Phosphatidylinositol transfer protein β displays minimal sphingomyelin transfer activity and is not required for biosynthesis and trafficking of sphingomyelin

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Mammalian phosphatidylinositol transfer proteins (PITPs) α and β , which share 77% identity, have been shown to exhibit distinct lipid-transfer activities. In addition to transferring phosphatidylinositol (PI) and phosphatidylcholine (PC), PTP β has been shown to transfer sphingomyelin (SM), and this has led to the suggestion that PTP β is important for the regulation of SM metabolism. In the present study, we have analysed the ability of human PTP β to transfer and regulate the metabolism of cellular SM. We report that, *in vitro*, the two PTP isoforms were comparable in mediating PI, PC or SM transfer. Using permeabilized HL-60 cells as the donor compartment, both PTP isoforms efficiently transferred PI and PC, and were slightly active towards SM, with the activity of PTP β being slightly greater. To identify which cellular lipids were selected by PITPs, PTP α and PTP β were exposed to permeabilized HL-60 cells, and subsequently repurified and analysed for their bound lipids. Both PITPs were able to select only PI and PC, but not SM. SM synthesis takes

place at the Golgi, and PTP β was shown to localize in that compartment. To examine the role of PTP β in SM biosynthesis, Golgi membranes were used. Purified Golgi membranes had lost their endogenous PTP β , but were able to recruit PTP β when added exogenously. However, PTP β did not enhance the activities of either SM synthase or glucosylceramide synthase. Further analysis in COS-7 cells overexpressing PTP β showed no effects on (a) SM and glucosylceramide biosynthesis, (b) diacylglycerol or ceramide levels, (c) SM transport from the Golgi to the plasma membrane, or (d) resynthesis of SM after exogenous sphingomyelinase treatment. Altogether, these observations do not support the suggestion that PTP β participates in the transfer of SM, the regulation of SM biosynthesis or its intracellular trafficking.

Key words: lipid metabolism, lipid transport proteins, lipid traffic, phosphatidylcholine.

INTRODUCTION

The phosphatidylinositol transfer protein (PTP) family is one of two families of transfer proteins that can bind and exchange one molecule of phosphatidylinositol (PI) or phosphatidylcholine (PC) and facilitate the transfer of these lipids between different membrane compartments; the other is the structurally unrelated Sec14p family [1]. In mammals, three soluble isoforms of PTP (α , β and RdgB β) have been identified. PTP α and PTP β are highly related, sharing 77% identity and 94% similarity at the amino acid level, and remain the best studied PTP isoforms [1]. In mice, a 80% reduction in PTP α levels leads to neurodegeneration [2], and PTP β knockout results in early death during embryogenesis [3]. Moreover, PTP β seems to be essential for stem cell viability [3]. PITPs are known to transfer PI with a high efficiency and, to a lesser extent, also transfer PC. PTP β has the additional property of possessing high transfer activity towards sphingomyelin (SM) [4,5].

PITP α and PTP β were identified as essential components for the formation of secretory vesicles from the Golgi [6], a function similar to that executed by Sec14p, the yeast PTP homologue [7]. In yeast, the mechanism by which Sec14p regulates vesicle formation appears to be dependent on the capacity of these proteins to modulate PC metabolism. Sec14p overexpression was shown to negatively regulate PC biosynthesis by inhibiting phosphocholine cytidylyltransferase activity, the rate-limiting

enzyme of the CDP-choline pathway. *In vitro* experiments showed that Sec14p inhibits this enzyme only when it binds a PC molecule [8]. Therefore Sec14p seems to be able to sense and maintain a reduced PC concentration at the Golgi, which may be required for the secretory pathway [9]. Sec14p was also reported to maintain a pool of diacylglycerol (DAG) at the Golgi [10].

In mammalian cells, previous reports have suggested a role for PITPs in the regulation of phosphoinositide metabolism [11]. Recent studies, however, suggest that PITPs positively modulate PC and SM biosynthesis in mammals. WRK-1 cells expressing antisense mRNA for PTP α exhibited a decrease in PC and SM *de novo* synthesis [12]. PTP β overexpression in NIH3T3 cells enhanced the resynthesis of SM upon its degradation by exogenous sphingomyelinase (SMase), indicating a function for PTP β in the regulation of SM synthase activity and/or SM transport [13]. SM synthesis occurs mainly in the lumen of the Golgi, from which it is transported to the outer leaflet of the plasma membrane by vesicular flow. SM synthase transfers the phosphocholine moiety of PC to ceramide to generate SM and DAG [14]. Therefore SM synthase might contribute to regulate, in opposite directions, the levels of PC and DAG in Golgi membranes. The function of PTP β in post-Golgi trafficking may be dependent on its ability to modulate SM synthase activity [15]. During the last few years, a crucial function of the SM/ceramide pathway has been documented in signal transduction. In response to various exogenous stimuli, SM is hydrolysed by a

Abbreviations used: C₆-NBD, N-6(7-nitrobenz-2-oxa-1,3-diazol-4-yl)aminohexanoyl; DAG, diacylglycerol; DMEM, Dulbecco's modified Eagle's medium; GluCer, glucosylceramide; PC, phosphatidylcholine; PC-PLC, inositol lipid-specific phospholipase C; PI, phosphatidylinositol; PTP, phosphatidylinositol transfer protein; SM, sphingomyelin; SMase, sphingomyelinase.

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SMase to ceramide, a well established second messenger molecule [16–18]. The role of PITP β in SM transport [4,5] and SM synthesis [13] may be crucial in the regulation of the SM/ceramide pathway.

The present study was undertaken to examine the functions of PITP β in SM metabolism. Herein, evidence is presented that (i) within a cellular context, PITP β fails to efficiently transfer SM; (ii) PITP β does not regulate SM synthase activity in Golgi membranes; and (iii) PITP β overexpression in COS-7 cells does not affect PC or SM metabolism.

EXPERIMENTAL

Materials

[γ - 32 P]ATP, [3 H]choline, [14 C]acetate, *myo*-[3 H]inositol and [3 H]dipalmitoyl-PC (1,2-dihexadecanoyl-*sn*-glycero-3-phosphocholine) were obtained from Amersham. [3 H]SM, a gift from Professor T. Levade (INSERM U466, Toulouse, France), was obtained by catalytic tritiation of bovine brain SM (Sigma, St. Quentin Fallavier, France). The purity of [3 H]SM corresponded to 97–99% [19]. Bacterial inositol lipid-specific phospholipase C (PC-PLC) and SMase and C $_6$ -NBD-ceramide [*N*-6(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino]hexanoyl-ceramide were purchased from Sigma. Recombinant His $_6$ -tagged human PITP α and PITP β were purified from *Escherichia coli* as described previously [20].

Cell lines and transfections

HL-60 and COS-7 cells were routinely grown in a humidified 5% CO $_2$ atmosphere at 37 °C in RPMI medium and Dulbecco's modified Eagle's medium (DMEM) (Gibco BRL) respectively, containing 10% (v/v) heat-inactivated fetal calf serum (Sera Laboratories International Ltd). COS-7 cells were transfected by electroporation (250 μ F, 500 V) with the empty pcDNA3 plasmid (Invitrogen) or with pcDNA3 carrying cDNA encoding human PITP α or PITP β .

In vitro assay for PI transfer

The PI transfer assay was monitored as described previously from [3 H]PI-labelled microsomes to unlabelled liposomes [21]. In microsomes, the major molecular species of PI is 1-stearoyl-2-arachidonoyl-*sn*-glycero-3-phosphoinositol [22]. The specific radioactivity of the [3 H]PI-labelled microsomes was approx. 6 mCi/mmol. [3 H]PI-labelled microsomes (50 nmol of microsome protein) were incubated with liposomes (50 nmol of phospholipid; 98 mol% PC/2 mol% PI), in the presence or absence of transfer protein, in a final volume of 125 μ l of SET buffer [0.25 M sucrose, 1 mM EDTA, 5 mM Tris/HCl (pH 7.4)]. After incubation at 27 °C for 30 min, microsomes were precipitated by the addition of 25 μ l of ice-cold sodium acetate (0.2 M, pH 5.0) and removed by centrifugation at 12000 *g* for 10 min. A 50 μ l aliquot of the supernatant was counted for radioactivity.

Assay for PC and SM transfer

PC and SM transfer assays were performed by using [3 H]PC- or [3 H]SM-labelled liposomes and rat liver mitochondria as previously described [23]. To monitor PC transfer, liposomes (40 nmol of phospholipid; 80 mol% PC/10 mol% PE/10 mol% phosphatidate) contained 0.2 μ Ci of [3 H]PC. The specific radioactivity of the [3 H]PC-labelled liposomes was 4.25 mCi/mmol.

To monitor SM transfer, liposomes (40 nmol of phospholipids; 10 mol% SM/70 mol% PC/10 mol% PE/10 mol% phosphatidate) contained 0.2 μ Ci of [3 H]SM. The specific radioactivity of the [3 H]SM-labelled liposomes was 34 mCi/mmol. Of note, the proportions of the different phospholipid classes in labelled liposomes were identical with those of donor liposomes used in previous studies [5]. Radiolabelled liposomes were incubated with rat liver mitochondria (2 mg of protein) in a final volume of 0.2 ml of SET buffer at 37 °C. After a 30 min incubation, mitochondria were sedimented by centrifugation at 12000 *g* for 10 min. The sedimented mitochondria were resuspended in 500 μ l of SET buffer and centrifuged (12000 *g* for 10 min) through 500 μ l of 14.3% (w/v) sucrose. The pellet was resuspended in 50 μ l of 10% SDS, boiled for 5 min and counted for radioactivity.

Alternatively, PC and SM transfer was monitored from [3 H]choline-labelled HL-60 cells to acceptor liposomes. HL-60 cells were labelled for 48 h with [3 H]choline (1 μ Ci/ml; 12 nM). After a 2 h chase, 5×10^6 HL-60 cells were incubated with liposomes (100 nmol of phospholipid; 98 mol% PC/2 mol% PI), in a final volume of 200 μ l of PBS, in the presence or absence of transfer protein. After a 1 h incubation at 37 °C, the cells were precipitated by the addition of 25 μ l of ice-cold sodium acetate (0.2 M, pH 5.0) and removed by centrifugation at 12000 *g* for 10 min. The supernatant containing the liposomes was collected and lipids were extracted. [3 H]Choline-labelled PC and SM were separated and quantified after alkaline hydrolysis treatment (0.125 M NaOH; 2 h at 37 °C) as previously reported [24]. Data are expressed as the percentage of the total radiolabelled PC or SM localized in the outer leaflet of the plasma membrane, as determined by the releasable amount of lipid after incubating cells in the presence of either exogenous PC-PLC (0.1 unit/ml; 1 h at 37 °C) or SMase (0.1 unit/ml; 30 min at 37 °C). In HL-60 cells, $31 \pm 4\%$ and $66 \pm 3\%$ of the total radiolabelled PC and SM respectively are localized in the outer leaflet of the plasma membrane.

Assay to monitor phospholipid transfer from permeabilized cells

Phospholipid transfers were monitored from permeabilized radiolabelled HL-60 cells to acceptor liposomes. HL-60 cells were labelled for 48 h with [3 H]choline (1 μ Ci/ml; 12 nM) or *myo*-[3 H]inositol (1 μ Ci/ml; 60 nM). Cells were permeabilized with 0.6 unit/ml streptolysin O for 10 min at 37 °C in Pipes buffer [20 mM Pipes, 137 mM NaCl, 3 mM KCl, 1 mM MgCl $_2$, 2 mM MgATP, 100 nM Ca $^{2+}$, 1 mg/ml glucose, 0.1 mg/ml BSA (pH 6.8)] to deplete the cells of endogenous PITPs. The cells were centrifuged at 1800 *g* for 10 min, washed and resuspended in Pipes buffer. Approx. 5×10^6 HL-60 cells were incubated with or without liposomes (100 nmol of phospholipid; 98 mol% PC/2 mol% PI) in a final volume of 200 μ l of Pipes buffer, in the presence or absence of different amounts of recombinant PITP α or PITP β . After a 1 h incubation at 37 °C, the reaction was stopped as described above. Data are expressed as percentages of the radiolabelled total phospholipids after subtracting the values obtained in the absence of liposomes.

Determination of phospholipids bound to PITPs

HL-60 cells were cultured for 48 h in the presence of [14 C]acetate (0.5 μ Ci/ml) to metabolically label the total cellular lipids to equilibrium. After a chase in fresh medium for 2 h, HL-60 cells (10^7 cells/ml) were permeabilized and prepared as described above. Then cells (5×10^7 /ml) were incubated at 37 °C for 15 min in the presence or absence of recombinant His $_6$ -tagged PITP α or

PITP β (600 μ g). After centrifugation at 1800 *g* for 5 min, supernatants were collected and PITPs were recovered using a Ni²⁺ column (Qiagen). Lipids were extracted and resolved by analytical TLC developed either in chloroform/methanol/acetic acid/water (75:45:12:2, by vol.) when PC and SM needed to be resolved, or in chloroform/methanol/acetic acid/water (75:45:3:1, by vol.) when PI and phosphatidylserine needed to be resolved. The plates were imaged using Fuji phosphorimaging screens and data were quantified by densitometry using AIDA software. Unlabelled lipid standards were used to identify the various metabolic products. Alternatively, HL-60 cells were labelled with [³H]choline, and SM and PC were separated and quantified after alkaline hydrolysis treatment.

Determination of SM synthase and glucosylceramide (GluCer) synthase activities in Golgi membranes

Golgi membrane were purified as described previously [25]. To recruit PITP β to Golgi membranes, membranes (10 μ g/assay tube) were preincubated in the presence or absence of recombinant PITP β (50 μ g or as indicated) for 15 min at 37 °C in a final volume of 200 μ l of reaction buffer (5 mM MgCl₂, 5 mM MnCl₂, 1 mM EDTA, 1 mM MgATP, 1.6 mg/ml phosphocreatine, 100 μ g/ml creatine kinase in 50 mM Hepes, pH 7.2). Membranes were ultracentrifuged (120 000 *g*) for 30 min and washed with 500 μ l of reaction buffer. Golgi membranes were resuspended in 100 μ l of reaction buffer, containing 100 μ g of rat brain cytosol where indicated. The substrate mixture consisted of 400 μ M egg PC, 20 μ M C₆-NBD-ceramide (complexed with BSA in a 1:1 molar ratio) and 400 μ M UDP-glucose in reaction buffer. Reactions were started by the addition of 100 μ l of substrate mixture to the Golgi membranes. After a 30 min incubation at 37 °C, reactions were quenched by adding 850 μ l of chloroform/methanol (2:1, v/v). After centrifugation (1000 *g*, 10 min), the lower phases were dried under vacuum and resolved by analytical TLC developed in chloroform/methanol/30% ammonia (70:30:5, by vol.). The plates were imaged and data were quantified by densitometry using AIDA software by comparison with known amounts of C₆-NBD-ceramide.

Determination of SM synthase and GluCer synthase activities in COS-7 cells

COS-7 cells were washed in cold PBS to remove serum and incubated at 4 °C for 1 h in cold DMEM containing 2.5 μ M C₆-NBD-ceramide. After incubation at 37 °C for the indicated times, cells were washed in PBS and sedimented at 4 °C by low-speed centrifugation, and cell pellets were immediately frozen at -20 °C. Cell pellets were suspended in 0.2 ml of distilled water, and cells were disrupted at 4 °C by brief sonication. After an aliquot had been taken for protein determination, lipids were extracted and quantified as described above.

Metabolic labelling of cellular lipids

For SM and PC quantification, HL-60 and COS-7 cells were incubated in their respective media containing 10% (v/v) fetal calf serum and [³H]choline (1 μ Ci/ml; 12 nM). After a 48 h incubation, cells were chased for 2 h in fresh medium.

To quantify *de novo* synthesis of SM and PC, COS-7 cells were incubated at 37 °C in DMEM containing 10% (v/v) fetal calf serum and [³H]choline (1 μ Ci/ml; 12 nM). At the indicated times, cells were sedimented at 4 °C by low-speed centrifugation, and cell pellets were immediately frozen at -20 °C.

Determination of SM resynthesis

[³H]Choline-labelled COS-7 cells (3 $\times$ 10⁶/assay) were incubated at 37 °C in the presence or absence of exogenous SMase (50 m-units/ml, 30 min), washed twice in PBS and further incubated in fresh medium. At the indicated times, cells were collected and the incubation was stopped as described above. [³H]Choline-labelled HL-60 cells (3 $\times$ 10⁶/assay) were incubated at 37 °C in the presence or absence of exogenous SMase (50 m-units/ml, 30 min) and washed twice in PBS. An aliquot was incubated in fresh RPMI medium to monitor SM resynthesis in intact cells. Another aliquot was permeabilized with 0.6 unit/ml streptolysin O for 10 min at 37 °C in Pipes buffer. Permeabilized cells were then incubated in Pipes buffer containing 2 mM MgATP, 100 nM Ca²⁺, 200 μ M GTP, 200 μ g/ml creatine kinase and 3.2 mg/ml phosphocreatine, in the presence or absence of PITP β (200 μ g/ml) and rat brain cytosol (4.5 mg/ml). After 5 h, incubations were stopped as described above.

Determination of SM transport

COS-7 cells, overexpressing PITPs or not, were incubated at 37 °C for 5 h with [³H]choline (5 μ Ci/ml; 60 nM) to label cellular SM. Then cells were incubated in the presence or absence of exogenous SMase (50 m-units/ml, 30 min, 37 °C) to hydrolyse cell surface SM. The quantity of SM that was accessible to SMase was used as a measure of SM transport from the Golgi to the cell surface [13,26].

Lipid extraction and analyses

Cell pellets were suspended in 0.2 ml of distilled water, and cells were disrupted at 4 °C by brief sonication. After an aliquot had been taken for protein determination, lipids were extracted, and [³H]choline-labelled SM and PC were quantified as described previously [24]. Ceramide and DAG masses were measured as previously reported [27]. Radioactive ceramide 1-phosphate and phosphatidate were isolated by TLC using chloroform/acetone/methanol/acetic acid/water (50:20:15:10:5, by vol.) as developing solvent.

Immunolocalization studies of PITPs in COS-7 cells

COS-7 cells were cultured on glass coverslips for 24 h. Cells were incubated with isoform-specific monoclonal antibodies (anti-PITP α antibody, clone 5F12; anti-PITP β antibody, clone 1C1) and a polyclonal anti-TGN38 antibody (a gift from Dr G. Banting, University of Bristol, U.K.). Cy3-labelled anti-mouse and Cy2-labelled anti-rabbit (Amersham, Little Chalfont, Bucks., U.K.) were used as secondary antibodies. The immunostained cells were observed using an LSR Digital Imaging system equipped with an Olympus 1 $\times$ 70 microscope, and images were captured with a digital 12-bit charge-coupled device camera.

Western blotting analyses

Cells were washed and harvested in PBS. Cells were lysed for 30 min on ice in a buffer containing 50 mM Hepes, pH 7.5, 150 mM NaCl, 10% glycerol, 1% Triton X-100 and 0.5% protease inhibitor cocktail (Sigma). Equal amount of proteins were separated in an SDS/10%-polyacrylamide gel and blotted on to nitrocellulose membranes (Hybond-C; Amersham Pharmacia Biotech). Proteins were detected using an ECL[®] detection

Figure 1 *In vitro* transfer of radiolabelled PI, PC and SM

(A) The indicated amounts of recombinant PITP α and PITP β were separated on an SDS/10%-polyacrylamide gel and stained with Coomassie Blue. *In vitro* transfer of PI (B), PC (C) and SM (D) by the indicated amounts of recombinant PITP α (●) or PITP β (○) was assessed as described in the Experimental section. Data are means $\pm$ S.E.M. of three independent experiments performed in duplicate.

system (Amersham Pharmacia Biotech). PITP α was detected using a rabbit antibody against the C-terminal part of PITP α , and PITP β was detected using the monoclonal antibody clone 1C1. Monoclonal anti-(human β -actin) antibody was purchased from Sigma.

RESULTS

Lipid-transfer properties of PITP β

PITP β was originally purified as a SM transfer protein using an assay that monitored the transfer of pyrenylacyl-labelled SM from donor to acceptor vesicles [4,5]. The ability to transfer SM was reported to be unique to PITP β [4,5]. We re-examined the transfer of SM by PITPs using natural radiolabelled SM. In order to compare the transfer activities of PITP β and PITP α , we produced in *E. coli* and purified recombinant PITP α and PITP β (Figure 1A). The lipid-transfer activities of these recombinant proteins were first determined using a donor membrane that contained radiolabelled phospholipids and an acceptor membrane compartment. In agreement with previous studies [5,20], PITP β efficiently transferred PI (20% transfer in 30 min), and its activity was marginally greater than that of PITP α (Figure 1B).

When transfer of choline-containing lipids was analysed, both PITP isoforms transferred PC and SM, with low and comparable efficiencies (1–2% transfer in 30 min), with PITP β being marginally better (Figures 1C and 1D). While low concentrations of PITP isoforms promoted PI transfer, higher concentrations were required for the transfer of choline-containing lipids (compare Figure 1B with Figures 1C and 1D).

To examine the transfer of SM in a different assay, we established a new protocol to monitor SM-lipid transfer using intact [3 H]choline-radiolabelled HL-60 cells as a donor compartment to acceptor liposomes. HL-60 cells were grown in the presence of [3 H]choline for 48 h to label both the SM and PC pools. SM is highly enriched in the outer leaflet of the plasma membrane [14,28]. The cell surface of HL-60 cells, which contains $66 \pm 3\%$ of the total radiolabelled SM (see the Experimental section), but not PI, appears to be the most appropriate cellular membrane for measuring SM transfer *in vitro*. Thus intact radiolabelled cells were incubated with PITP β in the presence of acceptor liposomes. After 1 h, the liposomes were isolated and monitored for the transfer of radiolabelled PC and SM. PC- and SM-transfer activity was expressed as a percentage of the pool of SM and PC (present in the external leaflet of the

Figure 2 Transfer of radiolabelled PC and SM from HL-60 cells by PITPβ

HL-60 cells were metabolically labelled for 48 h with [^3H]choline or [^3H]inositol. (**a, b**) [^3H]Choline-labelled cells were incubated with acceptor liposomes in the presence or absence of the indicated concentration of recombinant PITPβ. After a 1 h incubation at 37 °C, lipids present in the acceptor liposomes were extracted, and radiolabelled PC and SM were separated and quantified. (**b**) HL-60 cells were preincubated for 1 h at 37 °C in the presence (PC-PLC) or absence (None) of exogenous PC-PLC (0.1 unit/ml) before adding acceptor liposomes and recombinant PITPβ (200 μg/ml). Alternatively, cells were incubated with liposomes and 200 μg/ml heat-inactivated (H.I.; 95 °C for 5 min) PITPβ. Lipid transfer was then determined as described above. Data are means $\pm$ S.E.M. of two independent experiments performed in duplicate. (**c, d**) The same experiment was carried out using radiolabelled permeabilized cells as the donor compartment in the presence or absence of the indicated concentration of recombinant PITPβ (**c**) or PITPα (**d**). Data are means $\pm$ S.E.M. of one experiment carried out in duplicate, and are representative of three independent experiments.

plasma membrane) that was accessible to PITPβ. Under these experimental conditions, a robust transfer of PC, but not of SM, was observed (Figure 2a). Of note, preincubation of cells with exogenous PC-PLC reduced PC transfer without modifying SM-transfer activity. As a control, heat-inactivated PITPβ failed to transfer any phospholipid (Figure 2b).

To analyse the transfer activities displayed by the two PITP isoforms towards PI, PC and SM within a cellular context, [^3H]inositol- or [^3H]choline-labelled HL-60 cells were

permeabilized and incubated with liposomes in the presence or absence of recombinant PITPs (Figures 2c and 2d). After 1 h, the liposomes were isolated and monitored for the transfer of radiolabelled phospholipids. Both PITP isoforms efficiently transferred PI and PC, being more active towards the inositol-containing lipids. In addition, PITPs displayed weak SM transfer activity, with that of PITPβ being slightly greater than that of PITPα. These data are in agreement with previous studies showing that PITP from bovine brain and heart is slightly active

Figure 3 Determination of phospholipids bound to recombinant PITPs following incubation with permeabilized HL-60 cells

HL-60 cells were incubated for 48 h in the presence of [¹⁴C]acetate (**A** and **B**) or [³H]choline (**C**). After permeabilization with streptolysin *O*, labelled HL-60 cells were incubated for 15 min at 37 °C in the presence (PITPα, PITPβ) or absence (None) of recombinant PITPs. After centrifugation, the supernatants were collected, PITPs recovered, and the lipids associated with PITP extracted and separated by TLC. (**A**) Lanes 1 and 2 contain two different amounts of total extract (T.E.) from HL-60 cells (lane 1, 150 000 d.p.m.; lane 2, 60 000 d.p.m.); lane 3, lipids in control supernatant (None); lanes 4 and 5, lipids associated with PITPα and PITPβ respectively. PE, phosphatidylethanolamine; PS, phosphatidylserine. Data were quantified by densitometry (**B**) or after alkaline hydrolysis treatment in the case of [³H]choline-labelled SM and PC (**C**). Data are means ± S.E.M. of three independent experiments.

towards SM [29], but contrasts with a more recent study that reported that PITPβ was most efficient for transfer of SM even compared with PI [5].

Determination of phospholipids bound to PITPβ

A characteristic feature of PITPs is their ability to exchange their bound lipid with cellular lipids. To determine which phospholipid species are selected by PITPs for exchange when presented with a mixture of cellular lipids, permeabilized HL-60 cells were exposed to recombinant His₆-tagged PITPs. The cellular lipids were prelabelled by growing the cells in the presence of [¹⁴C]acetate to label total cellular lipids, or [³H]choline to label the choline-containing lipids only. PITPs were then recovered via their His₆-tag and the protein-bound lipids were extracted and analysed by TLC (Figures 3A and 3B) or quantified after alkaline methanolysis treatment to distinguish between SM and PC (Figure 3C). Under these experimental conditions, both PITP isoforms were able to select only PI and PC from cell membranes (Figure 3A). Neither PITPβ nor PITPα was capable of selecting SM. Taking into account that, in HL-60 cells, the total amount of SM is in the same range as that of PI, and considerably less than that of PC (Figure 3B), this finding indicates that both PITPs display a much higher affinity for PI than for any other phospholipid class. In the total cell extract the ratio of PI to PC was 0.19 ± 0.05 , while this ratio reached 0.6 ± 0.05 and 0.9 ± 0.14 ($n = 3$) in PITPα and PITPβ respectively (Figure 3). Thus 40 % of PITPα molecules are liganded with PI and 60 % with PC. In the case of PITPβ, the

percentages are slightly different; there is 50 % each of PI and PC. These data are in agreement with the higher transfer activity exhibited by PITPα and PITPβ towards PI compared with choline-phospholipids (Figures 2c and 2d).

Influence of PITPβ on SM synthase activity

PITPs have previously been reported to influence the metabolism of SM [12,13]. Since SM synthase and GluCer synthase are mainly localized at the Golgi [14,30,31], we monitored the effect of PITPβ on the synthesis of the two major classes of sphingolipids, SM and glycosphingolipids. The synthesis of most glycosphingolipids begins with glucosylation of ceramide to form GluCer, while SM is synthesized from ceramide and PC. In order to investigate whether PITPβ influences sphingolipid metabolism at the Golgi, we first monitored the localization of endogenous PITPβ (and, for comparison, PITPα) using isoform-specific monoclonal antibodies. In agreement with previous reports [5], PITPα was found mainly in the cytoplasm and the nucleus in COS-7 cells (Figure 4A), whereas PITPβ was associated with perinuclear structures (Figure 4B). PITPβ co-localized with the Golgi marker TGN38 (Figure 4E), indicating that PITPβ is Golgi-localized. A similar pattern of PITPα and PITPβ localization was observed in HL-60 cells and RBL-2H3 mast cells (results not shown).

For monitoring SM synthase and GluCer synthase activities *in vitro*, Golgi membranes were purified from rat liver [25]. No endogenous PITPβ was associated with the purified membranes, most probably due to loss of PITPβ during purification.

Figure 4 Localization of PITPs in COS-7 cells

Immunolocalization of PITP α (A), PITP β (B, C) and the Golgi marker TGN38 (D). (E) Co-localization of PITP β and TGN38 is indicated by the yellow colour in the combined image of (C) and (D).

However, PITP β was found to re-associate with the Golgi membranes when they were incubated for 15 min with recombinant PITP β (Figure 5A). As shown in Figures 5(B) and 5(C), production of SM and GluCer was not substantially modified by the presence of PITP β on Golgi membranes. We considered the possibility that cytosol might contain critical factors that allow PITP β action. However, when Golgi membranes were incubated in the presence of rat brain cytosol, PITP β slightly inhibited SM synthase activity ($27 \pm 8\%$ inhibition; $n = 3$). The GluCer synthase activity associated with purified Golgi membranes was low, probably due to loss during purification, as this enzyme is localized at the cytosolic face of the Golgi, unlike SM synthase, which is in the lumen [14,31]. In the presence of rat brain cytosol, GluCer synthase activity was also reduced ($39 \pm 9\%$ inhibition; $n = 3$), a phenomenon slightly increased when PITP β was added ($51 \pm 14\%$ inhibition; $n = 3$). Therefore our data indicate that PITP β might negatively regulate SM synthase and GluCer synthase activities at the Golgi.

PITPs do not regulate PC and SM metabolism in COS-7 cells

To further investigate the function of PITPs in the regulation of sphingolipid metabolism, we transiently transfected COS-7 cells with a plasmid encoding PITP α or PITP β . The transfection efficiency was evaluated by Western blotting analysis for expression of PITPs (Figure 6A). It was estimated by densitometry

that cells transfected with a plasmid encoding PITP α or PITP β contained 289 ± 7 ng of PITP α and 69.6 ± 14 ng of PITP β per $100 \mu\text{g}$ of total extract protein respectively, as compared with 12.3 ± 7 ng of PITP α and 11.2 ± 5 ng of PITP β for mock-transfected cells. In addition, microscopic analysis demonstrated that more than 50% of COS-7 cells were transfected with a plasmid encoding PITP α or PITP β in fusion with an enhanced green fluorescent protein tag, and that the localization of both isoforms was similar to that of the endogenous proteins (results not shown).

Overexpression of PITPs did not affect the time-dependent incorporation of [^3H]choline into PC (Figure 6B) or SM (Figure 6C), indicating that PITPs are not involved in the regulation of PC and SM *de novo* biosynthesis. To specifically measure the SM synthase and GluCer synthase activities localized at the Golgi, COS-7 cells were incubated at 4°C with C_6 -NBD-ceramide to label the Golgi compartment. Then cells were incubated at 37°C to allow the conversion of C_6 -NBD-ceramide into C_6 -NBD-SM and C_6 -NBD-GluCer. As shown in Figure 6(D), PITP β overexpression did not significantly modify the synthesis of fluorescent analogues of SM or GluCer. In addition, since SM synthase transfers phosphocholine (from PC) to ceramide to generate SM and DAG, we measured the intracellular ceramide and DAG amounts in control and PITP-overexpressing cells. As shown in Figure 6(E), intracellular levels of ceramide and DAG were not affected by overexpression of either PITP.

Figure 5 PITP β does not enhance SM synthase and GluCer synthase activities in Golgi membranes

(A) Rat liver Golgi membranes (2 μ g) were incubated with the indicated amount of PITP β in the presence of 100 μ g of BSA for 15 min at 37 °C, and membranes were recovered by ultracentrifugation. PITP β recruitment to Golgi membranes was visualized by Western blotting using an anti-PITP β antibody. (B, C) Rat liver Golgi membranes (10 μ g) were preincubated with or without PITP β (50 μ g) for 15 min at 37 °C, ultracentrifuged and recovered. Golgi membranes were assayed for SM (B) and GluCer (C) synthesis in the presence or absence of rat brain cytosol (100 μ g). Data are means $\pm$ S.E.M. of one experiment carried out in triplicate, and are representative of three independent experiments.

Since PITP β may only function when ceramide is produced at the plasma membrane [13], we evaluated the effects of PITP overexpression on the resynthesis of SM after exogenous SMase treatment. As shown in Figure 7(A), overexpression of PITP α or PITP β in COS-7 cells did not enhance the resynthesis of SM as compared with mock-transfected cells. In COS-7 cells, SM resynthesis upon degradation by exogenous SMase is rapid, suggestive of high SM synthase activity. Consistent with this observation, Luberto and Hannun [32] showed that simian virus 40 transformation enhances a SM synthase activity that is particularly pronounced when ceramide is produced in the plasma membrane.

To determine whether SM resynthesis took place intracellularly or at the plasma membrane, we examined the accessibility of newly synthesized SM to a second round of SMase treatment. After a first SMase treatment of [3 H]choline-labelled COS-7 cells

and a 3 h incubation in fresh medium, most of the neosynthesized SM was not accessible to a second SMase treatment, suggesting that the resynthesis of SM took place intracellularly. Of note, SM replenishment at the plasma membrane was not modified by PITP β overexpression, further indicating the lack of involvement of PITP β in the regulation of SM traffic (Figure 7B). In addition, the conversion of C₆-NBD-ceramide into C₆-NBD-SM was inhibited by exogenous SMase treatment (Figure 7C), whereas NBD-GluCer production was enhanced (Figure 7D), indicating a redistribution of the natural ceramide generated by SMase from the plasma membrane to the lumen of the Golgi. These findings support the conclusion that SM resynthesis does not occur at the plasma membrane, but takes place at the Golgi [33]. However, our data clearly indicate that, despite its Golgi localization, PITP β is not a key regulator of SM synthesis.

We next evaluated the effects of PITP overexpression on the transport of SM from the Golgi to the cell surface. COS-7 cells were pulse-labelled in the presence of [3 H]choline for up to 8 h. Then cells were incubated in the presence or absence of exogenous SMase to quantify the proportion of the radiolabelled SM localized in the outer leaflet of the plasma membrane [13,26]. After a 5 h pulse labelling followed by SMase treatment, the radioactive SM levels represented 76% of the values measured in the absence of phospholipase, indicating that 24% of the newly synthesized SM reached the cell surface. After an 8 h pulse labelling, 41% of the radioactive SM was accessible to the exogenous SMase. Of note, when cells were labelled at equilibrium, approx. 50% of the total radiolabelled SM was localized at the cell surface (see Figure 7A). COS-7 cells overexpressing PITPs or not were pulse-labelled with [3 H]choline for 5 h before exogenous SMase treatment. In mock-transfected cells, $23.5 \pm 1.5\%$ of the newly synthesized SM was accessible to the SMase. In PITP α - and PITP β -overexpressing cells, these values were $22.5 \pm 0.4\%$ and $19.5 \pm 2.7\%$ respectively. Therefore our data indicate that PITP overexpression in COS-7 cells does not affect the transport of SM from the Golgi to the plasma membrane.

DISCUSSION

PITPs are believed to play critical function(s) in choline-phospholipid metabolism, inositol-lipid signalling and vesicular traffic [1,6,34–37]. Determining the specific transfer activities of PITPs can help in clarifying the functions of these proteins. Previous studies reported that the PI transfer activity displayed by PITPs is required to restore PI-PLC signalling and exocytosis by virtue of their ability to maintain phosphoinositide synthesis [20,21,38,39]. Other studies have proposed a role for PITPs in PC and SM metabolism [12,13]. In the present study, evidence is presented that, within a cellular context, human PITP α and PITP β are able to transfer PI and PC efficiently, but SM only marginally. In a cell-free system, PITP β does not stimulate sphingolipid synthesis in purified Golgi membranes. Moreover, PITP overexpression in COS-7 cells does not modify SM synthesis or SM replenishment following SMase treatment, further indicating the lack of involvement of these proteins in SM traffic and metabolism.

PITPs are unable to transport SM efficiently

Using fluorescent phospholipid analogues, it was reported that, in contrast with PITP α , partially purified PITP β from bovine brain exhibited a higher transfer activity towards SM than even towards PI, suggesting that PITP β bound SM with high affinity

Figure 6 PC and SM metabolism in COS-7 cells overexpressing PITPs

(A) COS-7 cells were transiently transfected with empty vector (pcDNA3) or a vector encoding PITPα or PITPβ. Expression of PITPs was visualized by Western blotting. (B, C) Mock-transfected cells (●) and PITPα- (○) and PITPβ- (○) overexpressing cells were incubated in the presence of [³H]choline for the indicated times. Radiolabelled PC (B) and SM (C) levels were determined. Data are means ± S.E.M. of two independent experiments carried out in duplicate. (D) Mock-transfected COS-7 cells (●, ■, ▼) and PITPβ-overexpressing cells (○, □, ▽) were incubated at 4 °C in the presence of C₆-NBD-ceramide. Then cells were washed in PBS and incubated at 37 °C for the indicated times. Lipids were extracted and separated by TLC. C₆-NBD-ceramide (●, ○), C₆-NBD-GluCer (■, □) and C₆-NBD-SM (▼, ▽) were quantified by densitometry. Data are representative of two independent experiments. (E) Ceramide and DAG levels were monitored using the DAG kinase assay in mock-transfected cells (closed bars) and PITPα- (open bars) and PITPβ- (hatched bars) overexpressing cells. Data are means ± S.E.M. of two independent experiments carried out in duplicate.

[4,5]. In the present study, the lipid-transfer activities of recombinant PITPs were assessed by using a donor membrane that contained natural radiolabelled phospholipids. By these means, we clearly demonstrated that the two PITP isoforms have approximately the same phospholipid transfer specificity and efficiency. While our study was in progress, Li and co-workers [40] reported that the two rat PITP isoforms were capable of transferring SM with comparable efficiency, and that PITP transfer activity towards SM was lower than that towards PI and PC. PITPβ was found to be marginally more active than PITPα. In the present study we show that, in contrast with the transfer of

PI and PC, PITPs also fail to efficiently transfer SM from cellular membranes (see Figures 2 and 3). When permeabilized cells, but not intact cells, were used as the donor compartment, both PITP isoforms displayed some SM transfer activity, with PITPβ being slightly better. When the lipids associated with PITPs were analysed, the major bound lipids were PI and PC. SM is mainly associated with cholesterol and glycosphingolipids in plasma membrane microdomains [28]. Thus it is possible that biophysical and biochemical properties of microdomains might prevent PITPs from extracting SM from cellular membranes. Accordingly, PITP overexpression in COS-7 cells did not affect the

Figure 7 Influence of PITPs on SM resynthesis in COS-7 cells upon exogenous SMase treatment

(A) Mock-transfected (▼) and PITP α - (●) and PITP β - (○) overexpressing COS-7 cells were labelled with [3 H]choline for 48 h. After incubation of cells at 37 °C in the presence or absence of exogenous SMase (indicated by the arrow), cells were washed twice with PBS and incubated at 37 °C in fresh medium. At the indicated time points, incubations were stopped and SM levels determined. Data are representative of three independent experiments. (B) Mock-transfected cells (closed bars) and PITP β -overexpressing cells (hatched bars) were labelled with [3 H]choline for 48 h. After a 2 h chase, cells were incubated at 37 °C in the presence (SMase 1) or absence of exogenous SMase (50 m-units/ml, 30 min). SMase-treated cells were washed twice with PBS and further incubated at 37 °C in fresh medium. After 3 h, cells were incubated in the presence (SMase 2) or absence of exogenous SMase (50 m-units/ml, 30 min), and SM levels determined. Results are expressed as a percentage of the value measured in the absence of phospholipase at the corresponding time. Data are means $\pm$ S.E.M. of two independent experiments carried out in duplicate. (C, D) Mock-transfected COS-7 cells (▼, ●) and PITP β -overexpressing cells (▽, ○) were incubated at 4 °C in the presence of C₆-NBD-ceramide. Then, cells were washed in PBS and further incubated in DMEM at 37 °C in the presence (▽, ▼) or absence (●, ○) of exogenous SMase (50 m-units/ml, 15 min at 37 °C). Next, cells were washed in PBS and incubated in DMEM at 37 °C for the indicated times. Fluorescently labelled lipids were extracted, separated by TLC and quantified by densitometry. Data are expressed as a percentage of total NBD-sphingolipids, and are representative of two independent experiments.

transport of SM from the Golgi to the plasma membrane. Thus the SM transfer activity displayed by PITPs *in vitro* might not be essential for the intracellular trafficking of SM *in vivo*.

PITP β does not regulate SM metabolism

Previous reports based on the study of stably transfected NIH3T3 cells showed that PITP β -overexpressing cells exhibited an enhancement of SM replenishment upon its degradation by exogenous SMase [13]. SM resynthesis following exogenous SMase treatment of PITP β -overexpressing cells was not altered by monensin, a well known inhibitor of vesicular transport at the Golgi [13]. Thus it was proposed that PITP β might facilitate SM replenishment by removing SM from the site of synthesis via monomeric transport. Alternatively, it was suggested that

PITP β might be able to stimulate a SM synthase activity, different from the SM synthase acting at the Golgi, and possibly localized at the plasma membrane [41]. In both cases, PITP β relocalization from the Golgi to the plasma membrane might occur.

In COS-7 cells, PITP β overexpression did not stimulate SM resynthesis upon degradation by SMase, suggesting that the ability of PITP β to influence SM metabolism might be cell-specific. On the other hand, the amount of endogenous PITP β in COS-7 cells might be sufficient for stimulating SM synthase activity. Nevertheless, in purified Golgi membranes which are depleted of endogenous PITP β , recombinant PITP β did not increase SM production (see Figure 5). Moreover, PITP β failed to stimulate the resynthesis of SM in permeabilized HL-60 cells. After a 30 min incubation of intact HL-60 cells in the presence of

SMase (50 m-units/ml), the radioactive SM levels represented $35 \pm 2\%$ of the values measured in the absence of phospholipase. A further 5 h incubation in fresh medium resulted in a SM level of $56 \pm 4\%$ of the control, indicating SM resynthesis. However, when the exogenous SMase treatment was followed by permeabilization, SM resynthesis did not occur even in the presence of rat brain cytosol and recombinant PITP β (results not shown). Altogether, our data strongly suggest a lack of involvement of PITP β in SM metabolism.

PITP overexpression in COS-7 cells does not alter PC metabolism

Overexpression of Sec14p in *Saccharomyces cerevisiae* has been reported to inhibit *de novo* PC synthesis, most probably by inhibiting choline-phosphate cytidyltransferase activity [8]. The present study shows that PITP β overexpression in COS-7 cells does not affect *de novo* PC synthesis, suggesting that, in contrast with Sec14p in yeast, human PITP β has no effect on choline-phosphate cytidyltransferase activity. Accordingly, mammalian choline-phosphate cytidyltransferase isoforms are mainly localized in the endoplasmic reticulum [42], whereas PITP β is associated with the Golgi apparatus ([5,43]; see also Figure 4). By using an antisense strategy, PITP α s were described to positively modulate PC biosynthesis [12]. In that study, cells stably transfected with an antisense PITP construct had 20% less PITP α than control cells, and exhibited a 50% decrease in *de novo* PC synthesis. However, in the present study, a 30-fold increase in PITP α expression in COS-7 cells did not modify the incorporation of labelled precursors into PC, therefore making human PITP α unlikely to be a key biomodulatory molecule in PC synthesis.

We also examined the activity of phospholipases acting on PC in PITP-overexpressing cells. (i) In co-transfection experiments with plasmids encoding phospholipase D (phospholipase D1 or phospholipase D2), PITP overexpression did not modify basal phospholipase D activities nor phospholipase D activation upon stimulation with PMA. (ii) [3 H]Choline incorporation into lyso-PC was unchanged in PITP-overexpressing cells, suggesting that PITPs do not activate a phospholipase A acting on PC. (iii) The catabolism of radiolabelled PC, evaluated by chase experiments, remained unaffected in PITP-overexpressing cells, further indicating that PITPs do not play a critical role in the regulation of PC turnover (results not shown). Therefore, despite the ability of PITPs to transfer PC *in vitro*, the role of these proteins in PC metabolism and signalling remains to be established. In many ways, our results indicate that the function of PITP β in post-Golgi trafficking is unlikely to be dependent on its ability to influence either PC or SM metabolism.

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