

Fluorescently Labeled Phosphatidylinositol Transfer Protein Isoforms (α and β), Microinjected into Fetal Bovine Heart Endothelial Cells, Are Targeted to Distinct Intracellular Sites

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Upon permeabilization of Swiss mouse 3T3 fibroblasts, an isoform of phosphatidylinositol transfer protein (PI-TP) was preferentially retained, a major part of which was associated with the perinuclear Golgi system (K. J. de Vries, A. Momchilova-Pankova, G. T. Snoek, and K. W. A. Wirtz, *Exp. Cell Res.* 215, 109–113, 1994). In the present study, the intracellular localization of this isoform (PI-TP β) and the regular form (PI-TP α) was investigated in fetal bovine heart endothelial cells by microinjection of fluorescently labeled analogs followed by confocal laser scanning microscopy. The PI-TP α and PI-TP β used were purified from bovine brain cytosol and covalently labeled with sulfoindocyanine dyes. By this novel method it was found that PI-TP β was preferentially associated with perinuclear membrane structures whereas PI-TP α was predominantly present in the nucleus and in the cytoplasm. This intracellular localization was confirmed by indirect immunofluorescence indicating that the fluorescently labeled PI-TP α and PI-TP β were targeted to the same sites as their endogenous counterparts. © 1996

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INTRODUCTION

Phosphatidylinositol transfer protein (PI-TP) is a ubiquitous protein occurring in all eukaryotic cells investigated, including a great variety of mammalian, yeast, fungal and *Xenopus laevis* cells [2–4]. An inherent property of PI-TP is its ability to bind and transfer phosphatidylinositol (PI) and phosphatidylcholine (PC) between membranes [5, 6]. Yeast PI-TP encoded by the SEC14 gene [7, 8] acts as a sensor of the Golgi PI/PC ratio and specifically controls the enzyme choline-phosphate cytidylyltransferase, the rate-limiting step in PC biosynthesis [9]. Recently, a rat brain cDNA sequence encoding a novel isoform (designated PI-TP β) was published [10]. The amino acid sequence deduced from this cDNA is 77%

identical (94% similar) with that of rat brain PI-TP α [10]. PI-TP β was partially purified from bovine brain cytosol [11] and purified to homogeneity from chicken liver [12]. In addition to PI and PC, PI-TP β transfers sphingomyelin (SM) between membranes, an activity that PI-TP α lacks completely [11, 12].

By using permeabilized cytosol-depleted cells it was shown that PI-TP α is an essential cytosolic factor in the reconstitution of: (i) the GTP γ S-mediated phospholipase C- β (PLC- β) activity involved in phosphoinositide hydrolysis [13], (ii) the phospholipase C- γ activity as controlled by the epidermal growth factor receptor [14], and (iii) the priming of ATP-dependent, Ca²⁺-activated protein secretion [15, 16]. The reconstitution of these processes is most likely related to the stimulatory effect of PI-TP α on the biosynthesis of PI-4,5-bisphosphate [17, 18]. Despite differences in phospholipid transfer activity, bovine PI-TP β was as efficient as PI-TP α in reconstituting GTP γ S-mediated PLC- β activity [11]. Moreover, both isoforms were able to stimulate in a cell-free system the formation of secretory vesicles from the *trans*-Golgi network [19].

Given the ability of both isoforms to reconstitute these diverse cellular processes we considered it important to investigate their intracellular localization. By indirect immunofluorescence using antibodies which enabled us to discriminate between the isoforms, PI-TP α was detected in the nucleus and the cytoplasm of Swiss mouse 3T3 fibroblasts and PI-TP β associated with the perinuclear Golgi system [11]. In the present study PI-TP α and PI-TP β were purified to homogeneity from bovine brain cytosol, covalently labeled with the fluorescent dyes Cy3 and Cy5, and microinjected into fetal bovine heart endothelial (FBHE) cells. Their intracellular localization was established by confocal laser-scanning microscopy and compared with the localization determined by indirect immunofluorescence.

MATERIALS AND METHODS

Materials

Q-Sepharose, Sephacryl S100, and heparin Sepharose CL-6B were obtained from Pharmacia (Uppsala, Sweden). Hydroxyl apatite (Bio-

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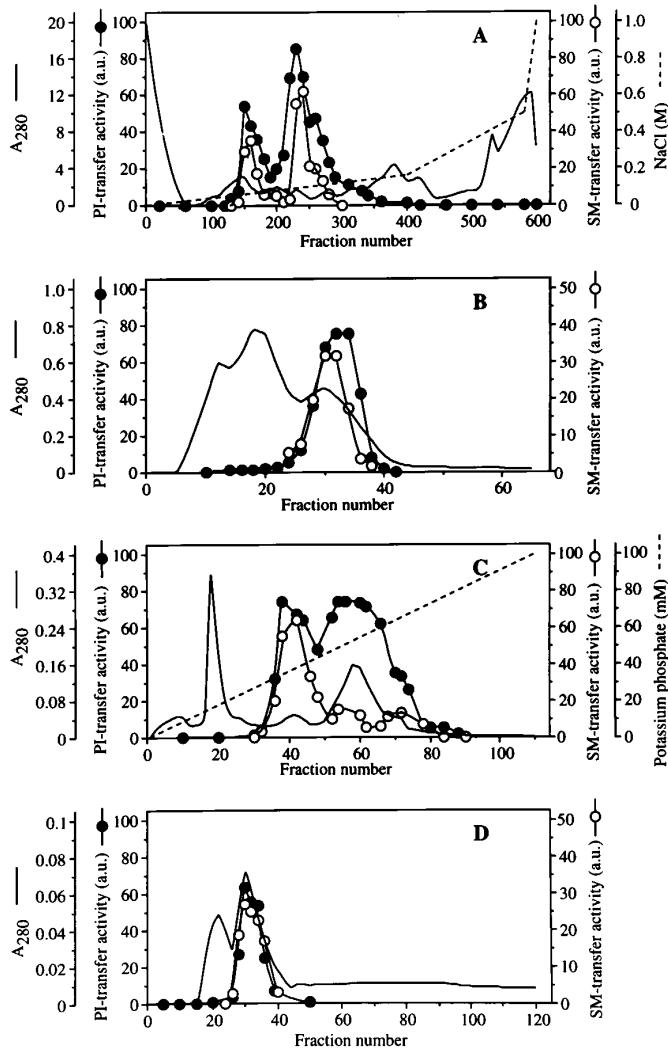


FIG. 1. Purification of PI-TP β from bovine brain cytosol. Fractionation was carried out by Q-Sepharose anion exchange chromatography (A), Sephacryl S100 gel filtration (B), chromatography on hydroxyl apatite (C), and heparin Sepharose chromatography (D). Column fractions were tested for SM transfer activity (open circles) and PI transfer activity (solid circles).

Gel, HTP) and P6DG desalting gel were from Bio-Rad (Hercules, CA). Egg yolk phosphatidylcholine (PC), phosphatidic acid (PA), trinitrophenyl-phosphatidylethanolamine (TNP-PE), and goat anti-rabbit IgG alkaline phosphatase conjugate (GAR-AP) were from Sigma (St. Louis, MO). *N*-pyrenylmyristoyl-SM (Pyr(14)SM) and 1-palmitoyl-2-pyrenyldecanoyl-PI (Pyr(10)PI) were a kind gift of Dr. P. J. Somerharju (Department of Basic Chemistry, University of Helsinki, Helsinki, Finland). The fluorescent sulfoindocyanine succinimidyl esters Cy3 and Cy5 were obtained from Biological Detection System, Inc. (Pittsburgh, PA). Centricon-10 ultrafiltration devices were from Amicon (Beverly, MA). An antibody cross-reactive with both PI-TP α and PI-TP β (antibody I) was raised in rabbits against a mixture of two synthetic peptides representing the predicted epitopes of rat brain PI-TP α (residues 40–60 and 240–260) [11, 20]. An antibody specific for PI-TP α was raised in rabbits against purified mouse PI-TP α expressed in *Escherichia coli* (antibody II) [21]. Goat anti-rabbit IgG (H + L)-peroxidase conjugate (GAR-PO), fluorescein isothiocyanate-conjugated goat anti-rabbit IgG (GAR-FITC), and tet-

ramethylrhodamine isothiocyanate-conjugated goat anti-rabbit IgG (GAR-TRITC) were from Nordic Immunological Laboratories (Tilburg, The Netherlands).

Methods

Purification of PI-TP Isoforms

PI-TP α was purified to homogeneity from bovine brain cytosol according to established procedures [5].

PI-TP β was purified from the same source, according to a slightly modified procedure developed for the purification of chicken liver PI-TP β (formerly designated PI/SM-TP; [12]). Active fractions in the various chromatography steps were identified by measuring SM and PI transfer activity (Fig. 1). All purification steps were done at 0–4°C.

A 25% (w/v) homogenate of the cerebral cortex (4800 g) was prepared in 20 mM Tris/HCl, pH 7.4, 5 mM EDTA, 5 mM EGTA, 1 mM dithiothreitol, 1 μ g trypsin inhibitor/ml, 3 mM KCl, 1 mM phenylmethylsulfonyl fluoride, 1 mM sodium pyrophosphate, and 10 mM sodium fluoride using a Waring blender. The homogenate was centrifuged at 14,000*g* for 1 h and the supernatant fraction was collected.

Step 1. A 40–70% saturated ammonium sulfate precipitate was prepared from the 14,000*g* supernatant fraction. The pellet was resuspended in buffer A (20 mM Tris/HCl, pH 7.4, 10 mM β -mercaptoethanol (β ME), 0.1 mM EDTA, 0.1 mM EGTA) containing 1 mM sodium pyrophosphate and 10 mM sodium fluoride and extensively dialyzed against buffer A.

Step 2. The protein solution was applied to a Q-Sepharose column (11.5 \times 21 cm) and eluted with two consecutive linear gradients of NaCl (0–0.17 M, 8 liters; 0.17–0.50 M, 4 liters) in buffer A (flow rate of 4 ml/min, fractions of 20 ml) (Fig. 1A). The major activity peak (fractions 222–280) was pooled (1170 ml), put into a dialysis bag, and concentrated against polyethylene glycol (PEG) 20,000 to 78 ml.

Step 3. The sample was loaded on a Sephacryl S100 gel filtration column (5 \times 94 cm) equilibrated with buffer B (10 mM potassium phosphate, pH 6.8, and 10 mM β ME) and eluted with the same buffer (flow rate of 4 ml/min, fractions of 18 ml) (Fig. 1B). The active fractions (26–35) were combined (180 ml).

Step 4. The sample was applied to a hydroxyl apatite column (2 \times 32 cm) equilibrated with buffer B. The column was eluted with a linear gradient of potassium phosphate (10–100 mM, 1 liter) in buffer B (flow rate of 0.6 ml/min, fractions of 9 ml) (Fig. 1C). The active fractions (35–47) were pooled (117 ml) and concentrated against PEG 20,000 to 7.5 ml.

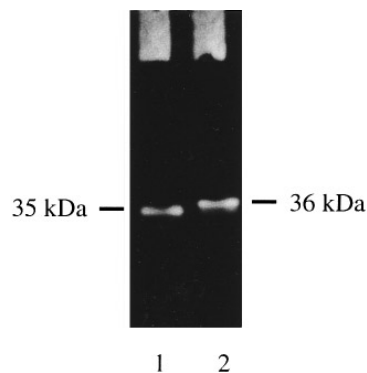


FIG. 2. SDS-polyacrylamide gel electrophoresis of Cy3-PI-TP α (lane 1) and Cy3-PI-TP β (lane 2). Fluorescence of labeled proteins was visualized by exciting the Cy3-label at 550 nm.

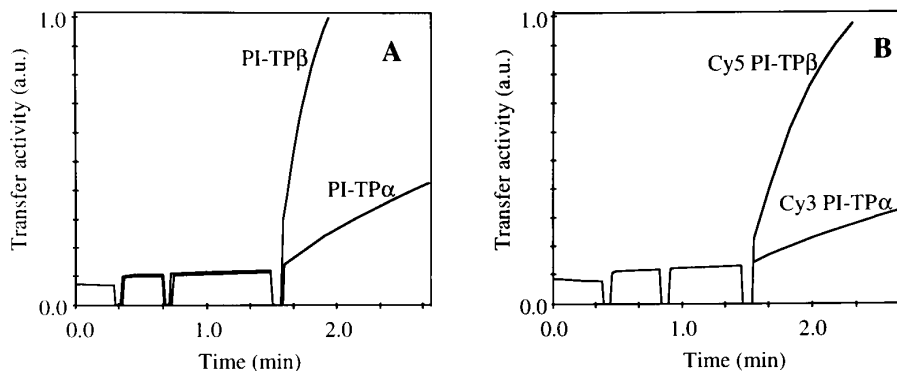


FIG. 3. PI transfer activity of unlabeled (A) and fluorescently labeled isoforms (B) of PI-TP. Transfer was initiated by adding 1 μg of either labeled or unlabeled protein to the donor-acceptor vesicle mixture. PI transfer activity was determined by recording the increase of the pyrenyl-monomer fluorescence at 378 nm resulting from the transfer of Pyr(10)PI from donor to acceptor vesicles (see Methods).

Step 5. The sample was applied to a heparin Sepharose column (2.5×21 cm), equilibrated with 20 mM Tris/HCl, pH 7.4, 10 mM β ME, and 25 mM NaCl, and eluted isocratically with the same buffer (flow rate of 0.3 ml/min, fractions of 4 ml) (Fig. 1D). PI-TP β eluted in fractions 28–42 with a yield of 1.27 mg. Analysis by SDS-polyacrylamide gel electrophoresis and Western blotting demonstrated that PI-TP β was purified to homogeneity. Based on SM transfer activity it was estimated that 54% of PI-TP β was recovered.

Phospholipid Transfer Activity Assay

PI and SM transfer activities were determined in a continuous assay by measuring the pyrene monomer fluorescence increase in a mixture of quenched donor vesicles and a 50-fold excess of unlabeled acceptor vesicles consisting of PC and PA (95:5, mol%) [6, 12]. The donor vesicles consisted of Pyr(10)PI:PC:TNP-PE (10:80:10, mol%) for measuring PI transfer activity and of Pyr(14)SM:PC:PA:TNP-PE (10:70:10:10, mol%) for measuring SM transfer activity. The transfer reaction was carried out in 2 ml 20 mM Tris/HCl, pH 7.4, 5 mM EDTA, and 200 mM NaCl at 37°C and initiated by addition of an active transfer protein fraction (10–50 μl). Measurements were performed on a SLM-Aminco SPF-500C fluorometer equipped with a thermostated cuvette holder and a stirring device. Excitation was set at 346 nm (slit 4 nm) and the pyrenyl-monomer fluorescence was monitored at 378 nm (slit 10 nm). The initial slope of the progress curve was taken as a measure of transfer activity.

Gel Electrophoresis and Blotting

Samples were analyzed by SDS-polyacrylamide gel electrophoresis and Western blotting as described in Ref. [11].

Fluorescence Labeling of PI-TP Isoforms

PI-TP α and PI-TP β (50 μg of protein) were introduced into 20 mM Bicine, pH 8.0, 0.5 mM EDTA, and 5 mM β ME by five cycles of concentration to 50 μl and redilution to 2 ml in a Centricon-10 ultrafiltration device at 3000g and 4°C. The reaction was initiated by adding Cy3 or Cy5 in dry dimethylformamid (DMF, stock solutions of 6 and 4.5 mM, respectively) to the protein solution (1 mg/ml) such that the final concentrations of the dyes were 10 times that of the protein. Care was taken that DMF did not exceed 1% (v/v) of the reaction mixture. The reaction was carried out at room temperature in the dark for 1 h and stopped by adding an aliquot of 1 M glycine (final concentration of 10 mM) followed by incubation for 5 min at 4°C. Excess dye was removed by gel filtration on a P6DG column (1 \times 9 cm) using a buffer consisting of 20 mM Tris/HCl, pH 7.4, and 120 mM KCl. Prior to use, the column was equilibrated with 20 mM

Tris/HCl, pH 7.4, 120 mM KCl, and 1 mg/ml bovine serum albumin to prevent aspecific adsorption. The fluorescently labeled PI-TPs eluting in the void volume (1.5 ml) were collected and concentrated to 50 μl by use of a Centricon-10 ultrafiltration device at 3000g and 4°C. The protein-dye ratios were determined by measuring the absorption at 280 nm (protein) and 554 (Cy3) or 650 nm (Cy5) with ϵ_{280} , 170,000 $M^{-1} \text{cm}^{-1}$; ϵ_{554} , 130,000 $M^{-1} \text{cm}^{-1}$; and ϵ_{650} , 200,000 $M^{-1} \text{cm}^{-1}$ [22].

Cell Culture

FBHE cells derived from the aorta were kindly provided by H. van Aken (Molecular Cell Biology, Utrecht University, Utrecht, The Netherlands). FBHE cells were grown on glass coverslips in Dulbecco's modified Eagle's medium (DMEM) containing 7.5% (v/v) fetal calf serum (FCS) buffered with 44 mM NaHCO_3 at 37°C and in a 7.5% CO_2 atmosphere and 95% humidity.

Microinjection of Fluorescent-Labeled PI-TP in FBHE Cells

Prior to microinjection, the medium of the FBHE cells was replaced with DMEM containing 7.5% (v/v) FCS buffered with 20 mM HEPES, pH 7.4. Cy3-labeled PI-TP α and Cy5-labeled PI-TP β in a 1:1 mixture (1 mg/ml) were microinjected in the cytosol of FBHE cells by using a combination of an Eppendorf Microinjector (Model 5244) at 80 hPa pressure (0.3 s) and an Eppendorf micromanipulator (Model 5170) under an inverted microscope with a 40X air objective. The cells were allowed to recover at 37°C in 5% CO_2 for 30 min. Upon being washed with PBS-0 (phosphate-buffered saline, 137 mM NaCl, 2.7 mM KCl, 6.5 mM Na_2HPO_4 , and 1.5 mM KH_2PO_4 , pH 6.8), the cells were fixed for 20 min at room temperature in 4% (w/v) paraformaldehyde in PBS-0 followed by washing in quenching solution (0.1 M NH_4Cl in PBS-0). After 10-min quenching at room temperature the cells were washed in PBS-0. The coverslips were mounted in PBS-0/glycerol containing Mowiol 4.88 (Hoechst). Cy3 and Cy5 fluorescence was visualized by using a confocal laser scanning microscope (LSM10, Zeiss); for Cy3, excitation 543-nm He-Ne laser line and emission 590 \pm 35 nm bandpass filter; for Cy5, excitation 633-nm He-Ne laser line and emission 665-nm longpass filter.

Immunofluorescence

The localization of PI-TP α and PI-TP β in FBHE cells was determined by indirect immunofluorescence using antibodies I and II as described in Ref. [11].

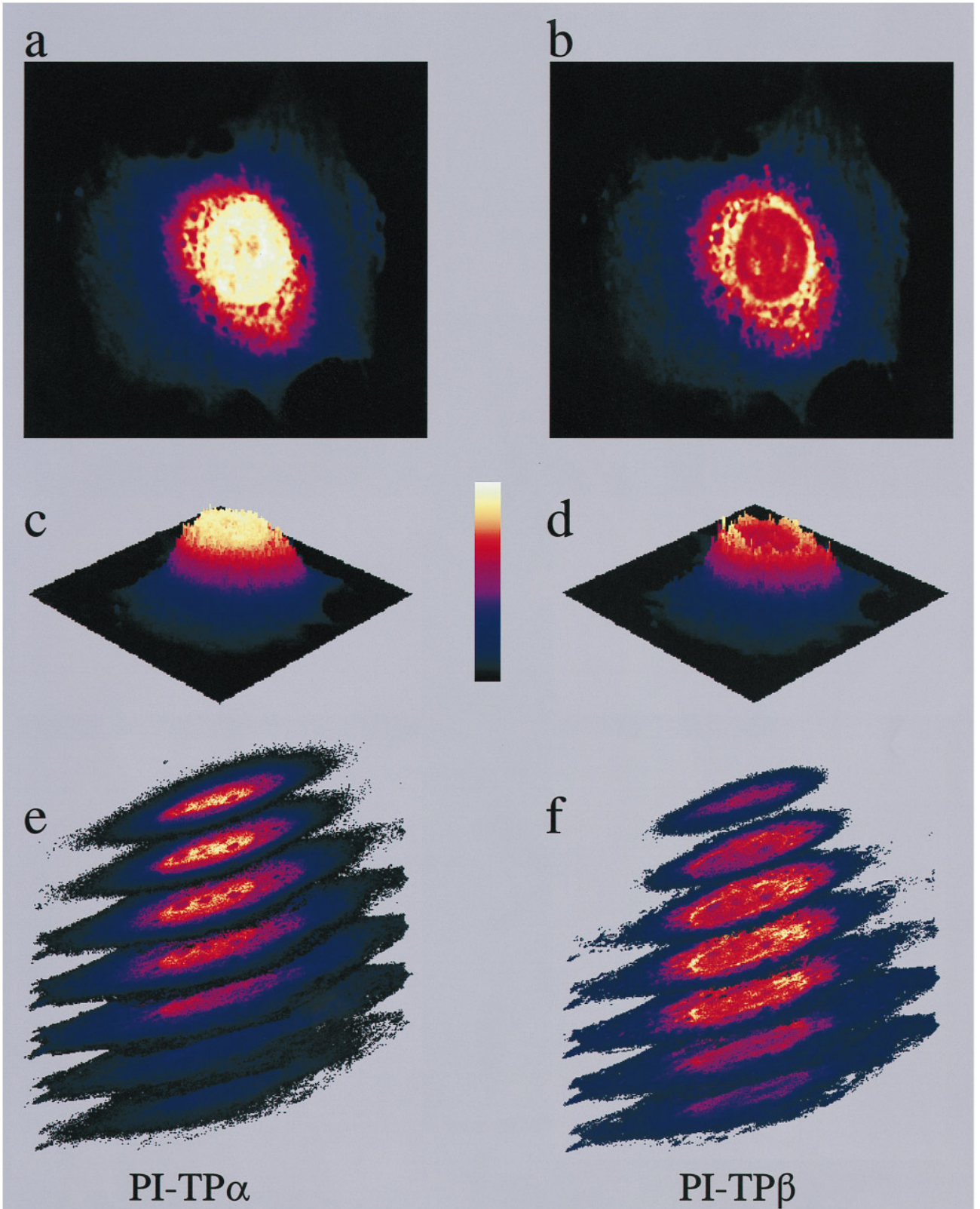


FIG. 4. Cellular localization of Cy3-PI-TP α (a, c, e) and CY5-PI-TP β (b, d, f) in fetal bovine heart endothelial cells after microinjection and fluorescence cross-scanning confocal microscopy. Intensity distributions of an optical section, at the equatorial plane (a and b), of the three-dimensional reconstruction (c and d), and of the optical cross sections of the cells (e and f). The intensity bar indicates the fluorescence signals from low (dark) to high (light).

RESULTS

Fluorescently Labeled PI-TP α and PI-TP β

PI-TP α and PI-TP β were labeled with Cy3 and Cy5. From the absorption at 280 nm (protein), at 552 nm (Cy3), and at 659 nm (Cy5), it was calculated that both PI-TP α and PI-TP β carried about one dye moiety per protein molecule. Analysis of Cy3-PI-TP α and Cy3-PI-TP β by SDS-polyacrylamide gel electrophoresis showed that the dye coincided with the protein stain, confirming the covalent coupling of the dye to the protein (Fig. 2). Similar results were obtained for Cy5-PI-TP α and Cy5-PI-TP β . Since the intracellular function of the PI-TPs most likely involves the binding and transfer of phospholipids, it was important to know whether the fluorescent labeling affected the lipid transfer activity. In order to be able to compare the lipid transfer activity before and after labeling, the solutions containing Cy3-PI-TP α and Cy5-PI-TP β were concentrated to a final volume similar to that of the unlabeled proteins (1 mg/ml). Equal aliquots of these solutions were taken for measuring PI transfer activity. As shown in Figs. 3A and 3B, the transfer activities of Cy3-PI-TP α and Cy5-PI-TP β were comparable to those of PI-TP α and PI-TP β , indicating that the labeling procedure had no adverse effect. From the activity curves it can be seen that PI-TP β was three to four times as active as PI-TP α in transferring PI. This is in agreement with the observations on chicken liver PI-TP α and PI-TP β [12].

Cellular Localization of Fluorescently Labeled PI-TP α and PI-TP β

The cellular distribution of the PI-TP isoforms was determined by microinjection of a 1:1 mixture of Cy3-PI-TP α and Cy5-PI-TP β into FBHE cells and fluorescence laser-scanning confocal microscopy (Fig. 4a-4f). Top views of the optical section at the equatorial plane and the three-dimensional reconstruction of the fluorescence signal demonstrate that Cy3-PI-TP α was preferentially distributed into the nucleus (Figs. 4a and 4c). In addition, the protein was associated with subcellular structures around the nucleus and diffusely present throughout the cytoplasm. Cy5-PI-TP β was preferentially associated with perinuclear structures (Figs. 4b and 4d). Relatively low levels of this protein were also found in the cytoplasm and nucleus. Optical cross sections also clearly show the preference of Cy3-PI-TP α for the nucleus (Fig. 4e) and of Cy5-PI-TP β for perinuclear membrane structures (Fig. 4f). By microinjection of increasing amounts of a Cy3-PI-TP α /Cy5-PI-TP β mixture it was verified that the concentration of labeled proteins did not affect this distribution in the cell. No evidence was obtained that either protein was associated with or preferentially localized near the plasma membrane.

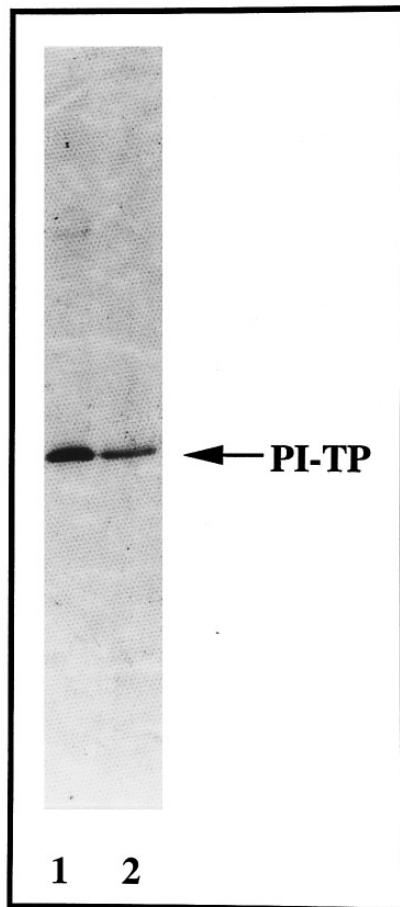


FIG. 5. Soluble and membrane-bound PI-TP in fetal bovine heart endothelial cells as analyzed by Western blot analysis using antibody I upon SDS-polyacrylamide gel electrophoresis. Lane 1, 100,000g supernatant fraction; lane 2, total membrane pellet.

Immunolocalization

The localization of PI-TP α and PI-TP β was also investigated by immunofluorescence using antibody I (cross-reactive with PI-TP α and PI-TP β) and antibody II (cross-reactive with PI-TP α) [11]. To demonstrate that these antibodies could be used, the membrane-free cytosol and total membrane fraction from FBHE cells were submitted to SDS-polyacrylamide gel electrophoresis followed by Western blotting. As shown in Fig. 5, both the cytosol (lane 1) and the membrane fraction (lane 2) contain one band at the position of PI-TP. Since the blot was developed with antibody I, this band represents a combination of PI-TP α and PI-TP β . To distinguish between these proteins in the immunolocalization, the cells were first labeled with antibody II and GAR-TRITC to label PI-TP α and then with antibody I and GAR-FITC to label PI-TP β [11]. As shown in Fig. 6A, PI-TP α is localized in the nucleus and distributed throughout the cytoplasm. PI-TP β is preferentially associated with perinuclear membrane structures (Fig.

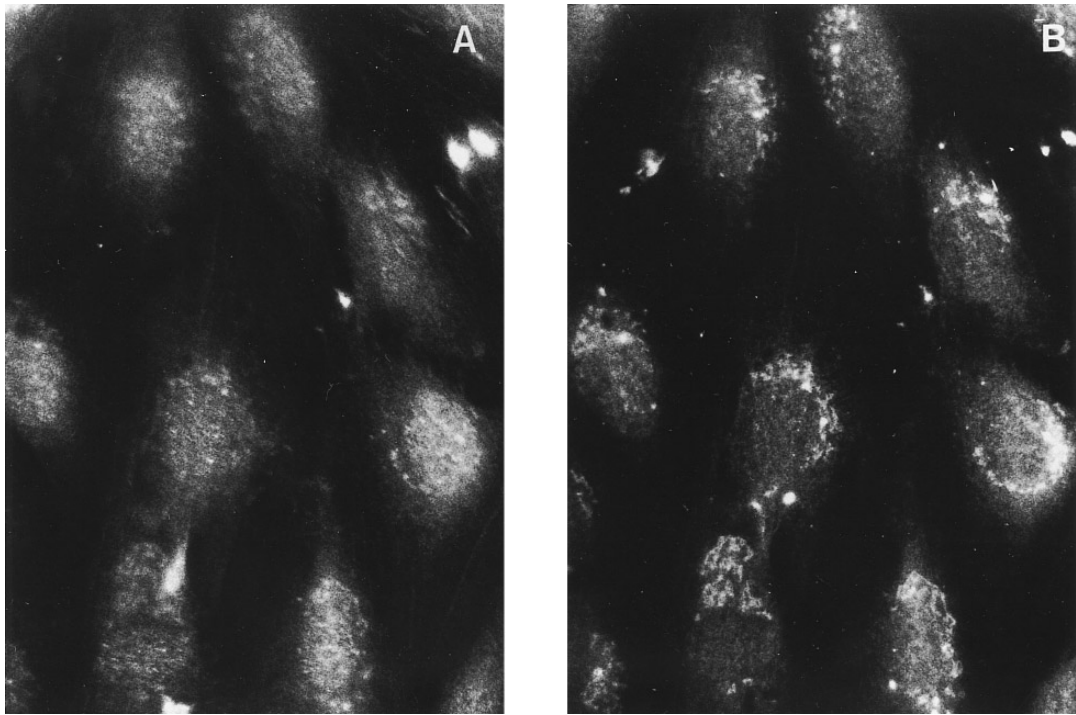


FIG. 6. Intracellular localization of PI-TP isoforms in fetal bovine heart endothelial cells determined by indirect immunofluorescence. PI-TP α (A) and PI-TP β (B).

6B). Comparing Figs. 4 and 6 we infer that Cy3-PI-TP α and Cy5-PI-TP β , when microinjected into the cell, are targeted toward the same intracellular sites as their endogenous counterparts. Previously, by immunolocalization using an antibody that is cross-reactive with both PI-TP α and PI-TP β , it was shown that the antibody labeling of the perinuclear structure coincided with the fluorescent Golgi marker [20]. In line with these results, we presume that the perinuclear structures labeled in the present study represent the Golgi system.

DISCUSSION

Western blot analysis of the cytosolic fraction of Swiss mouse 3T3 cells, using highly specific antibodies against PI-TP α , provided the first evidence for the occurrence of a 36-kDa immunoreactive protein [20]. This 36-kDa protein was partially purified from bovine brain cytosol [11]. Analysis of its N-terminal amino acid sequence (first 21 amino acid residues) indicated that this protein was most likely identical to PI-TP β , the cDNA of which was identified in rat brain [10]. In a parallel study, PI-TP β (formerly denoted PI/SM-TP) was purified to homogeneity from chicken liver [12]. Here, by adapting the procedure to bovine brain cytosol, we succeeded in purifying PI-TP β to homogeneity from this source.

In a previous immunolocalization study, evidence

was obtained that in Swiss mouse 3T3 fibroblasts PI-TP α was present in the cytoplasm and the nucleus whereas PI-TP β was associated with the perinuclear Golgi system [11]. This distinct localization appears to be at odds with the observations that both isoforms are equally effective in reconstituting GTP γ S-mediated PLC- β activity [11, 13] and in stimulating vesicle budding from the *trans*-Golgi network [17]. We have, therefore, reinvestigated the cellular localization by labeling PI-TP α and PI-TP β with the fluorescent sulfoindocyanine dyes Cy3 and Cy5 followed by microinjection in fetal bovine heart endothelial cells (Fig. 4). By this direct approach we have convincingly shown that PI-TP α was predominantly present in the cytoplasm and the nucleus and that PI-TP β was preferentially associated with the perinuclear Golgi. However, these distinctions are not absolute. Cy3-PI-TP α was also found associated with the Golgi system and Cy5-PI-TP β in the cytoplasm and nucleus, although at reduced levels. A similar picture of the intracellular distribution of PI-TP α and PI-TP β was obtained by immunofluorescence localization (Fig. 6). At present we are investigating what factors determine the preferential association of the isoforms with nucleus and Golgi. It is to be noted that neither isoform was found to be associated with the plasma membrane. This was unexpected given the proposed role of PI-TP α in delivering PI to sites of phosphorylation and the PI-4,5 biphosphate (PIP $_2$) formed to receptor-activated phospholipase C β (PLC β) [13].

Further studies are required to establish the intracellular localization of the PIP₂ pool which is susceptible to PLC β activity.

Transformation of yeast SEC14-1^{ts} mutant cells with a rat brain cDNA library yielded cells that grew at the nonpermissive temperature. The rescue of these transformants was due to the expression of the cDNA encoding PI-TP β [10]. By this approach the cDNA encoding PI-TP α was not identified. In agreement with this study, expression of the PI-TP α cDNA in yeast SEC14 null mutants failed to have an effect on viability [23]. Since rat PI-TP α did not associate with the Golgi in yeast in contrast to SEC14p which colocalized completely with the Golgi markers, it was suggested that a stable interaction of PI-TP/SEC14p with the Golgi membranes may be essential in rescuing the temperature-sensitive mutants [23]. In light of these and our own observations we think it likely that PI-TP β , and not PI-TP α , is the mammalian counterpart of yeast SEC14p. In this respect it will be of interest to investigate whether SEC14p is able to transfer SM.

In analogy with the proposed role of PI-TP α in delivering PI to sites of phosphorylation and PLC-catalyzed degradation [13, 14], PI-TP β may have a function in delivering SM to agonist-controlled sphingomyelinase. In this model both PI-TPs would play an important role in maintaining the delicate equilibrium between the PI and SM cycle which appears to be crucial in the regulation of several cellular functions [24]. It remains to be investigated whether the synthesis of SM and PI-4,5 bisphosphate in the Golgi system is regulated by the PI-TP isoforms interacting with this organelle [25, 26]. Recently, some evidence was presented on the possible relationship between Golgi function and signal transduction [27]. We are currently investigating whether the overexpression of PI-TP α and PI-TP β in 3T3 fibroblasts has an effect on phospholipid metabolism.

This research was carried out under the auspices of the Netherlands Foundation for Chemical Research (SON) and with financial aid from the Netherlands Organization for Scientific Research (NWO). We thank F. Brunink for his help in purifying PI-TP β and F. S. Wouters for his assistance in labeling proteins with fluorescent dyes.

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Received April 12, 1996

Revised version received June 3, 1996