# An isoform of the phosphatidylinositol-transfer protein transfers sphingomyelin and is associated with the Golgi system

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An isoform of the phosphatidylinositol-transfer protein (PI-TP) was identified in the cytosol fraction of bovine brain. This protein, designated PI-TP$\beta$, has an apparent molecular mass of 36 kDa and an isoelectric point of 5.4. The N-terminal amino acid sequence (21 residues) is 90 % similar to that of bovine brain PI-TP, henceforth designated PI-TP$\alpha$ (molecular mass 35 kDa and pI 5.5). As observed for PI-TP$\alpha$, PI-TP$\beta$ has a distinct preference for phosphatidylinositol over phosphatidylcholine. In addition, it expresses a high transfer activity towards sphingomyelin. PI-TP$\alpha$ lacks this activity completely. By indirect immunofluorescence we demonstrated that, in Swiss mouse 3T3 fibroblasts, PI-TP$\beta$ is preferentially associated with the Golgi system whereas PI-TP$\alpha$ is predominantly present in the cytoplasm and the nucleus. In cytosol-depleted HL60 cells, both PI-TP$\alpha$ and PI-TP$\beta$ were equally effective at reconstituting guanosine 5′-[$\gamma$-thio]triphosphate-mediated phospholipase C$\beta$ activity.

## INTRODUCTION

Proteins that facilitate the transfer of phospholipids between membranes *in vitro* have been purified and characterized from mammalian tissues, plants, yeast and fungi [1]. One of these proteins is the phosphatidylinositol-transfer protein (PI-TP), which has a distinct preference for phosphatidylinositol (PI), but also transfers phosphatidylcholine (PC) [2–4]. Given this dual specificity, PI-TP may be ideally suited to maintaining the PI/PC concentration ratio of membranes [5–10]. As anti-(bovine brain PI-TP) antibodies cross-react with a 35–36 kDa protein from mammals, birds, reptiles, amphibians and insects, it appears that PI-TP is highly conserved, suggesting an important function in the cell [11,12]. In line with this, the amino acid sequences of PI-TP from man, mouse and rat are 99 % identical [1,13].

PI-TP was initially purified from bovine brain and found to consist of two forms, PI-TP I, with an isoelectric point of 5.5 and containing a PI molecule, and PI-TP II, with an isoelectric point of 5.7 and containing a PC molecule [14]. Recently, the cDNA encoding mouse PI-TP was expressed in *Escherichia coli*. PI-TP purified from this source has an isoelectric point of 5.4 and contains a phosphatidylglycerol molecule [15]. The molecular mass of these PI-TP forms is 35 kDa [1,11]. A protein of 38 kDa purified from bovine brain and cross-reactive with anti-PI-TP antibodies is the possible precursor of PI-TP. This precursor has a very low transfer activity. However, on mild trypsin treatment, a 35 kDa protein is produced which shows a distinct ability to bind and transfer PI [16]. Recently, a novel form of PI-TP was found in exponentially growing Swiss mouse 3T3 fibroblasts, with a molecular mass of 36 kDa and an isoelectric point of 5.4 [17].

Since the first reports on the purification of PI-TP from bovine brain [2,18], speculations about its cellular function have been made. It took until 1990 before a clear indication of its function

in *Saccharomyces cerevisiae* was found. Then it was reported that the SEC14 gene encoding a protein required for the transport of secretory proteins from the Golgi membranes is identical with the gene encoding yeast PI-TP [5,6,9,10]. This protein has no amino acid sequence similarity to mammalian PI-TP [5,11]. Further clues to the function of PI-TP were provided by studies on protein secretion and inositol lipid signalling using permeabilized cells. Purification and characterization of cytosolic factors involved in these two cellular processes indicated that PI-TP represented the essential factor. Thus, in permeabilized PC12 cells, PI-TP was required for the ATP-dependent priming of $Ca^{2+}$-activated secretion [19,20]. Furthermore, addition of PI-TP to permeabilized HL60 cells greatly stimulated the guanosine 5′-[$\gamma$-thio]triphosphate (GTP[S])-mediated phospholipase C$\beta$ activity involved in phosphoinositide hydrolysis [21].

In the present paper we describe the partial purification, characterization and cellular localization of a PI-TP isoform. A very striking feature of this novel protein is that, in addition to PI and PC, it is also able to transfer sphingomyelin (SM). While this study was in progress, the cDNA sequence encoding an isoform of PI-TP (PI-TP$\beta$) in rat brain was published [22]. The first 21 N-terminal amino acid residues of the PI/SM-transferring protein were identical with this isoform. Henceforth, this protein will be referred to as PI-TP$\beta$ and the 35 kDa form of PI-TP as PI-TP$\alpha$.

## MATERIALS AND METHODS

### Materials

DEAE-cellulose DE52 was obtained from Whatman (Maidstone, Kent, U.K.). Sephacryl S100 and heparin–Sepharose CL-6B were from Pharmacia (Uppsala, Sweden). Egg yolk PC, phospha-

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tidic acid (PA), *p*-Nitro Blue Tetrazolium chloride, 5-bromo-4-chloro-3-indolylphosphate *p*-toluidine salt and goat anti-rabbit IgG–alkaline phosphatase conjugate (GAR–AP) were from Sigma (St. Louis, MO, U.S.A.). Trinitrophenyl-phosphatidyl-ethanolamine (TNP-PE) was prepared from egg yolk PE and trinitrobenzenesulphonic acid [23]. 2-Pyrenyldecanoyl-PI, 2-pyrenyldecanoyl-PC and *N*-pyrenyltetradecanoyl-SM were synthesized by established procedures [24]. Goat anti-rabbit IgG (H + L)–peroxidase conjugate (GAR–PO), fluorescein isothiocyanate-conjugated goat anti-rabbit IgG (GAR–FITC) and tetramethylrhodamine isothiocyanate-conjugated goat anti-rabbit IgG (GAR–TRITC) were from Nordic Immunological Laboratories (Tilburg, The Netherlands). High bonding microtitre plates were from Greiner (Alphen a/d Rijn, The Netherlands). Two different anti-PI-TP antibodies were used: one was raised against a mixture of two synthetic peptides representing the predicted epitopes of rat brain PI-TPα (residues 40–60 and 240–260) in rabbits (antibody I) [25] and the other was raised against purified mouse PI-TPα expressed in *E. coli* in rabbits (antibody II) [15].

## Partial purification of PI-TP and an isoform

PI-TP and an isoform were purified from bovine brain. All steps were carried out at 0–4 °C. A 25 % (w/v) homogenate of the cerebral cortex (750 g) was prepared in 20 mM Tris/HCl, pH 7.4, containing 5 mM EDTA, 5 mM EGTA, 1 mM dithio-erythritol, 1 μg/ml trypsin inhibitor, 3 mM KCl, 1 mM PMSF, 1 mM sodium pyrophosphate and 1 mM NaF using a Waring blender. After centrifugation at 14000 *g* for 1 h, a 40–60 %-satd. $(NH_4)_2SO_4$ precipitate was prepared from the supernatant. The pellet was resuspended in buffer A (20 mM Tris/HCl, pH 7.4, 10 mM 2-mercaptoethanol, 0.1 mM EDTA, 0.1 mM EGTA) containing 1 mM sodium pyrophosphate and 10 mM NaF, and extensively dialysed against buffer A. The protein solution was applied to a DEAE-cellulose column (300 ml) and eluted with a linear NaCl gradient (0–700 mM; 1 litre) in buffer A. The fractions (12.5 ml) were assayed for PI-transfer activity and analysed by ELISA using antibody I. After combining the active fractions, the protein solution (225 ml) was concentrated against poly(ethylene glycol) 20000 to 50 ml. This sample was loaded on a Sephacryl S100 gel-filtration column (2.2 litres) and eluted with 20 mM Tris/HCl, pH 7.4, containing 10 mM 2-mercaptoethanol and 25 mM NaCl. The fractions (5 ml) were tested for PI-transfer activity and immunoreactivity. The activity fractions were combined (200 ml) and concentrated against poly(ethylene glycol) 20000 to 9 ml. This active pool was applied to a heparin–Sepharose column (90 ml) equilibrated in 20 mM Tris/HCl, pH 7.4, containing 10 mM 2-mercaptoethanol and 25 mM NaCl, and eluted isocratically with the same buffer. Fractions (4 ml) were tested for PI-transfer activity and immunoreactivity and analysed by Western blotting. Active fractions were combined and concentrated against poly(ethylene glycol) 20000.

## Protein determination

The PI-TP concentration in the active fractions obtained by heparin–Sepharose affinity chromatography (fractions A and B, see Figure 1c) was determined by running a sample and a concentration range of BSA on an SDS/15 % polyacrylamide gel [26]. After Coomassie Brilliant Blue staining, the intensity of the PI-TP-containing band was compared with that of the BSA standard curve and the amount of PI-TP estimated.

## Assay of phospholipid-transfer activity

PI-transfer activity was determined in a continuous assay by measuring the pyrene monomer fluorescence increase in a mixture of quenched donor vesicles consisting of 2-pyrenyldecanoyl-PI/egg PC/TNP-PE (10:80:10, mol %) and an excess (20–100-fold) of unlabelled acceptor vesicles consisting of PC and PA (95:5 mol %) in 2 ml of 20 mM Tris/HCl, pH 7.4, containing 5 mM EDTA and 200 mM NaCl at 37 °C with stirring. Under similar conditions, PC- and SM-transfer activity were determined using donor vesicles consisting of 2-pyrenyldecanoyl-PC/egg PC/PA/TNP-PE (10:70:10:10, mol %) and *N*-pyrenyltetra-decanoyl-SM/egg PC/PA/TNP-PE (10:70:10:10, mol %) respectively. The transfer reaction was initiated by addition of fractions (10–50 μl) containing PI-TP. The initial slope of the progress curve was taken as an arbitrary unit of transfer activity [4]. Measurements were performed on an SLM-Aminco SPF-500C fluorimeter equipped with a thermostatically controlled cuvette holder and a stirring device.

## ELISA procedure

The ELISA for the qualitative detection of PI-TP was performed using high-bonding microtitre plates. Column fractions (0.1 ml) were added to the wells and incubated for 1 h at 37 °C. After the wells had been washed with PBS (137 mM NaCl, 2.7 mM KCl, 6.5 mM $Na_2HPO_4$ and 1.5 mM $KH_2PO_4$), they were incubated for 1 h at 37 °C with 0.11 ml of PBS containing 1 % (w/v) BSA as blocking agent. Further manipulations were carried out at room temperature. The wells were washed with PBS/Tween [PBS containing 0.05 % (v/v) Tween 20] and then incubated for 1 h with a solution (0.1 ml) containing antibody I diluted in PBS/Tween/BSA [PBS/Tween containing 0.1 % (w/v) BSA]. The wells were washed again with PBS/Tween and incubated with GAR–PO (0.1 ml; diluted 1:1000 in PBS/Tween/BSA) for 1 h. After the wells had been washed with PBS/Tween, the substrate solution (0.1 ml), containing 0.04 % (v/v) $H_2O_2$, 0.04 % (w/v) *o*-phenylenediamine, 25 mM citric acid and 50 mM di-sodium phosphate was added to each well. After incubation in the dark for 5–10 min, the reaction was stopped by addition of 2 M $H_2SO_4$ (0.1 ml) and the absorbance was determined at 492 nm.

## Amino acid sequence determination

Samples containing the isoforms of PI-TP were applied to a 15 % tricine/SDS/polyacrylamide gel [27] and then blotted onto poly(vinylidene difluoride) membrane (as described below). After Coomassie Brilliant Blue staining, the bands containing PI-TP were cut out and the N-terminal amino acid sequence was determined by automated Edman degradation using the 476A protein sequencer (Applied Biosystems).

## PAGE and blotting

Samples for SDS/PAGE were mixed with an equal volume of sample buffer containing SDS and 2-mercaptoethanol and analysed by electrophoresis on an SDS/15 % polyacrylamide gel [26]. Samples for isoelectric focusing (IEF) were applied to a gel containing 8.5 % polyacrylamide, 5.3 % (v/v) glycerol, 2.8 % (v/v) ampholines pH 3.5–10.0 and 2.8 % (v/v) ampholines pH 5.0–8.0 at 4 °C.

Proteins were electrophoretically transferred from the SDS/polyacrylamide gel at 1 mA/cm² for 75 min or from an IEF gel

at 1 mA/cm² for 45 min to nitrocellulose membranes. Gelatine (3%; w/v) in Tris-buffered saline (TBS; 20 mM Tris/HCl, pH 7.5, and 0.5 M NaCl) was used as a blocking agent. PI-TP was identified by incubating the nitrocellulose membranes with antibody I or II in TBS containing 1% (w/v) gelatine and then with GAR–AP in TBS containing 1% (w/v) gelatine. The GAR–AP was visualized with 5-bromo-4-chloro-3-indolyl-phosphate p-toluidine salt and p-Nitro Blue Tetrazolium chloride as colour-development substrate for alkaline phosphatase.

## Immunofluorescence

Swiss mouse 3T3 fibroblasts grown on glass coverslips [17] were washed with PBS and then fixed with methanol at −20 °C for 4 min. All other manipulations were carried out at room temperature. Cells were incubated with Dulbecco's modified Eagle's medium buffered with 25 mM Hepes containing 0.1% (w/v) BSA (DBH medium) as blocking agent for 30 min. They were then incubated with antibody II for 1 h before being washed with PBS and incubated for 1 h with GAR–TRITC (dilution 1:80 in DBH medium) to visualize PI-TP. Subsequently, these cells were washed with PBS and incubated for 1 h with antibody I before being washed in PBS and incubated for 1 h with GAR–FITC (dilution 1:80 in DBH medium) to visualize the isoform of PI-TP. Finally, the cells were washed with PBS and mounted in mowiol. The labelled cells were viewed with a Leitz inverted microscope equipped with barrier filters to prevent cross-over of TRITC and FITC fluorescence [17,25].

## Cell permeabilization and phospholipase Cβ reconstitution

HL60 cells prelabelled with [³H]inositol were depleted of cytosol by permeabilization and used to determine the ability of PI-TP and its isoform to reconstitute phospholipase Cβ activity, essentially as described previously [21]. The reconstituting activity is expressed as the percentage hydrolysis of inositol lipids calculated from the radioactivity of the [³H]inositol phosphates formed and the [³H]inositol lipids present in the permeabilized cells.

## RESULTS

### Partial purification of PI-TP and an isoform

Both PI-TP forms were purified from bovine brain cytosol by weak anion-exchange, gel-filtration and heparin-affinity chromatography (Figures 1a–1c). Column fractions were tested for PI-transfer activity and their immunoreactive response with antibody I. PI-transfer activity and immunoreactivity were co-eluted in a single peak from a DEAE-cellulose column (Figure 1a). Fractions 65–82 were combined and fractionated on a Sephacryl S100 column (Figure 1b). Two immunoreactive peaks were observed, the first of which (fractions 56–73) exhibited no transfer activity but the second (fractions 92–131) coincided with PI-transfer activity. Fractionation of the second peak on a heparin–Sepharose affinity column yielded three immunoreactive peaks (Figure 1c). The first (fractions 14–21) lacked transfer activity and contained two immunoreactive proteins with estimated molecular masses of 35 and 38 kDa (Figure 1d). The second (fractions 23–37) expressed PI-transfer activity. The major immunoreactive protein in this peak has an estimated molecular mass of 36 kDa (Figure 1d). The inactive 35 kDa protein, prominently present in the first peak, partially overlapped with the active 36 kDa protein in the second peak (see also Figure 3a, lane 1). The third peak (fractions 81–140) coincided with the regular PI-TP (molecular mass 35 kDa). The combined fractions
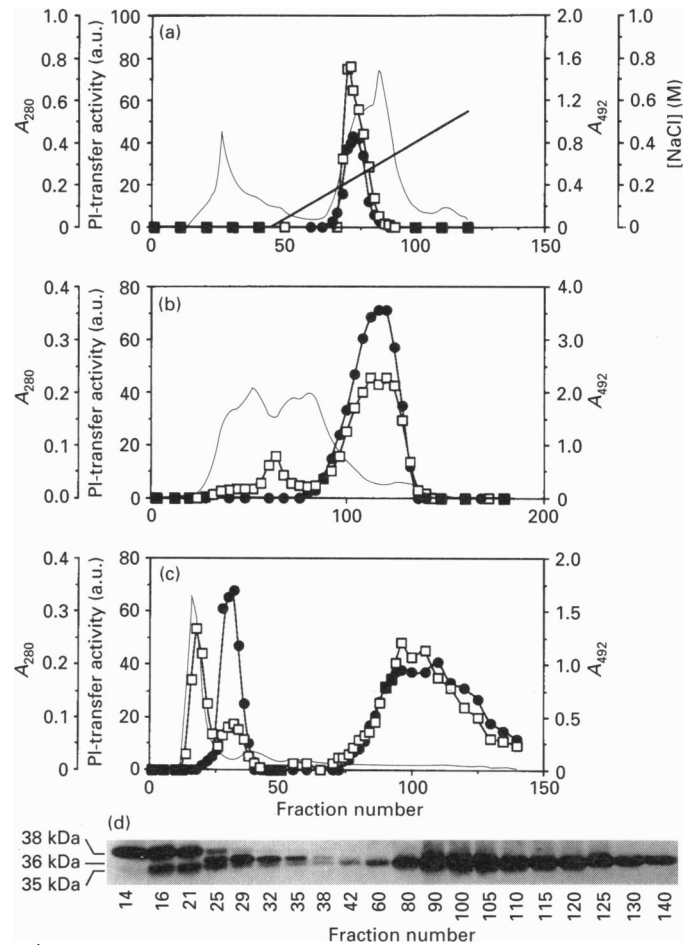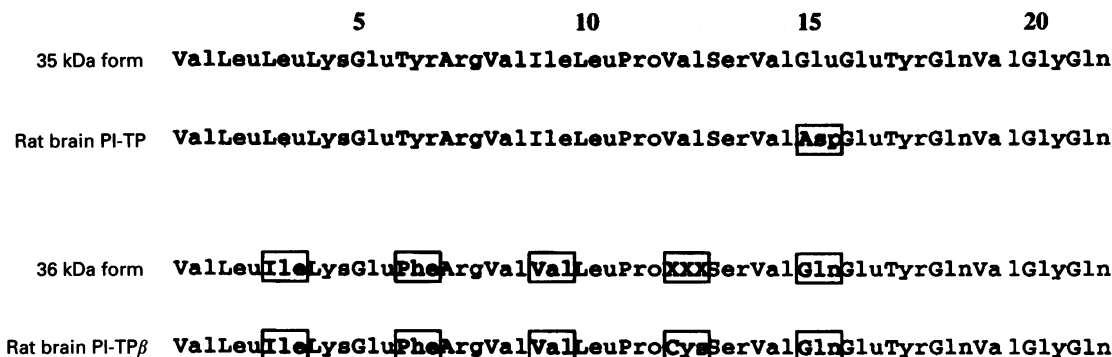


**Figure 1    Fractionation of the 35 and 36 kDa forms of PI-TP from bovine brain cytosol by DEAE-cellulose anion-exchange chromatography (a), Sephacryl S100 gel-filtration chromatography (b) and heparin–Sepharose chromatography (c)**

Column fractions were assayed for PI-transfer activity (●) and immunoreactivity by ELISA ($A_{492}$) (□). ——, $A_{280}$; ——, [NaCl]. Western-blot analysis of the fractions from the heparin–Sepharose column are shown in (d).

23–37 (fraction A) and 81–140 (fraction B) are taken to represent the 36 and 35 kDa forms of PI-TP respectively.

### Determination of the N-terminal amino acid sequence

Fractions A and B were submitted to SDS/PAGE to separate the bands representing the 36 and 35 kDa forms of PI-TP from contaminating proteins. These bands were analysed by automated Edman degradation. The first 21 residues of the N-terminal amino acid sequence were identified (Figure 2). The amino acid sequence of the 35 kDa form of PI-TP was found to be nearly identical with that of rat brain PI-TP except for the residue at position 15 where Asp is replaced by Glu [11]. The N-terminal amino acid sequence is in agreement with the sequence reported previously [21]. The amino acid sequence of the 36 kDa form of PI-TP very much resembled that of the 35 kDa form with conservative replacements at positions 3 (Ile for Leu), 6 (Phe for Tyr) and 9 (Val for Ile). The residue at position 12 was not identified and Gln replaced Glu at position 15. Recently, the cDNA encoding an isoform of rat brain PI-TP (designated PI-TPβ) was isolated and sequenced [22]. As indicated, the

|  |  | **5** | **10** | **15** | **20** |
|---|---|---|---|---|---|

35 kDa form    ValLeuLeuLysGluTyrArgValIleLeuProValSerValGluGluTyrGlnVa lGlyGln

Rat brain PI-TP    ValLeuLeuLysGluTyrArgValIleLeuProValSerVal[Asp]luTyrGlnVa lGlyGln

36 kDa form    ValLeu[Ile]LysGlu[Phe]rgVal[Val]euPro[XXX]erVal[Gln]luTyrGlnVa lGlyGln

Rat brain PI-TPβ    ValLeu[Ile]LysGlu[Phe]rgVal[Val]euPro[Cys]erVal[Gln]luTyrGlnVa lGlyGln

**Figure 2    N-terminal amino acid sequence of bovine brain PI-TP (35 kDa form) and its isoform (36 kDa form)**

For comparison, the sequences of rat brain PI-TPα and its isoform (PI-TPβ) are included. The amino acid residues in boxes represent differences from the 35 kDa form of bovine brain PI-TPα.

## Table 1    Phospholipid-transfer activity of PI-TPα and PI-TPβ

Aliquots of fractions A and B in the assay mix were equivalent to 1 $\mu$g of PI-TPβ and 1 $\mu$g of PI-TPα respectively. Activities are expressed relative to the PI-transfer activity of PI-TPα set at 1.0. PI-, PC- and SM-transfer activities were determined. Results are means $\pm$ S.D. ($n = 3$), representing fractions A and B from three purifications.

| Protein | Phospholipid-transfer activity | | |
|---|---|---|---|
|  | PI | PC | SM |
| PI-TPα | 1.00 | 0.12 ± 0.01 | 0.06 ± 0.01 |
| PI-TPβ | 0.96 ± 0.17 | 0.16 ± 0.06 | 1.48 ± 0.35 |

N-terminal amino acid sequence deduced from this cDNA is identical with the 36 kDa form of PI-TP. The 36 kDa form will be referred to as PI-TPβ and the 35 kDa form as PI-TPα.

## Lipid-transfer activity

In a continuous fluorescence-transfer assay with pyrenyl-labelled phospholipids as substrates, the lipid-transfer activities of PI-TPβ (fraction A) and PI-TPα (fraction B) were determined. As shown in Table 1, the PI-transfer activity of PI-TPβ is about equal to that of PI-TPα. In agreement with the observed dual specificity [2–4], PI-TPα has a low but distinct activity towards PC. A comparable PC-transfer activity is expressed by PI-TPβ. It was previously reported that PI-TP from bovine brain and heart is slightly active towards SM [28]. Because this earlier preparation of PI-TP may well have been contaminated with the isoform, we tested PI-TPα and PI-TPβ for SM-transfer activity. Here we show that PI-TPβ has a very distinct SM-transfer activity in contrast PI-TPα which is virtually devoid of this activity (Table 1). During the purification, the fractions of the heparin–Sepharose column were also assayed for SM-transfer activity. It was found that this activity coincided with the PI-transfer activity present in the second immunoreactive peak (Figure 1c). No other peaks with SM-transfer activity were observed. Work in progress to purify the PI/SM-transferring protein (PI-TPβ) to homogeneity confirms that the ratio of PI- to SM-transfer activity expressed by PI-TPβ is approximately one (K. J. de Vries, unpublished work).
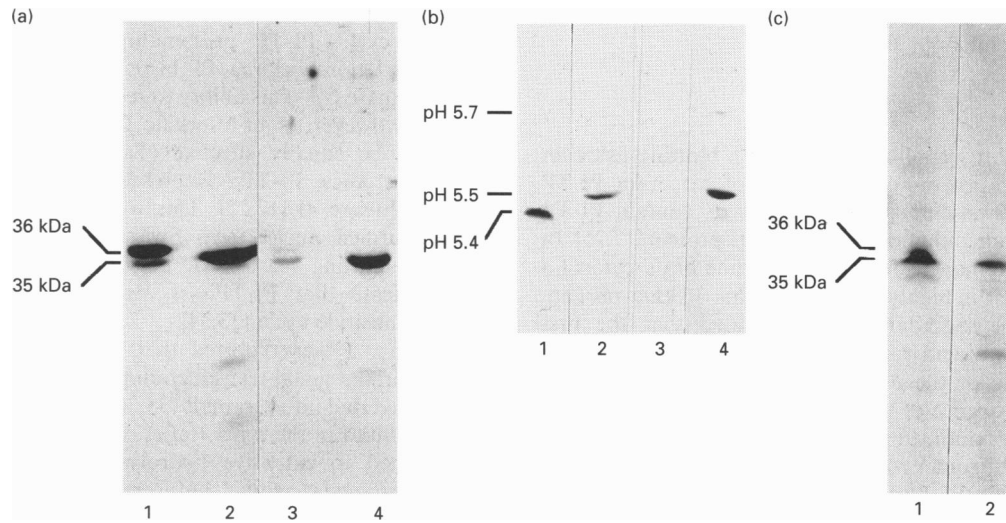
## Western-blot analysis

Fractions A and B were submitted to SDS/PAGE followed by Western blotting. Blots were analysed by using antibody I (raised against a mixture of synthetic peptides representing the predicted epitopes of rat brain PI-TPα) and antibody II (raised against purified mouse PI-TPα expressed in E. coli). As shown in Figure 3(a), antibody I reacted with both PI-TPβ in fraction A (lane 1) and PI-TPα in fraction B (lane 2). One can see also that fraction A is slightly contaminated with an immunoreactive 35 kDa protein which appears to be devoid of transfer activity (Figures 1c and 1d). Very interestingly, antibody II gave no response with PI-TPβ (lane 3) and a normal response with PI-TPα (lane 4). Antibody II reacted also with the 35 kDa band as a minor immunoreactive contaminant present in fraction A (lane 3).

In order to determine the isoelectric points of PI-TPβ and PI-TPα, fractions A and B were submitted to IEF followed by Western blotting. Using antibody I, PI-TPβ was found to have a pI of 5.4 and PI-TPα of 5.5 and 5.7 (Figure 3b, lanes 1 and 2). In agreement with Figure 3(a), antibody II gave no response with PI-TPβ (lane 3) and reacted with PI-TPα (lane 4). Recently, a novel form of PI-TP was detected in Swiss mouse 3T3 fibroblasts [17]. In view of it having a pI of 5.4 this form may well be similar to PI-TPβ (see below).

## Intracellular localization of PI-TPα and PI-TPβ

In agreement with previous results [17], Western blots of SDS/polyacrylamide gels using antibody I show that Swiss mouse 3T3 fibroblasts contain a 35 and a 36 kDa band (Figure 3c, lane 1). Similarly to the response with bovine brain PI-TPs (Figure 3a), antibody II is only reactive with the 35 kDa form of PI-TP (Figure 3c, lane 2). This strongly suggests that the 36 kDa band in the Swiss mouse 3T3 fibroblasts corresponds to PI-TPβ. In a previous study on the immunolocalization of PI-TP in exponentially growing cells, strong labelling was found to be associated with the Golgi system in addition to labelling of the cytoplasm and nucleus [17,25]. As in this study antibody I was used, the observed labelling may represent both PI-TPα and PI-TPβ. In order to be able to distinguish between the two proteins, the cells were first incubated with antibody II and GAR–TRITC to label PI-TPα (Figure 4a) and then with antibody I and GAR–FITC to label PI-TPβ (Figure 4b). It is evident from Figure 4(a) that PI-TP is distributed throughout the cytoplasm, is weakly associated with Golgi system (see arrow) and is localized

**Figure 3    Western-blot analysis of PI-TPβ and PI-TPα after SDS/PAGE (a), IEF (b) and Western-blot analysis of the 14000 g supernatant of exponentially growing Swiss mouse 3T3 fibroblasts on SDS/PAGE (c)**

In (a) and (b), lanes 1 and 3, PI-TPβ; lanes 2 and 4, PI-TPα. Antibody I was used in lanes 1 and 2 and antibody II in lanes 3 and 4. In (c), antibody I was used in lane 1 and antibody II in lane 2.
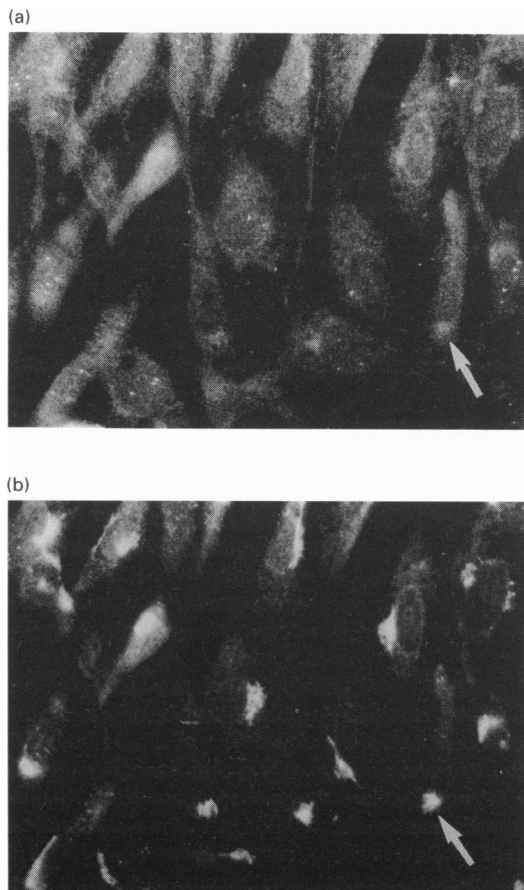


**Figure 4    Immunolocalization of PI-TPα (a) and PI-TPβ (b) in exponentially growing Swiss mouse 3T3 fibroblasts**

The arrow indicates the perinuclear Golgi system.

**Table 2    Reconstitution of phospholipase Cβ activity in permeabilized HL60 cells with PI-TPα and PI-TPβ**

Aliquots of fractions A and B in the assay mixture were equivalent to 100 μg/ml PI-TPβ and PI-TPα respectively. Each protein was assayed for phospholipase C reconstitution in the absence and presence of GTP[S] (10 μM). Results are means ± S.D. ($n = 3$)

| Protein | Hydrolysis (%) | |
| --- | --- | --- |
| | −GTP[S] | +GTP[S] |
| Control | 0.93 ± 0.08 | 5.59 ± 0.06 |
| PI-TPα | 1.08 ± 0.16 | 11.64 ± 0.14 |
| PI-TPβ | 1.00 ± 0.01 | 12.65 ± 0.47 |

in and around the nucleus. From Figure 4(b) it can be inferred that PI-TPβ is preferentially associated with the Golgi system (see arrow). Labelling of the cytoplasm and the nucleus was very slight. Identification of the Golgi system was based on labelling with TRITC–ricin (results not shown) carried out as in previous studies [25].

**Reconstitution of phospholipase Cβ activity**

PI-TPα and PI-TPβ were tested for their ability to reconstitute GTP[S]-mediated phospholipase Cβ activity in cytosol-depleted HL60 cells. As shown in Table 2, both proteins reconstituted this enzyme activity. This reconstitution was not observed when GTP[S] was absent. In agreement with PI-TPβ and PI-TPα being equally effective at transferring PI between membranes (Table 1), the two proteins (100 μg/ml) expressed about the same activity in the reconstitution assay. The ability to reconstitute phospholipase Cβ activity was also tested as a function of protein

concentration. Under all conditions PI-TP$\beta$ and PI-TP$\alpha$ were equally effective (results not shown).

## DISCUSSION

In a previous study it was shown by Western blotting using an antibody raised against epitope segments of rat brain PI-TP (antibody I) that Swiss mouse 3T3 fibroblasts contain PI-TP (35 kDa band) and an immunoreactive 36 kDa protein [17,25]. In the present study we have isolated from bovine brain cytosol a protein fraction that is highly enriched in this 36 kDa protein. Automated Edman degradation demonstrated that the first 21 N-terminal amino acid residues of the 36 kDa protein are identical with those of the $\beta$-isoform of rat brain PI-TP, the encoding cDNA sequence of which has recently been published [22]. This is taken to indicate that the 36 kDa protein is the $\beta$-isoform (PI-TP$\beta$). Alignment of the deduced amino acid sequence with that of rat brain PI-TP$\alpha$ gave a 77% identity and a 94% similarity [22]. Apart from the amino acid sequence, the isoelectric points of PI-TP$\alpha$ and PI-TP$\beta$ are very close with values of 5.5 and 5.4 respectively. Given these great similarities in primary structure, it is remarkable that, in addition to transferring PI and PC like PI-TP$\alpha$, this isoform very efficiently transfers SM, which PI-TP$\alpha$ cannot. It is quite striking that PI-TP$\beta$ clearly prefers SM over PC despite both lipids having an identical polar headgroup. This lipid specificity was confirmed for PI-TP$\beta$ purified to homogeneity from chicken liver [29].

By indirect immunofluorescence, it was shown that, in exponentially growing Swiss mouse 3T3 fibroblasts, PI-TP$\beta$ is predominantly associated with the Golgi system. In line with this, a pI-5.4 form of PI-TP, probably PI-TP$\beta$, is preferentially retained in these cells after their permeabilization by streptolysin O [17]. Recently, it was reported that the SEC14p required for the export of secretory proteins from the Golgi system in yeast is identical with yeast PI-TP [6,30]. In yeast, SEC14p is associated with the Golgi system [7,31]. This strongly suggests that in mammalian cells PI-TP$\beta$ is the counterpart of SEC14p. In support of this, rat brain PI-TP$\beta$ expressed in a temperature-sensitive SEC14 strain suppressed the SEC14 mutation, and the rescued strain grew at a rate comparable with that of the wild-type strain [22]. It has not been established whether this complementation of the SEC14 lesion correlated with the ability of PI-TP$\beta$ to associate with the yeast Golgi system. Expression of rat brain PI-TP$\alpha$ in SEC14$^{ts}$ strains also restored growth to essentially wild-type rates under conditions in which association with the Golgi system was not observed [31]. In permeabilized PC12 cells, rat PI-TP was shown to be one of the proteins required for the ATP-dependent priming of $Ca^{2+}$-activated secretion [20]. Given the difficulty of separating PI-TP$\alpha$ and PI-TP$\beta$, it is not known whether both PI-TP$\alpha$ and PI-TP$\beta$ or just one of these two proteins has priming activity. It is of note that, in this permeabilized cell system, SEC14p expressed priming activity [20].

In a previous study we observed that stimulation of semi-quiescent (serum-starved) Swiss mouse 3T3 fibroblasts produced a remarkable increase in Golgi-associated labelling [32]. This increased labelling probably reflects the enhanced association of PI-TP$\beta$ with the Golgi system. At this point it is not known what causes the rapid redistribution of PI-TP$\beta$ from cytosol to the Golgi system. It is unlikely that phosphorylation of PI-TP$\beta$ by protein kinase C is involved, as the isoform, in contrast with PI-TP$\alpha$, is not a substrate for this kinase *in vitro* (G. T. Snoek, unpublished work).

Recently, PI-TP$\alpha$ was shown to greatly stimulate the GTP[S]-mediated phospholipase C$\beta$-dependent hydrolysis of PI bis-

phosphate in cytosol-depleted HL-60 cells [21]. In this phospho-inositide cycle, PI-TP presumably transfers PI to sites of phosphorylation yielding PI bisphosphate as a substrate for phospholipase C$\beta$. This ability to reconstitute phospholipase C$\beta$ activity, however, is not specific for PI-TP$\alpha$, as PI-TP$\beta$ was found to be equally effective (Table 2). In agreement with previous studies, PI-TP$\alpha$ is present in both the cytosol and nucleus (Figure 4) [17,25]. This was confirmed by submitting highly purified nuclei from Swiss mouse 3T3 fibroblasts to Western blotting (L. Cocco, personal communication). This may indicate that PI-TP$\alpha$ is also involved in the nuclear phosphoinositide cycle [33,34].

Recently, it was reported that 10–20% of cellular SM is involved in the signal-transduction pathway (SM cycle) in which SM is converted into ceramide [35,36]. This distinct pool appears to be localized in the inner leaflet of the plasma membrane and is subjected to extensive hydrolysis by sphingomyelinase in response to extracellular inducers [35,36]. We are currently investigating whether PI-TP$\beta$ plays a role in this SM cycle.

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