Evidence that mammalian phosphatidylinositol transfer protein regulates phosphatidylcholine metabolism

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Phosphatidylinositol transfer proteins (PITPs) and their yeast counterpart (SEC14p) possess the ability to bind phosphatidylinositol (PtdIns) and transfer it between membranes *in vitro*. However, the biochemical function of these proteins *in vivo* is unclear. In the present study, the physiological role of PITP was investigated by determining the biochemical consequences of lowering the cellular content of this protein. WRK-1 rat mammary tumour cells were transfected with a plasmid containing a full-length rat PITP α cDNA inserted in the antisense orientation and the resultant cell clones were analysed. Three clones expressing antisense mRNA for PITP α were compared with three clones transfected with the expression vector lacking the insert. The three antisense clones had an average of 25% less PITP α protein than control clones. Two of the three antisense clones also exhibited a decreased rate of growth. All three antisense clones exhibited a significant decrease in the incorporation of labelled precursors into PtdCho during a 90-min incubation

period. Under the same conditions, however, there was no change in precursor incorporation into PtdIns. Further experimentation indicated that the decrease in precursor incorporation seen in antisense clones was not due to an increased rate of turnover. When choline metabolism was analysed more extensively in one control (2-5) and one antisense (4-B) clone using equilibrium-labelling conditions (48 h of incubation), the following were observed: (1) the decrease in radioactive labelling of PtdCho seen in short-term experiments was also observed in long-term experiments, suggesting that the total amount of PtdCho was lower in antisense-transfected clones (this was confirmed by mass measurements); (2) a similar decrease was seen in cellular sphingomyelin, lysoPtdCho and glycerophosphorylcholine; (3) an average two-fold increase in cellular phosphorylcholine was observed in the antisense-transfected clone; (4) cellular choline was, on average, decreased; and (5) cellular CDPcholine was not significantly altered.

INTRODUCTION

A variety of lipid-binding proteins have been described which can catalyse the transfer of lipids between membranes *in vitro* [1]. One such protein is phosphatidylinositol (PtdIns) transfer protein (PITP). In mammalian cells, two forms of PITP, designated PITP α and PITP β , have been isolated. PITP α transfers both PtdIns and phosphatidylcholine (PtdCho), whereas PITP β transfers PtdIns, PtdCho and sphingomyelin [2–5]. A functional homologue of the mammalian protein, SEC14p, has been characterized in yeast [6–8]. Recent reports have suggested a variety of functions for these proteins, including regulation of phospholipid metabolism [9–11], facilitation of vesicular traffic and secretion [7,12–19] and participation in agonist-induced phosphoinositide cycling [20–25]. The essential nature of this protein activity for cell survival has been suggested by experiments in both yeast [26] and mammalian cells [27].

WRK-1 rat mammary tumour cells have been extensively characterized with respect to growth [28,29] and phospholipid metabolism [30–32]. In order to characterize further the role of PITP α *in vivo*, WRK-1 cells were stably transfected with the mammalian expression vector, pcDNA3, containing a full-length rat PITP α cDNA in the antisense direction. As a control, cells were transfected with vector lacking the insert. Several clones from each transfection were isolated and expanded. Three control clones (2-2, 2-4 and 2-5), and three clones expressing the antisense cDNA (4-1, 4-3 and 4-B), were analysed. The results indicated

that a major function of PITP α involved regulation of PtdCho metabolism.

MATERIALS AND METHODS

Materials

Tissue culture media, trypsin, Dulbecco's PBS, antibiotics, antimycotics, glutamine, G418 and Lipofectin were purchased from Life Technologies. Plasticware for tissue culture was from Costar. Anion exchange resin (AG 1-X8, 200–400 mesh, formate form) and the Bio-Rad protein assay reagent were from Bio-Rad. [³H]inositol (15 Ci/mmol), [¹⁴C]choline (55 mCi/mmol) and [³H]choline (85 Ci/mmol) were from American Radiolabeled Chemicals. ³²P_i (carrier free) was purchased from ICN. Aquasol-2 was from DuPont–New England Nuclear. Vasopressin, bradykinin and G418 were purchased from Sigma. The expression vector, pcDNA3, was from Invitrogen. The full-length PITP α cDNA was generously provided by Dr. Lynwood Yarbrough (Department of Biochemistry and Molecular Biology, University of Kansas Medical Center, Kansas City, KS 66103, U.S.A.) [2].

Cell culture

WRK-1 cells were established from a dimethylbenz(a)anthracene-induced rat mammary tumour as described previously [29]. The variant (1B) used in these experiments arose spontaneously [33]. Monolayer cultures were maintained in Ham's F-12

medium supplemented with Earle's salts and fetal-calf serum (10% v/v), rat serum (2% v/v), penicillin (100 units/ml), Fungizone (0.25 µg/ml) and streptomycin (100 µg/ml). Cells were harvested using 0.05% (w/v) trypsin/0.02% (w/v) EDTA in 0.9% (w/v) NaCl and replicates were plated into 22-mm wells.

Preparation of plasmids containing PITP α cDNA

The full-length 1.67 kb rat PITP cDNA, subcloned into the *Eco*RI site of pGEM7Zf(+), was excised by *Eco*RI digestion and subcloned, in both the sense and anti-sense orientations, into the *Eco*RI site of the constitutive mammalian expression vector pcDNA3. Orientation of the insert was confirmed by restriction digestion.

Transfection of WRK-1 cells with PITP α cDNA

Plasmid DNAs were transfected into WRK-1 cells using the Lipofectin reagent. Briefly, 1×10^6 to 2×10^6 cells were seeded in a 100-mm tissue culture dish in Ham's F-12 medium supplemented with serum and antibiotics and were incubated overnight. Plasmid DNA (4 µg) in 200 µl of Opti-MEM medium was gently mixed with 20 µl of Lipofectin reagent in 200 µl of Opti-MEM medium and incubated at room temperature for 15 min. This mixture was mixed with 3.6 ml of growth medium without antibacterial reagents and then added to a dish containing cells. After incubation overnight at 37 °C, the medium containing plasmid DNA was removed and fresh medium was added. After 48 h, the cells were subcultured into selection medium (Ham's F-12 medium containing 400 µg/ml G418) at a ratio of 1:5. Two plasmids were transfected into WRK-1 cells: pcDNA3, and pcDNA3 containing PITP cDNA in the antisense direction. After incubation in selection medium for 3 weeks, the resultant clones were removed and expanded.

Measurement of PITP α protein

Cells were extracted with SET buffer (0.25 M sucrose/0.001 M EDTA/0.02 M Tris, pH 7.5) and homogenized using a Dounce homogenizer. Following centrifugation in an Eppendorf microfuge (Model 5415C) at 14000 *g* for 10 min, protein levels in the supernatants from each sample were measured spectrophotometrically by determining A_{260} and A_{280} and correcting for any nucleotide contamination. Equal amounts of protein from each sample were then precipitated with 5 vol. of ice-cold acetone. The protein samples were subjected to SDS/PAGE (15% gels) and analysed by Western blotting using an antibody raised against synthetic peptides representing epitopes in PITP α [13]. The Western blots were scanned with a Bio-Rad GS700 imaging densitometer. The PITP bands were scanned and the results were integrated. Total cytosol protein (20 µg and 40 µg) was analysed in duplicate from cells harvested in two separate experiments.

Extraction and analysis of phospholipids

Lipids and their breakdown products were extracted and analysed as described previously [34]. When cells were incubated with [3 H]inositol, the chloroform extractable radioactivity is referred to as labelled PtdIns, since > 95% of the radioactive lipids are PtdIns. When cells were incubated with radioactive choline, 85% of the labelled lipid was PtdCho, 10%, sphingomyelin and 5% lysoPtdCho. For identification of 32 P-labelled lipids the following chromatographic systems were used. Phospholipids were separated by TLC (silica-gel G) in chloroform/methanol/acetic acid/water (50:30:6:3, by vol.). Polyphosphoinositides were separated on Dowex following deacylation [34]. Separation

of [3 H]choline-labelled water soluble compounds was achieved by TLC (silica-gel G in either 0.9% NaCl/methanol/ammonia (50:70:5, by vol.) [35] or water/ethanol/ammonia (95:48:7, by vol.) [36]. Measurement of phospholipid mass was performed as described previously [37].

RESULTS AND DISCUSSION

PITP α protein levels in WRK-1 clones

Table 1 shows the levels of PITP α protein expressed in each clone. Compared with control clones there was a small but significant decrease in PITP α in each of the three antisense clones. When analysed as a group, the decrease in the antisense-transfected PITP level was 25% ($P = 0.0012$).

Growth of WRK-1 clones

It has been reported that a reduction in PITP results in a decrease in the growth rate of NIH 3T3 cells [27]. When the WRK-1 cell clones were examined, the growth curves shown in Figure 1 were

Table 1 PITP α protein levels in control and antisense-transfected WRK-1 clones

Cells were grown and analysed as described in the Materials and methods section. The values shown are the mean $\pm$ S.D. of two separate experiments.

Clone	PITP α protein level (relative intensity)
Control	
2-2	5.94 $\pm$ 0.47
2-4	6.87 $\pm$ 0.80
2-5	5.63 $\pm$ 0.39
Antisense	
4-1	4.67 $\pm$ 0.50
4-3	4.59 $\pm$ 0.03
4-B	4.65 $\pm$ 0.67

Figure 1 Growth curves for control and antisense-transfected WRK-1 clones

Cells were plated on day 0 at the same densities (approx. 150 000 cells/22-mm well). On the day indicated, cells were harvested with a solution of 5 mM EDTA in PBS, collected by centrifugation and the total protein concentration was determined. Protein concentration correlated exactly with cell number (results not shown). The values shown represent the means $\pm$ S.D. of sextuplicate determinations. The bottom two curves represent the antisense-transfected clones 4-1 and 4-3.

Table 2 Phospholipid metabolism in WRK-1 cell clones

*Cells were incubated with $^{32}\text{P}_i$ for 90 min and incorporation into PtdCho and PtdIns quantified as described in the Materials and methods section. The value for each clone represents the mean of sextuplicate determinations. S.D.s ranged from 8–21 % and averaged 12 %. †Alternatively, incorporation of $^{32}\text{P}_i$ into individual phosphoinositides is expressed as a percentage of the total radioactivity incorporated into all phosphoinositides in the absence and presence of 10^{-7}M vasopressin. Values shown are the means $\pm$ S.D. of sextuplicate determinations.

	Incorporation of $^{32}\text{P}_\text{i}$						<i>P</i>
	Control clones			Antisense clones			
	2-2	2-4	2-5	4-1	4-3	4-B	
$[^{32}\text{P}]\text{PtdCho}$ (c.p.m./cell)*	0.107	0.156	0.128	0.055	0.028	0.066	< 0.005
Mean $\pm$ S.D.	0.130 $\pm$ 0.02			0.050 $\pm$ 0.02			
$[^{32}\text{P}]\text{PtdIns}$ (c.p.m./cell)*	0.083	0.106	0.100	0.102	0.051	0.112	NS
Mean $\pm$ S.D.	0.096 $\pm$ 0.01			0.088 $\pm$ 0.03			
$[^{32}\text{P}]\text{PtdIns}$ (% of phosphoinositides)†	58.3 $\pm$ 2.5			55.3 $\pm$ 2.5			NS
+ Vasopressin	74.0 $\pm$ 6.2			75.3 $\pm$ 2.1			NS
$[^{32}\text{P}]\text{PtdInsP}$ (% of phosphoinositides)†	29.6 $\pm$ 0.58			32.3 $\pm$ 2.9			NS
+ Vasopressin	20.3 $\pm$ 5.8			19.6 $\pm$ 2.9			NS
$[^{32}\text{P}]\text{PtdIns}(4,5)\text{P}_2$ (% of phosphoinositides)†	11.3 $\pm$ 3.0			12.3 $\pm$ 1.5			NS
+ Vasopressin	5.6 $\pm$ 1.0			4.7 $\pm$ 0.3			NS

Table 3 Characterization of radioactive choline metabolites in WRK-1 cell clones

Cells were incubated for 48 h with $[^3\text{H}]\text{choline}$ (20 $\mu\text{Ci/ml}$). Following the incubation, radioactive metabolites were quantified as described in the Materials and methods section and expressed as c.p.m./mg of cell protein. The percentage increase or decrease in incorporation of the radioactively labelled precursor is indicated by arrows. Values shown are the means $\pm$ S.D. of sextuplicate determinations.

	Incorporation of radioactivity (c.p.m./mg of protein)		Change (%)	<i>P</i>
	Clone 2-5	Clone 4-B		
PtdCho	50709 $\pm$ 5999	31189 $\pm$ 2968	↓ 38	< 0.0005
Sphingomyelin	4595 $\pm$ 932	3034 $\pm$ 464	↓ 34	< 0.0025
LysoPtdCho	1027 $\pm$ 244	714 $\pm$ 181	↓ 30	< 0.0025
Glycerophosphorylcholine	20005 $\pm$ 4378	12538 $\pm$ 1841	↓ 37	< 0.0025
Choline	23275 $\pm$ 6443	10666 $\pm$ 1139	↓ 54	< 0.0005
Choline phosphate	15183 $\pm$ 3365	30290 $\pm$ 5323	↑ 99	< 0.0005
CDPcholine	1309 $\pm$ 216	1563 $\pm$ 378	↑ 19	NS

generated. Two of the three antisense-transfected clones (4-1 and 4-3) had significantly decreased rates of growth compared with control clones. A third antisense clone (4B) grew at the same rate as the control cells. In yeast, mutations in *sec14* are lethal but can be overcome by concomitant inactivation of the CDPcholine pathway of PtdCho synthesis [11], and defects in *Sac1p* result in expansion of the diacylglycerol pool in the Golgi [38]. Thus it is possible that, in WRK-1 cells, the growth-inhibitory effect of reducing PITP α is dependent on other biochemical parameters of the cell.

Phospholipid metabolism in WRK-1 clones

The metabolism of PtdCho and PtdIns was investigated in the transfectants. Cells were incubated with $^{32}\text{P}_i$ for 90 min, and incorporation of radioactivity into both PtdCho and the phosphoinositides was determined. Table 2 shows that antisense-transfected clones exhibited a significant reduction (62 %, $P < 0.005$) in the incorporation of precursor into PtdCho, whereas incorporation into PtdIns was the same in both experimental and control clones. Since clone 4-B, which grows at the same rate as control cells, exhibited the same reduction in incorporation of precursors into PtdCho as the slower growing clones, 4-1 and 4-3, the reduction could not be attributed to differences in growth

rate. With respect to the polyphosphoinositides, there was likewise no difference between control and experimental clones in incorporation of precursors into PtdInsP or PtdIns(4,5) P_2 .

When control and experimental cells were stimulated to cycle their phosphoinositides by the addition of vasopressin, the change in the percentage distribution of radioactivity in the phosphoinositides was identical in control and antisense-transfected clones. When the hormone-induced stimulation of phosphoinositide cycling was analysed by monitoring incorporation of $^{32}\text{P}_i$ into PtdIns, the fold increase in radiolabelled PtdIns was 2.18, 5.40 and 4.78 for control 2-2, 2-4 and 2-5 clones respectively, and for the antisense clones the fold increase was 5.74, 4.20 and 5.28 for 4-1, 4-3 and 4-B respectively. Each experiment was done in sextuplicate. The means $\pm$ S.D. were 4.12 ± 1.7 for controls and 5.07 ± 0.79 for antisense clones, and again the difference was not significant.

Analysis of choline metabolites in WRK-1 clones

In order to characterize differences in PtdCho metabolism further in control and antisense-transfected clones, one control clone (2-5) and one antisense-transfected clone (4-B) were incubated with $[^3\text{H}]\text{choline}$ or $[^{14}\text{C}]\text{choline}$ for 48 h, and the incorporation of radioactivity into choline metabolites was determined. Table 3

Table 4 Characterization of choline metabolites in WRK-1 cell clones: average percentage change determined from multiple experiments

The differences expressed represent the increase or decrease (shown by arrows) in clone 4-B cell metabolites relative to clone 2-5 cell metabolites.

	Change in individual experiments (%)	Average change (%)
PtdCho	↓ 35, ↓ 27, ↓ 24, ↓ 38	↓ 31 ± 7
Sphingomyelin	↓ 39, ↓ 26, ↓ 34	↓ 33 ± 7
LysoPtdCho	↓ 41, ↓ 35, ↓ 30	↓ 35 ± 6
Glycerophosphorylcholine	↓ 37, ↓ 54, ↓ 45	↓ 45 ± 8
Choline	↓ 54, 0, ↓ 41, ↓ 43	↓ 34 ± 23
Choline phosphate	↑ 99, ↑ 140, ↑ 131, ↑ 8, ↑ 81	↑ 91 ± 52
CDPcholine	↑ 19, ↑ 57, ↓ 20	↑ 19 ± 38

shows that the decrease in incorporation of radiolabelled precursor into PtdCho observed previously in a 90 min incubation (Table 2) was also found following a long-term incubation (Table 4). When lipid mass was measured directly, decreases of 22 and 33 % were observed in two separate experiments. A similar decrease was observed in sphingomyelin radioactivity (Tables 3 and 4) as well as in sphingomyelin mass (decreases of 14 and 21 % in two separate experiments). Radioactivity in lysoPtdCho and glycerophosphorylcholine was also decreased. When choline-containing metabolic precursors of PtdCho were examined, an average two-fold increase in radioactive choline phosphate was observed. A similar increase was seen in cells incubated for 90 min with radioactive choline ($91\,798 \pm 11\,500$ c.p.m./mg of protein for clone 2-5 versus $189\,313 \pm 34\,498$ c.p.m./mg of protein for clone 4-B). Since this could result from an increase in the rate of degradation of PtdCho, turnover of pre-labelled PtdCho was examined. The results shown in Figure 2(a) indicate that there was no significant difference in the turnover of PtdCho in the two clones. Under the same conditions, radioactivity in sphingomyelin actually increased in both clones (Figure 2b), suggesting that PtdCho is utilized as the precursor for sphingomyelin synthesis. Additionally, results from dual-label experiments (results not shown) confirmed that the increase in cellular choline phosphate did not result from an increase in PtdCho turnover. The decrease observed in radiolabelled choline might support the hypothesis that choline kinase activity is increased in antisense-transfected cells, but this would not explain the decrease in PtdCho. A decrease in the activity of the rate-limiting enzyme, phosphorylcholinecytidyl transferase, would explain the decrease in PtdCho. However, one would then expect CDPcholine levels to be decreased. In the experiments described, the results were equivocal and a non-significant rise in CDPcholine levels was observed. Incorporation of radioactivity into this particular metabolite was minimal (1 % of total incorporation) making it difficult to obtain reproducible results.

Thus antisense-transfected cells, which have only 25 % less PITP α than control cells, exhibit a greater than 50 % reduction in the amount of radioactive precursor incorporated into PtdCho during a short-term incubation, as well as a 30 % decrease following 48 h of incubation. The distribution of radioactivity in pathway intermediates suggests that the rate-limiting enzyme, phosphorylcholinecytidyl transferase, is the affected step. The increase in phosphorylcholine, combined with the decrease seen in both PtdCho and sphingomyelin, support this conclusion, although we can not rule out a role for choline phosphotransferase. Our inability to generate unequivocal results with

Figure 2 Turnover of [^3H]choline-labelled lipids in control and antisense-transfected WRK-1 cells

Cells were incubated with $5\,\mu\text{Ci/ml}$ [^3H]choline for 48 h, were then washed free of radioactivity and incubated in growth medium for an additional 24 h. Loss of radioactivity from PtdCho (a) and sphingomyelin (b) was determined at 9 h and 24 h. Values shown represent the means $\pm$ S.D. of sextuplicate determinations. Values at time 0 were normalized to 100 % for each clone.

respect to the levels of CDPcholine makes it difficult to be certain on this point.

These results are the opposite of those reported for yeast; in *Saccharomyces cerevisiae*, overexpression of SEC14p activity resulted in a decreased rate of PtdCho biosynthesis, suggesting that regulation of the PtdCho content of Golgi membranes by SEC14p somehow influences the secretory function of these organelles [9]. Furthermore, mutations in the CDPcholine pathway [disruption of the choline kinase (*CK1*) structural gene] can overcome the requirement for SEC14p [11]. Recent experiments in permeabilized mammalian cells suggest that both isoforms of PITP (α and β), as well as Sec14p, are capable of increasing guanosine 5'-[γ -thio]triphosphate-induced inositol phosphate accumulation, implying that transfer of PtdIns from internal membranes to the plasma membrane is required, although not sufficient, for continued cycling [25,39]. In addition, it was also shown that the PITPs augment the synthesis of PtdIns(4,5) P_2 , presumably by supplying plasma membrane lipid kinases with PtdIns [21–23,40,41]. In the experiments described in the present study, a small decrease in PITP α had no effect on basal or stimulated phosphoinositide metabolism; however, it is possible

that a further decrement in PITP α is required in order to observe an effect on phosphoinositide metabolism.

In summary, we have demonstrated that a reduction in cellular choline-containing lipids and an increase in cellular phosphorylcholine accompany a reduction in cellular PITP α in WRK-1 mammary tumour cells. In two of three experimental cell lines, a decreased growth rate was also observed. The precise mechanisms involved remain to be determined.

This work was supported by a Merit Review Award from the Veterans' Administration.

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