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Interaction between chicken protein tyrosine phosphatase 1 (CPTP1)-like rat protein phosphatase 1 (PTP1) and p60$^{v\text{-}src}$ in v-*src*-transformed Rat-1 fibroblasts

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Abbreviations: CPTP1, chicken PTP1; HPTP1B, human PTP1B; NC, nitrocellulose; PTK, protein tyrosine kinases; PTP1B, protein tyrosine phosphatase 1B

Abstract

CPTP1 is a nontransmembrane chicken protein tyrosine phosphatase having 92% sequence homology to the corresponding 321 amino acids of human protein tyrosine phosphatase 1B (HPTP1B). Using anti-CPTP1 antibody, we identified CPTP1-like rat PTP1 of 51 kDa in Rat-1 and v-*src*-transformed Rat-1 fibroblasts. Here we show that CPTP1-like rat PTP1 binds to p60$^{v\text{-}src}$ *in vivo* and CPTP1 also can associate with p60$^{v\text{-}src}$ in cell lysate of v-*src*-transformed Rat-1 fibroblasts. Interaction between HPTP1B-type PTPs, CPTP1-like rat PTP1 and CPTP1, and p60$^{v\text{-}src}$ was reduced by vanadate treatment for 13 h due to down regulation of the protein level of p60$^{v\text{-}src}$ *in vivo.* Interestingly, CPTP1-like rat PTP1 was coimmunoprecipitated with a 70-kDa protein which has a possibility to be tyrosine-phosphorylated by p60$^{v\text{-}src}$ in v-*src*-transformed Rat-1 fibroblasts. These results suggest that HPTP1B-type PTPs may play an important role in p60^{src} dependent signal pathway in eucaryotic cells.

Keywords: oncogene protein pp60 (v-*src*); phosphoprotein phosphatase; protein interaction mapping; protein-tyrosine-phosphatase

Introduction

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 eucaryotic cells can be regulated tightly by the two opposing enzymes, protein tyrosine kinases (PTKs) and protein tyrosine phosphatases (PTPs).

p60$^{v\text{-}src}$, the transforming principle of the Rous sarcoma virus, was discovered as the first PTK (Collett *et al.*, 1980; Hunter & Sefton,1980; Levinson *et al.*, 1980) and human protein tyrosine phosphatase 1B (PTP1B) (HPTP1B) has been known as the first PTP. HPTP1B, the prototype of nontransmembrane PTP, was originally identified as the major PTP activity in human placenta (Tonks *et al.*, 1988a and 1988b). Recent studies showed that SH2-PTPs are substrates of the p60^{src} both *in vitro* and *in vivo*, and the interaction between SH2-PTPs and p60^{src} has been observed (Moller *et al.*, 1994; Falet *et al.*, 1996). However, little is known about the interaction between HPTP1B-type PTPs and p60^{src} *in vivo*.

The product of the *c-src* protooncogene, p60$^{c\text{-}src}$, is a normal cellular counterpart of p60$^{v\text{-}src}$ (Collett *et al.*, 1978; Oppermann *et al.*, 1979), and this protein is implicated in various cell functions including cell to cell contact, neural differentiation, and cell proliferation (Cooper & Howell, 1993).

We have cloned a cDNA that encodes a cytosolic protein tyrosine phosphatase from chicken intestine cDNA library (CPTP1). Amino acid sequences among the CPTP1, HPTP1B, and rat PTP1 were very similar (Kim *et al.*, 1996). We demonstrated that both CPTP1 and HPTP1B were tyrosine-phosphorylated near their N-terminal region by p60$^{c\text{-}src}$ and p56^{lck}, and also phosphorylated on serine and threonine residues by casein kinase II *in vitro* (Jung *et al.*, 1998; Kang and Kim, 2000). This result prompted us to examine whether CPTP1-like rat PTP1 (Woodford-Thomas *et al.*, 1992) could bind to p60$^{v\text{-}src}$ in v-*src*-transformed Rat-1 fibroblasts.

In this paper, we show that CPTP1-like rat PTP1 associates with p60$^{v\text{-}src}$ in v-*src*-transformed Rat-1 fibroblasts and CPTP1 also binds to p60$^{v\text{-}src}$ *in vitro*, suggesting that HPTP1B-type PTPs may be involved in signal transduction cascades of p60^{src} in eucaryotic cells.

Materials and Methods

Production of anti-CPTP1 antibody

Female white rabbit was immunized with GST-CPTP1-1 fusion protein (deleted form of 72 amino acids in the C-terminal side of CPTP1) by subcutaneous injection (Kim *et al.*, 1996; Harlow and Lane, 1988). A whole serum of the CPTP1-immunized rabbit was absorbed four times for 4 days with GST (glutathione S-transferase) protein purified from *E. coli* to remove antibodies responding to GST. For immunoprecipitation experiments, anti-CPTP1 polyclonal antibody was coupled to CNBr-activated Sepharose 4B (Pharmacia Biotech) as described by the manufacturer.

Cell culture

Rat-1 and v-*src*-transformed Rat-1 fibroblasts were from Dr. Shin and cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum. Confluent cells (70-80%) were treated with or without 100 mM sodium vanadate for 13 h and then washed twice with cold phosphate-buffered saline (PBS). Cells were then lysed at 4°C for 20 min in cold lysis buffer (150 mM NaCl, 20 mM Tris, pH 8.0, 1% Triton X-100, 1 mM sodium vanadate, 1 mM NaF, 1 mM PMSF, and 1 μg/μl leupeptin) or radioimmunoprecipitation assay (RIPA) buffer (150 mM NaCl, 10 mM Tris, pH 7.4, 5 mM EDTA, 1% Triton X-100, 0.1% SDS, 1 mM sodium vanadate, 1 mM PMSF, and 100 μM aprotinin). After microcentrifugation, protein concentrations of the supernatants were measured with a protein assay kit (Bio-Rad) using bovine serum albumin (BSA) as a standard. Equivalent amount of proteins was used for immunoprecipitation, immunoblotting, and GST-pulldown assay.

Immunoprecipitation and immunoblotting

The cell lysates prepared as described above were precleared with CNBr-activated Sepharose 4B at 4°C for 1 h and immunoprecipitated at 4°C for 3 h using anti-CPTP1 antibody coupled to CNBr-activated Sepharose 4B. For the immunoprecipitation experiment using anti-p60$^{c\text{-}src}$ antibody, the cell lysates were precleared with 20 μl protein A agarose beads and then incubated with anti-p60$^{c\text{-}src}$ at 4°C for 3 h. Immune complexes were collected by adding protein A agarose beads at 4°C for 1 h.

For immunoblotting, cell lysates or immune complexes were separated by SDS-PAGE and subsequently transferred to the nitrocellulose (NC) membrane using a Mini Trans-Blot-cell system (Bio-Rad) for 1 h at 350 mA. The membrane was blocked for 1 h at room temperature in 5% skim milk/TBST (25 mM Tris-HCl, 137 mM NaCl, 2.7 mM KCl, and 0.1% Tween 20, pH 7.4) and incubated with the primary antibody (anti-CPTP1 and anti-p60$^{c\text{-}src}$ antibodies with 1:5000 titer dilution; anti-PY antibody (4G10) with 1:3000 titer dilution) in 5% skim milk/TBST for overnight. Anti-p60$^{c\text{-}src}$ antibody was from Upstate Biotechnology Incorporated and anti-PY antibody (4G10) was obtained from Dr. Shin. After washing three times with TBST, secondary antibody (HRP-conjugated goat anti-rabbit or anti-mouse antibody with 1:5000 titer dilution) was added and incubated for 1 h, and the blots were washed and visualized by the Amersham ECL system.

GST-pulldown assay

The cell lysates (2 mg) were precleared with glutathione agarose beads and incubated for 3 h at 4°C with 4 μg each of GST-CPTP1-2 (deleted form of 41 amino acids in the C-terminal side of CPTP1) purified as described by Jung *et al.* (1998). The complexes were collected by adding glutathione agarose beads for 1 h at 4°C. Aliquots from each sample were analyzed by 8% SDS-PAGE, transferred to NC membrane, and immunoblotted with anti-p60$^{c\text{-}src}$ antibody as previously described.

Results and Discussion

We previously cloned a nontransmembrane CPTP1 from chicken intestine cDNA library (Kim *et al.*, 1996). CPTP1 belongs to HPTP1B-type PTP and contains 92% sequence identity with the corresponding 321 amino acid residues of HPTP1B. Rat PTP1 was isolated from rat brain cDNA library as another HPTP1B-type PTP (Guan *et al.*, 1990) and shared 97% amino acid sequence identity with HPTP1B (Woodford-Thomas *et al.*, 1992).

SH2-PTPs such as PTP1C, Syp and PTP1D have been reported to be phosphorylated on tyrosine in cells transformed by v-*src* or over-expressing the β-PDGF receptor (Feng *et al.*, 1993; Vogel *et al.*, 1993; Matozaki *et al.*, 1994). Particularly, the tyrosine phosphorylation of PTP1D leads to increase of its catalytic activity (Vogel *et al.*, 1993).

To determine whether HPTP1B-type PTPs could interact with p60$^{v\text{-}src}$ *in vivo,* we firstly checked the expression of CPTP1-like rat PTP1 in Rat-1 and v-*src*-transformed Rat-1 fibroblasts. Immunoblot analysis showed that endogenous CPTP1-like rat PTP1 was detected by anti-CPTP1 antibody as a single protein band of 51 kDa in Rat-1 and v-*src*-transformed Rat-1 fibroblasts (Figure 1). This result indicates that endogenous 51 kDa CPTP1-like rat PTP1 corresponds to the rat PTP1 reported by Woodford-Thomas *et al.* (1992).

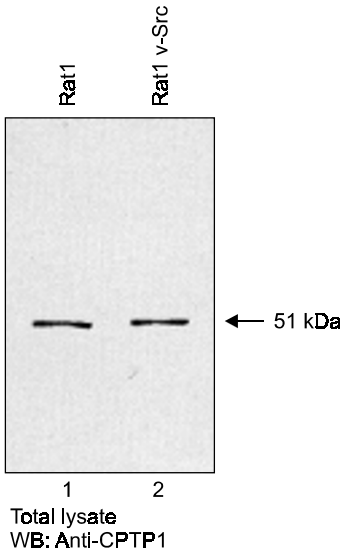

Figure 1. Immunoblot analysis of CPTP1-like rat PTP1 in Rat-1 and v-*src*-transformed Rat-1 fibroblasts. Rat-1 (lane 1) and v-*src*-transformed Rat-1 fibroblasts (lane 2) were lysed in cold lysis buffer at 4°C for 20 min and the cell lysates (15 μg each) were separated by 10% SDS-PAGE, transferred to NC membrane, and subjected to Western blot with anti-CPTP1 antibody. The protein was visualized by ECL detection system.

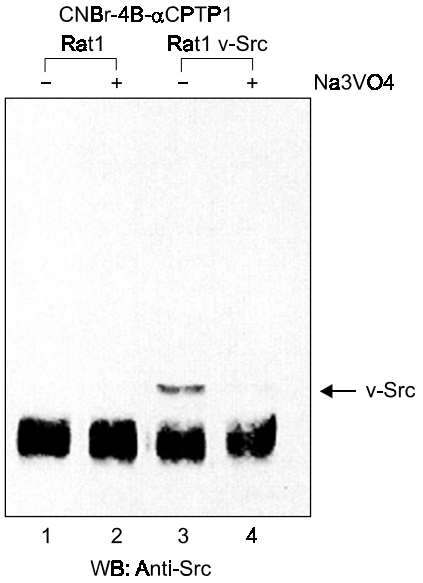

Figure 2. CPTP1-like rat PTP1 binds to $p60^{v\text{-}src}$ in v-*src*-transformed Rat-1 fibroblasts. Rat-1 (lanes 1 and 2) and v-*src*-transformed Rat-1 fibroblasts (lanes 3 and 4) treated without (lanes 1 and 3) and with vanatate (lanes 2 and 4) were precleared and immunoprecipitated at 4°C for 3 h using anti-CPTP1 antibody coupled to CNBr-activated Sepharose 4B. Immune complexes were separated by 8% SDS-PAGE, transferred to NC membrane, and immunoblotted with anti-$p60^{c\text{-}src}$ antibody.

To examine the association between CPTP1-like rat PTP1 and $p60^{v\text{-}src}$ *in vivo*, the cell lysates were immunoprecipitated with anti-CPTP1 antibody coupled to CNBr-activated Sepharose 4B and then immunoblotted with anti-$p60^{c\text{-}src}$ antibody. As shown in lanes 3 and 4 of Figure 2, endogenous CPTP1-like rat PTP1 associated with $p60^{v\text{-}src}$ in v-*src*-transformed Rat-1 fibroblasts, and long term treatment of vanadate, a phosphatase inhibitor, resulted in decrease of the binding affinity.

The interaction between CPTP1 and $p60^{v\text{-}src}$ was identified by GST-pulldown analysis using GST-CPTP1-2 fusion protein. The result showed that CPTP1 also bound to $p60^{v\text{-}src}$ in cell lysate of v-*src*-transformed Rat-1 fibroblasts and their binding affinity was decreased by long term treatment of vanadate (Figure 3, lane 3 and 4).

To address whether the decrease of binding affinity between these HPTP1B-type PTPs and $p60^{v\text{-}src}$ was caused by the tyrosine phosphorylation of $p60^{v\text{-}src}$ by long term treatment of vanadate, we tried to determine the tyrosine phosphorylation level of $p60^{v\text{-}src}$ by vanadate treatment. Cell lysates were immunoprecipitated with anti-$p60^{c\text{-}src}$ antibody and then immunoblotted with anti-PY antibody. We, however, could not detect the tyrosine-phosphorylated $p60^{v\text{-}src}$ in v-*src*-transformed Rat-1 fibroblasts (data not shown). Next we tested the protein expression level of $p60^{v\text{-}src}$ in cells treated with or without vanadate for 13 h. We

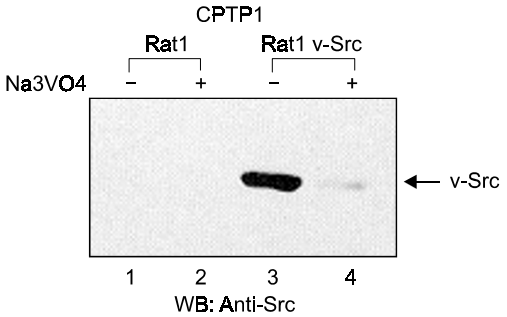

Figure 3. Interaction between CPTP1 and $p60^{v\text{-}src}$ in cell lysate of v-*src*-transformed Rat-1 fibroblasts. Rat-1 (lanes 1 and 2) and v-*src*-transformed Rat-1 fibroblasts (lanes 3 and 4) treated without (lanes 1 and 3) and with vanatate (lanes 2 and 4) were precleared and incubated at 4°C for 3 h with GST-CPTP1-2. The complexes were separated by 8% SDS-PAGE, transferred to NC membrane, and immunoblotted with anti-$p60^{c\text{-}src}$ antibody.

found that the protein expression level of $p60^{v\text{-}src}$ was severely down regulated by long term treatment of vanadate (Figure 4) and thus this led to decrease of binding affinity between HPTP1B-type PTPs, CPTP1-like rat PTP1 and CPTP1, and $p60^{v\text{-}src}$ (Figure 2 and 3). Vanadate has been known to induce massive apoptosis in mammary epithelial cells, although its mechanism is still unclear (Gallo-Hendrikx *et al.*,

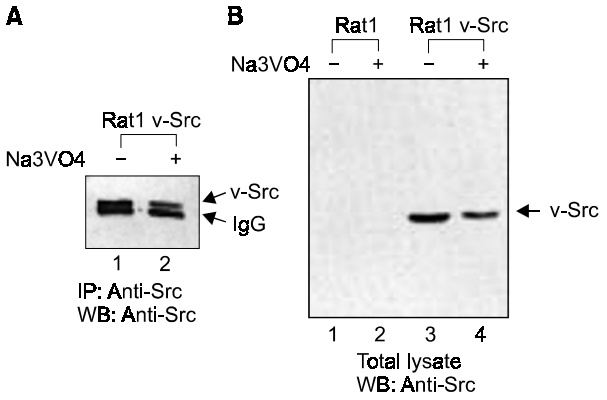

Figure 4. p60$^{v\text{-}src}$ protein is down regulated by long term treatment of vanadate *in vivo*. (A) v-*src*-transformed Rat-1 fibroblasts treated without (lane 1) and with vanatate (lane 2) were precleared, incubated with anti-p60$^{c\text{-}src}$ antibody for 3 h at 4°C, and separated by 8% SDS-PAGE. (B) Rat-1 (lanes 1 and 2) and v-*src*-transformed Rat-1 fibroblasts (lanes 3 and 4) treated without (lanes 1 and 3) and with vanatate (lanes 2 and 4) of 15 μg each were separated by 8% SDS-PAGE. Proteins of (A) and (B) were transferred to NC membrane and immunoblotted with anti-p60$^{c\text{-}src}$ antibody.

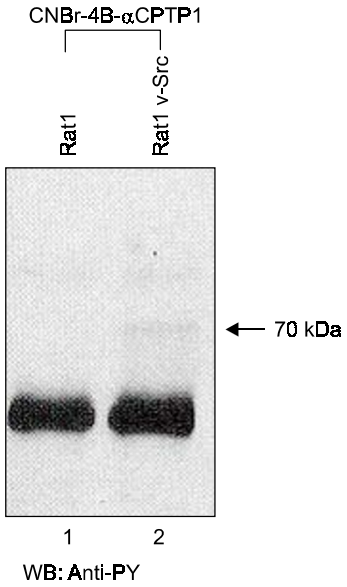

Figure 5. CPTP1-like rat PTP1 binds to a 70 kDa tyrosine-phosphorylated protein in v-*src*-transformed Rat-1 fibroblasts. Rat-1 (lane 1) and v-*src*-transformed Rat-1 fibroblasts (lane 2) were lysed in cold lysis buffer at 4°C for 20 min and the cell lysates (500 μg each) were precleared and immunoprecipitated at 4°C for 3 h using 30 μl of anti-CPTP1 antibody coupled to CNBr-activated Sepharose 4B. Immune complexes were separated by 8% SDS-PAGE, transferred to NC membrane, and immunoblotted with anti-PY antibody (4G10).

2001). This fact suggests that p60$^{v\text{-}src}$ may be a target of a degradation system in apoptotic signal transduction pathways activated by long term treatment of vanadate.

To examine whether CPTP1-like rat PTP1 is phosphorylated on tyrosine by p60$^{v\text{-}src}$ *in vivo*, the cell lysates were immunoprecipitated with anti-CPTP1 antibody coupled to CNBr-activated Sepharose 4B and then immunoblotted with anti-PY antibody (Figure 5). Immunoblot analysis showed that the tyrosine phosphorylation of CPTP1-like rat PTP1 was not shown because of being overlapped by heavy chain of anti-CPTP1 polyclonal antibody. Rather, we found that CPTP1-like rat PTP1 bound to a 70 kDa protein that could be phosphorylated on tyrosine by p60$^{v\text{-}src}$ in v-*src*-transformed Rat-1 fibroblasts (Figure 5, lane 2). To understand a physiological role of CPTP1-like rat PTP1 in the signal transduction cascades activated by p60$^{v\text{-}src}$, at present, it is required to investigate the function of this unknown 70 kDa protein in v-*src*-transformed Rat-1 fibroblasts.

Taken together, these results show that HPTP1B-type PTPs, CPTP1-like rat PTP1 and CPTP1, associate with p60$^{v\text{-}src}$, suggesting an important role of these proteins in p60$^{v\text{-}src}$-induced signal transduction cascades.

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References

Collett MS, Brugge JS, Erikson RL. Characterization of a normal avian cell protein related to the avian sarcoma virus transforming gene product. Cell 1978;15:1363-9

Collett MS, Purchio AF, Erikson RL. Avian sarcoma virus-transforming protein, pp60src shows protein kinase activity specific for tyrosine. Nature 1980;285:167-9

Cooper JA, Howell B. The when and how of Src regulation. Cell 1993;73:1051-4

Falet H, Ramos-Morales F, Bachelot C, Fischer S, Rendu F. Association of the protein tyrosine phosphatase PTP1C with the protein tyrosine kinase c-Src in human placenta. FEBS Lett 1996;383:165-9

Feng GS, Hui CC, Pawson T. SH-2 containing phosphotyrosine phosphatase as a target of protein-tyrosine kinases. Science 1993;259:1607-11

Gallo-Hendrikx E, Murray SA, Vonderhaar BK, Xiao ZX. Vanadate disrupts mammary gland development in whole organ culture. Dev Dyn 2001;222:354-67

Guan K, Haun RS, Watson SJ, Geahlen RL, Dixon JE. Cloning and expression of a protein-tyrosine-phosphatase.

Proc Natl Acad Sci USA 1990;87:1501-5

Harlow E, Lane D. Antibodies: a laboratory manual, Cold Spring Harbor, NY. 1988

Hunter T, Sefton BM. Transforming gene product of Rous sarcoma virus phosphorylates tyrosine. Proc Natl Acad Sci USA 1980;77:1311-5

Jung EJ, Kang Y-S, Kim CW. Multiple phosphorylation of chicken protein tyrosine phosphatase 1 and human protein tyrosine phosphatase 1B by casein kinase II and $p60^{v\text{-}src}$ *in vitro*. Biochem Biophys Res Comm 1998;246:238-42

Kang KR, Kim CW. Regulation of chicken protein tyrosine phosphatase 1 and human protein tyrosine phosphatase 1B activity by casein kinase II- and p56lck-mediated phosphorylation. Exp Mol Med 2000;32:47-51

Kim CW, Jung EJ, Kang Y-S. Cloning and characterization of a chicken protein tyrosine phosphatase, CPTP1. Exp Mol Med 1996;28:153-69

Levinson AD, Oppermann H, Varmus HE, Bishop JM. The purified product of the transforming gene of avian sarcoma virus phosphorylates tyrosine. J Biol Chem 1980;255:11973-80

Matozaki T, Uchida T, Fujioka Y, Kasuga M. Src kinase tyrosine phosphorylates PTP1C, a protein tyrosine phosphatase containing Src homology-2 domains that down-regulates cell proliferation. Biochem Biophys Res Commun 1994;204:874-81

Moller NP, Moller KB, Lammers R, Kharitonenkov A, Sures I, Ullrich A. Src kinase associates with a member of a distinct subfamily of protein-tyrosine phosphatases containing an ezrin-like domain. Proc Natl Acad Sci USA 1994;91:7477-81

Oppermann H, Levinson AD, Varmus HE, Levintow L, Bishop JM. Uninfected vertebrate cells contain a protein that is closely related to the product of the avian sarcoma virus transforming gene (*src*). Proc Natl Proc Sci USA 1979;76:1804-8

Tonks NK, Dilts CD, Fischer EH. Purification of the major protein-tyrosine-phosphatases of human placrenta. J Biol Chem 1988a;263:6722-30

Tonks NK, Dilts CD, Fischer EH. Characterization of the major protein-tyrosine phosphatases of human placrenta. J Biol Chem 1988b;263:6731-7

Vogel W, Lammers R, Huang J, Ullrich A. Activation of a phosphotyrosine phosphatase by tyrosine phosphorylation. Science 1993;259:1611-4

Woodford-Thomas TA, Rhodes JD, Dixon JE. Expression of a protein tyrosine phosphatase in normal and v-*src*-transformed mouse 3T3 fibroblasts. J Cell Biol 1992;117:401-14