Isolation and heterologous expression of a cDNA encoding bovine inositol polyphosphate 1-phosphatase

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ABSTRACT Inositol polyphosphate 1-phosphatase, an enzyme of the phosphatidylinositol signaling pathway, catalyzes the hydrolysis of the 1-position phosphate from inositol 1,3,4-trisphosphate and inositol 1,4-bisphosphate. The protein was isolated from calf brain and digested with trypsin or CNBr, and the amino acid sequence of several peptides was determined. Degenerate oligonucleotide primers were designed from amino acid sequence and used to synthesize an 80-base-pair (bp) fragment by the polymerase chain reaction. This product was used to isolate a 1.6-kbp cDNA with an open reading frame of 400 amino acids, 185 bp of 5’ untranslated region, and 171 bp of 3’ untranslated region followed by a putative poly(A) tail. The coding region of the cDNA was inserted into an expression vector that was used to obtain the recombinant protein from Escherichia coli cells. The recombinent enzyme (44 kDa) had a specific activity and other properties similar to those of native bovine brain inositol polyphosphate 1-phosphatase. It hydrolyzed both inositol phosphate substrates and was inhibited by lithium ions. The enzyme shows minimal sequence similarity to inositol monophosphate phosphatase, the other enzyme inhibited by lithium ions in the signaling pathway.

Inositol polyphosphate 1-phosphatase hydrolyzes the 1-phosphate from two substrates of the phosphatidylinositol signaling pathway, inositol 1,3,4-trisphosphate [Ins(1,3,4)P3] and inositol 1,4-bisphosphate [Ins(1,4)P2] (1–4). Other 1-phosphate-containing inositol polyphosphates are not substrates (1). The enzyme is likely to be involved in regulating intracellular levels of Ins(1,3,4)P3, Ins(1,4)P2, and their metabolites. It catalyzes one of three possible reactions involving Ins(1,3,4)P3, converting it to inositol 3,4-bisphosphate [Ins(3,4)P2]. Alternatively, Ins(1,3,4)P3 can be converted to inositol 1,3-bisphosphate or inositol 1,3,4,6-tetraakisphosphate by 4-phosphatase (5, 6) and 6-kinase (7–10) enzymes, respectively. Inositol polyphosphate 1-phosphatase is competitively inhibited by lithium ions [K, = 0.5 mM Ins(1,3,4)P3], a drug used to treat patients with psychiatric disorders (3, 4, 11, 12). The other substrate for this enzyme, Ins(1,4)P2, is hydrolyzed to yield inositol 4-phosphate [Ins(4)P]. A messenger role has also been proposed for Ins(1,4)P2 in that the low activity form of DNA polymerase α is stimulated by Ins(1,4)P2 (13). One way to discover functions for substrates and products of inositol polyphosphate 1-phosphatase is to change cellular levels of these substances. We hope to accomplish this by altering cellular levels of active enzyme. As a first step in this strategy, we have isolated a cDNA clone that encodes this enzyme and have expressed active recombinant enzyme in Escherichia coli. The recombinant enzyme is functionally similar to the native bovine enzyme.*

EXPERIMENTAL PROCEDURES

**Materials.** Bovine calf brains were from Pel-Freeze. [3H]Ins(1,4)P2 (4.5 Ci/mmol; 1 Ci = 37 GBq) and [3H]Ins(1,3,4)P3 (17 Ci/mmol) were from DuPont/New England Nuclear. Unlabeled Ins(1,4)P2, DEAE-Sepharose CL-6B, CNBr, and phenylmethylsulfonyl fluoride were from Sigma. Unlabeled Ins(1,3,4)P3 was from Calbiochem. AG 1-X8 ion-exchange resin (formate form, 200–400 mesh), Bio-Gel HTP hydroxyapatite, a Bio-Gel phenyl-5PW high performance liquid chromatography (HPLC) column, and protein molecular weight standards were from Bio-Rad. Aquapore BU-300 and RP-300 microscope HPLC columns were from Applied Biosystems. l-Tosylamido-2-phenyl ethyl chloromethyl ketone-treated trypsin was from Cooper. Nitrocellulose and an Elutrap electroelution device were from Schleicher & Schuell. [γ-32P]ATP and [α-35S]基金[d]ATP were from Amersham. A polymerase chain reaction (PCR) kit was from Perkin-Elmer/Cetus. A λZAP II MDBK (Madin–Darby bovine kidney cell) cDNA library, pBluescript KS(—) plasmid, and E. coli strain XL-1 Blue were from Stratagene. Phage T4 DNA polynucleotide kinase, T4 DNA ligase, restriction endonucleases, and a Sequenase version 2.0 sequencing kit were from United States Biochemical. Moloney murine leukemia virus reverse transcriptase was from BRL. All oligonucleotides were synthesized on an Applied Biosystems model 380B DNA synthesizer. All other materials were from Sigma or Fisher.

**Determination of Inositol Polyphosphate 1-Phosphatase Activity.** Enzymatic hydrolysis of Ins(1,4)P2 was assayed by one of two methods described previously (2). In assay 1, [3H]Ins(1,4)P2 and inositol polyphosphate 1-phosphatase were incubated in 50 mM Hapes, pH 7.5/3 mM MgCl2/100 mM KCl/0.5 mM EGTA with an excess of inositol monophosphate phosphatase (14). The reaction mixture was loaded onto a 0.6-ml AG 1-X8 formate column and [3H]inositol, the product of this coupled enzyme reaction, was eluted with buffer. Assay 2 was performed as above except that inositol monophosphate phosphatase was omitted. The product, [3H]Ins(4)P, was eluted with 0.05 M NH4HCOO containing 0.1 M HCOOH.

The assay for Ins(1,3,4)P3 hydrolysis by inositol polyphosphate 1-phosphatase was performed as described (2).

**Purification of Inositol Polyphosphate 1-Phosphatase.** The enzyme was obtained as described (3), with the following modifications. Phenylmethylsulfonyl fluoride (0.5 mM) was added to all column fractions immediately after their collection, to prevent proteolysis of the enzyme. After hydroxyapatite chromatography the sample was dialyzed against 33% (percent saturation) ammonium sulfate/50 mM Bistris, pH 7.0, and applied to a Bio-Gel phenyl-5PW HPLC column (75

Abbreviations: Ins(1,4)P2, inositol 1,4-bisphosphate; Ins(1,3,4)P3, inositol 1,3,4-trisphosphate; Ins(3,4)P2, inositol 3,4-bisphosphate; PCR, polymerase chain reaction; TFA, trifluoroacetic acid.

*The sequence reported in this paper has been deposited in the GenBank data base (accession no. M55916).

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× 7.5 mm) equilibrated in the same buffer. The column was washed with 5 column volumes of this buffer, and bound material was eluted with a decreasing linear gradient (50 ml) of 33% to 10% ammonium sulfate in 50 mM Bistris (pH 7.0). Fractions containing enzyme activity were pooled and precipitated in 75% ammonium sulfate. The pellet was resuspended and dialyzed against 0.1 M NH₄HCO₃, pH 7.8/0.01% SDS and was subjected to preparative SDS/PAGE (15). The 44-kDa protein corresponding to isoinositol polyphosphate 1-phosphatase was excised from the gel and electroeluted in SDS gel running buffer (15). Electroeluted enzyme was dialyzed against 0.1 M NH₄HCO₃, pH 7.8/0.01% SDS and precipitated with 10 volumes of acetone/1 mM HCl at −20°C.

**Protein Fragmentation and Sequencing. CNBr cleavage.** Inositol polyphosphate 1-phosphatase (15 µg) in 25 mM NH₄HCO₃/75% (vol/vol) trifluoroacetic acid (TFA) was incubated with 4 mg of CNBr at 25°C, under N₂, in the dark for 24 hr. The sample was then injected onto an Aquapore BU-300 microbore HPLC column (30 × 2.1 mm) equilibrated in 0.1% TFA. Peptides were eluted with a linear gradient (10 ml) of 0–100% acetonitrile in 0.1% TFA and protein sequencing was performed on an Applied Biosystems model 477A sequencer.

**Trypsin digestion.** Inositol polyphosphate 1-phosphatase (10 µg) was carboxamidomethylated and digested with 400 ng of trypsin as described (16). The digest was injected onto an Aquapore RP-300 microbore HPLC column (220 × 2.1 mm) equilibrated in 0.1% TFA and tryptic peptides were eluted and sequenced as described above.

**cDNA Cloning.** Completely degenerate sense and antisense oligonucleotides (1024-fold and 512-fold degenerate, respectively) corresponding to the amino-terminal and carboxy-terminal sequences of a CNBr peptide (Fig. 1) were synthesized as primers for PCR. Total RNA was prepared from fresh bovine (calf) cerebellum by a low-temperature guanidine isothiocyanate method (17). First-strand cDNA was made by incubating 50 µg of total RNA with 2 pmol of antisense oligonucleotide and 400 units of Moloney murine leukemia virus reverse transcriptase in 50 µl, using the manufacturer’s recommended buffer conditions, for 1 hr at 37°C. Following synthesis, the sample was treated with 160 mM NaOH for 10 min at 70°C, neutralized, and desalted on a small gel filtration column. The PCR was performed as described (18) for 30 cycles using 350 ng of sense and antisense oligonucleotides and 2% of the antisense-primed cDNA. In each cycle, denaturation was at 94°C for 1 min, annealing at 45°C for 1.5 min, and elongation at 72°C for 1 min. The PCR product was cloned into the Sma I site of pBluescript SK(−) and sequenced by the dideoxy chain-termination method (19). All DNA sequencing described here and throughout the manuscript was done using Sequenase version 2.0 (20).

The PCR product described above was end-labeled with T4 polynucleotide kinase and [γ-32P]ATP and used to screen an AZAP II MDBK cDNA library (106 recombinants) in *E. coli* XL-1 Blue. Duplicate filters were prepared and screened at 50°C in aqueous solution as described (21). Positive clones were retested at 100 plaques per plate, and 40 of these were used as templates for PCR using the primers in Fig. 1. Plasmids were excised from phage found to contain cDNA inserts by using the procedure described by Stratagene. The cDNA clones were sequenced entirely on both strands. Overlapping sequences were assembled using the Genetics Computer Group sequence-analysis software package (22).

**Construction of the Bovine Inositol Polyphosphate 1-Phosphatase Expression Vector.** A pBluescript SK(−) plasmid containing inositol polyphosphate 1-phosphatase cDNA in the orientational opposite to that of the β-galactosidase gene was digested with Sau I and religated, which removed all but 7 nucleotides of 3′ untranslated region. Digestion of this modified plasmid with EcoRV and HindIII liberated the 5′ untranslated region and codons for the first 3 amino acids of the enzyme. Replacement of this region with complementary oligonucleotides, 5′-AGCTTGAATATCCAGCATATG-3′ and 5′-ATCTGACATATTTGGAATT-3′, yielded a plasmid designated plTptase, containing only the coding region of inositol polyphosphate 1-phosphatase plus EcoRI and Nde I restriction sites. This plasmid was then digested with EcoRI and Nde I, and a synthetic trypophan promoter/operator (courtesy of G. P. Vlasuk, Merck Sharp & Dohme) with flanking 5′ EcoRI and 3′ Nde I linkers was inserted, yielding the expression plasmid pTrpTptase. A plasmid, pTrp, containing only the tryptophan promoter/operator was also constructed by inserting this promoter fragment into pBluescript SK(−). All of these plasmids were sequenced to verify the structure of the engineered regions. All cloning procedures used above were as described (21).

**Analysis of Recombinant Bovine Inositol Polyphosphate 1-Phosphatase.** Single colonies of *E. coli* XL-1 Blue transformed with each pTrpTptase, plTptase, or pTrp were used to inoculate 50 ml of LB medium (21) containing ampicillin (50 µg/ml) and were grown at 37°C to an OD₆₀₀ of 1.0. Cells were harvested and sonicated on ice in 1.0 ml of 20 mM Hepes, pH 7.5/3 mM MgCl₂/2 mM EGTA, pH 7.5/0.25 mM phenylmethylsulfonyl fluoride. Soluble and particulate fractions were separated by centrifugation at 10,000 × g for 10 min.

![Fig. 1. Partial cDNA sequence amplified by PCR. Line 1: sequence of 25 amino-terminal residues of a CNBr peptide released after cleavage of purified bovine brain inositol polyphosphate 1-phosphatase. Residue numbers are based on alignment with cDNA. Line 2: position and sequence of the sense and antisense degenerate oligonucleotide primers. The antisense oligonucleotide was used as a primer of bovine brain total RNA to make cDNA and both primers were used in the PCR of this cDNA. Single-letter codes represent multiple bases: N = G, A, T, or C; R = A or G; Y = C or T. Line 3: nucleotide sequence of one subcloned 80-base-pair (bp) PCR product. Line 4: nucleotide and amino acid sequence of the cDNA. Asterisk in line 1 denotes a difference between the amino acid encoded by the cDNA and that obtained from protein sequencing.](image-url)
min. The pellet was resuspended by vigorous Vortex mixing in 1.0 ml of the same buffer. Soluble and particulate fractions were analyzed by SDS/PAGE (15). Western blot analysis of SDS/PAGE-separated fractions used a rabbit polyclonal antibody (3) directed against bovine brain inositol polyphosphate 1-phosphatase and an alkaline phosphatase detection system from Promega.

**RESULTS**

**Sequence of Inositol Polyphosphate 1-Phosphatase.** Since attempts to obtain amino acid sequence from intact inositol polyphosphate 1-phosphatase failed, we digested the enzyme with CNBr and obtained the partial sequence of one peptide (Fig. 1, line 1). Sense and antisense oligonucleotides (Fig. 1, line 2) were used as primers to amplify an 80-bp PCR product from a single-stranded cDNA template as described in Experimental Procedures. The product was cloned and sequenced and found to encode the amino acids contained in the CNBr peptide between the primers (Fig. 1, line 3). The correct sequence of inositol polyphosphate 1-phosphatase cDNA in this region shows that the cloned PCR product contained 11 errors in the primer regions (Fig. 1, line 4). The PCR product was labeled and used to screen a ZAP II MDBK library (10^6 recombinants). Three identical clones

![Fig. 2. Nucleotide and encoded amino acid sequence of a bovine inositol polyphosphate 1-phosphatase cDNA. Numbering of the nucleotides (left side) and the amino acids (right side) begins at the initiation methionine. Amino acids confirmed by protein sequencing are underlined: solid line represents tryptic peptides; dashed line represents CNBr peptides. Boxed L (amino acid 84) indicates discrepancy from peptide sequence, where F was obtained at this position.](image-url)
were isolated. The cDNA insert of one of these was sequenced entirely on both strands (Fig. 2). The cDNA was 1572 bp with an open reading frame of 400 amino acids corresponding to a protein of 43,980 Da. The 3′ untranslated region contained the conserved polyadenylation signal sequence AAUAAA, 16 nucleotides upstream of the putative poly(A) tail. We confirmed 126 residues of amino acid sequence by sequencing CNBr and trypic peptides of bovine brain inositol polyphosphate 1-phosphatase. The only difference between the cDNA clone and the amino acid sequence was at residue 84, where phenylalanine was obtained by peptide sequencing and leucine was the amino acid predicted from the cDNA. We found no similarity between inositol polyphosphate 1-phosphatase and other proteins contained in the GenBank and EMBL databases (as of September 1996).

We compared the cDNA with three other proteins of the phosphatidylinositol signaling pathway—bovine inositol trisphosphate 3-kinase (23), human inositol 1,2-(cyclic)-phosphate 2-phosphohydrolase (24), and bovine inositol monophosphatase (25)—by using the program ALIGN (26). There was no significant similarity to either the 3-kinase or the cyclic hydrolase enzyme. Limited sequence similarity to inositol monophosphatase was observed (score = 3.8 SD). In one region of the molecule, 9 of 20 amino acids were identical (Fig. 3).

Expression of Inositol Polypolyphosphate 1-Phosphatase. We inserted the coding region of the cDNA into a bacterial expression vector driven by a tryptophan promoter/operator. Cultures of E. coli XL-1 Blue transformed with the plasmids pTrp, pLptase, or pTrpLptase were grown, harvested, and sonicated to yield soluble and particulate extracts. Extracts from cells transformed with pTrpLptase contained recombinant proteins of 44 kDa and 31 kDa (Fig. 4A). Both of these proteins are related to inositol polyphosphate 1-phosphatase, as demonstrated by immunoblotting using a polyclonal antibody against the enzyme (Fig. 4B). We attempted to sequence these intact proteins to further establish their identity. The recombinant proteins were separated by SDS/PAGE followed by electrophoretic transfer to poly(vinylidene difluoride) (Immobilon, Millipore) membranes (16). The 44-kDa protein had a blocked amino terminus and the 31-kDa protein yielded amino acid sequence corresponding to Met<sup>94</sup>-Gly<sup>131</sup> (Fig. 2).

Extracts from the transformed E. coli were also assayed for the ability to hydrolyze Ins(1,4)P<sub>2</sub>. Only extracts from pTrpLptase transformants contained activity. The soluble fraction hydrolyzed 0.02 μmol of Ins(1,4)P<sub>2</sub> per min per mg of protein (Fig. 5). There was also slight enzyme activity in the resuspended particulate fraction [0.001 μmol of Ins(1,4)P<sub>2</sub> hydrolyzed per min per mg of protein]. The relative lack of activity in the particulate fraction suggests that this insoluble form is denatured. Attempts to increase the activity of the particulate fraction by a variety of methods were unsuccessful (27). The recombinant inositol polyphosphate 1-phosphatase hydrolyzed Ins(1,4)P<sub>2</sub> (<i>K<sub>m</sub></i> 8.7 μM) and Ins(1,3,4)P<sub>3</sub> (<i>K<sub>m</sub></i> 29 μM) with similar maximum velocities. The bovine brain enzyme gave <i>K<sub>m</sub></i> values of 5 μM and 20 μM, respectively. Lithium ions uncompetitively inhibited hydrolysis of both substrates (Table 1), with apparent <i>K<sub>i</sub></i> values of 16.3 mM and 1.4 mM for Ins(1,4)P<sub>2</sub> and Ins(1,3,4)P<sub>3</sub>, respectively. These values compare to <i>K<sub>i</sub></i> values of 6 mM and 0.5 mM for the isolated bovine brain enzyme.

We determined that only the 44-kDa protein was active by separating it from the 31-kDa protein through anion-exchange
Table 1. Effect of lithium concentration on hydrolysis of Ins(1,4)P$_2$ and Ins(1,3,4,5)P$_4$ by recombinant inositol polyphosphate 1-phosphatase

<table>
<thead>
<tr>
<th>LiCl, mM</th>
<th>[3H]Ins(1,4)P$_2$ cpm</th>
<th>With 10 μM Ins(1,4)P$_2$ as substrate*</th>
<th>With 40 μM Ins(1,4)P$_2$ as substrate†</th>
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<tr>
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<tr>
<td>0</td>
<td>2330</td>
<td>0</td>
<td>1670</td>
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<td>5</td>
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<td>0.4</td>
<td>1530</td>
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<tr>
<td>10</td>
<td>1940</td>
<td>0.8</td>
<td>970</td>
</tr>
<tr>
<td>20</td>
<td>1240</td>
<td>1.6</td>
<td>870</td>
</tr>
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<td>1060</td>
<td>3.2</td>
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</tr>
<tr>
<td></td>
<td></td>
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</tr>
<tr>
<td></td>
<td></td>
<td>12.8</td>
<td>240</td>
</tr>
</tbody>
</table>

*Soluble protein from pTrp1pase extract (0.7 μg) was incubated with 10 μM [3H]Ins(1,4)P$_2$ (12,000 cpm/nmol) in the presence of the indicated concentrations of LiCl. The salt concentration of all reactions was adjusted to 100 mM by addition of NaCl. Results are expressed as cpm of [3H]Ins(1,4)P$_2$ produced after a 10-min incubation.

†pTrp1pase extract (1.2 μg) was incubated with 40 μM Ins(1,3,4,5)P$_4$ (3000 cpm/nmol). Results are expressed as cpm of [3H]Ins(3,4,5)P$_2$, formed after a 15-min incubation.

chromatography on a Mono Q FPLC column (Pharmacia). Only one peak of enzyme activity was eluted, and the fractions with activity contained only the 44-kDa protein as determined by immunoblotting. The 31-kDa protein was eluted in later fractions that did not have enzyme activity. In other immunoblotting experiments similar to that of Fig. 4, we determined that the specific activity of the soluble recombinant enzyme was the same as native bovine brain inositol polyphosphate 1-phosphatase (i.e., 25–50 μmol of Ins(1,4)P$_2$ hydrolyzed per min per mg of recombinant protein). The enzyme comprised about 0.05% of the total protein in soluble extracts from E. coli transformed with pTrp1pase.

**DISCUSSION**

We have isolated a cDNA encoding bovine inositol polyphosphate 1-phosphatase. We produced recombinant enzyme by inserting the open reading frame into a bacterial expression vector. The recombinant enzyme comigrates with purified bovine inositol polyphosphate 1-phosphatase in a SDS/polyacrylamide gel, and the apparent molecular mass, 44 kDa, is consistent with that predicted by the cDNA, suggesting that the native enzyme is not glycosylated. The properties and specific activity of the recombinant enzyme are similar to those of the bovine enzyme, implying that there are not major posttranslational modifications. We observed a 31-kDa protein in the recombinant protein extracts; this protein was determined to be a truncated form of inositol polyphosphate 1-phosphatase that was inactive. Immunoblotting of crude extracts from bovine brain did not show a 31-kDa protein, indicating that there is no truncated form of the enzyme in brain.

The amino acid sequence of bovine inositol polyphosphate 1-phosphatase is unique. No significant similarity to any other protein was found upon searching data bases. Alignment of this sequence with those of other enzymes in the inositol phosphate pathway showed a limited region of similarity only to bovine inositol monophosphate phosphatase; in one region of 20 amino acids, 9 were identical. Interestingly, inositol monophosphate phosphatase is the only other enzyme in this pathway known to be inhibited by lithium ions.

The ability to express active inositol polyphosphate 1-phosphatase in bacteria will facilitate studies aimed at identifying the catalytic and regulatory domains of the enzyme. Expression of this protein in various eukaryotic cells may allow us to understand the function(s) of Ins(1,4)P$_2$, Ins(1,3,4,5)P$_4$, and their metabolites within cells.

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