Isolation of Inositol 1,3,4-Trisphosphate 5/6-Kinase, cDNA Cloning, and Expression of the Recombinant Enzyme*

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Inositol 1,3,4-trisphosphate 5/6-kinase was purified 12,900-fold from calf brain using chromatography on heparin-agarose and affinity elution with inositol hexakisphosphate. The final preparation contained proteins of 48 and 36–38 kDa. All of these proteins had the same amino-terminal sequence and were enzymatically active. The smaller species represent proteolysis products with carboxyl-terminal truncation. The $K_m$ of the enzyme for inositol 1,3,4-trisphosphate was 80 nM with a $V_{max}$ of 60 nmol of product/min/mg of protein. The amino acid sequence of the tryptic peptide HSKLLARPAG-GLVGERCNAXP matched the protein sequence encoded by a human expressed sequence tag clone (GB T09063) at 16 of 22 residues. The expressed sequence tag clone was used to screen a human fetal brain cDNA library to obtain a cDNA clone of 1991 base pairs (bp) that predicts a protein of 46 kDa. The clone encodes the amino-terminal amino acid sequence obtained from the purified calf brain preparation, suggesting that it represents its human homologue. The cDNA was expressed as a fusion protein in Escherichia coli and was found to have inositol 1,3,4-trisphosphate 5/6-kinase activity. Remarkably, both the purified calf brain and recombinant proteins produced both inositol 1,3,4,6-tetrakisphosphate and inositol 1,3,4,5-tetrakisphosphate as products in a ratio of 2.3–5:1. This finding proves that a single kinase phosphorylates inositol in both the D5 and D6 positions. Northern blot analysis identified a transcript 3.6 kilobases in all tissues with the highest levels in brain. The composite cDNA isolated contains 3054 bp with a poly(A) tail, suggesting that 500–600 bp of 5' sequence remains to be identified.

The phosphatidylinositol signaling pathway involves a complex scheme in which cells use a series of kinases and phosphatases to interconvert the six known inositol lipids and the more than 20 inositol phosphates that exist in eukaryotic cells (1). These molecules have been implicated in a number of intracellular events, including calcium ion mobilization (2), nucleotide exchange (3), trafficking of intracellular vesicles (4), and cell proliferation in response to cytokines and growth factors (1, 2). Many of the reactions in this pathway are catalyzed by several different isoenzymes, most notably phospholipase C (5) and inositol polyphosphate 5-phosphatase isoenzymes (6, 7), where 8–10 isoforms of each have been discovered to date. We have now characterized an additional kinase of this pathway that utilizes inositol 1,3,4-trisphosphate as a substrate.

Inositol 1,3,4-trisphosphate (Ins(1,3,4)P$_3$) is at a branch point in inositol phosphate metabolism. It is dephosphorylated by specific phosphatases to either inositol 3,4-bisphosphate or inositol 1,3-bisphosphate. Alternatively, it is phosphorylated to inositol 1,3,4,6-tetrakisphosphate [Ins(1,3,4,6)P$_4$] or inositol 1,3,4,5-tetrakisphosphate [Ins(1,3,4,5)P$_4$] by inositol trisphosphate 5/6-kinase (Ins(1,3,4)P$_3$ 5/6-kinase) (8, 9). Ins(1,3,4,6)P$_4$ is the first intermediate in the pathway leading to the higher inositol phosphates including other tetrakisphosphates, pentakisphosphates, inositol hexakisphosphate, and pyrophosphate forms of these (10), all of which are ubiquitously found in tissues. Because the Ins(1,3,4)P$_3$ 5/6-kinase enzyme is at a branch point in metabolism leading to multiple different end products, it is likely to be regulated by its various end products. This enzyme has been partially purified from rat liver (8, 9), porcine brain (11), and bovine testes (11) and in each case was reported to phosphorylate Ins(1,3,4)P$_3$ on either the 5 or 6 position yielding a mixture of two products. This property seems remarkable since the 5 and 6 positions of myoinositol are on opposite faces of the ring. We therefore isolated the enzyme from calf brain and used the amino acid sequence that we determined to identify a human expressed sequence tag (EST) and to clone a cDNA encoding the human Ins(1,3,4)P$_3$ 5/6-kinase.

EXPERIMENTAL PROCEDURES

Materials—Heparin-agarose type I, bacterial ATP grade I, pepstatin A, soybean trypsin inhibitor type IIS, leupeptin, bestatin, diisopropyl fluorophosphate, benzamidine, phenylmethylsulfonyl fluoride, Coomasie Blue R250, isopropyl-$\beta$-D-galactopyranoside, calmodulin, calmodulin-agarose, phytic acid (inositol hexakisphosphoric acid), CHAPS, diithiothreitol, and ammonium sulfate were obtained from Sigma. Lysyl endopeptidase was purchased from Wako Chemicals (Dallas, TX). Calpain inhibitors I and II were from Calbiochem. Frozen calf brains were supplied by By Prod Corporation (St. Louis, MO). A Mono Q HR 5/5 fast protein liquid chromatography column was purchased from Pharmacia Biotech, Inc. Ins(1,3,4-trisphosphate, $\alpha$-Ins(1,3,4)-H(N), $\varepsilon$-Ins(1,3,4)-H(N), 21 Ci/mmole, inositol 1,4,5-trisphosphate, $\alpha$-inositol-1-$\varepsilon$-H(N), 15–30 Ci/mmole, and Colony/Plaque Screen$^\text{TM}$ filters were obtained from DuPont NEN. Escherichia coli expressing recombinant inositol 1,4,5-trisphosphate (Ins(1,4,5)P$_3$) 3-kinase were generously provided by S. G. Rhee (NIH). Dowex AG 1-X8 (formate form), protein assay dye reagent concentrate, and low molecular weight markers were obtained from Bio-Rad. ²$\text{P}$

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³ The abbreviations used are: Ins(1,3,4)P$_3$, inositol 1,3,4-trisphosphate; EST, expressed sequence tag; Ins(1,3,4)P$_3$, 5/6-kinase, inositol 1,3,4,5-tetrakisphosphate; Ins(1,3,4,5)P$_4$, inositol 1,3,4,5-tetrakisphosphate; Ins(1,4,5)P$_3$, inositol 1,4,5-trisphosphate; CHAPS, 3-(3-cholamidopropyl)dimethylammonio)-1-propanesulfonic acid; DTT, dithiothreitol; MES, 4-morpholineethanesulfonic acid; IP$_\varepsilon$, inositol hexakisphosphate; HPLC, high pressure liquid chromatography; bp, base pair(s); PKC(ε)psilon, epsilon isoform of protein kinase C.
Ins(1,3,4)P$_3$ 5/6-Kinase

Ins(1,4,5)P$_3$ 3-kinase was prepared as described previously (12). Immobilon-P membranes were purchased from Millipore (Bedford, MA). Human multitissue Northern blots were obtained from Clontech (Palo Alto, CA). Sequencing grade trypsin, T4 polynucleotide kinase, Quick Spin$^\text{TM}$ columns, calf intestinal alkaline phosphatase, and the random primed labeling kit were from Boehringer Mannheim. A $5/6$-kinase was mixed with SDS gel loading buffer for a final concentration of 10 mM urea to remove residual protein. The ammonium sulfate pellets were dissolved using a 1.0-ml Dowex-formate column equilibrated in 50 mM Tris-HCl, pH 7.5, 1 mM EDTA, 0.4 mM phenylmethylsulfonyl fluoride, 10 mM iodoacetamide, 1 mM pepstatin A, 10 mM bestatin, 1 mM DTT, 200 mM of diisopropyl fluorophosphatase, 17 µM of calpain inhibitor I/ml, and 17 µg of calpain inhibitor II/ml (14). Pooled fractions containing [32P]Ins(1,3,4,5)P$_4$ were lyophilized from substrate using a 1.0-ml Dowex-formate column equilibrated in 50 mM Tris-HCl, pH 7.5, 1 mM EDTA, 1 mM CaCl$_2$, and loaded onto a 1.2-ml calmodulin-agarose column equilibrated in 20 mM Tris-HCl, pH 7.5, 0.2 mM CaCl$_2$, 200 mM KCl, and 1 mM DTT (equilibration buffer). The column was washed with 15 ml of equilibration buffer, followed by a 6.5-ml wash of equilibration buffer containing 0.1% CHAPS. Ins(1,4,5)P$_3$ 3-kinase was eluted with 20 mM Tris-HCl, pH 7.5, 200 mM KCl, 1 mM MgCl$_2$, 1 mM DTT, and 0.5 mM EDTA. Fractions (1 ml) were assayed for 3-kinase activity using [3H]Ins(1,4,5)P$_3$ as substrate. Partially purified Ins(1,4,5)P$_3$ 3-kinase was incubated for 30 min at 37°C with [3H]Ins(1,4,5)P$_3$ in a reaction mixture containing 20 mM HEPES, pH 7.5, 0.1 mg of bovine serum albumin/ml, 5 mM ATP, 20 mM MgCl$_2$, 1 mM DTT, 1 mM calmodulin, 2.5 mM MgCl$_2$, 0.1 mM CaCl$_2$. Product was separated from substrate using a 1.0-ml Dowex-formate column equilibrated in water. Ins(1,4,5)P$_3$ was eluted with 0.8 mM ammonium formate, pH 3.5, and [32P]Ins(1,3,4,5)P$_4$ was eluted with 1.6 mM ammonium formate, pH 3.5. Pooled fractions containing [32P]Ins(1,3,4,5)P$_4$ were lyophilized prior to use.

RESULTS

Purification of Calf Brain Ins(1,3,4)P$_3$ 5/6-Kinase—Extracts of fresh frozen bovine tissues were assayed for Ins(1,3,4)P$_3$ 5/6-kinase activity to determine the best source for purification. Calf brain had the highest activity, with frozen calf brains from a commercial source (By Prod Corporation) having a specific activity of 25–30 min$^{-1}$mg protein and a freshly obtained calf brain frozen immediately in liquid nitrogen having a specific activity of 20 min$^{-1}$mg protein and a freshly obtained calf brain frozen immediately in liquid nitrogen having a specific activity of 15 min$^{-1}$mg protein.
activity of 48 min$^{-2}$/mg protein. Using supernatant from commercial calf brain as a reference of 100% activity, activities of other tissue supernatants were as follows: bovine brain (67%), rat brain (13%), bovine lung (10%), bovine thymus (5.7%), bovine kidney and liver (4.7%), bovine heart (1.1%), and bovine skeletal muscle (0.4%).

The purification of Ins(1,3,4)P$_3$ 5/6-kinase exploited the finding that various conditions altered the position of elution of enzyme from heparin-agarose columns. In the absence of MgCl$_2$, the Ins(1,3,4)P$_3$ 5/6-kinase activity elutes from heparin-agarose with 0.2 M NaCl (Fig. 1A). Upon rechromatography in the presence of 3 mM MgCl$_2$, Ins(1,3,4)P$_3$ 5/6-kinase activity bound more tightly and was eluted with 0.3 M NaCl (Fig. 1B).

Ins(1,3,4)P$_3$ 5/6-kinase activity was further purified on another heparin-agarose column eluted with an linear gradient of IP$_6$. In the absence of MgCl$_2$, Ins(1,3,4)P$_3$ 5/6-kinase activity elutes very early in the gradient (data not shown). In the presence of MgCl$_2$, Ins(1,3,4)P$_3$ 5/6-kinase activity elutes midway through the gradient at 0.4 mM IP$_6$ as shown in Fig. 1C. At this point, the preparation was divided into two parts based on the specific activity of the fractions. Pool 1 contained fractions with a specific activity of 5000–18,000 min$^{-2}$/mg, and pool 2 contained fractions with a specific activity of 18,000–52,000 min$^{-2}$/mg (Fig. 1C).

The two pools of enzyme activity were applied separately to a Mono Q column. Elution of pool 2 with a linear gradient of NaCl is shown in Fig. 1D. Ins(1,3,4)P$_3$ 5/6-kinase activity elutes at 0.12 M NaCl with a small peak of coincident protein. The bulk of protein elutes later in fractions 13–15. The specific activity of the peak fraction was 3.7 × 10$^5$ min$^{-2}$/mg, and pool 2 contained fractions with a specific activity of 18,000–52,000 min$^{-2}$/mg (Fig. 1C).

A summary of the purification is shown in Table I. From 36 calf brains, a total of 53 g of protein was obtained from the 48,000 × g supernatant (crude extract). The final material (pool 2) had a specific activity of 3.7 × 10$^5$ min$^{-2}$/mg protein. The overall yield for this fraction was 3%, with a 12,900-fold purification.

Gel Renaturation Assay of Partially Purified Ins(1,3,4)P$_3$ 5/6-Kinase—To determine which proteins on the SDS gel represented the Ins(1,3,4)P$_3$ 5/6-kinase, an SDS gel renaturation assay was done using material from an IP$_6$ affinity elution of a heparin-agarose column, with a specific activity of 12,000 min$^{-2}$/mg protein. The enzyme had some residual activity under these conditions if the sample was heated at no more than 40°C and if the gel slices were resuspended in a volume of assay buffer no smaller than 200 μl in order to dilute the SDS. Fig. 3 shows the result of a gel renaturation assay. Fig. 3A shows the Coomassie-stained gel with molecular mass markers and the lane of the sample that was used for the enzyme assay. The slices made in the gel can be seen at the far right of the Coomassie-stained gel piece. In this assay, two peaks of activity were found, corresponding to the 48- and 36–38-kDa proteins obtained in the final preparation. Both the 48- and the 36–38-kDa proteins were also shown to have the same amino-terminal amino acid sequence (data not shown).

Amino-terminal amino acid sequence analysis, obtained from sequencing excised bands from polyvinylidene difluoride membranes from early preparations of lower specific activity (350–550 min$^{-2}$/mg protein), indicated that a predominant
36-kDa band was aldolase type C (17), which was a major contaminant of the preparations (data not shown). Under non-reducing conditions on a series of gel filtration columns, aldolase elutes as a tetramer with an apparent molecular mass of 140 kDa, whereas Ins(1,3,4)P₃ 5/6-kinase activity was found only in fractions of lower molecular weight (data not shown). It was recently shown by Baron et al. (18) that aldolase type C binds Ins(1,4,5)P₃ and that this binding is inhibited strongly by Ins(1,3,4)P₃, which may explain its copurification with Ins(1,3,4)P₃ 5/6-kinase.

Cloning of a cDNA Encoding Ins(1,3,4)P₃ 5/6-Kinase—Amino acid sequence was obtained from amino-terminal sequencing of the intact calf brain enzyme, from several partially degraded proteins, and from tryptic and lysyl endopeptidase peptides. One of the peptides obtained from trypsin digestion had the sequence HSKLLARPAGGLVGERTCNAXP and was found to match the protein sequence predicted by the DNA sequence of a human EST clone in Genbank at 16 of 22 residues. This clone (GB T09063) was used to isolate additional clones from a human fetal brain library (see “Experimental Procedures”).

A schematic diagram of the EST clone GB T09063 and the overlapping human fetal brain clone is shown in Fig. 4. The consensus clone contains 3054 bp with an open reading frame of 1242 bp and 1700 bp of 3' untranslated region. A polyadenylation signal is located 23 bp upstream from a poly(A) tail. There is no in-frame stop codon in the sequence 5' of this, thus it is possible that initiation occurs in a 5' site not yet obtained. There is a weak Kozak consensus sequence (19) around bp 1 shown in Fig. 5. The open reading frame encodes a protein of 45.6 kDa with a pI of 6.1 (Fig. 5). The peptide sequences obtained from the calf brain preparation presumed to match the corresponding amino acids in the human protein are underlined. They were identical at 45 of 54 residues sequenced (83%).

The amino acid sequence of Ins(1,3,4)P₃ 5/6-kinase shares small regions of similarity to the epsilon isoform of protein kinase C (PKCe) from both rabbit and human sources, as determined from a BLAST search (20). The three regions are spaced throughout both the Ins(1,3,4)P₃ 5/6-kinase sequence and the PKCe sequences (Fig. 6A). In the first conserved region, comprised of 12 amino acids, there is 50% identity and 75% similarity between the two PKCe isoforms and Ins(1,3,4)P₃ 5/6-kinase. The second region contains 32% identity and 56% similarity, and the third region contains 72% identity and 81% similarity. In addition, the sequence obtained for Ins(1,3,4)P₃ 5/6-kinase has similarity to the predicted amino acid sequences of two other ESTs in the Genbank. Fig. 6B shows a comparison between the predicted amino acid sequence of human Ins(1,3,4)P₃ 5/6-kinase and the predicted amino acid sequences available for the EST clones GB Z5963 from Arabidopsis thali-

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**Table I**

| Step                              | Volume (ml) | Total activity (mg/ml) | Protein (mg) | Specific activity (min⁻¹ mg protein⁻¹) | Purification Fold | Yield (%) |
|-----------------------------------|-------------|------------------------|--------------|----------------------------------------|-------------------|-----------|
| Crude supernatant                 | 13,092      | 1,573,402              | 4.16         |                                        |                   | 100       |
| Pooled heparin eluate/NH₄SO₄ cut | 84          | 362,544                | 2.48         | 1,743                                  |                   | 60        |
| Affinity elution from heparin agarose<sup>a</sup> |            |                        |              |                                        |                   | 23        |
| Pool 1                            | 180         | 39,500                 | 0.022        | 9,975                                  | 345               | 2.5       |
| Pool 2                            | 280         | 188,200                | 0.022        | 30,954                                 | 1,071             | 12        |
| Mono Q                            | 1           | 13,915                 | 0.055        | 252,992                                | 8,754             | 0.9       |
| Pool 1                            | 1           | 47,644                 | 0.128        | 372,217                                | 12,879            | 3.0       |

<sup>a</sup> Fractions from the IP₆ elution of a heparin-agarose column were combined into two pools that were kept separate throughout the remainder of the purification. Pool 1, fractions 32–35 and 50–54. Pool 2, fractions 36–49.
ana and GB D46351 from rice. The predicted amino acid sequence of the Arabidopsis partial cDNA is 44% identical to and 79% similar to Ins(1,3,4)P3 5/6-kinase over 54 amino acids and contains a methionine near the putative initiation methionine. The predicted amino acid sequence of the rice partial cDNA is 32% identical and 76% similar to Ins(1,3,4)P3 5/6-kinase over 139 amino acids.

Northern Blot Analysis of Ins(1,3,4)P3 5/6-Kinase—Northern blot analysis of human tissues disclosed that there is a transcript of 3.6 kilobases that is most abundant in brain, followed by heart, skeletal muscle, kidney, pancreas, liver, placenta > lung (Fig. 7A). We also probed a Northern blot containing mRNA from various regions of the human brain and found expression of Ins(1,3,4)P3 5/6-kinase in all regions of the brain represented (Fig. 7B). In addition to the 3.6-kilobase transcript, a weakly hybridizing band of 5.3 kilobases was observed in some but not all of the regions of the brain. This may represent an alternatively spliced form or other isoform of Ins(1,3,4)P3 5/6-kinase.

Expression of Recombinant Ins(1,3,4)P3 5/6-Kinase—A bacterial expression construct was prepared using an EcoRI fragment of clone 15 containing the entire open reading frame inserted into a TrcHis vector with 6 histidine residues at the amino terminus. Bacterial lysates containing this construct were found to contain Ins(1,3,4)P3 5/6-kinase activity, whereas bacterially lysates expressing Ins(1,3,4)P3 5/6-kinase in an incorrect reading frame had no Ins(1,3,4)P3 5/6-kinase activity. The time course for phosphorylation of Ins(1,3,4)P3 5/6-kinase is shown in Fig. 8. The reaction is linear with time and is concentration-dependent.

Analysis of Products of Ins(1,3,4)P3 5/6-kinase—Previous analyses of phosphorylation of Ins(1,3,4)P3 5/6-kinase by partially
purified preparations of this kinase indicated two products, Ins(1,3,4,6)P₄ and Ins(1,3,4,5)P₄, in a ratio of 5:1 (9) or a ratio of 4:1 (11). We repeated the product analyses using the more highly purified protein (3.7 \times 10^5 \text{ min}^{-1}\text{mg}^{-1}) as well as the recombinant enzyme produced in bacteria. The result of HPLC analyses of the products of Ins(1,3,4)P₃ phosphorylation by purified calf brain Ins(1,3,4)P₃ 5/6-kinase and by recombinant human Ins(1,3,4)P₃ 5/6-kinase expressed in *E. coli* are shown in Fig. 9 (A and B, respectively). The minor product co-chromatographed with the internal standard of [32P]Ins(1,3,4,5)P₄ and the major product eluted just before this in a position previously shown for Ins(1,3,4,6)P₄ (21–24). The same ratio of products (2.3:1) was observed using either highly purified calf brain or recombinant enzyme in this experiment.

**DISCUSSION**

We have isolated an Ins(1,3,4)P₃ 5/6-kinase from calf brain that phosphorylates Ins(1,3,4)P₃ in bovine tissues the Ins(1,3,4)P₃ 5/6-kinase activity is most abundant in calf brain, constituting 0.13% of total soluble protein, and is 20- and 200-fold less prevalent in liver and in skeletal muscle, respectively. In the case of skeletal muscle, the lower amounts of Ins(1,3,4)P₃ 5/6-kinase activity correlate with the low levels of Ins(1,3,4,6)P₄ in skeletal muscle reported by Mayrand Thielemek (25). By contrast, inositol polyphosphate 4-phosphatase, which also utilizes Ins(1,3,4)P₃ as a substrate, is present in relatively high amounts in skeletal muscle (26), as is inositol 1,3-bisphosphate (25), the product of Ins(1,3,4)P₃ dephosphorylation by inositol polyphosphate 4-phosphatase (27). The relative abundance of these enzymes may contribute to the low level of Ins(1,3,4,6)P₄ found in this tissue.

Our purification of Ins(1,3,4)P₃ 5/6-kinase employed chromatography in the presence and the absence of MgCl₂ and affinity elution with IP₆. The final preparation had a peak specific activity of 3.7 \times 10^5 \text{ min}^{-1}\text{mg}^{-1} protein, with two sets of protein bands of apparent molecular masses of 48 and 36–38 kDa. Identification of these protein bands as Ins(1,3,4)P₃ 5/6-kinase was accomplished by gel renaturation assays, in which bands excised from an SDS gel were shown to phosphorylate...
Ins(1,3,4)P$_3$ 5/6-Kinase

The cloning of Ins(1,3,4)P$_3$ 5/6-kinase allowed definitive identification of the products of this enzyme. In enzyme preparations from tissues it was conceivable that two kinases were copurified that phosphorylated either the 5 or 6 position of Ins(1,3,4)P$_3$. The demonstration of both 5- and 6-kinase activities toward Ins(1,3,4)P$_3$ by the recombinant kinase rules out contamination of the protein preparation. This dual product formation from a single substrate makes Ins(1,3,4)P$_3$ 5/6-kinase unique among the inositol polyphosphate kinases and phosphatases. There is a constant ratio of the two tetrakisphosphate products formed by the purified calf brain enzyme and the recombinant human enzyme, with a preference for the 6 position, which might result from the formation and ultimate hydrolysis of a cyclic phosphate intermediate. If the two products result from hydrolysis of a cyclic intermediate, it is possible that the product ratio is different in cells. Ins(1,3,4)P$_3$ 5/6-kinase phosphorylates one of the two possible positions (i.e., P$_5$ is not a product). This finding would also be consistent with a cyclic 5/6 phosphate intermediate. Alternatively, the production of dual products may serve some as yet undiscovered cellular function. With Ins(1,3,4)P$_3$ 5/6-kinase available as a recombinant enzyme, the mechanism of its dual phosphorylation and studies of formation of higher phosphorylated inositol polyphosphates may be carried out.

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