Rapid replenishment of sphingomyelin in the plasma membrane upon degradation by sphingomyelinase in NIH3T3 cells overexpressing the phosphatidylinositol transfer protein β

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In order to study the *in vivo* function of the phosphatidylinositol transfer protein β (PI-TP β), mouse NIH3T3 fibroblasts were transfected with cDNA encoding mouse PI-TP β . Two stable cell lines were isolated (SPI β 2 and SPI β 8) in which the levels of PI-TP β were increased 16- and 11-fold respectively. The doubling time of the SPI β cells was about 1.7 times that of the wild-type (wt) cells. Because PI-TP β expresses transfer activity towards sphingomyelin (SM) *in vitro*, the SM metabolism of the overexpressors was investigated. By measuring the incorporation of [*methyl*-³H]choline chloride in SM and phosphatidylcholine (PtdCho), it was shown that the rate of *de novo* SM and PtdCho synthesis was similar in transfected and wt cells. We also determined the ability of the cells to resynthesize SM from ceramide produced in the plasma membrane by the action of bacterial sphingomyelinase (bSMase). In these experiments the cells were labelled to equilibrium (60 h) with [³H]choline. At relatively low bSMase concentrations (50 munits/ml), 50 % of [³H]SM in wt NIH3T3 cells was degraded, whereas the levels of [³H]SM in SPI β cells appeared to be unaffected. Since the release of [³H]choline phosphate into the medium was comparable

for both wt NIH3T3 and SPI β cells, these results strongly suggest that breakdown of SM in SPI β cells was masked by rapid resynthesis of SM from the ceramide formed. By increasing the bSMase concentrations to 200 munits/ml, a 50 % decrease in the level of [³H]SM in SPI β cells was attained. During a recovery period of 6 h (in the absence of bSMase) the resynthesis of SM was found to be much more pronounced in these SPI β cells than in 50 % [³H]SM-depleted wt NIH3T3 cells. After 6 h of recovery about 50 % of the resynthesized SM in the SPI β cells was available for a second hydrolysis by bSMase. When monensin was present during the recovery period, the resynthesis of SM in bSMase-treated SPI β cells was not affected. However, under these conditions 100 % of the resynthesized SM was available for hydrolysis. On the basis of these results we propose that, under conditions where ceramide is formed in the plasma membrane, PI-TP β plays an important role in restoring the steady-state levels of SM.

Key words: phospholipid transfer proteins, phospholipid metabolism, sphingomyelin synthase.

INTRODUCTION

Phosphatidylinositol transfer proteins (PI-TPs) and their role in phospholipid metabolism have been the subject of extensive investigation [1]. In mammals two isoforms have been identified, PI-TP α and PI-TP β , the amino acids of which share a 77 % identity and a 94 % similarity. Both proteins are able to transfer *in vitro* phosphatidylinositol and phosphatidylcholine (PtdCho) between membranes, whereas PI-TP β is also able to transfer sphingomyelin (SM) [2–5]. As shown for fibroblasts and endothelial cells, PI-TP α is mainly localized in the cytosol and nucleus, whereas PI-TP β is predominantly present at the Golgi complex [6].

So far, little is known about the function of these isoforms. In a cell-free system containing *trans*-Golgi membranes, both PI-TP α and PI-TP β stimulated the formation of constitutive secretory vesicles and immature granules [7]. In permeabilized HL60 cells, both isoforms were shown to restore guanosine 5'-[γ -thio]triphosphate-stimulated protein secretion in the presence of ARF [8]. These results indicate that PI-TP may be involved in intracellular membrane trafficking from the Golgi complex to the plasma membrane. However, their exact role in this process is not known. In analogy with PI-TP α which has been reported to regulate PtdCho metabolism [9] and to be involved in inositol

lipid signalling [10–13] it is possible that PI-TP β is involved in these or similar signalling pathways. Because of its affinity for SM, PI-TP β could also be involved in the metabolism of this phospholipid.

SM is formed by SM synthase, which transfers the choline-phosphate group from PtdCho to ceramide, thereby also generating diacylglycerol [14–16]. Recent studies have shown that SM synthase activity is localized in the (early) Golgi, the plasma membrane and possibly in recycling endosomes [16–24]. The involvement of SM synthase in the SM signalling pathway is the subject of current investigation [25].

To investigate the effect of PI-TP β on cellular function and to determine whether it is involved in SM metabolism, two stable cell lines (SPI β 2 and SPI β 8) have been isolated which expressed increased levels of this protein. Here we show that overexpression of PI-TP β in NIH3T3 cells resulted in altered growth characteristics and in an enhanced replenishment of SM in the plasma membrane upon its degradation by exogenous sphingomyelinase.

EXPERIMENTAL

Materials

The pBluescript SK[−] and the pBK-CMV (CMV stands for cytomegalovirus) vector were purchased from Stratagene, La

Abbreviations used: PI-TP, phosphatidylinositol transfer protein; PtdCho, phosphatidylcholine; SM, sphingomyelin; DMEM, Dulbecco's modified Eagle's medium; NCS, newborn-calf serum; RT, reverse transcriptase; bSMase, bacterial sphingomyelinase; TNF α , tumour necrosis factor α ; SV40, simian virus 40; MMLV, Moloney-murine-leukaemia virus; wt, wild-type; CMV, cytomegalovirus.

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Jolla, CA, U.S.A. The pGEX-2T vector (customized by Dr. M. Verhage, Rudolf Magnus Institute, Utrecht University, Utrecht, The Netherlands) and [*methyl-³H]*choline chloride were obtained from Amersham Pharmacia Biotech, Uppsala, Sweden. G418 (Geneticin), bacterial sphingomyelinase (bSMase; from *Staphylococcus aureus*) and monensin were obtained from Sigma, St. Louis, MO, U.S.A. Nitrocellulose membranes were purchased from Schleicher and Schuell, Dassel, Germany. The simian-virus-40 (SV40) RNA isolation kit was obtained from Promega, Madison, WI, U.S.A.

Moloney-murine-leukaemia-virus reverse transcriptase (MMLV RT) was purchased from New England Biolabs, Beverly, MA, U.S.A. and Silica-gel 60 TLC plates were obtained from Whatman, Clifton, NJ, U.S.A., and Silica-gel 60 high-performance-TLC plates from Merck KgaA, Darmstadt, Germany.

pBK-CMV-PI-TP β construct

The cDNA encoding mouse PI-TP β was isolated and cloned into the pGEX-2T vector. The PI-TP β cDNA contains an *Xba*I site upstream of the translational start codon, and a *Bam*HI and an *Sal*I site downstream of the translational stop codon. The *Xba*I–*Sal*I fragment (including the *Bam*HI site) was isolated and cloned into the corresponding restriction sites of the cloning vector pBluescript SK[–] in order to obtain a *Sac*I site upstream of the PI-TP β cDNA. The resulting *Sac*I–*Bam*HI fragment (containing the complete PI-TP β cDNA) was cloned into the corresponding restriction sites of the pBK-CMV expression vector. This construct will be referred to as ‘pBK-CMV-PI-TP β ’. The expression of PI-TP β will be regulated by the CMV immediate early promoter, and the SV40 poly(A) adenylation signal will provide the signal for termination of eukaryotic transcription and polyadenylation.

Transfection

Wild-type (wt) NIH3T3 fibroblast cells were transfected using the calcium phosphate precipitation method of Chen and Okayama [26]. Briefly, cells were seeded 5 h prior to transfection at a density of 1.3×10^4 cells/cm² and then transfected with 20 μ g of pBK-CMV-PI-TP β . Fresh medium was added 20 h after transfection and the next day the cells were reseeded at a density of 250 cells/cm². After another 24 h, G418 (0.4 mg/ml) was added for selection of G418-resistant cells. Fresh medium containing G418 was added every 4 days, and resistant clones (SPI β) were identified after 3 weeks of growth. NIH3T3 cells overexpressing PI-TP α were prepared by the same method.

Cell culture

wt NIH3T3 fibroblast cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% (v/v) newborn-calf serum (NCS) and buffered with 44 mM NaHCO₃. Cells were maintained at 5% CO₂ at 37 °C in a humidified atmosphere. The selected SPI β cells and PI-TP α overexpressors were maintained under the same conditions, except that G418 (0.4 mg/ml) was present in the medium. G418 was absent when SPI β cells and PI-TP α overexpressors were used for experiments.

Gel electrophoresis and Western blotting

The PI-TP β content of several G418-resistant clones was analysed by immunoblotting with anti-PI-TP β antibodies. Cells were washed twice with PBS and then incubated with 10 mM EGTA in PBS for 5 min at 37 °C to detach the cells from the dish. The cells were centrifuged and the cell pellet was stored at –20 °C. The cells were resuspended in 100 μ l of SET buffer (0.25 M

sucrose/1 mM EDTA/10 mM Tris/HCl, pH 7.4) and homogenized by sonication (1 min; 50 W; Branson sonifier). After centrifugation at 17 500 *g* for 15 min at 4 °C, the protein concentration of the supernatant was determined using the Bradford assay [27]. A 100 μ g portion of protein was subjected to SDS/PAGE on a 15% gel and analysed by Western blotting using an antibody raised against recombinant PI-TP β . The PI-TP β levels on the immunoblot were quantified using a Bio-Rad GS700 imaging densitometer equipped with an integrating program. Known PI-TP β concentrations were used as a standard.

RT-PCR

Total RNA was isolated from cells using the SV40 RNA isolation method, according to the instructions supplied with the kit. The amount and purity of the RNA samples were determined by measuring the *A*₂₆₀ and *A*₂₈₀. First-strand DNA was synthesized by MMLV RT from 2.5 μ g of the total RNA with a random decanucleotide mixture as primers. A 1.25 μ l portion of RT product was used as a template for PCR. The forward and reverse primers used in this reaction were specific for PI-TP β and have respectively the following sequences:

5'-AGAAGGGACAGTACACACAC-3'

and

5'-CTTGGTCTTGACTGACTGGA-3'.

These primers will amplify PI-TP β cDNA from nucleotide 158 to 508, resulting in a fragment of 351 bp. The PCR reaction was carried out in a thermocycler as follows: 5 min at 95 °C, followed by 30 cycles of 1 min at 95 °C, 1 min at 55 °C, 1 min at 72 °C and finally 10 min at 72 °C. The PCR products were analysed on a 1%-agarose gel.

Growth assay

Cells were seeded at a density of 3×10^4 cells/dish (9 cm²) in DMEM containing 10% NCS. Cell growth was determined by counting the cells (in duplicate) every day for 10 days. The medium was changed every 3 days.

Measurement of cellular SM and ceramide mass

Total lipids were extracted from cells (0.5 mg of protein) by the method of Bligh and Dyer [28]. SM was separated from the other lipids by TLC, the solvent being chloroform/methanol/acetic acid/water (50:30:8:5, by vol.). After staining with iodine the spots were scraped off and SM was extracted from the silica gel. SM levels were determined by measuring P₁ [29] in the SM sample and in the total lipid extract. Ceramide levels were determined as described in [25].

Labelling of cellular SM

The experiments on SM metabolism were performed as described before [25]. Briefly, cells were seeded at 0.8×10^5 cells/plate (wt NIH3T3 cells) and at 1×10^5 cells/plate (SPI β cells) in 10-cm-diameter Petri dishes, so as to obtain approximately the same cell density at the time of harvesting. After 2 days of growth, the medium was removed and cells were labelled for up to 60 h with 0.5 μ Ci/ml of [*methyl-³H]*choline chloride in DMEM containing 10% NCS. Cells were harvested by scraping them into PBS, sedimented by centrifugation at 350 *g* for 5 min and stored at –20 °C. Pellets were resuspended in distilled water and briefly sonicated to obtain a homogeneous lysate. An aliquot of the lysate was used for protein determination [27] and the remainder was used for lipid extraction with 2.5 ml of chloroform/methanol

(2:1, v/v) [30]. Total lipid extracts were subjected to mild basic hydrolysis [31]. The radioactivity in the aqueous phase representing the original [^3H]PtdCho pool was determined by liquid-scintillation counting. [^3H]SM in the organic phase was separated from remaining labelled lipids by TLC in chloroform/methanol/acetic acid/water (50:30:8:5, by vol.). The [^3H]SM-containing spot was scraped off and the radioactivity determined by liquid-scintillation counting. [^3H]SM was also determined by scanning the plate with a Berthold Tracemaster 20 Automatic TLC-Linear analyser.

Hydrolysis by bSMase

After labelling with [*methyl*- ^3H]choline chloride as described above, cell cultures were washed once with PBS and chased for 2 h. After washing with PBS again, cells were treated with bSMase for 30 min. The cells were washed twice with PBS to remove bSMase and the lipids were extracted to determine [^3H]SM and [^3H]PtdCho as described above. To determine [^3H]choline phosphate released into the medium, the medium was extracted by the method of Bligh and Dyer [28] and the aqueous phase was freeze-dried. Aliquots of the freeze-dried samples were applied to a high-performance TLC plate and developed in ethanol/water/ammonia (48:95:7, by vol.). [^3H]Choline phosphate was determined by scanning the plate with a Berthold Tracemaster 20 Automatic TLC-Linear analyser. In some experiments, after removal of the bSMase, the resynthesis of SM was determined by incubating the treated cells in fresh medium for up to 24 h. Resynthesis of SM was also determined in the presence of 10 μM monensin added to the medium.

RESULTS

Overexpression of PI-TP β in mouse NIH3T3 fibroblast cells

Mouse NIH3T3 cells were transfected with the pBK-CMV-PI-TP β vector by a modified calcium phosphate precipitation method [26]. Stable cell lines were selected using the antibiotic G418. From several hundred G418-resistant clones that appeared after 3 weeks of selection, 14 cell lines were analysed by Western blotting for the expression of PI-TP β . Two cell lines, designated as SPI β 2 and SPI β 8, were chosen for further experiments. The expression of PI-TP β in these cell lines was analysed by semi-quantitative immunoblotting of the cytosolic fraction using PI-TP β -specific antibodies. As shown in Figure 1(A), both the

Figure 1 Analysis of NIH3T3 cells transfected with pBK-CMV-PI-TP β

(A) Western blot. Aliquots of 100 μg of protein were applied to each lane. Lane 1, wt NIH3T3 cells; lane 2, SPI β 2 cells; lane 3, SPI β 8 cells. (B) RT-PCR analysis. Equal amounts of DNA obtained after the RT reaction were used in the PCR reaction with primers specific for PI-TP β . After the PCR, 10 μl of the reaction mixture was loaded on a 1% agarose gel. Lane 1, wt NIH3T3; lane 2, SPI β 2; lane 3, SPI β 8.

Table 1 Growth characteristics and SM/ceramide content of NIH3T3, SPI β 2 and SPI β 8 cells

The values are the means for three independent experiments performed in duplicate. The ceramide content is based on three determinations.

Cell line	Doubling time (h)	$10^{-5} \times$ Saturation density (cells/cm 2)	SM (pmol/nmol of total lipid P)	Ceramide (pmol/mol of total lipid P)
NIH3T3	21	1.0 ± 0.2	58.4 ± 9	4.1 ± 0.6
SPI β 2	34	0.67 ± 0.09	58.8 ± 5	3.4 ± 0.3
SPI β 8	36	0.69 ± 0.09	59.0 ± 4	n.d.

Figure 2 Synthesis of SM and PtdCho in transfected and wt NIH3T3 cells

Cells were labelled with [*methyl*- ^3H]choline chloride (0.5 $\mu\text{Ci}/\text{ml}$) for the times indicated. SM and PtdCho levels were determined as described in the Experimental section. The values represent the means $\pm$ S.D. for two independent experiments performed in duplicate. Δ , NIH3T3 (PC); $\blacktriangle$, NIH3T3 (SM); $\circ$, SPI β 2 (PC); $\bullet$, SPI β 2 (SM).

Figure 3 SM hydrolysis as a function of bSMase concentration

[^3H]Choline-labelled cells were incubated with different amounts of bSMase for 30 min and the SM levels were determined as described in the Experimental section. The extent of hydrolysis is expressed as the percentage of [^3H]SM/mg of cellular protein relative to untreated cells. Values are representative for two independent experiments performed in duplicate. $\blacktriangle$, NIH3T3 cells; $\bullet$, SPI β 2 cells U. unit.

SPI β 2 and SPI β 8 clones expressed an increased level of PI-TP β as compared with wt NIH3T3 cells. By scanning the immunoblot it was estimated that the transfected cell lines SPI β 2 and SPI β 8 contained 15.5 ± 0.7 ng and 10.6 ± 0.3 ng PI-TP β respectively per 100 μg of cytosolic protein as compared with 1.0 ± 0.3 ng PI-TP β for wt NIH3T3 cells. RT-PCR on RNA isolated from the SPI β

Figure 4 SM synthesis in NIH3T3 and SPIβ2 cells in response to bSMase treatment

[³H]Choline-labelled cells were incubated with 100 units/ml (wt NIH3T3 cells) or 200 units/ml (SPIβ2 cells) bSMase. After 30 min of treatment (indicated by the arrow) the cells were washed with PBS and incubated with fresh medium for up to 24 h. **(A)** The amount of protein per dish as a function of time. At zero time the number of cells/dish was about the same for both cell lines. The difference in protein content between NIH3T3 and SPIβ2 cells reflects the difference in cell size. The values are representative for two independent experiments performed in duplicate. **(B)** The amount of [³H]PC per mg cellular protein as a function of time. Values are representative for two independent experiments performed in duplicate. **(C)** The amount of [³H]SM per mg cellular protein as a function of time. Resynthesis of SM was determined as described in the Experimental section. The extent of resynthesis is expressed as the percentage of [³H]SM/mg of cellular protein relative to untreated cells. Values are means $\pm$ S.D. for three independent experiments performed in duplicate. Δ , Untreated NIH3T3 cells; $\blacktriangle$, bSMase-treated NIH3T3 cells; $\circ$, untreated SPIβ2 cells; $\bullet$, bSMase-treated SPIβ2 cells.

and wt cell lines using primers specific for PI-TPβ yielded results that were in agreement with the blotting experiment (Figure 1B). By indirect immunofluorescence it was shown that PI-TPβ was associated with the Golgi membranes in SPIβ cells (results not shown).

Growth characteristics of the SPIβ cells

The doubling time and the saturation density of the SPIβ cell lines were determined and compared with those of wt NIH3T3 cells. As shown in Table 1, the doubling time of the SPIβ cells was 1.5-times longer than that of wt NIH3T3 cells (about 35 h and 21 h respectively). This decreased growth rate was accompanied by a lower cell density at confluency. The maximal cell density decreased from $1.0 \times 10^5/\text{cm}^2$ for wt NIH3T3 cells to $0.67 \times 10^5/\text{cm}^2$ for SPIβ2 cells and $0.69 \times 10^5/\text{cm}^2$ for SPIβ8 cells. In line with this, the average size of the SPIβ cells was increased.

Mock-transfected cells had the same growth characteristics as wt NIH3T3 cells (results not shown).

Effect of PI-TPβ overexpression on SM synthesis

In order to investigate the effect of PI-TPβ overexpression on SM synthase activity, SPIβ2 and wt NIH3T3 cells were incubated with [³H]methyl-choleline chloride and the time-dependent incorporation of ³H label in PtdCho and SM was determined. As shown in Figure 2, the labelling pattern was similar for both cell lines, indicating that the level of PI-TPβ had no effect on the *de novo* synthesis of SM. In addition, lipid analysis indicated that about 6% of the total phospholipid was SM in both the SPIβ and wt cells (Table 1). Ceramide levels were also very similar, arguing against ceramide being the active agent in the observed growth arrest of the SPIβ cells [32,33]. As the bulk of SM is present in the outer leaflet of the plasma membrane, we treated

Figure 5 Availability of resynthesized SM to bSMase

[3 H]Choline-labelled cells were incubated with 100 munits/ml (wt NIH3T3 cells) or 200 munits/ml (SPI β 2 and SPI β 8 cells) bSMase. After 30 min of treatment the cells were washed with PBS, incubated with fresh medium for 6 h and again treated with bSMase as described above. Results are means $\pm$ S.D. for three independent experiments performed in duplicate.

the cells with bSMase to determine whether there is any difference in the size of the degradable SM pool. To this end, the cells were labelled to equilibrium for 60 h and incubated with different concentrations of bSMase for 30 min. As shown in Figure 3, 80 % of the SM in the SPI β 2 and wt cells can be hydrolysed. At low bSMase concentrations (50 munits/ml), the hydrolysis of SM in the wt cells was 50 %. The loss of [3 H]SM was comparable with the release of [3 H]choline phosphate into the medium (results not shown). Under these conditions of bSMase treatment, the SPI β cells appear to be less sensitive to hydrolysis. However, the release of [3 H]choline phosphate into the medium was similar for both wt NIH3T3 and SPI β cells (results not shown). This indicates that the apparent lack of hydrolysis in SPI β cells reflects a rapid replenishment of the degraded SM by newly synthesized SM, which does not appear to occur in wt NIH3T3 cells.

This difference in ability to resynthesize SM was further investigated by treating wt NIH3T3 cells with 100 munits/ml and SPI β 2 cells with 200 munits/ml bSMase so as to obtain the same extent of SM hydrolysis (about 50 %). After this treatment the cells were allowed to recover in fresh medium for up to 24 h. As shown in Figure 4(A), the amount of protein increased to the same extent in the treated and untreated cells during this recovery period. This is taken to indicate that cell growth was not affected by bSMase treatment. In line with the increased amount of cells the [3 H]PtdCho/mg of cellular protein decreased in treated and untreated cells (Figure 4B). During this period the [3 H]SM/mg of cellular protein in untreated cells also decreased, but the decrease was much less pronounced (Figure 4C). Resynthesis of SM in the bSMase-treated SPI β 2 cells was rapid, yielding basal levels of SM within 6 h. In contrast, in the bSMase-treated wt NIH3T3 cells, only a small increase of the [3 H]SM/mg of cellular protein was observed, suggesting that the resynthesis of SM was limited. In similar experiments where 25 % of the SM was hydrolysed, the basal level of SM in SPI β 2 cells was restored within 2 h (results not shown). As a control, experiments on degradation and resynthesis of SM were also carried out with NIH3T3 cells overexpressing PI-TP α [34]. Results obtained with these cells were similar to those of wt NIH3T3 cells (results not shown).

Figure 6 Effect of monensin on resynthesis of SM and subsequent hydrolysis by bSMase

[3 H]Choline-labelled cells were incubated with 100 munits/ml (NIH3T3 cells) or 200 munits/ml (SPI β 2 and SPI β 8 cells) bSMase. After 30 min the cells were washed with PBS, incubated with fresh medium for 6 h in the presence of 10 μ M monensin and again treated with bSMase as described above. Values are means $\pm$ S.D. for three independent experiments performed in duplicate.

This strongly suggests that the rapid resynthesis of SM in the treated SPI β cells is dependent on the increased levels of PI-TP β present.

It is important to note that the bSMase treatment did not induce cell death, nor could we observe any major morphological changes by microscopical analysis. This is consistent with the reported inability of exogenous bSMase to induce death in a great variety of cell types [25,35].

Localization of resynthesized SM

The above results indicate that, after bSMase treatment, SM synthesis proceeds much more rapidly in SPI β cells than in wt NIH3T3 cells. This raises the question as to where the resynthesized SM is localized. To this end, cells were treated with bSMase, allowed to recover with fresh medium for 6 h and again treated with bSMase to establish whether the resynthesized SM is present in the outer leaflet of the plasma membrane. As shown in Figure 5, in SPI β 2 and SPI β 8 cells about 50 % of the resynthesized SM was available for hydrolysis by bSMase. For comparison, SM resynthesis was very limited in wt NIH3T3 cells, and virtually no additional SM was hydrolysed during the second bSMase treatment.

The above experiment was repeated in the presence of 10 μ M monensin during the recovery period (Figure 6). In NIH3T3 cells, monensin had an effect neither on SM resynthesis nor on hydrolysis. Similarly, in SPI β 2 and SPI β 8 cells, monensin did not have a significant effect on the resynthesis of SM. However, in the presence of monensin, 100 % of the resynthesized SM was accessible to bSMase. This suggests that, under these latter conditions, the replenishment of SM in the outer leaflet occurs

more rapidly than in the experiments where monensin was absent (Figure 5).

DISCUSSION

Here we present data to show that NIH3T3 fibroblast cells overexpressing PI-TP β have the capacity to rapidly resynthesize SM upon degradation of this phospholipid by the action of exogenous bSMase (Figures 3 and 4). This resynthesis was barely observed in wt NIH3T3 cells. NIH3T3 cells overexpressing PI-TP α also lacked an enhanced capacity to resynthesize SM from ceramide produced at the cell surface. This strongly suggests that ceramide formed in the plasma membrane by the action of bSMase is rapidly converted into SM provided that sufficient PI-TP β is present. Accordingly, the very slow resynthesis of SM in wt NIH3T3 cells may well reflect the low level of PI-TP β in these cells (Figure 1). Since, as estimated from the release of choline phosphate in the medium, SM in wt NIH3T3 cells and overexpressors is equally susceptible to degradation by bSMase (50 munits/ml, 30 min), it can be seen from Figure 3 that, in SPI β cells, the 50% degradation of SM is masked owing to complete replenishment of SM by resynthesis (i.e. the rate of resynthesis is 100%/h). This very effective mechanism of SM replenishment was less apparent under conditions where the overexpressors were treated with an excess of bSMase (200 munits/ml, 30 min), followed by a recovery period in the absence of bSMase. As can be estimated from Figure 4, the rate of resynthesis was about 15%/h. Given that extensive SM resynthesis must have occurred in the SPI β cells during the 30 min of bSMase treatment, the relatively low rate of resynthesis during the subsequent recovery period could possibly be due to a depletion of a pool of [3 H]PtdCho used by SM synthase to convert ceramide into SM.

At this stage we do not know how PI-TP β is involved in maintaining the steady-state levels of SM in the overexpressors. In previous reconstitution studies *in vitro* PI-TP β was shown to be active in protein secretion and in the formation of constitutive secretory vesicles at the *trans*-Golgi [7,8]. In line with this activity, PI-TP β was found to be associated with the Golgi complex [4,6]. This suggests that PI-TP β may be involved in an active vesicle flow between the Golgi and the plasma membrane and that, by way of this vesicle flow and endocytosis, the ceramide generated in the plasma membrane is effectively delivered to the site of SM synthase activity. In agreement with other studies [17–19,21,23,24], this SM synthase may be localized in structures near the plasma membrane (e.g. endosomes) or in some other Golgi-related organelle with PI-TP β as a facilitator. On the other hand, it cannot be excluded that, given its ability to transfer PtdCho and SM *in vitro*, PI-TP β is directly involved in delivering PtdCho to the SM-synthase or removing SM from this site. Thus far, however, we have not been able to show *in vitro* that PI-TP β is able to stimulate SM synthesis using a homogenate of wt NIH3T3 cells.

Similar to what was observed with the wt NIH3T3 cells, the regeneration of SM was very slow in human lung fibroblasts (WI38) treated with bSMase [25]. In this case transformation by SV40 yielded cells that were also very active in SM resynthesis upon bSMase treatment. It was inferred that the enhanced resynthesis of SM was a direct consequence of the transformation and was possibly due to a form of SM synthase that resided in the plasma membrane or in a functional proximity to it so as to be able to act on plasma-membrane ceramide [25]. This SM synthase would be different from the enzyme involved in the *de novo* synthesis. Our results also indicate that the resynthesis of SM may involve a SM synthase activity different from the

activity involved in *de novo* SM synthesis. This conclusion is based on the following observations. First, resynthesis of SM after treatment of the SPI β cells with bSMase is not affected by monensin, which is known to block vesicular transport between the *cis*- and *trans*-Golgi cisternae [24,36,37]. Secondly, SM resynthesized in the presence of monensin is completely accessible for hydrolysis by bSMase. These observations are in agreement with similar studies on SM synthesis in BHK (baby-hamster kidney) cells [37] and indicate that the SM synthase involved in the resynthesis of SM is present at a site different from the *cis*-/*medial*-Golgi where the *de novo* synthesis occurs [20,25]. In the absence of monensin, about half of the resynthesized SM was available for hydrolysis by bSMase, suggesting that the other half had not yet reached the outer leaflet of the plasma membrane. This would imply that, in the presence of monensin, the replenishment of outer-leaflet SM proceeds faster, possibly owing to the fact that monensin interferes with the intracellular flow of endocytosed ceramide [38]. In line with other studies [19,25,39] we presume that the SM synthase acting on plasma-membrane ceramide plays a role in the SM/ceramide cycle. This also explains why the *de novo* SM synthesis is identical in wt NIH3T3 and SPI β cells under conditions where the SM/ceramide cycle is not stimulated (Figure 2).

There is convincing evidence for the existence of an agonist-sensitive pool of SM which may be resynthesized by the same SM synthase acting on the plasma-membrane ceramide [19,25]. It remains to be established whether PI-TP β plays a role in the regeneration of this SM pool. In the present study we have treated the SPI β cells with the tumour necrosis factor α (TNF α) in an attempt to activate the endogenous SMase. This was not successful, probably because most NIH3T3 mouse fibroblasts lack the receptor for this agonist [40]. Cells that do have the TNF α receptor are sometimes resistant to the action of TNF α [39]. This resistance was reversed by addition of a P-glycoprotein inhibitor, thereby making SM available in the inner leaflet of the plasma membrane. However, it is also possible that this resistance to TNF α inducing apoptosis is related to the activity of PI-TP β interfering with the production of ceramide. A striking observation was the decreased growth rate of the SPI β cells (Table 1). This growth arrest may be related to an altered ceramide metabolism, as has been observed for other cells [32,33]. In this respect it is to be noted that the levels of ceramide were similar in SPI β and wt NIH3T3 cells, but that in the overexpressors the composition of the ceramide was altered, with a shift from C_{16:0}/C_{18:0}-containing species to species with very-long-chain fatty acids (C. M. van Tiel, unpublished work). It remains to be established whether the observed growth arrest and the enhanced ability to convert plasma-membrane ceramide into SM are related.

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