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Regulation of chicken protein tyrosine phosphatase 1 and human protein tyrosine phosphatase 1B activity by casein kinase II- and p56^{lck}-mediated phosphorylation

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Abbreviations: CPTP1, chicken protein tyrosine phosphatase 1; HPTP1B, human protein tyrosine phosphatase 1B; CKII, casein kinase II

Abstract

Protein tyrosine phosphorylation and dephosphorylation are important in the regulation of cell proliferation and signaling cascade. In order to examine whether phosphatase activity of CPTP1 and HPTP1B, typical nontransmembrane protein tyrosine phosphatase, could be controlled by phosphorylation, affinity-purified PTPs were phosphorylated by CKII and p56^{lck} *in vitro*. Phosphoamino acid analysis revealed that CPTP1 was phosphorylated on both serine and threonine residues by CKII, and tyrosine residue by p56^{lck}. Phosphatase activity of CPTP1 was gradually increased by three-fold concomitant with phosporylation by CKII. Phosphorylation of HPTP1B by CKII resulted in quick two-fold enhancement of its phosphatase activity within 5 min of incubation and remained in that state. In the presence of CKII inhibitor, heparin or poly(Glu.Tyr), both phosphorylation and enhancement of phosphatase activity of CPTP1 and HPTP1B were mostly blocked. p56^{lck} catalyzed tyrosine phosphorylation of CPTP1 and HPTP1B was only observed by inhibiting the intrinsic tyrosine phosphatase activity. Taken together, these results indicate that CPTP1 or HPTP1B possesses a capability to regulate its phosphatase activity through phosphorylation processes and may participate in the cellular signal cascades.

Keywords: protein tyrosine phosphatase, CKII, p56^{lck}, phosphorylation

Introduction

The reversible phosphorylation of proteins on tyrosine residues regulates many cellular processes (Hunter, 1995; Tonks, 1996; Tonks and Neel, 1996). The tyrosyl phosphorylation status of a protein is controlled by the competing activities of the protein tyrosine kinases (PTKs) and the protein tyrosine phosphatases (PTPs). Over 100 PTKs and 50 PTPs have been identified, and the human genome may contain up to 500 of each (Hunter, 1995; Tonks and Neel, 1996). As were in the PTKs, the PTPs family are divided into two major classes, such as receptor-type (transmembrane) and nonreceptor-type (non-transmembrane) forms (Trowbridge, 1991).

PTP1B, the first isolated intracellular PTP from human placenta, has catalytic domain located at the N-terminal region (Tonks *et al*., 1988). This nonrecepter-type PTP is phosphorylated on serine residue(s) in growing HeLa cells (Frangioni *et al*., 1992). The HPTP1B is also phosphorylated *in vitro* at Ser 386 by p34^{cdc2} and at Ser 378 by protein kinase C (PKC) (Flint *et al*., 1993). These findings implicated that PTPs may participate in regulatory roles in various cellular signaling pathways, and the activity of PTPs are regulated by phosphorylation.

CPTP1, another non-transmembrane PTP present in chicken, has characteristic C-terminal 48 amino acid sequences distinguished from those of HPTP1B-type PTPs. Thus, CPTP1 does not contain C-terminal targeting or localization sequences to ER, indicating that CPTP1 might be purely cytosolic type of PTP (Frangioni *et al*., 1992; Woodford-Thomas *et al*., 1992). Rather CPTP1 contains several potential phosphorylation motifs of 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).

In the present study, we report that both CPTP1 and HPTP1B are phosphorylated by CKII or p56^{lck} *in vitro*, and induced enhancement of the phosphatase activity in chicken and human.

Materials and Methods

Materials

[γ-^{32}P]ATP (6,000 Ci/mmol) was purchased from New England Nuclear. CKII and p56^{lck} were from Upstate Biotechnology; *p*-nitrophenyl phosphate, glutathione, ATP, and heparin from Boehringer mannheim; sodium orthovanadate, poly(Glu.Tyr)(4:1), and thrombin from Sigma; hydro-

gen peroxide, and cellulose thin-layer chromatography (TLC) plates(without fluorescent indicator) from Merck. Glutathione sepharose 4B was obtained from Amersham Pharmacia Biotech., polyvinyldifluoride (PVDF) membrane was from Bio-Rad, and other chemicals were purchased as described in the text.

Purification of GST fusion proteins expressed in BL21(DE3) strain

GST-CPTP1-2 (truncated form of 41 amino acids in the C-terminal side of chicken PTP1, Jung *et al*., 1998), and GST-HPTP1B (truncated form coding for amino acids 1-321 of human PTP1B) plasmids were introduced into BL21 (DE3) cells and expressed in Luria-Bertani medium, supplemented with 50 μg/ml ampicillin. When the cell density in the culture medium reached an absorbance of 0.6 at 600 nm, isopropyl-1-thio-β-D-galactopyranoside (IPTG) was added to a final concentration of 1 mM. The cells were harvested by centrifugation (5,000 × g for 5 min) after 4 h of induction. The induced GST fusion proteins were purified by glutathione Sepharose 4B affinity column chromatography. To isolate CPTP1-2 and HPTP1B proteins, GST fusion proteins were incubated with thrombin and heparin at 25°C for overnight in the thrombin cleavage buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, and 5 mM $CaCl_2$) (Ausubel, *et al*., 1987).

In vitro phosphorylation

The phosphorylation reactions of CPTP1-2 and HPTP1B were carried out at 37°C for 30 min with CKII or p56^{lck} in a total reaction volume of 20 μl containing kinase reaction buffer (100 mM Tris-HCl, pH 7.2, 125 mM $MgCl_2$, 25 mM $MnCl_2$, 2 mM EGTA, 0.25 mM sodium orthovanadate, and 2 mM dithiothreitol (DTT)) in the presence of 50 μM of cold ATP or [γ-^{32}P]ATP. For time course phosphorylation, CPTP1-2 and HPTP1B proteins were reacted for 1, 5, 10, 30, 60, and 120 min. In case of sodium orthovanadate (Na_3VO_4) or hydrogen peroxide (H_2O_2) treatment, up to 3 mM of Na_3VO_4 and H_2O_2 were used to stimulate the phosphorylation reaction. The reaction was stopped by mixing with SDS sample buffer, boiled, and run on a 12% SDS-polyacrylamide gel. Stained gel was subjected to autoradiography after drying.

Phosphatase activity

The phosphatase reaction was performed at 37°C for 10 min in a total reaction mixture of 100 μl containing 0.1 M *p*-nitrophenyl phosphate (*p*NPP), 0.1M Tris-HCl, pH 8.0, 0.25 M NaCl, 5 mM EDTA, and 10 mM glutathione (Dunphy and Kumagai, 1991). At the end of the incubation, the reaction was terminated by the addition of 200 μl of 0.1 M NaOH. Absorbance at 410 nm was determined in Gilford spectrophotometer with a microvolume cell. The instrument was zeroed with assay buffer lacking PTP protein.

Phosphoamino acid analysis

CKII- and p56^{lck}-phosphorylated CPTP1-2 were prepared by *in vitro* phosphorylation as described above and then separated by SDS-polyacrylamide gel. ^{32}P-labeled proteins were transferred to PVDF membrane, and subjected to acid hydrolysis as described previously (Kang and Chung, 1999). The aqueous hydrolysate was dried in a speedvac concentrator, dissolved in pH 1.9 buffer (acetic acid/formic acid/water, 78:25:897, v/v/v) containing standard phosphoamino acids. Electrophoresis was done at pH 1.9 buffer (first dimension) followed by ascending chromatography in isobutyric acid/0.5 M ammonium hydroxide (5:3, v/v). Autoradiography was carried out after ninhydrin staining.

Results

CPTP1, a non-transmembrane chicken protein tyrosine phosphatase, contained multiple potential phosphorylation sequence motifs for CKII, p56^{lck}, and MAP kinase (Kim *et al*., 1996). The truncated form coding for amino acids 1-321 of human PTP1B, HPTP1B also contained several potential phosphorylation motifs for CKII and MAP kinase. By having such potential phosphorylation sites for these kinases, such modification may provide a mode of influencing their phosphatase activity. In order to examine whether such phosphorylation of PTP could be utilized, affinity-purified CPTP1-2 (deleted form of 41 amino acids in the C-terminal side of CPTP1) and HPTP1B were phosphorylated by a typical Ser/Thr kinase, CKII and Tyr kinase, p56^{lck} *in vitro*. As shown in Figure 1, thrombin-cleaved 36 kD CPTP1-2 and 37 kDa HPTP1B were phosphorylated by CKII (lane 1 and 2) and p56^{lck} (lane 3 and 4), respectively, and the degree of the phosphorylation of CPTP1-2 by CKII was much higher than that of HPTP1B.

CPTP1-2 and HPTP1B incubated with CKII in the presence of cold ATP for various time intervals (1~120 min) were used for phosphatase assay using *p*-nitrophenyl phosphate as a substrate. Phosphorylation of

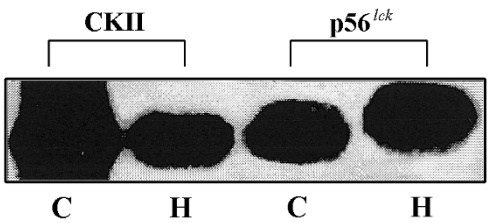

Figure 1. Phosphorylation of CPTP1-2 and HPTP1B by CKII or p56^{lck} *in vitro*. Affinity-purified CPTP1-2 (lane 1 and 3) and HPTP1B (lane 2 and 4) were incubated with CKII (lane 1 and 2) and p56^{lck} (lane 3 and 4), respectively, in the presence of [γ-^{32}P]ATP. After drying the SDS-polyacrylamide gel, autoradiography was performed with intensifying screen. Phosphorylated CPTP1-2 (36 kD) and HPTP1B (37 kD) were visible by autoradiography. C denotes CPTP1-2, and H denotes HPTP1B.

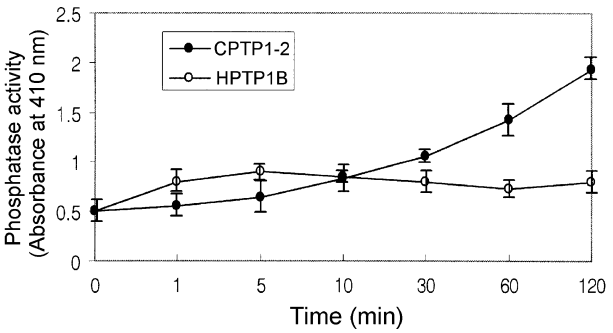

Figure 2. Effects of CKII-mediated phosphorylation on CPTP1-2 and HPTP1B activities. CPTP1-2 (closed circle) and HPTP1B (open circle) were phosphorylated by CKII at 37°C for 1 to 120 min. Subsequent phosphatase reaction was performed at 37°C for 10 min in *p*-nitrophenyl phosphate-containing buffer as described in 'Materials and Methods'.

CPTP1-2 by CKII resulted in more than three-fold enhancement of its phosphatase activity in a linear rate with time (Figure 2). However, phosphorylation of HPTP1B showed quick increment of its phosphatase activity during first 5 min and remained in a steady state during the rest of incubation period.

CPTP1-2 contained four CKII phosphorylation sequence motifs (S/TXXD/E) except one motif existed in deleted C-terminal region. CKII is ubiquitous Ser/Thr specific protein kinase implicated in a wide spectrum of cellular functions (Pinna, 1990).

Phosphorylation of CPTP1-2 and HPTP1B was blocked with the use of known CKII inhibitors, heparin, or poly(Glu.Tyr) (4:1) (Tawfic *et al*., 1995; Wang *et al*., 1999) in order to confirm whether the enhancement of phosphatase activity was mainly dependent on the phosphorylation process. The extent of CPTP1-2 phosphorylation was found to be about ten fold lower in the heparin (30 μg/ml) treated CPTP1-2 (Figure 3A, upper panel, lane 2) in comparison with control. Poly(Glu.Tyr)(3 mg/ml) also decreased ^{32}P-incorporation into CPTP1-2 in a similar extent as observed with heparin (Figure 3A, upper panel, lane 3). The CKII-mediated phosphorylation of HPTP1B in the presence of same inhibitors was blocked in a far lesser extent than those observed with CPTP1-2 (Figure 3A, lower panel). Figure 3B shows dose-dependent inhibition of heparin on phosphatase activity in the range of 10 μg/ml to 50 μg/ml concentration. The phosphatase activity started to decrease at 10 μg/ml of heparin, and at 50 μg/ml concentration, it was close to the activity level of the non-phosphorylated CPTP1-2. The presence of PTP activity was also blocked by treatment with 1 to 5 mg/ml of poly(Glu.Tyr) (4:1) (Figure 3C).

In addition to Ser/Thr kinase, CPTP1-2 and HPTP1B could be phosphorylated on tyrosine residue by p56^{lck}. The steady-state level of protein tyrosine phosphorylation in intact cells is tightly regulated by the opposing actions of PTKs and PTPs (Secrist *et al*., 1993). Heffetz

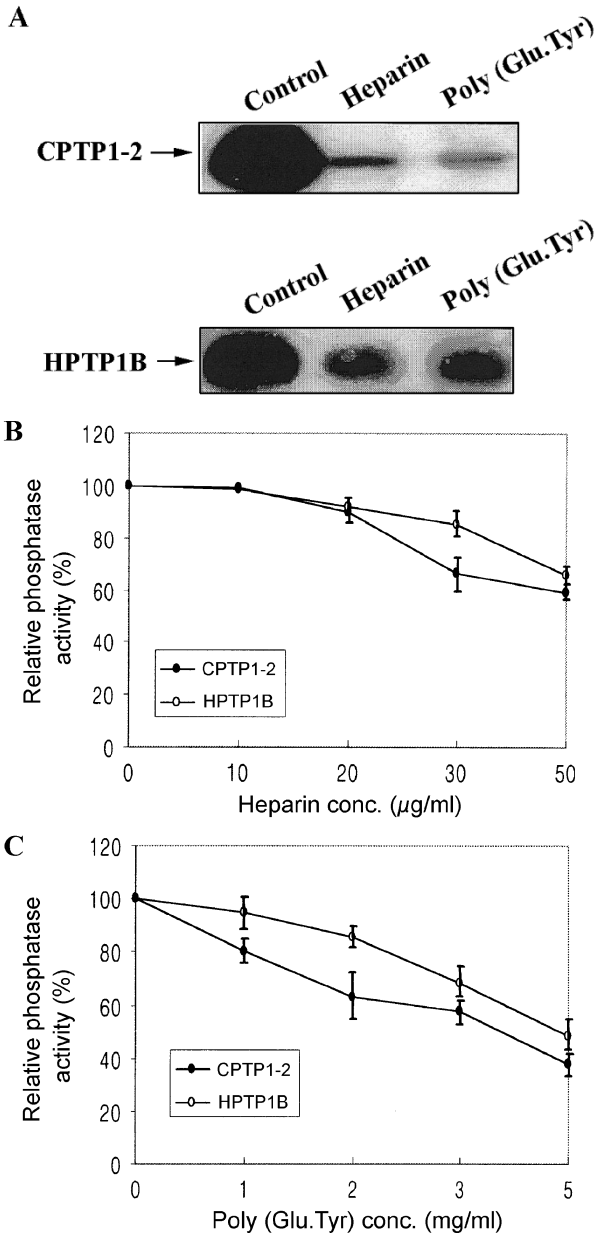

Figure 3. Dose-dependent inhibitory effects of heparin, and poly(Glu.Tyr) on the PTP phosphorylation by CKII. CPTP1-2 (A, upper panel) and HPTP1B (A, lower panel) were phosphorylated by CKII in the absence (lane 1) or presence of 30 μg/ml of heparin (lane 2), and 3 mg/ml of poly (Glu.Tyr)(4:1) (lane 3) at 37°C for 30 min. Phosphorylated PTPs were visualized by autoradiography after drying of SDS-polyacrylamide gel. CPTP1-2 and HPTP1B proteins were phosphorylated by CKII with various concentrations of heparin (B), and poly(Glu.Tyr) (C). Successive phosphatase reaction was carried out at 37°C for 10 min. Assays were performed in triplicate and the results are represented as percentage of the absorbance at 410 nm for each protein.

et al. 1990) reported that vanadate-induced accumulation of Tyr(P)-containing proteins is mediated by the inhibition of PTP activities. We evaluated the effects of vanadate and hydrogen peroxide (H_2O_2), well-known PTP inhibitor, on the phosphorylation of CPTP1 and

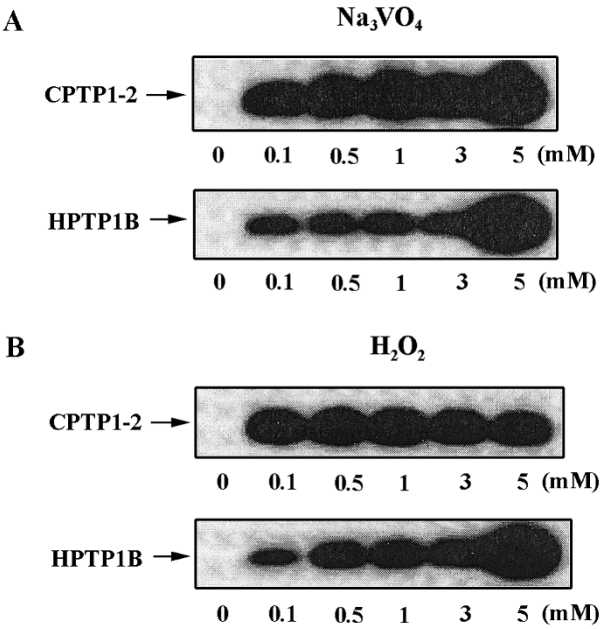

Figure 4. Effects of vanadate (Na_3VO_4) and hydrogen peroxide (H_2O_2) on the tyrosine phosphorylation of CPTP1-2 and HPTP1B by p56^{lck}. CPTP1-2 and HPTP1B were phosphorylated by p56^{lck} at 37°C for 30 min with the indicated concentrations (mM) of vanadate (A), and hydrogen peroxide (B).

HPTP1B by p56^{lck}. As shown in Figure 4A, the amount of phosphorylation in CPTP1-2 and HPTP1B by p56^{lck} started to increase at 0.1 mM vanadate (lane 2) and continued to increase up to 5 mM (lane 3 to 6), whereas it was autodephosphorylated by its phosphatase activity in the absence of vanadate (lane 1). H_2O_2, another PTP inhibitor, also was found to influence on the p56^{lck}-mediated phosphorylation of CPTP1-2 and HPTP1B. Figure 4B shows that CPTP1-2 and HPTP1B were phosphorylated by p56^{lck} in the presence of H_2O_2. The amount of ^{32}P-incorporation into CPTP1-2 started to increase at 0.1 mM H_2O_2 (lane 2, upper panel), and maintained in a steady-state up to 5 mM (lane 3 to 6, upper panel), whereas the degree of phosphorylation in HPTP1B increased continuously up to the 5 mM treatment of this PTP inhibitor (lower panel).

Phosphoamino acid analysis revealed that CPTP1-2 protein was phosphorylated on both serine and threonine residues by CKII *in vitro* (Figure 5A). Over two third of radioactivity was visible on threonine residue. Figure 5B showed that p56^{lck} absolutely phosphorylated CPTP1-2 on tyrosine residue.

Discussion

The primary sequence of CPTP1 had 92% sequence identity compared with the corresponding 321 amino acid residues of HPTP1B. By contrast, CPTP1 did not contain a hydrophobic C-terminal targeting or localization

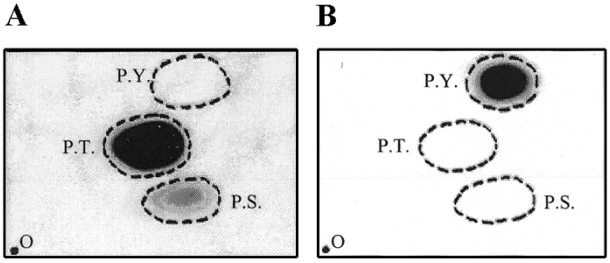

Figure 5. Phosphoamino acid analysis of CPTP1-2 phosphorylated by CKII and p56^{lck}. ^{32}P-labeled CPTP1-2 protein by CKII (A) and p56^{lck} (B) were separated by SDS-PAGE, transferred to PVDF membrane, and subjected to an acid hydrolysis and thin layer electrophoresis (first dimension)/ chromatography (second dimension). P.S., P.T., and P.Y. denote phosphoserine, phosphothreonine, and phosphotyrosine, respectively, and O denotes origin for sample.

sequences reported in the HPTP1B-type PTPs, such as HPTP1B (Frangioni *et al*., 1992) and rat PTP1 (Woodford-Thomas *et al*., 1992). We have shown that chicken PTP1 and human PTP1B are phosphorylated by CKII, p56^{lck}, and PKC (data not shown). Recent study revealed that these PTPs are phosphorylated by p60$^{c\text{-}src}$, but not phosphorylated by MAP kinase (mitogen-activated protein) (Jung *et al*., 1998).

In our study, CPTP1-2 phosphorylated by CKII enhanced its phosphatase activity more than three-fold during two hour experimental period. HPTP1B also increased the phosphatase activity by about two-fold after 5 min of incubation and remained in that level although phosphorylation was steadily increased during the experimental period (data not shown). These results are not in agreement with the observations of Jung *et al.* (1998) who showed declining effect of activity in CPTP1 phosphorylated by CKII through incubation period (up to 10 min). Jung *et al.* (1998) used CPTP1-1 (deleted form of 72 amino acids in the C-terminal side of CPTP1) as a substrate, and kinase buffer containing 10 mM dithiothreitol (DTT) in the phosphorylation reaction. It remains to be elucidated whether the discrepancy of the observed activities of CKII-phosphorylated CPTP1 was due to substrate difference or modulation by redox state.

Known CKII effectors such as polyanion heparin, and poly(Glu.Tyr)(4:1) diminished the enzyme activity of PTP phosphorylated by CKII in a dose-dependent fashion. These results confirmed the involvement of CKII in the regulation of the phosphatase activity by phosphorylation.

In recent years, more and more proteins are reported to be phosphorylated on multiple sites in hierarchical fashion (Roach, 1991; Stover and Walsh, 1994). In addition to CKII, CPTP1 and HPTP1B could be phosphorylated by Tyr kinase p56^{lck} and Ser/Thr kinase PKC. Vanadate and H_2O_2, typical protein tyrosine phosphatase inhibitor, induced tyrosine phosphorylation of CPTP1-2 and HPTP1B by p56^{lck} in a dose-dependent pattern. The phosphatase activity assay of the tyrosine phosphorylated CPTP1-2

and HPTP1B by $p56^{lck}$ was attempted but due to the presence of PTP inhibitor vanadate or H_2O_2, it was difficult to evaluate. However, the fact that the level of phosphatase activity in the PTPs phosphorylated by $p56^{lck}$ were lesser inhibited than those of unphosphorylated PTPs under the presence of vanadate and H_2O_2, suggest that some contribution of tyrosine phosphorylation in modulating its tyrosine phosphatase activity.

In conclusion, we have demonstrated that CPTP1-2 and HPTP1B could influence in the CKII-, and $p56^{lck}$-mediated signaling cascades as a results of changes in these PTP activities caused by phosphorylation.

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