Identification of rat liver phosphatidylinositol synthase as a 21 kDa protein

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Substantial purification of rat liver phosphatidylinositol (PtdIns) synthase has been achieved by a combination of Hecameg extraction, heat treatment, affinity chromatography and chromatography on PBE-94. The activity chromatographs as a single peak which has an apparent molecular mass between 150 and 200 kDa on Sepharose 4B. When analysed by SDS/PAGE, two major bands are seen. The enzyme activity is correlated with a protein band of 21 kDa. A second band, at 51 kDa, is eluted

from a PBE-94 column slightly ahead of the activity. Manganese is an absolute requirement for stabilization of activity in the presence of detergent. The effect of manganese is optimal at 0.5 mM; magnesium at a concentration of 10 mM is only minimally effective. Substrate K_m s are 1.3 mM and 9.5 μ M for inositol and CDP-diacylglycerol respectively. The activity eluting from the PBE-94 column is purified 5000-fold over the post-mitochondrial supernatant.

INTRODUCTION

Interest in lipid-metabolizing enzymes has increased in recent years with the elucidation of the lipid signal-transduction pathways, particularly the phosphoinositide cycle [1]. Many of the enzymes involved in cycling have been intensively studied, including phosphatidylinositol (PtdIns)-specific phospholipase C [2] and a variety of inositol lipid kinases [3]. However, the enzymes which function in the synthetic phase of the cycle, namely, phosphatidic acid cytidylyltransferase (PtdOHCT, EC 2.7.7.41) and PtdIns synthase (EC 2.7.8.11), have proven difficult to characterize in mammalian systems because of their resistance to purification. Several attempts have been made to purify mammalian PtdIns synthase, but with only limited success [4–7]. Recently, human placental PtdIns synthase was purified 8300-fold and determined to have a molecular mass of 24 kDa [8]. We report here a protocol for the purification of PtdIns synthase from rat liver which results in a 5000-fold purification of the protein, relative to a post-mitochondrial supernatant, and a tentative identification of a 21 kDa protein as the enzyme.

MATERIALS AND METHODS

Rat livers were obtained at the time of killing without regard to sex or age. The livers were frozen at -80°C until further use. The detergent, Hecameg [6-*O*-(*N*-heptylcarbamoyl)-methyl- β -glucopyranoside], was purchased from Vegatec (Villejuif, France). CDP-diacylglycerols (didecanoyl and egg lecithin) were purchased from Serdary Labs. Tritiated inositol (15 Ci/mmol) and CTP (15 Ci/mmol) were obtained from American Radio-labelled Chemicals. Aquasol 2 was purchased from New England Nuclear. The protein assay kit and silver stain kit were purchased from Bio-Rad.

Tissue homogenization

Rat livers were minced and homogenized in 8–10 vol. of 0.25 M sucrose with a Polytron homogenizer. All procedures were carried out at 4°C unless stated otherwise. The homogenate was centrifuged at 10000 *g* for 20 min to remove nuclei, mitochondria and cell debris. The resulting supernatant (10 S) was centrifuged at 100000 *g* for 60 min, and the pellet from this step was resuspended in the same volume of Buffer A [10 mM glycyl-

glycine, 1.6% (50 mM) Hecameg, 20% glycerol, 1 mM MnCl_2 , pH 8.2]. Following incubation at 4°C for 60 min, the membrane extract was centrifuged at 100000 *g* for 60 min. The resulting supernatant (Hex) was heated in a water-bath set at 65°C until a cloudy precipitate appeared. It was then centrifuged at 100000 *g* for 60 min; approx. 75% of the protein was precipitated. The supernatant (65 S) contained the active enzyme.

CDP-diacylglycerol affinity chromatography

Didecanoyl CDP-diacylglycerol affinity resin was prepared as previously described [9]. Prior to use, the resin was suspended in Buffer A. The 65 S enzyme preparation was reacted batchwise with the resin (1 ml of resin per 25 ml of extract) for 30 min at 4°C . The mixture was then poured on to a scintered-glass filter (coarse) at room temperature and washed with 10 vol. of Buffer A. The resin was then eluted twice, for 5 min each, with 5 vol. of Buffer A containing 2 mg/ml of CDP-diacylglycerol.

PBE-94 chromatography

The eluent from the affinity step was loaded on to a column of PBE-94 resin (1 ml of resin per 25 ml of eluent) previously equilibrated with Buffer B [50 mM glycylglycine, 0.33% (10 mM) Hecameg, 10 mM KCl, 20% glycerol, pH 8.2]. The enzyme activity was eluted with a linear gradient of KCl (10 mM to 500 mM) in Buffer B.

SDS/PAGE analysis

SDS/PAGE analytical gels were run according to the method of Laemmli [10] and stained with a silver stain [11].

Enzyme assays

PtdIns synthase was assayed at pH 8.6 in a solution containing 50 mM glycylglycine, 1 mM MnCl_2 , 1 mM inositol, 5 μ Ci of [^3H]inositol, 100 μ M CDP-diacylglycerol (from egg lecithin) and 0.3% Triton X-100 in a final volume of 100 μ l. Assays were carried out for various times at 37°C . Assays were terminated by the addition of 0.5 ml of methanol, 0.4 ml of water and 1.0 ml of

chloroform. Following vortexing and centrifugation to separate the layers, the amount of radioactivity in the chloroform fraction was determined. A unit of activity is defined as nmol of inositol incorporated into PtdIns in 1 min under the conditions described above.

The PtdOHCT assay was carried out at pH 7.2 in a solution containing 50 mM glycylglycine, 40 mM MgCl_2 , 40 μg of phos-

phatidic acid, 1 mM CTP and 10 μCi of $[^3\text{H}]\text{CTP}$ in a final volume of 100 μl . The assay was carried out for 30 min at 37 °C. Termination and extraction of the lipids were as described for PtdIns synthase, except that 2 M KCl was used instead of water, and the chloroform layer was washed once with 2 M KCl before determination of radioactivity.

Protein determinations

The method of Bradford was used to determine protein concentrations [12].

RESULTS

Purification of rat liver PtdIns synthase

Table 1 outlines the results of a typical purification. Following extraction with the non-ionic detergent, Hecameg, the sample was heated to 65 °C until a precipitate appeared. Removal of the precipitate by centrifugation resulted in a 4-fold purification of the enzyme activity.

The subsequent affinity chromatography step resulted in the greatest fold purification, as evidenced by SDS/PAGE (Figure 1). The eluent from the affinity step was subjected to chromato-

Table 1 Purification of rat liver PtdIns synthase

Abbreviations are as follows: 10S, the post-mitochondrial supernatant; 100P, the microsomal pellet suspended in buffer A; HEX, the detergent extract; 65S, the supernatant from the heat-treated extract; AE, the eluent from the CDP-diacylglycerol affinity column; PBE, the eluent from the PBE-94 column. Activity is expressed in units (nmol of inositol incorporated at 37 °C for 1 min).

Sample	Activity (nmol/min)	Protein (mg)	Specific activity (nmol/mg of protein per min)	Purification (fold)
10S	1808	3300	0.55	—
100P	1642	469	3.50	6.4
HEX	1586	314	5.05	9.2
65S	1478	74	19.86	36.3
AE	588	—*	—	—
PBE	633	0.227	2788	5070

* It was not possible to measure protein concentrations in this sample.

Figure 1 Analysis of rat liver proteins by SDS/PAGE at various stages in the purification of PtdIns synthase

A 10% acrylamide gel was run on samples as follows: lane A, 65 S; lane B, HEX; lane C, AE; lane D, PBE. Lanes A and B contained equal amounts of enzyme activity; lane C had 10 times as much activity as A or B; lane D had 100 times as much activity as A or B. See Table 1 for abbreviations.

Figure 2 Analysis of fractions from PBE-94 chromatography of the affinity eluent

An SDS/14% polyacrylamide gel was used to characterize the proteins eluted from the PBE column with a salt gradient. The activity of each sample is plotted above the gel. The enzyme eluted between 0.4 M and 0.5 M KCl.

Figure 3 Analysis of fractions from a second PBE-94 chromatography step

Fractions containing the peak activity eluting from a PBE-94 column were desalted, concentrated and rerun on a second PBE-94 column. The peak PtdIns-synthase-activity fractions from this second run were analysed as described for Figure 2. The activity loaded on each lane was as follows: A, 0.07 unit; B, 0.26 unit; C, 0.28 unit; D, 0.35 unit; E, 0.20 unit.

graphy on PBE-94. The peak of activity eluting from the PBE-94 column retained 35 % of the total activity and 0.007 % of the total protein present in the post-mitochondrial supernatant (10 S).

Figure 1 illustrates the results of a 10 % SDS/PAGE analysis of samples at different stages of purification following extraction with Hecameg. The lane containing the affinity eluent (AE) had 10 times, and the PBE-94 lane 100 times, the enzyme activity present in each of the first two lanes (HEX and 65 S). A prominent protein band of 51 kDa was present in both the AE and PBE-94 samples; however, the intensity of this band did not appear to be 10-fold greater in the PBE-94 sample than in the AE sample. On the other hand, we observed a band at 21 kDa which did appear more prominent in the PBE-94 sample.

To better observe the lower-molecular-mass band, 14 % gels were run of the peak samples from the PBE column. Figure 2 illustrates these results and compares the enzyme activity with the protein staining pattern in each sample. From these results, it is clear that the 21 kDa protein is most closely correlated with enzyme activity. In a separate experiment, the peak activity from a PBE-94 column was rechromatographed on a second PBE-94 column. Figure 3 shows that the activity continues to elute with a 21 kDa protein, and further purification is achieved.

Kinetic analyses of PtdIns synthase

Kinetic analyses of the partially purified enzyme (65 S) indicated that the K_m values observed for inositol and CDP-diacylglycerol were 1.3 mM and 9.5 μ M respectively (Figure 4). Maximal activity was observed with 0.5 mM manganese; 10 mM magnesium was much less effective (Figure 5). Addition of detergent to the assay mixture greatly enhanced the observed activity. Triton X-100 at a concentration of 0.1 % increased activity by 15-fold; Hecameg, at 0.7 % (22.5 mM), was considerably less effective (5-fold increase) (Figure 6a). In the presence of Triton X-100 (0.1 %), Hecameg was inhibitory above 0.16 % (5 mM) (Figure 6b).

Figure 4 Effect of substrate concentration on PtdIns synthase activity

The PtdIns synthase assay was done as described in the text. When the CDP-diacylglycerol concentration was varied (**a**), the inositol concentration was 1 mM. When the inositol concentration was varied (**b**), the concentration of CDP-diacylglycerol was 100 μ M.

DISCUSSION

The yeast PtdIns synthase has been purified to near homogeneity by Fischl and Carman [9,13]. It is stable up to 60 °C and has a molecular mass by SDS/PAGE of 34 kDa. The molecular mass of the yeast protein, as determined from the coding region of the yeast PtdIns synthase cDNA, is 24823 Da [14]. Previous attempts at purification of mammalian PtdIns synthase, however, have met with only limited success. The rat liver enzyme was purified 28-fold by Takenawa and Egawa [7]; the low extent of purification was attributed to the instability of the enzyme. The resulting preparation consisted of 3.3 % of the starting activity and 0.12 % of the total protein. The purified protein had a molecular mass of 60 kDa by SDS/PAGE. A 60-fold [4] and a 190-fold [5] purification of PtdIns synthase from rat brain have been reported. In addition, Parries and Hokin-Neaverson

Figure 5 Effect of manganese and magnesium on PtdIns synthase activity

Enzyme activity was assayed as described in the text. All the samples to which MgCl_2 was added contained 0.1 mM MnCl_2 .

reported the solubilization (by *n*-octylglucopyranoside) and stabilization (by manganese) of PtdIns synthase from canine pancreas [6]. Recently, Antonsson [8] was able to purify placental PtdIns synthase to near homogeneity using a CDP-diacylglycerol affinity column and a Mono Q column. The purified enzyme eluted as a fraction (5–10%) of the total activity in the fall-through from the Mono Q column, while the bulk of the activity was eluted with a 0–1 M NaCl gradient.

Using a relatively new non-ionic detergent, Hecameg [15], we were able to extract over 90% of the PtdIns synthase from rat liver membranes; Triton X-100 routinely extracted 60–70% of the activity. In addition, Hecameg has the advantage of a high critical micellar concentration (19.5 mM), which facilitates its rapid elimination from samples. Triton X-100 is much more difficult to remove, hampering the concentration of samples.

As was the case for the enzyme from dog pancreas [6], manganese was required for preservation of activity. Under these stabilizing conditions, the enzyme retained activity at temperatures up to 65 °C. Taking advantage of this property, we were able to remove a substantial amount of non-PtdIns synthase protein with a heat step. Subsequent affinity chromatography yielded the greatest fold purification, as shown by SDS/PAGE (Figure 1); however, greatest loss of activity was also incurred during this procedure. This step was also the least reproducible. Using the same batch of affinity gel, we often observed differences in binding capacity and/or recovery of bound enzyme. We have yet to determine the exact conditions which yield the best results. It has been well documented that the nature of the protein-detergent micelle can be affected by a variety of parameters including the detergent:protein ratio, pH, ionic strength, temperature and phospholipid content, making it difficult to achieve reproducible purifications of hydrophobic proteins [16]. A slight change in one or more of these parameters may explain the difficulty we observe with the affinity gel. It was not possible to calculate the protein concentration of the affinity eluent because

Figure 6 Effect of detergents on PtdIns synthase activity

Enzyme activity was assayed as described in the text. (a) ●, Triton X-100; ■, Hecameg. All the samples to which Triton was added contained 0.08% Hecameg. (b) All samples contained 0.1% Triton X-100.

the detergent interfered with the micromethods used, and the presence of CDP-diacylglycerol made concentration of the sample difficult.

The eluent from the affinity-chromatography step was then subjected to chromatography on PBE-94. The activity peak from this column yielded a sample which contained two major protein bands when subjected to SDS/PAGE. The enzyme activity correlated most closely with the 21 kDa protein (Figures 2 and 3). Since SDS destroys all of the enzyme activity, however, it was not possible to definitively identify the relevant protein band.

When the peak from the PBE-94 column was chromatographed on Sepharose 4B, the activity eluted as a single peak between markers of 150 and 200 kDa (results not shown). Since we do not know the size or composition of the Hecameg micelle, however, it is difficult to draw any conclusions regarding the size of the

enzyme. The eluent from this column contained both the 21 and 51 kDa proteins.

Results from kinetic analyses of the enzyme activity were similar to those reported previously for the rat liver enzyme, with the exception of the K_m value for CDP-diacylglycerol, which was approx. 14-fold higher (170 μ M) in previous studies [7,17]. Due to the lipid nature of this substrate, however, many factors, such as the concentration of detergent in the assay, can greatly affect the results. Figure 6 documents the effects of two different detergents on the activity of the rat liver enzyme. Triton X-100, at a concentration of 0.1 %, increases activity by 15-fold, while Hecameg (0.7 % or 22.5 mM) increases activity by 5-fold. In addition, in the presence of Triton, Hecameg is inhibitory. Previous studies from this laboratory have documented the differential effect of Triton X-100 on PtdIns synthase activity present in various subcellular fractions from WRK-1 rat mammary tumour cells [18]. In the same study, we determined that both the WRK-1 and the rat liver enzyme resided in the endoplasmic reticulum; no activity could be observed in the plasma membrane fraction [18].

CDP-diacylglycerol can potentially bind to a number of other liver enzymes, including PtdOHCT, phosphatidylserine synthase, cardiolipin synthase and phosphatidylglycerol synthase. Phosphatidylserine synthase probably does not exist in mammalian cells [19], and cardiolipin and phosphatidylglycerol synthases are mitochondrial enzymes [19]. We therefore did not test our samples for these activities. PtdOHCT, however, could have been present in the PtdIns synthase preparation. While there was substantial PtdOHCT activity in the 10 S sample, there was no activity in any detergent-treated sample (results not shown).

Rat liver mitochondrial cardiolipin synthase has recently been purified 598-fold, and it has been reported to have a molecular mass of 50 kDa [20]. The intracellular location (mitochondria),

and heat and detergent sensitivity of this enzyme, however, make it unlikely that our 51 kDa protein would exhibit cardiolipin synthase activity.

We are currently purifying the 21 kDa protein by preparative SDS/PAGE, and we hope to obtain its amino acid sequence as well as raise antibodies to it, allowing us to investigate further the nature of PtdIns synthase.

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