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Biochemical and Biophysical Research Communications 356 (2007) 38–44
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Identification of a novel PP2C-type mitochondrial phosphatase

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Received 2 February 2007
Available online 28 February 2007

Abstract

A novel phosphatase has been cloned and partially characterized. It has a mitochondrial leader sequence and its amino acid sequence places it in the PP2C family like two known mitochondrial phosphatases. Western blot analysis of subcellular fractions and confocal microscopy of 3T3L1 preadipocytes expressing the GFP-tagged protein confirm its mitochondrial localization. Western blot analysis indicates that the protein is expressed in several mouse tissues, with highest expression in brain, heart, liver, and kidney. The recombinant protein exhibits Mn^{2+}-dependent phosphoserine phosphatase activity against the branched-chain α-keto acid dehydrogenase complex, suggesting the enzyme may play a role in regulation of branched chain amino acid catabolism. Whether there are other mitochondrial substrates for the enzyme is not known.

Keywords: Protein phosphatase 2C; Mitochondria; Branched-chain α-keto acid dehydrogenase

Two enzymes are well established to be regulated by phosphorylation in mitochondria—the pyruvate dehydrogenase complex (PDC) and the branched chain α-keto acid dehydrogenase complex (BCKDC). Four of the five mitochondrial kinases cloned so far are PDC kinases (PDK1–4) [1] while a fifth (BDK) phosphorylates the BCKDC [2]. Two mitochondrial phosphatases have been cloned. Both are PDC phosphatases (PDP1 and 2) [3]. A phosphatase for BCKDC has been purified [4,5] but there are no reports of success in identification of a cDNA encoding this protein.

By amino acid sequence and some structural aspects the mitochondrial kinases are similar to bacterial histidine kinases [6]. The two mitochondrial phosphatases that have been cloned are of PP2C type [3], which bacteria likewise have been found to express [7]. The similarity between the enzymes that regulate the PDC activity in mitochondria and SpoIIAA activity in bacteria was pointed out by Adler et al. [8]. The PDKs responsible for regulation of PDC are related to the histidine kinases whereas the kinase (SpoIIAB) that regulates SpoIIAA activity is a histidine kinase. Likewise, the PDPs that dephosphorylate PDC are members of the PP2C family of phosphatases as is the phosphatase (SpoIIE) that dephosphorylates SpoIIAA. The similarity of mitochondrial and bacterial kinases and phosphatases reflects the bacterial origin of mitochondria. Based on the hypothesis that there may be additional members of the PP2C family of phosphatases in mitochondria, a database search was conducted for PP2C-type phosphatases with potential mitochondrial leader sequences. One such candidate was found and partially characterized, designated here as PP2C-type mitochondrial phosphoprotein phosphatase (PTMP).

Materials and methods

Expression and purification of recombinant protein. cDNAs for the full-length and mature forms of human PTMP (hPTMP) were subcloned into a pET-28a vector to encode proteins with a carboxy-terminal His_6 tag. The

Abbreviations: PTMP, PP2C-type mitochondrial phosphatase; PDC, pyruvate dehydrogenase complex; BCKDC, branched-chain α-keto acid dehydrogenase complex; PDP, PDC phosphatase; PDK, PDC kinase; BDK, BCKDC kinase; BDP, BCKDC phosphatase.

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0006-291X/$ - see front matter © 2007 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2007.02.108

vector was co-transformed with pGroEL/ES (which expresses the molecular chaperonins GroEL and GroES) into *Escherichia coli* BL21 Star™ (DE3)pLysS cells (Invitrogen, Carlsbad, CA) and selected for kanamycin and chloramphenicol resistance. Bacteria were grown in Luria–Bertani (LB) Broth containing antibiotics and 1 mM $MnCl_2$ at 37 °C until the OD of the culture at 600 nm reached 0.6. Expression of recombinant protein was induced by 0.3 mM isopropyl-β-D-thiogalactopyranoside. Incubation was continued for 6 h at 20 °C. Cells obtained by centrifugation were suspended in 10 volumes of buffer containing 20 mM Tris–HCl, pH 7.9, 0.1 M NaCl, 5 mM β-mercaptoethanol, 0.625% Triton X-100, 10% glycerol, and a mixture of protease inhibitors (400 μg/ml phenylmethylsulfonyl fluoride, 20 μg/ml leupeptin, and 1% (v/v) aprotinin). Cells were disrupted twice with a French press at 1500 psi. His-tagged PTMP was purified as previously described [3].

Subcellular localization studies. A cDNA for the full-length human PTMP (hPTMP) was subcloned into pEGFP-N2 vector (Clontech, Palo Alto, CA) to form a vector that encodes for full-length hPTMP tagged with green fluorescent protein (GFP) at its C-terminus. 3T3-L1 preadipocytes were transfected with pPTMP-GFP. Cells were incubated for 24 h after transfection and treated with 200 nM MitoTracker Red dye to stain mitochondria. Mitochondrial were visualized with a Zeiss LSM-510 confocal microscope in the Indiana Center for Biological Microscopy.

Mouse kidney mitochondria were isolated and 50 μg of proteins from tissue homogenates, mitochondria and cytosol were separated by SDS–PAGE and transferred to a nitrocellulose membrane. PTMP in these fractions was detected by Western blotting.

Assay of phosphatase activity with p-nitrophenyl phosphate. The assay was conducted at 30 °C in a reaction mixture (1 ml) containing 1–20 mM *p*-nitrophenyl phosphate in 20 mM Tris–HCl, pH 7.4, 10 mM $MnCl_2$, 0.5 mM EGTA, 3 mM β-mercaptoethanol, 0.2 mg/ml BSA, and 0.5 μg of recombinant hPTMP protein. The amount of *p*-nitrophenol formed was measured by absorbance at 410 nm. The millimolar extinction coefficient of *p*-nitrophenol was determined to be 12.4 under the conditions of these experiments.

Assay of phosphatase activity with ^{32}P-labeled PDC and BCKDC. Porcine heart PDC was obtained from Sigma (St. Louis, MO) and labeled with ^{32}Pi as described previously [3]. BCKDC was purified from rat liver [9] and labeled with ^{32}Pi by incubation with recombinant BDK and labeled ATP. Phosphorylated PDC and BCKDC (0.02–1.0 mg/ml) were incubated with 0.1 μg of hPTMP in 50 μl reaction mixture at 37 °C for 5 min in phosphatase buffer described above. Phosphatase activity was measured by methods described previously [3].

Western blot analysis of tissues. Antisera for detection of PTMP were raised in rabbits by Sigma Genosys (St. Louis, MO). A peptide (TDHTPERKDEKERIK) corresponding to the sequence of mPTMP but absent from other proteins in the genome database was conjugated with keyhole limpet hemocyanin and used as antigen. The antibody against the protein was purified by affinity chromatography on Affigel 15 beads (Bio-Rad laboratories, CA) cross-linked to the peptide according to the manufacturer's instructions. Western blot analysis of tissue extracts was done as described previously [10].

Results

Identification of a novel PP2C with a potential mitochondrial leader sequence

A systematic search of the databases for various mammalian genomes was conducted for a PP2C-type phosphatase with potential mitochondrial leader sequence. A novel phosphatase of the PP2C type was identified in the human database which was subsequently found entered in the NCBI database under GenBank Accession Nos. AY435431 and AK054678. Mouse and rat homologs (GenBank Accession Nos. AK044610 and XM_231833, respectively) are present in their respective genome databases. The protein can also be found in genome databases for chimpanzee, monkey, cattle, dog, chicken, zebra fish, and frog but not yeast.

Three different topology prediction programs (SignalP, MITOPROT, and Predator) predict a mitochondrial localization of the protein. A human EST (IMAGE: 4796865) encoding the hypothetical protein was obtained, completely sequenced and deposited as GeneBank Accession No. AY994097. This EST contains a cDNA of 2059 bp inserted into a pBluescriptR backbone with 221 bp of 5′ untranslated region, 1119 bp of coding region and 719 bp of 3′ untranslated region.

Sequence homology with other PP2C phosphatases

A multiple alignment program (www.ebi.ac.uk/clustalw) was used compare the sequence of hPTMP with known PP2C phosphatases. PTMP contains 10 out of 11 sequence motifs characteristic of PP2Cs (Fig. 1) [7]. Motif 10 is missing, but this region is not well conserved amongst members of the PP2C family, and is not essential for phosphatase activity. PP2Cs are classified as Group I or Group II enzymes based on the presence in motif 1 of consensus sequences RXXME/QD or KXXNED, respectively [11]. On this basis the protein identified belongs to Group II. Except for one aspartate (Asp 282), all residues shown previously to be involved in binding a divalent metal ion to PP2C enzymes [11] are conserved in PTMP. Although the primary sequences of PDP1 and PDP2 are quite similar (55% identity), PTMP is only 20% identical to the PDPs. On this basis PTMP is probably not a third PDP. Indeed, this relationship is similar to that observed for the PDKs versus BDK. The four PDKs are approximately 65% identical whereas BDK is only 25% identical in sequence to the PDKs.

Cloning and expression of the protein

Two forms of hPTMP were expressed in *E. coli*—the full-length protein and a truncated protein lacking the first 29 amino acids corresponding to the putative mitochondrial leader sequence. Both proteins were insoluble when expressed alone in *E. coli*. When co-expressed with GroEL/ES, the full-length protein was still insoluble whereas the truncated protein was mostly soluble. Phosphatase activity of hPTMP when expressed without Mn^{2+} in the culture medium was 50 times less than the activity of the protein when expressed with Mn^{2+}, suggesting that Mn^{2+} is required for proper folding of the enzyme. Calculated molecular weights of the full-length and the truncated protein are 40,982 and 37,690 Da, respectively. Calculated molecular weights of His-tagged full-length and truncated protein are 41,810 and 38,520 Da, respectively. On SDS–PAGE, full-length His-tagged protein runs at 48 kDa whereas the truncated His-tagged protein runs at 44 kDa. Running at a position higher than the predicted value is typical for members of the PP2C family [12,13]. As shown

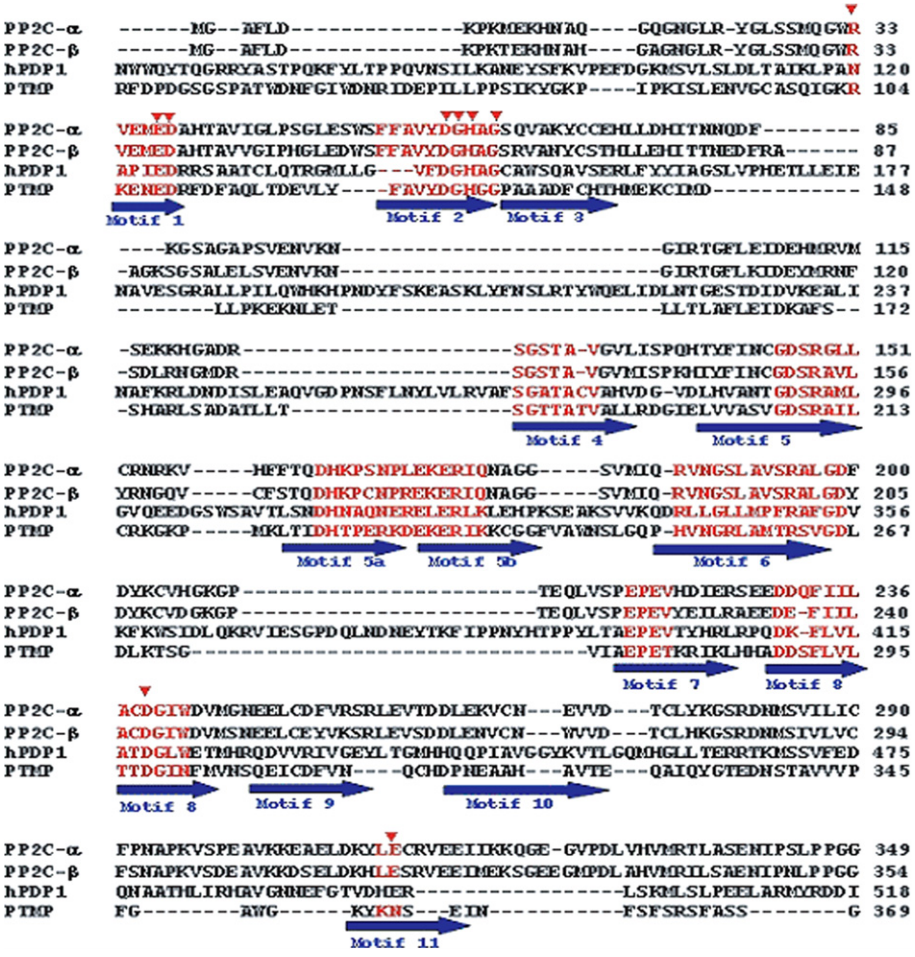

Fig. 1. Sequence alignment of PTMP with human PDP1, PP2C-α and PP2C-β (GenBank Accession Nos. Q9P0J1, P35813, and O75688, respectively). The catalytically important amino acids characteristic of PP2C phosphatases are indicated by inverted red triangles. Conserved sequence motifs are indicated by red amino acid residues and blue arrows.

below, the native mouse protein with a predicted molecular weight of nearly 38 kDa runs at 41 kDa on SDS–PAGE.

Subcellular localization of the phosphatase

The MITOPROT program predicted the mitochondrial leader sequence to be 30 amino acids long. The first 29 amino acids of the protein have typical characteristics of a mitochondrial leader sequence, including several hydrophobic and arginine residues, and the absence of acidic amino acids. Mitochondrial leader sequences cleaved by the two-step cleavage mechanism [14] are characterized by a serine at the −5 position, a hydrophobic amino acid at the −8 position, and an arginine at the −10 position relative to the cleavage site. As shown in Fig. 2A, cleavage of the leader sequence of PTMP should therefore occur between the 29th and 30th amino acid.

Confocal microscopy of 3T3L1 preadipocytes expressing the GFP-tagged protein also suggests that the phosphatase is localized to the mitochondria (Fig. 2B). GFP florescence is seen as elongated punctate objects that are characteristic of mitochondria. After staining with the mitochondria specific MitoTracker dye, the overlay image shows that the protein is localized in the mitochondria. The protein is highly enriched in the mitochondrial fraction as shown by Western blot analysis (Fig. 2C).

Phosphatase activity of the protein with p-nitrophenyl phosphate

Purified recombinant hPTMP exhibits phosphatase activity with *p*-nitrophenyl phosphate (Fig. 3A). The K_m for *p*-nitrophenylphosphate was 10.7 mM; the V_{max} 3.6 μmol/min/mg protein (Fig. 3A). No activity was seen in the absence of Mn^{2+} and the activity increased with increasing concentration of Mn^{2+} (half maximal effect at 3.7 mM). The enzyme exhibited no activity when Mg^{2+} was substituted for Mn^{2+}, in contrast to PDP1 and PDP2 and most other PP2Cs.

Activity with BCKDC and PDC

Recombinant hPTMP exhibits greater phosphatase activity with phosphorylated BCKDC than phosphory-

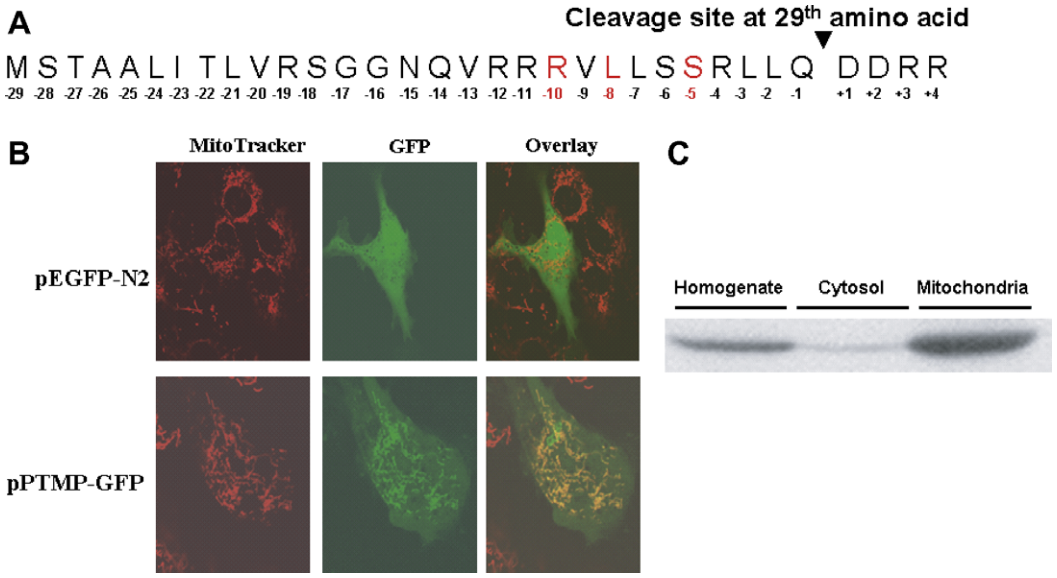

Fig. 2. Subcellular localization of the novel PP2C. (A) Mitochondrial leader sequence of PTMP. Conserved ser at −5, leu at −8, and arg at −10 positions corresponding to the putative cleavage site between the 29th and 30th amino acid are indicated in red. (B) Confocal microscopy image showing localization of the PTMP-GFP fusion protein in mitochondria of 3T3-L1 preadipocytes. (C) Western blot analysis of subcellular fractions.

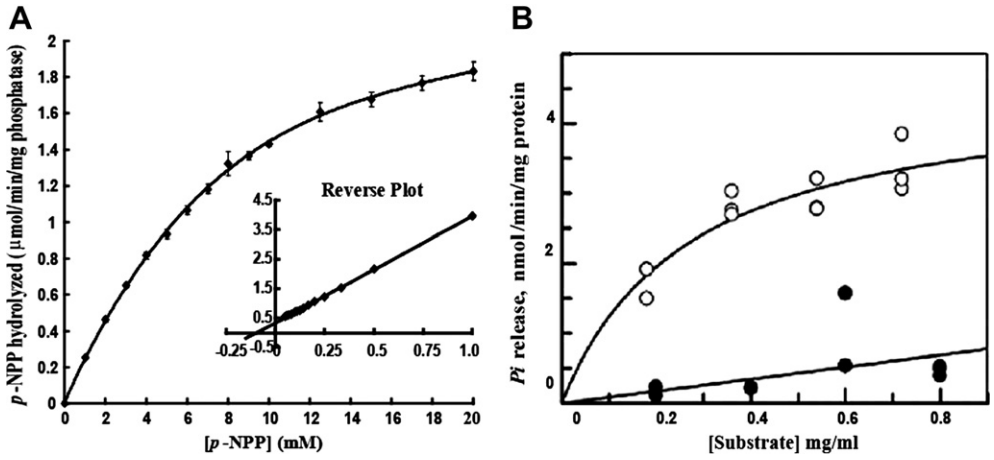

Fig. 3. (A) Phosphatase activity of PTMP with *p*-nitrophenyl phosphate. Lineweaver–Burk plot is given in the insert. (B) Phosphatase activity of PTMP towards BCKDC (○) and PDC (●).

lated PDC (Fig. 3B). Data points obtained with increasing concentrations of BCKDC defined a hyperbolic curve consistent with Michaelis–Menten kinetics. In contrast, data obtained with increasing concentrations of PDC could not be fitted to enzyme kinetics equations and kinetic values could not be estimated. The data suggest that PTMP is a not a third PDP but that it could be a BCKDC phosphatase. However, the specific activity of PTMP with BCKDC is relatively low, only 4 nmol/min/mg protein. This stands in contrast to specific activities of 35 nmol/min/mg protein for recombinant PDPs [3]. All of these values are markedly lower than the specific activity of 2 μmol/min/mg protein reported previously for BCKDC phosphatase [4,5].

Tissue specific expression

Western blot analysis of mouse tissue extracts using serum before and after immunization detected a protein at a molecular weight of about 41 kDa in antiserum-treated blot which was totally absent in the pre-immune serum treated blot, indicating that antibody was produced in the rabbit against the injected peptide (Fig. 4A). A position of 41 kDa on SDS–PAGE is too high for a protein with predicted molecular weight of 37 kDa and therefore raised the questions about the specificity of the antiserum. Recombinant hPTMP with a carboxy terminal histidine tag has a predicted molecular weight of about 39 kDa but ran at a position of about 44 kDa on SDS–PAGE.

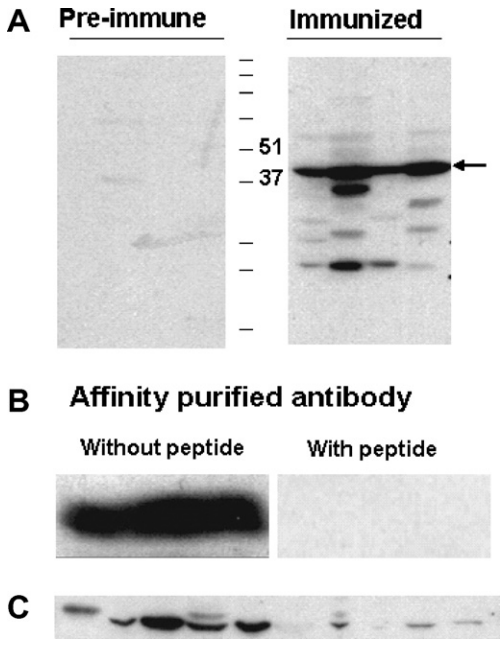

Fig. 4. (A) Western blot analysis with both pre-immune serum and serum obtained after immunization with the peptide. PTMP runs at apparent molecular weight of 41 kDa on SDS–PAGE. No corresponding band was observed at this molecular weight with pre-immune serum. Samples from left: whole liver extract, liver mitochondrial extract, whole kidney extract, and kidney mitochondrial extract. Signal corresponding to PTMP is indicated by the arrow. (B) Detection of PTMP in heart using affinity purified antibody. To test the specificity of the antibody, protein blot was incubated with antibody diluted 50 times in blocking buffer [5% milk powder in 50 mM Tris–HCl, 150 mM NaCl, and 0.1% (v/v) Tween 20] with and without 2 mg/ml peptide. The signal corresponding to PTMP was blocked by the peptide. (C) Tissue specific expression of PTMP in mice. Samples from left: recombinant hPTMP (runs slower because of his tag), brain, heart, liver, kidney, muscle, testis, spleen, lung, and adipose.

Even after accounting for a difference of 1 kDa in the molecular weights of endogenous mPTMP and His-tagged hPTMP due to the presence of six histidine residues, hPTMP was still running at much higher position than predicted. It was therefore clear that both hPTMP and mPTMP run aberrantly higher on SDS–PAGE gel. Furthermore, purification of the antibody against the peptide used for immunization specifically detected a protein at the same position. The signal obtained by using the affinity purified antibody was completely abolished if the blocking buffer contained excess of peptide used for immunization (Fig. 4B). The findings indicate that the antiserum detected PTMP and not any other protein. Western blot analyses of mouse tissues show that PTMP is highly expressed in the heart, kidney, brain, and liver and to a lesser extent in testis, lung, spleen, and adipose. Very low amount of PTMP was detected in muscle (Fig. 4C).

Discussion

A novel mitochondrial phosphatase found in the human genome database has been cloned and partially characterized. Mitochondrial proteins encoded by nuclear DNA have to be properly folded by chaperonins during their translocation into the mitochondrial matrix space. When these proteins are expressed in bacteria they often require co-expression of chaperonin proteins, e.g. GroES/EL, to obtain soluble protein. The requirement for GroES/EL for the expression of soluble protein observed in this study is consistent with its mitochondrial localization.

Sequence alignment of PTMP with consensus sequence for PP2C phosphatases shows that Asp 282 of the consensus sequence is replaced by asparagine (residue 364 in PTMP). Examination of the crystal structure of the PP2Cs reveals that the carbonyl group of this residue forms a co-ordinate bond with one of the two metal ions at the active site [11]. Since asparagine still has the carbonyl group required for metal ion binding at the active site, co-ordinate bonding with a metal ion may not be affected by this substitution.

Specific activity of hPTMP towards *p*-NPP is quite high. This is unusual for a serine/threonine phosphatase but there have been previous reports of PP2C type phosphatases with high specific activities against *p*-NPP [12,15,16]. The specific activity of hPTMP against *p*-NPP is comparable to the specific activities of the proteins described in those studies. Even though hPTMP shows phosphatase activity in presence of Mn^{2+}, unlike most other PP2Cs, hPTMP is not highly active in the presence of Mg^{2+}. The higher activity in the presence of Mn^{2+} could be attributed to greater affinity with which Mn^{2+} binds to the active site of the enzyme as compared to Mg^{2+}. In addition, when expressed in bacteria, PTMP might not be optimally folded in order to exhibit higher activity in presence of Mg^{2+}. This phenomenon of an absolute requirement for Mn^{2+} and lower activity in presence of Mg^{2+} has been observed with other PP2Cs expressed in bacteria [15].

The specific activity of recombinant PTMP is greater towards BCKDC than PDC but still relatively low towards BCKDC, which again could be due to improper folding of the protein. PTMP shows much lower activity when expressed without Mn^{2+} in the culture media. Proper folding of the protein may require other factors. It is also possible that post-translational modification or an accessory protein may be necessary for maximum enzyme activity. The possibility that BCKDC is not the most important substrate for this phosphatase can not be excluded.

Purification of a mitochondrial divalent cation-independent, spermine-stimulated phosphatase related to the PP2A family of phosphatases was reported by Damuni and Reed [17]. Damuni and Reed also reported the purification of BCKDC phosphatase, first in the form of a complex of 460 kDa [4], and subsequently as a 33 kDa catalytic subunit [5]. Metal ions were not required for activity and *p*-nitrophenyl phosphate was not hydrolyzed, clearly differentiating it from PTMP. Sequence information is not available, but clearly their enzyme was not a member of the PP2C family of phosphatases, and therefore was not related to the PDPs or PTMP.

Studies of mitochondrial phosphoproteins have been traditionally carried out by following the incorporation of ^{32}Pi into mitochondrial proteins. Owing to the higher activity of their kinases and the abundance of their expression in mitochondria, PDC and BCKDC are readily phosphorylated and were long thought to be the only proteins regulated by this mechanism in mitochondria. The failure of such methods to discover additional phosphoproteins in mitochondria could be attributed to lower expression levels and lower rates of covalently linked phosphate turnover. Recent studies suggest that second messenger molecules and the translocation of kinases may be involved in phosphorylation of mitochondrial proteins. The use of highly sensitive phosphospecific protein–dyes has lead to the detection of phosphoproteins that escaped previous investigations. The emergence of phosphorylation as a widespread mechanism of regulation of mitochondrial processes has been reviewed recently [18]. For example, the 18 kDa subunit of complex I is phosphorylated by protein kinase A and it has been suggested that a Mg^{2+}-dependent PP2C-type phosphatase must exist in the mitochondrial inner membrane for its dephosphorylation [19]. Hopper et al. [20] presented evidence for the potential phosphorylation of many proteins in mitochondria. The enzyme activity of only a few mitochondrial proteins other than PDC and BCKDC has been shown affected by phosphorylation. The growing list currently includes Bcl-XL/Bcl-2-associated death promoter [21,22] and a subunit of cytochrome oxidase [23,24]. Mutations in PDP1 cause lactic acidosis [27] while mutations in PTEN-induced kinase, recently localized to mitochondria, induce a rare form of Parkinson's disease [28]. These findings call for the identification and study of additional mitochondrial kinases and phosphatases to gain a detailed understanding of mitochondrial phosphorylation events and their physiological significance. With the genome databases largely complete, the characteristics of mitochondrial phosphatase and kinases such as sequence similarity and presence of mitochondrial leader sequences can be exploited to identify additional mitochondrial kinases and phosphatases.

Acknowledgments

This work was supported by grants from US Public Health Service (NIH DK 47844 and DK 61594, R.A.H.; DK 56898, K.M.P.) and the Midwest American Heart Association (M.J.).

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