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Isolation of a phosphatidylserine transfer protein from yeast cytosol

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A phospholipid transfer protein with a broad substrate specificity was isolated from yeast cytosol. The rate of transfer catalyzed by this protein in vitro is highest for phosphatidylserine; phosphatidylethanolamine, cardiolipin, phosphatidic acid and ergosterol are transported at a lower rate. In contrast to the yeast phosphatidylinositol transfer protein (Daum, G. and Paltauf, F. (1984) Biochim. Biophys. Acta 794, 385–391) the phosphatidylserine transfer protein does not catalyze the translocation of phosphatidylinositol or phosphatidylcholine. Using chromatographic methods the phosphatidylserine transfer protein was enriched approximately 3000-fold over yeast cytosol. The protein is inactivated by heat, detergents and proteinases. Divalent cations strongly inhibit the transfer of phosphatidylserine in vitro, and EDTA at low concentrations has a stimulatory effect.

Introduction

Lipid transfer proteins have been isolated from various types of cells including mammalian cells [1], plant cells [2] and microorganisms [3,4]. According to their substrate specificity in vitro phosphatidylcholine transfer proteins, phosphatidylinositol transfer proteins and non-specific lipid transfer proteins can be distinguished [5]. The latter species is characterized by its ability to translocate not only phospholipids, but also sterols between isolated membranes. The mammalian non-specific lipid transfer protein is identical with sterol carrier protein II [5].

A phosphatidylinositol/phosphatidylcholine specific transfer protein isolated from baker's yeast [6] closely resembles phosphatidylinositol transfer proteins from mammalian cells. A yeast mutant deficient in the gene coding for the phosphatidylinositol transfer protein (PIT-gene) was constructed by gene disruption and proved to be unable to grow [7]. Comparison of the nucleotide sequences of the PIT-gene and the previously described SEC 14 gene revealed their identity [7–9]. This finding demonstrates the essential role of the PIT-gene product as a component of the secretory pathway of proteins, but does not explain its function on a molecular level.

In the course of studies on intracellular lipid traffic we searched for additional lipid transfer proteins in yeast and detected phosphatidylserine transfer activity in the cytosol. This activity is associated with a protein whose isolation and partial characterization is described.

Materials and Methods

Yeast strain and culture conditions

Saccharomyces cerevisiae D 273-10B was grown under aerobic conditions at 30 °C on a semisynthetic medium with 2% lactate as a carbon source [10]. Cells were harvested in the mid-logarithmic phase with a yield of 7–10 g wet weight per liter.

Materials

Egg yolk phosphatidylcholine was from Lipid Products (Nutfield, U.K.), and bovine brain phosphatidylserine and ergosterol were from Sigma. Yeast phosphatidylethanolamine and phosphatidic acid [11], bovine heart cardiolipin and bovine heart sphingomyelin [12] were isolated from natural sources and purified by preparative thin-layer chromatography. Soybean phosphatidylinositol was a gift of R. Franzmair (Linz).

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1-Stearoyl-2-oleoyl-*sn*-glycero-3-phosphoethanolamine [13], *N*-trinitrophenylphosphatidylethanolamine [14], 1-palmitoyl-2-pyrenedecanoyl-*sn*-glycero-3-phosphoserine [15], 1-palmitoyl-2-pyrenedecanoyl-*sn*-glycero-3-phosphoethanolamine [15] and 1-palmitoyl-2-pyrenedecanoyl-*sn*-glycero-3-phosphocholine [16] were synthesized by published procedures. 1-Stearoyl-2-pyrenedecanoyl-*sn*-glycero-3-phosphoinositol was a gift of P.J. Somerharju (Helsinki).

Fluorescence assay of phospholipid transfer

Unilamellar phospholipid vesicles for fluorescence assays were routinely prepared by the ethanol injection method [17] described earlier. The final concentration of ethanol in the assay was 0.2–0.4% and had no influence on the activity of the transfer protein tested. In most cases donor vesicles contained 10 mol% *N*-trinitrophenylphosphatidylethanolamine as an internal quencher [18].

For a standard assay donor vesicles were obtained by injecting 20 μl of an ethanolic solution of 1-palmitoyl-2-pyrenedecanoylglycerophosphoserine (62.5 μM), *N*-trinitrophenylphosphatidylethanolamine (62.5 μM) and egg phosphatidylcholine (500 μM) into 500 μl 10 mM Tris-HCl (pH 7.0), 0.02% NaN_3 with stirring at 37 °C. The buffer was filtered (Sartorius membrane filters, 0.45 μm) prior to use. After an equilibration period of at least 10 min portions of 20 μl of the vesicle suspension were used in 0.5 ml Tris-HCl (pH 7.0), 0.02% NaN_3 to give a final concentration of 0.1 μM fluorescently labeled donor phospholipid in the assay. When the substrate specificity of the transfer protein was tested fluorescent phosphatidylserine was replaced by other pyrene-labeled phospholipids.

Acceptor vesicles for standard assays were prepared by mixing egg phosphatidylcholine (50 mol%) with 1-stearoyl-2-oleoyl-*sn*-glycero-3-phosphoethanolamine (50 mol%). Mixtures were dried under a stream of nitrogen and dissolved in ethanol to a final concentration of 5 mM. 24 μl of this solution was injected into 480 μl Tris-HCl (pH 7.0), 0.02% NaN_3 with stirring at 37 °C. For transfer assays 40 μl of this vesicle suspension were used in a total volume of 0.5 ml yielding a final concentration of 19 μM acceptor vesicle lipid.

The phospholipid transfer assay in vitro using fluorescently labeled phospholipids was similar to the method described by Somerharju et al. [18], Van Paridon et al. [19] and Szolderits et al. [20]. The excitation wavelength was 342 nm (2 nm slit), and the emission wavelength was 380 nm (10 nm slit). Donor vesicles containing 50 pmol pyrene-labeled phospholipid were incubated with a 20-fold excess of acceptor membrane vesicles in a total volume of 0.54 ml 10 mM Tris-HCl (pH 7.0), 0.02% NaN_3 at 35 °C. Transfer of fluorescently labeled phospholipids from donor to unlabeled acceptor membranes was followed continously by the increase of pyrene-monomer fluorescence intensity using a Shimadzu RF-540 spectrofluorimeter. Protein-catalyzed transfer was corrected for spontaneous transfer which occurs in the absence of the transfer protein.

Radioassay

As an alternative to the fluorescence assay transfer of radiolabeled phospholipids from unilamellar phospholipid vesicles to mitochondrial membranes was measured [6].

Radiolabeled phosphatidylserine was prepared by a biosynthetic method in vitro. The enzyme source was a yeast microsomal fraction (30000 × *g* pellet) [21]. Microsomal protein (5 mg) was incubated with 50 μCi [3-^{3}H]serine (30 Ci/mmol) in the presence of 0.1 M Tris-HCl (pH 8.0), 5 mM hydroxylamine, 0.2% Triton X-100, 0.6 mM $MnCl_2$ and 0.2 mM CDP-diacylglycerol in a total volume of 4 ml. After incubation for 2 h at 30 °C the reaction was stopped by the addition of 20 ml chloroform/methanol (2:1, v/v) and lipids were extracted by the method of Folch et al. [22]. Under these conditions phosphatidylserine was the only radiolabeled phospholipid as judged by thin-layer chromatography using the solvent chloroform/methanol/25% NH_3 (65:35:5, v/v).

[^{3}H]Phosphatidylethanolamine was synthesized in vitro by incubating 400 nmol [^{3}H]phosphatidylserine (approx. $7 \cdot 10^6$ cpm) obtained by the procedure described above with mitochondrial membranes (final protein concentration 1 mg/ml) in the presence of 50 mM Tris-HCl (pH 7.2), 0.12% Triton X-100, 10 mM dithiothreitol, 5 mM EDTA and 125 mM sucrose in a total volume of 2 ml. After incubating for 3 h at 30 °C the reaction was stopped by the addition of 10 ml chloroform/methanol (2:1, v/v) and lipids were extracted as described above. Radiolabeled phosphatidylserine and phosphatidylethanolamine were separated by preparative thin-layer chromatography using chloroform/methanol/25% NH_3 (50:25:6, v/v) as a developing solvent. Radioactively labeled phospholipids were extracted from silica gel with five 3 ml portions of chloroform/methanol (1:4, v/v).

Other radioactively labeled lipids used in this study were prepared biosynthetically following published procedures [11].

Radioactively labeled donor vesicles were prepared as described by Daum and Paltauf [6]. ^{3}H-labeled lipid (220 nmol, $8 \cdot 10^5$ cpm), traces of [^{14}C]triacylglycerol (10^5 cpm) and 162 μg unlabeled egg phosphatidylcholine were mixed, dried under nitrogen, suspended in 5 ml standard buffer and sonicated for 5 min with 70 W under a stream of nitrogen using a Braun Labsonic 2000 sonicator. Donor vesicles were used within 1–5 days after preparation.

Mitochondria used as acceptor membranes were isolated by the method described by Daum et al. [10].

Transfer assays were carried out by published procedures [6]. [^{14}C]Triacylglycerol was used as an internal non-transferable marker. 1 Unit (U) was defined as 1% of radioactively labeled lipid transferred to acceptor membranes within 30 min at 35 °C.

Chromatographic methods used for the isolation of the phosphatidylserine transfer protein from yeast cytosol

Details of the procedure are described in Results. Matrex Gel Red A was from Amicon, hydroxyapatite from Bio-Rad and DEAE-Sephacel from Pharmacia. All chromatographic steps were carried out at 4 °C.

Analytical procedures

Thin-layer chromatography of lipids [23], SDS PAGE of proteins [24], and quantitation of proteins [25] and lipid phosphorus [26] were carried out by published procedures. Pyrene-labeled phospholipids were quantitated by measuring the absorbance at 342 nm in ethanolic solution; the molar absorption coefficient is 42000 $mol^{-1} cm^{-1}$ [18].

Results

Assays of protein-catalyzed phosphatidylserine transfer

In order to measure the protein-catalyzed transfer of phosphatidylserine two types of assays were utilized. In a radioassay transfer of [^{3}H]phosphatidylserine from unilamellar vesicles to isolated mitochondrial membranes was measured [6]. In the second type of assay the transfer of pyrene-labeled phosphatidylserine from unilamellar donor vesicles to unlabeled unilamellar acceptor vesicles was monitored in a fluorescence assay as described by Szolderits et al. [20].

The radioassay is linear for 30 min, if less than 10% of donor-phosphatidylserine are transferred. The temperature optimum for this assay is 35 °C, the pH optimum 7.0–7.5. The fluorescence assay is linear for 5 min within a broad range of protein concentration. Temperature and pH optima are the same as for the radioassay.

Isolation of the phosphatidylserine transfer protein from yeast cytosol

Step 1: Preparation of yeast cytosol. Spheroplasts from yeast cells were prepared as described by Daum et al. [10] and homogenized in the presence of 0.6 M mannitol, 10 mM Tris-HCl (pH 7.4), 1 mM phenylmethylsulfonyl fluoride, using a Dounce homogenizer. The homogenate was cleared of membraneous particles by sequential centrifugation at 1000 × *g* (5 min), 10000 × *g* (10 min) and 100000 × *g* (60 min). Centrifugations as well as all the following steps were carried out at 0–4 °C. Prior to the following chromatographic steps NaN_3 was added to a final concentration of 0.02% and EDTA to a final concentration of 1 mM.

Step 2: Fractionated ammoniumsulfate precipitation (50–90% saturation). Solid ammoniumsulfate (29.1 g/100 ml) was added in small portions with gentle stirring to buffered yeast cytosol to give a 50% saturation. After 5–10 h on ice precipitated proteins were removed by centrifugation at 23000 × *g* for 12 min. The resulting supernatant was saved, and additional ammoniumsulfate (26.8 g/100 ml) was added for 90% saturation. Precipitated proteins were collected by centrifugation as described above and dissolved in 10 mM Tris-HCl (pH 7.4), 0.02% NaN_3, 1 mM EDTA (in the following referred to as standard buffer) at a concentration of 35 mg/ml. Insoluble material, most likely denaturated proteins, was then removed by centrifugation at 23000 × *g* for 12 min and discarded.

Step 3: Sephadex G-75 chromatography. Portions of 25 ml of the sample obtained in step 2 were applied to a Sephadex G-75 column (2.6 × 75 cm). Fractions of 5.4 ml were collected after elution with the standard buffer at a flow rate of 25 ml/h. Phosphatidylserine transfer activity was found in fractions 28–38.

Step 4: Hydrophobic chromatography using Matrex Gel Red A. Pooled active fractions of the previous step were adjusted to 10% glycerol (v/v) and applied to a Matrex Gel Red A column (2 × 8 cm) equilibrated with standard buffer containing 10% glycerol. Unbound proteins were eluted with 3–4 column volumes of the same buffer at a flow rate of 20 ml/h. Elution was continued with 500 ml of a linear salt gradient (0–700 mM NaCl), and fractions of 5.4 ml were collected. Transfer protein was eluted with 200–280 mM NaCl (fractions 27–37). Pooled active fractions were dialyzed against 5 mM KH_2PO_4 (pH 7.4), 0.02% NaN_3, 10% glycerol (v/v) prior to the next chromatographic step.

Step 5: Hydroxyapatite adsorption chromatography. Portions (50 ml, approximately 20 mg protein) of the dialyzed fraction were applied to a column (2 × 8 cm) filled with hydroxyapatite and equilibrated with the phosphate buffer mentioned above. Unbound proteins were eluted with 3–4 column volumes of the same buffer, and elution was continued with 200 ml of a linear potassium phosphate gradient (5–150 mM). Fractions of 4 ml were collected. Transfer protein appeared in fractions 29–36 corresponding to a phosphate concentration of 80–120 mM.

Step 6: Sephadex G-75 chromatography. Portions (20–25 ml) of pooled active fractions from the previous step were applied to a Sephadex G-75 column and eluted with standard buffer as described for step 3. Fractions of 5.4 ml were collected. The maximum of transfer activity was found in fractions 40–48. The increase in elution volume as compared to step 3 might be explained by dissociation of the protein either from homo- or heteroaggregates at this step of purification.

TABLE I

Purification of the phosphatidylserine transfer protein from yeast cytosol

Preparation of cytosol from the yeast, *Saccharomyces cerevisiae* D 273-10B, and chromatographic methods are described in Results. The starting material was 750 g yeast wet weight.

Purification step	Volume (ml)	Protein (mg)	Spec. act. (U/mg)	Recovery (%)	Purification factor
100000× *g* supernatant	4000	14400	15.3		1.0
Ammonium sulfate precipitation (50–90% sat.)	215	7525	71.4	100	4.7
Sephadex G-75	540	5778	113	122	7.4
Matrex Red A	1080	394	1370	83	90
Hydroxyapatite	1200	60	3400	31	222
Sephadex G-75	2400	16	13580	33	890
DEAE-Sephacel	2300	0.8	46875	7	3064

TABLE III

Inactivation of the yeast phosphatidylserine transfer protein by detergents and proteinases

Transfer of phosphatidylserine was measured both in the radioassay and the fluorescence assay. Highly enriched preparations of transfer protein were incubated with the respective detergents or proteinases. Excess of detergent was removed by dialysis prior to transfer assays. Trypsin was inhibited by soybean trypsin inhibitor, other proteinases by PMSF; inhibitors were used in a 20-fold (w/w) excess. Data are mean values of at least three experiments.

Pretreatment	Relative transfer rate (%)
None	100
Sodium deoxycholate (0.2%, 4 °C, 4.5 h)	70
Octyl glucoside (1%, 4 °C, 4.5 h)	40
Trypsin (24 μg/ml, 30 °C, 45 min)	5
Elastase (24 μg/ml, 30 °C, 45 min)	43
Proteinase K (24 μg/ml, 30 °C, 45 min)	3
Pronase (24 μg/ml, 30 °C, 45 min)	3

According to the elution profile an apparent molecular weight of 34–36 kDa was estimated for the phosphatidylserine transfer protein.

Step 7: DEAE-Sephacel chromatography. Combined active fractions (100 ml, approximately 1 mg protein) obtained in the previous step were applied to DEAE-Sephacel column (1.6 × 32 cm) equilibrated with standard buffer. Proteins were eluted with 500 ml of a linear salt gradient (0–100 mM NaCl) at a flow rate of 40 ml/h. Fractions of 5.4 ml were collected. Transfer activity was eluted with fractions 59–68 corresponding to 64–74 mM NaCl. At this stage the protein appeared as a single band on SDS-PAGE with an apparent molecular weight of 35 kDa. The purification factor was approximately 3000 as summarized in Table I.

Properties of the yeast phosphatidylserine transfer protein

The transfer protein isolated by the procedure described above exhibited a rather broad substrate specificity (Table II). The preferred substrate is phosphatidylserine; phosphatidylethanolamine, cardiolipin, phosphatidic acid and ergosterol are transferred at a lower rate. In contrast to mammalian non-specific lipid transfer proteins [5] the yeast phosphatidylserine transfer protein translocates neither phosphatidylcholine nor phosphatidylinositol. The protein does not immunologically crossreact with antibodies against the yeast phosphatidylinositol transfer protein (data not shown) nor against mammalian non-specific lipid transfer protein (Snoek, G., personal communication).

TABLE II

Substrate specificity of the yeast phosphatidylserine transfer protein

The transfer assays used to measure the substrate specificity are described under Materials and Methods. The transfer rate for phosphatidylserine was set at 100%. Data shown in the table are mean values of at least three experiments. n.d., not detectable.

	Relative transfer rate (%)
Phosphatidylserine	100
Phosphatidylethanolamine	30
Cardiolipin	14
Phosphatidic acid	10
Ergosterol	10
Phosphatidylinositol	n.d.
Phosphatidylcholine	n.d.

The temperature optimum for the reaction catalyzed by the phosphatidylserine transfer protein is 35 °C. The transfer protein is inactivated by heat. Incubation of the protein for 10 min at 40 °C leads to a 70% loss of activity; incubation for 10 min at 50 °C completely abolishes transfer activity. Freezing at −20 °C for 24 h with or without addition of 20–30% glycerol results in a 50% inactivation of the protein.

Detergents inactivate the phosphatidylserine transfer protein (Table III). Sodium deoxycholate and octyl glucoside were used for these experiments because these detergents could be removed rather efficiently by dialysis prior to transfer assays. Loss of activity therefore represents an irreversible inactivation of the transfer protein and not a disturbance of the assay system. As can be seen from Table III proteinase treatment at 30 °C led in most cases to an almost complete loss of activity. After treatment with proteinase inhibitors were added before assaying phosphatidylserine transfer.

Among the possible activators and inhibitors tested EDTA was the most effective (Table IV). Low concentrations (1–5 mM) of EDTA stimulated transfer up to 4-fold; for unexplained reasons higher concentrations of EDTA (20 mM) led to a marked (90%) inhibition. The stimulatory effect of 1–5 mM EDTA is consistent

TABLE IV

Effects of SH-reagents and chelate-forming reagents on protein-catalyzed transfer of phosphatidylserine

Data are mean values of at least three experiments.

Reagent	Concn. (mM)	Relative transfer rate (%)
Control		100
Iodoacetamide	5	87
	20	4
N-Ethylmaleimide	5	140
	20	140
Mercaptoethanol	5	85
	20	25
Dithiothreitol	5	60
	20	55
EDTA	1	300–400
	5	120
	20	10
o-Phenanthroline	5	95
	20	43

with the finding that divalent cations inhibit the transfer of phosphatidylserine. Mg^{2+} at a concentration of 0.5 mM reduces the rate of transfer to about the half, whereas Ca^{2+} or Mn^{2+} are more or less completely inhibitory (data not shown). The inhibitory effect of the SH-reagents β-mercaptoethanol and dithiothreitol are moderate at a concentration of 5 mM, but more pronounced at 20 mM.

In standard assays acceptor vesicles consisted of a 1:1 mixture of phosphatidylethanolamine and phosphatidylcholine. Since phosphatidylethanolamine is a substrate for the transfer protein (see Table II) an exchange of phospholipids between donor and acceptor membrane vesicles could occur under these conditions. When acceptor membrane vesicles consisted only of phosphatidylcholine the rate of phosphatidylserine transfer was exactly the same as to mixed acceptor membrane vesicles. This result indicates that the yeast phosphatidylserine transfer protein can catalyze an efficient net mass transfer. The presence of unlabeled phosphatidylserine in acceptor membrane vesicles even at low concentration (10 mol%) led to a dramatic decrease of the apparent rate of transfer. In this case competition between labeled and unlabeled phosphatidylserine as a substrate on one hand and inhibition of the transfer reaction by negative charges on the surface of acceptor membrane vesicles on the other hand might cause the observed overall effect.

Discussion

In this paper we describe the isolation of a new type of phospholipid transfer protein from the cytosol of the yeast, *Saccharomyces cerevisiae*. The protein has a broad substrate specificity, but shows the highest transfer rate for phosphatidylserine and is therefore termed phosphatidylserine transfer protein. This designation is preferred to 'non-specific lipid transfer protein' because the yeast protein, in contrast to the mammalian non-specific lipid transfer protein, does not catalyze the translocation of phosphatidylcholine and phosphatidylinositol, two major phospholipids of yeast membranes. Antibodies raised against the mammalian non-specific lipid transfer protein do not cross-react with the yeast phosphatidylserine transfer protein (Snoek, G., personal communication). The same is true for antibodies raised against the yeast phosphatidylinositol transfer protein [27]. Furthermore the yeast phosphatidylserine transfer protein is considerably larger (35 kDa) than the mammalian non-specific lipid transfer protein (14 kDa).

Yeast phosphatidylinositol transfer protein, as mammalian phosphatidylinositol transfer proteins, catalyzes phospholipid exchange between membranes by intermediate formation of a 1:1 complex with the respective phospholipids [20]. Non-specific lipid transfer proteins, on the other hand, do not form stable stoichiometric complexes with their substrates. Their proposed mode of action might be the formation of 'bridges' between adjacent membranes. We were unable to obtain stable complexes between phosphatidylserine and the yeast phosphatidylserine transfer protein (Lafer, G., unpublished results). Therefore we assume that the yeast phosphatidylserine transfer protein acts similarly to the mammalian non-specific lipid transfer protein. This assumption is corroborated by the finding that both proteins quite effectively promote net transfer of phospholipids between membranes in vitro.

Considering the participation of the yeast phosphatidylinositol transfer protein in the secretory pathway of proteins [7–9] a collection of secretory mutants [28] was screened for cytosolic phosphatidylserine transfer activity in vitro. None of these mutants showed a significant loss of transfer activity when cells were cultivated at the non-permissive temperature and/or when the assay temperature was 37 °C (Lafer, G., unpublished results). So the physiological role of the yeast phosphatidylserine transfer protein remains unknown. In principle it might mediate phosphatidylserine transport to and phosphatidylethanolamine transfer from mitochondria, since phosphatidylserine is synthesized extramitochondrially, whereas its conversion to phosphatidylethanolamine is catalyzed by the inner mitochondrial membrane enzyme phosphatidylserine decarboxylase [21]. However, in transfer assays in vitro using isolated mitochondria and microsomes the yeast phosphatidylserine transfer protein only marginally stimulates phosphatidylserine transport between the two organelles. In this context it is noteworthy that in mammalian cell mutants deficient in non-

specific lipid transfer protein interorganelle transport of phosphatidylserine is not affected [29]. With the help of yeast mutants defective in phosphatidylserine transfer protein it should be possible to obtain insight into the physiological role of this protein.

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