Cloning and characterization of a chicken protein tyrosine phosphatase, CPTP1

Choong Won Kim[1], Eun Joo Jung[1], and Yoon-Se Kang[1,2]

1 Department of Biochemistry and Gyeongsang Institute of Cancer Research, Gyeongsang National University College of Medicine, Chinju 660-280, Korea

2 Corresponding author

Abstract

Protein tyrosine phosphorylation and dephospho-rylation are an important regulatory reactions in cell physiology. We have cloned a cDNA that encode a cytosolic protein tyrosine phosphatase (CPTP1) from chicken intestine cDNA library. Amino acid sequence identity between the CPTP1 and low molecular weight form of human placenta enzyme (HPTP1B) was 92%. CPTP1 lacked 13 amino acids in N-terminal region, while it had an additional 48 amino acids in the C-terminal region in comparison with the truncated form of HPTP1B of 321 amino acids. This C-terminal sequence was different from those of all known PTPs. The CPTP1 does not have a membrane targeting or nuclear localization sequences at its C-terminus like other PTPs such as HPTP1B and murine homolog of the human T-cell protein tyrosine phosphatase (MPTP) do. The cloned cDNA has been expressed in *E. coli* and purified by affinity chromatography. Dephos-phorylation kinetics of this enzyme closely resembled those of the known PTPs. The dephosphorylation reaction required a reducing agent such as glutathione and dithiothreitol and was inhibited by sodium vanadate and formal-dehyde. Deletion of 72 amino acids from C-terminal side of CPTP1 gene resulted in higher expression in *E. coli* and more potent phosphatase activity than wild type CPTP1 gene product. This result suggests that the C-terminal region of the CPTP1 protein negatively regulates phosphatase activity. These results also imply that CPTP1 might be a nontransmembrane-type enzyme with a structure and localization specificity distinct from other known cytosolic PTP1B type homolog.

Keywords: chicken, protein tyrosine phosphatase

Introduction

Phosphorylation of proteins on tyrosyl residues is a mechanism by which multiple cellular events are regulated (Cantley *et al*., 1991). At steady state, the phosphorylation of a tyrosyl residue depends on the relative activities of protein tyrosine kinases (PTKs) and protein tyrosine phosphatases (PTPs). Most works on phosphorylation of a tyrosyl residue until 1988 had been focused on PTKs but with the determination of partial amino acid sequence of a PTP, which led to the cloning of a whole PTP gene in human by Tonks and his coworkers (Charbonneau *et al*., 1988), PTP begins to attract as much attention as PTKs. However, the function and substrate specificity of the PTPs are still unclear but there are many convincing line of reports that it might underlie the dephosphorylation of specific key regulatory proteins. On the basis of their function, structure and sequence, the PTPs can be grouped into transmembrane and nontransmembrane families (Fischer *et al*., 1991).

The prototype of nontransmembrane PTP, HPTP1B, was originally identified as the major PTP in human placenta (Tonks *et al*., 1988a, b). The sequence of this protein purified from placenta is truncated at amino acid 321. Thus it lacks the hydrophobic C-terminus. Molecular cloning of human (Chernoff *et al*., 1990; Brown-Shimer *et al*., 1990) and rat brain PTP (Guan *et al*., 1990) identified proteins of 435 and 432 amino acids, respectively, possessing two distinct structural motifs: the phosphatase catalytic domain (approxi-mately amino acid residues 40-276) and a highly hydrophobic stretch of amino acids near the C-terminus (amino acid residues 401-431 in human) (Charbonneau *et al*., 1989).

By analogy to the results that the known localization of non-transmembrane tyrosine kinase and their substrates, it seemed likely that the cytosolic PTPs would be found in plasma membrane, cytoskeleton, and/or nucleus. A PTP closely related to PTP1B from human T-cell (TPTP) has been cloned and when it is overexpressed in baby hamster kidney cell, the PTP associates exclusively with particular fraction (Cool *et al*., 1990). Further the association of the TPTP with the particulate has been shown to require its 98 C-terminal amino acids. Subsequently, using truncations and

domain swap experiments Frangioni *et al.* (1992) showed that C-terminal 35 amino acids of PTP1B were both necessary and sufficient for targeting to the endoplasmic reticulum. These discovery of the role of C-terminal hydrophobic region affords to propose the so-called localization specificity of these enzymes and contributes to elucidate the role of this region in PTP1B isoform.

To understand the role of a PTP in chicken undergoing rapid cell proliferation and differentiation in embryos, we cloned cDNA of a PTP called CPTP1 and deduced its amino acid sequences. The CPTP1 did not have a hydrophobic C-terminal targeting or localization sequences found in the PTP homologs of higher eukaryotes. This paper describes the expression of CPTP1 in *E.coli*, purification by affinity chromatography and its kinetic characterization.

Materials and Methods

Materials

Restriction enzymes, T4 DNA ligase, calf intestinal alkaline phosphatase, and Klenow DNA polymerase were purchased from Boehringer Mannheim (GmbH, Germany) or Bethesda Research Labo-ratories (Gaithersburg, MD). All chemicals, unless otherwise specified, were purchased from Sigma Chemical Company (St. Louis, MO). Yeast extract, tryptone, and bacto-agar were purchased from Difco. [α-^{32}P]dCTP and [α-^{35}S]dATP were from New England Nuclear and Amersham. All sequencing reactions were performed using Sequenase kit (United States Biochemical).

Isolation of cDNA clones from chicken intestine cDNA library

A chicken intestine cDNA library in λgt10 (provided by P. Matsudaira, Whitehead Institute for Biomedical Research, MIT), was plated at a density of 50,000 plaques/150-mm plate. Plaques were transferred to nitrocellulose filters according to the method of Grunstein and Hogness (1975). A total 500,000 recombinants were initially screened using a ^{32}P-labeled *Eco*RV-*Eco*RV 291 bp fragment (probe A) encoding amino acid sequences of the highly conserved catalytic domain within HPTP1B. Hybridization was carried out by a standard methods (Sambrook *et al.*, 1990). Nine positive clones were selected, subcloned into M13mp18/19, or pUC19 cloning vectors, and subjected to sequence analysis by the dideoxy nucleotide chain termination method (Sanger *et al.*, 1977).

Gel-directed Northern and Southern hybridization

For Northern analysis, 17 and 34 μg of total RNA or 10 μg of poly(A)$^{+}$ RNA isolated from 4 day chicken embryos were denatured in 25 μl of solution containing 1$\times$MNE (22 mM morpholinopro-panesulfonic acid, pH 7.0/5 mM sodium acetate/0.1 mM EDTA), 6.15 M formaldehyde, 17.5% formamide, and 0.01% bromo-phenol blue. The mixture was incubated at 60°C for 5 min, and then quick-chilled. Samples were electro-phoresed on 1% formaldehyde/agarose gel and soaked in distilled water for 2 h. The gel was washed in 6$\times$SSC (1$\times$SSC = 0.15 M sodium chloride/0.015 M sodium citrate, pH 7.0) for 10 min, and dried at 60°C for 1 h under the vacuum. Prior to prehybridization the dried gel was soaked in 6$\times$SSC for 5 min. The gel was prehybridized in prehybridization solution containing 6 x SSC/5$\times$Denhardt's solution/0.05% sodium pyrophos-phate/100 μg/ml herring sperm DNA/0.5% SDS at 65°C for 2 h, and hybridized in solution containing 6$\times$SSC/1$\times$Denhardt's solution/100 μg/ml yeast tRNA/0.05% sodium pyrophosphate/radiolabeled probe A (2-3$\times$10^{6} cpm/ml) at 65°C overnight. The gel was washed twice in 2$\times$SSC/0.1% SDS at room temperature for 5 min and three times for 30 min at 65°C in the same solution. The gel was dried at 60°C for 30 min under the vacuum and visualized by autoradiography with intensifier screen at -70°C.

For Southern hybridization, λgt10-cDNAs isolated from 9 positive clones were digested with *Eco*RI and *Not*I, and the insert DNA was analyzed on 1% agarose gel in TAE buffer (40 mM Tris, 12 mM sodium acetate, 2 mM EDTA, pH 8.3). The gel was dried under the vacuum at 60°C for 1 h. The gel was denatured in a solution containing 0.2 M NaOH/0.6 M NaCl at room temperature for 30 min prior to prehybridization, and then neutralized in a solution containing 0.2 M Tris (pH 7.4)/0.6 M NaCl at room temperature for 30 min twice. The other steps were done as described for the Northern analysis.

Construction of GST-PTP fusion plasmids

The CPTP1 cDNA in pUC19 was digested partially with *Eco*RI and linked in frame to the glutathione *S*-transferase (GST) gene of the pGEX-2T vector (Smith and Johnson, 1988) to generate the GST-CPTP1 fusion protein coding for amino acids 1-356 of CPTP1. For generation of the GST-CPTP1-1 fusion protein lacking in 72 amino acids in C-terminal side of CPTP1, pUC19-CPTP1 was digested with *Eco*RI and *Bgl*II and filled using the Klenow fragment of DNA polymerase I. This CPTP1-1 cDNA fragment was ligated to the *Sma*I-digested pGEX-1N vector. For generation of another GST-HPTP1B coding for amino acids 1-321 of HPTP1B, the truncated HPTP1B cDNA fragment was ligated to the *Sma*I-digested pGEX-2T. The resulting plasmids contained the coding sequence for CPTP1, CPTP1-1 and HPTP1B in frame with GST gene and

were transformed into *E. coli* strain BL21(DE3).

Expression and purification of GST-PTP fusion proteins in *E. coli*

Overnight cultures of *E. coli* transformed with parental or recombinant pGEX plasmids were diluted 1:10 in fresh LB medium containing ampicillin (100 µg/ml) and grown for 1 h at 37°C before adding isopropyl β-D-thiogalactopyranoside. When the cells had reached an OD_{600} of 0.6-1.0, isopropyl β-D-thiogalactopyranoside (1 mM) was added to the medium and the culture was incubated for 3 h. In a typical isolation of recombinant protein, 2.5 g of cells were resuspended in 15 ml of 130 mM NaCl/20 mM sodium phosphate (pH 7.4) containing 1% (v/v) Triton X-100 and were broken by sonication (Carlino *et al*., 1992). The lysate was centrifuged at 10,000 r.p.m. for 7 min, and the supernatant was mixed with 500 µl of glutathione agarose that had been equilibrated with the above buffer at 4°C. The beads were collected by centrifugation at 3,000 r.p.m. for 1 min and washed three times with 10 volumes of 50 mM Tris (pH 7.4)/150 mM NaCl buffer. Fusion proteins were eluted with 50 mM Tris (pH 8.0) containing 20 mM reduced glutathione. Absorption of fusion protein to the glutathione agarose beads and subsequent elution were done within 5 min.

Kinetic assay of CPTP1, CPTP1-1, and HPTP1B

Reactions were carried out in a volume of 100 µl containing 0.1 M p-nitrophenyl phosphate (pNPP), 0.1 M Tris (pH 8.0), 0.25 M NaCl, 5 mM EDTA, and 10 mM dithiothreitol or glutathione at 37°C for 10 min (Dunphy *et al*., 1991). At the end of the incubation, the reaction was terminated by the addition of 200 µl of 0.1 M NaOH. Absorbance of reaction mixture at 410 nm was measured with the spectrophotometer. Assay buffer lacking PTP in the reaction mixture was used as a blank.

Results and Discussion

Isolation of a CPTP cDNA

To study the expression of PTPs in chicken embryo, total RNA was isolated from 4 day embryos. Poly(A)+ RNA was also isolated from total RNA by oligo(dT)-cellulose. Total RNA and poly(A)+ RNA samples were electrophoresed and hybridized with the ^{32}P-labeled probe A. As shown Figure 1, a single RNA band of about 5 kb was detected in the chicken embryo extracts. This result suggested that a 5-kb transcript of PTP was expressed in chicken embryo.

We then screened chicken intestine cDNA library in λgt10 with probe A. Among 500,000 recombinants, 9 positive clones were selected finally. To determine whether the clones were related, they were digested with *Eco*RI and *Not*I, and analyzed by Southern hybridization with probe A (Figure 2) The result showed that cDNAs of all selected clones had two *Eco*RI fragments (960 bp, 780 bp) and one *Not*I fragment
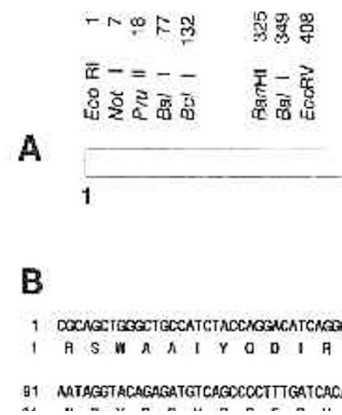
Figure 1. Expression of a CPTP mRNA in 4-day chicken embryos. Total RNA and poly (A)+ RNA were isolated from 4-day chicken embryos, and separated on 1% formaldehyde/agarose gel. Lane 1 and 2 contain 17 µg and 34 µg of the total RNA, respectively. By comparison, 10 µg of poly (A)+ RNA is loaded in lane 3. Expression of a CPTP mRNA was examined by hybridization with the ^{32}P-labeled probe A by gel-directed Northern hybridization method as described under "Materials and Methods".

Figure 2. Gel-directed Southern hybridization analysis. Nine positive λgt10-cDNAs were digested with *Eco*RI (E) and *Not*I (N), electrophoresed on 1% agarose gel, and hybridized with the ^{32}P-labeled probe A. M denotes a ^{32}P-labeled λ/*Hind* III size marker.

(1731 bp), suggesting they are the same cDNAs.

DNA sequence analysis

A diagrammatic representation of CPTP1 with restriction enzyme sites and deduced amino acid sequence are illustrated in Figure 3A and 3B, respectively. The isolated CPTP1 cDNA consisted of 1,718 nucleotides with an open reading frame coding for 356 amino acid residues. The open reading frame terminates with a TAA stop codon followed by 646 bp of 3' untranslated region. We did not find a polyadenylylation signal site or a 3' poly(A)$^+$ tail, suggesting that intact mRNA of CPTP1 contains more nucleotides than the isolated cDNA clone. The other PTPs without polyadenylylation signal and 3' poly(A)$^+$ tail have been reported (Cool *et al*., 1989; Guan *et al*., 1991). We note that Northern hybridization analysis showed a clear band of 5 kb (Figure 1). These results indicated that about 5-kb band could be another CPTP or CPTP1 may be a truncated form in the N-terminal region.

Analysis of amino acid of CPTP1 and comparison with other nontransmembrane-type PTPs

Deduced amino acid sequence of CPTP1 was aligned with yeast and mammalian nontransmembrane-type PTPs and the result is shown in Figure 4. Sequence identity between these enzymes was more than 95% in the conserved central region, but the sequences in the N- and C-terminal regions were distinct. The CPTP1 sequence contained a single highly conserved PTP catalytic sequence (VHCSAGIGRSG) and was shorter than those of other mammalian PTPs. In the catalytic sequence, there was a nucleotide-binding fold, GXGXXG, which was also found in various dehydrogenases, G-proteins and protein kinases (Pot *et al*., 1991). Nucleotide-binding fold was also located within the highly conserved active site region of other PTPs. A second absolutely conserved residue, aspartic acid, was observed in WPDF sequence. It was located at 34 N-terminal side of cysteine in catalytic motif. Located on a mobile loop in three-dimensional structure this aspartic acid can donate a proton acting as a general acid, enhancing catalytic activity of this enzyme.

CPTP1 contained another PTP motif FWXMVW and FKVRES, which are similar to FLVRES originally identified in the SH2 domain of the tyrosine kinase Src. The presence of SH2-like consensus sequence within CPTP1 suggested that CPTP1 binds to phospho-tyrosine of target proteins. In the CPTP1 sequence there were five casein kinase II (CKII) phosphorylation motifs (S/TXXD/E) and one mitogen-activated protein kinase phosphorylation site (P-E-S-P), suggesting that CPTP1 activity is regulated by phosphorylation at these sites.

The deduced amino acid sequence of isolated CPTP1 cDNA showed 92% sequence identity to the corresponding 321 residues of the HPTP1B (Figure 4). In comparison with truncated 321 amino acid HPTP1B, CPTP1 lacked 13 amino acids in N-terminal region, while it had an additional 48 amino acids in the C-terminal region. Amino acid sequence of this region completely differed from the C-terminal region of full
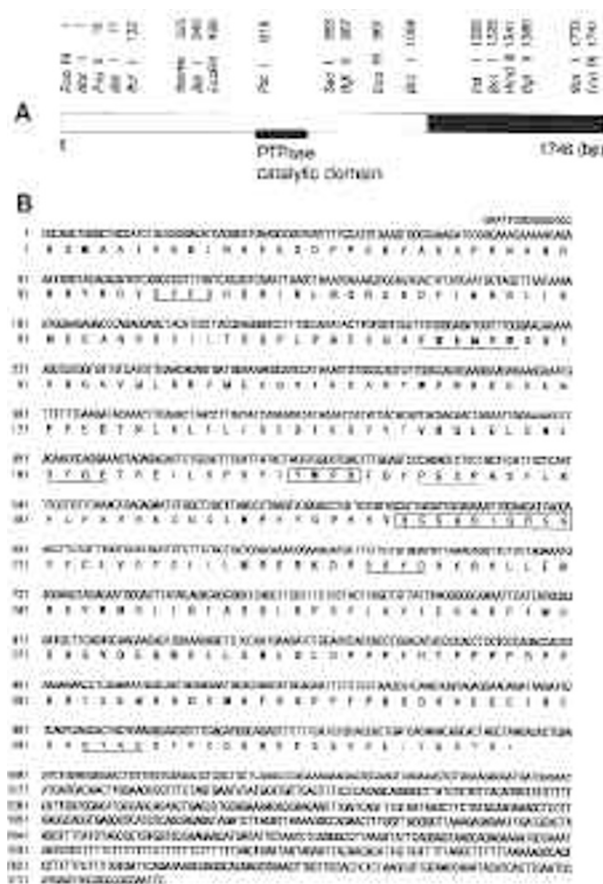

Figure 3. Restriction map, nucleotide and deduced amino acid sequences of the CPTP1 cDNA. (A) Restriction map of the CPTP1 cDNA. 3' untranslated regions are depicted by the black box, while the coding region is designated by the open box. The numbers at each restriction site in CPTP cDNA indicate numbers of base pair in the clone generated by cleavage and the scale of the map is shown in bp. (B) Nucleotide and deduced amino acid sequences of the CPTP1 cDNA. The nucleotide and the amino acid residues are numbered from the codon (CGC) and the arginine residue (R) is derived downstream from the linker sequence (GAATTCGCGGCCGC). Amino acid residues are denoted by the standard single letters underneath the nucleotide sequence. Termination codon is marked with asterisks. PESP (potential phosphorylation site by MAP kinase), S/TXXD/E(potential phosphorylation sequences by CKII), FKVRES (sequence found in SH2), GXGXXG (nucleotide-binding site) and another conserved motif of PTP (FWXMVW) are underlined. Catalytic domain of PTP (VHCSAGIGRSG) and TWPD are shown by open box.
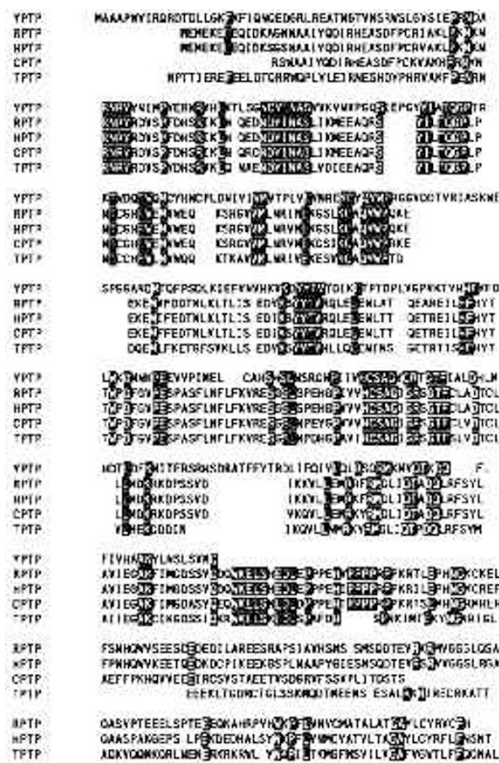
Figure 4. Amino acid sequence alignment of chicken PTP1 (CPTP), yeast PTP (YPTP), rat brain PTP (RPTP), human placenta PTP1B (HPTP), and human T-cell PTP (TPTP). The completely conserved amino acids are shown in black. Gaps (blank space) are introduced to maximize amino acid alignment. The underlined amino acid sequence of CPTP (position 309-356) is different from the PTPs found in higher eukaryotes

length 435 amino acids of HPTP1B. HPTP1B, TPTP and RPTP contain a C-terminal hydrophobic domain, which could function in targeting or localization of the protein to the cellular membrane. By contrast the C-terminus of CPTP1 was different from those of these nontransmembrane-type PTPs of higher eukaryotes. Therefore, CPTP1 does not have a C-terminal targeting sequences as other mammalian PTPs. From this result it was suggested that the CPTP1 belongs to another nontransmembrane-type PTP localized in cytoplasmic compartment.
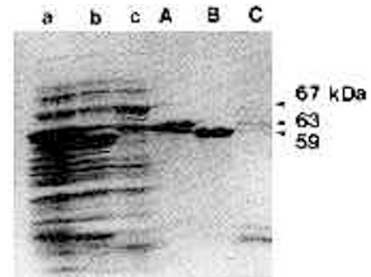

Figure 5. Affinity-purification of CPTP1-1, CPTP1, and HPTP1B GST-fusion proteins expressed in *E. coli*. Bacterial lysates shown in lanes a, b, and c were affinity-purified on S-hexylglutathione agarose bead and the purified proteins are shown in lanes A, B, and C lanes. Lanes a and A, HPTP1B; lanes b and B, CPTP1-1; lanes c and C, CPTP1. Molecular weights of isolated CPTP1-1, CPTP1 and HPTP1B fusion proteins are identified with 59, 67, and 63 kDa marker proteins.
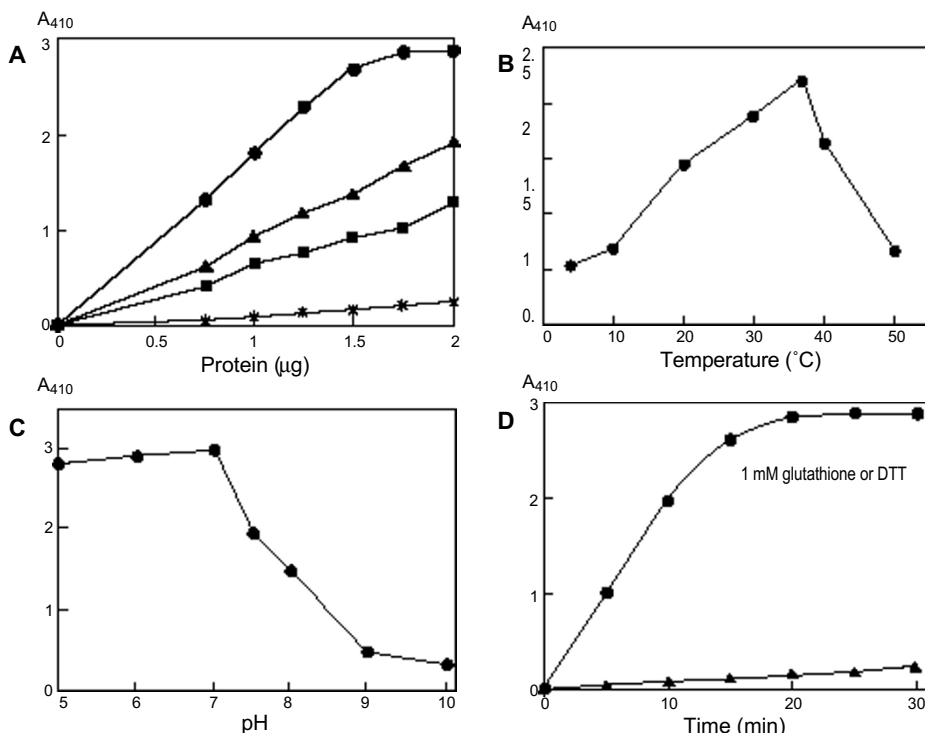

Figure 6. Determination of phosphatase activity. (A) Comparison of phosphatase activities of CPTP1-1, CPTP1, and HPTP1B. CPTP1-1 (●), CPTP1 (■), and HPTP1B (▲) were assayed at the indicated concentrations for their ability to hydrolyze pNPP as described in Materials and Methods. As a control, GST protein was assayed under the same conditions (✳). (B) Effect of temperature on phosphatase activity of CPTP1-1. (C) Effect of pH on phosphatase activity of CPTP1-1. (D) pNPP hydrolytic activities of CPTP1-1 in the presence (●) and absence (▲) of 1 mM glutathione or dithiothreitol.

Enzyme activity of CPTP1

To characterize the activity of CPTP1, the GST-CPTP1 fusion cDNA construct was expressed in *E. coli* and purified by the method using glutathione-agarose affinity chromatography (Figure 5). On an analysis by SDS/PAGE, the isolated CPTP1-1, a C-terminal truncated form of CPTP1, and HPTP1B showed single bands, but the full-length CPTP1 showed several faint bands with different mobilities, suggesting that the product was degraded. In addition its recovery was low. Since thrombin treatment did not result in the cleavage of GST-CPTP1 and GST-HPTP1B fusion proteins, carrier-free PTP proteins could not be obtained.

To assay phosphatase activity of the purified recombinant PTPs, the fusion proteins were added to the reaction mixture containing phosphatase substrate, pNPP. As shown in Figure 6A, hydrolysis of pNPP was dependent on concentration of these PTPs and phosphatase activity of CPTP1-1 was shown to be higher (2.6 fold at 1 μg of PTP) than those of full-length CPTP1 and HPTP1B, suggesting that C-terminal part of this enzyme had negative regulatory function or it might be cleaved for full enzyme activity in the cell. The optimum temperature of CPTP1-1 phosphatase activity was 37°C (Figure 6B) and hydrolysis of pNPP occurred easily under acidic conditions, although the reaction displayed a broad pH optimum between 5 and 7 (Figure 6C). This optimal pH profile also distinguished CPTP1-1 from HPTP1B, which hydrolyzed pNPP optimally at pH 5.0-5.5 (Tonks *et al*., 1988b). As observed in other phosphatases, PTP-dependent hydrolysis of pNPP absolutely required reducing agent such as dithiothreitol or glutathione (Figure 6D).

Inhibition of phosphatase activity

Sodium vanadate, a potent inhibitor of the known PTPs, strongly inhibited the pNPPase activity of the CPTP1-1: half and complete inhibition occurred at 300 mM and 10 mM, respectively (Figure 7A). All of the known PTPs contain a critical cysteine residue in catalytic site that is proposed to act as the nucleophile, forming a thioester intermediate and initiates hydrolysis of the phosphate ester linkage with the phenyl side chain (Streuli *et al*., 1989, 1990; Guan and Dixon, 1990; Guan *et al*., 1991). When the enzyme is treated with alkylating agents, the phosphatase activity was completely blocked by modification of this cysteine residues. The CPTP1-1 was also sensitive to formaldehyde, undergoing the same inhibition mechanism (Figure 7B). The inhibitory action of formaldehyde to CPTP1-1 was stronger than that of sodium vanadate: half maximal inhibition by formaldehyde occurred at 3 μM and total inhibition occurred at 100 μM. The result confirmed that the cysteine residue is critical to this tyrosine phosphatase activity.
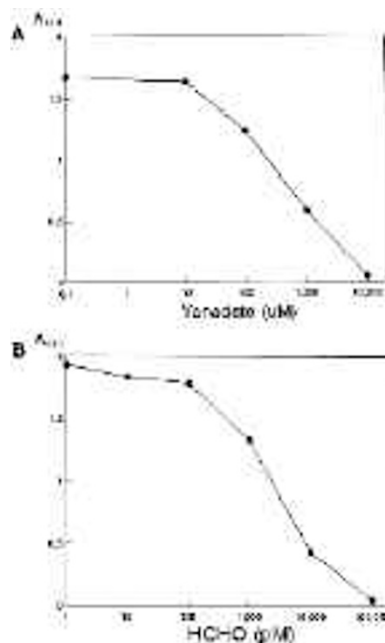

Figure 7. Inhibition of phosphatase activity of CPTP1-1. (A) sensitivity by sodium orthovanadate. (B) Alteration of a conserved cysteine residue by treatment with formaldehyde.
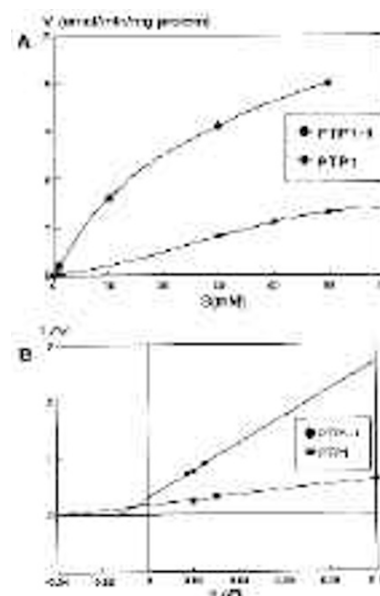

Figure 8. Kinetic analysis of CPTP1-1 and CPTP1 -dependent hydrolysis of pNPP. (A) Plot of the initial reaction velocity versus substrate concentration. (B) Lineweaver-Burk plot of the data in (A).

Kinetic analysis of pNPP cleavage by CPTP1 and CPTP1-1

Hydrolysis of pNPP was measured in the presence of CPTP1 and CPTP1-1 with various concentrations of substrate (Figure 8A). A Lineweaver-Burk plot of the data revealed that the K_m for the CPTP1 was 83.3 mM using pNPP as substrate and V_{max} was 3.6 μmol per

min per mg of the enzyme (Figure 8B). By contrast, K_m value of CPTP1-1 was 25 mM pNPP and V_{max} was 6 μmol per min per mg of the protein. Thus, deletion of 72 amino acids in C-terminal side resulted in higher substrate affinity and phosphatase activity than full CPTP1 protein. This result indicated that the C-terminal region of the CPTP1 protein acts as negative regulator domain for phosphatase activity.

Acknowledgement

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