Human L-3-phosphoserine phosphatase: sequence, expression and evidence for a phosphoenzyme intermediate

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Abstract **We report the sequence of the cDNA encoding human L-3-phosphoserine phosphatase. The encoded polypeptide contains 225 residues and shows 30% sequence identity with the *Escherichia coli* enzyme. The human protein was expressed in a bacterial expression system and purified. Similar to known L-3-phosphoserine phosphatases, it catalyzed the Mg^{2+}-dependent hydrolysis of L-phosphoserine and an exchange reaction between L-serine and L-phosphoserine. In addition we found that the enzyme was phosphorylated upon incubation with L-[^{32}P]phosphoserine, which indicates that the reaction mechanism proceeds via the formation of a phosphoryl-enzyme intermediate. The sensitivity of the phosphoryl-enzyme to alkali and to hydroxylamine suggests that an aspartyl- or a glutamyl-phosphate was formed. The nucleotide sequence of the cDNA described in this article has been deposited in the EMBL data base under accession number Y10275.**

1. Introduction

L-3-Phosphoserine phosphatase is the enzyme responsible for the third and last step in L-serine formation. This enzyme not only catalyzes the Mg^{2+}-dependent hydrolysis of L-phosphoserine but also an exchange reaction between L-serine and L-phosphoserine [1,2], suggesting that its reaction mechanism proceeds through the formation of a phosphoryl-enzyme intermediate; the latter has, however, never been directly demonstrated. As L-3-phosphoserine phosphatase has been reported to be deficient in one case of L-serine deficiency [3], it was of interest to determine the sequence of the human enzyme. Databanks contain DNA sequences of several bacteria and of two eukaryotes (*Saccharomyces cerevisiae* and *Schistosoma mansoni*) that are homologous to *SerB*, the gene encoding *Escherichia coli* L-3-phosphoserine phosphatase [4].

In this paper we report the sequence of the human enzyme. We have also expressed an active protein and show that it forms a phosphoenzyme when incubated with its substrate.

2. Material and methods

2.1. Materials

L-[^{32}P]Phosphoserine was synthesized by incubating 1 mM L-serine with 10 μM ^{32}P-labeled inorganic pyrophosphate (0.10 mCi), 2 mM magnesium acetate, 1 mM dithiothreitol, 25 mM Tris-HCl (pH 7.1), and 0.03 U pyrophosphate: L-serine phosphotransferase [5] for 40 min at 30°C in a final volume of 3 ml; it was purified as in [6]. Radioactive compounds and Thermosequenase were from Amersham and *Pwo* polymerase from Boehringer. Chemicals were from Sigma or Merck. Clones R14208, N23530, N42133, W05752, H38879 and T82144 from the IMAGE consortium [7], were kindly provided by the UK HGMP Resource Centre.

2.2. Amplification of cDNA

DNA obtained from human cDNA libraries (from renal cell carcinoma line LE9211-RCC [8], from the urinary bladder transitional-cell carcinoma LB831-BLC, or from EBV-transformed lymphoblastoid cell line LG2-EBV [9]) was amplified with *Pwo* polymerase (a polymerase with proofreading activity) using two primers chosen as described in Section 3. The first (GTGCATATGGTCTCCCACTCAGAGCTG) had a start codon (underlined) inserted in a *Nde*I site and the second (ACGGATCCTCATCTGAAGTTGTTTGGAGC) corresponded to nucleotides 882–902 of the sequence that we report in the EMBL databank, flanked by a *Bam*HI site. The PCR-amplified product obtained with the human kidney library was purified by agarose gel electrophoresis, inserted in the *Eco*RV site of pBluescript and sequenced.

2.3. Expression and purification of recombinant human L-3-phosphoserine phosphatase

The insert of the resulting plasmid was excised with *Nde*I and *Bam*HI restriction enzymes and ligated in the expression vector pET3a [10]. BL21(DE3)pLysS cells were transformed with this plasmid and were aerobically grown in M9 medium at 37°C until A_{600} reached 0.5–0.6. The culture was then maintained on ice for 15 min, after which time isopropylthiogalactoside was added to a final concentration of 0.4 mM. After 20 h of incubation at 37°C, bacterial extracts were prepared as described [11], the lysis buffer being supplemented with 5 μg/ml of antipain and 5 μg/ml of leupeptin. The extract was centrifuged for 40 min (20000×*g* at 4°C). The resulting supernatant (25 ml) was diluted 3-fold with buffer A (10 mM Tris-HCl (pH 8.5), 1 mM dithiothreitol, 1 μg/ml leupeptin, 1 μg/ml antipain) and applied onto a DEAE-Sepharose column (1.6×10 cm). The column was washed with 100 ml of buffer A and protein was eluted with a NaCl gradient (0–400 mM in 250 ml of buffer A). L-3-Phosphoserine phosphatase came out at a Na^+ concentration of 250 mM. The active fractions were pooled and concentrated 4-fold by ultrafiltration in an Amicon cell (YM-10 membrane). Two milliliters of this preparation were further purified by gel filtration on Sephacryl S-200 (1.6×50 cm) in buffer B (20 mM Hepes (pH 7.5), 1 mM dithiothreitol, 100 mM KCl, 0.5 μg/ml leupeptin and 0.5 μg/ml antipain) at a flow rate of 0.25 ml/min.

2.4. Purification and sequencing of rat liver L-3-phosphoserine phosphatase

L-3-Phosphoserine phosphatase was purified from rat liver by a previously described procedure [12], followed by a gel filtration on Sephacryl S-200 equilibrated with buffer B. The active fractions were submitted to SDS-PAGE. Staining with Coomassie Blue revealed the presence of about 10 bands with M_r 20000–70000. The gel was sliced, and the polypeptides were extracted and subjected to a renaturation procedure [13]. Phosphoserine phosphatase activity was observed only in the fraction corresponding to a 25000 M_r polypeptide. Tryptic peptides were obtained from this protein and sequenced as described [14].

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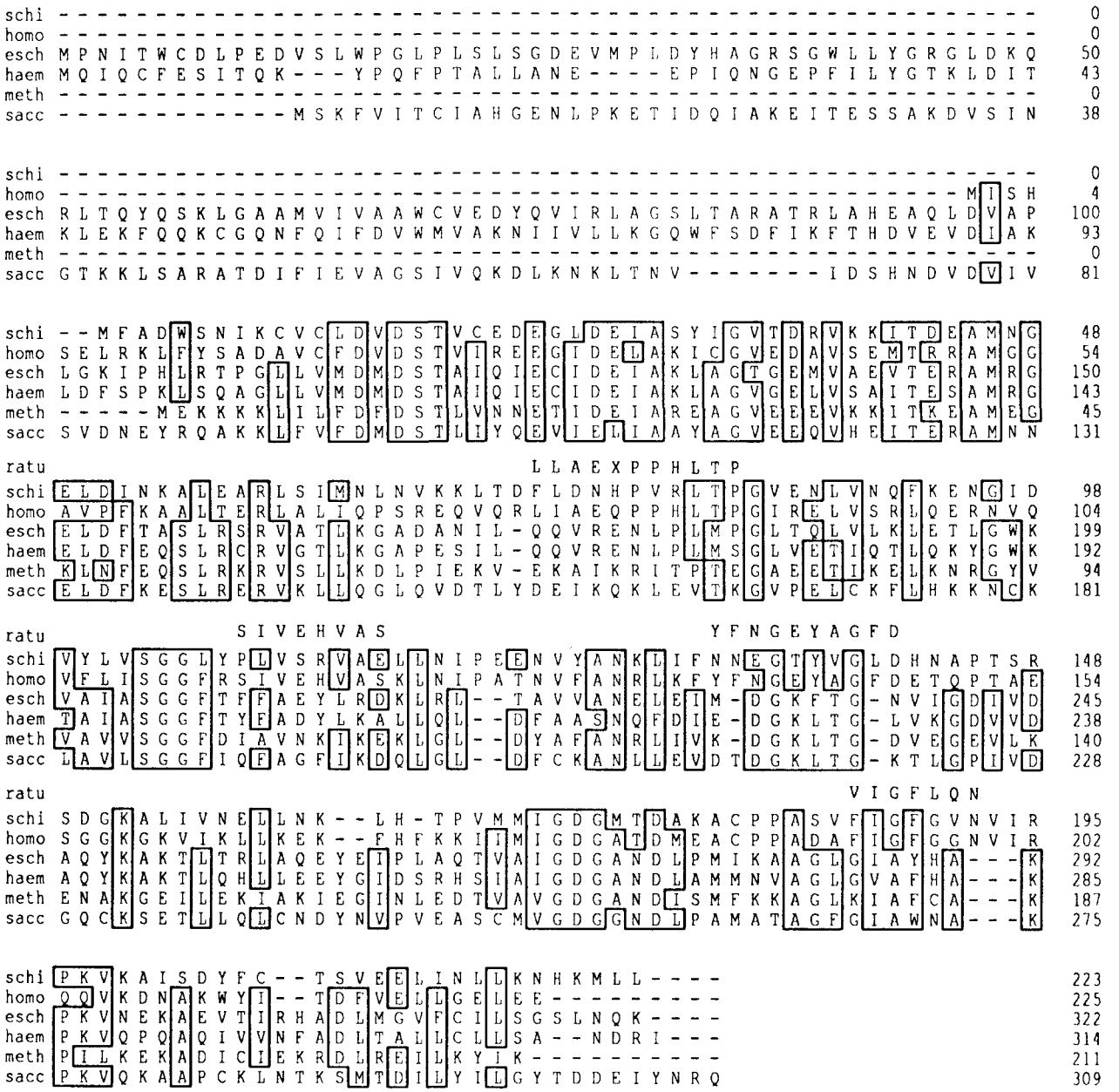

Fig. 1. Alignment of l-3-phosphoserine phosphatase sequences from various species (ratu: *Rattus norvegicus*; schi: *Schistosoma mansoni*; homo: *Homo sapiens*; esch: *Escherichia coli*; haem: *Haemophilus influenzae*; meth: *Methanococcus jannaschii*; sacc: *Saccharomyces cerevisiae*). The tryptic peptides are shown for the rat enzyme. Similar and conserved residues are shown in boxes.

2.5. Enzyme assays

In the course of its purification, L-3-phosphoserine phosphatase was assayed at 30°C by the release of Pi from unlabelled L-phosphoserine in an assay mixture (250 μl) containing 25 mM Mes (pH 6.5), 5 mM $MgCl_2$, 1 mM dithiothreitol, 5 mM L-phosphoserine, 0.1 mg/ml bovine serum albumin and 1–10 mU L-3-phosphoserine phosphatase. Reactions were stopped by the addition of 250 μl of 10% trichloroacetic acid (TCA) and Pi was measured [15]. One unit of enzyme is the amount that catalyzes the conversion of 1 μmol of substrate per minute under these conditions. For kinetic studies, the enzyme was assayed by the release of [^{32}P]Pi from L-[^{32}P]phosphoserine [16] in the assay mixture described above. The exchange reaction was measured by the incorporation of L-[^{14}C]serine into L-phosphoserine in an assay mixture containing 25 mM Hepes (pH 7.5), 1 mM dithiothreitol, 1 mM $MgCl_2$, 1 mM L-phosphoserine, 0.5 mM L-serine and 20000 cpm L-[^{14}C]serine in a final volume of 100 μl. The incubation was arrested by spotting 25 μl of the reaction mixture on Whatman DE-81 papers, which were washed in water, dried and counted for radioactivity in the presence of 5 ml of HiSafe 2 scintillant.

2.6. Demonstration of the formation of the phosphoenzyme

Unless otherwise indicated, about 0.15 U of recombinant L-3-phosphoserine phosphatase was incubated at 0°C in a mixture containing 50 mM Hepes (pH 7.5), 1 mM dithiothreitol, 1 μM L-phosphoserine, 60000 cpm of L-[^{32}P]phosphoserine, 5 mM $MgCl_2$, 20 mM L-serine and 1 mg/ml bovine serum albumin in a volume of 100 μl. The reaction was stopped at the indicated times by the addition of 250 μl of 5% TCA and filtration on a polyethersulfone membrane (Supor® 200, from Gelman). The membrane was washed with 10 ml of TCA 5% and counted for radioactivity with 5 ml of HiSafe 2 scintillant.

2.7. Other methods

Protein was measured according to Bradford [17] with bovine gamma-globulin as a standard. Sequencing was performed using T7 Ther-

mo Sequenase (Amersham), fluorescent primers and the LI-COR automated DNA sequencer 4000L.

3. Results

3.1. Identification and sequencing of human cDNAs

The predicted amino acid sequence of the *S. mansoni* enzyme, most likely the closest to that of the human enzyme, was used to perform a Blast search [18]. Several human ESTs (expressed-sequence tags) were identified, which corresponded to the 5′ (IMAGE Consortium [LLNL] cDNA clones R14208; N23530; N42133; W05752) or the 3′ end (clones H38879 and T82144) of the parasite enzyme's open reading frame. Oligonucleotide primers derived from these sequences were used in PCR reactions to amplify human cDNAs derived from bladder or kidney tumor cells, or of lymphocytes. In all cases one single fragment of 725 bp was obtained. The fragment obtained by amplification of the kidney cell cDNA was cloned in pBluescript and sequenced. Several of the above-mentioned clones were also sequenced. The composite sequence of the reconstituted human cDNA is reported in the EMBL databank. Note that clones N42133 and W05752 lacked nucleotides 281 to 757, and that in clone N23530, there was a 122 bp insert homologous to the *Alu* I family between nucleotides 280 and 758.

The sequence totals 1600 bp; the 5′ and 3′ non-coding regions amount to 187 and 735 bp, respectively. The ATG codon opens a reading frame of 675 bp, encoding a protein of 225 amino acids. This protein shows 30% and 40% sequence identity with the enzymes from *E. coli* and *S. mansoni*, respectively. It shows also a high degree of identity with several peptides derived from L-3-phosphoserine phosphatase purified from rat liver (Fig. 1).

3.2. Expression and characterization of a recombinant protein

To check that the sequence encoded L-3-phosphoserine phosphatase, the open reading frame was inserted in pET3a [10] and expressed in *E. coli*. Induction of the cells with isopropylthiogalactoside led to the appearance of a 25 000 M_r polypeptide which after 20 h represented about 5% of the soluble proteins. L-3-Phosphoserine phosphatase was purified by chromatography on DEAE-Sepharose and Sephacryl S-200, from which it was eluted with an apparent molecular mass of 50 000 *M*r, indicating a dimeric structure (not shown). The purified protein had a specific activity of 6 U/mg protein, the yield of the purification being 25%.

The purified protein was dependent for its activity on the presence of Mg^{2+}, which half-maximally stimulated the enzyme at 5 mM. It displayed a K_m of 20 μM for L-phosphoserine in the presence of 1 mM Mg^{2+}, was non-competitively inhibited by L-serine (K_i = 0.5 mM) and catalyzed an exchange reaction (incorporation of L-[^{14}C]serine into L-phosphoserine) at a rate about 50% that of the hydrolysis (3 μmol/min/mg protein). These properties are similar to those of other L-3-phosphoserine phosphatases [1,2,19]. Other phosphate esters (glucose 6-phosphate, fructose 6-phosphate, glycerol 2- or 3-phosphate and 3-phosphoglycerate, all tested at 5 mM) were not significantly hydrolyzed by the purified preparation.

3.3. Demonstration of the formation of a phosphoenzyme

Upon incubation of human L-3-phosphoserine phosphatase with L-[^{32}P]phosphoserine, a phosphoenzyme was formed.

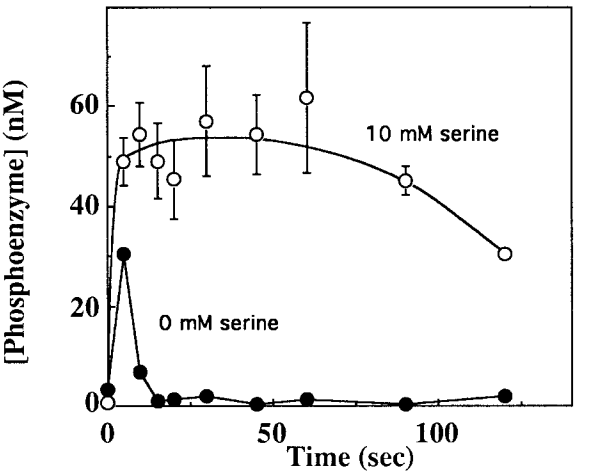

Fig. 2. Time-course of the labelling of the rat liver enzyme with L-[^{32}P]phosphoserine. L-3-Phosphoserine phosphatase (0.15 U) was incubated at 0°C in the presence of radiolabelled L-phosphoserine with (○) or without (●) 10 mM L-serine.

This could be demonstrated using a method in which protein was precipitated in acid and immediately isolated by filtration. Other methods in which filters are washed for prolonged time (30–60 min) [20,21] yielded insignificant incorporation, presumably because of the lability of the phosphoenzyme (see below). As shown in Fig. 2, the formation of the phosphoenzyme was greatly favored by L-serine which acted most likely by slowing down the degradation of L-phosphoserine. In the presence of 10 mM L-serine, the K_m for the formation of this phosphoenzyme was 5 μM and a maximum of 2.5 nmol of phosphate was incorporated per milligram of protein, indicating a stoichiometry of 0.06 mole per mole of enzyme subunit (not shown). SDS-PAGE [22] under denaturing conditions (at 0°C, to minimize hydrolysis) confirmed that the phosphate was only incorporated into the 25 000 M_r polypeptide (not shown). The phosphoenzyme was acid labile, being 50% hydrolyzed after 50 min of incubation in 5% TCA at 50°C. It was extremely labile in alkali, since it was completely lost when resuspended in 1 M NaOH at 0°C and reprecipitated in TCA. It was also completely hydrolyzed when resuspended in 0.2 M NH_2OH at pH 5.3 (not shown) and maintained for 10 min at 20°C.

4. Discussion

We report here the identification of clones encoding human L-3-phosphoserine phosphatase. This identification rests on the similarity of the encoded protein with the *E. coli* enzyme as well as with tryptic peptides derived from the purified rat liver enzyme. The identity was confirmed by expression of the cDNA and demonstration that the encoded protein behaved as a specific L-3-phosphoserine phosphatase with properties similar to those of the enzyme present in mammalian tissues.

The fact that L-3-phosphoserine phosphatase catalyzes an exchange reaction between L-serine and L-phosphoserine and that it is non-competitively inhibited by L-serine suggested that the mechanism of this enzyme involved the formation of a phosphoenzyme. This is also consistent with its sensitivity to vanadate [23,24]. We show in this work that, indeed, a

phosphorylated enzyme is formed upon incubation with the substrate.

Many phosphotransferases proceed through the formation of a phosphoenzyme. Four different types of residues have been shown to be implicated in covalently binding phosphate in these enzymes: serine, as in alkaline phosphatase [25]; histidine, as in acid phosphatase [26], fructose 2,6-bisphosphatase [27] and phosphoglycerate mutase [28]; cysteine in tyrosine phosphatases [29]; and aspartate in ATPases of the Na^+/K^+ ATPases family [30,31]. Histidine and cysteine are most likely not involved since there is no conserved cysteine or histidine in L-3-phosphoserine phosphatase (see Fig. 1). Furthermore the lability of the phosphoenzyme to alkali and to NH_2OH indicates an acyl-phosphate linkage, as in phosphoaspartate or phosphoglutamate. The low stoichiometry of phosphorylation and the lability of the phosphoenzyme bond prevented us from isolating a tryptic phosphopeptide suitable for sequencing. The identity of the phosphorylated residue remains therefore to be determined.

Two highly conserved motifs containing aspartate residues are found in L-3-phosphoserine phosphatases: DXDST and GDGXXD. The first one shares two residues (DXXXT) with the consensus phosphorylation site of ATPases of the Na^+/K^+ ATPase family. The second motif is also found in the same family of ATPases, where it is highly conserved (e.g., residues 713 to 718 of the alpha subunit of human Na^+/K^+ ATPase). These considerations indicate that there may be functional homology between phosphoserine phosphatases and ATPases.

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