that p58^{GTA} was a functional serine/threonine protein kinase, and that it co-purified with the bovine GalTase proteins. Elevated expression of the p58^{GTA} kinase in CHO cells resulted in a sequestering of these cells at the late telophase/early G_1-phase cell cycle boundary, as well as a 40-fold increase in mitotic abnormalities (3). Conversely, diminished expression of the p58^{GTA} kinase in CHO cells was found to enhance DNA replication and was accompanied by decreased GalTase enzyme activity (4). These results suggested that the p58^{GTA} kinase might function to control some aspect of cell cycle regulation, and that it may also act upon the GalTase proteins with which it co-purifies.

The abbreviations used are: GalTase, β1-4 galactosyltransferase; GTA, galactosyltransferase associated; CAT, chloramphenicol acetyltransferase; CHO, chinese hamster ovary fibroblasts; MMTV-LTR, mouse mammary tumor virus-long terminal repeat.

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Here, we report that the transient expression of the p58^{GTA} kinase in COS cells results in a substantial increase in GalTase enzyme activity. This enhancement in GalTase activity is apparently not due to increased steady-state GalTase mRNA or protein levels, suggesting that the p58^{GTA} kinase may post-translationally modify GalTase. Further support for this notion comes from evidence that the p58^{GTA} kinase phosphorylates the GalTase proteins in vitro, that dephosphorylation of the GalTase proteins in vitro results in a substantial loss of enzyme activity, and that a specific p58^{GTA} kinase antibody inhibits GalTase enzyme activity in vitro.

Materials and Methods

DNA constructs and expression analysis. A 4.22 kb Hind III fragment from the pMSG-p58^{GTA} construct (3), which contains the mouse mammary tumor (MMTV) LTR promoter, was ligated to Hind III digested pSV$_2$ CAT (5). Proper orientation of 4.22 kb pMSG-p58^{GTA} fragment replaces the SV40 enhancer/origin sequence that was disrupted in pSV$_2$ CAT by Hind III digestion (Figure 1). The final construct, pSV$_2$CAT-hGTA, contains both the human p58^{GTA} cDNA and the CAT gene, which are expressed independently of one another, and as separate transcripts. The inclusion of the MMTV-LTR promoter allows induction of the p58^{GTA} cDNA upon addition of 10^{-6}M dexamethasone (6). However, we and others have found that even in the absence of exogenous dexamethasone this promoter is active (3, 7). COS M-6 cells were elctroporated as previously described (3). RNA and protein were extracted from the cells 60 hrs. post-electroporation for further analysis. Northern blots were performed as described previously (3) using human p58^{GTA}, murine GalTase, and human γ-actin cDNA probes (3, 4, 8). Western blot analysis was performed as described previously using a rabbit anti-GalTase antibody (4).
Enzyme activity measurements. CAT activity was quantitated from 15 μg of total cellular protein from the electroporated COS cells essentially as described by others (5). GalTase enzyme assays were performed as described previously (3) using 10 μg of total cellular protein from the same cells examined above. All experiments were performed at least three different times.
In vitro phosphorylation, dephosphorylation and antibody inhibition studies. In vitro kinase assays using purified bovine p58^{GTA} kinase and GalTase were performed as described previously (3). Two-dimensional gel electrophoresis and autoradiography was performed as described by Strous et al. (9). Bovine Galtase was treated with either 0.12 units of potato acid phosphatase for 15 minutes at 37°C or 0.12 units of potato acid phosphatase plus 100 mM 4-nitrophenylphosphate prior to GalTase enzyme assay. For antibody inhibition studies, increasing amounts of affinity purified p58^{GTA} peptide antibody (10 mg/ml) were incubated with 0.2 units of GalTase at 37°C for 30 minutes prior to GalTase enzyme assay. Additionally, increasing amounts of competing p58^{GTA} peptide (starting at 100 nM and increasing in log$_{10}$ increments) was incubated with 1 mg/ml of p58^{GTA} antisera and 0.2 units of bovine GalTase at 37°C for 30 minutes prior to GalTase enzyme assay.

Results and Discussion

Transient expression of the p58^{GTA} cDNA and a reporter gene. To assess the possible influence of the p58^{GTA} kinase in vivo on GalTase activity quantitatively, we designed an expression vector construct containing both the p58^{GTA} cDNA and the E. coli chloramphenicol acetyltransferase (CAT) reporter gene (Figure 1). This vector allowed us to avoid potential problems with the efficiency of co-transfection of the two genes as separate plasmids.

Expression of the E.coli CAT gene and p58^{GTA} cDNA was examined by functional assay and Northern blot analysis, respectively. In Figure 2, we

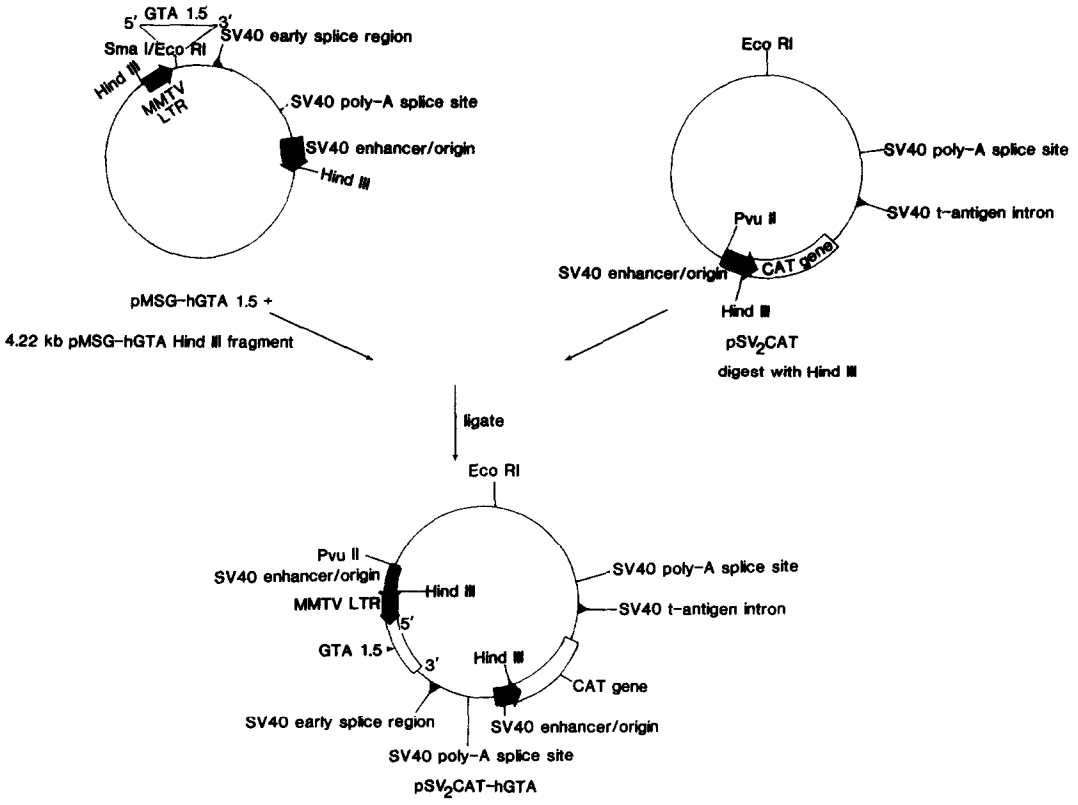


Figure 1. Schematic diagram demonstrating the construction of the pSV$_2$CAT-hGTA expression plasmid from pMSG-hGTA and pSV$_2$CAT. Promoters are shown as filled arrowheads. The p58^{GTA} cDNA and CAT gene are indicated. Additional features of the expression construct essential for function are also shown.

demonstrate that the CAT gene contained in pSV$_2$CAT and pSV$_2$CAT-hGTA is functional in electroporated COS cells. CAT activity is indicated by the presence of both acetylated and di-acetylated chloramphenicol in the pSV$_2$CAT positive controls and the pSV$_2$CAT-hGTA COS cells 60 hours post-electroporation, while no enzyme activity is present in negative control cells (Figure 2). The results of these experiments assured us that the reporter gene in our expression vector was functional, and that it would enable us to quantitate the effect of p58^{GTA} expression on GalTase activity. Analysis of total RNA from COS cells electroporated with either pSV$_2$CAT or pSV$_2$CAT-hGTA plasmids was then performed by Northern blot using the human p58 cDNA probe. A hybrid 3.0 kb MMTV-p58^{GTA} transcript, containing approximately 1,350 bp of the MMTV-LTR, 1,500 bp of the human p58^{GTA} cDNA and 150 bp of SV40 derived sequences upstream from the poly-A addition site was observed in the pSV$_2$CAT-hGTA electroporated COS cells (Figure 3, panel A, lane 2), but not in the pSV$_2$CAT electroporated cells (Figure 3, panel A, lane 1). The 4.0 and 1.7 kb transcripts in both lanes are the endogenous african green monkey p58^{GTA} transcripts. Equal loading of RNA samples was confirmed by

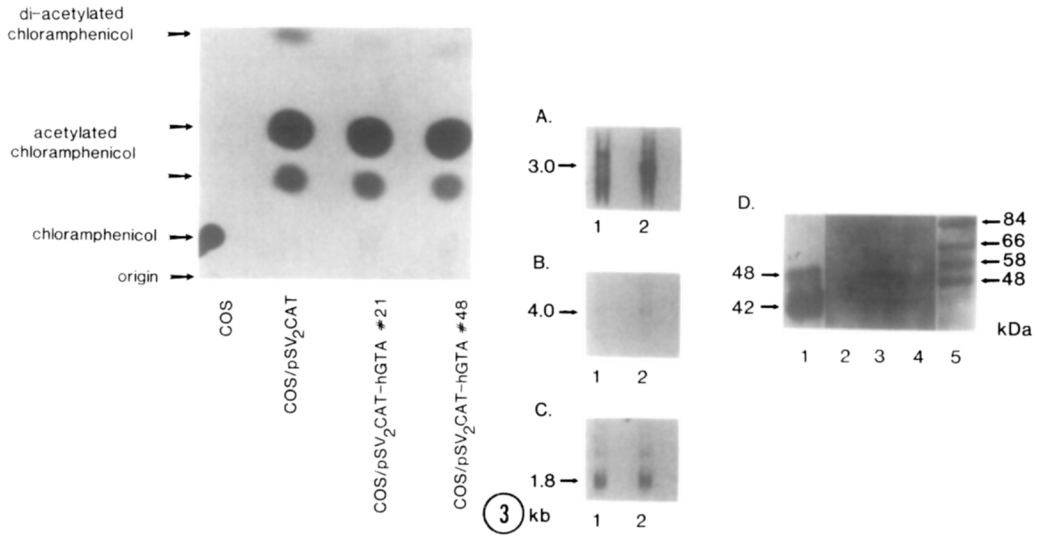


Figure 2. Autoradiogram of a representative CAT assay. The origins and migration of chloramphenicol, acetylated chloramphenicol, and di-acetylated chloramphenicol are shown. The origin of the cellular extracts tested for CAT enzyme activity are shown below. Two different pSV_2CAT-hGTA constructs were initially tested, however pSV_2CAT-hGTA #48 was used in all subsequent experiments.

Figure 3. Northern and Western blot analysis of cellular products. (Panel A) Northern blot analysis of 30 μg of total cellular RNA using the $p58^{GTA}$ cDNA (3) as probe. Lane 1, pSV_2CAT electroporated COS cells. Lane 2, pSV_2CAT-hGTA electroporated COS cells. (Panel B) Rehybridization of the same Northern blot shown in panel A with a murine GalTase cDNA probe (4). (Panel C) Rehybridization of the same Northern blot shown in panels A and B with a γ-actin cDNA probe (8). (Panel D) Western blot analysis of cellular proteins and bovine GalTase using a polyclonal GalTase antibody. Lane 1, 0.1μg of semi-purified bovine GalTase, demonstrating the ability of the GalTase antibody to recognize the 42 and 48 kDa bovine GalTase proteins. Lane 2, 50 μg of fetal calf serum proteins. Lane 3, 50 μg of total cellular protein from pSV_2CAT-hGTA electroporated COS cells. Lane 4, 50 μg of total cellular protein from pSV_2CAT electroporated COS cells. Lane 5, molecular weight markers. The sizes of the molecular weights of the markers are shown on the right; the molecular weights of the two bovine GalTase proteins is shown on the left.

hybridization with a γ-actin cDNA probe (Figure 3, panel C). Previously, we have shown that this hybrid $p58^{GTA}$ mRNA leads to increased p58 protein expression by both Western blot and indirect immunofluorescence analysis (3).

Effects of $p58^{GTA}$ expression on endogenous GalTase enzyme activity. We next assayed GalTase enzyme activity in the various electroporated COS cells. Each electroporation experiment was performed on three different occasions, using identical DNA constructs. The results of the CAT assays for each of the DNA constructs are shown in column one of Table 1. The results of GalTase enzyme activity assays are shown as total cpm in column two of Table 1. To obtain an accurate quantitative difference we used the CAT activity values to establish a baseline value which was representative of transfection efficiency. This was done by normalizing the CAT values for all DNA constructs from each experiment to the lowest CAT assay value for that experiment, thereby generating a correction factor.

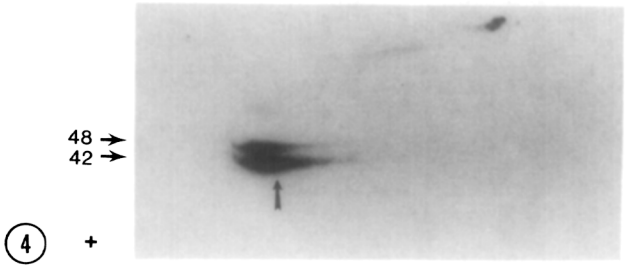
TABLE 1

DNA Sample	CAT Assay (Total cpm)			4β-GT Assay (Total cpm)			4β-GT Assay (Corrected total cpm)			Increase in 4β-GT Enzyme Activity#		
	1	2	3	1	2	3	1	2	3	1	2	3
COSM-6 pSV_2CAT	180,200	47,551	37,946	2,592	3,367	1,972	2,435	1,885	1,631	1.0	1.0	1.0
COSM-6 pSV2CAT-GTA1.5(+) (- Dex)	169,256	35,613	31,381	6,423	4,983	4,729	6,423	3,724	4,829	2.6	2.0	3.0
COSM-6 pSV2CAT-GTA1.5(+) (+ Dex)	175,688	26,622	64,638	7,908	4,873	11,894	7,619	4,873	5,947	3.1	2.6	3.6

The relative fold-increase in 4β-GT activity is based on the pSV_2CAT vector containing cells equaling 1.0.

The total cpm from the GalTase assays for the corresponding experiment was then corrected using this factor, and the values are shown in column three of Table 1. The relative-fold increase in GalTase activity in the pSV_2CAT-hGTA containing cells is shown in the final column of Table 1. The pSV_2CAT containing cells from each experiment were assigned a baseline value of one. These experiments demonstrate that expression of the $p58^{GTA}$ kinase in the absence of exogenous dexamethasone enhances GalTase activity approximately 2.5-fold, while in the presence of 10^{-6}M dexamethasone it is enhanced approximately 3.1-fold. Assays for changes in α2, 6 sialyltransferase enzyme activity from the same cell extracts demonstrated no substantial change (data not shown). Thus, either directly or indirectly, expression of this kinase is capable of modulating GalTase enzyme activity.

Finally, to crudely determine whether or not this effect was potentially post-transcriptional or post-translational, we analyzed the RNA and protein from these cells with specific GalTase cDNA and antibody probes (4, 10). Rehybridization of the Northern blot in Figure 3 with a murine GalTase cDNA probe revealed that the steady-state level of GalTase mRNA was not affected by $p58^{GTA}$ expression (Figure 3, panel B, lanes 1 and 2). Similarly, the steady-state level of GalTase protein in these cells was also invariable using a previously well characterized GalTase antibody (Figure 3, panel D). However, we detected two different molecular weight forms of GalTase, approximately 48 kDa and 52 kDa in size in the pSV_2CAT electroporated COS cells (Figure 3, panel D, lane 4). These sizes are consistent with the reported size of the GalTase proteins found in HeLa cells (11). The smaller 48 kDa molecular weight form of GalTase was not as abundant in the pSV_2CAT-hGTA electroporated cells, while the 52 kDa form was slightly enhanced (Figure 3, panel D, lane 3). The 42 kDa and 48 kDa bovine GalTase proteins seen by other GalTase antibodies (12) were also readily detected with this antibody (Figure 3, panel D, lane



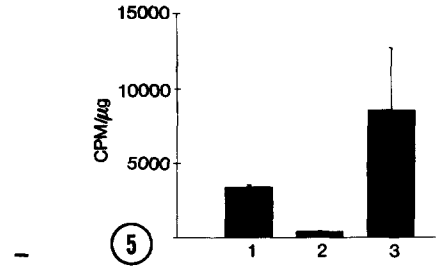


Figure 4. Two dimensional gel analysis of the 42 and 48 kDa bovine GalTase proteins after phosphorylation by the purified bovine p58^{GTA} kinase. The pH gradient was between 3.5 and 10.0. The position of bovine serum albumin (pH 4.7) is indicated by the arrow.

Figure 5. GalTase enzyme assays in the absence and presence of exogenously added potato acid phosphatase. Lane 1, 0.2 units of bovine GalTase. Lane 2, 0.2 units of bovine GalTase after treatment with potato acid phosphatase . Lane 3, same as lane 2 with the addition of 100 mM phosphatase substrate during the potato acid phosphatase incubation. All experiments were performed in triplicate. Standard deviations are indicated above each bar.

1), while no immunoreactivity is seen when fetal calf serum is blotted (Figure 3, panel D, lane 2).

Bovine GalTase is phosphorylated by p58^{GTA} in vitro and its activity is diminished by dephosphorylation or p58^{GTA} antibody. The experiments shown above suggested that phosphorylation of GalTase might influence enzyme activity. This is not without precedent since Banerjee and colleagues have demonstrated that mannosylphosphodolichol synthase activity is enhanced by cAMP-mediated protein phosphorylation (13). Various isoenzyme forms of GalTase with acidic isoelectric focusing points have also been described (14). Additionally, a serine phosphorylated form of GalTase has been isolated from both HeLa and HepG2 cells (9). Therefore, we examined the ability of the p58^{GTA} kinase to phosphorylate bovine GalTase in vitro. The phosphorylated 42 and 48 kDa GalTase proteins were examined by two-dimensional gel electrophoresis and autoradiography (Figure 4). We found that both the 42 and 48 kDa bovine GalTase proteins were phosphorylated by p58^{GTA}, and that the ^{32}P-labelled isoenzyme forms were identical to those published by Strous and colleagues (9). Next, we treated bovine GalTase with potato acid phosphatase. In Figure 5 (lane 2) we show that in the presence of potato acid phosphatase, GalTase activity is diminished approximately 6-fold as compared to native bovine GalTase (lane 1). To demonstrate that this effect was not due to proteolysis, the same experiment was performed in the presence of 100 mM phosphatase substrate, 4-nitrophenylphosphate. There was no similar decrease in GalTase activity (Figure 5, lane 3). The observed increase in GalTase activity in the presence of phosphatase substrate may reflect the presence of a contaminating phosphatase in the semi-purified bovine GalTase proteins. This was confirmed by incubating the bovine GalTase in the presence of 100 mM 4-nitrophenylphosphate alone (data not shown). Finally, we used a monospecific

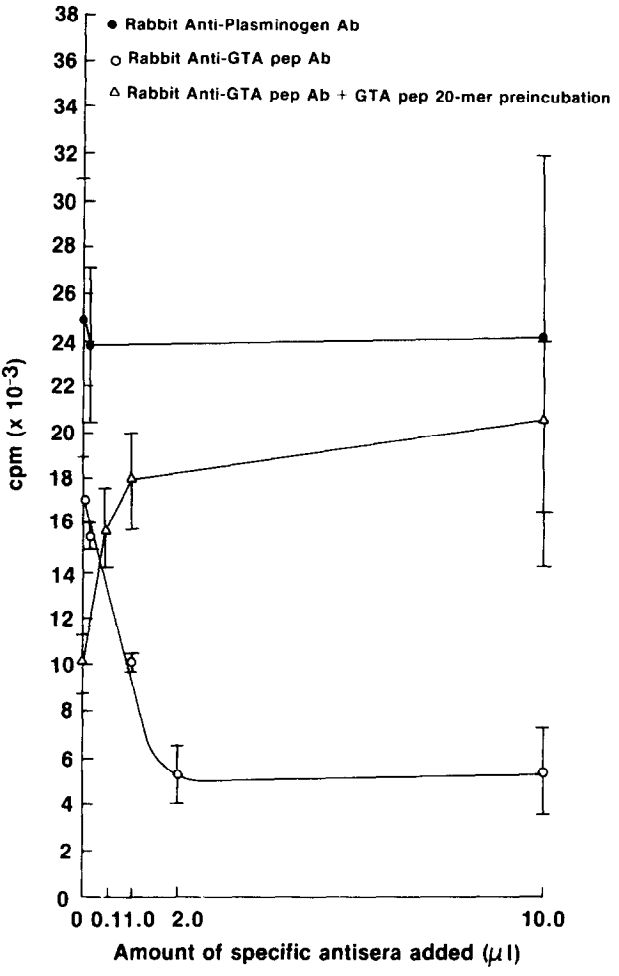


Figure 6. p58^{GTA} antibody inhibition of GalTase enzyme activity. Increasing amounts of a p58^{GTA} peptide antibody (○) were preincubated with bovine GalTase before enzyme assay analysis. Similarly, a fixed amount of p58^{GTA} peptide antibody (1 mg/ml) was preincubated with increasing amounts of competing p58^{GTA} peptide (100 nM, 1,000 nM and 10 mM) and bovine GalTase (△) before analysis. A non-specific antibody control (●) was also tested.

antibody to the p58^{GTA} kinase (3, 4) to determine whether pre-incubation of the antibody with bovine GalTase affected GalTase enzyme activity. We found that the p58^{GTA} antibody decreased GalTase activity, and that this inhibition could be competed with the peptide to which the antibody was made (Figure 6). In addition, a non-specific antibody control did not effect GalTase enzyme activity (Figure 6).

Our results suggest that GalTase activity is influenced by p58^{GTA} kinase expression, possibly via phosphorylation of GalTase itself. Further, more definitive experiments are now underway using re-constituted in vitro systems and in vivo labeling to determine whether or not the p58^{GTA} kinase functions to regulate cellular GalTase activity.

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