Phosphorylation of chicken protein tyrosine phosphatase 1 by casein kinase II *in vitro*

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Abbreviations: CPTP, chicken protein tyrosine phosphatase; HPTP1B, human placenta protein tyrosine phosphatase 1B; CKII, casein kinase II; MAP kinase, mitogen-activated protein kinase; GST, glutathione S-transferase; pNPP, *p*-nitrophenyl phosphate; EGF, epidermal growth factor

Abstract

The phosphorylation and dephosphorylation of proteins on tyrosyl residues are key regulatory mechanisms of cell growth and signal transduction and are controlled by opposing activities of protein tyrosine kinases and phosphotyrosyl phosphatases (PTPs). We have previously cloned and characterized a nontransmembrane chicken protein tyrosine phosphatase 1 (CPTP1) similar to human placental PTP1B (HPTP1B). CPTP1 contains several phosphorylation sequence motifs (S/T-X-X-D/E) for casein kinase II (CKII), [(I>E>V)-Y-(E>G)-(E>D>P>N)-(I/V>L)] for p56^{lck}, and (P-E-S-P) for MAP kinase. To examine whether phosphatase activity of CPTP1 could be controlled by phosphorylation, CPTP1 and HPTP1B fusion proteins purified from *E. coli* were subjected to the *in vitro* phosphorylation by CKII. Phosphoamino acid analysis revealed that CPTP1 was phosphorylated on both serine and threonine residues by CKII *in vitro*. In addition, the degree of the phosphorylation of CPTP1 by CKII was shown to be five times higher than that of HPTP1B. Phosphorylation on both serine and threonine residues of CPTP1 *in vitro* results in an inhibition of its phosphatase activity. This result suggests that phosphorylation of CPTP1 and HPTP1B by CKII might be implicated in the regulation of their catalytic activities in the cell.

Keywords: chicken protein tyrosine phosphatase 1, casein kinase II

Introduction

The phosphorylation of protein tyrosine residues is an early event in signal transduction initiated by binding of growth factors and hormones to their cognate receptors and it leads to regulation of cellular activities which include proliferation, differentiation, and also malignant transformation of cells (Hunter, 1989; Ullrich and Schlessinger, 1990; Cantley *et al.*, 1991). Under normal conditions, the level of tyrosine phosphorylation within a cell is determined by a balance between the actions of protein tyrosine kinases (PTKs) and protein tyrosine phosphatases (PTPs) (Hunter, 1989; Fischer *et al.*, 1991; Trowbridge, 1991). PTPs do not simply reverse the action of tyrosine kinases, but rather, PTP itself may play a central role in cellular regulation. PTPs are generally classified as transmembrane (receptor-type) and nontransmembrane (nonreceptor-type) enzymes based on the presence or absence of extracellular and transmembrane portions of their predicted sequence (Fischer *et al.*, 1991). Because the activity of tyrosine kinase can be controlled by phosphorylation, it has been postulated that PTP activity may be regulated by phosphorylation as well. For example, protein tyrosine phosphatase activity of CD45, a transmembrane PTP, is activated by sequential phosphorylation by *v-abl* and casein kinase II (CKII) *in vitro* (Stover and Walsh, 1994). CD45 activates the src family protein-tyrosine kinases, p56^{lck} and p59^{lyn}, by dephosphorylating a negative regulatory tyrosine in the carboxyl terminus (McFarland and Thomas, 1995). A direct interaction between p56^{lck} and CD45 has been demonstrated upon phosphorylation of CD45 by p50^{csk} *in vitro* (Autero *et al.*, 1994).

HPTP1B, a nontransmembrane PTP, is phosphorylated on serine residue(s) in growing HeLa cells (Frangioni *et al.*, 1992). *In vitro*, HPTP1B is phosphorylated at Ser-386 by the mitotic protein serine/threonine kinase p34^{cdc2}, and also phosphorylated at Ser-378 by protein kinase C (PKC) (Flint *et al.*, 1993). These findings have strengthened our view that PTPs play important regulatory roles in, various cellular signaling pathways, and the activity of PTPs are regulated by phosphorylation.

To investigate possible mechanisms regulating CPTP1 protein activity, we searched for potential kinase target sites in its primary sequence. CPTP1 contains five CKII phosphorylation sequence motifs (S/T-X-X-D/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) (Kim *et al.*, 1996). Until now, little is known about the regulation of PTP by CKII-mediated phosphorylation. In this study we demonstrated that CPTP1 is phosphorylated by CKII *in vitro* and this phosphorylation causes a decrease in the CPTP1

phosphatase activity.

Materials and Methods

Materials

Casein kinase II (CKII), reduced glutathione, T4 DNA ligase, calf intestinal alkaline phosphatase (CIAP), Klenow enzyme, and most of all restriction enzymes were purchased from Boehringer Mannheim (Mannheim, Germany). Yeast extract, trypton, and bacto-agar were from Difco (Detroit, MI). [γ - 32 P]ATP and Kodak X-ray film were from Amersham (Buckinghamshire, UK). S-hexylglutathione agarose beads and general chemicals were from Sigma (St. Louis, MO).

Bacterial expression and purification of CPTP and HPTP1B fusion proteins

For generation of GST-CPTP, CPTP1 cDNA was digested with *Eco*RI and *Bgl*II and filled with the Klenow enzyme. This CPTP DNA fragment, deleted in 72 amino acids in C-terminal side of CPTP1 coding for 356 amino acids, was ligated to the *Sma*I-digested pGEX-1N vector (pGEX1N-CPTP). For generation of GST-HPTP1B, HPTP1B cDNA fragment coding for amino acids 1-321 of HPTP1B was ligated to the *Sma*I-digested pGEX-2T vector (pGEX2T-HPTP1B). The resulting pGEX1N-CPTP and pGEX2T-HPTP1B plasmids were expressed by induction with 1 mM IPTG and purified by affinity chromatography using S-hexylglutathione agarose beads as described previously (Kim *et al.*, 1996).

In vitro phosphorylation

The phosphorylation reaction of CPTP and HPTP1B fusion proteins was carried out at 30°C for 10 min by CKII in a total reaction volume of 20 μ l containing kinase buffer [50 mM Tris (pH 7.5), 150 mM NaCl, 10 mM MgCl₂, and 10 mM DTT], 1 mM sodium vanadate and 5 mCi of [γ - 32 P]ATP. For time course phosphorylation, CPTP and HPTP1B were reacted for 1, 5, 10, 30, and 60 min. The reaction was terminated by mixing with SDS sample buffer, boiled, and run on a 12% SDS-PAGE. The gel was stained in staining solution (0.125% Coomassie brilliant blue R-250/40% methanol/10% glacial acetic acid) and destained in a solution of 40% methanol/10% glacial acetic acid. After drying, the gel was exposed to a film (Kodak x-ray) to visualize phosphorylated proteins.

Phosphoamino acid analysis

32 P-labeled CPTP1 was prepared by *in vitro* phosphorylation as described above and then separated by SDS-PAGE. Transfer to the PVDF membrane was carried out using Tris-glycine buffer (25 mM Tris/192 mM glycine/0.1% SDS/20% methanol), and protein was visualized by staining with Coomassie blue R-250 (0.1% in 50%

methanol) and destained several times with destaining solution (50% methanol/10% glacial acetic acid). The membrane was rinsed with several changes of H₂O, and then dried on Whatman 3MM filter paper. 32 P-labeled CPTP1 band was excised, transferred to corkscrew tube, and hydrolyzed at 110°C for 2 h in 6 N HCl under nitrogen gas. Membranes were mixed with 100 ml methanol and 1 ml H₂O and allowed to soak for 30 min. The aqueous hydrolysate was lyophilized in a SpeedVac concentrator, dissolved in 10 ml thin layer chromatography (TLC) solvent (glacial acetic acid: pyridine: n-butanol: H₂O = 15:50:75:60), and resolved by TLC. Three μ g each of phosphoserine, phosphothreonine and phosphotyrosine were used as a TLC standard marker. After fluorography with ninhydrin solution, phosphorylated residue(s) was identified by autoradiography.

Determination of the tyrosine phosphatase activity

To investigate the regulation of the phosphatase activity of CPTP1 by CKII-mediated phosphorylation, GST-CPTP was phosphorylated at 30°C for 30 s 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 μ 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 (Dunphy *et al.*, 1991). At the end of the incubation, the reaction was terminated by the addition of 200 μ l of 0.1 M NaOH. Absorbance of reaction mixture at 410 nm was measured in spectrophotometer with a microvolume cell.

Results and Discussion

We have previously cloned and characterized a non-transmembrane chicken protein tyrosine phosphatase (CPTP1) similar to HPTP1B (Kim *et al.*, 1996). CPTP1 contains multiple potential phosphorylation sequence motifs for CKII, p56^{lck}, and MAP kinase. This suggests that CPTP1 and HPTP1B could be phosphorylated on serine and threonine as well as tyrosine by these kinases.

Bacterial expression vectors for generation of GST-CPTP and GST-HPTP1B fusion proteins were constructed as described in "Materials and Methods". The resulting pGEX1N-CPTP and pGEX2T-HPTP1B plasmids were expressed with IPTG induction in *E. coli* (Figure 1, lanes 2 and 3) and purified by affinity chromatography using glutathione agarose beads (Figure 1, lanes 5 and 6). Additionally, GST protein as a control was expressed and purified from pGEX-2T vector (Figure 1, lanes 1 and 4).

To examine whether phosphatase activity of CPTP1 could be controlled by phosphorylation, CPTP1 and HPTP1B fusion proteins purified from *E. coli* were subjected to the *in vitro* phosphorylation by CKII. As shown in Figure 2, both CPTP1 and HPTP1B were phosphorylated by

Figure 1. Affinity purification of CPTP and HPTP1B fusion proteins expressed in *E. coli*. pGEX-2T, pGEX1N-CPTP and pGEX2T-HPTP1B plasmids were expressed with IPTG induction (1 mM) and purified by affinity chromatography using glutathione agarose beads. Bacterial lysates are located in lanes 1, 2 and 3 and the purified proteins are shown in lanes 4, 5 and 6. Molecular weights of isolated GST, GST-CPTP and GST-HPTP1B proteins are identified with 26, 59 and 63-kDa, respectively.

Figure 2. Phosphorylation of CPTP1 and HPTP1B by CKII *in vitro*. To investigate the *in vitro* phosphorylation of CPTP1 and HPTP1B by CKII, the phosphorylation reaction of GST-CPTP and GST-HPTP1B (200 ng each) was carried out at 30°C for 10 min in a total reaction volume of 20 µl containing kinase buffer [50 mM Tris (pH 7.5), 150 mM NaCl, 10 mM MgCl₂, and 10 mM DTT], 1 mM sodium vanadate and 5 µCi of [γ -³²P]ATP. The reaction was terminated by mixing with SDS sample buffer, boiled, and run on a 12% SDS-PAGE. After staining and destaining, the gel was exposed to a film to obtain the autoradiography.

Figure 3. Amino acid sequence comparison between the truncated CPTP and HPTP1B. Four CKII phosphorylation motifs (S/T-X-X-D/E) for CPTP and HPTP1B are underlined. Conserved catalytic domain (VHCSAGIGR SG) is indicated by bold letter. The sequence marked with double underline in the C-terminal region of HPTP1B shows the proline rich sequence.

CKII, and the degree of the phosphorylation of CPTP1 was shown to be five times higher than that of HPTP1B. The deduced amino acid sequence of CPTP1 showed 92% sequence identity to the corresponding 321 residues of the HPTP1B. In comparison with truncated form of HPTP1B, CPTP1 lacked 13 amino acid residues in N-termination region, while it had an additional 48 residues in C-terminal region. Unfortunately, full-length CPTP1 protein was not isolated by considerable degradation of intact CPTP1 expressed in *E. coli*. Thus we used the truncated CPTP protein (deleted in 72 amino acids in C-terminal side of CPTP1) for *in vitro* phosphorylation reaction by CKII. Figure 3 shows the amino acid sequence

comparison between the truncated CPTP and HPTP1B. Both CPTP and HPTP1B have four CKII phosphorylation motifs (S/T-X-X-D/E) and conserved catalytic domain of ~230 amino acid residues. Therefore, the 13 amino acid residues in N-terminal region and the 23 amino acid proline rich sequence in C-terminal region somehow lead to the decrease in phosphorylation of HPTP1B by CKII kinase.

Figure 4 shows that CPTP1 and HPTP1B are phosphorylated in a time-dependent manner by CKII. This result suggested that four different CKII phosphorylation motifs in the CPTP protein has different reactivities with CKII enzyme. Otherwise the conformational change elicited by the one phosphorylation motif can increase

Figure 4. Time course of phosphorylation of CPTP1 and HPTP1B by CKII. The kinase reaction of GST-CPTP and GST-HPTP1B (300 ng each) was carried out at 30°C for various times in a total reaction volume of 20 μ l containing kinase buffer. The reaction was terminated by mixing with SDS sample buffer, boiled, and run on a 12% SDS-PAGE. After staining with Coomassie blue, the gel was dried and exposed to a X-ray film.

Figure 5. Phosphoamino acid analysis of CPTP1 phosphorylated by CKII. 32 P-labeled CPTP1 by CKII was separated by SDS-PAGE, transferred to the PVDF membrane, visualized by staining with Coomassie blue R-250, and destained several times. The membrane was rinsed with several changes of H₂O, and then dried on Whatman 3MM filter paper. 32 P-labeled CPTP1 band was excised and subjected to an acid hydrolysis at 110°C for 2 h in 6 M HCl, and then the aqueous hydrolysate was lyophilized and resolved by thin layer chromatography. Three μ g each of phosphoserine, phosphothreonine and phosphotyrosine were used as a standard marker (A). After fluorography with ninhydrin solution, phosphorylated residues were visualized by autoradiography (B).

Figure 6. Regulation of the phosphatase activity of CPTP1 by CKII-mediated phosphorylation. GST-CPTP was phosphorylated at 30°C for 30 s to 10 min in the absence or presence of CKII. Phosphatase reaction was performed at 37°C for 10 min in a total reaction mixture of 100 μ l containing phosphatase buffer. The reaction was terminated by the addition of 200 μ l of 0.1 M NaOH. Absorbance of reaction mixture was measured at 410 nm with a microvolume cuvette.

the phosphorylation of the other motif. This sequential conformational change by initial phosphorylation might accelerate later phosphorylation of PTP when time elapses.

Phosphoamino acid analysis revealed that CPTP1 was phosphorylated on both serine and threonine residues by CKII *in vitro* (Figure 5). Figure 6 shows that phosphorylation of CPTP1 on these residues resulted in an inhibition of its phosphatase activity (Figure 6). In recent years, more and more proteins are reported to be phosphorylated on multiple sites in hierarchical fashion (Roach, 1991; Stover and Walsh, 1994). This multiple protein phosphorylation extends the repertoire of structural changes that might allow more intricate regulatory circuits

to alter enzyme activity. Recently, the 3D-structure of HPTP1B was determined by X-ray crystallography (Barford *et al.*, 1994; Jia *et al.*, 1995). The catalytic site, formed from residues Cys²¹⁵ to Arg²²¹ corresponding to the conserved PTP signature motif, is located at the base of the phosphotyrosine binding pocket. Main chain nitrogens of Ser²¹⁶ to Arg²²¹ and the guanidinium side chain of Arg²²¹ form six hydrogen bonds and two salt bridges, respectively, with the three terminal phosphate oxygen. When CPTP1 is phosphorylated on serine residue corresponding to Ser²¹⁶ of HPTP1B by CKII, it may change the structure of CPTP1 and inhibit enzyme activity.

Until now, there has been no report on the regulation

of PTP by CKII-mediated phosphorylation in the cell. This paper shows that CPTP1 and HPTP1B are phosphorylated by CKII *in vitro*, and the phosphatase activity of CPTP1 decreases by its serine and threonine phosphorylation. This result indicates that changes in CPTP1 activity caused by phosphorylation could be implicated in its regulation by physical interaction with protein kinases.

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