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Inhibition of Src by direct interaction with protein phosphatase 2A

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Abstract In this study, we report that Src kinase is inhibited by protein phosphatase 2A (PP2A), a serine/threonine phosphatase. We carried out experiments in vitro using purified PP2A (AC dimer) and full-length v-Src or truncated forms of v-Src. The inhibition of v-Src by PP2A is concentration- and time-dependent. Addition of okadaic acid, a PP2A phosphatase inhibitor, abolished the PP2A-dependent inhibition of v-Src. When experiments were carried out at 4°C under conditions where PP2A activity is inhibited, Src activity was unaffected by the presence of PP2A, suggesting that PP2A binding alone is insufficient to block Src activity. These results imply that PP2A activity is essential for inhibition of v-Src. We also demonstrate that PP2A binds to the catalytic and the regulatory domains of v-Src. © 2001 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.

Key words: Protein phosphatase 2A; v-Src; Tyrosine kinase activity; Inhibition; SH2 domain; Catalytic domain

1. Introduction

Members of the Src kinase family have important roles in controlling growth, proliferation, and differentiation [1,2]. Src kinases are composed of an N-terminal membrane binding region, a unique domain, an SH3 domain that binds proline-rich sequences, a phosphotyrosine binding SH2 domain, a tyrosine kinase catalytic domain, and a C-terminal tail [2,3]. Src family kinase activity is negatively regulated by intramolecular interactions involving the SH3 and SH2 domains [4,5]. Src kinase activity is also regulated positively or negatively by tyrosine phosphorylation in the kinase activation loop and C-terminal tail, respectively [4,5].

Protein phosphatase 2A (PP2A) is a widely expressed serine/threonine phosphatase involved in the regulation of signal transduction, growth and development [6–8]. The PP2A holoenzyme is composed of catalytic (C), structural (A) and regulatory (B) subunits [7–9]. The core heterodimer (AC) forms complexes with various regulatory subunits, giving rise to different substrate specificities and intracellular localizations. PP2A is also regulated by posttranslational modifications to the C subunit such as phosphorylation [10–12] and carboxymethylation [13,14].

Various serine/threonine protein kinases are known to form stable complexes with PP2A and to be regulated by PP2A [6,7]. However, relatively few tyrosine kinases have been reported to associate with PP2A. Tyrosine phosphorylation of PP2A occurs in response to growth factor stimulation and v-Src transformation of fibroblasts. Src, $p56^{lck}$, the epidermal growth factor (EGF) receptor, and insulin receptor can phosphorylate the carboxy-terminal region of the C subunit of PP2A (Tyr-307), resulting in inhibition of phosphatase activity [10,11]. Replacement of this tyrosine with an acidic residue abolishes the interaction between the AC heterodimer and the B subunit [15]. Recently, we have reported that Jak2, a member of a different family of tyrosine kinases, also phosphorylates the C subunit of PP2A and inhibits PP2A [16]. Thus, tyrosine phosphorylation of the C subunit is one of the regulatory mechanisms of PP2A.

Src kinase has been reported to form a complex with PP2A [17–21]. PP2A has also been found to associate with SV40 small T-antigen and polyoma virus middle T-antigen (MT), resulting in changes in PP2A activity or substrate specificity [22–24]. Both small T-antigen and MT form complexes with the 65-kDa putative regulatory subunit (PR65)/C subunit dimer of PP2A [17,25]. MT induces cell transformation by associating with and modulating the activities of cellular proteins involved in cell proliferation. MT binds three members of the Src family kinase, $pp60^{c-src}$ [26], $pp62^{c-yes}$ [27] and $pp59^{c-fyn}$ [28,29]. MT/Src complexes contain equimolar amounts of PP2A [20]. However, it is not clear whether Src associates directly with PP2A. The physiological functions of Src–PP2A complexes have not been completely elucidated. In particular, the effect of complex formation on Src kinase activity has not been investigated. In this study, we analyzed the effect of the PP2A–Src association using purified protein components. We show that the interaction is direct, that PP2A binding decreases Src tyrosine kinase activity, and that PP2A binds to SH2 and SH3 domains in addition to the catalytic domain of Src.

2. Materials and methods

2.1. Materials

PP2A protein (heterodimer of C subunit and A subunit), PP2A C subunit antibody and phosphotyrosine antibody (4G10) were obtained from Upstate Biotechnology (Lake Placid, NY, USA). Okadaic acid (OA) and protein A-agarose were from Sigma. [γ-^{32}P]ATP was from Amersham Pharmacia Biotech (Piscataway, NJ, USA). Ni-NTA resin was from Qiagen. E_4IYM_4 (KKEEEEIYMMMM) peptide was synthesized on an Applied Biosystems automated peptide synthesizer and purified by reverse-phase high performance liquid chromatography. Glutathione-agarose and glutathione $_S$-transferase (GST) antibody were from Molecular Probes (Eugene, OR, USA). The $(HA)_3$-tagged PP2A C subunit expression plasmid was a gift from Dr. David Brautigan (University of Virginia, Charlottesville, VA, USA). The Ts-72 cell line (NIH 3T3 fibroblasts transformed by a temperature-

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sensitive mutant of Src) was obtained from Dr. Steven M. Anderson (University of Colorado Health Science Center, Denver, CO, USA). Ts-72 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% of fetal bovine serum and antibiotics at 39°C. For activation of Src, cells were cultured at 34°C for 2 days.

2.2. Expression and purification of v-Src

Expression of the full-length 60-kDa Rous sarcoma virus v-Src gene product was carried out in *Spodoptera frugiperda* (Sf9) cells infected with a recombinant baculovirus harboring the v-Src gene [30]. Immunoaffinity purification of v-Src was performed as described previously [30].

To produce the truncated form of v-Src lacking the unique region, v-Src DNA from the Schmidt-Ruppin strain of Rous sarcoma virus (encoding amino acids 77–526, comprising the SH3, SH2 and catalytic domain of v-Src) was subcloned into pFastBacHTb (Gibco BRL) as a *Bam*HI/*Eco*RI fragment. Truncated v-Src was expressed in Sf9 cells and purified using Ni-NTA resin, followed by a hydroxyapatite column, as described previously [31].

The following domains of v-Src were expressed in *Escherichia coli* as GST fusion proteins: SH2, SH3–SH2, catalytic, and SH2–catalytic. The SH2 domains of Nck and SHP2 were also expressed in *E. coli* as GST fusion proteins. Expression of the GST fusion proteins was carried out in bacterial strain NB42, and the constructs were purified on glutathione-agarose as described previously [32].

2.3. Src kinase assay

Src activity was measured using the phosphocellulose binding assay. Assays were carried out in 20 μl at either or 30°C or 4°C, as described previously [31]. The reaction mixtures contained 20 mM Tris–HCl (pH 7.4), 10 mM $MgCl_2$, 0.1 mM Na_3VO_4, 5 mg/ml bovine serum albumin (BSA), 0.25 mM ATP, 0.5 mM E_4IYM_4 peptide (KKEEEEIYMMMM) and [γ-^{32}P]ATP (200–300 cpm/pmol) with or without PP2A (heterodimer of C subunit and A subunit, Upstate Biotechnology). Reactions were terminated by addition of 50% acetic acid and spotted on P81 paper. After washing with 0.5% phosphoric acid, incorporation of ^{32}P into peptides was determined by liquid scintillation counting.

2.4. Pull-down experiments using GST fusion proteins

Ts-72 cells were harvested, washed twice with ice-cold phosphate-buffered saline, and lysed in lysis buffer (10 mM Tris–HCl (pH 7.5), 5 mM EDTA, 50 mM NaF, 2 mM Na_3VO_4, 10 mM dithiothreitol, 1% Nonidet P-40 (NP-40), 0.05% SDS, 0.25% sodium deoxycholate, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, 10 μg/ml aprotinin) at 4°C for 30 min. Cell lysates were centrifuged at $15\,000\times_g$ for 10 min. The resulting supernatants (1 mg cellular proteins) were used for GST-catalytic, GST-SH2–catalytic, GST-SH2 or GST-SH3–SH2 pull-down experiments. After addition of 10 μl of GST fusion proteins coupled to glutathione-agarose (or GST-agarose gel as a control), incubations were continued 5 h at 4°C. The resin was collected and washed four times with phosphate-buffered saline plus 0.5% NP-40. The precipitated proteins were analyzed on a 7.5% SDS–polyacrylamide gel. For Western blot analysis, proteins in the gel were transferred to Immobilon membrane (Millipore, Bedford, MA, USA) in the presence of 0.1% SDS. The membranes were blocked using 5% milk in Tris-buffered saline plus 0.1% Tween 20, then probed with GST, phosphotyrosine or PP2A antibodies. Blots were visualized the horseradish peroxidase-conjugated second antibody with ECL (Enhanced Chemiluminescence, Amersham). Pull-down experiments with the GST-SH2 domains of Nck and SHP2 were performed in a similar manner.

2.5. Pull-down experiments using immobilized PP2A

Immobilized PP2A was prepared as described previously [33]. Briefly, an expression vector encoding $(HA)_3$-tagged PP2A C subunit was transfected into COS-7 cells. Cells were harvested after 24 h and HA antibody was added to the cell lysates. Protein A-agarose was added and then incubated overnight. For some experiments, the immobilized PP2A C subunit was phosphorylated by addition of v-Src (0.04 μg) at 30°C for 1 h in kinase assay buffer. As a control, non-phosphorylated PP2A C subunit was prepared by addition of v-Src without ATP in kinase buffer. Pull-down reactions were carried out by incubating Src constructs with immobilized PP2A (or mouse IgG protein agarose as a control) overnight at 4°C.

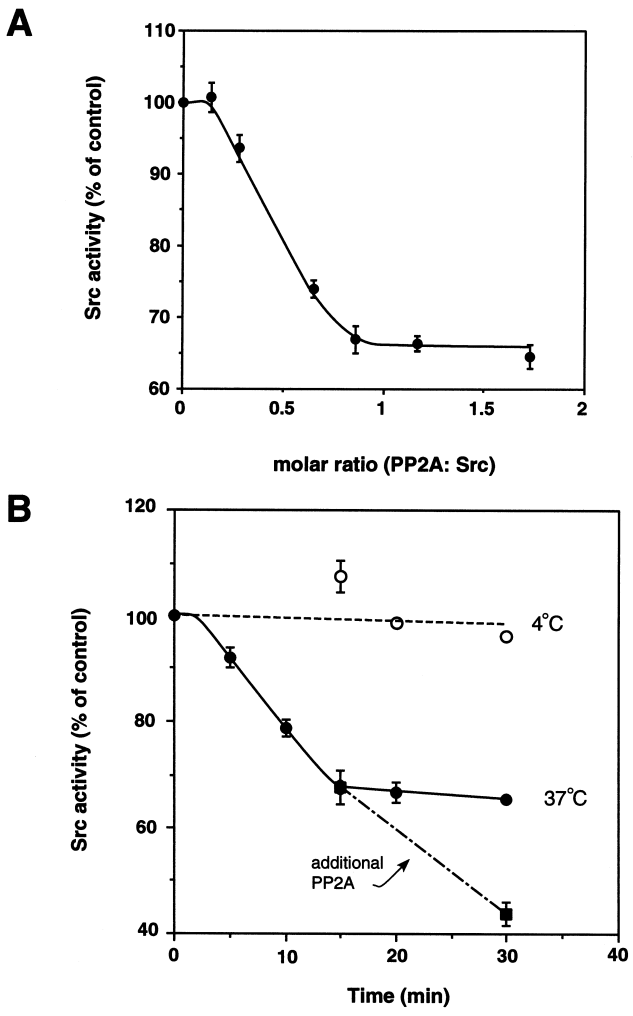

Fig. 1. Inhibition of Src by PP2A. A: The activity of v-Src was measured after 30 min of incubation with various amounts of PP2A. Src activity was measured using 0.5 mM E_4IYM_4 peptide. The activity of Src in the absence of PP2A was taken as 100%. B: Src activity was measured after different times of incubation with 1.2 mol PP2A per mol Src. The activity of Src alone was taken as 100%. Closed circles: assays performed at 30°C; open circles: assays performed at 4°C. Closed square: addition of 2.4 mol of PP2A at 15 min, followed by an additional 15 min incubation.

3. Results and discussion

3.1. Inhibition of Src activity by PP2A in vitro

To analyze the effect of PP2A on Src activity, truncated v-Src was incubated with various concentrations of PP2A for 30 min. As shown in Fig. 1A, Src activity becomes inhibited with incubation of exogenous PP2A. Maximum inhibition of Src activity occurred at a ratio of about 0.9 mol of PP2A to one mol of Src, and inhibition seems to plateau at molar ratios higher than 1:1 (see below). Similar results were obtained using full-length v-Src (result not shown).

We next analyzed the time course of v-Src inhibition using a PP2A:Src ratio of 1.2 (Fig. 1B). The time-dependent inhibition of v-Src reached a plateau after 15 min. One possible explanation for the plateau is that tyrosine phosphorylation of PP2A is likely to occur in the reaction mixture. Tyrosine phosphorylation of the C subunit of PP2A by Src and other tyrosine kinases has previously been shown to inhibit PP2A

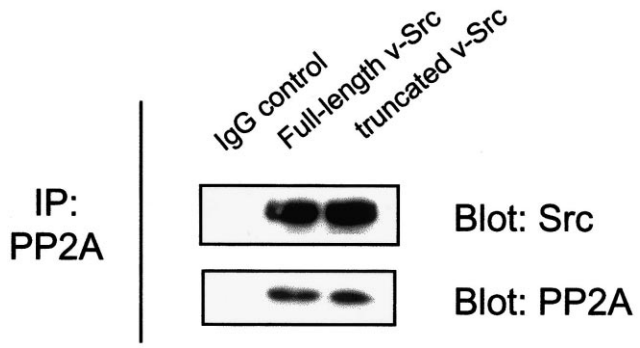

Fig. 2. Immunoprecipitation of full-length and truncated v-Src by PP2A in vitro. Purified PP2A (dimer of A and C subunits, 0.1 μg) was incubated with full-length v-Src (0.37 μg) or histidine-tagged truncated v-Src (0.37 μg) in the presence of 500 μg BSA. Immunoprecipitation reactions were carried out using PP2A antibody or mouse IgG as control. Bound proteins were subjected to SDS–PAGE and analyzed with polyclonal Src antibody or PP2A antibodies. The results are representative of four independent experiments.

[10,11,16]. Thus, even at higher concentrations of PP2A, the available PP2A activity for Src inhibition may become limited. We tested this by adding additional PP2A to a Src–PP2A reaction mixture after 15 min. This treatment resulted in further inhibition of Src (Fig. 1B).

We investigated whether the phosphatase activity of PP2A is necessary for inhibition of v-Src. (An alternative possibility is that PP2A binding alone is sufficient to inhibit v-Src.) It is well established that PP2A activity is not required for binding to Src [20,21,33]. Thus, we carried out experiments under two conditions in which PP2A activity is inhibited. First, we performed experiments in the presence of OA, a specific PP2A phosphatase inhibitor. As shown in Table 1, OA abolished the inhibitory effect of PP2A. These data imply that PP2A activity is necessary for inhibition of v-Src. We also performed experiments at 4°C; PP2A is completely inhibited under these conditions, but Src retains significant activity (results not shown). Under these assay conditions, PP2A did not show any inhibition of Src activity (Fig. 1B). Thus, using two different approaches, we demonstrated that PP2A activity is required to inhibit Src activity in vitro. We speculate tyrosine-phosphorylated PP2A would be released from the Src–PP2A complex in vivo, and would subsequently regain its activity by autodephosphorylation [10,11].

Table 1
Effect of OA on Src inhibition by PP2A

OA (nM)	Src activity (% of control)
0	66.7 ± 9.6
25	79.6 ± 8.3
50	91.2 ± 8.4
100	98.0 ± 7.6

Src activity in the presence of PP2A (1.2-fold molar excess) was determined after 30 min incubation with different concentrations of OA in the assay. Src activity without incubation with PP2A was taken as 100%.

3.2. Mapping of PP2A binding sites on Src

The binding sites for PP2A on Src have not previously been determined. The principal sites for serine/threonine phosphorylation of Src lie in the N-terminal unique region [1]. The unique region contains protein kinase (PK) A (Ser-17) and PKC (Ser-12, 48) phosphorylation sites, although the role of serine/threonine phosphorylation is currently not clear [1]. The presence of these phosphorylation sites suggested that the unique region might be the point of contact with PP2A. To investigate the importance of the Src unique region in complex formation with PP2A, a construct consisting of the SH3, SH2, and catalytic domains of v-Src was incubated with PP2A in vitro. The reaction was subjected to immunoprecipitation with PP2A antibody and analyzed by Western blotting with Src antibody. As shown in Fig. 2, the SH3–SH2–catalytic domain construct was immunoprecipitated by PP2A antibody as well as full-length v-Src. These data show a direct interaction between the two molecules in vitro, and suggest that the N-terminal membrane binding and unique domains of Src are dispensable for binding to PP2A. When purified from Sf9 cells, the SH3–SH2–catalytic domain construct has no detectable serine phosphorylation (as judged by Western blotting with anti-phosphoserine antibody). Thus, inhibition of Src activity by PP2A is most likely not related to dephosphorylation of serine/threonine residues on Src. At present, the mechanism of inhibition of Src activity by PP2A is not clear.

To analyze the potential involvement of the Src SH2 domain on PP2A binding, we prepared GST fusion proteins containing the catalytic domain and the SH2–catalytic do-

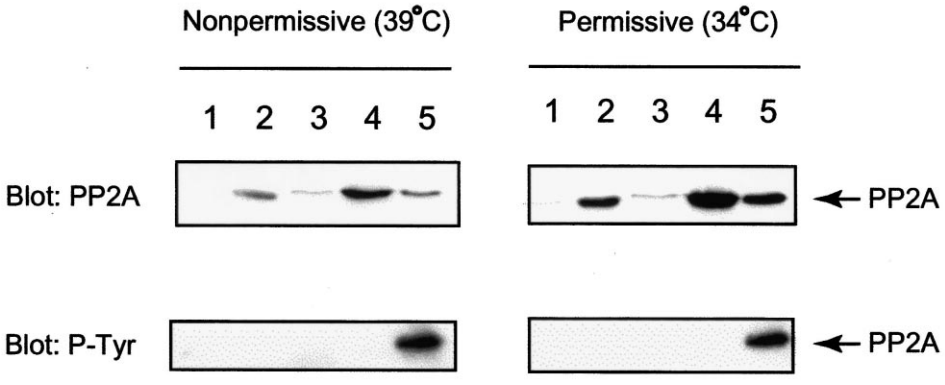

Fig. 3. GST pull-down experiments. Immobilized GST fusion proteins were incubated with lysates (1 mg protein) from Ts-72 cells grown at the permissive and non-permissive temperatures. Bound proteins were separated by SDS–PAGE and analyzed by immunoblotting with PP2A and phosphotyrosine antibodies. Lane 1: GST; lane 2: GST-Nck SH2; lane 3: GST-SHP2 SH2; lane 4: GST-Src catalytic domain; lane 5: GST-SH2–catalytic domains of Src. The results are representative of three independent experiments.

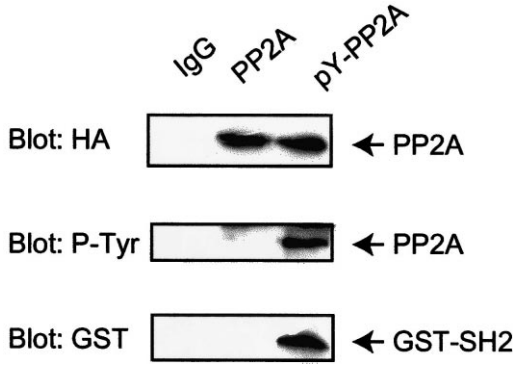

Fig. 4. Pull-down experiments with $(HA)_3$-tagged PP2A C subunit. Immobilized PP2A (or tyrosine-phosphorylated PP2A, designated pY-PP2A) was washed twice with PBS containing 0.5% NP-40, then incubated with a GST fusion protein containing the SH2 domain of Src (1.5 μg) at 4°C overnight. Bound proteins were analyzed by SDS–PAGE and Western immunoblotting with anti-HA, phosphotyrosine and GST antibodies. The results are representative of three independent experiments.

mains of v-Src. We used these fusion proteins to analyze proteins in Ts-72 cell lysates that specifically bound to the Src domains. The tsNY72-4 mutant form of v-Src expressed in these cell lines results in a tumorigenic phenotype only when cells are cultured at the permissive temperature [34]. Experiments were carried out at both the Src permissive (34°C) and Src non-permissive (39°C) temperatures. We have previously reported that tyrosine phosphorylation of PP2A was increased by shifting from the non-permissive to the permissive temperature in Ts-72 cells [33]. Bound proteins were analyzed by immunoblotting with phosphotyrosine and PP2A antibodies (Fig. 3). In all experiments, we used similar amounts of GST fusion proteins (results not shown).

PP2A bound to the catalytic domain of Src, and the binding of PP2A was stronger when lysates were prepared from cells grown at the Src permissive temperature (Fig. 3). PP2A also interacts with the SH2–catalytic construct. In contrast, tyrosine-phosphorylated PP2A interacted with the SH2–catalytic construct, but not with the catalytic domain alone, as shown by reblotting with phosphotyrosine antibody (Fig. 3). The lack of binding between tyrosine-phosphorylated PP2A and the catalytic domain of Src is consistent with the speculation that tyrosine-phosphorylated PP2A is released from the complex with Src. Tyrosine-phosphorylated PP2A also interacted with a GST fusion protein containing the isolated SH2 domain of Src (results not shown).

We investigated the specificity of PP2A binding to the Src SH2 domain using SH2 domains from the Nck adapter protein and from the tyrosine phosphatase SHP2. PP2A interacted with the Nck-SH2, but in this case the bound PP2A did not react with anti-phosphotyrosine antibody (Fig. 3). The SH2 domain of SHP2 showed only a weak interaction with PP2A. These results suggest that tyrosine-phosphorylated PP2A preferentially binds to the SH2 domain of Src.

We carried out additional experiments to investigate the binding of tyrosine-phosphorylated PP2A to the Src SH2 domain. Immobilized PP2A C subunit (tyrosine-phosphorylated or unphosphorylated) was incubated with the GST-SH2 domain. The Src SH2 domain bound to the phosphorylated PP2A C subunit, but not to unphosphorylated PP2A (Fig. 4). Tyrosine phosphorylation of the PP2A C subunit was confirmed by phosphotyrosine immunoblotting. In parallel experiments with a construct containing the SH3–SH2–catalytic domains of Src, unphosphorylated PP2A bound more strongly than tyrosine-phosphorylated PP2A (data not shown). This suggests that an intramolecular interaction between SH3, SH2 and catalytic domains may limit the access of tyrosine-phosphorylated PP2A to the SH2 domain.

The PP2A C subunit contains no typical Src SH2 binding sequence (pY-E/D-E/D-hydrophobic). Tyr-307 at the carboxy-terminus of the C subunit of PP2A (in the sequence TRRTPDYFL) is the site reported to be tyrosine-phosphorylated by Src, $p56^{lck}$, epidermal growth factor receptors and insulin receptors [10,11]. The result in Fig. 4 implies that Tyr-307 is involved in binding to the SH2 domain of Src. At present, there are no other reported sites of tyrosine phosphorylation on the C subunit. However, we cannot exclude the possible involvement of another tyrosine phosphorylation site. The carboxy-terminus of the PP2A C subunit is highly conserved and has been shown to be methylated in vivo at Leu-309 [13,15,35]. Methylation could potentially regulate the association between Src and PP2A. The PP2A C subunit contains a phosphorylation site at Thr-304 in addition to Tyr-307. The carboxy-terminus is important in specific binding to B subunits [15], raising the possibility that the phosphorylation status of the C-terminus may influence holoenzyme assembly of PP2A.

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