Molecular cloning and expression of a rat hepatic multiple inositol polyphosphate phosphatase

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The characterization of the multiple inositol polyphosphate phosphatase (MIPP) is fundamental to our understanding of how cells control the signalling activities of 'higher' inositol polyphosphates. We now describe our isolation of a 2.3 kb cDNA clone of a rat hepatic form of MIPP. The predicted amino acid sequence of MIPP includes an 18 amino acid region that aligned with approximately 60 % identity with the catalytic domain of a fungal inositol hexakisphosphate phosphatase (phytase A); the similarity encompassed conservation of the RHGXRXP signature of the histidine acid phosphatase family. A histidine-tagged, truncated form of MIPP was expressed in *Escherichia coli* and the enzymic specificity of the recombinant protein was characterized: Ins(1,3,4,5,6)P_5 was hydrolysed, first to Ins(1,4,5,6)P_4 and then to Ins(1,4,5)P_3, by consecutive 3- and 6-phosphatase activities. Inositol hexakisphosphate was catabolized without specificity towards a particular phosphate group, but in contrast, MIPP only removed the β-phosphate from the 5-diphosphate group of diphosphoinositol pentakisphosphate. These data, which are consistent with the substrate specificities of native (but not homogeneous) MIPP isolated from rat liver, provide the first demonstration that a single enzyme is responsible for this diverse range of specific catalytic activities. A 2.5 kb transcript of MIPP mRNA was present in all rat tissues that were examined, but was most highly expressed in kidney and liver. The predicted C-terminus of MIPP is comprised of the tetrapeptide SDEL, which is considered a signal for retaining soluble proteins in the lumen of the endoplasmic reticulum; the presence of this sequence provides a molecular explanation for our earlier biochemical demonstration that the endoplasmic reticulum contains substantial MIPP activity [Ali, Craxton and Shears (1993) J. Biol. Chem. **268**, 6161–6167].

INTRODUCTION

The multiple inositol polyphosphate phosphatase (MIPP) provides the cell with the only known means of dissipating the cellular pools of Ins(1,3,4,5,6)P_5 and InsP_6 [1,2]. These polyphosphates are now recognized to have several important cellular actions, both in terms of their being functionally active in themselves, and also as receptor-mobilized precursor pools for intracellular signals [3]. Some quite substantial changes in levels of Ins(1,3,4,5,6)P_5 and InsP_6 occur during several physiologically and pathologically important events: progression through the cell cycle [4,5]; differentiation of HL60 promyelocytic leukaemia cells [6]; v-*src* transformation [7]; invasion of intestinal epithelial cells by *Salmonella* (L. Eckmann, A. E. Traynor-Kaplan and S. B. Shears, unpublished work); and erythrocyte development [3]. A greater understanding of the control of these MIPP-catalysed metabolic events is essential to determining their cause/effect relationship to the accompanying physiological changes.

Not only do the catalytic activities of MIPP have the capacity to regulate the cellular activities of Ins(1,3,4,5,6)P_5 and InsP_6, but in addition MIPP yields some downstream metabolites that are themselves physiologically active. For example, we have purified MIPP activity from rat liver, and we have shown it to convert Ins(1,3,4,5,6)P_5 into Ins(1,4,5)P_3, the Ca^{2+}-mobilizing signal [8], via Ins(1,4,5,6)P_4 [1], which is a potent competitive ligand for some pleckstrin homology domains [9]. These preparations of MIPP also attack PP-InsP_5 ('InsP_7') and $(PP)_2$-InsP_4 ('InsP_8') [10], compounds that are receiving attention as potential high-energy donors and/or molecular 'switches' [11–13]. It is particularly valuable to characterize the enzymes that metabolize these diphosphorylated compounds, so as to determine what physiological advantage is gained from the considerable free-energy change resulting from their hydrolysis. However, all these data on substrate specificity were obtained using preparations of MIPP that were not purified to homogeneity [2].

A fundamental step in developing insight into the cellular regulation of MIPP activity is to characterize the enzyme at the molecular level. We now describe the isolation and analysis of a cDNA clone of this enzyme from rat liver. In addition, the expression of a catalytically active product of this clone has provided us with the first opportunity to determine whether a single enzyme is responsible for the diverse range of phosphatase activities previously ascribed to preparations of native MIPP [1,2,8].

MATERIALS AND METHODS

Protein purification and amino acid sequence determination

Our purified MIPP migrates on SDS/PAGE as a doublet [2]. Approximately 10 μg [in 10 mM triethanolamine/1 M KCl/0.05 % (w/v) CHAPS, pH 7.5] was either digested *in situ* with endoproteinases Asp-N or Lys-C or alternatively chemically cleaved with cyanogen bromide [14]. For endoproteolytic digestions, MIPP was applied to a mini-octadecyl-silica hydrophobic sequencing column (Hewlett Packard) according to the manufacturer's protocol, digested with either Asp-N or Lys-C, and individual peptides were subsequently resolved by reverse-phase HPLC [15]. *In situ* pyridylethylation of cysteine residues was performed as previously described [15]. Peptide sequences were

Abbreviations used: ER, endoplasmic reticulum; MIPP, multiple inositol polyphosphate phosphatase; NTA, nitrilotriacetate.

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1                                              TCGGTGCTTAGCCCCTACTTCGGCACGAAGACACGCTACGAAGATGTCAACCCCTGGCTG
   1  S  L  P  G  R  G  D  P  V  A  S  V  L  S  P  Y  F  G  T  K  T  R  Y  E  D  V  N  P  W  L
            H
61    CTGGGCGACCCGGTGGCGCCGCGACGGGACCCGGAGCTGCTGGCGGGGACTTGCACCCCGGTGCAGCTGGTCGCCCTCATCCGTCACGGC
  31  L  G  D  P  V  A  P  R  R  D  P  E  L  L  A  G  T  C  T  P  V  Q  L  V  A  L  I  R  H  G
151   ACCCGCTACCCTACGACCAAGCAGATCCGCAAGCTGAGGCAGCTGCAGGGGCTGCTGCAGACCCGCGAGTCCGTGGATGGCGGGAGCCGA
  61  T  R  Y  P  T  T  K  Q  I  R  K  L  R  Q  L  Q  G  L  L  Q  T  R  E  S  V  D  G  G  S  R
241   GTGGCCGCCGCTCTGGACCAATGGCCGCTGTGGTACGATGACTGGATGGACGGGCAGCTGGTGGAAAAGGGGCGGCAGGACATGCGACAG
  91  V  A  A  A  L  D  Q  W  P  L  W  Y  D  D  W  M  D  G  Q  L  V  E  K  G  R  Q  D  M  R  Q
331   CTGGCCCTGCGTCTGGCCGCCCTCTTCCCTGACCTCTTCTGCCGGGAGAACTACGGCCGCCTGCGGCTGATCACCAGCTCCAAGCACCGC
 121  L  A  L  R  L  A  A  L  F  P  D  L  F  C  R  E  N  Y  G  R  L  R  L  I  T  S  S  K  H  R
421   TGTGTGGACAGCAGCGCCGCCTTCCTCCAAGGGTTGTGGCAACATTACCACCCAGGATTGCCACCTCCCGACGTCTCAGACATGGAGTGT
 151  C  V  D  S  S  A  A  F  L  Q  G  L  W  Q  H  Y  H  P  G  L  P  P  P  D  V  S  D  M  E  C
511   GACCCTCCGAGAGTTAATGATAAGCTAATGAGGTTCTTCGATCACTGTGAGAAGTTTTTAACCGAAGTCGAAAGAAACGCCACGGCTCTT
 181  D  P  P  R  V  N  D  K  L  M  R  F  F  D  H  C  E  K  F  L  T  E  V  E  R  N  A  T  A  L
601   TATCATGTGGAAGCCTTCAAAACCGGGCCAGAAATGCAGACAGTTTTAAAGAAAGTTGCAGCCACTTTGCAAGTGCCAGTGAACAATTTA
 211  Y  H  V  E  A  F  K  T  G  P  E  M  Q  T  V  L  K  K  V  A  A  T  L  Q  V  P  V  N  N  L
691   AATGCAGACTTAATTCAGGTAGCCTTTTTCACCTGTTCGTTTGACCTGGCAATTCAAGGTGTCCATTCTCCCTGGTGCGATGTGTTTGAC
 241  N  A  D  L  I  Q  V  A  F  F  T  C  S  F  D  L  A  I  Q  G  V  H  S  P  W  C  D  V  F  D
781   GTAGATGATGCGAAGGTTCTGGAATACTTAAATGATCTGAAACAGTACTGGAAACGAAGTTATGGCTATGCCATTAACAGCCGGTCCAGC
 271  V  D  D  A  K  V  L  E  Y  L  N  D  L  K  Q  Y  W  K  R  S  Y  G  Y  A  I  N  S  R  S  S
871   TGCAACCTGTTTCAGGACATTTTTCTACACCTGGACAAAGCAGTTGAGCAGAAGCAAAGGTCTCAGCCGGTCTCTTCTTCAGTCATCCTC
 301  C  N  L  F  Q  D  I  F  L  H  L  D  K  A  V  E  Q  K  Q  R  S  Q  P  V  S  S  S  V  I  L
                                                                                   P
961   CAGTTTGGTCATGCGGAGACCCTCCTACCCCTGCTCTCGCTCATGGGCTACTTCAAGGACAAGGAGCCCCTGACAGCATACAATTTTGAG
 331  Q  F  G  H  A  E  T  L  L  P  L  L  S  L  M  G  Y  F  K  D  K  E  P  L  T  A  Y  N  F  E
1051  GAGCAGGTGCATCGCGAGTTCCGAAGTGGTCACATCGTACCATATGCTTCAAACCTAATATTTGTGCTTTACCATTGTGAAGACGCACAG
 361  E  Q  V  H  R  E  F  R  S  G  H  I  V  P  Y  A  S  N  L  I  F  V  L  Y  H  C  E  D  A  Q
1141  ACCCCTCAAGAAAAATTCCAGATACAAATGCTGCTGAATGAAAAGGTGTTACCCTTAGCTCACTCGCAGAAAACTGTTGCCTTGTATGAG
 391  T  P  Q  E  K  F  Q  I  Q  M  L  L  N  E  K  V  L  P  L  A  H  S  Q  K  T  V  A  L  Y  E
1231  GATCTGAAGAACCACTACCAGGACATTCTTCAGAGCTGTCAAACTAGTAAAGAATGTAACCTACCCAAGGTGAACATCACGTCCGACGAG
 421  D  L  K  N  H  Y  Q  D  I  L  Q  S  C  Q  T  S  K  E  C  N  L  P  K  V  N  I  T  S  D  E
1321  CTCTGAGGACTCATCAGTGCTCTGCTGAGGGCGCTTGTTGCCAATAGGTAGCCACTCTAAAGGCAGCAACAGGAGGATCTCTGTGAGCTC
 451  L  *

1411  AAGGCCAACCTGTTCTACATAGTGAGTTCCAGGCCAGCCAAGGCTGCGTAGAGAAATAAAGTTTGGTCCTTTTGTCTTTTCACAGAAAAT
1501  GATAGTTTCTTTTAGAATCTGGACATACGGGTAAGACATGACTCTCCCTGGAGCAGCTCTCTTCAGAAAAACTAATTCAGCAAAACAGCT
1591  GTCCCTCCCAGTGTTTGCAGAGCTGAAATTTTCCTAATGACCTAAGAAAATGCTGATGTAGAATGGTATTAGAAAATAACACTTCAAAAG
1681  TGTTGGATACCAAAGCACAGTGGCAGCTGGGTGAGCCGCAGTGAGTGACTGAGATGGGGACTTGAGTGATCATGTTGGGTTCTTTCCTTC
1771  TCCTTCACGAAGGACACAAAGAAGGAAGTCTAATAACGTATCCATCCAGACAGGAAATCAACTCGATATTAAGAACCAGGCTGAAGTAAA
1861  ACTGAAAGTGTGGGCTATTTTTGTTGATGTTATTTACAAAAAGATTTAAACACTGTCAGTAATTGCCTTTAACCTCCAAGTAGGTCTTGC
1951  AGAACCACCTCCATCCCTCGGACCTGTTTGAGGCGCGCAGTTATAATGGGGCCCAGCCTGGTACAGAGCCGACTTCCTTGACTGTTGCCT
2041  GGTTATCTTTCGTTCCATCATGGCTCCCCTTTTTATATCTTGATATTACATAAAGTTTATCTTTTGGTGGCTTGGATTTTTTTTTAAATA
2131  AAGACTTATCTGCCTAATTTAATTGTAGAGATTCGAACCTGATTCAAAGAAATTTTGAGTTCTTTCAAATACCATAAAAATGTTTGCTAC
2221  AATAAATAAATAAAATTCTTGTGGCTTTACTACCAAAAAAA
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Figure 1 Nucleotide and deduced amino acid sequence of recombinant MIPP

The underlined residues of the predicted amino acid sequence of MIPP were also derived by microsequencing native MIPP; the broken-line portions of the underscoring designate residues in the microsequence that could not be identified unequivocally and independently. Two places are indicated where the microsequenced peptides either do not match each other (residue 3), or do not match the deduced sequence (residue 327); the alternative microsequence is indicated. Potential N-glycosylation sites (Asn-216 and Asn-445) are shown in bold type. Several domains containing serine/threonine residues comprise the minimum requirements for permitting phosphorylation by either casein kinase 2 (Ser-176, Thr-201, Thr-218, Thr-391, Thr-435 and Thr-447) or protein kinase C (Thr-65, Ser-146, Ser-412 and Thr-435). In the 3′ untranslated region, there were five polyadenylation signals (underlined) and three ATTTA sequences (bold type) which may mediate mRNA instability [29]. The nucleotide sequence described in this Figure has been added to the GenBank database under the accession number AF012714.

determined using an Applied Biosystems 477A pulsed-liquid sequencer with a 120A phenylthiohydantoin (PTH) analyser.

Generation of DNA probes by PCR amplification

To generate a homologous probe for identification of putative MIPP cDNAs, degenerate sense (S) and antisense (A) oligonucleotide primers were synthesized that corresponded to peptide sequences of 7–9 amino acid residues in length. Sense and antisense primers were synthesized with *Eco*RI or *Hin*dIII restriction sites at their respective 5′ termini for subsequent cloning into *Eco*RI/*Hin*dIII sites of pBluescript(SK+). Deoxyinosine was utilized as a 'neutral' base at positions of 3- or 4-fold degeneracy to significantly reduce overall primer degeneracy. PCR reactions were performed using 1 ng of rat liver cDNA (Clontech) as a template, and 0.2 μM of each oligonucleotide primer. Each amplification cycle (40 in total) consisted of 1 min at 94 °C, 2 min at 48–51 °C and 3 min at 72 °C. Products were resolved on 4% NuSieve GTG agarose gels. Three products were observed, designated 1S-1A (90 bp), 2S-1A (210 bp) and 2S-2A (240 bp), using the following primers:

1S, 5′-TT(CT)CA(AG)ATICA(AG)ATG(CT)TI(CT)TIAA-(CT)GA-3′

2S, 5′-GCITA(CT)AA(CT)TT(CT)GA(AG)GA(AG)CA(AG)-GTICA-3′

1A, 5′-(AG)TT(AG)TT(CT)TTIA(AG)(AG)TC(CT)TC(AG)-TA-3′

2A, 5′-(CT)TT(CT)TG(AG)CAIII(CT)TGIA(AG)IAT(AG)T-C(CT)TG-3′

Screening of cDNA libraries and isolation of MIPP cDNA clones

A size-selected cDNA library (inserts less than 4 kb) was constructed from rat liver poly(A)$^+$ RNA using a cDNA synthesis kit (Pharmacia LKB Biotechnology Inc.), and inserted into a Lambda ZAPII insertion vector at an *Eco*RI site using a Gigapack II Gold packaging extract according to the manufacturer's instructions. An aliquot of the amplified rat liver cDNA library (5×10^5 recombinants) was plated on *Escherichia coli* XL-1 Blue at a density of 10^4 plaque-forming units per dish (10×10 cm NZY agar plates), and plaques were transferred to nitrocellulose membranes. Filters were prehybridized and hybridized under standard conditions, using [α-^{32}P]dCTP-labelled 2S-2A (as above). Positive clones were plaque-purified and rescued as recombinant plasmids from Lambda phage by co-infection with ExAssist helper phage, according to the supplier's instructions (Stratagene).

DNA sequencing of PCR products and cDNA clones

All PCR products and fragments of MIPP cDNA were subcloned into pBluescript SK(+). DNA sequencing was performed by the dideoxynucleotide chain-termination method using the Sequenase system, Version 2.0 (US Biochemical Corporation) and [α-^{35}S]dATP. The sequencing strategy involved both sequencing subcloned *Sac*I, *Spe*I or *Rsa*I restriction fragments with T3 or T7 universal primers, and a series of custom-synthesized primer walks. An automated fluorescent dye sequencing method (MacConnell Research Corporation, CA, U.S.A.) was used to confirm the nucleotide sequence. DNA sequences were analysed using the GCG Wisconsin package.

Expression of His$_6$MIPP

The coding sequence of MIPP from Ser-11 (Figure 1) to the C-terminus was amplified by PCR with *Pfu* DNA polymerase (Stratagene), using primers containing restriction enzyme sites for *Bam*HI (5′ primer) and *Sal*I (3′ primer). The 1.3 kb PCR product was then cloned into the *Bam*HI and *Sal*I sites of the His-tag bacterial expression vector pQE-30 (Qiagen). The resulting construct, pHis$_6$MIPP, therefore encodes a truncated form of MIPP with the His$_6$ epitope at the N-terminus. The sequence of pHis$_6$MIPP was confirmed by automated sequencing (MacConnell Research Corporation) and by manual sequencing. Cultures of *E. coli* strain JM109, transformed with either pHis$_6$MIPP or pQE-30, were grown at 37 °C until $A_{600} = 0.7$–0.9. Cultures were then induced with 1 mM isopropyl β-D-thiogalactoside and grown at 28–30 °C for 6–8 h. Cells were harvested by centrifugation and the pellets were stored at −30 °C. All subsequent procedures were conducted at 0–4 °C. Cells were lysed for 30 min in 5 vol. of lysis medium [150 mM NaCl/10 mM Tris/HCl (pH 7.5)/1 mM PMSF/10 μM *trans*-epoxysuccinyl-L-leucylamido-(4-guanidino)butane (E-64)/5 μg/ml aprotinin] plus 1 mg/ml lysozyme. The suspensions were sonicated, and particulate material was removed by centrifugation at 12000 ***g*** for 10 min. The supernatant was added to 0.05 vol. of a 50% slurry (in lysis buffer) of Ni-nitrilotriacetate (NTA) Superflow (Qiagen). After 1 h of gentle agitation, the Ni-NTA matrix was pelleted and washed with 20 bed vols. of lysis buffer alone, followed by three washes with 20 bed vols. of lysis buffer which was supplemented first with 0.5 M NaCl (wash 1), then 1 M NaCl (wash 2), followed by 10 mM imidazole (wash 3). Matrix-bound proteins were eluted with 10 bed vols. of lysis buffer plus 0.5 M imidazole; purity was assessed by SDS/PAGE and Western blotting using an anti-$^{\text{RGS}}$His antibody (Qiagen) according to the manufacturer's instructions. Finally, 30% (v/v) glycerol and 1 mg/ml BSA were added to the preparation of recombinant enzyme, which was stored in aliquots at −30 °C.

Assay of His$_6$MIPP

His$_6$MIPP activity was assayed in 100 μl aliquots of buffer containing 100 mM KCl, 25 mM Hepes (pH 7.4), 1 mM EDTA, 2 mM CHAPS, 0.02% (w/v) BSA and 0.1–30 μM [^{3}H]Ins(1,3,4,5)P_4 (12000 d.p.m). In some experiments, [^{3}H]Ins(1,3,4,5)P_4 was substituted by either [^{3}H]Ins(1,3,4,5,6)P_5, [^{3}H]InsP_6 or [5-β-^{32}P]PP-InsP_5. Reactions that were analysed by gravity-fed ion-exchange chromatography [2] were quenched with 1 ml of 0.2 M ammonium formate/0.1 M formic acid/0.05% (w/v) InsP_6. Other samples were quenched with perchloric acid, neutralized with K_2CO_3 and analysed by either Adsorbosphere SAX HPLC [8] or Partisphere SAX HPLC [10].

Additional materials

[^{3}H]Ins(1,3,4,5)P_4, [^{3}H]InsP_6 and [β-^{32}P]PP-InsP_5 were obtained from New England Nuclear. [^{3}H]Ins(1,3,4,5,6)P_5 and [^{14}C]-labelled inositol phosphates were prepared from avian erythrocytes labelled with either [^{3}H]inositol or [^{14}C]inositol respectively [8]. Non-radioactive Ins(1,3,4,5)P_4 was purchased from Cell Signals Inc, Lexington KY, U.S.A. The Adsorbosphere 5 μm HPLC columns were purchased from Krackeler Scientific, Durham, NC, U.S.A.

RESULTS

Isolation and analysis of a cDNA clone of MIPP

No sequence information was generated after direct N-terminal sequencing of purified hepatic MIPP, but 19 internal peptides were obtained by proteolytic and chemical digestion (Figure 1, and see below). cDNA from rat liver was used as a template for PCR amplification using multiple permutations of degenerate sense and antisense oligonucleotide primers designed from primary peptide information (see Materials and methods section). The longest PCR product (240 bp) was used as a probe to screen a size-selected rat liver cDNA library (see Materials and methods section). A single clone of 2.26 kb was isolated and completely sequenced (Figure 1). This clone comprises an open reading frame of 1323 bp, followed by a 938 bp potential 3′-untranslated region, which contains five putative polyadenylation signals and a poly(A)$^+$ tail (Figure 1). Since we did not identify a translation-initiation signal, this cDNA represents a near full-length clone of MIPP, comprising 90% of the nucleotide sequence (see below). We were unable to obtain further unambiguous upstream sequences from cloned PCR products obtained by rapid amplification of cDNA ends (5′ RACE).

The 19 peptide sequences obtained from native MIPP contained 199 residues that matched precisely the deduced amino acid sequence of MIPP; eight additional microsequenced residues could not be unequivocally identified (Figure 1). Two microsequenced peptides which matched the predicted N-terminus of the MIPP clone also extended beyond it by an additional ten residues (Figure 1). Intriguingly, this region of the microsequence contains an RGD protein–protein interaction motif [16]. These two peptides were of additional interest in that they differed in the residue present at position 3, which was Pro in one case and His in the other (Figure 1). The fact that these two peptides were not identical is confirmed by their being baseline resolved from a single HPLC run (results not shown). There was one additional difference between the deduced sequence and the microsequence: Ser-327, deduced from the MIPP clone, aligned with a Pro

2A

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MIPP     115  RQDMRQLALRLAALFPDLFCRENYGRLRLITSSKHRCVDSSAAFLQG
Band 17    1  RRDMEHLARRLAARFPALFAARR--RLALASSSKHRCLQSGAAFRRG

MIPP     162  LWQHYHPGLPPPDVSDMECDPPRVNDKLMRFFDHCEKFLTEVERNAT
Band 17   46  L----GPSL-SLGADETEIE---VNDALMRFFDHCDKFVAFVEDNDT

MIPP     209  ALYHVEAFKTGPEMQTVLKKVAATLQVPVNNLNADLIQVAFFTCSFD
Band 17   85  AMYQVNAFKEGPEMRKVLEKVASALCLPASELNADLVQVAFLTCSYE

MIPP     256  LAIQGVHSPWCDVFDVDDAKVLEYLNDLKQYWKRSYGYAINSRSSCN
Band 17  132  LAIKNVTSPWCSLFSEEDAKVLEYLNDLKQYWKRGYGYDINSRSSCI

MIPP     303  LFQDIFLHLDKAVEQKQRSQPVSSSVILQFGHAETLLPLVSLMGYFK
Band 17  179  LFQDIFQQLDKAVDESRSSKPISSPLIVQVGHAETLQPLLALMGYFK

MIPP     350  DKEPLTAYNFEEQVHREFRSGHIVPYASNLIFVLYHCEDAQTPQEKF
Band 17  226  DAEPLQANNYIRQAHRKFRSGRIVPYAANLVFVLYHCEQ-KTSKEEY

MIPP     397  QIQMLLNEKVLPLAHSQKTVALYEDLKNHYQDILQSCQTSKECNLPK
Band 17  272  QVQMLLNEKPMLFHHSNETISTYADLKSYYKDILQNCHFEEVCELPK

MIPP     444  VNIT-SDEL
Band 17  319  VNGTVADEL
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2B

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Rat Acid Pase 31  RSLRFVTLLYRHGDRSPV
  PhyB        71  CEVDQVIMVKRHGERYPS
 PhyA (Myc)   73  VTFAQ-V-LSRHGARAPT
 PhyA (Asp)   71  VTFAQ-V-LSRHGARYPT
  MIPP        48  CTPVQLVALIRHGTRYPT
  PPA3        64  CEMKQLQMLARHGERYPT
  PPA2        58  CAIKQVHLLQRHGSRNPT
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Figure 2 Amino acid sequences in MIPP that share significant similarity with other proteins

(**A**) The predicted amino acid sequence of the Band 17 gene product (accession number U59421) was aligned with the predicted amino acid sequence of MIPP. (**B**) The catalytic sites of some histidine acid phosphatases [20] were aligned with MIPP. The accession numbers are as follows: rat acid phosphatase, M27893; phytases A and B from *Aspergillus awamori*, P34753 and P34755 respectively; phytase A from *Myceliophthora thermophilia*, U59806; acid phosphatase PPA2 from *Schizosaccharomyces pombe*, Q01682; acid phosphatase PPA3 from *Saccharomyces cerevisiae*, P24031. In (**A**) and (**B**) dashes represent spaces that were introduced to optimize alignments.

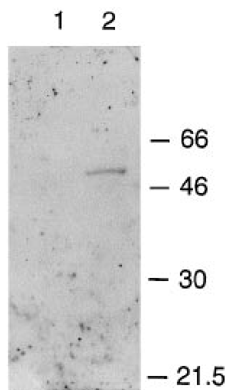

Figure 3 Bacterial expression of recombinant pHis$_6$MIPP and detection by Western blotting

Equal aliquots of the Ni-NTA–agarose-purified proteins from cultures of *E. coli* expressing pQE30 (lane 1) or pHis$_6$MIPP (lane 2) were resolved by SDS/PAGE using 10% polyacrylamide Ready Gels (Bio-Rad). Western blotting onto nitrocellulose membranes (Schleicher and Schuell) was performed with a Bio-Rad Mini Trans-Blot apparatus for 30–60 min at 4 °C in buffer containing 25 mM Tris/HCl/192 mM glycine/20% (v/v) methanol, pH 8.3. Membranes were probed with an anti-^{RGS}His antibody (Qiagen) according to the manufacturer's instructions. Antibody–antigen complexes were detected with a chemiluminescent assay (Clontech) followed by autoradiography. The positions of molecular-mass markers (marked in kDa) are also indicated.

present in two peptides generated from independent chemical and enzymic digests (Figure 1). These two amino acid discrepancies at positions 3 and 327 may result from there being two closely related proteins in the original digest.

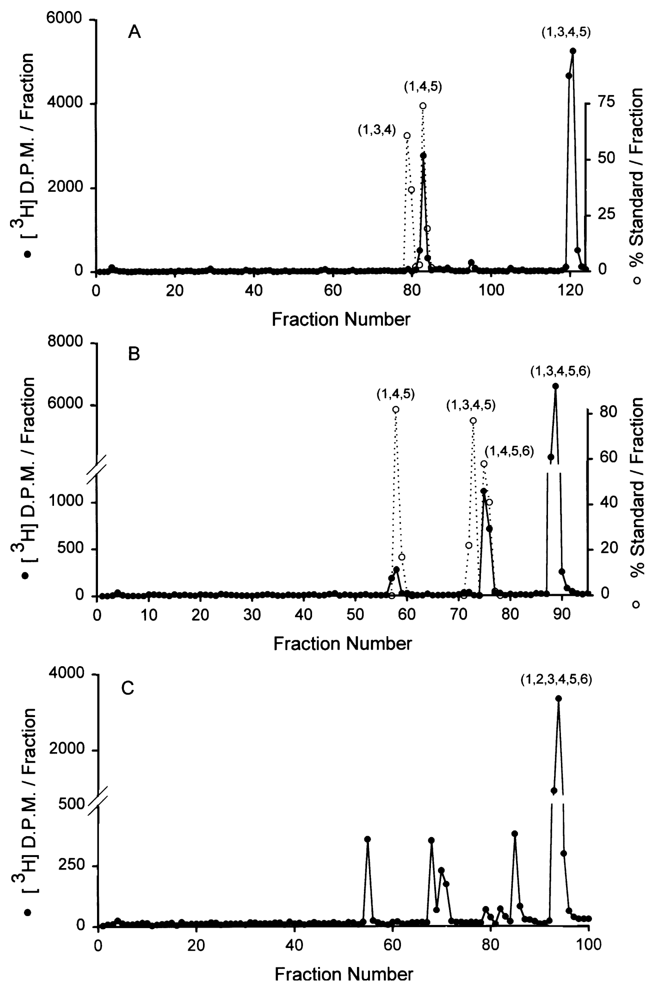

Figure 4 HPLC analysis of the products of hydrolysis of Ins(1,3,4,5)P_4, Ins(1,3,4,5,6)P_5 and InsP_6 by recombinant MIPP

His$_6$MIPP was incubated with either [^{3}H]Ins(1,3,4,5)P_4 (**A**), [^{3}H]Ins(1,3,4,5,6)P_5 (**B**) or [^{3}H]InsP_6 (**C**) and reactions were analysed (●) by Adsorbosphere SAX HPLC (see Materials and methods section). In (**A**), fractions 1–44 are 1 min, and the remainder were 0.5 min; (**A**) also shows an internal standard of [^{14}C]Ins(1,3,4)P_3 and a standard of [^{3}H]Ins(1,4,5)P_3 (both ○), obtained from a sequential HPLC run. In (**B**) and (**C**), 1 min fractions were collected throughout; (**B**) includes internal standards of [^{14}C]Ins(1,4,5,6)P_4 and [^{14}C]Ins(1,3,4,5)P_4, plus a standard of [^{3}H]Ins(1,4,5)P_3 obtained from a sequential HPLC run (all ○). In (**A**) and (**B**), the isomers of inositol phosphates are identified; in (**C**) only the total number of phosphates is known (see [1]): from left to right, an InsP_3 peak, two InsP_4 peaks and three InsP_5 peaks.

A hydropathy plot [17] of the deduced amino acid sequence of MIPP indicated it to be a highly hydrophilic protein with no candidate transbilayer helices (results not shown). A BLAST search of the protein databases revealed that MIPP was unrelated to other mammalian inositol phosphate metabolizing enzymes. In fact, MIPP only showed significant overall identity with one other protein, namely, a partly-sequenced product [18] of the Band 17 gene in chick chondrocytes (Figure 2a). The similarity between these two proteins is considerable (64%) and apparently extends to their intracellular location, since both have an XDEL tetrapeptide at their C-terminus that is a signature of luminal endoplasmic reticulum (ER) resident proteins [19]. The expression of the chick protein is up-regulated during cellular hypertrophy [18].

Another illuminating feature of the sequence of MIPP is that amino acids 48–65 aligned with 61 and 55% identity with

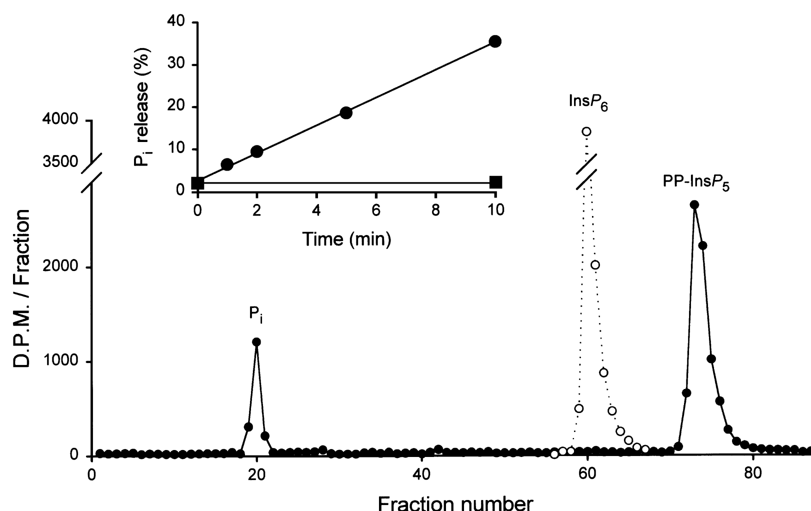

Figure 5 HPLC analysis of [β-^{32}P]PP-InsP_5 hydrolysis by recombinant MIPP

His$_6$MIPP was incubated with [^{32}P]PP-InsP_5 and reactions (●) were analysed by Partisphere SAX HPLC (see Materials and methods section) with an internal standard of [^{3}H]InsP_6 (○). The inset shows a time-course of [^{32}P]P$_i$ release by recombinant MIPP (●) compared with vector controls (■).

corresponding regions of two isoforms of phytase A from *Aspergillus* and *Myceliophthora* respectively (Figure 2b). This similarity in part reflects the shared presence of the strictly conserved catalytic motif of histidine acid phosphatases (RHGX-RXP; see Figure 2b and [20,21]). There was no significant similarity of any of these other phosphatases to MIPP outside this 18 amino acid domain.

Enzymic activity of recombinant MIPP

One of the goals of this work was to investigate whether a single protein was responsible for the quite different catalytic activities seen in preparations of native MIPP isolated from rat liver. We therefore expressed an N-terminal His$_6$-tagged truncated version of MIPP (His$_6$MIPP) in *E. coli* (see Materials and methods section), which was partially purified from a crude cell extract by affinity chromatography. The estimated molecular mass of His$_6$MIPP was 49 ± 2 kDa ($n=6$), as determined by SDS/PAGE and Western blotting using an antibody to the His-tag (Figure 3). The predicted molecular mass is 51.8 kDa.

His$_6$MIPP activity was assayed in the absence of Mg^{2+} (see Materials and methods section); the native enzyme is also Mg^{2+}-independent [1]. In a typical preparation, His$_6$MIPP activity against its prototypical substrate, Ins(1,3,4,5)P_4 [1], was 302 ± 6 pmol/μg of protein per min ($n=3$); the rate of Ins(1,3,4,5)P_4 dephosphorylation in extracts prepared from vector-transformed controls was 0.13 ± 0.03 pmol/μg of protein per min ($n=3$). The K_m of His$_6$MIPP for Ins(1,3,4,5)P_4 was 6.9 ± 1.7 μM ($n=3$), which is approximately an order of magnitude lower in affinity than native MIPP [1].

The specificity of His$_6$MIPP activity was studied by using an HPLC analytical procedure previously established to resolve many of the naturally-occurring inositol polyphosphates ([1] and Figure 4): [^{3}H]Ins(1,3,4,5)P_4 was dephosphorylated to a single [^{3}H]InsP_3 peak that co-eluted precisely with a standard of Ins(1,4,5)P_3, which in turn was baseline resolved from a standard of Ins(1,3,4)P_3 (Figure 4A). These data are entirely consistent with the recombinant enzyme duplicating the well-characterized Ins(1,3,4,5)P_4 3-phosphatase activity of native MIPP [1], with the caveat that we did not exclude the possibility that Ins(1,3,5)P_3 and Ins(3,4,5)P_3 might also be formed by the recombinant enzyme, although for this to be the case these alternative InsP_3 isomers would have to co-elute with Ins(1,4,5)P_3.

In separate incubations, His$_6$MIPP dephosphorylated [^{3}H]Ins(1,3,4,5,6)P_5 to a single [^{3}H]InsP_4 peak that co-eluted with an internal [^{14}C]Ins(1,4,5,6)P_4 standard (Figure 4B). Our HPLC system resolves Ins(1,4,5,6)P_4 from all other potential products of Ins(1,3,4,5,6)P_5 hydrolysis, with the exception of Ins(3,4,5,6)P_4 [1,8]. The results described in Figure 4 are therefore compatible with recombinant MIPP catalysing the Ins(1,3,4,5,6)P_5 3-phosphatase activity that is known to be performed by native MIPP [1,8]. The identity of the Ins(1,4,5,6)P_4 product was consistent with it being further dephosphorylated by His$_6$MIPP to an InsP_3 that co-eluted with a standard of Ins(1,4,5)P_3 (Figure 4B); native MIPP also dephosphorylates Ins(1,4,5,6)P_4 to Ins(1,4,5)P_3 [8].

InsP_6 was hydrolysed non-specifically by His$_6$MIPP; from the published elution properties of this HPLC column [1] we ascertained that the following classes of inositol polyphosphates were formed: three InsP_5 peaks, two InsP_4 peaks and an InsP_3 (Figure 4C). The exact isomers were not identified, but the same pattern of products was previously seen in experiments with native MIPP [1].

We also studied the activity of His$_6$MIPP towards the 5-diphosphate group present [13] in the mammalian forms of the diphosphorylated inositol polyphosphates. The activity against [5-β-^{32}P]PP-InsP_5 was assayed by HPLC analysis (Figure 5). The recombinant enzyme catalysed a time-dependent release of [^{32}P]P$_i$ that was not evident in extracts from cells transformed with vector only (Figure 5). There was no accumulation of [5-β-^{32}P]PP-InsP_4, indicating that there was no significant hydrolysis of the monoester phosphates of PP-InsP_5 (Figure 5). Recombinant His$_6$MIPP also attacked the 5-diphosphate group in (PP)$_2$-InsP_4 (results not shown).

As is the case with native MIPP [1], the recombinant enzyme did not hydrolyse either Ins(1,4)P_2, Ins(1,4,5)P_3 or Ins(1,3,4)P_3 (results not shown). Even though His$_6$MIPP shares the catalytic domain of phytases (see above), it did not hydrolyse those phosphate esters that are typically attacked by this family of

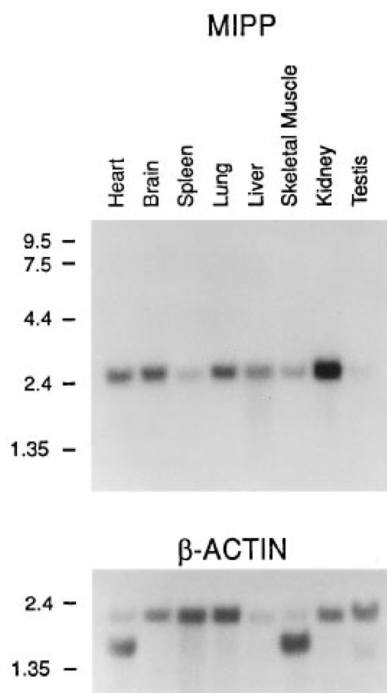

Figure 6 Northern blot of rat mRNA isolated from various tissues

A Northern blot (Clontech) containing mRNA from various rat tissues was hybridized at 42 °C for 18 h in 5 × SSPE [0.15 M NaCl/10 mM sodium phosphate (pH 7.4)/1 mM EDTA], 10 × Denhardt's solution (0.02% Ficoll 400/0.02% polyvinylpyrrolidone/0.002% BSA)/50% (v/v) formamide/2% (w/v) SDS/100 μg/ml denatured salmon sperm DNA plus a random-hexamer primed ^{32}P-labelled probe of MIPP cDNA. The blot was washed with 2 × SSC (0.5 M NaCl/0.015 M sodium citrate)/0.05% (w/v) SDS at ambient temperature for 3 × 10 min and subsequently washed with 0.1 × SSC/0.1% (w/v) SDS at 50 °C for 2 × 20 min. The blot was exposed to autoradiographic film for 6 h at −70 °C with two intensifying screens. The blot was subsequently stripped according to the manufacturer's recommendations and reprobed under identical conditions with a human β-actin control probe. To compensate for non-equivalent loading of Clontech blots (see [30]), the data were scanned using an imaging densitometer and the ratios of the signals from MIPP compared with β-actin were as follows: kidney, 4.6; liver, 4.2; brain, 2.5; lung, 1.3; heart, 1.0; spleen, skeletal muscle and testis, 0.5.

enzymes, such as *p*-nitrophenol phosphate, pyrophosphate, glucose 6-phosphate, fructose 1,6-bisphosphate and ATP (results not shown).

Relative levels of expression of MIPP mRNA in various tissues

The entire MIPP cDNA was used to probe a Northern blot containing poly(A)$^+$ RNA isolated from eight rat organs and tissues (Figure 6). A single 2.5 kb mRNA transcript was observed (Figure 6), suggesting that our cDNA clone (2.26 kb) is about 90% complete. Figure 6 also shows that the relative expression levels of MIPP in several tissues (adjusted relative to the expression of the β-actin controls) were as follows: kidney $\approx$ liver > brain > lung $\approx$ heart > spleen $\approx$ skeletal muscle $\approx$ testis. The same rank-order was obtained on a second multiple tissue Northern blot when a 460 bp digoxigenin-labelled probe from the extreme 5′ end of the MIPP cDNA was used and detected with a chemiluminescent assay (results not shown).

DISCUSSION

In this paper we describe the molecular cloning and expression of a catalytically active form of MIPP. These are important steps forward towards our goal of understanding this enzyme at both a structural and a functional level. For example, we have obtained the first definitive evidence that a single enzyme is responsible for all the inositol polyphosphate catabolic activities associated with native MIPP, which has not previously been purified to homogeneity. That is, the 3-phosphates are specifically hydrolysed from Ins(1,3,4,5)P_4 and Ins(1,3,4,5,6)P_5 (Figure 4 and [1]), the 6-phosphate is specifically removed from Ins(1,4,5,6)P_4 (Figure 4 and [8]), yet it is the 5-β phosphate that is cleaved from the diphosphate group on PP-InsP_5 (Figure 5 and see [10,13]). Despite these precise positional specificities, every phosphate on InsP_6 is susceptible to hydrolysis (Figure 4 and [1]); 'lower' inositol polyphosphates such as Ins(1,4,5)P_3, Ins(1,3,4)P_3 and Ins(1,4)P_2 were not substrates (see Results section).

This remarkable set of hydrolytic activities is unique among mammalian inositol polyphosphate metabolizing enzymes. It is particularly significant for us to demonstrate that a single recombinant enzyme is responsible, because our previous preparations of native enzyme migrated as a doublet on SDS/PAGE [2]. Indeed, during microsequencing we generated two peptides that differed from each other by a single amino acid, and we also isolated two further peptides that both differed at a single residue from the deduced amino acid sequence (see Figure 1 and Results section). It is therefore possible that our native preparations contain two closely-related isoforms of MIPP. However, even though the mRNA for the MIPP we have cloned is widely distributed in rat tissues, we did not detect multiple mRNA transcripts (Figure 6). The search for possible MIPP isoforms will be an important future direction.

A BLAST search revealed that MIPP was unrelated to all the other mammalian inositol phosphate metabolizing enzymes that have been sequenced to date. Indeed, MIPP only showed significant identity with one protein of the animal kingdom, a partly characterized chick chondrocyte protein, the expression of which is up-regulated when these cells become hypertrophic [18]. Hypertrophic chondrocytes are part of a specialized developmental structure, namely, the advancing ossification front that divides newly synthesized bone from the remaining cartilage [22]. When the full-length chick protein becomes available, it may be useful to determine whether it shares the catalytic activities of MIPP; it is an intriguing possibility that up-regulation of an MIPP-like protein, and a corresponding increase in the capacity of the cell to hydrolyse Ins(1,3,4,5,6)P_5 and InsP_6, are functionally important during a key period of bone development. Even if the chick protein does not itself hydrolyse inositol phosphates, the (as yet unknown) function of the very similar C-termini of these two proteins is likely to be shared.

The similarity between MIPP and the chick chondrocyte protein apparently extends to a common subcellular location, since both proteins have an XDEL tetrapeptide at their C-terminus (X may be either Ala, His, Lys, Arg or Ser; see [19]). This functions as a retrieval signal, salvaging for return to the ER those luminal proteins that inevitably escape in the bulk flow out of this organelle [19]. This new molecular evidence that MIPP is a soluble constituent of the ER is underscored by the results of a hydropathy plot of the deduced amino acid sequence [17], which indicates a highly hydrophilic protein with no candidate transmembrane helices (results not shown). Thus we have confirmed at a molecular level our earlier biochemical data which indicated that the ER of rat liver contains MIPP activity [23]. Resolving the so-far unknown mechanism by which MIPP gains access to its substrates will be an important aspect of future research.

Our data (Figure 1) indicate that MIPP contains an RGD protein–protein interaction motif that typically binds to clusters of Asp and Glu [16]. The ER does contain proteins that possess

these targets: the luminal portion of the ER-based Ins(1,4,5)P_3 receptor [24] and some ER chaperones [25]. The presence of MIPP in the ER also addresses the long-standing anticipation that there is some cellular compartmentalization of both the Ins(1,3,4,5,6)P_5 and InsP_6 substrates [26] and at least one of their downstream metabolites, Ins(1,4,5)P_3; see [8,26,27]. Since our cDNA clone of MIPP is an estimated 10% short of full-length at the 5′ end, the corresponding N-terminus that is missing from our expressed protein can be anticipated to contain the signal that would normally target the protein to the ER *in vivo* [28]. This targeting sequence would be cleaved from the newly synthesized protein [28].

A further notable region of MIPP is the 18 amino acid sequence that aligns with 61% and 55% identity with a corresponding region of phytase A isozymes from, respectively, *Aspergillus* and *Myceliophthora* (Figure 2b). Outside of this region the MIPP sequence does not show any significant similarity to the fungal enzymes. These aligned sequences include the histidine acid phosphatase RHGXRXP catalytic motif (Figure 2b), which is a feature not yet found in any other mammalian inositol phosphate metabolizing enzyme. An interesting evolutionary question concerns the nature of the selective pressures that led to the specific conservation in MIPP of only this 18 amino-acid portion of phytase A.

Now that we have obtained a cDNA clone of MIPP that encodes a catalytically active protein, we can begin to unravel the structure of the catalytic site and the molecular mechanisms that underlie the quite different enzymic reactions it catalyses. We are also now an important step closer to analysing the metabolic and physiological consequences of manipulating the expression of MIPP activity inside cells. Such studies should improve our insight into the actions of both the substrates and the products of this versatile and important enzyme.

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