Molecular cloning of rat phosphatidylinositol synthase cDNA by functional complementation of the yeast *Saccharomyces cerevisiae pis* mutation

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Abstract Phosphatidylinositol synthase (CDP-1,2-diacyl-*sn*-glycerol: 3-phosphatidyltransferase, EC 2.7.8.11) catalyzes the formation of phosphatidylinositol and CMP from CDP-diacylglycerol and *myo*-inositol. We have cloned a phosphatidylinositol synthase cDNA from rat brain by functional complementation of the yeast *pis* mutation, which is defective in phosphatidylinositol synthase. The deduced protein comprised 213 amino acids with a calculated molecular mass of 23 613 Da. The predicted protein sequence is highly homologous to the previously determined yeast phosphatidylinositol synthase sequence. The cDNA hybridized to a 1.7-kb mRNA that was abundantly expressed in rat brain and kidney.

Key words: Phosphatidylinositol synthesis; Functional cloning; Rat brain

1. Introduction

Phosphatidylinositol (PI) occurs widely in amounts usually corresponding to 2–12% of the total phospholipids in cells [1]. Besides being an essential component of the cell membrane, PI plays an important role in anchoring proteins to the membrane and is the precursor to signaling molecules such as polyphosphoinositides and diacylglycerol [2,3]. The enzyme responsible for the final step of de novo biosynthesis of PI is PI synthase (CDP-1,2-diacyl-*sn*-glycerol: 3-phosphatidyltransferase, EC 2.7.8.11), which catalyzes the reaction of CDP-diacylglycerol and *myo*-inositol (inositol) to form PI and CMP [4,5]. This enzyme is membrane-associated and, in animal tissues, is localized primarily in the endoplasmic reticulum and the Golgi [6,7]. Recently, however, enzyme activity was measured in plasma membranes isolated from GH_3 pituitary cells [8]. Moreover, the existence of two forms of PI synthase in pituitary cells has been postulated on the basis of different kinetic characteristics between the endoplasmic-reticulum and the plasma-membrane enzymes [8,9].

The first purification of PI synthase was performed in the yeast *Saccharomyces cerevisiae* by Fischl and co-workers [10], who also characterized its enzymatic properties [11]. Purification was achieved by affinity chromatography with CDP-diacylglycerol after solubilization of the microsomes with Triton X-100. The purified enzyme was shown to have a molecular weight of 34 000 as determined by SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Several attempts have been made to purify mammalian PI synthase, but with limited success [12–15]. Recently, PI synthases have been purified from human placenta [16] and rat liver [17], and determined to have molecular masses of 24 000 Da and 21 000 Da, respectively, when analyzed by SDS-PAGE.

In spite of the obvious importance of PI synthase in mammalian cells, attempts to isolate cDNA clones that encode this enzyme have been unsuccessful. However, in the yeast *S. cerevisiae*, Nikawa and Yamashita [18] have isolated a gene (*PIS*) encoding PI synthase. The *PIS* open reading frame encoded 220 amino acids with a calculated molecular weight of 24 823 and was indispensable for cell growth [19].

Here we report the cloning of the first mammalian PI synthase cDNA by functional complementation of a yeast *pis* mutant defective in PI synthase activity [20] with a rat brain cDNA library. The cDNA encoded 213 amino acids with a calculated molecular mass of 23 613 Da. Comparison of the predicted sequence with the yeast *PIS* gene product [19] revealed 39% identity.

2. Materials and methods

2.1. Yeast strains, media and transformation

The PI synthase mutant strain D278-2A (*MATα pis leu2 his4*) and the wild-type strain D451-3 (*MATa leu2 ura3 can1*) have been described [18,21]. Cloning host strain D501-1 (*MATa pis leu2 ura3*) was derived from a cross of D278-2A and D451-3. Cells were grown aerobically with shaking at 30°C unless otherwise stated. The composition of inositol-depleted minimum medium (M-i) was as described [22]. Inositol-supplemented minimum medium (M20i) was prepared by supplementing M-i medium with 20 μg of inositol/ml. When necessary, L-leucine and uracil were added to the culture medium at a concentration of 20 μg/ml. Yeast transformation was performed by the lithium acetate method [23].

2.2. cDNA library, DNA manipulations, and sequencing

A rat brain cDNA library was prepared as described previously [24]. *Escherichia coli* plasmids were prepared using Wizard Minipreps (Promega). Yeast plasmids were extracted as described [18]. DNA sequencing was performed by the dideoxy chain-termination method, using a Taq dye primer cycle sequencing kit and an ABI 373A DNA sequencer (Applied Biosystem). The final sequence was confirmed from both strands.

2.3. PI synthase assay

PI synthase activity was assayed by measuring the incorporation of [^{3}H]inositol (Amersham) into PI in the presence of CDP-diacylglycerol. The standard reaction mixture contained 50 mM Tris-HCl (pH 8.0), 20 mM $MgCl_2$, 100 μM CDP-diacylglycerol, 0.3% Triton X-100, 0.57 mM [^{3}H]inositol ($\sim 4 \times 10^4$ dpm/nmol), and membrane pro-

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Abbreviations: PI, phosphatidylinositol; inositol, *myo*-inositol; PAGE, polyacrylamide gel electrophoresis; PCR, polymerase chain reaction; RACE, rapid amplification of cDNA ends

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tein (10–50 μg) in a total volume of 0.1 ml. The reaction was allowed to proceed for 10 min at 37°C. The extraction and determination of PI were performed according to Parries and Hokin-Neaverson [14]. The membrane fraction of yeast cells, which had been cultured in medium M20i at 36°C, was prepared as previously described [25]. An enzyme unit is defined as the amount of enzyme that catalyses the formation of 1 μmol of PI in 1 min.

2.4. Rapid amplification of cDNA ends

5′ rapid amplification of cDNA ends (RACE) was carried out using the 5′ Ampli FINDER RACE kit (Clontech) and rat brain 5′-RACE Ready cDNA (Clontech) according to recommendations given by the supplier. For the first and second polymerase chain reaction (PCR), 5′-GGAAATGATGGCGAAAACAATCCGG-3′ (primer 1, complementary to nt 193–217) and 5′-GCAAGCTTAAGATTAGGCACGAACAGGAAG-3′ (primer 2, complementary to nt 160–181+GCAAGCTT) were used, respectively. A *Hin*dIII site (AAGCTT) was engineered at the 5′ end of the primer 2. The second PCR products were fractionated on an agarose gel and visualized by staining with ethidium bromide. An intensely stained band of ~250 bp was excised from the gel, digested with *Eco*RI and *Hin*dIII, and ligated into the *Eco*RI/*Hin*dIII site of Bluescript II KS$^-$ (Stratagene) to produce plasmid pBS/race. Three independent inserts were sequenced.

3. Results and discussion

3.1. Cloning strategy

Previously, we isolated a K_m mutant of *S. cerevisiae* with a lesion in PI synthase [20]. The mutant, strain 423, requires a high concentration of inositol for growth and possesses an enzyme with an apparent K_m for inositol over 200-times higher than that of wild-type enzyme. To clone mammalian PI synthase cDNA, we used strain D501-1 (*MATa pis leu2 ura3*), a derivative of strain 423, which was constructed using standard genetic methods. The strain can grow in inositol-supplemented minimum medium M20i, but not in inositol-depleted minimum medium M-i, like the original 423 strain. Transformation of the mutant with PI synthase cDNA should restore growth in medium M-i. Thus, we thought that a PI synthase cDNA could be identified by complementation of the *pis* mutation.

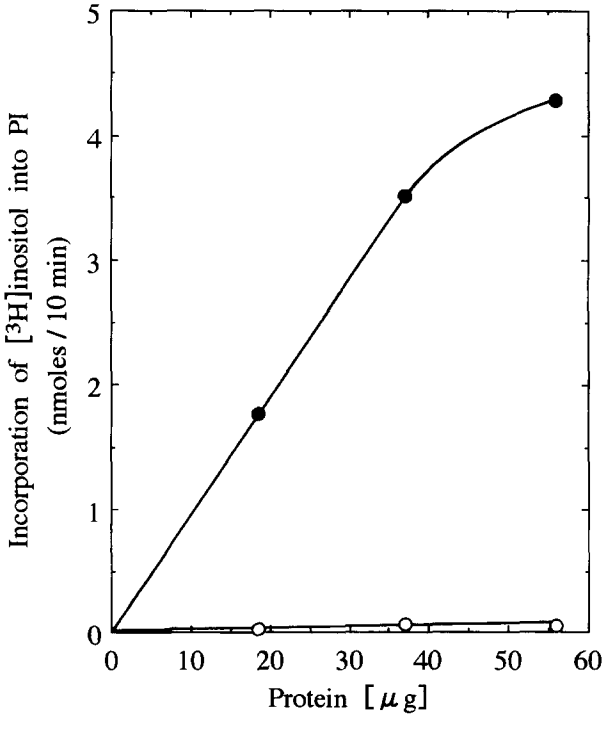

Fig. 1. PI synthase activity of the *pis* transformant expressing rat PI synthase. D501-1 cells harboring pPIS3 (●) and D501-1 cells harboring pDB20 (○) were grown at 36°C in 250 ml of M20i medium supplemented with leucine to the mid-logarithmic phase. The cells were harvested by centrifugation and membrane fractions were obtained. PI formation was determined with the indicated amounts of membranes under standard assay conditions.

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GTGGATCGCCCGGGAGAACTGGGCCCAGGGCTCAGGTGGAGCTGCCGATGCCCCGGGCTG   60
AAAGGTGAGACCACGCTGGTGAGGCTGGGACAAGACCTTGCACCTCCAGACCTGGGCCGA  120
GACCACAAGGGGAGCCGCCAAGATGCCAGAGGAAAATATCTTCCTGTTCGTGCCTAATCT  180
                    M  P  E  E  N  I  F  L  F  V  P  N  L

TATTGGTTATGCCCGGATTGTTTTCGCCATCATTTCCTTCTACTTTATGCCCTGCTGCCC  240
  I  G  Y  A  R  I  V  F  A  I  I  S  F  Y  F  M  P  C  C  P

CTTCACGGCCTCCTCCTTCTATCTACTCAGCGGACTTCTAGACGCTTTCGATGGACACGC  300
  F  T  A  S  S  F  Y  L  L  S  G  L  L  D  A  F  D  G  H  A

AGCTCGAGCCCTTAATCAAGGAACCCGATTTGGGGCCATGCTCGACATGCTGACGGACCG  360
  A  R  A  L  N  Q  G  T  R  F  G  A  M  L  D  M  L  T  D  R

TTGCGCCACCATGTGTCTCTTGGTCAACCTGGCCCTGCTCTACCCTCGGGCCACTCTTCT  420
  C  A  T  M  C  L  L  V  N  L  A  L  L  Y  P  R  A  T  L  L

GTTCCAGCTCAGCATGAGCCTGGACGTGGCCAGTCACTGGCTGCATCTGCACAGTTCTGT  480
  F  Q  L  S  M  S  L  D  V  A  S  H  W  L  H  L  H  S  S  V

GGTGCGAGGCAGTGAGAGCCACAAGATGATTGACCTGTCTGGGAACCCTGTGCTTCGAAT  540
  V  R  G  S  E  S  H  K  M  I  D  L  S  G  N  P  V  L  R  I

CTACTATACTTCCAGGCCTGCTCTGTTCACCCTGTGTGCTGGAAATGAACTCTTCTACTG  600
  Y  Y  T  S  R  P  A  L  F  T  L  C  A  G  N  E  L  F  Y  C

CCTCCTGTACCTGTTCAATTTCTCCGAGGGACCACTAGTCGGCTCTGTGGGGCTTTTCCG  660
  L  L  Y  L  F  N  F  S  E  G  P  L  V  G  S  V  G  L  F  R

AATGGGCCTCTGGATCACAGCTCCCATTGCCCTGCTCAAGTCCATCATTAGTGTCATCCA  720
  M  G  L  W  I  T  A  P  I  A  L  L  K  S  I  I  S  V  I  H

CCTCGTCACGGCTGCAAGGAACATGGCTGCCCTCGATGCAGCAGACCGCGCCAAGAAGAA  780
  L  V  T  A  A  R  N  M  A  A  L  D  A  A  D  R  A  K  K  K

ATGATCCTCTCCCATCCCCAGCTGCCCACCTGCCCTGGGTATCTACTGTGCCACATGGCT  840
  *

CCTCTCCTCCTAAGAGGTCCCAGTGTCATGTCTTTCTCATGTGTTCTCTAACCTGCTGGG  900
ATTGGGGGTCACCCTCTGTTTGGTGTTGTCACTGTCTTTTTACTCCTGATAACTAGACCC  960
AACCCGTGACCCCAAGGACCTGGCACTGCAAAGCAGGAAGGGTGCTCAGGAGGCCCACTC 1020
ATCCAGCCGGTGCTCCAGGAGGCCTGTCTGAAGACTGGGTGTTGCCACATATCCTGCTGG 1080
ATCAGCCTGGGGTCTGGCCTGAGACTTTGGGAACTTATACGGGCAAAGGAGTCAGGCTGA 1140
TGGGCCAAGAAGGCTGGGGTACCAGGCCAGGGAGGGAAGGGGCCAAGGGCCAACTAGATC 1200
TTTCCCTTAGGCAAGCTCCTCTATCCTGTCCTGCTCTGGAAACCTCCATAATAAAGGGGG 1260
GCACCTTCACTCTTTTTGGATTTGTTTACTTTGGGACACTCTTAGATGATGGCCTAGGGT 1320
TCAAAGGAGCTGGGTAAAAAAAAAAGAAATCCACCTGGATGCTTTTCCCACAAAGGTGGT 1380
TGGCTCCTCCCTGTCATTCCAGTGACCTCAGAGAGGCCTCGAGGTTCACAAGCGTTGTCC 1440
TGCCCACCTGCTCTATGTCCTGGCTGGCCACTTTCACTGTAACCTTGATGTTTTTGACCC 1500
TCATGAGAATGTAAGCTTCACAAGAGCAGATTTAGATCTTTCTCGTCTCTGCCTGGAGTG 1560
AAAAGTAGCCTGGCATGTGGTAACTGCTTAATAAATATTCATGGAAAGATGAAAAAAATA 1620
C                                                              1621
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Fig. 2. Nucleotide sequence of PI synthase cDNA and the deduced amino acid sequence. Isolated clones (pPIS3 and pPIS5) and the 5′ RACE product are combined to construct the composite cDNA. The largest open reading frame is translated into an amino acid sequence and shown below the nucleotide sequence in the single-letter code. The numbers at the right denote the positions of the last nucleotide of each line. The stop codon is indicated by an asterisk. The sequence reported in this paper has been deposited in the GenBank data base (accession no. D82928).

3.2. Cloning of rat PI synthase cDNA

Competent cells were prepared and transformed with a rat brain cDNA library [24] by the lithium acetate method [23]. The library had been constructed in a yeast expression vector, pDB20 [25], containing the *ADH1* promoter and terminator, a *Not*I cloning site, and *URA3* as a selectable marker. Thus the cDNA could be expressed under the control of *ADH1* promoter in yeast cells. We used about 50 μg DNA of the library per 10^9 cells. The transformants were cultured on medium M-i agar plates (diameter, 9 cm) supplemented with leucine at 30°C for 12 h, followed by further incubation at 36°C for 5 days. From 10 such plates, we obtained 8 independent colonies. Seven plasmids recovered from these transformants complemented the *pis* mutation after retransformation of strain D501-1. Restriction analysis revealed that the plasmids could

be classified into two groups (plasmids pPIS3 and pPIS5) and that the two clones contained overlapping cDNA inserts of 1.5 and 1.2 kbp, respectively.

To confirm that the cDNA clone pPIS3 which contained a longer insert cDNA than pPIS5 encoded PI synthase, we assayed the enzyme activity. Membrane fractions were prepared from D501-1[pDB20] and D501-1[pPIS3] cells which had been cultured in medium M20i, and were used for a PI synthase assay (Fig. 1). The membranes from the *pis* mutant harboring control plasmid pDB20 synthesized very low amounts of PI (spec. act. <0.1 mU/mg protein) under standard assay conditions, since the mutant enzyme possesses a very low affinity for inositol [20]. However, the pPIS3 transformant expressed considerable PI synthase activity. The specific activity was 9.21 mU/mg protein, which was comparable to or even higher than that detected in rat tissue [12,17]. The enzyme activity of the pPIS3 transformant was absolutely dependent on CDP-diacylglycerol (specific activity in the absence of CDP-diacylglycerol, <0.01 mU/mg protein). The apparent K_m for inositol was estimated to be 0.6 mM. This value was two orders of magnitude lower than that of the enzyme in the host *pis* mutant cells [20]. When the enzyme was assayed in the presence of 10 mM EDTA in stead of $MgCl_2$, its activity decreased by 82% of that assayed under standard assay conditions. Thus, we concluded that the cloned cDNA encoded a considerable length, if not the full-length, of rat PI synthase.

3.3. Sequence analysis of the rat brain PI synthase cDNA

We next sequenced the two inserts in plasmids pPIS3 and pPIS5. Plasmid pPIS3 contained 1496 nt from positions 136 to 1631, and plasmid pPIS5 contained 1187 nt from positions 91 to 1277 (Fig. 2). The nucleotide sequences overlapped, indicating that they were derived from an identical transcript. Within the sequences there existed an open reading frame of 213 amino acids with a predicted molecular mass of 23 613 Da (Fig. 2). However, as there were no stop codons upstream of the predicted start methionine, it was not clear that the cDNAs encoded the full-length protein. In order to extend the insert cDNAs in the 5′ direction, we used RACE PCR [26,27]. This resulted in the identification of an additional 91 nt preceding the 5′ end of the pPIS5 clone. Two in-frame stop codons were found in the 5′ RACE product. We constructed the composite cDNA by combining pPIS3, pPIS5 and the 5′ RACE product. The nucleotide and deduced amino acid sequences for the coding region of the composite cDNA are presented in Fig. 2. We assumed the ATG codon at positions

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          MPEENIFLFVPNLIGYARIVFAIISFYFMPCCPFTASSFYLLSGLLDAFDG   51'
          ...:... ..:: :::.:.. : .::. :   : . . .: : ::::.::
MSSNSTPEKVTAEHVLWYIPNKIGYVRVITAALSFFVMKNHPTAFTWLYSTSCLLDALDG   60"

HAARALNQGTRFGAMLDMLTDRCATMCLLVNLALLYPRATLLFQLSMSLDVASHWLHLHS  111'
  :: :: ...::.:::.:::..:  :.  : . ::.  ..::: ..::..::..:...
TMARKYNQVSSLGAVLDMVTDRSSTAGLMCFLCVQYPQWCVFFQLMLGLDITSHYMHMYA  120"

SVVRGSESHKMIDLSGNPVLRIYYTSRPALFTLCAGNELFYCLLYLFNPSEGPLVGSVGL  171'
:.  :..::: .. .....:..:::.: .:::.:: ::::: ::: :::... :.
SLSAGKTSHKSVGEGESRLLHLYYTRRDVLFTICAFNELFYAGLYLQLFSNSATFGK---  178"

FRMGLWITAPIALLKSIISVIHLVTAARNMAALDAADRAKKK                    213'
. .. :. :  ..: . .:..:  :: .:. :: . ..:.
-WTTI-ISFPGYVFKQTANVVQLKRAALILADNDAKNANEKNKTY                  221"
```

Fig. 3. Sequence comparison. The deduced amino acid sequence of the rat brain PI synthase (upper line) is compared with that of the *S. cerevisiae* enzyme (lower line). Colons indicate identical amino acids, and dots conservatively substituted amino acids. The figures at the right denote the positions of the right-end amino acids.

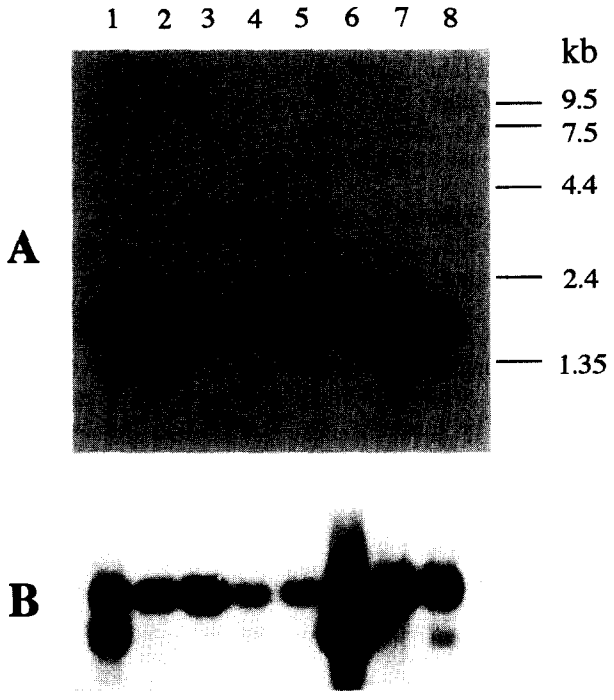

Fig. 4. Expression of PI synthase mRNA in rat tissues. (A) A nylon membrane containing poly(A)$^+$ RNA from various tissues (Clontech) was used for the Northern analysis [35]. The blot was hybridized with a 1.5-kbp cDNA fragment from pPIS3. (B) Same blot was rehybridized with a manufacturer-supplied β-actin probe. ^{32}P-labeled probes were prepared using a Megaprime DNA labeling kit (Amersham International). Lanes 1–8 contain, in order, 2 μg of RNA from rat heart, brain, spleen, lung, liver, skeletal muscle, kidney, and testis. Size markers are indicated on the right.

143–145 was the translational initiation codon for the following reasons. First, two in-frame stop codons were located at positions 59–61 and 80–82. Second, the predicted protein molecular weight compares fairly well to those estimated by SDS-PAGE of purified human placenta and rat liver enzymes [16,17].

3.4. Structural properties of rat PI synthase

The predicted rat PI synthase is an extremely hydrophobic protein, composed of 60% hydrophobic, 20% hydrophilic and 20% neutral amino acid residues. A more detailed analysis of its hydrophobicity was carried out using the computer program of Kyte and Doolittle [28]. Its grand average hydropathy score was 0.60, comparable to the scores of most integral membrane proteins [28]. The PI synthase contains several hydrophobic stretches within its sequence. Among them, stretches encompassing residues 17–35 (average hydropathy index, 1.62), 77–98 (average hydropathy index, 1.50) and 165–195 (average hydropathy index, 1.68) are sufficiently extended and hydrophobic to be membrane-associated. We searched the protein data base for similar sequences using the FASTA program [29]. As expected, *S. cerevisiae* PI synthase was found to bear significant sequence similarity to the predicted rat PI synthase (Fig. 3). 39% of the amino acids are identical and 73% are similar in the two predicted sequences. Furthermore, rat PI synthase had a region showing a high degree of local homology to the conserved domains in phospholipid-synthesizing enzymes and amino alcohol phosphotransferases of *E. coli*, *S. cerevisiae*, and *Bacillus subtilis*

[30–34]. The consensus sequence, $DGX_2ARX_8GX_3DX_3D$, in the four enzymes of *S. cerevisiae* (PI synthase, phosphatidylserine synthase, cholinephosphotransferase and ethanolaminephosphotransferase) [31], *E. coli* phosphatidylglycerophosphate synthase [30], and *B. subtilis* phosphatidylserine synthase [34] was found in residues 50–72 of rat PI synthase.

3.5. Tissue distribution of PI synthase mRNA

The distribution of rat PI synthase mRNA in various tissues was investigated by probing a gel blot of size-fractionated poly(A)$^+$ mRNA from rat heart, brain, spleen, lung, liver, skeletal muscle, kidney and testis with a radiolabeled fragment of the rat brain cDNA. As shown in Fig. 4, a single transcript of 1.7-kb was detected in all tissue types tested with varying abundance. The transcript was expressed in the brain and kidney abundantly and to much lesser extent in the testis. There may be an other form (isozyme) of PI synthase in tissues which expressed a low amount of the 1.7-kb transcript, since the enzyme is considered to be essential for the growth of all cell types.

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