BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS **246,** 238–242 (1998)
ARTICLE NO. RC988605

Multiple Phosphorylation of Chicken Protein Tyrosine Phosphatase 1 and Human Protein Tyrosine Phosphatase 1B by Casein Kinase II and p60$^{c\text{-}src}$ *in Vitro*

Eun Joo Jung, Yoon-Se Kang, and Choong Won Kim[1]
Department of Biochemistry and Gyeongsang Institute of Cancer Research, College of Medicine, Gyeongsang National University, Chinju 660-280, Korea

Received March 4, 1998

We have cloned a soluble chicken protein tyrosine phosphatase, named CPTP1, from the cDNA library of chicken intestine. The CPTP1 showed 92% sequence identity to the corresponding 321 amino acid residues of human PTP1B (HPTP1B). CPTP1 lacked 13 amino acids of the N-terminal region compared with HPTP1B, while the C-terminal 48 amino acid sequence of this protein was distinct from those of other PTPs. *In vitro* phosphorylation and phosphoamino acid analysis showed that both CPTP1 and HPTP1B were phosphorylated on serine and threonine residues near their N-terminus by casein kinase II (CKII). Furthermore, phosphorylation of CPTP1 by CKII resulted in an inhibition of its phosphatase activity *in vitro.* Interestingly, both CPTP1 and HPTP1B were also tyrosine-phosphorylated near their N-terminus by p60$^{c\text{-}src}$. When we examined the vanadate effect, in the absence of vanadate, the tyrosine-phosphorylated CPTP1 by p60$^{c\text{-}src}$ was autodephosphorylated by its own phosphatase activity. These results suggest that both CPTP1 and HPTP1B might play an important role in CKII- and p60$^{c\text{-}src}$-induced signal transduction cascades.

The protein tyrosine phosphorylation and dephosphorylation have been known to play a central role in signal transduction cascades of cell growth, differentiation, malignant transformation, and cell cycle. Under normal condition, the level of phosphotyrosine of proteins in eukaryotic cells can be regulated tightly by the two opposing enzymes, protein tyrosine kinases (PTKs) and protein tyrosine phosphatases (PTPs). As were in the PTKs, the family of PTPs are divided into two major classes, such as transmembrane (or receptor-type) and nontransmembrane (or nonreceptor-type) forms (1).

[1] To whom reprint requests should be addressed. Fax: +82 (591) 759-8005. E-mail: cwkim@gshp.gsnu.ac.kr.

The prototype of nontransmembrane PTP, HPTP1B, was originally identified as the major PTP in human placenta (2,3). Flint *et al.* (4) reported that HPTP1B is phosphorylated *in vitro* at Ser-386 by the mitotic protein serine and threonine kinase p34^{cdc2} and at Ser-378 by protein kinase C (PKC). They further demonstrated that in nocodazole-arrested cells HPTP1B was phosphorylated at Ser-352 and Ser-386. Until now, HPTP1B has been known to be phosphorylated on serine residues near its C-terminus both *in vitro* and *in vivo* (4-6). Nothing has been reported about the phosphorylation of HPTP1B by CKII and p60$^{c\text{-}src}$.

To understand possible regulatory role of PTP in chicken, we have cloned a nontransmembrane chicken protein tyrosine phosphatase1 (CPTP1). The CPTP1, however, did not have a hydrophobic C-terminal targeting or localization sequences reported in the HPTP1B-type PTPs, such as HPTP1B (5) and rat PTP1 (7). Instead CPTP1 contained several potential phosphorylation motifs of casein kinase II (CKII) (S/T-X-X-D/E), p56^{lck} [(I>E>V)-Y-(E>G)-(E>D>P>N)-(I/V>L)], and MAP kinase (P-E-S-P). Since p56^{lck} is a subfamily of p60^{src}, we tested whether CPTP1 could be also phosphorylated by p60^{src}. In this report, we demonstrate that both CPTP1 and HPTP1B are phosphorylated multiply on threonine and tyrosine residues as well as serine near their N-terminus by CKII and p60$^{c\text{-}src}$ *in vitro,* suggesting a possible involvement of CPTP1 and HPTP1B in signaling cascades of CKII and p60$^{c\text{-}src}$ *in vivo.*

MATERIALS AND METHODS

Isolation of PTP cDNA clones from cDNA library of chicken intestine. A chicken intestine cDNA library in l gt 10 was plated at a density of 50,000 plaques/150-mm plate. Five hundreds thousand recombinants were screened by a standard hybridization method (8) using ^{32}P-labeled *Eco*RV-*Eco*RV 291 bp fragment (probe A) encoding amino acids of the highly conserved catalytic domain within HPTP1B. 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 (9).

0006-291X/98 $25.00

Construction of expression vectors. The CPTP1 cDNA in pUC19 (pUC19-CPTP1) was digested with *Not*I, and the fragment was filled with Klenow fragment and linked into *Sma*I-digested pGEX-2T vector to generate the GST-CPTP1 fusion protein coding for amino acids 1-356 of CPTP1 (pGEX2T-CPTP1). For generation of GST-CPTP1-1 fusion protein (deleted form of 72 amino acids in the C-terminal side of CPTP1), pUC19-CPTP1 was digested with *Eco*RI and *Bgl*II, and the fragment was filled with the Klenow fragment and ligated to *Sma*I-digested pGEX-1N vector (pGEX1N-CPTP1-1). For generation of GST-CPTP1-2 (deleted form of 41 amino acids in the C-terminal side of CPTP1), pGEX2T-CPTP1 was digested with *Eco*RI and then filled by ligation (pGEX2T-CPTP1-2). For generation of GST-HPTP1B (truncated form coding for amino acids 1-321 of HPTP1B), the truncated HPTP1B cDNA fragment was filled with Klenow fragment and then ligated to *Sma*I-digested pGEX-2T vector (pGEX2T-HPTP1B).

Bacterial expression and purification of GST fusion proteins. pGEX2T, pGEX1N-CPTP1-1, pGEX2T-CPTP1-2, and pGEX2T-HPTP1B plasmids were transformed into BL21(DE3) *E. coli* and expressed by induction with 1 mM IPTG. The resulting GST, GST-CPTP1-1, GST-CPTP1-2, and GST-HPTP1B proteins were purified by affinity chromatography using S-hexylglutathione agarose beads as described by Carlino *et al.* (10). For thrombin cleavage, the beads absorbing GST-CPTP1-2 and GST-HPTP1B fusion proteins were reacted with human thrombin (Sigma) at 25°C for overnight in the thrombin cleavage buffer containing 150 mM NaCl and 2.5 mM $CaCl_2$.

In vitro phosphorylation. The phosphorylation reaction of CPTP1 and HPTP1B was carried out at 30°C for 10 min by CKII or 30 min by $p60^{c\text{-}src}$ kinase in a total reaction volume of 20 mkl containing kinase buffer (50 mM Tris (pH 7.5), 150 mM NaCl, 10 mM $MgCl_2$, and 10 mM DTT), 1 mM sodium vanadate, and 5-10 maCi of [gi-^{32}P]ATP. For a kinetic study of phosphorylation rate following a time course, CPTP1 and HPTP1B were reacted for 1, 5, 10, 30, and 60 min. The reaction was terminated following the time course by mixing each sample with SDS sample buffer, boiled, and run on a 12% SDS-PAGE. After staining and destaining, the gel was exposed to an x-ray film to obtain autoradiography.

Phosphoamino acid analysis. ^{32}P-labeled CPTP1 and HPTP1B were prepared by *in vitro* phosphorylation as described above and then separated by SDS-PAGE. ^{32}P-labeled proteins were transferred to the PVDF membrane and subjected to an acid hydrolysis as described by Matsudaira (11). The aqueous hydrolysate was lyophilized and resolved by thin layer chromatography (TLC) in a solvent system of glacial acetic acid: pyridine: n-butanol: H_2O = 15: 50: 75: 60.

Determination of the tyrosine phosphatase activity. To investigate the regulation of the phosphatase activity of CPTP1 by CKII-mediated phosphorylation, GST-CPTP1-1 was phosphorylated at 30°C for 30 sec to 10 min with or without CKII. The phosphatase reaction was performed at 37°C for 10 min in a total reaction mixture of 100 m l 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 glutathione (12). At the end of the incubation, the reaction was terminated by the addition of 200 m l of 0.1 M NaOH. Absorbance of reaction mixture at 410 nm was measured in spectrophotometer with a microvolume cell.

RESULTS

Sequence analysis of CPTP1 cDNA. The isolated CPTP1 cDNA had 1718 nucleotides with the open reading frame coding for 356 amino acid residues, and showed 92% sequence identity to the corresponding 321 residues of the HPTP1B (Figure 1). The initiator methionine was not present within the CPTP1. Neither a polyadenylation signal nor a 3′ poly(A)$^+$ tail was observed. CPTP1 lacked 13 amino acids in N-terminal region compared with HPTP1B, and its C-terminal 48 amino acids sequence marked with box was different from those of known PTPs as well as HPTP1B (Figure 1).

To investigate the regulatory mechanism of CPTP1, we examined the potential kinase targeting sites by analyzing amino acids sequence. CPTP1 contained five CKII phosphorylation sequence motifs (S/TXXD/E), one $p56^{lck}$ site [(I>E>V)-Y-(E>G)-(E>D>P>N)-(I/V>L)], and one MAP kinase site (P-E-S-P) as indicated by underline (Figure 1). Since $p56^{lck}$ is subfamily of $p60^{src}$, CPTP1 was assumed to be phosphorylated by $p60^{src}$. As we analyzed the truncated form coding for amino acids 1-321 of HPTP1B, HPTP1B also contained several potential phosphorylation motifs of CKII and MAP kinase. Thus our sequence analysis indicated that both CPTP1 and HPTP1B could be phosphorylated by these kinases and thus their phosphatase activity might be regulated by this modification.

Expression and purification of CPTP1 and HPTP1B in E. coli. To perform *in vitro* phosphorylation experiment on CPTP1 and HPTP1B, first we purified GST, GST-CPTP1-1, GST-CPTP1-2, and GST-HPTP1B in *E. coli* as described in "Materials and Methods" (Figure 2A). The proteins were then digested with thrombin to isolate the GST-deleted CPTP1 and HPTP1B proteins. As shown in Figure 2B, 36 kDa CPTP1 and 37 kDa HPTP1B were generated from GST-CPTP1-2 and GST-HPTP1B fusion proteins, respectively. The 66 kDa band of BSA protein was originated from human thrombin.

CPTP1 and HPTP1B are phosphorylated on serine and threonine residues by CKII in vitro. Since we could not isolate full-length CPTP1 protein due to a considerable degradation of intact CPTP1 when it was expressed in *E. coli,* we used GST-CPTP1-1 protein (deleted form of 72 amino acids in the C-terminal side of CPTP1) in *in vitro* phosphorylation reaction by CKII. Figure 3A indicated that CPTP1 and HPTP1B were phosphorylated in a time-dependent manner by CKII, and the degree of the phosphorylation of CPTP1 was much higher than that of HPTP1B. Phosphoamino acid analysis revealed that CPTP1 and HPTP1B were phosphorylated on both serine and threonine residues by CKII *in vitro* (Figure 3B). Both CPTP1 and HPTP1B have four CKII phosphorylation motifs (S/TXXD/E) and conserved catalytic domain of ~230 amino acid residues (data not shown). Therefore, 13 amino acid residues in N-terminal region and 23 proline rich sequence in C-terminal region of HPTP1B might be relevant to the decrease in its phosphorylation by CKII. Interestingly, serine and threonine phosphorylation of CPTP1 by CKII resulted in an inhibition of its

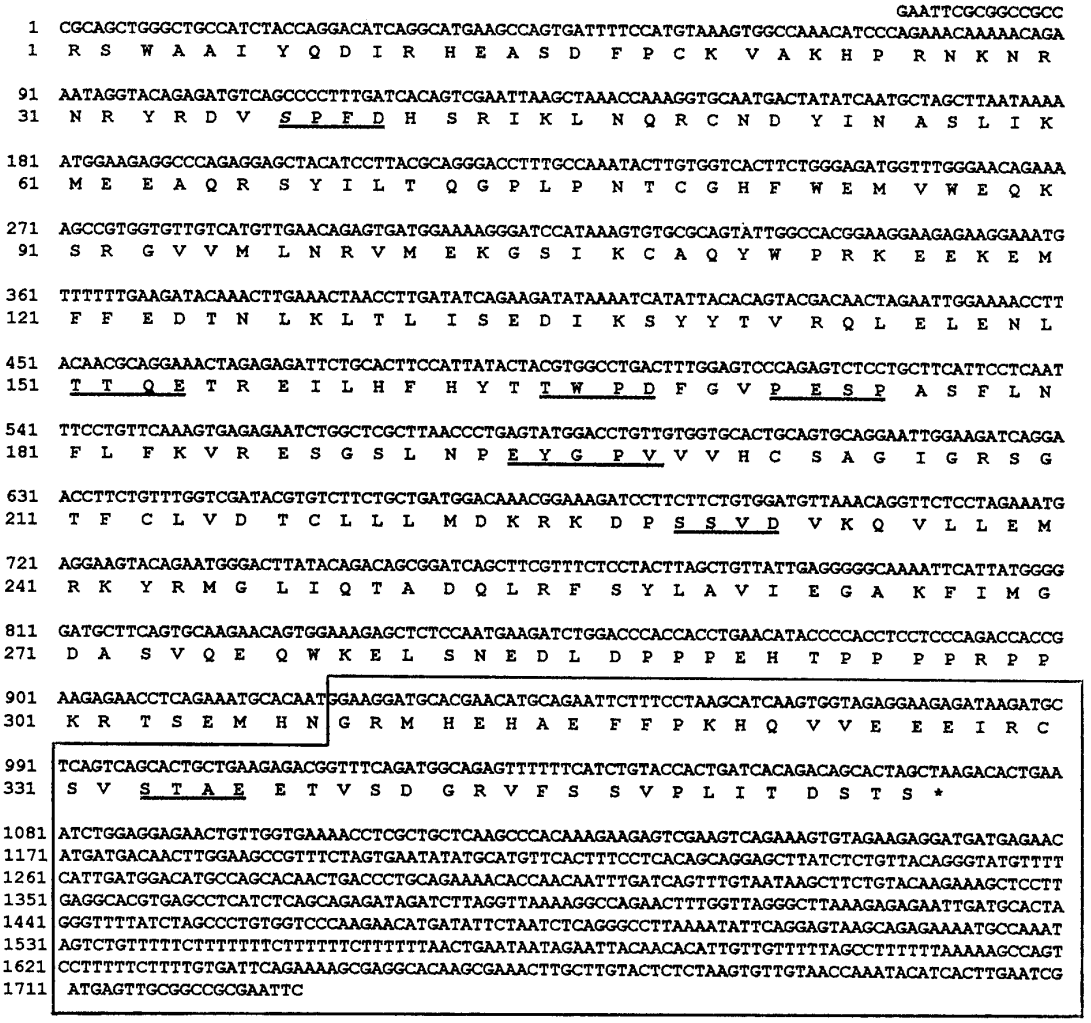

FIG. 1. Nucleotide and deduced amino acid sequences of CPTP1 cDNA. The nucleotide sequence starts from downstream linker sequence (GAATTCGCGGCCGC). First amino acid residue in the sequence is arginine (R). Termination codon is marked with asterisk. The region marked with box indicates distinct sequence from known PTPs as well as HPTP1B. The sequences marked with underline indicate the potential phosphorylation motifs of CKII (S/TXXD/E), p56^{lck} [(I>E>V)-Y-(E>G)-(E>D>P>N)-(I/V>L)], and MAP kinase (PESP) within the CPTP1.

phosphatase activity, suggesting that CKII may function as a regulator of the phosphatase activity of CPTP1 *in vivo* (Figure 4).

CPTP1 and HPTP1B are phosphorylated on tyrosine(s) by p60$^{c\text{-}src}$ in vitro. To examine whether CPTP1 could be phosphorylated by p60^{src}, we tested the effect of vanadate on *in vitro* phosphorylation of CPTP1 by p60$^{c\text{-}src}$. Figure 5A showed that GST-CPTP1-1 was phosphorylated in a dose-dependent manner by p60$^{c\text{-}src}$ in the presence of vanadate, whereas it was autodephosphorylated by its phosphatase activity in the absence of vanadate. The GST-CPTP1-1 expressed in *E. coli* migrated on the same band with p60$^{c\text{-}src}$ in SDS-PAGE. To resolve their ambiguity in SDS-PAGE, we performed *in vitro* phosphorylation by p60$^{c\text{-}src}$ using 36 kDa CPTP1-2 and 37 kDa HPTP1B cleaved by thrombin. As shown in Figure 5B, both CPTP1 and HPTP1B were phosphorylated by p60$^{c\text{-}src}$, and the stoichiometry of phosphorylation indicated that CPTP1 was less than HPTP1B. Phosphoamino acid analysis revealed that both CPTP1 and HPTP1B were exclusively phosphorylated on tyrosine residue(s) by p60$^{c\text{-}src}$ (Figure 5C). These results suggest that both CPTP1 and HPTP1B could be phosphorylated by p60$^{c\text{-}src}$ *in vivo*, and thus may play an important role in signal transduction cascades of p60$^{c\text{-}src}$.

DISCUSSION

The isolated CPTP1 from chicken intestine cDNA library had 92% sequence identity compared with the corresponding 321 amino acid residues of HPTP1B, while

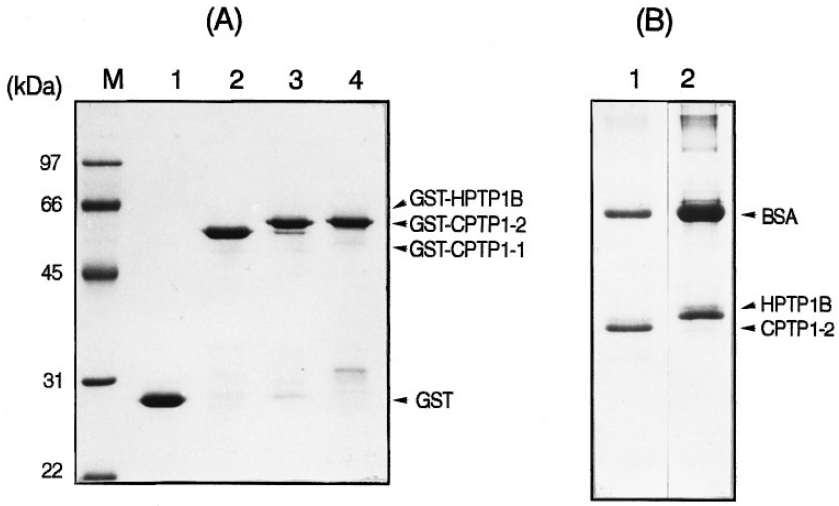

FIG. 2. Purification of CPTP1 and HPTP1B in *E. coli*. (A) Affinity purification of GST, GST-CPTP1-1, GST-CPTP1-2, and GST-HPTP1B proteins. Bacterial lysates of 26 kDa GST (lane 1), 59 kDa GST-CPTP1-1 (lane 2), 62 kDa GST-CPTP1-2 (lane 3), and 63 kDa GST-HPTP1B (lane 4) proteins were purified by affinity chromatography using S-hexylglutathione agarose beads. (B) Thrombin cleavage of GST-CPTP1-2 and GST-HPTP1B fusion proteins. For thrombin cleavage, the beads absorbing GST-CPTP1-2 and GST-HPTP1B were reacted with human thrombin at 25°C for overnight in the thrombin cleavage buffer containing 150 mM NaCl and 2.5 mM $CaCl_2$. The resulting 36 kDa CPTP1 (lane 1) and 37 kDa HPTP1B (lane 2) were electrophoresed on 12% SDS-PAGE. The 66 kDa band of BSA protein was originated from human thrombin.

C-terminal 48 amino acids sequence of this protein was distinct from those of HPTP1B-type PTPs (5,7). Thus CPTP1 does not have C-terminal targeting or localization sequence to ER found in HPTP1B-type PTPs, indicating that CPTP1 might be purely cytosolic type of PTP.

In this study, we show that both CPTP1 and HPTP1B are phosphorylated by CKII, $p56^{lck}$ (data not shown), and $p60^{c\text{-}src}$, but not phosphorylated by MAP kinase (data not shown). Recent studies revealed that the transgenic mice of CKII a subunit developed lymphoma in stochastic manner (13), and this as subunit bound to PP2A, serine and threonine phosphatase, *in vitro* and in mitogen-starved cells (14). Up to now, little is known about the phosphorylation of PTPs by CKII, except that the receptor-type PTP CD45 is a target protein of CKII *in vitro* (15) and is phosphorylated in response to mitogenic stim-

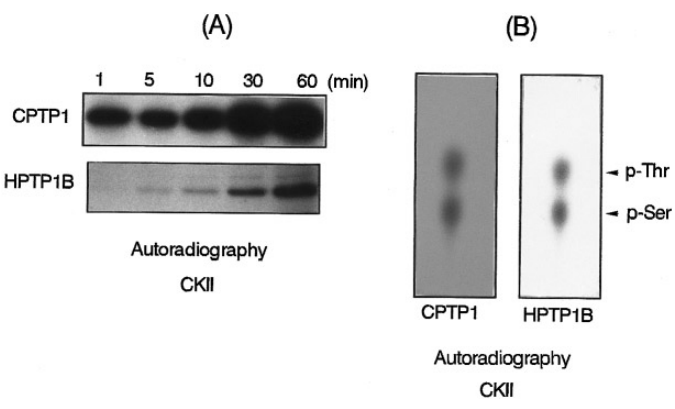

FIG. 3. *In vitro* phosphorylation and phosphoamino acid analysis of CPTP1 and HPTP1B by CKII. (A) Time course phosphorylation of CPTP1 and HPTP1B by CKII. The phosphorylation reaction of 300 ng each of GST-CPTP1-1 (upper panel) and GST-HPTP1B (low panel) was carried out as described in "Materials and Methods". (B) Phosphoamino acid analysis. ^{32}P-labeled CPTP1 (left panel) and HPTP1B (right panel) by CKII were separated by SDS-PAGE, transferred to the PVDF membrane, and subjected to an acid hydrolysis and thin layer chromatography.

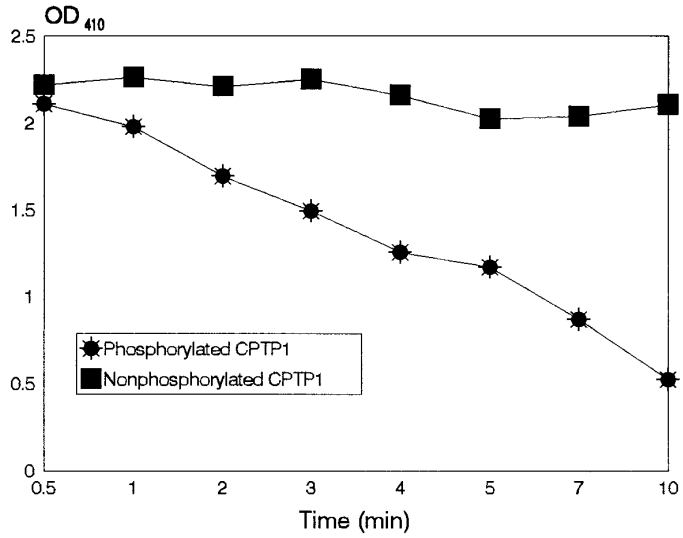

FIG. 4. Regulation of the phosphatase activity of CPTP1 by CKII-mediated phosphorylation. GST-CPTP1-1 was phosphorylated at 30°C for 30 sec to 10 min with or without CKII and followed by phosphatase reaction in phosphatase buffer containing pNPP as described in Materials and Methods.

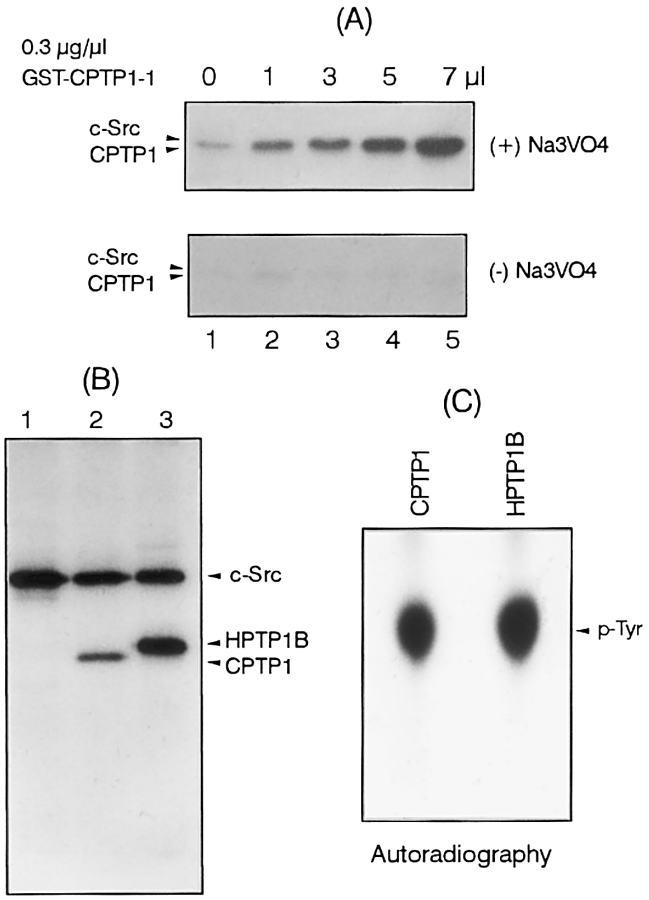

FIG. 5. *In vitro* phosphorylation and phosphoamino acid analysis of CPTP1 and HPTP1B by p60$^{c\text{-}src}$. (A) Effect of vanadate on the tyrosine phosphorylation of CPTP1 by p60$^{c\text{-}src}$. The phosphorylation reaction using the indicated amounts of GST-CPTP1-1 was performed with (upper panel) or without (low panel) vanadate in kinase buffer containing [g-^{32}P]ATP and p60$^{c\text{-}src}$ as described in Materials and Methods. The reaction was terminated by mixing with SDS sample buffer, boiled, and run on a 12% SDS-PAGE. After drying, the gel was exposed to an x-ray film to obtain autoradiography. (B) Phosphorylation of CPTP1 and HPTP1B by p60$^{c\text{-}src}$ *in vitro.* The phosphorylation reaction of the thrombin-cleaved CPTP1-2 and HPTP1B was carried out as described in Materials and Methods. (C) Phosphoamino acid analysis. ^{32}P-labeled CPTP1 (left panel) and HPTP1B (right panel) by p60$^{c\text{-}src}$ were separated by SDS-PAGE, transferred to the PVDF membrane, and subjected to an acid hydrolysis and thin layer chromatography.

ulation of T cells on four serine residues which are the potential phosphorylation motifs of CKII (16). Our data reveal that CPTP1 is phosphorylated on serine and threonine residues by CKII *in vitro* and this leads to an inhibition of its phosphatase activity. From these results it was suggested that CPTP1 could be phosphorylated *in vivo* on serine and threonine residues which are present within the potential phosphorylation motifs of CKII and thus CKII may function as a regulator of phosphatase activity of CPTP1.

HPTP1B has been known to be phosphorylated at Ser-352, Ser-378, and Ser-386 near its C-terminus *in vitro* or *in vivo* (4-6). These results indicate that HPTP1B function may be regulated by its C-terminal phosphorylation *in vivo.* In this study, we demonstrate that HPTP1B are multiple phosphorylated on threonine and tyrosine as well as serine near its N-terminus by CKII and p60$^{c\text{-}src}$ *in vitro.*

Taken together, these results suggest that both CPTP1 and HPTP1B may affect signaling cascades induced by CKII and p60$^{c\text{-}src}$, and may function as an important coordinator of CKII- and p60$^{c\text{-}src}$-induced intracellular signals.

ACKNOWLEDGMENTS

We thank Dr. Paul Matsudaira, Whitehead Institute, for his generous gift of the chicken intestine cDNA library and Dr. Axel Ullrich, Max-Planck Institut fur Biochemie, for providing the human PTP1B cDNA. We also thank Dr. Yunbae Pak, Gyeongsang National University, and Byung Hak Jhun, Pusan National University, for critical reading of the manuscript. This work was supported by the Research Aid Program for Basic Medicine (1996 and 1997), Ministry of Education, Republic of Korea.

REFERENCES

1. Trowbridge, I. S. (1991) *J. Biol. Chem.* **266,** 23517–23520.
2. Tonks, N. K., Dilts, C. D., and Fischer, E. H. (1988) *J. Biol. Chem.* **263**, 6722–6730.
3. Tonks, N. K., Dilts, C. D., and Fischer, E. H. (1988) *J. Biol. Chem.* **263,** 6731–6737.
4. Flint, A. J., Gebbink, M. F., Franza, B. R., Hill, D. E., and Tonks, N. K. (1993) *EMBO J.* **12,** 1937–1946.
5. Frangioni, J. V., Beahm, P. H., Shifrin, V., Jost, C. A., and Neel, B. G. (1992) *Cell* **68,** 545–560.
6. Schievella, A. R., Paige, L. A., Johnson, K. A., Hill, D. E., and Erikson, R. L. (1993) *Cell Growth Differ.* **4,** 239–246.
7. Woodford-Thomas, T. A., Rhodes, J. D., and Dixon, J. E. (1992) *J. Cell Biol.* **117,** 401–414.
8. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1990) Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
9. Sanger, F., Nicklen, S., and Coulson, A. R. (1977) *Proc. Natl. Acad. Sci. USA* **74,** 5463–5467.
10. Carlino, A., Toledo, H., Skaleris, D., Delisio, R., Weissbach, H., and Brot, N. (1992) *Proc. Natl. Acad. Sci. USA* **89,** 2081–2085.
11. Matsudaira, P. (1993) A Practical Guide to Protein and Peptide Purification for Microsequencing, 2nd ed., Academic Press, San Diego.
12. Dunphy, W. G., and Kumagai, A. (1991) *Cell* **67,** 189–196.
13. Seldin, D. C., and Leder, P. (1995) *Science* **267,** 894–897.
14. Heriche, J.-K., Lebrin, F., Rabilloud, T., Leroy, D., Chambaz, E. M., and Goldberg, Y. (1997) *Science* **276,** 952–955.
15. Autero, M., Saharinen, J., Pessa-Morikawa, T., Soula-Rothhut, M., Oetken, C., Gassmann, M., Bergman, M., Alitalo, K., Burn, P., Gahmberg, C. G., and Mustelin, T. (1994) *Mol. Cell Biol.* **14,** 1308–1321.
16. Stover, D. R., and Walsh, K. A. (1993) Techniques in Protein Chemistry (Angeletti, R. H., Ed.), Vol. IV, pp. 193–204, Academic Press, San Diego.