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Review

Overexpression of phosphatidylinositol transfer protein β in NIH3T3 cells has a stimulatory effect on sphingomyelin synthesis and apoptosis

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This article is dedicated to Prof. Henk van den Bosch. As a colleague, friend and teacher he has been an example of scientific integrity and dedication to me (K.W.A.W.) for a period of nearly 40 years

Abstract

Phosphatidylinositol transfer proteins (PI-TPs) consist of two isoforms (PI-TPα and PI-TPβ), which differ in phospholipid transfer properties and intracellular localization. Both PI-TP isoforms are substrates for protein kinase C and contain a minor phosphorylation site (Ser166 in PI-TPα; Ser165 in PI-TPβ). Only PI-TPβ contains a major phosphorylation site at Ser262, which must be phosphorylated for PI-TPβ to be associated with the Golgi. The PI-TP isoforms are completely conserved between mammals. Although their function is still not clear, their importance follows from knock-out studies, showing that mice lacking PI-TPα die soon after birth and that embryonic stems cells lacking PI-TPβ cannot be generated [Mol. Biol. Cell 13 (2002) 739].

We determined the levels of the PI-TP isoforms in various mouse tissues by immunoblotting. PI-TPα is present in all tissues investigated, with highest levels in brain (167 ng/100 μg total protein). The levels of PI-TPβ are 50–100 times lower than those of PI-TPα, with relatively high levels found in liver and brain (1.2 and 1.8 ng/100 μg of total protein, respectively). In contrast to NIH3T3 cells overexpressing PI-TPα, cells overexpressing PI-TPβ (SPIβ cells) were able to maintain steady-state levels of sphingomyelin in plasma membrane under conditions where this lipid is degraded by exogenous sphingomyelinase. This process of rapid sphingomyelin replenishment is dependent on PI-TPβ being associated with the Golgi as cells overexpressing a mutant PI-TPβ in which the major phosphorylation site is replaced (PI-TPβ(S262A) behave as wild-type NIH3T3 cells. Since the SPIβ cells display a decreased growth rate (35 h as compared to 21 h for wtNIH3T3 cells), we have investigated the sensitivity of these cells towards UV-induced apoptosis. We have found that the SPIβ cells, but not the cells overexpressing PI-TPβ(S262A), are very sensitive. We are currently investigating whether a relationship exists between PI-TPβ being involved in maintaining plasma membrane sphingomyelin levels and the enhanced sensitivity towards apoptosis.

Keywords: Phosphatidylinositol transfer protein; Tissue distribution; Phospholipase A_2 activation; Sphingomyelin metabolism; Apoptosis

1. Introduction

Phosphatidylinositol transferring proteins (PI-TPs) have been detected in all mammalian cells investigated to date [2]. The PI-transfer activity has also been detected in a variety of other organisms like yeast, plant and *Dictyostelium discoideum* [3–5]. In vitro PI-TP is able to transfer phosphatidylinositol (PI) and, to a lesser extent, phosphatidylcholine (PC) between membranes [6,7]. When PI-TP was first purified to homogeneity from bovine brain cytosol, two forms were detected based on a difference in isoelectric point. One form with an isoelectric point of 5.5 carried one molecule of PI, whereas the other form, with an isoelectric point of 5.7, was shown to contain a PC molecule [8]. The yeast homologue Sec14p is also able to transfer PI and PC between membranes in vitro, yet it shares no primary sequence homology with the mammalian PI-TPs [3,9,10]. Studies on *D. discoideum* have revealed that this organism contains homologues of both mammalian PI-TPs and *Saccharomyces cerevisiae* Sec14p [5]. To date, it has been clearly demonstrated that Sec14p plays an essential role in vesicle budding from the Golgi [3].

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2. The discovery of a PI-TP isoform

By use of indirect immunofluorescence, it was shown that in exponentially growing Swiss mouse 3T3 fibroblasts PI-TP is localized in the nucleus, throughout the cytoplasm and associated with the Golgi system [11]. Upon stimulation of semi-quiescent Swiss moue 3T3 cells with phorbol 12-myristate 13-acetate (PMA), a redistribution of PI-TP from the cytoplasm to the Golgi system was observed. This redistribution was accompanied by an increased phosphorylation of PI-TP [12]. Analysis of the cytoplasm of these cells by isoelectric focusing showed that two forms of PI-TP were present. One form (with an isoelectric point of 5.5) was the PI-carrying PI-TP that is commonly purified from mammalian tissue, whereas the second PI-TP (with an isoelectric point of 5.4) had not previously been detected. This new form was preferentially associated with the Golgi system and suggested to be a phosphorylated form of PI-TP [13].

Subsequently this acidic form of PI-TP was purified from bovine brain cytosol and the amino acid sequence was determined. It turned out that this 36-kDa PI-TP was not the phosphorylated form but instead was identical to a novel PI-TP cloned from rat brain [14]. This isoform was denoted as PI-TPβ, in distinction of PI-TPα initially purified from bovine brain. Surprisingly, bovine brain PI-TPβ was found to be highly homologous to a sphingomyelin (SM)-transferring protein from chicken liver. This activity was determined by measuring the transfer of pyrene-labeled SM (Pyr-SM) from donor to acceptor vesicles [15]. By use of this vesicle assay, we confirmed that, in contrast to PI-TPα, PI-TPβ expressed a very distinct SM transfer activity [16]. Similar to the chicken liver protein, the SM transfer activity of bovine brain PI-TPβ was dependent on the *N*-pyrenylacyl chain length of the SM with a preference for longer (i.e. tetradecanoyl) *N*-pyrenylacyl chains (Fig. 1). This is in contrast to what is observed with pyrenylacyl-PI and -PC species, transfer of which decreases with increasing acyl chain length. In recent studies measuring the transfer of radiolabelled phospholipids between membranes, rat PI-TPβ expressed SM transfer activity similar to or slightly lower than that of PC transfer activity. However, in both studies PI-TPα also displayed a low but distinct SM transfer activity [17,44]. In measuring transfer between radiolabelled permeabilized HL60 cells and liposomes, PI-TP-mediated transfer of SM was minimal [44]. We believe that these discrepancies are largely due to the type of transfer assay used. Given that these transfer activities are measured in vitro, one cannot be sure that the PI-TPs express similar activities in situ.

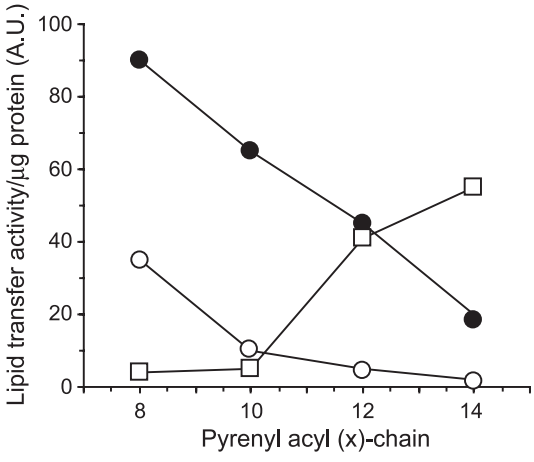

Fig. 1. Phospholipid transfer activity of bovine brain PI-TPβ. Phospholipid transfer activity was determined in the continuous fluorescence transfer assay using pyrene labeled PI (●), PC (○) and SM (□) as substrates [8]. Substrates with different pyrenyl-labeled acyl chain lengths were used where 'x' indicates the number of carbon atoms of the acyl chain.

3. Tissue distribution and expression levels

As shown by Hamilton et al. [18], the so-called mouse vibrator mutation results in an 80% decrease of PI-TPα levels in the brain. This leads to severe neurodegeneration and juvenile death. Attempts to generate PI-TPβ knock-out mice or mouse embryonic stem cells lacking PI-TPβ failed, whereas specific genetic ablation of PI-TPα function did not compromise stem cell viability [1]. This strongly suggests that PI-TPα and PI-TPβ probably have different functions and that PI-TPβ is an essential protein in murine cells.

To gain insight into the function of PI-TP isoforms, mRNA levels were determined in human tissue (heart, brain, placenta, lung, liver, skeletal muscle, kidney and pancreas). It was shown that PI-TPα mRNA was present in all tissues examined, with highest levels in pancreas and brain. On the other hand, PI-TPβ mRNA levels were highest in liver and the lowest level was detected in skeletal muscle [19]. In similar studies on rat tissue, high levels of PI-TPα and PI-TPβ mRNA were detected in brain [14,20]. The expression of PI-TP mRNA was also determined in testes showing that this organ contained two times more PI-TPα mRNA than brain [20], whereas PI-TPβ mRNA was expressed at very low levels [14].

To establish whether tissue mRNA levels reflect the amount of protein, PI-TPα and PI-TPβ were quantified by Western blotting and densitometric analysis. The antibodies used were raised against recombinant mouse PI-TPα and PI-TPβ in rabbits. Antibodies raised against PI-TPα were specific for this isoform [21]. However, the antibodies against PI-TPβ were slightly cross-reactive with PI-TPα. To remove the antibodies that recognized both isoforms, the antiserum was purified on a column consisting of a glutathione *S*-transferase-PI-TPα fusion protein bound to glutathione agarose beads. The anti-PI-TPβ antibody in the run-through was shown to be specific for PI-TPβ; amounts of PI-TPα as high as 0.1 μg were not detected by Western blotting. These isoform-specific antibodies were used to determine the amount of PI-TPα and PI-TPβ in

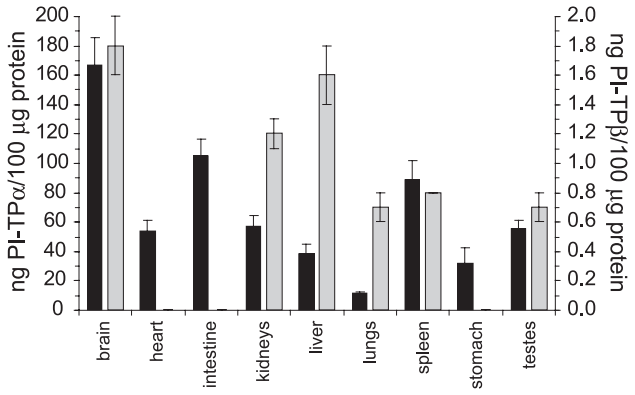

Fig. 2. Levels of PI-TPα and PI-TPβ in mouse tissues. The amount of PI-TPα and PI-TPβ was determined by isolation of the tissues from male Balb/c mice starved overnight. A 25% homogenate was prepared in SET buffer (0.25 M sucrose, 1 mM EDTA, 10 mM Tris–HCl, pH7.4) using a Dounce homogenizer. Prior to homogenization, the content of the intestine was removed by rinsing with SET buffer. The homogenates were centrifuged at 17,500 × *g* for 10 min and the pellet discarded. The protein content of the supernatant fractions was determined using the Bradford assay [43]. The supernatant proteins from the various tissues were subjected to SDS-PAGE on a 12% gel and analyzed by Western blotting using PI-TPα- or PI-TPβ-specific antibodies. On each gel, a concentration range of PI-TPα or PI-TPβ was included as to be able to quantify the levels of these proteins in the tissue samples, using a Bio-Rad GS700 imaging densitometer equipped with an integrating program. The values are the means of three independent experiments performed in duplicate.

mouse tissues. In agreement with the expression of rat PI-TPα mRNA [14], PI-TPα was present in all tissues analyzed with high levels in brain, intestine and spleen (i.e. 169, 105 and 89 ng/100 µg of tissue protein) (Fig. 2). PI-TPα was also detected in testes, but levels were lower than expected from the studies on mRNA expression. When compared to PI-TPα, tissue levels of PI-TPβ were approximately 50–100-fold lower. The highest levels were found in brain, liver and kidney (i.e. 1.8, 1.6 and 1.2 ng/100 µg of tissue protein, respectively). PI-TPβ could not be detected in heart, intestine and stomach.

4. The role of PI-TP in inositol lipid signaling, vesicle budding and secretion

To date, little is known about the exact cellular function of the PI-TP isoforms. Although the primary structures of PI-TPα and PI-TPβ are very similar with a sequence identity of 77% [14], the PI-TPs not only display a difference in phospholipid transfer activity and tissue distribution, but are also localized in different compartments of the cell. As was shown by indirect immunofluorescence and microinjection of fluorescently labeled proteins, PI-TPα is localized in the cytosol and nucleus, whereas PI-TPβ is mainly found at the Golgi membranes [16,22]. In a recent study it was suggested that PI-TPβ is also present in the cytosol available for dynamic interaction with the plasma membrane [23].

Despite this difference in cellular distribution, PI-TPα and PI-TPβ were shown to behave similarly in reconstitution assays used to establish PI-TP function. In cytosol-depleted permeabilized HL60 cells, total inositol phosphate production was greatly diminished, indicating a loss of the GTPγS-mediated phospholipase C (PLC) hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP_2). Screening of partially purified cytosol fractions for the presence of a factor that could reconstitute the GTPγS-mediated activity of PLC identified PI-TPα as an active factor in restoring the hydrolysis of PIP_2 [24]. In this assay, not only PI-TPα, but also PI-TPβ and the structurally completely different yeast homologue of PI-TP, Sec14p, were active [25].

In permeabilized PC12 cells, the PI-TPs are not only able to enhance the activity of PLC, but were also able to prime the ATP-dependent, Ca^{2+}-regulated fusion of secretory vesicles. Purified Sec14p was also active in this system, again showing that proteins lacking sequence similarity can promote the same process [26]. A similar role for PI-TP was observed when protein secretion was studied in permeabilized HL60 cells. In this system, both PI-TPα and PI-TPβ were able to restore GTPγS-stimulated secretion of proteins from preformed granules [27]. In a cell-free system using isolated Golgi membranes, both PI-TP isoforms were able to stimulated the formation of constitutive secretory vesicles and immature granules from the trans-Golgi network [28]. These studies show that Sec14p, being a crucial factor in yeast Golgi function, is also active in mammalian systems, replacing PI-TPs.

A temperature-sensitive mutant yeast strain (sec14-1^{ts}) was generated in which the PI transfer activity and cell growth are inhibited when the cells are grown at 37 °C, but not at 25 °C. Under non-permissive conditions, lack of PI-transfer activity resulted in an expansion of the Golgi complex and an accumulation of secretory granules within the cytoplasm [3]. Surprisingly, the Golgi secretory activity in these mutants was restored when the structural genes of the PC biosynthesis via the CDP-choline pathway were disrupted, implying a connection between Sec14p function and the CDP-choline pathway [29]. These results gave rise to the hypothesis that the relative PI/PC content of the Golgi membranes is directly dependent on Sec14p activity. According to this hypothesis, Golgi membranes must maintain a relatively high PI/PC ratio to sustain normal protein secretion. This PI/PC ratio was altered in sec14-1^{ts} at the non-permissive temperature, resulting in a loss of secretion [30]. Since secretion was restored by inhibiting the PC biosynthesis via the CDP-choline pathway in these mutants, it was proposed that indeed the PI/PC ratio was important for Golgi function. It was suggested that Sec14p could act as a sensor of the Golgi membrane phospholipid composition and regulate the activity of the CDP-choline pathway in such a way that the PC content required for secretion is maintained [30].

5. The role of PI-TP in PLA_2 activation and SM synthesis

The experiments described above were all performed on reconstitution systems. Another approach to gain insight into the cellular function of PI-TP was the overexpression of mouse PI-TPα or mouse PI-TPβ in NIH3T3 fibroblast cells. In one of these studies, NIH3T3 cells overexpressing PI-TPα two- to threefold (denoted as SPIα cells) and mock-transfected NIH3T3 cells were labeled to equilibrium with *myo*-[^{3}H]inositol. Analysis of the inositol metabolites showed increased levels of glycerophosphoinositol, inositol 1-phosphate, inositol 2-phosphate and lysophosphatidyl inositol in the SPIα cells. These results strongly suggested that in these mouse fibroblast cells, it is not a PLC that is activated but rather a PI-specific phospholipase A_2 [31]. As a control, similar experiments were carried out on NIH3T3 cells overexpressing PI-TPβ 10–15-fold (denoted SPIβ cells). In these cells, however, there was no significant change in inositol metabolites [32].

Since PI-TPβ is able to bind and transfer SM, we have investigated whether in these SPIβ cells this protein could be involved in SM metabolism. To this end, the SPIβ, SPIα and NIH3T3 cells were incubated with [methyl-^{3}H]choline chloride for 60 h to label the SM and PC pool. Subsequently the cells were treated for 30 min with sphingomyelinase (from *Staphylococcus aureus*), washed and incubated in fresh medium for 6 h. Although SM degradation (based on the release of [^{3}H]cholinephosphate) was comparable in all three cell lines, we observed that the levels of [^{3}H]SM remained constant in the SPIβ cells. This indicated that in these cells the degradation of SM was masked by a rapid synthesis of SM. This was confirmed by the finding that when SPIβ cells were treated with an excess of sphingomyelinase, the level of SM was back to basal levels after 6 h of recovery. In contrast, in NIH3T3 cells and SPIα cells this resynthesis of SM was very limited [32]. Similar experiments carried out in control COS cells and cells overexpressing PI-TPβ showed that SM resynthesis was rapid in both cell types [33]. However, the level of PI-TPβ in control COS cells (11.2 ng/100 μg total protein) was comparable to that in our SPIβ cells (10.6 ng/100 μg total protein). Hence, we presume that the replenishment of SM in COS cells is already maximal and cannot be enhanced by increasing PI-TPβ levels.

It is as yet unclear how PI-TPβ is involved in this rapid replenishment of SM. Apparently the decrease of SM in the plasma membrane is sensed by PI-TPβ. Given its in vitro transfer activity of PI, PC and SM, PI-TPβ may play a role in delivering substrate (i.e. PC) to SM synthase, which has been proposed to reside in the Golgi [34–36] and possibly also in removing the product (i.e. SM) from the synthase. On the other hand, PI-TPβ has been shown to be active in protein secretion and the formation of constitutive secretory vesicles [27,28]. Hence, PI-TPβ may also act on SM synthesis by promoting vesicle flow between the Golgi system and the plasma membrane. In analogy to Sec14p acting as a sensor of the PI/PC ratio in the Golgi, PI-TPβ could be involved in SM synthesis by a similar mechanism. As shown in the model (Fig. 3), vesicles flowing from the plasma membrane to the Golgi and in the opposite direction have a constant SM/PC ratio under steady-state conditions. Under these conditions PI-TPβ is enriched in SM and has little effect on SM synthase. Upon degradation of SM in the plasma membrane, vesicles with a low SM/PC ratio flow to the Golgi resulting in a PI-TPβ enriched in PC. Then PI-TPβ could stimulate SM synthase, which would lead to increased production of SM in the Golgi and an increased SM/PC ratio in the vesicles going to the plasma membrane, thereby maintaining the SM/PC ratio in the plasma membrane.

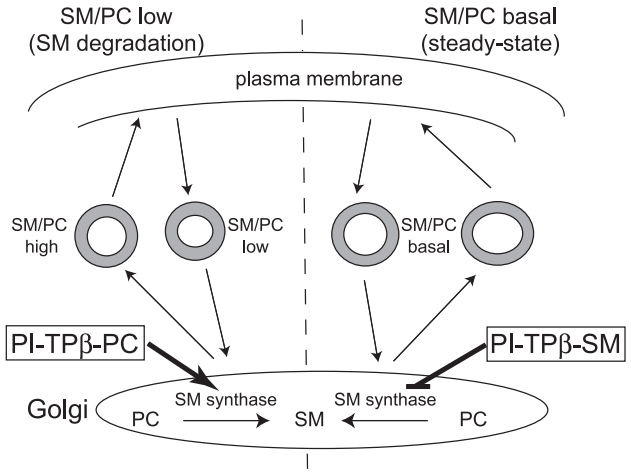

Fig. 3. Model for the regulatory role of PI-TPβ in maintaining plasma membrane SM levels. In the model, a continuous vesicle flow between plasma membrane and Golgi network ensures that the SM level in the plasma membrane remains constant. PI-TPβ associated with the Golgi acts as a sensor, stimulating SM synthesis when vesicles derived from the plasma membrane have a low SM/PC ratio (as a result of sphingomyelinase treatment), leading to an increased SM/PC ratio in the vesicles derived from the Golgi and thereby restoring SM levels in the plasma membrane. Under steady-state conditions, we envisage that PI-TPβ has little effect on SM synthesis. However, under both conditions PI-TPβ may stimulate vesicle flow from the Golgi.

Use of mouse fibroblast cells overexpressing the two PI-TP isoforms has provided the first clear evidence that the PI-TP isoforms have different cellular functions. This is in line with the fact that embryonic stem cells lacking PI-TPα are viable whereas attempts to generate PI-TPβ deficient stem cells have failed [1].

6. PI-TP as a substrate of protein kinase C

It was described in 1993 by Snoek et al. [12] that PI-TPα purified from bovine brain was a substrate for protein kinase C (PKC). Subsequent studies on recombinant

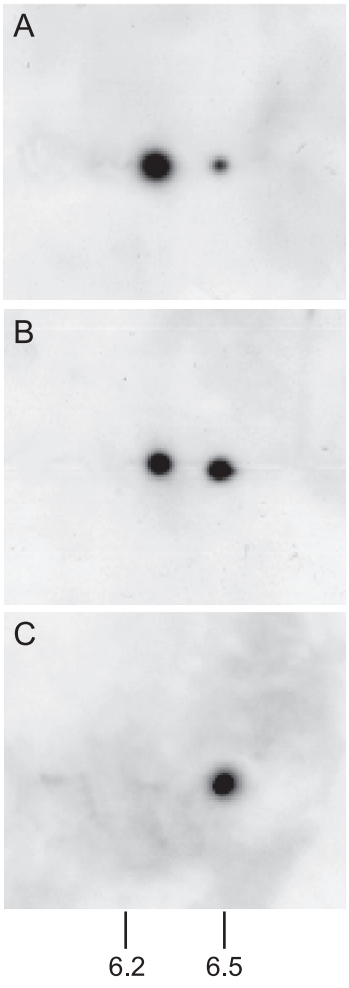

Fig. 4. PI-TPβ is mainly present in its phosphorylated form in SPIβ cells. Cell lysate protein from SPIβ cells (panel A), SPIβ cells incubated for 16 h with 5 μM GF 109203X (panel B) and SPIβS262A (panel C) were analyzed by two-dimensional PAGE followed by Western blotting using a PI-TPβ-specific antibody. In the first dimension, an immobilized pH gradient strip was (pH 5–8) was used. In the second dimension, a 12% SDS-gel was used. Phosphorylated PI-TPβ runs at pH 6.2 and its non-phosphorylated form at pH 6.5.

mouse PI-TPα and PI-TPβ showed that PI-TPβ was a much better substrate for PKC than PI-TPα [21,37]. By site-directed mutagenesis it was established that PI-TPα is exclusively phosphorylated on Ser166 [38]. Identification of the phosphorylation site in PI-TPβ showed that in vitro the major site is Ser262, which is absent in PI-TPα whereas residue Ser165 (identical to Ser166 in PI-TPα) is a minor site. Ser165 is present in virtually all members of the large family of PI-TPs [39]. In contrast, PI-TPβ is unique in that it is the only member of this family which contains Ser262. Surprisingly, PI-TPβ purified from bovine brain was a poor substrate for PKC [16]. We now believe that this is due to the fact that this bovine brain PI-TPβ is already phosphorylated (see below).

To determine whether in situ PI-TPβ is phosphorylated, two-dimensional polyacrylamide gel electrophoresis was carried out using lysates from SPIβ cells followed by Western blotting using the PI-TPβ-specific antibody. This showed that PI-TPβ is present in these cells in two forms with different isoelectric points. By densitometric analysis it was estimated that about 15% of the PI-TPβ collected in a spot at pH 6.5 whereas 85% collected in a spot at pH 6.2 (Fig. 4A). When the same analysis was carried out on lysates from SPIβ cells incubated with the PKC inhibitor GF109203X, a shift of PI-TPβ from the major spot at pH 6.2 to the minor spot at pH 6.5 was observed, indicating that PI-TPβ with an isoelectric point of 6.2 represented the phosphorylated form (Fig. 4B). This was corroborated by analysis of NIH3T3 cells overexpressing PI-TPβ(S262A). In line with the major phosphorylation site being absent, all the PI-TPβ collected at pH 6.5 (Fig. 4C). These results clearly showed that in situ PI-TPβ is mainly present as a phosphorylated protein [37].

Phosphorylation of PI-TPβ does not affect its SM transfer activity; this is also true for the PI and PC transfer activities. Similarly, the transfer activities of PI-TPβ(S262A) were the same as that of PI-TPβ [37]. As mentioned above, PI-TPβ is important in maintaining SM levels under conditions where this lipid is hydrolyzed by sphingomyelinase. Surprisingly, NIH3T3 cells overexpressing PI-TPβ(S262A) seven- to eightfold are virtually unable to resynthesize SM upon sphingomyelinase treatment [37]. This implies that SM transfer activity alone is not sufficient; rather that PI-TPβ must be phosphorylated to be active in SM replenishment. Phosphorylation is required for PI-TPβ to be associated with the Golgi, since incubation of SPIβ cells with a PKC inhibitor resulted in a relocation of PI-TPβ from the Golgi to the cytoplasm. Similarly, the mutant PI-TPβ(S262A) is also localized in the cytoplasm [37]. In conclusion, as for the model in Fig. 3, it appears that PI-TPβ has to be localized at the Golgi for it to be active in SM replenishment, being both a regulator of SM synthesis and a trigger for vesicle budding.

7. PI-TPα and PI-TPβ: a role in apoptosis?

In generating cells that overexpress PI-TPα and PI-TPβ, we noticed a striking difference in growth rates. As shown in Table 1, SPIα cells grow much faster than

Table 1
Growth characteristics of NIH3T3, SPIα, SPIβ and SPIβS262A cells

Cell line	Doubling time (h)	Fold increase in PI-TP (α or β) level
SPIα	13	2–3
NIH3T3	21	–
SPIβS262A	23	8–9
SPIβ	35	10–15

The doubling time was determined by counting the number of cells during growth and upon confluency. The amount of PI-TPα and PI-TPβ in the cells was determined by Western blotting using antibodies specific for either PI-TPα or PI-TPβ.

NIH3T3 cells, with a doubling time of 13 and 21 h, respectively, whereas SPIβ cells grow slower with a doubling time of 35 h [31,32]. Also in this case, SPIβS262A cells resemble NIH3T3 cells with a doubling time of 23 h.

Since there is a correlation between high growth rates and resistance towards apoptosis, we considered the possibility that the overexpression of the PI-TP isoforms could affect the sensitivity of the cells towards UV-induced apoptosis. To investigate this, the cell lines were incubated for 4 h with Dulbecco's modified Eagle's medium containing 1% (w/v) bovine serum albumin (DBB medium) to sensitize the cells. Subsequently the cells received a dose of 200 J/m^2 of UV light, were incubated for 3 h in DBB medium, followed by staining with Hoechst 33342 to visualize chromatin condensation as a measure for apoptosis [40]. Under these conditions, NIH3T3 cells show extensive chromatin condensation and cell blebbing (Fig. 5A–C). The extent of apoptosis for the various cell lines was determined by counting the number of blebbing cells (Fig. 5D). As compared to the NIH3T3 cells, the SPIα cells were almost completely resistant towards UV-induced apoptosis whereas the SPIβ cells were highly sensitive. This is in line with the observed decreased growth rate of these SPIβ cells. This increased sensitivity can be attributed to increased levels of PI-TPβ associated with the Golgi, since SPIβS262A cells behave as NIH3T3 cells after UV-irradiation (Fig. 5D). A similar apoptotic sensitivity was observed after treatment of cells with hydrogen peroxide and tumor necrosis factor α. In the absence of UV-irradiation, apoptosis of the various cell lines was observed due to the incubation with DBB medium, yet the extent of apoptosis was a factor of 10 lower. These experiments strongly suggest that PI-TPα protects cells against apoptosis, whereas PI-TPβ makes cells more apoptosis-sensitive. We are currently investigating which signaling pathways are involved in the decreased survival, i.e. whether the stimulatory effect of PI-TPβ on SM synthesis is related to the increased apoptosis sensitivity of SPIβ cells. In this respect, it is to be noted that D609, which inhibits SM synthase activity is anti-apoptotic [41,42], suggesting a positive function of SM synthase on apoptosis.

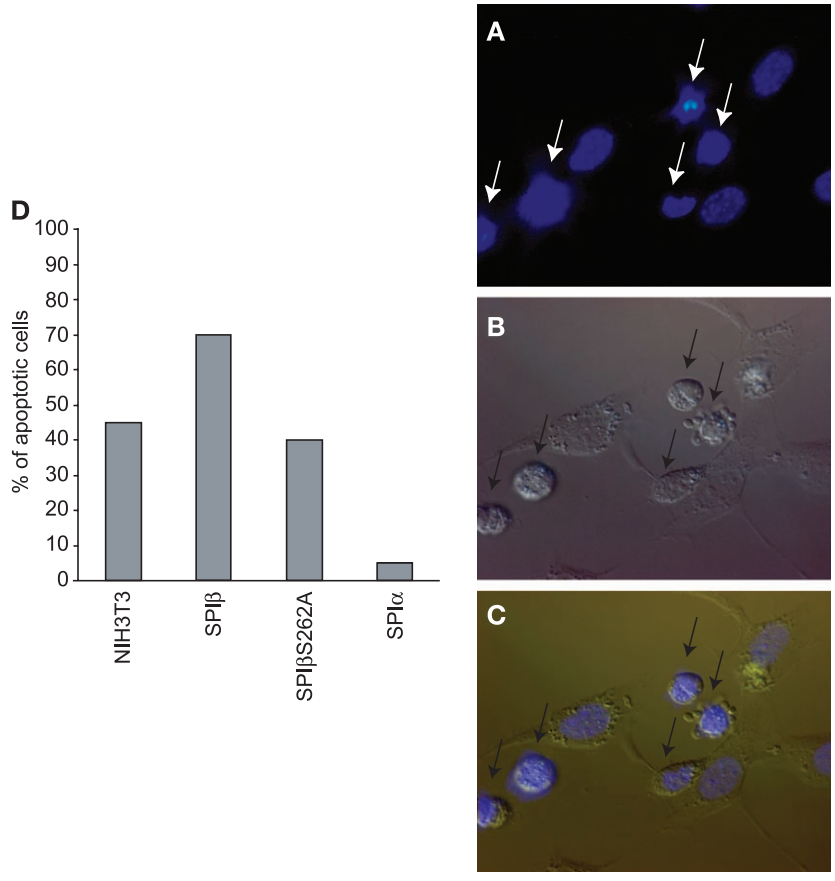

Fig. 5. UV-induced apoptosis as determined by chromatin condensation and cell blebbing. Cells were incubated with Dulbecco's modified Eagle's medium containing 1% (w/v) BSA for 4 h to sensitize the cells. Then they were exposed to a dose of 200 J/m^2 of UV light, incubated for 3 h and stained with Hoechst 33342 to visualize the nuclei. The images were taken on a Nikon TE2000 U inverted microscope. Differential interference contrast was measured in combination with fluorescence microscopy. (A) Hoechst staining of NIH3T3 cells; (B) differential interference contrast of NIH3T3 cells; (C) merge of figures A and B; (D) percentage of apoptotic cells in the various cell lines. Arrows indicate apoptotic cells. This presents a representative experiment.

Acknowledgements

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