Overexpression of Phosphatidylinositol Transfer Protein α in NIH3T3 Cells Activates a Phospholipase A*

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In order to investigate the cellular function of the mammalian phosphatidylinositol transfer protein α (PI-TPα), NIH3T3 fibroblast cells were transfected with the cDNA encoding mouse PI-TPα. Two stable cell lines, i.e. SPI6 and SPI8, were isolated, which showed a 2- and 3-fold increase, respectively, in the level of PI-TPα. Overexpression of PI-TPα resulted in a decrease in the duration of the cell cycle from 21 h for the wild type (nontransfected) NIH3T3 (wtNIH3T3) cells and mock-transfected cells to 13–14 h for SPI6 and SPI8 cells. Analysis of exponentially growing cultures by fluorescence-activated cell sorting showed that a shorter G1 phase is mainly responsible for this decrease. The saturation density of the cells increased from 0.20 × 10^5 cells/cm^2 for wtNIH3T3 cells to 0.53 × 10^5 cells/cm^2 for SPI6 and SPI8 cells. However, anchorage-dependent growth was maintained as shown by the inability of the cells to grow in soft agar.

Upon equilibrium labeling of the cells with myo-[3H]inositol, the relative incorporation of radioactivity in the total inositol phosphate fraction was 2–3-fold increased in SPI6 and SPI8 cells when compared with wtNIH3T3 cells. A detailed analysis of the inositol metabolites showed increased levels of glycerophosphoinositol, Ins(1)P, Ins(2)P, and lysophosphatidylinositol (lyso-PtdIns) in SPI8 cells, whereas the levels of phosphatidylinositol (PtdIns) and phosphatidylinositol 4,5-bisphosphate were the same as those in control cells. The addition of PI-TPα to a total lysate of myo-[3H]inositol-labeled wtNIH3T3 cells stimulated the formation of lyso-PtdIns. The addition of Ca^{2+} further increased this formation. Based on these observations, we propose that PI-TPα is involved in the production of lyso-PtdIns by activating a phospholipase A acting on PtdIns. The increased level of lyso-PtdIns that is produced in this reaction could be responsible for the increased growth rate and the partial loss of contact inhibition in SPI6 and SPI8 cells. The addition of growth factors (platelet-derived growth factor, bombesin) to these overexpressers did not activate the phospholipase C-dependent degradation of phosphatidylinositol 4,5-bisphosphate.

Phospholipid transfer proteins are proteins that are able to transfer phospholipids between membranes in vitro. A major phospholipid transfer protein in mammalian tissues is the phosphatidylinositol transfer protein alpha (PI-TP) (1). Recently, two isoforms of PI-TPα have been identified (i.e. PI-TPα and PI-TPβ) that demonstrate differences in cellular localization and in specific lipid transfer activity (2–5).

PI-TPα has been purified from both rat and bovine brain (6, 7). Cloning of the cDNA encoding rat brain PI-TPα showed that the protein consists of 271 amino acid residues (8). The subsequent isolation of the cDNAs encoding mouse and human PI-TPα revealed a high homology between the different mammalian PI-TPα (about 99% amino acid sequence identity) (9, 10). Furthermore, the cross-reactivity of the antibodies raised against bovine PI-TPα with a 35-kDa protein from other animals (e.g. rat, mouse, chicken, frog, and lizard) indicates an extensive conservation of the amino acid sequence between species (11). An exception is PI-TPα from yeast (i.e. SEC14p) that has the same molecular weight as mammalian PI-TPα and comparable phospholipid transfer activities yet shows no homology in the amino acid sequence (12–14).

So far, very little is known about the precise cellular role of mammalian PI-TPα. Since PI-TPα is able to transfer in vitro PtdIns between membranes in exchange for phosphatidylcholine, it was proposed that PI-TPα has a function in the transfer of PtdIns from its site of synthesis in the endoplasmic reticulum to other cellular membranes in order to maintain the level of PtdIns upon metabolism (15–17). PtdIns is a precursor molecule for several intracellular (and possibly also extracellular) lipid messengers, the best characterized of which are 1,2-diacylglycerol and inositol 1,4,5-trisphosphate (Ins(1,4,5)P_3) (18, 19). These messengers are formed when PtdIns is phosphorylated by PtdIns 4-kinase and phosphatidylinositol 4-phosphate 5-kinase to phosphatidylinositol 4,5-bisphosphate (PtdIns (4,5)_P_2), which subsequently is degraded by PLC. Other PtdIns derivatives of potential biological significance include those formed in the PtdIns 3-kinase pathway (20, 21), the inositol polyphosphates (22), the cyclic inositolphosphates (23), the glycerophosphoinositols (24–28), and lysophosphatidylinositol.

The abbreviations used are: PI-TP, phosphatidylinositol transfer protein; PtdIns, phosphatidylinositol; GroPIns, glycerophosphoinositol; Ins(4)P, inositol 4-phosphate; PtdIns(4)P, phosphatidylinositol 4-phosphate; PtdIns(4,5)P_2, phosphatidylinositol 4,5-bisphosphate; Ins(1,4,5)P_3, inositol 1,4,5-trisphosphate; Ins(1,4)P_2, inositol 1,4-bisphosphate; PLA, phospholipase A; PLC, phospholipase C; PDK, platelet-derived growth factor; NCS, newborn calf serum; DMEM, Dulbecco’s modified Eagle’s medium; DF, DMEM supplemented with 10% newborn calf serum; NCS, newborn calf serum; PBS, phosphate-buffered saline; PBS0, PBS without Ca^{2+} and Mg^{2+}; GTPγS, guanosine 5′-3′-(thio)triphosphate; Ins(1,2)cycP, inositol 1,2-cyclic phosphate; PIP_2, total inositol phosphate.

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(lyso-PtdIns) (29–32). A number of recent studies suggest a role of PI-TPα in the production of several of these derivatives. Thomas et al. (33) showed that PI-TPα is an essential cytosolic factor to stimulate PLCβ activity in permeabilized HL60 cells. Furthermore, Cunningham et al. (34) showed that PI-TPα promotes the synthesis of PtdIns(4,5)P2. Recently, it was shown in permeabilized human neutrophils that PI-TPα stimulates the formylmethionyl leucylphenylalanine-dependent production of phosphatidylinositol 3,4,5-trisphosphate in the presence of PtdIns 3-kinase γ (35). Moreover, in permeabilized PC12 cells, PI-TPα was found to be one of the three essential factors needed for the ATP-dependent, Ca2+-regulated fusion of secretory granules with the plasma membrane (36). An additional effect on secretion was shown in permeabilized HL60 cells, where PI-TPα and PI-TPβ were able to restore GTPγS-stimulated protein secretion in the presence of ADP-ribosylation factor (37). In a cell-free system containing trans-Golgi membranes it was shown that PI-TPα (as well as PI-TPβ) stimulates the formation of constitutive secretory vesicles and immature secretory granules (38). These results indicate that PI-TP has a function in intracellular membrane traffic from the Golgi to the plasma membrane that may be linked to the production of intracellular lipid messengers. The above studies, using semi-intact cells and in vitro systems, would indicate that PI-TP acts in different compartments of the cell, in particular at the plasma membrane and at the Golgi membranes. Localization studies by indirect immunofluorescence and by microinjection of fluorescently labeled purified PI-TPα and PI-TPβ into intact mammalian cells have shown that PI-TPα is mainly localized in the nucleus and in cluster-like structures in the cytosol and that PI-TPβ is mainly associated with the Golgi membranes (3, 4, 39, 40). However, upon stimulation of the cells by different growth factors (bombesin, PDGF) that stimulate the phospholipase C-dependent degradation of PtdIns(4,5)P2, accumulation of PI-TPα near the plasma membrane was not observed. Thus, no correlation was found between the cellular localization of PI-TPα and its proposed sites of action.

In order to gain further insight in the function and the mechanism of action of PI-TPα, we have established stable cell lines that overexpress PI-TPα. In this paper, we show that overexpression of PI-TPα in NIH3T3 cells affects the phenotype, the growth characteristics, and the inositol lipid metabolism of these cells.

**EXPERIMENTAL PROCEDURES**

**Materials**

The pBluescript vector SK+ was from Stratagene (La Jolla, CA). The anti-PI-TP antibodies were raised in rabbits against synthetic peptides representing the amino acid sequence of predicted epitopes in rat brain anti-PI-TP antibodies were raised in rabbits against synthetic peptides. The recombinant PI-TPα and PI-TPβ clones were isolated (including the NcoI restriction site around the translational stop codon and an XhoI site downstream of the translational stop codon. The Neol–Xhol fragment was isolated (including the EcorI site) and ligated into the cloning vector pUC21 (41) in the corresponding restriction sites in order to introduce an extra EcoRI site upstream of the PI-TPα cDNA. The resulting EcoRI fragment (containing the complete coding cDNA) was cloned into the unique EcoRI site of the pBSG expression vector (42). A construct was selected with the cDNA encoding PI-TPα in the sense direction. This construct will be denoted as pSG5-PI-TPα. The expression of PI-TPα will be regulated by the SV40 early promoter, and polyadenylation will be provided by the SV40 poly(A)-adenylation signal (42).

**Cell Culture**

All cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% newborn calf serum (NCS) and buffered with NaHCO3 (44 mM) in a 7.5% CO2 humidified atmosphere at 37 °C.

**Transfections**

wtNIH3T3 fibroblasts were seeded 5 h prior to transfection at a density of 1.3 × 10⁴ cells/cm². Cells were co-transfected with 30 μg of pSG5-PI-TPα and 10 μg of pSV2-neo (43) using a modified calcium phosphate precipitation technique at a CaO concentration of 7.5% (44). Fresh medium was added 20 h after transfection, and the next day the cells were seeded in new flasks at a density of 2500 cells/10 cm². After 24 h, neomycin (400 μg/ml Genetizin G418) was added for the selection of neomycin-resistant cells. Fresh medium containing neomycin was added every 4 days, and resistant clones were identified after 2 or 3 weeks of growth.

**Gel Electrophoresis and Immunoblotting**

The PI-TPα content of several neomycin-resistant clones was analyzed by immunoblotting with anti-PI-TP antibodies. Confluent cell cultures were washed twice with PBS and then incubated in 0.1 M lineal without Ca2+ and Mg2+ and removed from the dish by incubation with 8 mM EGT @ PBS 0 min at 37 °C. The cells were centrifuged, and the pellet was stored at −20 °C. A cell homogenate in 0.1 ml of SET buffer (10 mM Tris-HCl, pH 7.4, 1 mM EDTA, and 0.25% sucrose) was prepared in a Dounce homogenizer, followed by sonication (1 min at 50 watts). The homogenate was centrifuged for 10 min at 17,000 × g, and the supernatant (10% of the supernatant was used to calculate the saturation density of 17.5 μg of supernatant protein was loaded on an SDS-polycrylamide gel, and gel electrophoresis was performed as described (39). The proteins were electrophoretically transferred to a nitrocellulose sheet in a MultiPhor II Nova Blot electrophoretic transfer unit (Amersham Pharmacia Biotech) at room temperature applying 1 mA/cm² of gel for 2 h, and PI-TPα was detected as described (39). Quantification of the PI-TPα levels on an immunoblot was performed by scanning with a Bio-Rad GS 700 imaging densitometer equipped with an integrating program, with known PI-TPα concentrations as a standard.

**Growth Assay**

Cells were seeded at a density of 5 × 10⁴ cells/dish (9 cm²) in DMEM containing 10% NCS. Cell growth was determined by counting the cells every 9 days (in duplicate). The saturation density of the different cell lines was determined by seeding 10⁶ cells/dish (9 cm²), and the number of cells was determined after 7 days (in triplicate). The medium was changed every 3 days in both assays.

To determine the ability of the different cell lines to grow in soft agar, 2 × 10³ cells were suspended in 0.3% agar in DMEM containing 10% NCS and layered on 0.5% agar in the same medium. Fresh medium was added every 5 days. The colony growth was determined after 3 weeks.

**Analysis of the Cell Cycle by Fluorescence-activated Cell Sorting**

Cells were resuspended in 10 ml EDTA in PBS and washed once with PBS. The cells were fixed in PBS/methanol (3:1, v/v) for 20 min at 4 °C. A 9-fold excess of PBS was added, and the suspension was centrifuged for 5 min at 2500 rpm. The cell pellet was resuspended in 100 μl of PBS containing RNase A (1 mg/ml). Propidium iodide (50 μg/ml) was added, and the amount of the cells were incubated for 30 min at 37 °C. The samples were diluted 10-fold with PBS before analysis by fluorescence-activated cell sorting.

**Labeling of the Cells: Extraction and Analysis of Inositol Metabolites**

Two methods were used to analyze the inositol metabolites. The first method was used to obtain a quantitative preparation of the water-soluble total inositol phosphate (IP₃) fraction. The second method was used to analyze the composition of the inositol phosphate fraction and of the inositol phospholipids.

**Method 1**—The cells were grown in a six-well plate. 60–70% confluent cell cultures were incubated for 48 h with 1 μCi of myo-[3H]inositol in HEPES-buffered DP medium without inositol, containing 2% diazylated NCS. Cultures were washed twice with PBS and scraped in 20
mm Tris buffer, pH 7.4, containing 0.25 mM sucrose, 1 mM EDTA, 0.1% Nonidet P-40, and 10 mM LiCl. The cells were sonicated for 1 min in a sonication bath (Branson 1200), and a small sample was removed for protein determination. The cells were extracted by a modified Bligh and Dyer method (45). Upon phase separation, the organic phase was washed twice with MeOH/CHCl₃, and the water phases were combined and loaded on a Sep pak column (Waters Accel™ Plus QMA Cartridges) Free myo-[³H]inositol was eluted from the column by water, and the total IP₃ fraction was eluted with 500 mM triethyl ammonium hydrogen carbonate buffer. Radioactivity was determined by liquid scintillation counting. The organic phase, including the protein layer, was acetylated by the addition of 0.03 N HCl and washed twice with H₂O/MeOH/0.03 N HCl. The inositol phospholipids were separated by thin layer chromatography.

**Method 2**—The cells were grown in a 12-well plate and labeled as in method 1 except that 5 μCi of myo-[³H]inositol was used. After labeling, the cells were stimulated for 10 min with PDGF (20 ng/ml) or bombesin (10 nM). Prior to stimulation, 0.5 ml of DF medium without inositol, containing 0.3% bovine serum albumin and 10 mM LiCl, was added to the cells. After 10 min at 37 °C, the incubation was continued for 15 min in the absence or presence of the growth factors. Cultures were washed twice with PBS0 and harvested by scraping in 1 ml of 50 mM phenylmethanesulfonil fluoride and homogenized by 10 strokes in a Dounce homogenizer. The homogenate was centrifuged at 400 rpm for 2 min in an Eppendorf centrifuge at 4 °C to remove unbroken cells and cell debris, and 0.4 ml of the supernatant, containing about 600,000 dpm in [³H]inositol deriv-

## RESULTS

**Mouse NIH3T3 Fibroblast Cells Stably Transfected with the DNA Encoding PI-TPα**—The cDNA encoding mouse PI-TPα (9) was cloned into the expression vector pSG5, and the vector with PI-TPα in the sense orientation is denoted as pSG5-PI-TPα.

Mouse NIH3T3 fibroblast cells were co-transfected with both the pSG5-PI-TPα and pSV2-neo vectors or with only the pSV2-neo vector (control) by a modified calcium phosphate precipitation technique (44). Stable clones were selected by using the antibiotic Geneticin G418. Several hundred positive neomycin-resistant clones appeared after 2 weeks. From these clones, we selected three stable clones transfected with only the control pSV2-neo vector and 15 clones co-transfected with both the pSG5-PI-TPα and the pSV2-neo vector.

The stable clones transfected with the control vector (pSV2-neo) are denoted as OPIx (control vector, clone x), and the clones co-transfected with both pSG5-PI-TPα and pSV2-neo are denoted as SPIx (sense PI-TPα, clone x). The level of PI-TPα in the immunostained PI-TPα bands was compared with the density of the immunostained PI-TPα bands indicated that the trans-

**TABLE I**

| Cell line | Cell cycle duration (h) | Saturation density (%h) | Cells in S phase/mitosis | Duration of G₁ (h) |
|-----------|-------------------------|-------------------------|-------------------------|-------------------|
| NIH3T3    | 21                      | 0.20 ± 0.02             | 38                      | 13                |
| OPI3      | 21                      | 0.16 ± 0.02             | 35                      | 13                |
| SPI6      | 14                      | 0.53 ± 0.03             | 43                      | 8                 |
| SPI8      | 13                      | 0.53 ± 0.02             | 46                      | 7                 |

The cell cycle duration and saturation density were determined by counting the number of cells during growth and upon confluency; the duration of the G₁ phase and the percentage of cells in the S phase/mitosis were determined by fluorescence-activated cell sorting analysis of exponentially growing cell cultures. The values are the means of three independent experiments, performed in duplicate.

**FIG. 1A.** Analysis of the levels of PI-TPα by Western blotting (A) and the morphology of wtNIH3T3 cells, mock-transfected cells, and cells that are transfected with cDNA encoding PI-TPα (B). A, 17.5 μg of protein of the 100,000 × g supernatant of the cell lysates was applied in each lane. Cell lysis, electrophoresis, and Western blotting were performed as described under "Experimental Procedures." The density of the PI-TPα bands was compared with the density of known concentrations of PI-TPα. Gray bars, 10, 20, and 40 ng of purified recombinant mouse PI-TPα; white bars, wtNIH3T3 (1), SPI8 (2), OPI3 (3), and SPI6 (4). B, morphology of wtNIH3T3 (1), SPI6 (2), and SPI8 (3).
SPI8 cells, this percentage was 43 and 46%, respectively (Table I). From these values, and from the duration of the full cell cycle, it was calculated that the G1 phase is significantly shorter in SPI6 cells and SPI8 cells (7–8 h) than the G1 phase in wtNIH3T3 cells or in SPI3 cells (13 h, Table I).

Confluent monolayers of wtNIH3T3 and SPI6/SPI8 cells were different. At full confluency, wtNIH3T3 cultures shed dead cells, while SPI6/SPI8 monolayers started to curl up from the edges of the culture dish, forming a “solid” piece of tissue.

To investigate whether increased expression of PI-TPα led to anchorage-independent growth of the cells, the capacity of the cells to grow in soft agar was investigated. However, none of the cell lines were able to form colonies in soft agar. Therefore, increased expression of PI-TPα does not lead to a loss of contact inhibition or to transformation.

Incorporation of myo-[3H]inositol—In order to investigate whether overexpression of PI-TPα affects the metabolism of the inositol phospholipids, the SPI8/SPI6 cells and wtNIH3T3 cells were labeled with myo-[3H]inositol, and the relative incorporation in the inositol derivatives was determined as described in method 1. After equilibrium labeling of the cells (experimentally established by comparing various periods of labeling) the 3H label in the total IPn fraction was determined (Fig. 2). In wtNIH3T3 cells, 4.4% of the myo-[3H]inositol was incorporated in the IPn fraction. The addition of LiCl had no significant effect on the incorporation. However, incubation with bombesin, in the presence of LiCl, led to a 2–3-fold increase in the level of myo-[3H]inositol (the endogenous level) in the IPn fraction (Fig. 2). In the SPI6 and SPI8 cells, the percentage of incorporation in the IPn fraction was 2–3-fold higher as compared with wtNIH3T3 cells. Incubation with LiCl or with bombesin (in the presence of LiCl) did not further increase the level of incorporation. This indicates that the stimulation of the PLC-mediated degradation of PtdIns(4,5)P2 is impaired in the cells overexpressing PI-TPα.

TLC analysis of the inositol phospholipid fraction showed that the relative incorporation of myo-[3H]inositol in PtdIns, PtdIns(4)P or PtdIns(4,5)P2 was similar for wtNIH3T3, SPI6, and SPI8 cells (data not shown).

Analysis of Inositol Metabolites in wtNIH3T3 and SPI8 Cells—Since the water-soluble IPn fraction consists of a great number of inositol-containing metabolites, it is very possible that the composition of the IPn fraction from the SPI6 and SPI8 cells is different from that of the growth factor-stimulated wtNIH3T3 cells. This possibility was investigated by analysis of the water-soluble inositol metabolites by a high pressure liquid chromatograph connected to an on-line scintillation counter. The labeling and extraction procedures were adapted so as to increase the incorporation of myo-[3H]inositol into the metabolites and to optimize the recovery of lyso-PtdIns, which tends to disappear from the organic phase by frequent wash steps. In agreement with Fig. 2, the initial analyses of metabolites from SPI6 and SPI8 cells gave comparable results. We therefore restricted the detailed analysis to the wtNIH3T3 and SPI8 cells.

As shown in Table II, the total incorporation of myo-[3H]inositol in SPI8 cells was about 70% of that observed in wtNIH3T3 cells, whereas the total amount of protein per well, reflecting the number of cells, was twice as high. Despite this lower total incorporation, the absolute amount of label in the water-soluble inositol phosphates from the SPI8 cells was about twice as high, while the absolute amount of label in the inositol phospholipids was similar to that in the wtNIH3T3 cells.

The relative incorporation of myo-[3H]inositol in the inositol phosphate and inositol phospholipid derivatives is shown in Table III. The analysis of the water-soluble inositol phosphates indicates that in SPI8 cells the levels of Ins(1)P and Ins(2)P are significantly (p < 0.05) increased. There was also a 2-fold increase in the level of GroPIns and a small change in the level of Ins(4)P. Low levels of labeled Ins(1,4)P2 and Ins(1,4,5)P3 were also detected, showing no significant difference between wtNIH3T3 and SPI8 cells. Analysis of the inositol phospholipids showed that in SPI8 cells the relative incorporation of myo-[3H]inositol in lyso-PtdIns was clearly increased (p = 0.06). In these cells, the incorporation in PtdIns(4)P was significantly decreased; no changes were observed in the relative labeling of PtdIns and PtdIns(4,5)P2.

As shown in Fig. 2, the stimulation of the overexpressers with bombesin (10 nm) did not result in an increased incorporation of myo-[3H]inositol in the total IPn fraction, in contrast to what was observed with the wild type cells. In order to investigate whether the PLC-mediated degradation of PtdIns(4,5)P2 is operative in the overexpressers, SPI8 and wild type cells were stimulated with bombesin (10 nm) or with PDGF (20 ng/ml). Analysis of the inositol phosphate fractions showed that stimulation of the wtNIH3T3 cells mainly resulted in an increased incorporation of myo-[3H]inositol in Ins(1,4)P2 and Ins(4)P; there was no significant effect on Ins(1)P and Ins(2)P (Table IV). However, no effect was seen on the level of Ins(4)P or Ins(1,4,5)P3 in SPI8 cells, indicating the loss of growth factor-stimulated PtdIns(4,5)P2 degradation in these cells.

The Effect of PI-TPα on the Formation of Lyso-PtdIns in Vitro—The increased level of lyso-PtdIns in the SPI8 cells suggests the activation of a PLA1 and/or PLA2. In order to investigate whether PI-TPα was able to stimulate the formation of lyso-PtdIns in vitro, a homogenate of myo-[3H]inositol-labeled wtNIH3T3 cells was incubated with different amounts of this protein in the absence and presence of Ca2+ (5 mM). Each incubation contained about 0.1 μg of endogenous PI-TPα. As shown in Fig. 3, incubation with 0.5 μg of PI-TPα in the presence of Ca2+ led to a 2-fold increase in the level of lyso-PtdIns. The formation of lyso-PtdIns was further enhanced at a higher PI-TPα concentration (2.0 μg). In the presence of Ca2+, the stimulatory effect of PI-TPα was more pronounced. The increased levels of lyso-PtdIns were accompanied by a significant decrease in the level of PtdIns (Fig. 3). Under the assay conditions, there was no change in the absolute levels of [3H]PtdIns(4)P and [3H]PtdIns(4,5)P2. The level of lyso-PtdIns in incubations with 0.1 μg of PI-TPα (the endogenous level)
Overexpression of PI-TPα and the Production of Lyso-PtdIns

The values are the means of three independent experiments, performed in duplicate, ± S.E.

Table II

| Cell line | Total cellular \(^{3}\)H | Free myo-\(^{3}\)Hinositol | Inositol phosphates | Inositol phospholipids | Protein |
|-----------|-----------------|----------------|------------------|----------------------|---------|
|           | \(\text{dpm} \times 10^3\) | \(\text{dpm} \times 10^3\) | \% \(\text{dpm} \times 10^3\) | \% \(\text{dpm} \times 10^3\) | mg/well |
| wtNIH3T3  | 12.54 ± 1.80    | 7.35 ± 1.60    | 59               | 0.51 ± 0.13          | 4.68 ± 0.53 | 37      |
| SPI8      | 7.96 ± 1.56     | 3.47 ± 0.91    | 44               | 1.05 ± 0.59          | 3.45 ± 1.32 | 43      |

Table III

The percentages are the means of three independent experiments, performed in duplicate.

| Inositol derivatives | Percentage of total incorporated cellular \(^{3}\)H label |
|----------------------|---------------------------------|
|                      | wtNIH3T3 | SPI8 |
| myo-\(^{3}\)Hinositol | 58.14 ± 2.82 | 43.55 ± 3.23 |
| GroPIns              | 2.26 ± 0.23 | 4.72 ± 1.28 |
| Ins(1)P              | 1.15 ± 0.03 | 6.27 ± 1.71 |
| Ins(2)P              | 0.47 ± 0.03 | 2.66 ± 0.72 |
| Ins(1,4,5)P\(_3\)    | 0.06 ± 0.01 | 0.06 ± 0.01 |
| Ins(4)P              | 0.01 ± 0.005 | 0.02 ± 0.005 |
| PtdIns               | 0.07 ± 0.01 | 0.14 ± 0.04 |
| Lyso-PtdIns          | 29.97 ± 2.69 | 27.29 ± 3.16 |
| PtdIns(4)P\(_2\)     | 4.44 ± 1.11 | 12.74 ± 3.42 |
| PtdIns(4,5)P\(_2\)   | 2.34 ± 0.17 | 1.38 ± 0.21 |

**a** \(p < 0.05\), **b** \(p < 0.01\).

Table IV

The incorporation is expressed as a percentage of the total \(^{3}\)H label.

| Inositol phospholipids | wtNIH3T3 | SPI8 |
|------------------------|----------|------|
| \(\text{dpm} \times 10^3\) | 0.06 ± 0.01 | 1.15 ± 0.03 |
| \(\text{dpm} \times 10^3\) | 1.55 ± 0.03 | 6.02 ± 0.50 |
| \(\text{dpm} \times 10^3\) | 2.75 ± 0.05 | 0.65 ± 0.03 |
| \(\text{dpm} \times 10^3\) | 0.06 ± 0.01 | 6.27 ± 1.71 |
| \(\text{dpm} \times 10^3\) | 0.08 ± 0.02 | 4.10 ± 0.65 |
| \(\text{dpm} \times 10^3\) | 4.46 ± 0.18 | 1.95 ± 0.14 |

**a** \(p < 0.05\), **b** \(p < 0.01\).

reflected the level observed in intact cells (compare Fig. 3 and Table III). These results indicate that wtNIH3T3 cells contain a PLA\(_2\)/PLA\(_3\) activity acting on PtdIns that can be activated by PI-TPα in a Ca\(^{2+}\)-sensitive fashion.

**DISCUSSION**

The cellular function of PI-TPα has been extensively investigated using permeabilized cells and cell-free systems (33, 36, 38, 46). From these studies it was inferred that PI-TPα is involved in the synthesis of PtdIns(4,5)P\(_2\), possibly by delivering PtdIns to PtdIns 4-kinase. A more direct approach is to study the PtdIns metabolism in cell lines in which the expression level of PI-TPα is changed. In the present study we have established stable mouse NIH3T3 fibroblast cell lines that express a 2–3-fold increase in the levels of PI-TPα. These cells were chosen for the transfection experiments because they have a well defined PtdIns metabolism, several well known growth factor receptors, and a significant level of endogenous PI-TPα.

As shown in Table I, enhanced levels of PI-TPα lead to a dramatic increase in the growth rate of the cells. The cause of the increased growth rate can be manifold. However, since PI-TPα is involved, we have investigated the production of PtdIns metabolites. Two well known PtdIns-derived mitogenic signals are Ins(1,4,5)P\(_3\) and 1,2-diacylglycerol, which are formed when a PtdIns(4,5)P\(_2\)-specific PLC is activated by binding of growth factors to their receptors (18, 19). Another PtdIns derivative with mitogenic activity is lyso-PtdIns that is produced by the PLA\(_2\)-dependent pathway (24, 25, 29, 30). In the present study, we show that the incorporation of myo-\(^{3}\)Hinositol into the water-soluble inositol phosphate fraction was increased in the SPI8 cells when compared with wtNIH3T3 cells. An increase of \(^{3}\)H-labeled inositol phosphates was also observed in permeabilized PC12 cells upon the induction of purified PI-TPα (33, 34, 37). However, in a number of these studies the expression level of the IP\(_{3}\) fraction was not established; an increased "IP\(_{3}\)" fraction may include Ins(1)P and Ins(2)P as well as glycerophosphoinositol (products of PLA\(_2\)/lyso-PLA activation) and does not necessarily indicate that levels of Ins(4)P (and hence PLC activity) have increased. Hence, detailed analysis of the inositol phosphate fraction indicated that in SPI8 cells the levels of Ins(1)P and Ins(2)P were significantly decreased, whereas the levels of Ins(4)P and Ins(1,4,5)P\(_3\) were similar to that in wtNIH3T3 cells. This indicates that overexpression of PI-TPα in intact wtNIH3T3 cells has no effect on PtdIns(4,5)P\(_2\)-specific PLC. Rather, the identified inositol phosphate derivatives are characteristic for the degradation of PtdIns by PLA\(_2\) (24, 25, 27). In the latter studies, it was shown that the lyso-PtdIns that is produced upon activation of PLA\(_2\) can be degraded by a lysophospholipase to GroPIns. Alternatively, lyso-PtdIns can be degraded by a PLC to Ins(1,2)cycP, which, due to the acidic extraction conditions used, may be converted into Ins(1)P and Ins(2)P (23, 47). The activation of a potentially PtdIns-specific PLA in SPI8 cells was confirmed by the analysis of the inositol phospholipid fraction, showing that the level of lyso-PtdIns was 2–3-fold increased as
compared with control cells. In line with the enhanced level of lyso-PtdIns, the level of GroPins was also increased in SPI8 cells.

As for the other inositol phospholipids in SPI8 cells, the relative incorporation of myo-[3H]inositol was significantly decreased in PtdIns(4)P, while there were no changes in PtdIns and PtdIns(4,5)P2. Furthermore, the addition of purified PI-TPα to a crude lysate of [3H]inositol-labeled wtNIH3T3 cells induced a 2–3-fold increase in the level of lyso-PtdIns most probably derived from PtdIns, since the level of [3H]-labeled PtdIns decreased. No significant change was observed in the relative labeling of PtdIns(4)P and PtdIns(4,5)P2. These data strongly suggest that in cells with an increased expression of PI-TPα, a potentially PtdIns-specific PLA is constitutively activated.

Based on former studies, the activation of a PLA2 is most likely (25, 26). However, activation of a PtdIns-specific PLA cannot as yet be excluded. The procedures generally used to extract inositol phospholipids may lead to a loss of the rather water-soluble lyso-PtdIns.

This may explain why the PLA-mediated signal transduction pathway has not been detected in the semi-intact cells or in isolated membrane systems upon the addition of PI-TP (33–37). Furthermore, and as indicated above, the detailed analysis of inositol phosphates is required to ascertain whether PLC and/or PLA is activated.

Lyso-PtdIns has been shown to be a signaling molecule itself and could therefore be responsible for the increased growth rate of SPI6 and SPI8 cells. The mechanism by which this molecule acts is not yet fully clear. It has been proposed that lyso-PtdIns can either be released and act by binding to a membrane receptor analogous to the reported membrane receptor for lysophosphatidic acid or act intracellularly by interacting with target proteins (29, 30, 47, 48). Furthermore, lyso-PLA activity on lyso-PtdIns can produce GroPIns, which can itself be phosphorylated to glycerophosphoinositol 4-phosphate, which has been reported to be a novel intracellular messenger of the Ras pathway (28). The increased levels of GroPIns, lyso-PtdIns, and Ins(1)P could also be associated with different stages of differentiation rather than being characteristic of the malignant transformation process.

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